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ORIGINAL ARTICLE

Survey of antibiotic resistance traits in strains of *Lactobacillus casei/paracasei/rhamnosus*

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Abstract In this study the occurrence of antibiotic resistance (AR) traits was evaluated in 184 lactic acid bacteria (LAB) strains belonging to the species *Lactobacillus casei/paracasei/rhamnosus* and isolated from various sources. The strains were tested for resistance at the cut-off values fixed by the European Food Safety Authority (EFSA) for the antibiotics ampicillin, chloramphenicol, clindamycin, gentamicin, erythromycin, kanamycin, streptomycin and tetracycline, in order to distinguish resistant from susceptible strains. The strains that were not inhibited at the cut-off concentrations for one or more antibiotics, namely 27 *L. paracasei* and 50 *L. rhamnosus* strains, were further examined for minimum inhibitory concentration (MIC) and presence of acquired genes encoding resistance to the specific antibiotics by PCR assays. A minority of these strains exhibited MIC values that indicated a potentially acquired AR for ampicillin (one *L. paracasei* strain), clindamycin (two *L. paracasei* and one *L. rhamnosus* strains), gentamicin (two *L. rhamnosus* strains) and tetracycline (two *L. paracasei* strains and one *L. rhamnosus* strain); however, the genetic determinants responsible for resistance could not be identified. This study highlighted a low frequency of AR phenotypes and the absence of the most frequently acquired AR genes in the *L. casei/paracasei/rhamnosus* strains examined, thus evidencing a low risk related to AR dissemination by these bacteria.

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Introduction

Lactobacillus casei/paracasei/rhamnosus are closely related species that naturally occur in different fermented products, usually dairy products, wine, and sourdough (Rojo-Bezares et al. 2006; Reale et al. 2011), feed products, and in the intestines of humans and animals. These bacteria reach high numbers and persist in cheeses, where they represent a major component of the nonstarter microbiota, even in the advanced stage of ripening (Coppola et al. 2003; Rossi et al. 2012). For their technological characteristics, strains of these species are very often used as adjunct cultures in many foods, mainly dairy products (Briggiler-Marcó et al. 2007; Gupta et al. 2013).

Given the ability of some strains of these species to colonize the human gut (Jacobsen et al. 1999; Ya et al. 2008), the possible presence of acquired antibiotic resistance (AR) genes must be investigated to prevent the risk of their horizontal spread to bacterial pathogens in the intestinal ecosystem. These species have a Qualified Presumption of Safety (QPS) status, according to the European Food Safety Authority (EFSA), but their intentional addition to food and feed should be carried out after verification that single strains do not harbour acquired genes conferring resistance to clinically relevant antibiotics (EFSA 2011; 2013).

Most studies regarding the distribution of AR genes in *L. paracasei* have analyzed the presence of genes for erythromycin resistance, primarily *erm* genes encoding ribosomal methyltransferases that confer a macrolide-lincosamide-streptogramin (MLS) resistance phenotype (Sutcliffe et al.

1996), and tetracycline resistance conferred by ribosomal protection proteins or membrane efflux pumps (Ng et al. 2001). The *erm*B, *tet*M, and *tet*W genes were found in some strains of *L. paracasei* species (Cataloluk and Gogebakan 2004; Huys et al. 2008; Zonenschain et al. 2009; Comunian et al. 2010; Ishihara et al. 2013). Toomey et al. (2010) reported also the detection of the macrolide resistance gene *mrs*A/B in *L. paracasei* isolates from pork meat.

In the species *L. rhamnosus*, Cataloluk and Gogebakan (2004) identified several strains harbouring the *erm*B and *tet*M genes, while other studies reported also the presence of *tet*W in a few strains (Zonenschain et al. 2009; Ishihara et al. 2013) or the absence of the AR genes tested (Korhonen et al. 2010).

The presence of genes encoding resistance to antibiotic classes other than macrolides and tetracyclines was never demonstrated in *L. casei/paracasei/rhamnosus,* and no data are available on the occurrence of acquired AR genes in the species *L. casei*.

The aim of this study was to evaluate the occurrence of antibiotic resistance (AR) in bacteria belonging to the species considered and the presence of AR genes commonly associated with mobile genetic elements. For this purpose, the susceptibility to the antibiotics recommended for testing by the EFSA, namely ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, and tetracycline (EFSA 2012), was determined for 184 lactic acid bacteria strains belonging to the species *Lactobacillus casei/paracasei/rhamnosus* isolated from various sources. Most strains, whose complete list and provenance was reported by Zotta et al. (2014) and Iacumin et al. (2015), were isolated from food products and samples of human faeces collected in Italy and have been previously characterized for their tolerance to technological and in vivo stresses (Reale et al. 2015). Moreover, the presence of AR genes most frequently found and/or previously detected in lactobacilli (Tannock et al. 1994; Villedieu et al. 2003; Cataloluk and Gogebakan 2004; Rojo-Bezares et al. 2006; Hummel et al. 2007; Huys et al. 2008; Rosander et al. 2008; Ouoba et al. 2008; Egervärn et al. 2009; Zonenschain et al. 2009; Toomey et al. 2010; Ishihara et al. 2013) was analyzed by PCR assays for the strains that were resistant to the antibiotic cut-off levels.

Materials and methods

Bacterial strains and culture conditions

Bacteria analyzed in this study are the same as those reported in the study of Zotta et al. (2014), which comprised the reference strains *L. casei* LMG 6904, DSMZ 20178, *L. paracasei* LMG 9191, 9192, 9438, 5622, 4905, 11459, 23511 and *L. rhamnosus* DSMZ 20021 and GG. All the newly isolated strains were identified to the species level using a polyphasic approach (species specific-PCR, multiplex-PCR, High Resolution Melting Analysis) (Iacumin et al. 2015).

All strains were maintained as frozen (−80 °C) stocks in 11 % (*w*/*v*) reconstituted skim milk (RSM) (Oxoid, Milan, Italy) containing 0.1 % (*w*/*v*) ascorbic acid in the Culture Collection of the Agriculture Environment and Food Department, Università degli Studi del Molise, Campobasso, Italy. Strains were propagated in MRS broth (Oxoid, Milan, Italy) for 24 h at 37 °C.

Phenotypic and genetic AR testing

Susceptibility to antibiotics was determined in lactic acid bacteria susceptibility test medium (LSM), composed of 90 % MRS broth (Oxoid) and 10 % Iso-Sensitest broth (Oxoid) (Klare et al. 2005). The following eight antimicrobial agents were used: ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline. All the antibiotic powders were obtained from Sigma (Milan, Italy) and used as aqueous solutions or as a solution in methanol for tetracycline, and in ethanol for erythromycin and clindamycin. Antibiotics were used, as suggested by the EFSA (2012), at the cut-off levels for *L. casei/paracasei* (1 μg/ml for erythromycin and clindamycin, 4 μg/ml for ampicillin, tetracycline, and chloramphenicol, 32 μg/ml for gentamicin, and 64 μg/ml for kanamycin and streptomycin) and for *L. rhamnosus* (4 μg/ml for ampicillin and chloramphenicol, 16 μg/ml for gentamicin, 64 μg/ml for kanamycin, 32 μg/ml for streptomycin, 1 μg/ml for erythromycin and clindamycin, and 8 μg/ml for tetracycline).

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of all antibiotics was determined with the broth microdilution method in LSM broth containing the antibiotics at different concentrations in the range 0.125–256 μg/ml. Antibiotics were added as described above. The inoculum was adjusted to a turbidity equivalent to 0.5 McFarland standard ($\approx 5 \times 10^5$ CFU/ml). The inoculum was derived from a broth culture which was incubated for 16 h at 37 °C in anaerobic conditions. The MIC was defined as the lowest antibiotic concentration giving a complete inhibition of visible growth in comparison to an antibiotic-free control well. Resistance was assessed by reference to the respective cut-off values.

Molecular methods

Genomic DNA of the strains exhibiting phenotypic resistance was extracted as described by Rossi et al. (2006), but with incubation times with lysozyme and proteinase K both increased to 1 h.

Plasmid extraction was carried out from 4 ml of fresh cultures in MRS broth (Oxoid) using the Qiagen Plasmid Mini Kit according to instructions, and preceded by incubation for 1 h with 10 mg/ml lysozyme at 37 °C in P1 buffer (Qiagen Srl., Milan, Italy). DNA extracts were run on 0.8 % (w/v) agarose gels.

In this study PCR tests for AR genes were carried out with the primers reported in Table 1 and according to the respective references. Positive controls for the PCR reactions were constructed in this study using extra-long primers constituting a 3′ moiety identical to the primers used to amplify a 295 bp region of the *erm*C gene, and a 5′ moiety identical to each primer pair used for the detection of all the other AR genes considered. These primer pairs were used for the amplification of positive control PCR fragments from *L. plantarum* F87, a strain belonging to the Culture Collection of the Agriculture Environment and Food Department, University of Molise, and carrying the *erm*C gene. The positive-control PCR fragments were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co, Düren, Germany) according to the instructions, and used in the respective AR gene PCR tests. The positive-control fragments were added at a final concentration of about 0.1 ng/μl in the amplification reactions.

PCR products were separated at 120 V on 1.5 % (w/v) agarose gels stained with 0.5 μg/ml ethidium bromide in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA). All AR gene PCR tests were run in three repetitions.

Sequencing

The PCR fragments from the AR gene-specific assays were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel), adjusted to the required concentration, and sent together with the appropriate primers to Beckman Coulter Genomics GB (UK). Sequence identification was done by BLAST alignment with the GeneBank database.

Results

Phenotypic and genetic assessment of resistance

The initial screening of all the 184 strains at the antibiotic cut-off levels allowed us to select 27 *L. paracasei* and 50 *L. rhamnosus* strains for MIC determination (Table 2). MIC

Table 1 AR gene-targeted primers used in this study and respective references

Target gene	Sequence (5′→3′)	Amplicon size (bp)	Reference
*aad*A	ATCCTTCGGCGCGATTTTG GCAGCGCAATGACATTCTTG	282	Ouoba et al. 2008
*aad*E	ATGGAATTATTCCCACCTGA TCAAAACCCCTATTAAAGCC	385	Ouoba et al. 2008
aac(6′)*aph*(2″)	CCAAGAGCAATAAGGGCATA CACTATCATAACCACTACCG	219	Rojo-Bezares et al. 2006
aph(3″)-IIIa	GCCGATGTGGATTGCGAAAA GCTTGATCCCCAGTAAGTCA	291	Rojo-Bezares et al. 2006
bla	CATARTTCCGATAATASMGCC CGTSTTTAACTAAGTATSGY	297	Hummel et al. 2007
*cat**	TTAGGTTATTGGGATAAGTTA GCATGRTAACCATCACAWAC	300	Hummel et al. 2007
*erm*A	TCAAAGCCTGTCGGAATTGG AAGCGGTAAACCCCTCTGAG	441	Jensen et al. 1999
*erm*B	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	425	Jensen et al. 1999
*erm*C	ATCTTTGAAATCGGCTCAGG CAAACCCGTATTCCACGATT	295	Jensen et al. 1999
*erm*T	TATTATTGAGATTGGTTCAGGG GGATGAAAGTATTCTCTAGGGATTT	395	Tannock et al. 1994
*lnu*A	TGGAAAACAACAAAGAGAACACA CCAGAATGAAAAAGAAGTTGAGC	317	Rosander et al. 2008
*mef*A/E	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	348	Sutcliffe et al. 1996
*mrs*A/B	GCAAATGGTGTAGGTAAGACAACT ATCATGTGATGTAAACAAAAT	399	Sutcliffe et al. 1996
*str*A	CTTGGTGATAACGGCAATTC CCAATCGCAGATAGAAGGC	547	Ouoba et al. 2008
*str*B	ATCGTCAAGGGATTGAAACC GGATCGTAGAACATATTGGC	508	Ouoba et al. 2008
tetK	TCGATAGGAACAGCAGTA CAGCAGATCCTACTCCTT	169	Ng et al. 2001
tetL	TCGTTAGCGTGCTGTCATTC GTATCCCACCAATGTAGCCG	267	Ng et al. 2001
tetM	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	406	Ng et al. 2001
tetO	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTC	515	Ng et al. 2001
tetS	CATAGACAAGCCGTTGACC ATGTTTTTGGAACGCCAGAG	667	Ng et al. 2001
tetW	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	Aminov et al. 2001

*It was verified by BLAST analysis that the primers targeted on the chloramphenicol resistance encoding gene *cat* also anneal on the *cat*-TC gene variant (Lin et al. 1996)

Table 2 Strains resistant to the antibiotic cut-off values and respective isolation source

Antibiotic	Species	
	L. paracasei	*L. rhamnosus*
Ampicillin	Dairy: LMG9192, S1, W11, HSG09, DBPZ0318, M354, R61, F17, N24, P71	Dairy: CF1350, D44
	Human: LMG9438, LMG24101	Human: N25, N95, N178, N715
	Sourdough: Q4, DBPZ572	Coffee: DIAL 40
Chloramphenicol	Dairy: R61, F17, N24, SP57	Dairy: CF1350, D44, PRA331, O14
		Unknown: DBPZ524
Clindamycin	Dairy: M308, M354	Dairy: P1E4
	Wine: B350	
Erythromycin	Dairy: LMG9192,	Dairy: D44, M307, L9, P1E4, LACcas13,
	M308, R61, F17, H12,	Human: N21, N22, N26, N201, N131, N132, N173
	TMW 1.1259, P71 Wine: LMG11961, LMG11963	Coffee: DIAL 40
	Fermented beverage:	
	LACcas29	
Gentamicin	Dairy: LMG9192, H12, SP57, P71, TH1229	Dairy: FSG01, M307, O14, PRA331, CI4362, P1E4, PRA204, PRA232, CF377, DBPZ430, DBPZ446, DBPZ449
	Wine: B169, B195 Fermented beverages:	Human: GG, DBTC4, N171, N94, N83, N201, N202, N209, N2011, N2012, N2013, N22, N26, N812, N172, N173, N176, HA111
	LACcas29	
Kanamycin	Dairy: LMG9192, R61, F17, H12, SP57, P71, TH1229,	Dairy: DBPZ430, DBPZ446, CF377, CI4362, D44, H25, PRA204, LACcas13
	Wine: LMG11961, LMG11963, B169, B195	Human: N95, N132, N21, N22, N812
	Fermented meat: LMG12164	Fermented meat: CTC1676, 2220
	Fermented beverage: LACcas29 Sourdough: DBPZ572	Coffee: DIAL 40
Streptomycin		Dairy: O14, CF377, L47, P1E4, P2P3,
		Human: N131, N173, N176, N715, DSMZ 20021, HA111
		Wine: B172
		Fermented meat: 2220
Tetracycline	Dairy: LMG9192, R61, P71, F17, N24, TMW 1.1259, M359	Dairy: P1E4
	Wine: LMG11963, B169	Human: N132, N22
	Fermented meat: LMG12164	Wine: B172

values evidenced that a few strains isolated from cheese (*L. paracasei* M308, M354, M359), wine (*L. paracasei* B169 and *L. rhamnosus* B172), milk (*L. rhamnosus* CF377), and sourdough (*L. paracasei* DBPZ572) were proven to be actually resistant to ampicillin, clindamycin, gentamicin and tetracycline above the cut-off level; the MIC values for these strains were low to moderate, being at most eightfold the cut-off level for *L. paracasei* DBPZ572 resistant to ampicillin (Table 3).

The PCR-based screening for the presence of acquired AR genes was carried out for all the strains that were resistant to the cut-off level for any of the antibiotics tested.

Amplification products of apparently expected size were obtained in the PCR tests targeted on genes *tet*S, *aph*(3″)-IIIa for all the *L. paracasei* strains tested, but these were proven to be aspecific products derived from gene loci LSEI_0973 and LSEI_0981, respectively, referring to the genome of strain *L. paracasei* ATCC 334 (GenBank Acc. no. CP000423). For the *cat* targeted PCR assay, two *L. rhamnosus* strains gave amplicons of the expected size, but these were also aspecific PCR products originating from gene LGG_00127, referring to the genome of *L. rhamnosus* GG (GenBank Acc. no. FM179322). No amplification products were obtained with the other primer pairs.

The acquisition of resistance determinants to antimicrobials of clinical importance could not be demonstrated in the bacteria examined in this study, and the genetic basis for the phenotypic resistance observed in some strains remains to be investigated. Moreover, the strains with AR phenotype either did not harbour plasmids or possessed large plasmids that in

Table 3 MIC (μg/ml) for the *L. paracasei* and *L. rhamnosus* strains with a resistance phenotype

Antibiotic	MIC (μg/ml) of AR strains	
	L. paracasei	*L. rhamnosus*
Ampicillin	DBPZ572 (32)	
Clindamycin	M308 (4); M354 (4)	P1E4 (2)
Gentamicin		N2011 (32); CF377 (64)
Tetracycline	M359 (8); B169 (16)	B172 (32)

L. casei/*paracasei*/*rhamnosus* are not associated with AR traits (Zhou et al. 2005; Douillard et al. 2013), thus the genetic determinants of resistance are most probably chromosomally encoded.

Discussion

In this study, the distribution of AR phenotypes and AR genes commonly associated with mobile genetic elements was examined in strains of *L. casei*/*paracasei*/*rhamnosus* to evaluate if these species might contribute to the spread of AR traits. Therefore, the analysis of many isolates from close geographical sites was avoided in order to obtain data not biased by the prevalence of some AR genes in specific locations. Indeed, previous studies reported that in environments where *erm*B and *mrs*A/B were harboured by different species of lactic acid bacteria, *L. paracasei* strains had also acquired those genes (Toomey et al. 2010). Similarly, the same AR genes were found in genotypically different strains of *L. paracasei* isolates from the same food product type and close geographical origin in Italy (Zonenschain et al. 2009; Comunian et al. 2010). The identity of the mobile genetic element carrying the *tet*M gene was also defined in one study on *L. paracasei* strains isolated from milk and whey cultures for the manufacture of Mozzarella di Bufala Campana. This was found, as in other instances (Clementi and Aquilanti 2011), to be a Tn*916* family transposon proven to be transferable with low frequency (Devirgiliis et al. 2009).

On the contrary, the uneven distribution of *tet*M and *tet*W in *L. casei* strains was reported by Ishihara et al. (2013), who found those genes in isolates from imported cheeses, but not in cheeses produced in Japan. In addition, resistance to erythromycin and clindamycin was recently reported for an *L. rhamnosus* strain isolated from a clinical setting that had caused a bloodstream infection (Bartalesi et al. 2012).

Differently than found in previous investigations regarding strains also isolated in Italy, AR genetic determinants previously described in *L. paracasei*/*rhamnosus*, i.e. *tet*M, *tet*W and *erm*B (Cataloluk and Gogebakan 2004; Huys et al. 2008; Zonenschain et al. 2009; Comunian et al. 2010; Ishihara et al. 2013), were not detected in this study.

In contrast with previous reports carried out on different strains, in this study, resistance to erythromycin was not observed; moreover, the genetic background of resistance to tetracycline could not be elucidated.

Moderate resistance to ampicillin, rare in lactobacilli, not conferred by the *bla* gene (Hummel et al. 2007), was found in a strain isolated from sourdough. Moreover, new findings obtained in this study were resistance to clindamycin not associated with an MLS phenotype and not conferred by the gene *lnu*A (Rosander et al. 2008) in *L. paracasei* strains isolated from cheese and resistance to gentamicin not due to the *aac*(6′)*aph*(2″) determinant in *L. rhamnosus* strains isolated from cheese and human faeces. Indeed, gentamicin resistance was previously reported for *L. paracasei,* but not for *L. rhamnosus* (Dušková and Karpíšková 2013). However, the gentamicin-resistant strains were identified on the basis of the cut-off value fixed by the EFSA (EFSA 2012), while these strains could be considered not truly resistant when taking into account the microbiological breakpoint value defined by Danielsen and Wind (2003) of 128 μg/ml. Cases in which AR phenotypes in these species were not corroborated by a genetic background were reported by Delgado et al. (2005), Klare et al. (2007), Korhonen et al. (2010) and Bartolesi et al. (2012), and regarded high resistance to streptomycin in *L. paracasei*, to oxytetracycline, clindamycin, and erythromycin, and multi-resistance to streptomycin, erythromycin, and clindamycin, to ampicillin and tetracycline and to streptomycin and tetracycline in *L. rhamnosus*.

Results obtained in this study indicated a low frequency of antibiotic-resistant phenotypes in *L. casei* and *L. rhamnosus* and the absence of the transferable AR genes found in other instances in Italian food products (Garofalo et al. 2007; Aquilanti et al. 2007; Flórez et al. 2014) that can be a consequence of the tendency of *L. casei*/*paracasei*/*rhamnosus* to lose easily AR genes in the absence of selective pressure.

Further investigations could be carried out by PCR tests that target additional AR genes possibly responsible for the AR phenotypes observed and occurring in the ecological niches from which the resistant strains were isolated, namely dairy products, human faeces, wine, and sourdough. The identification of the mobile genetic elements involved could provide clues for the identification of the environments where the possible horizontal gene transfer events took place and that can pose a risk for AR dissemination.

Some of the PCR tests used in this study, and previously adopted to analyze the presence of AR genes in lactobacilli, originated non-specific amplification products from *L. paracasei* and *L. rhamnosus,* and therefore, should not be used in future investigations on these species. This finding suggests that the specificity of the AR gene-targeted PCR

assays available should be assessed in many different bacterial taxonomic groups.

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