

Antibiotic Resistance of Lactobacilli Isolated from Two Italian Hard Cheeses

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ABSTRACT

One hundred forty-one lactobacilli strains isolated from natural whey starter cultures and ripened Grana Padano and Parmigiano Reggiano cheeses were tested for their susceptibility to 13 antibiotics, in particular, penicillin G, ampicillin, amoxicillin, oxacillin, cephalotin, cefuroxime, vancomycin, gentamicin, tetracycline, erythromycin, clindamycin, co-trimoxazole, and nitrofurantoin. The strains belonged to the species *Lactobacillus helveticus*, *L. delbrueckii* subsp. *lactis*, *L. rhamnosus*, and *L. casei*. The strains of the first two species were isolated from whey starter cultures, and the strains of the last two species were from the ripened cheeses. Significant differences among the strains in their antibiotic resistance were found in relation to the type of cheese and, especially, the strains from Parmigiano Reggiano were more resistant to gentamicin and penicillin G. The strains isolated in the ripened cheese were generally more resistant than those isolated from natural whey starter cultures; in particular, significant differences regarding oxacillin, vancomycin, cephalotin, and co-trimoxazole were observed. Finally, no significant difference in relation to the type of cheese was found among the thermophilic lactobacilli isolated from whey cultures, while the facultatively heterofermentative lactobacilli isolated from Parmigiano Reggiano showed higher resistance toward gentamicin and penicillin G than did the same species isolated from Grana Padano.

Since their introduction more than 50 years ago, the use of antibiotics caused the selection of resistant strains among the microbial species against which they were used. For a long time, the interest of the researchers in studying such resistance onset was limited to the human pathogenic species (37).

However, in the last decades the attention has been focused also on commensal, usually nonpathogenic, bacteria for two fundamental reasons: (i) the dramatic increase of antibiotic use in livestock farming to control the diseases deriving from intensive animal husbandry and as growth promoters (2, 48); (ii) the knowledge acquired in the field of gene transmission in bacteria, which pinpoints the wide possibilities of exchanges of genetic material encoding antibiotic resistance among diverse bacterial species (24, 49). The genetic nature of antibiotic resistance can be intrinsic (innate) or acquired. Intrinsic resistance is chromosomally controlled, is related to the general physiology or anatomy of a microorganism, and stems from preexisting mechanisms or properties. It is not horizontally transferable. Acquired resistance results from genetic changes that occur through mutation of the antimicrobial's target site within the bacterium or acquisition of genetic material encoding resistance via mobile genetic elements (plasmids or transposons) (34, 37). These aspects lead to consider also

the food chain as a fundamental reservoir for the transmission of antibiotic resistance and as a source of contamination (51). This effect has been deeply investigated for foodborne pathogenic bacteria, but recently the attention has been focused also on other bacteria. Among these, lactic acid bacteria (LAB) are particularly important because of their presence in several fermented foods. In this framework, the case of enterococci is the most studied; the members of this microbial group, in fact, can easily exchange mobile genetic material with bacteria not even belonging to their genus and can accumulate resistance to several antimicrobials under environmental pressure (27, 37). Moreover, under certain conditions, they can act as opportunistic pathogens and their control with antibiotic therapy can be extremely difficult (15, 20, 46). The spread of vancomycin-resistant enterococci is the most representative example from this point of view (18, 19). In several fermented foods the dominant bacteria often belong to the genus *Lactobacillus*, which can both derive from starter cultures and be selected by the conditions imposed during ripening. Moreover, lactobacilli are often a relevant microbial component of probiotic food and can interact with gut microflora (2). In spite of the large consumption of live lactobacilli, the presence of acquired antibiotic resistance is not crucial for their classification as generally recognized as safe by the U.S. Food and Drug Administration, while more stringent is the European Union position, which asks for the presence of transmissible antibiotic

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resistance markers; in fact, there is no barrier between pathogenic (e.g., streptococci), potentially pathogenic (e.g., enterococci), and commensal (e.g., lactobacilli and lactococci) LAB regarding acquired antibiotic resistance, and identical genes responsible for resistance are found among these organisms (37).

A more thorough investigation of the antibiotic resistance profiles of lactobacilli is motivated by three fundamental reasons. First is the possibility of exchange of resistance factors with other microorganisms, especially those belonging to the gut microflora, even if the propensity of lactobacilli to exchange mobile genetic elements is limited compared to that of other LAB such as enterococci (37, 47). Second, lactobacilli have been reported as the etiological agents in some cases of endocarditis that can be controlled only by antibiotic therapy (46). Finally, the optimization of the use of probiotic lactobacilli in cases of gastrointestinal disorders requires the knowledge of their antibiotic resistance to reinforce the concomitant antibiotic therapy (45). In this framework, while an increasing number of studies on the antibiotic resistance of probiotic lactobacilli used for fermented milk production have been published (13, 15, 35, 41, 47, 53), information regarding lactobacilli involved in cheese production and ripening is limited. Cheese, especially if produced from raw milk by using natural starter cultures, is an environment in which lactobacilli derive mainly from milk. The antibiotic resistance of these microorganisms can be affected by antibiotic treatment of the milk-producing animals. Antibiotic resistance has been tested in lactobacilli isolated from Armada (30), Cabrales (17), and Karst (6) cheeses and in *Lactobacillus rhamnosus* from Parmigiano Reggiano cheese (12).

The objective of this work was to compare the antibiotic resistance of lactobacilli isolated from natural whey starter cultures used to produce Grana Padano and Parmigiano Reggiano and from the ripened cheeses. Both of these cheeses are Protected Designation of Origin products and are obtained from raw milk with the addition of natural whey starter cultures. Natural whey starters are artisanal starter cultures obtained from whey from the previous day's cheesemaking and incubated at a gradually decreasing temperature, and they are used for the production of both of these Protected Designation of Origin cheeses (14, 44). Grana Padano and Parmigiano Reggiano are characterized by similar cheesemaking and ripening conditions, while the milk is produced in farms located in different geographical areas and with different dimensions and feeding and environmental conditions (23). In spite of similar technological conditions during cheesemaking and ripening, differences have been observed in the phenotypic and genotypic characteristics of natural whey starter microbial populations (21, 23, 25). The investigation of the differences among the antibiotic resistance profiles of 141 lactobacilli has been carried out in relation to the step of production (whey starter cultures or after ripening) and the type of cheese (Grana Padano or Parmigiano Reggiano) from which the strains were isolated.

MATERIALS AND METHODS

Strains. One hundred forty-one strains of lactobacilli isolated from Grana Padano and Parmigiano Reggiano cheeses between 1980 and 2008 were tested for their antibiotic resistance. Fifty-six lactobacilli were isolated from Grana Padano, and 85 strains were isolated from Parmigiano Reggiano. Forty-five strains were isolated from natural whey starter cultures sampled in 10 dairies producing Grana Padano (20 strains) and 10 dairies producing Parmigiano Reggiano (25 strains). The former were located in Lombardia Region, the latter in Emilia Romagna Region (Italy). The remaining 96 lactobacilli were isolated from Grana Padano (36 strains) and Parmigiano Reggiano 12-month-ripened cheeses (60 strains). These strains were isolated by sampling 25 cheese wheels (10 of Grana Padano and 15 of Parmigiano Reggiano). The strains were isolated onto de Man Rogosa Sharpe (MRS) agar (Oxoid, Basingstoke, UK) and incubated at 42°C for 48 h for the whey starter culture samples and cheese agar medium (39) and incubated at 37°C for 72 h for the ripened cheese samples. The isolates were then purified using MRS agar. After purification, the strains were maintained at -80°C in MRS broth (Oxoid) supplemented with 30% glycerol (Merck, Rome, Italy).

Identification by 16S rRNA gene sequencing. Genomic DNA of the isolated strains was extracted from overnight cultures (anaerobic incubation at 42°C) by a Chelex 100 (Sigma-Aldrich Co., St. Louis, MO), using the procedure described by Giraffa et al. (26). The presence of DNA was verified on 1.5% (wt/vol) agarose ethidium bromide gel, and DNA concentration and purity were determined spectrophotometrically at 260 and 280 nm (Jasco V-530, Tokyo, Japan). The phylogenetic positions of the strains at species level by subunit ribosomal 16S-rRNA gene sequencing were studied. DNA amplification and sequencing were performed as previously described by Giraffa et al. (26), and each sequence obtained was confirmed manually and searched for sequence homology, using Basic Local Alignment Search Tool (BLAST) (4).

Antibiotics. Antibiotic-susceptible disks (Oxoid) were stored in sealed containers with a desiccant at -18°C and were maintained at 4°C the day of the trials. The antibiotics tested were amoxicillin (10 µg), ampicillin (10 µg), cefuroxime (30 µg), cephalotin (30 µg), clindamycin (2 µg), co-trimoxazole (25 µg), erythromycin (15 µg), gentamicin (10 µg), nitrofurantoin (300 µg), oxacillin (1 µg), penicillin G (10 IU), tetracycline (30 µg), and vancomycin (5 µg).

In vitro antimicrobial susceptibility testing. Antibiotic susceptibility was determined by disk diffusion using a modification of the overlay diffusion method of the National Committee for Clinical Laboratory Standards (now the Clinical and Laboratory Standards Institute) (38) as described by Charteris et al. (7). The strains were grown twice in MRS broth for 24 h at 37°C. Then, the concentration of the inoculum was standardized as described by Huys et al. (33) at an optical density at 590 nm of 0.1 ± 0.02 (Shimadzu UV-1601 UV-Visible Spectrophotometer). Fifty milliliters of semisolid MRS agar that was maintained at 45°C was inoculated with 1.25 ml of the standardized inoculum. Eight milliliters of the inoculated MRS was poured on top of 15-ml MRS petri plates containing the disks (four disks for each plate). Plates were incubated anaerobically at 37°C for 48 h. Inhibition zone diameters were measured using a sliding caliper, and results were interpreted as resistant (R), intermediate (I), or sensitive (S) in accordance with the method of Charteris et al. (7). The interpretation of R, I, or S for oxacillin was based on the data

reported by the National Committee for Clinical Laboratory Standards (38) for staphylococci, as no other data of inhibition zone diameters for *Lactobacillus* were found in the literature. All the lactobacillus strains that were R or I against at least five of the antibiotics tested were considered to be multiresistant. The precision and accuracy of the procedure were monitored using *Enterococcus faecalis* ATCC 29212 (and *Staphylococcus aureus* ATCC 25923 for oxacillin).

Data analysis. Chi-square analysis was performed using statistical analysis software Statistica (StatSoft Inc., Tulsa, OK). Differences were considered significant at *P* values of <0.05.

RESULTS AND DISCUSSION

A total of 141 lactobacilli strains isolated from natural whey starter cultures and during ripening of Grana Padano and Parmigiano Reggiano cheeses were tested for their resistance to 13 antibiotics.

The analysis of the 16S rRNA gene of the isolates allowed the identification of all the isolates collected from natural whey starters and ripened cheeses. Eighty-five strains, all isolated from ripened cheeses, belonged to the species *L. rhamnosus* (28 and 57 strains from Grana Padano and Parmigiano Reggiano, respectively). Thirty-five strains isolated from natural whey starter cultures (13 from Grana Padano and 22 from Parmigiano Reggiano) belonged to the species *Lactobacillus helveticus*. Among the remaining 21 lactobacilli, 11 strains were isolated from ripened cheeses and classified as *L. casei* (8 strains from Grana Padano and 3 strains from Parmigiano Reggiano). Finally, 10 strains isolated from natural whey starter cultures were identified as *L. delbrueckii* subsp. *lactis* (7 strains from Grana Padano and 3 strains from Parmigiano Reggiano).

L. helveticus and *L. delbrueckii* subsp. *lactis* are starter lactic acid bacteria (SLAB) and are the dominant species in natural whey starter cultures of Grana Padano and Parmigiano Reggiano (22, 44). *L. rhamnosus* and *L. casei* are the lactobacilli mainly isolated from ripened Grana Padano and Parmigiano Reggiano. They derive from raw milk (11, 14) and can be defined as nonstarter lactic acid bacteria (NSLAB) according to Cogan et al. (10).

Table 1 shows the number of strains for each species observed to be S, R, and when applicable, I to the antibiotic tested. In addition the total percentages of S, R, and I are reported. All the isolates, regardless of their origin, were susceptible to ampicillin. Susceptibility was widespread also against amoxicillin (98%), tetracycline (98%), erythromycin (97%), nitrofurantoin (97%), and cefuroxime (97%). High percentages of susceptible strains were also observed in the presence of penicillin G (90%), cephalotin (86%), and clindamycin (84%).

Generally, lactobacilli are sensitive to the inhibitors of cell wall synthesis, such as penicillins, but more resistant to cephalosporins (2). A widespread sensitivity toward penicillins has already been observed in lactobacilli used as probiotic or starter cultures (13), in lactobacilli from the human intestinal tract (15), and in probiotic strains (7). No lactobacilli from Norwegian starter cultures were resistant to penicillin G and ampicillin (36). A slightly higher resistance was observed in lactobacilli isolated from ewe's cheese (6),

while resistance toward penicillin G was found to be widespread among probiotic lactobacilli belonging to the species *L. rhamnosus*, *L. reuteri*, and *L. plantarum* (47). Also, *L. plantarum* strains isolated from wine were sensitive to penicillin G (43), with some exceptions, confirming the findings of Zarazaga et al. (52). A high frequency of resistance to penicillin G of the species *L. plantarum* was observed in isolates from cheeses (17). Furthermore, high percentages of penicillin G resistance have been observed among lactobacilli isolated from sausages (24), Nigerian fermented foods (40), and European probiotic products (47).

In spite of their susceptibility to penicillins, the isolates from Grana Padano and Parmigiano Reggiano were rather resistant to oxacillin (about 46% were resistant and 25% showed intermediate resistance). This characteristic has already been described by Danielsen and Wind (13). Similar results were reported for *L. rhamnosus* strains isolated from Parmigiano Reggiano (12) and in lactobacilli from Armada cheese (30).

A general susceptibility to the cephalosporins (cephalotin and cefuroxime) was observed in this study, even if less pronounced with respect to penicillins. Other authors (13, 28) found a higher degree of resistance against these antibiotics. This susceptibility was higher in particular if compared with that found in *L. rhamnosus* isolated from Parmigiano Reggiano, whose resistance was assessed by the same method (12). By contrast, Temmerman et al. (47) found that almost all the probiotic strains belonging to the species *L. reuterii* and *L. crispatus* were resistant to this antibiotic, while this resistance was observed with >20% of *L. rhamnosus* strains.

The strains tested in this study showed also a high susceptibility toward the inhibitors of protein synthesis such as erythromycin and tetracycline and, to a lesser extent, clindamycin. These observations confirmed the data reported by Danielsen and Wind (13) and Ammor et al. (2). This sensitivity was also observed in *L. rhamnosus* strains isolated from Parmigiano Reggiano (12) and lactobacilli from Armada (30) and Cabrales (17) cheeses.

The high susceptibility toward nitrofurantoin of the isolates from Grana Padano and Parmigiano Reggiano confirmed the observation of Charteris et al. (7), while Danielsen and Wind (13) observed in lactobacilli different levels of susceptibility to this antibiotic among strains of the same species. By contrast, many lactobacilli isolated from Armada cheese were resistant to nitrofurantoin (30) and a high resistance of lactobacilli toward this antibiotic was described also by Halami et al. (29), even though these authors used a different method to test the antibiotic susceptibility.

The resistance of many *Lactobacillus* species toward glycopeptides (among which vancomycin) has been often described as intrinsic (2). However, in spite of the diffusion of this resistance among lactobacilli, it has also been suggested that it cannot be an intrinsic property of the species, since variability occurred among *L. delbrueckii* subsp. *bulgaricus* (7), *L. acidophilus*, *L. johnsonii*, and *L. crispatus* strains (47). This resistance was widespread also among the strains considered here (about 68% resistant and only 21% susceptible), and particularly among the faculta-

TABLE 1. Number of lactobacilli strains for each species considered susceptible (S), intermediately resistant (I), and resistant (R) to the antibiotic tested^a

Antibiotic		<i>L. helveticus</i> (n = 35)	<i>L. delbrueckii</i> subsp. <i>lactis</i> (n = 10)	<i>L. rhamnosus</i> (n = 85)	<i>L. casei</i> (n = 11)	Total percentage (n = 141)
Ampicillin	S	35	10	85	11	100
	I	0	0	0	0	0
	R	0	0	0	0	0
Amoxicillin	S	35	9	84	11	98
	I	0	0	1	0	1
	R	0	1	0	0	1
Cefuroxime	S	35	10	81	11	97
	I	0	0	1	0	1
	R	0	0	3	0	2
Cephalotin	S	34	10	67	11	86
	I	0	0	12	0	9
	R	1	0	6	0	5
Clindamycin	S	29	6	73	10	84
	I	2	0	5	0	5
	R	4	4	7	1	11
Co-trimoxazole	S	3	3	0	0	4
	I	1	1	1	0	2
	R	31	6	84	11	94
Erythromycin	S	34	8	85	10	97
	R	1	2	0	1	3
Gentamicin	S	5	1	12	3	15
	R	30	9	73	8	85
Nitrofurantoin	S	35	7	84	11	97
	R	0	3	1	0	3
Oxacillin	S	30	5	6	3	29
	I	3	2	23	4	25
	R	2	3	56	4	46
Penicillin G	S	33	10	74	11	90
	I	2	0	11	0	9
	R	1	0	0	0	1
Tetracycline	S	35	8	84	11	98
	I	0	1	1	0	1
	R	0	1	0	0	1
Vancomycin	S	27	2	1	0	21
	I	7	6	1	0	10
	R	1	2	83	11	69

^a All values except those in the last column represent numbers of strains.

tively heterofermentative species (*L. rhamnosus* and *L. casei*), but it cannot be considered characteristic of a species.

High resistance was observed toward gentamicin (85%) and co-trimoxazole (94%). Lactobacilli are generally resistant to aminoglycosides (2, 8) and to inhibitors of nucleic acid synthesis, such as co-trimoxazole (7, 13). Similar results were observed also in strains isolated from cheese (12, 17, 30).

Table 2 reports the percentages of S, I, and R lactobacilli in relation to the cheese from which they were isolated. The presence of significant differences in the resistance related to the origin was evaluated using the chi-square test. The *P* value associated with the chi-square test was not significant for almost all the antibiotics considered. Only gentamicin resistance showed a highly significant ($P < 0.0001$) difference in relation to the type of cheese. In fact, it was

generally present (96%) among the isolates from Parmigiano Reggiano and to a lesser extent (68%) among isolates from Grana Padano. Also penicillin G resistance showed a significant ($P < 0.0005$) difference, even if at a lower level, caused by a more widespread intermediate resistance characterizing the isolates from Parmigiano Reggiano (14%).

The chi-square test was also applied after grouping lactobacilli according to their presence and role during the production process: SLAB, added to the milk, are dominant during the first part of cheesemaking, but their number decreases in a few weeks, while NSLAB, naturally present in milk, increase after autolysis of SLAB in a successive phase of the cheese ageing and dominate the microflora in ripened cheese (5). Several significant differences were found between SLAB (*L. helveticus* and *L. delbrueckii* subsp. *lactis*) isolated from natural whey starter and NSLAB

TABLE 2. Differences in antibiotic resistance depending on the cheese and the production step from which lactobacilli were isolated^a

Antibiotic		Cheese			Production step		
		Grana Padano (n = 56)	Parmigiano Reggiano (n = 85)	P	SLAB (n = 45)	NSLAB (n = 96)	P
Amoxicillin	S	100	98	0.512577	98	99	0.271659
	I	0	1		0	1	
	R	0	1		2	0	
Cefuroxime	S	100	95	0.257651	98	96	0.752472
	I	0	1		0	1	
	R	0	4		2	3	
Cephalotin	S	91	84	0.313969	98	81	0.022443
	I	7	9		0	13	
	R	2	7		2	6	
Clindamycin	S	89	80	0.25696	78	87	0.256633
	I	2	7		4	5	
	R	9	13		18	8	
Co-trimoxazole	S	3	5	0.921459	13	0	0.000456
	I	2	2		5	1	
	R	95	93		82	99	
Erythromycin	S	95	99	0.143444	93	99	0.060743
	R	5	1		7	1	
Gentamicin	S	32	4	3.02 × 10⁻⁰⁶	13	16	0.721624
	R	68	96		87	84	
Nitrofurantoin	S	98	96	0.541709	93	99	0.060743
	R	2	4		7	1	
Penicillin G	S	96	86	0.023481	93	88	0.358351
	I	2	14		5	11	
	R	2	0		2	1	
Oxacillin	S	27	30	0.122066	78	6	2.48 × 10⁻¹⁷
	I	34	19		11	31	
	R	39	51		11	63	
Tetracycline	S	96	99	0.443529	96	99	0.290740
	I	2	1		2	1	
	R	2	0		2	0	
Vancomycin	S	21	21	0.965339	64	1	0.52 × 10⁻²⁶
	I	11	9		29	1	
	R	68	70		7	98	

^a Results (other than significance values) are expressed as percentages of S, I, and R strains. The significance of the differences is expressed by *P* values derived by the chi-square test (*P* values of ≤0.05 are in boldface type).

(*L. rhamnosus* and *L. casei*) isolated from the ripened cheese (Table 2). Vancomycin resistance was more widespread among NSLAB (98%) than among SLAB (7%) (*P* < 0.0001). While vancomycin resistance among mesophilic homofermentative lactobacilli is well known (37), no data are reported, to our knowledge, for *L. delbrueckii* subsp. *lactis*. However, Temmerman et al. (47) found that two of three strains of *L. delbrueckii* subsp. *bulgaricus* were resistant to vancomycin. Also Charteris et al. (7) found *L. casei* and *L. rhamnosus* to be resistant to vancomycin, while *L. delbrueckii* subsp. *bulgaricus* resistance was variable.

Highly significant differences were also found in the resistance to oxacillin (*P* < 0.0001): it was higher between the NSLAB (63% versus 11%), which even if to a lesser extent, were also more resistant toward cephalothin (*P* < 0.05) and co-trimoxazole (*P* < 0.001). Even though the relatively low number of the isolates belonging to the species *L. casei* and *L. delbrueckii* subsp. *lactis* did not allow a reliable statistical

comparison with the two dominant mesophilic and thermophilic species, the data reported in Table 1 indicate that the behavior was similar within the two groups.

Consequently, the antibiotic resistance profiles of SLAB and NSLAB lactobacilli were compared by the chi-square test to detect significant differences within these groups in relation to the cheese from which they were isolated (Table 3). In general, NSLAB were more resistant to antibiotics than were SLAB. In fact, considering the number of R and I strains, a mean value of 4.3 and 2.8 was observed for NSLAB and SLAB, respectively. Furthermore, if the strains showing resistance or intermediate resistance against at least five of the antibiotics tested here are considered, these multiresistant lactobacilli represent 9 and 34% of SLAB and NSLAB lactobacilli, respectively. Moreover, while no significant difference in antibiotic resistance was observed for SLAB in relation to the cheese from which they were isolated, the NSLAB isolated from

TABLE 3. Differences in antibiotic resistance of SLAB and NSLAB depending on the type of cheese^a

Antibiotic		SLAB			NSLAB		
		Grana Padano (n = 20)	Parmigiano Reggiano (n = 25)	P	Grana Padano (n = 36)	Parmigiano Reggiano (n = 60)	P
Amoxicillin	S	100	96	0.365712	100	98	0.225350
	I	0	0		0	2	
	R	0	4		0	0	
Cefuroxime	S	100	100	0.193355	100	93	
	I	0	0		0	2	
	R	0	0		0	5	
Cephalotin	S	92	100	0.258195	89	77	0.089995
	I	0	0		11	13	
	R	8	0		0	10	
Clindamycin	S	70	84	0.506300	100	79	0.008549
	I	5	4		0	8	
	R	25	12		0	13	
Co-trimoxazole	S	10	16	0.850016	0	0	0.225350
	I	5	4		0	2	
	R	80	80		100	98	
Erythromycin	S	90	96	0.422678	97	100	0.113734
	R	10	4		3	0	
Gentamicin	S	15	12	0.768624	42	0	6.37 × 10⁻⁰⁸
	R	85	88		58	100	
Nitrofurantoin	S	95	92	0.688499	100	98	0.225350
	R	5	8		0	2	
Oxacillin	S	70	84	0.532590	3	8	0.053456
	I	15	8		44	23	
	R	15	8		53	69	
Penicillin G	S	90	96	0.517405	100	82	0.004617
	I	5	4		0	18	
	R	5	0		0	0	
Tetracycline	S	95	96	0.358587	97	100	0.113734
	I	0	4		3	0	
	R	5	0		0	0	
Vancomycin	S	60	68	0.695680	0	1	0.373902
	I	30	28		0	1	
	R	10	4		100	98	

^a Results (other than significance values) are expressed as percentages of S, I, and R strains. The significance of the differences is expressed by *P* values derived by the chi-square test (*P* values of ≤0.05 are in boldface type).

Parmigiano Reggiano were characterized by a significantly higher resistance toward gentamicin ($P < 0.0001$). In fact, all of the strains from the latter cheese were resistant to this antibiotic, while more than 40% of the strains from Grana Padano were susceptible. In addition, significantly higher resistance ($P < 0.05$) in the NSLAB Parmigiano Reggiano strains was observed also toward penicillin G and clindamycin. Also the resistance to oxacillin and cephalotin was higher in the strains isolated from Parmigiano Reggiano, but the difference was not significant ($P > 0.05$).

A high incidence of gentamicin-resistant lactobacilli was observed also in a study by Hummel et al. (32) that considered probiotic strains and lactobacilli used as starter cultures for sausage manufacture. Some authors indicated that the use of gentamicin in animal farms can induce in bacteria a resistance transmittable via mobile genetic elements. In particular, Hershberger et al. (31) demonstrated that the use of gentamicin in swine and dairy farms caused a

diffuse resistance toward this antibiotic in enterococci. This resistance was related to the presence of genes such as *aph(2'')* or *aac(6')-aph(2'')*, which can be carried by plasmids (9) or transposons (1). An increased resistance to this antibiotic among streptococci was also observed in a Swiss dairy farm following therapeutic treatment of mastitis (42). Hershberger et al. (31) demonstrated also the transfer of resistant enterococci from animals to humans through the food chain. Donabedian et al. (16) found that when a gentamicin-resistant gene was present in resistant enterococci from animals, that gene was also present in enterococci isolated from food products of the same animal species. Moreover, these authors suggest similarities in gentamicin resistance among enterococci isolated from humans, retail food (meat and dairy), and farm animals from geographically diverse areas of the United States.

Antibiotics have been used in animal farms for more than 50 years both to control infectious diseases and to

improve efficiency of food utilization (34). About one-half of the total worldwide antibiotic production is used as animal therapeutic agents, prophylactics, and growth promoters (48, 50). The selective pressure induced by this use of antibiotics can make the bacteria in the food chain, even if not pathogenic or opportunistic microorganisms, an extremely important reservoir of resistance genes.

The species of lactobacilli considered in this work are of great technological interest in Grana Padano and Parmigiano Reggiano production and ripening. The species usually isolated from natural whey starters (*L. helveticus* and *L. delbrueckii* subsp. *lactis*) are thermophilic obligately homofermentative species belonging to group I lactobacilli, while the lactobacilli isolated from ripened cheeses, *L. rhamnosus* and *L. casei*, belong to group II, i.e., facultatively heterofermentative lactobacilli with lower optimum temperature (3). Also the antibiotic resistance profiles of the lactobacilli belonging to these two groups showed significant differences for vancomycin, oxacillin, co-trimoxazole, cephalotin, and to a lesser extent, nitrofurantoin and erythromycin.

The comparison of the antibiotic resistance profiles of the lactobacilli in relation to the two cheeses they come from pointed out a highly significant difference for gentamicin resistance. The reason for this difference can be explained by considering the two different groups of lactobacilli in relation to the cheese from which they were isolated. In fact, while the farming and milking conditions for the cows utilized for Grana Padano and Parmigiano Reggiano production are different, according to the Protected Designation of Origin protocols, the cheesemaking procedures are very similar. Nevertheless, relevant phenotypic and genotypic differences have been observed among the SLAB, even within the various biotypes of the same species of the starter microbiota (23). However, the SLAB present in the whey cultures are selected by at least two stringent conditions driven by the technology applied to produce these two cheeses: cooking curd temperature and the high acidity reached in fermented whey (23). The antibiotic resistance profiles reported in this work for the SLAB did not show any relevant difference likely to discriminate them in relation to the cheese type. This indicates that these microbial populations, which are daily propagated in the dairies with new processed milk, are not subjected in these conditions to the selective pressure caused by the presence of different amounts of antibiotics related to the cheese type.

By contrast, highly significant differences in the antibiotic resistance in relation to the type of cheese were found among the NSLAB lactobacilli isolated from ripened cheeses. In particular, important factors driving the different antibiotic profiles could be found in the farming conditions, which differ as to geographical area, farm size, animal feeding, and pasture. These differences can induce the need for different therapeutic antibiotic treatments. In this scenario, the raw milk can be considered to be only the carrier to the cheese of resistant NSLAB that developed their resistance in the livestock farm. The different antibiotic resistance profiles in relation to the cheese type observed for oxacillin, clyndamicin, penicillin G, and above all, gentamicin can be explained in this framework.

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