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To cite this version:

Barbara M. Pisano, Francesca Patrignani, Sofia Cosentino, M. Elisabetta Guerzoni, Charles M. A. P. Franz, et al.. Diversity and functional properties of Lactobacillus plantarum-group strains isolated from Italian cheese products. Dairy Science & Technology, 2011, 91 (1), pp.65-76. 10.1051/dst/2010037 . hal-00874091

HAL Id: hal-00874091

https://hal.science/hal-00874091v1

Submitted on 17 Oct 2013

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Dairy Sci. & Technol. (2011) 91:65–76
DOI 10.1051/dst/2010037

ORIGINAL ARTICLE

Diversity and functional properties of *Lactobacillus plantarum*-group strains isolated from Italian cheese products

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Received: 25 January 2010 / Revised: 11 June 2010 / Accepted: 11 June 2010 /
Published online: 2 August 2010
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Abstract The aim of this study was to characterize dairy *Lactobacillus plantarum*-group strains on the basis of their phenotypic and genotypic profiles in order to increase the knowledge on the ecology and biodiversity within this wild *Lactobacillus* group. In addition, their in vitro potential probiotic properties were evaluated with a view to identifying potential interesting applications. Among the strains, both physiological and biochemical characteristics differed noticeably, indicating biodiverse phenotypic differences. Genotyping experiments using randomly amplified polymorphic DNA (RAPD)-PCR with primer M13 also showed a remarkable heterogeneity among the strains and allowed the strains to be grouped into the species *L. plantarum* and *L. pentosus*/*L. paraplantarum*. With regard to probiotic functional characteristics, the *L. plantarum* strains 31C and 143C and the *L. plantarum* strains 64FS and 61FS, isolated from Caciotta and Fiore Sardo cheeses, respectively, survived simulated gastrointestinal conditions and were considered to be acid and bile

This article should be cited as: Pisano M.B. et. al., Diversity and functional properties of *Lactobacillus plantarum*-group strains isolated from Italian cheese products, Dairy Science & Technology (2010), doi:10.1051/dst/2010037

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tolerant. The majority of the strains exhibited antagonistic activity towards *Escherichia coli* ATCC 43895 and *L. sakei* DSM 20017, but only one of these strains was found to produce a bacteriocin-like compound. The results of this study suggest the presence of both phenotypic and genotypic variation within the *L. plantarum* group isolated from the two different Italian cheeses. Further investigation and development as potential probiotic strains is required.

摘要 – 意大利干酪中*Lactobacillus plantarum* 菌群的多样性和功能特性。为了增加对野生 型乳杆菌(*Lactobacillus*)菌群的生态学和生物多样性的了解， 本文利用表型和基因鉴定法研 究了意大利干酪 (Italian cheese) 中植物乳杆菌 (*Lactobacillus plantarum*) 菌群的分布。并通 过体外实验探讨了这些菌株的潜在益生菌功能特性。结果显示不同菌株的生理和生化特征 完全不同。以引物 M13 进行 RAPD-PCR 实验，发现不同菌株之间的RAPD图谱有显著差 异，可以归 为干酪乳杆菌 (*L. plantarum*), 戊糖乳杆菌 (*L. pentosus*) 和副干酪乳杆菌 (*L. paraplantarum*)。关于益生菌的功能特性，源于Caciotta 干酪中的菌株 *L. plantarum* 31C 和 143C 及 Fiore Sardo 干酪中的 *L. plantarum* 64FS 和 61FS 菌株都能在模拟肠道环境中 存活，意味着这些菌是耐酸和耐胆汁的。干酪中大部分乳杆菌能抑制大肠杆菌ATCC 43895 和 *L. sakei* DSM 20017 的生长，但只有一株菌能产生类细菌素的化合物。结果显示表型和基因鉴 定法均能得到两种不同意大利干酪中植物乳杆菌群的变化。但是其中的潜在益生菌特性则 需要进一步研究。

Keywords *Lactobacillus plantarum*-group · Caciotta cheese · Fiore Sardo cheese · RAPD-PCR · probiotics

关键词 植物乳杆菌群 · Caciotta 干酪 · Fiore Sardo 干酪 · RAPD-PCR · 益生菌

1 Introduction

The genus *Lactobacillus* is the largest and, perhaps, the most important genus of lactic acid bacteria, representatives of which play a significant role in the human and animal gastrointestinal tract. Moreover, lactobacilli represent one of the major groups involved in desirable fermentation and contribute to food preservation (Coeuret et al. 2003; Holzapfel 2002).

Within the genus *Lactobacillus*, *L. plantarum* is a heterogeneous and versatile species that is encountered in a variety of environmental niches, including dairy, meat and many vegetable or plant fermentations. It is one of a group of mesophilic lactobacilli which may become the dominant microorganism in several types of cheese during ripening (Hynes et al. 2003; Rantsiou et al. 2008; Sánchez et al. 2005). Its predominance has also been documented in different African traditional fermented milk products such as *Ititu* (Kasseye et al. 1991), *Gari* (Kostinek et al. 2005b) and *Kule naoto* (Mathara et al. 2004, 2008).

Lactobacillus plantarum strains are characterized by a highly variable phenotype (Mathara et al. 2008) and a relatively large genome (Kleerebezem et al. 2003), which explain their wide distribution and high interstrain diversity. This, unfortunately, complicates their discrimination from the closely related species of *L. pentosus* and *L. paraplantarum* on the basis of phenotypic methods alone (Kostinek et al. 2005b). Species-specific primers were designed to obtain a clear distinction among these species but unfortunately they did not guarantee a sufficient level of specificity (Berthier and Erlich 1998; Torriani et al. 2001). Determination of the diversity of the

L. plantarum population to strain level necessitates the use of a rapid method suitable for handling large number of isolates. Randomly amplified polymorphic DNA (RAPD) analysis has been used to investigate the diversity of *L. plantarum* strains isolated from different sources (Spano et al. 2002) to both the species or the strain level.

Lactobacillus plantarum also has a role as a potential probiotic organism. Even though definitions of probiotic bacteria originally included intestinal source strains, currently, many non-starter lactic acid bacteria, some non-lactics (Holzapfel et al. 1998) and also some yeasts, especially *Saccharomyces boulardii*, are used in commercial probiotic products. For this reason, the search for strains which show resistance to biological barriers of the human gastrointestinal tract, and which possess physiological characteristics compatible with probiotic properties may eventually lead to the finding of new probiotic strains for functional dairy foods.

Therefore, this work aimed to study the diversity of *L. plantarum*-group strains, isolated from artisanal dairy products by phenotypic characterization and RAPD-PCR techniques and to evaluate their potential probiotic properties.

2 Materials and methods

2.1 Bacterial strains and growth conditions

A total of 57 strains phenotypically assigned to the *L. plantarum*-group were examined in this study. Thirty-two strains were isolated from Fiore Sardo, a PDO raw milk cheese (Pisano et al. 2007) and twenty-five from Caciotta cheeses obtained from raw, pasteurized or high-pressure-homogenized (HPH) cow milk (Lanciotti et al. 2006). The strains were grown in de Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C for 24 h. Pure cultures were obtained by streaking out onto MRS agar (Merck, Darmstadt, Germany). Stock cultures were stored at −18 °C in MRS broth containing 15% glycerol. Working cultures were prepared from frozen stocks and were transferred at least twice in MRS broth before use in experiments.

2.2 Phenotypic characterization

Growth at 15 °C and 45 °C was determined in MRS broth after incubation for 7 and 2 days, respectively. Salt tolerance was determined using MRS broth containing 6.5% NaCl incubated for 48 h at 37 °C. CO2 production from glucose, hydrolysis of arginine and determination of the presence of *meso*-di-aminopimelic acid (*m*-DAP) in the cell wall were determined according to the chromatographic method by using thin-layer chromatography on cellulose plates (Schillinger and Lücke 1987).

Carbohydrate fermentation patterns were determined in microtiter plates as previously described (Jaine-Williams 1976). Sugars tested during growth at 30 °C for 48 h included amygdalin, arabinose, cellobiose, esculin, galactose, glucose, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and xylose (Merck, Darmstadt, Germany). The type strains *L. plantarum* ATCC 20174 T, *L. plantarum* ATCC 8014, *L. paraplantarum* LTH 5200 T and *L. pentosus* DSM 20314 T were included as reference species. Strain ATCC 8014 was received from Agrotechnical Research Institute, Wageningen, The

Netherlands. The production of both D(−) and L(+) lactate enantiomers from glucose fermentation was enzymatically determined in the cell-free supernatant from 24 h cultures in MRS broth, using an UV enzymatic kit (Boehringer-Mannheim, Mannheim, Germany).

2.3 Genotypic characterization

2.3.1 DNA isolation

Total genomic DNA was isolated using the guanidium thiocyanate extraction method (Pitcher et al. 1989), as modified for Gram-positive microorganisms (Björkroth & Korkeala 1996).

2.3.2 Genetic fingerprinting by RAPD-PCR

Randomly amplified polymorphic DNA (RAPD)-PCR analysis was used to determine the heterogeneity of the *L. plantarum* strains and to compare genotypes isolated from different sources. *L. plantarum* ATCC 8014, *L. paraplantarum* LTH 5200 T, *L. arizonensis* DSM 13273 T, *L. pentosus* DSM 20314 T, *L. plantarum* DSM 20174 T, *L. plantarum* CNRZ 1228 and *L. plantarum* BFE 617 type or reference strains were included in the study for comparison purposes. Amplification was performed in a Primus 96 plus Thermal Cycler (MWG Biotech, Ebersberg, Germany) using primer M13 (5′-GAG GGT GGC GGT TCT-3′) (Andrighetto et al. 2001). PCR was performed in 50 μL reaction mixture volumes each containing 250 $\mu mol \cdot L^{-1}$ dNTPs, 1 $\mu mol \cdot L^{-1}$ primer, 2.5 $mmol \cdot L^{-1}$ $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Amersham, Buckinghamshire, UK), 1 X PCR buffer and 10 μL of template DNA. PCR products were analyzed by gel electrophoresis on 1.8% (w/v) agarose gels using 1 X TBE buffer. The gels were run for 16 h at 48 V, stained with ethidium bromide and visualized with UV transilluminator. Photographs of RAPD-PCR gels were scanned and the electrophoretic profiles were analyzed by BioNumerics (version 2.5) software (Applied Maths, Sint-Martens-Latem, Belgium). Grouping of the RAPD-PCR fingerprints was performed by means of the Pearson product-moment correlation coefficient (r) and UPGMA clustering algorithm.

2.4 Antagonistic activity

The agar spot test was used for monitoring the antagonistic activity of the tested strains against *L. sakei* subsp. sakei DSM 20017^T and *E. coli* ATCC 43895 (Schillinger and Lücke 1989). In addition, the agar spot test method used by Uhlman et al. (Uhlman et al. 1992) was further used in order to test the activity of cell-free-neutralized supernatants. Briefly, cell-free-neutralized supernatants were obtained from overnight producer cultures grown in MRS broth at 37 °C. After centrifuging the culture (7,200 × g, 10 min), the supernatants were neutralized with sterile 5 $mol \cdot L^{-1}$ NaOH and then boiled for 5 min to inactivate residual viable cells. The supernatants were tested against the same indicator strains used above. In order to establish the proteinaceous nature of the inhibitory compounds, sensitivity to the proteolytic enzyme protease (Sigma, Milan,

Italy) of the cell-free supernatants was tested. Samples of 100 μL were incubated for 2 h in the presence of 1 mg·mL^{-1} (final concentration) enzyme and tested for antimicrobial activity by using the agar spot testmethod as described before.

2.5 Survival under gastric and intestinal conditions

2.5.1 Acid resistance and bile tolerance: preliminary selection of strains

Acid resistance was determined according to the method described by Hydrominus et al. (Hydrominus et al. 2000). The *Lactobacillus* overnight cultures were centrifuged (13,000× g, 4 min), resuspended in MRS broth adjusted to pH 2.5 and 2.0 with 5 mol L^{-1} HCl and cultured for 2 h at 37 °C. The survival was evaluated by determining viable counts after 0 and 2 h of incubation at 37 °C by plate counting on MRS agar (incubation for 48 h at 37 °C, anaerobic conditions). Bile salt tolerance was assessed by resuspending cells in MRS broth (pH 6.5, 2.5 and 2.0) containing 0.3% (w/v) Oxgall (Sigma, Munich, Germany), and determining their growth at 37 °C for 48 h. The positive control comprised inoculated MRS broth without bile salts.

2.5.2 Response in simulated stomach duodenum-passage

To evaluate the ability of selected strains to survive the gastrointestinal barriers, a simulated stomach duodenum-passage (SSDP) test was performed similar to that of Vizoso Pinto et al. (2006). Overnight cultures of the tested strains were diluted (1:10) in quarterstrength Ringer solution and their absorbance was determined at 600 nm. The strains were inoculated to a final concentration of 2×10^8 cell mL^{-1} in 10 mL of MRS broth adjusted to pH 3.0 with 5 mol·L^{-1} HCl. Initial viable counts were determined on MRS agar (incubated at 37 °C for 24–48 h under anaerobic conditions). After 1 h, 4 mL of 10%Oxgall (Sigma) and 17 mL of synthetic duodenal secretion (pH 7.4), consisting of 6.4 g·L^{-1} $NaHCO_3$, 0.239 g·L^{-1} KCl and 1.28 g·L^{-1} NaCl (Merck, Darmstadt, Germany), were added to the cell suspensions contained in the flask. After 1, 2 and 3 h of incubation at 37 °C, the survival rate was determined by the plate method at the conditions described above.

3 Results and discussion

3.1 Phenotypic characterization of *L. plantarum*-group strains

All 57 strains were presumptively assigned to the *L. plantarum*-group on the basis of the phenotypical tests (Table 1). These strains were all catalase-negative, facultatively heterofermentative rods, which contained *m*-DAP in their cell walls and which produced both the D and L lactate enantiomers. Moreover, all were able to grow at 15 °C. None of the strains from Fiore Sardo cheese was able to hydrolyze arginine, while one strain from Caciotta cheese was positive for this trait. Seven strains from Caciotta cheese were able to grow at 45 °C. Despite their mesophilic

Table 1 Physiological and biochemical characteristics of the *L. plantarum* group strains isolated from the two different cheeses.

Characteristics		Fiore Sardo strains		Caciotta strains	
		No. and % of positive strains		No. and % of positive strains	
m-DAP		32	100	25	100
Growth at	15 °C	32	100	25	100
	45 °C	0	0	7	28
	6.5% NaCl	32	100	25	100
Hydrolysis of Arginine		0	0	1	4
Hydrolysis of Aesculin		32	100	25	100
Fermentation of:	Amygdaline	29	90.6	25	100
	L-arabinose	32	100	16	64
	Cellobiose	29	90.6	25	100
	Galactose	32	100	25	100
	Lactose	32	100	25	100
	Maltose	32	100	25	100
	Melezitose	5	15.6	21	84
	Melibiose	32	100	21	84
	Raffinose	15	46.8	4	16
	Ribose	32	100	21	84
	Salicine	32	100	25	100
	Sorbitol	12	37.5	21	84
	Sucrose	5	15.6	25	100
	Trehalose	32	100	25	100
	Xylose	3	9.4	7	28
	Mannitol	32	100	25	100
	Amygdaline	32	100	25	100
Lactate isomer produced		DL (all strains tested)		DL (all strains tested)	

aptitude, some *L. plantarum* strains have already been reported to grow at this high temperature (Kostinek et al. 2008; Stiles and Holzapfel 1997).

With respect to the sugar fermentation patterns, it was noted that a high number of strains from both types of cheese were able to ferment pentoses such as ribose and L-arabinose, which is consistent with their characterization as facultatively heterofermentative *L. plantarum* strains. In contrast, some variations were also observed between the strains stemming from the two different cheeses. In particular, a very low percentage (15.6%) of strains from Fiore Sardo cheese was able to ferment melezitose and sucrose when compared with the 84% melezitose-positive and 100% sucrose-positive strains from Caciotta. Raffinose was fermented by 46.8% and 16% of strains isolated from Fiore Sardo and Caciotta cheeses, respectively. Furthermore, 10 strains (three from Fiore Sardo and seven from Caciotta) fermented xylose, which is a typical characteristic of *L. pentosus* strains.

3.2 Genotypic characterization

The RAPD profiles of the cheese strains generated with primer M13 produced the UPGMA dendrogram shown in Fig. 1. RAPD-PCR was done in duplicate for some reference strains to determine the reproducibility of the method. Cluster analysis showed correlation values of r=90%, 87% and 80% for the duplicates of the strains *L. paraplantarum* LTH 5200 T, *L. plantarum* ATCC 8014 and *L. arizonensis* DSM 13273 T, respectively, and 77.8% correlation between the duplicates of the strain *L. pentosus* DSM 20314^{T}. For *L. pentosus* DSM 20314 three separate profiles were actually done, as the correlation value obtained initially with two fingerprint sets of the same type strain was considered low. A third fingerprint clustered better one of the initial two prints at 77.8% and we took this value as the cutoff value for possible clonal relationships. A high diversity was revealed among the strains tested according to the heterogeneity in the fingerprint patterns obtained (Fig. 1). The strains were grouped in four clusters (I, II, III and IV) at r=15%. Cluster I included the *L. plantarum* type strains DSM 20174^{T}, *L. plantarum* CNRZ 1228, *L. plantarum* ATCC 8014 from duplicate DNA extractions, *L. plantarum* BFE 617, 16 isolates from Caciotta cheese and 14 from Fiore Sardo cheese. Cluster II included 17*L. plantarum*-group strains isolated from Fiore Sardo and three strains from Caciotta cheese. In addition, this cluster also contained the type strain of *L. arizonensis* DSM 13273^{T}. The species *L. arizonensis* was previously rejected by Kostinek et al. (2005a) as a later heterotypic synonym of *L. plantarum*. Cluster III included the type strains of *L. pentosus* DSM 20314^{T} and *L. paraplantarum* LTH 5200^{T}, three strains from Caciotta and one from Fiore Sardo. Cluster IV consisted of three strains from Caciotta cheese. Four out of seven strains included in these later two clusters were able to ferment xylose, a typical feature of *L. pentosus* species.

The RAPD analysis also revealed the occurrence of several subclusters poorly related to each other (similarity 30%), confirming the high genetic diversity of *L. plantarum* previously reported by other authors (Kostinek et al. 2008; Sánchez et al. 2005). Some strains had very similar fingerprinting patterns and showed a correlation coefficient >77.8%. This suggested that these strains could be multiple isolates of the same strain, since they were isolated from the same cheese. Although most subclusters were mainly constituted by strains isolated from Fiore Sardo or Caciotta cheese, no correlation between grouping and origin of the strains was found and RAPD types showing a high level of similarity were isolated from the two cheeses. For example, strains 75C, 64FS and 32FS clustered together at 83% similarity. Similar findings were obtained for strains 57C, 37C and 66FS.

3.3 Antagonistic activity

Most of the strains tested showed an inhibitory activity toward *E. coli* and *L. sakei* DSM 20017 (Table 2), most likely due to the production of organic acids. Only the supernatant of strain 143C was able to inhibit *E. coli* after neutralization and heating suggesting a possible production of a heat stable bacteriocin (data not shown). Complete inactivation in antimicrobial activity was observed after treatment of the cell-free supernatant with protease confirming its proteinaceous nature.

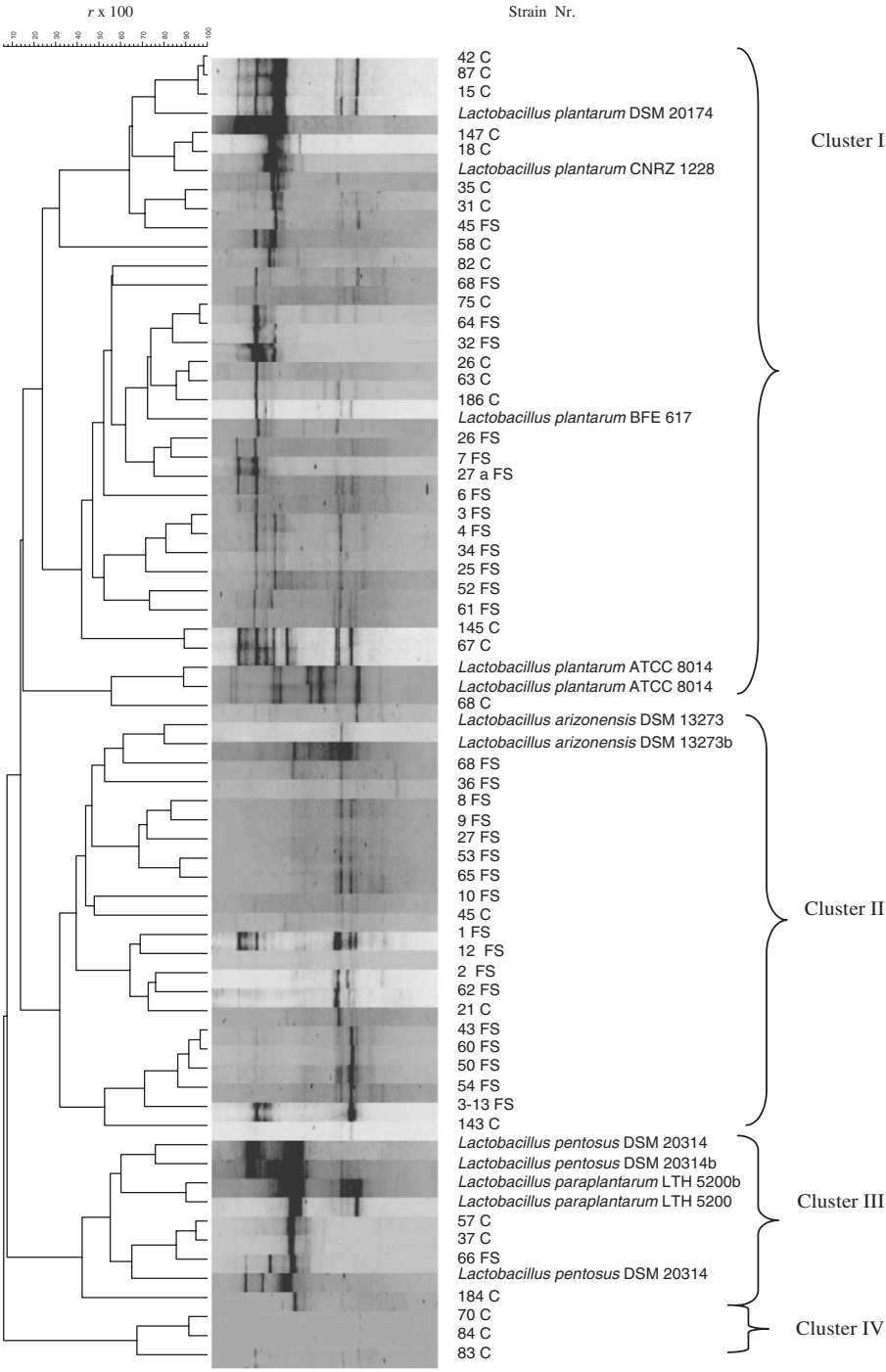

Fig. 1 Dendrogram obtained by UPGMA of correlation value r of RAPD-PCR fingerprint patterns with primers M13 of facultatively heterofermentative *L. plantarum* group isolates from Fiore Sardo and Caciotta cheese and reference strains

Table 2 Antagonistic activity of *L. plantarum* strains isolated from Fiore Sardo and Caciotta cheeses

Strains tested	Antimicrobial activity against	
	E. coli ATCC 43895	*L. sakei* DSM 20017
Fiore Sardo cheese		
1FS; 3—13FS; 52FS	+++	+
10FS; 3FS	+++	++
25FS	+++	−
45FS; 27FS; 6FS; 32FS; 53FS; 43FS; 61FS; 36FS; 81FS; 65FS; 50FS; 54FS	++++	++
2FS; 8FS	−	−
12FS	++++	−
26FS; 60FS; 9FS	++++	+
7FS	+++	−
64FS; 66FS; 34FS	++++	++
4FS	+++++	+
68FS; 27FS; 62FS	+++++	++
Caciotta cheese		
37C	++	++
18C	++	+
75C	+++	+
42C	+++	++
84C; 87C	−	−
15C; 147C; 45C; 21C; 82C; 35C; 186C; 145C; 67C; 184C; 68C; 57C; 70C; 83C	++	−
26C	++	++
31C	+	−
58C	++	++
63C	+++	−
143C	+++++	+++++

Inhibition was scored according to the width of the zone of clearing around the colonies of the test strain according these criteria: clear zone > 8 mm (+++++); clear zone 8-6 mm (++++); 4 mm < clear zone < 6 mm (+++); 2 mm < clear zone < 4 mm (++); 0.5 mm < clear zone < 2mm (+).

3.4 Resistance to simulated gastrointestinal conditions

Results suggested that generally these strains had a good resistance to pH 2.5 (> 75% of strains) (data not shown). On the contrary, only few strains were able to survive after exposure to pH 2.0 for 2 h. These included strains 31C, 184C and 143C from Caciotta cheese and the strains 10FS, 61FS, 64FS and 50FS from Fiore Sardo cheese (Table 3). All the strains grown in MRS broth at pH 2.0 and 2.5 were re-inoculated in MRS broth in the presence of 0.3% (w/v) bile salts and their growth was evaluated after 24 and 48 h of incubation at 37 °C (data not shown). Stresses to

Table 3 Tolerance of *L. plantarum* group strains to pH 2.0 for 2 h at 37 °C

Strains[a]	Initial count (log10 CFUs·mL)	Bacterial counts after 2 h incubation at 37 °C in MRS broth at pH 2.0 (log10 CFUs·mL)
10FS	9.34	4.48
64FS[b]	9.00	5.54
61FS[b]	8.90	4.25
50FS	8.98	4.55
31C[b]	9.68	5.14
184C	8.30	3.39
143C[b]	8.90	4.47

[a] The remainder of strains tested in this study exhibited a viability lower than 4 log CFU·mL^{-1} (data not shown). [b] Strains resistant to 0.3% bile.

microorganisms begin in the stomach, which has a pH between 1.5 and 3.0, and in the upper intestine which contains bile. Survival at pH 3.0 for 2 h and at a bile concentration of 1000 mg·L^{-1} is considered optimal acid and bile tolerance for potentially probiotic strains (Usman 1999). The *L. plantarum*-group strains 31C and 143C, isolated from Caciotta cheese manufactured from raw and HPH treated milk, respectively, and 64FS and 61FS, isolated from Fiore Sardo, seem particularly interesting, because they were able to survive 2 h incubation at pH 2.0 and in the presence of 0.3% bile. The same strains were also able to survive to SSDP, confirming their good adaptation capabilities (Table 4).

Table 4 Survival of *Lactobacillus* strains isolated from Fiore Sardo and Caciotta cheese in simulated stomach duodenum-passage at 37 °C. Results are means of three independent experiments

	Viable cell numbers ($\log_{10}$ CFUs·mL^{-1})				Growth (after 24 h)
	Time (h) of incubation in simulated stomach-duodenum juice				
Strain	0 h	1 h	2 h	3 h	24 h
		Fiore Sardo cheese			
64FS	8.87±0.04	6.39±0.13	6.39±0.07	6.34±0.16	W
61FS	8.98±0.04	6.54±0.33	7.03±0.60	6.27±0.26	+
50FS	8.00±0.06	6.73±0.06	6.60±0.05	6.30±0.06	−
10FS	8.23±0.04	6.39±0.13	5.99±0.12	6.05±0.21	−
		Caciotta cheese			
143C	8.14±0.30	7.10±0.35	6.81±0.15	6.00±0.30	+
31C	8.09±0.15	6.80±0.20	5.54±0.25	4.90±0.20	+
184C	8.13±0.25	5.05±0.05	−	−	−

W = very weak, + = growth detected, − = no growth detected.

4 Conclusion

This study is another contribution to the knowledge on the ecology and biodiversity of strains belonging to the *L. plantarum* group that were isolated from two different Italian cheeses. Phenotypic and genotypic characterization methods were used to study the diversity among these strains. Genotyping data obtained by RAPD-PCR analysis confirmed the previously observed high degree of heterogeneity of *L. plantarum* strains, and allowed to discriminate between the phenotypically closely related species *L. plantarum* and *L. pentosus*/*L. paraplantarum*. The in vitro study of some functional characteristics related to probiotic properties allowed to screen some *L. plantarum* group strains possessing good potential for further studies on their probiotic capacity. Clearly, to select strains for use as starter cultures or as probiotic requires more in vitro and in vivo trials. This study does, however, allow a pre-selection of potentially interesting cultures which can be further investigated for their probiotic activity.

Acknowledgements The authors are particularly grateful to Frau E. Lubecki and Dr J. Maina Mathara for their technical support and to all the staff of the BFEL Institute.

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