Antimicrobial Resistance of Lactobacilli Isolated from Food

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Abstract

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Bacteria of the genus *Lactobacillus* are of great benefit in many areas of life. They are widely used in food industry, in particular as part of starter cultures for fermented dairy and meat products, but also in human and veterinary medicine as probiotics. The increasing global problem of antimicrobial resistance may also involve lactic acid bacteria because of the possible risk of resistance genes transfer. We determined the antimicrobial susceptibility of lactobacilli isolated from food. Ninety facultatively heterofermentative lactobacilli isolated from retail dairy and meat products were tested. The resistance to antimicrobials was screened by the disk diffusion method and the minimum inhibitory concentrations were determined by the broth microdilution method. Fifteen strains (17%) were resistant to at least one antimicrobial agent and one strain was multiresistant.

Keywords: *Lactobacillus* spp.; disk diffusion method; broth microdilution method; minimum inhibitory concentration (MIC); susceptibility to antibiotics; dairy products; meat products

Bacterial resistance to antimicrobial agents is a major global public health problem, affecting not only human and veterinary medicine (Ammor *et al*. 2008) but also food production. The food chain is becoming a possible way of dissemination of antibiotic resistance among bacterial populations of animals and humans (Witte 2000). Many species of lactobacilli, previously generally recognised as safe (GRAS), may become vectors of antibiotic resistance genes. These bacteria are usually consumed in high quantities and close contact with other bacteria in the human gastrointestinal tract provides perfect conditions for horizontal transfer of conjugative plasmids and transposons with genes encoding resistance to antimicrobial agents (Mathur & Singh 2005; Jacobsen *et al*. 2007; Ammor *et al*. 2008; Nawaz *et al*. 2011). The absence of the acquired antimicrobial resistance has become an important criterion for evaluating the safety of lactobacilli used as starter cultures or probiotics (Mayrhofer *et al.* 2008).

Although the minimum inhibitory concentrations (MIC) are defined for clinically important microorganisms, internationally valid MICs for lactobacilli have not been determined yet. To distinguish the strains with the acquired antimicrobial resistance from the susceptible ones, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) defined the microbiological breakpoints used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. The breakpoint data were derived from the published body of research and from national and European monitoring programmes (EFSA 2008).

Following a request from EFSA, the Panel on Biological Hazards (BIOHAZ) was asked to review the list of the Qualified Presumption of Safety (QPS) microorganisms and to update the antimicrobial resistance criteria used to judge the safety of food/feed use microorganisms. If a defined

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taxonomic unit does not raise safety concerns or if any possible concerns can be excluded, the QPS approach can be applied and the taxonomic unit can be recommended to be included in the QPS list. The QPS list is reviewed and updated annually by the Panel on Biological Hazards. This QPS list includes 36 species of *Lactobacillus* (EFSA 2011).

The aim of this study was to determine the antimicrobial susceptibility of lactobacilli isolated from food.

MATERIAL AND METHODS

Bacterial strains. The susceptibility to antimicrobial agents was monitored in 90 facultatively heterofermentative lactobacilli isolates. They originated from 68 food samples collected in the Czech Republic from retail meat products (n = 11) and dairy products (n = 57), and seven strains were obtained from the Czech Collection of Microorganisms (CCM; Brno, Czech Republic). The isolates were cultivated on MRS agar (Oxoid, Basingstoke, UK) at 30°C for 48–72 h microaerophilically. Cell morphology in the suspect colonies was studied microscopically (Gram staining), while the cultures were also tested for the presence of catalase and oxidase (JK Trading, Prague, Czech Republic). Genotype confirmation of lactobacilli isolates was performed with the use of polymerase chain reaction (PCR) with genus-specific primers LbLMA 1-rev and R16-1 (Dubernet *et al.* 2002). DNA was extracted from bacterial cultures by the boiling procedure with Chelex® 100 (Bio-Rad, Hercules, USA). Amplification took place in a PTC-200 thermocycler (MJ Research, Watertown, USA). A two-step multiplex PCR method was used for species identification in the selected isolates and subsequently, following the classification of lactobacilli into groups, PCR with species-specific primers was also used based on the detection of nucleotide sequences of the 16S-23S rRNA intergenic spacer region and adjacent 23S rRNA gene differing for individual species of lactobacilli (Berthier & Ehrlich 1998; Ward & Timmins 1999; Song *et al*. 2000; Walter *et al.* 2000). Amplicons were detected by agarose gel electrophoresis, stained with ethidium bromide, and visualised using a UV transilluminator (λ = 305 nm). The distribution of 90 lactobacilli isolates by species and origin is summarised in Table 1.

Antimicrobial susceptibility testing. The resistance was determined using two methods, broth microdilution and disk diffusion. Inocula of the isolates tested were prepared in a sterile saline by suspending the colonies from MRS agar plates (Oxoid, Basingstoke, UK) incubated at 30°C for 24 h under microaerophilic conditions.

The broth microdilution method (Trios, Prague, Czech Republic) was the first method of choice. The following antimicrobials were tested (in MRS broth): ampicillin (AMP; 0.015–2 mg/l), trimethoprim (TRI; 0.25–32 mg/l), gentamicin (GEN; 1–128 mg/l), chloramphenicol (CMP; 0.5–64 mg/l), oxacillin (OXA; 0.25–32 mg/l), streptomycin (STR; 2–256 mg/l), tetracycline (TET; 2–256 mg/l), erythromycin (ERY; 0.031–4 mg/l), and vancomycin (VAN; 2–256 mg/l). Microtiter plates inoculated with the bacterial suspension with a McFarland standard turbidity of 0.5 were incubated at 30°C for 24 h under microaerophilic conditions. The minimum inhibitory concentrations were established. The strains were classified as susceptible or resistant based on the minimum inhibitory concentration required to inhibit the growth of 90% of organisms (MIC_{90}).

Bacterial suspensions with a turbidity equivalent to a McFarland standard of 1 were swabbed evenly onto MRS agar plates with a sterile cotton swab for the disk diffusion method. Antibiotic disks containing 10 µg ampicillin, 5 µg trimethoprim, 10 µg gentamicin, 30 µg chloramphenicol, 5 µg

Table 1. Distribution of 90 lactobacilli isolates by species and origin

Species	No. of isolates	Origin of isolates			
		CCM	meat products	BIO milk products	cheeses
L. casei	2	2	–	–	–
L. curvatus	18	2	1	14	1
L. paracasei	16	-	–	8	8
L. plantarum	10	1	5	4	–
L. rhamnosus	38	2	–	35	1
L. sakei	6	–	6	-	–
Total	90	7	12	61	10

oxacillin, 10 µg streptomycin, 30 µg tetracycline, 15 µg erythromycin, and 30 µg vancomycin (Oxoid, Basingstoke, UK) were placed on MRS agar plates. The plates were incubated at 30°C for 24 h under microaerophilic conditions and then the inhibition zone diameters (IZ_D), including the diameter of the disk, were measured.

Statistical analysis. To the correlation between the broth microdilution method (MICs in µg/ml) and the disk diffusion method (IZ_Ds in mm), logistic or linear (for AMP) regression analysis was applied after logarithmic conversion (log_2) of the MICs. Overall statistical significance was assessed using χ^2-test for linear regression and *F*-test for logistic regression. *P* values of < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The minimum inhibitory concentrations of 90 facultatively heterofermentative lactobacilli and MIC_{90} ranges are shown in Table 2. Based on the MIC_{90}, 15 isolates (17%) were determined as resistant to at least one antibiotic. These strains and their resistance phenotypes are listed in Table 3. *Lactobacillus plantarum* A54 isolated from fermented sausage was resistant to five antibiotics of four antimicrobial groups. The resistance to gentamicin was found to be the most frequent (in 7.8% of isolates). Danielsen and Wind (2003) and Nawaz *et al.* (2011) have also shown a high resistance to gentamicin. Frequent resistance of lactobacilli to aminoglycosides has been reported by Katla *et al.* (2001).

In this study, the resistance to trimethoprim and vancomycin was not evaluated by the broth microdilution method. The MIC_{90} values for these antibiotics were above the range determined in the microdilution plate used.

Based on the statistical analysis of the MIC results, the discriminatory inhibition zones IZ_D (in mm) for the disk diffusion method were established to categorise the lactobacilli isolates as susceptible ($IZ_{isolate} > IZ_D$) or resistant ($IZ_{isolate} \leq IZ_D$) to antimicrobials. The rate of complete agreement between the two methods ranged from 63.3% to 97.8%. The sensitivity of the disk diffusion method for the assessment of resistant isolates ranged between 98.1% and 100% and its specificity (ability of correct detection of susceptible isolates) was 55.0–97.7%, except for vancomycin with only 2.9%. This low specificity was caused by the value of $MIC_{90} \geq 250$ mg/l and 98.9% of isolates showed an inhibition zone of 6 mm. The IZ_D, percentage of the resistant isolates and rates of complete agreement between the two antimicrobial susceptibility testing methods are presented in Table 4.

According to the discriminatory inhibition zones, a high percentage of isolates resistant to vancomycin and trimethoprim was determined. The resistance of facultatively heterofermentative lactobacilli to vancomycin is intrinsic, due to the presence of D-Ala-D-lactate in their peptidoglycan instead of the normal dipeptide D-Ala-D-Ala (Ammor *et al.* 2008). Although relatively rare in Gram-positive bacteria, the acquired trimethoprim resistance has been occasionally detected (Young *et al.* 1987; Charpentier & Courvalin 1997). The data available (Korhonen *et al.* 2007) indicate that within the species of lactobacilli, the range of the apparent trimethoprim resistance can be wide with no clear breakpoint values. Therefore,

Table 2. Minimum inhibitory concentratio (MIC) ranges determined by the microdilution method

Antimicrobial	MIC range (mg/l)	MIC_{90} (mg/l)	No. of resistant isolates	Breakpoints (mg/l)
AMP	< 0.015–0.5	0.5	0 (0%)	4
TRI	0.25–> 32	> 32	ND	ND
GEN	< 1–64	16	7 (7.8%)	16
CMP	< 0.5–4	2	3 (3.3%)	4
OXA	2–32	16	3 (3.3%)	ND
STR	4–256	128	4 (4.4%)	64
TET	< 2–32	8	3 (3.3%)	8
ERY	< 0.031–0.5	0.25	2 (2.2%)	1
VAN	16 –> 256	> 256	ND	ND

MIC_{90} – number of resistant isolates according to MIC_{90} and EFSA breakpoints; AMP – ampicillin; TRI – trimethoprim; GEN – gentamicin; CMP – chloramphenicol; OXA – oxacillin; STR – streptomycin; TET – tetracycline; ERY – erythromycin; VAN – vancomycin; ND – not determined

Table 3. Resistant isolates according to MIC_{90} and resistance phenotypes

Strain	Species	Origin of isolate	Resistance phenotype
A54	*L. plantarum*	meat product	**GEN**, CMP, **STR**, TET, ERY
C16	*L. plantarum*	meat product	GEN
BIO I 16	*L. plantarum*	BIO milk product	TET
CCM 7039T	*L. plantarum*	collection strain	CMP, ERY
D16	*L. sakei*	meat product	TET
M I 9	*L. sakei*	meat product	OXA
M I 13	*L. sakei*	meat product	OXA
M I 19	*L. sakei*	meat product	OXA
US 27	*L. curvatus*	cheese	**GEN, STR**
BIO III 67	*L. curvatus*	BIO milk product	STR
BIO III 70	*L. curvatus*	BIO milk product	STR
CCM 7558T	*L. curvatus*	collection strain	GEN
BIO II 65	*L. paracasei*	BIO milk product	GEN
BIO III 53	*L. paracasei*	BIO milk product	GEN
CCM 7088T	*L. casei*	collection strain	GEN, CMP

in bold – antibiotics belonging to the same group (aminoglycosides); AMP – ampicillin; TRI – trimethoprim; GEN – gentamicin; CMP – chloramphenicol; OXA – oxacillin; STR – streptomycin; TET – tetracycline; ERY – erythromycin; VAN – vancomycin

the MIC testing of trimethoprim for lactic acid bacteria was not considered relevant (EFSA 2008).

The results of trimethoprim susceptibility testing may be distorted by reading at 80% inhibition of the growth (broth microdilution method) and ignoring the slight growth within the inhibition zones (disk diffusion method). The difficulty in reading at 80% inhibition of growth has also been reported by Mayrhofer *et al.* (2008).

The resistance to chloramphenicol was 3.3%, similar results have been reported also by Katla *et al.* (2001) and Ammor *et al.* (2008). Testing for chloramphenicol resistance would efficiently cover for the hazard of acquiring the resistance to linezolid since non-mutational resistance to linezolid is encoded by the *cfr* gene, which also confers the resistance to chloramphenicol (Toh *et al.* 2007; Arias *et al.* 2008; EFSA 2008).

Table 4. Inhibition zones for nine antimicrobials, percentage of resistant *Lactobacillus* isolates, and sensitivity and specificity of the method

Antimicrobial	IZ_D (mm)	Resistant isolates (%)	Complete agreement between BM and DD (%)	DD sensitivity[a] (%)	DD specifity (%)
AMP	19	22.2	77.8	–	77.8
TRI	8	87.8	90.0	100.0	55.0
GEN	8	76.7	82.2	98.2	57.1
CMP	27	24.4	78.9	100.0	78.2
OXA	8	5.6	97.8	100.0	97.7
STR	7	62.2	95.6	100.0	89.5
TET	22	11.1	92.2	100.0	92.0
ERY	25	8.9	93.3	100.0	93.2
VAN	6	98.9	63.3	100.0	2.9

AMP – ampicillin; TRI – trimethoprim; GEN – gentamicin; CMP – chloramphenicol; OXA – oxacillin; STR – streptomycin; TET – tetracycline; ERY – erythromycin; VAN – vancomycin; IZ_D – dicriminatory inhibition zone; BM – broth microdilution method; DD – disk diffusion method

[a]100.0% – all resistant isolates (based on the MIC) were determined as resistant based on the IZ

A crucial factor for antimicrobial testing of lactobacilli is the selection of suitable cultivation medium. Lactobacilli have specific nutritional and atmospheric requirements for their growth, therefore standardised susceptibility test media such as Mueller-Hinton broth and Iso-Sensitest (IST) broth were not used. Charteris *et al.* (2001) and Delgado *et al.* (2005) used MRS medium in their studies. Ocaña *et al.* (2006) evaluated MRS agar as a suitable medium to study antimicrobial susceptibility of microaerophilic or anaerobic lactobacilli. However, there are indications that the medium MRS may exhibit antagonistic effects with supplemental antimicrobials in susceptibility testing (Huys *et al.* 2002). Klare *et al.* (2005) developed lactic acid bacterium susceptibility test medium (LSM), a mixed formulation of IST broth (90% v/v) and MRS broth (10% v/v). This medium was used by Huys *et al.* (2010) for antimicrobial susceptibility testing of non-enterococcal lactic acid bacteria (NELAB) and bifidobacteria in eight European countries and this study has further validated the standard use of LSM and formed the basis for the ISO 10932/IDF 223 from 2010 (standard for susceptibility testing of NELAB and bifidobacteria). Our study had begun before the publication of the International Standard, so the applied method is different from the ISO 10932:2010.

For the purpose of distinguishing the strains harbouring acquired antimicrobial resistance from susceptible strains, the FEEDAP Panel defines the microbiological breakpoints (or epidemiological or cut-off values). These breakpoints (Table 2) have been set by studying the distribution of MICs of antimicrobials in bacterial populations belonging to a single taxonomical unit. The data used for the definition of microbiological breakpoints have been derived from the published body of research and from national and European monitoring programmes. Our values of $MICs_{90}$ were obtained by testing 90 isolates only, so they may be different from the FEEDAP breakpoints.

The isolates with MICs above the breakpoint require further investigation to make the distinction between the intrinsic and acquired resistance (through the gain of exogenous DNA or the mutation of indigenous genes). The presence of the acquired antimicrobial resistance genes on mobile elements poses the highest risk of antimicrobial resistance dissemination. The FEEDAP Panel considers that the strains of bacteria carrying the acquired resistance to antimicrobials should not be used as feed additives, unless it can be demonstrated that it is a result of chromosomal mutation (EFSA 2008).

CONCLUSIONS

The study results show that lactobacilli may be resistant to antimicrobial agents. Resistant strains were detected in all food categories examined. Although considered non- pathogenic, lactobacilli commonly occur in large numbers in foods, especially fermented foods. Lactobacilli enter into human gastro-intestinal tract in large numbers where they interact with the intestinal microflora. When a bacterial strain demonstrates the resistance to antimicrobials by phenotypic methods, it is desirable to monitor the molecular basis of this resistance and to distinguish whether it is intrinsic or acquired. The strains with the mobile genetic elements carrying genes encoding resistance should not be used as starter cultures. They might contribute negatively to an uncontrolled horizontal spread of the resistance to antibiotics throughout the human food chain.

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