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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