Assessment of antibiotic resistance in starter and non-starter lactobacilli of food origin

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Abstract

The absence of acquired resistance to antimicrobials has become an important criterion in evaluation of the biosafety of lactobacilli used as industrial starter or probiotic cultures. The aim of this study was to assess antibiotic resistance in starter and non-starter lactobacilli of food origin. Minimal inhibitory concentrations of ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin were established in 81 strains of lactobacilli (*L. acidophilus, L. animalis, L. brevis*, *L. curvatus, L. delbrueckii, L. fermentum, L. helveticus, L. paracasei, L. plantarum, L. rhamnosus* and *L. sakei*) by the microdilution method. The strains were classified as susceptible or resistant to antimicrobials based on the cut-off values according to the EFSA guideline. Sixty-two strains (77% food isolates, 76% starter or adjunct cultures) were resistant to at least one antimicrobial agent (the most frequently to aminoglycosides). Adjunct cultures showed a higher antibiotic resistance (80%) than starters (60%). Four multiresistant strains (3 food isolates, 1 adjunct culture) were analyzed by whole genome sequencing. One potentially transferable *aad*E gene (responsible for streptomycin resistance) was detected only in one multi-drug resistant strain of *L. animalis* originating from an adjunct culture. Thus, there is a risk of horizontal transmission of this gene. It is necessary to eliminate such strains from use in the food industry. This study provides relevant data concerning the use of lactobacilli in safe food production. To ensure food safety, detailed characterization of resistance to antimicrobials is necessary not only in starter strains but also in non-starter lactic acid bacteria isolated from food products.

Broth microdilution method, minimal inhibitory concentration, antimicrobial susceptibility, aadE gene

The emergence of antibiotic-resistant microbiota is a worldwide problem primarily caused by inappropriate and excessive use of antimicrobials in veterinary and human medicine and as growth promoters in farm animals (Bernardeau et al. 2008; Bardon et al. 2018). The development of bacterial resistance to antimicrobial agents is an increasing problem for public health. It affects not only the treatment in the human and veterinary medicine sector but it also influences the production and quality of food (Ammor et al. 2008; Verraes et al. 2013). The food chain constitutes one of the routes of spreading antibiotic resistance (Mathur and Singh 2005; Nawaz et al. 2011). Antimicrobial-resistant bacteria may be found in soil, water and in samples of animal or human origin. Food products of animal origin may contain resistant microorganisms due to faecal contamination during the slaughter and meat processing. Products of plant origin may be contaminated during their production, e.g. by contaminated irrigation water, untreated manure or other sewage discharges. Furthermore, microbiota added during food processing as a starter culture, probiotics and bioconserving microbiota may act as reservoirs of transmissible genes of antibiotic resistance. There is also the possibility of cross-contamination with antimicrobial-resistant microbiota during food processing. As a consequence, transfer of

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antibiotic resistance encoding genes between microorganisms after ingestion by humans may occur (Verraes et al. 2013).

Bacteria from the genus *Lactobacillus* are used as adjunct or starter cultures in the production of various fermented foods. A starter culture can be defined as a microbial suspension of large numbers of cells of at least one microorganism to be added to a pasteurized or raw food matrix intended for production of fermented food by steering and accelerating the fermentation process. These microorganisms cause rapid acidification of the raw material, production of organic acids, mainly lactic acid (Leroy and Vuyst 2004; Hati et al. 2013). Adjunct cultures are used for acceleration and intensification of flavour development in food products and for their probiotic characteristic (Ortigosa et al. 2006). In this way, they not only improve the texture and contribute to the acceptable sensory profile of the final product, but also enhance its microbial safety and shelf life. Lactobacilli are also able to colonize fermented food products and represent a predominant part of non-starter lactic acid bacteria (LAB) (Leroy and Vuyst 2004; Ortigosa et al. 2006).

This genus with over 200 described species is characterized by high heterogeneity, which is reflected in its complex phylogeny (Abriouel et al. 2015). Lactobacilli reach the gastrointestinal tract via ingestion of a high number of representatives of this bacterial genus in the fermented food (typically > 8 log CFU/ml), where they interact with the resident gut microbiota of the host (Mathur and Singh 2005; Abriouel et al. 2015). However, some strains are able to carry antibiotic resistance genes and may be phenotypically resistant to antimicrobials. Some of them may even carry the mobile genetic elements containing the resistance genes and transfer them to other LAB or even to pathogens, thus threatening human health (Mathur and Singh 2005; Ammor et al. 2008; Toomey et al. 2010; Nawaz et al. 2011; Devirgiliis et al. 2013; Verraes et al. 2013; Rossi et al. 2014; Guo et al. 2017).

One possibility to reduce antibiotic resistance spread via food is testing the sensitivity of lactobacilli used in the food production to selected antibiotics. The European Food Safety Authority recommends that bacterial strains carrying mobile genetic elements containing the antibiotic resistance determinants should not be used in feeds, for the preparation of fermented products, and as probiotics. In 2018, the EFSA-FEEDAP Panel updated the criteria for the evaluation of antimicrobial resistance (AMR) in LAB. The established microbiological (epidemiological) breakpoints (cut-offs) facilitate the differentiation between resistant strains and strains susceptible to antimicrobials (EFSA 2018).

It is distinguished between two types of resistance, intrinsic (primary) and acquired (secondary). The primary resistance of bacteria to an antimicrobial agent is an integral, genetically determined component typical of this bacterial species. Intrinsic AMR is not considered to be of safety concern. In contrast, when a strain of a typically susceptible species is resistant to antimicrobial agent, it is considered to be acquired resistance (EFSA 2018). This resistance can be the result of either mutations in chromosomal genes or due to the acquisition of external genes from other bacteria. Mobile genetic elements that allow horizontal resistance gene transfer between microorganisms present the greatest risk factor in the spread of resistance (Mathur and Singh 2005; Nawaz et al. 2011; EFSA 2012, 2018). Identification of AMR genes is crucial to understanding the issue of resistance, for identification of resistant strains when genes are not or weakly expressed *in vitro* (Zankari et al. 2012). Therefore, the aim of this study was to assess the antibiotic resistance in starter and non-starter lactobacilli of food origin.

Materials and Methods

Bacterial strains

Susceptibility to antimicrobial agents was monitored in 81 lactobacilli of different species: *L. acidophilus, L. animalis, L. brevis*, *L. curvatus, L. delbrueckii, L. fermentum, L. helveticus, L. paracasei, L. plantarum, L. rhamnosus* and *L. sakei* (Table 1). The strains were identified based on the 16S rRNA gene sequencing

(Ehrmann et al. 2003; Atashpaz et al. 2010; Yoon et al. 2017), polymerase chain reaction with genus- and species-specific primers and MALDI-TOF MS analysis in previous studies (Dušková et al. 2012). Twenty-five strains from starter or adjunct cultures and fifty-six strains originating from the collection of the Department of Animal Origin Food and Gastronomic Sciences (University of Veterinary and Pharmaceutical Sciences Brno) were isolated during routine analysis of fermented dairy products from retail, raw cow's milk and goat's colostrum (n = 39) obtained at the farm level, meat products and swab samples of semi-finished food products and the processing environment (n = 17). *Lactobacillus paracasei* LMG12586 and *Lactobacillus plantarum* LMG6907, obtained from Belgian collections of microorganisms (LMG, Ghent, Belgium), were used as quality control strains for controlling the precision of susceptibility testing according to the ISO10932/IDF223 guideline (2010).

Antimicrobial susceptibility testing

Resistance/susceptibility to antimicrobials was determined using a broth microdilution method based on the international methodologies of the Clinical Laboratory Standards Institute (CLSI 2016), ISO10932/IDF223 guideline (2010) and the EFSA recommendations (2018). Bacterial cultures were tested on a microtitre plate with 100 ml of LSM culture medium (90% ISO-Sensitest broth + 10% MRS broth; Oxoid Ltd., Basingstoke, UK) with the addition of cysteine (0.3 g of cysteine per litre of medium, Sigma-Aldrich Corp., St. Louis, MO, U.S.A.). The following antimicrobials were tested: ampicillin (AMP; 0.125-16 µg/ml), streptomycin (STR; 2-256 µg/ml), tetracycline (TET; 0.5-64 µg/ml), erythromycin (ERY; 0.063-8 µg/ml), clindamycin (CLI; 0.063-8 µg/ml), chloramphenicol (CMP; 0.25-32 µg/ml), kanamycin (KAN; 0.5-2050 µg/ml), gentamicin (GEN; 0.125-512 µg/ml) and vancomycin (VAN; 0.25-32 µg/ml). Microtitre plates inoculated with 5 µl of the bacterial suspension with a McFarland standard turbidity of 1.2–1.3 were incubated at 30 °C (*L. animalis, L. brevis*, *L. fermentum, L. plantarum, L. rhamnosus, L. sakei*) or at 37 °C (*L. acidophilus, L. curvatus, L. delbrueckii, L. helveticus, L. paracasei*) over a period of 24 h under aerobic (*L. paracasei, L. plantarum*), anaerobic (*L. acidophilus, L. animalis, L. delbrueckii, L. helveticus*) or microaerophilic conditions (*L. brevis*, *L. curvatus, L. fermentum, L. rhamnosus, L. sakei*). The minimum inhibitory concentrations (MIC, µg/ml) were established. Based on the EFSA guidelines (2018), the strains were classified as susceptible or resistant. A tested strain was recorded to be resistant to an antibiotic, if its MIC value was higher than the reference cut-off value. If its MIC value was equal to or lower than the reference cut-off value, the strain was considered susceptible. A strain was considered as multidrug-resistant (MDR), if it was resistant to three or more antimicrobial groups. Each strain was tested repeatedly.

Whole genome sequencing analysis of bacterial strains

Based on the phenotypic results, four MDR strains were selected and subjected to whole genome sequencing (WGS) to determine firstly the acquired AMR genes. Total genomic DNA was extracted from the strains using cetyl trimethylammonium bromide (CTAB) according to Pavlik et al. (1999). Only high-quality DNA (A260/280 ratio of ~1.8) was used for the following analyses.

DNA libraries for whole genome sequencing were constructed using the NEBNext® Fast DNA LibraryPrep Set for Ion Torrent™ (New England Biolabs Inc., Ipswich, MA, U.S.A.) and quantified by a KAPA Library Quantification Kit (KAPA Biosystems Inc., UK). Whole-genome sequencing was performed using the Ion Torrent Proton platform (Thermo Fisher Scientific, Waltham, MA, U.S.A.) by the genome research company SEQme (Dobříš, Czech Republic). The analysis was conducted using Torrent Suite software, version 5.0.4. The reads were assembled and annotated by PATRIC, version 3.5.20 (Wattam et al. 2017). The presence of acquired antibiotic resistance genes was investigated using ResFinder, version 2.1 (Zankari et al. 2012) and CARD (Comprehensive Antibiotic Resistance Database; McArthur and Wright 2015).

Results

Phenotypic profiles of antimicrobial resistances

AMR testing of 81 lactobacilli is shown in Table 1. Only 19 of 81 tested strains (23.5%) were susceptible to all antimicrobials tested. Sixty-two strains (76.5%) were resistant to at least one antimicrobial agent. These strains and their phenotypes of resistance are listed in Table 2.

Identification of acquired antimicrobial resistance genes

Four phenotypically determined MDR strains (3 food-derived strains of *L. brevis*, 1 adjunct culture of *L. animalis*) were analysed by whole genome sequencing. However, an antibiotic resistance determinant on a mobile element was confirmed in only one strain of *L. animalis*. Other genes of resistance to antibiotics could be located on the chromosome. In this strain originating from adjunct culture, *aad*E gene was detected on the mobile element using ResFinder. Also, the presence of ANT(6) gene in this strain was detected

Table 1. The species of lactobacilli tested by microdilution method and the number of strains resistant to at least one antimicrobial agent classified based on the cut-off values according to the EFSA guideline (2018).

Origin of isolates	Species (no. of isolates)	Antimicrobials									No. ofresistant isolates
		AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CMP	
Starter/adjunct cultures	*Lactobacillus acidophilus* (1)			1	1	1					1 (100%)
	Lactobacillus animalis (1)	1	1	1	1	1			1		1 (100%)
	Lactobacillus brevis (1)		n.r.		0						0 (0%)
	Lactobacillus delbrueckii (3)				2	2					2 (66.7%)
	Lactobacillus fermentum (1)		n.r.	1	1						1 (100%)
	Lactobacillus helveticus (9)				8						8 (88.9%)
	Lactobacillus paracasei (4)		n.r.	3	3	3					3 (75%)
	Lactobacillus plantarum (2)		n.r.	2	2	n.r.					2 (100%)
	Lactobacillus rhamnosus (3)		n.r.	1	1	1					1 (33.3%)
Food isolates	*Lactobacillus brevis* (11)	2	n.r.	7	11	8		2	5		11 (100%)
	Lactobacillus curvatus (3)		n.r.	2	1	2		0			2 (66.7%)
	Lactobacillus fermentum (7)		n.r.	4	4	4					4 (57.1%)
	Lactobacillus plantarum (8)		n.r.	8	7	n.r.					8 (100%)
	Lactobacillus rhamnosus (21)		n.r.	2	12	2					12 (57.1%)
	Lactobacillus sakei (6)	2	n.r.	6	4	6					6 (100%)
Total	*Lactobacillus* spp. (81)	5 (6.2%)	1 (7.1%)[I]	38 (46.9%)	58 (71.6%)	30 (42.3%)[II]	0 (0%)	2 (2.5%)	6 (7.4%)	0 (0%)	62 (76.5%)

AMP – ampicillin, VAN – vancomycin, GEN – gentamicin, KAN – kanamycin, STR – streptomycin, ERY – erythromycin, CLI – clindamycin, TET – tetracycline, CMP – chloramphenicol, n.r. – not required; [I] – without 67 strains n.r.; [II] – without 10 strains n.r.

Table 2. Resistant lactobacilli classified based on the cut-off values according to the EFSA guideline (2018) and their phenotypes of resistance.

Species	Strains	Phenotype of resistance[I, II]
Lactobacillus acidophilus	LBC 01	*GEN-KAN-STR*
Lactobacillus animalis	LBC 02*	AMP-VAN-*GEN-KAN-STR*-TET
Lactobacillus brevis	BIO I 44*, BIO III 62*	*GEN-KAN-STR*-CLI-TET
	ML 438, BIO II 67	*GEN-KAN-STR*-TET
	BIO II 72*	AMP-*KAN-STR*-TET
	ML 177, ML 71	*GEN-KAN-STR*
	ML 165	*GEN-KAN*
	MLZ 334	*KAN-STR*
	ML 74	AMP-*KAN*
	BIO II 60	*KAN*
Lactobacillus curvatus	C 44	*GEN-KAN-STR*
	KAS 575	*GEN-STR*
Lactobacillus delbrueckii	LBC 04, LBC 06	*KAN-STR*
Lactobacillus fermentum	KAS 578, BIO II 57, BIO IV 14,	*GEN-KAN-STR*
	LBC 07	*GEN-KAN*
Lactobacillus helveticus	LBC 08, LBC 09, LBC 10, LBC 11,	*KAN*
	LBC 13, LBC 14, LBC 15, LBC 16	
Lactobacillus paracasei	LBC 17, LBC 18, LBC 19	*GEN-KAN-STR*
Lactobacillus plantarum	A 54, LBC 21, BIO I 16, C 16, C 33,	*GEN-KAN*
	D 42, KAS 521, KAS 594, LBC 22	
	KAS 526	*GEN*
Lactobacillus rhamnosus	BIO II 7, LBC 23	*GEN-KAN-STR*
	BIO III 25	*GEN-KAN*
	BIO III 39	*KAN-STR*
	BIO I 5, BIO II 5, BIO II 13, BIO II 15,	*KAN*
	BIO III 15, BIO III 21, BIO IV 2,	
	BIO IV 3, BIO IV 4,	
Lactobacillus sakei	KAS 1099, KAS 1105	AMP-*GEN-KAN-STR*
	KAS 881, KAS 885	*GEN-KAN-STR*
	KAS 462, KAS 473	*GEN-STR*

* Multidrug-resistant strain (resistant to at least three groups of antimicrobial agents); [I] AMP – ampicillin, VAN – vancomycin, GEN – gentamicin, KAN – kanamycin, STR – streptomycin, CLI – clindamycin, TET – tetracycline; [II] in italics – antibiotics belonging to the same group (aminoglycosides)

by CARD. Based on the analysis of the surrounding *aad*E sequences in the studied strain, it has been found that a gene for the transposase is present (the transposase is part of the mobile elements and allows the transfer of genes present in these elements). Therefore, a risk of horizontal transmission of the *aad*E gene exists. Based on these results, this strain may act as a reservoir of antibiotic resistance for other bacteria including the pathogenic ones.

Resistance to antibiotics of lactobacilli based on origin of strains

The least resistant strains were found in starter cultures where 40% of strains were susceptible to all tested antibiotics (Table 3). Adjunct cultures showed a higher percentage of resistance (80% of tested strains) than starter cultures (60% of tested strains). Yogurt-derived strains, which were predominantly non-starter LAB or adjunct cultures, also showed almost 40% sensitivity to antibiotics.

Table 3. Resistance to antibiotics by origin of lactobacilli isolates.

Origin of isolates (no. of isolates)	Antimicrobials									No. of resistant isolates
	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CMP	
Cheeses (10)[a]	1 (10%)	n.r.	5 (50%)	7 (70%)	5 (55.6%)[I]	0 (0%)	1 (10%)	3 (30%)	0 (0%)	7 (70%)
Yogurt (22)b	0 (0%)	n.r.	4 (18.2%)	14 (63.6%)	4 (18.2%)	0 (0%)	1 (4.6%)	1 (4.6%)	0 (0%)	14 (63.6%)
Raw milk and colostrum (7)[c]	1 (14.3%)	n.r.	4 (57.1%)	6 (85.7%)	4 (57.1%)	0 (0%)	0 (0%)	1 (14.3%)	0 (0%)	6 (85.7%)
Meat products (9)[d]	2 (22.2%)	n.r.	9 (100%)	9 (100%)	5(100%)[II]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	9 (100%)
Production environment (8)[e]	0 (0%)	n.r.	7 (87.5%)	3 (37.5%)	4 (80%)[III]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	7 (87.5%)
Starter cultures (5)[f]	0 (0%)	0 (0%)	1 (20%)	3 (60%)	3 (60%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (60%)
Adjunct cultures (20)[g]	1 (5%)	1 (11.1%)[IV]	8 (40%)	16 (80%)	5 (27.8%)[V]	0 (0%)	0 (0%)	1 (5%)	0 (0%)	16 (80%)

AMP – ampicillin, VAN – vancomycin, GEN – gentamicin, KAN – kanamycin, STR – streptomycin, ERY – erythromycin, CLI – clindamycin, TET – tetracycline, CMP – chloramphenicol, n.r. – not required; [I] – without 1 strain n.r.; [II] – without 4 strains n.r.; [III] – without 3 strains n.r.; [IV] – without 11 strains n.r.; [V] – without 2 strains n.r.; [a] – *Lactobacillus brevis* (n = 4), *L. curvatus* (n = 1), *L. fermentum* (n = 3), *L. plantarum* (n = 1), *L. rhamnosus* (n = 1); [b] – *L. brevis* (n = 1), *L. fermentum* (n = 1), *L. rhamnosus* (n = 20); [c] – *L. brevis* (n = 6), *L. fermentum* (n = 1); [d] – *L. curvatus* (n = 1), *L. plantarum* (n = 4), *L. sakei* (n = 4); [e] – *L. curvatus* (n = 1), *L. fermentum* (n = 2), *L. plantarum* (n = 3), *L. sakei* (n = 2); [f] – *L. acidophilus* (n = 1), *L. delbrueckii* (n = 3), *L. helveticus* (n = 1); [g] – *L. animalis* (n = 1), *L. brevis* (n = 1), *L. fermentum* (n = 1), *L. helveticus* (n = 8), *L. paracasei* (n = 4), *L. plantarum* (n = 2), *L. rhamnosus* (n = 3)

Table 4. Distribution of minimal inhibitory concentrations (MICs) of 81 *Lactobacillus* spp. determined by microdilution method.

Anti-	No. of isolates with the respective MIC values (µg/ml) total (starter or adjunct cultures/food isolates)															
microbials	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2050	n.r.
AMP		5[I](4[I]/1[I])	7(3/4)	18(8/10)	12(2/10)	26(4/22)	10(3/7)	3(1/2)									
VAN			1[I](1[I]/0)	7(7/0)	5(5/0)					1[II](1[II]/0)							67(11/56)
GEN		1[I](1[I]/0)			2(2/0)	3(3/0)	3(1/2)	11(8/3)	23(1/22)	16(3/13)	14(3/11)	8(3/5)					
KAN				1[I](1[I]/0)			1(1/0)	1(0/1)		3(3/0)	25(9/16)	26(4/22)	11(3/8)	10(3/7)[III]	2(1/1)[III]	1(0/1)[III]	
STR						10[I](9[I]/1[I])		12(2/10)	7(1/6)	7(3/4)	10(4/6)	11(1/10)	14[II](3[II]/11[II])				10(2/8)
ERY	27[I](15[I]/12[I])	29(6/23)	17(1/16)	8(3/5)													
CLI	33[I](9[I]/24[I])	11(6/5)	18(3/15)	9(6/3)	4(1/3)	2(0/2)	2(0/2)	2[II](0/2[II])									
TET				35[I](7[I]/28[I])	18(10/8)	7(3/4)	2(1/1)	13(4/9)	3(0/3)	3(0/3)							
CMP			3[I](0/3[I])	15(2/13)	39(15/24)	20(7/13)	4(1/3)										

AMP – ampicillin, VAN – vancomycin, GEN – gentamicin, KAN – kanamycin, STR – streptomycin, ERY – erythromycin, CLI – clindamycin, TET – tetracycline, CMP – chloramphenicol; n.r. – not required; [I] – minimal inhibitory concentration equal to or lower than the value; [II] – minimal inhibitory concentration equal to or higher than the value; [III] – high-level kanamycin resistance (HLKR) with minimum inhibitory concentration values > 500 µg/ml

The largest number of resistant strains originated from meat products. Each of these strains was phenotypically resistant to at least one antimicrobial agent. A low percentage of susceptible strains were isolated from the food processing environment (12.5%) and from raw milk and colostrum (14.3%).

Lactobacilli from our study showed high level of kanamycin resistance (HLKR) with minimum inhibitory concentration values >500 μg/ml in 13 strains (Table 4). Four strains with HLKR originated from adjunct cultures (*L. animalis*, *L. paracasei*, *L. plantarum*), four strains from meat products (*L. plantarum*), two strains from cheeses (*L. brevis*, *L. fermentum*), one strain from the product processing environment (*L. plantarum*), one strain from goat's milk yogurt (*L. brevis*) and one strain from raw cow's milk (*L. brevis*). *Lactobacillus brevis* from raw cow's milk belongs to non-starter LAB. Other strains with HLKR were either included in the starter and adjunct cultures, or isolated from fermented products and food production environments. High-level gentamicin resistance (HLGR) and high-level streptomycin resistance (HLSR) were not detected in any tested strain.

Discussion

Phenotypic profiles of antimicrobial resistance

In our study, resistance to aminoglycosides was detected most frequently. Sixty-two strains (76.5%) were resistant to at least one aminoglycoside antibiotic (streptomycin, kanamycin or gentamicin). Increased resistance to aminoglycosides has been described in lactobacilli in a number of other studies (Nawaz et al. 2011; Zhou et al. 2012; Jaimee and Halami 2016; Guo et al. 2017; Li et al. 2019). Nawaz et al. (2011) and Zhou et al. (2012) most often observed resistance to kanamycin in the tested lactobacilli. In our study, resistance to kanamycin was found in 71.6% of the strains. Curragh and Collins (1992) considered that in most cases, aminoglycoside resistance may be explained by chromosomal mutations. In spite of that, specific genes associated with aminoglycoside resistance have been described in LAB (Ouoba et al. 2008; Zhou et al. 2012). Resistance to erythromycin and tetracycline in lactobacilli is often tested. Both types of resistance are often demonstrated in association with horizontally transmissible *erm* and *tet* genes (Nawaz et al. 2011; Devirgiliis et al. 2013). Nawaz et al. (2011) confirmed the transfer of *erm*(B) and *tet*(M) genes from *L. fermentum, L. salivarius, L. plantarum* and *L. brevis* to *Enterococcus faecalis*. Resistance to erythromycin was not detected in our tested strains. Generally, most species of lactobacilli were susceptible to antimicrobials that are able to inhibit protein synthesis (clindamycin, erythromycin, chloramphenicol and tetracycline) (Katla et al. 2001; Ammor et al. 2007; Ammor et al. 2008; Guo et al. 2017; Li et al. 2019). Most studies showed susceptibility of lactobacilli to ampicillin (Nawaz et al. 2011; Georgieva et al. 2015; Guo et al. 2017; Li et al. 2019).

This is in accordance with our study where resistance to ampicillin was demonstrated in only five strains (6.2%) and resistance to clindamycin in two strains (2.5%). The resistance to chloramphenicol was not detected. Chloramphenicol resistance testing would efficiently cover for the hazard of acquired resistance to linezolid, encoded by the *cfr* gene, conferring the resistance to chloramphenicol (Toh et al. 2007; Arias et al. 2008; EFSA 2008).

It is recommended to consume foods where lactobacilli are a typical ingredient. Due to the possibility of antibiotic resistance genes transfer from lactobacilli to commensal and pathogenic bacteria in the intestine, the potential risk of spreading resistance increases. Therefore, it is necessary to monitor resistance also in bacteria that are considered to be generally recognized as safe. The isolates with MICs above the cut-off values recommended by the FEEDAP Panel for antibiotics require further investigation to determine the nature of the resistance and to make a distinction between intrinsic and acquired resistance. Presence of acquired resistance on mobile genetic elements poses

the highest risk of resistance dissemination. The FEEDAP Panel considers not using the strains of bacteria that carry the acquired resistance to antimicrobials as feed additives (EFSA 2012, 2018).

Antimicrobial resistance genes

Whole genome sequencing should be introduced/used for the detection of genes coding for or contributing to resistance to antibiotics relevant to their use in animals and human. For this purpose, a comparison against up-to-date databases should be performed (EFSA 2018). ResFinder and CARD are web servers providing a user experience way of identifying acquired antibiotic resistance genes on mobile elements in sequenced strains (Zankari et al. 2012; EFSA 2018). The low number of available genome sequences to date are limiting a precise understanding of the origin of phenotypic resistances, either induced or uninduced by the presence of antibiotics (Abriouel et al. 2015).

The *aad*E gene detected in *L. animalis* from adjunct culture encodes aminoglycoside O-nucleotidyltransferase ANT(6) which determines resistance to streptomycin (Ramirez and Tolmansky 2010). This gene has already been reported in lactobacilli (Shao et al. 2015; Dec et al. 2017). On the basis of comparison of the observed sequence with the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), *aad*E gene was confirmed, for example, in *Streptococcus suis*, *Staphylococcus aureus*, *Enterococcus faecium*, *E. faecalis* and *L. salivarius*. The gene can be found on both the chromosome and the plasmids.

High-level kanamycin resistance (HLKR) and high-level gentamicin resistance (HLGR) with minimum inhibitory concentration values > 500 μg/ml have been associated with the bifunctional *aac*(6′)Ie-*aph*(2″)Ia gene (Jaimee and Halami 2016). Although HLKR was found in one *L. animalis* strain (1024 μg/ml) and one *L. brevis* strain (2050 μg/ml), this bifunctional gene was not detected in our study. If no known AMR gene linked to the phenotype is detected, no further studies are required (EFSA 2018).

Phenotypic resistance to tetracycline was detected in six strains of two species (*L. animalis*, *L. brevis*), out of which four multiresistant strains were analysed by WGS. The *tet* genes together with *erm* genes belong to the most widespread antibiotic resistance determinants in LAB which are commonly associated with horizontal gene transfer. However, based on the analysis by CARD and ResFinder, any known acquired gene determinants for tetracycline resistance were not identified. This discrepancy between phenotypic and molecular analysis may be explained by the results of Egervärn et al. (2009), who observed an association of the occurrence of *tet* genes in *L. reuteri* with a certain level of MIC, particularly *tet*(W) determinants were detected only in strains with MIC over 64 μg/ml. In the current study, all strains had MIC under 64 μg/ml. The absence of the *tet* gene in strains with tetracycline resistance may also be caused by other mechanisms such as mutations (Chopra and Roberts 2001). The mechanisms and genetic basis of resistance to certain antimicrobials are still largely unknown in LAB (Li et al. 2019).

The failure of AMR gene transfer in complex gastrointestinal environments indicates the interference from indigenous gut microbiota of the hosts (Feld et al. 2008; Egervärn et al. 2010; Ma et al. 2017). However, it is still not clear whether or not the foodborne lactobacilli worsen the problem of AMR of bacteria in the gastrointestinal tract of human (Ma et al. 2017). Therefore, the public health safety of lactobacilli species should be considered, even though they are commonly present in the indigenous microbiota (the oral cavity, the female genital tract, and the gastrointestinal tract).

Resistance to antibiotics of lactobacilli based on origin of strains

Although resistance to tetracycline and erythromycin in *L. plantarum* from cheeses and yogurts has already been described (Nawaz et al. 2011; Zago et al. 2011), our strains of *L. plantarum* from these commodities were sensitive to both antibiotics.

In the study of Zonenschain et al. (2009), 75% of lactobacilli (*L. curvatus*, *L. plantarum* and *L. sakei*) isolated from Italian fermented dry sausages were phenotypically resistant to tetracycline, 50% to erythromycin, and 45% were resistant to both antibiotics. In our case, any resistance to erythromycin and tetracycline was determined in strains of *L. curvatus*, *L. plantarum,* and *L. sakei* isolated from fermented salami or other meat products.

In animal husbandry, inappropriate use of aminoglycosides has led to the selection of high-level aminoglycoside resistance (HLAR) in LAB (Jaimee and Halami 2016). One hundred percentage of our strains isolated from meat products were resistant to kanamycin, gentamicin and streptomycin. Jaimee and Halami (2016) assessed the presence of aminoglycoside resistant LAB in meat products and farm animals. In their study, lactobacilli isolated from meat products were resistant to kanamycin (19%) and gentamicin (13%). Thirty-four percent of lactobacilli showed MIC values of ≥ 128 μg/ml for streptomycin. Meat products such as non-fermented sausages were contaminated with aminoglycoside-resistant lactobacilli. In our study, even 71% of samples of meat products (cooked ham, hot smoked dry sausages and fermented salami) and food production environment contained lactobacilli with MIC of ≥ 128 μg/ml for streptomycin.

In conclusion, the results of this study show that starter or adjunct cultures and lactobacilli naturally present in food are resistant to antimicrobials and may act as reservoirs of AMR genes. Due to food consumption, the safety of these bacteria is of high importance as their resistance to antimicrobials can be one of the many potential risks to public health. When resistance to antibiotics is demonstrated in a bacterial strain by the phenotypic method, it is desirable to check the molecular basis of such resistance to determine whether it is intrinsic or acquired. In our study, a horizontally transmissible gene (*aad*E) was confirmed in *L. animalis* from an adjunct culture used for fermented dairy products. Horizontal transfer of resistant genes should be tested not only in strains used as starter cultures but also in adjunct cultures. Although *Lactobacillus* species are not considered as pathogenic bacteria, they occur frequently and in large numbers in food, especially fermented one. This fact may negatively contribute in the spread of genes encoding resistance to antibiotics through the human food chain.

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