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Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use

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***Objectives*: To determine MICs of 16 antimicrobials representing all major classes for 473 taxonomically well-characterized isolates of lactic acid bacteria (LAB) encompassing the genera *Lactobacillus*, *Pediococcus* and *Lactococcus*. To propose tentative epidemiological cut-off (ECOFF) values for recognizing intrinsic and acquired antimicrobial resistances in numerically dominant species.**

***Methods*: On the basis of depositors' information, LAB were grouped in categories of probiotic, nutritional, probiotic or nutritional research, human and animal isolates and tested for their antibiotic susceptibilities by broth microdilution using LAB susceptibility test medium (LSM). Tentative ECOFFs were defined according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing. Isolates showing acquired antimicrobial resistance(s) were selected for PCR-based detection of resistance gene(s) and *in vitro* conjugative transfer experiments.**

***Results*: Tentative ECOFF values of 13 antibiotics were determined for up to 12 LAB species. Generally, LAB were susceptible to penicillin, ampicillin, ampicillin/sulbactam, quinupristin/dalfopristin, chloramphenicol and linezolid. LAB exhibited broad or partly species-dependent MIC profiles of trimethoprim, trimethoprim/sulfamethoxazole, vancomycin, teicoplanin and fusidic acid. Three probiotic *Lactobacillus* strains were highly resistant to streptomycin. Although erythromycin, clindamycin and oxytetracycline possessed high antimicrobial activities, 17 *Lactobacillus* isolates were resistant to one or more of these antibiotics. Eight of them, including six probiotic and nutritional cultures, possessed *erm*(B) and/or *tet*(W), *tet*(M) or unidentified members of the *tet*(M) group. *In vitro* intra- and interspecies filter-mating experiments failed to show transfer of resistance determinants.**

***Conclusions*: Finding of acquired resistance genes in isolates intended for probiotic or nutritional use highlights the importance of antimicrobial susceptibility testing in documenting the safety of commercial LAB.**

Keywords: lactic acid bacteria, antimicrobial susceptibility testing, broth microdilution, MIC ranges, epidemiological cut-off values

Introduction

The genera *Lactobacillus*, *Pediococcus* and *Lactococcus* belong to the lactic acid bacteria (LAB) and are part of the commensal intestinal flora of humans and animals.[1,2] Strains of these genera are frequently used on a large-scale as starter cultures in food industries (e.g. in the production of fermented milk products or sausages) or as probiotics.[3–6]

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In probiotic applications, selected LAB strains mainly belonging to the genera *Lactobacillus* and *Bifidobacterium* are used as food supplements that may favourably influence the intestinal flora of human and animal hosts, e.g. by competitive exclusion of gastrointestinal pathogens, stimulation of the immune response or antimutagenic and anticancerogenic activities.[7–9] On the other hand, lactobacilli, pediococci and lactococci have also been implicated in rare cases of human infections.[10–14]

Because of their long-time use in various food and feed preparations, LAB have been given the so-called GRAS status (generally recognized as safe).[15–19] In practice, this means that such LAB strains are food-grade organisms without imposing a health risk for the consumers or the environment. However, there are several studies that have documented the presence and expression of virulence genes and/or antibiotic resistance genes in food-associated LAB.[16,19–23] When located on mobile genetic elements such as plasmids or (conjugative) transposons, antibiotic resistance traits can potentially be transferred to the human or animal commensal flora and to pathogenic bacteria temporarily residing in the hosts. Therefore, it is very important to verify that probiotic and nutritional LAB strains consumed on a daily basis worldwide lack acquired antimicrobial resistance properties prior to considering them safe for human and animal consumption.

Antimicrobial susceptibility testing of LAB can be performed by several methods, including agar disc diffusion and agar overlay disc diffusion, Etest, agar dilution and broth macro- and microdilution.[24] In general, dilution methods and the Etest are preferred over diffusion tests providing inhibition zones, as the former techniques allow determination of MICs of antimicrobials that result in a more reliable indication of the intrinsic or acquired nature of a given resistance phenotype. However, since many LAB require special growth conditions in terms of medium acidity and carbohydrate supplementation, conventional media such as Mueller–Hinton and Iso-Sensitest (IST) agar or broth are often not suitable for susceptibility testing of lactobacilli, pediococci and lactococci. Previously, we developed a broth formula referred to as the LAB susceptibility test medium (LSM) for determining MICs of antibacterial agents of all major antibiotic classes for *Lactobacillus*, *Pediococcus* and *Lactococcus* species.[24] The aim of the present study is to further validate the use of the LSM formulation for susceptibility testing by broth dilution of an extensive collection of 473 well-characterized isolates encompassing 24 species of the genera *Lactobacillus*, *Pediococcus* and *Lactococcus* against 16 antimicrobials. Isolates were of human, animal or nutritional category and also included several cultures that are currently under investigation as probiotic candidates or that are already on the market in commercial probiotic products. Secondly, from the large data set obtained from this study, tentative species- or group-specific epidemiological cut-off (ECOFF) values of MICs were defined for most of the antimicrobial agents tested, to allow better differentiation between wild-type (WT) isolates (lacking acquired antimicrobial resistance traits) and non-wild-type (NWT) isolates (containing one or more acquired antibiotic resistance traits). Recognition of isolates with acquired antibiotic resistances is very important because of the potential transferability of resistance traits to other bacteria, including pathogenic microbes.

Materials and methods

Bacterial isolates

We tested 473 isolates belonging to the genera *Lactobacillus* (416 isolates of 21 species) and *Pediococcus* (49 isolates of 2 species) and to *Lactococcus lactis* (8 isolates). Species designations and categories of the 473 non-enterococcal LAB isolates included in this study are listed in Table 1. Isolates were obtained in the framework of the EU project 'Biosafety Evaluation of Probiotic Lactic Acid Bacteria Used for Human Consumption' (PROSAFE) and were classified into probiotic (i.e. isolates effectively used in probiotic products; $n = 129$), nutritional (i.e. isolates used in food products as starter cultures without a specific probiotic claim; $n = 27$), research (i.e. isolates under investigation as probiotic or nutritional candidates; $n = 24$), human (i.e. isolates from healthy humans and human clinical isolates; $n = 288$) and animal (i.e. isolates from healthy animals; $n = 5$) categories based on the descriptive information provided by the respective depositors.[25] The full official designations of the PROSAFE (PRSF-) isolates were abbreviated for practical reasons in the present paper and only the abbreviations of the corresponding genus (e.g. L, *Lactobacillus*) and the running number of the isolates are named here. For instance, the short designation of the *Lactobacillus crispatus* isolate 'L-295' stands for 'PRSF-L-295'. All isolates were re-identified up to the species level using a polyphasic identification strategy as previously described.[26] Isolates were routinely cultured at 37°C on de Man, Rogosa, Sharpe (MRS) agar plates (Oxoid) under aerobic conditions (but a 5% CO_2-enriched atmosphere can be favourable for some isolates) from which fresh cultures were prepared for inoculation of the broth microdilution test.[24]

Antimicrobial susceptibility testing

MICs of 16 antimicrobial agents encompassing nearly all important classes were determined by microdilution using the newly developed and standardized LSM broth formulation essentially consisting of a mixture of IST broth (90%) and MRS broth (10%) adjusted to pH 6.7 as previously described.[24]

The following antimicrobials were tested in the concentration ranges (mg/L) given in parentheses: penicillin G (0.032–64), ampicillin (0.032–64), sulbactam/ampicillin (sulbactam was tested as fixed concentration of 8 mg/L: 0.032–64), gentamicin (1–2048), streptomycin (2–4096), vancomycin (0.125–256), teicoplanin (0.125–256), quinupristin/dalfopristin (tested as 30:70 ratio: 0.032–64), erythromycin (0.016–32), clindamycin (0.032–32), oxytetracycline (0.063–128), chloramphenicol (0.125–256), fusidic acid (0.063–128), linezolid (0.016–32), trimethoprim (0.25–512) and trimethoprim/sulfamethoxazole (tested as 1:19 ratio: 0.25–512). In Table S1 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)], concentrations that were outside the test range of the corresponding antimicrobial are marked in grey.

Epidemiological MIC cut-off values for differentiation of WT and NWT isolates

MIC_{50}s, MIC_{90}s and tentative ECOFF values for differentiation between WT and NWT isolates were determined to the antimicrobials named above for the following 12 LAB species represented by 10 or more isolates: *Lactobacillus acidophilus*, *L. crispatus*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Pediococcus acidilactici* and *Pediococcus pentosaceus*.

Table 1. Distribution of species and categories of 473 LAB isolates included in the present study

Species	Total number of isolates tested	Distribution of isolate numbers per category					
				research			
		probiotic	nutritional	probiotic	nutritional	human	animal
Lactobacillus acidophilus	20	15	2	—	—	1	2
Lactobacillus amylovorus	3	1	—	—	—	2	—
Lactobacillus brevis	1	1	—	—	—	—	—
Lactobacillus buchneri	1	1	—	—	—	—	—
Lactobacillus casei	3	—	—	—	—	3	—
Lactobacillus crispatus	13	—	—	2	—	11	—
Lactobacillus curvatus	3	—	2	—	—	1	—
Lactobacillus delbrueckii[a]	11	5	5	—	—	1	—
Lactobacillus fermentum	25	2	—	4	—	19	—
Lactobacillus gasseri	24	2	—	—	—	22	—
Lactobacillus helveticus	5	5	—	—	—	—	—
Lactobacillus jensenii	1	—	—	—	—	1	—
Lactobacillus johnsonii	12	5	1	—	—	5	1
Lactobacillus paracasei	90	23	5	4	1	57	—
Lactobacillus paraplantarum	1	—	1	—	—	—	—
Lactobacillus pentosus	4	4	—	—	—	—	—
Lactobacillus plantarum	46	25	2	4	—	15	—
Lactobacillus reuteri	11	2	1	—	—	6	2
Lactobacillus rhamnosus	131	19	5	7	—	100	—
Lactobacillus sakei	4	—	2	—	—	2	—
Lactobacillus salivarius	7	3	—	1	—	3	—
Lactobacillus spp. (total *n*, %)	416 (100.0)	113 (27.2)	26 (6.3)	22 (5.3)	1 (0.2)	249 (59.8)	5 (1.2)
Lactococcus lactis (*n*, %)	8 (100.0)	4 (50.0)	1 (12.5)	—	—	3 (37.5)	—
Pediococcus acidilactici	29	11	—	1	—	17	—
Pediococcus pentosaceus	20	1	—	—	—	19	—
Pediococcus spp. (total *n*, %)	49 (100.0)	12 (24.5)	—	1 (2.0)	—	36 (73.5)	—
Total LAB spp. (*n*, %)	473 (100.0)	129 (27.3)	27 (5.7)	23 (4.9)	1 (0.2)	288 (60.9)	5 (1.0)

[a]Encompassing subsp. *bulgaricus* ($n = 9$) and subsp. *lactis* ($n = 2$).

MIC_{50} and MIC_{90} are defined as MICs inhibiting 50% and 90% of the isolates tested, respectively, and ECOFF values were determined from MIC distributions for each species–drug combination as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[27–30] (Table S1). WT and NWT isolates are characterized by their MIC values as follows: WT $\leq z$ mg/L, NWT $> z$ mg/L; z is the ECOFF value of the corresponding antibiotic for the species in question. According to EUCAST definitions, a WT isolate of a microbial species is characterized by the absence of acquired and/or mutational resistance mechanisms to the antimicrobial agent in question, whereas a NWT isolate is defined by the presence of such resistance mechanisms. WT and NWT microorganisms may or may not respond clinically to treatment with antimicrobial agents.[27]

Detection of antibiotic resistance genes in NWT LAB isolates

Isolates displaying MICs that were situated in the NWT subpopulation of the MIC distribution profiles were selected for PCR-based detection of genes conferring resistance to the aminoglycoside streptomycin [*aad*(E) gene], to erythromycin (*erm* genes) and oxytetracycline (*tet* genes), respectively (Table 2). DNA of LAB isolates was isolated by using a DNeasy Tissue Kit (Qiagen) and amplification of the corresponding gene fragments was performed in a DNA Engine Thermal Cycler (PTC-200, MJ Research). The PCR mixtures consisted in each case of 0.25 μL of the two primers, 0.25 μL of DNA-containing DNeasy beads and 24.25 μL of Aqua bidest. The primer sequences, annealing temperatures and amplicon sizes are listed in Table 2. PCR-based detection of the genes *aad*(E), *erm*(A), *erm*(B), *erm*(C), *tet*(K), *tet*(L) and *tet*(M), and of further members of the *tet*(M) group, was performed under the following conditions: 95°C for 1 min; 94°C for 30 s, 55°C for 30 s and 72°C for 30 s (30 cycles); and 72°C for 4 min. Detection of the genes *tet*(O), *tetB*(P), *tet*(Q), *tet*(S), *tet*(T) and *tet*(W) was conducted using the PCR conditions previously described.[33] Amplification products were detected by electrophoresis in a 1.4% agarose gel (Type II: Medium EEO, Sigma) and subsequent staining in ethidium bromide solution.

In vitro experiments on intra- and interspecies transfer of antibiotic resistance genes

Selected LAB isolates of probiotic, nutritional and research categories from the PROSAFE strain collection (marked in Table 5 by footnote e) that represented NWT isolates with acquired antibiotic

Table 2. Primers and PCR conditions for selected antibiotic resistance genes tested in the LAB strains

Resistance gene	Primers	Primer sequence	T_a (°C)	Amplicon size (bp)	Reference(s)
aad(E)	aadEI	5′-GCAGAACAGGATGAACGTATTCG-3′	55	369	this study
	aadEII	5′-ATCAGTCGGAACTATGTCCC-3′			
erm(A)	ermAI	5′-AAGCGGTAAACCCCTCTGA-3′	55	190	Strommenger *et al.*[31]
	ermAII	5′-TTCGCAAATCCCTTCTCAAC-3′			
erm(B)	ermBI	5′-TTTTGAAAGCCGTGCGTCTG-3′	55	202	this study
	ermBII	5′-CTGTGGTATGGCGGGTAAGTT-3′			
erm(C)	ermCI	5′-AATCGTCAATTCCTGCATGT-3′	55	299	Strommenger *et al.*[31]
	ermCII	5′-TAATCGTGGAATACGGGTTTG-3′			
tet(K)	tetKI	5′-CAATACCTACGATATCTA-3′	50	352	this study
	tetKII	5′-TTGAGCTGTCTTGGTTCA-3′			
tet(L)	tetLI	5′-TGGTCCTATCTTCTACTCATTC-3′	53	385	Werner *et al.*[32]
	tetLII	5′-TTCCGATTTCGGCAGTAC-3′			
tet(M)	tetMI	5′-GGTGAACATCATAGACACGC-3′	55	401	Werner *et al.*[32]
	tetMII	5′-CTTGTTCGAGTTCCAATGC-3′			
tet(O)	tetOI	5′-AGCGTCAAAGGGGAATCACTATCC-3′	55	1723	this study
	tetOII	5′-CGGCGGGGTTGGCAAATA-3′			
tetB(P)	TetB/P-FW	5′-AAAACTTATTATATTATAGTG-3′	46	169	Aminov *et al.*[33]
	TetB/P-RV	5′-TGGAGTATCAATAATATTCAC-3′			
tet(Q)	TetQ-FW	5′-AGAATCTGCTGTTTGCCAGTG-3′	63	169	Aminov *et al.*[33]
	TetQ-RV	5′-CGGAGTGTCAATGATATTGCA-3′			
tet(S)	tetS-FW	5′-ATCAAGATATTAAGGAC-3′	55	573	Gevers *et al.*[23] and Charpentier[34]
	tetS-RV	5′-TTCTCTATGTGGTAATC-3′			
tet(T)	TetT-FW	5′-AAGGTTTATTATATAAAAGTG-3′	46	169	Aminov *et al.*[33]
	TetT-RV	5′-AGGTGTATCTATGATATTTAC-3′			
tet(W)	tetWI	5′-GGMCAYRTGGATTTYWTIGC-3′	TD[a]	1187	Aminov *et al.*[33]
	tetWII	5′-TCIGMIGGIGTRCTIRCIGGRC-3′			
tet(M) group	tetMgrI	5′-GAYACICCIGGICAYRTIGAYTT-3′	45	1100	Clermont *et al.*[35]
	tetMgrII	5′-GCCCARWAIGGRTTIGGIGGIACYTC-3′			

[a]TD, touch down PCR, T_a 72–50°C.

resistance(s) were used as donors for intra- and interspecies *in vitro* transfer experiments by conjugation (filter-mating). Intraspecies recipients were generated by a multi-step approach to obtain strains with high-level resistance to rifampicin and fusidic acid, starting from suitable susceptible isolates of the corresponding *Lactobacillus* species from the PROSAFE collection. The well-documented strains *Enterococcus faecium* 64/3 and *Enterococcus faecalis* JH2-2 displaying high-level resistance to rifampicin/fusidic acid were chosen as recipients for interspecies gene transfer experiments. Possible transconjugants were identified in several steps, selecting for the selective and non-selective markers.

In each case, 1 mL of the fresh logarithmic growth phase cultures of the donor and recipient strains were mixed and filtered (sterile 0.45 μm Millipore membrane filter). The filtrate was filtered again by the addition of 2 mL of sterile peptone physiological saline (PPS = 0.85% saline with 0.1% neutralized bacterial peptone; useful for tight contact of the cells[36]) using the same membrane filter. Subsequently, the bacteria-loaded filter was cultured overnight on an antibiotic-free nutrient agar plate optimized for the recipient's growth. Bacteria were washed from the filter with 2 mL of PPS, and suitable dilutions of this mating mixture were spread on selective agar plates (containing oxytetracycline/rifampicin or erythromycin/rifampicin or streptomycin/rifampicin, respectively). After incubation for up to 72 h, single colonies were streaked on agar plates containing the selective antimicrobial (oxytetracycline, erythromycin or streptomycin) and thereafter on agar plates with the non-selective antibiotic (fusidic acid). The colonies grown on the latter nutrient medium were considered possible transconjugants and were further characterized by MIC determination, PCR-based detection of resistance genes, PFGE and fingerprinting by PCR-based amplification of repetitive bacterial DNA elements using $(GTG)_5$ primers [$(GTG)_5$-PCR].[37,38]

Results

MIC distribution profiles

The complete distribution of MICs of 16 antimicrobial agents tested for 473 LAB isolates has been made available as online supplementary data (Table S1). Additionally, MIC_{50}s, MIC_{90}s, tentative ECOFF values and the presence of intrinsic and/or acquired antibiotic resistance(s) in the corresponding LAB species are indicated in Table S1. Table 3 summarizes the data from Table S1 for the 12 *Lactobacillus* and *Pediococcus* species represented by 10 or more isolates. For some of these species, ECOFFs could not be defined because the MIC distributions were poorly delineated (e.g. for trimethoprim and trimethoprim/sulfamethoxazole) or truncated at the low end of dilutions (Table S1).

Table 3. MIC data and tentative ECOFF values for *Lactobacillus* and *Pediococcus* species represented by 10 or more isolates determined in LSM broth by microdilution

Antimicrobials	Species (no. of isolates tested)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Tentative ECOFF (mg/L)
Penicillin	*Pc. acidilactici* (29)	0.125–1	0.25	0.5	1
	Pc. pentosaceus (20)	0.25–0.5	0.5	0.5	1
	Lb. rhamnosus (131)	0.125–2	0.25	1	2
	Lb. paracasei (90)	0.063–1	0.25	0.5	1
	Lb. plantarum (46)	0.5–2	1	2	2
	Lb. fermentum (25)	0.063–0.5	0.25	0.25	0.5
	Lb. gasseri (24)	≤0.032–0.063	≤0.032	0.063	0.125
	Lb. acidophilus (20)	≤0.032–0.125	0.125	0.125	0.25
	Lb. crispatus (13)	≤0.032–0.25	0.125	0.25	0.25
	Lb. johnsonii (12)	0.063–0.125	0.125	0.125	0.25
	Lb. delbrueckii (11)[a]	≤0.032–0.125	≤0.032	0.063	0.125
	Lb. reuteri (11)	0.063–16	0.5	4	IE
Ampicillin	*Pc. acidilactici* (29)	1–2	1	2	4
	Pc. pentosaceus (20)	1–2	2	2	4
	Lb. rhamnosus (131)	0.25–4	1	2	4
	Lb. paracasei (90)	0.125–2	0.5	1	2
	Lb. plantarum (46)	0.125–2	0.25	1	2
	Lb. fermentum (25)	0.063–0.25	0.125	0.25	0.5
	Lb. gasseri (24)	0.063–0.25	0.125	0.25	0.5
	Lb. acidophilus (20)	0.125–0.5	0.25	0.5	0.5
	Lb. crispatus (13)	0.125–1	0.5	1	1
	Lb. johnsonii (12)	0.125–0.5	0.5	0.5	1
	Lb. delbrueckii (11)[a]	≤0.032–0.5	0.063	0.25	0.5
	Lb. reuteri (11)	0.125–4	0.5	4	2
Ampicillin/ sulbactam	*Pc. acidilactici* (29)	1–2	1	2	4
	Pc. pentosaceus (20)	1–2	2	2	4
	Lb. rhamnosus (131)	0.25–4	1	1	4
	Lb. paracasei (90)	0.125–2	0.5	1	2
	Lb. plantarum (46)	0.125–1	0.25	0.5	2
	Lb. fermentum (25)	0.063–0.25	0.125	0.25	0.5
	Lb. gasseri (24)	≤0.032–0.25	0.125	0.25	0.5
	Lb. acidophilus (20)	0.125–0.5	0.25	0.5	0.5
	Lb. crispatus (13)	0.063–1	0.5	0.5	1
	Lb. johnsonii (12)	0.063–0.5	0.25	0.5	1
	Lb. delbrueckii (11)[a]	≤0.032–0.25	0.063	0.125	0.5
	Lb. reuteri (11)	0.125–2	0.5	2	2
Gentamicin	*Pc. acidilactici* (29)	2–8	4	4	8
	Pc. pentosaceus (20)	2–4	2	2	4
	Lb. rhamnosus (131)	≤1–8	1	2	8
	Lb. paracasei (90)	≤1–8	2	4	8
	Lb. plantarum (46)	≤1–8	1	2	8
	Lb. fermentum (25)	≤1–2	1	1	IE
	Lb. gasseri (24)	≤1–4	1	2	4
	Lb. acidophilus (20)	≤1–2	1	2	4
	Lb. crispatus (13)	≤1–4	2	4	4
	Lb. johnsonii (12)	≤1–8	4	4	8
	Lb. delbrueckii (11)[a]	≤1–4	1	2	4
	Lb. reuteri (11)	≤1	1	1	IE
Streptomycin	*Pc. acidilactici* (29)[b]	16–128	64	128	128
	Pc. pentosaceus (20)[b]	32–128	32	64	128
	Lb. rhamnosus (131)[b,c]	≤2–>4096	4	16	32

Continued

Table 3. *Continued*

Antimicrobials	Species (no. of isolates tested)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Tentative ECOFF (mg/L)
	Lb. paracasei (90)[b,c]	≤2– > 4096	16	32	64
	Lb. plantarum (46)[b]	4–128	16	32	64
	Lb. fermentum (25)	4–64	8	16	64
	Lb. gasseri (24)	≤2–8	2	4	8
	Lb. acidophilus (20)	≤2–8	2	4	8
	Lb. crispatus (13)	≤2–32	4	16	16
	Lb. johnsonii (12)	≤2–16	4	8	16
	Lb. delbrueckii (11)[a]	≤2–16	4	8	16
	Lb. reuteri (11)	≤2–16	4	8	16
Vancomycin	*Pc. acidilactici* (29)[b]	>256	>256	>256	IE
	Pc. pentosaceus (20)[b]	>256	>256	>256	IE
	Lb. rhamnosus (131)[b]	≥256	>256	>256	IE
	Lb. paracasei (90)[b]	≥256	>256	>256	IE
	Lb. plantarum (46)[b]	≥256	>256	>256	IE
	Lb. fermentum (25)[b]	32– > 256	256	>256	IE
	Lb. gasseri (24)	0.25–1	0.5	1	1
	Lb. acidophilus (20)	0.25–0.5	0.5	0.5	1
	Lb. crispatus (13)	0.25–0.5	0.5	0.5	1
	Lb. johnsonii (12)	0.25–1	0.5	1	1
	Lb. delbrueckii (11)[a]	0.25–0.5	0.25	0.5	1
	Lb. reuteri (11)[b]	128–256	256	256	IE
Teicoplanin	*Pc. acidilactici* (29)[b]	64– > 256	>256	>256	IE
	Pc. pentosaceus (20)[b]	≥256	>256	>256	IE
	Lb. rhamnosus (131)[b]	64– > 256	>256	>256	IE
	Lb. paracasei (90)[b]	64– > 256	256	>256	IE
	Lb. plantarum (46)[b]	32– > 256	256	>256	IE
	Lb. fermentum (25)[d]	2– > 256	128	>256	IE
	Lb. gasseri (24)	≤0.125–0.25	≤0.125	≤0.125	IE
	Lb. acidophilus (20)	≤0.125	≤0.125	≤0.125	IE
	Lb. crispatus (13)	≤0.125–0.25	≤0.125	≤0.125	IE
	Lb. johnsonii (12)	≤0.125–0.25	≤0.125	≤0.125	IE
	Lb. delbrueckii (11)[a]	≤0.125–0.25	≤0.125	≤0.125	IE
	Lb. reuteri (11)[b]	64–256	128	256	IE
Quinupristin/ dalfopristin	*Pc. acidilactici* (29)	0.063–1	0.5	1	2
	Pc. pentosaceus (20)	0.5–2	1	2	2
	Lb. rhamnosus (131)	≤0.032–1	0.25	0.5	1
	Lb. paracasei (90)	≤0.032–1	0.25	0.25	1
	Lb. plantarum (46)	0.063–1	0.25	1	1
	Lb. fermentum (25)	0.063–0.125	0.125	0.125	0.25
	Lb. gasseri (24)	≤0.032–0.25	0.125	0.125	0.25
	Lb. acidophilus (20)	≤0.032–0.5	0.125	0.25	0.5
	Lb. crispatus (13)	≤0.032–0.25	0.063	0.25	0.25
	Lb. johnsonii (12)	0.063–0.25	0.125	0.25	0.5
	Lb. delbrueckii (11)[a]	≤0.032–0.25	0.063	0.063	0.25
	Lb. reuteri (11)	≤0.032–0.125	0.125	0.125	0.25
Erythromycin	*Pc. acidilactici* (29)	0.063–0.25	0.063	0.5	0.25
	Pc. pentosaceus (20)	0.032–0.25	0.063	0.125	0.25
	Lb. rhamnosus (131)[c]	≤0.016– >32	0.032	0.063	0.25
	Lb. paracasei (90)	≤0.016–0.25	0.032	0.063	0.25
	Lb. plantarum (46)	≤0.016–0.5	0.125	0.25	0.5
	Lb. fermentum (25)	≤0.016–0.125	0.032	0.063	0.125
	Lb. gasseri (24)	≤0.016–0.032	≤0.016	0.032	0.063

Continued

Table 3. *Continued*

Antimicrobials	Species (no. of isolates tested)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Tentative ECOFF (mg/L)
	Lb. acidophilus (20)	≤0.016–0.063	0.032	0.063	0.063
	Lb. crispatus (13)[c]	≤0.016– > 32	0.032	>32	0.063
	Lb. johnsonii (12)	≤0.016–0.063	≤0.016	0.032	0.063
	Lb. delbrueckii (11)[a]	≤0.016–0.125	≤0.016	0.032	0.063
	Lb. reuteri (11)	0.032–0.25	0.032	0.125	0.25
Clindamycin	*Pc. acidilactici* (29)[e]	≤0.032–0.5	≤0.032	0.063	IE
	Pc. pentosaceus (20)	≤0.032	≤0.032	≤0.032	IE
	Lb. rhamnosus (131)[c]	≤0.032–8	0.063	0.125	0.5
	Lb. paracasei (90)	≤0.032–0.25	≤0.032	0.063	0.25
	Lb. plantarum (46)	≤0.032–1	0.125	0.5	0.5
	Lb. fermentum (25)	≤0.032–0.125	≤0.032	≤0.032	0.125
	Lb. gasseri (24)	≤0.032–2	0.5	2	IE
	Lb. acidophilus (20)	≤0.032–0.5	0.125	0.5	0.5
	Lb. crispatus (13)[c]	≤0.032– > 32	0.063	>32	0.25
	Lb. johnsonii (12)	≤0.032–1	0.063	0.25	0.5
	Lb. delbrueckii (11)[a]	≤0.032–0.063	≤0.032	≤0.032	IE
	Lb. reuteri (11)	≤0.032–0.063	≤0.032	≤0.032	IE
Oxytetracycline	*Pc. acidilactici* (29)[b]	4–16	8	16	32
	Pc. pentosaceus (20)[b]	8–16	16	16	32
	Lb. rhamnosus (131)[c]	0.125–16	0.5	0.5	1
	Lb. paracasei (90)[c]	0.25–16	0.5	1	2
	Lb. plantarum (46)[b,c]	4– > 128	8	16	32
	Lb. fermentum (25)	0.5–4	2	4	8
	Lb. gasseri (24)	0.125–4	1	2	4
	Lb. acidophilus (20)	0.25–2	0.5	1	2
	Lb. crispatus (13)[c]	0.25–64	1	64	2
	Lb. johnsonii (12)[c]	0.5–16	0.5	16	2
	Lb. delbrueckii (11)[a]	≤0.063–2	0.5	2	2
	Lb. reuteri (11)[c]	2– > 128	4	>128	8
Chloramphenicol	*Pc. acidilactici* (29)	2–4	2	4	8
	Pc. pentosaceus (20)	1–4	2	4	4
	Lb. rhamnosus (131)	0.5–8	2	4	8
	Lb. paracasei (90)	1–8	2	4	8
	Lb. plantarum (46)	2–8	4	4	8
	Lb. fermentum (25)	2–4	2	4	8
	Lb. gasseri (24)	0.5–4	2	2	4
	Lb. acidophilus (20)	0.5–4	2	4	8
	Lb. crispatus (13)	1–4	1	4	4
	Lb. johnsonii (12)	1–4	2	4	4
	Lb. delbrueckii (11)[a]	1–4	2	2	4
	Lb. reuteri (11)	1–4	2	4	4
Fusidic acid	*Pc. acidilactici* (29)[d]	0.5–16	4	4	8
	Pc. pentosaceus (20)	2–4	2	4	8
	Lb. rhamnosus (131)[b]	2– > 128	128	>128	IE
	Lb. paracasei (90)[b]	16– > 128	64	128	256
	Lb. plantarum (46)[b]	2–32	16	16	32
	Lb. fermentum (25)	0.25–1	0.5	1	1
	Lb. gasseri (24)[b]	8– > 128	64	128	256
	Lb. acidophilus (20)[b]	32– > 128	64	128	256
	Lb. crispatus (13)[b]	4–128	32	64	128
	Lb. johnsonii (12)[b]	16–128	64	128	128
	Lb. delbrueckii (11)[a,b]	32–128	64	128	128
	Lb. reuteri (11)	0.125–2	0.5	2	2

Continued

Table 3. *Continued*

Antimicrobials	Species (no. of isolates tested)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Tentative ECOFF (mg/L)
Linezolid	*Pc. acidilactici* (29)	0.5–2	1	2	4
	Pc. pentosaceus (20)	0.5–2	1	1	2
	Lb. rhamnosus (131)	0.125–2	1	1	2
	Lb. paracasei (90)	0.25–2	1	1	4
	Lb. plantarum (46)	1–2	1	2	4
	Lb. fermentum (25)	0.5–2	1	1	2
	Lb. gasseri (24)	0.5–2	1	2	2
	Lb. acidophilus (20)	0.5–4	1	4	4
	Lb. crispatus (13)	0.5–2	1	2	2
	Lb. johnsonii (12)	0.5–2	1	2	2
	Lb. delbrueckii (11)[a]	0.5–1	0.5	1	1
	Lb. reuteri (11)	0.5–2	1	2	2
Trimethoprim[f]	*Pc. acidilactici* (29)	8– > 512	32	128	IE
	Pc. pentosaceus (20)	8–128	16	32	IE
	Lb. rhamnosus (131)	1– > 512	32	64	IE
	Lb. paracasei (90)	≤0.25– > 512	2	16	IE
	Lb. plantarum (46)	≤0.25– > 512	2	32	IE
	Lb. fermentum (25)	≤0.25–256	4	64	IE
	Lb. gasseri (24)	≤0.25– > 512	8	>512	IE
	Lb. acidophilus (20)	0.5– > 512	4	512	IE
	Lb. crispatus (13)	≤0.25– > 512	8	256	IE
	Lb. johnsonii (12)	≤0.25– > 512	256	>512	IE
	Lb. delbrueckii (11)[a]	128– > 512	>512	>512	IE
	Lb. reuteri (11)	16– > 512	64	>512	IE
Trimethoprim/ sulfamethoxazole[f]	*Pc. acidilactici* (29)	64–512	256	512	IE
	Pc. pentosaceus (20)	128–512	256	256	IE
	Lb. rhamnosus (131)	16– > 512	256	512	IE
	Lb. paracasei (90)	0.5– > 512	16	128	IE
	Lb. plantarum (46)	0.5– > 512	4	256	IE
	Lb. fermentum (25)	2–512	16	128	IE
	Lb. gasseri (24)	0.5– > 512	128	>512	IE
	Lb. acidophilus (20)	4– > 512	64	512	IE
	Lb. crispatus (13)	≤0.25– > 512	128	>512	IE
	Lb. johnsonii (12)	1– > 512	512	>512	IE
	Lb. delbrueckii (11)[a]	256– > 512	512	>512	IE
	Lb. reuteri (11)	32– > 512	256	>512	IE

Pc., *Pediococcus*; *Lb.*, *Lactobacillus*. MIC_{50} and MIC_{90}, MICs (mg/L) that inhibited 50% and 90% of the number of isolates tested, respectively. ECOFF, epidemiological cut-off for differentiation into WT and NWT isolates (WT: z mg/L ≤ ECOFF; NWT: z mg/L > ECOFF). IE, insufficient evidence noted when several isolates showed MICs outside (above or below) the corresponding test ranges and/or no bimodal distribution curve of the MIC profiles was observed in the corresponding species.

[a]Encompassing subsp. *bulgaricus* ($n = 9$) and subsp. *lactis* ($n = 2$).

[b]Occurrence of isolates that possessed intrinsic resistance to the corresponding antimicrobial.

[c]Occurrence of isolates that possessed acquired resistance to the corresponding antimicrobial.

[d]Occurrence of isolates that possessed probably intrinsic resistance to the corresponding antimicrobial.

[e]Occurrence of isolates that possessed probably acquired resistance to the corresponding antimicrobial.

[f]Antagonists of trimethoprim (thymidine) and sulfamethoxazole (*p*-aminobenzoic acid) in LSM broth led to non-evaluable MIC profiles of both antimicrobials (see the Discussion section).

Altogether, of those 12 LAB species that are represented by at least 10 isolates, acquired antibiotic resistances were only found to 4 out of 16 antimicrobials tested (streptomycin, erythromycin, clindamycin and oxytetracycline). Of the 383 *Lactobacillus* isolates listed in Table 4 belonging to different categories, in each case, 3 isolates (0.8%) were resistant to streptomycin, erythromycin or clindamycin and 12 isolates (3.1%) were resistant to oxytetracycline. In contrast, none of the pediococci and lactococci tested showed acquired antibiotic resistances (Table S1 and Table 4). In the following text, these data are described in more detail.

The three penicillins tested in this study exhibited comparable antimicrobial activities against all LAB species examined. In general, most of the LAB isolates were inhibited by a maximum

Table 4. Phenotypically detected acquired antibiotic resistances in LAB species

LAB species[a] (no. of isolates tested)	Number (%) of acquired resistances to important antimicrobials tested[b]					
	streptomycin	vancomycin teicoplanin	erythromycin	clindamycin	oxytetracycline	fusidic acid
Lactobacillus rhamnosus (131)	2[c,d] (1.5)	0 (IR)	1[d] (0.8)	1[d] (0.8)	3[e] (2.3)	0 (IR)
Lactobacillus paracasei (90)	1[f] (1.1)	0 (IR)	0	0	1[g] (1.1)	0 (IR)
Lactobacillus plantarum (46)	0	0 (IR)	0	0	1 (2.2)	0 (IR)
Lactobacillus fermentum (25)	0	0 (IR)	0	0	0	0
Lactobacillus gasseri (24)	0	0	0	0	0	0 (IR)
Lactobacillus acidophilus (20)	0	0	0	0	0	0 (IR)
Lactobacillus crispatus (13)	0	0	2[h] (15.4)	2[h] (15.4)	2[h] (15.4)	0 (IR)
Lactobacillus johnsonii (12)	0	0	0	0	3 (25.0)	0 (IR)
Lactobacillus delbrueckii (11)	0	0	0	0	0	0 (IR)
Lactobacillus reuteri (11)	0	0 (IR)	0	0	2 (18.2)	0
Lactobacillus spp., total (383)	3[c,d,f] (0.8)	0	3[d,h] (0.8)	3[d,h] (0.8)	12[e,g,h] (3.1)	0
Pediococcus acidilactici (29)	0 (IR)	0 (IR)	0	0	0 (IR)	0 (IR)
Pediococcus pentosaceus (20)	0 (IR)	0 (IR)	0	0	0 (IR)	0
Pediococcus spp., total (49)	0 (IR)	0 (IR)	0	0	0 (IR)	0
LAB spp., total (432)	3[c,d,f] (0.7)	0	3[d,h] (0.7)	3[d,h] (0.7)	12[e,g,h] (2.8)	0

0 (IR), no acquired resistance but intrinsic antibiotic resistance was observed.
[a]Only species represented by 10 or more isolates were evaluated for epidemiological breakpoints (ECOFF values).
[b]No NWT isolates were observed for the three penicillins, gentamicin, quinupristin/dalfopristin, chloramphenicol and linezolid; trimethoprim and trimethoprim/sulfamethoxazole were not evaluable (see the Discussion section).
[c]One *L. rhamnosus* was high-level resistant to streptomycin.
[d]One *L. rhamnosus* was resistant to streptomycin (high-level), erythromycin and clindamycin.
[e]Three *L. rhamnosus* were only resistant to oxytetracycline.
[f]One *L. paracasei* was high-level resistant to streptomycin.
[g]One *L. paracasei* was only resistant to oxytetracycline.
[h]Two *L. crispatus* were resistant to erythromycin, clindamycin and oxytetracycline (see also Table 5).

of 2 or 4 mg/L of the corresponding penicillin and no NWTs were identified among the isolates examined. However, species of the *L. acidophilus* group (*L. acidophilus*, *L. gasseri*, *L. crispatus*, *L. johnsonii*, *L. delbrueckii* and *Lactobacillus amylovorus*) were relatively more susceptible to penicillins in comparison with other LAB species. The *L. acidophilus* group was also relatively more susceptible to penicillin than to aminopenicillins when compared with pediococci, *L. rhamnosus*, *L. paracasei*, *L. plantarum* and, to a lesser extent, also with *L. reuteri*. The aminoglycosides gentamicin and streptomycin showed well-defined MIC distributions between $\leq$1 and 8 mg/L for gentamicin and between $\leq$2 and 128 mg/L for streptomycin. However, most isolates displayed MICs at the low end of the concentration ranges ($\leq$1 mg/L for gentamicin and $\leq$2 mg/L for streptomycin, respectively), whereas three probiotic *Lactobacillus* isolates (*L. rhamnosus* L-015 and L-455 and *L. paracasei* L-005) displayed high-level resistance to streptomycin with MICs of $\geq$2048 mg/L. Species of the *L. acidophilus* group were relatively more susceptible to streptomycin than other *Lactobacillus* species. In contrast, pediococci and members of several *Lactobacillus* species (including *L. paracasei* and *L. plantarum* and to some extent also *L. rhamnosus* and *L. fermentum*) appeared to be less susceptible to streptomycin. The glycopeptides vancomycin and teicoplanin exhibited heterogeneous profiles in their *in vitro* activities. Intrinsic high-level resistance to glycopeptides was found in pediococci (MICs: vancomycin $>$256 mg/L and teicoplanin $\geq$64 mg/L) and in several *Lactobacillus* species such as *L. rhamnosus*, *L. paracasei*, *L. plantarum*, *L. reuteri* (MICs: vancomycin $\geq$128 mg/L and teicoplanin $\geq$32 mg/L) and *L. fermentum* with a broader spectrum of susceptibilities to teicoplanin (MICs: vancomycin 32– $>$ 256 mg/L and teicoplanin 2– $>$ 256 mg/L). In contrast, the tested lactococci and species of the *L. acidophilus* group were clearly susceptible to these antibiotics, displaying MICs between 0.25 and 1 mg/L for vancomycin and between $\leq$0.125 and 0.25 mg/L for teicoplanin, respectively. Quinupristin/dalfopristin exhibited activities against all LAB tested in this study, with MIC ranges of $\leq$0.032–2 mg/L. Erythromycin and clindamycin were also very active against most LAB isolates examined, and clindamycin showed high activities especially against *P. acidilactici*, *P. pentosaceus*, *L. lactis* and several *Lactobacillus* species for which most isolates generated MICs of $\leq$0.125 mg/L (erythromycin) and $\leq$0.25 mg/L (clindamycin). However, there were also three isolates that showed MICs of erythromycin and clindamycin outside their corresponding ranges for WT organisms, namely, the probiotic *L. rhamnosus* strain L-455 and the two research isolates *L. crispatus* L-295 and L-296 (Table 5). Oxytetracycline was generally active against the majority of LAB isolates examined, especially against *L. lactis*. In contrast, pediococci showed relatively high MICs of 4–16 mg/L of this antibiotic. However, some isolates of different *Lactobacillus* species exhibited MICs higher than their corresponding ECOFF values for this antibiotic, i.e. the probiotic isolates *L. plantarum* L-437, *L. reuteri* L-285 and

Table 5. Characteristics of 19 NWT *Lactobacillus* isolates detected in the present study

Species	PRSF isolate	Category[a]	Phenotypic resistance (MIC in mg/L) to				Resistance gene(s) detected by PCR
			streptomycin	erythromycin	clindamycin	oxytetracycline	
Lactobacillus rhamnosus	L-015	P	2048				[b]
	L-455	P	>4096	>32	8		[b,c]
	L-016	P				16	[d]
	L-341	P				16	[d]
	L-078	H				8	[d]
Lactobacillus paracasei	L-005	P	>4096				[b]
	L-343	P				16	[d]
Lactobacillus crispatus	L-295[e]	R(P)		>32	>32	64	*erm*(B), *tet*(W)
	L-296[e]	R(P)		>32	>32	64	*erm*(B), *tet*(W)
Lactobacillus plantarum	L-437[e]	P				>128	*tet*(M) group
Lactobacillus johnsonii	L-073	H				16	*tet*(W)
	L-155	H				16	[d]
	L-153	A				8	*tet*(W)
Lactobacillus reuteri	L-285[e]	P				>128	*tet*(W)
	L-285-2[e]	P				>128	*tet*(M) group
Lactobacillus curvatus[f]	L-344	N				16	[d]
	L-377[e]	N				128	*tet*(M)
Lactobacillus brevis[f]	L-405	P				8	[d]
Lactobacillus buchneri[f]	L-366	P				8	[d]

[a]Categories of isolates: P, probiotic; R(P), research (probiotic); N, nutritional; A, animal; H, human (see also the Materials and methods section).
[b]Negative in PCR for *aad*(E).
[c]Negative in PCR for *erm*(A), *erm*(B), *erm*(C).
[d]Negative in PCR for *tet*(K), *tet*(L), *tet*(M) group.
[e]P, N and R(P) NWT isolates used as donors in *in vitro* transfer experiments.
[f]Species represented by less than 10 isolates tested (no ECOFFs determinable).

L-285-2, *L. rhamnosus* L-016 and L-341 and *L. paracasei* L-343, the research isolates *L. crispatus* L-295 and L-296, the human isolates *L. rhamnosus* L-078, *L. johnsonii* L-073 and L-155 and the animal strain *L. johnsonii* L-153. Additionally, four isolates with MICs of oxytetracycline between 8 and 128 mg/L were observed in three species that were presented by less than 10 isolates and for which no ECOFF could be determined, i.e. the probiotic isolates *Lactobacillus brevis* L-405 and *Lactobacillus buchneri* L-366 and the two nutritional isolates *Lactobacillus curvatus* L-377 and L-344 (Table 5 and Table S1). Chloramphenicol showed efficient *in vitro* antibacterial activities, nearly all LAB isolates tested were inhibited by ≤4 mg/L and no NWT isolates were observed. *In vitro* susceptibilities to fusidic acid were very different among the LAB species tested. Within the genus *Lactobacillus*, members of *L. fermentum* and *L. reuteri* appeared to be susceptible to fusidic acid (MICs ≤2 mg/L), whereas other species (e.g. *L. rhamnosus*, *L. paracasei*, *L. plantarum* and those of the *L. acidophilus* group) seem to be intrinsically resistant. Although two isolates of *P. acidilactici* displayed an MIC of fusidic acid of 16 mg/L, pediococci and *L. lactis* were mostly inhibited by 2 or 4 mg/L. The oxazolidinone antibiotic linezolid showed a unique pattern of high antibacterial activities against the different LAB species tested in this study. Nearly, all strains were inhibited by 1–2 mg/L, and only three isolates of *L. acidophilus* possessed an MIC of linezolid of 4 mg/L. Trimethoprim and trimethoprim/sulfamethoxazole showed broad spectra and no Gaussian curves of their MIC profiles, which did not allow determination of ECOFF values for the LAB species tested; the MICs for many LAB species ranged between ≤0.25 (or 0.5) and >512 mg/L for both antibiotics (Table S1).

Detection of antimicrobial resistance genes

The NWT *Lactobacillus* isolates that possessed MICs higher than the corresponding species-specific ECOFF values of the tested antibiotics were selected for PCR detection of the corresponding resistance gene(s). Species for which no ECOFF values were determined, but containing isolates displaying high MICs when compared with NWT ranges in other *Lactobacillus* species, were also tested. Altogether, 19 out of 416 (4.6%) *Lactobacillus* isolates tested in the present study displayed MICs exceeding the respective ECOFF values. However, only some of these NWT isolates also possessed the corresponding antibiotic resistance gene(s) (Table 5). Three probiotic isolates (*L. paracasei* L-005 and *L. rhamnosus* L-015 and L-455) displaying high-level resistance to streptomycin were negative for the *aad*(E) gene. The latter isolate was also resistant to erythromycin and clindamycin, but did not contain the corresponding resistance genes *erm*(A), *erm*(B) or *erm*(C) (Table 5). Eight, mostly probiotic *Lactobacillus* isolates that generated MICs of oxytetracycline between 8 and 16 mg/L, did not possess any of the tested *tet* genes. However, two isolates of *L. johnsonii* with MICs of this antibiotic in the range of 8–16 mg/L were *tet*(W)-positive. All *Lactobacillus* with higher MICs of oxytetracycline (64–>128 mg/L) contained *tet*(M), *tet*(W) or a non-specified member

of the *tet*(M) group. In addition, the research isolates *L. crispatus* L-295 and L-296 that showed MICs of erythromycin and clindamycin of >32 mg/L were positive for the *erm*(B) gene (Table 5).

In vitro transfer experiments

Probiotic, nutritional or research NWT isolates containing one or two antibiotic resistance genes (Table 5; isolates are marked by footnote e) were selected as donors for experiments. Rifampicin- and fusidic acid-resistant isolates of the corresponding *Lactobacillus* species and the strains *E. faecium* 64/3 and *E. faecalis* JH 2-2 served as recipients. However, none of the intra- and interspecies donor–recipient combinations tested produced transconjugants under the experimental *in vitro* conditions applied (data not shown).

Discussion

In the present study, MICs of 16 antimicrobial agents of nearly all important classes were determined for a collection of 473 isolates encompassing 24 species of the genera *Lactobacillus*, *Pediococcus* and *Lactococcus* by microdilution test using LSM broth.[24] Because most isolates were identified by fingerprinting techniques that also allow determination of the relationship of isolates at the individual strain level, there is reasonable evidence to assume that the human, animal and research isolates were not duplicate isolates of the same strain. Within the probiotic or nutritional categories, however, a number of isolates received from different depositors produced very similar fingerprints (data not shown). In these cases, it cannot be ruled out that duplicate cultures were included in the present study.

Overall, our results are in good agreement with data from other studies for a broad range of LAB species and antibiotics, although different nutrient media, incubation conditions and/or susceptibility testing methods were used.[22,23,39–47] In some studies, higher MICs of the aminoglycosides gentamicin and streptomycin were reported for LAB, which is probably due to the fact that susceptibility testing was performed on MRS agar (e.g. by Etest).[22,48] In the latter two studies, streptomycin-resistant *Lactobacillus* spp. were encountered with MICs of streptomycin of >256 mg/L. The reason for the increased MICs of the aminoglycosides on MRS agar may be due to the medium's low pH (6.2 ± 0.2), because the pH optimum of aminoglycosides is in the alkaline range (pH 7.8).[49] In contrast, the pH value of LSM broth is adjusted to pH 6.7, which appears to favour the antibacterial activities of aminoglycosides, resulting in lower MICs. The observed difficulties in determining MICs of trimethoprim and trimethoprim/sulfamethoxazole, in contrast, are obviously linked to the presence of antagonistic components such as *p*-aminobenzoic acid (against sulfamethoxazole) and/or thymidine (against trimethoprim)[50] in LSM broth as the consequence of its 10% portion of MRS broth.[24] The synthetic IST broth as the main component (90%) of LSM broth[24] is known for its poor content of antagonists and, therefore, the portion of MRS broth in LSM broth obviously caused the non-evaluable MIC profiles of trimethoprim and trimethoprim/sulfamethoxazole. However, we did not find negative effects of LSM broth on the activities of other antibiotics examined and the LAB species tested did not show growth problems.[24]

On the basis of the MIC data obtained for 16 antimicrobial agents, we were able to define tentative ECOFF values for 13 antibiotics in up to 12 LAB species represented by at least 10 isolates. These tentative ECOFFs may offer an objective basis to update and/or modify specific guidelines, such as those outlined by the Feed Additives and Products (FEEDAP) panel of the European Food Safety Authority (EFSA).[51] Among the 432 LAB isolates tested (Table 4), low resistance frequencies were found to oxytetracycline (2.8%) and to streptomycin, erythromycin and clindamycin (in each case 0.7%), resulting in a total frequency of 4.6% NWT isolates (19 out of 416) for the genus *Lactobacillus* (Tables 1, 4 and 5). However, when looking in more detail at the frequencies of *Lactobacillus* isolates with acquired antibiotic resistances according to their categories, striking differences were observed (Tables 1, 4 and 5), e.g. 11 isolates among 113 (9.7%) probiotic, 2 among 23 probiotic/nutritional research (8.7%), 2 among 26 nutritional (7.7%), 1 among 5 ('20%') animal isolates, but only 3 among 249 human isolates (1.2%) possessed acquired antibiotic resistances. However, duplicate cultures of probiotic or nutritional isolates cannot be ruled out as discussed above. Furthermore, several intrinsic antibiotic resistances (or probable intrinsic resistances) were recorded in different LAB species tested, as indicated in Table S1 and Tables 3 and 4. Although 44% of the human isolates originated from blood cultures and 41% from faecal and vaginal flora of healthy and hospitalized persons, the low frequency of acquired antibiotic resistance found in human *Lactobacillus* isolates was surprising. But in a retrospective review on pathogenic relevance of over 200 *Lactobacillus*-associated infections, the isolates were also mostly susceptible to antibiotics such as erythromycin and clindamycin, except the species-dependent intrinsic resistance to vancomycin.[14] Additionally, the low frequency of NWT in human isolates may be due to the biased selection of human isolates within the PROSAFE project. The probiotic NWT isolates investigated mainly belong to species most commonly used in probiotic applications, i.e. *L. plantarum*, *L. paracasei* and *L. rhamnosus*. This finding reinforces the antimicrobial susceptibility testing in the safety assessment procedure of strains intended for probiotic or nutritional use, especially in humans.[17,52]

Several studies have reported the prevalence of *tet* genes in *Lactobacillus* isolates. The most widespread of these genes, *tet*(M), has been detected in members of *L. plantarum*, *L. curvatus*, *Lactobacillus casei*, *L. acidophilus*, *L. gasseri* and *L. crispatus*.[23,53,54] The *tet*(W) gene appears to be less widely distributed in lactobacilli and has so far only been reported in the probiotic strain *L. reuteri* SD 2112.[55] In the present study, *tet*(M), *tet*(W) and unidentified members of the *tet*(M) group were detected in five *Lactobacillus* species. Of these, the occurrence of *tet*(W) in strains of *L. crispatus* and *L. johnsonii* is a novel finding. Erythromycin resistance displayed by two isolates of *L. crispatus* was attributed to the presence of the *erm*(B) gene. This gene has previously been detected in various *Lactobacillus* species including *L. reuteri*, *L. fermentum*, *L. casei*, *L. plantarum*, *L. acidophilus*, *L. gasseri*, *L. rhamnosus* and *L. johnsonii*.[54,56–58] The gene *aad*(E) responsible for high-level streptomycin resistance was not detected in the three *Lactobacillus* isolates displaying MICs of streptomycin of ≥2048 mg/L. Possibly, another mechanism may be responsible for the high-level resistance to this antibiotic, such as mutations in genes encoding ribosomal proteins and/or rRNA. Previously, a *Lactobacillus* isolate from yoghurt was reported with an MIC

of streptomycin of 1024 mg/L, but no details were provided on species identity and the presence of resistance genes.[45]

None of the *in vitro* transfer experiments using NWT isolates with confirmed presence of *erm*(B) or different *tet* genes as donor strains and susceptible isolates of the corresponding *Lactobacillus* species and *Enterococcus* laboratory strains (*E. faecium* 64/3 and *E. faecalis* JH 2-2), respectively, as recipient strains yielded confirmed transconjugants. However, interspecies transfer experiments from the control strains *L. plantarum* LMG 21684 [containing *erm*(B) and *tet*(M)] and *L. plantarum* LMG 21687 [containing *tet*(M)] to *E. faecalis* JH2-2 produced transconjugants but in low frequencies of $\sim 10^{-8}$ per recipient cell (data not shown), compared with higher frequencies of $10^{-4} - 10^{-6}$ previously reported.[59] Possibly, higher transfer frequencies could be obtained with the NWT isolates tested under modified *in vitro* conditions or as demonstrated recently under *in vivo* conditions.[60,61]

In conclusion, the finding of acquired resistance genes in isolates intended for probiotic use or in isolates that are already used as probiotics for a longer time raises the question whether such cultures should be deliberately released in the food chain. The precautionary principle would plead against the use of such strains, independent of the fact of whether intra- and interspecies transfer can be proven or not. According to the opinion of the FEEDAP panel on the updating of criteria used in the assessment of bacteria for resistance to antibiotics of human and veterinary importance, strains carrying an acquired resistance to antimicrobial(s) should not be used as feed additives, unless it can be demonstrated that it is a result of chromosomal mutation(s).[51] In this respect, however, it is important to realize that acquired antibiotic resistances can be transferred not only by conjugation but also by other mechanisms, such as transformation or transduction, that are even more difficult to study under controlled laboratory conditions.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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