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Response of *Leuconostoc* strains against technological stress factors: Growth performance and volatile profiles

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ABSTRACT

The ability of twelve strains belonging to three *Leuconostoc* species (*Leuconostoc mesenteroides, Leuconostoc lactis* and *Leuconostoc pseudomesenteroides*) to grow under diverse sub-lethal technological stress conditions (cold, acidic, alkaline and osmotic) was evaluated in MRS broth. Two strains, *Leuconostoc lactis Ln* N6 and *Leuconostoc mesenteroides Ln* MB7, were selected based on their growth under sub-lethal conditions, and volatile profiles in RSM (reconstituted skim milk) at optimal and under stress conditions were analyzed. Growth rates under sub-lethal conditions were strain- and not species-dependent. Volatilomes obtained from the two strains studied were rather diverse. Particularly, *Ln* N6 (*Ln. lactis*) produced more ethanol and acetic acid than *Ln* MB7 (*Ln. mesenteroides*) and higher amounts and diversity of the rest of volatile compounds as well, at all times of incubation. For the two strains studied, most of stress conditions applied diminished the amounts of ethanol and acetic acid produced and the diversity and levels of the rest of volatile compounds. These results were consequence of the different capacity of the strains to grow under each stress condition tested.

1. Introduction

Leuconostoc are heterofermentative lactic acid bacteria (LAB) that use lactose and citrate to produce lactic acid, ethanol, acetate and CO_2. Strains belonging to this genus are used as primary starter in butter and cream fermentation, because of their capacity to produce diacetyl, acetoin and 2, 3-butanediol. They can also be added as adjuncts (generally using *Lactococcus* strains as primary starter) in traditional cheeses, contributing to their distinctive flavors (Vedamuthu, 1994; McSweeney and Sousa, 2000; Hemme and Foucaud-Scheunemann, 2004; Montel et al., 2014; Pogačić et al., 2016). On the other side, *Leuconostoc* are naturally present in raw milk as non-starter LAB (NSLAB) and give, together with other NSLAB, special characteristics to cheeses manufactured with raw milk (Montel et al., 2014). The continuous selection of new strains in the design of starters to be used in diverse types of cheeses is a subject of permanent research (Johnson, 2014). With this aim, determination of volatile compound profiles is essential during the screening of *Leuconostoc* strains, since high variations can be observed from strain to strain potentially used as adjuncts (Pogačić et al., 2016).

On the other hand, strains used as starter cultures could be exposed to many adverse conditions (stress factors) during their preparation and storage and throughout the product manufacture as well. These stress factors are diverse and include pH variation (acidity or alkalinity), temperature (heat and cold), oxidative and osmotic changes, among others (van de Guchte et al., 2002; Zotta et al., 2008; Serrazanetti et al., 2009). As a general definition, stress could be considered any transition of a bacterial cell from one condition to another that causes alterations to the cell's genome, transcriptome, proteome, and/or metabolome leading to reduced growth or survival potential (Papadimitriou et al., 2016). The intensity of the stress applied could lead to cell death (lethal stress) or to cell adaptation by appropriate molecular responses in an attempt to ameliorate the negative effects and restore the growth or the survival potential (sub-lethal or mild stress conditions) (van de Gutche et al., 2002; Serrazanetti et al., 2009; De Angelis and Gobbetti, 2011; Papadimitriou et al., 2016). The study of the diversity in LAB response against stress conditions has a high practical relevance because aids in the comprehension of response

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mechanisms and would allow a better starter selection, resulting in higher performance and improved survival level during the process (Desmond et al., 2004).

In a previous work carried out by our group (D'Angelo et al., 2017), some *Leuconostoc* strains revealed good resistance to technological lethal stress conditions. The aim of this study was selecting, among this pool of "intrinsic resistant" strains, those showing the best potential for industrial use. The selection was based on the capacity of these strains to grow under diverse technological sub-lethal stress conditions and on their volatile profiles, determined in optimal and under diverse stressor conditions.

2. Materials and methods

2.1. Strains and conservation conditions

In a previous study, 12 out of 29 *Leuconostoc* strains were selected based on their good resistance to diverse technological stress factors (D'Angelo et al., 2017). Their taxonomic identification and source is shown in Table 1. The strains were stored frozen at −20 °C and −80 °C in MRS broth (Biokar, Beauvais, France) added of 15% (v/v) of glycerol as cryoprotective agent. For routine use, they were cultured in MRS broth for 24 h at 30 °C and stored at 8 °C.

2.2. Growth rates under sub-lethal stress conditions

Fresh cultures (MRS broth, 24 h at 30 °C) were harvested and washed twice with phosphate buffer 10 mM pH 7 (PB7). The pellets were suspended in PB7 to the same initial volume and inoculated (2%, v/v) in MRS broth prepared diversely according to the stress factor studied (Reale et al., 2015) as follows: i) MRS broth at pH 5.0 and 5.5 (adjusted with lactic acid, after sterilization), incubating at 30 °C during 24 h (acidic stress); ii) MRS broth at pH 8 (adjusted with NaOH, after sterilization), incubating at 30 °C during 24 h (alkaline stress); iii) MRS broth added of NaCl 4% (w/v), incubating at 30 °C during 24 h (osmotic stress) and iv) MRS broth incubating at 10 °C during 24 and 48 h (cold stress). As control, the strains were grown in MRS broth at 30 °C (optimal growth conditions) for the respective time of each experience. Growth rates (GR, %), defined as $O.D._s/O.D._c \times 100$ ($O.D._s$ = optical density of the strain under stress conditions and $O.D._c$ = optical density of the control, both measured at the end of the experience), were calculated. The assays were performed in three independent experiments.

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

Strain[a]	Taxonomic identification[b]	Source
Ln MB7	*Leuconostoc mesenteroides*	Soft cheese
Ln N19	*Leuconostoc mesenteroides*	Soft cheese
Ln N12	*Leuconostoc mesenteroides*	Semi-hard cheese
Ln D2	*Leuconostoc mesenteroides*	Soft cheese
Ln D11	*Leuconostoc mesenteroides*	Soft cheese
Ln L79-1	*Leuconostoc mesenteroides*	Commercial strain
Ln LcR-1	*Leuconostoc mesenteroides*	Commercial strain
Ln D5	*Leuconostoc lactis*	Whey cream
Ln LS	*Leuconostoc lactis*	Pasteurized milk
Ln N6	*Leuconostoc lactis*	Pasteurized milk
Ln D1	*Leuconostoc lactis*	Pasteurized milk
Ln D16	*Leuconostoc pseudomesenteroides*	Soft cheese

[a] *Leuconostoc* strains belong to the INLAIN collection.
[b] Taxonomic identification performed by sequence analysis of 16S rRNA gene (DNA sequencing).

2.3. Volatilome determination

Volatile profiles were performed for *Leuconostoc lactis Ln* N6 and *Leuconostoc mesenteroides Ln* MB7, selected based on their good survival and growth capacity under sub-lethal stress conditions. Fresh cultures (MRS broth, 24 h at 30 °C), were harvested and washed twice with PB7. The pellets were suspended in the same initial volume with PB7 and inoculated (2%, v/v) in reconstituted skim milk (RSM) 10% (w/v), prepared and/or incubated diversely according to the stress factor studied, as follows: i) RSM, incubated at 30 °C (optimal growth conditions); ii) RSM added of 0.5% of glucono delta-lactone (GDL), incubated at 30 °C (acidic stress); iii) RSM, incubated at 10 °C (cold stress); iv) RSM added of NaCl 4% (w/v), incubated at 30 °C (osmotic stress) and v) RSM at initial pH 8 (adjusted with NaOH after sterilization), incubating at 30 °C (alkaline stress). Samples were collected at 24 h, 5 d and 11 d of incubation. Non-inoculated RSM tubes incubated in the same conditions were used as controls. Assays were performed in two independent experiences.

Solid-phase microextraction (SPME) technique was employed for the isolation of volatile compounds from samples. Prior to analysis, frozen samples were thawed at 4 °C overnight. Aliquots of 10 ml of each culture were transferred to 40 ml screw-top glass vials sealed with a Teflon-lined silicone rubber septum. The septum was pierced with a sharp needle to allow the insertion of the SPME syringe. A microstirring bar was also introduced into the vials, which were placed on an aluminum block maintained at 40 °C and stirred at 250 rpm, using IKA heater/stirrer (Instrumentalia SA, Buenos Aires, Argentina). Then, a SPME fiber (DVB/Car/PDMS 50/30 μm) from Supelco (Bellefonte, PA, USA) was inserted into the headspace of the vial. After 10 min, it was exposed at 40 °C for 30 min. Analytes retained in the fiber were thermally desorbed in splitless mode at 250 °C during 5 min into the injector port of the GC (Perkin Elmer Model 9000 gas chromatograph) equipped with a split/splitless injector and a flame ionization detector (FID). The compounds were separated on a HP-Innowax capillary column (60 m × 0.25 mm × 0.25 μm) (Agilent J&W, Agilent Technologies, USA). The oven temperature, initially held at 45 °C for 5 min, was programmed to 250 °C at a heating rate of 10 °C/min, and then was finally held at 250 °C for 5 min. The FID temperature was set at 290 °C. Carrier gas was hydrogen at a flow rate of 2 m/min.

Tentative identification of peaks from chromatograms was performed by comparing the retention time with those of authentic standards, when available (Sigma Aldrich, Milan, Italy). Besides, linear retention indices (LRI) were calculated for the GC peaks by interpolation of the retention times of the volatile compounds with those of saturated alkanes (C7–C30) (Supelco, Bellefonte, PA, USA) analyzed under the same analytical conditions. Calculated LRI were compared with those values reported in the literature for pure standards analyzed with the same kind of stationary phase (Bianchi et al., 2007). Confirmation of the tentative identification of volatile compounds performed with GC-FID was made by mass spectrometry (MS) using a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 2000 ion trap mass detector (Varian Inc., Palo Alto, CA, USA). The GC conditions were the same as those of the chromatographic analysis by FID. MS was operated in the electron impact mode (EI, 70 eV) and mass spectra were obtained over a mass range from 40 to 350 amu (scan rate, 0.5 scan/s). The transfer line was held at 230 °C. Helium was the carrier gas, at flow rate of 1 ml/min. Volatile compounds were identified by comparing their mass spectra with mass spectra libraries (NIST 98, Gaithersburg, MD, USA; Wiley libraries, Hoboken, NJ, USA) and standard compounds (Sigma-Aldrich) (when available). The GC-MS analysis was only used for verification of peak identification. Thus, peak absolute areas for those compounds confirmed by GC-MS were obtained by

the GC-FID and used for the semi-quantitative analysis. Results were expressed as means of peak area values of duplicate analysis from two independent experiments.

2.4. Viable cell counts and pH determination

Cell counts and pH determination were performed to follow up the growth of strains throughout the assays described in section 2.3. Samples were enumerated at same times of volatile determinations by the traditional viable cell count method in MRS agar (72 h at 30 °C). Determinations were performed in two independent experiences.

2.5. Statistical analysis

Data were processed by applying one-way ANOVA (Tukey's test, *pos hoc*) and multivariate analysis by Principal Component Analysis (PCA), using IBM SPSS Statistics Version 21 (IBM Corp. 2012). Also, Matrix Hierarchical Cluster Analysis (Euclidean distance, Ward's minimum variance method) was performed with the PermutMatrix program v.1.9.3 (LIRMM, France).

3. Results

3.1. Growth rates under sub-lethal stress conditions

Growth rates under initial alkaline conditions (pH = 8) were similar to those obtained in optimal growth conditions (data not shown). ANOVA analysis of the results obtained under the others sub-lethal stress conditions (Table 2) allowed a good discrimination between the strains. Depending on the stress factor studied, a minimum of four and a maximum of nine subgroups were obtained. Each subgroup is represented by a superscript letter (Table 2) and assembles the strains according to their similar intrinsic resistance against the stress factor considered. Strains *Ln* N6, *Ln* N19, *Ln* N12, *Ln* D2, *Ln* D11 and *Ln* MB7 showed the best performance under most of stress factors applied. *Ln. lactis Ln* N6 and *Ln. mesenteroides Ln* MB7 were selected for further study of their volatile profiles. This selection was based on the good growth rate under sub-lethal stress conditions and also on their high resistance against diverse technological lethal stress factors, according to results reported in a previous work carried out by our group (D'Angelo et al., 2017).

3.2. Volatilome determination

Volatile compounds (excepting ethanol and acetic acid) produced by *Ln. lactis Ln* N6 and *Ln. mesenteroides Ln* MB7 for all treatments and all times are shown in Tables 3 and 4, respectively. For a better analysis, data obtained for ethanol and acetic acid are presented separately (Fig. 1A and B, respectively), because these compounds are produced in amounts substantially higher than the rest of volatile compounds.

In optimal growth conditions, *Ln* N6 produced approximately 1.5 times more ethanol than *Ln* MB7, at all incubation times. On the other hand, the level of acetic acid produced by *Ln* N6 was 9 times higher than that detected for *Ln* MB7 at 24 h, and of approximately 4.5 times higher at 5 d and 11 d of incubation. Besides ethanol and acetic acid, *Ln* N6 showed the production of 6 (24 h and 5 d) and 13 (11 d) diverse volatile compounds. Specifically, after 11 d of incubation, 2-octanol, hexanoic acid, octanoic acid, acetaldehyde and esters (ethylacetate and isoamylacetate) were the most abundant (Table 3). Alternatively, *Ln* MB7 showed a total of 6, 9 and 6 diverse volatile compounds at 24 h, 5 d and 11 d of incubation, respectively. After 11 d of treatment, *Ln* MB7 produced significantly lower amounts and diversity of compounds than those of strain *Ln* N6. In fact, acetaldehyde, esters, alcohols (in particular 2-octanol) and some ketones were not detected for this strain at this incubation time (Table 4).

The production of ethanol and acetic acid was affected differently when the strains grew under stress conditions. For both strains, the levels of ethanol produced under initial alkaline conditions (pH 8) were similar to those obtained in optimal growth conditions, being slightly higher for *Ln* MB7 (Fig. 1, state −4). The production of acetic acid did not change for *Ln* MB7, although it was diminished (approximately 1.5 times) for *Ln* N6. Instead, the gradual decrease of pH values caused by the addition of GDL, disfavored ethanol production for both strains analyzed (Fig. 1, state −1). The level of acetic acid diminished for *Ln* N6 at 5 d and 11 d of incubation, but not for *Ln* MB7, since the latter showed always higher amounts of this compound than those obtained in optimal conditions. Under cold and osmotic stress conditions, *Ln* N6 always showed higher production of ethanol and acetic acid than *Ln* MB7 and, for both strains, the production of acetic acid was always more affected than that of ethanol.

The effect of stress conditions on for the production of the rest of volatile compounds was unclear (Tables 3 and 4). Despite this, some general assessments could be made in the case of fatty acids such as

Table 2
Growth rates (mean ± standard deviation) obtained for *Leuconostoc* strains growing in MRS broth, under diverse stress conditions.

Strain	Growth rate (GR, %) in the following conditions[a]				
	NaCl 4% (w/v)	pH 5.0	pH 5.5	10 °C − 24 h	10 °C − 48 h
Ln D11	$37.9 \pm 11.1^{c,d}$	$15.1 \pm 1.5^{b,c}$	$79.7 \pm 0.7^{a,b,c}$	8.7 ± 0.5^{e}	52.2 ± 0.4^{b}
Ln N19	$55.5 \pm 0.3^{a,b,c}$	$15.9 \pm 0.2^{b,c}$	82.1 ± 1.0^{a}	8.5 ± 0.4^{e}	45.4 ± 8.5^{b}
Ln L79-1	$54.8 \pm 6.0^{a,b,c}$	$10.5 \pm 1.1^{c,d}$	$80.2 \pm 3.2^{a,b}$	$6.9 \pm 0.1^{e,f}$	18.8 ± 2.8^{f}
Ln LcR-1	15.4 ± 1.9^{d}	9.3 ± 0.1^{d}	42.7 ± 0.1^{i}	1.9 ± 0.2^{g}	2.8 ± 0.6^{g}
Ln N12	74.5 ± 2.8^{a}	27.2 ± 2.4^{a}	$74.7 \pm 2.6^{c,d}$	3.2 ± 1.5^{g}	21.1 ± 7.7^{e}
Ln MB7	41.1 ± 5.5^{c}	$13.0 \pm 0.7^{b,c,d}$	$52.0 \pm 2.0^{g,h}$	13.5 ± 1.9^{d}	24.8 ± 14.9^{d}
Ln LS	$44.9 \pm 10.5^{b,c}$	$13.0 \pm 0.4^{b,c,d}$	$58.7 \pm 0.1^{f,g}$	25.3 ± 0.6^{b}	36.6 ± 16.6^{b}
Ln D5	$38.4 \pm 11.7^{c,d}$	$13.0 \pm 0.5^{b,c,d}$	$63.6 \pm 1.3^{e,f}$	13.5 ± 0.1^{d}	34.9 ± 8.1^{c}
Ln D1	$38.1 \pm 5.7^{c,d}$	$11.9 \pm 0.1^{c,d}$	$48.1 \pm 1.0^{h,i}$	22.2 ± 0.1^{b}	52.3 ± 0.1^{b}
Ln N6	$70.1 \pm 0.1^{a,b}$	$14.4 \pm 0.6^{b,c,d}$	59.8 ± 0.2^{f}	33.4 ± 1.4^{a}	53.7 ± 4.4^{a}
Ln D16	$30.8 \pm 3.1^{c,d}$	$15.7 \pm 3.5^{b,c}$	$69.6 \pm 1.8^{d,e}$	17.6 ± 0.2^{c}	48.5 ± 4.4^{b}
Ln D2	78.0 ± 3.1^{a}	17.8 ± 0.4^{b}	$72.6 \pm 3.3^{c,d}$	$4.1 \pm 0.1^{g,f}$	35.0 ± 3.7^{d}

MRS broth was used as base medium.
[a]GR (%) = $O.D._{.s}/O.D._{.c}$ x 100 ($O.D._{.s}$ = optical density of the strain under stress condition; $O.D._{.c}$ = optical density of the control, both measured at the end of the experience). Different superscript letters (subgroups) in the same column indicate significant difference between mean RI values, using one-way ANOVA ($a < 0.05$). Subgroups (superscript letters "a" to "h") correspond to decreasing resistance to the respective stress factor analyzed.

Table 3
Production of volatile compounds (excluding ethanol and acetic acid) for *Ln* N6 growning under diverse sub-lethal stress conditions.

Compound	LRI[a]	Control[b]			RSM − acidic[c]			RSM −10 °C[d]			RSM − NaCl[e]			RSM − alkaline[f]		
		24 h	5 d	11 d	24 h	5 d	11 d	24 h	5 d	11 d	24 h	5 d	11 d	24 h	5 d	11 d
Aldehydes																
Acetaldehyde	668	−	−	+++	+	−	−	−	−	−	−	−	+	−	−	−
Benzaldehyde	1537	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Esters																
Ethyl acetate	882	−	++	++	+	+	−	+	−	++	++	−	+	+	+	+
Ethyl butanoate	1034	−	−	−	−	−	−	−	+	+	−	+	−	++	−	−
Isoamyl acetate	1118	−	−	++	+	+	++	−	−	−	−	−	−	−	+	−
Ethyl hexanoate	1236	−	−	−	−	+	−	−	+	+	−	−	−	++	−	−
Alcohols																
1-Propanol	1041	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
3-Methyl-1-butanol	1210	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
2-Heptanol	1323	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
2-Octanol	1427	−	++	++++	+++	+	−	−	+	−	−	−	+	+	++	−
1-Octanol	1567	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Phenyl ethyl alcohol	1892	+	−	+	−	−	−	−	+	+	−	−	−	−	−	−
Ketones																
2-Hexanone	1079	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3-Hydroxy butanone (Acetoin)	1291	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
2-Heptanone	1180	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2-Nonanone	1391	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2-Undecanone	1603	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Acids																
Butyric	1640	+	++	++	+	+	−	+	+	+	−	+	+	+	+	−
3-Methyl butanoic	1682	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Hexanoic	1861	++	+++	+++	++	++	++	+	++	++	−	+++	+++	+	+++	++
Octanoic	2073	++	+++	+++	++	++	++	+	++	+++	−	+++	+++	+	++	++
Decanoic	2286	++	++	++	−	+	+	+	++	++	−	++	++	+	++	+

[a] LRI: linear retention index. The strains were inoculated in: [b] reconstituted skim milk (RSM) incubated at 30 °C; [c] RSM incubated at 10 °C; [d] RSM + glucono delta-lactone (GDL, 0.5% w/v) incubated at 30 °C; [e] RSM + NaCl (4% w/v) incubated at 30 °C; [f] RSM + NaOH (pH 8 as initial value) incubated at 30 °C. (++++) Very high, (+++) high, (++) medium and (+) weak production; (-) not detected. Results are the difference between the values obtained for inoculated and non-inoculated samples incubated in the same conditions.

Table 4
Production of volatile compounds (excluding ethanol and acetic acid) for *Ln* MB7 growing under diverse sub-lethal stress conditions.

Compound	LRI[a]	Control[b]			RSM − acidic[c]			RSM-10 °C[d]			RSM-NaCl[e]			RSM-alkaline[f]		
		24 h	5 d	11 d	24 h	5 d	11 d	24 h	5 d	11 d	24 h	5 d	11 d	24 h	5 d	11 d
Aldehydes																
Acetaldehyde	668	−	+	−	−	−	−	−	−	−	−	++	++	−	+	++
Benzaldehyde	1537	−	−	−	−	++	−	−	−	−	−	−	−	−	−	−
Esters																
Ethyl acetate	882	+	+	−	−	−	−	+	−	−	−	−	+	−	−	+
Ethyl butanoate	1034	−	−	−	−	−	+	−	−	−	−	−	−	++	−	−
Isoamyl acetate	1118	−	−	−	−	−	−	−	−	−	+	+	++	−	+	++
Ethyl hexanoate	1236	−	−	−	−	−	+	−	−	−	−	+	−	+	−	−
Alcohols																
1-Propanol	1041	−	+	+	−	−	+	−	−	+	+	+	+	−	−	−
3-Methyl-1-butanol	1210	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
2-Heptanol	1323	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2-Octanol	1427	+++	++++	−	−	−	+++	−	−	−	−	++++	+++	−	+++	++++
1-Octanol	1567	−	−	+	+	+	−	−	−	−	+	−	−	+	+	−
Phenyl ethyl alcohol	1892	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Ketones																
2-Hexanone	1079	−	−	−	−	−	−	−	+++	−	−	−	−	−	−	+
3-Hydroxy butanone (Acetoin)	1291	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2-Heptanona	1180	−	−	−	+	−	−	+	−	++	++	−	−	+	−	−
2-Nonanone	1391	−	−	−	−	−	−	++	−	+	+	−	−	−	−	−
2-Undecanone	1603	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+
Acids																
Butyric	1640	+	++	++	+	+	+	−	−	+	−	+	−	+	++	+
3-Methyl butanoic	1682	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−
Hexanoic	1861	+	+++	+++	++	++	+++	−	−	+	−	++	+	+	+++	+++
Octanoic	2073	+	+++	+++	++	+	+++	−	−	+	+	+++	−	+	+++	+++
Decanoic	2286	+	++	+++	++	+	++	−	−	+	+	++	−	+	+++	++

[a] LRI: linear retention index. The strains were inoculated in: [b] reconstituted skim milk (RSM) incubated at 30 °C; [c] RSM incubated at 10 °C; [d] RSM + glucono delta-lactone (GDL, 0.5% w/v) incubated at 30 °C; [e] RSM + NaCl (4% w/v) incubated at 30 °C; [f] RSM + NaOH (pH 8 as initial value) incubated at 30 °C. (++++) Very high, (+++) high, (++) medium and (+) weak production; (-) not detected. Results are the difference between the values obtained for inoculated and non-inoculated samples incubated in the same conditions.

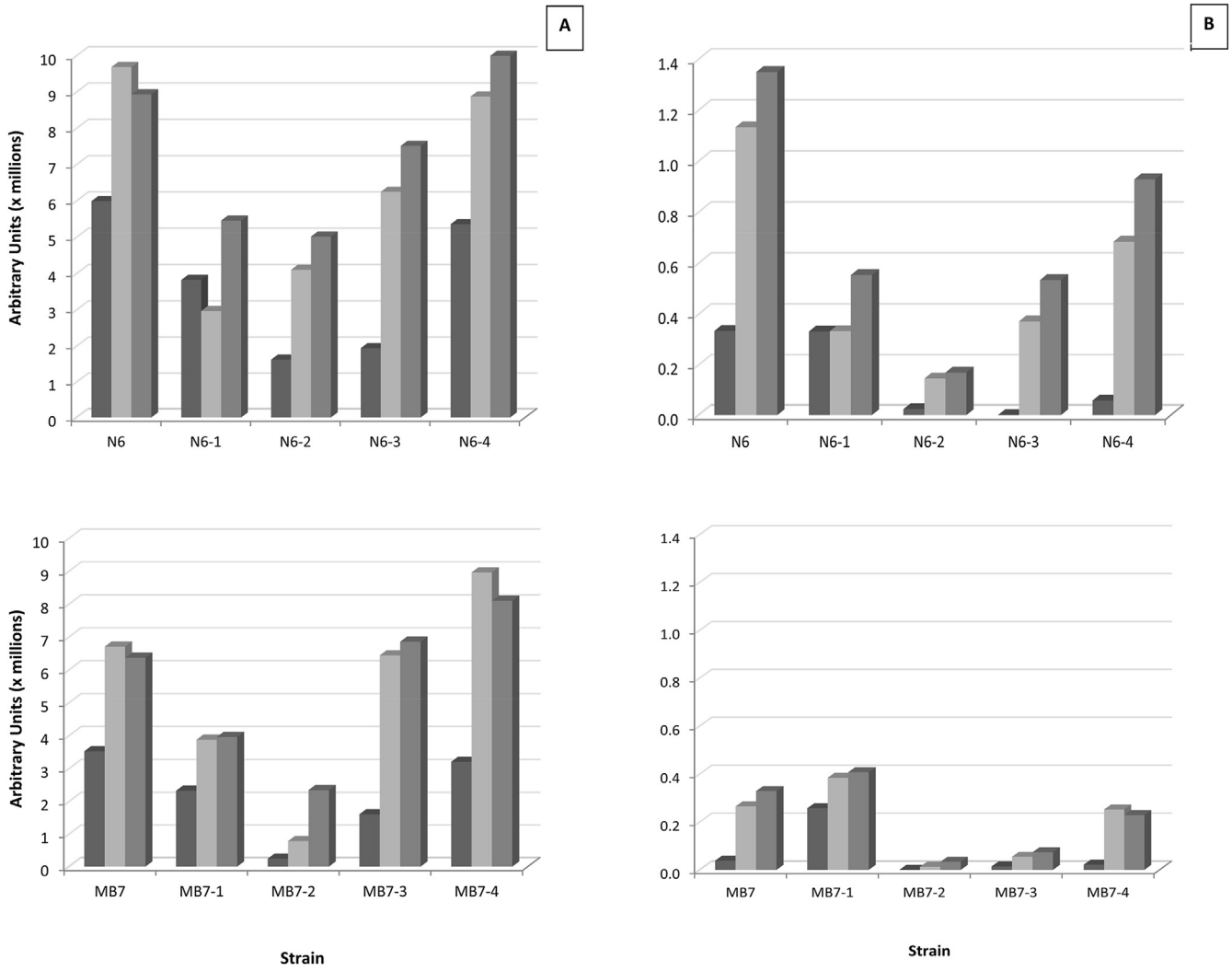

Fig. 1. Production of ethanol (A) and acetic acid (B) of *Ln* N6 and *Ln* MB7 growing in diverse sub-lethal stress conditions at 24 h (■), 5 d (□) and 11 d (▩) of incubation. Strains inoculated (2%, v/v) in reconstituted skim milk (RSM) and incubated at 30 °C were used as controls (indicated as N6 and MB7). Diverse stress conditions are indicated by: −1, RSM + glucono delta-lactone (GDL, 0.5% w/v) incubated at 30 °C; −2, RSM incubated at 10 °C; −3, RSM + NaCl (4% w/v) incubated at 30 °C and −4, RSM + NaOH (pH 8 as initial value).

butyric, hexanoic, octanoic and decanoic acids. In this sense, a net production of these acids was observed, with a few exceptions, at all incubation times for the different stress conditions. For both strains, the levels of fatty acids decreased, at different degrees, in comparison to those obtained in optimal growth conditions. In particular, for strain *Ln* MB7, cold stress led to severe diminished fatty acid production. On the other hand, diacetyl and/or acetoin (its reduction product) were usually not detected.

Multivariate analysis by principal component analysis (PCA) of volatile compounds performed for the strains growing in optimal conditions at diverse times of incubation is shown in Fig. 2. The results are described in two dimensions (PC 1 and PC 2), obtaining an accumulative percentage of variance of 74.6% (PC 1, 49.5% and PC 2, 25.1%). According to this analysis, strain *Ln* N6 was undoubtedly the best producer of volatile compounds at all times of incubation. At the end of the incubation (Fig. 2, N6 3 point), higher production of acetic acid, acetaldehyde, 3-methyl 1-butanol or isoamyl alcohol, isoamyl acetate, ethyl acetate and 2-octanol was observed, compared with that of *Ln* MB7 (Fig. 2, MB7 3 point), being the latter characterized by the presence of primary alcohols such as 1-octanol and 1-propanol. Fig. 3 shows the matrix hierarchical cluster analysis performed to compare the behavior of the strains in optimal and under stress growth conditions. Four clusters named I, II, III and IV, were clearly identifiable. Cluster I grouped strain *Ln* N6 in diverse growth conditions (the more favorable ones) and *Ln* MB7 growing in alkaline initial conditions at 5 d of incubation. This group showed the highest amounts of volatile compound production. Particularly, it was observed that *Ln* N6 grown in optimal conditions, produced low amount of acetoin (1.6×10^4 arbitrary units), which it was not detected in other growth conditions. In general, cluster II grouped strain *Ln* N6 in the more benevolent growth conditions (optimal, alkaline and osmotic ones) and strain *Ln* MB7 in optimal and the advanced incubation times under alkaline and osmotic stress conditions. This group showed lower production of some alcohols (1-propanol, 1-octanol, 2-octanol and 2-heptanol) and acids (acetic and butyric) in comparison to cluster I. Cluster III grouped both strains, mostly in acidic conditions, and particularly *Ln* MB7 in optimal growth conditions at 24 h of incubation. Finally, cluster IV included the strains growing in the more restrictive conditions (low temperature and osmotic) during the early times of incubation. This group showed a remarkable lack of ethanol and acetic acid production in particular and of other

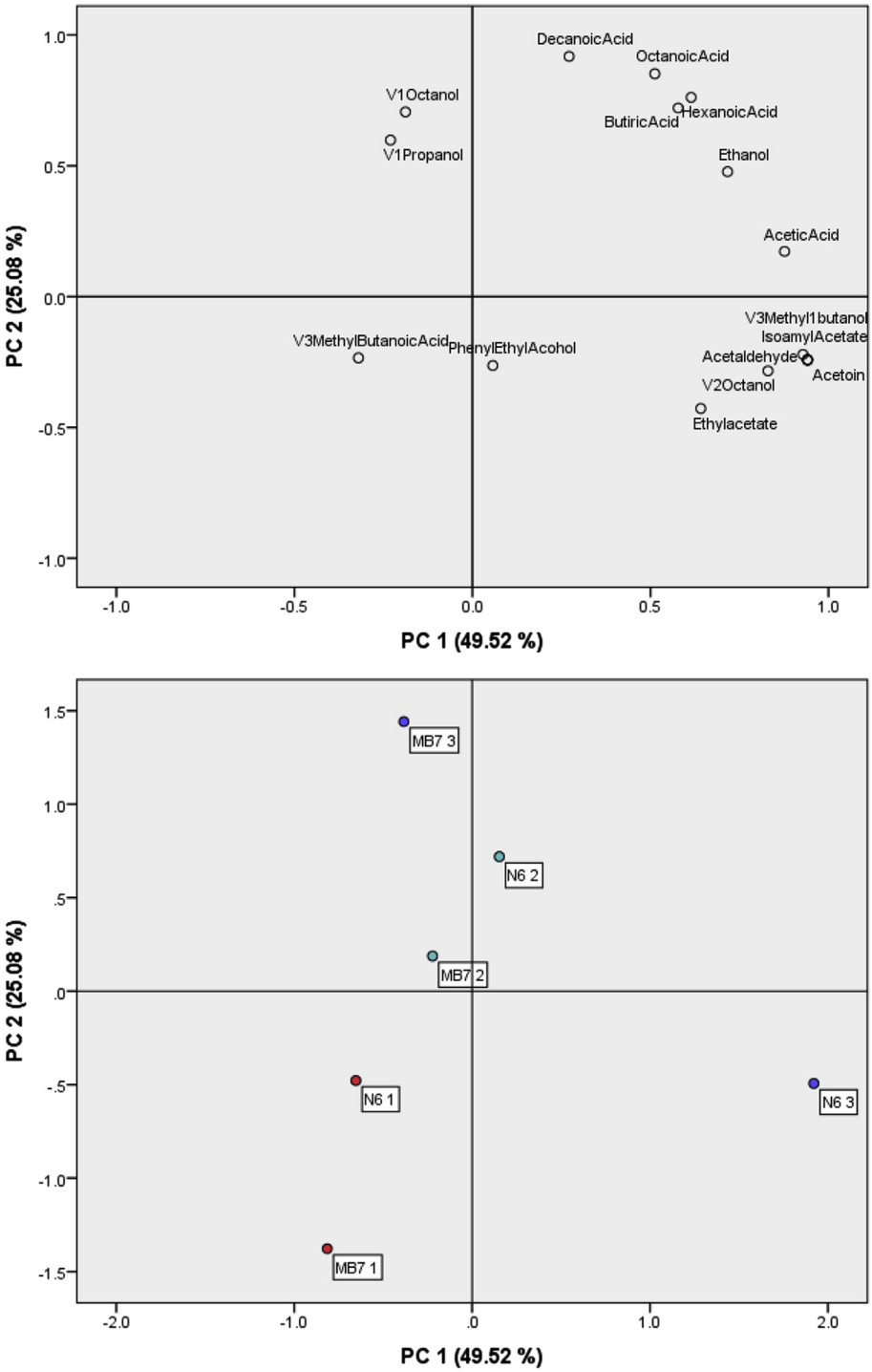

Fig. 2. Score plots obtained by Principal Component Analysis (PCA) for volatile compounds produced by strains *Ln* N6 and *Ln* MB7 growing in optimal conditions (RSM, 30 °C) at 24 h (1), 5 d (2) and 11 d (3) of incubation.

alcohols, acids and esters in general. In contrast, larger amounts of ketones (2-hexanone, 2-heptanone and 2-nonanone) were detected in these cases.

3.3. Viable cell counts and pH determination

Results are shown in Table 5. In optimal growth conditions, both strains showed an increase of 1.5 logarithmic orders (log CFU/ml) in viable cell counts after 24 h of incubation. Although both strains achieved similar viable cell counts, pH values were lower for *Ln* N6 than for *Ln* MB7. At the end of the experience (11 d), viable cell counts decreased from 8.5 to 5.1 log orders and from 8.5 to 6.7 log orders for *Ln* N6 and *Ln* MB7, respectively. After incubation at 10 °C, strains *Ln* N6 and *Ln* MB7 reached similar viable cell counts at 5 d and 11 d of incubation, respectively. The acidic conditions disfavored the growth of both strains, showing *Ln* N6 higher cell counts than *Ln* MB7 at 24 h of incubation. After 11 d, both strains reached similar pH values, while the decrease in cell counts was

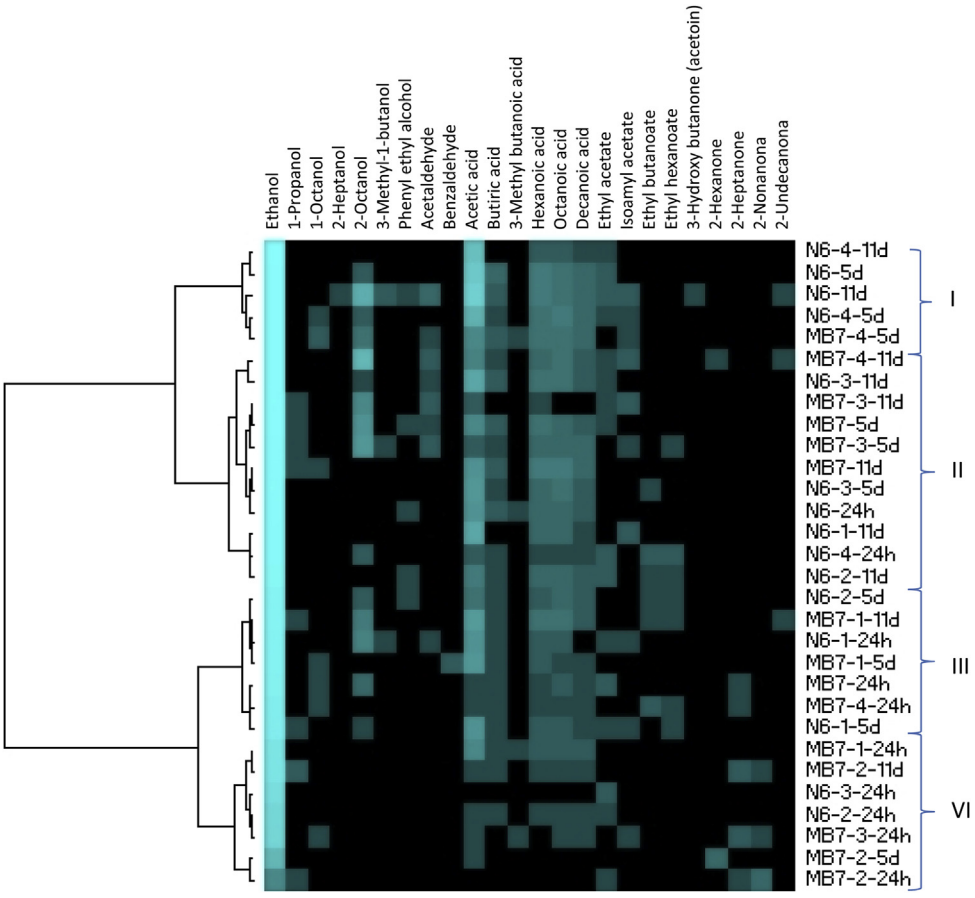

Fig. 3. Dendrogram obtained using Matrix Hierarchical Cluster Analysis (Euclidean distance, Ward's minimum variance method) of volatile compounds produced by *Ln* N6 and *Ln* MB7 growing in diverse sub-lethal stress conditions at 24 h, 5 d and 11 d of incubation. Strains inoculated (2%, v/v) in reconstituted skim milk (RSM) and incubated at 30 °C were used as controls (indicated as N6 and MB7). Diverse stress conditions are indicated by: −1, RSM + glucono delta-lactone (GDL, 0.5% w/v) incubated at 30 °C; −2, RSM incubated at 10 °C; −3, RSM + NaCl (4% w/v) incubated at 30 °C and −4, RSM + NaOH (pH 8 as initial value). Incubation times are indicated as −24 h, -5 d and −11d at the end of each sample. Colours ranging from dark to light turquoise indicate low to high abundance of volatile compounds; absence of detection is indicated in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Assessment of pH values and viable cell counts of *Leuconostoc* strains growing under diverse sub-lethal stress conditions.

Strain and growth conditions	Determination of							
	pH[f]				Viable cell count[f] (log CFU/ml)			
	0 h	24 h	5 d	11 d	0 h	24 h	5 d	11 d
***Ln* N6**								
Control[a]	6.46 ± 0.02	5.13 ± 0.14	4.56 ± 0.13	4.57 ± 0.07	7.15 ± 0.09	8.45 ± 0.11	7.15 ± 0.05	5.10 ± 0.10
RSM − 10 °C[b]	6.42 ± 0.04	6.25 ± 0.05	5.56 ± 0.03	5.32 ± 0.05	7.08 ± 0.08	8.04 ± 0.08	8.36 ± 0.07	7.28 ± 0.07
RSM − acidic[c]	6.27 ± 0.05	4.72 ± 0.08	4.48 ± 0.09	4.48 ± 0.10	7.08 ± 0.10	8.15 ± 0.08	6.18 ± 0.11	6.08 ± 0.08
RSM −NaCl[d]	6.18 ± 0.02	5.81 ± 0.04	4.90 ± 0.05	4.81 ± 0.04	7.11 ± 0.05	7.89 ± 0.12	7.67 ± 0.10	6.36 ± 0.12
RSM- alkaline[e]	8.07 ± 0.03	5.70 ± 0.03	4.71 ± 0.08	4.72 ± 0.11	7.11 ± 0.06	8.57 ± 0.09	6.59 ± 0.12	5.65 ± 0.05
***Ln* MB7**								
Control	6.47 ± 0.01	5.61 ± 0.08	4.82 ± 0.03	4.72 ± 0.07	7.23 ± 0.10	8.49 ± 0.12	7.34 ± 0.05	6.67 ± 0.11
RSM − 10 °C	6.46 ± 0.10	6.40 ± 0.09	6.27 ± 0.15	5.48 ± 0.05	6.61 ± 0.12	7.58 ± 0.08	8.23 ± 0.01	8.34 ± 0.06
RSM − acidic	6.39 ± 0.09	4.94 ± 0.12	4.62 ± 0.09	4.53 ± 0.10	7.08 ± 0.09	7.73 ± 0.05	6.99 ± 0.11	5.13 ± 0.08
RSM −NaCl	6.22 ± 0.05	5.89 ± 0.08	5.02 ± 0.18	4.81 ± 0.04	7.25 ± 0.08	8.04 ± 0.05	8.04 ± 0.09	7.48 ± 0.11
RSM- alkaline	7.95 ± 0.06	6.43 ± 0.11	5.01 ± 0.10	4.85 ± 0.07	7.61 ± 0.10	8.50 ± 0.07	8.04 ± 0.07	5.20 ± 0.05

The strains were inoculated in: [a] Reconstituted skim milk (RSM), incubated at 30 °C; [b] RSM, incubated at 10 °C; [c] RSM + glucono delta-lactone (GDL, 0.5 % w/v), incubated at 30 °C; [d] RSM + NaCl (4 % w/v), incubated at 30 °C; [e] RSM + NaOH (pH 8 as initial value), incubated at 30 °C. Values are the mean and standard deviation of two independent trials.

alike as well (of approximately 2.3 log orders). Similarly, bacterial growth in the presence of NaCl was diminished, and the evolution of pH was comparable for both strains. Finally, growth of the strains was not affected by the alkaline medium and viable cell counts were similar to those obtained for the controls.

4. Discussion

Leuconostoc, as other microorganisms used as starters in food elaboration, is frequently subjected to diverse adverse growth conditions during the manufacture process. The stress factors

usually involved include pH variations (acidity or alkalinity), temperature (heat and cold), oxidative and osmotic changes, between others (van de Guchte et al., 2002; Zotta et al., 2008). In a previous work, we studied the response of 29 *Leuconostoc* strains (survival aptitude) against diverse lethal stress treatments (D'Angelo et al., 2017), which allowed us the selection of twelve strains for further studies. The aptitude of each strain to grow under sub-lethal stress conditions (adaptation) depends on their ability to express genes whose products are required to oppose to injurious events (Serrazanetti et al., 2009). The responses of the selected strains against thermal- (cold), acidic-, alkaline- and osmotic-mild stress conditions were, in effect, strain but not species dependent. Adaptation to mild changes of the environment may allow bacteria not only to survive, but also to grow when confronted to environmental conditions which may normally be unfavorable (Kim et al., 1999; Reale et al., 2015). Mechanisms involved in adaptation are very complex and they act in sense to improve bacterial stress resistance. The integration of these stress responses is accomplished by networks of gene regulators that allow the cell to react to various and complex environmental shifts (Serrazanetti et al., 2009).

As known, *Leuconostoc* strains are included in adjunct starters not as acidifying but as gas (CO_2) and volatile compounds producers. For this reason, *Leuconostoc* isolates are usually used in combination with acid-producing lactococci as starters or starter adjuncts (Server-Busson et al., 1999; Hemme and Foucaud-Scheunemann, 2004). In this sense, it is important to study the potential of strains to produce aroma compounds, not only in optimal growth conditions but also when they are subjected to stress factors commonly present during the manufacture of fermented foods. Moreover, the selection of new strains based not only on their production of aromatic compounds but also on their resistance to various stress conditions, is indispensable.

The production of volatile compounds from LAB involves three major metabolic pathways: (1) metabolism of lactate and citrate, (2) liberation of free fatty acids and their subsequent metabolism, (3) degradation of the proteins to a range of peptides, followed by degradation to free amino acids, and ultimately involves the catabolism of free amino acids (Sgarbi et al., 2013). The potential to produce flavor compounds is a strain-specific capacity (Pogačić et al., 2016). *Leuconostoc* genus is known for its particular metabolism that includes the utilization of carbohydrates and citrate, leading to typical compounds such as diacetyl and related compounds (acetoin, 2,3-butanediol), ethanol, acetaldehyde, acetic acid, among others (Keenan, 1968; Hemme and Foucaud-Scheunemann, 2004). In this work, optimal growth conditions (RSM, 30 °C) allowed *Ln. lactis Ln* N6 showed faster transformation of lactose in comparison to *Ln. mesenteroides Ln* MB7, since higher ethanol production and lower pH values (due mainly to lactic acid production) were revealed for the former. Similar behavior was observed regarding citrate utilization, as acetic acid level was higher for *Ln* N6 than for *Ln* MB7. In this sense, it is reported that *Ln. lactis* shows more rapid acidification than other species and such a difference is also stated for the rate of citrate utilization (Hache et al., 1999). The maintenance of the homeostasis of internal pH is essential for growth and survival of LAB. *Ln. lactis* species is able to regulate better this parameter than *Ln. mesenteroides* and, in consequence, its growth is less self-limited by acid production (Hemme and Foucaud-Scheunemann, 2004). Conversely, strain *Ln* N6 showed also higher production of other aroma compounds such as acetaldehyde, ethyl acetate, isoamyl acetate, 3-methyl 1-butanol, 2-heptanol and 2-octanol than *Ln* MB7, mainly at the end of incubation time. Alegría et al. (2013) studied the production of volatile compounds of 14 strains belonging to *Ln. citreum* (eight strains), *Ln. mesenteroides* (four strains) and *Ln. lactis* (two strains) incubated in milk during 24 h, 48 h and 5 days. These authors reported the production of eight volatile compounds, being ethanol the major one for all strains. On the other hand, acetic acid, 2-propanone and ethyl acetate were produced by almost all strains studied, whereas butyric acid, 2-butanone, 2-heptanone and diacetyl were rarely detected. Guglielmotti et al. (2012) evaluated the effect of high pressure homogenization and heat treatment on volatile profile of adventitious *Leuconostoc* strains isolated from Cremoso Argentino cheese. They reported the presence of numerous volatile compounds including aldehydes, alcohols, hydrocarbons and diverse acids, which levels depended on the applied treatment. Nieto-Arribas et al. (2010) tested 27 *Leuconostoc* isolates from Manchego cheese regarding their ability to produce diacetyl and acetoin, revealing little or no production of these compounds by the analyzed strains. Pogačić et al. (2016) studied the potential of *Leuconostoc* to synthesize volatile compounds. The different strains were incubated in a curd-based slurry medium under conditions mimicking cheese ripening. They found that *Leuconostoc* strains produced high concentrations of primary and secondary alcohols, esters and sulphur compounds. Regarding fatty acids, we detected net production of these compounds at similar levels for both strains in optimal growth conditions, at all times assayed. The presence of fatty acids is associated with esterolytic and lipolytic activity of strains, which have been reported in *Leuconostoc* species (Nieto-Arribas et al., 2010; Pedersen et al., 2013).

Although diacetyl and acetoin are considered essential for flavor development in cheese and cream, they were not detected in this study. These results are in agreement with several reports supported by other authors for *Leuconostoc* strains (Cogan, 1987; Levata-Jovanovic and Sandine, 1996; Schmitt et al., 1997; Sánchez et al., 2005; Nieto-Arribas et al., 2010; Pogačić et al., 2016). The inability of the strains to produce these compounds may be due to incubation conditions that did not favor the corresponding metabolic pathways (Pogačić et al., 2016).

It is worth noting that, in optimal growth conditions and at the end of the experience (11 d), reduction in viable cell counts of *Ln* N6 was higher than that of *Ln* MB7 (almost 3.5 versus 1.8 log orders, respectively). These data could be indicating not only cell death but potential cellular lysis and thus bacterial cytoplasmic enzymes could have been releasing into the food matrix. Proteins, oligopeptides, lipids, fats, and fatty acids are the main substrates in food matrices for cytoplasmic enzymes released by fermenting LAB, which have a potential impact on the aroma profile of the fermented food product (Smid and Kleerebezem, 2014; Lazzi et al., 2016).

This work revealed that diverse sub-lethal stress conditions affected growth ability of the strains, as it was shown by decreased viable cell counts (Table 5). Most of stress conditions applied (excluding alkaline medium) diminished ethanol and acetic acid production because they caused a delay in cell growth. In particular for strain *Ln* MB7, reduction of pH by addition of GDL, slightly increased the production of acetic acid, in comparison to that obtained in optimal growth conditions. This could be due to the fact that citrate is better metabolized at pH values close to 5.3 (Cogan et al., 1981; Schmitt and Diviès, 1991). Thus, the drop of pH by GDL hydrolysis would allow a faster citrate use, even when there was a delayed cell growth, with the consequent production of acetic acid. In this condition, both strains demonstrated to produce similar amounts of acetic acid.

Even though the strains belonging to *Ln. lactis* have previously demonstrated higher resistance to lethal stress conditions than *Ln. mesenteroides* strains (D'Angelo et al., 2017), this work revealed similar behavior for both species when they were subjected to sub-lethal stress conditions. In this sense, adaptation was undoubtedly a strain - dependent phenotype. On the other hand, the production of

volatile compounds depended on the growth capacity shown by the strains against the stress factor applied. In optimal and under growth stress conditions as well, *Ln. lactis Ln* N6 showed richer volatile profiles than those obtained for *Ln. mesenteroides Ln* MB7. These results could be indicating a relationship between *Leuconostoc* species and volatile production. Due to the low number of strains analyzed in this work, more studies to verify the strain- and/or the species-dependency of volatile compound production are mandatory.

Acknowledgments

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