Characterisation and use in food matrices of *Lactobacillus casei* group strains

A Ph.D. dissertation presented by

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to the

University of Udine

for the degree of Ph.D. in the subject of

Food Science (Cycle XXIX)

Department of Agricultural, Food,
Environmental and Animal Science

UNIVERSITY OF UDINE

Italy

March 2016

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"Under the most rigorously controlled conditions of pressure, temperature, volume, humidity, and other variables, any experimental organism will do as it damn well pleases."

(Harvard Law, in Bloch, Arthur (1980 edition).Murphy's Law, and Other Reasons Why Things Go WRONG, Los Angeles: Price/Stern/Sloan Publishers, Inc.)

ABSTRACT

Different strains from international collections and universities (University of Udine, University of Basilicata, University of Molise, University of Stellenbosch-South Africa, etc.) have been subjected to technological characterisation and the study of safety traits. Specifically it has been evaluated: their ability to growth at different temperatures or pH values and different concentrations of NaCl or ethanol; ability to produce biogenic amines, by qualitative analysis and the search of genes involved in their production (*hdc, agdi, odc* and *tyrdc*); presence of gene implicated in the production of ethyl carbamate (*arcABC* genes); ability to produce bacteriocins and monoamine oxidase; antibiotic resistance; hemolytic activity.

After that, the effect of anaerobic, aerobic, respiratory and acidic condition of growth on the cellular structure was investigated. Strains previously selected were analysed using Transmission Electron Microscopy (TEM). Due to the results obtained during this study, the project focused on assessing the effect of two conditions of adaptation (anaerobic and respiratory) and technological stress (milk fermentation) applied to two selected strains, previously identified as belonging to the species *L. casei* and *L. paracasei*. The following analysis were carried out: fermentative capabilities of the strains; samples shelf life; antibiotic resistance; hemolytic activity; bacteriocins production; competition with pathogens; resistance of the stressed cells to *in vitro* digestion. In the simulated gastro-intestinal tract the viable but non-colturable cells (VBNC) were also investigated. Also the proteolysis in fermented milk and the antimicrobial ability of the soluble fraction have been further investigated.

Moreover, the study was also aimed at evaluating the activity into salami of a selected strain of *L. casei* adapted in the two different conditions (anaerobic and respiratory). The following characteristics were analysed: fermentative capabilities of the strain; evaluation of the chemical/physical parameters (aw, pH, color, weight loss); changes in salami micro - flora depending on inocula; presence during the ripening of the inoculated strain (DGGE); antioxidant activity and volatile molecules profile (GC-MS / SPME); proteolysis (SDS - page); evaluation of VBNC.

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Introduction

Lactic Acid Bacteria

Lactic acid bacteria (LAB) are Gram-positive, non-spore-forming, catalase negative bacteria. The shape of the cells can occur as rods or coccobacilli. They are microaerophilic, chemo-organotrophic and fermentative, that produce lactic acid and are often involved in food and feed fermentation (Von Wright and Axelsson, 2012). The presence of the same specific enzyme of the energetic metabolism in different genera of LAB, demonstrate the existence of a strong natural affinity and a close phylogenetic relation among them (Figure 1)

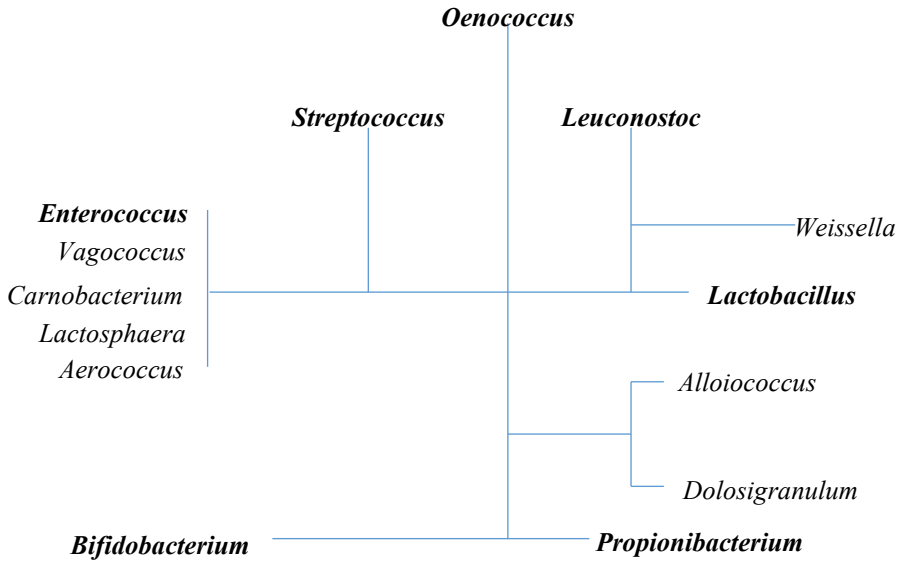

Figure 1 Major phylogenetic groups of the LAB. In bold are the genera that are involved in fermented food. (Zambonelli et al., 2001)

The classification of the LAB is based on the type of fermentation (Barrangou et al., 2012):

- obligate homofermentative: are able to ferment hexoses almost exclusively to lactic acid by the Embden-Meyerhof-Parnas (EMP) pathway, while pentoses and gluconate are not fermented as they lack phosphoketolase;
- facultative heterofermentative: they degrade pentoses and often gluconate as they possess both aldolase and phosphogluconate pathway producing lactate, ethanol or acetic acid and carbon dioxide; moreover, pentoses are fermented by this pathway;
- heterofermentative: they convert about the 50 % of glucose in lactic acid, producing a considerable amount of acetic acid, ethanol and carbon dioxide (Felis and Dellaglio, 2007).

Heterofermentative species are considered important for their capabilities to form, during fermentation, aromatic compounds, like acetaldehyde and diacetyl that contribute to the formation of flavour and aroma.

The relevance of their role in promoting human health, as well as their impact in food and beverage fermentation have been studied in recent years (Matsuzaki et al., 2007). This bacteria are ubiquitous and they are present in a wide range of environments such as: milk and dairy

products, meat, fermented foods, fish products, surface of some plants and fruits, gastro intestinal tract of humans and animals. Moreover, LAB are extensively used in food production and preservation such as yogurt, cheese, fermented milk and meat (Ringø and Gatesoupe, 1998; Konings et al., 2000; Solieri et al., 2012). They are traditionally used to extent shelf-life and enhanced hygienic quality and safety of foods. The natural preserving capacity of food of LAB, has gained increasing interest during the recent years, due to consumers demand for reduced use of chemical preservatives (Voulgari et al, 2010). Also, their antagonistic capabilities against potential pathogens had an important impact on fermented foods and intestinal microflora (Klein et al., 1995; de Vrese and Marteau, 2007).

The genus *Lactobacillus*, with 170 species (Goldstein et al., 2015) and subspecies, represents the largest group of LAB. Members of the genus are strictly fermentative and aerotolerant, but they grow well under anaerobic conditions. Because the main end product is lactic acid, lactobacilli prefer relatively acidic conditions (pH 5.5 and 6.5) (Giraffa et al., 2010; Salvetti et al., 2012). Considering DNA base composition of the genome, they usually show GC content lower than 54 mol % (Felis and Dellaglio, 2007). Species of this genus has been widely studied because of their use in improving the quality or health aspects of many foods (production, preservation, inhibition of pathogens), their importance in human health (probiotics) and queries by legislative industry, bodies and consumers about strain integrity, patents, safety and labelling (Shu et al., 1999; Holzapfel and Schillinger, 2002; Singh et al., 2009, Doherty et al., 2010; Douillard et al., 2013c).

Lactobacillus casei group

Lactobacillus spp. includes the *L. casei* group. The strains of this group are extensively used in various commercial and traditional fermented foods. The first classification of this group consisted in a single species (*Lactobacillus casei*) with five subspecies: *casei*, *galactosus*, *pseudoplantarum*, *tolerans* and *rhamnosus* (Singh et al., 2009). Nowadays, the classification used is the one proposed by Collins et al., (1989), that is composed by three species:

- *L. casei:* strains previously belonging to the *L. casei* subsp. *casei*;
- *Lactobacillus paracasei* with two subspecies: *L. paracasei* subsp. *paracasei* (including the previous subspecies *L. casei* subsp. *alactosus* and *L. casei* subsp. *pseudoplantarum*) and *L. paracasei* subsp. *tolerans* (including the previous subspecies *L. casei* subsp. *tolerans*);
- Lactobacillus rhamnosus, previously classified as L. casei subsp. rhamnosus.

As reported by several authors, discrimination among the species of this group is not easy. Different strains can have the same phenotypical response, but different genotype (Felis and Dellaglio 2007; Singh et al., 2009; Salvetti et al., 2012). Small differences at nucleotide level in 16S rRNA gene create taxonomic disputes and ambiguity (Iacumin et al., 2015), not only among *L. casei* group species, but also in other groups, such as *Lactobacillus delbrueckii, Lactobacillus acidophilus* and *Lactobacillus plantarum* (Singh et al., 2009; Salvetti et al., 2012). Salvetti et al., (2012) gave a taxonomic update of the *genus Lactobacillus*, confirming that many species of *L. casei* group share a 16S rRNA sequence identity higher than 98.8%. Therefore, in 2008 the Judical commission of the International Committee on Systematics of Bacteria reclassified *Lactobacillus zeae* as *L. casei*, excluding it as a single specie.

The correct identification of the species is very important and probiotic producers have to be very careful with the nomenclature of the strains used. Several techniques have been used to identify and characterize *Lactobacillus* spp. isolates based on their physiological characteristics (peptidoglycan analysis, studies of the fermentative pathways, carbohydrates assay, etc.). The

use of biochemical and phenotypical test can be applied as preliminary identification test. However, this analysis often gives conflicted results, because of the strong similarities among species (Richard et al., 2001; Dubernet et al., 2002; Huang et al., 2011). The advancement in molecular assay increased the performance of identification and discrimination of species belonging to *L. casei* group (Bernardeau et al., 2008). Several authors proposed different couples of polymerase chain reaction (PCR) primers and various methodologies (specie specific PCR, Rep - PCR, DGGE, TGGE, PFGE, RAPD). The primers used have different target, such as V1 region of 16S rRNA gene, 16S-23S rRNA intergenic spacer region or 16S rRNA gene sequences, fragment of the *rec*A gene (Ward and Timmins, 1999; Walter et al., 2000; Felis et al., 2001; Vasquez et al., 2001; Berthier et al., 2001; Desai et al., 2006, Huang and Lee, 2009). Moreover, 10 *L. casei*, 7 *L. paracasei* and 8 *L. rhamnosus* strains have been completely sequenced and assembled. Other strains have been sequenced, but the assembly is not finished yet (http://www.ncbi.nlm.nih.gov). Finally, the existing correlations between strains behaviours and their genomic characteristics were studied in several comparative genomic studies (Douillard et al., 2013a; Douillard et al., 2013b; Douillard et al., 2013c; Smokvina et al., 2013; Boonma et al., 2014; Yu et al., 2014; Rasinkangas et al., 2014; Kant et al., 2014; Nadkarni et al., 2014).

Stress response in *Lactobacillus casei* group

Depending on their environmental origins and employment, lactobacilli may encounter several kind of stress factors (pH, a_w, starvation, oxygen, osmotic pressure, etc.). The microbial response to stress could stimulate several defence mechanisms (Hosseini Nezhad et al., 2015). Common stress factors, that *Lactobacillus* strains can deal with their industrial or health employment, are displayed in Figure 2.

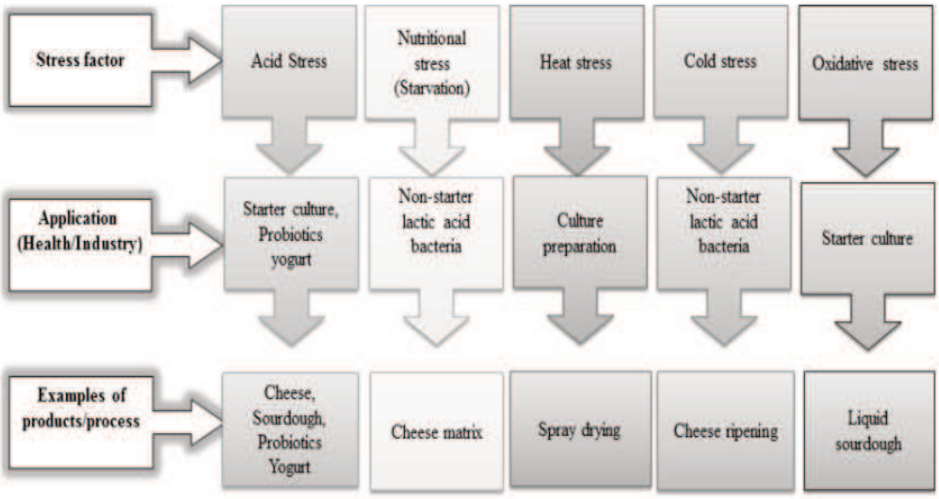

Figure 2: Example of some stress factors that *Lactobacillus* spp. may encounter (Hussain

LAB strains can use two defensive systems to survive and respond to stresses. The first one is activated by the cell in order to remove the chemical or physical stress source, while the second defensive system work through the activation of metabolic pathways allowing the cells to adapt to the adverse environment (De Angelis and Gobbetti, 2004). Microorganism that have specific regulators to each of its regulated genes and adapt expression depending on habitat condition could quickly respond to stresses, which is essential (Serrazanetti et al., 2009).

Acid stress

The acid stress response mechanism of LAB have a great relevance, because of their capability to survive to their own by-products of metabolism and for their use in low-pH foods. The production of organic acids as end-products of fermentation, that determine an acidification of the environment enabling the survival of many other organisms, are one of the main characteristic of LAB. Moreover, their use as probiotics subjected strains to extreme acidic conditions, as they reach the stomach. Passive diffusion allows the acids to enter into the cells, where they are rapidly dissociated. This causes a cytoplasmic acidification that damage peptides and nucleic acids and reduces the activity of acid-sensitive enzymes. In order to maintain the pH homeostasis and survive, the cells developed several protection mechanisms. Common mechanism include: a) decarboxylation reactions, with the formation of biogenic amines; b) desamination/amidation delivering ammonium; c) ATPase triggered proton pumping; d) malolactic fermentation; e) conversion of glutamine to glutamate and f) deviations of pyruvate methabolism towards C-4 compounds (Serrazanetti et al., 2009). Also, acid tolerance response (ATR) mechanism could be possessed by lactobacilli. This last mechanism permits the adaptation of their physiology to low pH, involving the production of acid shock proteins (ASPs), protecting microorganisms not only from acid stress but from heat, osmotic or oxidative stress, too. Furthermore, several studies confirmed that membrane fatty acid adaptation is involved in acid stress response. In fact, strains of *L. casei*, grown under low pH conditions, alter their membrane composition to contain increased levels of long-chained, mono-unsaturated fatty acids (Fozo et al., 2004; Wu et al., 2012; 2014). Another study focused on the behaviour of *L. casei* Zhang acid-resistant mutants revealing that as response to acid stress, the membrane fluidity was enhanced and a higher proportion of unsaturated fatty acids was found (Zhang et al., 2012).

Proteomic approaches revealed several changes in the relative levels of protein expression among strains growth at the optimal condition compared to the same growth at low pH. The adaptation of an ATR strategy by the strain to adapt itself to acidic condition is assumed (Hossein Nezhad et al., 2010). In another study, the authors found the impact of acidic pH on growth rate of *L. casei* strain GCRL 12, confirming that the adaptation to acid environments is associated to surface proteins of this strain (Hossein Nezhad et al., 2012).

Other defence mechanism is the ATP dependent expulsion of protons by a proton-translocating ATPase. This expulsion induce the production of basic compounds by the arginine deaminase pathway, composed by three enzymes that catabolise arginine producing NH_3 and ATP, or urease activity, that catalyses the hydrolysis of urea to CO_2 and ammonia (Van de Guchte et al., 2002; Champomier-Vergès et al., 2002; De Angelis and Gobbetti, 2004).

Yanez et al., (2008) studied how acid stress can interfere with changings in growth rate and lactate production in *L. rhamnosus* cells. Their findings demonstrated that the acid toxicity caused a decrease of the maximum growth rate, but in this environment the cells were still able to produce lactate.

On the other hand, under acidic condition *L. rhamnosus* GG upregulate the expression of F(0)F(1)-ATP synthase genes (involved in the ADI pathway). This strain modulates its pyruvate metabolism as response to environmental pH changings (Koponen et al., 2012).

Acid stress induces the production of Autoinducer-2 signalling molecules, mediated by LuxS enzyme. The AI-2 activity and transcription of the *luxS* gene were evaluated after exposure of *L. rhamnosus* GG to different low pH conditions (pH 5.0, 4.0 and 3.0) and to pH 6.5 (control) in adapted and non-adapted cells by Moslehi-Jenabian et al., (2009). In non adapted cells, AI-2 activity increased as the pH was low in a dose-dependent manner and was negatively influenced by acid pre-adaptation. Results showed that the *luxS* gene expression augmented

over time. It reached a maximum expression level and decreased subsequently in non-adapted cells, while the acid adaptation determined a decrease in transcription levels confirming the involvement of this gene in acid stress response.

Heat stress

Heat tolerance of LAB is a composite process, that involves several proteins with different function, such as ribosome stability and ribosomal activity, severe response mediation and chaperone function. Heat stress typically occurs during the production of dairy products and dried cultures used as technological starters or probiotics. The effects of high temperature on *Lactobacillus* spp. have been extensively studied (Derrè et al., 1999; Prasad et al., 2003; De Angelis et al., 2001; De Angelis and Gobbetti, 2004; G-Alegría et al., 2004; Spano et al., 2005; Sugimoto et al., 2008; Suokko et al., 2008; Parente et al., 2010; Ricciardi et al., 2012). Time occurring to initiate the stress response is different, reliant on the treatment (Serrazanetti et al., 2009).

When cells are exposed to high temperatures, the main effect is the denaturation of proteins, membrane and nucleic acids. Cells start increasing the synthesis of molecular chaperones known as heat shock proteins (HSPs) as response to the heat shock. The role of these proteins is to refold the stress-denatured and aggregated proteins, mediate the correct folding of newly synthetized peptides, translocate proteins across membranes, prevent protein aggregation and assembling or disassembling oligomeric structures. Main groups of HSPs are: 70 kd HSPs and 60 kd HSPs. The first is also known as DnaK chaperon, composed by DnaK, DnaJ and GrpE as main components, involved in the general stress response mechanisms. The second HSP is known as GroEL complex, consisting in GroEL and GroES proteins (Van de Guchte et al., 2002; De Angelis and Gobbetti, 2004; Sugimoto et al., 2008).

Heat shock (HS) response in three *Lactobacillus* strains, including *L. casei* LC301 were studied by Broadbent (1997). Thermo-tolerance experiments showed that HS enhance the synthesis of the heat shock proteins. Moreover, an increase in GroEL expression were revealed, by two-dimensional polyacrylamide electrophoresis gel, also in probiotic *Lactobacillus paracasei* NFBC338 when treated at 52 °C for 15 min (Desmond et al., 2004).

The importance of GroESL in stress tolerance were underline by Corcoran et al., (2006), that also suggested that the selection of strains overproducing this HSP could be useful for the production of probiotic cultures. This because of their study was a comparison of the viability of an overproducting GroESL strain of *L. paracasei* NFBC 338 after spry and freeze-drying, and controls demonstrated that the overproducing GroESL strain showed a better survival.

L. paracasei NFBC 338 pre-treated in a 0.3 M NaCl environment demonstrated an higher resistance to heat stress (60 °C for 30 min) than the non-adapted control (Desmond et al., 2001). Other study performed on the same strain, demonstrated that the *GroEL* expression was increased after the exposure to 52 °C for 15 min as heat adaptation conditions (Desmond et al., 2004).

Pre-treated cells of *L. rhamnosus* HN001 (microbial starter) showed an enhanced tolerance against heat stress. When this strain was pre-stressed with either heat (50 °C) or salt (0.6 M NaCl), it showed a significant ($P < 0.05$) improvement in viability compared with the non-stressed control culture after storage at 30 °C in the dried form (Prasad et al., 2003).

Other studies demonstrated that the use of protective agents can increase the heat tolerance of the strains (Gardiner et al., 2000).

Cold stress

The entire protein expression process is closely related to modification of cellular metabolism (reduction of the membrane fluidity, DNA supercoiling and formation of stable secondary structures in the nucleic acids that cause a reduced efficiency of replication, transcription and protein synthesis), that occurs during the adaptation to a new and/or hostile environment. Cold stress in lactobacilli is mostly related to industrial processes, such as refrigerated storage of starter and probiotic cultures, fermentation at low temperatures during cheese ripening, temperatures far below the optimum of grow during food preparation process or chill storage of fermented products. Lactobacilli are capable to grow at a reduced rate after a decrease of about 20 °C below their optimal growth temperature. The adaptation of the microorganisms to the cold shock consists in the synthesis of Cold-Induced Proteins (CIPs). These proteins were found in a wide range of bacteria and they have a fundamental role in maintaining the membranes fluidity by increasing the proportion of shorter and/or unsaturated fatty acids in lipids composition. They can also reduce the DNA super-coiling and stimulate the transcription and translation necessary by the cell for the adaptation to low temperature (Champomier-Vergèsa et al., 2002; van De Guchte et al., 2002; De Angelis and Gobbetti, 2004; Phadtare, 2004). Cold-shock proteins (CSPs) are the main among CIPs proteins. Cold induction of this last proteins is composite but seems to be regulated mainly at the post-transcriptional level (Serrazanetti et al., 2009).

Sauvageot et al., (2006) studied the presence of a Csp-like protein in *L. casei* BL23, cloning and characterizing it using Northern blot analysis. They observed that *cspA* expression was induced after a decrement of the temperature from 37 °C to 20 °C. Additionally, they constructed a mutant (Δ*cspA*), and a decreased growth rate compared to the wild type was observed.

A proteome analysis on *L. casei* mutants was performed by Beaufils et al., (2007), observing that the cold shock protein CspA was significantly overproduced compared to the wild-type strain. Moreover, the temperature shift, as well as medium composition, were related to mutant growth rate. Mutants grown in medium with glucose showed higher sensitivity to cold stress, but in the presence of ribose or maltose medium resistance to freezing and thawing was similar to the wild-type strain suggesting a direct interaction of HPr, or one of its phosphor-derivatives, with cspA and/or another undetected cold shock protein in *L. casei*.

Osmotic stress

In their natural environment, lactobacilli are often exposed to changing in the osmolarity. Also, during their application in food industries can incur in osmotic stress, due to the presence of a high concentration of salt and sugars in some production. The general response observed in lactobacilli in a hyperosmotic environment is the accumulations of solutes like glycine betaine, proline glutamate (not interfering with the cell physiology) or the synthesis of general stress proteins (including HSPs GroES/GroEl and DnaK). On the other hand, when an increase of the osmolarity occurs the release of water from the cytoplasm to the outside, causing loss in the cellular turgor, is the main effect. An intracellular accumulation of compatible solutes can preserve turgor of the cells (Van de Guchte et al., 2002; De Angelis and Gobbetti, 2004; Hörmann et al., 2006).

Several papers have been reported, as response to osmotic stress, the modification in the membrane composition of *L. casei* strains. Changes in the chemical and structural properties of the membrane and other mechanisms occur in *L. casei* ATCC 393 when exposed to hyperosmotic conditions, finding that the hydrophobicity and the bile salt sensitivity of the cultures were increased after the stress exposure (Machado et al., 2004). Other strains of *L.*

casei grown under high salt conditions presented changes of cell wall components, such teichoic acids and the contents of the cell wall polymer lipoteichoic acid (LTA) that can increase the ability of these microorganisms to form biofilms (Piuri et al., 2003,2005; Palomino et al., 2013).

L. rhamnosus VTT E-97800 (E800), probiotic strain, was studied to investigated the response to sucrose osmotic stress. Under the conditions selected for the tests, the strain was able to adapt and survive with no significant loss of culturability/viability. This results confirm the capability to accumulate sucrose of these cells, as response to changes in their environmental osmotic conditions, in order to protect both the membranes and internal organs (Sunny - Roberts et al., 2008).

Bile salt tolerance

Bile salts display a strong antimicrobial capability, due to its capability to disassemble biological membranes. *L. casei* group members are potentially probiotic microorganisms, so it is important for them to possess the resistance at the antimicrobial action of bile salts. Lactobacilli survive to the bile salt action thanks enzymes that inactivate the bile acids deconjugating them (bile salt hydrolases, BSH) (Van de Guchte et al., 2002; De Angelis and Gobbetti, 2004). *L. casei* strain Zhang, isolate from home-made koumiss in Inner Mongolia of China, showed high resistance to bile salts, due to this capability the action of its BSH were investigated. The predicted BSH gene was significantly up-regulated under the stress of bile salts (Zhang, 2009). A subsequent study on the same strain compared the growth and protein expression patterns when exposed to bile salt stress. Twenty-six (26) proteins differentially expressed were found, using a 2-dimensional gel electrophoresis, in strain tested under normal growth condition and in the presence of bile salt. Real-time, quantitative reverse transcription-PCR and bioinformatics analysis were used for further verification. The obtained results showed that bile stress response is characterized by the activation of genes involved in cell membrane structure (*nagA*, *galU*, and *PyrD*), in cell protection (*dnaK* and *groEL*) and in other housekeeping genes (*pepC*, *cysK*, *pfK*, *pgM*, *luxS* and *ef-Tu*) (Wu et al., 2010).

Koskenniemi et al., (2011) studied bile stress response in probiotic *L. rhamnosus* GG. Their studies revealed that this kind of stress determines changes in the transcriptome, which seems to strengthen the cell envelope against bile-induced stress and indicating to the strain cells the gut entrance. Changing in the transcriptome were detected in another strain, *L. casei* BL 23 after bile stress exposure. The stress carry out by bile salts induced changes in several genes and proteins involved in the stress response and in other cellular pathways (metabolism of carbohydrates, fatty acid and cell wall biosynthesis, transport of peptides, coenzyme syntesis, membrane H(+)-ATPase activity) (Alcántara and Zùñiga, 2012).

Oxidative stress

Lactobacilli are facultative anaerobic bacteria, as they can grow both under anaerobic and aerobic environments. Reactive oxygen species (ROS), such as superoxide (O_2^-), hydroxyl radicals (HO) and hydrogen peroxide (H_2O_2), can generate oxidative stress. Among LAB, there are species possessing enzymes allowing them to degrade, tolerate, or survive to the toxicity of oxygen and reactive oxygen species. Enzymes such as pyruvate, lactate and NADH oxidase have been found in several LAB. They allow the microorganisms to eliminate oxygen from the cell. Manganese superoxide dismutase (SOD), manganese catalase and NADH peroxidase have been extensively studied in *Bacillus subtilis*, *Lactococcus lactis* and *Lactobacillus plantarum* (Van de Guchte et al., 2002; De Angelis and Gobbetti, 2004; Talwalkar and Kaliasapathy, 2003; Pedersen et al., 2008; Zotta et al., 2012; 2013). As reported by Serrazanetti et al., (2009), some strains or species in the presence of heme can express an active respiratory chain and/or have

NADH oxidase. Recently, the oxidative stress mechanisms of *L. casei* group were investigated. Zotta et al., (2014) focused their study on the capability of 184 strains belonging to the species *L. casei*, *L. paracasei* and *L. rhamnosus*, to grow under aerobic condition. To perform the analysis, the tested strains were cultured in media containing heme and menaquinone and/or compounds generating reactive oxygen species (ROS). The authors evaluated the presence of strains with oxygen-tolerant phenotypes. As a surprising result, they found that most of the analysed microorganisms were able to survive the aerobic conditions. Moreover, many strains showed the capability to grow under respiratory conditions. Further, Ricciardi et al., (2014) developed a rapid method to evaluate the oxygen consumption in strains belonging to *L. casei* group grown under anaerobic, aerobic and respiratory conditions. Ianniello et al., (2015) investigate how the aeration and supplementation with heme and menaquinone can affect the antioxidant capability and their survival. On the other hand, implementation of *L. casei* group strains growth under respiratory condition in fermented food were investigated by Reale et al., (2016a/b). The first study investigated the effect of respiratory metabolism of *L. casei* species on qualitative characteristics of sourdough. The selected respirative strain influenced the acidification, the biomass quantity, proteolytic profiles and volatile production compared with the anaerobic cultures. In the second study, two *L. casei* strains cultivated in anaerobic and respiratory conditions, were added in the production of Cheddar-type cheeses. The addition of anaerobic or respirative cultures did not affect cheese composition and primary proteolysis. However, respirative cells increased the production of peptides and free amino acids, acetoin and diacetyl, while the content of free radicals as well as lipid and protein oxidation were reduced.

Potential application of the stress response in food and probiotic industry

As described before, food processing generates several kind of stress sources for LAB. Improving resistance in strains is possible with the adjunction of protective agents or using pre – treatment that can enhance the survival capabilities. As example, *L. paracasei* NFBC 338 survival to several stress sources (heat, bile and H_2O_2) were increased by the addition of an exudate gum from gum acacia (a tree). The addition of this compound to the growth medium also enhanced survival during spray-drying (Desmond et al., 2002). Furthermore, on the basis of stress response studies performed in the recent years, it can be assumed that microbial stress factors can induce the activation of metabolic pathways and can lead to several changes in metabolites production. These metabolites can significantly affect the sensory characteristic, the texture and the general quality of foods in which they are used. Also, they may bring to an increase of the resistance of the microorganism to technological stresses.

Other method to develop stress resistance in microorganism is the novel techniques of bioengineering, that allow the production of mutant strains with enhanced stress resistance capabilities. However, the European Union has very strict condition for the utilization of "artificially" mutated microorganisms (Directive 2001/18/EC). On the other hand, the use of "naturally selected mutants" is not forbidden by the European law. In the last years, several comparative genomic studies have been made, and thanks to the genome sequencing techniques (Heather and Chain, 2016; Loman and Pallen, 2015) a comparative genomics approach could be an efficient tool to discover biochemical pathways for the selection of strains with industrial potential (Zhu et al., 2009). Besides, when it is not possible to artificially manipulate the bacterial genome in order to obtain a strain with implemented technological performances, is possible to exploit stress response in selected strains, in order to enhance their defences against a certain kind of stress (Mills et al., 2011; Reale et al., 2015).

The knowledge of the stress responses in LAB is fundamental for the selection of strains that showing the best industrial performances, not only regarding strains survival but also for their capabilities to improve the quality of the final product.

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Safety traits, genetic and technological characterisation of *Lactobacillus casei* group strains

Introduction

Lactic acid bacteria (LAB) comprise a wide range of genera and include a considerable number of species (Goldstein et al., 2015), like the *Lactobacillus casei* group. They are usually catalase negative and Gram-positive, they growth under strictly anaerobic to microaerophilic conditions and they are able to produce lactic acid (von Wright et al., 2011). These bacteria are the major component of the starters used in fermentation, especially for dairy products, and some of them are also natural components of the gastrointestinal microflora (Coeuret et al., 2003).

Lactobacillus casei, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* are closely related species belonging to the *L. casei* group. However, it is not easy to differentiate *L. casei* from *L. paracasei* strains, whereas *L. rhamnosus* could be easily identified (Klein et al., 1998).

L. casei and *L. paracasei* are normally found in the human and animal gastro intestinal tract and in several environments like milk, meat, vegetables, cereals, beer and wine (Coudeyras et al., 2008). They are extensively used in food industries as starter cultures, for example, in milk fermentation their addition cause an intensification and acceleration of flavor development (Toh et al., 2013), on the other hand their addition can enhance the quality and the hygienic properties of the products. They can be use even as probiotics, like the most studied and first commercially used *L. casei* Shirota used as starter culture in the diary drink Yakult (Capra et al., 2014).

L. rhamnosus have the capability to colonize multiple habitats (Lebeer et al., 2010; Pitino et al., 2012 and Douillard et al., 2013) and it has been isolated from a large variety of ecological niches, e.g. oral cavity, human intestinal tract, vaginal cavity and cheese, showing its adaptability. However, few studies focused on the characterisation of *L. rhamnosus* strains, in spite of its wide use in the production of several kind of foods (Douillard et al., 2013). Of this species the most used and documented strain is *L. rhamnosus* GG, a strain isolated from human intestine, is the most studied strain belonging to this species because of its probiotic features (Douillard et al., 2013).

The strains of *L. casei* group should resist to stressful condition during the industrial process, but also in the natural habitat they had to respond to stress to survive. This species were subjected to particular interest cause of their wide use in food industries and their presence as non-starter lactic acid bacteria (NSLAB) in fermented products. Many intrinsic and extrinsic properties of food, such as water activity, temperature of process and storage, pH, oxygen level, influence viability of this microorganisms.

The strains of *L. casei* and *L. paracasei* are obviously closely related, but is important to understand the differences. There were also few studies focused on the characterisation of *L. rhamnosus* strains, in spite of its wide use in the production of several kind of foods (Douillard et al., 2013).

Moreover, the capabilities of this microorganism to survive under stressful condition is not completely understand. It was established that this species evolved a stress-sensing system against stresses (van de Guchte et al., 2002). The response to several stresses are characterized by physiological changes that, normally, help the microorganism to survive in adverse condition (Hussain et al., 2013)

Even if some strains were largely studied, an extensive knowledge of strains belonging to the *L. casei* group could open new possibilities of application.

However, despite the generally recognized as safe (GRAS) status, *Lactobacillus* spp. must be genetically, phenotypically and taxonomically characterize. Moreover, to find strains with interesting functional and taxonomic traits, that can be used in technological or for probiotic application a fully characterisation is necessary. Among the safety traits, it is necessary investigate the production of biogenic amines (BAs) and the capabilities to degrade them. Also the formation of ethyl carbamate must be examined. Biogenic amines (BAs) are mainly generated by microbial decarboxylation of amino acids, which can be found in fishes, cheeses, meat products, fermented vegetables, wine, beer, etc. (Silla Santos, 1996). The products quality is not altered by their presence, but the ingestion of large quantities of food containing BAs can have toxicological effect (Coton and Coton, 2005; Landete et al., 2007a; Ladero et al., 2011; Costantini et al., 2013). In particular, histamine can induce allergen type reaction, while tyramine can cause vomiting, hypertension and headaches. (Naila et al., 2010; Landete et al., 2007a/b). Putrescine and cadaverine are not considered toxic. However, putrescine can interfere with the detoxification of this compounds, whereas cadaverine can potentiate the toxicity of histamine (Landete et al., 2007a/b; Garcià – Ruiz et al., 2011). A quick detection of bacteria that could produce BAs is fundamental (Coton and Coton, 2005; Landete et al., 2007b). There are several methods to control the formation of BAs in food, including temperature and pH control, use of additives and preservatives, addition of enzymes able to degrade amines such as mono - and diamine - oxidase (AO and DAO) and the use of bacteria which possess these enzyme (Suzzi and Gardini, 2003; Naila et al., 2010; Callejon et al., 2014). Other factor need to be considered in order to guarantee the safety of the strains. FAO/WHO (2006) and European Commission (2004) highlighted the importance to study hemolytic capability and antibiotic resistance. Hemolysis activity promotes iron accessibility to bacteria causing edema and anemia in the host. Moreover is a frequent virulence factor among pathogens (Rodrigues da Cunha et al., 2012). Acquired resistance to antibiotics may results by acquired genes via plasmid or transposon, while intrinsic resistance is not transferable (Ammor et al., 2007; Belletti et al., 2009). Horizontally transfer of these acquired genes concern also *L. casei, L. paracasei* and *L. rhamnosus*. Their large use in food industries and their consequentially presence in different environment without barrier among them and pathogens could lead to a transfer of antibiotics resistant genes.

The aim of this study was to investigate the characteristics of 8 *Lactobacillus casei*, 121 *Lactobacillus paracasei* and 60 *Lactobacillus rhamnosus* type strains and isolates. The microorganisms, previously identified, were evaluated for the presence in their genome of *arcABC* genes and genes involved in BAs production. Moreover, growth capability at different concentration of NaCl, EtOH and different pHs, antibiotic resistance, hemolysis/biogenic amine/antimicrobial compounds production, biogenic amines (BAs)-degrading activity were evaluated.

Materials and methods

Strains

Eight (8) *L. casei*, 121 *L. paracasei* and 60 *L. rhamnosus* strains obtained by isolation from different samples or from international collections by University of Udine, University of Basilicata and by the University of Molise were previously identified by Iacumin et al., 2015 (Table 1). They were stored at the temperature of - 80°C in cryovials containing DeMan, Rogosa, and Sharp broth (MRS, Oxoid, Milan, Italy) supplemented with 2% glycerol.

At the time of use, the cultures were streaked on MRS Agar (Oxoid, Milan, Italy) and incubated at 30°C, in order to check their purity and prepare them for the following stages of the experiment by inoculating a single colony in 2 ml of MRS broth (Oxoid, Milan, Italy).

Table 1 Strains used in this work and their origins and identification

Origin	Identification
Humans (saliva, dental caries, blood, urethra, faeces of infants and adults)	***L. casei***: LMG23516[1], N87[16], N811[16], N2014[16]
	L. paracasei: DSM20020[2], LMG9438[1], LMG11459[1], LMG23511[1], LMG23518[1], LMG23523[1], LMG23538[1], LMG23543[1], LMG24098[1], LMG24101[1], LMG24132[1], DBTA34[18], N161[16], N42[16], N44[16], N76[16]
	L. rhamnosus: DBTA86[18], DBTC4[18], N171[16], N178[16], N715[16], N94[16], N95[16], N83[16], N201[16], N209[16], N2012[16], N132[16], N22[16], N26[16], N812[16], N173[16], N1110[16], N131[16], N21[16], N172[16], N2010[16], N2013[16], N202[16], N25[16], N176[16], N2011[16], TMW 1.1538[6], Mo2[16], N1710[16], N175[16]
Green, creamy and seasoned cheeses (Italian cheeses: Scamorza, Parmigiano Reggiano, Grana Padano, Spressa, Asiago, Montasio, Canestrato di Moliterno, Morlacco, Bellunese, Pecorino, Caciocavallo, Provolone, Emmenthal, Raclette de Savoie; Chinese and Tunisian cheeses)	***L. casei***: CI4368[12]
	L. paracasei: LMG 6904[1], LMG25880[1], LMG25883[1], LMG12164[1], DBPZ0420[8], DBPZ0421[8], DBPZ0422[8], DBPZ0424[8], DBPZ0434[8], DBPZ0435[8], DBPZ0450[8], DBPZ0451[8], DBPZ0472[8], DBPZ0475[8], DBPZ0476[8], DBPZ0477[8], DBPZ0478[8], DBPZ0635[8], DBPZ0733[8], DBPZ0734[8], M266[8], M268[8], M299[8], M308[8], M348[8], M354[8], M359[8], S1[8], S3[8], V3[8], W11[8], DSG03[8], DSG05[8], DSG07[8], ESG10[8], HSG09[8], PSG06[8], PSG09[8], PSG10[8], P71[9], TH1229[9], SP57[9], L24[9], TH406[9], FSL436[10], FSL451[10], DBPZ0436[8], TMW1.1444[6], TMW1.1259[6], LACcas7[7], Cst7[11], 3LC[11], DBPZ0718[8], CF143[12], R61[13], F17[13], N24[13], H12[13],
	L. rhamnosus: M15[9], O14[8], PRA204[5], PRA232[5], PRA331[5], DBPZ0430[8], DBPZ0445[8], DBPZ0446[8], DBPZ0448[8], DBPZ0449[8], FSG01[8], CI230[12], CI4362[12], CF1350[12], CF377[12], D44[13], H25[13], 5A9T[9], 5D9T[9], L9[9], L47[9], LACcas13[7], DBPZ0428[8],
Raw and heat treated milk, yoghurt, milking machines	***L. paracasei***: LMG9192[1], DSM5622[2], P1E6[3], P2P3[3] , DIALIac1[5], DIALIac2[5], DIALIac3[5], DIALIac4[5], DIALIac5[5], DIALIac6[5], DIALIac7[5], DIALIac8[5] , DIALdan1[15] , DIALdan2[15], DIALdan3[15], DIALdan4[15], DIALdan5[15], DIALdan6[15], DIALdan7[15], DIALdan8[15] ***L. paracasei* subsp. *tolerans***: LMG9191[1], DSM20258[2]
	L. rhamnosus:, HA111[4], PRA152[5]
Fermented sausages	***L. paracasei***: CTC1675[14]
	L. rhamnosus: CTC1676[14], 2220[15]
Sourdoughs	***L. casei***: DBPZ0571[8],
	L. paracasei: DBPZ0561[8], DBPZ0572[8], Q2[8], Q4[8], I1[4], I2[16], DBPZ0563[8], DBPZ0564[8], DBPZ0579[8], I3[16]
Wine, must and cellar equipment's	***L. paracasei***: LMG11961[1], LMG11963[1], LMG13717[1], LMG13731[1], B061[17], B161[17], B169[17], B171[17], B195[17], B196[17], B350[17]
Bier, malt	***L. paracasei***: LACcas25[7], LACcas29[7], TMW 1.300[6]
Coffee	***L. casei***: DSM20178[2]
	L. rhamnosus: DIAL40[15]
Unknown	***L. casei***: DSM4905[2]
	L. paracasei: NRRL B-456[19]
	L. rhamnosus: NRRL B-176[19] , DSMZ20021[2]

[1]LMG: BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms (BCCM™), Belgium.
[2]DSMZ: DSMZ, Deutsche Sämmlung von Mikroorganismen und Zellkülturen, Braunschweig, Germany
[3]Dipartimento di Agraria, Università degli Studi di Sassari, Sassari, Italy.
[4]Harmonium International Inc., Mirabel, Canada.
[5]Dipartimento di Scienze Agrarie e degli Alimenti, Università degli Studi di Modena e Reggio Emilia, Italy
[6]Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany
[7]Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Italy
[8]Scuola di Scienze Agrarie, Alimentari e Ambientali, Università degli Studi della Basilicata, Potenza, Italy
[9]Università degli Studi di Verona, Dipartimento di Biotecnologie, Strada le Grazie 15, Verona, Italy
[10]Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy
[11]Istituto sperimentale Lattiero Caseario - I.L.C., Lodi, Italy.
[12]Dipartimento di Scienze e Tecnologie Agro-Alimentari, Università degli Studi di Bologna, Bologna, Italy
[13] Dipartimento di Scienze delle Produzioni Agrarie e Agroalimentari , Università degli Studi di Catania, Catania, Italy.
[14]Institut de Recerca I Technologia Agroalimentaries (IRTA), Lleida, Spain
[15]Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine, Udine, Italy.
[16]Dipartimento di Agricoltura, Ambiente e Alimenti, Università degli Studi del Molise, Campobasso, Italy.
[17] Institute for Wine Biotechnology Department of Viticulture and Oenology, Stellenbosch University, South Africa
[18] Dipartimento di Biotecnologie, Università degli Studi di Verona, Verona, Italy
[19] ARS Culture (NRRL) Collection, United States Department of Agriculture, USA

Growth capability in the presence of different NaCl, EtOH concentration and different pHs.

The growth capabilities of the strains were evaluated. Different concentrations of NaCl (2, 4, 6.5 % w/v) and ethanol (12 – 15 % v/v) were tested, as well as different pH value (3.2, 3.8, 4.2 and 4.6). The strains were grown on MRS broth for 24 h at 30 °C. The cultures were centrifuged at 5000 rpm for 5 min, and the pellets were washed two times with peptone water 0.1 % w/v at pH 7. Then the *inocula* were standardized at 0.1 optical density at 600 nm corresponding to 10^8 CFU/ml, and used to inoculate 200 µl of MRS broth prepared with the addition of NaCl (Sigma-Aldrich, Milan, Italy), or ethanol (EtOH, Sigma-Aldrich, Milan, Italy) to achieve the concentration previously specified, or brought to the required pH by addition of HCl (Sigma-Aldrich, Milan, Italy) at a final concentration of 10^2 CFU/ml.

The growth of the strains was monitored by a spectrophotometer (OD 600 nm) after the inocula and after 3, 24 and 48 h at 30 °C. Strains were defined as sensible (S) strains (from 0 to 0.3 of OD value after 48 h), intermediate (I) strains (from 0.3 to 0.9 of OD value after 48 h) and resistant (R) strains (from 0.9 to 1.5 of OD value after 48 h).

Antibiotic resistance

The strains were grown in MRS broth for 24 h at 37 °C. The inocula were standardized at 0.1 optical density at 600 nm. Then, the antibiotic-susceptible disk diffusion assay was performed according with Belletti et al., (2009) onto plates of MRS agar. After anaerobic incubation for 24 h at 37 °C, inhibition zone diameters were measured using a caliper and results were interpreted as described by Charteris et al. (1998b) as sensitive (S, inhibition zone diameter 19-30 mm), intermediate (I, inhibition zone diameter 10-18 mm) or resistant (R, inhibition zone diameter 1-9 mm). The disk diffusion assay were performed with the following antibiotics: cefoperazone 30 µg (CFP30), cefazolin 30 µg (KZ30), chloramphenicol 10 µg (C10), clindamycin 10 µg (DA10), erythromycin 30 µg (E30), kanamycin 30 µg (K30), ofloxacin 5 µg (OFX5), quinupristin/dalfopristin 15 µg (QD15), rifampicin 30 µg (RD30), streptomycin 25 µg (S25), tetracycline 10 µg (TE10), tobramycin 10 µg (TOB10), vancomycin 30 µg (VA30).

Hemolysis

The strains were grown in MRS broth at 37 °C for 24 h. Then, the cells were recovered by centrifugation at 13,000 rpm for 5 min and resuspended in physiological solution (8.5 % NaCL, Sigma-Aldrich, Milan, Italy). Finally, strains were streaked onto Columbia agar plates containing 5% defibrinated horse blood (Oxoid, Milan, Italy). After 24 and 48 h, the plates were examined for the presence of α, β, γ hemolysis.

Antimicrobial capabilities (Bacteriocin production)

Agar well diffusion assay was performed. As described by Schillinger and Lucke, (1989), the strains were overnight cultured on MRS broth at 37 °C. The cultures were centrifuged for 10 minutes at 10,000 rpm to obtain a cells-free supernatant, followed by filtration through a 0.2 µm pore-size cellulose acetate filter. The hydrogen peroxide inhibitory activity was eliminated adjusting pH to 6.5 and proteinase K (2 mg/ml) was added to the supernatants to inhibit bacteriocins. Then, Brain Heart Infusion (Oxoid, Milan, Italy) soft agar plates (1 % agar) were inoculated with the pathogen (*Listeria monocytogenes* ATCC7644*, Staphilococcus aureus* DSM 4910*, Escherichia coli* DSA*, Salmonella enteritidis* DSA). Four (4) wells of 5 mm in diameter were made into each plate and filled with 100 µl of strain cultures (competition) , 100 µl of pH 6.5 culture supernatant (presence of bacteriocins) and 100 µl of bacteriocin free

supernatant (presence of hydrogen peroxide and/or undissociated lactic acid, low pH) . The plates were incubated for 24 h at 37 °C and examined for inhibition zones.

DNA extraction

DNA extraction from pure colonies was carried out using 2 ml of a 48 h culture in MRS broth, that where centrifuged at 13000 rpm for 10 minutes at 4°C to pellet the cells, which were subjected to DNA extraction by using MasterPureTM Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, USA).

Biogenic Amines (BAs) production

To promote the enzyme production, before the screening test, strains were grown 5 times in MRS broth added with 0.1 % of each precursor amino acid precursor (tyrosine free base, histidine monohydrochloride, ornithine monohydrochloride, arginine hydrochloride and lysine hydrochloride) in addition with 0.005 % of pyridoxal-5-phosphate. After that, the strains were inoculated in plates and broths of decarboxylase media (Bover-cid et al., 1999). A change in purple color in both, plates and broth, gave clear evidence of a positive reaction. Strains were also screened for the presence in their genome of 4 BAs related genes (*hdc*, *agdi*, *odc* and *tyrdc*) using the primers developed by Coton et al., 2010. Positive control strains were also used. In particular, *Enterococcus faecalis* EF37 (strains collection of DISTAL, University of Bologna, Italy), positive for *tyrdc* and *agdi* genes, and *Streptococcus thermophilus* PRI60 positive for *hdc* gene (Rossi et al., 2011). *Lactobacillus rhamnosus* N132, resulted positive in a previous trial (unpublished data) for *odc* gene presence, was used as positive control.

arcABC presence

The genomes of the strains were screened for the presence of *arcABC* genes, coding for enzymes involved in the arginine dihydrolase system (ADI) pathway in *L. casei* group. To detect the presence of *arc* genes, degenerate primers *arcA* (arginine deiminase), *arcB (*ornithine transcarbamylase) and *arc* (carbamate kinase), that produces amplicons of 266, 181, 343 bp, respectively, were used as described by Araque et al., 2009. *L. plantarum 64* and *L. plantarum 70* (strains collection of the Viticulture and Oenology Department, Stellenbosch University, South Africa) were used as positive controls. After PCR, amplicons were resolved by electrophoresis in 1.5 % (w/v) agarose gels 0.5X TBE added with EtBr 0.025 % (v/v).

Biogenic Amines (BAs)-degrading activity: Diaminobenzidine (DAB) assay and Multi

Copper Oxidase (MCO) detection

The strains were cultured overnight at 37 °C on modified MRS supplemented with L-cysteine 0.5 g/L and putrescine, tyramine, histamine at 10 mg/L each. According to the method described by Callejon et al., (2014), after the cell-free extracts were obtained, samples were electrophoresed for an hour at 30 mA in a PolyAcrylamide Gel Electrophoresis (Native - PAGE). After protein separation, the gel was used for in-gel aminedegrading activity (DAB) or multicopper oxidase (MCO) activity detection. The presence of amine-degrading activity was revealed by the apparition of a brown color on the active band after 1–2 h, due to the precipitated product of DAB oxidation. MCO activity was revealed after 10 min by the presence of an orange-yellow band.

Statistical analysis

Average, standard deviation and percentage were calculated in R v 3.3.2 (R Development Core Team, 2011).

Results and discussion

Growth capability in the presence of different NaCl, EtOH concentration and different pHs.

Growth abilities of strains could reveal interesting functional aspects for probiotic or technological employment. The strains did not show significant differences, demonstrating that the resistance to different stresses was strain specific. Only 13 strains showed to be resistant to all the applied stresses (NaCl, EtOH and acidic), two were *L. casei* (DBPZ0571 and N2014) and eleven were *L. paracasei* (LMG11961; LMG23518; LMG24101; DBPZ0324; DBPZ0563; DBPZ0579; Q4; M354; I3; DialDan5 and DialYak5). Most of the strains were sensitive to low pH, on the other hand the strains were resistant to the presence of NaCl at 2% and 4%. There were no significant difference between *L. casei* and *L. paracasei* (Table 2).

Table 2 Results of stress resistance of *L. casei* and *L. paracasei* strains in percentage.

	pH				**NaCl**			**EtOH**	
	3.2	**3.8**	**4.2**	**4.6**	**2%**	**4%**	**6.5%**	**12%**	**15%**
Sensitive	77.7	7.7	3.8	3.1	3.8	1.5	6.2	18.5	22.3
Intermediate	22.3	17.7	1.5	3.1	0	0.8	12.3	16.9	37.7
Resistant	0	74.6	94.6	93.8	96.2	97.7	81.5	64.6	40

Only one strain, *L. rhamnosus* DBTC4 showed to be resistant at 6.5 % NaCl, 15 % of ETOH and resistant at pH 3.8, but intermediate resistance at pH 3.2. The 90 % of the strains showed to be sensitive to pH 3.2, on the other hand the 80 % of the strains were resistant to the presence of 6.5% of NaCl. The results were show in the Table 3.

Table 3 Results of stress resistance of *L. rhamnosus* strains in percentage.

	pH				**NaCl**			**EtOH**	
	3.2	**3.8**	**4.2**	**4.6**	**2%**	**4%**	**6.5%**	**12%**	**15%**
Sensitive	90	13.3	5.0	0	1.7	0	13.3	36.7	55.0
Intermediate	10	18.3	13.3	1.7	0	3.3	6.7	36.7	31.7
Resistant	0	68.4	81.7	98.3	98.3	96.7	80.0	26.6	13.3

Several studies were carried out considering the stress factor that could affect the growth *L. casei/paracasei* species (Hussain et al., 2013). Wu et al., (2014) found that the survival rate of *L. casei* cells at pH 3.5 was 4.5 %, confirming the sensitivity to low pH value. Also Rubio et al., (2014) reported a sensitivity to 3.5 pH value. On the other hand, other studies demonstrated the capabilities of survival and growth of *L. casei*/*paracasei* at lower pH values (Jacobsen et al., 1999; Federici et al., 2014). Regarding NaCl tolerance, the resistance to osmotic stress could be ascribed to cell wall structural properties modification as found by Piuri et al., (2005). The osmotic stress produced by 2% of NaCl did not affect the growth of these species (Rubio et al., 2014). Ethanol is produced during alcoholic fermentation in wine and added as preservative in various food. The information about ethanol sensitivity are dissimilar. However, the majority of the *Lactobacillus* spp. can bear with 15 % of ethanol concentration and *L. casei* are described to be more tolerant (du Toit et al., 2011). This findings are in complete agreement with our results, which showed that the 40 % of the strains was resistant to 15 % of EtOH, whereas the 37.7 was intermediate resistant to 12 % of EtOH.

Regarding *L. rhamnosus* strains different studies were carried out about the capabilities of this specie to survive to a 2 - 3 pH value (Charteris et al., 1998a; Jacobsen et al.,1999; Vinderola et al., 2003; Corcoran et al., 2005; Federici et al., 2014), probably for their wide use in probiotic formulations. Some strains demonstrated good capabilities of survival, but as found by Prasad et al., (1998) out of 200 strains of lactobacilli and bifidobacteria, only a few strains with satisfactory acid resistance were discovered. Rubio et al., (2014) investigated the capabilities of few strains of *L. rhamnosus* to survive to 2.5 % of NaCl, which demonstrate good capabilities of growth in this condition. No alcohol sensitivity range is available, because literature is scarce of studies evaluating this feature in the species analysed in this work. However, *L. rhamnosus* strains were found in continuous wet mill fuel ethanol facility (Skinner et al., 2004), demonstrating the capability of this species to tolerate alcoholic environments. Our results give a first clear and unexpected evidence that confirms the findings of Skinner et al., (2004), in fact the 26.67 % of the strains resulted resistant to the 12 % of EtoH, and the 13.33 % even to the 15 % of EtOH. Moreover, the percentage of strains demonstrating an intermediate resistance was 36.67 % and 31.67 % to the 12 % and 15 % of EtOH, respectively.

Antibiotics resistance

Lactobacillus spp. have a generally recognize as safe (GRAS) status. However, they can cause endocarditis or bacteremia (Belletti et al., 2009; Salminen et al., 2002; 2004; 2006). The attention to their antibiotics resistance increased according to the rise of antibiotics distribution and the knowledge about gene transmission in bacteria. Intrinsic resistance is not horizontally transferable, while acquired resistance can results by an acquisition of genes via plasmids or transponson (Ammor et al., 2007; Belletti et al., 2009). The European Commission, 2004 proposed a qualified presumption of safety (QPS), the safety assessment of food LAB could be limited to the presence of transmissible antibiotic resistance markers. The interest in the resistance profiles of these group is related to their wide use in food industries as starter and their extensive presence in several niches.

There were no significance difference between species. According to Sharma et al., (2014) and Charteris et al., (1998b) the strains of *L. casei* and *L. paracasei* were resistant (>80%) to kanamycin, streptomycin, tobramycin and vancomycin. On the other hand they demonstrated a variable behaviour against ofloxacin. The strains were sensitive to all the other tested antibiotics (Sharma et al., 2014; Ammor et al., 2007; Charteris et al., 1998b; Belletti et al., 2009). A different behavior was observed in 8 strains. *L. paracasei* DBPZ0478 showed a high resistance to the most of the tested antibiotics, except for clindamycin, quinupristin/dalfopristin and rifampicin. Some strains isolated from yogurt showed sensitivity to tobramycin, but they were resistant to erythromycin. *L. paracasei* DialDan8 was sensitive against all the tested antibiotics, with some exceptions. It was kanamycin resistant and erythromycin and tobramycin intermediate resistant. *L. paracasei* M308 was sensitive to vancomycin. These results are in disagreement with the results of other studies, describing the vancomycin resistance as an intrinsic characteristic of this species (Sharma et al., 2014).

According with several authors (Charteris et al., 1998b; Mathur & Singh, 2005; Ammor et al., 2007; Belletti et al., 2009 and Sharma et al., 2014) *L. rhamnosus* tested strains resulted resistant to kanamycin 30 µg, ofloxacin 5 µg, streptomycin 25 µg, tobramycin 10 µg and vancomycin 30 µg.

The strains were sensitive to the other tested antibiotics. Only three strains demonstrated a different attitude towards antibiotics. *L. rhamnosus* PRA331 was intermediately resistant to kanamycin 30 µg, ofloxacin 5 µg, tetracycline 10 µg, tobramycin 10 µg and vancomycin 30 µg and sensible to streptomycin 25 µg. *L. rhamnosus* CI230 was susceptible to ofloxacin 5 µg and vancomycin 30 µg and intermediately resistant to kanamycin 30 µg, streptomycin 25 µg,

tetracycline 10 µg. *L. rhamnosus* N2010 was sensitive to kanamycin 30 µg, streptomycin 25 µg and vancomycin 30 µg and intermediately resistant to ofloxacin 5 µg and tobramycin 10 µg. These results are in disagreement with the results of other studies, describing the vancomycin resistance as an intrinsic characteristic of this species (Sharma et al., 2014).

In the Table 4 is possible to notice the differences between a standard strain *L. casei* LMG23516 and the others.

Table 4 Differences in antibiotic resistance using *L. casei* LMG23516 as reference strain

Strain	**Origins**	**CFP30**	**KZ30**	**C10**	**DA10**	**E30**	**K30**	**OFX5**	**QD15**	**RD30**	**S25**	**TE10**	**TOB10**	**VA30**
L. casei LMG 23516 (reference strain)	**Human faeces**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**R**
L. paracasei DBPZ0478	Caciocavallo	R	R	R	S	R	R	R	S	S	R	R	R	R
L. paracasei M308	Canestrato Moliterno	S	S	S	I	S	R	R	I	S	I	S	R	S
L. paracasei DialDan8	Yogurt	S	S	S	S	I	R	S	S	S	S	S	I	S
L. paracasei DialYak4	Yogurt	S	S	S	S	R	R	I	S	S	R	S	S	R
L. paracasei DialYak5	Yogurt	S	S	S	S	R	R	I	S	S	R	S	S	R
L. paracasei DialYak6	Yogurt	S	S	S	S	S	R	I	S	S	R	S	R	R
L. paracasei DialYak7	Yogurt	S	S	S	S	R	R	I	S	S	R	S	S	R
L. paracasei DialYak8	Yogurt	S	S	S	S	R	R	I	S	S	R	S	S	R
L. rhamnosus PRA331	Cheese	S	S	S	S	S	I	I	S	S	S	I	I	I
L. rhamnosus CI230	Parmigiano Reggiano	S	S	S	S	S	I	S	S	S	I	I	R	S
L. rhamnosusN2010	Body excreta	S	S	S	S	S	S	I	S	S	S	S	I	S

Legend: CFP30, Cefoperazone 30 µg; KZ, Cefazolin 30 µg; C10, Chloramphenicol 10 µg; DA10, Clindamycin 10 µg; E30, Erythromycin 30 µg; K30, Kanamycin 30 µg; OFX5, Ofloxacin 5 µg; QD15, Quinupristin/Dalfopristin 15 µg; RD30, Rifampicin 30 µg; S25, Streptomycin 25 µg; TE10, Tetracycline 10 µg; TOB10, Tobramycin 10 µg; VA30, Vancomycin 30 µg.

Hemolysis

Hemolytic activity facilitates iron accessibility to the bacteria, causing edema and anemia in the host. It is a common virulence factor among pathogens. However, lactobacilli do not require iron to grow (Rodrigues da Cunha et al., 2012). The pyrimidine and purine metabolism of *Lactobacillus* spp. may be the cause of the iron demand, inducing hemolysis activity (Elli et al., 2000).

Hemolysis activity for *L. casei* strains was equally divided in α/β hemolysis, whereas in *L. paracasei* group, the strains showed 63 % of α-hemolysis, 29 % of β-hemolysis and 8 % of γ-hemolysis. Several studies demonstrated the capabilities of *Lactobacillus* spp. to perform α and β hemolysis (Maragkoudakis et al., 2006; Baumgartner et al., 1998). *L. rhamnosus* tested strains showed mainly β hemolysis (59.6 %) then α hemolysis (25.9 %) and only the 14.5 % of the strains showed γ hemolysis (no hemolysis). These results are in contrast with the results found by Baumgartner at al. (1998), who demonstrated that all their 53 strains studied express α hemolysis

On the other hand, Vesterlund et al., 2007 analyzed 111 strains of *Lactobacillus* spp. isolated from different sources, and did not found any strain capable to degrade red blood cells.

Comparing these results with the results of other scientific papers, it is possible to suppose that this characteristic is not related to the belonging species, nor to the source of isolation.

Antimicrobial capabilities (bacteriocins production)

Antimicrobial ability of LAB was reported by several authors (Sameshima et al., 2002; Lozo et al., 2007; Verdenelli et al., 2009; Reis et al., 2012; Georgieva et al; 2015; Peng et al., 2015). This capability can be advantageous in food preservation, preventing the growth of foodborne pathogens. Production of lactic acid and hydrogen peroxide by LAB can obstruct the growth of pathogens due to their bactericidal effect. The bactericidal/bacteriostatic mechanism of lactic acid is probably related to the cytotoxic properties of non-dissociated lactic acid and insolubility of dissociated lactate, which causes acidification of cytoplasm and collapse of proton impulse forces. Furthermore, it could reflect on trans-membrane pH gradient and reduce the quantity of accessible energy for cells to grow (Reis et al., 2012; Capra et al., 2014).

L. paracasei strains showed a good competition capability versus pathogens except for *St. aureus* DSM 4910. *L. casei* strains demonstrate antagonistic activity versus *E. coli* DSA and *S. enteritidis* DSA but not against *St. aureus* DSM 4910 and *L. monocytogenes* ATCC7644. Sameshima et al., (2002) found a strain of *L. paracasei* that inhibited the growth of some pathogenic bacteria, such as *L. monocytogenes, S. enteritidis* and *E. coli.* Antagonistic activity of *L. paracasei* strains versus *E. coli* was also found by Caridi, (2002).

Good ability of competition (Table 2), especially versus *E. coli,* but no one strain was able to do more than compete with *St. aureus*. Sameshima et al., (2002) found a strain of *L. rhamnosus* that inhibited the growth of some pathogenic bacteria, such as *L. monocytogenes, S. enteritidis* and *E. coli.* Also Georgieva et al., (2015) found a strain, which have antagonistic activity against *E. coli*, but they also found a high antagonistic activity against *St. aureus* that is in disagreement with our results.

None of our strains showed bacteriocin production (Table 5).

Table 5 Percentage distribution of the capabilities of competition of the strains

	Competition %	Organic acid %	Bacteriocins %
***L. casei* strains**			
L. monocytogenes	37,5	12,5	0
St. aureus	25	0	0
S. enteritidis	50	37,5	0
E. coli	62,5	50	0
***L. paracasei* strains**			
L. monocytogenes	71,96	17,75	0
St. aureus	31,78	0	0
S. enteritidis	71,03	57,01	0
E. coli	76,64	59,81	0
***L. rhamnosus* strains**			
L. monocytogenes	47,62	16,19	0
St. aureus	33,33	0	0
S. enteritidis	43,81	30,48	0
E. coli	55,24	40	0

Biogenic Amines production

Biogenic amines (BAs) are organic bases with aromatic, aliphatic or heterocyclic structure, which can be found in different food and beverage (fishes, cheeses, meat products, fermented vegetables, wine, beer ecc.), mainly generated by microbial decarboxylation of amino acids (AA) (Silla Santos, 1996). Decarboxylation of AA can be an important energetic source for microorganism in poor nutritional environment (Suzzi and Gardini, 2003). Consumption of high quantity of these amines can have toxicological effect, but their presence did not affect the sensory quality of the product (Coton and Coton, 2005; Landete et al., 2007a/b; Ladero et al., 2011; Costantini et al., 2013). Tyramine can cause vomiting, hypertension and headaches. while histamine can induce allergen type reaction, such as low blood pressure, edema, heart palpitation, vomiting, rash, difficulty in breathing. Putrescine and cadaverine are not considered toxic, but they can interfere with the detoxification of histamine and tyramine or potentiate the toxicity of histamine, respectively (Landete et al., 2007a/b; Garcià – Ruiz et al., 2011). It is essential to use starter cultures which not produce BAs (Suzzi and Gardini, 2003). Moreover an early detection of potential BAs former bacteria can prevent BAs production and accumulation in food products (Coton and Coton, 2005; Landete et al., 2007b).

Using the method described by Bover-Cid et al., 1999, there were no strain of *L. casei* able to produce BAs in the decarboxylase medium, whereas 2 strains (B169 and B195) of *L. paracasei* were able to produce tyramine. This result is in agreement with the results of Bover-Cid et al., (1999) and Landete et al., (2007a). Moreover, one *L. casei* strains (N2014) was positive for two of the target genes (*agdi* and *hdc*). *L. paracasei* PSG10, was positive for *tyrdc* and *hdc* genes. Looking at the results obtained from the decarboxylase medium and compared with the results obtained from the PCR, there was only one strains (*L. paracasei* B169) that showed congruent results. Also *L. paracasei* B195 and *L. paracasei* PSG10 showed a positive reaction for tyramine and arginine, respectively. But the PCR results shown that B195 had *agdi* gene and PSG10 had *tyrdc* plus *hdc* genes (Table 6).

Four out of the 60 tested strains of *L. rhamnosus* were able to produce tyramine and one strain, *L. rhamnosus* CTC1676, was able to produce putrescine and cadaverine but the detected gene is the *agdi* (Table 6). The detection of the genes related to BAs production showed a presence of four strains positive for *odc* gene, two strains positive for *agdi* gene and four strains positive for *odc* and *hdc*

genes. Strain N202 showed *odc* gene and not the *tyrdc* gene as expected, on the other hand, strain D44, N178 and N812, despite demonstrating tyramine production capability in decarboxylase medium, resulted negative for the presence of *tyrdc* gene.

Looking at the results obtained from the decarboxylase medium, there were no strains able to produce histamine, whereas seven strains were positive to the *hdc* gene (Table 6).

In these cases of non-correspondence among PCR and decarboxylase medium, a false positive due to the formation of chemicals with alkaline reaction in medium has to be taken into consideration and further investigated (Bunkovà et al., 2009). On the other hand, the strains positive for the presence of the BAs related genes that did not show a positive reaction in the medium, probably did not achieve the prerequisites for biogenic amine formation, as conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity (Silla Santos, 1996

Therefore, the PCR is an accurate method to reveals the potential of the strains to produce the biogenic amines, detecting the strains that had the gene for a corresponding enzyme production.

Table 6 Differences between detected gene and positive reaction in decarboxylase medium

	Genes tested				**BAs on decarboxylase medium**			
***L. casei* strain**	***tyrdc***	***odc***	***agdi***	***hdc***	**Tyramine**	**Putrescine**	**Agmatine or Putrescine**	**Cadaverine**
N2014	-	-	+	+	-	-	-	-
***L. paracasei* strains**	***tyrdc***	***odc***	***agdi***	***hdc***	**Tyramine**	**Putrescine**	**Agmatine or Putrescine**	**Cadaverine**
DBPZ0525	+	-	-	-	-	-	-	-
B161	-	-	+	-	-	-	-	-
B169	-	-	+	-	+	-	+	-
B195	-	-	+	-	+	-	-	-
Mo4	-	-	+	-	-	-	-	-
LacCas7	-	-	+	-	-	-	-	-
B350	-	-	-	+	-	-	-	-
PSG10	+	-	-	+	-	-	+	-
***L. rhamnosus* strains**	***tyrdc***	***odc***	***agdi***	***hdc***	**Tyramine**	**Putrescine**	**Agmatine or Putrescine**	**Cadaverine**
D44	-	-	-	-	+	-	-	-
N178	-	-	-	-	+	-	-	-
N812	-	-	-	-	+	-	-	-
N132	-	+	-	-	-	-	-	-
N21	-	+	-	-	-	-	-	-
N2013	-	+	-	-	-	-	-	-
N202	-	+	-	-	-	-	-	-
L47	-	-	+	-	+	-	-	-
CTC1676	-	-	+	-	-	+	-	+
CI4362	-	+	-	+	-	-	-	-
N22	-	+	-	+	-	-	-	-
N26	-	+	-	+	-	-	-	-
N131	-	+	-	+	-	-	-	-

Tyrdc: tyrosine decarboxylase gene; *odc:* ornithine decarboxylase gene; *agdi:* agmantine decarboxilase gene; *hdc*: histidine decarboxylase gene.

arcABC presence

Citrulline and carbamyl phosphate released during the arginine deiminase (ADI) pathway of LAB and urea can react naturally with ethanol forming ethyl carbamate, which is a possible carcinogenic compound present in several fermented foods, mainly in wine (Tonon and Lonvaud-Funel, 2002; Fang et al., 2013). In this part of the study, the three genes encoding for three main enzimes involved

in this pathway were investigated(*arcA,* arginine deiminase; *arcB,* ornithine transcarbamylase and *arcC,* carbamate kinase).

Among all the tested strains for the presence of *arcA*, *arcB* and *arc* genes in the genome, only the ones reported in Table 7 resulted to be positive for one of the investigates genes.

Table 7 Stains which resulted to be positive for *arcA*, *arcB* or *arcC* genes

	Genes		
L. paracasei strains	***arcA***	***arcB***	***arcC***
P71	-	+	-
P2P3	-	+	-
B169	-	-	+
B195	-	-	+
DSM5622	-	-	+
L. rhamnosus strains			
DBPZ0446	-	-	+
N1710	-	+	-
N812	-	+	-
L47	-	-	+

Nine strains out of the total (7.44 %), resulted to be positive for one of the three genes. Statistically, none were positive for *arcA*, 3.30 % were positive for *arcB* and 4.13 % for *arcC.* No *L. casei* strain had these genes. Only five strains of *L. paracasei* and 4 of *L. rhamnosus* resulted to be positive for almost one of the genes. Two strains, *L. paracasei* P71 and P2P3, were positive for *arcB* gene*,* while other three for *arcC* gene (*L. paracasei* B169, B195 and DSM5622). *L. rhamnosus* N1710 and N812 were positive for *arcB* gene, while DBPZ0446 and L47 for *arcC.*

Citrulline derived from LAB metabolism, are the main precursors for Ethil Carbamate (EC. Heterofermentative LAB have been shown to be active arginine degradative (Liu et al., 1994; Mira de Orduña et al., 2000; Uthurry et al., 2006). There are no other studies about the presence of these genes in *L. casei* group. There are some studies about other facultative or obligate heterofermentative LAB. Especially *L. hilgardii*, *L. plantarum* and *O. oeni* were found to degrade arginine (Arena et al., 1999; Tonon and Lonvaud-Funel, 2002; Ribéreau-Gayon et al., 2006). Also *L. buchneri* strains can excrete citrulline (Mira de Orduña et al., 2000). Araque et al., (2009) investigated the genes involved in the ADI pathway and found some strains belonging to *L. brevis*, *L. hilgardii*, *L. buchneri, L. plantarum, O. oeni*, *P. pentosaceus* and *Leu. Mesenteroides* which had the *arcABC* genes in their genome.

Biogenic Amines (BAs) degrading activity: DAB assay and MCO detection

As reported before, presence of BAs could be toxic, with different manifestation. People who lack in natural mechanism of detoxification are more susceptible (Naila et al., 2010; Landete et al., 2007a/b). Methods to control the formation of BAs in food include the use of additives and preservatives, addition of enzymes able to degrade amines such as mono- and diamine-oxidase (AO and DAO) and the use of bacteria which possess these enzyme (Suzzi and Gardini, 2003; Naila et al., 2010; Callejon et al., 2014).

In this study, no extracts showed bands with DAB assay. The same extracts were tested with 2,6-dimethoxyphenol (DMP) staining to investigate the MCO activity. Three, twelve and eighteen extracts from *L. casei, L. paracasei* and *L. rhamnosus* respectively, showed positive reaction. These results show that some strains of *L. casei* and *L. paracasei* had multicopper oxidase activity. Also the capability of some strains of *L. rhamnosus* to use histamine, tyramine, putrescine and DMP as substrates was confirmed. In all the three species this capability are responsible for the oxidation of these compounds (Callejon et al., 2014). In the gels, the yellow-orange bands appeared at the same

heights, suggesting that could be the same enzymes. The source of isolation of the strains did not reveal a trend in the presence of these enzymes (Table 8). On the other hand, the same authors, described how the two methods were closely related, but also in their results they suggest that is it possible that different enzymes may catalyze amine and DMP degradation. Also a low production of the enzymes could be responsible of no reaction in the DAB assay.

Table 8 MCO positive strains.

Strains	**Source**
L. casei DSM20178	Unknow
L. casei N87	Body excreta
L. casei N2014	Body excreta
L. paracasei NRRL B-456	Unknow
L. paracasei DBPZ0564	sourdough Altamura
L. paracasei DBPZ0572	sourdough Altamura
L. paracasei V3	Canestrato Moliterno
L. paracasei ESG10	Parmigiano Reggiano
L. paracasei R61	Pecorino cheese
L. paracasei F17	Pecorino cheese
L. paracasei H12	Pecorino cheese
L. paracasei N76	Body excreta
L. paracasei B195	Wine
L. paracasei LACcas25	Elisir
L. paracasei TMW 1.1444	Pecorino cheese
L. paracasei TMW 1.1259	Parmesan cheese
L. rhamnosus DBPZ0446	Caciocavallo Di Leo
L. rhamnosus NRRL B-176	Unknow
L. rhamnosus PRA331	Dairy industry
L. rhamnosus L9	Formaggio Asiago
L. rhamnosus L47	Formaggio Asiago
L. rhamnosus N171	Body excreta
L. rhamnosus N2012	Body excreta
L. rhamnosus N812	Body excreta
L. rhamnosus N1110	Body excreta
L. rhamnosus N21	Body excreta
L. rhamnosus N176	Body excreta
L. rhamnosus N2011	Body excreta
L. rhamnosus DSMZ20021	Unknow
L. rhamnosus Mo2	Unknow
L. rhamnosus LACcas13	Grana
L. rhamnosus HA111	Probiotic starter
L. rhamnosus 2220	Unknow
L. rhamnosus TMW 1.1538	faeces

CONCLUSIONS

In this study, the evaluation of the safety traits and the technological properties showed a high variability in the behaviour of the strains. This confirms that these capabilities are strain-specific.

There were some strains that showed particular capabilities.

L. rhamnosus PRA331 showed low resistance to antibiotics and MCO presence. Otherwise *L. casei* N2014 had *agdi* and *hdc* genes and showed a MCO abilities.

L. paracasei B169 and *L. rhamnosus* L47 had *arcC* gene and *agdi* gene. *L. paracasei* B195 had *arcC* gene, *agdi* gene and showed a MCO abilities, on the other hand *L. rhamnosus* N812 had *arcB* gene and no gene related to BAs, but showed MCO activities.

In order to better understand the distribution of the various characteristic of the strains, the results of *L. casei* and *L. paracasei* were grouped in a single matrix, while another were created for *L. rhamnosus* strains. In particular, different colours in heat map are a measure of the significance of the correlation. In particular, in Figure 3 all the strains are connected in subgroups, but *L. paracasei* TMW 1.1444, TMW 1.1259 and V3 that are the most different strains considering all the tested characteristics. Moreover, it is possible to observe how the studied traits form two clusters (I and II). Cluster I showed correlation among almost all growth capabilities (pH value and different concentration of NaCl and ethanol) and intrinsic resistance to antibiotics (vancomycin and tobramycin). Cluster II grouped the presence/absence of BAs and ethyl carbamate genes, BAs production, hemolysis ability, MCO detection and the most of the antibiotic resistance capabilities. Also, the growth capability at pH 3.2 clustered in this second group. The heat map of *L. rhamnosus* strains (Figure 4) showed the presence of several subgroups. Strain CI4362 clustered alone. On the other hand, 3 clusters (I, II, III) were formed by the investigated characteristics, but it was not possible to make a differentiation of the features present in cluster I and II. Whereas cluster III includes features absent in all strains (*tyrdc* and *arcA* genes and resistance to rifampicin 30 μg).

The deep knowledge of the strains of this group is still far from being complete

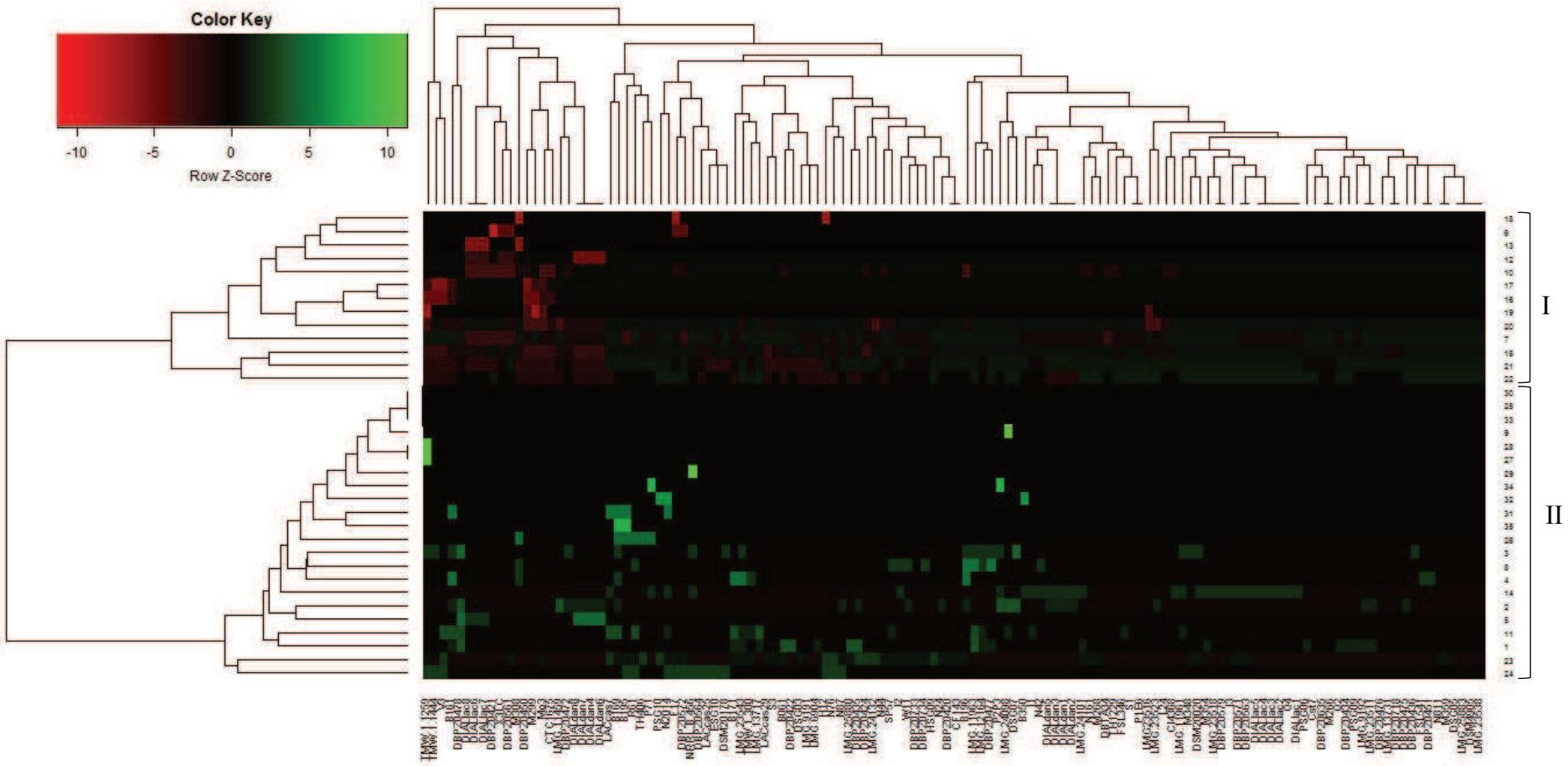

Figure 3 Heat map of *L. casei* and *L. paracasei* strains obtained by the comparison of the several characteristic studied. Each number indicate a characteristic: 1 to 13: antibiotic resistance (cefoperazone 30 µg, cefazolin 30 µg, chloramphenicol 10 µg, clindamycin 10 µg, erythromycin 30µg, kanamycin 30 µg, ofloxacin 5 µg, quinupristin/dalfopristin 15 µg, rifampicin 30 µg, streptomycin 25 µg, tetracycline 10 µg, tobramycin 10µg and vancomycin 30 µg); 14 to 22 growth abilities (pH 3.2, 3.8, 4.2; 4.6; NaCl 2%, 4%, 6.5% and ethanol 12%, 15%); 23 = hemolysis capabilities, 24 = MCO production; 25 to 28 BAs production evaluated by Bover – Cid et al., 1999 method (histamine, tyramine, putrescne or agmantine, cadaverine); 29 to 32 genes involved in BAs production (*tyrdc, odc, agdi, hdc* genes) and 33 to 35 genes involved in ethil carbamate production (*arcA, arcB, arcC* genes).

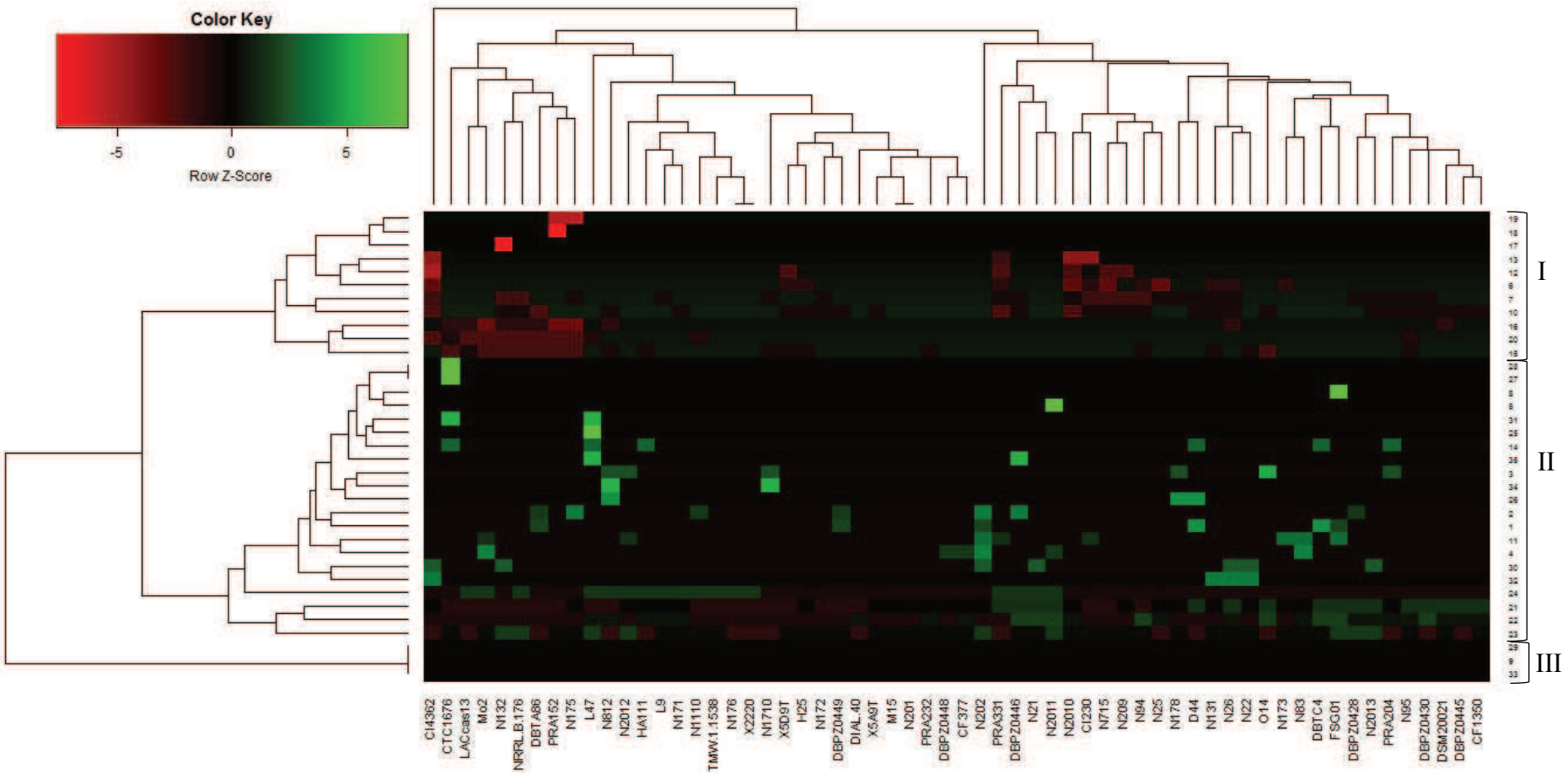

Figure 4 Heat map of *L. rhamnosus* strains obtained by the comparison of the several characteristic studied. Each number indicate a characteristic: 1 to 13: antibiotic resistance (cefoperazone 30 µg, cefazolin 30 µg, chloramphenicol 10 µg, clindamycin 10 µg, erythromycin 30µg, kanamycin 30 µg, ofloxacin 5 µg, quinupristin/dalfopristin 15 µg, rifampicin 30 µg, streptomycin 25 µg, tetracycline 10 µg, tobramycin 10µg and vancomycin 30 µg); 14 to 22 growth abilities (pH 3.2, 3.8, 4.2; 4.6; NaCl 2%, 4%, 6.5% and ethanol 12%, 15%); 23 = hemolysis capabilities, 24 = MCO production; 25 to 28 BAs production evaluated by Bover – Cid et al., 1999 method (histamine, tyramine, putrescne or agmantine, cadaverine); 29 to 32 genes involved in BAs production (*tyrdc, odc, agdi, hdc* genes) and 33 to 35 genes involved in ethil carbamate production (*arcA, arcB, arcC* genes).

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Effects of anaerobic, aerobic and respiratory adaptation on the physiological response

Introduction

Lactic Acid Bacteria (LAB) incorporate a various and heterogeneous group of bacteria. The growth of LAB under respiratory condition could determinate the expression of the phenotype, determining/causing an increase in the technological and stress response properties, adding haeme and menaquinone, in presence of oxygen (Pedersen et al., 2012, Zotta et al., 2012; 2013; 2014).

Lactobacillus casei group strains have been recognized as capable of growth under aerobic and respiratory condition (Ianniello et al., 2015; Zotta et al., 2014). Comparative genomic studies underscore the heterogeneity of this group, suggesting that this differentiation is the cause of their capabilities to adapt to different niches (Douillard et al., 2013, Smokvina et al., 2013, Toh et al., 2013 Zotta et al., 2014). Furthermore, metabolic profiles and physiological response of respirative strains grown in aerobic respiration changed (Ianniello et al., 2015, Pedersen et al., 2008, Ricciardi et al., 2015). Typically, stresses had a negative connotation, but in bacterial evolution is one of the most important forces (Serrazanetti et al*.,* 2009). In order to respond to complex and diverse environmental changes, bacterial stress responses rely on gene coordinated expression that modify several cellular processes to enhance stress tolerance (van de Guchte et al*.,* 2002).

The effect of the aerobic and respiratory metabolism on the physiological response of some *Lactobacillus* species has been investigated in synthetic media. However, few studies (Reale et al., 2016a/b) tested respirative strains in fermented foods. In order to enhance the knowledge that is necessary in the design of functional foods, human health and food fermentation new biotechnologies are needed to improve the robustness and functional properties of the strains.

L. casei group are able to colonize a large variety of niches and fermented foods, several strains of this group had found an application in industrial or home made production as starter cultures. In fermented milk production, starter cultures are added in the first phase of manufacture, immediately after milk pasteurization. Usually, a mixture of *Lactobacillus delbrueckii* subs. *bulgaricus* and *Streptococcus thermophilus* are normally used as main starter cultures in yogurt, as well as *L. casei* group strains were added in some fermented milk to obtain an acceleration of fermentation and/or an intensification of flavour (De Angelis and Gobbetti 2011, Ortigosa et al., 2006) and for their probiotic properties (Verdenelli et al., 2009). There are numerous fermented milk products which are manufactured in many countries of the world. These kind of production are one of the oldest methods used by man to extended the shelf-life of milk (Tamime and Marshall, 1997). However, about 200 traditional fermented milks and several hundred of non traditional ones were manufactured in the world (Kroger et al., 1992). The classification of this products is based on several factors such as: growth requirements of the starter cultures, metabolite produced by the microorganism involved, raw material used and manufacturer method (Mucchetti and Neviani, 2006). Fermented milk are define as milk and/or milk products fermented by specific microorganism with a subsequential reduction of pH value and coagulation. Also, this microorganism must be active, abundant (at least 10^7 CFU/g) and viable during shelf life of products (International Dairy Federation, 1997). In fermented milks starter cultures occurs in the early phase of manufacture. Usually, a mixture of *Lactobacillus delbrueckii* subs. *bulgaricus* and *Streptococcus thermophilus* are used as main starter cultures, as well as *Lactobacillus helveticus, Lactococcus* spp., *Leuconostoc cremoris* and *Leuconostoc dextranicum,* (Tamime, 2002).

Nowadays consumers pay attention in preventing diseases and improving their wellness, also they are concerned about chemical preservatives. Functional foods contains compounds that promote health, reduce or prevent some diseases and/or generically contribute to the organism function (Granato *et al*., 2010).

Proteins present in milk have biofunctional properties. This capabilities are mainly due to bioactive peptides, that are part of some proteins and have demonstrate different biological active such as: opioid agonist (casomorphins and lactorphins) and antagonist (lactoferroxins and casoxins); antithrombotic (casolplatelins); immunostimulants (immunopeptides); antihypertensive (casokinins); mineral carriers (phosphopeptides); antimicrobial (lactoferricin and casoicidin) and others (Korhonen *et al*., 1998; Silva and Malcata, 2005; Möller *et al*., 2008). Bioactive peptides can be liberated during digestion of the proteins, but also during the fermentation of the milk (Hafeez at al., 2014). During fermentation the proteolysis conducted by LAB can lead to the formation of this peptides, that were deeply investigated by several authors during the past year (Korhonen and Pihlanto, 2006). Therefore, functionalized fermented milk can be obtained using the proteolysis carried out by the LAB proteolytic system without supplementation of additives. The benefits that could be acquired using selected LAB strains include health benefit for consumers and shelf – life extension of the product, as well as improved safety due to the antimicrobial peptides.

Also foods containing probiotics strains are important for health maintenance and diseased prevention. Several studies reported the benefit obtained by the consumption of probiotics even if they are not viable (Saarela et al., 2000; Drisko et al., 2003; Senok et al., 2005; Nagpal et al., 2012; Shiby and Mishra, 2013). As reported by Holzapfel and Schillinger, (2002) several microorganism, mainly LAB and few others species, have probiotic properties. Furthermore, the capability of some strains to produce bacteriocin such as nisin, sakacins, pediocins, leucocins, curvacins, lactacins and other is well documented (Quinto et al., 2014). Finally, several dairy products were used as carrier for probiotic strains (Dave and Shah, 1996; Patrignani et al., 2009; Nagpal et al., 2012).

Moreover, presence of LAB that promote health and preserve food were easily accepted by customer.The aim of this study was to investigate the effects of anaerobic, aerobic, respiratory and acidic conditions of growth on the cellular structure of strains of the *L. casei* group. Then, two strains (*L. casei* N87 and *L. paracasei* V3) were selected in order to understand the effects of two different adaptation conditions (anaerobiosis and respiration) and technological stresses derived form a fermentation (fermented milk) and storage process on the antibiotic resistance, hemolytic activity, bacteriocins production, competition with pathogens and resistance to *in vitro* digestion (bile salt stress) on their cells. Also, antimicrobial capability of the soluble fraction and proteolysis of the fermented milk samples were investigated.

Materials and methods

Strains adaptation

The strains *L. casei* N87 and CI4368 and *L. paracasei* V3 were previously isolated and identify by Iacumin et al., (2015), then selected for their tolerance to aerobic and/or respiratory growth from the results obtained from previous study (Zotta et al., 2014). They were stored at the temperature of - 80° C in cryovials containing DeMan, Rogosa and Sharp broth (MRS, Oxoid, Milan, Italy) supplemented with 2% glycerol.

At the time of use, the cultures were streaked onto MRS Agar plates (Oxoid, Milan, Italy) and incubated at 37 °C, in order to check their purity and prepare them for the following stages of the experiment by inoculating a single colony in 2 mL of MRS broth (Oxoid, Milan, Italy). All strains were cultivated (24 h, 37 °C) as described by Zotta et al., (2014):

1) anaerobiosis (AN; MRS broth);

2) aerobiosis (AE; M17 broth, shaking with a magnetic stirrer);

3) respiratory promoting conditions (RS; AE with 2.5 μg/ml hemin and 1 μg/ml menaquinone supplementation)

After these adaptation steps the medium was acidified at pH 3.2 and incubated for 16 h at 37 °C. Part of the cells grown under AN condition were used as control (no acidification).

Transmission Electron Microscopy (TEM)

The cultures were centrifuged at 13000 rpm for 7 min, and the pellets were washed twice with PBS buffer 0.1 M, pH 7.2 (this buffer was used throughout the procedures). Then the inocula were standardized at 0.1 optical density at 600 nm. The standardized suspension were centrifuge again and the sample were subjected to chemical fixation, dehydration, embedding, inclusion, polymerization, sectioning and staining (Musetti et al., 2011).

Milk fermentation and sampling

L. casei N87 and *L. paracasei* V3 demonstrated better fermentation performance respect *L. casei* CI4368, hence these two strains were selected to carry out the procedures described in Figure 5. The strains were adapted as described before and inoculated in milk. Tubes filled with 50 ml of semi skimmed milk treated with Ultra High Temperature (UHT) were inoculated with the adapted cells of the strains, final concentration 10^8 CFU/ml. The fermentations were carried out in triplicate. To promote the fermentation, the inoculated milk samples were placed for 48 h at 42 °C, then the samples were put under refrigerated storage for 72 h at 4 °C. The fermentations were carried out in triplicate.

The strains were monitored before (adaptation of the strain), during (after 0, 24 and 48 h from the inoculum) and at the end of the fermentation process and 3 days of refrigerate storage (cold stress for the bacteria). Microbial counts, using MRS agar (Oxoid, Milano, Italy) with the addition of DELVOCID®INSTANTANT (DSM Food Specialities b.v., Netherland) 0.01 mg/ml, Gelatin Sugar Free Agar (Oxoid, Milano, Italy) and Malt Agar (Oxoid, Milano, Italy) with the addition of tetracycline 0.025 mg/ml and evaluation of the pH were performed. After the inoculum, the MRS agar plates were incubated at 37 °C for 36 h, the Gelatin Sugar Free Agar and the Malt Agar plates at 30 °C for 48 h. In order to evaluate the survival of the strains to a prolonged time of storage and evaluate a possible shelf – life additional counts were

performed at the end of the fermentation process (0) and after 3, 7, 14 and 21 days of refrigerated storage using the same technique explained previously.

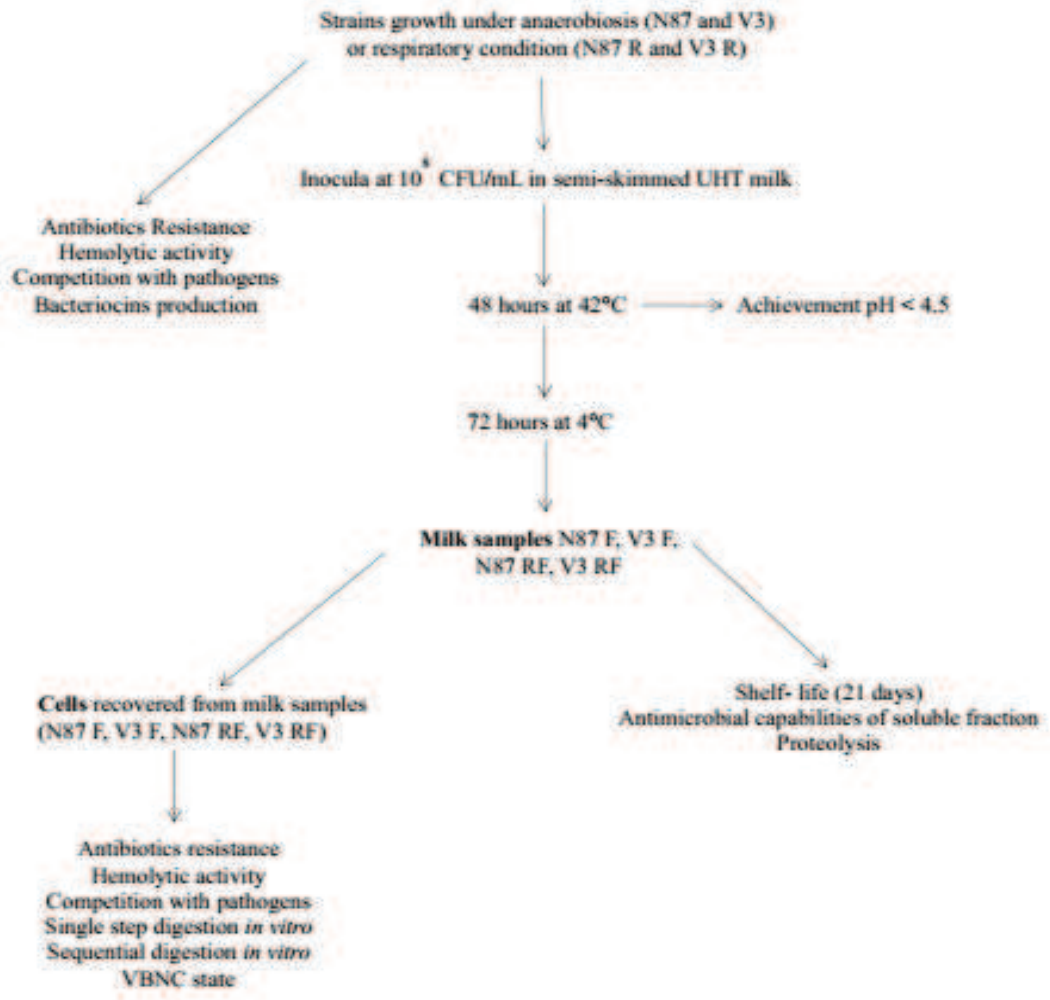

Figure 5 Workflow of the study

Evaluation of the physiological response

Before the fermentation process, the *L. casei* N87 and *L. paracasei* V3 adaptated in anaerobiosis and respiratory condition of growth were tested for their antibiotics resistance, hemolytic activity, competition with pathogen and bacteriocin production.

After the fermentation process, the cells of the strains were tested for: antibiotics resistance, hemolytic activity and antimicrobial capabilities of the soluble fractions. The recovered cells were also evaluated for their capabilities of competition with pathogens and survival ability in the gastro-intestinal tract *in vitro*, initially using single steps digestion and then sequential digestion. During the sequential *in vitro* digestion Viable But Not Culturable (VBNC) cells were also monitored. (Figure 5)

Antibiotics resistance

Antibiotics resistance was evaluated against the following antibiotics: ampicillin (AMP 0.016-256 μg/ml), clindamycin (CD 0.016-256 μg/ml), erythromycin (E, 0.016-256 μg/ml), chloramphenicol (C, 0.016-256 μg/ml), gentamicin (GN, 0.016-256 μg/ml), tetracycline (TE, 0.016-256 μg/ml), kanamycin (K, 0.016-256 μg/ml) and streptomycin (S, 0.064-1024 μg/ml), using MIC Strip test (Liofilchem®, Teramo, Italy). The fermented milk were diluted, in order to obtain an inoculum of 10^6 CFU/ml of the selected strains. The plates were inoculated and the inoculum was spread with a spatula. After that the MIC strip test was placed on the plate surface. The plates were incubated at 37 °C and checked at 24 and 48 h.

Hemolytic activity

The hemolytic activity was performed striking the strains on Columbia Blood Agar (5% defibrinate horse blood) in triplicate. To avoid false positive results, due to the acidity of the fermented milk, the cells were separated from the fermented milk by chemical/mechanical separation prior to inoculation (Garcia-Cayuela et al., 2009).

Antimicrobial capabilities of the soluble fraction

The antimicrobial capabilities of the soluble fraction of fermented milk were verified using Millette et al., (2007) method with some modifications. The fermented milks were centrifuged at 7000 rpm for 10 min at 4 °C, and the supernatants were filter-sterilized in three step, 0.8 μm - 0.45 μm – 0.2 μm pores filters. Each cell-free supernatant was aliquoted into three groups: 1) the unaltered state supernatants (competition), 2) supernatants were treated with proteinase K, 2 mg/ml for 30 minutes at 37 °C (acidity, presence of hydrogen peroxide and/or undissociated lactic acid, low pH) 3)the pH was adjusted to 6.5 in order to evaluate te presence of antimicrobial compounds.. The agar well diffusion assay was performed on plates of Brain Heart Infusion (Oxoid, Milano, Italy) supplemented with 0.2 % of agar, inoculated with strains of *Listeria monocytogenes* ATCC7644*, Staphylococcus aureus* DSM 4910*, Escherichia coli* DSA*, Salmonella enteritidis* DSA and *Bacillus cereus* DSA, 100 μL of each supernatant were poured in the wells. The plates were incubated at 37 °C for 24 h. Also, a similar technique was performed to test the antimicrobial capabilities and the bacteriocins production of the strains adapted in anaerobiosis and respiratory condition of grow (Schillinger and Lucke, 1989). The cells were overnight cultured on MRS broth (Oxoid, Milan, Italy) at 37°C. A cells-free solution was obtained by centrifuging the culture for 10 minutes at 10000 rpm, followed by filtration through a 0.2 μm pore-size cellulose acetate filter (cell - free supernatant). The inhibitory activity from the hydrogen peroxide was eliminated adjusting pH to 6.5 (antimicrobial compounds), the supernatants antimicrobial compounds free were obtained by the addition of proteinaseK (2 mg/ml) (acidity).

Competition with pathogens

The adapted strains *L. casei* N87 and *L. paracasei* V3 were also evaluated for their capabilities of competition with pathogens: *Listeria monocytogenes* ATCC7644*, Staphylococcus aureus* DSM 4910*, Escherichia coli* DSA*, Salmonella enteritidis* DSA and *Bacillus cereus* DSA. In order to evaluate this characteristic, co-inocula in test tubes of MRS and incubated at 37 °C for 20 hours were set up. As a control, the stressed cells of the strains were inoculated in MRS broth without the presence of the pathogen. The pathogens were inoculated at a concentration of 10^2 CFU/ml and *L. casei* N87 or *L. paracasei* V3 cells, that were recovered from fermented milk as described before, were inoculated at a concentration of 10^8 CFU/ml. A first evaluation of the growth curves was performed in MRS broth (Oxoid, Milano, Italy) during 48 h at 37 °C

and monitored through optical density (600 nm) in order to define the different growth phases and choose the correct time ranges in which sampling. Samplings were performed at 0, 8, 10, 12, 14, 16, 18 e 20 h from the inocula, on the following medium: MRS Agar (Oxoid, Milano, Italy) for *L. casei* N87 and *L. paracasei* V3 cells (37 °C for 24 h), *Bacillus cereus* Agar base (BCA, Oxoid, Milano, Italy) for *B. cereus* (37 °C for 24 - 48 h), *Listeria* Selective Agar (LSA, Oxoid, Milano, Italy) for *L. monocytogenes* (37 °C for 24 h), Baird Parker (BP, Oxoid, Milano, Italy) for *St. aureus* (37 °C for 24 h), Brilliant green Agar (BGA, Oxoid, Milano, Italy) for *S. enteritidis* (37 °C for 24 h) and Coli ID (Bio-Merieux, Firenze, Italia) for *E. coli* (37 °C for 24 h).

in vitro digestion

The effects of the digestion process was evaluated *in vitro*, initially using a single step approach (effect of saliva, stomach and intestine passage was evaluate independently from each other) and then in a simulated gastro-intestinal tract (sGIT) (the strains were subjected to the entire sGIT passage in a consecutive way). The method described by García-Ruiz et al., (2014) was used to evaluate the lysozyme resistance (saliva). Stomach and intestines digestion steps were carried out with the method proposed by Corcoran *et al*. (2005) with some modification. The simulated gastric juice was formulated using 3.5 g/L of glucose, 2.05 g/L of NaCl, 0.60 g/L of KH_2PO_4, 0.11 g/L of $CaCl_2$ and 0.37 g/L of KCl, adjusted to pH 2.0 using 1 M HCl or to pH 8 using 1 M NaOH, and filter-sterilized. Porcine bile (0.05 g/L), pepsin (13.3 mg/L) and pancreatin (1 g/L) were added as stock solutions, filter-sterilized, prior to analysis. Samplings were performed at 1, 5, 10 minutes after the addition of fermented milk in saliva; at 1, 30, 60, 120, 180, 240 minutes in stomach after additions in simulating solution; and at 1, 30, 60, 120, 180, 240 minutes in intestine after additions in simulating solution for the single steps of the digestion. For sGIT after each step, the mix was centrifuged at 7000 rpm for 10 minutes, in order to separate the fermented milk and the bacteria from the digestion solution. Then the digestion solution was discharged and replaced with the successive solution. The sampling times were as follows: at 1, 5 minutes for saliva, 1, 60, 90, 120, 180 minutes for stomach and 1, 60, 120, 180 minutes for intestine (cells-fermented milk/digestion solution 1:1 v/v).

At each sampling point viable and viable but not culturable cells (VBNC) were evaluated.

The VBNC cells were investigated during the simulated gastro-intestinal tract. Before the digestion two aliquots of the samples were collected, and during the *in vitro* digestion two aliquots of each samples were collected after 5 minutes in saliva solution, 60 and 180 min in the stomach solution and after 60 an 180 min in the intestine solution. One of this aliquots were treated with propidium monoazide (PMA) and the others was not treated. The method described by Vendrame et al., (submitted to Food Microbiology) were performed.

Proteolysis assessment

The fermented milk (N87 F, N87 RF, V3 F and V3 RF) and control sample (MILK) after 48 h of fermentation (0) and during the shelf life (3, 7, 14, 21 days) were investigated. The instrument used was a Mini Protean II (Bio – Rad, Milano, Italy). A 12 % acrylamide/bis - acrylamide running gel and 5% acrylamide/bis - acrylamide stacking gel were used. Proteins and peptides extracts were prepared as described by Vannini et al., (2008) with some modification: 10 g of sample were homogenized with 20 ml of distilled water for 3 min at 20 °C and incubated for 1 h at pH 4.6 at 40 °C. Samples were centrifuged at 5000 rpm for 20 min at 4 °C. The obtained supernatants (soluble protein fraction) were filtered through a 0.22 µm filter (LLG, Meckenheim, Germany) and frozen until the time of use. On the other hand, the pellets (insoluble protein fraction) were frozen until the time of use, at the time of use 10 mg were

resuspended in 5 ml of NaOH 0.1 M. Before the run, Bradford assay was performed. Fifteen microliter of sample were mixed with 15 µl of Laemmli sample buffer 2X (Bio-Rad, Milano, Italy) containing β – mercaptoethanol. The mixtures were incubated at 100 °C for 5 min. Precision Plus Standard Protein (Bio – Rad, Milano, Italy) and a mixture of α-casein, β-casein and lactoferrin (Sigma-Aldrich, Milano, Italy) were used as standards. The wells of the gels were loaded with 25 µl of the denatured protein samples. The run was performed at 50 V for 15 min and 120 V for 90 min.

Staining was performed in 1 h with Comassie Blue 1 % (0.1 %), Methanol (50 %) and Acetic Acid (7 %), while de-staining was made for 2 h or overnight in a solution of Methanol (5 %) and Acetic Acid (7 %).

Alignments of the bands were performed using GelAnalyzer 2010 Software (Lazar and Lazar, 2010).

Bioactive peptides detection

After protein extraction and quantification, 500 µl of the extracted soluble proteins of each sample were subjected to filtration using Spin – X ® 10 kDa (Corning®, Flintshire, England) according to the operator instructions of the manufacturer. Proteins and peptides with a weight lower than 10 kDa were collected and subsequently concentrated in Concentrator 5301 (Eppendorf, Milano, Italy) for 3 h at room temperature. Then they were resuspended in 100 µl of distilled water.

For the denaturation of protein, 25 µl of the sample were mixed with 10 µl of Laemmli sample buffer 2X (Bio-Rad, Milano, Italy) containing β – mercaptoethanol. The mixtures were incubated at 100 °C for 5 min. Analysis were carried out using 20 % polyacrylamide gels. Ten (10) µl of Color Marker Ultra Low Range (Sigma-Aldrich, Milano, Italy) was used as reference. The wells of the gels were loaded with 30 µl of the denatured protein samples.

Gels were run in a Mini Protean II (Bio – Rad, Milano, Italy) with a Tris – Glicine SDS buffer, 50 V for 60 min and 75 V for 3 h and 20 min. Staining and de – staining were performed as described before.

Alignments of the bands were made using GelAnalyzer 2010 Software (Lazar and Lazar, 2010).

All the bands detected in the various electrophoretic patterns were groupedin a single matrix, which was submitted to a two-way hierarchical analysis, a heatmap was obtained. In the heatmap, values are represented by cells coloured according to the Z-values, which represent the the significance of the correlation.

Statistical analysis

Anova analysis with Tukey's test and the two way hierarchical analysis were performed in R v 3.3.2 (R Development Core Team, 2011).

Results and discussion

TEM

The analysis performed using TEM (Figure 6) demonstrated that all the strains, adapted under anaerobiosis or aerobiosis and subjected to acidic stress (pH 3.2) (panel B and Figure 6C), the shape of the cells was not affected by the different adaptation methodologies (panel A). Conversely, the strains grown under respiratory condition and acidic stress (panel D) showed squared shape and the presence of ghosts appeared in the cultural matrix. The morphological changes that occured when the *L. casei* strains were exposed to different stresses were related to their viability and capability of surviving. The response to acid stress is mostly important for the industrial application of this group (Hosseini Nezhad et al., 2015), not only for their survival capabilities, but also to obtain an improvement of their fermentative ability as well as of products quality (Serrazanetti et al., 2009).

In the light of the obtained results, the subsequent tests were performed subjecting the strains to anaerobiosis vs respiratory condition of growth, which were the optimal and the most stressful conditions of growth, respectively.

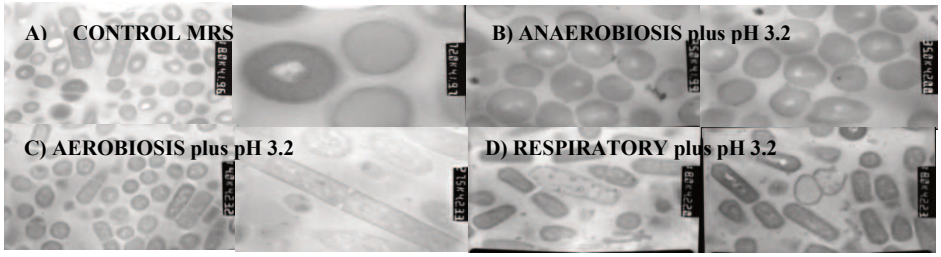

Figure 6 A, B, C, D *L. casei* CI4368, used as example, TEM view

Milk fermentation and sampling

During the fermentation process the inoculated strains were able to adapt to the environment and ferment the milk reducing the pH from 6.6 to value below 4.5 units (4.1, 3.7, 3.9 and4.2 value in N87 F, N87 RF, V3 F and V3 RF, respectively, data not shown). The bacteria concentration, reached during fermentation, remained stable and viable during the 21 days of refrigerated storage, the same trend was observed for pH values (Figure 7). No significant differences in the fermentation process were observed considering the two different adaptation conditions. Also there were no growth on the Gelatin Sugar Free Agar and Malt Agar plates, so the aerobic microorganism and the yeast were under the limit of detection (<1 Log CFU/ml).

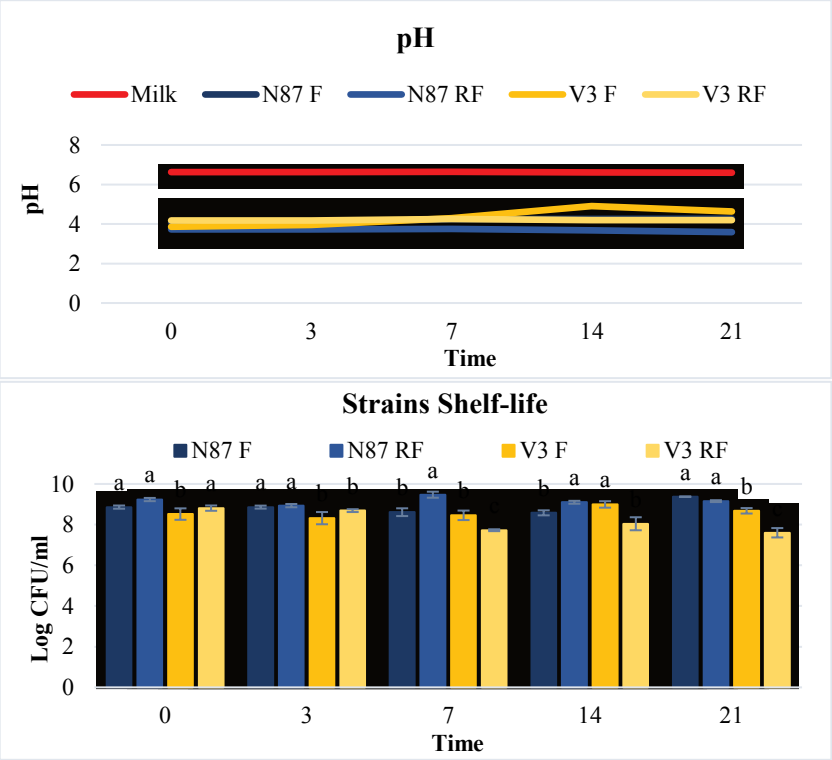

Figure 7 pH value and LAB counts of the fermented milks during a 21 days shelf – life.

Antibiotic resistance

Also the antibiotic resistance capabilities of the cells can be subjected to changing after adaptation and technological stress.

The antibiotics resistance of the *L. casei* N87 strain changed depending on the adaptation and the technological process. In fact, the resistance of the strain versus kanamycin, streptomycin and to a small extent to gentamicin increased. No differences were found on the others antibiotics tested.

Also, condition of growth and technological stresses modified the resistance of the V3 strain. Regarding V3 samples, against kanamycin the resistance increased in V3 F (64 to 96 µg/ml), while gentamycin resistance increased for both the samples (1.5 to 12 µg/ml, respectively). On the other hand the resistance versus streptomycin decreased (64 to 48 µg/ml) (Figure 8).

Both the strains showed changes in the resistance towards aminoglycosides, that binding in the 30S ribosomal subunit. The increase of the resistance could be attributed to a lower permeability of the cells wall due to the acidic conditions (Bender and Marquis, 1987).

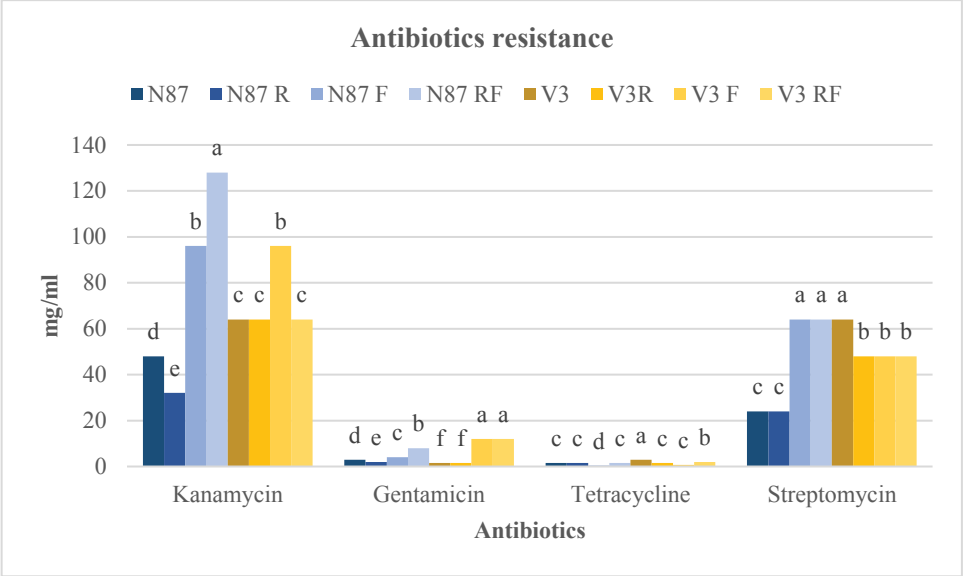

Figure 8 Antibiotics resistance results. The strains (N87 and V3) grown under anaerobic condition were considered as control. Anova with Tukey's test were performed comparing the results of the different adaptation and after stresses of the same antibiotic, the results were show in the labels on the bars

Hemolytic activity

Hemolytic activity facilitate iron accessibility to the bacteria, causing edema and anemia in the host. It is a common virulence factor among pathogens. Changing in the capabilities of the strains after stress exposure could be an important factor when choosing strains to use in foods.

The cells of *L. casei* N87 not subjected to stresses showed ß hemolysis ability. Differently, after subjecting the strain to the fermentation process as well as to the oxidative stress, its ability changed from ß to γ hemolysis. *L. paracasei* V3 proved γ hemolysis capability, in fact no evidence of haemolytic zone was present on Columbia Blood Agar, and no changes in this behaviour appeared after the application of the various stresses. Many authors investigate the capability of *Lactobacillus* spp. to perform hemolysis (Baumgartner et al., 1998, Maragkoudakis et al., 2006, Rodrigues de Cunha et al., 2012, Vesterlund et al., 2007), suggesting a variability among the species or genera considered. Moreover, correlation between the isolation sources and haemolytic activity was not found. However, no literature was found about changes in the hemolytic activity of *Lactobacillus* spp. after stress exposure.

Antimicrobial capabilities of the soluble fraction

Bioactive peptides can be liberated during milk fermentation thanks to proteolysis performed by LAB (Korhonen and Pihlanto, 2006; Hafeez et al., 2014). Through an accurate selection of strains could be possible obtain antimicrobial peptides who preserve the products from pathogens. However, during the fermentation process several stress were applied to strains and the physiological response of the cells could change.

The antimicrobial capability was tested using the soluble fraction of the milk after fermentation to evaluate the possible residual activity of some metabolites.

In Table 9 the changes are shown. Without technological stresses the cell-free culture supernatant was not effective in reducing/stopping the pathogens growth, whereas the milk soluble fraction (supernatant) obtained with the strains adapted under both the conditions was capable to inhibit *L. monocytogenes* and *S. enteritidis*. Moreover, the soluble fraction obtained from the milk, fermented with the strain N87 growth under anaerobiosis condition (N87 F), was able to inhibit the two pathogens also after pH neutralization treatment, suggesting the presence of antimicrobial compounds. Regarding the soluble fraction (supernatant) obtained from the milk, fermented with the strain V3 grown under anaerobic conditions, an inhibition of *L. monocytogenes* and *Salmonella enteritidis* was observed, but, after pH neutralization treatment, only in the trial V3 F an effect against *S. enteritidis* was observed, suggesting the presence of an antimicrobial compound in the soluble fraction. On the other hand, the soluble fraction (supernatant) obtained from the milk fermented with the strain V3 growth under respiratory condition (V3 RF), was able to inhibit that two pathogens, but after pH neutralization treatment only *L. monocytogenes* was inhibited. These results suggested the production of an antimicrobial compound produced by the strain when subjected to oxidative stress (Table 10)

Lactic acid bacteria produce proteolytic enzymes during yogurt manufacturing, which cleave peptide bonds of milk proteins leading to generation of bioactive peptides and free amino acids (Meisel and Bockelmann 1999; Donkor et al., 2007; Korhonen, 2009). Also different authors found that some strains of *L. casei* was capable to produce lacticin 705 (Vignolo et al., 1993) or *casei*cin A (Osalupo et al., 1995).

Table 9 Antimicrobial capability of the soluble fraction for *L. casei* N87 cells in all the trials performed.

	N87			**N87 R**			**N87 F**			**N87 RF**		
	Cell – free Supernatant	**Acidity**	**Antimicrobial compound**	**Cell – free Supernatant**	**Acidity**	**Antimicrobial compound**	**Supernatant**	**Acidity**	**Antimicrobial compound**	**Supernatant**	**Acidity**	**Antimicrobial compound**
L. monocytogenes	-	-	-	-	-	-	+	+	+	+	+	-
S. enteritidis	-	-	-	-	-	-	+	+	+	+	+	-
S. aureus	-	-	-	-	-	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus	-	-	-	-	-	-	-	-	-	-	-	-

Table 10 Antimicrobial capability of the soluble fraction for *L. paracasei* V3 cells in all the trial performed.

	V3			**V3 R**			**V3 F**			**V3 RF**		
	Cell – free Supernatant	**Acidity**	**Antimicrobial compound**	**Cell – free Supernatant**	**Acidity**	**Antimicrobial compound**	**Supernatant**	**Acidity**	**Antimicrobial compound**	**Supernatant**	**Acidity**	**Antimicrobial compound**
L. monocytogenes	+	-	-	+	-	-	+	+	-	+	+	+
S. enteritidis	+	+	-	+	+	-	+	+	+	+	+	-
S. aureus	-	-	-	-	-	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus	-	-	-	-	-	-	-	-	-	-	-	-

Competition with pathogens

L. casei N87, *L. paracasei* V3 and the pathogens were further monitored to evaluate their simultaneous growth in MRS broth (Oxoid, Milano, Italy). All of them reached the exponential phase after 8 h from the inoculum and after 14 h they gained 10^8 CFU/ml. On these bases the effect of the competition was evaluate.

N87 cells not subjected to the fermentation and 4 °C storage, under both the adaptation condition, was not able to compete with pathogens. Conversely, strain V3 in the same conditions was able to compete with *L. monocytogenes* and *S. enteritidis*.

The cells of *L. casei* N87 (Figure 9) after the fermentation process were able to compete with *S. aureus* (panel F) and *S. enteritidis* (panel E), in both case the pathogens tried to increase their load but they never reached more than 2 Log CFU/ml. Regarding *E. coli* (panel C) and *B. cereus* (panel D), it was able to compete, in this case the pathogens were not able to increase their load, but their cells decreased until they reached the limit of determination (<1 cell/ml). Different behaviour where found in the co-inocula with *L. monocytogenes* (panel B). In this case N87 cells, growth under both the adaptation, were not able to compete during the first twelve hours. After twelve hours the load of the listeria cells decreased, meanwhile the load of the N87 cells remained stable.

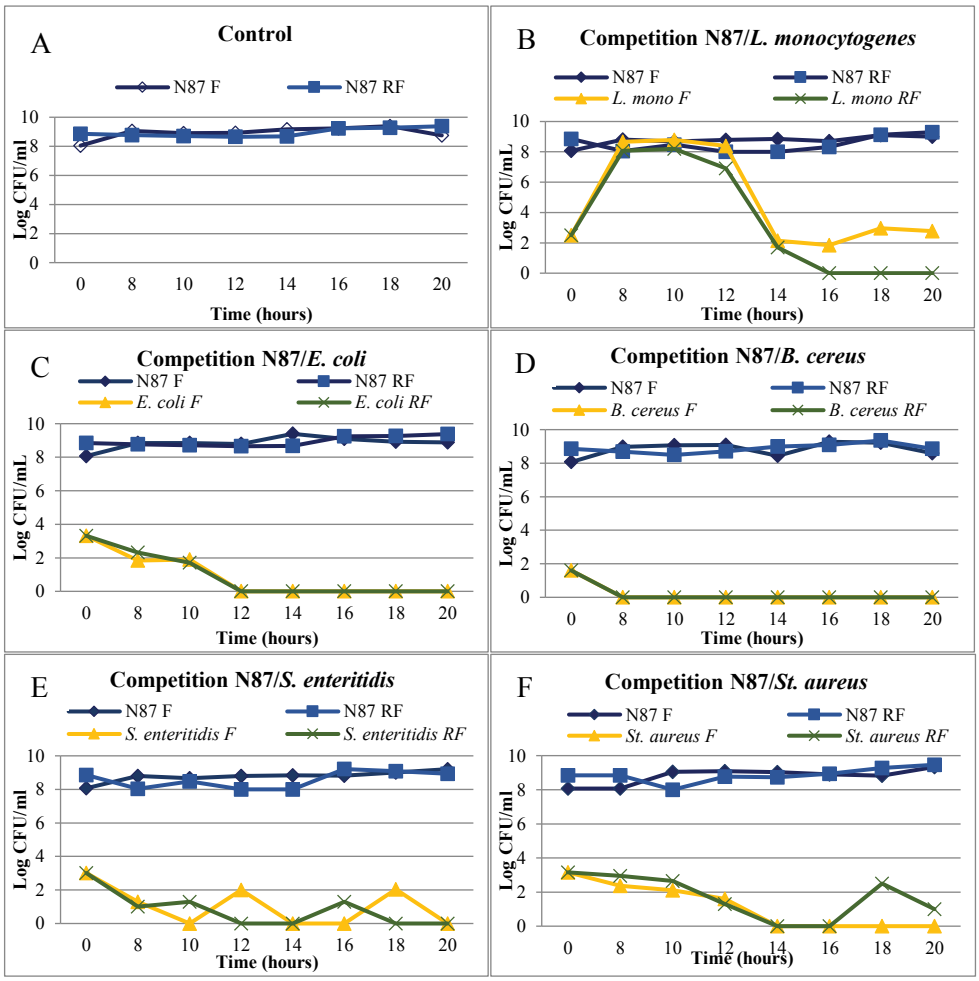

Figure 9 Panel A, B, C, D, E, and F. Competition between *L. casei* N87 and the pathogens, after both the adaptation and the technological stresses.

N87 F, strain grown under anaerobic conditions and subjected to technological stresses; N87 RF, strain grown under respiratory conditions and subjected to technological stresses. Letters F and RF after the pathogens name means the inoculation with N87 F or N87 RF, respectively.

On the other hand, *L. paracasei* V3 cells showed a different behaviour (Figure 10). This strain grown under both the adaptation conditions and subjected to the technological stresses maintained the ability to compete with *S. enteritidis* (panel E). On the other hand, in the case of *St. aureus* (panel F) in co-inoculum with V3 cells grown under anaerobic condition, the pathogen decreased until the limit of detection (<1 Log CFU/ml). Considering the co-inocula with *B. cereus* (panel D), the strain grown under anaerobic condition was not able to compete and after 16 h the pathogen reached 8 Log CFU/ml, while in the case of the strain grown under respiratory conditions, the pathogen growth was contained under the value of 4 Log CFU/ml. Despite the concentration of the V3 cells remained stable, in both the adaptation, they were unable to compete with *L. monocytogenes* (panel B) and *E. coli* (panel C), in fact both the pathogens were able to reach 7 Log CFU/ml after 8 h from the inoculum.

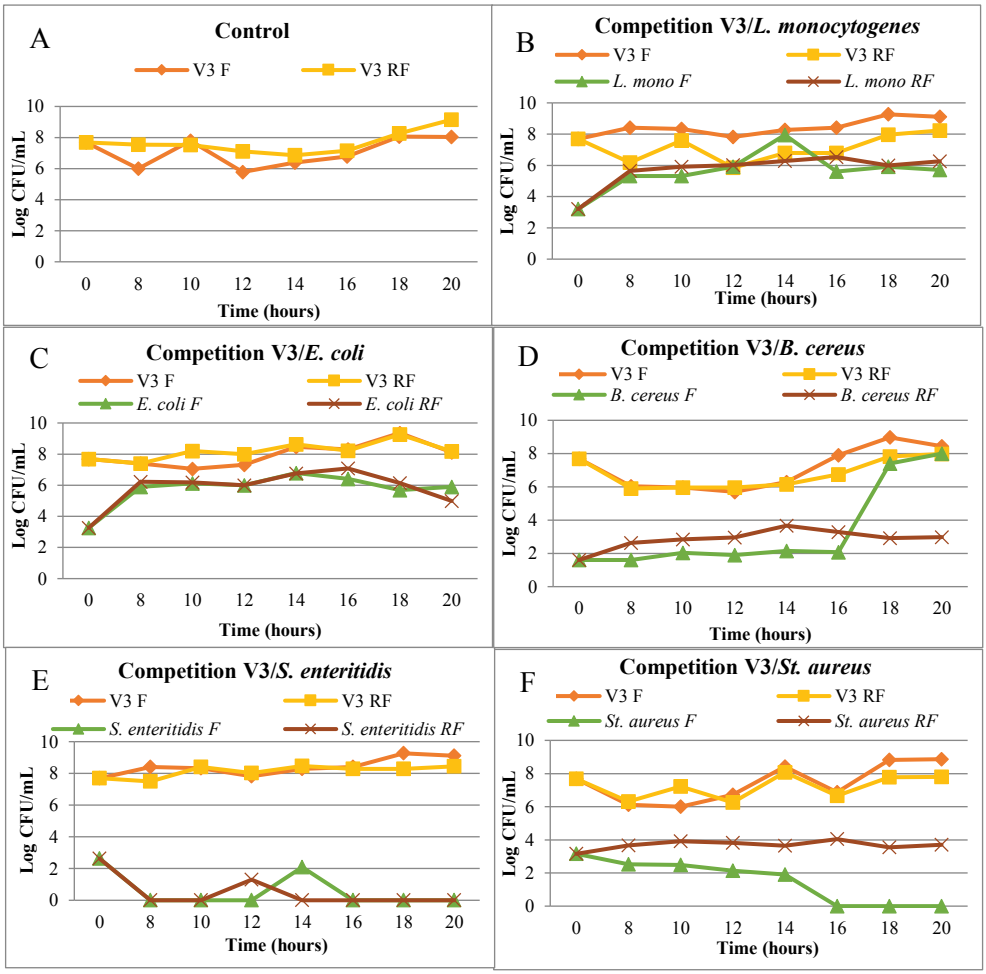

Figure 10, Panel A, B, C, D, E, and F. Competition between *L. paracasei* V3 cells after both the adaptation and the technological stresses and the pathogens strains.

V3 F, strain grown under anaerobiosis conditions and subjected to technological stresses ; V3 RF, strain grown under respiratory conditions and subjected to technological stresses. Letters F and RF after the pathogens name means the inoculation with N87 F or N87 RF, respectively.

Iranmanesh and Ezzatpanah (2015) suggested the presence of bacteriocin like substances, also Saarela et al., (2000) indicated different low molecular weight metabolites for the main role in the antagonistic properties of the strains.

Comparing these the results with findings previously reported about the antimicrobial capability of the milk soluble fraction is possible to say that probably the presence of a rich proteins or precursor environment can lead the formation of compound with an inhibitory/antimicrobial activity, that the strains are not able to form in MRS broth. On the other hand, for the same reason, some pathogen can be fortified by the nutrient present in the same niche, which leading an increment of the resistance to potentially antimicrobial compounds producted.

in vitro digestion

The results of the single steps digestion *in vitro*, showed the ability of the selected strains to survive to the saliva and intestine. Regarding the survival ability in the stomach, *L. paracasei* V3, adapted in both anaerobic and respiratory condition, showed the capability to survive whereas *L. casei* N87 strain after 30 minutes in the stomach decreased its viability, and the adaptation under respiratory conditions amplified this trend, decreasing the viable cells from 8.7 and 8.5 Log CFU/ml of the inoculum, to 4.3 and non detectable Log CFU/ml after the time of exposure in N87 F and N87 RF respectively (data not shown).

In the sGIT, strain V3 did not show changes in the behaviour of the cells, that survive during the passages. On the other hand, N87 after a decrement during the stomach passage, an increase in the cellular load was observed in the intestinal tract (Figure 11). This findings are in agreement with Corsetti et al., (2008), who found that some strains are sensitive to acid stress, but after an incubation in simulated intestinal fluid there could be a recovery of the viability.

The cells of N87 did not showed the same behaviour in the single steps digestion and in the sGIT suggesting that the passage in the saliva solution could protect the cells.

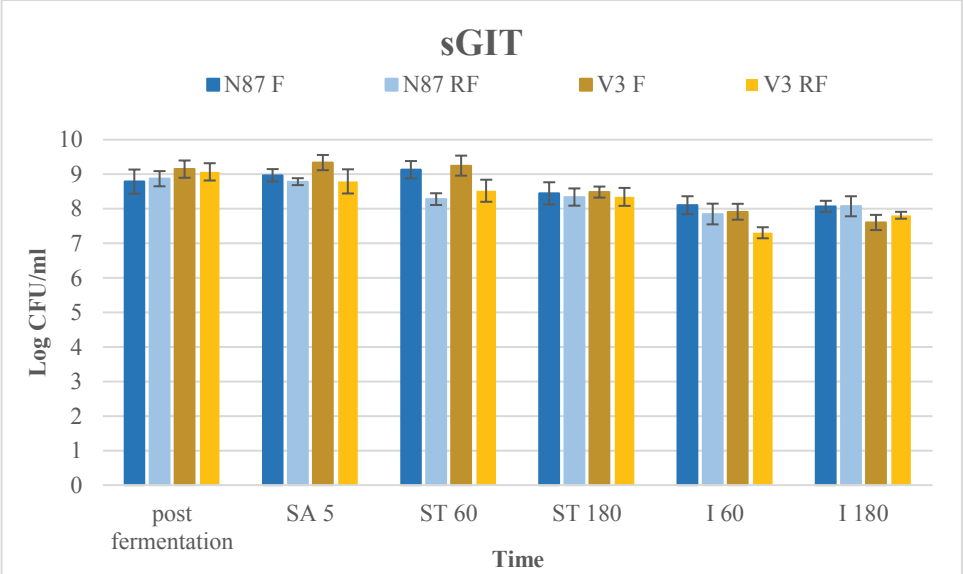

Figure 11 Effect of the sGIT passage on the survival of the adapted cells. Sa, saliva; St, stomach; I, intestine.

When subjected to environmental stress during food processes and digestion, constituent microbes could pass to a VBNC state, in which they are dormant yet metabolically active (Davis, 2014). Some differences could be found in cells of the same strain growth in diversified condition.

The VBNC cells were calculated during the sGIT passage. Looking at the cells value obtained through the method performed, it was possible to confirm that during the stomach passage the cells of both the strains became not colturable but they are still viable (Figure 12).

VBNC cells were defined as cells physiologically active, but that could not be cultured on medium (Fakruddin et al., 2013). The PMA – qPCR is a promising technique in the control of the VBNC, already tested for the detection of pathogens (Nocker et al., 2006). The obtained results show the capability of this strains to enter in this stasis state, as found by Casalta et al., (2009) and Rantsiou et al., (2008) for *Lactococcus lactis.* The cells of N87 did not showed the same behaviour in the single steps digestion and in the sGIT suggesting that the passage in the saliva solution could protect the cells

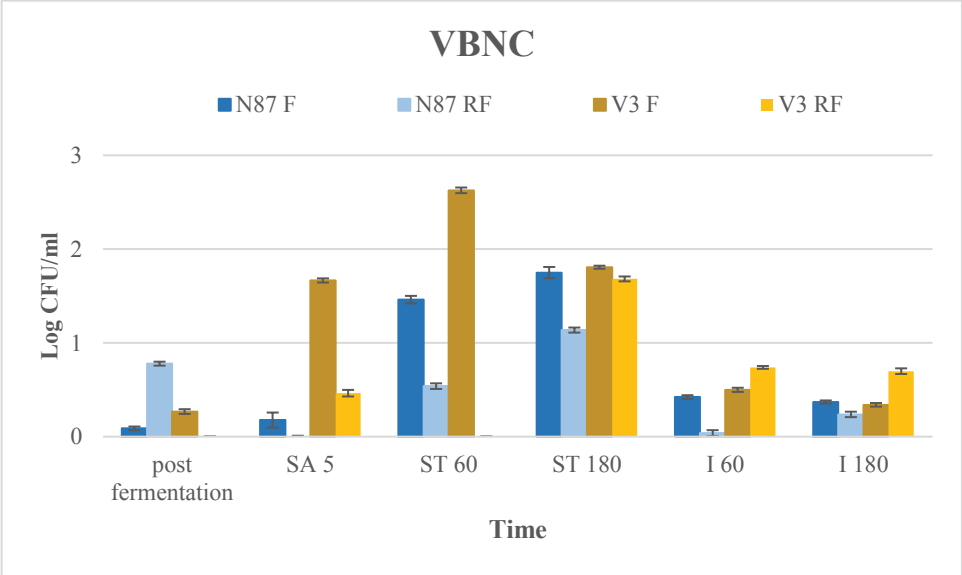

Figure 12 Presence of VBNC cells during sGIT passage. Sa, saliva; St, stomach; I, intestine.

Proteolysis assessment and bioactive peptides detection

The LAB proteolytic activity system is complex, because is composed of a multitude of intracellular peptidases and cell-wall-bound proteinase. In the milk fermentation processes, the proteolytic system of LAB has an industrial importance; it contributes to the fermentation under which the formation of peptides know to contribute to the flavour and texture of the products. Also it has a key role because it allows these bacteria to grow in milk (Savijoki et al., 2006). Moreover, bioactive peptides with different function (antimicrobial, immunostimulants, antihypertensive etc.) can be liberated during the fermentation of the milk (Hafeez at al., 2014). Proteolysis conducted by LAB during the fermentation process can lead to the formation of this peptides (Korhonen and Pihlanto, 2006).

The SDS – page, performed during the fermentation and the shelf life showed high similarity in the proteolysis. Unfermented milk showed 4 bands 83, 27, 17 and 14 kDa until 21 days of shelf life (MILKT4), where the bands were 71, 23, 20, 19 and 18 kDa demonstrating a degradation of the proteins. In the fermented milk samples the bands weight changed at the end of the fermentation when compared to initial unfermented milk. Bands of 83, 27, 17, 14, 9 and 3-1 kDa were found. After 14 days of shelf – life the band at 27 kDa disappeared, while the band at 83 kDa decreased in intensity (data not shown).

Bands at 83, 27 kDa were aligned with Lactoferrin and α – Casein respectively, bands at 18-17 kDa seemed corresponding to β – Lactoglobulin. The bands lower than 10 kDa seemed to be peculiar of the fermented milk from T1 to T4 (bands of 9 and 3-1 kDa) and the milk at the end of the shelf life (9 kDa).In the heat map (Figure 13), it is possible to see three main cluster, cluster I that grouped T2,T3,T4 samples, cluster II T0 and T1 and cluster III milk samples

except for MILKT4. Only N87FT0, that had a band at 18 kDa and not at 17 as the others, and MILKT4 remained out of the clusters.

A release of active peptides from milk proteins was reported by several authors (Gifford et al., 2005; Hernández-Ledesma et al., 2008, Lahov and Regelson, 1996, Meisel, 1998, Meisel 2004, Meisel and Bockelmann, 1999, Pellegrini et al., 2001, Phelan et al., 2009, Schanbacher et al., 1998, Zucht et al., 1995), according with these authors there are different bioactive peptides with a molecular weight included between 4.8 and 0.5 kDa that have antimicrobial ability. These peptides could derive from different protein like Lactoferrin, α-Casein and β-Lactoglobulin. Dionysius and Milne (1997) found that lactoferricin (3.12 kDa) had antibacterial activity against *E. coli* and *St. carnosus*, while Lahov and Regelson (1996) investigated the capacity of Iracidin (1.33 kDa) to inhibit *St. aureus* in lethal infection in mice. β-Lactoglobulin derived fragment showed bactericidal activity towards Gram-positive bacteria (Pellegrini et al., 2001) and bacteriostatic capability against E. coli, B. subtilis and St. aureus (Hernández-Ledesma et al., 2008). The antimicrobial activity of these peptide was determined by increasing cell membrane permeability (Gifford et al., 2005; Meisel, 2004; Meisel and Bockelmann, 1999). The presence of these peptides in the samples could be the answer to the antimicrobial capability of the soluble fractions discussed before. Also the increment of the competition with pathogen could potentially be linked to these results.

As proposed by Gobbetti et al., (2002) LAB, specifically *L. casei* N87 and *L. paracasei* V3, could generae bio - functional peptides.

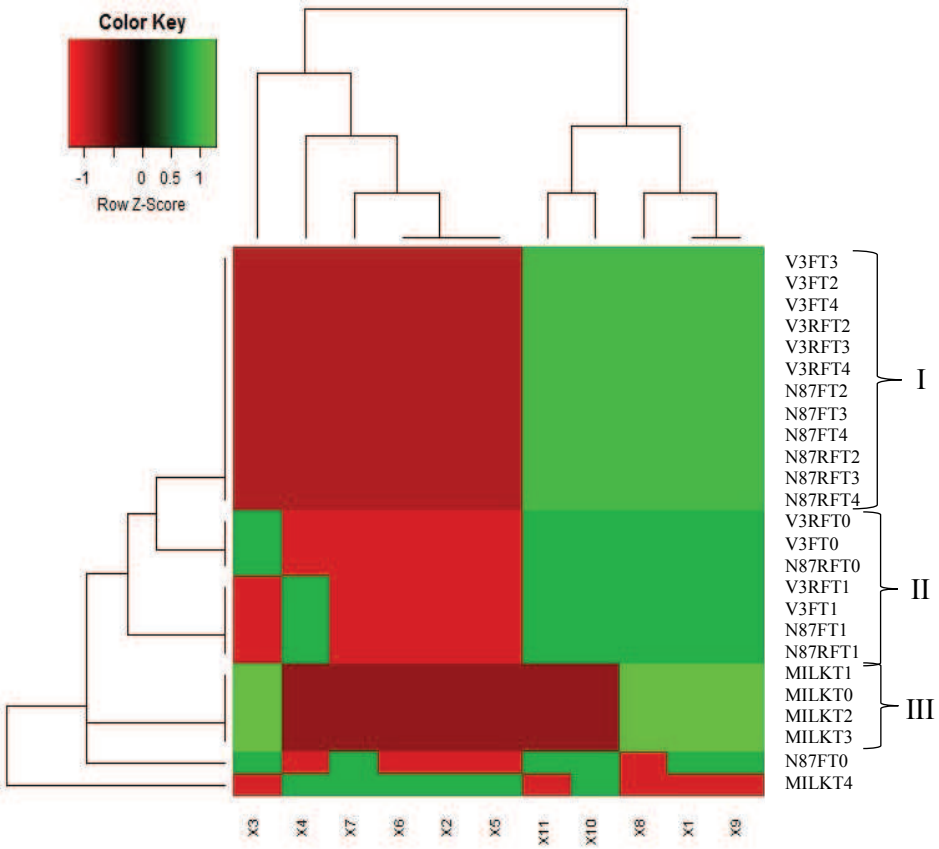

Figure 13. Heatmap. Legends of the molecular weight: X1, 82 kDa; X2, 71 kDa; X3, 27 kDa; X4, 22 kDa; X5, 20 kDa; X6, 19 kDa; X7, 18 kDa; X8, 17 kDa; X9, 14 kDa; X10, 9 kDa; X11, 3-1 kDa.

CONCLUSIONS

This study proved that the respiratory condition of growth can change the physiological response of the cells without effect on the fermentation capability of the selected strains. The mutation in the antibiotics resistance, the hemolytic activity and competition with pathogens was evident compared to the non-stressed cells. On the other hand, there were no significant differences in the proteolysis and production of peptides considering the two adaptations. However, further investigation will be needed on the peptides production ability and the changes in the physiological response.

The variation observed in the response could be helpful in probiotics investigation and design of functional foods.

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Effects of anaerobic and respiratory adaptation of *Lactobacillus casei* N87 on fermented sausages production.

Introduction

Fermentation is a method to preserve meat from ancient times. Europe is the major consumer and producer of fermented sausage, usually produce with pork or beef. Fermented sausages are obtained from the fermentation of chopped fresh pork meat, mixed with lard and other ingredients, such as sugars, NaCl and additives (nitrate, nitrite and spices), and stuffed into natural or synthetic casings (Comi et al., 2005). The manufacturing is conducted in three stages (formulation, fermentation and ripening) with the addition of starter cultures if required (Urso et al., 2006).

European fermented sausages can be divided in two main categories (North and South Europe manufacture), which differs in final pH values and/or water activity (a_w) and process condition (Talon et al., 2007).

The most important constituent of the sausages is meat, which is the muscular part more or less infiltrated from fats. The meat and fat part may come from different parts of the carcass. After sectioning the cuts, both lean that fats are cooled as quickly as possible, at of 0-2°C, to control microbial growth. Finally, they are ready for the preparation of sausages (Zambonelli *et al.*, 1992).

During ripening, protein degradation causes changes in the aroma and texture of the sausages. Initially peptides and free amino acids are formed. Amino acids can be decarboxylated and deaminated forming amines and organic acids, respectively. These compounds are substrates for subsequent reactions leading to the formation of many other aromatic compounds (Hierro et al., 1999). Muscle proteinase were the main enzymes involved in the initial breakdown of sarcoplasmic and myofibrillar proteins (Fadda et al., 2010). Several authors have demonstrated the existence of an intense proteolytic activity of cathepsins (Demeyer et al., 1992; Verplaetse et al., 1992; Toldrà et al., 1993). Toldrà et al., (1992) studied the influence of various agents on the activity of cathepsins B, H, L and D. These authors concluded that cathepsins B, L and D are active in the dough and during fermentation while, cathepsin L activity is greater in the ripening phase. However, also enzymes originating from bacteria occur in this process (Savijoki et al., 2006). In dry fermented sausages *Lactobacillus* enzymes seems to be responsible for the production of amino acid and small peptides, which provide as precursor of flavour molecules (Fadda et al., 1999; Sanz et al., 1999a,b). Therefore, *in vitro* and *in vivo* studies describe the proteolytic activity of lactic acid bacteria (LAB) on meat proteins (Demeyer et al., 2000; Fadda et al., 1999; 1998; 2001; 2002, 2010; Hierro et al., 1999; Hughes et al., 2002; Sanz et al., 1999a/b;Spaziani et al., 2009; Aro Aro et al., 2010). These studies suggest that, since the initial breakdown of the muscle proteins is due to meat proteinases, the microbial enzyme are more involved in the latter stages of ripening. Also, mould, *Micrococcaceae* and catalase positive and negative cocci could be implicated in protein degradation (Montel et al., 1992 ;1996; Selgas et al., 1993; Hammes et al., 1995; Lucke, 1997; Rouhi et al., 2013).

Main agents of lipolysis were tissue enzymes and lipases derived from bacteria, yeast and mould (Lucke, 2000; Rouhi et al., 2013). Bacterial lipase of salami derived mainly from *Staphylococcus* spp. and *Micrococcus* spp. (Iacumin et al., 2006; Cocolin et al., 2009; Pisacane et al., 2015; Aquilanti et al., 2016).

Aroma and flavour development in fermented meat is a complex process involving biochemical and chemical conversion of several components, as proteins, lipids and carbohydrates (Meynier

et al., 1999; Lucke, 2000). Lipolysis and proteolysis have a key role in the aroma formation (Rohui et al., 2013).

The use of starter cultures in fermented meat product is increasing. They are necessary to ensure the hygienic safety, standardize property as the aroma and colour, to decrease the curing period. High sensory properties and good quality of the final product can be obtained using selected microorganism as starter cultures (Coppola et al., 1997; Lucke, 2000; Leroy and De Vuyst, 2004).

Commercial starter cultures preparations contain a combination of coagulase-negative staphylococci (*Staphylococcus carnosus* and *Staphylococcus xylosus),* LAB strains, mainly *Lactobacillus* (*Lactobacillus sakei, Lactobacillus curvatus* and *Lactobacillus plantarum*) or *Pediococcus* (*Pediococcus pentosaceus* and *Pediococcus acidilactici*) genus, with members of *Micrococcaceae* (*Kocuria)* (Gounadaki et al., 2009). These starter are often selected for their capabilities of acidifying, reddening and flavouring (technological profiles) and to enhance the microbiological safety, shelf - life and obtain an homogeneous casing (Bedia et al., 2011).

Lactic acid bacteria had a central role in fermented sausages. Their use as starter cultures can cause rapid acidification, inhibition of pathogens and production of precursors of aroma compounds (Leroy and Vuyst, 2004; Ammor and Mayo, 2007; El Adab et al., 2015; Mati et al., 2015; Pisacane et al., 2015). About 10^6 cells/g of LAB cultures are added in European – style sausage (Lucke, 2000). LAB are used as starter due to their ability to generate lactic acid, improving sensory quality and having a bio - protective role (Bagdatli and Kundakci, 2016).

The dry or fermented meat is not a usual carrier for probiotic bacteria (Ammor and Mayo, 2007; Arihara, 2006). In addition it is thought that the array of sausages can protect these bacteria from the gastro-intestinal tract (Klingberg and Budde, 2006). However, the potential negative impact of meat environment as carrier has to take into account, in particular with respect to the action of salt content, low pH and a_w, acidification and drying (Rouhhi et al., 2013). Therefore the choice of the appropriate microorganism to be applied as a probiotic is important. An obvious choice is to use bacteria commonly associated with meat and possessing the physiological characteristics and health promoters properties (Pennacchia et al., 2004 and 2006).

Lactobacillus casei is a LAB used in several fermented foods, such as dairy, bakery and fermented meat products (Ammor et al, 2007; Bedia et al., 2011). This microorganism can be use as starter to ferment meat and to improve health benefit, considering the possibility to use this matrix as probiotic carrier (Cenci-Goga et al., 2008; Jaworska et al., 2011; Pidcock et al., 2002; Rubio et al., 2014; Rouhi et al., 2013; Sidira et al., 2015, 2014; Trzaskowska et al., 2014; Wojciak et al., 2012).

Lactobacillus casei group were recently examined to better understand the adaptive response to stresses and adverse conditions (Reale et al., 2015; Zotta et al., 2014). Several authors studied the response of strains of this group and others, subjected to respiratory growth and the changing in metabolism and physiological response (Ianniello et al., 2015; Pedersen et al., 2008; Ricciardi et al., 2015). Although not many studies tested *L. casei* strains growth under respiratory condition in foods (Reale et al., 2016a/b).

The adjunction of *L. casei* strains*,* adapted or not under respiratory condition of growth, to fermented sausage can affect the physiological response of cells, influencing growth, survival and capabilities. The variation in metabolic pathway can influence the microbial population, the chemical/physical parameters, the aroma and flavour development and the proteolysis of the final product. For this reasons, the aim of this study was to investigate the effects of anaerobic and respiratory conditions of growth on *L. casei* N87 during salami ripening. Specifically, the evolution of the microflora, the chemical/physical parameters (pH, a_w, weight

loss, colour), the production of volatile compounds, the antioxidant capabilities, the proteolysis and the survival of the strain, were evaluated.

Materials and methods

Strain and growth conditions

Lactobacillus casei N87 strain was used to promote the fermentation process in this work. The strain was stored at the temperature of - 80° C in cryovials containing DeMan, Rogosa and Sharp broth (MRS, Oxoid, Milan, Italy) supplemented with 2 % glycerol.

At the time of use, the strain was streaked on MRS Agar (Oxoid, Milan, Italy) plates and incubated at 30° C for 48 h, in order to check the purity and prepare the inoculum for the following stages of the experiment by inoculating a single colony in 2 mL of MRS broth (Oxoid, Milan, Italy).

The strain was adapted under anaerobic and respiratory conditions for 18 hours at 37 °C, than adapted cells were used as starter cultures for fermented sausages production. The respiratory condition was performed according to Zotta et al. (2014): the strain was cultivated in M17 broth (Oxoid, Milan, Italy) added with 2.5 µg/ml hemin and 1 µg/ml menaquinone at 30 °C for 48 h. Three subcultures were set up.

Fermented sausages production

The production involved the following ingredients: pork meat 64.8 %, pork fat 25.2 %, sodium chloride 2.5 %, black pepper, sugar and red wine mixed with garlic (360 ml in 45 kg). No E252 was added. The meat batter was minced and divided in three aliquots of 15 kg each, for the production of three different lots of fermented sausages: 1) control (C, fermented sausages without the addition of starter culture); 2) fermented sausages with the addition of *Lactobacillus casei* N87 adapted under anaerobic conditions (A); 3) fermented sausages with the addition of *Lactobacillus casei* N87 adapted under respiratory conditions (R). The inoculum was at a concentration of 6 Log CFU/ml. After the addition of the cells each meat batters were minced and stuffed into pork casings.

Sausages were fermented for 90 days as described in Table 11. Sampling was performed at 0, 2, 5, 7, 15, 30, 60 and 90 days. Analysis were performed in triplicate on three biological replicate per thesis.

Table 11 Fermentation condition used during the process.

Time	*Temperature*	*Relative Humidity*
24 h	4°C	
24 h	23°C	99%
36 h	19°C	65%
24 h	17°C	70%
24 h	15°C	75%
36 h	14°C	80%
7 days	14°C	75%
76 days	13°C	75%

Microbial analysis

Twenty-five grams of each sample were homogenized in 225 mL Buffered-Peptone Water (BPW) and in Fraser Half broth, in order to investigate the presence of *Salmonella spp.* and *Listeria monocytogenes* respectively, using a Stomacher machine (Stomacher Lab-Blender 400, A.J. Seward Lab. London, UK) for 3 min. Decimal dilutions were then prepared from the sample diluted in BPW. The following analysis were carried out: (i) total aerobic count of bacteria on Gelatin Sugar Free Agar, at 30 °C for 48 h; (ii) lactobacilli on MRS with the addition of DELVOCID®INSTANTANT (DSM Food Specialities bv., Netherland) 0.01 mg/ml, at 30 °C for 48 h; (iii) lactococci on M17 Agar at 30 °C for 48 h (iv) Coagulase negative - Catalase positive (CNCPC) on Mannitol Salt Agar at 30°C for 48 h; (v) total coliforms and *Escherichia coli* on ChromID Coli (Biomerieux, Firenze, Italy), at 37 °C for 48 h; (vi) *Staphylococcus aureus* on Baird-Parker agar supplemented with egg yolk tellurite emulsion, at 37°C for 48 h; (vii) molds and yeasts on Malt Extract Agar, with the addition of tetracycline 0.025 mg/ml, incubated at 30 °C for 48 h; (viii) enterococci on Kanamycin Aesculine Azide Agar, at 37°C for 48 h. MRS and M17 plates were incubated under restricted oxygen conditions achieved using AnaeroGen (Oxoid, Milano, Italy). The presence of *Salmonella spp.* was investigated using the ISO 6579:2002/Corr.1:2004 method, for *Listeria monocytogenes* the ISO 11290-1 was carried out. Unless otherwise specified, all media and ingredients were obtained from Oxoid (Milano, Italy). Colonies were counted, and the results were calculated as the means and standard deviation of three determinations per each replicate.

L. casei enumeration and bulk cell collection

After counting, all MRS plates were used for bulk formation as previously described (Ercolini et al., 2001). In brief, bulk formation was performed using all plates from the serial dilutions (-2 to the last). For each dilution, all colonies present on the plate surface were suspended in a suitable volume of quarter strength Ringer's solution, harvested with a sterile pipette and frozen at -20 °C. To minimize the effects of different concentrations, all suspensions were standardized at 1 unit of optical density (600 nm). Then, 1 ml of the bulk suspension was used for DNA extraction as described below and subjected to molecular analysis.

DNA extraction from bulk cultures

One millilitre of each bulk suspension was centrifuged at 14,000x g for 10 min at 4 °C to pellet the cells, and the pellet was subjected to DNA by using MasterPure™ Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, USA).

PCR amplification

Amplification was performed with a C1000 Touch™ Thermal Cycler (Bio - Rad, Milano, Italy) in a final volume of 50 µl containing: 10 mM TrisHCl pH 8·3, 50 mM KCl, 3 mM MgCl2, 0.2 mM of each dNTPs, 1.25 IU *Taq* – polymerase (Applied Biosystems™ AmpliTaq™ ,Thermo Fisher Scientific, Milano, Italy), 10 µmol of the primers and the DNA template (DNA extracted from standard strains and from MRS plates samples). The samples were processed using the following protocol: 95°C for 5min, 30 cycles of 94°C for 1 min, 42°C for 1 min and 68°C for 1 min, and finally 68°C for 7 min (Cocolin at al., 2000). The primers used were (Muyzer et al., 1993, Ampe et al., 1999):

- forward primer 338f, with GC clamp (underlined): 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG–3';

- reverse primer 518 r: 5'-ATT ACC GCG GCT GCT GG-3'.

Five microliters of the amplification products were analysed first by electrophoresis in 1.5 % (w/v) agarose gels 0.5X TBE added with EtBr 0.025% (v/v) before denaturing gradient gel electrophoresis (DGGE) analysis.

DGGE analysis

For the sequence specific separation of the PCR products the Dcode Universal Mutation Detection System™ (Bio – Rad, Milano, Italy) was used. Electrophoresis was performed in 8% [wt/v] acrylamide/bisacrylamide 37.5:1 (Bio – Rad, Milano, Italy) gel containing a 40–60% urea-formamide (Sigma – Aldrich, Milano, Italy) denaturing gradient. The gels were subjected to a constant voltage of 120 V for 5 h at 60°C. After electrophoresis, they were stained for 20 min in 1.25X Tris-acetate-EDTA containing 1X SYBR Green (final concentration; Molecular Probes, Eugene, Oreg.). Pictures of the gels were digitally captured by a GeneGenius BioImaging System (SynGene). A reference pattern was established consisting of amplicons from 5 different bacterial species: *L. casei* N87 and *L. curvatus* DSMZ 20019, *L. brevis* DSMZ 20054, *L. plantarum* DSMZ 20174, *L. sakei 6333* from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (Braunschweig, Germany). By including this standard reference pattern three times on each DGGE gel, resulting DGGE fingerprint band profiles from the different sausages were digitally normalised using Gel Compare 4.1 software (Applied Maths, Kortrijk, Belgium). Additionally, this reference pattern was used to obtain a preliminary identification of *L. casei* specie. DGGE analyses were performed at least twice.

Sequence analysis of DGGE bands

Blocks of polyacrylamide gels containing selected DGGE bands corresponding to the reference position of *L. casei* were excised with gel cutting tips. Blocks were then transferred to 50 µl sterile water, and the DNA in the bands was left to diffuse overnight at 4 °C. Two microliters of the eluted DNA were used for re-amplification, and PCR products generated with a GC-clamped primer were verified by DGGE. Only products migrating as a single band and at the same position with respect to the control were amplified, as described above, with the primer without the GC clamp. Products were then cloned into the pGEM-T Easy vector (Promega, Milan, Italy) following the manufacturer's instructions. Clones were checked as described above (co-migration with control), and the inserts in appropriate clones were sequenced at a commercial facility (Eurofins Genomics GmbH, Ebensdorf, Germany). Sequence comparisons were performed using a BLAST program (Altschul et al., 1997).

Total, viable and viable but not colturable (VBNC) *L. casei group* quantification

Total, viable and VBNC cells of *L. casei* group were also quantified during fermentation using a culture independent approach using PMA-qPCR. Two aliquots of 1 ml both of first dilution of each sample were collected. One of these aliquots were treated with propidium monoazide (PMA, Biotium, Inc., Hayward, CA, USA) and the other was not treated. Before the process with PMA, samples were centrifuged at 13,000 rpm for 10 min, the pellets were washed twice and resuspended in 300 µl of sterile peptone water (Maximum recovery diluent, Oxoid), after that 1.4 µl PMA 20 mM in 20% dimethyl sulfoxide (Sigma-Aldrich, Oakville, Ontario, Canada) were added. Each tube was incubated on ice in the dark for 5 min. For the photo-activation process, the tubes were inserted in the PhAST Blue instrument (GenIUL, Barcelona, Spain) for 15 min at room temperature.

DNA extraction

The suspensions treated with PMA were centrifuged at 14,500 rpm for 7 min to pellet the cells. DNA was extracted from the pellets using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, USA). DNA was resuspended in 40 µL of sterile bidistilled water. After extraction, the DNA concentration and purity was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Rodano, Italy) and standardised to 25 ng/µl.

qPCR protocol

Primers LCgprpoA-F2 (5'-CACTCAARATGAAYACYGATGA -3') and LCgprpoA-R2 (5'-CGTGGTGAGATTGAGCCAT -3') (Huang et al., 2011), targeting the *rpoA* gene of *L. casei* group, were chosen to perform qPCR. The qPCR mixture contained 10 µl of 2X SsoFast EvaGreen Supermix (Biorad, Milan, Italy), 300 nM of each primer, and 2.5 ng/µl of DNA, and the volume was adjusted to 20 µl with sterile DNA-free Milli-Q water. RotorGene Q system (Qiagen, Milan, Italy) was used to perform qPCR with one cycle of initial denaturation of template DNA and activation of *Taq* – polymerase at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 20 s, primer annealing at 50 °C for 20 s and extension at 72 °C for 20 s. Fluorescence signal acquisition was performed during the extension step. To determine whether non-specific products or primer dimers had formed, the dissociation curves of the final products of each PCR were analysed from 55 to 95 °C at 1 °C intervals.

Construction of standard curves

L. casei N87 growth in MRS broth (Oxoid, Milano, Italy) were enumerated at the exponential phase on MRS Agar (Oxoid, Milano, Italy) to determine the exact Log CFU/ml. The signals produced in the qPCR reactions (threshold cycle, C_t) by the serial dilution were plotted against the CFU/ml, and standard curves were constructed. The construction of standard curves was performed using three replicates per each standard point, and each reaction was carried out in triplicate.

pH and a_w measurements

The pH potentiometric measurements were carried out with a pin electrode pH-meter (Basic20 pH, Crison, Bacelona, Spain) that was inserted directly into the sample. The water activity (a_w) was determined with a water activity meter (AquaLab 4TE, Decagon Devices, USA). For both the analysis three independent measurements per each replicate were performed.

Colorimetric analysis and weight loss

Also the color changing of the samples were investigated, using a ChromaMeter CR-200 (Minolta, Japan), for every sample three independent measurement were performed, in three different part of the sausages. For the weight loss evaluation, 10 samples per each lot of production were weighed at every time of sampling.

Antioxidant abilities and volatiles profiles

Antioxidant abilities of the strain and volatiles profiles of the samples were monitored at the end of ripening using a GC-MS-SPME (gas chromatograph - mass spectrometry coupled with solid phase micro extraction) technique. Five (5) grams of each sample were placed in 10 ml vials sealed by PTFE/silicon septa. The samples were equilibrated for 10 min at 45 °C and

volatiles adsorbed for 40 min at 45 °C on a fused silica fiber SPME-DVB-Carboxen/PDMS (85 μm, Supelco, Bellefonte, PA, USA). Adsorbed molecules were desorbed in GC for 10 min. For peak detection, an Agilent Hewlett – Packard 7890 GC equipped with MS detector 5977A and Autocamp PAL RSI 85 (Hewlett – Packard, Geneva, Switzerland) was used. A 30 meter for 0.25 mm of internal diameter Chrompack VF-Wax MS (Chrompack, Middelburg, Netherlands) was used. The conditions were the same described in Serrazanetti et al. (2011). Volatile peak identification was carried out by computer matching of mass spectral data with those of the compounds contained in the Agilent Hewlett – Packard NIST 2014 mass spectral database and literature (Procida et al., 1999; Meynier et al., 1999; Bianchi et al., 2007; Di Cagno et al. 2008; Spaziani et al., 2009; Serrazanetti et al., 2011; Tabanelli et al., 2012 and 2013; Sidira et al., 2015; Montanari et al., 2016). The quantitative data were normalized respect an internal standard (4-Methyl-2-pentanol, which was used at a final concentration of 50 ppm).

Proteolysis assessment

All samples were investigated in order to evaluate the proteolysis using Mini Protean II (Bio – Rad, Milano, Italy). A 13.5 % acrylamide/bis - acrylamide running gel and 4 % acrylamide/bis - acrylamide stacking gel were used. Total proteins were extracted as described by Còrdoba et al., (1994) with some modification: 2 g of samples were homogenized for 3 min with 40 mL of 1.1 M potassium iodide, 0.1 M sodium phosphate buffer pH 7.4. Samples were centrifuged at 5000 rpm for 15 min at 4 °C. The obtained supernatants were filtered through a 0.45 μm and 0.22 μm filter and frozen until the time of use. Before the run, Bradford assay was performed. Fifteen microliter of sample were mixed with 15 μl of Laemmli sample buffer 2X (Bio-Rad, Milano, Italy) containing β – mercaptoethanol. The mixtures was incubated at 100 °C for 5 min. The standards used were Precision Plus Standard Protein (Bio – Rad, Milano, Italy). The wells of the gels were loaded with 25 μl of the denatured protein samples. The run was performed at 50 V for 15 min and 120 V for 90 min.

Staining was performed in 1 h with the following solution:

Blue di Comassie 1%	0.1 %
Methanol	50 %
Acetic Acid	7 %

De-staining was made for 2 h or overnight in a solution of:

Methanol	5 %
Acetic Acid	7 %

Alignments of the bands were made using GelAnalyzer 2010 Software (Lazar and Lazar, 2010). The molecular weights of the bands were used to build up a single matrix, which was submitted to a two-way hierarchical analysis, a heatmap was obtained. In the heatmap, values are represented by cells coloured according to the Z-values, which represent the observed values/standard deviation.

Statistical analysis

Anova analysis with Tukey's test and the two way hierarchical analysis were performed using R v 3.3.2 (R Development Core Team, 2011).

Results and discussion

Evolution of the microflora

A variety of microbial species are able to grow in fermented sausages. Samples were subjected to microbiological analysis to investigate the changes in the populations, considering the inoculum previously made. From the beginning of the process it was possible to see that the composition of the microbial flora within the sausages was various (Table 12).

Yeast population grow regardless the previous inocula, only samples collected at 7 days inoculated with *L. casei* N87 adapted under respiratory condition (R) show a significant difference according to the Tukey test. In this lot (R) an initial yeast count of 3 – 4 Log CFU/g was found increasing over 5 Log CFU/g during ripening. Several authors in their studies observed different values concerning the yeast population in their salami samples, starting from 1 Log CFU/g in the raw material to 7.7 Log CFU/g at the end of the casing (Cocolin et al., 2009; Cocolin et al., 2006; Comi et al., 2005; Aquilanti et al., 2007; Coppola et al., 2000; Gardini et al., 2001; Moretti et al., 2004; Di Cagno et al., 2008).

Total aerobic counts reached the maximum concentration in the first 5 days of ripening and then remained stable, and no significant differences were observed among the three different thesis. This result is in according on what found by different authors (Ceci – Goga et al., 2008; Pisacane et al., 2015; Comi et al., 2005).

Concerning enterococci, the different fermentations differs between initial and final count, showing a non-linear behaviour during the period monitored, although N87 samples showed lower values after 15 days of ripening. This outcome are in correspondence with previous studies (Comi et al., 2005; Di Cagno et al., 2008; Pisacane et al., 2015).

Regarding CNCPC, in all the fermentation they had a similar behaviour and, after an initial gain they remained stable through the ripening. In accordance with various authors (Coppola et al., 2000; Iacumin et al., 2006; Aquilanti et al., 2007; Silvestri et al., 2007; Di Cagno et al., 2008; Cocolin e al., 2009; Francesca et al., 2013) the trend of this microbial population can fluctuate between 3-6 Log CFU/ml.

LAB population at time 0 in control samples was detected at about 4.08 Log CFU/g, reaching 6 Log CFU/g after 5 days and remaining stable until the end, this is similar to what was found by other authors (Urso et al., 2006; Pisacane et al., 2015; Cenci – Goga et al., 2008; Aquilanti et al., 2007; Polka et al., 2015; Coppola et al., 2000). On the other hand, Comi et al., 2005 results showed an higher quantity of LAB in naturally fermented sausages (8 Log CFU/g). Looking at the results of time 0, the inocula of A and R samples supposedly went successful.

The lactococci counts did not give evidence of differences depending on the inocula after five days of ripening, except for the control samples at 0 and 2 days, when they were at 4.8 ± 0.08 and 3.8 ± 0.34 Log CFU/g, whereas A and R samples had from the beginning a microbial count of about 6-7 Log CFU/g. Also Cenci-Goga et al., (2008) and Ranucci et al., (2013), in their studies found that the not inoculated samples showed an high numbers of lactococci for the 21 and 6 days of ripening, respectively. Similar findings were obtained by Francesca et al., 2013, at the end of the ripening (42 days) their samples had about 5 Log CFU/g of lactococci.

Total coliforms showed a high variability during the ripening. They were under the limit of detection (<1 Log CFU/g) on the raw materials of the sample R, on the other hand in control samples and A samples had a count of 2.5 and 1.49 Log CFU/g respectively. Throughout the period monitored, coliforms increased their count during the first 15 days of ripening (4 Log CFU/g) before falling back to the raw material values, where A samples remained under the detection limit. This result are in agreement with the data observed by Cocolin et al., (2009)

and Silvestri et al., (2007), however different authors found coliforms in their studies, but not at the end of the fermentation process (Pisacane et al., 2015; Aquilanti et al., 2007; Moretti et al., 2004).

S. aureus and *E. coli* counts were always below the limit of detection, 2 and 1 Log CFU/g respectively. Also, *Salmonella* and *Listeria* investigated using ISO methods resulted absent in 25 g of product at every time of sampling. The results regarding these microbial populations, despite the presence of coliforms, underlined a good hygienic quality and safety of the raw materials. Moreover, some differences could be attributed at the matrix inhomogeneity.

Table 12 Results of microbiological analysis carried out on the three samples (Log CFU/g).

Microbiological analysis		0		2		5		7		15		30		60		90	
	Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Yeast	C	3,89	0,23^{a}	3,53	0,21^{a}	5,31	0,29^{a}	5,10	0,62^{a}	4,41	0,58^{a}	4,43	0,42^{a}	4,85	0,02^{a}	4,68	0,34^{a}
	A	3,93	0,32^{a}	3,49	0,18^{a}	5,05	0,53^{a}	5,15	0,32^{a}	4,98	0,08^{a}	5,01	0,53^{a}	4,45	0,61^{a}	3,73	0,34^{b}
	R	3,51	0,15^{a}	3,37	0,36^{a}	6,18	0,50^{a}	4,83	0,93^{a}	5,07	0,34^{a}	4,41	0,73^{a}	4,60	0,43^{a}	4,81	0,13^{a}
Total Aerobic Count	C	5,02	0,11^{ab}	4,48	0,16^{a}	6,43	0,46^{a}	6,30	0,40^{a}	6,84	0,16^{a}	6,51	0,15^{a}	6,30	0,23^{a}	6,08	0,12^{a}
	A	4,74	0,04^{b}	4,52	0,09^{a}	6,44	0,13^{a}	5,72	0,60^{a}	6,41	0,17^{a}	6,43	0,50^{a}	5,61	0,13^{b}	5,48	0,31^{b}
	R	5,14	0,21^{a}	4,46	0,38^{a}	6,73	0,60^{a}	6,24	0,43^{a}	6,57	0,30^{a}	6,42	0,31^{a}	6,17	0,23^{a}	5,88	0,19^{ab}
Enterococci	C	6,00	0,10^{b}	5,00	0,10^{b}	4,00	0,22^{b}	4,20	0,21^{a}	5,91	0,25^{a}	4,78	0,55^{a}	4,41	0,63^{a}	5,43	0,21^{a}
	A	6,67	0,18^{a}	5,49	0,32^{a}	5,46	0,29^{a}	4,10	0,70^{b}	3,70	0,14^{b}	2,76	0,60^{b}	3,12	0,62^{b}	4,64	0,60^{b}
	R	6,00	0,01^{b}	4,90	0,01^{b}	5,30	0,01^{a}	4,50	0,11^{a}	5,77	0,17^{a}	5,34	0,10^{a}	4,19	0,47^{a}	5,41	0,27^{a}
CNCPC	C	5,93	0,29^{a}	3,84	0,12^{a}	6,28	0,07^{a}	6,15	0,19^{a}	6,02	0,46^{a}	6,36	0,15^{a}	6,33	0,16^{a}	5,93	0,18^{a}
	A	3,87	0,11^{c}	4,02	0,36^{a}	6,40	0,09^{a}	5,84	0,50^{a}	6,14	0,23^{a}	6,68	0,65^{a}	5,91	0,64^{a}	5,12	0,56^{a}
	R	4,94	0,39^{b}	3,75	0,18^{a}	6,23	0,33^{a}	6,09	0,46^{a}	6,38	0,41^{a}	6,52	0,60^{a}	6,14	0,21^{a}	5,73	0,13^{a}
LAB	C	4,08	0,04^{c}	3,68	0,19^{c}	6,00	0,51^{a}	6,04	0,73^{b}	6,97	0,65^{a}	6,51	0,48^{b}	6,48	0,29^{a}	6,82	0,65^{a}
	A	7,18	0,04^{a}	7,13	0,11^{a}	7,07	0,63^{a}	7,67	0,10^{a}	7,89	0,16^{a}	7,69	0,14^{a}	7,33	0,32^{a}	7,75	0,16^{a}
	R	6,43	0,06^{b}	5,70	0,22^{b}	6,23	0,29^{a}	6,33	0,15^{b}	7,14	0,68^{a}	6,01	0,39^{b}	6,79	0,63^{a}	6,63	0,43^{a}
Lactococci	C	4,79	0,08^{c}	3,78	0,34^{c}	6,32	0,22^{a}	6,23	0,27^{a}	7,31	0,25^{a}	6,85	0,60^{ab}	6,61	0,08^{ab}	7,10	0,56^{ab}
	A	7,25	0,16^{a}	7,19	0,22^{a}	7,07	0,58^{a}	7,21	0,70^{a}	7,78	0,18^{a}	7,84	0,14^{a}	7,18	0,41^{a}	7,83	0,14^{a}
	R	6,57	0,13^{b}	5,60	0,21^{b}	6,44	0,14^{a}	6,15	0,33^{a}	7,69	0,34^{a}	6,11	0,70^{b}	6,75	0,60^{b}	6,56	0,23^{b}
Coliforms	C	2,50	0,35^{a}	1,62	0,15^{a}	4,30	0,48^{a}	5,06	0,41^{a}	4,78	0,61^{a}	3,80	0,70^{a}	1,95	0,29^{a}	1,70	0,70^{a}
	A	1,49	0,20^{b}	1,62	0,28^{a}	4,31	0,15^{a}	3,62	0,73^{b}	3,90	0,72^{a}	1,99	0,11^{a}	1,36	0,32^{a}	<1	n.a.
	R	<1	n.a.	1,49	0,43^{a}	4,84	0,67^{a}	4,84	0,27^{ab}	4,56	0,16^{a}	2,99	0,62^{a}	1,39	0,12^{a}	1,78	0,14^{a}
E. coli	C	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.
	A	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.
	R	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.	<1	n.a.

Abbreviation: SD, standard deviation; n.a., not applicable.

ANOVA analysis with Tukey's test were performed in samples, clustered according to microbial analysis and time of sampling. The results are showed as top letters next to SD value.

Evaluation of the physical/chemical parameters.

In the Mediterranean area, salami are dry or semi dry, ripening higher or lower than 4 weeks and a_w lesser than 0.90 or between 0.90 and 0.95, respectively (Lucke, 2000). The final pH can be about 5 or comprised between 5.3 and 6.3 (Aquilanti et al., 2016). The results of this study are in agreement with those range (Figure 14). The pH value of C and R samples are in accordance with the several authors that investigate fermented sausages production in this part of Italy (Friuli - Venezia Giulia) (Comi et al., 2005; Urso et al., 2006; Cocolin et al., 2009). On the other hand, A was lower, similar to the pH values found by other authors with or without the adjunction of starter (Aquilanti et al., 2007; Ranucci et al., 2013;. Coppola et al., 2000; Tabanelli et al., 2016; Wojciak et al., 2012; Trzaskowska et al., 2014) (panel A). The water activity values during and at the end of the ripening did not show significant differences (panel B).

The weight losses averaged over inoculum type were 36.45% ± 2.45[a], 35.48% ±1.14[a] and 40.26 ±1.79[a] respectively for C, A and R samples. This weight loss was similar to data reported by other studies (Coffey et al., 1998; Moretti et al.,2004; Tabanelli et al., 2012; Bagdatli and Kundakci, 2016).

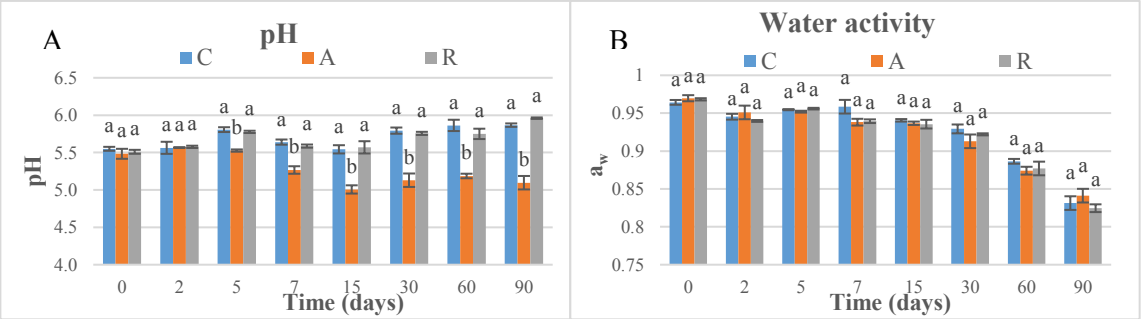

Figure 14 – Panel A pH and Panel B water activity of the samples during ripening.

ANOVA analysis with Tukey's test were performed in samples, clustered according to the analysis and time of sampling. The results are showed as top letters.

In this study, different colour parameters were considered. However no notable divergences were found. Examined samples showed stable red index (a*) and chroma rate, while the hue angle (red-purplish color) and the lightness (L*) decrease (Figure 15). The weight loss influence the L* value as found by Bagdatli and Kundakci, 2016, even so the L* decrease were reported by other author (Olivares et al., 2010; Ercoskun and Ozkal, 2011). The stability of a* during ripening suggest that the formation of nitrosylmiyoglobin was not affected by microbial and enzymatic degradation.

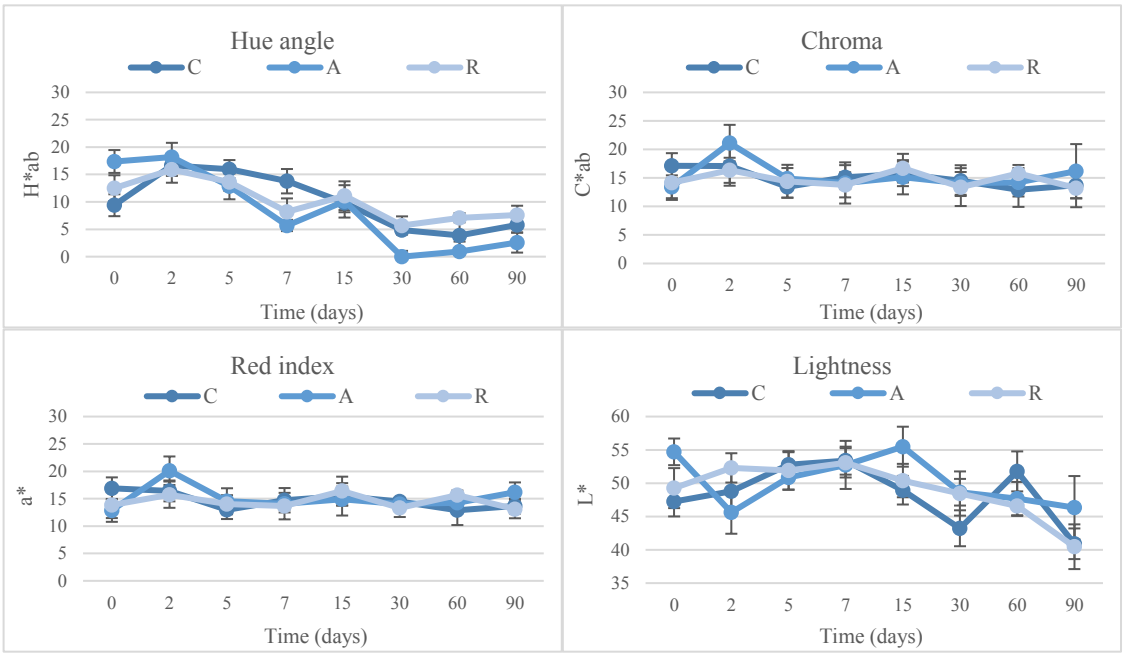

Figure 15 Colour change in samples during ripening.

Presence of *L. casei* N87 in samples

DGGE was performed in order to investigate the presence of the strain during the ripening. The band of *L. casei* N87 was identified by comparing its relative position of migration in the gels with the DGGE profile in the ladder. In Figure 16 is possible to see an example of the DGGE profile. In sample from fermentation C, the band corresponding to *L. casei* was never present. On the other hand, the strain was found in all the A samples and band was visible up to a dilution of 10^{-6}. Also the cells of *L. casei* N87 adapted under respiratory condition were found at every time of sampling. On the other hand, band presence in R samples has been identified at dilution 10^{-6} at the beginning of the production, by day 7 there was a decrease (10^{-4}), which fall further at 15 days of ripening (dilution 10^{-3}), after that was stable until the end of the seasoning. *L. casei* strains inoculated in fermented sausages were investigated by several authors using molecular techniques (RAPD – PCR or DGGE), their findings showed the capabilities of this species to survive until the end of ripening (Sidira et al., 2014; Rubio et al., 2014; Bagdatli and Kundakci, 2016). However, other authors found *L. casei* or *L. paracasei* strains in natural fermented salami up to 40 days of casing (Coppola et al., 2000; Andrighetto et al., 2001; Pisacane et al., 2015).

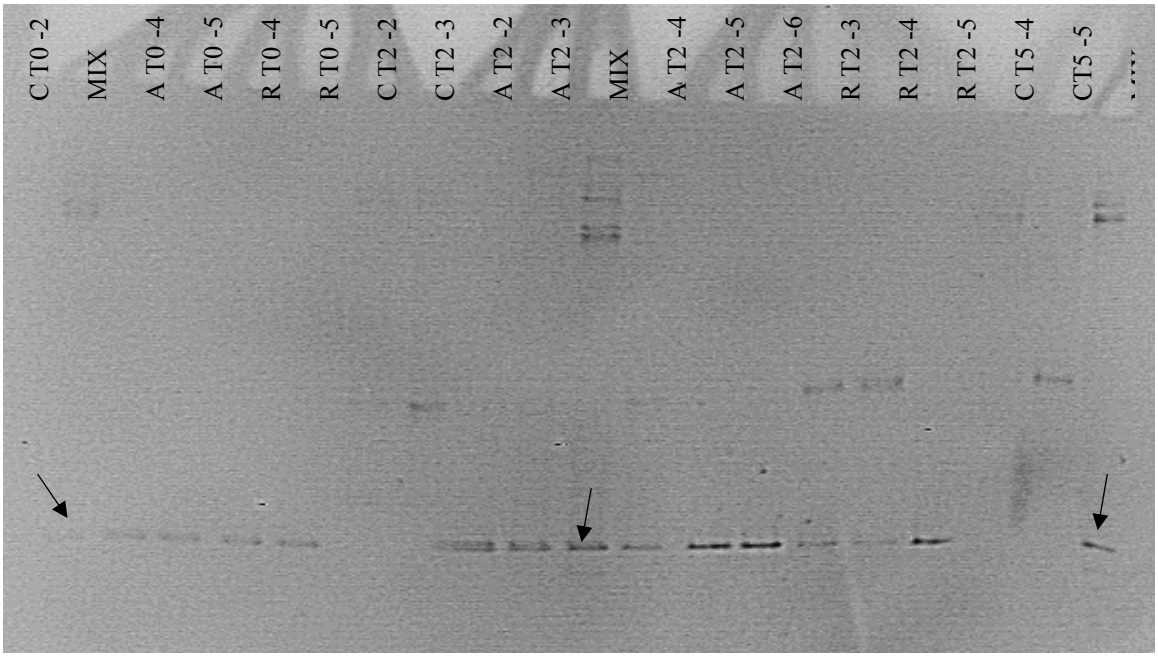

Figure 16 Example of DGGE profiles, obtained by the DNA extracted from patinas of MRS petri dishes at different time of sampling. Arrows indicates *L. casei* N87 amplicons in identification ladder.

Total, Viable and VBNC cells

Results showed in Figure 17A describe the presence of viable and total cells belonging to the *L. casei* group. In control samples, the viable cells remain between 2.5 – 6 Log CFU/ml during the ripening. It has to be underlined that using this technique all the three species of the *L. casei* group can be detected. For this reason the concentration obtained can probably be attributed to *L. rhamnosus* or *L. paracasei,* remembering that using DGGE analysis *L. casei* was never detected. On the other hand A and R samples at time zero showed 6 Log CFU/ml achieved through the inoculum. During the ripening the total count (about 2-Log CFU/ml above viable cells, data not show), detecting dead or VBNC cells.

L. casei group viable cells had high load in inoculated sample, while in control samples the amount was variable (Figure 17A). Considering the presence of viable cells of this group is important to understand if they were the main LAB group in the samples. In Figure 17B is possible to see that the principal LAB group in control samples was not the *L. casei* group. On the other hand, in A and R the cells detected by PMA – qPCR and the number of colonies counted on MRS plates were statistically similar (Figure 17C, D). The non-linear trend of analysed amples may be caused to the fact that salami examined at each sampling were not the same.

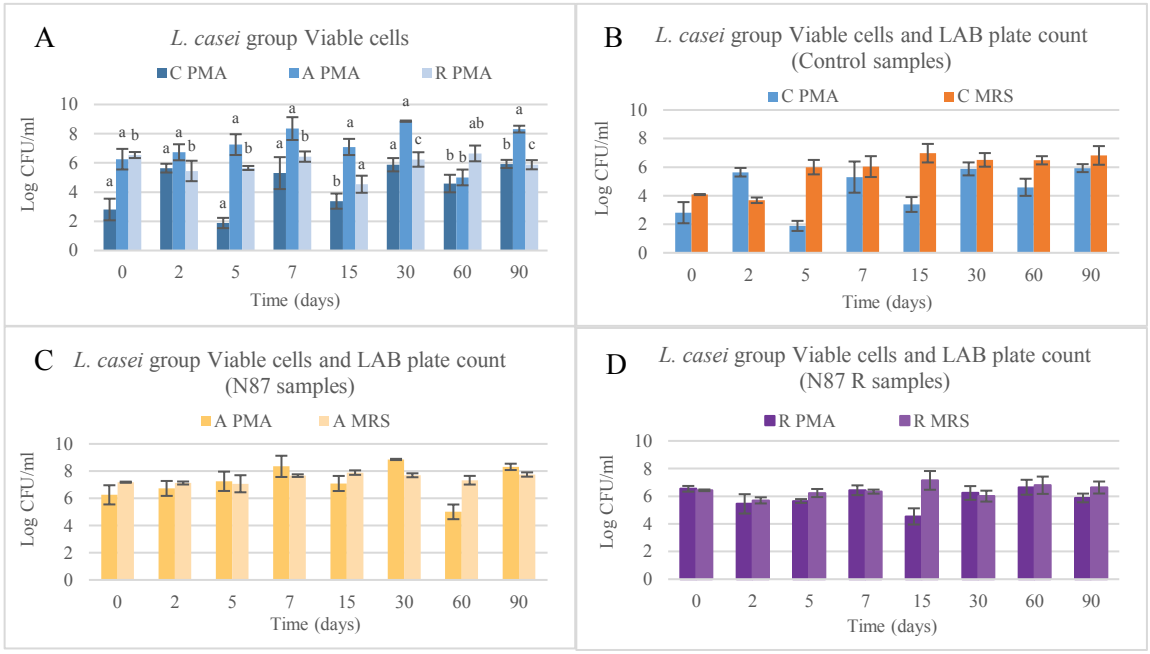

Figure 17 A, B, C, D Comparison of *L. casei* group viable cells and MRS plate count in all the samples during ripening. A *L. casei* group viable cells in all the samples; B Control samples; C N87 samples; D N87 R samples.

The VBNC state in bacteria is relevant in food safety. Many detection systems have been used, like flow cytometry, fluorescent staining, RT – qPCR, cDNA mycroarrays with RT – qPCR and DNA hybridization (Nicolò and Guglielmino, 2012; Li et al., 2014; Sohier et al., 2014). More than 60 genera and 100 species have been recognized as capable to enter VBNC state (Su et al., 2013; Pinto et al., 2015; Oliver, 2016; Ayrapetyan and Oliver, 2016), but a few studies were carried out about LAB and *Bifidobacterium* (Millet and Lonvaud-Funel, 2000; Oliver, 2005; Suzuki et al., 2006; Lahtinen et al., 2008), but strains of the *L. casei* group were not investigated.

Regarding the results obtained in this studies is not possible to presume that the *L. casei* N87 cells could enter in the VBNC state in a rich substrates as fermented sausages, because the major cause of the induction of this state is starvation (Fakruddin et al., 2013; Pinto et al., 2015).

Antioxidant abilities and volatiles profiles

Volatile compounds of raw material and final products were reported in Table 13. Terpenes and terpenoids detected and deriving form pepper and garlic were not considered (Meynier et al., 1999; Sidira et al., 2015; Montanari et al., 2016). Forty-eight compounds were identified, in particular 13 aldehydes, 3 ketons, 9 esters, 8 acids, 12 alcohols, 3 hydrocarbons.

Alcohols were the most represented group, mainly ethanol, also (E) – 2 –hexen – 1- ol and 1-hexanol were in the raw material, while ethanol, 1- octen – 3 – ol and phenylethyl alcohol in the samples at the end of ripening. Alcohol biosynthesis could be carried out by many metabolic pathways, that occur in fermented meat, such as lipid oxidation, amino acid metabolism and methyl ketone reduction (Sidira et al., 2015). Ethanol is a product of carbohydrate metabolism, however the adjunction of wine (0.8% v/w) during the manufacture could affected the amount detected. 1 – octen 3 – ol is produced by bacterial metabolism (lipid β – oxidation) (Marco et al., 2006) and imparts mushroom flavour (Tabanelli et al., 2012). As found by Gardini et al., (2013) phenylethyl alcohol decreased during ripening.

The second group occurred in samples was aldehydes, saturated and unsaturated produced during lipids auto – oxidation (Marco et al., 2006; Bianchi et al., 2007), especially hexanal, octanal and nonanal are considered as marker of the oxidation process (Procida et al., 1999; Bianchi et al., 2007; Tabanelli et al., 2013; Sidira et al., 2015). In samples, hexanal was the most abundant conferring an odour of green leaves (Meynier et al., 1999; Di Cagno et al., 2008), but there were not significant differences between the analysed fermented sausages. Among the identified compounds, benzene-acetaldehyde derived from phenylalanine metabolism and imparts a floral aroma (Tabanelli et al., 2012).

Using aldehydes (hexanal, octanal and nonanal) as marker for the oxidation of the final product, is possible to see that the control and R samples were less oxidize in respect of A. Similar findings were obtained by Reale et al., (2016b). In their study cheddar cheese was inoculated with *L. casei* N87 after adaptation under anaerobiosis and respiration conditions, observing a lower oxidation of lipids in samples inoculated with the cells growth under respiratory condition.

Other compounds present in abundance were hydrocarbons, these compounds were found by other authors (Meynier et al., 1999; Tabanelli et al., 2012), but they does not contribute significantly to the odour profile due to their high odour thresholds (Marco et al., 2006).

Mainly ethyl ester were identified, they are often present in fermented sausages or salami, providing a fruity note to the products and a very low detection outset (Demeyer et al., 2000; Marco et al., 2006). However, they could be formed by alcohol oxidation or esterification of acids and alcohol (Meynier et al., 1999; Bianchi et al., 2007). Nine esters were determined, among them ethyl acetate is formed by *Staphylococci* esterase activity (Marco et al., 2006).

The two groups with less abundance were acids and ketones. Acids are not responsible for sensorial enhancements of the products, while they can be precursor of compounds altering aroma and texture (Sidira et al., 2015). As reported by Ansorena et al., (2000), the lipolytic activity of micrococci attack long chain fatty acids, producing these compounds. Acetic acid is a fermentation product (Sidira et al., 2015).

The ketones group is composed by acetone, 2,3 – octanedione and 2 – nonanone, all found in samples at the end of casing. Diketones (2,3 – octanedione) were produced during Maillard reaction (Bianchi et al., 2007) and are characterized by a butter-creamy flavour. Acetone was the most abundant, it has fermentative origins (Procida et al., 1999) but the involved pathway is not clear (Tabanelli et al., 2012) .2 – nonanone is produced by bacterial metabolism through

lipid β – oxidation (Marco et al., 2006), but it was identified only in control samples at the end of ripening.

Table 13 Volatile compounds identified in samples, expressed in ppm (mg/kg), grouped in chemical families (aldehydes, ketons, esters, acids, alcohols, hydrocarbons). CT0 = meat mixture; CT90 = control sample; AT90 = salami inoculated with *L. casei* N87 adapted in anaerobiosis condition; AT90 = salami inoculated with *L. casei* N87 adapted in respiratory condition.

Compound	CT0	CT90	AT90	RT90
Aldehydes				
Hexanal	0,084	0,225	0,267	0,225
(E)-2-hexenal	0,011	0,010	n.d.	n.d.
Octanal	n.d.	n.d.	n.d.	0,025
(z)-2-heptenal	n.d.	0,024	0,054	0,026
Nonanal	0,066	0,045	0,048	0,033
(E)-2-octenal	n.d.	0,020	0,020	0,014
(E)-2-Nonenal	n.d.	n.d.	0,029	0,019
(E)-2-decenal	0,018	0,010	0,024	0,013
Benzenacetaldehyde	n.d.	0,056	n.d.	0,023
2,4-Nonadienal	n.d.	n.d.	0,009	0,004
2-undecenal	n.d.	0,008	0,008	0,005
(E,E)- 2,4-decadienal	0,010	n.d.	n.d.	n.d.
Octadecanal	n.d.	0,010	0,010	0,010
TOTAL ALDEHYDES	**0,189**	**0,407**	**0,470**	**0,397**
Ketones				
Acetone	n.d.	0,048	0,020	0,052
2,3- Octanedione	n.d.	0,023	0,036	0,038
2-Nonanone	n.d.	0,004	n.d.	n.d.
TOTAL KETONS	**0,000**	**0,075**	**0,056**	**0,091**
Esters				
Ethyl acetate	n.d.	0,024	0,034	0,020
Ethyl ester hexanoic acid	n.d.	0,018	0,020	0,028
Ethyl ester-2-hydroxy-propanoic acid	n.d.	0,003	0,015	0,002
ethyl ester octanoic acid	n.d.	0,024	0,018	0,022
ethyl ester decanoic acid	n.d.	0,029	0,032	0,036
ethyl ester dodecanoic acid	n.d.	n.d.	n.d.	0,003
methyl ester 10-octadecenoic acid	0,023	n.d.	n.d.	n.d.
ethyl ester hexadecanoic acid	n.d.	n.d.	n.d.	0,003
2-octyl-cyclopropanetetradecanoic acid methyl ester	0,005	n.d.	n.d.	n.d.
TOTAL ESTERS	**0,028**	**0,098**	**0,119**	**0,113**
Acids				
Acetic acid	0,580	0,225	0,319	0,139
Butanoic acid	n.d.	0,036	0,034	0,026
3-methyl-butanoic-acid	0,017	0,059	0,022	0,013
Hexanoic acid	0,023	0,043	0,056	0,031
2-hexanoic acid	0,029	n.d.	n.d.	n.d.

Compound	CT0	CT90	AT90	RT90
Octanoic acid	n.d.	0,040	0,037	0,030
Nonanoic acid	0,045	0,043	0,025	0,021
n-decanoic acid	n.d.	0,028	0,034	0,021
TOTAL ACIDS	**0,045**	**0,071**	**0,058**	**0,042**
Alcohols				
Ethanol	0,377	0,402	0,266	0,403
1-pentanol	n.d.	0,011	0,017	0,015
2-hexanol	n.d.	0,053	0,061	n.d.
1-butoxy-2-propanol	n.d.	0,019	0,028	0,021
1-hexanol	0,118	0,006	0,024	0,008
(Z)-3-hexen-1-ol	0,047	0,004	n.d.	n.d.
(E)-2-hexen-1-ol	0,163	n.d.	n.d.	n.d.
1-octen-3-ol	n.d.	0,033	0,043	0,029
2,3-butanediol	n.d.	0,021	n.d.	0,002
3-methyl-2-butanol	n.d.	0,033	0,012	0,008
Phenylethyl alcohol	0,043	0,021	0,013	0,016
TOTAL ALCOHOLS	**0,748**	**0,603**	**0,464**	**0,502**
Hydrocarbons				
3-ethyl-1,5-octadiene (?)	0,019	n.d.	n.d.	n.d.
1,3,3-trimethyl-tricyclo(2,2,1,0(2,6))heptane	0,136	0,274	0,239	0,167
(E)-2,7-dimethyl-3-octen-5-yne	0,040	0,024	0,014	0,000
TOTAL HYDROCARBONS	**0,196**	**0,298**	**0,253**	**0,167**

n.d. not detectable

To better demonstrate the connection between the aroma profile and the inocula, two principal component analysis (PCA) were performed using the concentration of the volatiles reported in Table 13. Figure 18A shows the distance among control and inoculated samples, while Figure 18B reports the coordinates of the variable factor. Meat mixture (CT0) is positioned in the sector characterised by 4 alcohol (phenyethyl alcohol, (Z)- 3 – hexen – 1 – ol; 1 – hexanol and (E)- 2 – hexen – 1 –ol), 2 acids (2 – hexanoic acid and acetic acid) and 2 aldehydes (nonanal and (E;E)- 2,4 – decadienal). Control samples after 90 days of ripening is in another section, divided from CT0 and the inoculated samples. Five molecules outline CT90, 1 ketone (2-nonanone), 1 aldehyde (benzenacetaldehyde) and 2 alcohols (3 – methyl – 2 – butanol and 2,3 – butanediol). However, in this sample the alcohols were the main representative compounds. Also considering the molecules present between CT0 and CT90 samples is noticeable the presence of ethanol, that characterize both the samples. As said before the addition of wine in the mixture affected the presence of alcohols in samples. AT90 and RT90 are clustering together, classified in relation to 3 aldehydes (2,4 – nonadienal, (E)- 2 – nonenal and octanal) and 3 esters (ethyl ester-2-hydroxy-propanoic acid; ethyl ester dodecanoic acid and ethyl ester hexadecanoic acid). All the other compounds are located among samples at the end of ripening.

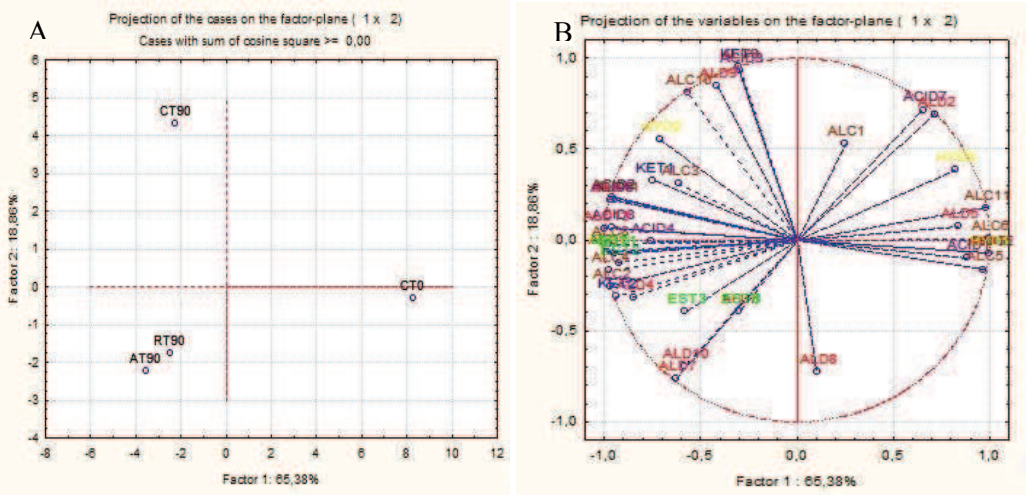

Figure 18 PCA plots. A, Loading plots on the two first factors of the analysed samples. B Loading plot of the aroma compounds on the first two factors obtained from the PCA.

ALD1 = Hexanal; ALD2 = (E)-2-hexenal; ALD3 = Octanal; ALD4 = (z)-2-heptenal; ALD5 = Nonanal; ALD6 = (E)-2-octenal; ALD7 = (E)-2-Nonenal; ALD8 = (E)-2-decenal; ALD9 = Benzenacetaldehyde ; ALD10 = 2,4-Nonadienal; ALD11 = 2-undecenal; ALD12 = (E,E)- 2,4-decadienal; ALD13 = Octadecanal; KET1 = Acetone; KET2 = 2,3- Octanedione; KET3 = 2-Nonanone; EST1 = Ethyl acetate; EST2 = Ethyl ester hexanoic acid; EST3 = Ethyl ester-2-hydroxy-propanoic acid; EST4 = ethyl ester octanoic acid; EST5 = ethyl ester decanoic acid; EST6 = ethyl ester dodecanoic acid; EST7 = methyl ester 10-octadecenoic acid; EST8 = ethyl ester hexadecanoic acid; EST9 = 2-octyl-cyclopropanetetradecanoic acid methyl ester; ACID1 = Acetic acid; ACID2 = Butanoic acid; ACID3 = 3-methyl-butanoic-acid; ACID4 = Hexanoic acid; ACID5 = 2-hexanoic acid; ACID6 = Octanoic acid; ACID7 = Nonanoic acid; ACID8 = n-decanoic acid; ALC1 = Ethanol; ALC2 = 1-pentanol; ALC3 = 2-hexanol; ALC4 = 1-butoxy-2-propanol; ALC5 = 1-hexanol; ALC6 = (Z)-3-hexen-1-ol; ALC7 = (E)-2-hexen-1-ol; ALC8 = 1-octen-3-ol; ALC9 = 2,3-butanediol; ALC10 = 3-methyl-2-butanol; ALC11 = Phenylethyl alcohol; HYD1 = 3-ethyl-1,5-octadiene; HYD2 = 1,3,3-trimethyl-tricyclo(2,2,1,0(2,6))heptane; HYD3 = (E)-2,7-dimethyl-3-octen-5-yne.

Another PCA analysis was performed considering the total amount of each chemical class. The distinction among meat mixture and samples at the end of casing, as is possible to see in Figure 19AB, is still clear and characterized by the alcohols present in the CT0 samples.

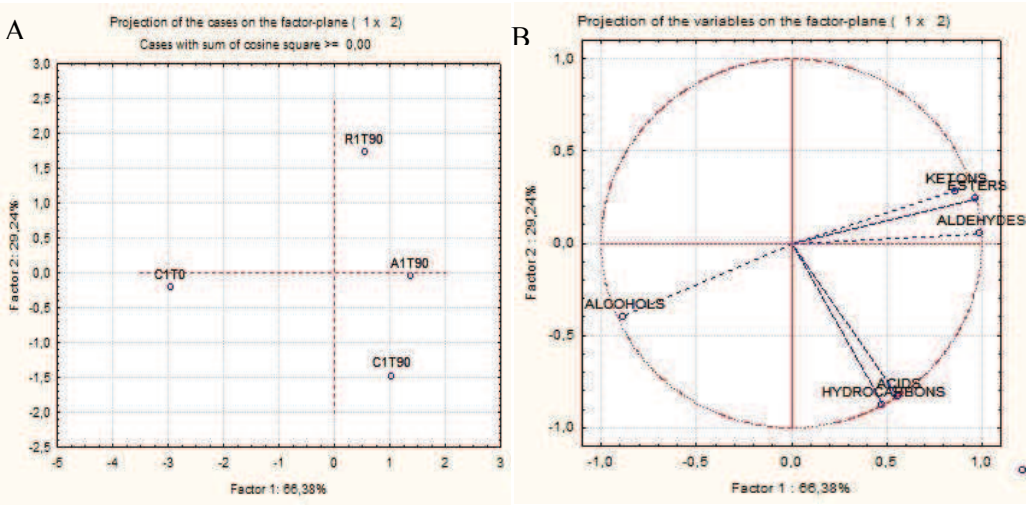

Figure 19 PCA plots. A, Loading plots on the two first factors of the analysed samples. B Loading plot of the total groups compounds selected on the first two factors obtained from the PCA

Proteolysis assessment

During meat fermentation the main enzymes involved in the initial breakdown of sarcoplasmic and myofibrillar proteins were muscle proteinase (Fadda et al., 2010). However, LAB also occur in this process. Their proteolytic activity system, composed by various intracellular peptidases and cell-wall-bound proteinase, is complex (Savijoki et al., 2006). In dry fermented sausages *Lactobacillus* enzyme seems to be responsible for the production of amino acid and small peptides, which provide as precursor of flavour molecules (Fadda et al., 1999; Sanz et al., 1999a,b).

The SDS – page, performed during the fermentation showed changing in the proteolytic profile. Presence of bands higher than 140 kDa was detected in meat mixture, while lower bands were found during all the period of ripening. Bands lower than 18 kDa were discovered in C and A samples, except for 15 and 13 kDa bands that were detected only in A samples.

In the heatmap (Figure 20) is possible to see 7 main cluster, which group together or the three kind of samples or the bands. Inside cluster III is possible to differentiate two other classifications, samples from 0 to 7 and from 15 to 90 days of ripening. Samples C and R had a similar proteolytic profile. A lot, due to the effects of lower pH on the proteolytic characteristic (Candogan et al., 2009), differs from the other. This also for the presence of bands with a low molecular weight.

However, similar results were found in cluster I, except for samples at 2 and 15 days of casing that clustering in a different way. Clusters concerning bands were 4, including heterogeneous molecular weight bands.

After 15 days in all the samples the band at 146 kDa disappeared, several author reported a lower presence of myosin heavy chain concentration during fermented sausage ripening (Casaburi et al., 2008; Fadda et al., 2002; Spaziani et al., 2009) Cathepsin D, aspartic and cysteine proteinases derived from meat, were considered as the main involved enzymes in the

production of small fragments of myofibrillar proteins (122, 38, 29, 13 kDa) (Demeyer et al., 2000; Sanz et al., 1999). In agreement with the study of Fadda et al., 2010, the 45 kDa band (probably actin) were detected in all the samples until the end of ripening. Also bands with a molecular weight lower than 24 kDa were found, they are presumably myosin light chain (Hughes et al., 2002; Fadda et al., 2010). At the end of the ripening 17 kDa band (myoglobin), with the exclusion of C samples, was completely degraded. Other bands were detected indiscriminately during the 90 days.

Therefore, the proteolytic activity of LAB on meat proteins had been described by several authors with *in vitro* and *in vivo* studies (Demeyer et al., 2000; Fadda et al., 1999; 1998; 2001; 2002, 2010; Hierro et al., 1999; Hughes et al., 2002; Sanz et al., 1999a/b;Spaziani et al., 2009; Aro Aro et al., 2010). These studies suggested that the microbial enzyme are more involved in the latter stages of ripening, since the initial breakdown of the muscle proteins is due to meat proteinases. On the other hand, mould and catalase positive and negative cocci could be implicated in protein degradation (Lucke, 1997; Rouhi et al., 2013).

Acids, alcohols and aldehydes developed form the degradation of several amino acids (methionine, leucine, valine, phenylalanine) have a very low threshold. Amino acids conversion in volatile compounds is important to induce a characteristic flavour in salami or fermented sausage (Rouhi et al., 2013).

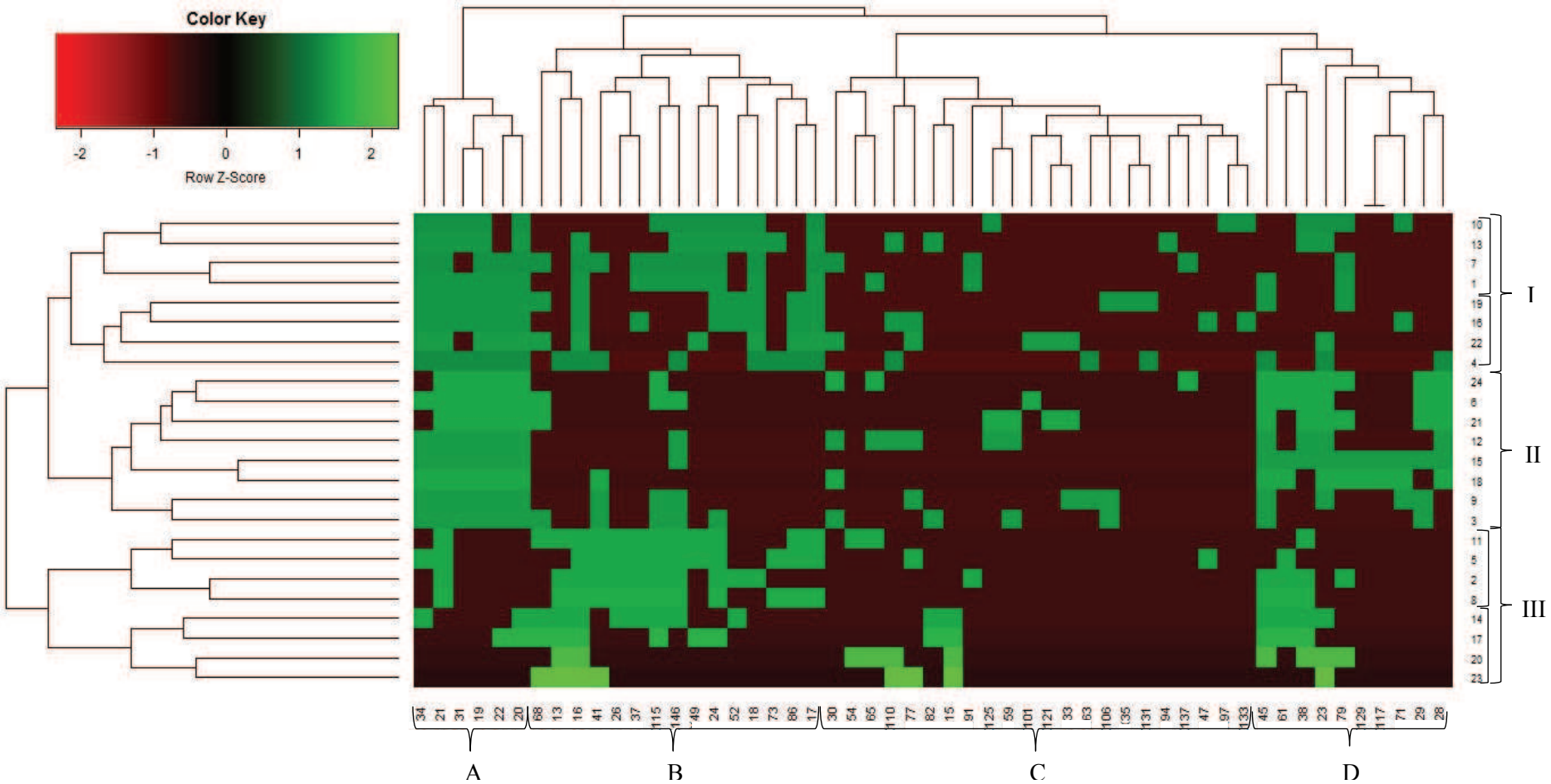

Figure 20 Heatmap of bands molecular weight and samples.

Legends samples: 1 = CT0; 2 = AT0, 3 = RT0; 4 = CT2; 5 = AT2; 6 = RT2; 7 = CT5; 8=AT5; 9 = RT5; 10 = CT7; 11 = AT7; 12 = RT7; 13 = CT15; 14 = AT15; 15 = RT15; 16 = CT30; 17 = AT30; 18 = RT30; 19 = CT60; 20 = AT60; 21 = RT60; 22 = CT90; 23 = AT90; 24 =RT90.

CONCLUSIONS

Despite the performed inocula, the LAB count in control samples reached the same Log CFU/ml at the end of ripening. Regarding physical/chemical parameters, the a_w did not show differences among samples, on the other hand pH was lower in A (5.2) samples and the weight loss in R samples (2.5% less compared to other).

Detection of *L. casei* N87 in samples were performed using DGGE. As expected the presence was not detected in control samples, while in A and R samples was found at 6 and 3 Log CFU/ml at the end of ripening.

As reported before, the PMA – qPCR performed was able to detect the viable cells of *L. casei* group. A and R salami showed a stable load of 8 and 6 Log CFU/ml respectively, whereas C samples rise from 2.5 to 6 Log CFU/ml. The comparison of viable cells with MRS plate count reported that the majority of LAB is composed by *L. casei* group in A and R salami, however the control samples showed an increase of this group during ripening. This result can be compared with findings obtained with DGGE, where the viable and colturable cells of *L. casei* N87 were detected. Probably the viable count of R samples obtained through PMA – qPCR is due to the natural increase of this group, as found in C samples.

The proteolysis occurred during the ripening indicate a differentiation of A salami from the other. Despite this, the volatile profiles of the inoculated salami is similar, although volatiles molecules developed form the degradation of several amino acids.

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Conclusion

Food processing lead the creation of several adverse condition for microbial growth and survival, such as extreme temperature, acid, oxygen, and osmotic stresses. An ideal LAB strain with industrial potential should resist to these difficult conditions. Response and defense mechanisms in LAB have been extensively studied during the past years (van de Guchte et al., 2002). Genomic investigation is a valid method to understand the molecular mechanism of stress responses (Zhu et al., 2009). Genotypic, phenotypic and physiological response of strains can perhaps result in design strategies to improve the robustness of LAB for application purpose.

The taxonomy of species belonging to *L. casei* group, especially *L: casei* and *L. paracasei* species, is strict related. However, this group are able to colonize a large variety of niches and fermented foods and several strains of this group had found an application in industrial or production as starter cultures or probiotics (Ortigosa et al., 2006; Ammor et al, 2007; Verdenelli et al., 2009; Bedia et al., 2011; De Angelis and Gobbetti 2011,). As said before, strain-specific properties knowledge open new possibilities of application.

Due to their capability to growth and colonize several niches, the results of strains isolation from Montasio cheese are not surprising. Mainly *L. paracasei* and a few strains of *L. rhamnosus* were found in this environment. On the other hand, most of the isolated strains showed the same fingerprints, for this reason strains from other collection were added to the study.

During the evaluation of the safety traits and the technological properties strains showed a high variability. Furthermore, some strains (*L. rhamnosus* PRA331; *L. casei* N2014; *L. paracasei* B169; *L. paracasei* B195; *L. rhamnosus* N812 and *L. rhamnosus* L47) showed particular capabilities or presence of gene related to BAs or ethyl carbamate production, that confirms that often abilities are strain-specific.

Also, new possibilities of use of these strains can be related to the capability of this group strains to growth under aerobic and respiratory promoting condition (Ianniello et al., 2015; Zotta et al., 2014). Several authors find changing in metabolic profiles and physiological response of respirative strains growth in aerobic/respiration circumstances (Pedersen et al., 2008, Ianniello et al., 2015, Ricciardi et al., 2015). Effects of this condition in *L. casei* group strains inoculated in fermented foods were investigated in few studies (Reale et al., 2016a/b).

The investigation conducted in this project proved that the respiratory condition of growth can change the physiological response of the cells without effect on the fermentation capability of the selected strains (*L. casei* N87 and *L. paracasei* V3) in milk. On the other hand, changing in antibiotics resistance, hemolytic activity and competition with pathogens were evident compare to the non-stressed cells. Proteolysis and peptides production were not affected by the promoting condition of growth.

Furthermore, *L. casei* N87 was inoculated in salami. During the ripening, the a_w did not show differences among samples (control and salami inoculated with the strain grow under anaerobiosis or respiratory condition), on the other hand pH was lower in samples inoculated with the strain grow in anaerobiosis, while weight loss in samples manufactured with the strain growth under respiratory condition were lower compared to other. Also, the detection of the strain showed that the inoculated cells were able to survive until 90 days of casing, with 3 Log CFU/g of difference among cells grow under anaerobiosis and the ones in respiration condition. Differences were obtained also in the detection of the viable cells of *L. casei* group using PMA – qPCR protocol. Comparing the results gather with the methodologies adopted during the study, it is possible to assume that in inoculated samples the *L. casei* group was the major LAB group, while in control samples this group increase naturally during ripening. Likewise, proteolysis occurred during the ripening indicate a differentiation of salami inoculated with the cells growth under anaerobiosis condition from the other. Despite this, the volatile profiles of the inoculated salami is near to each other, although volatiles molecules developed form the degradation of several amino acids.

Stress applied during foods manufacturing can affect the response of the selected strains, as well as the microflora that naturally occur in raw materials. The application of different stress (heat, cold or osmotic) to pre-adapt strains were used by several authors (Desmond et al., 2001; Lorca and de Valdez, 2001; Derzelle et al., 2003; Sanz, 2007; Mathipa and Thantsha, 2015). However, as said before few studies were made about the physiology and the use in food matrices of strains of *L. casei* group grow under respiratory condition. The results obtained demonstrate a variation in cells physiology, including safety traits (antibiotic resistance and hemolysis). A deeper knowledge is necessary to better understand the mechanism beyond these changing, the possibly application in foods processes and the resulting safety, sensory and quality properties of the final product.

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