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ORIGINAL ARTICLE

Phenotypic typing, technological properties and safety aspects of *Lactococcus garvieae* strains from dairy environments

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Abstract

Aims: To characterize *Lactococcus garvieae* strains of dairy origin and to determine their technological properties and safety for their possible use in starter culture preparation.

Methods and Results: Forty-seven *L. garvieae* isolates, recovered from two artisanal Italian cheeses were studied, in comparison with 12 fish isolates and the type strain of the species. Phenotypic typing revealed that the strains could be differentiated on the basis of their ecological niche of origin in lactose positive strains (all isolated from dairy sources) and lactose negative strains (all isolated from fish). Furthermore, the strains exhibited a high degree of physiological variability, showing the presence of 26 different biotypes. The strains possessed moderate acidifying and proteolytic activities and did not produce bacteriocins. A safety investigation revealed that all strains were sensitive to vancomycin and moderately resistant to kanamycin; some biotypes were tetracycline resistant. Production of biogenic amines or presence of genes encoding virulence determinants occurred in some isolates.

Conclusions: The prevalence of *L. garvieae* in some artisanal Italian cheeses can be linked to the typicity of the products. Although in a few cases an antimicrobial resistance or a presence of virulence determinants may imply a potential hygienic risk, most of the strains showed positive properties for their possible adjunction in a starter culture preparation, to preserve the natural bacterial population responsible for the typical sensorial characteristics of the traditional raw milk cheeses.

Significance and Impact of the Study: *L. garvieae* strains can be considered an important part of the microbial population associated with the natural fermentation of artisanal Italian cheeses. A deepened characterization of the strains may aid in understanding the functional and ecological significance of their presence in dairy products and in selecting new strains for the dairy industry.

Introduction

Lactococcus garvieae, an elder subjective synonym of *Enterococcus seriolicida*, is a well-recognized bacterial fish pathogen and it is considered a serious problem in cultured marine and freshwater fish species such as yellowtail in Japan (Kawanishi *et al.* 2005) and rainbow trout in Europe and Australia (Prieta *et al.* 1993; Eldar *et al.* 1999). The septicaemic infection produced by *L. garvieae*, termed lactococcosis, is actually a worldwide bacterial disease with sanitary and economic impact on the fishery farming industry (Ghittino and Prearo 1992; Vela *et al.* 2000; Eyngor *et al.* 2004). *L. garvieae* has also been recovered from clinical specimens of human blood, urine and

skin (Fefer *et al.* 1998; Furushita *et al.* 2003). Therefore, this micro-organism is regarded as an emerging pathogen of increasing clinical significance in the fields of veterinary and human medicine.

The earliest description of *L. garvieae* dates back to 1984, when it was isolated in the UK from a mastitic cow udder (Collins *et al.* 1984). However, despite of its limited importance as a mastitogenic agent, this organism has been isolated as nondominant species in the dairy environment, such as natural starter cultures, raw milk, curd and cheeses (Klijn *et al.* 1995; Teixeira *et al.* 1996; Parente *et al.* 1997; Hatzikamari *et al.* 1999; Prodromou *et al.* 2001; An *et al.* 2004). Recently, our research group discovered in some traditional Italian cheeses, from raw cow and goat milk, high counts of *L. garvieae* strains that were sometimes predominant (Fortina *et al.* 2003; Foschino *et al.* 2006).

Various questions arise from the above-mentioned observations:

i Is the dairy environment a natural reservoir for this species? The isolation of *L. garvieae* in milk and cheeses relies on the use of molecular techniques with high discriminatory power, compared with cultural methods such as phenotypical tests, which in the past led to a misidentification with *L. lactis* group.

ii Is the prevalence of *L. garvieae* in certain cheeses directly linked to the typicity of the products? It is well-known that nonstarter Lactic Acid Bacteria, particularly raw milk microbial population, increase the diversity of cheese flavours and may also be involved in producing the typical sensorial traits of cheeses during ripening.

iii What is the relationship between the presence of *L. garvieae* in dairy and food safety? In particular, which factors normally related to virulence are present in *L. garvieae* and which could potentially develop into pathogenic strains in raw milk cheese? The presence of this species in food environments needs further investigation to recognize a potential hazard for the consumers.

In the literature, no data on physiological properties of *L. garvieae* strains of dairy origin are available. For this reason, in this study we carried out a primary phenotypic, biotechnological and genetic characterization of *L. garvieae* strains isolated from different dairy sources, in comparison with diseased fish strains, with the aim of obtaining discrimination between biotypes with respect to their source and an understanding of the functional and ecological significance of their presence in dairy products.

Materials and methods

Bacterial strains

A total of 59 isolates of *L. garvieae*, in comparison with the type strain of the species, were studied: their source and origin are listed in Table 1. Samples of milk, whey, curd and cheeses were obtained from different dairy environments in two Northern Italian regions. Thirty-two strains were collected from the manufacture of the artisanal protected denomination origin (PDO) Italian cheese, Toma Piemontese, a semi-cooked cheese produced from raw cow-milk without the addition of any selected cultures. Fifteen isolates were recovered from the productive chain of Caprino Lombardo cheese, a fresh goat cheese traditionally curdled by acidification with natural whey cultures. The twelve fish isolates, collected from 1999 to 2004 from diseased rainbow trouts and catfish, were kindly provided by Dr Marino Prearo (Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle D'Aosta, Torino, Italy – LG isolates) and by Dr Amedeo Manfrin (Istituto Zooprofilattico Sperimentale delle Venezie, National Reference Lab. for Fish Diseases, Legnaro, Italy – V isolates). The type strain, *L. garvieae* DSM 20684^T was also included in the study. All isolates, stored frozen (−80°C) in broth cultures supplemented with 15% (w/v) glycerol, were grown at 37°C for 24 h in M17 (Difco, Detroit, MI, USA) supplemented with 5 g l^{-1} glucose.

Genotypic identification

The taxonomic position of the isolates was established through a PCR amplification of the 16S-23S rRNA spacer region (RSA), as previously reported (Fortina *et al.* 2003), and through a species-specific PCR assay, as reported by Zlotkin *et al.* (1998). The primers were synthesized by PRIMM srl (Milano, Italy; Table 2).

Biochemical and enzymatic characterization

Biochemical and enzymatic tests were performed with the Rapid ID 32 Strep and API CH50 system (bioMérieux, Marcy l'Etoile, France) following the manufacture's instructions, except for the temperature of incubation, which was always 37°C. Sugar-fermentation patterns were also determined by using a microtitre plate assay and a basal M17 medium containing chlorophenol red as indicator and the desired filter-sterilized carbohydrate at a final concentration of 0·2% (w/v). Additional tests included growth at 10, 40 and 45°C; growth in the presence of 4% and 6·5% NaCl; growth at pH 9·6.

Acidifying activity

Fresh milk cultures of each strain were inoculated at 1% in 100 ml sterile reconstituted (10% w/v) skimmed milk prewarmed at 37°C and the pH was measured and recorded automatically, throughout at 48 h incubation period.

Table 1 *L. garvieae* strains analysed in this study and their characteristics*

				Phenotypic characteristics										Virulence factors				
Strain(s)	Source†		Year	LAC	SAC	C-DEX	GTA	NAG	TAG	L-ARA	HIP	MEL	Biotype	Tetracycline resistance‡	Tyramine production	PCR amplification of virulence genes		
G1, G2, G3, G4, G5,G6	Cow curd	-DF1	2001	+	+	+	−	+	+	−	+	−	A1	S	−			
G7		-DF2	2001	+	+	−	−	+	−	−	−	−	A2	S	−			
G8		-DF2	2001	+	+	+	−	+	+	−	+	−	A1	S	−			
G9	Cow cheese	-DF1	2001	+	+	−	−	−	+	−	−	−	A3	S	−			
G10, G11		-DF1	2001	+	+	+	−	−	−	−	+	−	A4	S	−			
G12, G13, G14		-DF3	2001	+	+	+	−	+	+	−	+	−	A1	S	−			
G15		-DF4	2001	+	+	+	−	−	+	−	−	−	A5	S	−			
G16		-DF4	2001	+	+	+	−	+	+	−	−	−	A6	S	−			
G17, G21, G22, G24	Cow milk	-DF1	2002	+	+	+	−	+	+	−	+	−	A1	S	−			
G18		-DF1	2002	+	+	+	−	+	+	−	−	−	A5	S	−			
G19		-DF1	2002	+	+	+	−	+	+	−	−	−	A6	S	−			
G20, G23		-DF1	2002	+	+	+	−	−	+	−	+	−	A7	S	−			
G25		-DF1	2003	+	+	−	−	−	+	−	−	−	A3	S	−			*tetS*
G30		-DF1	2003	+	+	−	−	−	+	−	−	−	A3	R	−	*tetM*	Int-Tn	
G26,		-DF1	2003	+	+	+	−	+	+	−	−	−	A6	S	−			*tetS*
G27, G28		-DF1	2003	+	+	+	−	+	+	−	−	−	A6	R	−	*tetM*	Int-Tn	
G31		-DF1	2003	+	+	+	−	+	+	−	−	−	A6	Int-R	−			*tetS*
G29		-DF1	2003	+	+	−	−	+	−	−	−	−	A2	R	−	*tetM*	Int-Tn	
G32		-DF1	2003	+	+	+	−	+	+	−	+	−	A1	S	−			
M4	Goat milk	- DF5	2001	+	+	+	+	−	−	−	−	−	A8	S	+			
M41		-DF5	2001	+	+	+	−	−	+	−	−	−	A5	S	+			
M43		-DF5	2001	+	+	+	−	+	+	+	−	−	A12	S	−			
M241		-DF6	2001	+	+	−	+	−	+	−	−	−	A15	S	+			
M249		-DF6	2001	+	+	+	−	+	−	−	−	−	A16	S	+			
M27	Goat whey	-DF7	2001	+	+	+	+	−	−	−	−	+	A10	S	+			
M75		-DF8	2001	+	+	−	−	−	+	−	−	−	A3	S	−			
M7	Goat cheese	- DF5	2001	+	+	+	+	+	+	+	−	+	A9	S	−			
M29		-DF7	2001	+	+	+	+	+	+	+	−	+	A9	S	+			
M31		-DF7	2001	+	+	−	−	−	−	−	−	−	A11	S	−			
M35		-DF7	2001	+	+	+	+	−	−	−	−	−	A8	S	+			
M48		DF5	2001	+	+	+	−	−	−	−	−	−	A13	R	+			*tetS*
M84		DF9	2001	+	+	−	+	−	−	−	−	−	A14	S	+			
M115		DF8	2001	+	+	+	+	−	−	−	−	+	A10	S	+			
M300		DF10	2001	+	+	+	−	−	−	+	−	+	A17	S	+			
LG9	Rainbow trout Italy		2000	−	−	−	−	+	−	−	−	−	B1	S	−			
LG19, LG20			2000	−	−	+	−	+	−	−	−	−	B2	S	−			
LG28			2001	−	−	−	−	−	−	−	−	−	B4	S	−			
LG30			2001	−	−	−	−	+	−	−	−	−	B1	S	−			
V61 (213)			2003	−	+	+	+	+	+	−	+	−	B6	S	−			

Table 1 Continued

Strain(s)	Source†	Year	Phenotypic characteristics									Virulence factors		
			LAC	SAC	C-DEX	GTA	NAG	TAG	L-ARA	HIP	MEL	Biotype	Tetracycline resistance‡	Tyramine production	PCR amplification of virulence genes
LG23	Rainbow trout Spain	2000	–	+	–	–	+	+	–	+	–	B3	S	–	
V32 (PP60)		1999	–	+	+	–	+	+	–	+	–	B5	S	–	
V63 (00021UK)	Rainbow trout UK	2003	–	+	+	+	+	+	–	+	–	B6	S	–	
V69 (091UNIUD)	Trout Italy	2004	–	–	–	+	+	–	–	–	–	B7	S	–	
V72 (85F)		2002	–	–	+	+	+	–	–	–	–	B8	S	–	
V79 (297/8C2)	Catfish Italy	1999	–	+	+	+	–	+	–	+	–	B9	R	–	*tetM* Int-Tn
DSM 20684^T	Cow UK	1984	–	–	–	–	+	–	–	–	–	B1	S	–	

*All strains were negative for vancomycin resistance, *β*-haemolysis, gelatinase production, detection of *vanA*, *vanB*, *gelE* and *cylI* genes; they showed a intermediate resistance to kanamycin

† DF are different Dairy Farms

‡ resistance level to tetracycline: high, R > 16 μg ml^{-1}; intermediate, Int-R from 5 to 16 μg ml^{-1}; S < 5 μg ml^{-1}.

Values of ΔpH after 6, 12, 24 and 48 h were used to evaluate the acidifying activity of the strains. Two trials for each strain were carried out.

Proteolytic activity

The amino acids/peptides accumulated in milk after 24–48 h of incubation at 37°C, as a consequence of the proteolytic activity of the tested strains were determined using the *o*-phthaldialdehyde method (Church *et al.* 1983). Results were expressed in L-glycine equivalent (μg ml^{-1}).

Bacteriocin activity

Bacteriocin activity was assessed by physiological methods. The agar well diffusion assay (AWDA) using *Listeria monocytogenes* NCTC 10527 as indicator strain, was performed as described by Schillinger and Lücke (1987).

Antibiotic susceptibility

Vancomycin, kanamycin and tetracycline-resistances were tested. The minimum inhibitory concentration (MIC) of the antibiotics was calculated after growth in M17 broth at 37°C, using 10^5 cells ml^{-1} as initial inoculum. Interpretative criteria for susceptibility status were the microbiological breakpoints defined by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) for *Enterococcus* genus (FEEDAP Panel 2005). High-level resistance to vancomycin was defined as a MIC > 8 μg ml^{-1}, and to kanamycin and tetracycline as a MIC > 1024 and 16 μg ml^{-1} respectively.

Production of gelatinase and haemolysin

Production of gelatinase was tested on agar plates containing gelatine as reported previously (Franz *et al.* 2001). Haemolytic test was performed at 37°C in Columbia Agar with sheep defibrinated blood (Oxoid, Unipath, Basingstoke, UK).

Biogenic amine determination

Screening plate method to investigate the ability of biogenic amine production by *L. garvieae* strains was performed as reported by Bover-Cid and Holzapfel (1999). Positive reactions for decarboxylase activity of strains producing tyramine or histamine were recorded when a purple colour halo occurred in response of the bromocresol purple indicator to a pH shift or when tyrosine precipitate disappeared around the colonies.

Table 2 PCR primers used in this study and their products

Gene and primers	Sequence	Product size (bp)	Reference
rRNA spacer region	5′-GAAGTCGTAACAAGG-3′ 5′-CAAGGCATCCACCGT-3′	410	Fortina *et al.* 2003
16S rRNA	5′-CATAACAATGAGAATCGC-3′ 5′-GCACCCTCGCGGGTTG-3′	1100	Zlotkin *et al.* 1998
vanA	5′-GGGAAAACGACAATTGC-3′ 5′-GTACAATGCGGCCGTTA-3′	732	Dutka-Malen *et al.* 1995
vanB	5′-ATGGGAAGCCGATAGTC-3′ 5′-GATTTCGTTCCTCGACC-3′	635	Dutka-Malen *et al.* 1995
tetM	5′- GTTAAATAGTGTTCTTGGAG-3′ 5′- CTAAGATATGGCTCTAACAA-3′	656	Kim *et al.* 2004
tetS	5′-CATAGACAAGCCGTTGACC-3′ 5′-ATGTTTTTGGAACGACAGAG-3′	667	Kim *et al.* 2004
Int-Tn	5′-TGACACTCTGCCAGCTTTAC-3′ 5′-CCATAGGAACTTGACGTTCG-3′	579	De Barbeyrac *et al.* 1996
gelE	5′-ACCCCGTATCATTGGTTT-3′ 5′-ACGCATTGCTTTTCCATC-3′	419	Eaton and Gasson 2001
Cytolysin genes			
$cylL_L$	5′-GATGGAGGGTAAGAATTATGG-3′ 5′GCTTCACCTCACTAAGTTTTATAG-3′	253	Semedo *et al.* 2003
$cylL_S$	5′-GAAGCACAGTGCTAAATAAGG-3′ 5′-GTATAAGAGGGCTAGTTTCAC-3′	240	
cylM	5′-AAAAGGAGTGCTTACATGGAAGAT-3′ 5′-CATAACCCACACCACTGATTCC-3′	2940	
cylB	5′-AAGTACACTAGTAGAACTAAGGGA-3′ 5′-ACAGTGAACGATATAACTCGCTATT-3′	2020	
cylA	5′-TAGCGAGTTATATCGTTCACTGTA-3′ 5′-CTCACCTCTTTGTATTTAAGCATG-3′	1282	

Detection of virulence factors

For each strain, total chromosomal DNA from overnight broth cultures was extracted according to Fortina *et al.* (2003). To verify whether the quality of the extracted DNA was PCR grade, a PCR amplification of 16S rRNA gene from all the bacterial strains, using the primers described by Lane (1991), was carried out.

PCR reactions were performed in a 25 μl reaction mixture containing 100 ng bacterial DNA, 2·5 μl 10x reaction buffer (MBI-Fermentas, Vilnius, Lithuania), 200 μmol l^{-1} of each dNTP, 2·5 mmol l^{-1} $MgCl_2$, 0·5 μmol l^{-1} of each primer, and 0·5 U *Taq* polymerase (MBI-Fermentas, Vilnius, Lithuania). Amplifications were carried out using a Gene Amp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA), programmed as follows: an initial denaturation step at 94°C for 2 min, 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 45 s, followed by a final extension at 72°C for 7 min. Amplification products were separated on a 1·5%agarose gel stained with ethidium bromide in 1x TAE (40 mmol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA, pH 8·0) buffer and photographed. The specific primers used for the detection of virulence genes and their amplification products are reported in Table 2, with relevant references. For the detection of glycopeptide resistance genotypes primers targeting *vanA* and *vanB* genes, respectively, were used; occurrence of tetracycline resistance genes was examined with primers for *tetM*, *tetS* and for the integrase gene(*Int-Tn*) of the transposon Tn*1545*-Tn*916*; the presence of gelatinase determinants was investigated with primers for *gelE*; the detection of cytolysin genes was carried out on the whole operon, comprising the structural genes ($cylL_L$ and $cylL_S$) and genes involved in the modification (*cylM*), secretion (*cylB*) and activation (*cylA*) of cytolysin subunits.

Results

PCR identification

All isolates gave the typical RSA profile of the *L. garvieae* species, characterized by a unique band migrating at approximately 410 bp, previously described for the species (Fortina *et al.* 2003), and the expected 1100 bp species-specific PCR amplification product (Zlotkin *et al.* 1998).

Phenotypic characterization

All the strains had several common characteristics: growth at 40°C, at pH 9·6 and in the presence of 4%

NaCl, arginine deamination and acetoin production. They were positive for the presence of the enzymes arginine dihydrolase (ADH), β-glucosidase (βGLU), alanyl-phenylalanyl-proline arylamidase (APPA), pyroglutamic acid arylamidase (PyrA), and for the acidification of D-ribose (RIB), D-mannitol (MAN), D-trehalose (TRE), methyl-β-D-glucopyranoside (MBDG) and D-maltose (MAL). They were negative for the presence of the enzymes α- and β-galactosidase (αGAL), alkaline phosphatase (PAL), β-glucuronidase (βGUR), β-mannosidase (βMAN), urease (URE), and for the acidification of D-raffinose (RAF), D-sorbitol (SOR), D-arabitol (DARL), glycogen (GLYG), pullulan (PUL) and D-melezitose (MLZ).

For other phenotypic traits a great variability among the *L. garvieae* isolates was found. The ability of growing at 10°, 45°C and in presence of 6·5% NaCl was present in 58%, 88% and 56% of the isolates respectively, with no apparent correlation with the origin. The acidification of lactose allowed the grouping of the isolates in two main clusters: the lactose positive strains, all isolated from dairy sources (cluster A) and the lactose negative strains, all isolated from fish (cluster B). This difference permitted to discriminate the strains on the basis of their principal ecological niche of origin. It is noteworthy that lactose utilization by commercial kits gave contradictory results; for this reason this determination was carried out by microtitre plate assay. Within cluster A some characteristics allowed a further discrimination between cow and goat biotypes: the isolates coming from cow samples were able to acidify D-tagatose, and failed to utilize L-arabinose, D-melibiose and to synthesize glycyl-tryptophan arylamidase. For the isolates from goat samples these characteristics seemed to be variable, with about 30% of the strains being positive for L-arabinose and D-melibiose, 53% positive for glycyl-tryptophan arylamidase, while D-tagatose was fermented only by 40% of the strains. The hydrolysis of hippurate, negative for all goat isolates, resulted positive for 60% of the cow isolates tested. Finally, within cluster A additional biotypes were obtained when the ability to acidify α-cyclodextrin (84% for cow isolates and 73%for goat isolates) and to produce *N*-acetyl-β-glucosaminidase (72% for cow isolates and 20% for goat isolates) were considered. All dairy isolates were able to utilize saccharose. As shown in Table 1, 17 different biotypes were distinguishable, with biotype A1 grouping 32% of the dairy strains. Within cluster B, grouping fish isolates, only two characteristics were common to all isolates, the failure to utilize *L*-arabinose and D-melibiose. For the other characteristics analysed, there was a wide heterogeneity, with the possibility to obtain nine different biotypes (Table 1).

Technological characterization

The results of the acidification experiments were consistent with those previously found for the lactose utilization. On average dairy isolates showed a quicker and higher capability of acidification with ΔpH values of 0·30 ± 0·12 (mean ± SD) at 6 h, 0·58 ± 019 at 12 h, 0·91 ± 0·25 at 24 h and 1·35 ± 0·17 at 48 h, allowing the curdling of the skim milk at the end of the incubation. Conversely fish isolates achieved ΔpH values of 0·23 ± 0·06 at 6 h, 0·37 ± 0·06 at 12 h, 0·64 ± 0·18 at 24 h and 0·89 ± 0·05 at 48 h; none of these strains was able to coagulate milk within the test incubation time. Data of ΔpH at 6, 12, 24 and 48 h were submitted to Analysis of Variance to infer the effect of some factors, such as source of isolation and time of incubation; the results confirmed the presence of significant differences between the acidifying activities of dairy and fish isolates either at 12 h ($P < 0·005$), 24 h ($P < 0·001$) and 48 h ($P < 0·001$).

The proteolytic activity was low for all strains, without any difference linked to the source of isolation. The mean value, in L-glycine equivalent, was 42·7 ± 33·3 μg ml^{-1}, with the only exception of the LG19 strain that showed a value of 840·6 μg ml^{-1}. None of the strains tested showed antagonistic activity against the target strain.

Virulence factors

All strains were susceptible to vancomycin, except for a goat isolate (M84) that showed a reduced resistance (MIC 4 μg ml^{-1}). An intermediate resistance to kanamycin was commonest and it was observed in all isolates, with MIC values ranging from 120 to 500 μg ml^{-1}. A high level of resistance to tetracycline was detected in seven strains (12%), most of which of food origin. Within these strains, only one isolate (G31), was inhibited at the breakpoint level of tetracycline (MIC = 16 μg ml^{-1}), whereas four isolates from cow milk (G27, G28, G29, G30) showed MIC values >150 μg ml^{-1}. β-hemolysis, as well as gelatinase activity, was not observed in any of the tested strains. Eleven isolates (18%) from Caprino Lombardo samples produced detectable quantities of tyramine, while no production of histamine was revealed. The results obtained are reported in Table 1.

Detection of virulence determinants

All strains were analysed for the presence of several virulence determinants by using PCR with specific primers already published. A good congruence between phenotypic traits and molecular screening was obtained (Table 1). The *vanA* and *vanB* genes were not present in any of the

tested strains, according to the phenotypic susceptibility to vancomycin. Four cow milk isolates showing high level of tetracycline resistance carried *tetM* genes and where positive for *Int-Tn*, together with one isolate from diseased fish that showed a MIC value of 32 μg ml^{-1}. Other two tetracycline resistant strains carried *tetS* genes. No *gelE* genes were detected in the tested strains, suggesting that gelatinase production by this species is not common. Agreement between phenotypic and genotypic traits was also observed between blood agar haemolysis and detection of *cyl* genes: the strains were not β-haemolytic and absence of amplification was observed for all genes of the *cyl* operon.

Discussion

All over the world, the manufacture of artisanal cheeses with typical features represents a significant part of the food culture of various people, and it plays an important role in economics for millions of farmers and cheese-makers. To keep on these traditions it is important to establish the microbiological characteristics of the product and to provide more deepened information for starter culture design at industrial level. With this study, we contribute to the characterization of *Lactococcus garvieae* strains associated with artisanal cheeses from Italy, in consideration of the preservation and promotion of typical products. While the association of enterococci with dairy fermented foods is well-known, little is known about the presence and role of *L. garvieae.* In our previous studies this species was shown to represent a relevant component of the microbial population of two artisanal Italian cheeses (Fortina *et al.* 2003; Foschino *et al.* 2006), as the organism could be isolated from raw milk employed for cheese production and throughout the technological process. In the same way, other authors found *L. garvieae* as a part of the autochthonous bacterial population in different Italian and Greek cheeses, although at a lower incidence. Thus, this species appears to be often associated with fermented dairy products. On the contrary, *L. garvieae* is considered as an emerging pathogen of increasing clinical significance in the fields of fish farming. The presence of this species in foods and the increasing report of *L. garvieae* associated with disease, led us to investigate the characteristics of this micro-organism, by studying food isolates collected from two Italian cheeses and clinical isolates from animal origin. Indeed, despite the increased clinical significance of this species and its involvement in the manufacture of dairy products, deepened studies on phenotypic and genetic traits and pathogenic relationship among isolates from different environments have not yet been carried out.

The investigation on phenotypic characteristics of *L. garvieae* strains showed that the strains tested could be divided into two major clusters, according to the ecological niche of origin. The first cluster, encompassing dairy isolates, was characterized by the ability to acidify lactose, while the second included fish isolates, unable to acidify this carbon source. This physiological difference reflected the inability of the fish isolates to coagulate milk within 48 h and to produce significant amount of lactic acid. As this trait could be used as phenotypic epidemiological marker, it is remarkable to underline that the results obtained with the Rapid ID 32 Strep and API CH50 systems for the acidification trials were subjected to variation and did not agree with the ones obtained by use of conventional biochemical characterization tests. These results should be taken into account when rapid commercial systems are used for routine identification of the isolates. Moreover, the occurrence of different *L. garvieae* biotypes emphasizes the difficulties of a definitive identification based on phenotypic traits alone.

Regarding technologically important traits, cheeses isolates showed a slow rate of acidifying ability during the first 6–12 h of incubation, in comparison with dairy starters (International Dairy Federation FIL-IDF 1995) and a low proteolytic activity in skim milk. In this context, the strains were similar to wild lactococcal population isolated from other cheeses (Requena *et al.* 1991; Prodromou *et al.* 2001). None of the *L. garvieae* strains produced antimicrobial compounds. Only eleven strains from goat samples produced the biogenic amine tyramine.

It appears from our study that the incidence of known virulence factors in *L. garvieae* isolates, either from dairy samples or diseased fish is generally low, no strain carrying more than one virulence factor. All strains lacked the *van*, *cyl* and *gel* virulence determinant, according to phenotypic traits. Only seven strains, six of them being of dairy origin, showed tetracycline resistance and the relative genes, *tetM* and *tetS*. The *tetM* gene is often associated with conjugative transposons belonging to the Tn*1545*-Tn*916* family, which appear to play a significant role in the dissemination of antibiotic resistance among clinically important species (Clewell *et al.* 1995), while *tetS* gene is normally involved in the synthesis of another ribosomal protection protein related to tetracycline resistance (Aminov *et al.* 2001). In addition, *tetS* gene was detected in two food isolates, (G25, G26), but no tetracycline resistance could be found for these strains. The presence of silent genes is known and could be explained by low levels or downregulation of gene expression or by an inactive gene product (Eaton and Gasson 2001).

As the tested food strains do not seem to possess high virulent profiles, their use in long-established artisanal cheeses may be pursued as a low risk is involved.

However, as the incidence of virulence factors seems to be strain specific, for safety considerations each strain should be tested for the different virulence traits, as well as for antibiotic resistance. One problem that remains is the possibility of transfer of virulence determinants from a strain, which harbour these genes to a starter strain. The possibilities of gene transfer *in vitro* and *in vivo* conditions, as well as further genotypic and epidemiological characterization requires further investigation.

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