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Leuconostoc strains isolated from dairy products: Response against food stress conditions

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ABSTRACT

A systematic study about the intrinsic resistance of 29 strains (26 autochthonous and 3 commercial ones), belonging to *Leuconostoc* genus, against diverse stress factors (thermal, acidic, alkaline, osmotic and oxidative) commonly present at industrial or conservation processes were evaluated. Exhaustive result processing was made by applying one-way ANOVA, Student's test (*t*), multivariate analysis by Principal Component Analysis (PCA) and Matrix Hierarchical Cluster Analysis. In addition, heat adaptation on 4 strains carefully selected based on previous data analysis was assayed. The strains revealed wide diversity of resistance to stress factors and, in general, a clear relationship between resistance and *Leuconostoc* species was established. In this sense, the highest resistance was shown by *Leuconostoc lactis* followed by *Leuconostoc mesenteroides* strains, while *Leuconostoc pseudomesenteroides* and *Leuconostoc citreum* strains revealed the lowest resistance to the stress factors applied. Heat adaptation improved thermal cell survival and resulted in a cross–resistance against the acidic factor. However, all adapted cells showed diminished their oxidative resistance. According to our knowledge, this is the first study regarding response of *Leuconostoc* strains against technological stress factors and could establish the basis for the selection of "more robust" strains and propose the possibility of improving their performance during industrial processes.

1. Introduction

Leuconostoc is a heterofermentative lactic acid bacteria (LAB) genus, gas (CO_2), aroma compound (diacetyl, acetoin, acetate, ethanol and 2, 3-butylenglycol) producer, used in diverse fermentative food manufactures (Björkroth and Holzapfel, 2006; Hemme and Foucaud-Scheunemann, 2004; Macedo and Malcata, 1997; van Hylckama Vlieg and Hugenholtz, 2007; Vedamuthu, 1988). In the dairy industry, *Leuconostoc* is commonly used in combination with *Lactococcus* (mixed starters) for stimulation of flavor compound production (Server-Busson et al., 1999) and improvement of the texture by gas production.

LAB used as starter cultures are exposed to many adverse factors (stress factors) during their preparation and storage as well as during the fermented product manufacture. The stress factors are diverse and include pH variation (acidity or alkalinity), temperature (heat and cold), oxidative and osmotic changes, between others (van de Guchte et al., 2002; Zotta et al., 2008). As other bacteria, LAB have developed sophisticated defense mechanisms against stress factors, allowing them to survive under adverse growth conditions and/or sudden environmental changes. Some of the stress–induced genes seem to be genuinely specific, while others are induced by a wide variety of stress factors and are thus thought to be general stress response genes (cross – resistance) (Capozzi et al., 2016; Serrazanetti et al., 2009; van de Guchte et al., 2002). On the other hand, when a strain is subjected to sub-lethal stress conditions for some time (adaptation) a transient induction of stress proteins, specific and/or general is produced. In particular, thermal adaptation of bacterial cells is characterized by induction of general stress proteins and, consequently, this situation could be a resource to improve the robustness of strains face to diverse stress conditions (De Angelis and Gobbetti, 2011). The study of the

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diversity responses of LAB to stress conditions has a high practical relevance because aids the comprehension of response mechanisms and would allow a better starter selection, resulting in higher performance and improved survival level during the process (Desmond et al., 2004).

There are several studies regarding the response of LAB of industrial importance against diverse stress factors. In this sense, the most studied species were *Streptococcus thermophilus* (Arena et al., 2006; Giliberti et al., 2002; Thibessard et al., 2001; Zotta et al., 2008), *Lactobacillus delbrueckii* (Monnet et al., 2003; Streit et al., 2007), *Lactobacillus helveticus* (Di Cagno et al., 2006; Guerzoni et al., 2001) and *Lactobacillus plantarum* (De Angelis et al., 2004; Derzelle et al., 2000; Ferrando et al., 2015, 2016; Glaasker et al., 1998; Parente et al., 2010; Zotta et al., 2012). On the contrary, response to stress factors of *Leuconostoc* genus has not been studied relevantly. There are, however, several studies reported on *Oenococcus oeni*, a genus highly related to *Leuconostoc* and used as starter in wine fermentation (Bourdineaud et al., 2003; Grandvalet et al., 2005; Guzzo et al., 2000; Jobin et al., 1999; Le Marrec et al., 2007).

The aim of this study was to investigate the response of *Leuconostoc* strains to several unfavorable growth and conservation conditions. Systematic analysis of the results would allow performing a correct selection of the strains with better intrinsic resistance to be used for industrial purposes.

2. Material and methods

2.1. Strains and culture conditions

A total of twenty nine (29) *Leuconostoc* strains isolated from cheese-making ingredients (whey protein concentrate, pasteurized milk and whey cream) and soft Cremoso Argentino cheeses, were used in this work. The source and taxonomic identification of these strains are shown in Table 1. They were stored frozen at −20 °C and −80 °C, in MRS broth (Biokar, Beauvois, France), added of 15% (v/v) of glycerol as cryoprotective agent. For their routinely use, the strains were reactivated in MRS broth (Biokar) for 24 h at 30 °C and stored at 8 °C.

2.2. Genetic studies

2.2.1. Analysis of the 16 S rRNA gene (DNA sequencing)

Total DNA of the *Leuconostoc* strains were obtained by using the GeneElute™ Bacterial Genomic DNA kit (Sigma, St. Louis, MO, USA), following the manufacturer's instructions. The species was confirmed by sequencing a 1500 bp fragment within the 16 S rRNA gene (Edwards et al., 1989). PCR reactions were performed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA) following conditions previously published (Pujato et al., 2014). Nucleotide sequences of purified amplicons were determined at the DNA Sequencing Service of Macrogen (Seoul, Korea). The identity of isolates was established by nucleotide-nucleotide BLAST of the NCBI database (www.ncbi.nlm.nhi.gov/blast).

2.2.2. Random amplification of polymorphic DNA-PCR (RAPD-PCR)

Bacterial DNA was extracted by the chelex method for Gram-positive (and acid-fast) bacteria according to Giraffa et al. (2000). Two primers (Biotez, Berlin, Germany) were used for generation of the PCR profiles: the M13 minisatellite core sequence (Huey and Hall, 1989) (5′-GAGGGTGGCGGTTCT - 3′), and primer R5 (Aymerich et al., 2006) (5′-AACGCGCAAC - 3′). 1 Kb Plus DNA Ladder (Invitrogen, Milan, Italy) was used as DNA molecular weight marker. Gels were photographed under UV illumination using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290, Celbio, Milan, Italy) equipped with the EDAS 290

Table 1
Source and taxonomic identification of the *Leuconostoc* strains studied.

Strain	Taxonomic identification*	Source
Ln MB7	*Leuconostoc mesenteroides*	Soft cheese [a]
Ln LT-3	*Leuconostoc mesenteroides*	Soft cheese [a]
Ln N18	*Leuconostoc mesenteroides*	Whey protein concentrate (WPC) [b]
Ln MB8	*Leuconostoc mesenteroides*	Soft cheese [a]
Ln D4	*Leuconostoc mesenteroides*	Pasteurized milk [b]
Ln D14	*Leuconostoc mesenteroides*	Whey protein concentrate (WPC) [b]
Ln N4	*Leuconostoc mesenteroides*	Soft cheese [a]
Ln N19	*Leuconostoc mesenteroides*	Soft cheese [b]
Ln N12	*Leuconostoc mesenteroides*	Semi-hard cheese [c]
Ln D2	*Leuconostoc mesenteroides*	Soft cheese [b]
Ln D10	*Leuconostoc mesenteroides*	Whey protein concentrate (WPC) [b]
Ln D11	*Leuconostoc mesenteroides*	Soft cheese [b]
Ln L79-1	*Leuconostoc mesenteroides*	Commercial strain
Ln LcR-1	*Leuconostoc mesenteroides*	Commercial strain
Ln R707	*Leuconostoc pseudomesenteroides*	Commercial strain
Ln N17	*Leuconostoc pseudomesenteroides*	Soft cheese [b]
Ln N1	*Leuconostoc pseudomesenteroides*	Soft cheese [a]
Ln LN-B	*Leuconostoc pseudomesenteroides*	Pasteurized milk [a]
Ln MB2	*Leuconostoc pseudomesenteroides*	Soft cheese [a]
Ln D6	*Leuconostoc pseudomesenteroides*	Pasteurized milk [b]
Ln N14	*Leuconostoc pseudomesenteroides*	Petit Suisse cheese [d]
Ln LT-1	*Leuconostoc pseudomesenteroides*	Soft cheese [b]
Ln MB4	*Leuconostoc pseudomesenteroides*	Soft cheese [a]
Ln D16	*Leuconostoc pseudomesenteroides*	Soft cheese [a]
Ln D5	*Leuconostoc lactis*	Whey cream [b]
Ln LS	*Leuconostoc lactis*	Pasteurized milk [e]
Ln N6	*Leuconostoc lactis*	Pasteurized milk [a]
Ln D1	*Leuconostoc lactis*	Pasteurized milk [b]
Ln MB1	*Leuconostoc citreum*	Soft cheese [a]

* Taxonomic identification was made by 16 S rRNA gene sequence analysis (DNA sequencing). Superscript letters indicate the origin of isolation.

imaging cabinet. Images were saved as TIFF files and analyzed with the pattern analysis software package BioNumerics™ (version 5.0; Applied Maths BVBA, Saint-Martens-Latem, Belgium). Calculation of similarity of band profiles was based on the Pearson correlation coefficient *r*. A dendrogram was deduced from the matrix of similarities by the unweighted pair group method using arithmetic average clustering algorithm (Vauterin and Vauterin, 1992).

2.3. Determination of incubation conditions – growth kinetics

Fresh strain cultures were inoculated (2%, v/v) in MRS broth and incubated 24 h at 30 °C. Absorbance ($O.D._{570\ nm}$) values were determined at intervals of 30 min, using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific Inc.). For each strain, the incubation time needed to reach the same cell physiology state (early stationary phase) was determined from their respective curves ($O.D._{570nm}$ values against time). Assays were performed by triplicate in independent trials.

2.4. Stress treatments

All stress treatments were applied at the exponential and the stationary phase growth of the strains. For each strain, cells at the stationary phase were obtained from cultures grown in MRS broth and incubated at 30 °C for the period of time calculated in section 2.3. Cells in exponential phase were obtained from cultures incubated (30 °C) until optical density of 0.5–0.6, measured at 560 nm ($O.D._{560\ nm}$). After respective incubation time, cells were centrifuged at 6000 × *g* for 5 min. To reach the same cell density in both conditions (stationary and exponential phase), the pellets were washed twice with sodium phosphate buffer 10 mM pH 7.0 (PB 7) and suspended in diverse media (depending the stress factor studied) until the same initial volume, for stationary phase cells, and concentrating 5 times for exponential phase cells (Ferrando et al., 2015; Parente et al., 2010; Zotta et al., 2008).

The stress treatments assayed were: i) MRS broth, 15 min–55 °C (thermal shock), ii) sodium-lactate buffer (1 M) pH 4, 30 min - 30 °C (acidic shock), iii) NaOH - glycine buffer (0.1 M) pH 9.8, 24 h - 30 °C (alkaline shock), iv) NaCl aqueous solution (30% w/v), 24 h - 30 °C (osmotic shock) and v) hydrogen peroxide (H_2O_2) 0.3% (p/v) solution, 30 min - 30 °C (oxidative shock). Cells suspended in PB 7 buffer and maintained at 30 °C for the corresponding incubation time, were used as controls. Resistance index (RI), defined as $RI = \log N_0/N_f$ (N_0 = initial cell count; N_f = final cell count), was calculated at each case. Cell counts were made in surface, using MRS agar and incubating 72 h at 30° C, in microaerophilia. Assays were performed by triplicate in independent trials.

2.5. Thermal pre-treatment and further stress treatments

Four (4) strains, selected on the basis of their general stress resistance in stationary phase (section 2.4), were subjected to thermal adaptation. With this aim, cells grown until stationary growth phase were washed twice with PB 7 and suspended in fresh MRS broth. Thermal pre-treatment was performed in water bath for 30 min at 40 °C (10 °C over the optimal growth temperature) (De

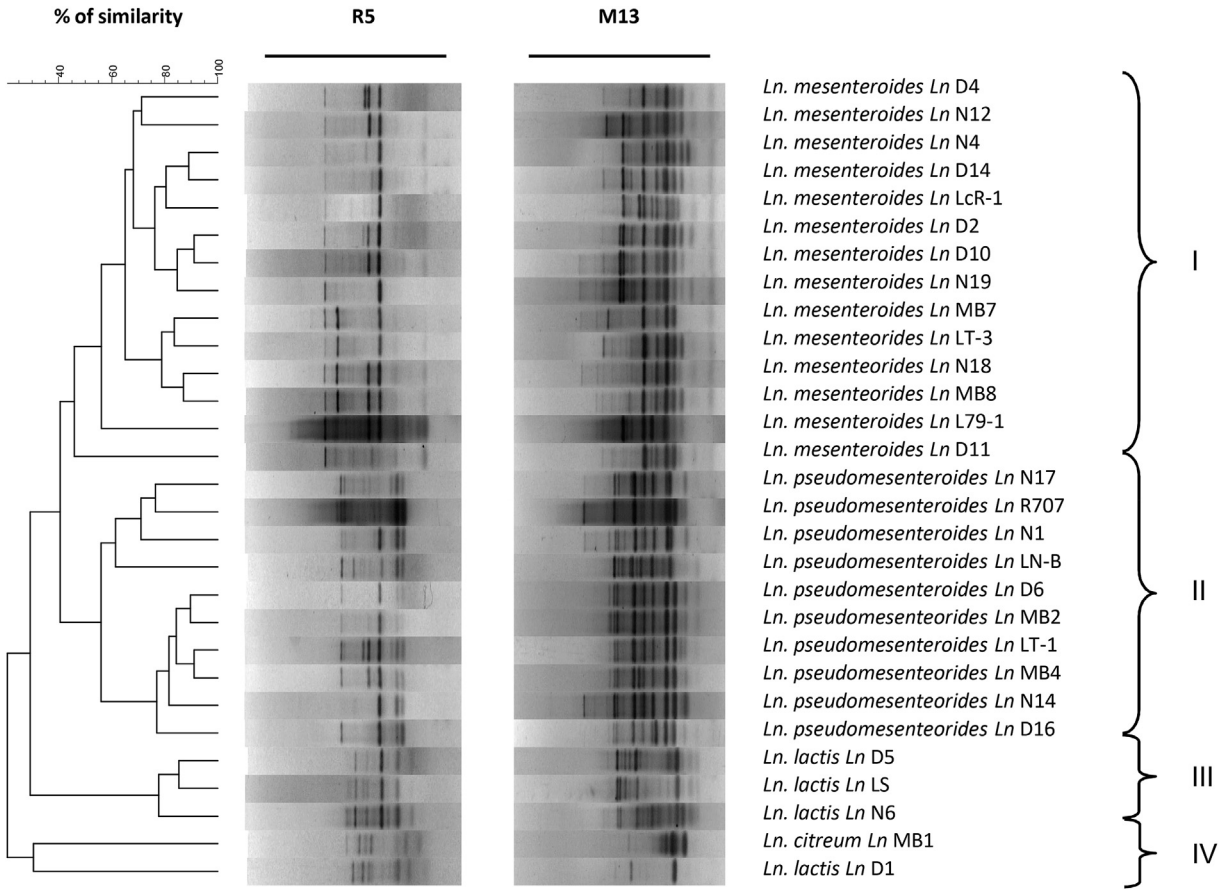

Fig. 1. RAPD-PCR profiles obtained for *Leuconostoc* strains using primers M13 and R5, and the corresponding dendrogram derived from the unweighted pair group average linkage of Pearson correlation coefficients (expressed as a percentage value). Four clusters (I, II, III and IV) obtained by similarity are also shown.

Angelis and Gobbetti, 2004; Ferrando et al., 2015). The cells thus obtained ("thermal adapted cells") were subjected to thermal, acidic and oxidative stress, as previously described (section 2.4). New RIs values were calculated and compared with those obtained for non-adapted cells.

2.6. Statistical analysis

Data was processed by applying one-way ANOVA, Student's test (t) and multivariate analysis by Principal Component Analysis (PCA), using IBM SPSS Statistics Version 21 (IBM Corp. 2012). Also, Matrix Hierarchical Cluster Analysis (Euclidean distance, Average linkage UPGMA method) was performed with the PermutMatrix program v.1.9.3 (LIRMM, France).

3. Results

3.1. Genetic studies

Sequencing of a 1500 bp fragment of the 16 S rRNA gene allowed identification of all *Leuconostoc* strains studied (Table 1). On the other hand, the genetic diversity was studied by RAPD-PCR analysis, using M13 and R5 as oligonucleotides. The dendrogram obtained showed four (4) clusters named I, II, III and IV (Fig. 1). Within some clusters, sub-clusters with diverse percentages of similarity were distinguished. Cluster I included all strains identified as *Leuconostoc mesenteroides* and three sub-clusters sharing 66% of similarity were detected. Strains *Ln* L79-1 and *Ln* D11 showed a lower similarity, 55% and 45% respectively, with the other sub-clusters. All the strains included in Cluster II belonged to *Leuconostoc pseudomesenteroides* species and two sub-clusters, with 56.1% of similarity between them, were observed. Finally, Cluster III included three strains belonging to *Leuconostoc lactis* species (*Ln* D5, *Ln* LS and *Ln* N6), while cluster IV grouped two strains, *Ln. lactis Ln* D1 and *Leuconostoc citreum Ln* MB-1. In general, wide genetic diversity among the strains used in this study was observed.

3.2. Determination of incubation conditions (stationary phase) – growth kinetics

According to growth kinetics obtained, *Leuconostoc* strains studied were split into five (5) different incubation conditions to reach the same physiologic state (early stationary phase). Fig. 2 shows a representative strain of each group: I) 12 h (*Ln* D2), II) 14 h (*Ln* N4), III) 16 h (*Ln* N18), IV) 18 h (*Ln* 79–1), and V) 24 h (*Ln* LN-B) of incubation. According to this classification, group I included *Ln* D2, *Ln* MB1, *Ln* D10 and *Ln* D14; group II, *Ln* LN4, *Ln* D4, *Ln* N12, *Ln* N6, *Ln* MB7, *Ln* MB8, *Ln* D5, *Ln* LS and *Ln* LT-3; group III, *Ln* N18, *Ln* N19, *Ln* D1, *Ln* N1 and *Ln* D11; group IV, *Ln* 79–1, *Ln* D16, *Ln* LT-1, *Ln* MB2, *Ln* D6, *Ln* LcR1, *Ln* N14 and *Ln* MB4; and group V, *Ln* LN-B, *Ln* R707 and *Ln* N17.

3.3. Stress treatments

A wide variability among the strains regarding resistance to all stress factors studied, both in stationary and exponential phase, was found (Tables 2 and 3). A high number of subgroups, with significant differences for each stress factor, were obtained when one-way ANOVA ($\alpha < 0.05$, Tukey's test *pos hoc*) was used as statistical analysis method. Strains *Ln* MB7, *Ln* D5, *Ln* N6, *Ln* D1 and *Ln* LS were the most resistant against thermal stress in both physiological states, with mean RI values between 0.36 and 0.91 for stationary phase, and between 0.93 and 2.25 for exponential phase. Strains with highest resistant to thermal shock were also the most resistant to acidic shock at both growth phases, with mean RI values between 0.04 and 0.81 for stationary state, and between 0.08 and 1.24 for exponential state. Oxidative stress produced the highest diversity of responses among the strains, observing 15

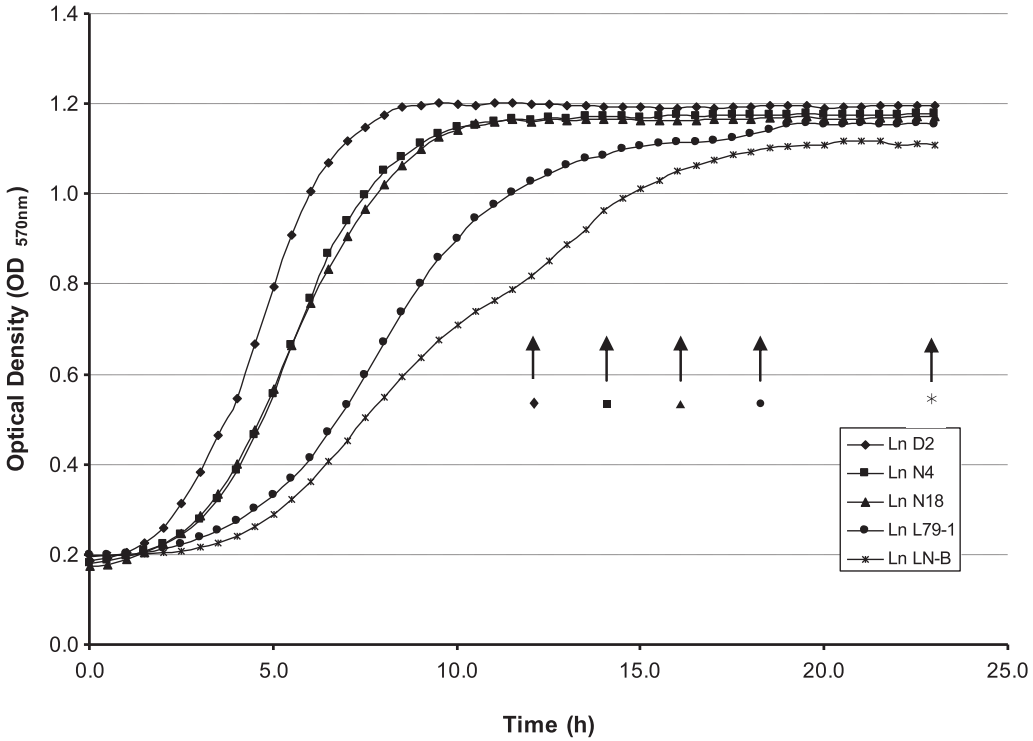

Fig. 2. Growth kinetics (24 h–30 °C) of *Leuconostoc* strains to determine the incubation time (arrows) to reach the same cell physiology state. The figure shows a selected strain for each type of growth kinetics.

Table 2
Resistance Index (RI) values of *Leuconostoc* strains in stationary phase of growth after thermal-, acidic-, oxidative-, osmotic- and alkaline shocks.

Strain	Resistance Index [a] after shock				
	Thermal	Acidic	Oxidative	Osmotic	Alkaline
Ln MB7	0.36 ± 0.25^{A}	0.26 ± 0.05^{AB}	4.42 ± 0.04^{GHI}	0.99 ± 0.28^{CDEFG}	3.73 ± 0.01^{EFGH}
Ln LT3	6.56 ± 0.60^{EFGH}	4.94 ± 0.68^{GHIJ}	3.95 ± 0.30^{EFGH}	0.16 ± 0.12^{AB}	2.43 ± 0.07^{BCDE}
Ln N18	5.36 ± 0.83^{CDEFGH}	6.66 ± 0.54^{IJKLM}	2.60 ± 0.16^{BCD}	0.84 ± 0.02^{ABCDEFG}	3.42 ± 0.14^{EFG}
Ln MB8	6.18 ± 0.66^{EFGH}	6.05 ± 1.45^{GHIJKLM}	2.87 ± 0.08^{CDE}	1.31 ± 0.10^{FGHI}	2.34 ± 0.21^{BCDE}
Ln D4	6.11 ± 0.72^{DEFGH}	8.20 ± 0.65^{M}	3.77 ± 0.15^{DEFGH}	1.31 ± 0.15^{FGHI}	4.08 ± 0.88^{FGHI}
Ln D14	5.22 ± 0.38^{CDEFGH}	7.26 ± 0.00^{KLM}	3.95 ± 0.04^{EFGH}	1.49 ± 0.40^{GHI}	3.24 ± 0.40^{EFG}
Ln N4	5.62 ± 0.15^{CDEFGH}	6.31 ± 1.04^{HIJKLM}	6.66 ± 0.81^{LM}	1.32 ± 0.04^{FGHI}	2.67 ± 0.07^{BCDEFG}
Ln N19	4.61 ± 0.29^{CDEFG}	4.20 ± 0.39^{EFGH}	3.23 ± 0.02^{CDEFG}	0.66 ± 0.04^{ABCDEFG}	1.66 ± 0.41^{ABCD}
Ln N12	4.87 ± 0.04^{CDEFG}	5.82 ± 0.02^{GHIJKL}	2.97 ± 0.16^{CDE}	0.28 ± 0.10^{ABCD}	3.52 ± 0.91^{EFGH}
Ln D2	4.35 ± 1.90^{CDEF}	5.55 ± 0.73^{GHIJKL}	2.12 ± 0.01^{BC}	0.70 ± 0.40^{ABCDEF}	2.61 ± 0.25^{BCDEF}
Ln D10	5.06 ± 1.64^{CDEFGH}	7.15 ± 0.22^{JKLM}	4.22 ± 0.39^{FGHI}	0.35 ± 0.06^{ABCD}	5.36 ± 0.08^{I}
Ln D11	3.46 ± 0.06^{BC}	5.32 ± 1.23^{GHIJK}	1.44 ± 0.09^{AB}	0.45 ± 0.29^{ABCDE}	0.52 ± 0.20^{A}
Ln L79-1	4.00 ± 0.01^{CDE}	5.77 ± 0.04^{GHIJKL}	3.04 ± 0.52^{CDEF}	1.02 ± 0.19^{DEFG}	3.00 ± 0.01^{CDEFG}
Ln LCR-1	3.47 ± 0.56^{CDE}	7.77 ± 0.36^{LM}	0.90 ± 0.10^{A}	1.13 ± 0.04^{EFG}	2.27 ± 0.55^{BCDE}
Ln R707	5.75 ± 0.33^{CDEFGH}	7.02 ± 0.85^{JKLM}	6.93 ± 0.34^{LMN}	1.99 ± 0.25^{IJ}	4.16 ± 0.21^{GHI}
Ln N17	5.21 ± 0.51^{CDEFGH}	4.16 ± 0.37^{EFGH}	8.48 ± 0.00^{O}	2.54 ± 0.10^{JK}	3.56 ± 0.47^{EFGH}
Ln N1	4.75 ± 0.97^{CDEFG}	2.49 ± 0.21^{CDEF}	3.99 ± 0.23^{EFGH}	1.32 ± 0.11^{FGHI}	5.00 ± 1.10^{HI}
Ln LNB	6.72 ± 1.04^{FGH}	4.09 ± 0.10^{EFGH}	8.11 ± 0.00^{NO}	2.93 ± 0.18^{K}	3.55 ± 0.21^{EFGH}
Ln MB2	5.84 ± 0.53^{CDEFGH}	2.64 ± 0.08^{DEF}	8.46 ± 0.00^{O}	1.18 ± 0.15^{EFGH}	1.45 ± 0.10^{AB}
Ln D6	4.90 ± 0.02^{CDEFG}	2.32 ± 0.35^{BCDE}	5.35 ± 0.22^{IJK}	0.90 ± 0.05^{BCDEFG}	3.21 ± 0.26^{DEFG}
Ln N14	7.19 ± 0.10^{GH}	4.63 ± 0.24^{FGHI}	7.67 ± 0.00^{MNO}	2.62 ± 0.16^{JK}	3.24 ± 0.00^{EFG}
Ln LT1	7.65 ± 0.35^{H}	2.20 ± 0.23^{ABCDE}	5.90 ± 0.91^{KL}	1.35 ± 0.04^{FGHI}	2.50 ± 0.44^{BCDE}
Ln MB4	6.52 ± 1.15^{EFGH}	3.95 ± 0.26^{EFG}	4.25 ± 0.01^{GHI}	1.91 ± 0.13^{HIJ}	2.28 ± 0.04^{BCDE}
Ln D16	4.20 ± 0.87^{CDEF}	2.18 ± 0.16^{ABCDE}	4.69 ± 0.30^{HIJ}	0.92 ± 0.04^{CDEFG}	1.39 ± 0.18^{AB}
Ln D5	0.54 ± 0.33^{A}	0.22 ± 0.12^{AB}	5.85 ± 0.21^{JKL}	2.26 ± 0.17^{JK}	1.44 ± 0.43^{AB}
Ln LS	0.91 ± 0.22^{AB}	0.81 ± 0.19^{ABCD}	6.40 ± 0.36^{KL}	1.89 ± 0.04^{HIJ}	1.39 ± 0.16^{AB}
Ln N6	0.73 ± 0.47^{A}	0.36 ± 0.32^{ABC}	6.20 ± 0.34^{KL}	0.55 ± 0.07^{ABCDE}	1.58 ± 0.13^{ABC}
Ln D1	0.47 ± 0.14^{A}	0.04 ± 0.03^{A}	4.36 ± 0.11^{GHI}	0.25 ± 0.21^{ABC}	2.49 ± 0.09^{BCDE}
Ln MB1	6.24 ± 0.57^{EFGH}	4.05 ± 0.62^{EFG}	2.54 ± 0.34^{BC}	0.14 ± 0.08^{A}	4.18 ± 0.11^{GHI}

[a] $RI = \log N_0/N_f$ (N_0 = initial cell count; N_f = final cell count) expressed as mean ± standard deviation; Different superscript letters in the same column indicate significant difference between mean RI values, using one-way ANOVA ($\alpha < 0.05$).

Table 3
Resistance Index (RI) values of *Leuconostoc* strains in exponential phase of growth after thermal-, acidic-, oxidative-, osmotic-c and alkaline shocks.

Strain	Resistance Index [a] after shock				
	Thermal	Acidic	Oxidative	Osmotic	Alkaline
Ln MB7	1.50 ± 0.04^{A}	1.24 ± 0.01^{A}	7.06 ± 0.87^{FGHIJ}	1.64 ± 0.25^{ABCDE}	3.58 ± 0.16^{BCDEFGH}
Ln LT3	6.83 ± 0.12^{CDEFGH}	8.30 ± 0.09^{IJK}	4.43 ± 0.29^{ABCDE}	1.32 ± 0.65^{ABCD}	3.08 ± 0.19^{BCD}
Ln N18	8.00 ± 0.28^{FGHI}	7.89 ± 0.21^{GHIJ}	6.55 ± 0.19^{FGHIJ}	1.82 ± 0.37^{ABCDE}	3.95 ± 0.17^{CDEFGHIJ}
Ln MB8	8.43 ± 0.42^{HI}	8.73 ± 0.00^{JK}	4.46 ± 0.49^{ABCDE}	2.25 ± 0.21^{BCDEFG}	3.67 ± 0.04^{BCDEFGHI}
Ln D4	6.17 ± 0.10^{BCDEF}	8.21 ± 0.34^{IJK}	7.90 ± 0.60^{J}	3.20 ± 0.07^{EFGHI}	5.57 ± 0.16^{LM}
Ln D14	4.89 ± 0.08^{B}	7.83 ± 0.20^{GHIJ}	5.71 ± 0.21^{DEFGH}	2.68 ± 0.27^{CDEFGH}	3.20 ± 0.76^{BCDE}
Ln N4	5.82 ± 0.08^{BCDE}	8.27 ± 0.00^{IJK}	5.41 ± 0.01^{CDEFG}	2.67 ± 0.59^{CDEFGH}	3.43 ± 0.08^{BCDEFG}
Ln N19	6.38 ± 0.08^{BCDEFG}	7.95 ± 0.09^{HIJK}	4.03 ± 0.09^{ABCD}	1.98 ± 0.45^{ABCDEF}	4.19 ± 0.12^{FGHIJ}
Ln N12	6.08 ± 0.64^{BCDE}	9.14 ± 0.51^{K}	7.00 ± 0.52^{FGHIJ}	0.96 ± 0.23^{ABC}	4.74 ± 0.14^{JKL}
Ln D2	5.08 ± 0.16^{BC}	7.28 ± 0.18^{GHI}	4.22 ± 0.32^{ABCDE}	1.90 ± 0.37^{ABCDE}	4.42 ± 0.39^{HIJK}
Ln D10	6.10 ± 0.00^{BCDE}	7.85 ± 0.71^{GHIJ}	3.30 ± 1.12^{A}	3.80 ± 0.23^{FGHIJ}	5.76 ± 0.34^{M}
Ln D11	7.09 ± 0.13^{DEFGHI}	5.93 ± 0.14^{DEF}	5.24 ± 0.30^{BCDEF}	1.58 ± 0.03^{ABCDE}	3.90 ± 0.01^{CDEFGHIJ}
Ln L79-1	7.28 ± 0.51^{EFGHI}	8.28 ± 0.12^{IJK}	6.05 ± 0.74^{EFGHI}	2.60 ± 0.19^{CDEFGH}	5.60 ± 0.16^{LM}
Ln LCR-1	6.45 ± 0.49^{BCDEFG}	8.01 ± 0.48^{HIJK}	3.57 ± 0.05^{ABC}	1.79 ± 0.04^{ABCDE}	5.64 ± 0.14^{LM}
Ln R707	5.80 ± 0.98^{BCDE}	8.78 ± 0.22^{JK}	8.39 ± 0.00^{J}	4.04 ± 0.41^{GHIJ}	5.29 ± 0.06^{KLM}
Ln N17	8.89 ± 0.00^{I}	5.46 ± 0.49^{D}	8.35 ± 0.01^{J}	2.16 ± 0.23^{BCDEF}	4.39 ± 0.21^{GHIJK}
Ln N1	5.60 ± 1.22^{BCDE}	6.93 ± 0.42^{FGH}	8.24 ± 0.00^{J}	2.24 ± 0.14^{BCDEFG}	4.57 ± 0.58^{IJK}
Ln LNB	8.71 ± 0.08^{I}	6.70 ± 0.08^{EFG}	8.19 ± 0.01^{J}	5.26 ± 0.55^{J}	4.81 ± 0.28^{JKLM}
Ln MB2	8.53 ± 0.00^{HI}	5.39 ± 0.05^{D}	8.31 ± 0.01^{J}	1.66 ± 0.05^{ABCDE}	4.15 ± 0.53^{EFGHIJ}
Ln D6	5.75 ± 1.39^{BCDE}	3.38 ± 0.35^{B}	7.83 ± 0.00^{IJ}	1.66 ± 0.10^{ABCDE}	3.20 ± 0.04^{BCDE}
Ln N14	6.06 ± 0.55^{BCDE}	5.55 ± 0.06^{DE}	7.93 ± 0.01^{J}	4.62 ± 0.40^{IJ}	4.51 ± 0.00^{HIJK}
Ln LT1	6.17 ± 0.03^{BCDEF}	3.90 ± 0.08^{BC}	7.21 ± 1.47^{GHIJ}	2.73 ± 0.06^{CDEFGH}	3.24 ± 0.09^{BCDEF}
Ln MB4	8.44 ± 0.00^{HI}	3.71 ± 0.14^{BC}	5.98 ± 0.77^{EFGH}	0.97 ± 0.29^{ABC}	2.98 ± 0.06^{BC}
Ln D16	5.29 ± 0.29^{BCD}	4.71 ± 0.06^{CD}	3.45 ± 0.05^{AB}	2.15 ± 0.06^{ABCDEF}	2.85 ± 0.11^{B}
Ln D5	1.91 ± 0.06^{A}	1.24 ± 0.45^{A}	6.65 ± 0.00^{FGHIJ}	4.15 ± 0.28^{HIJ}	1.05 ± 0.02^{A}
Ln LS	2.07 ± 0.11^{A}	0.13 ± 0.01^{A}	7.04 ± 0.00^{FGHIJ}	2.95 ± 0.17^{DEFGHI}	1.21 ± 0.05^{A}
Ln N6	2.25 ± 0.00^{A}	0.67 ± 0.02^{A}	4.07 ± 0.11^{ABCD}	3.27 ± 1.92^{EFGHI}	0.79 ± 0.03^{A}
Ln D1	0.93 ± 0.22^{A}	0.08 ± 0.01^{A}	8.24 ± 0.12^{J}	0.34 ± 0.14^{A}	4.01 ± 0.06^{DEFGHIJ}
Ln MB1	8.08 ± 0.34^{GHI}	5.90 ± 0.03^{DEF}	7.33 ± 0.00^{HIJ}	0.67 ± 0.20^{AB}	4.48 ± 0.12^{HIJK}

[a] $RI = \log N_0/N_f$ (N_0 = initial cell count; N_f = final cell count) expressed as mean ± standard deviation; Different superscript letters in the same column indicate significant difference between mean RI values, using one-way ANOVA ($\alpha < 0.05$).

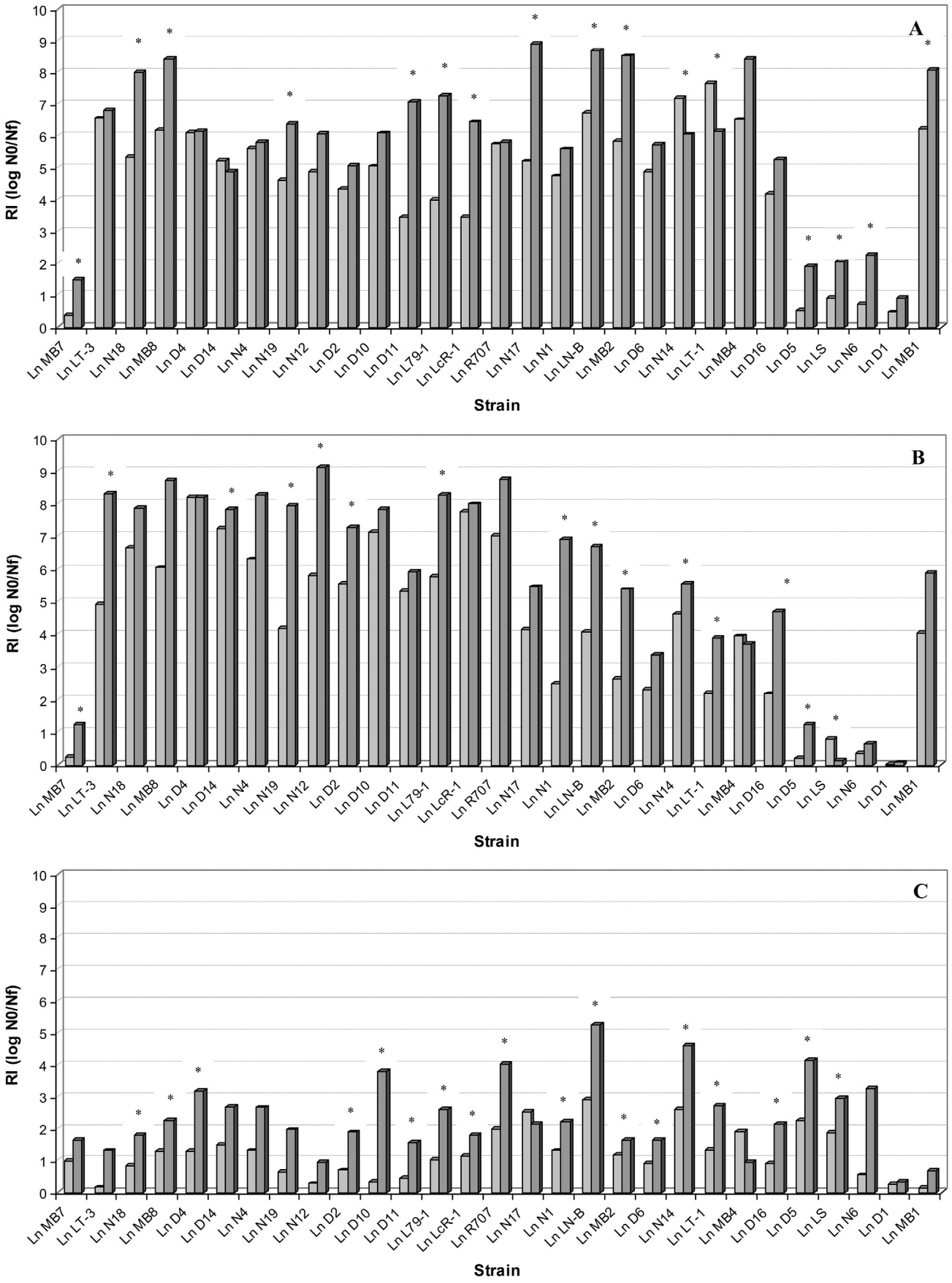

Fig. 3. Resistance Index (RI) (mean ± standard deviation) obtained for strains in stationary (□) and in exponential (■) phase of growth for thermal (A), acidic (B), osmotic (C), alkaline (D) and oxidative (E) shocks. Asterisks (*) correspond to strains with mean RI values significantly different (Test *t* de Student, $\alpha = 0.05$) between both growth phases.

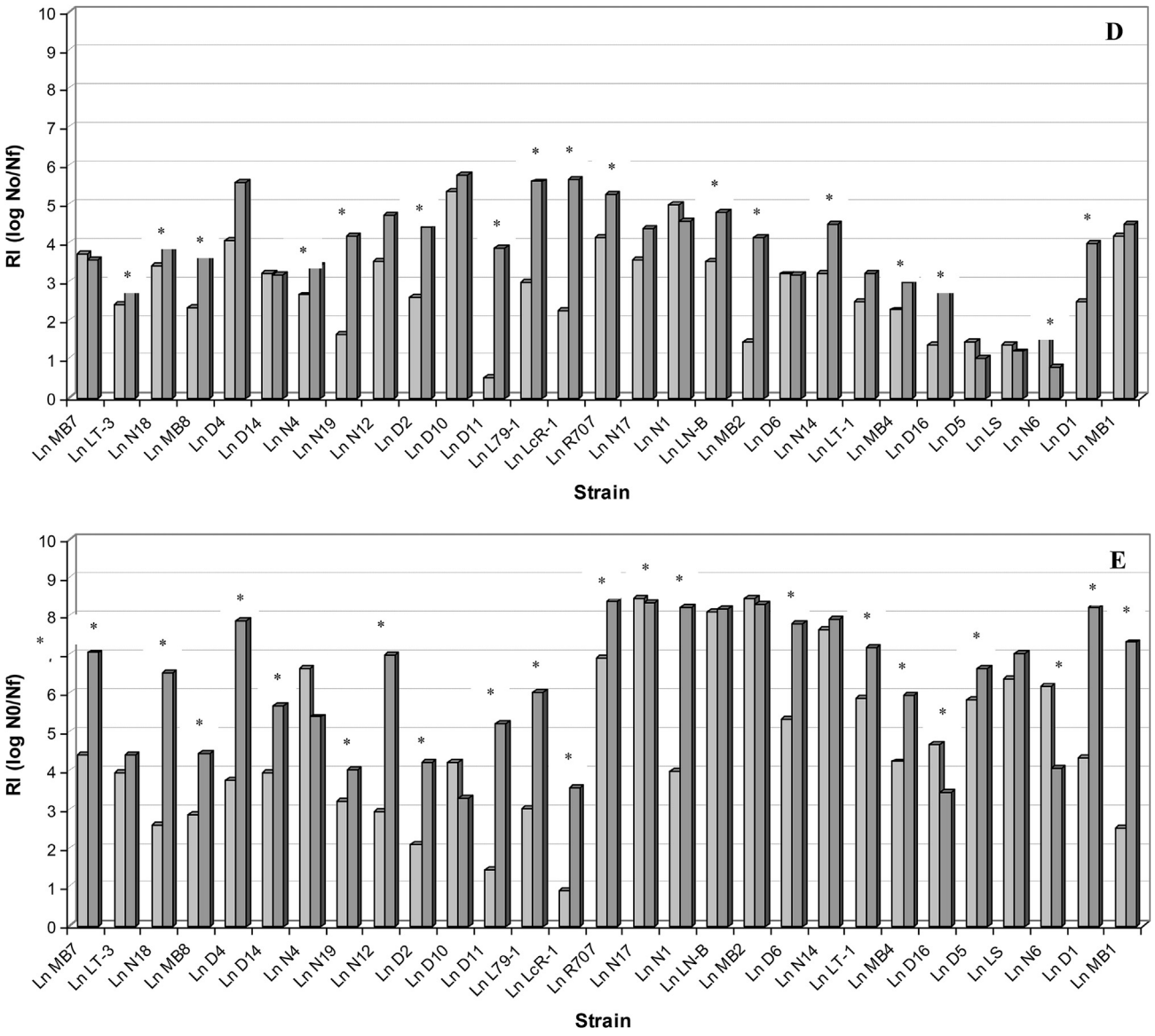

Fig. 3. (*continued*).

subgroups in stationary phase and 10 subgroups in exponential phase. The subgroup of most resistant strains regarding oxidative stress in stationary state was formed by *Ln* LcR-1, *Ln* D11, *Ln* D2 and *Ln* MB1, with mean RI values between 0.90 and 2.54. At exponential growth state, the most resistant strains were *Ln* D10, *Ln* LcR-1and *Ln* D16, with mean RI values between 3.30 and 3.57. The most resistant strains to osmotic shock at stationary state were *Ln* LT-3, *Ln* MB1, *Ln* D1, *Ln* N12 and *Ln* D10, with mean RI values between 0.16 and 0.35, while *Ln* D1, *Ln* MB1, *Ln* N12 and *Ln* MB4 revealed the highest resistance at exponential growth state, with mean RI values between 0.34 and 0.97. Finally, alkaline shock discriminated *Ln* D11, *Ln* D16, *Ln* D5, *Ln* MB2, *Ln* N6 and *Ln* N19 as the most resistant strains at the stationary state, with mean RI values between 0.52 and 1.66, while strains *Ln* N6, *Ln* D5, *Ln* LS, *Ln* D16, *Ln* MB4 and *Ln* LT-3 were the most resistant at the exponential state, with mean RI values between 1.05 and 3.08.

Statistical analysis (Test *t* de Student, $\alpha = 0.05$) between mean RI values obtained for each strain in stationary and exponential phase growth and for each stress factor, can be observed in Fig. 3 (A, thermal; B, acidic; C, osmotic; D, alkaline and E, oxidative shock, respectively). Eighty-nine out of 145 total treatments applied (29 strains subjected to five stress shocks) showed significant differences between both physiological states (61.4%). Usually, cell resistance against stress factors was higher in stationary than in exponential growth phase. However, in a low number of treatments (6), a higher resistance was found for cells grown at the exponential phase. The acidic shock showed statistically different responses between both growth phases for 51.7% of the strains, while for oxidative shock this value was of 79.3%. Percentages obtained for other factors studied were 55.2% for thermal, 58.6% for alkaline and 62.0% for osmotic shock.

Multivariate analysis performed by principal component analysis (PCA) of mean RI values obtained for stationary and exponential phase growth could be described in two dimensions by two components (PC1 and PC2), with cumulative percentage of variance of 83.7% (PC 1, 53.4% and PC2, 30.3%) and 80.1% (PC1, 62.7% and PC2, 17.4%) for stationary and exponential phase growth, respectively. The influence of all factors was similar for both cell growth phases. In fact, in relation to PC1, thermal, acidic and alkaline (positive values) and oxidative (negative value) and for PC2, thermal,

oxidative, alkaline (positive values) were the most determining factors (data not shown). Representation in two dimensions for PC1 and PC2 scores (Fig. 4) allowed the visualization of three clusters (I, II and II) regarding the behavior against the stress factors studied. Cluster I grouped all *Ln. lactis* strains and *Ln. mesenteroides* strain *Ln* MB7 in both growth phases. Cluster II included most *Ln. mesenteroides* strains, while cluster III most *Ln. pseudomesenteroides* strains on both growth phases. Decreasing resistance for strains grouped in Custer I, II and III was observed; being those contained in Cluster I the most "robust" strains and those in Cluster III the most sensitive ones.

To investigate the possible relationship between response to stress factor and strain sources, a multivariate analysis by matrix hierarchical cluster was performed. This analysis included results from both stationary and exponential growth phases. The results revealed three clusters (Fig. 5) that included almost identical strains than those obtained by principal component analysis (PCA). Cluster I grouped most resistant strains (all *Ln. lactis* strains and *Ln. mesenteroides Ln* MB7 as an exception); Cluster II included all *Ln. mesenteroides* strains studied and *Leuconostoc pseudomesenteroides Ln* R707 as an exception; while Cluster III was formed by the rest of *Ln. pseudomesenteroides* strains and *Ln. citreum Ln* MB1. A clear relationship between strain origin and resistance against the stress factors studied for pasteurized milk and soft cheese was not found, since the strains isolated from these sources were included indistinctly in the three clusters. On the contrary, all the strains isolated from whey protein concentrate (WPC) belonged to *Ln. mesenteroides* species and are included in Cluster II (intermediate resistance).

3.4. Thermal pre-treatment and further shock treatments

Four strains (*Ln* D11, *Ln* N19, *Ln* N12 and *Ln* D2) showing good resistance against all stress factors studied but with thermal mean RI values low enough to observe a possible improvement in their resistance against the same factor (thermal) or against others (cross resistance), were selected. The results are shown in Fig. 6. Thermal mean RI values of three strains (*Ln* D11, *Ln* N12 and *Ln* D2) decreased (meaning higher resistance) between 1.43 and 1.84 when thermal adaptation was applied. The same behavior was observed when these strains were subjected to acidic shock. In this case, strains *Ln* N12 and *Ln* D2 diminished their acidic mean RI values in 4.85 and 2.79, respectively, while for *Ln* D11 the decrease was of 1.26. For thermal and acidic shock, the mean RI values of strain *Ln* N19 did not significantly change when thermal pre-treatment was applied. Regarding the oxidative effect, all strains showed the same behavior and increased their mean RI values (meaning lower resistance) between 1.30 and 3.05. These results showed the negative impact of thermal adaptation on the oxidative shock.

4. Discussion

All *Leuconostoc* strains studied were isolated from dairy products, and they were classified as *Ln. lactis* (4 strains), *Ln. mesenteroides* (14 strains), *Ln. pseudomesenteroides* (10 strains) and *Ln. citreum* (1 strain). Similar *Leuconostoc* species were isolated by other authors from several dairy products and starters (Cibik et al., 2000; Cuesta et al., 1996; Gonçalves de Almeida Júnior et al., 2015; Jokovic et al., 2008; Nieto-Arribas et al., 2010; Voidarou et al., 2011). Wide genetic diversity between strains studied was revealed by RAPD-PCR, even for the species with a low number of samples (*Ln. lactis*). The only *Ln. citreum* strain studied was grouped with *Ln. lactis* strains, showing the highest similarity with *Ln* D1 (Cluster IV). This grouping is not surprising because these two species are very closed phylogenetically (Holzapfel and Wood, 2014).

Survival and good technological performance of strains used as starter and/or adjuncts after exposition to unfavorable processing conditions are essential to obtain the expected results from them. In this sense, a good selection based on their intrinsic resistance against diverse stress conditions is crucial for an accurate choice (Ferrando et al., 2015). The resistance and adaptation capacity of a microorganism to environmental adverse conditions is a main force in the biological evolution, allowing more "robust" cells in unfavorable growth and survival conditions (De Angelis and Gobbetti, 2011; Ferrando et al., 2015; Serrazanetti et al., 2009; van de Guchte et al., 2002). In this work, a wide variability in stress resistance of the 29 *Leuconostoc* strains studied was found, especially for temperature, acidic and oxidative factors. With this regard, and as can be seen in Table 2, mean RI values were ranged

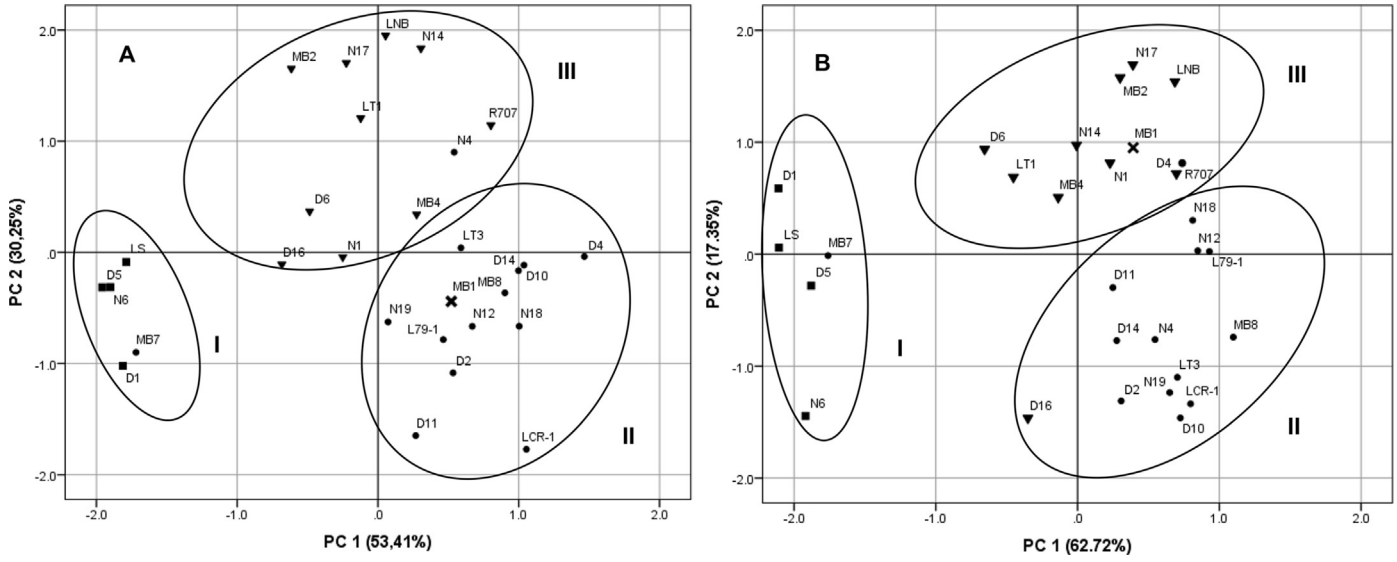

Fig. 4. Score plots of Principal Component Analysis (PCA) carried out on inactivation results (Resistance Index, RI) on stationary (A) and exponential (B) phase cells of *Leuconostoc lactis* (■), *Leuconostoc mesenteroides* (●), *Leuconostoc pseudomesenteroides* (▼) and *Leuconostoc citreum* (✖) strains. Clusters I, II and III were obtained for each growth phase, with decreasing resistance from I to III.

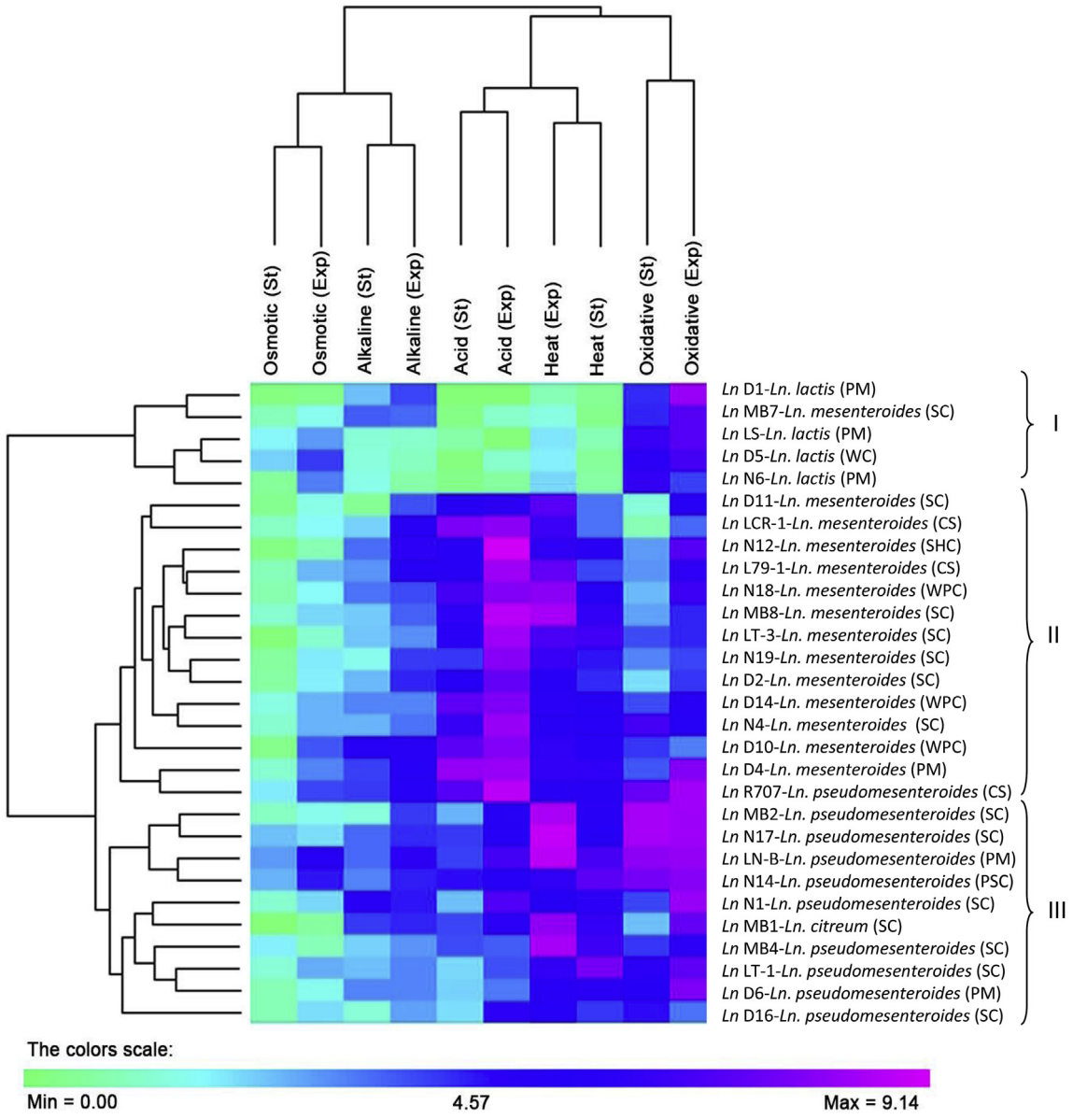

Fig. 5. Dendrogram obtained using Matrix Hierarchical Cluster Analysis (Euclidean distance, Average linkage UPGMA method) with data of *Leuconostoc* strain resistance against different stress factors and including both physiological states (exponential-Exp and stationary-St phases). Clusters I, II and III were obtained, with decreasing resistance from I to III. PM: Pasteurized Milk, SC: Soft Cheese, WC: Whey Cream, SHC: Semi Hard Cheese, CS: Commercial Strain, PSC: Petit Suisse Cheese and WPC: Whey Protein Concentrate.

between 0.36 and 7.65 (thermal shock), 0.26 and 8.20 (acidic shock), 3.30 and 8.39 (oxidative shock), all of them obtained in stationary growth phase. Similar results were found by Zotta et al. (2008), when they screened 56 strains of *Streptococcus thermophilus*, *Streptococcus salivarius* and *Streptococcus macedonicus* and related dairy streptococci for their tolerance against acidic, osmotic, oxidative and heat stress factors. *S. thermophilus* is a species confined almost exclusively to dairy environments and has also remarkably large diversity in stress tolerance for the factors enumerated. Parente et al. (2010) reported the screening of 63 strains of the taxonomically related species *Lactobacillus plantarum* subsp. *plantarum*, *Lactobacillus plantarum* subsp. *argentoratensis*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus* regarding diverse stress conditions (acidic, alkaline, heat, oxidative, osmotic, detergent and starvation stress factors). Remarkably, these authors used the same thermal treatment (55 °C - 15 min) applied in this work; although they harvested cells at the exponential growth state. Unlike our results, they reported a high acidic resistance for the strains, similar to that obtained with osmotic and alkaline treatments. On the other hand, Reale et al. (2015) studied 108 *Lactobacillus paracasei*, 68 *Lactobacillus rhamnosus* and 8 *Lactobacillus casei* strains, isolated from different sources (dairy and meat products, sourdoughs, wine, beverages, vegetables and human body) and geographical regions against their tolerance to acidity, bile salts, high osmolarity and capability to grow at different non-optimal temperatures and survival after exposure to refrigeration or freezing. These authors obtained two clear groups of strains, all isolated from dairy products, regarding to their resistance against osmotic, acidic and bile factors. One of these groups included the more resistant strains and it was mostly composed by *L. paracasei* strains. The other one included the more sensitive strains and it was composed mostly by *L. rhamnosus* strains. The response of our strains against the osmotic and alkaline stress did not differentiate nor strains neither species. The effect of these two stress factors

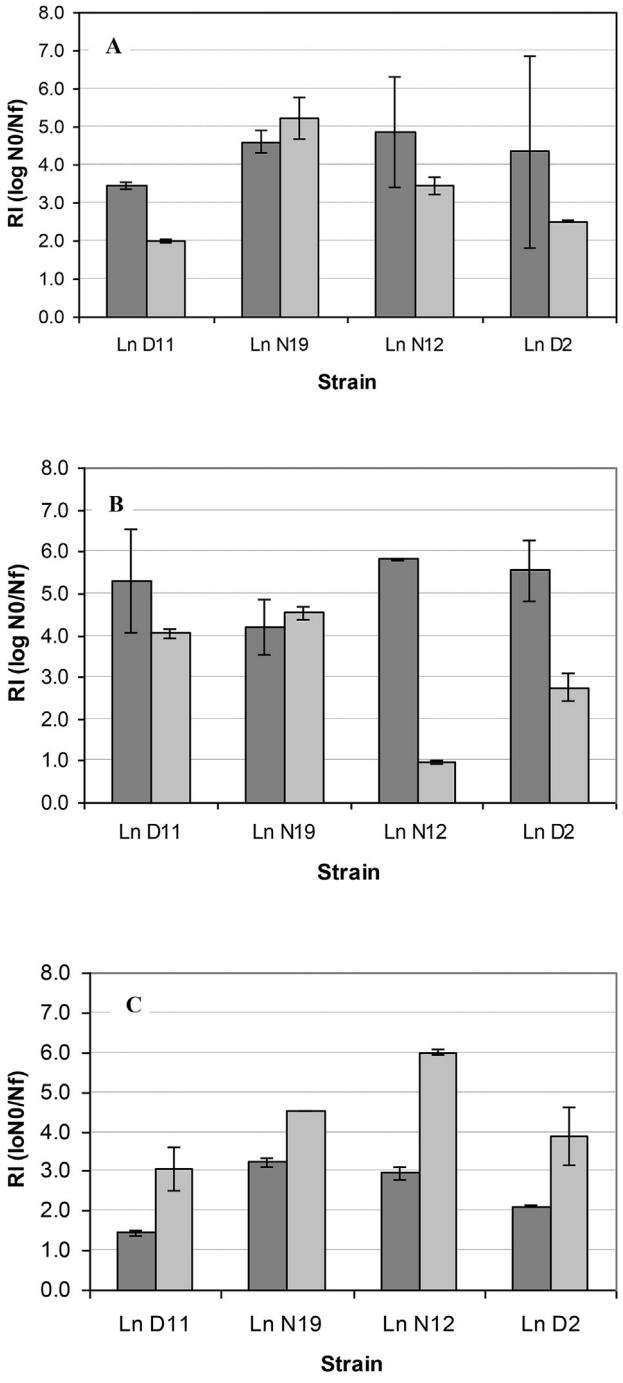

Fig. 6. Resistance Index (RI) (mean ± standard deviation) for *Leuconostoc* strains subjected to thermal adaptation (40 °C – 30 min) and further thermal (A), acidic (B) and oxidative (C) shocks (□), compared with results obtained for non-adapted cells (■).

was really lower than thermal, acidic and oxidative ones. In general, for all LAB species, osmotic factor do not influence cell viability (Ferrando et al., 2015; Kuda et al., 2014; Parente et al., 2010; Zotta et al., 2008). As it is known, the bacterial response to hyper-osmolarity is related to the ability of the cells to accumulate osmoprotective compounds (Le Marrec et al., 2007; Pichereau et al., 2000). Osmotic stress can lead to accumulation of humectant molecules (for example, sugar) or synthesis of osmoregulatory compounds to maintain osmotic balance. The mechanism behind this effect remains to be fully elucidated, but increased levels of compatible solutes play positive roles in cell survival and enzyme activity (Carvalho et al., 2004).

In this study, we found a clear relationship between stress resistance and the species using matrix hierarchical cluster analysis (Fig. 5). Specifically, all *Ln. lactis* strains (4) were included in the more resistant group (Cluster I). Cluster II grouped all strains belonged to *Ln. mesenteroides* species, with the exception of *Ln* MB7 which was included into Cluster I. Finally, and clearly more sensitive, *Ln. pseudomesenteroides* strains were grouped in Cluster III. Another exception was *Ln pseudomesenteroides Ln* R707, which was included in Cluster II. Even if these findings need to be confirmed using a larger number of strains, these results show a marked trend regarding different stress resistance depending on the species. Heat resistance in *Leuconostoc* is related, as for other LAB genera, to the induction of a protein set that includes chaperons DnaK and GroEL, Clp multimeric complex and small heat – shock proteins (sHSPs) (Bourdineaud et al., 2003; Capozzi et al., 2016; De Angelis and Gobbetti, 2011; Grandvalet et al., 2005; Salotra et al., 1995; Serrazanetti et al., 2009). The difference of heat response found among *Leuconostoc* species could be related with a higher expression of these stress proteins for *Ln lactis* than that for *Ln. mesenteroides* and *Ln. pseudomesenteroides*. Regarding acidic stress, homeostasis of the internal pH is essential for growth and survival of all biological cells. However, bacterial growth is a self-limiting process through the acidification of the external medium and acid accumulation (Hemme and Foucaud-Scheunemann, 2004). Hache et al. (1999) have reported that *Ln. mesenteroides* has low capacity to control the homeostasis of pH in comparison to *Ln. lactis*. On the other hand, some authors have reported a direct relationship between heat and oxidative resistance (against H_2O_2 as oxidative agent) in lactobacilli (Ferrando et al., 2015; Parente et al., 2010), but this association was not found in this study. However, it is reported that general stress resistance mechanisms may also confer resistance to oxidative stress (Rallu et al., 2000). Moreover, it was not possible to find a clear relationship between stress resistance and the strain source. Strains of diverse *Leuconostoc* species were found in different dairy products, independently of the previous technological process and/or their physical conditions. These conclusions were coincident with the results informed by Parente et al. (2010). Diversely, Zotta et al. (2008) found a statistically significant association between the source of isolation and stress tolerance for acid and oxidative stresses, obtaining that the more resistant strains were isolated from yogurt and natural starters.

The adaptive response during exponential phase usually involves the induction of specific groups of genes or "regulons" to cope with a specific stress condition, while the stress response during the stationary phase is mediated by numerous regulons that cope with numerous stress conditions (De Angelis and Gobbetti, 2011; van de Guchte et al., 2002). In consequence, cells in stationary phase are, in general, more resistant against all stress factors than cells grown at the exponential phase. The same behavior was observed for *Leuconostoc* strains studied in our work, with some few exceptions. In addition, we found that cells grown in both physiological growth phases were influenced similarly by the applied stress factors, as shown in Fig. 4.

Frequently, strain adaptation by exposure to sub-lethal conditions of diverse stress factors lead to the increment, not only of the resistance against the specific factor but also against others (cross-resistance). This is due to the fact that adaptation could involve the induction of several stress proteins, which are commonly observed when strains respond to a variety of stresses (van de Guchte et al., 2002). In this sense, the strains selected in this study for thermal adaptation showed, as expected, an increment of heat and acidic resistance compared to the non-adapted strains, except for strain

Ln N19, for which mean RI values remained similar. The cross-acidic resistance of thermal adapted cells could be due to the synthesis, during thermal adaptation, of a group of proteins that are synthesized throughout acidic stress as well, and participates on cell protection against both heat and acidic stress. This group of proteins includes small heat shock proteins (Hsp1 and 3) and chaperonins (DnaK, GrpE, GroEL, and GroES), and plays a role in protein folding (Cotter and Hill, 2003; Fernandez et al., 2008; Heunis et al., 2014; Wu et al., 2012). Surprising results in relation to acid resistance were observed for *Ln* N12 and *Ln* D2, because the increase of the resistance for thermal-adapted cells of this phenotype, was higher than that previously reported (De Angelis et al., 2004), especially considering that the adaptation was carried out on cells grown at the stationary phase. Regarding oxidative stress, all strains exhibited a lower resistance when thermal adapted-cells were studied. The oxidative response seems to be uncoupled from synthesis of the two major heat shock proteins (DnaK and GroEL chaperons) (Flahaut et al., 1998), even if some studies reported an over-expression of these proteins when strains were induced by H_2O_2 (Arena et al., 2006; Zotta et al., 2008, 2012). According to our knowledge, there are no studies describing a reduction in the oxidative resistance after thermal adaptation of the cells.

Taking into account the scarce data available, results obtained in this study provide valuable information regarding the general behavior of strains belonging to diverse *Leuconostoc* species subjected to technological stress conditions. In this work, all strains included in Cluster I (Principal Components and Matrix Hierarchical Cluster Analysis) could be proposed as adjuncts for fermentative food industry, such as dairy, vegetable and meat products. Studies concerning to volatile profiles and CO_2 production of them are currently in course.

Acknowledgments

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