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Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products

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***Objectives*: The aim of this study was to assess the antimicrobial susceptibility of a taxonomically diverse set of *Bifidobacterium* strains to different classes of antimicrobial agents using a recently described medium.**

***Methods*: The susceptibility of 100 strains encompassing 11 bifidobacterial species originating from humans, animals and probiotic products to 12 antimicrobial agents was tested by agar overlay disc diffusion. Based on these results, one or two strains per species were selected for susceptibility testing to nine antibiotics by broth microdilution using the Lactic acid bacteria Susceptibility test Medium (LSM) supplemented with cysteine. The genotypic basis of atypical tetracycline resistance was further characterized using PCR, Southern blotting and partial sequencing.**

***Results*: Based on the distribution of inhibition zone diameters and MIC values, all strains tested were susceptible to amoxicillin, chloramphenicol, erythromycin, quinupristin/dalfopristin, rifampicin and vancomycin. Our data also reinforce earlier observations indicating that bifidobacteria are intrinsically resistant to gentamicin, sulfamethoxazole and polymyxin B. Susceptibility to trimethoprim, trimethoprim/sulfamethoxazole, ciprofloxacin, clindamycin, tetracycline and minocycline was variable. The *tet*(W) gene was responsible for tetracycline resistance in 15 strains including 7 probiotic isolates belonging to the taxa *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum*. This gene was present in a single copy on the chromosome and did not appear to be associated with the conjugative transposon Tn*B1230* previously found in *tet*(W)-containing *Butyrivibrio fibrisolvens*.**

***Conclusions*: The use of the LSM + cysteine medium allowed us to discriminate between intrinsic and atypical resistance properties of bifidobacteria and sets the scene for future definition of epidemiological cut-off values for all important *Bifidobacterium* species. The presence of an acquired *tet*(W) gene in several probiotic product isolates stresses the need for a minimal safety evaluation during the selection of *Bifidobacterium* strains for probiotic use.**

Keywords: disc diffusion, MICs, LSM, tetracyclines, *tet*(W)

Introduction

Bifidobacteria are Gram-positive, bifid-shaped anaerobes that constitute a major group of the human and animal gastrointestinal microbiota. Because these organisms are known to play a pivotal role in maintaining the microbial balance of a healthy intestinal tract, they are frequently applied as probiotics in health-promoting dairy products and dried food supplements.[1] Therapeutic administration of antimicrobial agents is likely to affect the intestinal microbial balance, e.g. by suppressing bacterial groups such as bifidobacteria that are beneficial to the host, and often results in intestinal disorders. In co-administration with antibiotics in order to restore the intestinal health of the host, the presence of antimicrobial resistance in probiotic *Bifidobacterium* strains might be regarded as a desirable trait to allow their survival in the gastrointestinal tract. On the other hand, there is also the growing concern that these antimicrobial resistances, if encoded by genes located on mobile elements, may be potentially transferable from probiotic strains to commensal flora or human opportunists. For this reason, the presence of acquired

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antimicrobial resistances is one of the first safety criteria to be checked during the selection process of a potentially probiotic strain.

Bifidobacteria are generally considered to be food-grade organisms that do not impose health risks on the consumer or the environment. Nevertheless, it should be noted that rare cases of *Bifidobacterium*-associated gastrointestinal and extra-intestinal infections have been described.[2,3] In contrast to susceptibility testing of clinically important bacteria,[4,5] no standard procedures are specifically dedicated to the determination of resistance phenotypes in *Bifidobacterium* strains. To date, a large variety of methods and protocols have been described for antimicrobial susceptibility testing of bifidobacteria, including agar (overlay) disc diffusion (DD),[6–8] broth dilution[9,10] and agar dilution.[8] In addition, various growth media have been used primarily on the basis that they meet the complex growth requirements of bifidobacteria. As opposed to conventional susceptibility test media such as Mueller–Hinton (CLSI) and Iso-Sensitest medium (BSAC), none of these *Bifidobacterium*-specific media are well-defined in terms of minimal interaction between specific antimicrobial agents and growth medium components.

Recently, a newly defined medium formulation referred to as the Lactic acid bacteria (LAB) Susceptibility test Medium supplemented with cysteine (LSM + cysteine) was proposed for susceptibility testing of bifidobacteria.[11] The LSM + cysteine medium was tested for a minimal set of *Bifidobacterium* reference strains and was not found to display significant antagonistic effects with any of the tested agents. In the present study, the LSM + cysteine medium was used to determine the susceptibility profile of 100 bifidobacterial isolates to 15 common antimicrobial agents, including inhibitors of cell wall synthesis, protein synthesis, nucleic acid synthesis and cytoplasmic membrane function using the agar overlay DD method and the broth microdilution method. The bifidobacterial isolates under investigation represent 11 species encompassing strains of human and animal origin, strains previously isolated from probiotic products[12] as well as strains isolated from dental caries[13] and clinical sources.[14] For a subset of strains, the genotypic basis of tetracycline resistance was characterized.

Materials and methods

Bacterial strains

A total of 100 *Bifidobacterium* strains were investigated in this study, including 50 type and reference strains obtained from the BCCM™/LMG Bacteria Collection, Ghent University, Belgium (http://bccm.belspo.be/index.php), and 50 isolates obtained from a variety of probiotic products.[11] The strain selection included representatives of the following species: *Bifidobacterium adolescentis* (n = 6), *Bifidobacterium angulatum* (n = 2), *Bifidobacterium animalis* subsp. *animalis* (n = 2), *B. animalis* subsp. *lactis* (n = 44), *Bifidobacterium bifidum* (n = 8), *Bifidobacterium breve* (n = 7), *Bifidobacterium catenulatum* (n = 2), *Bifidobacterium dentium* (n = 3), *Bifidobacterium gallicum* (n = 1), *Bifidobacterium longum* biotype infantis (n = 7), *B. longum* biotype longum (n = 11), *Bifidobacterium pseudocatenulatum* (n = 5) and *Bifidobacterium scardovii* (n = 2).

Agar overlay disc diffusion testing

Susceptibility testing was based on the agar overlay DD method described by Charteris *et al.*[6] with slight modifications as described by Huys *et al.*[15] Initially, 10 strains were used to compare the performance of two complex growth media for DD testing, i.e. LSM + cysteine [i.e. 90% Iso-Sensitest broth, 10% MRS broth and 15 g/L agar, supplemented with 0.3 g/L L-cysteine–HCl (Sigma, C-4820)][11] and Modified Columbia Agar (MCA) [i.e. 23 g of special peptone (Oxoid, L72), 1 g of soluble starch, 5 g of NaCl, 0.3 g of L-cysteine–HCl, 5 g of glucose and 15 g of agar dissolved in 1 L of distilled water]. The 10 strains used for the comparison of both media represented the species *B. animalis* subsp. *animalis* and subsp. *lactis*, *B. bifidum*, *B. breve*, *B. dentium*, *B. longum* biotype infantis and biotype longum and *B. scardovii*. Subsequently, the most suitable medium was used for antimicrobial susceptibility testing of all 100 bifidobacterial strains. Strains were grown overnight in the corresponding broth medium at 37°C under anaerobic conditions (84% N_2, 8% H_2, 8% CO_2). Cell suspensions with an inoculum density (OD_{590}) of 1.0 ± 0.05 were prepared using a vitalab 10 spectrophotometer (Vital Scientific). Further manipulations were performed as described by Huys *et al.*[15] All plates were subsequently incubated under anaerobic conditions at 37°C during 24 h. In the exceptional case that inhibition zones could not be measured accurately after 24 h of incubation, plates were incubated for another 24 h. Susceptibility was tested against antimicrobial agents (Oxoid) representing inhibitors of cell wall synthesis (i.e. amoxicillin, AMX10), protein synthesis (i.e. gentamicin, GEN10; tetracycline, TET30; chloramphenicol, CHL30; erythromycin, ERY15; clindamycin, CLI2 and quinupristin/dalfopristin, Q/D15), nucleic acid synthesis (i.e. rifampicin, RIF5; ciprofloxacin, CIP5; sulfamethoxazole, RL100 and trimethoprim, TMP5) and inhibitors of cytoplasmic membrane function (i.e. polymyxin B, PB300). Inhibition zones were measured using digital callipers (Mauser digital 2). Partial inhibition was defined as a slightly turbid inhibition zone close to the disc compared with areas of no inhibition further away from the disc. In these cases, inhibition zone diameters were measured as far as the turbid zone. *B. animalis* subsp. *animalis* strain LMG 10508^T was included as a control strain in every DD assay.

Determination of the MIC

Strains were grown overnight in LSM + cysteine broth under anaerobic conditions at 37°C. Fresh inocula with a density of OD_{590} 0.1 ± 0.01 were prepared using a Biolog™ reader (Biolog). In order to obtain a 1/100 dilution, 100 μL of this suspension was transferred to 9.9 mL LSM + cysteine broth. For each agent, two sterile stock solutions were prepared from which a 2-fold dilution series was prepared in LSM + cysteine broth each encompassing a range of four concentrations. Subsequently, 50 μL of each agent dilution was added to the wells of a microtitre plate and mixed with 50 μL of the 1/100 diluted cell suspension. Each plate also included a well only containing 50 μL LSM + cysteine broth as a negative control. Inoculated plates were incubated for 24 h under anaerobic conditions at 37°C. For a selection of strains, the MICs of the following antimicrobial agents were determined: tetracycline (Sigma, T-3383), minocycline (Sigma, M-9511), clindamycin (Sigma, C-5269), ciprofloxacin (Fluka, 17850), polymyxin B (Sigma, P-4932), vancomycin (Sigma, V-2002), trimethoprim (Sigma, T-0667)), sulfamethoxazole (Sigma, S7507) and sulfamethoxazole/trimethoprim (20/1). The MIC was determined as the lowest concentration of antimicrobial agent at which no visible growth was recorded. The MIC_{90} was defined as the lowest concentration of a given agent that inhibited growth of 90% of the tested strains. For each agent tested, a control strain for which the MIC was located within the concentration range tested was included for reproducibility assessment.

Molecular detection of tet genes and TnB1230 in strains showing atypical tetracycline resistance

Total genomic DNA was extracted as described previously.[16] The 50 μL PCR assay mix used for detection of tetracycline resistance genes contained 32.8 μL of sterile Milli-Q water, 5 μL of 10× PCR buffer including 15 mM $MgCl_2$ (Applied Biosystems), 200 μM of each of four dNTPs (dATP, dCTP, dGTP and dTTP), 3 μL of oligonucleotide primer (10 pmol/μL) (Table 1) and 1 U of AmpliTaq DNA polymerase (Applied Biosystems). A 50 ng/μL dilution of total genomic DNA was used as the template. All PCR amplifications were performed using a Perkin Elmer 9600 thermal cycler. In a first PCR assay, the presence of tetracycline resistance genes encoding a ribosomal protection (RP) mechanism was investigated with the group-specific degenerate primer pairs DI/DII and Ribo-2-FW/Ribo-2-RV. The following temperature program was used for primer pair DI/DII: initial denaturation (95°C, 5 min); 35 cycles of denaturation (95°C, 45 s), annealing (45°C, 45 s) and extension (72°C, 1 min); and final extension (72°C, 10 min). For the degenerate Ribo2 primers, a touchdown PCR was performed as follows: initial denaturation (95°C, 5 min); 22 cycles of denaturation (94°C, 30 s), annealing (30 s with 1°C decrements from 72 to 50°C), and extension (72°C, 30 s); 20 cycles of 94°C for 30 s, 50°C for 30 s and 7°C for 30 s; and final extension (72°C, 7 min). Strains containing an RP-type *tet* gene were subjected to additional PCR assays with primers specific for individual genes of the RP group, i.e. *tet*(M), *tet*(O), *tet*(S), *tet*(T) and *tet*(W). In addition, strains with atypical resistance for tetracycline were also tested for the presence of the tetracycline efflux genes *tet*(K) and *tet*(L). For the detection of the RPP *tet* genes as well as the tetracycline efflux genes, the following temperature program was used: initial denaturation (95°C, 5 min), followed by 25 cycles of denaturation (94°C, 45 s), annealing [primer-specific temperature, 1 min (Table 1)], extension (72°C, 1 min) and a single final extension step (72°C, 10 min). PCR products were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide.

The presence of transposon Tn*B1230* was verified by PCR using primers designed by Dr Katarzyna Kazimierczak (personal communication) based on the published sequence of Tn*B1230* (accession number: AJ222769),[17] as well as by hybridization of the DNA samples of all *tet*(W)-positive strains with a Tn*B1230*-specific PCR product, derived from *Butyrivibrio fibrisolvens* (DNA of this organism was kindly provided by Dr Karen Scott).

Localization and copy number determination of the tet(W) gene

Isolation of plasmid DNA was based on the alkaline lysis method of Anderson and McKay.[18] *B. breve* strain LMG 13194, which is known to possess one plasmid of size 5.6 kb,[19] was used as a positive control for plasmid DNA extraction. Plasmids were separated after electrophoresis on a 0.7% agarose gel during 3.5 h at 100 V and visualized by ethidium bromide staining. Total genomic DNA was prepared *in situ* in agarose blocks and digested with endonucleases *Spe*I and *Xba*I and subsequently separated using PFGE as described previously.[12] Probe labelling and Southern hybridization were performed using the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences) according to the manufacturer's instructions. A 1100 bp *tet*(W)-specific amplicon generated with PCR primers DI/DII was used as probe.

Partial sequencing of the tet(W) gene

The *tet*(W) gene of a selection of strains, including two *B. pseudocatenulatum* strains, two *B. animalis* subsp. *lactis* strains and one *B. adolescentis* strain, was amplified using the degenerate primer pair DI/DII as described above. PCR products were purified using a QIAquick PCR Purification kit (Qiagen) according to the instructions of the manufacturer. Sequence analysis was performed using the Big Dye™ Termination RR Mix V3.1 (Applied Biosystems) on an ABI 3100 automated DNA sequencer (Applied Biosystems). For each sequencing reaction, a 10 μL reaction mixture was prepared containing 0.67 μL of Big Dye™, 1.66 μL of 5× sequencing buffer (Applied Biosystems), 3 μL of DI or DII (5 μM), 3.67 μL of sterile Milli-Q water and 1 μL of the purified PCR product. The temperature program consisted of 30 cycles of denaturation (96°C, 15 s), annealing (35°C, 1 s) and extension (60°C, 4 min). PCR products were purified using the Genesis workstation 200 (Tecan Customized Solutions). Sequence analysis was performed using the software package Kodon (Applied Maths) and sequences were BLASTed against the EMBL sequence database to confirm the *tet*(W) identity of the amplicons.

Nucleotide sequence accession numbers

The *tet*(W) sequences determined in this study have been submitted to the EMBL database under the following accession numbers: *B. pseudocatenulatum* LMG 11593, AM181315; *B. pseudocatenulatum* LMG 10505^T, AM181316; *B. animalis* subsp. *lactis* LM 624, AM181317; *B. animalis* subsp. *lactis* LMG 18314, AM181318; *B. adolescentis* LMG 11579, AM181319.

Results

Agar overlay disc diffusion testing

The effect of the growth medium on the inhibition zone sizes of 12 antimicrobial agents determined with the agar overlay DD method was assessed for 10 *Bifidobacterium* strains. For this purpose, the MCA medium was compared with the LSM + cysteine medium. For all disc types tested, differences in inhibition zones between both media increased with the zone diameter thus indicating that the diffusion gradient of the antimicrobial agent is mostly affected by the medium composition at lower concentrations. Differences in inhibition zones between both media of more than 3 mm (40 of 120 strain–disc combinations) were mostly found for zone diameters >20 mm (29 of 40 strain–disc combinations). In 72.4% of these 29 strain–disc combinations a larger inhibition zone was found on LSM + cysteine medium, which suggests that this medium exerts lower overall antagonistic effects compared with the MCA medium. Taken together with the fact that LSM + cysteine medium was able to sustain growth of all bifidobacterial strains so far tested,[11] it was decided to use this formulation as the standard medium in all subsequent DD and MIC assays.

In order to evaluate the reproducibility of the agar overlay DD method, reference strain LMG 10508^T was included in each series of antibiogram determinations. An overall mean standard deviation of ±1.9 mm with a maximum variation of 3 mm was obtained for all agents tested. For a subset of strains mainly encompassing one strain per species, antibiotic susceptibility profiles were compared after 24 and 48 h of incubation. In most cases, diameters of inhibition zones measured after 48 h coincided with those obtained after 24 h of incubation (data not shown). Between both incubation times, an overall mean standard deviation of ±0.4 mm with a single maximum variation of 4 mm was obtained for all agents tested. The results of DD susceptibility testing of 100 *Bifidobacterium* strains to 12 antimicrobial

Table 1. PCR primers and conditions used for detection of *tet* genes

Primer pair	Gene(s) targeted	Sequence[a]	Annealing temp. (°C)	Amplicon size (bp)	Positive control plasmids (reference)	Primers reference
DI	RPP type	5′-GAYACNCCNGGNCAYRTNGAYTT-3′	45	1083	pJI3 (26)	30
DII		5′-GCCCARWANGGRTTNGGNGGNACYTC-3′				
Ribo-2-FW	RPP type	5′-GGMCAYRTGGATTTYWTIGC-3′	touchdown[b]	1187	pJI3 (26)	31
Ribo-2-RV		5′-TCIGMIGGIGTRCTIRCIGGRC-3′				
TetK-FW1	*tet*(K)	5′-TTATGGTGGTTGTAGCTAGAAA-3′	55	382	pAT102 (27)	27
TetK-RV1		5′-AAAGGGTTAGAAACTCTTGAAA-3′				
TetL-FW3	*tet*(L)	5′-GTMGTTGCGCGCTATATTCC-3′	55	717	pAT103 (27)	27
TetL-RV3		5′-TGAAMGRWAGCCCACCTAA-3′				
TetM-FW	*tet*(M)	5′-ACAGAAAGCTTATTATATAAC-3′	55	171	pJI3 (26)	31
TetM-RV		5′-TGGCGTGTCTATGATGTTCAC-3′				
DI/TetM-RV	*tet*(M)		45	1513	pJI3 (26)	30
TetO-FW1	*tet*(O)	5′-AATGAAGATTCCGACAATTT-3′	55	801	pAT121 (27)	32
TetO-RV1		5′-CTCATGCGTTGTAGTATTCCA-3′				
TetS-FW	*tet*(S)	5′-GAAAGCTTACTATACAGTAGC-3′	50	169	pVP2 (28)	31
TetS-RV		5′-AGGAGTATCTACAATATTTAC-3′				
TetT-FW	*tet*(T)	5′-AAGGTTTATTATATAAAAGTG-3′	55	169	Total DNA	31
TetT-RV		5′-AGGTGTATCTATGATATTTAC-3′				
TetW-FW	*tet*(W)	5′-GAGAGCCTGCTATATGCCAGC-3′	64	168	pGEM-tetW (29)	31
TetW-RV		5′-GGGCGTATCCACAATGTTAAC-3′				

[a]N = A, C, G and T; R = A and G; W = A and T; Y = C and T.
[b]PCR conditions are described in the Materials and methods section.

Table 2. Inhibition zone diameters recorded for 100 *Bifidobacterium* strains in disc diffusion testing of 12 antimicrobial agents on LSM + cysteine medium

Species (number of strains)	Inhibition zone diameter range (mm)											
	AMX10	GEN10	TET30	ERY15	CLI2	Q/D15	CIP5	RL100	TMP5	CHL30	RIF5	PB300
B. adolescentis (6)	26–44	6–11*	10–41	34–38	16–36	32–43	20–23	6–35*	6–18*	31–41	29–39*	6–12*
B. angulatum (2)	38–43	6–9*	35–38	34–35	30–31	37–38	20–21	6	17–23*	35–37	28–29	6
B. animalis subsp. *animalis* (2)	29–39*	6–10*	28–36	30–38	13–16*	30–36	12–14*	6	35–44*	29–40	23–31*	6–10*
B. animalis subsp. *lactis* (44)	26–44	6–13*	11–37*	30–50	26–43	33–46	6–18*	6	21–46*	28–40	21–35*	6–16*
B. bifidum (8)	29–50	6–15	26–50	31–50	28–50	27–40	6–14*	6–29*	6–30*	29–50	23–40	6–22*
B. breve (7)	25–30	6–13*	28–34	28–33	18–30	29–35	9–12*	6	16–23*	27–33	26–32	6–10
B. catenulatum (2)	35–37	6–7*	31–32	31–32	15–18	30–33	18*	6–12*	16–20*	31–34	23*	6–8*
B. dentium (3)	33–38	6–9*	34–41	36–39	32–34	38–41	18–21	6	19–22*	37–40	27–33	6
B. gallicum (1)	31	8	40	39	31	35	17	6	6	42	37	10
B. longum biotype infantis (7)	31–50	12–17	27–50	32–50	25–50	34–50	12–16*	6–50*	9–50*	32–50	24–50	6–12*
B. longum biotype longum (11)	32–44	8–12*	33–48	34–47	31–43	38–50	6–14*	6–27*	6–22*	35–52	29–43	6–18*
B. pseudocatenulatum (5)	32–42*	8–11*	14–35*	32–42*	28–40*	31–44*	19–21*	6–27*	11–19*	32–40*	22–30*	7–10*
B. scardovii (2)	27–31*	8–9*	30–33*	29–35*	21–25*	25–28*	14–15*	6	17*	32–35*	30–31*	7

AMX, amoxicillin; GEN, gentamicin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; Q/D, quinupristin/dalfopristin; CIP, ciprofloxacin; RL, sulfamethoxazole; TMP, trimethoprim; CHL, chloramphenicol; RIF, rifampicin, PB, polymyxin B.
*Partial inhibition.

compounds are summarized in Table 2. A unimodal distribution of large inhibition zones was observed for amoxicillin (≥25 mm), chloramphenicol (≥27 mm), erythromycin (≥28 mm), quinupristin/dalfopristin (≥25 mm) and rifampicin (≥21 mm). This type of distribution was noticed for all species tested, which implies that the overall susceptibility to these compounds probably is characteristic for the genus *Bifidobacterium*. Conversely, small unimodally distributed inhibition zone diameters were measured for gentamicin, sulfamethoxazole and polymyxin B, suggesting that bifidobacteria are intrinsically resistant to these agents. However, it should be noted that partial inhibition was occasionally noted for some of the tested species. In the case of gentamicin, slightly larger inhibition zones were observed for strains belonging to *B. longum* biotype infantis compared with the other species tested (Table 2). A relatively broad distribution of inhibition zone diameters was noticed for tetracycline (10–50 mm) and trimethoprim (6–50 mm). For these two compounds, levels of resistance appeared to be strain-specific in particular species. In the case of tetracycline, a large number of *B. animalis* subsp. *lactis* and *B. pseudocatenulatum* strains as well as one *B. adolescentis* strain displayed smaller inhibition zones compared with the other strains tested. In the trimethoprim DD assay, smaller inhibition zones were measured for *B. gallicum* and several representatives of *B. adolescentis*, *B. bifidum* and *B. longum* biotype longum. In this regard, it should be noted that the larger zones were mainly observed in cases of partial inhibition. Finally, bimodally distributed inhibition zone diameters were observed for clindamycin and ciprofloxacin. Strains displaying smaller inhibition zones for ciprofloxacin belonged to the species *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve* and *B. longum* biotype longum and can thus be considered as resistant to this agent. In the case of clindamycin, smaller inhibition zones pointed to reduced susceptibility to this compound within *B. animalis* subsp. *animalis* and *B. catenulatum* and for some strains of *B. breve* and one *B. adolescentis* strain.

Determination of the MIC

MICs were determined for nine antimicrobial compounds. This selection included four agents for which bifidobacteria showed a broad or bimodal distribution of DD inhibition zone diameters, i.e. ciprofloxacin, trimethoprim, tetracycline and clindamycin, as well as two antibiotics to which bifidobacteria are presumed to be intrinsically resistant, i.e. polymyxin B and sulfamethoxazole. In addition, MICs were also determined for three compounds not included in the DD assays, i.e. minocycline, vancomycin and the therapeutic combination trimethoprim/sulfamethoxazole. For reproducibility testing, a control strain was included in every series of MIC determinations. A maximum deviation of one $\log_2$ dilution step was recorded for all antimicrobial agents tested. A selection of strains displaying a broad range of inhibition zone diameters, usually encompassing two strains per species, was subjected to MIC determination. Based on the broad zone diameter distribution obtained for tetracycline (Table 2), an extended selection of strains was included for MIC measurements of this agent. In addition, strains that possessed a *tet*(W) gene as well as some *tet*(W)-negative strains were also subjected to determination of minocycline MIC. Because vancomycin was not included in the DD assay, 78 strains were subjected to MIC determination for this antimicrobial agent. MIC analysis of clindamycin was restricted to *B. animalis* subsp. *animalis* and subsp. *lactis* in order to substantiate the differences observed among inhibition zones (Table 2). MIC values could be classified in three categories (Table 3). In a first category, the tested

Table 3. MICs for *Bifidobacterium* strains of different antimicrobial agents determined by broth microdilution in LSM broth + cysteine

	MIC range (mg/L)								
Species (no. of strains)	polymyxin B	sulfamethoxazole	ciprofloxacin	trimethoprim	tetracycline (no. of strains)*	minocycline (no. of strains)*	trimethoprim/ sulfamethoxazole	vancomycin (no. of strains)*	clindamycin (no. of strains)*
B. adolescentis (2)	32	8, 512	1, 2	2, 64	≤0.5–32 (6)	32 (1)	4	≤0.125 (6)	ND
B. angulatum (2)	16, 32	64, >1024	1, 8	≤0.5, 1	≤0.5	ND	≤0.5, 2	≤0.125	ND
B. animalis subsp. *animalis* (2)	>64	>512, >1024	4, 8	≤0.5	1	2 (1)	≤0.5, 8	≤0.125, 0.5	0.5, 1
B. animalis subsp. *lactis* (2)	>64	>1024	4	≤0.5, 1	4–16 (10)	4–16 (10)	≤0.5	≤0.125–0.250 (24)	≤0.125 (10)
B. bifidum (2)	1, >64	64, >1024	16	4, 8	≤0.5, 4	4 (1)	2, 4	≤0.125–1 (8)	ND
B. breve (2)	64	≥1024	8, 16	2, 8	≤0.5	ND	≤0.5, 8	≤0.125–0.250 (7)	ND
B. catenulatum (2)	>64	64, >1024	2	8	≤0.5	ND	2, 4	≤0.125	ND
B. dentium (3)	32–64	>1024	≤0.5–1	1–2	≤0.5	ND	1–2	≤0.125	ND
B. gallicum (1)	32	>512	2	≤0.5	≤0.5	4	≤0.5	≤0.125	ND
B. longum biotype infantis (2)	>64	64, >512	4	4, 16	1, 2	1 (1)	2, 16	≤0.125–0.250 (7)	ND
B. longum biotype longum (2)	32, >64	64, >1024	4, 16	8	≤0.5	ND	2, 16	≤0.125–0.5 (9)	ND
B. pseudocatenulatum (2)	>64	64	1, 4	16, 32	1–32 (5)	1–2 (3)	1, 2	≤0.125 (5)	ND
B. scardovii (2)	>64	>512, >1024	2, 4	2, 8	≤0.5	ND	2	≤0.125, 0.250	ND
MIC_{90} (all strains)	>64	>1024	16	16	16	16	8	0.250	0.5

ND, not determined.
*MICs of tetracycline, minocycline, vancomycin and clindamycin were determined for an extended set of strains per species. Deviating numbers are indicated within brackets.

bifidobacterial strains displayed high overall MIC values indicating intrinsic resistance of all members of the genus to the compound. This was the case for polymyxin B (MIC_{90} >64 mg/L) and sulfamethoxazole (MIC_{90} >1024 mg/L) and these findings thus confirm the results obtained with the DD assay. As could be predicted from the large zone diameter measured in DD testing, the type strain of *B. bifidum* displayed a much lower MIC of 1 mg/L for polymyxin B. In a second category, MIC values were more variable and broadly distributed within the strain set tested. In line with DD results, broad MIC distributions were obtained for trimethoprim (MIC range ≤0.5–64 mg/L; MIC_{90} 16 mg/L) and tetracycline (MIC range ≤0.5–32 mg/L; MIC_{90} 16 mg/L). In the case of trimethoprim, most strains displayed an MIC of 8 mg/L although some strains of *B. adolescentis*, *B. longum* biotype infantis and *B. pseudocatenulatum* exhibited higher MIC values up to 64 mg/L. In agreement with DD data, higher MIC values of tetracycline were observed for *B. adolescentis*, *B. animalis* subsp. *lactis* and *B. pseudocatenulatum.* Overall, most strains (42.5%) displayed an MIC value ≤0.5 mg/L for this compound. MIC values of the second-generation tetracycline compound minocycline (MIC range: 1–32 mg/L; MIC_{90}: 16 mg/L) were usually distributed in a similar way as those of tetracycline. However, for all three *B. pseudocatenulatum* strains tested, considerably lower MIC values (1–2 mg/L) were obtained compared with those observed for tetracycline (32 mg/L) (Table 4). In contrast to the intrinsic sulfamethoxazole resistance observed in DD testing, MIC values of the combined therapeutic preparation trimethoprim/sulfamethoxazole (1/20) were broadly distributed and comparable to or lower than the MIC values observed for trimethoprim. Although to a lesser extent, a relatively broad MIC distribution was also recorded for ciprofloxacin (MIC_{90} 16 mg/L) for which the majority of the strains tested displayed an MIC of 4 mg/L. A third category was represented by overall low MIC values of clindamycin and vancomycin. The latter compound is known to diffuse poorly in agar media[20] for which reason vancomycin resistance of bifidobacteria was only tested by means of the broth microdilution method. The highest MIC value of vancomycin (1 mg/L) was observed for some strains of the species *B. bifidum*, whereas the majority of the strains tested were found to be inhibited at even lower concentrations (MIC_{90}: 0.250 mg/L). These data indicate that most members of the genus *Bifidobacterium* are susceptible to this compound. As was also noticed from the DD results, MIC values of clindamycin for *B. animalis* subsp. *animalis* were found to be slightly higher (MIC range 0.5–1 mg/L) than for *B. animalis* subsp. *lactis* (≤0.125 mg/L).

Table 4. Phenotypic and genotypic characterization of tetracycline resistance for 29 *Bifidobacterium* strains

Species (number of strains)	MIC (mg/L)		*tet* gene
	tetracycline	minocycline	
B. adolescentis (*n* = 1)	32	32	*tet*(W)
B. pseudocatenulatum (*n* = 3)	32	1–2	*tet*(W)
B. animalis subsp. lactis (*n* = 5)	16	4–8	*tet*(W)
B. animalis subsp. lactis (*n* = 3)	8	4–16	*tet*(W)
B. animalis subsp. lactis (*n* = 2)	4	4	*tet*(W)
B. bifidum (*n* = 1)	4	4	*tet*(W)
B. longum biotype infantis (*n* = 1)	2	1	NF
B. animalis subsp. animalis (*n* = 2)	1	2	NF
B. pseudocatenulatum (*n* = 2)	1	ND	NF
B. longum biotype infantis (*n* = 1)	1	ND	NF
B. breve (*n* = 1)	≤0.5	ND	NF
B. scardovii (*n* = 2)	≤0.5	ND	NF
B. dentium (*n* = 2)	≤0.5	ND	NF
B. gallicum (*n* = 1)	≤0.5	4	NF
B. bifidum (*n* = 1)	≤0.5	ND	NF
B. adolescentis (*n* = 1)	≤0.5	ND	NF

ND, not determined; NF, not found.

Genetic basis of tetracycline resistance

A subset of 29 strains, covering a broad tetracycline MIC range (≤0.5–32 mg/L), was subjected to PCR detection of tetracycline resistance genes (Table 4). In 15 strains displaying MICs in the range of 4–32 mg/L, the presence of the *tet*(W) gene conferring RP against tetracycline was detected. These strains belonged to *B. adolescentis*, *B. pseudocatenulatum*, *B. animalis* subsp. *lactis* and *B. bifidum*. The identity of the *tet*(W) amplicons was confirmed by partial sequence analysis (positions 319–1263, i.e. 49% of the 1921 bp *tet*(W) gene of *B. fibrisolvens*, Accession No. AJ427421). These analyses included three strains with comparable MIC values for both tetracycline and minocycline and two *B. pseudocatenulatum* strains for which lower minocycline MIC values (1–2 mg/L) were recorded compared with those observed for tetracycline (32 mg/L). At two positions, base substitutions resulting in a mutation at the protein level were detected in the partial *tet*(W) sequence. At amino acid positions 262 and 265 of the TetW protein of *B. fibrisolvens*, glycine and arginine were substituted in both *B. pseudocatenulatum* strains by aspartic acid and leucine, respectively.

None of the 29 strains tested was positive for the efflux genes *tet*(K) and *tet*(L)

In all 15 *tet*(W)-positive strains, the *tet*(W) gene was found to be present in a single copy on the chromosome, since no plasmids could be isolated. The presence of Tn*B1230* could not be demonstrated by PCR or by Southern hybridization in any of these strains.

Discussion

In contrast to clinically relevant bacteria for which resistance monitoring is indispensable,[4,5] no standard procedures or interpretive breakpoints have been established for antimicrobial susceptibility testing of bifidobacteria. In this regard, several test media have been used that meet the complex growth requirements of bifidobacteria, including tryptic soy broth supplemented with 0.2% yeast extract and 0.06% L-cysteine–HCl,[10] TPY medium[6,7,9] and Brucella agar supplemented with 5% laked sheep blood and vitamin K1.[8] However, the susceptibility test medium should not only sustain growth of the tested organisms but should also provide a non-interfering matrix exerting minimal

antagonistic effects against a wide range of antimicrobial agents. Although the defined and universally applied test media Iso-Sensitest agar (ISA) (BSAC) and Mueller–Hinton agar (CLSI) meet the latter requirement, it has been shown that they do not always support growth of any given LAB food strain.[15] Recently, the newly developed LSM + cysteine medium formulation was found to provide sufficient growth support of bifidobacterial reference strains.[11] Furthermore, the use of this formulation in a microdilution method resulted in correct indications of known MICs for a set of international control strains. In an initial phase of the present study, the performance of the LSM + cysteine medium was compared with the undefined MCA medium which is routinely used to culture bifidobacteria for DD susceptibility testing of 10 *Bifidobacterium* reference strains. Especially at the lower concentrations of the gradients, it was found that inhibition zones gradually decreased on MCA compared with those recorded on LSM + cysteine agar. This observation substantiates the previous finding that the latter medium formulation is much more effective in minimizing antagonistic effects between antimicrobial agents and growth medium components.[11]

Using the LSM + cysteine medium, the antibiogram of 100 *Bifidobacterium* strains belonging to 11 species and representing animal and human strains as well as isolates from probiotic products was recorded using the agar overlay DD method. A selection of these strains was also included for MIC determination using a broth microdilution assay. Owing to the lack of published cut-off values that allow separation of strains with and without an acquired antimicrobial resistance mechanism in *Bifidobacterium*, susceptibility data were interpreted largely on the basis of histogram analyses. Depending on the relative position and the type of distribution (unimodal, bimodal or broad) of DD and/or MIC data in these histograms, strains were classified as resistant or susceptible. Ideally, 10 or more strains belonging to the same taxon need to be investigated in order to delineate epidemiological cut-off values at the species level. Because this condition was fulfilled for some but not all species in the present study, interpretation of susceptibility data was mainly restricted to the genus level. In general, anaerobes such as bifidobacteria possess a natural resistance to aminoglycosides due to the lack of cytochrome-mediated drug transport.[21] Accordingly, overall resistance was observed to gentamicin, which confirms earlier findings.[6–10] Likewise, our data also indicate that bifidobacteria are generally resistant to polymyxin B, a compound that is almost exclusively active against Gram-negatives.[22] Strains were generally resistant to sulfamethoxazole as a separate compound. However, the therapeutic combination trimethoprim/sulfamethoxazole showed activity against most bifidobacterial strains due to their synergic inhibitory effect on thymidine synthesis. This points to the fact that the reduced resistance towards the therapeutic combination trimethoprim/sulfamethoxazole is mainly due to the action of trimethoprim. All tested strains appeared to be uniformly susceptible to chloramphenicol, erythromycin, rifampicin and amoxicillin, which is in agreement with data from previous studies.[6–10] The overall susceptibility to the β-lactam antimicrobial amoxicillin may be explained by the lack of β-lactamase activity in *Bifidobacterium*.[8] Although not yet reported, quinupristin/dalfopristin was also found to be an active antimicrobial combination. Susceptibility to trimethoprim, ciprofloxacin, tetracycline and minocycline was variable and strain-specific. The range of MIC values for tetracyclines may be specific for some taxa (e.g. *B. pseudocatenulatum*), but clearly more strains need to be tested to substantiate this observation. Except for some *B. bifidum* strains, all tested *Bifidobacterium* strains were considered to be susceptible to vancomycin. This finding contradicts the conclusion of Charteris *et al.*[6] stating that vancomycin resistance is a general characteristic of bifidobacteria. Possibly, this discrepancy may be due to the limited reliability of the DD method used by the latter authors considering the fact that vancomycin is known to diffuse poorly in agar media.[20] Although our data suggest that bifidobacteria are susceptible to clindamycin, comparison of MIC data indicated reduced susceptibilities for some strains. In support of their recent taxonomic description,[23] strains of *B. animalis* subsp. *animalis* and subsp. *lactis* included in this study could also be differentiated on the basis of quantitative differences in clindamycin MIC values.

The *tet*(W) gene is known to be responsible for acquired tetracycline resistance in several rumen anaerobes and in human *B. longum* strains.[24] Recently, this gene was also detected in single strains of tetracycline-resistant *B. pseudocatenulatum* and *B. bifidum*.[8] In the present study, *tet*(W) was found in 15 strains encompassing the species *B. pseudocatenulatum*, *B. bifidum, B. animalis* subsp. *lactis* and *B. adolescentis*. To our knowledge, this is the first report on the presence of *tet*(W) in the latter two *Bifidobacterium* species. All *tet*(W)-positive strains showed an MIC of tetracycline in the range of 4–32 mg/L, whereas all strains with lower MIC values contained none of the tested *tet* genes. These findings indicate that an MIC of ≤2 mg/L can be proposed as the epidemiological cut-off value for defining tetracycline susceptibility in bifidobacteria, but more strains need to be analysed to substantiate this. The observation that resistance towards tetracycline was not always joined by resistance to minocycline initiated partial *tet*(W) gene sequence analyses and revealed two amino acid substitutions. However, whether these substitutions are responsible for the difference in susceptibility remains to be investigated.

Previously, a sequence similarity of >99.9% was reported between the *tet*(W) gene of a rumen isolate of *B. fibrisolvens* and human *B. longum* isolates, suggesting that the gene is potentially exchangeable between animals and humans.[24] In *B. fibrisolvens*, the *tet*(W) gene is integrated in the conjugative transposon Tn*B1230* which is thought to be responsible for the environmental dissemination of *tet*(W).[17] In contrast, none of the 15 *tet*(W)-positive *Bifidobacterium* strains in this study was found to contain Tn*B1230* using both PCR-based and Southern blotting detection. Similarly, Scott *et al.*[24] were not able to identify this mobile element in human *B. longum* isolates. This finding suggests that a different genetic support exists for the *tet*(W) gene in bifidobacteria and merits further investigation. Previously, it has been shown that the *tet*(W) gene is transferable between genotypically diverse *B. fibrisolvens* strains.[25] In this study, preliminary conjugation experiments between *B. animalis* subsp. *lactis* LMG 11615 and *B. adolescentis* LMG 10734 by filter mating did not result in successful transconjugants (L. M., unpublished data), but do not rule out the possibility that the gene is transferable using other recipients under different selective conditions.

Interest in the issue of antimicrobial resistance as a safety criterion for lactic acid bacteria used in probiotic applications is growing at a steady pace. In this context, interpretive reading of bifidobacterial resistance phenotypes has been significantly hampered by the lack of a validated method tested on a taxonomically diverse set of strains. The use of the recently

developed LSM + cysteine medium formulation allowed us to discriminate between intrinsic and atypical resistance properties of bifidobacteria. Together with reduced susceptibilities to trimethoprim and/or ciprofloxacin in several strains, resistance to tetracyclines appears to occur in multiple *Bifidobacterium* species. In all cases, the tetracycline resistance phenotype was linked to the presence of an acquired non-plasmid located *tet*(W) gene. In follow-up studies, the LSM + cysteine medium needs to be tested using an extended strain panel (≥10 strains per species) which will allow epidemiological cut-off values (http://www.srga.org/eucastwt/WT_EUCAST.htm) for all major *Bifidobacterium* species to be defined. This will not only lead to a more widespread acceptance of the method, but will also result in the definition of interpretive standards for use in the food industry and by regulatory agencies.

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Transparency declarations

We have no conflicts to declare.

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