Lactobacillus casei group: identification, characterization and genetic evaluation of the stress response

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I declare that my PhD thesis has been amended to address all the Referee's comments.

To my parents

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Summary

Lactic acid bacteria (LAB) are an heterogeneous group of microorganisms used as starter and/or adjunctive cultures in the production of fermented foods to improve the shelf-life, the organoleptic properties and promote health. Many of these species, in fact, have been extensively characterized as probiotics several studies and clinical tests to substantiate health claims are available for several of them. During use, formulation and preservation as starter, adjunctive and/or probiotic cultures LAB are subjected to physical and chemical stresses that can influence growth, cell viability and fermentation capabilities, changing the technological fitness of the strains.
This thesis focused on *Lactobacillus casei*, *L. rhamnosus* and *L. paracasei* with the aim to encrease the knowledge on this strains. These microbial species are involved in the fermentation of various food matrices (meat, vegetable and dairy products) and they could be used as probiotics or for the development of new functional products, as many studies reported the presence of several strains belonging to these species with probiotic features.
Considering the hardness existing in the correct identification of these strains, so genetically close to each other, a wide collection of strains isolated from several sources and previously identified as belonging to *L. casei* group was collected (201 total strains), and a species-specific PCR, multiplex PCR, group-specific PCR and HRM analyses have been performed or developed to identify *L. casei*, *L. rhamnosus* and *L. paracasei* strains. Almost all the techniques resulted effective for the identification, but the group-specific PCR had to be applied as the first step of identification. Anyway, the attribution to one of the species of the group remained uncertain for some strains.
Therefore, all the strains were characterized using three different assays (Rep-PCR, RAPD-PCR and Sau-PCR), in order to have a complete point of view of their genotype. As a result, a high variability was found among strains with all the three performed assays, confirming the presence of a high number of genotipes.
Therafter 45 strains were selected for the development of an MLST scheme based on the analysis of internal fragments (*loci*) of stress related genes. The results confirmed the presence of a high variability among strains and it was not surprising to found strains with different STs. Considering that the analysis was performed on 45 strains, and the high number of STs that has been found, it would be interesting to charachterize other strains, in order to find possible correlation between the source of isolation or a specific stress response behaviour. Four-teen strains were selected for the whole genome sequencing and core-genome and pan-genome comparisons were performed, obtaining a clear distinction among *L. paracasei* and *L. rhamnosus* strains. Nonetheless, a *L. paracasei* and two *L. casei* strains grouped in *L. rhamnosus* cluster,

confirming how hard is the discrimination among these species, because of their high level of similarity.
SAPD-PCR analysis were also performed, confirming a high genomic variability among strains.
The same strains where then subjected to the evaluation of their mucus binding capabilities. The binding assays were performed *in vitro* and the strains were tested before and after oxidative stress exposure. The behaviour of the microorganisms analysed resulted strain-dependent and analysis allowed the selection of few strains with interesting binding properties, that will be further analysed for their probiotic characteristics before to do *in vivo* analysis and to understand their adhesion mechanisms.
At least, a strain of *L. paracasei* was selected for its ability to conduct malolactic fermentation under different EtOH and pH conditions studing how the expression of stress related genes was affected in the different tested conditions. The strain resulted able to performe MLF under all the tested conditions, and as expected, the lower concentration of glucose, corresponding to the lower final concentration of alcohol, demonstrated to be less stressful. Considering that the strain was able to conduct MLF also when in the presence of a concentration of glucose, corresponding to 15% v/v of ethanol, it can be considered as a potential good MLF starter culture. Another topic for the future research could be the evaluation of the stress response of this strain during MLF when adapted in a respiratory envinronment before the inoculation.

1. Stress response in *Lactobacillus casei* group

1.1 Introduction

Lactic acid bacteria (LAB) are among the most important bacteria for the food industry because of their essential role in food and beverage fermentations, as well as in promoting human health and have therefore been the focus of scientific studies in recent years (Matsuzaki et al., 2007). These microorganisms are characteristic of many habitats: the gastro-intestinal tracts of various animals and humans; milk and dairy products; poultry; fish products; fermented products; and the surfaces of certain plants and fruits. Moreover, LAB are widely used in the production and preservation of food products such as cheese, meat and yogurt (Ringø and Gatesoupe, 1998; Konings et al., 2000; Solieri et al., 2012). Their important impact on fermented foods and intestinal microflora is due to their antagonistic activity against potential pathogens (Klein et al., 1995; de Vrese and Marteau, 2007).

The *genus Lactobacillus* has been extensively studied because of several factors: the importance of these microorganisms in human health; their use in improving the quality or health aspects of many foods; and queries by legislative bodies, industry and consumers about safety, labelling, patents and strain integrity (Shu et al., 1999; Holzapfel and Schillinger, 2002; Singh et al., 2009, Doherty et al., 2010). *Lactobacillus* spp. includes the *L. casei* group, which consists of *L. casei*, *L. paracasei, L. rhamnosus* and *L. zeae*. These species are widely used in various commercial and traditional fermented foods. These species are genetically closely related to each other (Holzapfel and Schillinger, 2002; Ong et al., 2007; Sakai et al., 2010) and, because of their use as microbial starters or probiotics, stress response of these microorganisms is the topic of a huge number of scientific papers describing their behaviours under osmotic stress, heat and cold shock, starvation, acidic and oxidative stress.

1.2 *Lactobacillus casei* group

The *genus Lactobacillus* is one of the largest of the Lactic Acid Bacteria (LAB), counting 212 species (http://www.bacterio.net/lactobacillus.html).

For several years, the taxonomy of LAB has been based on the phenotypic properties of the strains (Klein et al., 1998). Actually LAB are divided, on the basis of the type of fermentation, into three groups. The first one consists of obligate homofermentative microorganisms fermenting hexoses via glycolysis

with mainly production of lactic acid and unable to ferment pentose and gluconate. The second group includes obligate heterofermentative bacteria, fermenting hexoses via 6 - phospho - gluconate / phosphoketolase (6PG / PK) with ethanol production (acetic acid), carbon dioxide and lactic acid. The third group consists in facultative heterofermentative microorganisms (fermenting hexoses through the route of the Embden - Meyerhof or glycolysis and pentose with ethanol production (acetic acid), carbon dioxide and lactic acid via 6PG / PK) (Hammes and Vogel, 1995). In the last decades, the availability of novel techniques of molecular biology caused several changes and disputes in the taxonomy of lactobacilli, since the availability of 16S rRNA gene sequence allowed the first phylogenetic analysis of the *genus* (Singh et al., 2009; Salvetti et al., 2012). *Lactobacillus casei* group, at the beginning, consisted in a single species (*casei*) with five subspecies: *casei*, *galactosus*, *pseudoplantarum*, *tolerans* and *rhamnosus* (Singh et al., 2009). Collins et al. (1989) proposed a classification of the strains into three species. The first one was *L. casei* (including strains previously belonging to the *L. casei* subsp. *casei*). The second one consisted in *L. 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*) and at least *L. rhamnosus* (including the previously classified as *L. casei* subsp. *rhamnosus*).
Discrimination among the species of this group is not always easy, as revealed from several authors (Felis and Dellaglio 2007; Singh et al., 2009; Salvetti et al. 2012) because several strains can have the same phenotypical response, but different genotype. There are several cases of taxonomic disputes due to the presence of slight differences at nucleotide level in 16S rRNA gene creating ambiguity, not only among species belonging to *L. casei* group, but also in other groups such as *L. acidophilus*, *L. plantarum*, and *L. delbruekii* (Shing et al 2009; Salvetti et al., 2012). As example, the case of the type strain *L. casei* ATCC 393 that from a genotypic point of view is more similar to *L. zeae* than other *L. casei* species. Regarding this strain, in 2008 *L. zeae* has been reclassified as *L. casei* from the Judical commission of the International Committee on Systematics of Bacteria. Recent work of Salvetti et al. (2012) gave a taxonomic update of the *genus Lactobacillus* based on the comparison of 16S rRNA sequences, confirming that many species of *L. casei* group share a 16S rRNA sequence identity higher than 98.8%.

1.3 Identification of species belonging to *L. casei* group

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; these techniques include the study of the fermentative pathways, assays on carbohydrates, lactic acid configuration or peptidoglycan analysis.

A preliminary identification throught the use of biochemical and phenotypical test can be applied. *L. casei* strains are known for their inability to ferment lactose and sucrose and their capability to grow at 10 °C but not at 45 °C. Strains belonging to *L. paracasei* subsp. *paracasei* can ferment lactose and sucrose, but not rhamnose and, as *L. casei*, they are able to grow at 10 °C but not at 45 °C. A few strain produce racemic lactic acid, due to the activity of L(+) and D(-) lactate dehydrogenases. *L. paracasei* subsp. *tolerans* is capable to survive at 72 °C for 40 s, but it has a different fermentation profile compared with the other *L. casei* group members. Finally, *L. rhamnosus* is distinguishable from the other species members of the group because of its capability to ferment rhamnose and its ability to grow at 45 °C (Holzapfel and Wood, 2014). However, because of the strong similarities among species, such analyses can often give ambiguous results (Richiard et al., 2001; Dubernet et al., 2002; Huang et al., 2011). For this reason, several methods have been proposed for the discrimination among species belonging to *L. casei* group and, the improvement of molecular assays increased the quality and the efficiency of the identification (Bernardeau et al., 2008). Ward and Timmins (1999), as an example, proposed three couples of polymerase chain reaction (PCR) primers, which were specific for each species based on differences in the V1 region of the 16S rRNA gene obtaining a good discrimination among species. Walter et al. (2000) identified several *Lactobacillus* strains isolate from human and porcine gastrointestinal samples to the species level by using Denaturing gradient gel electrophoresis (DGGE) and species-specific PCR primers having as target the 16S-23S rRNA intergenic spacer region or 16S rRNA gene sequences. DGGE analysis gave good discrimination of the strains belonging to *L. casei* group, but did not give significant results at species level, while species-specific primers allowed the identification of the individual species. Felis et al. (2001) performed a phylogenetic study among strains belonging to the *L. casei* group collected from international collection or isolated, and identified by API50 CHL kit and amplifying a 318 bp fragment of the *rec*A gene. The PCR products, were sequenced and phylogenetic trees were designed. The molecular marker *rec*A gave a clear distinction of all the type strains. Ryu et al. (2001) used ECO RI rybotyping to discriminate among 91 type strains belonging to *L. casei* group. This technique resulted at species level discriminating, but the identification of two of the analysed reference strains

resulted wrong. In the same year, a paper published by Vasquez et al. (2001) proposed to use the TGGE technique on amplicons of the 16S rRNA region to discriminate among reference and type strains belonging to international collection and strains isolated from human mucosae. This technique was efficient to discriminate among *L. casei-paracasei* and *L. rhamnosus* strains, but not between *L. casei* and *L. paracasei*. Comparative analysis of partial *tuf* sequences was evaluated for the identification and differentiation of lactobacilli by Chavagnat et al. (2002). The technique resulted in a good discrimination among *Lactobacillus* groups. In the case of *L. casei* group, it was not possible to distinguish between *L. casei* and *L. paracasei*, while *L. casei* ATCC 393 and NCDO 173 were identified as *L. zeae* and *L. paracasei* respectively, similarly to other previous works (Felis at al., 2001). In the same year, Bertier et al. (2001) used Rep-PCR and species-specific PCR primers (having as target 16S rRNA gene sequences) to successfully discriminate among strains belonging to *L. casei* group. Dobson et al. (2004) tried to discriminate among *L. casei* group strains by amplifying the first 3 variable regions of the 16S rRNA gene, the 16S-23S rRNA interspacer region, and part of the chaperonin 60 gene. Comparing the sequences, they obtained three main isolated clusters for *L. casei-paracasei*, *L. rhamnosus* and *L. zeae*. The strain ATCC 393 grouped with other isolates identified as *L. zeae*, confirming what reported in previously described studies. Desai et al. (2006) used a set of species-specific PCR primers targeting on 16S rRNA gene sequences, coupled with PFGE, that allowed to distinguish *Lactobacillus* strains belonging to the *L. casei* group. Their results confirmed that the usefulness of sugar fermentation tests was limited to the identification of *L. paracasei* and *L. rhamnosus* only.

Huang and Lee (2009) developed three pairs of species-specific primers based on RAPD fingerprinting, useful for the identification of *L. rhamnosus*, *L. paracasei* subsp. *tolerans* and *L. zeae* species among the *L. casei* group, but not for *L. casei* and *L. paracasei* subsp. *paracasei*. In the same year, the same authors compared the use of 16S rRNA and *dnaK* genes to discriminate among members of the *L. casei* group using sequencing and RFLP. The results showed that *dnaK* gene was effective to distinguish *L. casei*, *L. paracasei*, *L. zeae* and *L. rhamnosus.* For this reason, the authors proposed *dnaK* gene as an “additional molecular phylogenetic marker” (Huang and Lee, 2011). Huang et al. (2011) designed a *L. casei* group-specific PCR primer pair using the *rpoA* gene sequence and performed a SNaP shot minisequencing assay useful for the discrimination among the species belonging to the *L. casei* group. In 2011, Shevtsov et al. studied the polymorphisms of the nucleotide sequences of the *groEL*, *rpoB*, and *rplB* genes in *Lactobacillus* strains isolated fron fermented milk. A good intraspecific differentiation was observed, expecially among

strains belonging to the *L. casei* group (as *L. paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans*).
In 2015 Huang et al. used *dnaJ* as target for developing two-plex species-specific SNP primers able to distinguish among strains. Recently, a technique to characterize *L. casei-paracasei* strains was developed by Diancourt et al. (2007). The authors analysed a collection of strains previously identified as *L. casei* or *L. paracasei* by *rplB* gene sequencing comparing the sequences with reference strains of *L. zeae*, *L. rhamnosus*, and other species. After the identification made by the construction of a phylogenetical tree, they genotyped the strains using amplified fragment length polymorphism, MultiLocus Sequence Typing (MLST) selecting *loci* from seven housekeeping genes (*fusA*, *ileS*, *lepA*, *leuS*, *pyrG*, *recA*, and *recG*), and tandem repeat variation obtaining concordant results.
Moreover, the new genome sequencing techniques (whole-genome shotgun sequencing, high-throughput sequencing and single-molecule long-read sequencing) allowed the knowledge of the whole genome of several strains and comparative genomic studies among sequenced strains have been made. Actually, 9 strains of *L. casei*, 5 *L. paracasei* and 6 *L. rhamnosus* have been completely sequenced and assembled. Other strains have been sequenced, but the assembly is not finished yet (http://www.ncbi.nlm.nih.gov). Several comparative genomic studies have been made on isolates belonging to the species of the *L. casei* group, allowing to study the existing correlations between strains behaviors and their genomic characteristics (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).

1.4 Technological and probiotic utilization of *L. casei* group strains

Among LAB, *L. casei* group strains are known to be the dominant species of non-starter LAB (NSLAB) in ripened cheese, but they can be found as a minor microbial component in the microflora of fermented sausages as well (Samelis et al., 1994; Cocolin et al., 2000; Coppola et al., 2000). NSLAB are lactobacilli that are not used like starter cultures, but they are able to survive pasteurization in low number becoming a significant part of the microflora of most cheese varieties during ripening. Their proteolytic and lipolytic activity is responsible for the production of aromatic compounds (Bernardeu et al., 2008; Giraffa et al., 2010; Hosseini Nezhad et al., 2015). In fact, *L. casei* and *L. paracasei*

produce aminotransferase (AT) enzymes similar to those produced by strains belonging to *genus Lactococcus*, often used as starter cultures (Hosseini Nezhad et al., 2015). In literature, there is a huge amount of papers demonstrating that strains belonging to *L. casei* group are able to improve the cheese quality giving pleasant aromas and flavours. Scolari et al. (2005) demonstrated that the adjunct of the *L. casei* 5 Mn 373 strain to cheese-milk shortened the ripening time with no negative effects on the quality of the final product, underlining the importance of this species for the casein proteolysis. The utilization of starters belonging to *L. casei* group has been studied by Menéndez et al. (2000). They evaluated the effects of the inclusion of four homofermentative mesophilic *Lactobacillus* strains isolated from Arzúa-Ulloa cheese (normally consumed at 4 weeks ripening) selected for their high proteolytic activity. As results, the acidification during ripening was improoved and, due to the high production of diacetyl–acetoin during the late cheese ripening stage, cheese had a pleasant yogurt and butter aroma; spicy and slightly rancid aromas; and the texture was soft. The lipolytic and proteolytic activities of strains belonging to *L. casei* group, due to the aminotransferase activity of the strains (Thage et al. 2004), and the consequent production of aromatic compounds was confirmed also by other authors (Madkor et al. 2000; Weinrichteret al., 2004, Milesi et al., 2010; Barouei et al., 2011). Recent studies on *L. casei* and *L. rhamnosus* isolated from pecorino cheese demonstrated the capability of these species to generate key volatile compounds (Randazzo et al., 2007) and another recent study confirmed the capability of some *L. rhamnosus* strains to produce γ-glutamyl and lactoyl-amino acids (responsible for "kokumi" flavor in several seasoned cheeses) *in vitro* (Sgarbi et al., 2013).
Because of their potential therapeutic and prophylactic attributes, probiotic lactobacilli have also been proposed as starter cultures for the production of probiotic functional foods. Several *L. casei*, *L. paracasei* and *L. rhamnosus* strains can be used as non starter coltures for the production of probiotic cheese, without having a negative impact on cheese organoleptic and sensory quality (Stanton et al.,1998; Lynch et al., 1999; Dimitrellou et al., 2014; Tamime et al., 2005; Hong-Xin et al., 2015). Other studies focused on the utilization of *L. casei* group probiotic strains as starter cultures for the productions of dry fermented sausages, obtaining promising results (Erkkilä et al., 2001; De Vuyst et al., 2008).

1.5 Probiotic effects of *L. casei* group strains

In the early '900, Elie Metchnikoff, a Russian biologist, demonstrated for the first time that LAB can produce several benefits on human health. In 2001, an Expert Consultation of the United Nations Food and Agriculture Organization and the World Health Organization (FAO/WHO) adopted a consensus definition of probiotics as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (Anukam et al., 2007). Among lactobacilli, strains belonging to *L. acidophilus* and *L. casei* groups are the most frequently microorganisms used as probiotics (Hosseini Nezhad et al., 2015). Probiotics have been used to control viral and bacterial enteric infections as norovirus gastroenteritis (Nagata et al., 2011), diarrhoea caused by *E. coli* O157:H7 (Lema et al., 2001) and *H. pylori* infections (Sgouras et al., 2004). Thanks to the competition for mucosal surface binding sites mechanism, *L. casei* Shirota and *L. rhamnosus* GG are able to displace bound enterovirulent *E. coli* and *S. enterica* var. *typhimurium* from human intestinal mucus (Lee et al., 2003). A strong antimicrobial activity was also found in *L. rhamnosus* GG against *S. enterica* serovar *typhimurium*, due to the accumulation of lactic acid produced by this strain (De Keersmaecker, 2006). Other probiotics have been widely tested to control *S. enterica* colonization and infection. It has been demonstrated that the continued administration of *L. casei* CRL diminished *Salmonella* counts in the intestine and the extra intestinal spread (de LeBlanc et al., 2010), while *L. casei* Shirota strain resulted effective in the protection of mice against lethal infection with multi-drug resistant *S. typhimurium* DT104 (Asahara et al., 2011). Furthermore, probiotic strains could have a positive effect in the stimulation of the immune system enhancing intestinal integrity and protection against enteric infection (Kato et al., 1999; Matsuguchi et al., 2003; Matsumoto et al., 2005; Christensen et al., 2002; Tsai et al., 2010). Several studies focused on the immune-modulating capabilities of the probiotic strain *L. rhamnosus* GG studying *in vitro* how this microorganism can modulate signaling pathways, resulting in beneficial effects (Cabot et al., 2001; Pen˘a and Versalovic, 2003; Cho, 2008; Di Caro et al., 2005; Foligne et al., 2007; Iliev et al., 2005; Miettinen et al., 2000; Perea V´elez et al., 2007; Roessler et al., 2008). Moreover, it has been demonstrated that the lypopolisaccharides produced by some *L. casei* strains could be used as adjuvant in the oral vaccination against enteric pathogens (Khan et al., 2014). Another interesting effect has been observed in *L. rhamnosus* PL60, a starter strain producing conjugated linoleic acid having anti-obesity effect (Lee et al., 2006). Furthermore, beneficial effects on the circulatory system, in particular in decreasing blood pressure and the bleeding risk, were observed when *L.*

casei was administered in combination with other probiotic species (De Santis et al., 2000). *L. rhamnosus* GG is one of the most studied strains belonging to *L. casei* group because of its probiotic effects. It has been demonstrated that this strain is capable to inhibit cytokine-induced apoptosis in IEC lines by activating the Akt/protein kinase B signalling pathway, giving an important help to maintain the barrier function of the intestinal epithelium of the patients affected by chronic intestinal inflammation and in patients infected by pathogens (Gupta et al., 2000; Yan and Polk, 2002; Goyal et al., 2013). A similar effect have been observed in *L. casei* ATCC 334 and ATCC 393 (Yan et al., 2007).
Another positive effect, correlated to the consumption of *L. casei* Shirota, is the improving of the mood in depressed people after a constant consumption of fermented milk containing this strain (Benton et al., 2007). *L. paracasei* subsp. *paracasei* consumption could bring to an improvement in memory and concentration in people affected by Chronic Fatigue Syndrome (CFS) (Sullivan et al., 2009). Chainging in the behaviour have been observed also in mice treated with *Lactobacillis rhamnosus* (JB-1). This strain induced changes in GABA receptor expression in the brain, having an anti anxiety- and depression effects in mice (Bravo et al. 2011).
At least, an important antioxidant and anticancer activity (expecially against ColoRectal Cancer) have been observed in several *L. casei* group strains with probiotic features (Lee et al., 2004; Baldwin et al., 2010; Escamilla et al., 2012). Moreover, *L. casei* Shirota (LcS) showed strong anti-metastatic effects on transplantable tumor cells (Takagi et al., 2001).

1.6 Stress response in *L. casei* group strains

Depending on their employment, lactobacilli may encounter several kind of stress factors and, as a result, the microbial response to stress may activate several defense mechanisms (Hosseini Nezhad et al., 2015).
Figure 1 shows the most common stress factors that *Lactobacillus* strains can encounter during their industrial or health employment.

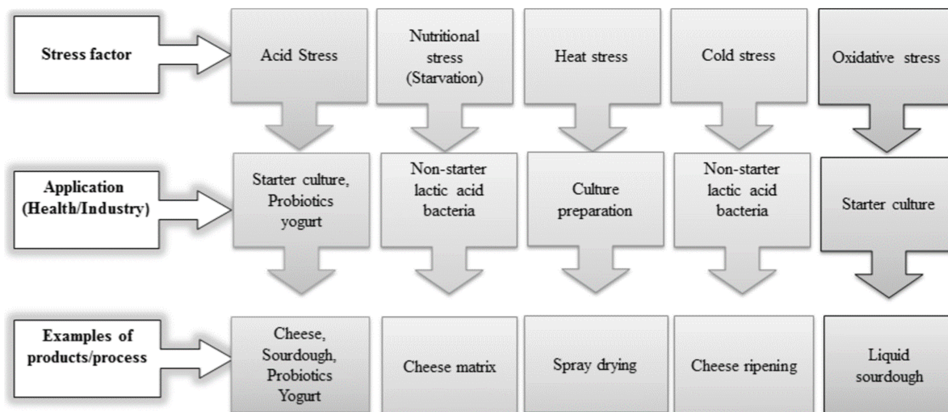

Figure1. Example of some stress factors that *Lactobacillus* spp. may encounter (Hussain et al., 2013).

There are two defensive systems against stress. The first one is activated by the microbial cell in order to eliminate the chemical or physical stress source, while the second defensive system is characterized by the activation of several metabolic pathways allowing the cells to adapt themselves to the hostile environment (De Angelis and Gobbetti, 2004).
The physiological and molecular mechanisms of technological and environmental stress response in lactobacilli will be described below.

Heat stress

Heat stress typically occurs during the production of dairy products and dried cultures to be used as technological starters or probiotics. The effects of the temperature changes on *genus Lactobacillus* have been extensively studied (Derrè et al., 1999; Prasad et al., 2003; De Angelis et al., 2001; De Angelis and Gobbetti, 2004; G-Alegria et al., 2004; Spano et al., 2005; Sugimoto et al., 2008; Suokko et al., 2008; Parente et al., 2010; Ricciardi et al., 2012).
When cells are exposed to high temperatures, the main effect is the denaturation of proteins, membrane and nucleic acids. As response to the heat shock, cells start increasing the synthesis of molecular chaperones known as heat shock proteins (HSPs). The role of these proteins is to mediate the correct folding of newly synthetized peptides, refolding the stress-denatured and aggregated proteins, to prevent protein aggregation, translocate proteins across membranes and assembling or disassembling oligomeric structures. The two major groups of HSPs are: 70 kd HSPs and 60 kd HSPs. The first HSP is also known as DnaK chaperon, consisting in three main components (DnaK, DnaJ and GrpE), involved in the general stress response mechanisms. The second HSP is also 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).
Broadbent (1997) investigated the heat shock (HS) response in tree *Lactobacillus* strains, including *L. casei* LC301. Thermotolerance experiments showed that heat shock (HS) improved the synthesis of the heat shock proteins DnaK, GroEL, ClpB, and GrpE. DnaJ. Two-dimensional polyacrylamide gel electrophoresis revealed that GroEL expression was increased also in probiotic *Lactobacillus paracasei* NFBC338 when treated at 52 °C for 15 min (Desmond et al., 2004).
Corcoran et al. (2006) compared the viability of an overproducting GroESL strain of *L. paracasei* NFBC 338 after spry and freeze-drying, with that of controls. As result, the overproducing GroESL strain showed a better survival, underlining the importance of GroESL in stress tolerance and suggesting that the selection of strains overproducting this HSP coul be useful for the production of probiotic cultures.
The enhanced tolerance against heat stress of a strain adapted with a pre-treatment was also investigated in a study performed on the microbial starter *L. rhamnosus* HN001. It was observed that, when pre-stressed with either heat (50°C) or salt (0.6 M NaCl), the tested strain 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).
Studies on *L. paracasei* NFBC 338 demonstrated that, when pretreated in a 0.3 M NaCl environment, the cells were more resistant to heat stress (60 °C for 30 min) than the non-adapted control (Desmond et al., 2001). A subsequest study performed on the same strain, demonstrated that the *GroEL* expression was ehnanced after the exposure to heat adaptation conditions (52 °C for 15 min) (Desmond et al., 2004).
Other studies demonstrated that the heat tolerance of the strains were enhanced by the utilization of protective agents (Gardiner et al., 2000).

Cold stress

Cold stress in lactobacilli is related to the storage of the starter and probiotic cultures, the fermentation at low temperatures during the cheese ripening or the refrigerated storage of the fermented products ready for consumption. Lactobacilli are able to grow at a reduced rate after a decrease of about 20 °C below their optimal growth temperature. The physiological changes that can occur in the cells are: 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. 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 the lipids. They can also reduce the DNA supercoiling and stimulate the transcription and translation needed 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).
Sauvageot et al. (2006) studied the presence of a Csp-like protein in *L. casei* BL23, cloning and characterizing it. Doing a Northern blot analysis they discovered that *cspA* expression was induced after a decrease of the temperature from 37°C to 20°C. Additionally, they constructed a Δ*cspA* mutant, and a decreased growth rate compared to the wild type was observed.
Beaufils et al. (2007) made a proteome analysis on mutants of *L. casei*, observing that the cold shock protein CspA was significantly overproduced compared to the wild-type strain. Moreover, it was observed that the mutant growth rates were related to the shift of temperatures and to the composition of the growth medium. Mutants grown in medium with glucose, showed higher sensitivity to cold stress, but when grown on 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*.

Acid stress

LAB are famous because of their production of organic acids that determine an acidification of the environment, enabling the survival of many other organisms. These bacteria, nonetheless, can encounter extreme acidic conditions if used as probiotic strains, as they reach the stomach. Acids enter into the cells by passive diffusion and they rapidly dissociate it. This causes a cytoplasmic acidification that reduces the activity of acid-sensitive enzymes and damages the peptides and the nucleic acids. In order to survive and maintain the pH homeostasis, the cells developed several protection mechanisms. Lactobacilli possess the capability to adapt their physiology to low pH thanks to the acid tolerance response (ATR) mechanism, involving the production of acid shock proteins (ASPs), protecting microorganisms not only from acid stress but from heat, osmotic or oxidative stress too. Fozo et al. (2004) confirmed that membrane fatty acid adaptation is envolved in acid stress response. They observed, in fact, that in low pH conditions, *L. casei* 4646 alters its membrane composition to contain increased levels of long-chained, mono-unsaturated fatty acids.
In a recent study, the effect of pH on growth and phenomic characterization of acid stress responses was investigated on a typical strain of the *L. casei* group by electron microscopy observations. Alterations on cell surface were detected in cells grown under acid stress. Proteomic approaches revealed several changes in the relative levels of protein expression at low pH compared to the optimal conditions. It is assumed that the analysed strain adopted an ATR

strategy to adapt itself to the acidic conditions (Hosseini Nezhad 2010). In anoher study, the impact of acidic pH on growth rate of *L. casei* strain GCRL 12 was determined, confirming that surface proteins of this strain are associated with adaptation to acid environments (Hossein Nezhad et al., 2012). 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 proportions of unsaturated fatty acids was found (Wu et al., 2012).
Other defence mechanisms are the ATP dependent expulsion of protons by a proton-translocating ATPase and 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-Vergèsa et al., 2002; De Angelis and Gobbetti, 2004).
In *L. rhamnosus* GG the expression of F(0)F(1)-ATP synthase genes (involved in the ADI pathway) is upregulated under acidic conditions. Moreover, this strain modulates its pyruvate metabolism as response to envinronmental 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 by Moslehi-Jenabian et al. (2009) after exposure of *L. rhamnosus* GG to different low pH conditions (pH 5.0, 4.0 and 3.0) and to pH 6.5 as control in adapted and non-adapted cells. 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, reaching a maximum level and decreased subsequently, in non adapted cells, while the acid adaptation determined a decrease in the transcription levels confirming the involvment of *luxS* gene in acid stress response.
Yanez et al. (2008) studied how acid stress can interfere with changings in growth rate and lactate production in *L. rahamnosus* cells. The acid toxicity caused a decrease of the maximum growth rate, but in this environment the cells were still able to produce lactate.

Bile salt tolerance
Bile salts display a strong antimicrobial activity: acting as detergents, they are able to disassemble biological membranes. Since *L. casei* group members are potentially probiotic microorganisms, they must possess the ability to resist at the antimicrobial action of bile salts. Lactobacilli survive to the bile salt action thanks to the bile salt hydrolases (BSH), enzymes that inactivate the bile acids deconjugating them (Van de Guchte et al., 2002; De Angelis and Gobbetti, 2004). The action of BSH in *L. casei* was investigated in the strain Zhang, an isolate from home-made koumiss in Inner Mongolia of China, which showed

high resistance to bile salts. The predicted BSH gene was significantly up-regulated under the stress of bile salts (Zhang, 2009). A subsequent study, compared the growth and protein expression patterns of *L. casei* Zhang when exposed to bile salt stress. A 2-dimentional gel electrophoresis, permitted to reveal the presence of 26 proteins differentially expressed by the strain tested under normal growth condition and in presence of bile salt. Further verification made by using real-time, quantitative reverse transcription-PCR and bioinformatics analysis showed that bile stress response is characterized by the activation of genes envolved in cell protection (*dnaK* and *groEL*), in cell membrane structure (*nagA*, *galU*, and *PyrD*), and in other housekeeping genes (*pfK*, *pgM*, *cysK*, *luxS*, *pepC*, and *ef-Tu*) (Wu et al., 2010).
A study of Koskenniemi et al. (2012) on bile stress response in probiotic *L. rhamnosus* GG, revealed that this kind of stress determines changes in the transcriptome that seems to strengthen the cell envelope against bile-induced stress and signal the GG cells of gut entrance. Changing in the transcriptome were detected also in *L. casei* BL 23 after bile stress exposure. Bile stress induced changes in several genes and proteins involved in the stress response and in other cellular pathways (fatty acid and cell wall biosynthesis, metabolism of carbohydrates, transport of peptides, coenzyme syntesis, membrane H(+)-ATPase activity) (Alcantara and Zùñiga, 2012).

Osmotic stress

In their natural environment, lactobacilli are often exposed to changing in the osmolarity. During their industrial use, for example, they can incur in osmotic stress, in the production of food characterized by the presence of a high concentration of salt and sugars. The general response observed in lactobacilli when an increase of the osmolarity occurs, is the release of water from the cytoplasm to the outside, causing loss in the cellular turgor. Vice versa, in a hyperosmotic environment, the response of the cells 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) (Van de Guchte et al., 2002; De Angelis and Gobbetti, 2004; Hörmann et al., 2006).
As response to osmotic stress, in several papers have been reported the modification in the membrane composition of *L. casei* strains. Changing in the membrane structure were reported from Machado et al., (2004). They studied the changes in the chemical and structural properties of the membrane and other mechanisms occurring in *Lactobacillus 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.
L. casei strains grown in 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; Palomino et al., 2013).
Another study investigated the response to sucrose osmotic stress of the probiotic strain *L. rhamnosus* VTT E-97800 (E800). Under the investigated conditions, the tested strain adapted itself and survived with no significant loss of culturability/viability proving that these cells responded to sudden changes in their environmental osmotic conditions by accumulating sucrose in order to protect both the membranes and internal organs (Sunny Roberts et al., 2008).

Oxidative stress
Oxidative stress occurs in presence of reactive oxygen species (ROS) such as superoxide (O_2^-), hydroxyl radicals (HO) and hydrogen peroxide (H_2O_2). Lactobacilli are facultative anaerobic bacteria, as they can grow both under anaerobic and aerobic environments. Among LAB, there are species possessing enzymes allowing them to degrade, tolerate, or survive to the toxicity of oxygen and reactive oxygen. Enzymes such as NADH oxidase, pyruvate oxidase and lactate oxidase allow the microorganisms to eliminate oxygen from the cell, and have been found in several LAB. Manganese superoxide dismutase (SOD), manganese catalase and NADH peroxidase have been extensively studied in *Bacillus subtilis* and *Lactococcus lactis* (Van de Guchte et al., 2002; De Angelis and Gobbetti, 2004; Talwalkar and Kaliasapathy, 2003). Only in the last years, the oxidative stress mechanisms of *L. casei* group were investigated. Zotta et al. (2014) focused their study on one hundred eighty four strains belonging to the species *L. casei*, *L. paracasei* and *L. rhamnosus*, with the purpose to investigate their capability 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 to aerobic conditions. Moreover, many strains showed the capability to grow under respiratory conditions.

1.7 Potential application of the stress response in food and probiotic industry

As mentioned in the previous paragraphs, during food processing, LAB can encounter several kind of stress sources. The novel techniques of bioengineering allow the production of mutant strains with enhanced stress resistance capabilities, but the European Union has very strict condition for the utilization of "artificially" mutated microorganisms (Directive 2001/18/EC). However, the use of "naturally selected mutants" is not forbidden by the

European low. For this reason, the knowledge of the stress responses in LAB is fundamental for the selection of the strains showing the best industrial performances. In the last years, several comparative genomic studies have been made, and thanks to the genome sequencing techniques (whole-genome shotgun sequencing, high-throughput sequencing and single-molecule long-read sequencing) (Heather and Chain, 2015; Loman et al., 2015) a comparative genomics approach would be an efficient tool to discover biochemical pathways for the selection of strains with industrial potential (Zhu et al., 2009). Besides, when is not possible to artificially manipulate the bacterial genome in order to obtain a strain with better 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., 2014).
Improving resistance in strains is possible also by the activation of other microbial mechanisms, like the adjunct of a protectant to the growth medium prior to exposure of the strain to environmental stress. As example, a study demonstrated that an exudate gum from gum acacia (a tree), enhanced survival of *L. paracasei* NFBC 338 to several stress sources (heat, bile and H_2O_2) when added to the growth medium and also enhanced survival during spray-drying (Desmond et al., 2002). On the basis of the studies on stress response performed in the recent years, it can be stated that microbial stress factors induce the production of a wide variety of metabolites and the activation of metabolic pathways. These metabolites, may lead to an increase of the resistance of the microorganism to technological stresses, and can significantly influence the sensory quality and texture of food in which they are used.

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2. Collection and identification of strains belonging to the *Lactobacillus casei* group

2.1 Aim of the study

The correct identification and characterization of bacteria is essential for several reasons: the classification of lactic acid bacteria (LAB) has changed significantly over the years, and it is important to distinguish and define them correctly according to the current nomenclature. Incorrect classification can create problems in the interpretation of literature. Furthermore, because LAB are increasingly used as probiotics in foods, food industries must define these bacteria accurately on the labels.
Consequently, there is increased use of molecular techniques to identify strains belonging to the *L. casei* group and to classify them into *L. casei*, *L. paracasei* and *L. rhamnosus*. In this study, two such molecular techniques were developed and optimised: PCR using species-specific primers and HRM (high-resolution melting) analysis. Two hundred one strains attributed to the *L. casei* group were examined using these techniques and the results were compared with consolidated molecular methods already published.

2.2 Materials and methods

2.2.1 Strains and culture conditions

Two hundred one (201) strains belonging to the species *L. casei*, *L. paracasei* and *L. rhamnosus* isolated from different sources (Table 2.1) were used in this study. The strains were previously isolated and identified by the respective Universities or Research Institutes using biochemical and morphological tests or different molecular techniques.
All strains were maintained as frozen stocks in reconstituted 11 % (w/v) skimmed milk containing 0.1 % (w/v) ascorbic acid (RSM) in the Culture Collection of the Department of Food Science, University of Udine. The isolates were routinely propagated (1 % w/v) in MRS broth (pH 6.8) (Oxoid, Italy) for 16 h at 37 °C.
L. casei (DSM 20178), *L. paracasei* (DSM5622) and *L. rhamnosus* (DSM20021) were used as reference strains for optimisation of all the molecular methods used for identification. The following strains were used as negative controls: *L. fermentum* (DSM 20049), *L. pontis* (DSM 8475), *L. sanfranciscensis* (DSM 20451), *L. brevis* (DSM 20054), *L. reuteri* (DSM 20053), *L. plantarum* (DSM 20174), *L. sakei* (DSM 6333), *Lactococcus lactis* (DSM 20481), *Leuconostoc citreum* (DSM 5577), *Leuc. gasicomitatum* (DSM

15947), *Leuc. mesenteroides* subsp. *mesenteroides* (DSM 20343) and *Pediococcus pentosaceus* (DSM 20336).

Table2.1 List of the strains used in this study and their original identification.

Origin	Given identification
Raw and heat treated milk, yogurt, milking machines	***L. paracasei***: LMG9192[1], DSM5622[2]
	L. casei/paracasei**:** P1E5[3], P1E6[3], P2P3[3]
	L. paracasei* subsp. *tolerans: LMG9191[1], P1E4[3], DSM20258[2]
	L. rhamnosus:, HA111[4], PRA152[5]
Green, creamy and seasoned cheeses	***L. casei***: LMG6904[1], TMW1.1444[6], TMW1.1259[6], LACcas13[7] , LACcas7[7]
(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. paracasei***: LMG25880[1], LMG25883[1], LMG12164[1], DBPZ0421[8], DBPZ0422[8], DBPZ0424[8], DBPZ0434[8], DBPZ0435[8], DBPZ0450[8], DBP0451[8], DBPZ0472[8], DBPZ0475[8], DBPZ0476[8], DBPZ0477[8], DBPZ0478[8], DBPZ0635[8], DBPZ0733[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], DBPZ0428[8], M335[8], M290[8],M303[8], H12[13]
	L. casei/paracasei**:** Cst7[11], 3LC[11], DBPZ0718[8], M307[8]
	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], CI4368[12] ,DBPZ0420[8], DBPZ0734[8], CF143[12] ,R61[13], F17[13], N24[13]
Fermented sausages	***L. casei/paracasei***: CTC1675[14]
	L. casei/rhamnosus: CTC1676[14], 2220[15]
Sourdoughs	***L. paracasei***: DBPZ0561[8], DBPZ0571[8], DBPZ0572[8], Q2[8], Q4[8], I1[4], I2[16]
	L. casei/paracasei: 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],B082[17], B083[17], B085[17], B087[17], B161[17], B169[17], B171[17], B174[17], B195[17], B196[17], B350[17], B166[17] ,B084[17], B086[17], B163[17], B164[17], B167[17], B168[17], B170[17], B172[17], B173[17], B175[17] ,B176[17], B177[17], B179[17]
Beer, malt	***L. casei***: LACcas25[7], LACcas29[7], TMW 1.300[6]
Coffee	***L. casei***: DSM20178[2]
	L. rhamnosus: DIAL40[15]
Humans	***L. casei***: LMG23516[1]
(saliva, dental caries, blood, urethra, faeces of infants and adults)	***L. zeae:*** N87[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], DSM4905[2]
	L. casei/paracasei: N161[16], N42[16], N44[16], N76[16], N1710[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], N811[16], N2014[16], N175[16]
Unknown	***L. paracasei***: NRRL B-456[19], DSM5622[2] ***L. rhamnosus***: NRRL B-176[19], NRRL B-442[19], DSM20021[2]

[1]LMG: BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms (BCCM™), Belgium.
[2]DSM: DSM, 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 delgi 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, Unversità 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, Unversità degli Studi del Molise, Campobasso, Italy.
[17] Institute for Wine Biotechnology Department of Viticulture and Oenology, Stellenbosh 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

2.2.2 DNA extraction from pure cultures

Two millilitres of a 48-h culture in MRS broth were centrifuged at 13,000*g* for 10 minutes at 4 °C to pellet the cells, which were then subjected to DNA extraction using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, USA). The DNA concentration and purity were measured using an absorbance ratio of 260/280 nm and verified by agarose gel electrophoresis.

2.2.3 *L. casei* group-specific PCR

The *L. casei* group-specific PCR primer pair, LCgprpoA-F2 (5'-CACTCAARATGAAYACYGATGA-3') and -R2 (5'-CGTGGTGAGATTGAGCCAT-3') was used as described by Huang et al. (2011). The reactions were performed in a final volume of 25 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 U of *Taq*-polymerase (Applied Biosystems, Italy). The thermal cycling protocol was as follows: initial strand denaturation at 94 °C for 5 min followed by 25 cycles of 94 °C for 1 min, 61 °C for 1 min and 72 °C for 1.5 min, and a final extension step at 72 °C for 7 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad, Italy). The PCR products were analysed by 2 % agarose gel electrophoresis with ethidium bromide staining, and the expected amplicon size was 364 bp.

2.2.4 Species-specific PCRs

Three different primer pairs were used to identify strains by species-specific PCRs, as described by Ward and Timmins (1999) (Table 2.2). The reactions were performed in a final volume of 25 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 U of *Taq*-polymerase (Applied Biosystems, Italy). The amplification was performed for 30 cycles at 95 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad, Italy). An initial denaturation step (95 °C for 5 min) and a final extension step (72°C for 5 min) were used. The PCR products were verified by electrophoresis in a 2 % agarose gel using 0.5X TBE as the running buffer. Ethidium bromide (0.5 µg/ml) was added to the gel before solidification. After electrophoresis, the gels were examined using the BioImaging System GeneGenius (*SynGene*, United Kingdom).

Table 2.2 Primers used in this study.

Target microorganism	Primer name	Sequence (5′–3′)	Amplicon Size (bp)	Temperature of annealing (°C)	Reference
L. casei	casei	TGCACTGAGATTCGACTTAA	290	53 °C	Ward and Timmins (1999)
	Y2	CCCACTGCTGCCTCCCGTAGGAGT			
L. paracasei	para	CACCGAGATTCAACATGG	290	53 °C	Ward and Timmins (1999)
	Y2	CCCACTGCTGCCTCCCGTAGGAGT			
L. rhamnosus	rham	TGCATCTTGATTTAATTTTG	290	53 °C	Ward and Timmins (1999)
	Y2	CCCACTGCTGCCTCCCGTAGGAGT			
L. rhamnosus	dnaKRHf dnaKRHr	GAACAGCAGGGATCC GATCTTTCCGGTGTGA	235	58 °C	This study
L. paracasei/casei	dnaKCPf	AAACTGTGCCCGCGT	281	59 °C	This study
	dnaKCPr	GCGACGGGGTCTTTG			
L. casei	dnaJPAf dnaJPAr	CGGCTGCGAACTGCATTA TTCCTGCTGGCACCCAAA	162	64 °C	This study

In this study, a second set of species-specific PCRs was developed, using a different part of the genome as a target sequence for primer annealing compared to the region used by Ward and Timmins (1999). The *dnaJ* and *dnaK* genes were targeted. All of the sequences of these genes available in GenBank for species of the *L. casei* group were aligned using the MultAlin software (Corpet, 1988), and the primer pairs designed were dnaKRHf/dnaKRHr, dnaKCPf/dnaKCPr, and dnaJCPf/dnaJCPr (Table 2.2), which were specific to the *L. casei* group for *L. rhamnosus*, *L. paracasei/L. casei* and *L. paracasei*, respectively. Before optimisation of the amplification protocol, primer specificity was tested *in silico* using the FastPCR 6.1 software (Kalendar et al., 2009) and *in vivo* using *L. fermentum* (DSM 20049), *L. pontis* (DSM 8475), *L. sanfranciscensis* (DSM 20451), *L. brevis* (DSM 20054), *L. reuteri* (DSM 20053), *L. plantarum* (DSM 20174), *L. sakei* (DSM 6333), *Lactococcus lactis* (DSM 20481), *Leuconostoc citreum* (DSM 5577), *Leuc. gasicomitatum* (DSM 15947), *Leuc. mesenteroides* subsp. *mesenteroides* (DSM 20343) and *Pediococcus pentosaceus* (DSM 20336) as negative controls.
The reactions were performed in a final volume of 25 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 U of *Taq*-polymerase (Applied Biosystems, Italy). PCR was performed using the thermal cycling protocol described above, with the annealing temperatures shown in Table 2.2.

2.2.5 *tuf* multiplex PCR

Amplification reactions were performed with a 50 µl (total volume) solution containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 200 µM each dNTP, 10 pmol each of primers PAR (5'-GACGGTTAAGATTGGTGAC-3'), CAS (5'-ACTGAAGGCGACAAGGA-3'), and RHA (5'-GCGTCAGGTTGGTGTTG-3'), 50 pmol of primer CPR (5'-CAANTGGATNGAACCTGGCTTT-3') (Ventura et al., 2003), 25 ng of template DNA, and 2.5 U of *Taq*-DNA polymerase (Applied Biosystems, Italy). Amplification reactions were performed using a thermocycler (Perkin-Elmer Cetus 9700) with the following temperature profiles: 1 cycle at 95 °C for 5 min; 30 cycles at 95 °C for 30 s, 54 °C for 1 min, and 72 °C for 1.5 min; and 1 cycle at 72 °C for 7 min, in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad, Italy). PCR amplicons were analysed by 2% (w/v) agarose gel electrophoresis in TBE 0.5X buffer at a constant voltage of 7 V/cm, visualised with ethidium bromide (0.5 µg/ml), and photographed under UV light at 260 nm, using the BioImaging System GeneGenius (*SynGene*, United Kingdom).

2.2.6 Development and optimisation of High-Resolution Melting (HRM) analysis

The variable regions V1 to V3 flanked by highly conserved sequences within the 16S rRNA were selected for HRM analysis. Three consolidated primer pairs that have been used to discriminate different species by DGGE analysis were used to discriminate *L. casei*, *L. paracasei* and *L. rhamnosus* by HRM analysis: P1V1 and P2V1 (Klijin at al., 1991), BA-338f and UN-518r (Muyzer et al., 1993), Y1 and Y2 (Young et al., 1991). The analyses were performed in a 25-µl reaction volume containing 2X HRM PCR Master mix (Qiagen, Italy), 0.7 µM each primer and 100 ng of DNA. The PCR amplifications were performed in a Rotor-Gene Q (Qiagen, Italy) with the following conditions: 95 °C for 1 min followed by 45 cycles at 95 °C for 10 s, 55 °C for 30 s 72 °C for 10 s. After amplification, HRM analysis was performed from 65 to 90 °C with increments of 0.1 °C/2 sec. The Rotor-Gene Q series software version 2.2.2 (Qiagen, Italy) was used to analyse the HRM data. The melting profiles were subjected to fluorescence normalisation to minimise inter- and intra-run variability. Difference plots were generated by normalising the melting profiles of strains to a negative control strain whose melting profile was converted to a horizontal line. Three difference graphs were obtained for the *L. casei, L. paracasei* and *L. rhamnosus* strains using the fluorescence of each reference strain (per each graph) set as the baseline (confidence level of 90 %)

(Andersson et al., 2009; Gurtler et al., 2012). The ScreenClust program (Qiagen, Italy) was used for Principal Component Analysis (PCA).
All analyses were performed in triplicate; positive/negative controls and non-template controls (NTC) were included in each run. For the validation assay, 10 strains were used for each species tested.

2.3 Results and discussion

2.3.1 Preliminary identification by *L. casei* group-specific PCR

A total of 201 strains belonging to the *L. casei* group were collected from national and international collections (Table 2.1). The strains were isolated from sources including raw and heat-treated milk, yogurt, milking machines, green/creamy and seasoned cheeses, fermented sausages, sourdoughs, wine, must and cellar equipment, beer, malt, coffee and humans; the source of some strains were unknown. These strains were isolated over several years. Therefore, in some cases, there was no information on the origin or method of identification used. In other cases, biochemical tests or molecular analyses were performed for strain identification. To uniformly identify strains, a preliminary *L. casei* group-specific PCR was performed. The expected amplicon was obtained from 194 strains (Figure 2.1), confirming that these strains belonged to the *L. casei* group. The amplicon was not obtained from 7 strains, and they were excluded from subsequent analyses. None of the negative control strains yielded the amplicon, confirming the specificity of the primers.

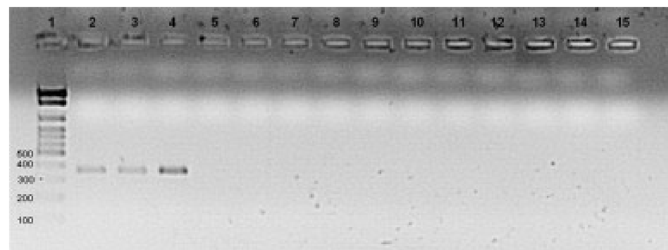

Fig. 2.1. Specific PCR for *Lactobacillus casei* group (amplicon size 364 bp): line 1: ladder, 100 bp low ladder (Sigma–Aldrich, Italy); line 2: DSM20178, *L. casei*; line 3: DSM20021, *L. rhamnosus*; line 4: DSM5622, *L. paracasei*; line 5: DSM 20451, *L. sanfranciscensis*; line 6: DSM 20054, *L. brevis*; line 7: DSM 20053, *L. reuteri*; line 8: DSM 20174, *L. plantarum*; line 9: DSM 6333, *L. sakei*; line 10: DSM 20481, *Lactococcus lactis*; line 11: DSM 5577, *Leuconostoc citreum*; line 12: DSM 15947, *Leuc. gasicomitatum*; line 13: DSM 20343, *Leuc. mesenteroides* subsp. *mesenteroides*; line 14: DSM 20336, *Pediococcus pentosaceous*; line 15: negative control.

2.3.2 Species identification by species-specific PCRs and *tuf* multiplex PCR

The identification methods were tested on the three reference strains and were able to discriminate *L. casei*, *L. paracasei* and *L. rhamnosus* species (Figure 2.2). Species-specific PCRs yielded an amplicon of the expected size (290 bp) only for the targeted species, and no amplification product was obtained for the other two *L. casei* group species (Figure 2.2, panel A). Similarly, the *tuf* multiplex PCR profiles yielded different numbers of bands for *L. casei, L. paracasei* and *L. rhamnosus*, which enabled the discrimination of these species. The amplification profile of *L. casei* comprised five bands of approximately 350, 450, 500, 900 and 1100 bp, which was not completely consistent with the profile obtained by Ventura et al. (2003). The *L. paracasei* amplification profile comprised a strong band of approximately 200 bp and a thinner band of 500 bp, which was not always visible (Figure 2.2, panel B, lines L5 and L10); Ventura et al. (2003) obtained strong amplification products corresponding to these sizes. The amplification profile of *L. rhamnosus* comprised a single amplicon of approximately 500 bp, consistent with Ventura et al. (2003). Although both these techniques discriminated species within the *L. casei* group, amplification products were also obtained for specific negative control LAB strains (data not shown); these strains yielded a 290-bp amplicon in the species-specific PCR analysis and profiles comparable to the *L. casei* group species in the *tuf* multiplex PCR analysis. Therefore, a preliminary screening step comprising the *L. casei* group-specific PCR is required for the identification of LAB isolates using these techniques.

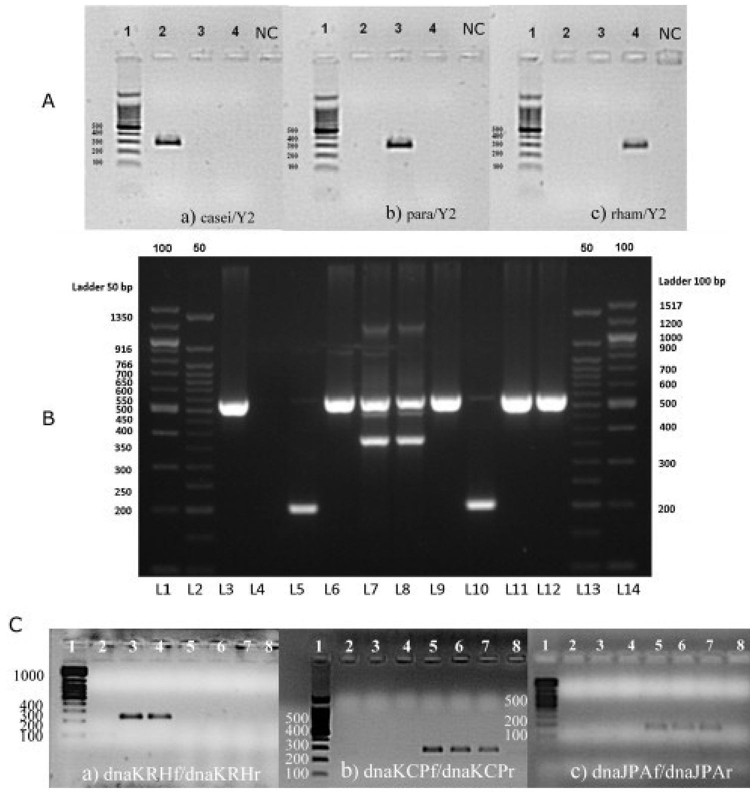

Fig. 2.2 Species identification by species–specific PCRs and tuf multiplex PCR. Panel A, Species-specific PCRs by Ward and Timmins (1999). a) Amplification specific for *L. casei*; b) Amplification specific for *L. paracasei*; c) Amplification specific for *L. rhamnsosus*. Line 1, Ladder, 100 bp low ladder (Sigma–Aldrich, Italy); line 2, DSM20178, *L. casei*; line 3, DSM5622, *L. paracasei*; line 4, DSM20021, *L. rhamnosus*; NC, negative control. Panel B,tuf multiplex PCR by Ventura et al. (2003). Lanes L1, L14: Ladder 100 bp (New England Biolabs); Lanes L2, L13: Ladder 50 bp (New England Biolabs); Lane L3: DSM20021, *L. rhamnosus*; Lane L4: negative control; Lane L5: DSM5622, *L. paracasei*; Lane L6: FSG01, *L. rhamnosus*; Lane L7: DSM20178, *L. casei*; Lane L8: N87, *L. casei*; Lane L9: D44, *L. rhamnosus*; Lane L10: Cst7, *L. paracasei*; Lane L11: N202, *L. rhamnosus*; Lane L12: N1110, *L. rhamnosus*. Panel C, Species-specific PCRs, this study. a) Amplifican specific for *L. rhamnosus*. Line 1, ladder, 100 bp low ladder (Sigma–Aldrich, Italy); line 2, negative control; line 3, DSM20021, *L. rhamnosus*; line 4, N202, *L. rhamnosus*; line 5, DSM20178, *L. casei*; line 6, N87, *L. casei*; line 7, DSM5622, *L. paracasei*; line 8, Cst7, *L. paracasei*. b) Amplification specific for *L. paracasei*/*L. casei*. Line 1, ladder, 100 bp low ladder (Sigma–Aldrich, Italy); line 2, negative control; line 3, DSM20021, *L. rhamnosus*; line 4, N202, *L. rhamnosus*; lines 5–6, DSM20178, *L. casei*; line 7, N87, *L. casei*; line 8, DSM5622, *L. paracasei*. c) Amplification specific for *L. paracasei*. Line 1, ladder, 100 bp low ladder (Sigma–Aldrich, Italy); line 2, negative control; line 3, DSM20021, *L. rhamnosus*; line 4, N202, *L. rhamnosus*; line 5, DSM5622, *L. paracasei*; line 6, Cst7, *L. paracasei*; line 7, LMG13087, *L. paracasei*; lines 8, DSM20178, *L. casei*.

Inconsistent results were obtained using the two techniques on some of the collected strains (Table 2.3).

Table 2.3 Comparison of the results obtained using the different techniques on 6 out of the 194 strains: inconsistent results.

Strain	ID Specific PCR (Ward and Timmins, 1999)	ID Specific PCR (this study)	ID HRM	ID Multilpex
LMG 6904	*L. paracasei*	*L. paracasei*	*L. paracasei*	*L. casei*
DSM4905	*L. casei*	*L. casei*	*L. casei*	*L. paracasei*
DBPZ0420	*L. paracasei*	*L. paracasei*	*L. paracasei*	*L. rhamnosus*
DBPZ0571	*L. casei*	*L. casei*	*L. casei*	*L. paracasei*
DBPZ0734	*L. paracasei*	*L. paracasei*	*L. paracasei*	*L. rhamnosus*
N2014	*L. casei*	*L. casei*	*L. casei*	*L. rhamnosus*

For 6 strains, the two methods yielded different results. For some strains, the obtained results were not unexpected. LMG6904 (synonyms ATCC393, DSM20011, CCUG21451) is a well-known strain whose taxonomic classification has been repeatedly modified and is under debate; the Judicial Commission of the International Committee for Systematics of Prokaryotes ruled the following: i) The designation of ATCC334, a strain of *L. paracasei*, as the neotype of *L. casei* contravenes rules 51b (1) and (2); ii) Typification of *L. casei* (Orla-Jensen 1916) Hansen and Lessel 1971 is based on ATCC393; iii) The proposal to revive the name *L. zeae* contravenes rules 51b (1) and (2); iv) The name *L. paracasei* has not been rejected by the Judicial Commission and is legitimate, validly published and may be used as a correct name. This ruling confirms the deliberations (Waynes, 1994) that followed a previous Request for Opinion by Dellaglio et al. (1991) (Dellaglio et al., 1991; Waine, 1994; Dicks et al., 1996; Mori et al., 1997; Chen et al., 2000; Biavati, 2001; Klein, 2001; Dellaglio et al., 2002; Judicial Commission Of The International Committee On Systematics Of Prokaryotes, 2008). Identification of the strain DSM4905 (synonym ATCC1158) is also ambiguous based on the species classification provided by the DSM and ATCC collections. In the DSM collection, this strain is considered the reference strain for the *L. paracasei* species, whereas the ATCC considers this strain the reference strain for the *L. casei* species. Four strains could not be accurately identified because the two techniques yielded inconsistent results (Table 2.3); therefore, taxonomic classification of these strains requires further studies.
For the remaining 188 strains, the two identification methods yielded consistent results, but for 46 out of the 188 strains, the results were in disagreement with the original identification.
To confirm these results, two different methods were developed in this study: alternate species-specific PCRs and HRM analysis.
The species-specific primer pairs designed for the *dnaK* and *dnaJ* genes were specific within the *L. casei* group; amplicons were obtained exclusively from *L. rhamnosus*, *L. paracasei/L. casei* and *L. casei* using the primer pairs

dnaKRHf/dnaKRHr (Figure 2, panel C, a), dnaKCPf/dnaKCPr (Figure 2, panel C, b) and dnaJPAf/dnaJPAr (Figure 2, panel C, c), respectively. The results were consistent with the species-specific PCRs and *tuf* multiplex PCRs for the 188 strains. The data for the 6 unidentified strains LMG6904, DSM4905, DBPZ0420, DBPZ0571, DBPZ0734, and N2014 are shown in Table 2.3.

2.3.3 High-resolution melting (HRM) analysis

HRM analysis was used to resolve inconsistencies between the species-specific PCR and *tuf* multiplex PCR analyses compared to the original identification.
HRM analysis is a novel technique that enables the identification of point mutations in a DNA sequence. It has been previously used to characterize nonstarter LAB (Porcellato et al., 2012a, 2012b), and the results seemed to be promising in discriminating among the *L. casei* group species. This technique involves the amplification of a specific DNA sequence using a primer pair that allows annealing and DNA amplification in all the three species considered. The amplicons were produced using the qPCR technique and SYBR Green as an intercalating fluorescent dye and then subjected to a thermal gradient with temperature increments of 0.1 °C/sec using sensitive instrumentation that enables absolute precision of the temperatures used. By continuously monitoring the fluorescence emitted by SYBR Green, it is possible to assess the exact melting temperature of the amplicon, with a precision of 0.1 °C. Base differences and/or insertions or deletions of one or more bases is revealed, and this enables discrimination between amplicons and, consequently, between species.
Before using HRM analysis, a preliminary optimisation step was performed to determine the most effective primer pair among three candidate pairs. For optimisation, six strains whose original identification was confirmed by both species-specific PCRs and *tuf* multiplex PCRs were used: *L. casei* DSM20178 and LACcas7; *L. rhamnosus* DSM20021 and 2220; *L. paracasei* DSM20258 and DSM5622. HRM analysis on these strains revealed that only the primers P1V1-P2V1 were effective in discriminating among the three species (Figure 2.3). The primer pairs BA-338f/UN518r and Y1/Y2 yielded amplicons with highly similar melting curves comprising the following melting peaks: DSM20178 *L. casei*, 85.95 °C; DSM5622 *L. paracasei*, 85.55 °C; DSM20258 *L. paracasei*, 85.38 °C; 2220 *L. rhamnosus*, 85.47 °C; DSM20021 *L. rhamnosus*, 85.40 °C; LACcas7 *L. casei*, 84.30 °C (using BA-338f/UN518r) and DSM20178 *L. casei*, 84.90 °C; LACcas7 *L. casei*, 86.40 °C; DSM5622 *L. paracasei*, 84.85 °C; 2220 *L. rhamnosus*, 84.30 °C; DSM20021 *L. rhamnosus*, 84.30 °C; DSM20258 *L. paracasei*, 84.67 °C (using Y1/Y2). Considering these data and the melting curves (Figure 2.3, panel A, a; panel B, a), the normalised melting curves (Figure 2.3, panel A, b; panel B, b) and the principal component

analysis (PCA) graphs (Figure 2.3, panel A, c; panel B, c), these primer pairs could not be used to discriminate among the three species. However, the melting profiles and the normalised fluorescence curves as well as the PCA of the amplicons obtained using P1V1/P2V1 allowed to group the strains into 3 species-specific clusters (Figure 2.3, panel C, a, b, and c).

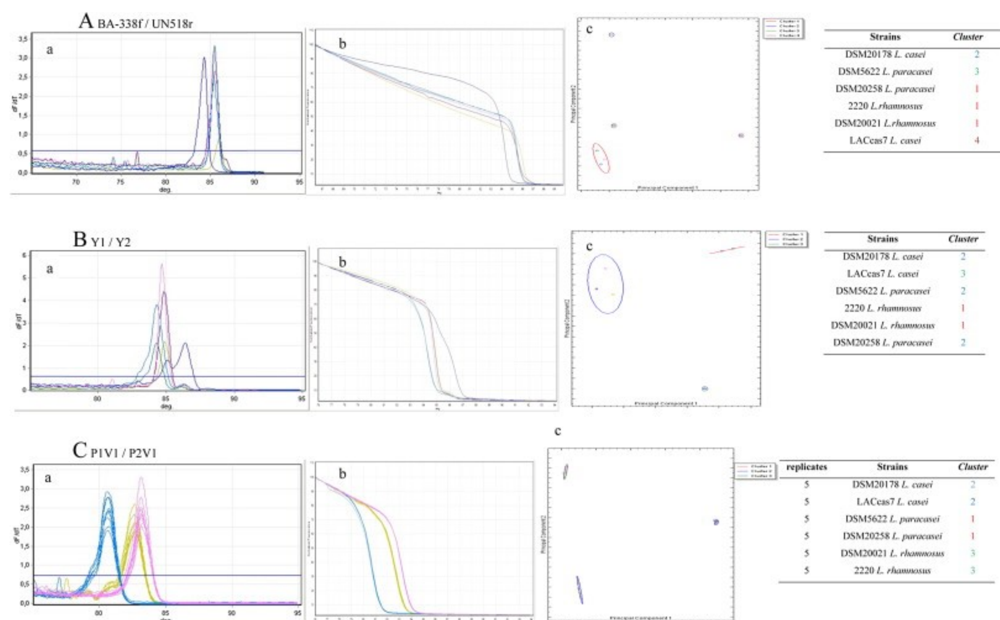

Fig. 2.3. HRM results obtained using the three different couples of primers. Panel A, primers BA-338f/UN518r; Panel B, primers Y1/Y2; Panel C, primers P1V1/P2V1. a) Melting curves profiles; b) Normalized melting curves; c) Principal component analysis (PCA).

To examine the reproducibility of these data, HRM analysis was performed on five replicates for each strain, and the curves overlapped completely. The average melting temperature of the standard strains tested was 83.69 ± 0.03 °C for *L. casei* (DSM20178 and LACcas7, 5 replicates per strain); 81.66 ± 0.06 °C for *L. rhamnosus* (DSM20021 and 2220, 5 replicates per strain), and 84.16 ± 0.04 °C for *L. paracasei* (DSM20258 and DSM5622, 5 replicates per strain). Therefore, HRM analysis yielded reproducible results. To highlight the differences among the three species, 3 difference graphs were generated using *L. casei* (DSM20171), *L. paracasei* (DSM20258) and *L. rhamnosus* (DSM20021) (confidence level of 90 %) as baselines (Figure 2.4, panel A, B, and C).

The blue, green and pink curves indicate ten replicates of the two *L. rhamnosus*, *L. casei* and *L. paracasei* strains, respectively. When one species was used as the baseline, the fluorescence values for that species were almost

a flat line, whereas the other two species had different performance curves. These graphs indicate the difference in the amplitudes of the curves and that this technique clearly discriminated the three species. Furthermore, the replicates yielded overlapping normalised curves, confirming the reproducibility of this technique. The different graph amplitudes are derived from melting curves that are always normalised to the same number of arbitrary fluorescence units by the Corbett 6000 software; therefore, these amplitudes can be compared across different runs using *L. casei* (DSM20178), *L. paracasei* (DSM20258) and *L. rhamnosus* (DSM20021) as standard controls in each run.

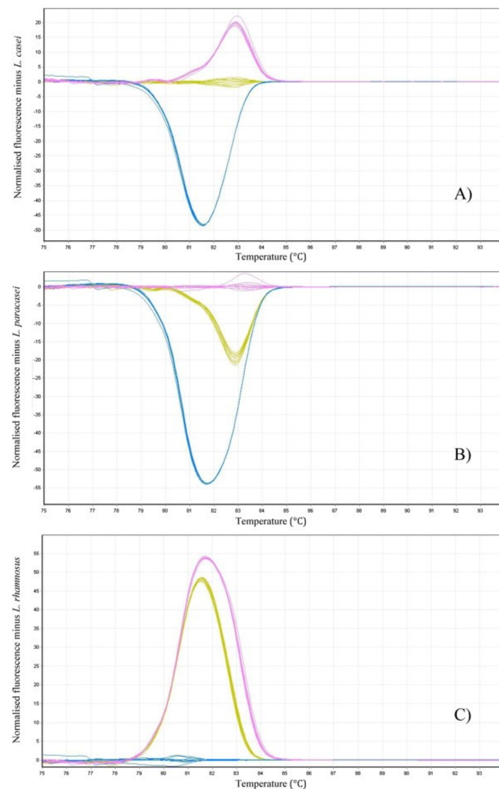

Fig. 2.4 Difference graphs obtained for the ten replicates of the three standard species. Panel A) *L. casei* was used as the baseline; panel B) *L. paracasei* was used as the baseline; panel C) *L. rhamnosus* was then used as the baseline..

After optimisation of HRM analysis, all the 194 strains, confirmed to belong to the *L. casei* group, were analysed using this method. Because of the large number of strains, more runs were required, and standard controls were included to reveal any changes and to compare all the tested strains at the end of the analysis. Therefore, after PCA, it was possible to identify the strains according to the cluster in which they were grouped (Figure 5). The example

shown in Figure 5 demonstrates that the three species were grouped in three well-defined and distant clusters (Figure 2.5, panel A). The normalised fluorescence curves overlapped completely (Figure 2.5, panel B); the difference graphs showing the normalised fluorescence curves vs. the control strains, also overlapped completely (Figure 2.5, panels C).

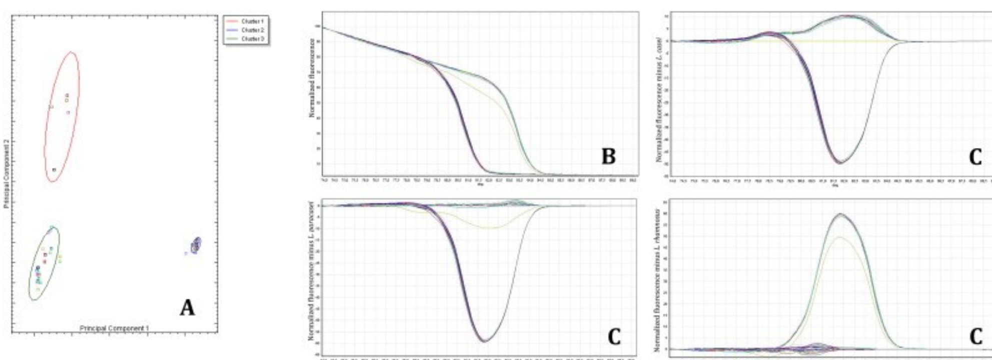

Fig. 2.5 HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, *L. casei*; cluster 2, *L. paracasei*; cluster 3, *L. rhamnosus*.

The results of the HRM analysis were consistent with the other methods used in this study, confirming the identity of 188 strains; inconsistent results were obtained only for the 6 strains shown in Table 2.3. Further studies such as whole-genome sequencing are required to elucidate the taxonomic classification of these strains. For 46 of the remaining 188 strains, the strain identity obtained using this method was inconsistent with the original identification (Table 2.4). Notably, the strain DIALYac was isolated from a commercial probiotic yogurt and identified as *L. casei* (Shirota); however, in this study, all methods classified this strain as *L. paracasei*, consistent with Sutula et al. (2012). Therefore, there is significant ambiguity in the use of the correct taxonomic name in industrial and scientific settings. In fact, also in recent studies the old classification name has been used (Douillard et al., 2013).

Table 2.4 Final identification of the tested strains.

Origin	Given identification
Raw and heat treated milk, yogurt, milking machines	***L. paracasei***: LMG9192[1], DSM5622[2] , P1E6[3], P2P3[3] P1E5[3], DialYac[15], DialDan[15] ***L. paracasei* subsp. *tolerans***: LMG9191[1], DSM20258[2], P1E4[3] ***L. rhamnosus***:, HA111[4], PRA152[5]
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***: LMG25880[1], LMG25883[1], LMG12164[1], 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], 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], M290[8], M303[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], M335[8], M307[8] ***L. rhamnosus***: M15[9], O14[8], PRA204[5], PRA232[5], PRA331[5], DBPZ0420[8],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]
Fermented sausages	***L. paracasei***: CTC1675[14] ***L. rhamnosus***: CTC1676[14], 2220[15]
Sourdoughs	***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], B082[17], B083[17], B085[17], B087[17], B161[17], B169, B171[17], B174[17], B195[17], B196[17], B350[17], B084[17], B086[17], B163[17], B164[17], B167[17], B168[17], B170[17], B172[17], B173[17], B175[17], B179[17], B166[17]
Bier, malt	***L. paracasei***: LACcas25[7], LACcas29[7], TMW 1.300[6]
Coffee	***L. casei***: DSM20178[2] ***L. rhamnosus***: DIAL40[15]
Humans (saliva, dental caries, blood, urethra, faeces of infants and adults)	***L. casei***: LMG23516[1], N87[16], N811[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]
Unknown	***L. paracasei***: NRRL B-456[19] ***L. rhamnosus***: NRRL B-176[19] , NRRL B-442[19], DSMZ20021[2]

***Strains with uncertain identification**: LMG 6904[1], DSM4905[2], DBPZ0420[8], DBPZ0571[8], DBPZ0734[8], N2014[16]

The strains underlined in red did not result to belong to L. casei group. The identification of the strains underlined in black was in disagreement with the original identification. The new identification has been reported

[1]LMG: BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms (BCCM™), Belgium.

[2]DSM: DSM, 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 delgi 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
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[15]Dipartimento di Scienze degli Alimenti, Università degli studi di Udine, Udine, Italy.
[16]Dipartimento di Agricoltura, Ambiente e Alimenti, Unversità degli Studi del Molise, Campobasso, Italy.
[17] Institute for Wine Biotechnology Department of Viticulture and Oenology, Stellenbosh 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

2.4 Conclusions

Accurate strain classification is critical for strains that are important for industrial purposes, including strains belonging to the *L. casei* group, which have probiotic properties. There is significant ambiguity in strain names within the *L. casei* group because some authors use the new classification system (Dellaglio et al., 2002; Dobson et al., 2004), whereas others do not (Mori et al., 1997; Ward and Timms, 1999; Vásquez et al., 2005; Desai et al., 2006). Furthermore, commercial strains are often described as "*L. casei"*, and this description is used for strains of any of these species. Furthermore, these species share close genetic relationships, and accurate identification is difficult (Nour, 1998; Beale et al., 2002; Klijn et al., 1991). The use of multiple coupled techniques can elucidate the taxonomic position of some strains; therefore, we proposed two new molecular tools to identify species belonging to the *L. casei* group: species-specific PCRs and HRM analysis. Both methods yielded accurate results, and considering the large number of strains tested (194), these methods were effective in discriminating among the three species within the *L. casei* group. For some strains, the results obtained using these methods were inconsistent with the original identification and the results obtained using other molecular methods. This discrepancy is not unexpected because in most cases, the original identification was performed using phenotypical and biochemical tests. These tests are often based on colour changes, which can be misinterpreted because colour changes are rarely precise and sharp. Misinterpretation of these results often leads to an incorrect identification. Furthermore, many strains were identified at a time when only one species, *L. casei,* and the subsp. *paracasei* were classified. Therefore, the classification of these strains was not consistent with the current strain taxonomy.

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3. Genetic characterization of *Lactobacillus casei* group strains

3.1 Aim of the study

The aim of this study was to investigate the genetic characteristics of the collected and previously identified strains belonging to *L. casei* group and of 14 more *L. paracasei* strains, identified by HRM technique. The strains analized consisted in 60 *L. rhamnosus*, 8 *L. casei* and 121 *L. paracasei*. The microorganisms were characterized for their genetic aspects using tree different techniques: Rep-PCR, RAPD and Sau-PCR.

3.2 Materials and methods

3.2.1 Strains

Sixty *L. rhamnosus*, eight *L. casei* and one hundred and twenty one *L. paracasei* strains collected and identified as described in chapter 2, and stored at the temperature of - 80° C in cryovials containing De Man and Rogosa Sharp broth (MRS, Oxoid, Italy) supplemented with 2% glycerol, were used as target strains.
At the time of use, the cultures were streaked on MRS Agar (Oxoid, 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, Italy).

3.2.2 DNA extraction from pure colonies

Two millilitres of a 48-h culture in MRS broth where centrifuged at 13,000*g* 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).

3.2.3 REP-PCR analysis

One hundred nanograms of the DNA extracted from the strains were subjected to rep-PCR analysis using primer (GTG)5 (5’-GTGGTGGTGGTGGTG-3’) as previously reported (Gevers et al., 2001). Reactions were carried out in a final volume of 25 μl containing: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTPs, 1 μM primer (GTG)5 and 1.25 U L *Taq*-polymerase (Applied Biosystem, Italy). The tubes were placed in a Euroclone Thermal Cycler (Celbio, Italy) and amplified for 31 cycles of denaturation at 94 °C for 3 s followed by a step at 92 °C for 30 s, annealing at 40 °C for 1 min

and extension at 65 °C for 8 min. The initial denaturation was at 95 °C for 2 min and the final extension at 65 °C for 8 min. Amplicons were separated in a 1.8% (w/v) agarose gel in TBE 0.5X at 120 V for 6 h. After the run, gels were stained with ethidium bromide 0.25 µl/ml (Sigma-Aldrich, Italy) from 20 to 30 min. Pictures of the gels were analysed by using the pattern analysis software package Gel Compare II Version 4.1 (Applied Maths, Belgium). Calculation of similarity in the profiles of bands was based on Pearson product-moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithms (Vauterin & Vauterin, 1992).

3.2.4 RAPD-PCR analysis

Amplifications were performed in a 25 µl reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl,1.5 mM MgCl2, 0.2 mM each dNTPs, 1 µM primer M13(50-GAG GGT GGC GGT TCT-30) (Huey & Hall, 1989), 1.25 U L *Taq*-polymerase (Applied Biosystem, Italy) and 100 ng of the DNA. PCR reactions were carried out with a Euroclone Thermal Cycler (Celbio, Italy) using the primer M13. The amplification cycle was as follows: 35 repetitions of 94°C for1 min, 38°C for 1 min, ramp to 72°C at 0.6°C/s, 72°C for 2 min. An initial denaturation at 94°C for 5 min, and a final extension at 72°C for 5 min, were also carried out. RAPD-PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gels in 0.5X TBE at 120 V for 4 h.Gels were stained in 0.5X TBE buffer containing 0.25 µl/ml ethidium bromide (Sigma) from 20 to 30 min. Pictures of the gels were digitally captured using the BioImaging System GeneGenius (SynGene) and the pattern analysis software package Gel Compare II Version 4.1 (AppliedMaths, Belgium) was used for the analysis. Calculation of similarity in the profiles of bands was based on Pearson product-moment correlation coefficients. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithms (Vauterin & Vauterin, 1992).

3.2.5 Sau-PCR analysis

One microliter of Sau3 A, restriction endonuclease (10U/µl), was used to digest 200 ng of DNA, extracted as previously described. The restriction reactions were carried out in a final volume of 20 µl. Amplification reaction was performed in a 50 µl reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTPs, 2 µM primer SAG1 (50-CCGCCGCGATCAG-30) (Corich et al., 2002), 1.25 U L *Taq*-polymerase (Applied Biosystem) and 1 µl DNA digested as previously described. PCR reactions were carried out with a Euroclone Thermal Cycler (Celbio, Italy)

using the following amplifications conditions: 25 °C for 5 min, ramp to 60 °C at 0.1 °C/s, 60 °C for 30 s, 2 cycles of 95 °C for 1 min, 50 °C for 15 s, ramp to 25 °C at 0.1 °C/s, ramp to 50 °C at 0.1 °C/s, 50 °C for 30 s, 35 cycles of 94 °C for 15 s, 46 °C for 1 min, 65 °C for 2 min, and the final extension at 65 °C for 2 min. Amplicons were separated in a 1.5% (w/v) agarose gel in TBE 0.5X at 120 V for 4 h. After the run, gels were stained with ethidium bromide 0.25 µl/ml (Sigma-Aldrich, Italy) from 20 to 30 min. Pictures of the gels were analysed by using the pattern analysis software package Gel Compare II Version 4.1 (Applied Maths, Belgium). Calculation of similarity in the profiles of bands was based on Pearson product–moment correlation coefficients. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithms (Vauterin & Vauterin, 1992).

3.3 Results and discussion

3.3.1 Rep-PCR Analysis

Lactobacillus rhamnosus

The profiles obtained with the Rep-PCR analysis of the 60 *L. rhamnosus* strains were analysed and compared using a coefficient of similarity of 83 %. The analysis led to the formation of eight main groups while nine strains did not group with others (Figure 3.1).

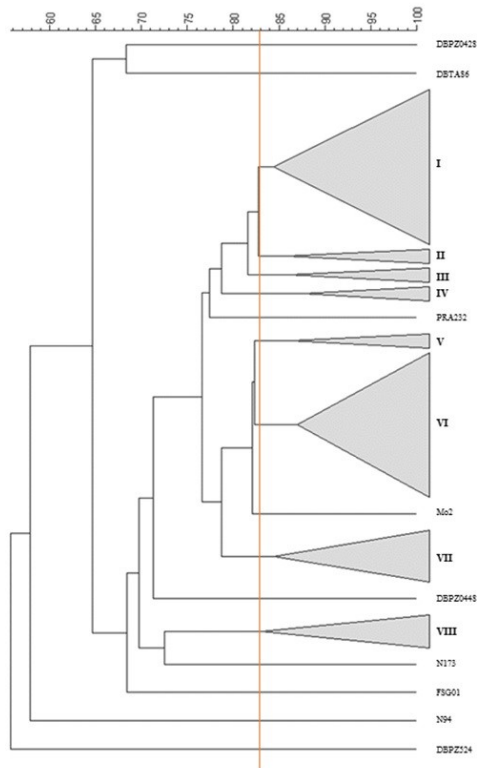

Figure 3.1 Cluster analysis resulting from the analysis Rep-PCR on *L. rhamnosus* using a coefficient of similarity of 83%. Clusters are numbered from I to VIII, while 9 strains are not grouped with other, remaining separate.

Table 3.1 Composition of clusters obtained using Rep-PCR analysis and application of UPGMA and Pearson's correlation coefficient with a similarity coefficient of 83%.

Cluster	**N. of strains**		**Source**	
		Human	**Food**	**Unknown**
I	17	14	3	0
II	2	1	1	0
III	2	0	2	0
IV	2	0	1	1
V	2	0	2	0
VI	16	9	7	0
VII	6	2	3	1
VIII	4	1	3	0

Table 3.1 shows the composition of the cluster obtained. It is possible to observe that almost all of the strains of human origin (23/26) are divided into two main groups: cluster I (14 strains) and cluster VI (9 strains).
The way the strains clustered was not strictly related to their source of isolation. Otherwise, at a level of similarity of 83%, nine strains did not cluster with others.

Lactobacillus casei* and *L. paracasei

129 strains of *L. paracasei* and *L. casei* were subjected to molecular characterization Rep-PCR. The results of fingerprinting analysis, carried out with a coefficient of similarity of 83%, led to the formation of 21 clusters, while 11 strains did not group with others (Figure 3.2). The composition of the clusters is summarized in Table 3.2.

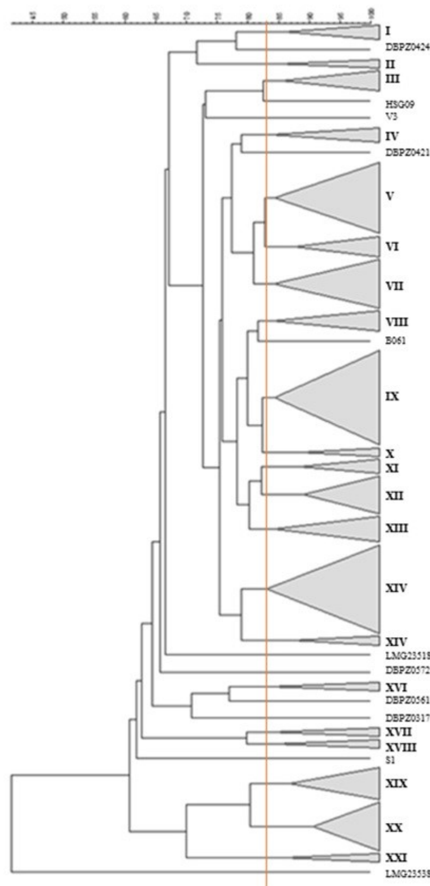

Figure 3.2 Cluster analysis resulting from the Rep-PCR analysis on *L. paracasei* and *L. casei* using a coefficient of similarity of 83%. The clusters are numbered, while 11 strains did not group with other, remaining separate.

Table 3.2 Composition of clusters obtained by Rep-PCR analysis and application of UPGMA and Pearson's correlation with a similarity coefficient of 83%.

Cluster	**Number of strains**	**Source**			
		Human	**Animal**	**Food**	**Unknown**
I	3	1	0	2	0
II	2	0	0	2	0
III	4	0	0	4	0
IV	3	1	0	2	0
V	13	5	0	8	0
VI	4	2	0	2	0
VII	9	2	1	6	0
VII	4	4	0	0	0
IX	17	3	0	14	0
X	2	0	0	2	0
XI	3	0	0	3	0
XII	7	0	0	7	0
XIII	5	0	0	4	1
XIV	16	0	0	16	0
XV	2	0	0	2	0
XVI	2	0	0	1	1
XVII	2	0	0	2	0
XVIII	2	0	0	2	0
XIX	6	2	0	4	0
XX	9	1	0	8	0
XXI	2	1	0	1	0

Observing the data reported in Table 3.2, it is possible to observe the presence of 3 principal clusters (V, IX, XIV). One of them was composed only by strains isolated from food matrixes. In general, strains are not grouped according to the source of isolation. The geographical origin of the strains was also taken into consideration, but no correlation was found between the strain's origin and the formation of the cluster. The high number of clusters is related to the presence of high variability among strains, as found by other authors that used this technique to discriminate among bacterial isolates (Comi et al., 2005; Iacumin et al., 2007).

3.3.2 RAPD-PCR Analysis

Lactobacillus rhamnosus

The fingerprint profile RAPD-PCR analysis was performed using a percentage of similarity of 88%. The UPGMA method allowed the formation of 10 clusters, while 10 strains did not grouped with the others. Figure 3.3 shows the clusters obtained, while in Table 3.3 the composition of the clusters and the source of isolation of the strains was described.

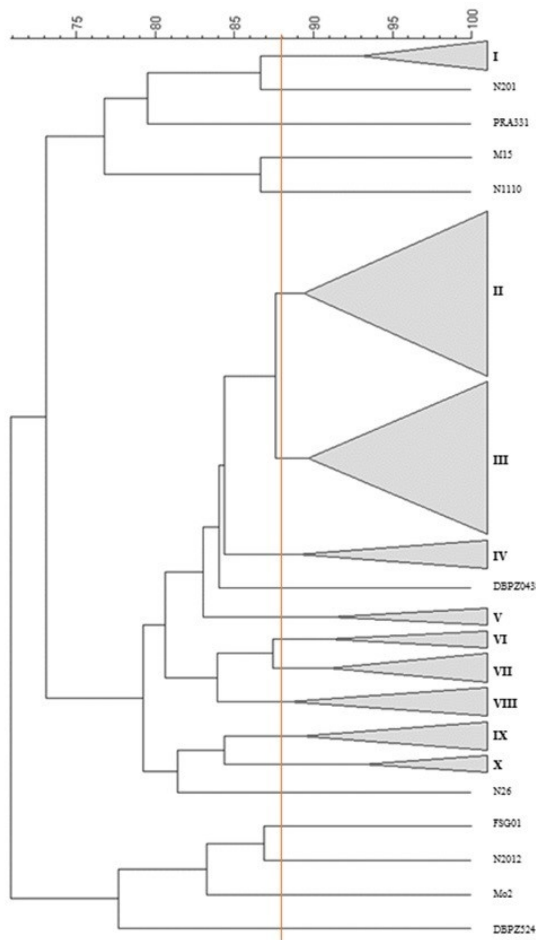

Figure 3.3 Cluster analysis resulting from RAPD-PCR analysis on *L. rhamnosus* using a coefficient of similarity of 88%. Clusters are numbered from I to X and there are 10 strains that are not grouped with other.

Table 3.3 Composition of clusters obtained using RAPD-PCR Analysis and application of UPGMA and Pearson's correlation coefficient, with a similarity coefficient of 88%.

Cluster	N. of strains	Source		
		Human	Food	Unknown
I	3	1	2	0
II	15	8	7	0
III	14	8	5	1
IV	3	1	2	0
V	2	0	1	1
VI	2	1	1	0
VII	3	3	0	0
VIII	3	1	2	0
IX	3	1	2	0
X	2	2	0	0

Observing the clusters composition, the presence of a high variability among strains was evident. However, this technique revealed a similar genetic profile for 15 strains in cluster II and 14 strains in cluster III. Clusters VII and X are composed only by strains isolated from human sources. In some cases, strains grouped on the basis of their origin. The high number of clusters and the presence of 10 strains forming a unique-strain cluster were related to the heterogeneity of the sources of isolation. Such a high level of variability has been previously observed by Martin et al. (2008). In their study, they compared the RAPD profiles of *Enterococcus* isolates from fermented sausages made in different factories. Also Turková et al. (2012) examined the RAPD profiles of several *Lactobacillus* spp. and they used a level of similarity of the 80% among *L. rhamnosus* strains isolated from several sources (human, silage, dairy products).

Lactobacillus casei **and** ***L. paracasei***

Figure 3.4 shows the cluster analysis obtained by RAPD-PCR analysis using a percentage of similarity of 83% that led to the formation of 16 clusters. Thirteen strains did not cluster with the others. In Table 3.4 the composition of the clusters is described by indicating the source of isolation of the analysed strains.

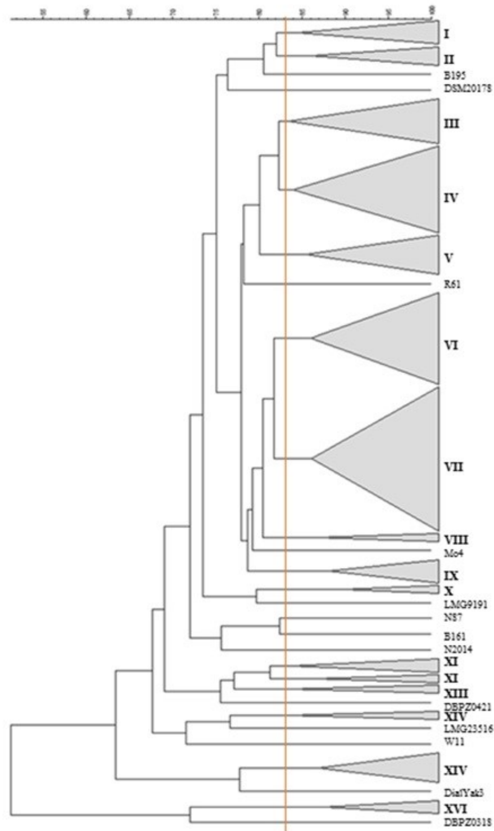

Figure 3.4 Cluster analysis resulting from the analysis RAPD-PCR on *L. paracasei* and *L. casei* using a coefficient of similarity of 83%. Clusters are numbered from I to XVI, while there are 13 strains not grouped with other.

Table 3.4 Composition of clusters obtained from RAPD-PCR analysis and application of UPGMA and Pearson's correlation coefficient with a similarity coefficient of 83%.

Cluster	**N. of strains**	**Source**			
		Human	**Animal**	**Food**	**Unknown**
I	5	0	0	5	0
II	4	0	0	4	0
III	9	2	0	6	1
IV	17	3	0	14	0
V	8	0	1	7	0
VI	18	2	0	16	0
VII	28	4	0	24	0
VIII	2	1	0	1	0
IX	5	0	0	5	0
X	2	1	0	1	0
XI	3	1	0	2	0
XII	2	1	0	1	0
XIII	2	1	0	1	0
XIV	2	0	0	2	0
XV	6	1	0	5	0
XVI	3	0	0	3	0

In this case, it is possible to observe the presence of 3 big clusters (IV, VI, VII) mainly comprensive of strains isolated from foods. Smaller clusters as I, IV, IX, XIV and XVI included few strains isolated all from food sources. In general, the distribution of the strains among clusters resulted to be heterogeneous. Turková et al. (2012), examined the RAPD profiles of several *Lactobacillus* spp. and used a level of similarity higher than 80% to group *L. casei-paracasei* strains isolated from human feaces. Another study reported similar values of similarity among *Lactobacillus* spp. isolated from different diary products from different geographical area, but, in that case, the clusters included mainly strains isolated from products of the same geographical area (Rossetti and Giraffa, 2005).

3.3.3 Sau-PCR Analysis

Lactobacillus rhamnosus

The last technique used for the characterization was Sau-PCR.
Figure 3.5 shows the clusters obtained using a percentage of similarity of 88%. UPGMA analysis allowed the formation of 10 cluster. At least, Table 3.5 showed the strains grouping in each cluster and their source of isolation.

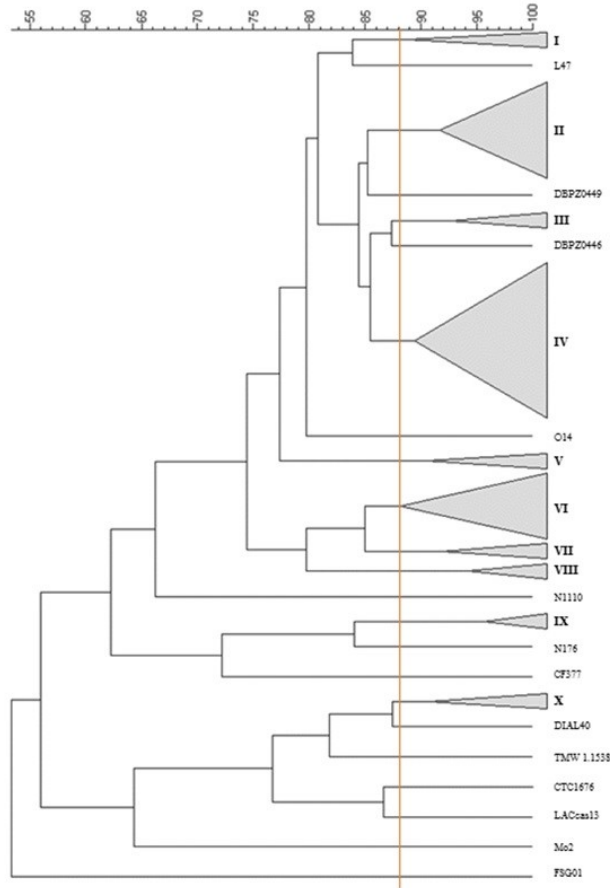

Figure 3.5 Cluster analysis resulting from Sau-PCR analysis on *L. rhamnosus* using a coefficient of similarity of 88%. Clusters are numbered from I to X. Thirteen strains had a genetic profile different from the others and did not group with others.

Table 3.5 Composition of clusters obtained using Sau-PCR analysis and application of UPGMA and Pearson's correlation coefficient, with a similarity coefficient of 88%.

Cluster	**Strains**	**Source**		
		Human	**Food**	**Unknown**
I	2	1	0	1
II	10	6	4	0
III	2	1	1	0
IV	16	10	6	0
V	2	2	0	0
VI	7	2	5	0
VII	2	1	1	0
VIII	2	0	1	1
IX	2	2	0	0
X	2	0	2	0

Observing Table 3.5 is evident that in cluster II and in cluster IV were grouped the highest number of strains, isolated from different sources. Only clusters V, IX and X were comprensive of strains with the same origin. Thirteen strains did not share any similarity with the others analysed, forming single-strain clusters.

Lactobacillus casei* and *L. paracasei

The clusters obtained after Sau-PCR analysis are shown in Figure 3.6. The results of the analysis, carried out with a coefficient of simitarity of 86%, led to the formation of 22 clusters, and 16 ungrouped strains. Table 3.6 defines the number of strains grouped in each cluster and their source of isolation.

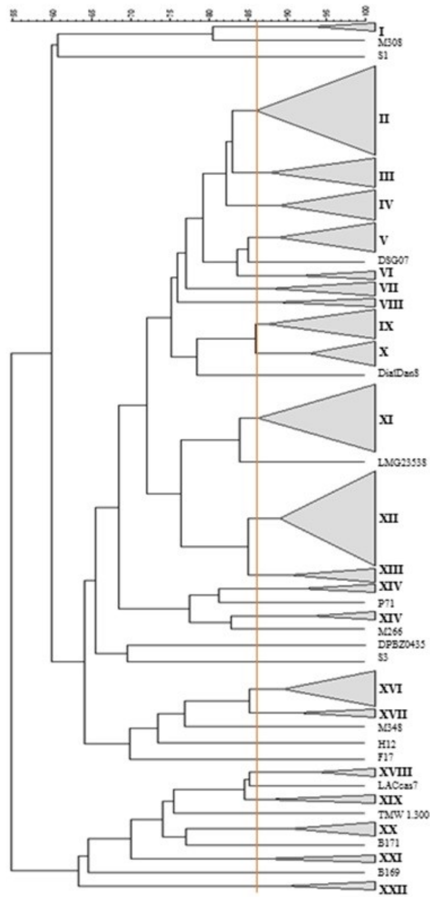

Figure 3. 6 Cluster analysis resulting from the analysis Sau-PCR on *L. paracasei* and *L. casei* using a coefficient of similarity of 85.4%. Clusters are numbered, while there are 18 strains not grouped with other, remaining separate.

Table 3.6 Composition of clusters obtained using Sau-PCR analysis and application of UPGMA and Pearson's correlation coefficient with a similarity coefficient of 85.4%.

Cluster	N. of strains	Source			
		Human	Animal	Food	Unknown
I	2	0	0	2	0
II	17	6	0	11	0
III	6	3	0	3	0
IV	6	1	0	5	0
V	6	0	1	5	0
VI	2	0	0	2	0
VII	3	0	0	3	0
VIII	2	0	0	2	0
IX	6	0	0	6	0
X	5	0	0	5	0
XI	13	3	0	9	1
XII	18	5	0	13	0
XIII	3	2	0	1	0
XIV	2	0	0	2	0
XV	2	0	0	2	0
XVI	7	0	0	7	0
XVII	2	0	0	2	0
XVIII	2	0	0	2	0
XIX	2	0	0	2	0
XX	3	0	0	3	0
XXI	2	0	0	2	0
XXII	2	0	0	1	1

The genetic profiles obtained using this assay demonstrated the presence of a great differentiation among strains, producing a high number of clusters, grouping only few strains, except for cluster II and XII, which included 17 and 18 strains respectively, composed mainly by strains isolated from food. Sixteen clusters included strains from the same origin. The presence of such a high number of clusters is synonymous of the presence of strains with high genetic variability, as confirmed also by other authors (Corich et al., 2002).

3.4 Conclusions

Within the *genus Lactobacillus*, the *L. casei* group has been the subject of several studies, due to the high similarity existing between the species belonging to this group and the complexity related to the correct identification of the strains. Strains belonging to this group have a declared probiotic activity, and several studies focused on the identification of other strains suitable for the production of probiotics and functional foods. A problem for the manufacturers of these products is the statement on the label of the microorganisms present in the product (species and the strain used).

The results obtained give important information regarding the genetic variability of the analysed strains.

Considering the analysis made on the *L. rhamnosus* strains, Rep-PCR resulted the less discriminant technique, as found by Turkova et al. (2012), allowing the formation of only eight clusters. Otherwise, strains isolated from human sources grouped all together in two big clusters. RAPD and Sau-PCR allowed the formation of ten clusters each, in which strains, in some cases, grouped following their source of isolation. For this reason, it is possible to affirm that these two techniques demonstrated a higher variability among strains, as confirmed by Corich et al. (2002).

In the case of *L. casei* and *L. paracasei*, all the techniques gave a high degree of variability, resulting in a high number of clusters. In particular, Sau-PCR UPGMA tree revealed the presence of few strains clustering together based on their isolation source. It is assumed that this high level of diversification is related on the heterogeneous origin of the strains (Iacumin et al., 2006).

The results of the analysis revealed the presence of a high variability among the tested strains, for all the speacies and with all the techniques used, as confirmed also from the high number of strains forming single cluster.

This result can be considered a good starting point in the research of potentially probiotic strains, as it would be interesting to assess if the high genotypic variability observed in this study corresponds to a high phenotypic variability.

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4. MLST and SAPD analysis to characterize the stress response in *Lactobacillus casei* group

4.1 Aim of the study

Because of their use as Non Starter Cultures (NSC) and their potential probiotic utilization, strains belonging to *Lactobacillus casei* group (*L. casei*, *L. paracasei*, *L. rhamnosus* and *L. zeae*) are becoming the focus of several scientific papers.
The misunderstandings regarding the correct identification of these strains at species level (as demonstrated in chapter 2) is due to the high level of similarity existing among these three species. Usually, molecular characterization of the strains using techniques such as RAPD, Rep-PCR, Sau-PCR, DGGE, TGGE, RLFP, allow to study the genetic variability existing among strains, but in order to know the differences existing among strains at level of single gene, it is necessary to examine the genome in a deepened way. In this work, a MultiLocus Sequence Typing method for the genetic characterization of strains belonging to *L. casei* group considering genes envolved in the general stress response was proposed. The obtained results were then compared with a new fingerprinting technique: SAPD-PCR (Specific amplified polymorphic DNA-PCR).

4.2 Materials and methods

4.2.1 Bacterial strains

A total of 45 strains (Table 4.1) belonging to the *L. casei* group identified as described in chapter 2 and characterized as described in chapter 3, were studied. They were chosen on the basis of the results obtained by Sau-PCR, as representative of each obtained cluster. Cultures were retrieved from storage (MRS broth with 30% glycerol at -80 °C) and routinely grown in MRS medium (Oxoid, Italy), for 48-h at 30 °C before the analysis.

4.2.2 DNA extraction

The DNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, USA). The concentration and purity of the extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) by recording its optical density at 260 and 280 nm, respectively.

Table 4.1 List of the strains tested in this work.

	SOURCE	SPECIES
LMG25883	Dairy product, China	*L. paracasei*
DBPZ0435	Caciocavallo Cheese (CS)	*L. paracasei*
NRRL B - 456	Unknown	*L. paracasei*
S1	Moliterno Cheese	*L. paracasei*
S3	Moliterno Cheese	*L. paracasei*
V3	Moliterno Cheese	*L. paracasei*
DSG07	Emmenthal Cheese	*L. paracasei*
ESG10	Parmigiano Reggiano Cheese	*L. paracasei*
PSG09	Provolone Torrealta Cheese	*L. paracasei*
M268	Canestrato Moliterno Cheese	*L. paracasei*
M348	Canestrato Moliterno Cheese	*L. paracasei*
R61	Pecorino Cheese	*L. paracasei*
F17	Pecorino Cheese	*L. paracasei*
I2	Sourdough Molise	*L. paracasei*
B161	Wine or must	*L. paracasei*
B169	Wine or must	*L. paracasei*
B171	Wine or must	*L. paracasei*
B195	Wine or must	*L. paracasei*
B196	Wine or must	*L. paracasei*
LacCas7	Grana Cheese	*L. paracasei*
TMW 1.300	Spoiled beer	*L. paracasei*
DIALDAN8	Fermented milk	*L. paracasei*
DIALYAK1	Fermented milk	*L. paracasei*
DIALYAK3	Fermented milk	*L. paracasei*
DIALYAK6	Fermented milk	*L. paracasei*
DBPZ0430	Provolone Cheese	*L. rhamnosus*
DBPZ0446	Caciocavallo Cheese	*L. rhamnosus*
DBPZ0448	Caciocavallo Cheese	*L. rhamnosus*
DSM20021	Unknown	*L. rhamnosus*
80	Grana Padano Cheese	*L. rhamnosus*
O14	Parsley Cheese	*L. rhamnosus*
CF377	Parmigiano Reggiano Cheese	*L. rhamnosus*
L47	Asiago Cheese	*L. rhamnosus*
N95	Body excreta	*L. rhamnosus*
N132	Body excreta	*L. rhamnosus*
N26	Body excreta	*L. rhamnosus*
N1110	Body excreta	*L. rhamnosus*
N202	Body excreta	*L. rhamnosus*
Mo2	Human	*L. rhamnosus*
CTC1676	Fermented sausage	*L. rhamnosus*
DSM4905	DSM4905	*L. casei*
CI4368	Parmigiano Reggiano Cheese	*L. casei*
N87	Body excreta	*L. casei*
N811	Body excreta	*L. casei*
N2014	Body excreta	*L. casei*

4.2.3 Selection of the genes for the MLST protocol

In order to characterize the strains for their stress response, *in silico* comparisons were made comparing the sequences of the stress related genes of the *L. casei* group strains that were enterely sequenced and deposited in GeneBank. The analysed genes are listed in Table 4.2. Comparisons were obtained using Mega6.06 software: first the sequences were aligned with Clustal W and then UPGMA trees were obtained (Appendix 1).

Table 4.2 List of the genes analized *in silico* and their correlation with different applied stresses.

Genes			**Stress**			**Reference**
	Oxidative	**Acid**	**Alkaline**	**Thermal**	**Osmotic**	
accC		X	X			Fernandez et al., 2008
clpB		X	X			van de Gutche et al., 2002; Sugimoto et al., 2006
clpC		X		X	X	van de Gutche et al., 2002; Fernandez et al., 2008
clpE		X		X		van de Gutche et al., 2002; Fernandez et al., 2008
clpL	X					Derrè et al., 1999; Suokko et al., 2005; Ricciardi et al., 2012; Wall et al., 2007; Fernandez et al., 2008; Vogel et al., 2005
clpP	X					Fernandez et al., 2008; Ricciardi et al., 2012
ctsR	X					Derrè et al., 1999; Ricciardi et al., 2012
cydA	X					Das et al., 2005
cydB		X		X		Das et al., 2005
cydC			X			Zhang et al., 2007
cydD		X	X	X	X	Zhang et al., 2007
dnaJ		X	X	X		Fernandez et al., 2008
dnaK		X				van de Gutche et al., 2002; Chavez de Paz 2007; Fernandez et al., 2008; Ricciardi et al., 2012
ef-g		X				Burns et al., 2010
groEL		X				De Angelis et al., 2001; Desmon et al., 2004; Prasad et al., 2003; Fernandez et al.,2008; Prasad et al., 2003; Vogel et al., 2005; van de Gutche, 2002
groES		X				Ricciardi et al., 2012; Burns, et al., 2010; Fernandez et al., 2008; Ricciardi et al., 2012
grpE	X					De Angelis et al., 2001; van de Gutche, 2002; Fernandez et al., 2008
hrcA	X					van de Gutche, 2002; Fernandez et al., 2008;

						van de Gutche, 2002
hpR						Prasad et al., 2003; Chavez de Paz et al., 2007
hprK	X	X				Burns et al., 2010
htrA		X		X		van de Gutche, 2002
luxS		X	X	X		Moslehi-Jenabian, 2009; Wu et al., 2012
nagA				X	X	Wu et al., 2012
nox		X		X		van de Guchte, 2002, Kang . et al., 2013
npr		X				Kang et al., 2013
pox	X					Burns et al., 2010
sod	X					Amanatidou et al., 2001
uvrA	X			X		van de Guchte, 2002; Cappa et al., 2005

accC, Acetyl-CoA carboxylase; ***clpB***, ATP-dependent chaperone ClpB; ***clpC***, ATP-dependent Clp protease, ATP-binding subunit ClpC; ***clpE***, ATP-dependent Clp protease, ATP-binding subunit ClpE; ***clpL***, ATP-dependent Clp protease ATP-binding subunit; ***clpP***, ATP-dependent Clp protease proteolytic subunit; ***ctsR***, transcription repressor of class III stress genes, ***cydA***, cytochrome d ubiquinol oxidase subunit I; ***cydB***, cytochrome d ubiquinol oxidase subunit II; ***cydC***, cytochrome D ABC transporter ATP-binding and permease; ***cydD***, ABC transporter ATP-binding protein; ***dnaJ***, molecular chaperone DnaJ; ***dnaK***, chaperonine dnaK; ***ef-g***, elongation factor; ***gro-EL***, chaperonin GroEL; ***groES***, co-chaperonin GroES; ***grpE***, protein grpE; ***hrcA***, heat-inducible transcription repressor; ***hpR***, phosphocarrier protein HPr; ***hprK***, HPr kinase/phosphorylase; ***htrA***, serine protease HtrA; ***luxS***, S-ribosylhomocysteinase; ***nagA***, N-acetylglucosamine-6-phosphate deacetylase; ***nox***, NADH oxidase; ***npr***, NADH peroxidase; ***pox***, pyruvate oxidase; ***sod***, superoxidase dismutase; ***uvrA***, excinuclease ABC subunit A.

On the basis of the results obtained from the *in silico* analysis, eight genes related to stress response were selected: *ctsR*, *hrcA*, *cydD*, *cydA*, *nox*, *npr*, *pox* and *dnaK*. Eight couples of primers (listed in Table 4.3) were designed using *Lactobacillus casei* ATCC 334, *Lactobacillus rhamnosus* GG and *Lactobacillus paracasei* N1115 as reference genomes, available on NCBI (http://www.ncbi.nlm.nih.gov/) and tested *in silico* using FastPCR 6.1 software (Kalendar et al., 2009).

Table 4.3 List of the primers designed in this work.

Gene	Primers	Sequence (5'-3')	PCR product lenght (bp)	Annealing Temperature
nox	NOXf	gttggtgatcatgtcagtga	951bp	65 °C
	NOXr	tggaagagcatgtcgaccat		
npr	NPRf	cgcgacacgaagaacg	970bp	63.5 °C
	NPRr	cctggctggaagaagaagtc		
hrcA	HRCAf	cgagtgctaaaggtgaggtga	957bp	64 °C
	HRCAr	ccgatcatcttggagtacggcat		
ctsR	CTSRf	gtgtaatagtcaagattggt	547bp	49.5 °C
	CTSRr	gcactaggggtaaataagtgg		
cydA	CYD A2f	gccggaatttttgcatgtttt	877bp	64.5 °C
	CYD A2r	aggcatgactgaacgcctcc		
pox	POX2f	ttcccattccagccwtactt	750bp	55 °C
	POX2f	agttgttcaaccggaatt		
cydD	CYD D2f	atgccgattcgtgattttgg	951bp	64.5 °C
	CYD D2f	cattgaattcacctcgcatttc		
dnaK	DNAK2f	cggaaacatccttgtagtcac	813bp	55 °C
	DNAK2f	gcatyaacccagacgaagc		

PCR were performed in a final volume of 50 μL containing 10mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) dNTPs, 1 μM primer, and 1.25 U of *Taq*-polymerase (Applied Biosystems, Italy). The amplification cycle was performed as follows: initial strand denaturation at 95 °C for 5 min; then 35 cycles of 95 °C for 1 min, annealing step for 1 min (annealing temperatures are listed for each couple of primers in Table 4.3) and 72 °C for 1.5 min; and a final extension step at 72 °C for 7 min. All the reactions were performed using a Celbio Thermocycler (Euroclone, Italy). The PCR products were checked by electrophoresis in 2% agarose gel using as running buffer TBE 0.5X. Etidium bromide (0.5 μg/ml) was added to the gel before solidification. After the run, the gels were examined using the BioImaging System GeneGenius (SynGene, United Kingdom). Subsequently, PCR products were purified using QIAquick PCR Purification Kit (Quiagen, Italy) and sent to MWG Eurofin Genomics Company (Germany) for sequencing.

4.2.4 Data analysis

For each *locus*, the sequences obtained from all the strains were compared and allele numbers were assigned to each unique sequence. For each strain it was established a Strain Type (ST) Number, deriving from the combination of the numbers corresponding to the allelic version of each *locus*. Sequences, which resulted in a difference, even at a single nucleotide site level, were considered as distinct alleles.

Sequences alignments and comparisons between sequences were performed using MEGA version 6.06 software (http://www.megasoftware.net). Phylogenetic trees of each *locus* and concatenated sequences of the analysed *loci* were obtained using the same program by the Unweight Pair Group Method with Arithmetic averages (UPMGA). Minumum spanning tree was created with the BioNumeric Software (Applied Maths, Belgium). DnaSP software version 5.10.1 (http://www.ub.edu/dnasp) was used to perform the descriptive analysis such as fragment size, mol % G+C content, number of polymorphic sites and alleles, π (nucleotide diversity per site), average number of nucleotide difference per site), θ (average number of nucleotide difference per site) and Tajima's D value.

4.2.5 Whole genome sequencing

On the basis of the MLST analysis results, 14 strains (I2, DialYak1, NRRL-B 176, TMW 1.300, DSM 4905, LMG 25883, M268, N1110, B 196, O14, N202, No2, N2014, CI 4368) were selected for the whole genome sequencing using MiSeq (Illumina, UK) at GenProbio srl (Parma, Italy) following the supplier's protocol (Illumina, UK). Fasta files obtained from targeted genome sequencing of the isolated strains were used as input for assembly with MIRA software. Protein-encoding ORFs were predicted using Prodigal (BMC Bioinformatics) and assignment of protein function to predicted coding regions was performed using a custom script based on RapSearch2 software, PFAM database and the non-redundant protein database provided by the National Center for Biotechnology Information. Whole genome alignments between contigs obtained from assembly of metagenomics and isolate datasets were obtained using MAUVE software and the metagenomic genome as reference. After the analysis of the row data, comparisons among the core genome of the strains have been made, while pangenomes were compared using the Genome-To-Genome Distance Calculator (GGDC) (http://ggdc.dsmz.de/distcalc2.php).

4.2.6 Specifically Amplified Polymorphic DNA (SAPD) PCR

SAPD-PCR was performed as previously described by Pfannebecker and Fröhlich (2008). Briefly, four separate primers were used, with every primer consisting of an adenine deoxyribonucleotide at 5′ end, followed by the NotI recognition sequence (5′-GCGGCCGC-3′), followed by a further desoxyribonucleotide (A, C, G or T) at 3′ end of the primer. Amplification reactions were performed in 25 μl reaction mixtures containing 5 μl template DNA (50 ng μl−1), 1 μl *Taq* DNA polymerase (1 U) (Applied Biosystems, Italy), 1 μl dNTP mix (0.1 mM each dNTP) (Applied Biosystems, Italy), 1 μl of primer (5 μM) (Sigma-Aldrich, Italy), 2 μl of $MgCl_2$ (2,5 mM) (Applied

Biosystems, Italy), 2.5 µl of 10× reaction buffer (containing 2 mM $MgCl_2$) (Applied Biosystems, Italy) and 12.5 µl of water (Applied Biosystems, Italy). The PCR protocol was performed as follows: 5 min at 95 °C for initial denaturation, followed by 35 cycles consisting of 1 min at 94 °C for denaturation, 1 min at 35 °C for annealing, followed by a prolonged ramp (15 temperature increments of 0.5 °C for 12 s, 1 min at 42.5 °C, 7 temperature increments of 1.5 °C for 12 s), followed by 5 min at 72 °C for elongation. The final elongation step was carried out at 72 °C for 10 min. SAPD-PCR amplifications were performed in a Celbio Thermocycler (Euroclone, Italy).

4.2.7 Gel electrophoresis

PCR products were analysed on a 1.5% agarose gels containing 0.035% sodiumsilicate (Na_2SiO_3) (Sigma-Aldrich, Italy) in 1×TBE buffer and separated in a horizontal electrophoresis system (BioRad, Munich, Germany) at 120 V. The GeneRuler™ DNA Ladder Mix SM0331 (Thermo Scientific, Italy) was used as molecular size marker. Agarose gels were stained with a 0.002 mg/ml ethidium bromide solution (Sigma-Aldrich, Italy). Visualization of gel bands under UV light was carried out in a BioImaging System GeneGenius (SynGene, United Kingdom).

4.2.8 Cluster analysis of SAPD-PCR patterns

After electrophoresis, the SAPD-PCR patterns were analysed using the Gel Compare II version 4.1 (Applied Maths, Belgium). The calculation of similarities in the profiles of bands was based on the Pearson product correlation coefficient. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm and by the Composite Database Analysis method (Vauterin and Vauterin, 1992).

4.3 Results

In order to characterize the strains for the stress response, *in silico* comparisons were made comparing the sequences of the stress related genes of the *L. casei* group strains that were totally sequenced and deposited in GenBank. The sequences were aligned with Clustal W and then UPGMA trees have been designed. After the *in silico* analysis, eight genes related to stress response were selected: *ctsR*, *hrcA*, *cydD*, *cydA*, *nox*, *npr*, *pox* and *dnaK*. The sequences of the eight chosen *loci* were determined for all the examined strains by the sequencing of the PCR products obtained from each *locus*.

Among the fifteen *L. rhamnosus* strains, from ten to fifteen alleles per *locus* were found (Table 4.4).

Table 4.4 Allelic variants and Strain Types (STs) in *L. rhamnosus*.

	SOURCE	ALLELIC PROFILE								STs
		nox	***npr***	***hrcA***	***ctsr***	***cydA***	***pox***	***cydD***	***dnaK***	
DBPZ0430	Provolone Cheese	1	1	1	1	1	1	/	1	1
DBPZ0446	Caciocavallo Cheese	2	2	2	2	2	2	1	2	2
DBPZ0448	Caciocavallo Cheese	1	3	3	3	3	3	/	3	3
DSM20021	Unknown	1	1	4	4	4	4	/	4	4
FSG01	Grana Padano Cheese	/	4	/	5	/	/	/	/	5
O14	Parsley Cheese	3	5	5	6	5	5	2	5	6
CF377	Parmigiano Reggiano Cheese	4	6	6	7	6	6	3	6	7
L47	Asiago Cheese	5	7	7	8	7	7	4	7	8
N95	Body excreta	6	8	8	9	8	8	5	8	9
N132	Body excreta	7	9	9	10	9	9	6	9	10
N26	Body excreta	8	10	10	11	10	10	7	10	11
N1110	Body excreta	9	1	11	12	4	11	8	11	12
N202	Body excreta	10	11	12	13	11	12	9	12	13
Mo2	Human	11	11	13	14	12	13	10	7	14
CTC1676	Fermented sausage	12	12	14	15	13	14	/	13	15
		ALLELIC VARIANT								
		12	12	14	15	13	14	10	13	15

cydD locus was not amplified in strains DBPZ0430, DBPZ0448, DSM20021, FSG01, and CTC1676. Strain FSG01 give the production of the amplicon only only for *npr* and *ctsR loci*. The mean G + C content of the different gene fragments varied from 0,432 (*ctsR*) to 0,527 (*npr*) mol %. The number of polymorphic sites ranged from 6 (*hrcA*) to 57 (*cydD*). π and θ values varied respectively from 0.00511 (*hrcA*) to 0.02674 (*cydD*) and from 0.00830 (*hrcA*) to 0.02934 (*cydD*). Tajima's D value observed ranged from -1.44827 (*hrcA*) to 0.33663 (*npr*) and none of the observed values was statistically significant (Table 4.5).

Table 4.5 Results of the descriptive analysis of the strains divided by species.

LOCUS	**FRAGMENT SIZE**	**MEAN DNA G + C CONTENT (mol%)**	**No. of polimorphic sites**	**Nucleotide diversity per site π**	**Average number of nucleotide differences per site θ**	**Tajima's D value**	**No. of alleles**
			Lactobacillus casei				
nox	951	0,515	165	0,11601	0,13171	-0,90886	5
npr	970	0,535	224	0,15229	0,16561	-0,61371	4
hrcA	957	-	-	-	-	-	2
ctsR	547	0,454	90	0,07091	0,08601	-1,33401	4
cydA	877	0,514	185	0,09694	0,13775	-0,93870	5
pox	750	-	-	-	-	-	1
cydD	951	0,532	194	0,12248	0,13384	-0,89020	1
dnaK	813	-	-	-	-	-	1
			Lactobacillus rhamnosus				
nox	951	0,503	22	0,01149	0,01163	-0,05037	13
npr	970	0,527	55	0,02052	0,01906	0,33663	13
hrcA	957	0,518	6	0,00511	0,00830	-1,44827	15
ctsR	547	0,432	12	0,00758	0,00976	-0,90591	16
cydA	877	0,480	17	0,01946	0,01965	-0,04082	14
pox	750	0,489	40	0,01826	0,01788	0,09195	15
cydD	951	0,506	57	0,02674	0,02934	-0,43453	11
dnaK	813	0,493	32	0,01680	0,01694	-0,03587	14
			Lactobacillus paracasei				
nox	951	0,498	125	0,10692	0,08694	0,90553	23
npr	970	0,511	214	0,10839	0,19107	-1,71777	22
hrcA	957	0,505	103	0,07724	0,05916	1,19788	22
ctsR	547	0,449	79	0,06055	0,05216	0,62621	23
cydA	877	0,531	195	0,08176	0,06913	0,71217	24
pox	750	0,485	62	0,01947	0,06182	0,67003	22
cydD	951	0,477	28	0,07197	0,06780	0,23137	20
dnaK	813	0,467	54	0,04847	0,05CF377	-0,19454	22

Among the twenty-five strains of *L. paracasei*, from twenty to twenty-four alleles per *locus* were found (Table 4.6).

Table 4.6 Allelic variants and Strain Types (STs) in *L. paracasei.*

	SOURCE	ALLELIC PROFILE								STs
		nox	***npr***	***hrcA***	***ctsR***	***cydA***	***pox***	***cydD***	***dnaK***	
LMG25883	Dairy product	1	1	1	1	1	1	1	1	1
DBPZ0435	Caciocavallo Cheese	2	2	2	2	2	2	2	2	2
NRRL B – 456	NRRL B-456	3	3	3	3	3	3	3	3	3
S1	Moliterno Cheese	4	3	4	4	4	4	4	4	4
S3	Moliterno Cheese	5	4	5	5	5	5	5	5	5
V3	Moliterno Cheese	6	3	6	6	6	3	6	6	6
DSG07	Emmenthal Cheese	7	5	7	7	7	6	7	7	7
ESG10	Parmigiano Reggiano Cheese	8	6	8	8	8	7	8	8	8
PSG09	Provolone Torrealta Cheese	9	7	9	9	9	8	9	9	9
M268	Canestrato Moliterno Cheese	10	8	10	10	10	9	10	10	10
M348	Canestrato Moliterno Cheese	11	9	4	11	11	10	1	11	11
R61	Pecorino Cheese	12	10	1	12	12	11	11	12	12
F17	Pecorino Cheese	13	11	11	13	13	12	12	13	13
I2	Sourdough Molise	14	12	1	14	14	13	6	14	14
B161	Wine or must	15	13	12	15	15	14	13	15	15
B169	Wine or must	16	14	13	16	16	15	14	16	16
B171	Wine or must	17	15	14	1	17	16	15	17	17
B195	Wine or must	18	16	15	17	18	17	16	14	18
B196	Wine or must	17	17	16	18	19	18	17	18	19
LacCas7	Grana Granarolo	19	2	17	19	19	18	18	19	20
TMW 1.300	Spoiled beer	20	18	18	1	20	7	19	20	21
DIALDAN8	Fermented milk	20	19	19	20	21	19	7	21	22
DIALYAK1	Fermented milk	21	20	20	21	22	20	20	22	23
DIALYAK3	Fermented milk	22	21	21	22	23	21	7	23	24
DIALYAK6	Fermented milk	23	22	22	23	24	22	7	22	25
		ALLELIC VARIANT								
		23	22	22	23	24	22	20	22	25

The mean G + C content of the different gene fragments varied from 0.449 (*ctsR*) to 0.531 (*cydA*) mol %. The number of polymorphic sites ranged from 28 (*cydD*) to 214 (*npr*). π and θ values varied respectively from 0.01947 (*pox*) to 0.10839 (*npr*) and from 0.05105 (*dnaK*) to 0.19107 (*npr*). Tajima' D value observed ranged from -1.71777 (*npr*) to 1.19788 (*hrcA*) and none of the observed value was statistically significant (Table 4.5).
Among the *L. casei* analysed strains, strains N87, N811, N2014 did not produce PCR products for *hrcA locus*. *pox* and *dnaK loci* were amplified only in strain DSM4905. In strain N811 also *cydD locus* did not allowed the

production of amplicons, while in strain N2014 no PCR product was found for *npr locus* (Table 4.7).

Table 4.7 Allelic variants and Strain Types (STs) in *L. casei.*

	SOURCE	ALLELIC PROFILE								STs
		nox	***npr***	***hrcA***	***ctsr***	***cydA***	***pox***	***cydD***	***dnaK***	
DSM4905	DSM4905	1	1	1	1	1	1	1	1	1
CI4368	Parmigiano Reggiano Cheese	2	2	2	2	2	/	2	/	2
N87	Body excreta	3	3	/	3	3	/	3	/	3
N811	Body excreta	4	3	/	3	4	/	/	/	4
N2014	Body excreta	5	4	/	4	5	/	4	/	5
		ALLELIC VARIANT								
		5	4	2	4	5	1	1	1	5

From five to one alleles per *locus* were found. The mean G + C content of the different gene fragments varied from 0.454 (*ctsr*) to 0.535 (*npr*) mol %. The number of polymorphic sites ranged from 90 (*ctsr*) to 224 (*npr*). π and θ values varied respectively from 0.07091 (*ctsr*) to 0.15229 (*npr*) and from 0.08601 (*ctsR*) to 0.16561 (*npr*). Tajima' D value observed went from -1.33401 (*ctsR*) to -0.61371 (*hrcA*). Only for *cydD locus*, the observed value was statistically significant (Table 4.5).

4.3.1 UPGMA tree based on MLST data

Phylogenetic trees were constructed by MEGA version 6.06 software using the Unweight Pair Group Method with Arithmetic averages (UPMGA) for each *locus* grouping the sequences divided per species and considering all the species together.
Among *L. rhamnosus*, minimal differences among strains were observed in all the analysed *loci*. The results are shown for each *locus* in Figure 4.1. In *ctsR* tree there are 3 clusters with a percentage of divergence of the 2.3%, while in *cydA*, it is possible to observe 2 clusters with a percentage of divergence of the 4%. In *cydD* UPGMA tree, the differences observed were very low; the percentage of divergence among strains was only 0.14%. Regarding *dnaK locus* the analysis resulted in the formation of tree clusters using a percentage of divergence of the 1.7%. Concerning *nox,* the differences observed among strains were of the 3.9%, with tree clusters. Whereas among the *npr locus* the divergence was 2.15%. and 1.4% was the percentage of divergence observed among clusters.

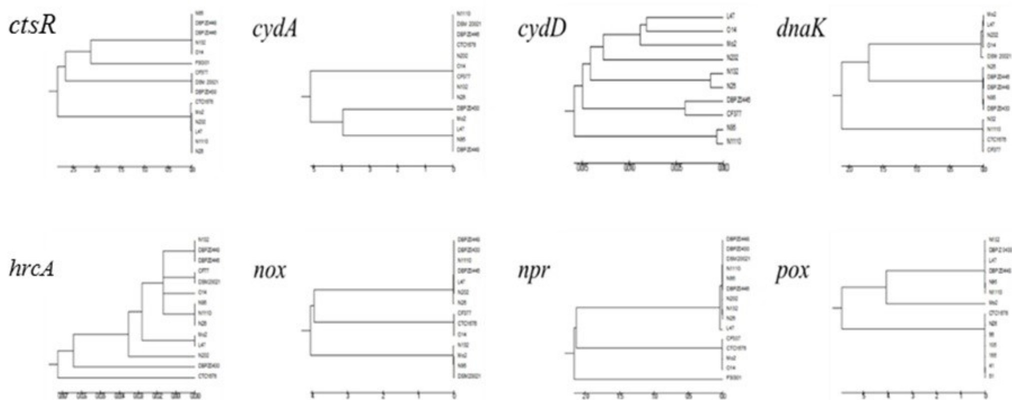

Figure 4.1 UPGMA trees obtained comparing the sequences of *L. rhamnosus* strains analysed in this work (Appendix 2 for a more detailed view).

In figure 4.2 the results obtained comparing strains belonging to the *L. paracasei* species are shown. As far as *ctsR*, *cydA*, *hrcA*, *dnaK*, *npr* and *nox* are concerned, it is possible to observe the presence of 2 clusters, while considering *cydD*, the divergence observed among strains was 1.15%, forming 3 clusters. The analysis of the concatenated sequences led to the formation of 5 clusters. Strains B161, LacCas7 and DBPZ0416 always grouped together in all the dendograms.

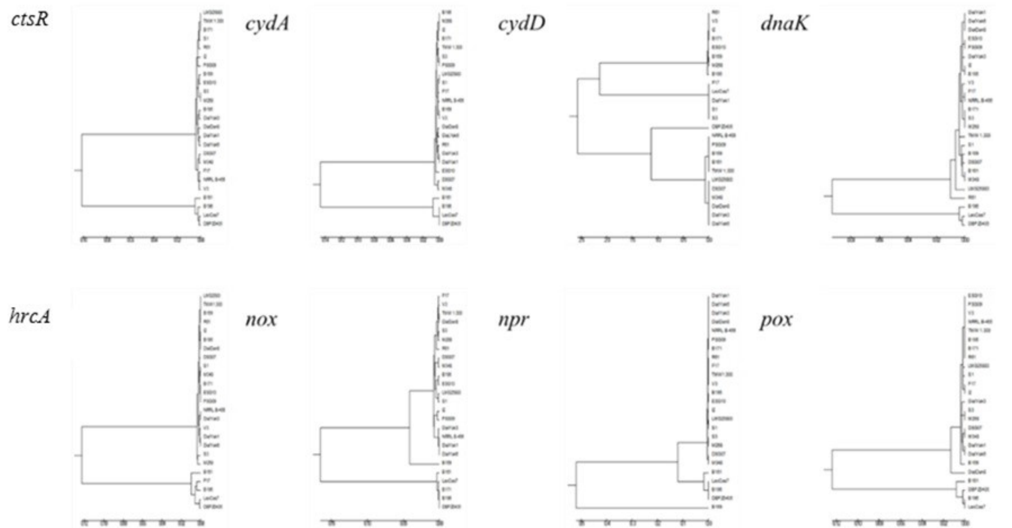

Figure 4.2 UPGMA trees obtained comparing the sequences of *L. paracasei* strains analysed in this work (Appendix 2 for a more detailed view).

Among the *L. casei* strains examined (Figure 4.3) it is possible to observe that, for all the *loci*, the strain DSM4905 always clustered alone. The highest percentage of divergence observed was 3.4% for *ctsR locus*, the lowest was 0.12% for *cydA locus*.

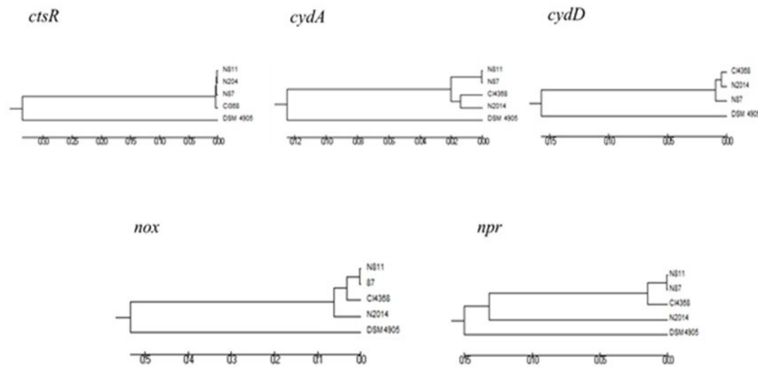

Figure 4.3 UPGMA trees obtained comparing the sequences of *L. casei* strains analysed in this work (Appendix 2 for a more detailed view).

After, the analysis of the sequences devided per species was performed for each *locus*, all the sequences of the three species were aligned and UPGMA trees were realized (Figure 4.4).

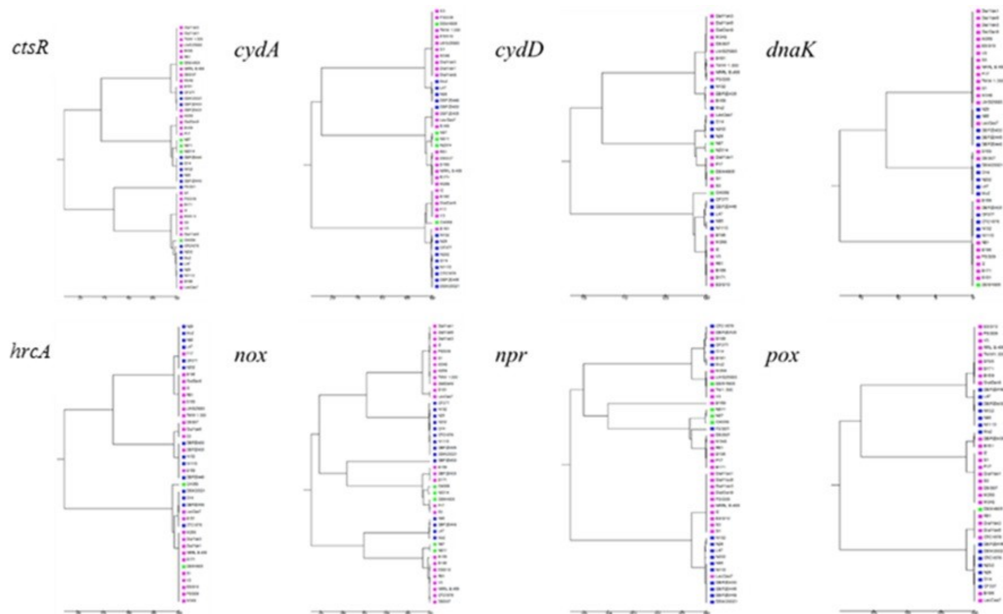

Figure 4.4 UPGMA trees obtained comparing the sequences obtained for each *locus* in all the species (Appendix 2 for a more detailed view).

After that, UPGMA trees were realized comparing the concatenated sequences (Figure 4.5).

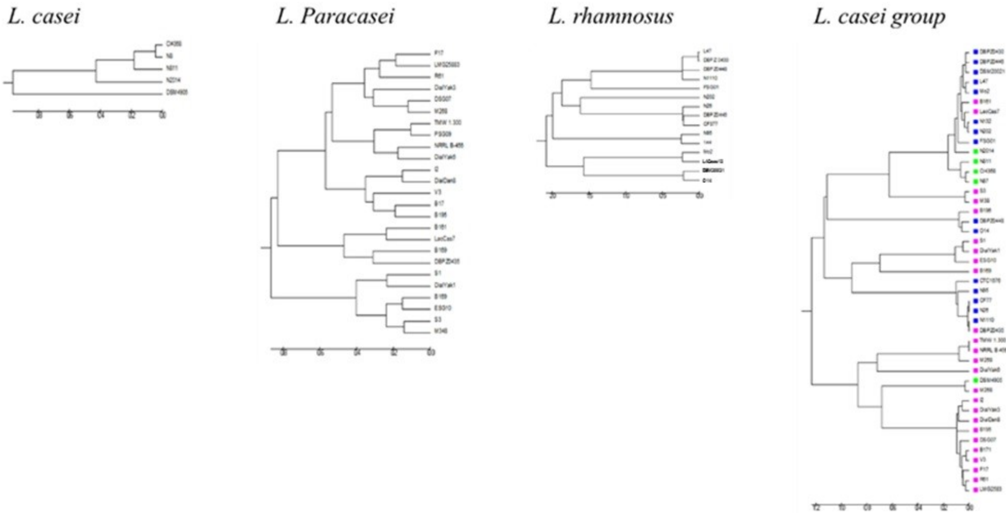

Figure 4.5 UPGMA trees obtained comparing the concatenated sequences obtained for each species and of all the species (Appendix 2 for a more detailed view).

Looking at the pictures, it is interesting to observe how some strains belonging to different species grouped together. In particular, *L. casei* DSM4905 always clustered with other *L. paracasei*.

The STs sequences obtained for each species were put all toghether and the minimum spanning tree was created. Fig. 4.6 shows how strains grouped together.

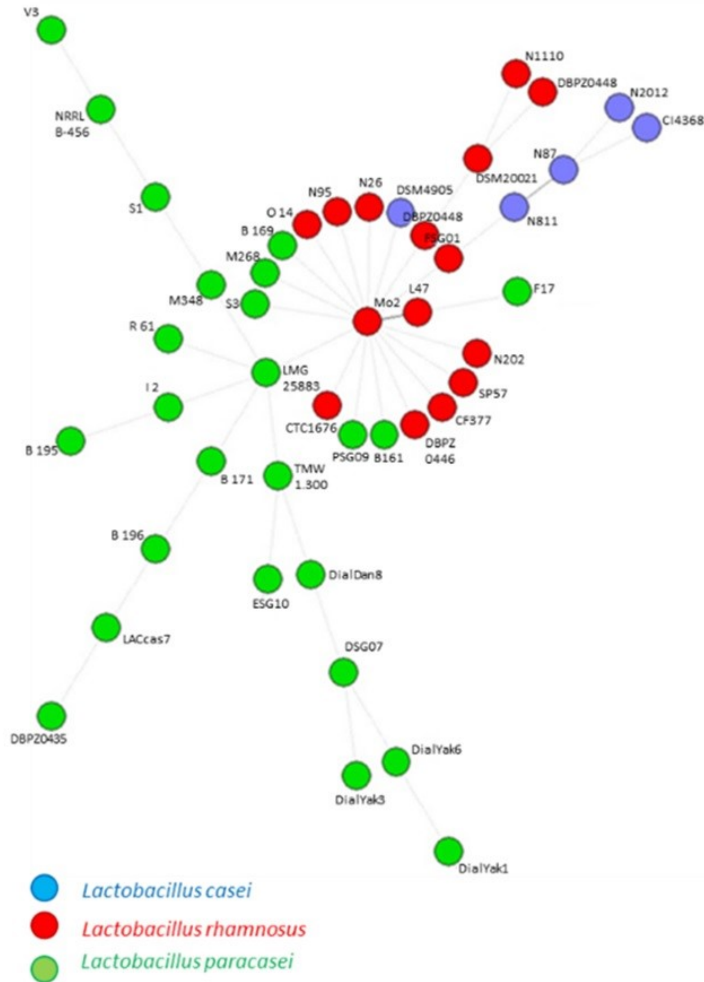

Figure 4.6 Minimum spanning tree analysis of 45 *L. casei* group isolates based on allelic profiles of 8 gene fragments and according to species affinity. Each circle represents the sequence type, the size of the circle is proportional to the number of isolates within any given ST.

4.3.2 Whole genome sequences comparison

The results obtained by the comparison of the core-genome (Figure 4.7) were characterized by the formation of two cluster: one including all the *L. paracasei* strains plus the strain DSM 4509 (confirming the results of the MLST UPGMA trees) and another one including *L. rhamnosus* and the other two *L. casei* strains. Strain B196 also clustered in this group, confirming how

hard is to find a phylogenetic marker for the discrimination between strains belonging to *L. casei* group, without misunderstandings.

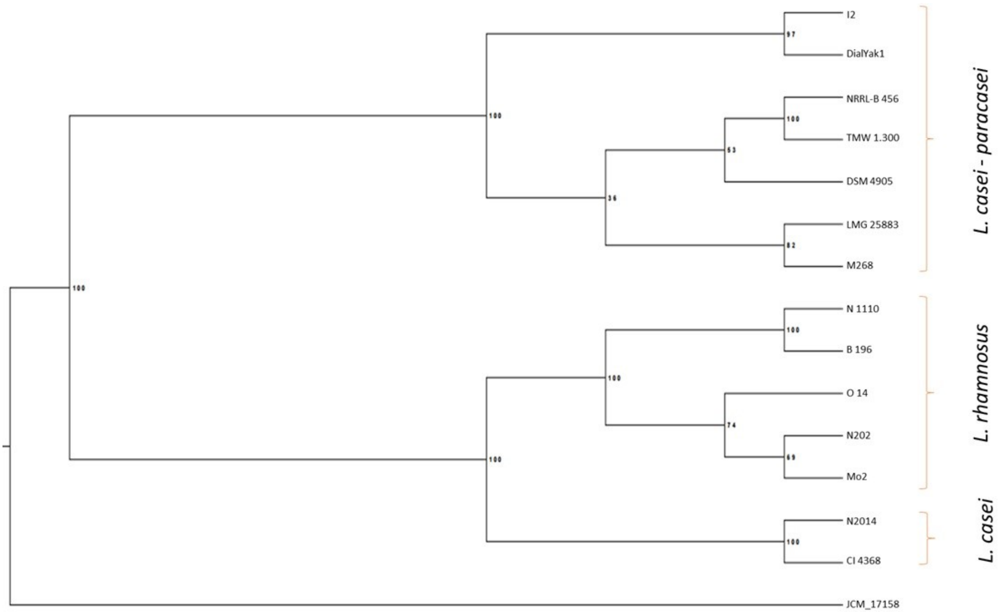

Figure 4.7 Core-genome phylogenetic tree for the four-teen sequenced genomes. The genomes were compared with two *L. nasuensis* JCM 17158.

The comparison of the pan-genomes (Figure 4.8) revealed very similar results to the core genome analysis: is possible to observe two main clusters divided per species, one composed by *L. paracasei* strains plus *L. casei* DSM4509 and one composed by *L. rhamnosus* strains plus the other two *L. casei* strains analysed.

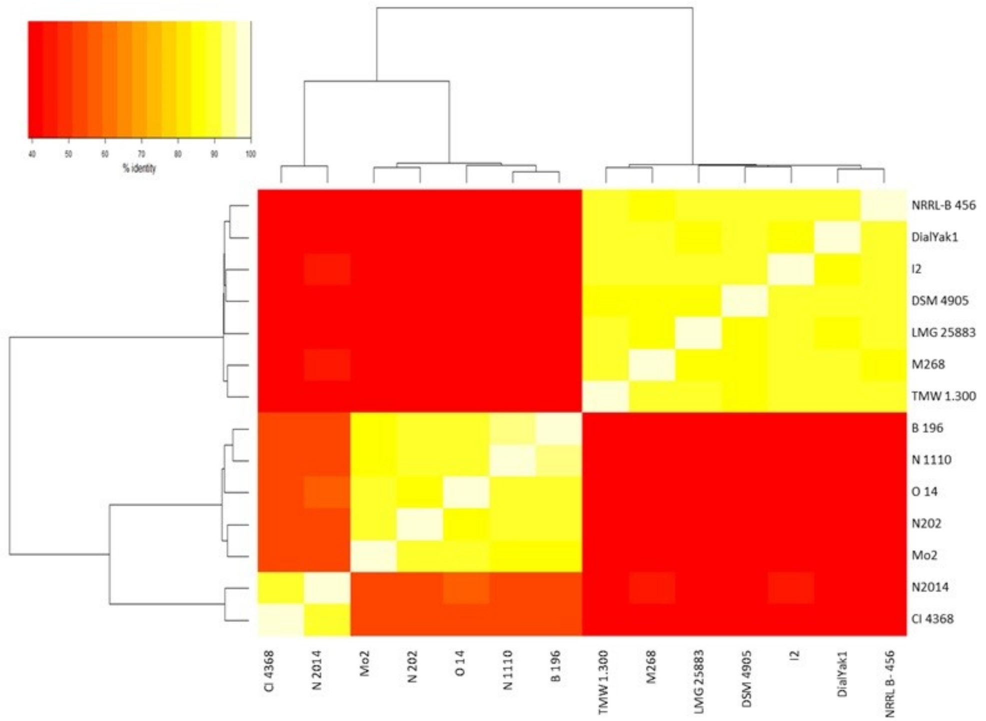

Figure 4.8 Pan-genome phylogenetic tree compareson for the four-teen sequenced genomes (GGDC compareson).

4.3.3 UPGMA tree based on SAPD-PCR data

Lactobacillus casei

The profiles obtained with the A-not primer were analysed and compared using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm. The analysis led to the formation of a main group, composed by four strains (Figure 4.9). Based on the data obtained no correlation according to the source or provenience was observed.

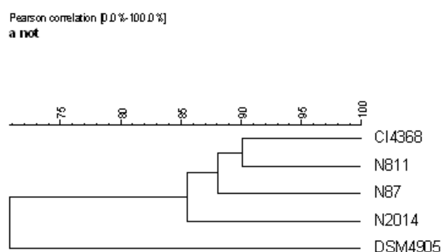

Figure 4.9 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. casei*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (A- not primer).

The analysis of the fingerprint profiles obtained by SAPD-PCR with C-not primer, led to the formation of 1 main cluster (including strains with 80% of similarity) (Figure 4.10). Also in this case it was not possible to define a correlation according to the source of isolation of the strains.

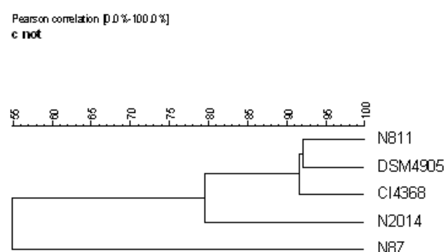

Figure 4.10 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. casei*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (C- not primer).

Figure 4.11 shows the clusters resulting from the comparison among G- not fingerprinting. In this case there were two foring clusters.

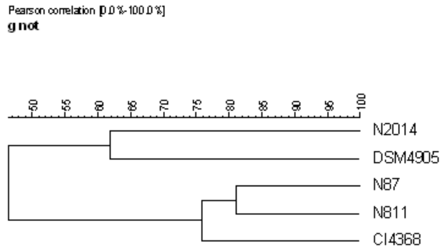

Figure 4.11 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. casei*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (G- not primer).

The profiles obtained with the T-not primer were analysed and compared using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm. The analysis led to the formation of 1 group and two unclustered strains (Figure 4.12). No amplification product was obtained for strain N2014 using this primer.

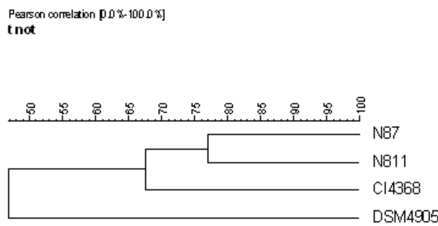

Figure 4.12. Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. casei*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (T- not primer).

At least the composite dataset analysis led to the formation of 2 clusters with a percentage of similarity of 67% and 74% (Figure 4.13).

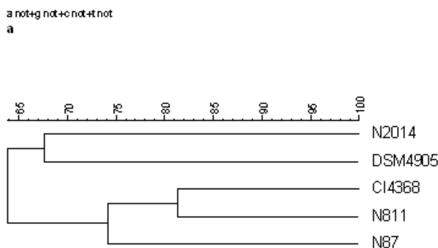

Figure 4.13. Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. casei*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (Composite Dataset Analysis).

Lactobacillus paracasei

The fingerprint profiles obtained with the A-not primer were analysed by the Unweighted Pair Group Method with Arithmetic averages clustering algorithm. Looking at the dendograms is evident that 19 strains had a level of similarity higher than 80% (Figure 4.14). Based on the data obtained is not possible to say that the strains grouped according to the source of isolation. Strain S1 did not give PCR products with this primer.

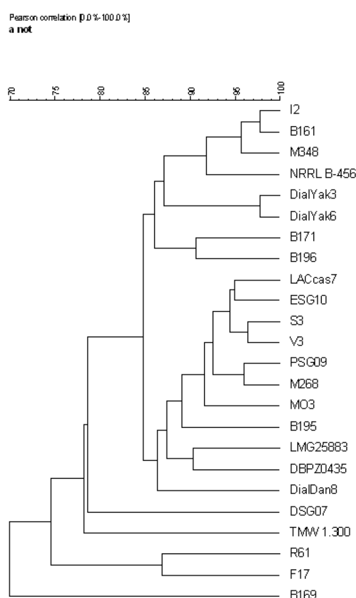

Figure 4.14 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. paracasei*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (A- not primer).

The analysis of the fingerprints obtained by SAPD-PCR with C-not primer, carried out to the formation of tree clusters with a coefficient of similarity higher than 80% (Figure 4.15). Moreover, in this case no correlation was found, according to the source of isolation of the strains. Strains B195 did not give PCR products with this primer.

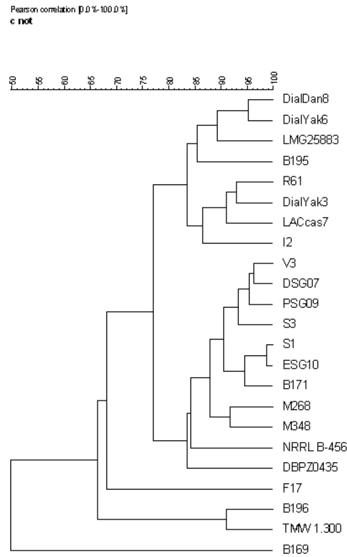

Figure 4.15 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. paracasei*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (C- not primer).

Figure 4.16 shows the clusters resulting from the comparison among the fingerprints obtained afer the PCR reaction with G- not primer. Strains DialDan8 and DialYak3 did not produce PCR products using this primer.

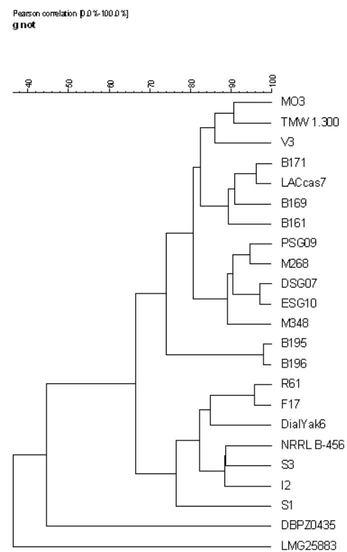

Figure 4.16 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. paracasei*. Dendrograms were obtained usng the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (G- not primer).

The profiles obtained with the T-not primer were analysed and compared by the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (Figure 4.17). Strains B161, LACcas7, Mo3, TMW1.300, B195, B196, DialDan8, F17 and B169 did not amplify with this primer.

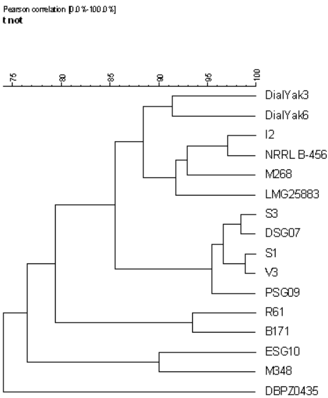

Figure 4.17. Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. paracasei*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (T- not primer).

At least observing the dendograms obtained by the composite dataset analysis, is evident the presence of strains grouping with a percentage of similarity higher than 80% (Figure 4.18).

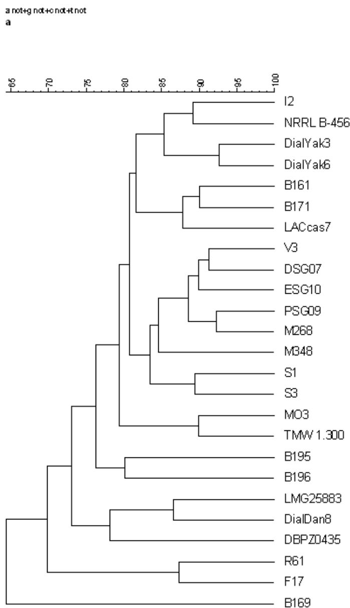

Figure 4.18. Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. paracasei* (Composite Dataset Analysis).

Lactobacillus rhamnosus

The profiles obtained with the A-not primer were analysed and compared using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm. The analysis led to the formation of two main groups (Figure 4.19).

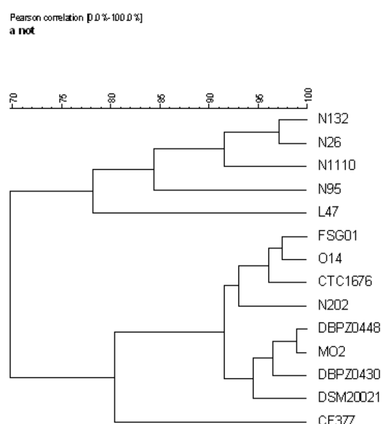

Figure 4.19 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. rhamnosus*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (A- not primer).

Based on the data obtained, is not possible to say that the strains of *L. rhamnosus* grouped according to their source of isolation. Strain DBPZ0446 did not amplify with this primer.
The analysis of the fingerprint obtained by SAPD-PCR with C-not primer, led to the formation of two main clusters (Figure 4.20). Strains DPBZ0448, N202, Mo2, DSM20020 and CF377 did not amplify with this primer.

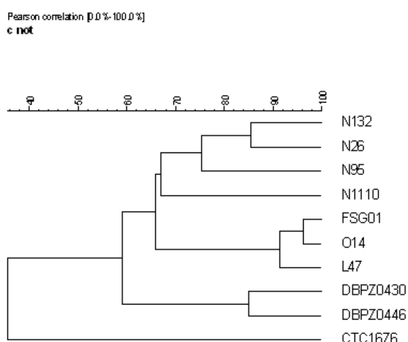

Figure 4.20 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. rhamnosus*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (C- not primer).

Figure 4.21 shows the clusters resulting from the comparison among fingerprints obtained performing the SAPD-PCR with G- not primer. In this case there are two main clusters clusters, while a strain unclustered.

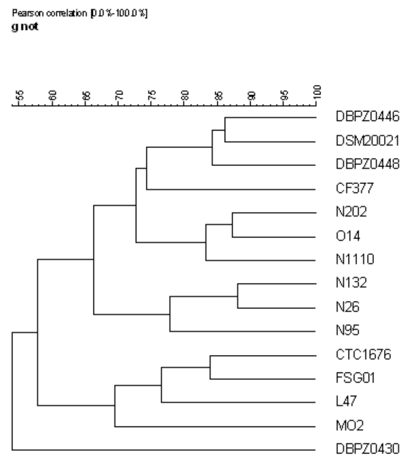

Figure 4.21 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. rhamnosus*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (G- not primer).

The profiles obtained with the T-not primer were analysed by the Unweighted Pair Group Method with Arithmetic averages clustering algorithmand. The analysis led to the formation of a main cluster (Figure 4.22), but only strains FSG01, Mo2, DSM20020 and L74 amplified with this primer.

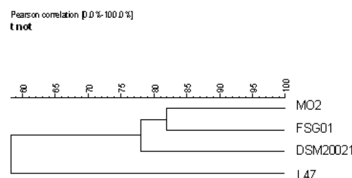

Figure 4.22 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. rhamnosus*. Dendrograms were obtained using the Unweighted Pair Group Method with Arithmetic averages clustering algorithm (T- not primer).

At least the composite dataset analysis led to the formation of two main clusters including strains having a coefficient of similarity higher than 85% (Figure 4.23).

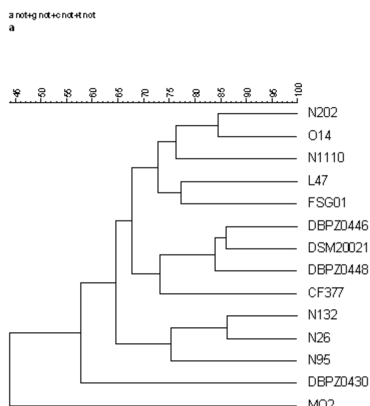

Figure 4.23 Cluster analysis of the profiles obtained performing the SAPD-PCR analysis on *L. rhamnosus* (Composite Dataset Analysis).

4.4 Discussion and Conclusions

In this work we analysed the diversity of eight stress related genes. The internal fragments of the eight selected *loci* (*npr*, *nox*, *hrcA*, *ctsR*, *cydA*, *cydD*, *dnaK*, *pox*) were amplified in all the examined strains with the exception of some *L. casei* strains. In particular, internal fragments of *hrcA*, *ctsR*, *cydD*, *dnaK* and *pox* genes could not be amplified in strain N811. In strain N87 there were not PCR product in the amplification of *hrcA*, *pox*, and *dnaK loci*. Finally, for strain N2014, *npr*, *hrcA*, *pox* and *dnaK loci* could not be amplified. From these data, we could take in consideration the possibility of a deletion of these *loci* or the presence of a non-homologous gene copy in these strains. The examined *loci* are correlated to stress response and it's also possible that there is a relation between a low stress resistance and the absence of the *locus*.

At least, for all the analysed species, we did not observe the presence of strains with the same ST, but some strains presented the same allelic variant for one *locus* or more.

The mean G + C content of the different gene fragments was different among species. The lower values were observed for the genes of *L. paracasei* analysed strains, the higher were observed in *L. casei*. All the G+C mol% value observed for the *ctsR* gene, except in *L. rhamnosus*, were higher than 0.45, this means that the analysed genes are stable. This results are not so far from what was observed before from other authors performing MLST an other *Lactobacillus* spp. Parolo et al. (2011) performed MLST analysis on 75 *L. paracasei* strains isolated from human oral cavity and for the analysed *loci* (*fusA, ileS, lepA, leuS, pyrG, recA* and *recG*) they obtained G+C mol% values that ranged from 46.9% to 50%. Analog values have been observed in *L. planctarum* (from

4.10% to 51%) and in *L. fermentum* (from 48.26% to 60.44%) (Xu et al., 2015; Dan et al., 2015).
Among the analysed strains, a higher number of polimorphic sites was observed in *L. casei* and *L. paracasei*. In general, lowers values were observed from other authors developing MLST schemes on *L. casei* and *L. paracasei* strains. Diancour et al. (2007) developed a MLST scheme for *L. casei* based on the analysis of seven housekeeping genes (*fusA, ileS, lepA, leuS, pyrG, recA*, and *recG*), founding lower values of nucleotide variation (π ranging from 0.0038 to 0.0109) and polimorphic sites (fron 2 to 17). Parolo et al. (2011) performed MLST analysis on 222 strains of *L. paracasei* using the same scheme performed by Diancourt (2007) and observing similar values. However, other authors developed an MLST scheme for the *L. acidophilus* complex, comparing *loci* from the same selected genes proposed by Diancourt et al. finding up to 148 polimorphic sytes for a single *locus* (Ramachandran et al., 2013).
Surprisingly, *L. casei* strains N87, N811, N2014 did not give PCR products for *hrcA*, *pox* and *dnaK loci*. PCR products were obtained only for strain DSM4905. In strain N811 also *cydD locus* did not give PCR products, while in strain N2014 no PCR product was obtained for *npr locus*. After the alignment and comparison on GeneBank of the obtained sequences for the amplification of the *loci nox*, *cydA* and *ctsR*, we discovered that the sequences were homologous to whom of *L. casei* ATCC 393. Considering that the identity of that strain is still under discussion (Felis et al., 2001; Desai et al., 2006) it could be assumed that the primers did not anneal on their target sequences because of the differences existing between *L. casei* and *L. zeae* species. Among strains, no one had the same strain type, but grouping the STs of all the analysed strains and creating a Minimum spanning tree, a clear distintion among species was observed.
MLST analysis was more efficient than SAPD analysis in underlining similarities among the strains. This depends on the fact that SAPD-PCR is a technique that allows to analyse polymorphisms at whole genome level, while in MLST we analysed only 8 *loci*, a small part of the entire genome. Anyway, the source of isolation was heterogeneous and this etereogenity was confirmed using both the techniques: even if the strains were grouped on different basis, no evidence of similarities related to the source of isolation or the geographical origin was found. Heterogenity in strain clustering related to the source of isolation, was found also from Parolo et al. (2011) among *L. paracasei* strains. Otherwhise, the core-genome and pan-genome comparisons, presenting two distinct clusters grouping *L. paracasei* and *L. rhamnosus* strains, confirmed how hard is the discrimination among species belonging to this group, because of the presence of a *L. paracasei* and two *L. casei* strains grouping in *L. rhamnosus* cluster.

Concluding, in this work we developed a MLST scheme for *L. rhamnosus*, *L. paracasei* and *L. casei* considering genes envolved in the general stress response. A total of 45 strains coming from several isolation sources were used. As aspected, a huge number of STs was observed, without finding strains having the same ST. This could be due to the fact that the strains were originally isolated from different matrices and on the basis of their Sau-PCR fingerprint. Some strains, previously classified as *L. casei*, did not gave PCR products for all the examined *loci* and resulted to have homologous sequences to *L. casei* ATCC 393, whom identification is still under discussion because of its higher level of similarity with *L. zeae* (Iacumin et al., 2015). The whole genome sequencing analysis supported this theory. Two *L. casei* strains, which showed higher similarity to *L. casei* ATCC 393, grouped with *L. rhamnosus* strains and their 16s rRNA gene had a higher affinity to *L. zeae* than to *L. casei*. On this basis, the classification of these strains should be revisited.

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5. Mucus binding properties of *Lactobacillus casei* group strains before and after oxidative stress

5.1 Aim of the study

The capability of a strain to adhere to mucus, is one of the features required from the Whorld Healt Organization for a strain to be considered as a potential probiotic. Several studies demonstrated that binding mucus is an efficient way to inhibit the Gastrointestinal (GI) colonization by pathogens. Moreover, the capability of some *Lactobacillus* spp. strains to bind mucus has been already studied, demonstrating that this property is not dependent on the species, but is strain specific. The stress exposure can modify the behaviours of several microorganisms and the aim of this study was to evaluate how the exposure to oxidative stress could modify the capability of *L. casei* group strains to bind mucus

5.2 Material and methods

5.2.1 Bacteria and growth conditions

The strains tested in this study where selected among a wide range of strains belonging to *Lactobacillus casei* group, previously identified and subjected to molecular characterization (chapters 2, 3, 4). The strains tested in this study are listed in Table 5.1. The strains were isolated from various fermented products of animal or plant origin or from human body excreta or obtained from culture collections by University of Udine, University of Basilicata and by the University of Molise.

Table 5.1 List of the strains examined in this chapter.

	SOURCE	SPECIES
LMG25883	Dairy product, China	*L. paracasei*
DBPZ0435	Caciocavallo Cheese (CS)	*L. paracasei*
NRRL B - 456	Unknown	*L. paracasei*
S1	Moliterno Cheese	*L. paracasei*
S3	Moliterno Cheese	*L. paracasei*
V3	Moliterno Cheese	*L. paracasei*
DSG07	Emmenthal Cheese	*L. paracasei*
ESG10	Parmigiano Reggiano Cheese	*L. paracasei*
PSG09	Provolone Torrealta Cheese	*L. paracasei*
M268	Canestrato Moliterno Cheese	*L. paracasei*
M348	Canestrato Moliterno Cheese	*L. paracasei*
R61	Pecorino Cheese	*L. paracasei*
F17	Pecorino Cheese	*L. paracasei*
I2	Sourdough Molise	*L. paracasei*
B161	Wine or must	*L. paracasei*
B169	Wine or must	*L. paracasei*
B171	Wine or must	*L. paracasei*
B195	Wine or must	*L. paracasei*
B196	Wine or must	*L. paracasei*
LacCas7	Grana Cheese	*L. paracasei*
TMW 1.300	Spoiled beer	*L. paracasei*
DIALDAN8	Fermented milk	*L. paracasei*
DIALYAK1	Fermented milk	*L. paracasei*
DIALYAK3	Fermented milk	*L. paracasei*
DIALYAK6	Fermented milk	*L. paracasei*
DBPZ0430	Provolone Cheese	*L. rhamnosus*
DBPZ0446	Caciocavallo Cheese	*L. rhamnosus*
DBPZ0448	Caciocavallo Cheese	*L. rhamnosus*
DSM20021	Unknown	*L. rhamnosus*
80	Grana Padano Cheese	*L. rhamnosus*
O14	Parsley Cheese	*L. rhamnosus*
CF377	Parmigiano Reggiano Cheese	*L. rhamnosus*
L47	Asiago Cheese	*L. rhamnosus*
N95	Body excreta	*L. rhamnosus*
N132	Body excreta	*L. rhamnosus*
N26	Body excreta	*L. rhamnosus*
N1110	Body excreta	*L. rhamnosus*
N202	Body excreta	*L. rhamnosus*
Mo2	Human	*L. rhamnosus*
CTC1676	Fermented sausage	*L. rhamnosus*
DSM4905	DSM4905	*L. casei*
CI4368	Parmigiano Reggiano Cheese	*L. casei*
N87	Body excreta	*L. casei*
N811	Body excreta	*L. casei*
N2014	Body excreta	*L. casei*

The strains were stored at the temperature of - 80° C in cryovials containing MRS broth (Oxoid, Italy) supplemented with 2% glycerol. At the time of use, the cultures were streaked on MRS Agar (Oxoid, 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 5 mL of MRS broth (Oxoid, Italy).

5.2.2 Mucin binding assay

Partially purified type III porcine gastric mucin (Sigma-Aldrich, Italy) was dissolved in phosphate-buffered saline (PBS, Sigma-Aldrich, Italy) (pH 7.4) to a final concentration of 5 mg/mL. 150 µl were immobilized in polystyrene microtiter 96 wells plates (Maxisorp Nunc, Denmark) by overnight incubation at 4 °C at slow rotation.
The wells were washed three times with PBS and saturated with a 2% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, Italy) solution for 2 h and washed again three times with PBS. The bacterial cells capability to adhere to porcine gastric mucin type III was tested after a preliminary cell adaptation under anaerobic (16 h in MRS broth) and respiratory (48 h in M17 medium containing heme 2.5 µg/ml and vitamin K 1 µg/ml) conditions.
The cultures were centrifuged at 6000*g* for 5 min and the pellets were washed twice with sterile PBS, resuspended in the same buffer and adjusted to the optical density (OD) (600 nm) of 0.1. 100 µl of the bacterial suspension were added to each well ($\approx 10^6$ cfu/mL). *L. plantarum* WCFS1 and *L. rhamnosus* GG were used as positive control. The microplates were incubated for 2 h at 37 °C. The wells were washed five times with sterile PBS to remove unbound bacteria. The wells were then treated with 200 µl of a 0.5% (v/v) Triton X-100 solution to desorb the bound bacteria. Plates were then incubated for 20 min at room temperature under orbital agitation. 100 µl of the content of each well were removed, diluted in PBS and plated on MRS agar plates.

5.2.3 Adhesion to Matrigel Matrix

Adhesion properties of the strains were tested also on reconstituted basement membranes using Basement Membrane Matrigel (Corning, MA, USA) with a protocol from Tallon et al. (2007). Corning Matrigel Basement Membrane Matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin/nidogen. In breaf, the Matrigel basement membrane preparation was diluted 1/20 in ice-cold phosphate buffered saline (PBS). 150 µl of the suspension were placed onto a glass

chamber slide (Lab-Tek, four Chamber SlidesTMserif) at 4 °C and allowed to gel for 90min at 37 °C. The slides were then washed three times for 5 min with PBS (Sigma-Aldrich, Italy) containing 0.1 % BSA (0.1 % BSA-PBS), quenched for 2 h at room temperature with 2% BSA-PBS and the bacterial cell suspension was then placed into the well at a concentration of 10^8 cells/mL. After 2 h of incubation at room temperature, each slide was washed three times for 5 min with 0.1% BSA-PBS. The adherent bacteria were than stained with Gram's crystal violet solution (Sigma-Aldrich, Italy). 14 randomly chosen fields were photographed using light microscopy (Zeiss Axiphot microscope, Axiocam). The number of bacteria in 14 fields was counted and the results expressed as means ± SD.

5.2.4 Mucin binding assay after *in vitro* digestion

Mucin binding assay was performed after *in vitro* digestion on the strains that showed the best binding performances. The selected strains were: S1, O14, N95, N26 and N202 adapted under respiratory conditions. After 48 hours in M17 medium containing heme 2.5 µg/ml and vitamin K 1 µg/ml at 37°C, the cultures were centrifuged at 6000*g* for 5 min and the pellets were washed twice with sterile PBS and resuspended in 5 ml of PBS buffer at the concentration of 4 x 10^9 cfu/ml. The in vitro digestion was performed as follows: 30 s in simulated saliva, 30 min in artificial gastric juice and 2 h in artificial intestinal solution. The saliva solution was composed as described by Garcìa Rulez et al. (2014). In brief, the solution (pH 6.5) was composed by 0.22 g/l $CaCl_2$ (Sigma-Aldrich, Italy), 6.2 g/l NaCl (Sigma-Aldrich, Italy), 2.2 g/l KCl (Sigma-Aldrich, Italy), 1.2 g/l $NaHCO_3$ (Sigma-Aldrich, Italy), sterilized by filtration and added of 0.1 g/l of lysozyme (Sigma-Aldrich, Italy) before the assay. Simulated gastric juice was formulated as reported by Corcoran et al. (2005). The solution was composed by glucose 3.5 g/l (Sigma-Aldrich, Italy), 2.05 g/l NaCl (Sigma-Aldrich, Italy), .0.60 g/l KH_2PO_4 (Sigma-Aldrich, Italy), 0.11 g/l $CaCl_2$ (Sigma-Aldrich, Italy), KCl 0.37 g/l (Sigma-Aldrich, Italy), adjusted to pH 2.0 using 1M HCl (Sigma-Aldrich, Italy) and autoclaved at 121 °C for 15 min. 0.0133 g/L of pepsine (Sigma-Aldrich, Italy) were adjunted prior to analysis. Simulated intestine juice was formulated using the same solution of the gastric juce, but adjusted at pH 8. 0.05 g/l of bile (Sigma-Aldrich, Italy) and 1 g/l of pancreatine (Sigma-Aldrich, Italy) were added before to perform the analysis.
After the in vitro digestion, 100 µl of intestinal solution were used to perform the mucin-binding assay as previously described.

5.3 Results

5.3.1 Bacterial adhesion to mucin

The adhesion of the probiotic strains *L. plantarum* WCFS1 and *L. rhamnosus* GG, that are well known for their adhesion properties (de Wouters et al., 2015), reached a level of 4.3 log cfu/well and 4.2 log cfu/well, respectively (Table 5.2).

Among the 5 tested strains belonging to *L. casei* species, no one of the strains showed such a high adhesion properties neither before the oxidative stress exposure nor after the stress exposure. A lower but significant level of adhesion was observed for the strain CI4368 (3.6 log cfu/well before stress exposure and 3.2 log cfu/well after stress exposure) and in strain N87 after the stress exposure (3.0 log cfu/well). In strains: DSM4905, N87 and N2014 the binding levels were higher after the stress exposure. In particular, the binding level of the strain N87 was almost doubled.

Among the 25 *L. paracasei* strains tested in this assay, before the stress exposure strain B169 showed an adhesion value very close to the one obtained from the positive controls (3.8 log cfu/well). Lower but significant levels of adhesion were observed for the strains NRRL B – 456, DSG07, M348, B195, B196, TMW 1.300: the adhesion levels of these strains before stress exposure ranged from 2.7 log cfu/well to 2.9 log cfu/well. Oxidative stress exposure enhanced the binding capabilities of the strains LMG25883, DBPZ0435, S1, S3, V3, ESG10, M268, F17, I2, B161, B171, TMW 1.300, DIALDAN8, DIALYAK1, DIALYAK3, DIALYAK6. In particular, looking at the data showed in Table 5.2, is possible to observe that in seven of the previously listed strains, the mucus binding level was doubled respect to the values obtained in the assay done before stress exposure. The most interesting adhesion results were obtained for strains S1 and B171, with levels of binding of 3.9 log cfu/well and 4.4 log cfu/well respectively.

Very interesting results were obtained from the analysis made on the 15 *L. rhamnosus* strains tested in this study. Before the stress exposure, no one of the strains showed the same adhesion properties of the strain used as positive control. Anyway, a lower but significant level of adhesion was observed for the strain N1110 (3.0 log cfu/well). Stress exposure to oxidative stress enhanced the binding capabilities of the strains DBPZ0448, O14, CF377, L47, N95, N26, N202, CTC1676. In particular, looking at the data showed in Table 5.2, is possible to observe that in three of the previously listed strains, the mucus binding level was doubled respect to the values obtained before stress exposure and in strains O14 and N95 this value increased three times. The most interesting adhesion results were obtained for strains O14 (4.0 log cfu/well), N95 (5.3 log cfu/well), N26 (4.7 log cfu/well) and N202 (4.2 log cfu/well).

Strains N95 and N26 reached adhesion values one log higher than the positive control strains.

5.3.2 Adhesion to Matrigel Matrix

After the adhesion to mucin, the capability of the strains to bind Matrigel matrix was tested. The results are shown in Table 5.2. It's interesting to observe that strains that showed a good level of adhesion to mucin had a low level of Matrigel solution binding and viceversa. Among the 5 tested strains belonging to of *L. casei* species, no one of the strains showed such a high adhesion properties before the oxidative stress exposure nor after the stress exposure. Among the 25 *L. paracasei* strains, before the stress exposure strains S1 and DIALDAN8 showed an adhesion value very close to 3.0 log cfu/glass. Stress exposure to oxidative stress enhanced the binding capabilities of strains LMG25883, M268, R61, F17, B169, B171, LacCas7, TMW 1.300. The most interesting adhesion result was obtained for strains S1 and B195, with levels of binding of 3.1 and 2.9 log cfu/glass. The behaviour of the strains seems not to be related to the mucin binding properties observed in the previous experiment.
Among the 15 *L. rhamnosus* strains that have been tested before the stress exposure only strains DBPZ0466 and O14 showed an adhesion value higher than 3.0 log cfu/glass). Stress exposure to oxidative stress enhanced the binding capabilities of 6 strains. Strain N132 did not bind Matrigel before stress exposure, but after the oxidative stress treatment it increased its binding properties reaching a level of binding of 1.8 log cfu/glass. The most interesting adhesion result after stress esposure was obtained for strain DBPZ0466, with levels of binding of 3.0 log cfu/glass respectively. Even in this case, the behaviour of the strains did not seem to be related to the mucin binding properties observed in the previous experiment.

Table 5.2 Results obtained for the Mucin binding assay (on the left) and Matrigel binding assay (on the right) before and after oxidative stress exposure.

		MUCIN BINDING ASSAY				**MATRIGEL BINDING ASSAY**			
		Anaerobiosis		**Respiration**		**Anaerobiosis**		**Respiration**	
		Mean (log cfu /ml)	**St. Dev.**	**Mean (log cfu /ml)**	**St. Dev.**	**Mean (log cfu/ field)**	**St. Dev.**	**Mean (log cfu/ field)**	**St. Dev.**
DSM4905	*L. casei*	2.1	1.7	2.6	1.6	-	-	2.3	1.3
CI4368	*L. casei*	3.6	2.3	3.2	2.7	-	-	0.0	0.0
N87	*L. casei*	1.7	0.9	3.0	1.3	1.0	0.7	0.8	0.6
N811	*L. casei*	2.0	0.6	1.7	0.6	1.7	0.9	1.4	1.1
N132	*L. casei*	1.7	0.9	-	-	3.0	2.1	2.2	1.0
LMG25883	*L. paracasei*	1.2	0.4	2.3	1.8	2.1	1.4	0.0	0.9
DBPZ0435	*L. paracasei*	1.1	0.8	2.8	2.0	2.1	1.5	2.3	0.9
NRRL B - 456	*L. paracasei*	2.9	2.7	2.1	1.8	-	-	-	-
S1	*L. paracasei*	1.7	0.9	3.9	2.8	3.3	1.9	3.1	0.8
S3	*L. paracasei*	1.6	1.2	3.1	2.7	2.3	1.2	2.2	1.0
V3	*L. paracasei*	1.9	1.0	2.6	2.1	1.1	0.9	0.6	0.4
DSG07	*L. paracasei*	2.7	2.2	2.3	1.3	1.5	0.7	0.1	0.2
ESG10	*L. paracasei*	1.5	1.4	1.69	0.36	-	-	-	-
PSG09	*L. paracasei*	1.5	1.1	1.8	1.2	1.6	0.8	-0.2	0.1
M268	*L. paracasei*	1.4	0.4	3.1	2.3	2.4	1.1	2.3	1.0
M348	*L. paracasei*	2.8	2.1	2.9	2.7	1.1	0.5	1.9	1.0
R61	*L. paracasei*	1.9	0.8	2.0	1.4	2.4	1.3	-0.5	-0.2
F17	*L. paracasei*	1.4	1.2	2.1	1.6	2.5	1.1	1.8	0.5
I2	*L. paracasei*	1.8	1.0	1.5	0.9	2.7	0.8	2.1	1.1
B161	*L. paracasei*	1.5	0.9	2.0	1.2	0.5	0.4	0.3	0.5
B169	*L. paracasei*	3.8	2.7	3.2	2.1	0.1	0.2	1.1	1.0
B171	*L. paracasei*	2.2	1.8	4.4	3.3	2.1	1.5	2.5	0.8
B195	*L. paracasei*	3.0	2.4	2.9	1.8	2.1	1.2	2.9	1.4
B196	*L. paracasei*	2.8	2.1	1.9	0.9	2.4	1.1	2.2	0.8
LacCas7	*L. paracasei*	1.9	1.6	2.4	1.0	1.6	0.3	1.8	0.8
TMW 1.300	*L. paracasei*	2.9	1.8	3.2	2.3	2.2	1.4	1.7	0.6
DIALDAN8	*L. paracasei*	0.8	1.1	1.9	1.5	3.1	1.6	2.2	0.9
DIALYAK1	*L. paracasei*	1.3	0.6	0.11	-0.22	0.0	0.0	1.8	0.8
DIALYAK3	*L. paracasei*	0.1	0.4	0.8	0.8	0.0	0.2	-0.5	-0.2

Strain	Species								
DIALYAK6	*L. paracasei*	1.2	1.4	1.8	0.8	2.7	1.5	2.9	1.1
DBPZ0430	*L. rhamnosus*	1.4	0.4	1.2	0.7	1.3	0.8	2.4	1.2
DBPZ0446	*L. rhamnosus*	2.3	1.6	2.1	1.4	3.2	1.7	3.0	0.9
DBPZ0448	*L. rhamnosus*	1.5	1.0	2.5	1.8	2.4	1.3	2.1	0.8
DSM20021	*L. rhamnosus*	2.5	1.9	2.8	1.7	2.5	1.2	2.5	1.3
FSG01	*L. rhamnosus*	1.5	1.4	1.7	0.4	0.1	0.4	1.4	0.9
O14	*L. rhamnosus*	1.3	1.3	4.0	3.9	3.1	1.5	-0.5	-0.2
CF377	*L. rhamnosus*	1.7	0.6	2.4	1.0	2.0	0.9	1.1	1.0
L47	*L. rhamnosus*	0.1	0.4	1.9	1.1	2.0	0.8	2.1	1.1
N95	*L. rhamnosus*	2.5	1.3	5.3	4.4	2.1	1.4	-0.5	-0.2
N2014	*L. rhamnosus*	2.4	1.7	2.9	2.3	2.0	0.8	2.4	0.9
N26	*L. rhamnosus*	2.3	1.0	4.7	4.8	1.0	0.4	2.3	1.2
N1110	*L. rhamnosus*	3.0	1.6	2.8	1.8	1.9	1.0	1.8	1.0
N202	*L. rhamnosus*	1.8	1.0	4.2	2.6	2.3	0.7	2.0	0.8
Mo2	*L. rhamnosus*	2.3	2.0	1.8	1.3	2.1	1.2	1.3	0.6
CTC1676	*L. rhamnosus*	1.6	1.1	2.1	1.3	2.4	1.8	1.2	1.0
L. plantarum WFSC1		4.3	2.6			3.2	1.4		
L. rhamnosus GG		4.2	2.6			3.0	1.5		

5.3.3 Mucin binding assay after *in vitro* digestion

Mucin binding assay was performed after *in vitro* digestion on the strains that showed the best binding performances. The selected strains were: S1, O14, N95, N26 and N202 adapted in respiratory conditions. After 48 hours in M17 medium containing heme 2.5 µg/mL and vitamin K 1 µg/mL at 37 °C, the strains were digested *in vitro* as follows: 30 s in simulated saliva, 30 min in artificial gastric juice and 2 h in artificial intestinal solution. The results are reported in Table 5.3.

All the experiments were made in triplicate, with three technical replicates each. The results are listed as means of the replicates, in Table 5.3. After the digestion, a decrease in the adhesion was observed in strains N 95 (1.6 log cfu/ml) and in strain B171 (2.34 log cfu/ml). Strain O14 lost its binding capability. Strains N26 and N202 manteined their binding capabilities. The decreasing binding capabilities of three strains after the in vitro digestion could be due to the fact that this strains were mainly isolated from food matrices,

with the exception of the strain N95, isolated from human excreta. Strains N26 and N202 were isolated fron human excreta as well, and, as expected, they give the higher values of binding.

Table 5.3 Mucin adhesion properties of the six selected strains before and after *in vitro* digestion.

		MUCIN BINDING ASSAY			
		Before *in vitro* digestion log cfu/ml		**After *in vitro* digestion log cfu/ml**	
		Mean	**St. Dev**	**Mean**	**St. Dev**
S1	*L. paracasei*	3.9	2.8	3.8	3.4
O14	*L. rhamnosus*	4.0	3.9	-	-
N95	*L. rhamnosus*	5.3	4.4	3.8	3.1
N26	*L. rhamnosus*	4.7	4.8	5.1	4.2
N202	*L. rhamnosus*	4.2	2.6	4.1	3.9
B171	*L. paracasei*	4.4	3.3	2.1	1.4

5.4 Discussion and Conclusions

The role of probiotics in the prevenction of gastrointestinal infection has wildely been studied in several works. They are able to compete with pathogens by the mean of several mechanisms, such as the production of antimicrobial compound, the competition for the metabolites and the competition for the adhesion site (Van Tassel and Miller, 2011). Mucus-binding assay is currently one of the main *in vitro* tests for the study of the probiotic traits strains as reported in the guidelines for the evaluation of probiotics in foods (FAO/WHO, 2002).

In this work, the adhesion properties, on two different biological matrix (PGM type III and MatriGel), before and after stress exposure, were evaluated.

The results suggested that these properties are strain and matrix dependent, in agreement with those obtained by Tallon et al. (1999), who tested the ability of 31 *L. plantarum* strains to adhere to several biological matrixes. A similar phenomenon was observed in other species, such as *L. casei* (Tuomola and Salminen 1998). Collado et al. (2007) evaluated the adhesion capacity of specific LAB isolates from *dadih*, an Indonesian traditional fermented milk, to intestinal mucus and they found that the adhesion of tested LAB strains was strain-dependent, as well. In a recent rewiew, Sengupta et al. (2013) confirmed that different cell surface characteristics of lactobacilli are not only species-specific, but also strain-dependent, confirming the results obtained in this work. The results obtained for the Matrigel assay are comparable to whom obtained by Bouzaine et al. (2005), that studied selected LAB isolated from

intestinal tract of chicken to investigate their ability to adhere in vitro to Basement Membrane Matrigel (BMM). Afters those preliminary analysis, they selected a strain showing a good adherence in BMM for other studies.
On the basis of the results obtained, the strains giving the best adhesion results in this study, were selected to investigate their binding capabilities after *in vitro* digestion. As expected, the strains isolated from food matrices decreased their binding capabilities. One of these strains lost completely its binding features. Considering that several studies demonstrated that food matrices can enhance the capability of a strain to resist against stress factors (Charteris et al., 1998; Ross et al., 2005), further studies will be made on these isolates in order to evaluate their binding capability after in vitro digestion, when used to produce fermented milk.
In conclusion, this work allowed the selection of several potentially probiotic strains, to be tested to evaluate if they possess some other useful charatcteristics, such as ability to degrade biogenic amines, anti-inflammatory activity, ability to degrade food addittives (ie. sorbate, sodium benzoate..). Moreover, the binding mechanisms of these strains need to be investigated, in order to have a complete view about their probiotic features before to eventually start the analysis *in vivo*.

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6. Effect of different pH level at different EtOH concentration on the expression of stress response related gene of *Lactobacillus paracasei* B171 in wine

6.1 Aim of the study

In wine industry the interest in LAB is related to their capability in conducting malolactic fermentation (MLF), thanks to their ability to metabolize malic acid and convert it into lactic acid. This capability is related to the presence of the β-glucosidase enzyme (Boido et al., 2002; Matthew set al., 2004; Wightman et al., 1997). Many studies focused on MLF because of its positive impact on wine flavour, expecially on *Oenococcus oeni*, a microorganism tipically used in wine making to lead MLF, while few works evaluated this ability in other LAB, such as in *L. paracasei*.
The purpose of this work was to evaluate the gene expression of stress related genes in a strain of *L. paracasei* for its ability to conduct malolactic fermentation under different EtOH and pH conditions. The fermentation was performed in co-inoculation of *Saccharomyces cerevisiae* and *L. paracasei* B 171 in sterile must of Muscat and maintained at the temperature of 19 °C. Two pH values and two different concentrations of glucose/EtOH were tested. Bacterial and yeast populations were monitored during fermentation and RT-qPCR was performed.

6.2 Material and methods

6.2.1 Inoculation of must samples

The juice used was Muscat, from the "Cantine La Delizia," Casarsa della Delizia (PN), chosen for the feature of being rich in aromatic precursors, in particular β-glucosides. In order to evaluate the capability of *Lactobacillus paracasei* B171, belonging to the collection of Stellenbosh University, to perform malolactic fermentation in co-inoculation with *Saccharomyces cerevisiae*, two defined pH at different concentrations of ethanol, were used. Free SO_2 and total SO_2 values of the must were 5.0 mg/l and 45 mg/l respectively.
Sterile must was obtained by filtration at decreasing porosity, up to reach 0.2 µm. pH was adjusted to values of 3.2 and 3.8 units, by addition of a solution of 2M NaOH (Carlo Erba, Italy), starting from a value of 3.48.
Subsequently, sterile glass bottles with a capacity of 1 L were filled with 800 ml of steril must. The starting EtOH content of the must was 2.46% v/v. Sugar concentration of the must was adjusted using Glucose (Sigma, Italy) in order to obtain two series of samples: one with a concentration of EtOH in the final

wine of 11% v/v (pH 3.2 and pH 3.8) and one with a final concentration of ethanol of 15% (pH 3.2 and pH 3.8). The fermentations were conducted in triplicate, for each of the four conditions tested.
After the distribution of must to the flasks and after glucose and pH adjustement, *Saccharomyces cerevisiae*, strain P. Supertuscan (Wine Vason, Italy), prepared according to manufacturer's directions, at a concentration of 10^6 cfu/ml was added to the musts. Samples were taken to confirm the exact yeasts inoculation cell numbers: a ten fold serial dilution was made in sterile saline solution (8.5 g/l NaCl (Sigma-Aldrich, Italy) and 1 g/l bacteriological peptone (Oxoid, Italy)) and 100 µl of suspension was spread plted onto Yeast Extract agar (Oxiod, Italy) and incubated at 30°C for 48 h. Next, the bottles were placed in an incubator set at 19 ° C for 24 hours.
The inoculation of *L. paracasei* B171, was performed after 24 h from the yeast inoculation, to avoid competition effects that could inable the yeasts to start the alcoholic fermentation. *L. paracasei* strain was grown in MRS broth (de Man, Rogosa, Sharpe, Oxoid, Italy) at 30 °C until the cell mumber was ± 10^8 cfu/ml. The must was finally inoculated with the suspension of *L. paracasei* at a concentration of 10^6 cfu/ml.
Samplings were taken at 0, 1, 2, 3, 7, 9, 10, 13 days and ten days after the end of the fermentation. All analyses were performed in triplicate.
At each sampling point, the following analyses were performed.

6.2.2 Microbial enumeration

Microbial counts of *S. cerevisiae* and *L. paracasei* were performed as follows. One ml of the sample taken was diluted in 9 ml of sterile saline (8.5 g/l NaCl (Sigma-Aldrich, Italy) and 1 g/l bacteriological peptone (Oxoid, Italy)) and, further decimal dilutions were performed.
S. cerevisiae was enumerated onto WL nutrient Agar (Oxoid, Milan, Italy). The spreading was carried out for pouring, after mixing the tube, and smearing, 0.1 ml of each dilution directly on the agar medium. Thus the inoculated plates were put in a thermostat at 30 °C until the growth of the colonies (48 hours), which were then counted.
L. paracasei was enumerated in MRS agar added of Delvocid (Instant DSM, USA). From each serial dilution, 1 ml of suspension was collected, placed in in a sterile empty Petri dish and subsequently included in a double layer of medium. Even in this case, the plates were placed in thermostat set at 30 °C for 48 hours and then the colonies were counted.

6.2.3 Determination of pH

The pH determination was performed using a pH meter Basic20pH (Crison, Spain), suitably calibrated according to the protocol indicated by the manufacturer of the instrument.

6.2.4 Determination of lactic and malic acid by the use of enzymatic commercial kits

Malic and lactic acid were monitored at each sampling point in order to assess the performance of the malolactic fermentation. For their evaluation, the analysis were carried out by the of enzymatic colorimetric detection kit, following the manufacturer's instructions (Biogamma, Italy).

6.2.5 Determination of alcohol content

The alcohol content (% v/v) was measured on 2 mL of each sample, taken at each sampling point, using an analyzer model Alcolyzer Plus (Anton Paar, Austria), in order to monitor the progress of alcoholic fermentation.

6.2.6 Determination of β-glucosidase activity with p-NPG

At each sampling point, the β-glucosidase activity of *L. paracasei* was evaluated, discriminating between the activities of the cell pellet and the supernatant devoid of cells using the method performed by Grimaldi et al. (2000). To do this, 2 ml of must were collected, filtered using syringe filter with porosity 0.45 μm (Millipore Corporation, Italy) to remove the yeast cells. One ml aliquot of the filtrate was then collected in Eppendorf tubes and centrifuged at 14500 rpm for 7 minutes, in order to separate the cellular pellet from the liquid phase. The supernatant was recovered and placed in another Eppendorf tube. Then, a 250 mM citrate-phosphate (Sigma-Aldrich, Italy) and 10mM p-nitrophenyl β-D-glucopyranoside (P-NPG, Sigma-Aldrich, Italy) buffer solution at pH 5.0 was added and the solution was incubated at 25 ° C for two hours.

The cellular pellet was first washed in 1 ml of a NaCl solution 150 mM (Sigma-Aldrich, Italy), centrifuged at 14500 rpm for 7 minutes and the supernatant was removed. Then, the pellet was resuspended in 125 mM citrate-phosphate (Sigma-Aldrich, Italy) and 5mM p-nitrophenyl β-D-glucopyranoside (P-NPG, Sigma-Aldrich, Italy) buffer solution at pH 5.0. This reaction mixture was then incubated for two hours at 25 °C.

After incubation, samples were added with a carbonate/bicarbonate solution (Sigma-Aldrich, Italy) pH 10.2, in order to stop the raction, and centrifuged at

14500 rpm for 7 minutes. An aliquot of 1 ml of supernatant was transferred into cuvettes, and the absorbance was read at 400 nm by NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). The molar extinction coefficient was set at 17100 M/cm.
The units of β-glucosidase enzyme activity were defined as the μmol of ρ-nitrophenol liberated per minute per milligram of sample. A calibration curve with known increasing concentrations of ρ-nitrophenol (Sigma-Aldrich, Italy), has been constructed for this purpose, by determining the absorbance at 400 nm.

6.2.7 Determination of gene expression by RT-qPCR

The expression of *L. paracasei* genes coding for ATPaseA and pstS proteins was evaluated. The first one is a proton translocating ATPase envolvend in the acid stress response, while pstS is an ABC transmembrane proton transporter envolvend in both acid and osmotic stress (Van de Guchte, 2002; De Angelis and Gobbetti, 2004; Wallenius et al., 2011). The samples taken at each sampling point during the fermentations were subjected to filtration with syringe and sterile filter porosity 0.45 μm filter (Millipore Corporation, USA) in order to remove the yeast cells. Next, 1 ml of the collected filtrate was centrifuged at 14,500 rpm for 7 minutes and, after removing the supernatant, the cellular pellet was covered with 200 μl of RNAlater® (Sigma-Aldrich, Italy). Finally, the samples were stored at -20 ° C, waiting to proceed with the subsequent analysis.

6.2.8 RNA extraction

Once the samples were taken from the freezer where they were stored, they were centrifuged for 10 minute at 13400 rpm and RNA was extracted from the cellular pellet with "Master Pure ™ Complete DNA & RNA Purification Kit" (Epicentre, USA), according to the protocol provided by the manufacturer. The only change to the indications corporate covered the expected time for the incubation with DNase at 37 °C, since, in order to have greater safety on the elimination of contaminant DNA, the time was increased from 30 minutes to 3 hours.

6.2.9 Standardization and reverse transcription of RNA

The concentration of extracted RNA for each sample was measured by the use of a NanoDrop spectrophotometer (Thermo Scientific, USA), standardized at

20 ng/µl, adding sterile MilliQ water beforehand Private RNase by treatment with DEPC.
Standardised RNA samples were converted into cDNA. Reverse transcription reactions were carried out using the ImProm-IITM Reverse Transcription System (Promega, Italy) according to the manufacturer's instructions. cDNA samples were then used as templates for qPCR reactions.

6.2.10 Primer design

The expression of *ATPaseA* and *pstS* stress genes was evaluated. Two couple of primers were designed using as template the sequences of the strain *L paracasei* N1111 avaliable on NCBI (http://www.ncbi.nlm.nih.gov/). To optimize the amplification protocol, primer specificity was tested *in silico* using the FastPCR 6.1 software (Kalendar et al., 2009) and *in vivo* using *Lactobacillus paracasei* B169 and B171.
The reactions were performed in a final volume of 25 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 U of *Taq*-polymerase (Applied Biosystems, Italy). The amplification was performed for 35 cycles at 95 °C for 1 min, 64 °C for 1 min and 72 °C for 1 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad, Italy). An initial denaturation step (95 °C for 5 min) and a final extension step (72 °C for 5 min) were used. The PCR products were verified by electrophoresis in a 2 % agarose gel using 0.5X TBE as the running buffer. Ethidium bromide (0.5 µg/ml) was added to the gel before solidification. After electrophoresis, the gels were examined using the BioImaging System GeneGenius (*SynGene*, United Kingdom).

6.2.11 Real Time quantitative PCR

The quantitative analysis of the expression of the *ATPaseA* and *pstS* coding gene was performed by quantitative PCR on the cDNA obtained through the reverse transcription.
The reference genes used in the following analysis were *ldhd* and *ftsz*. Therefore each cDNA sample was amplified in 4 different qPCR reactions using 3 couples of primers: atpAf (5'- CCACAGTCGCTTGTTGGAAC -3') and atpAr (5'- TTAGTCGGGATGTACGCGG -3'), targeting the *ATPa* coding gene; pstS *f* (5'- TGTCGGCAGTACGCCTG -3') and pstS *r* (5'-GCCATTGATTGGTCGTGACATT -3'), targeting the *pstS* coding gene; LdhdF (5'-GCCGCAGTAAAGAACTTGATG- 3') and LdhdR (5'-TGCCGACAACACCAACTGTTT-3'), targeting the D-lactate dehydrogenase enzyme coding gene; paraftsZF (5'-GACCCGCGGCCTAGGTGC-3') and

paraftsZR (5'-CCACGCCGACAGTCAAGGC-3'), targeting the phosphate acetiltransferase enzyme coding gene.
Real-time PCR mixtures contained 10 µl of 2x SsoFast EvaGreen Supermix (Biorad, Italy), 0.4 µM of each primer, 2 ng/µL of cDNA and the reaction mixture's volume was adjusted to 20 µL with sterile DNA-free Milli-Q water. Real-time PCRs were performed using a RotorGene Q system (QIAGEN, Italy), using the time/temperature settings described as follows: 50 repetitions of 98° for 30 sec, 60 ° C for 20 sec, 72 ° C for 20 sec (with fluorescence signal acquisition); an initial denaturation at 95 ° C for 3 min and a final melt step (56> 99 ° C, with a temperature increase of one every 5 seconds).
Fluorescence signal acquisition was performed during the extension step. To verify that formation of non-specific products or primer dimers had not occurred, the dissociation curves of the final products for each PCR were analysed from 55 to 95 °C at 1 °C intervals. Each qPCR reaction was performed in triplicate for each sample.
Data obtained by qPCR were analysed using qbaseplus software (Biogazelle Belgium) in order to identify the most stable reference genes and to calculate the expression values of *ATPaseA* and *pstS* coding gene by normalization against the two selected internal controls.

6.3 Results and discussion

6.3.1 Microbial enumeration

pH 3.2

Microbiological sampling was performed every 48 hours, starting from the must, till the end of fermentation. Taking into consideration that the must was coinoculated, alcoholic fermentation finished before the MLF. The results of the microbial counts are described in the Figures 6.1 and 6.2 and represent the average plate count value of the performed analysis.

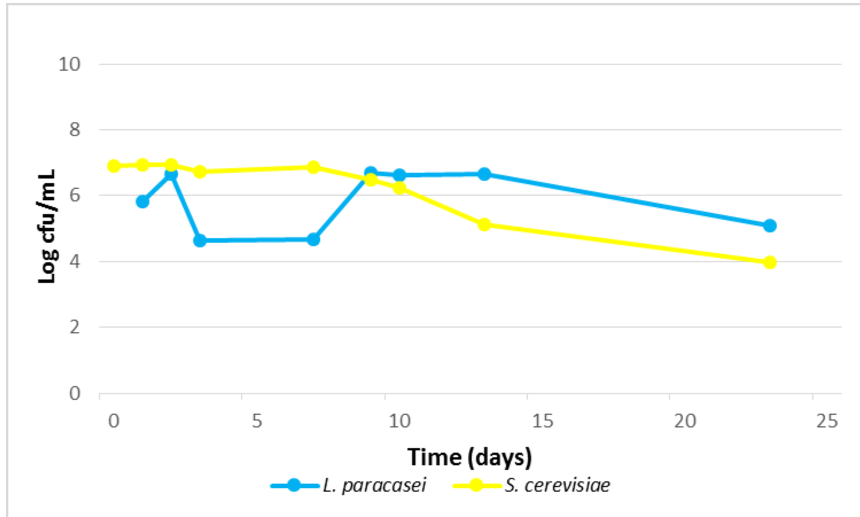

Figure 6.1 Microbial count of *L. paracasei* and *S. cerevisiae* at EtOH 11% final concentration after alcoholic fermentation.

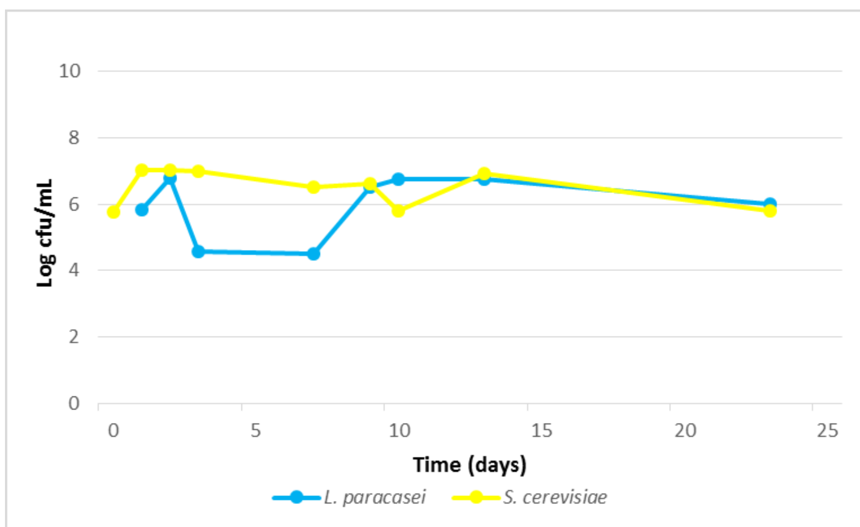

Figure 6.2 Microbial count of *L. paracasei* and *S. cerevisiae* at EtOH of 15% final concentration after alcoholic fermentation.

Both the microorganisms were inoculated at a concentration of 6 log ufc/ml. As it can be seen from the growth curve of both the tested conditions, *S. cerevisiae* and *L. paracasei* had a slightly different pattern. In Figure 6.2, it is possible to observe that from T0 to T7 *S. cerevisiae* showed a constant amount of cells, and started decreasing at T9. This decrease could be due to the consumption of fermentable sugars into the must. *L. paracasei* increased in

concentration at the beginning, then at T2 the count decreased due to an adaptation phase of the LAB to the harsh conditions of the must (low pH, high concentration of sugar, competition with yeasts). In correspondence of the decrease in cell number of the yeasts, the growth curve of the LAB shows an increase, being after that, stable. This trend is probably due to the decreasing competition with yeasts and the beginning of autolysis of dead *S. cerevisiae* cells, which determined a release of free amino acids, mannoproteins, vitamins and other substances that have been associated with the stimulation of bacterial growth in wine (Guillox-Benatier et al. 1995).
In Figure 6.2, plate count values for the other tested condition (15% EtOH final concentration after alcoholic fermentation) are reported. In this case, *S. cerevisiae* viable cells increased after inoculation maintaining a stable result until TVI, when they started decreasing. *L. paracasei* showed a trend similar to the one obtained during the fermentation carried out in presence of a lower concentration of sugar, and the same considerations already discussed can be made.
Therefore there was not a significant difference between the two different experimental conditions tested.

pH 3.8

The results of the sampling are described in the Figures 6.3 and 6.4 and represent the average plate count value of the performed analysis.

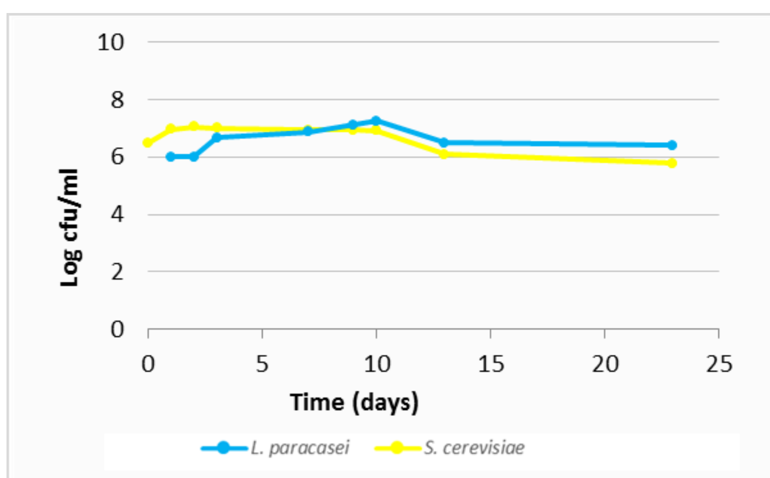

Figure 6.3 Microbial count of *L. paracasei* and *S. cerevisiae* at EtOH 11% final concentration after alcoholic fermentation.

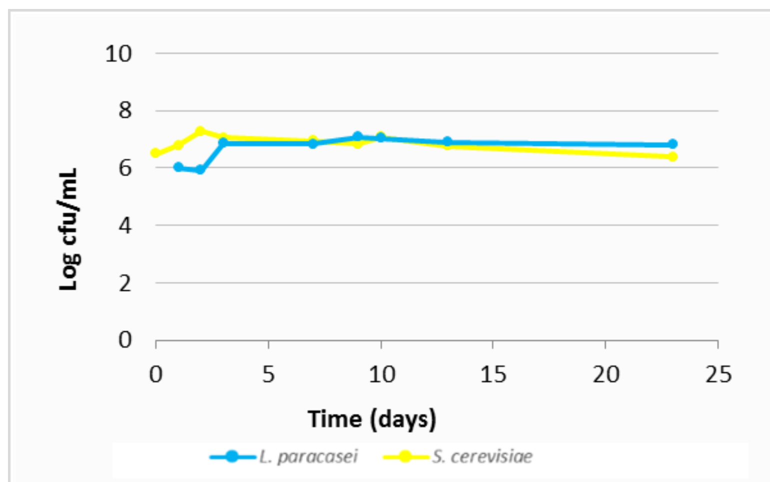

Figure 6.4 Microbial count of *L. paracasei* and *S. cerevisiae* at EtOH 15% final concentration after alcoholic fermentation.

Looking at the enumerations of *S. cerevisiae*, it is possible to note the achievement of maximum microbial count after to two days in both the tested conditions. Thus, different concentration of sugar did not affect the adaptation of yeasts, nor theyr growth in both cases. No latency phases were observed and the increase of the number of microorganisms occurred fast (from 6.4 to 7.1 log cfu/ml and from 6.5 to 7.3 cfu/mL for samples coming from the fermentations respectively to 11 and 15% v/v of EtOH). Stationary phase was reached after 10 days, followed by an increase of the number of viable cells between day 10 and 13. This is could be due to the decrease of fermentable substrate, connected as the end of the alcoholic fermentation, logically more pronounced for the final 11% v/v EtOH, because less rich in sugars.
L. paracasei had a short latency phase, between days 1 and 2, followed by a growth that continues until day 10, the point of maximum microbial load detected. After that, in both the thesis, a decrease in bacterial concentration was observed.
In general, comparing the growth curves, is possible to affirm that the different sugar content and the resulting alcohol content did not seem to affect the microbial growth.
Substantial differences were not found even between the fermentations conducted at different pH.

6.3.2 pH measurements

Must had an initial pH of 3.4, adjusted with the addition of Cloridric acid (Carlo Erba, Italy), at a values of 3.2 and 3.8 before the beginning of the experiments. Figures 6.5 and 6.6 show how pH changed in the four thesis analysed.

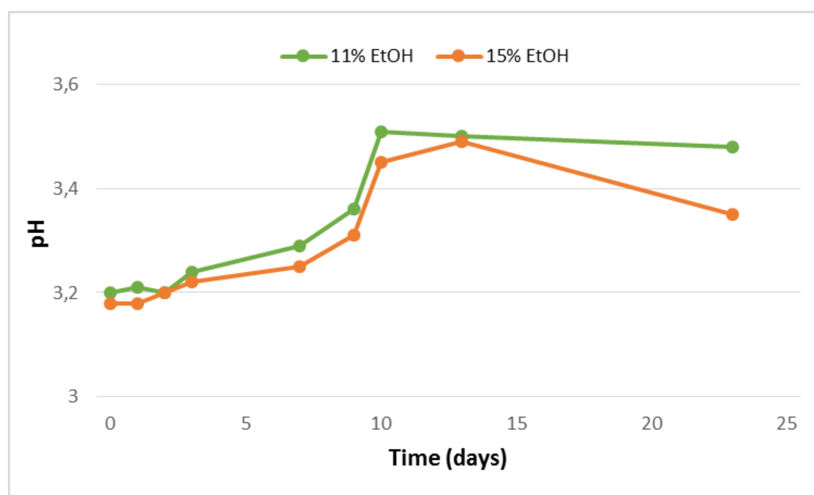

Figure 6.5 Values of the determination of the pH for the alcoholic percentages of 11 and 15% (v/v) after alcoholic fermentation in must with initial pH value of 3.2.

The inoculated must started from a pH value of 3.2. This value increased to 3.29 and 3.25 average. This change is related to the beginning of the MLF that started at T3 (as wil be reported below). According to other authors (Margalit, 1997), at the end of the MLF the increase in pH was between 0.3 and 0.4 units.

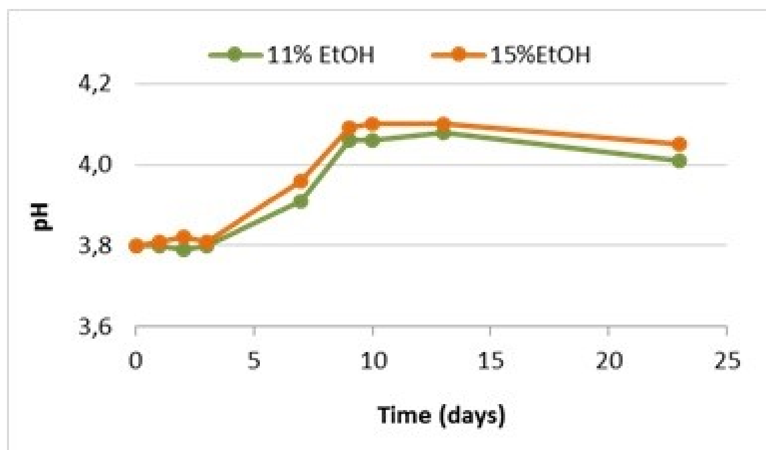

Figure 6.6 Values of the determination of the pH for the alcoholic percentages of 11 and 15% (v/v) after alcoholic fermentation in must with initial pH value of 3.8.

In the second thesis, the pH of the original must was 3.8 at the biginning. An increase occurred presumably due to the expiration of the MLF, from the day 7, where the pH values observed were close to 4.1.

6.3.3 Determination of lactic and malic acid by the use of enzymatic commercial kits

The falling level of L-malic acid and rising level of L-lactic acid were monitored by the use of an enzymatic kit (Biogamma, Italy). The results found are described in Figures 6.7, 6.8, 6.9 and 6.10 according to starting pH and the wine alcoholic percentage.

pH 3.2

In the trial where 11%v/v ethanol had to be developed, MLF has been completed after 6 days since the inoculation of the must, while in presence of a higher concentration of sugar, leading to an increased concentration of ethanol (15% v/v), MLF was over in 8 days. Both cases demonstrate the ability of the selected strain (B171) to perform MLF in coinoculation with *S. cerevisiae*, in presence of low pH values (3.2) and both in presence of high and low sugar/ethanol concentration.

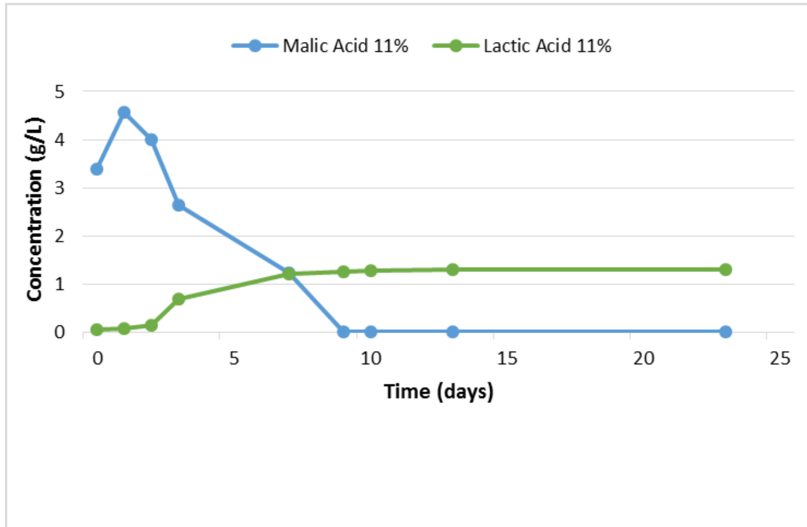

Figure 6.7 Concentration of Malic and Lactic acid performed by Biogamma kit at EtOH of 11% (v/v).

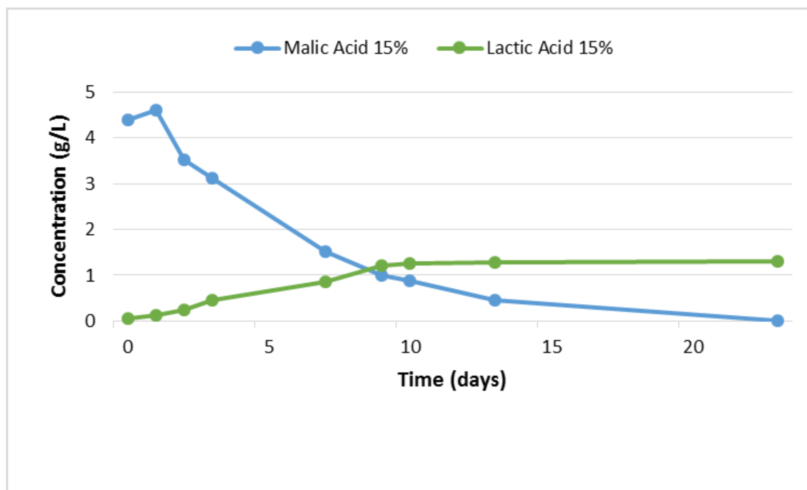

Figure 6.8 Concentration of Malic and Lactic acid performed by Biogamma kit at EtOH of 11% (v/v).

pH 3.8

Figures 6.9 and 6.10 show that in both the tested conditions, the MLF started at day 2, corresponding to the time following inoculation of *L. paracasei*. At this point, a minimal decrease in the malic acid amount was detected. In day 9 a peak of the production of lactic acid was observed, corresponding to the depletion of the malic acid. Malic acid ranged from an initial value of 3.90 g/L to 0.04 g/L for the samples with 11% v/v of alcoholic degree potential, and to

0.05 g/L to n those with 11% v/v of alcoholic degree potential. Vice versa, the lactic acid started from a value of 0.04 g/L and reached the concentrations of 1.36 g/L and 1.35 g/L in the samples with 11% and 15% v/v of alcoholic degree potential respectively.

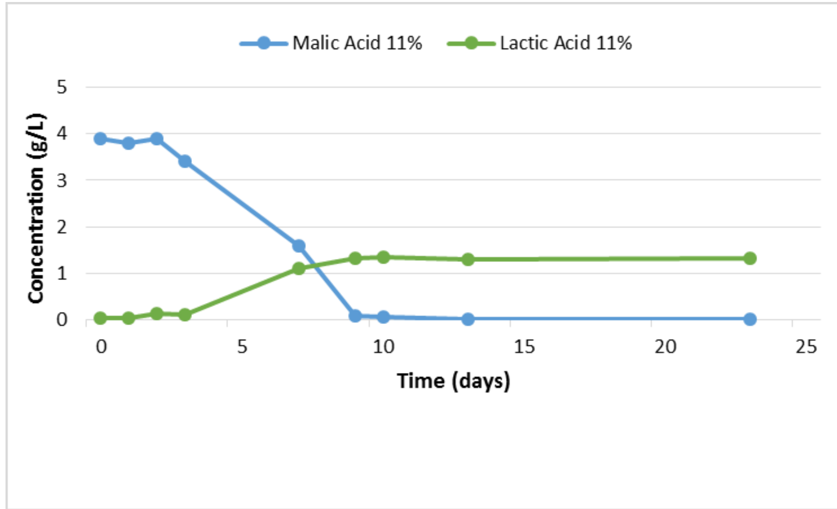

Figure 6.9 Concentration of Malic and Lactic acid performed by Biogamma kit at EtOH of 11% (v/v).

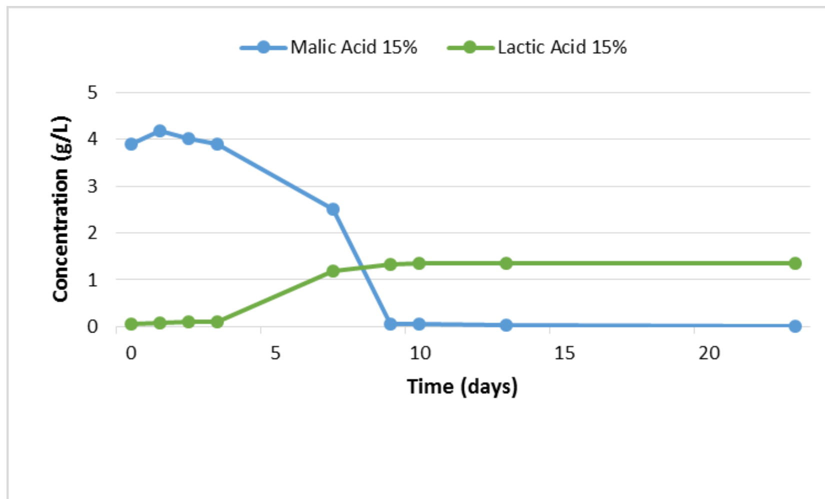

Figure 6.10 Concentration of Malic and Lactic acid performed by Biogamma kit at EtOH of 15% (v/v).

The ninth day of sampling, the values of both organic acids remained constant, indicating the end of MLF. This consideration was confirmed by the trend of

pH values previously reported, in which you see the same pH level off from the day 9, another sign of the conclusion of the conversion of malic acid into lactic acid.
Then, significant differences among the four condition tested were not found.

6.3.4 Determination of alcohol content

Figure 6.11 and Figure 6.12 show the trend of the alcohol content in the samples analysed.

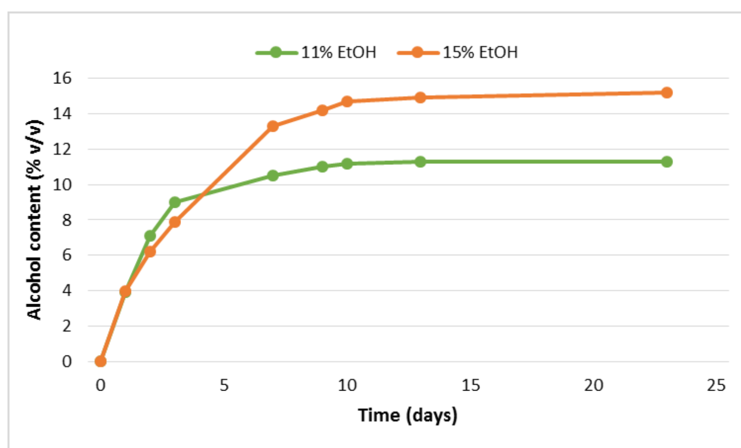

Figure 6.11 Trend of the alcohol content % v/v musts with starting pH value of 3.2.

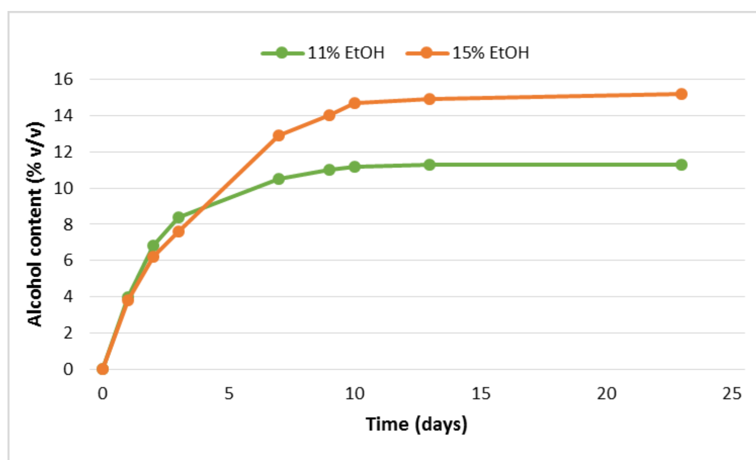

Figure 6.12 Trend of the alcohol content % v/v musts with starting pH value of 3.8.

The alcohol content grew uniformly during the alcoholic fermentation and reached the expected level after 10 and 13 days of fermentation, in both the

different condition tested, confirming how the co-presence of *S. cerevisiae* and *L. paracasei*, if properly managed, allow a regular fermentation course, regardless of the sugar content and the pH of the original must.

6.3.5 Determination of β-glucosidase activity with p-NPG

β-glucosidase is an enzyme responsible for enhancing wine aroma during fermentation and aging by releasing the aromatic compounds from their glycosilated precursors. Fungal and plant β-glucosidases may be aroma liberators, but these enzymes do not display their effectiveness on aroma during winemaking due to low pH and temperature, and high ethanol conditions (Wanapu et al., 2012).

pH 3.2

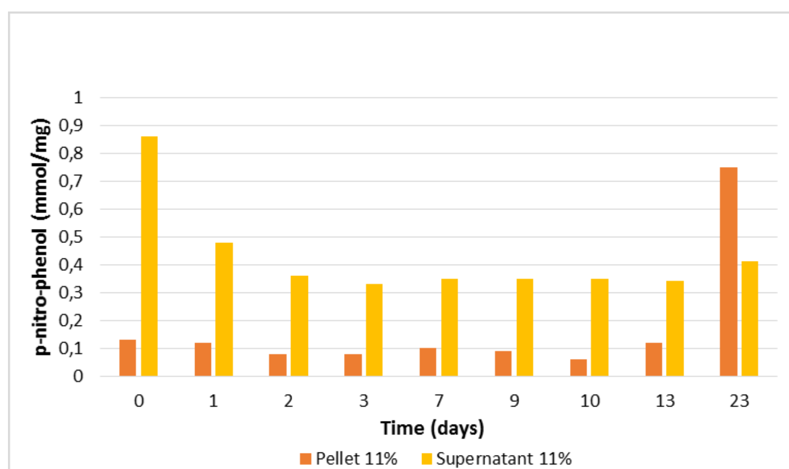

Figure 6.13 Concentration of Pellet and Supernatant at EtOH of 11% (v/v) with p-NPG.

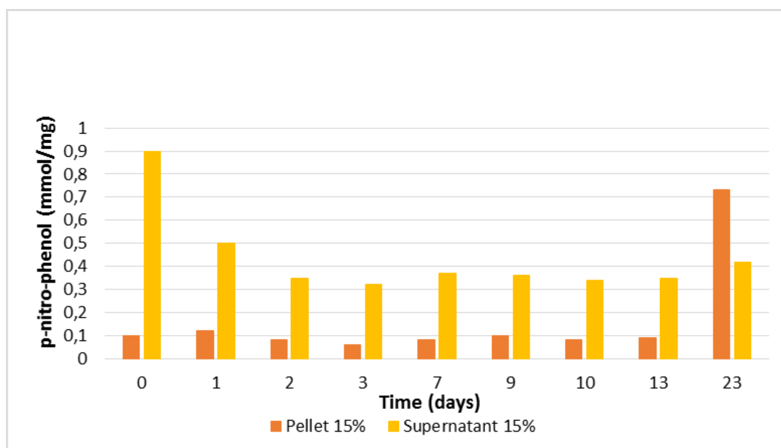

Figure 6.14 Concentration of Pellet and Supernatant at EtOH of 15% (v/v) with p-NPG.

The β-glucosidase activity was evidenced by hydrolysis of the substrate (ρNPG) and release of ρ-nitrophenol. The readings were made at the spectrophotometer at a wavelength of 400 nm, and both the cell pellet and the supernatant were analysed. Figures 6.13 and 6.14 show a decrease of the enzymatic activity in the supernatant after inoculation, maintaining then the same levels till the end of fermentation, when (at T9) an increase is shown. On the other hand, considering the enzymatic activity in the pellet, an almost constant result until T9, when there is an increase. There are no large differences between the results obtained in presence of 11% and 15% (v/v) ethanol. This demonstrates that ethanol is not affecting the enzymatic activity of β-glucosidase, thus not influencing the aroma releasing capacity. It must also be said that Grimaldi et al. (2000) demonstrated the negative effect of glucose on the activity of β-glucosidase enzyme, and this could explain the increase in activity in corrispondence of the last sampling point, when sugar concentration was very low.

pH 3.8

The results are shown in Figures 6.15 and 6.16 starting from T1, the day of inoculation of *L. paracasei* in the following tested conditions.

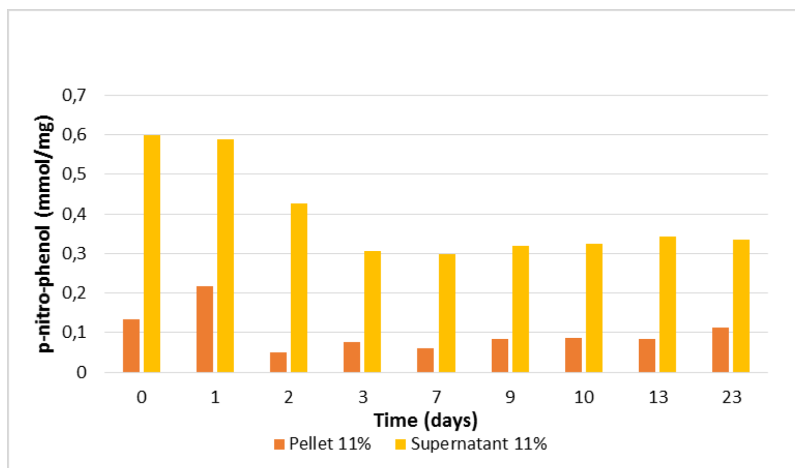

Figure 6.15 Concentration of Pellet and Supernatant at EtOH of 11% (v/v) with p-NPG.

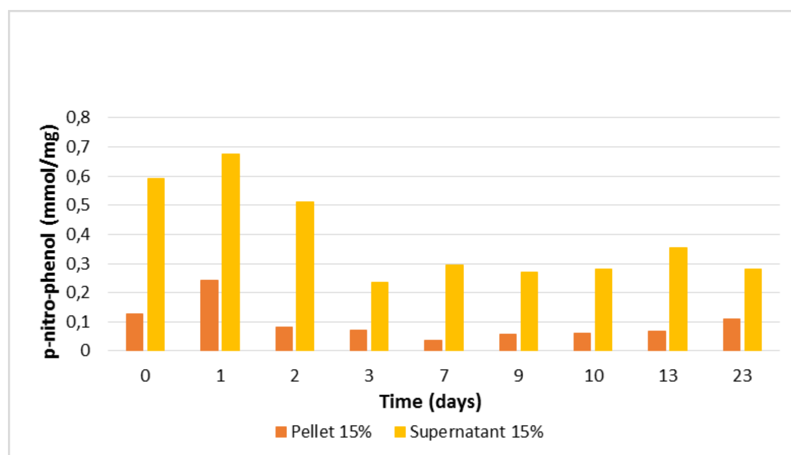

Figure 6.16 Concentration of Pellet and Supernatant at EtOH of 15% (v/v) with p-NPG.

The highest enzyme activity of the supernatant was observed in the days following the inoculation of bacteria (days 1, 2, 3), then a decrease was detected, reaching values which remained stable during the entire monitoring time, in both theses considered.
In the pellet a lower enzymatic activity was found, compared to the supernatant, for each sampling point, for each of the examined thesis. Furthermore, in this case, the values remained more constant.
In contrast with Grimaldi et al., (2000), the values of the highest enzymatic activity were observed in presence of high sugar concentrations and, in addition, nothing influenced the different values of sugar concentration before and alcoholic degree then had no effects on β-glucosidase enzyme activity, the two theses in fact had comparable values.

6.3.6 Determination of gene expression by RT-qPCR

Observing the gene expression of the samples at ethanol concentration of 11% (Fig. 6.17), is possible to observe increase in the expression of all the analysed genes afer the end of the alcoolic fermentation. In this case, the expression was higher as the pH increased. This fact is more evident in Figure 6.18, where the semple were take from a must of a higher initial pH.

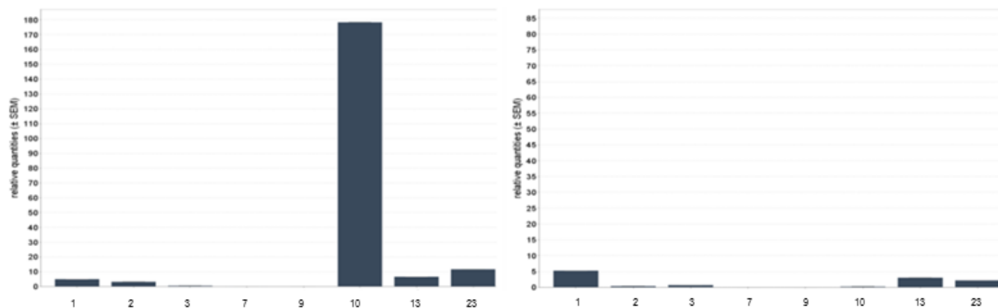

Figure 6.17. Gene espression of *atpA* (on the left) and *pstS* (on the right) gene in EtoH 11% and pH 3.2.

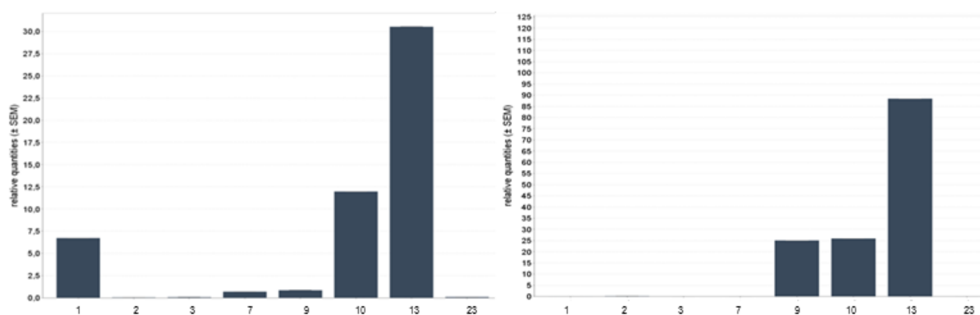

Figure 6.18. Gene espression of *atpA* (on the left) and *pstS* (on the right) gene in EtoH 11% and pH 3.8.

Gene expression of *atpA* gene was almost inhesistent during the fermentetion of the must at 15% of EtOH and pH 3.2 (Fig. 6.19), while was encreased in must at 15% of EtOH and pH 3.8 (Fig. 6.20).

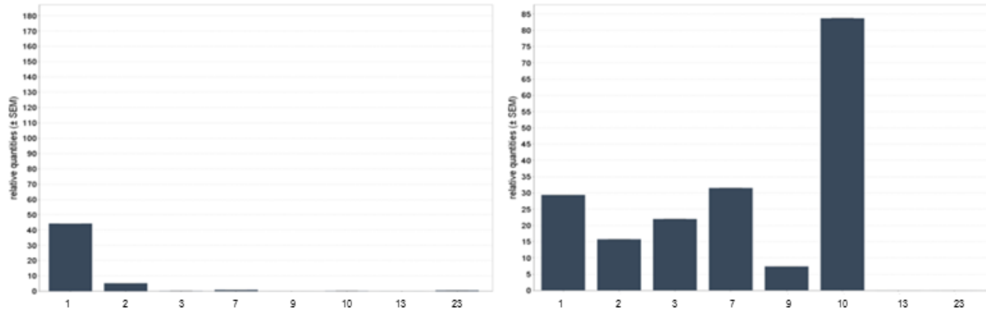

Figure 6.19 Gene espression of *atpA* (on the left) and *pstS* (on the right) gene in EtoH 15% and pH 3.2.

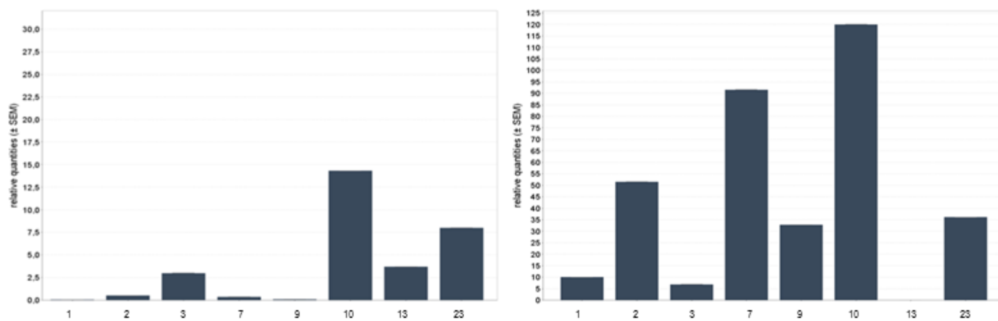

Figure 6.20 Gene espression of *atpA* (on the left) and *pstS* (on the right) gene in EtoH 15% and pH 3.8.

This confirms the expression values obtained for *atpA* gene in figure 6.17. The expression of *pstS* gene was higher in the samples at EtOH 15%, and, also in this case, the expression increased as the alcholic fermentation finished.

6.4 Conclusions

The use of MLF in winemaking consists in the conversion of malic acid to lactic acid and CO_2 by lactic acid bacteria (*Oenococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*). The use of MLF is required since it can improve the body or mouth feel of the wine, without significantly diminishing the fruity component (Lerm et al., 2010).
The practice to control MLF by inoculatin selected bacterial strains, has various advantages, including increased chances of the successful completion of MLF and reduced risks associated with spontaneous MLF by spoilage LAB. The most of the studies on MLF focused on *Oenococcus oeni*, bacterium typically used for this kind of fermentation, while few studied other LAB,

including *Lactobacillus* species and *Pediococcus* (Cho et al., 2011; Michlmayr et al., 2009).
In this study *L. paracasei* was analysed to assess its capability as starter for MLF in co-inoculation with *S. cerevisiae*.
This species was able to carry out the MLF, converting in few time all the malic acid into lactic acid. The malolactic activity was not affected by different concentrations of the sugar to which the fermentations were conducted (11 and 15% v/v potential alcoholic degree) nor from the two different pH condition tested (pH 3.2 and 3.8). It seems, therefore, possible to define *L. paracasei* a possible replacement of (or adjunct to) *O. oeni* as regards the conduction of the MLF in wines with characteristics comparable to those considered in this project.
In order to evaluate if different EtOH concentration and different pH values could affect the gene expression of stress related genes of the strain B 171, the expression of *atpA* and *pstS* genes was evaluated.
Observing the obtained results, it is evident that the expression of these genes was higher when the malolactic fermentation started. It is plausible that this increase in the expression of both genes occurred in response to a first stage of adaptation of the same strain. However, gene expression was higher for both genes in the semples growth with an ethanol concentration of the 15% and pH 3.8. At the same concentration of ethanol, but at lower pH, there was a greater expression of the gene *pstS*, in disagreement with what was observed by Wallenius et al. (2012) about gene expression of these genes in *L. plantarum* at different pH values. These results suggest that the conditions less stressful for the strain studied are found to be those of the theses characterized by a lower concentration of ethanol.

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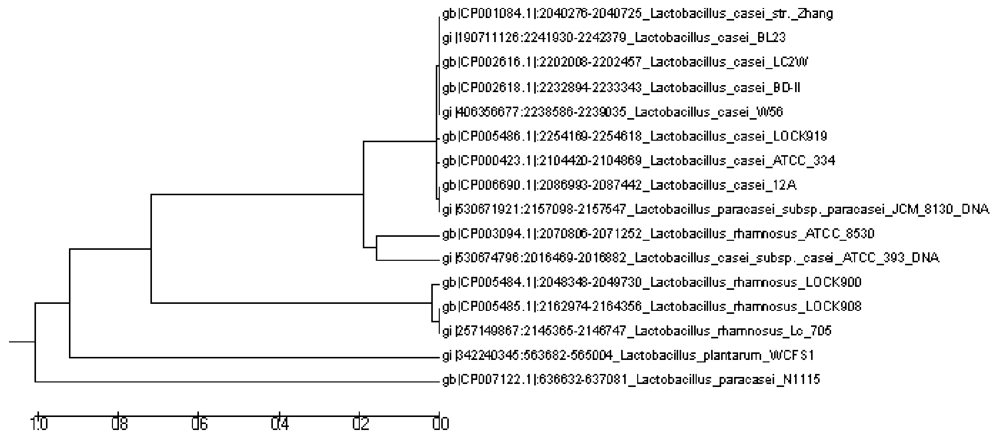
Appendix 1

Stress related genes *in silico* analysis - UPGMA trees



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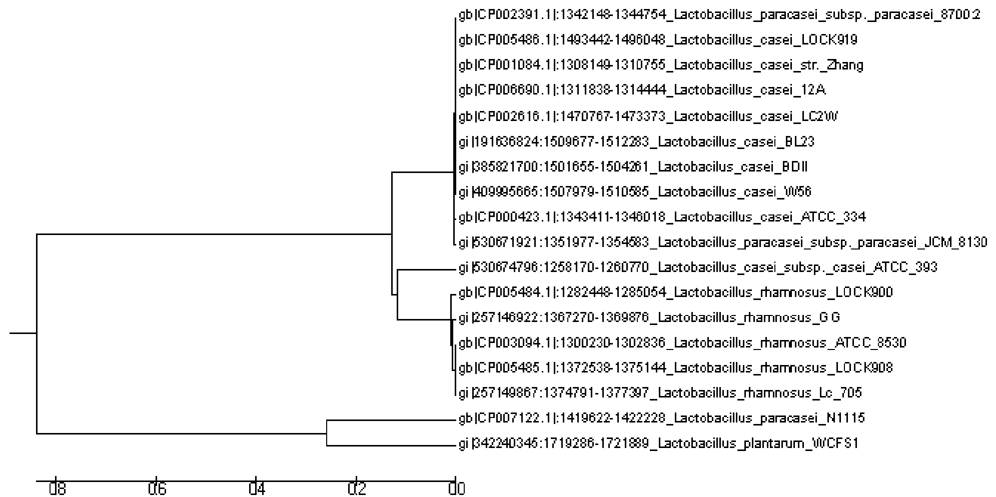

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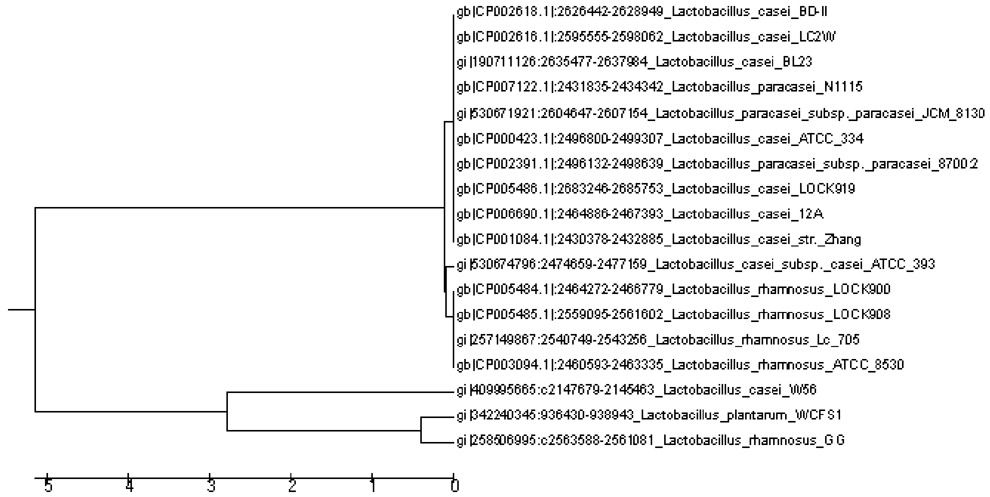

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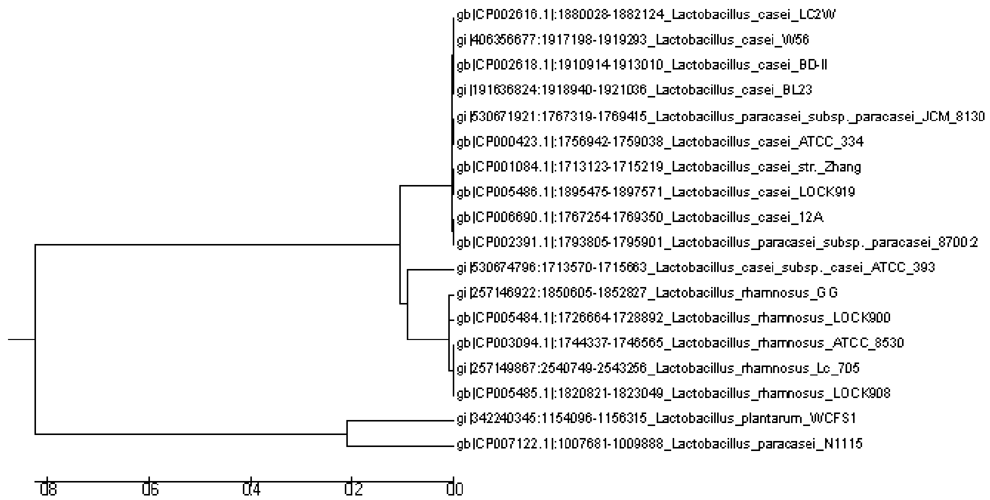

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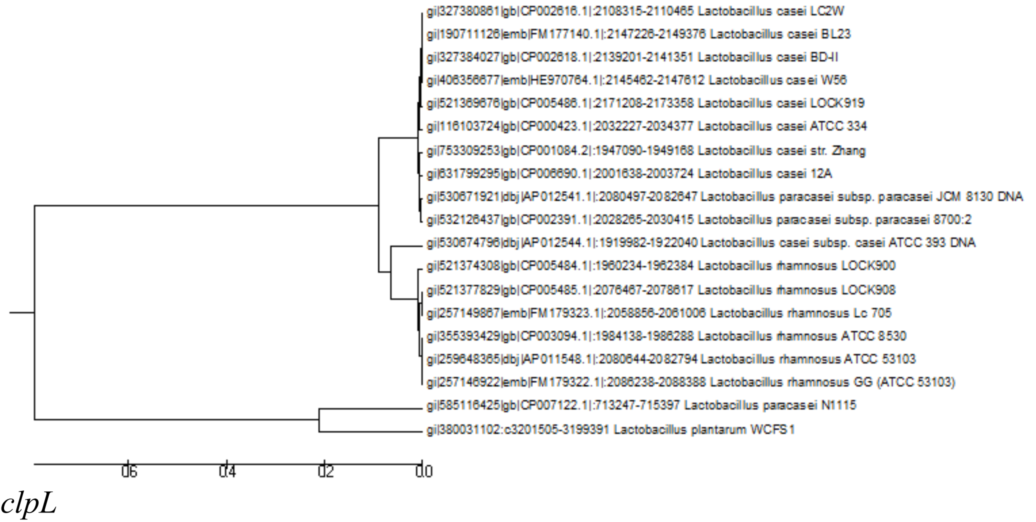

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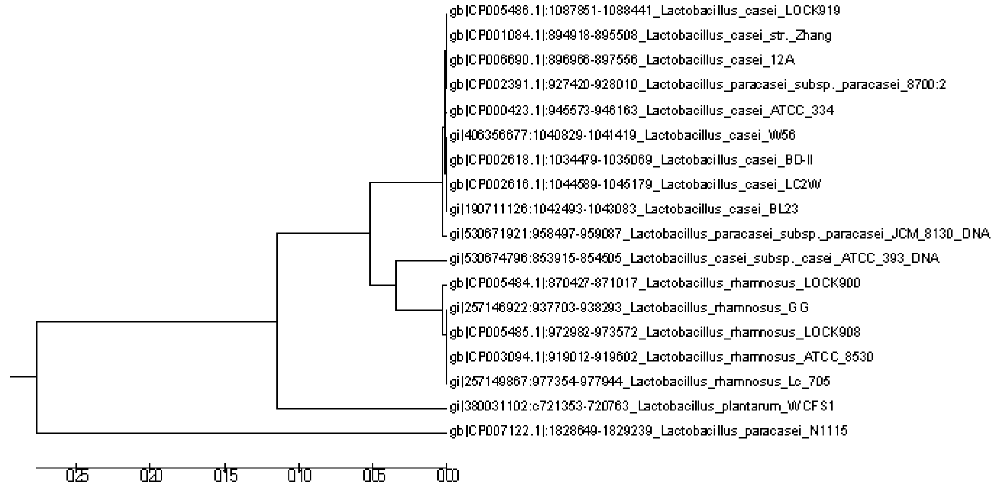

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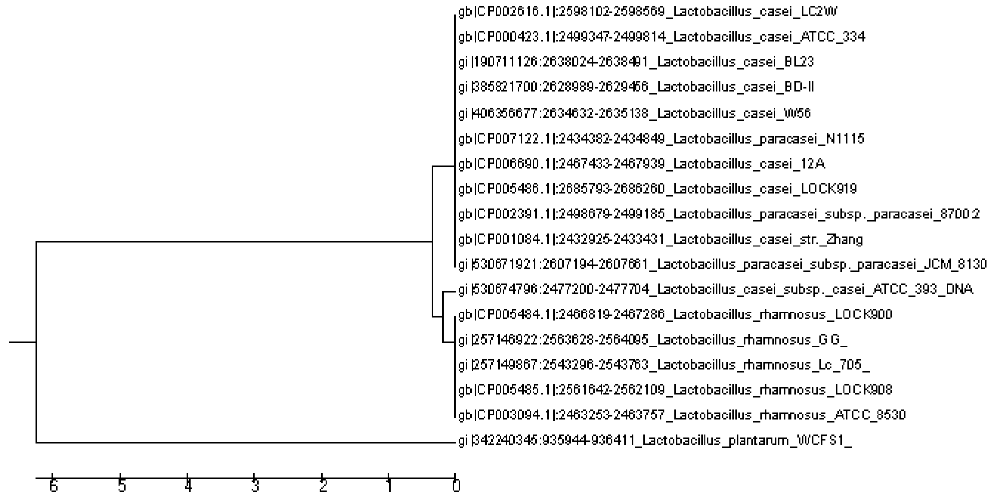

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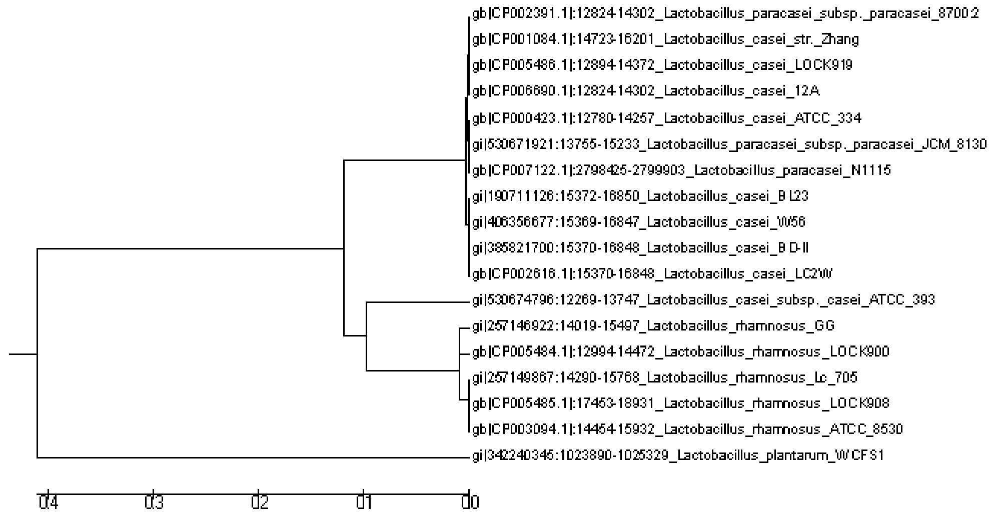

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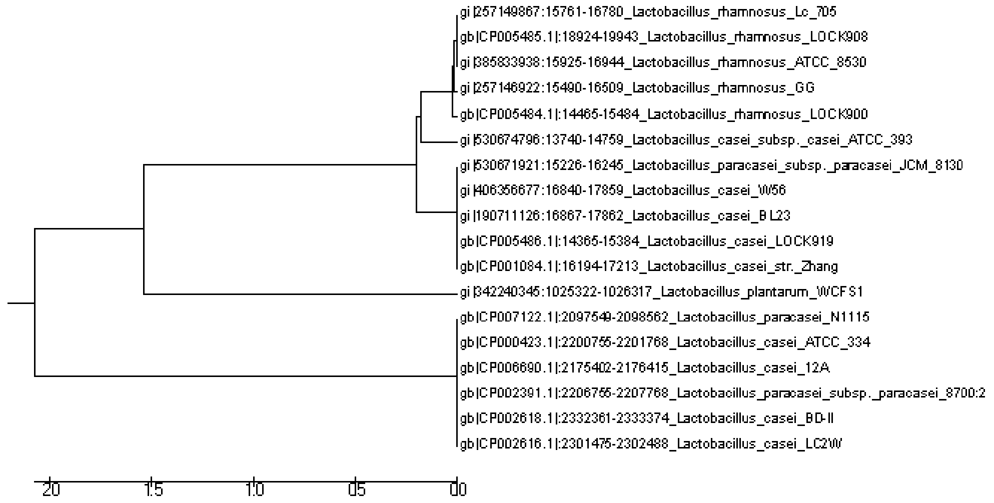

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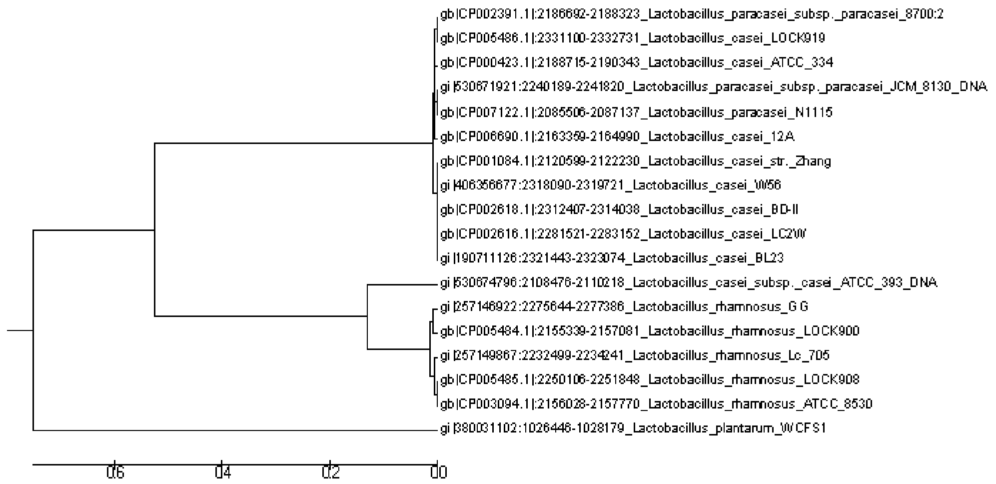

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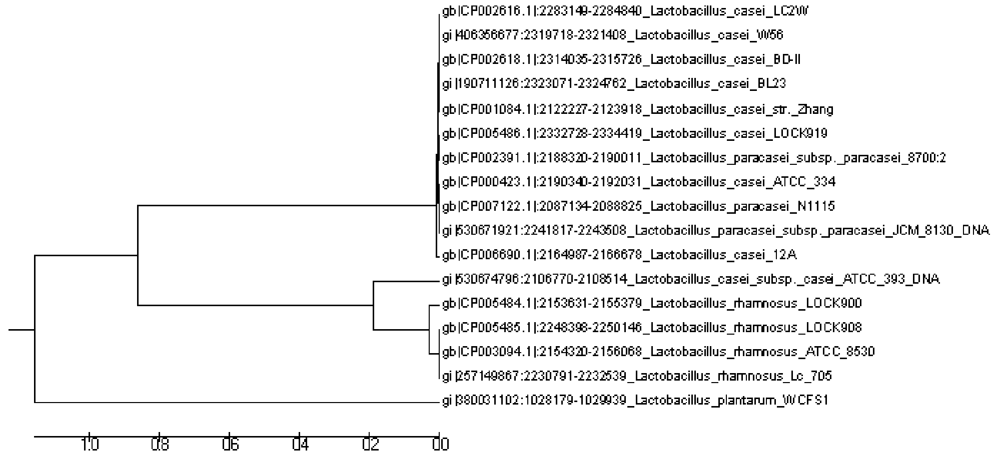

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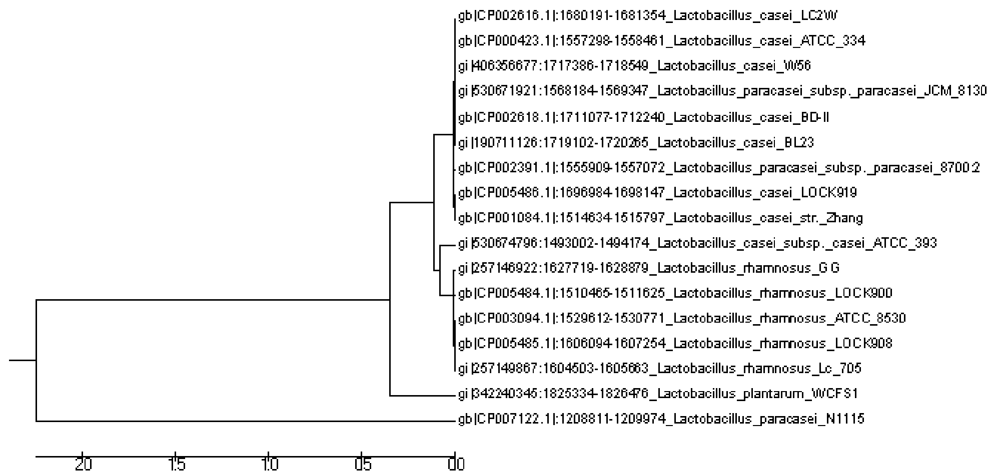

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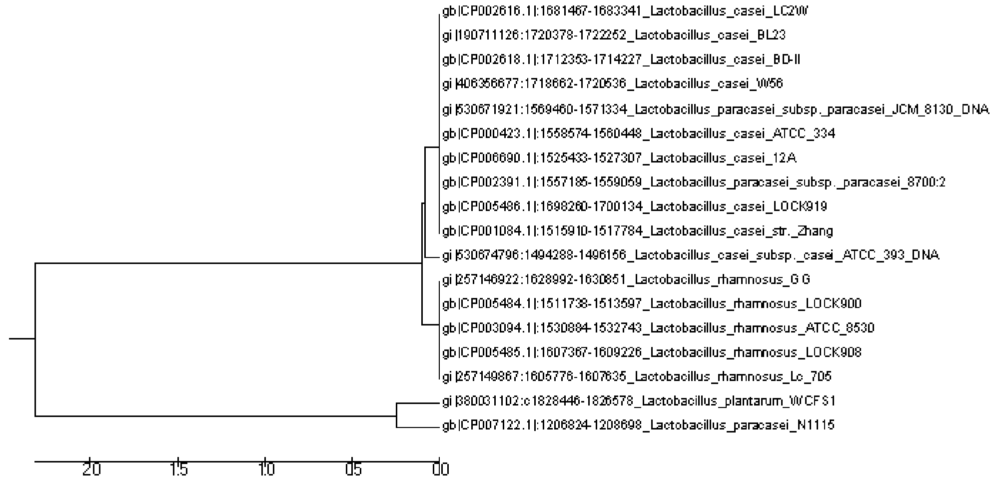

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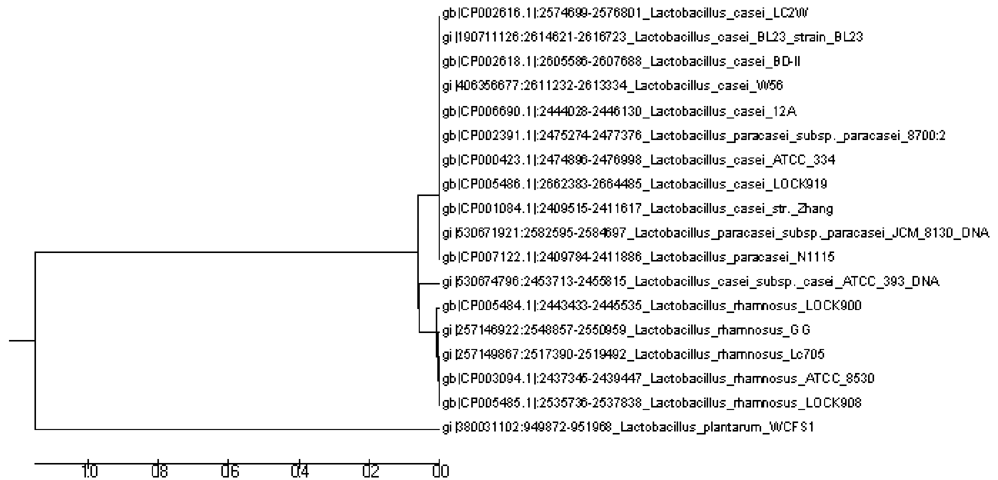

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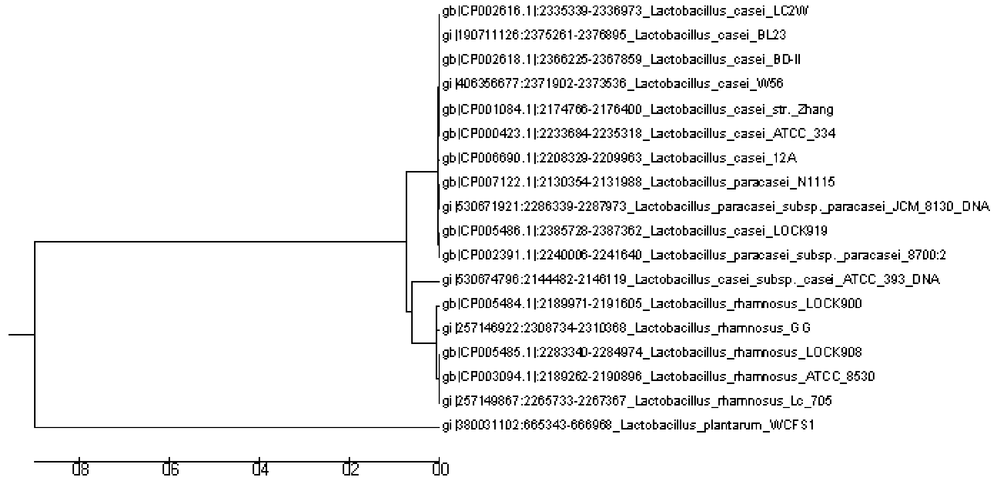

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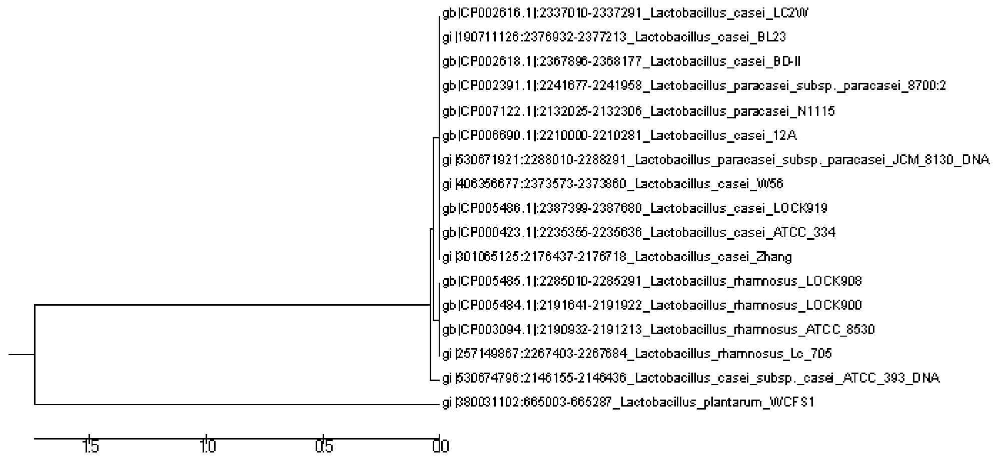

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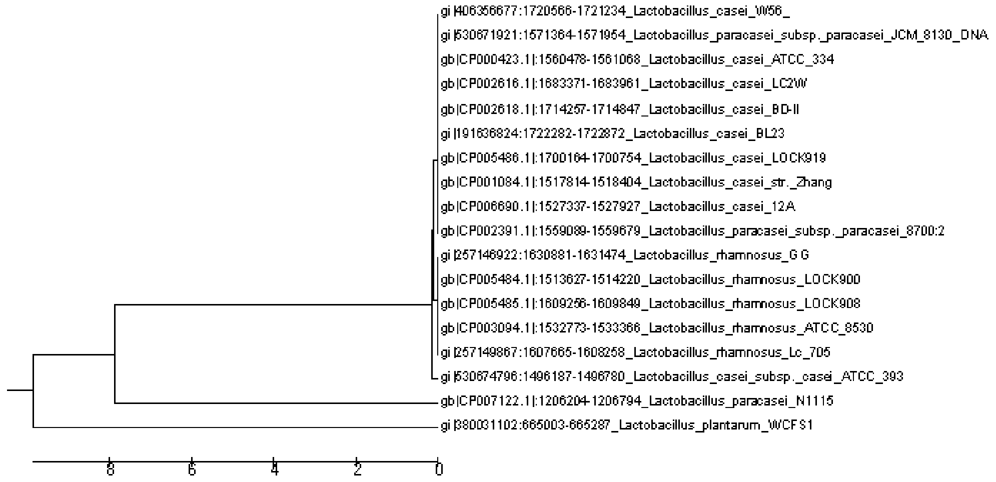

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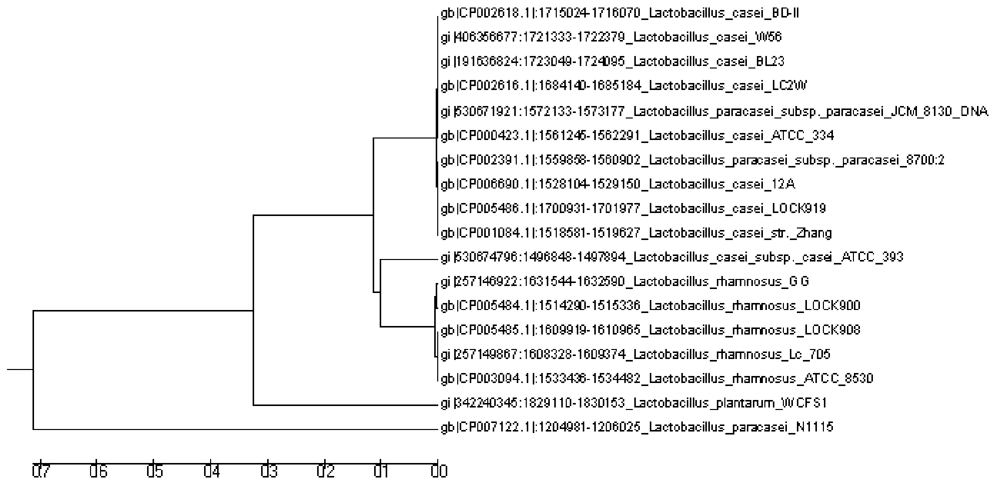

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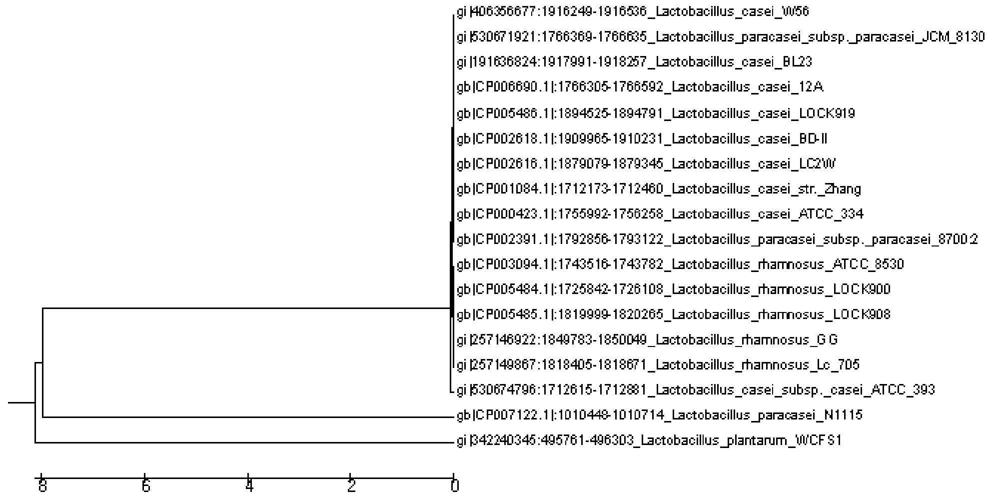

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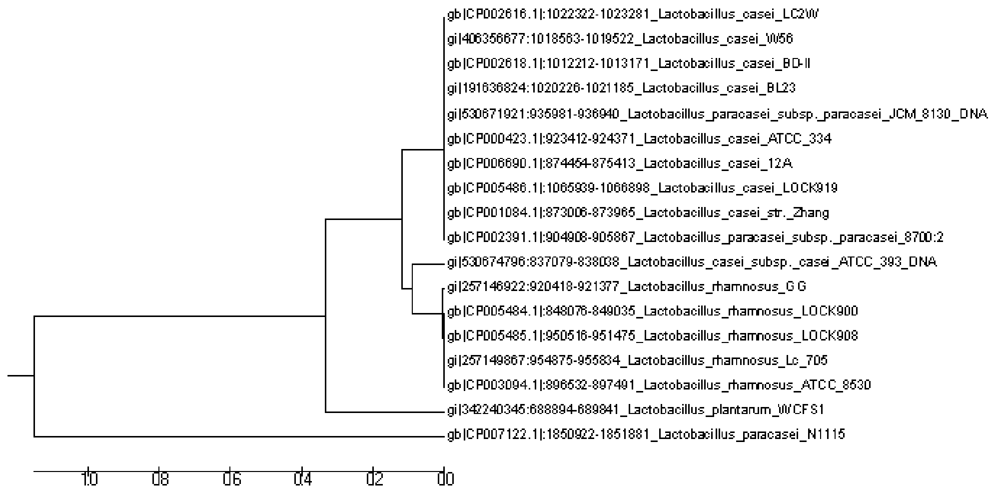

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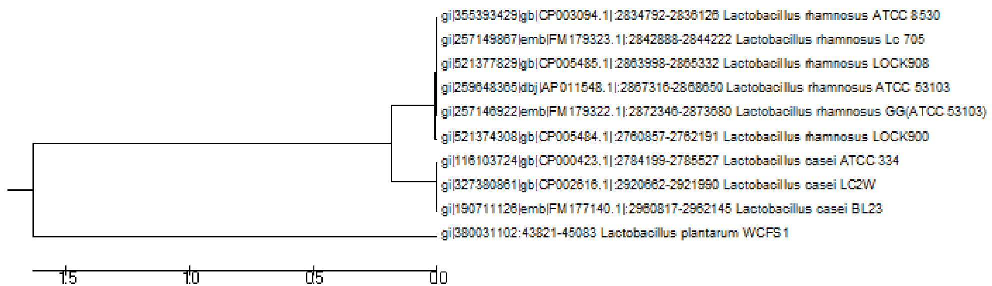

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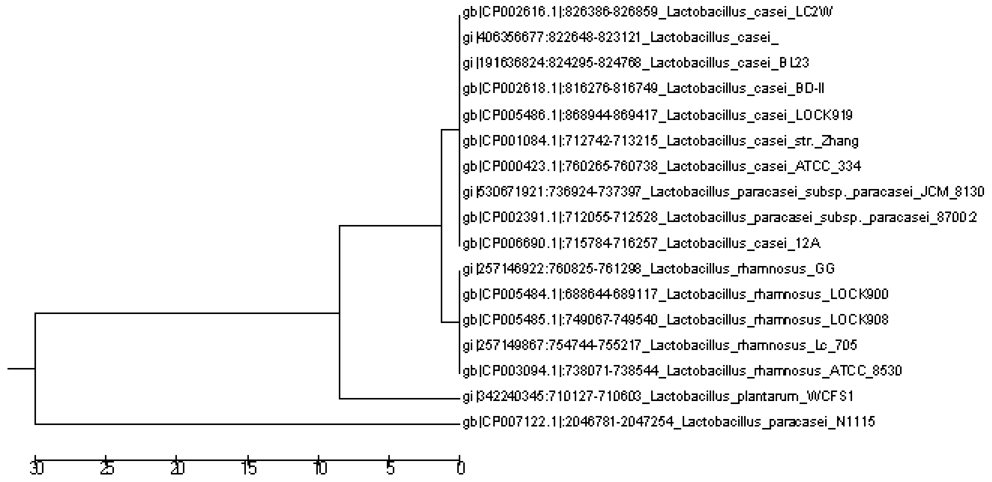
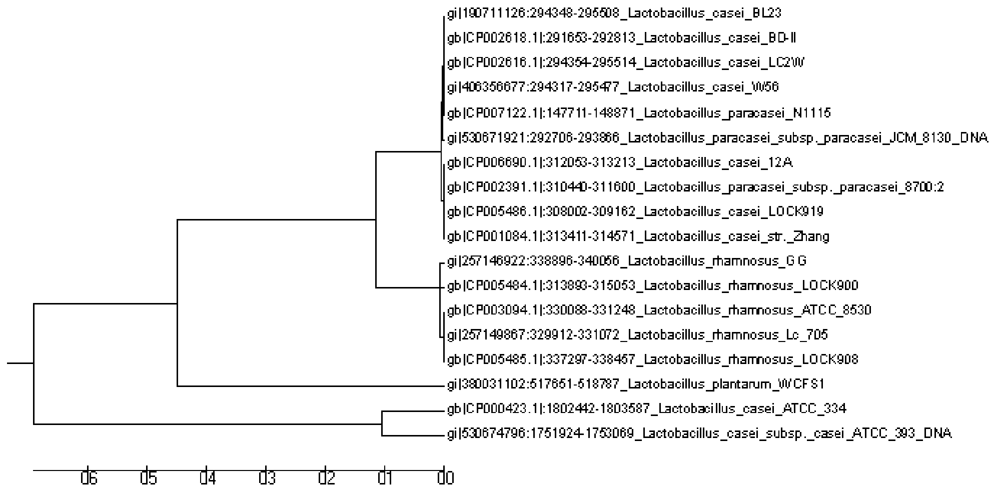
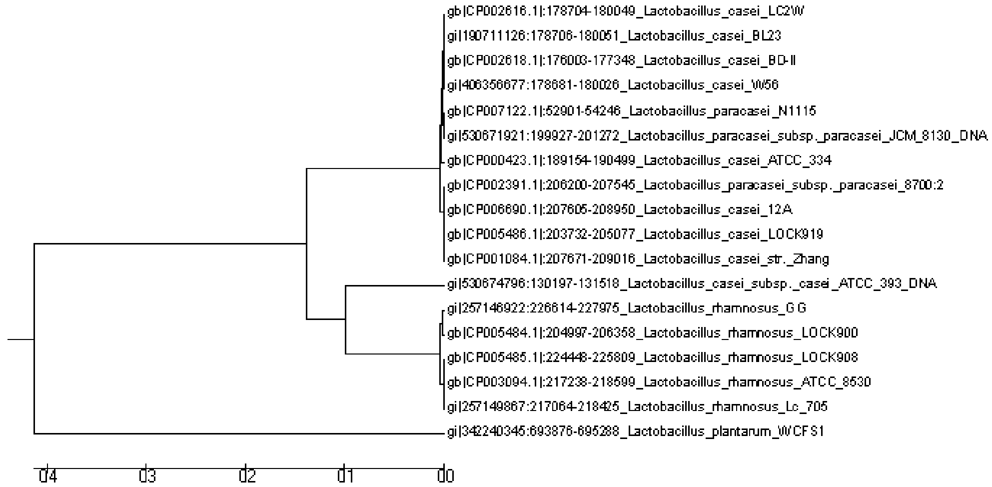


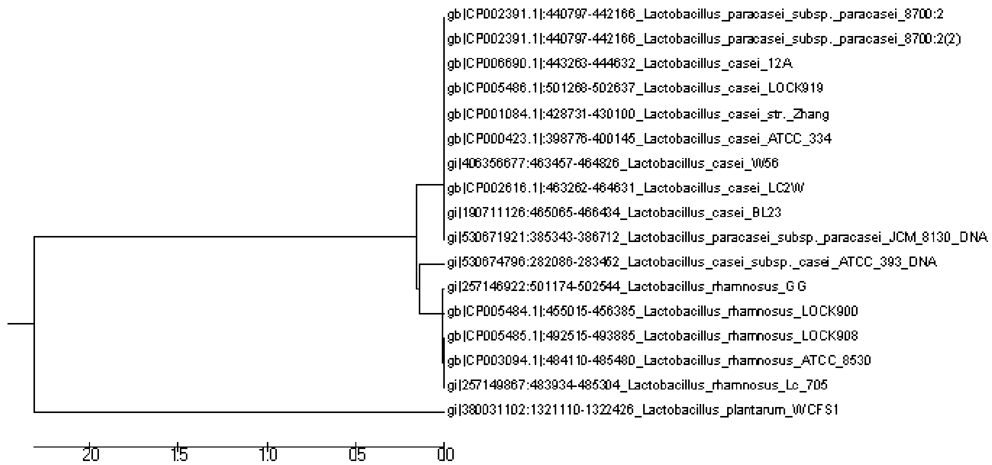
luxS



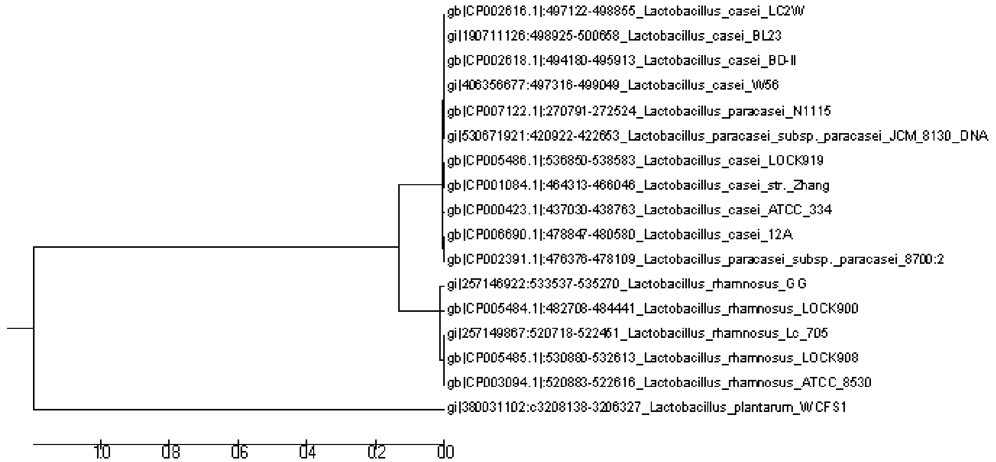
nagA



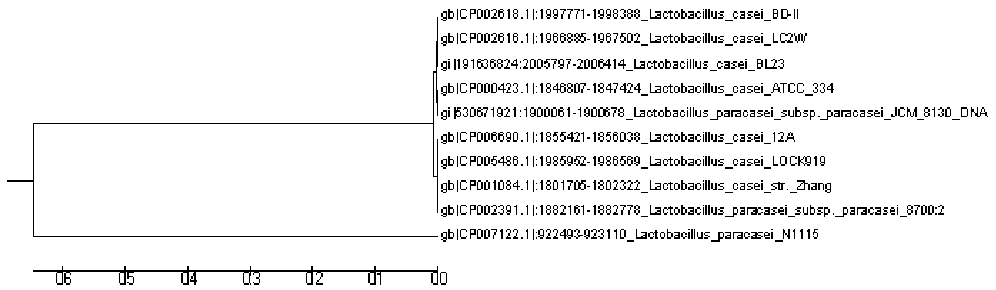
nox



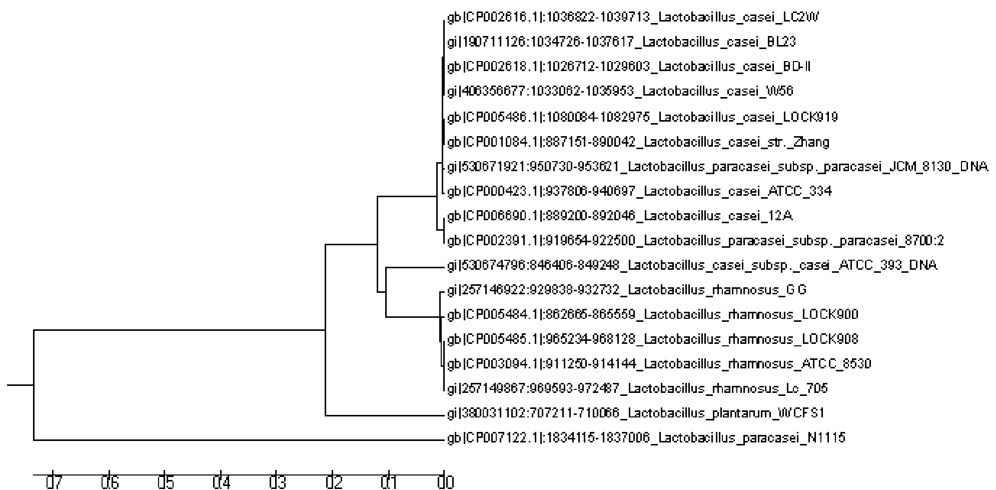
npr



pox



sod



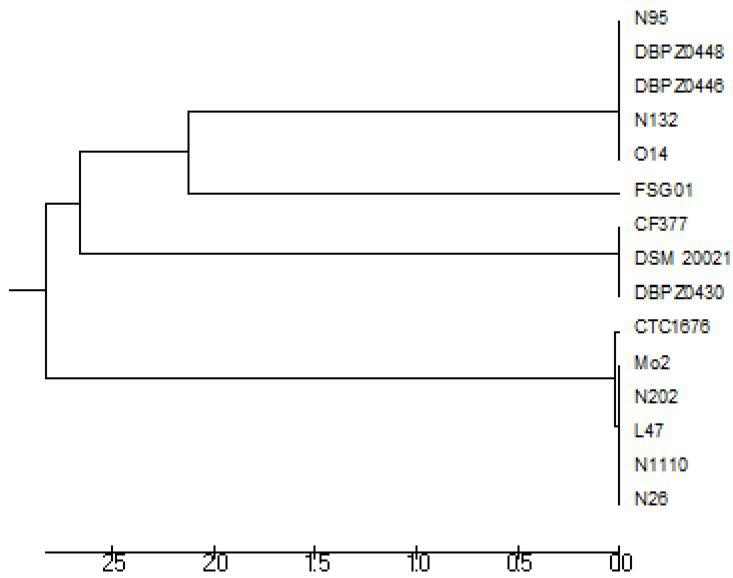
uvrA

Appendix 2

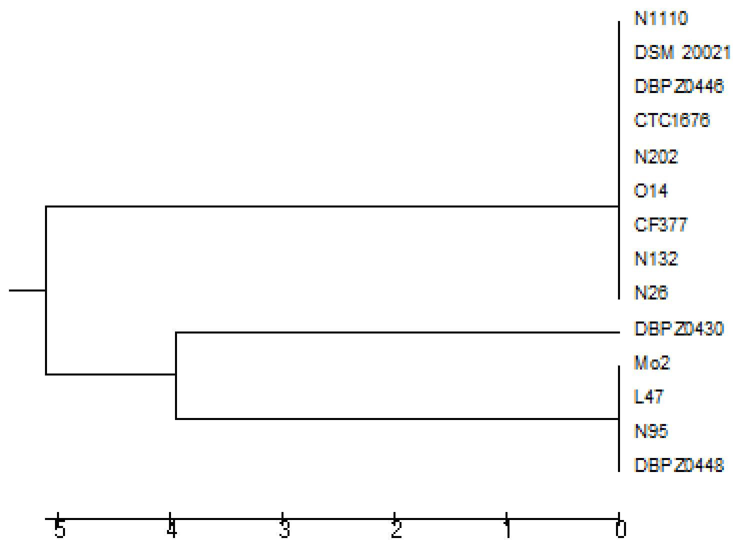
MultiLocus Sequence Typing UPGMA trees

UPGMA trees obtained comparing the sequences of the *L. rhamnosus* analysed strains.

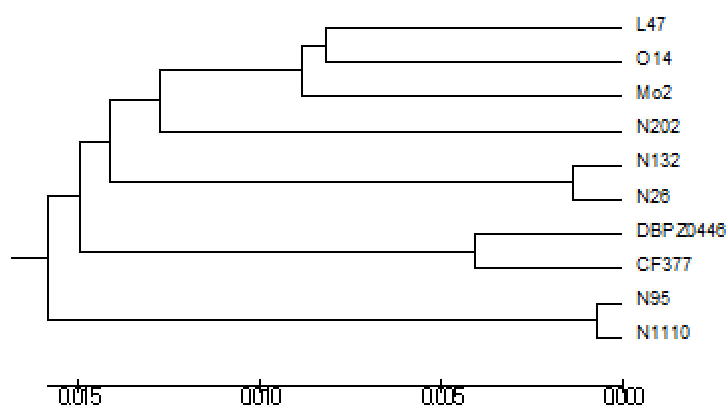
ctsR



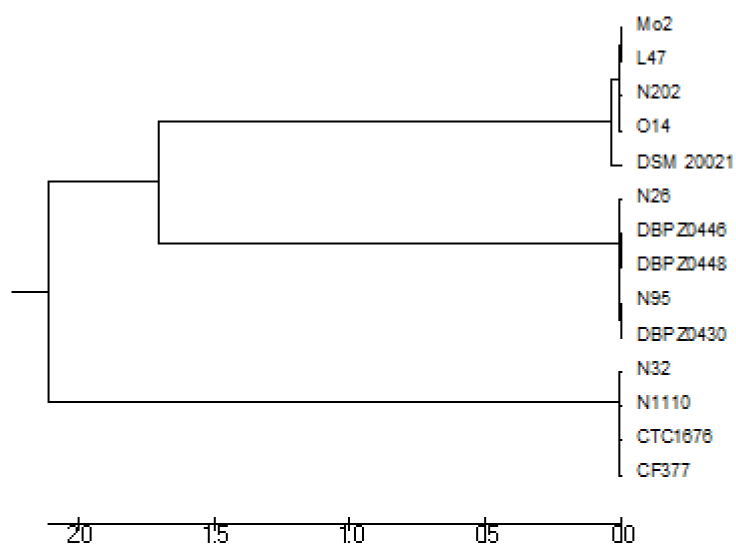
cydA



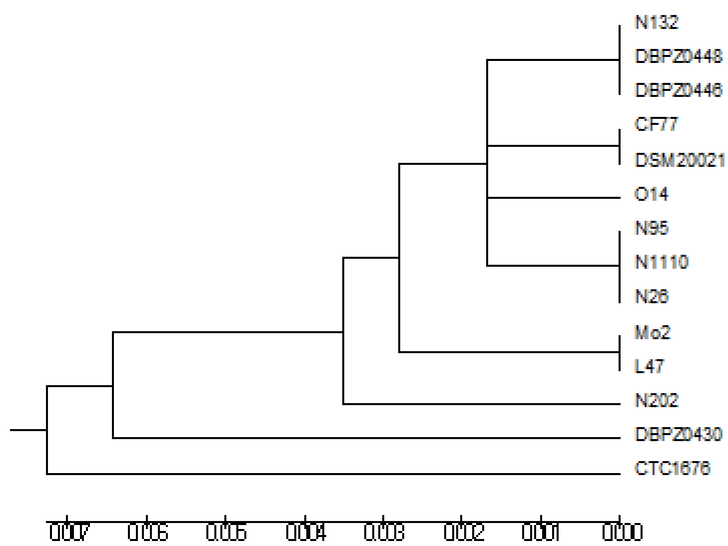
cydD



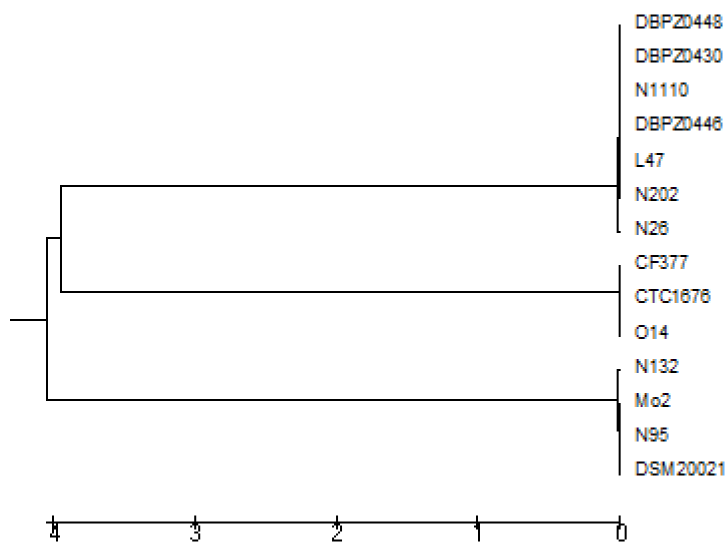
dnaK



hrcA



nox



npr

DBPZ0448
DBPZ0430
DSM20021
N1110
N95
DBPZ0446
N202
N132
N26
L47
CF337
CTC1676
Mo2
O14
FSG01

2.0 1.5 1.0 0.5 0.0

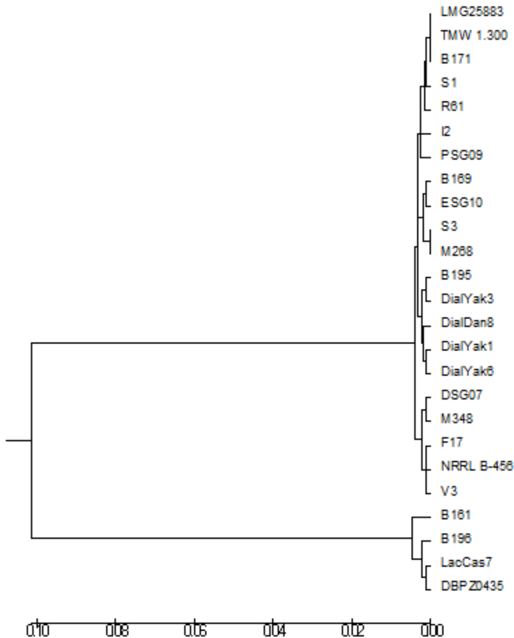
pox

N132
DBPZ0430
L47
DBPZ0448
N95
N1110
Mo2
CTC1676
N26
O14
CF377
N202
DBPZ0446
DSM 20021

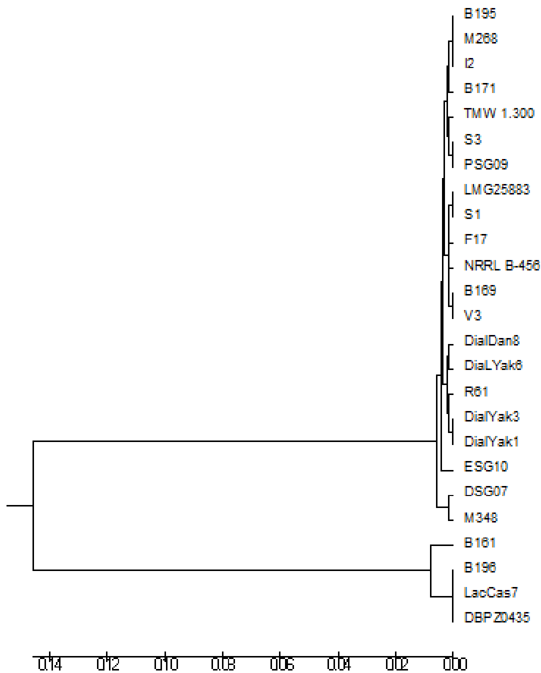
5 4 3 2 1 0

UPGMA trees obtained comparing the sequences of the *L. paracasei* analysed strains.

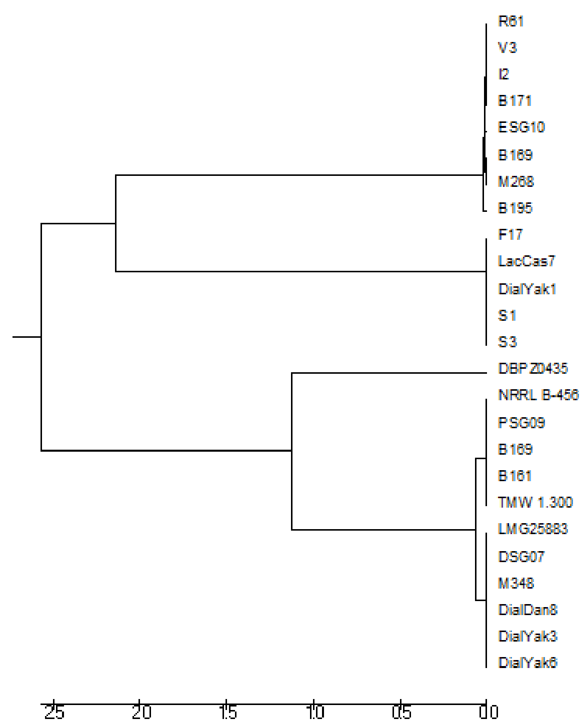
ctsR



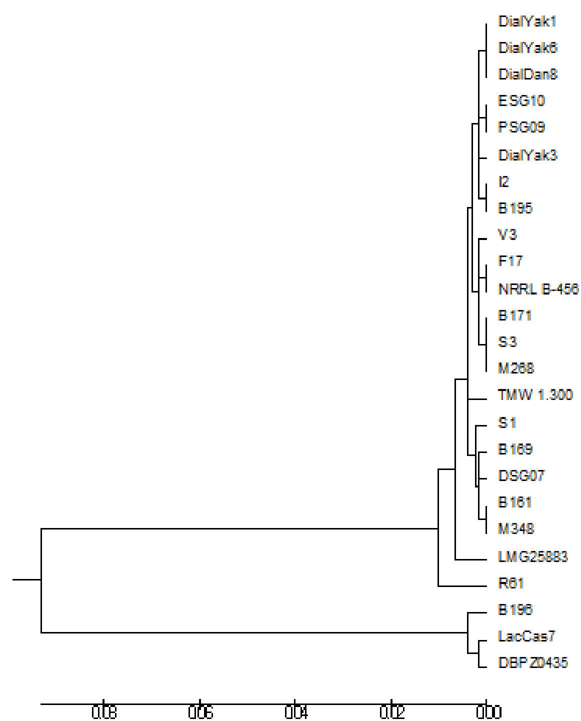
cydA



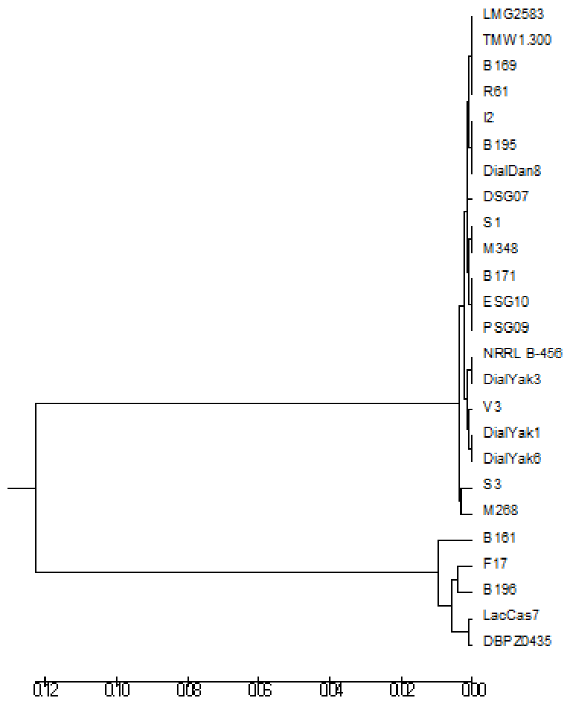
cydD



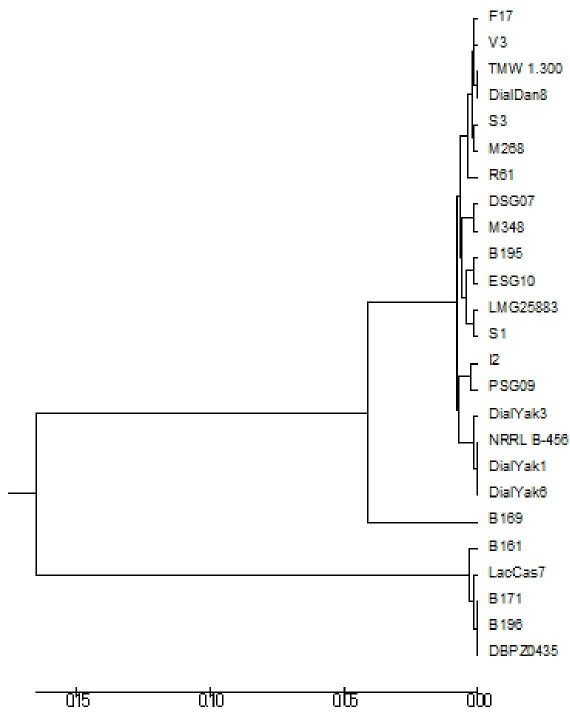
dnaK



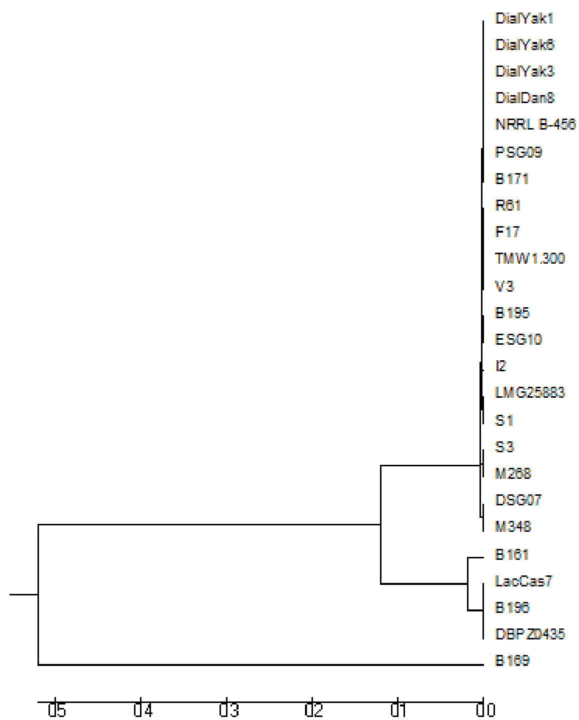
hrcA



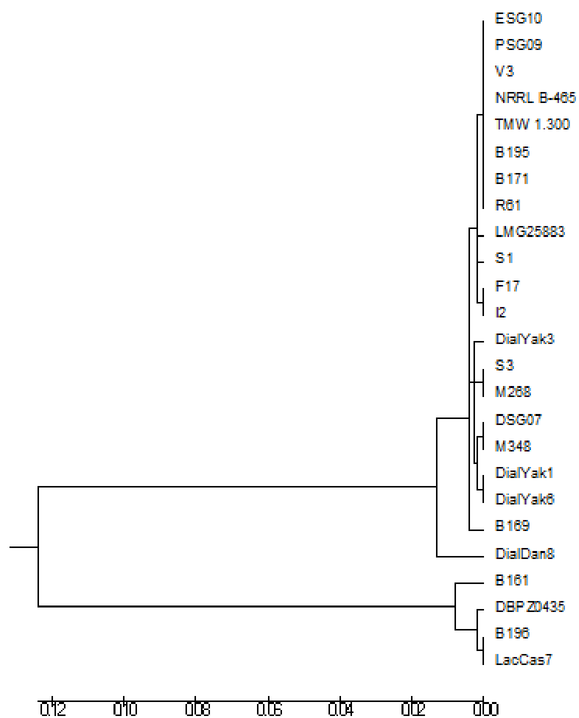
nox



npr

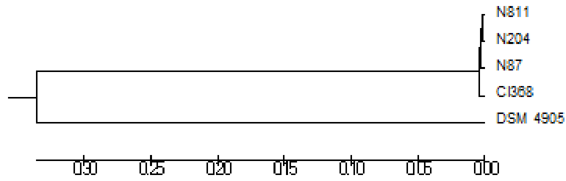


pox

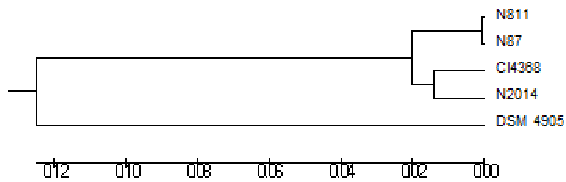


UPGMA trees obtained comparing the sequences of the *L. casei* analysed strains.

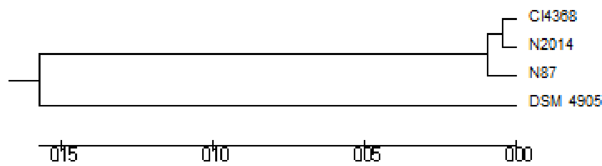
ctsR



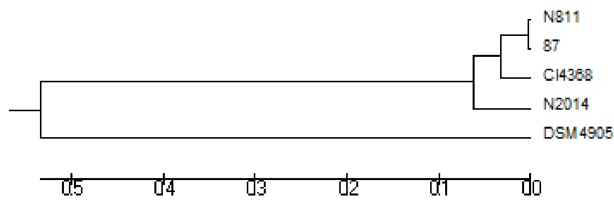
cydA



cydD



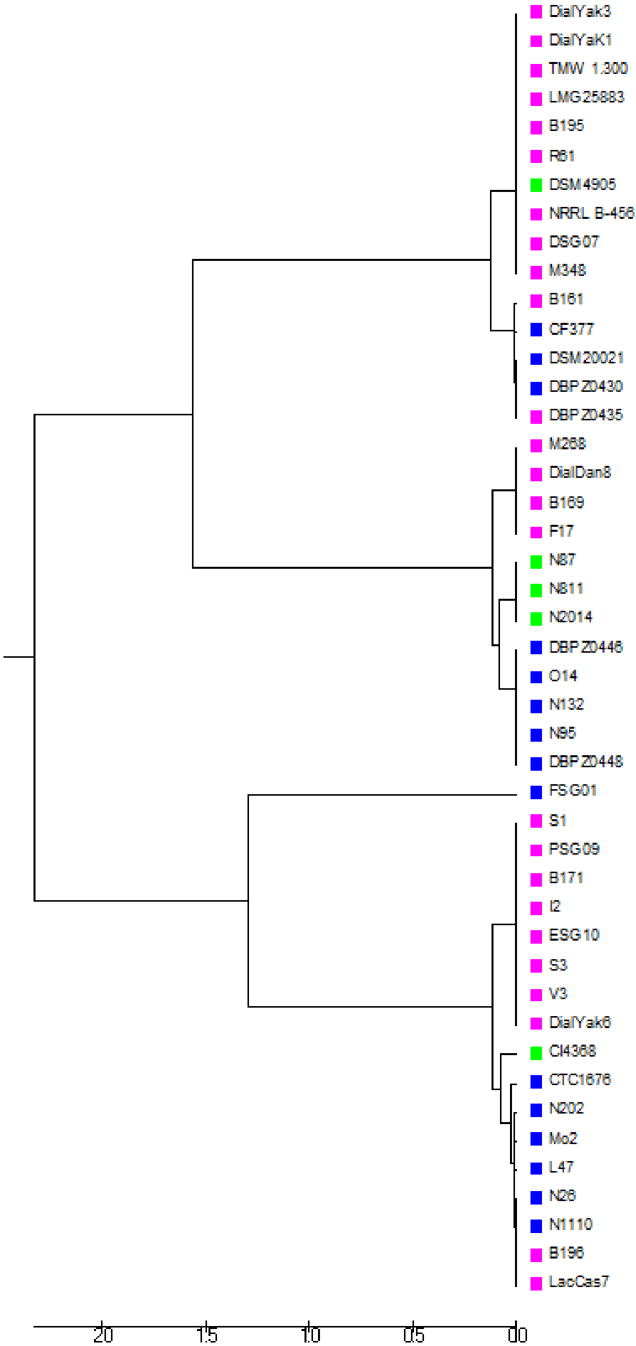
nox



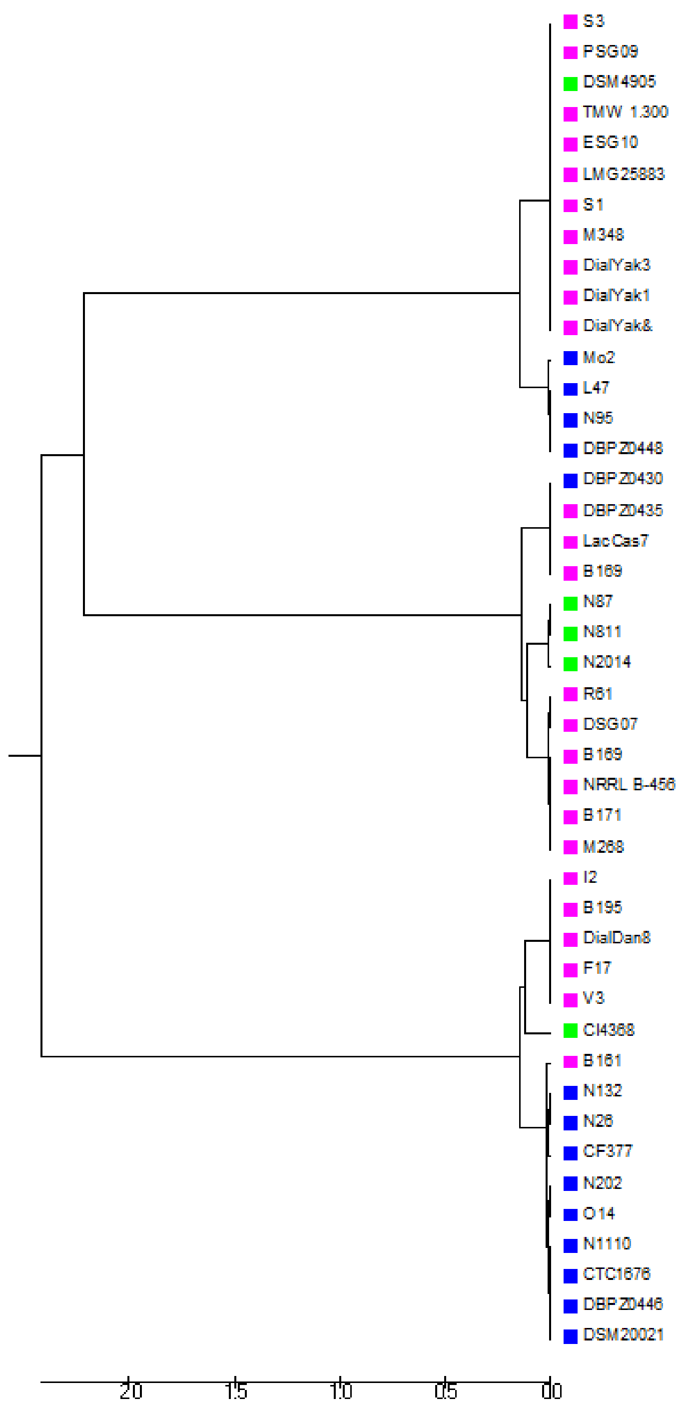
npr

UPGMA trees obtained comparing the sequences obtained for each *locus* in all the analysed species.

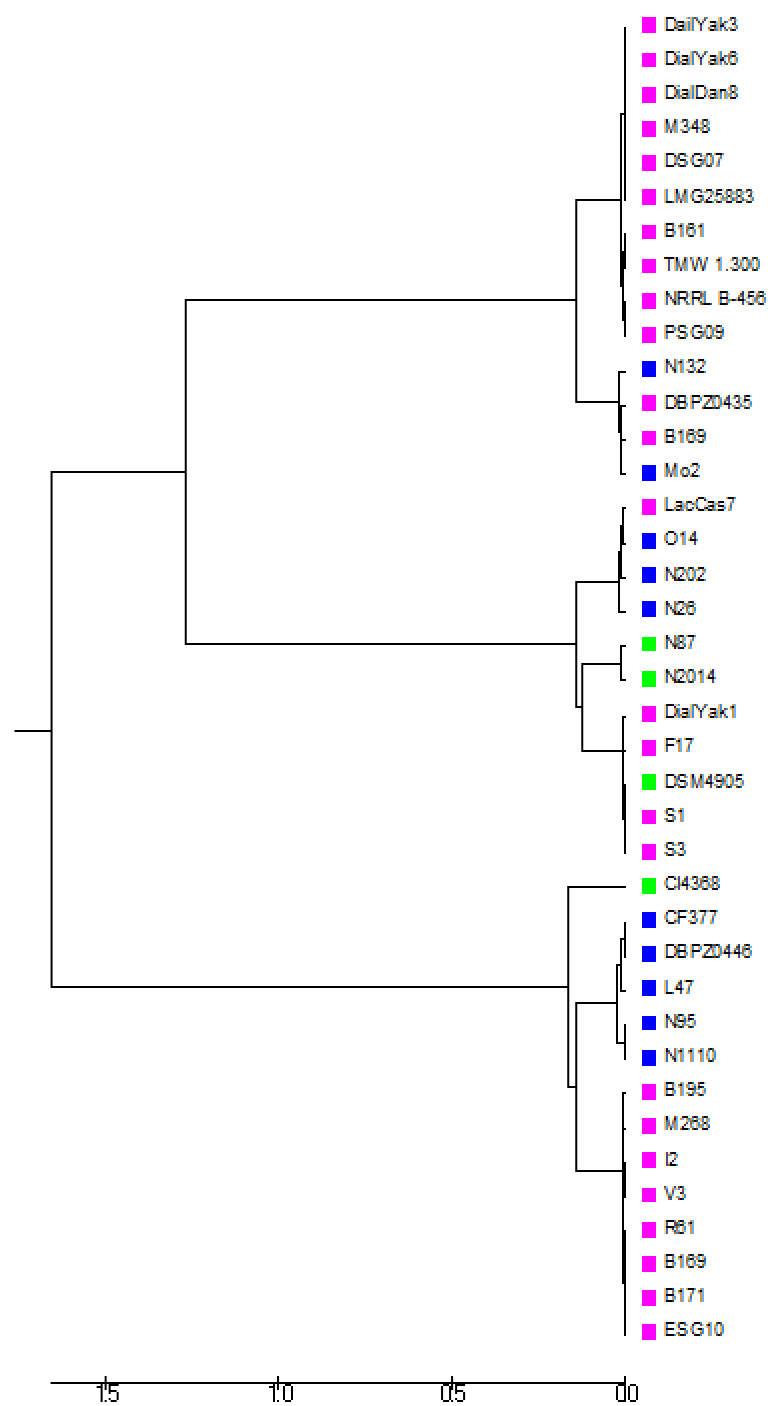
ctsR



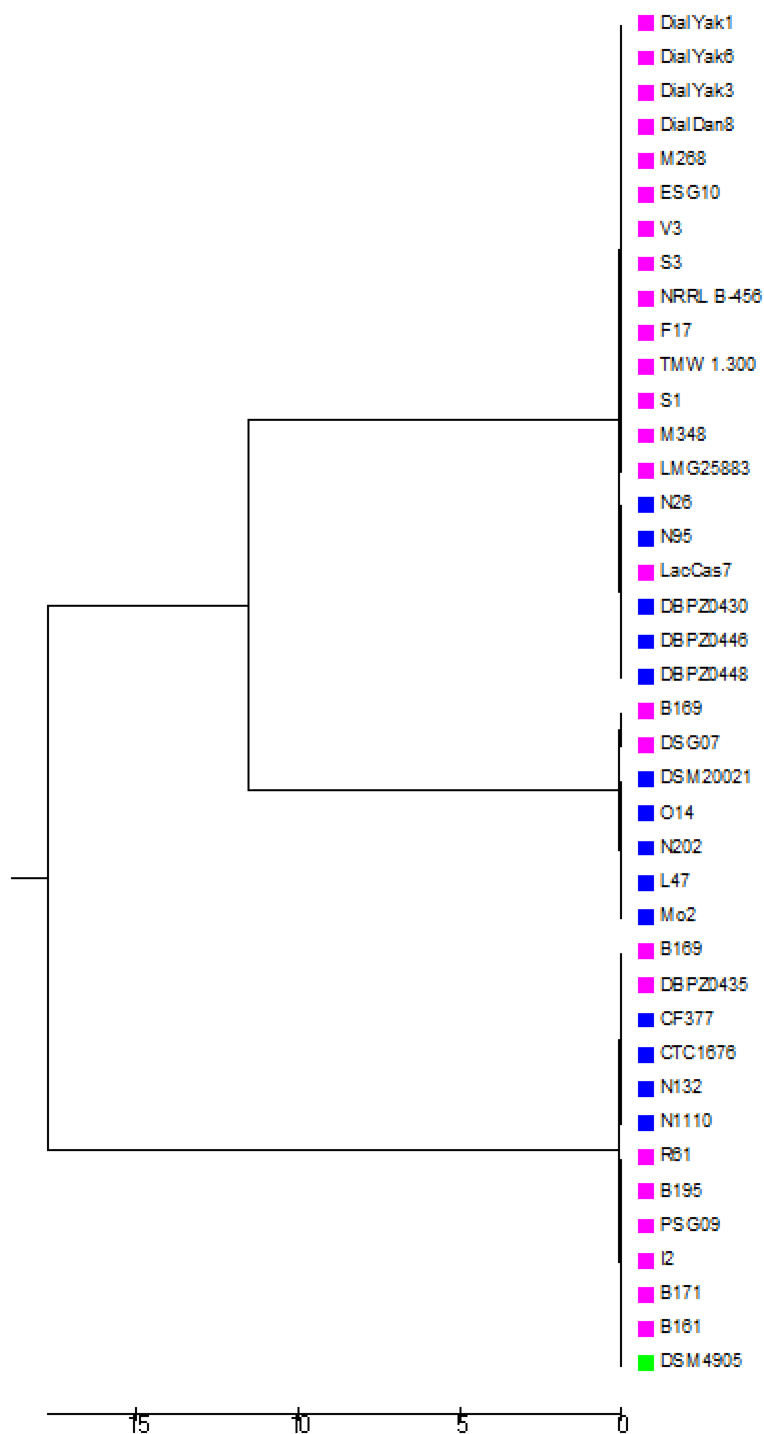
cydA



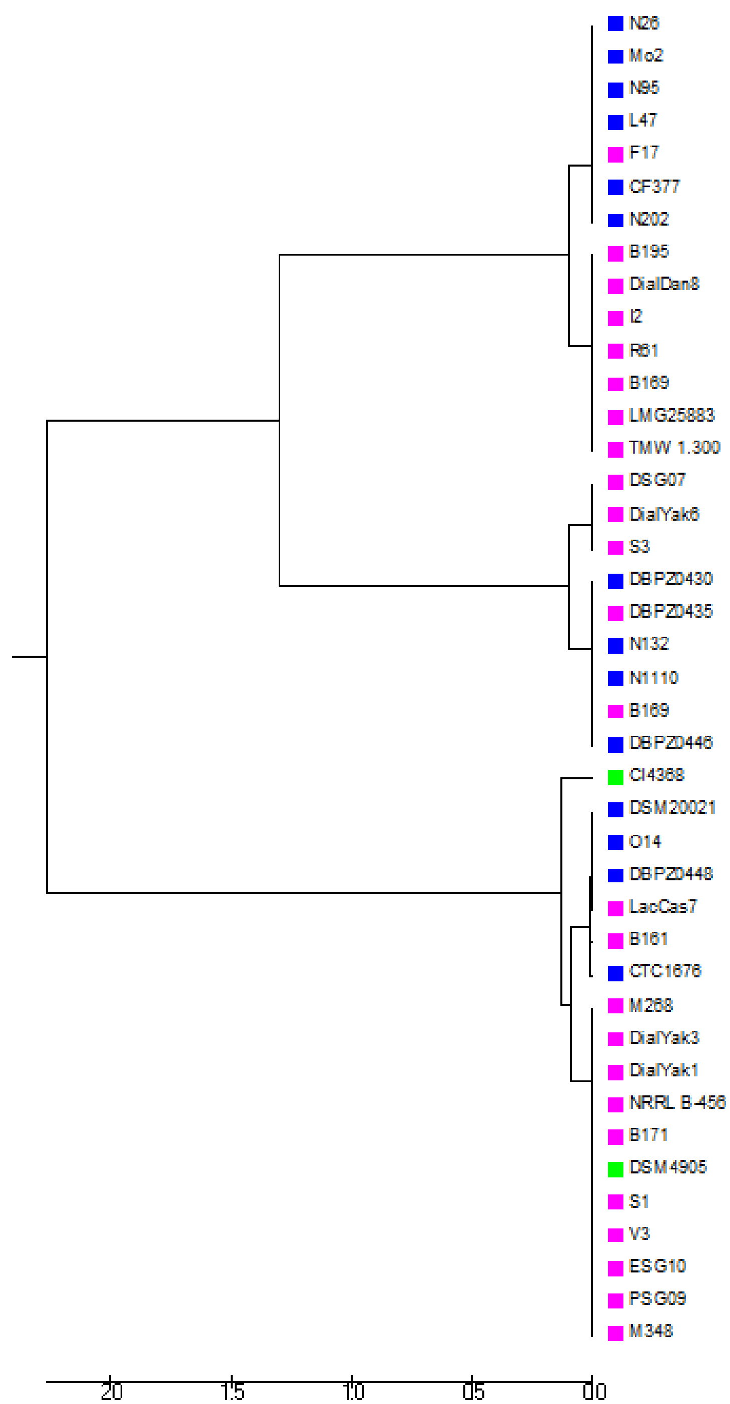
cydD



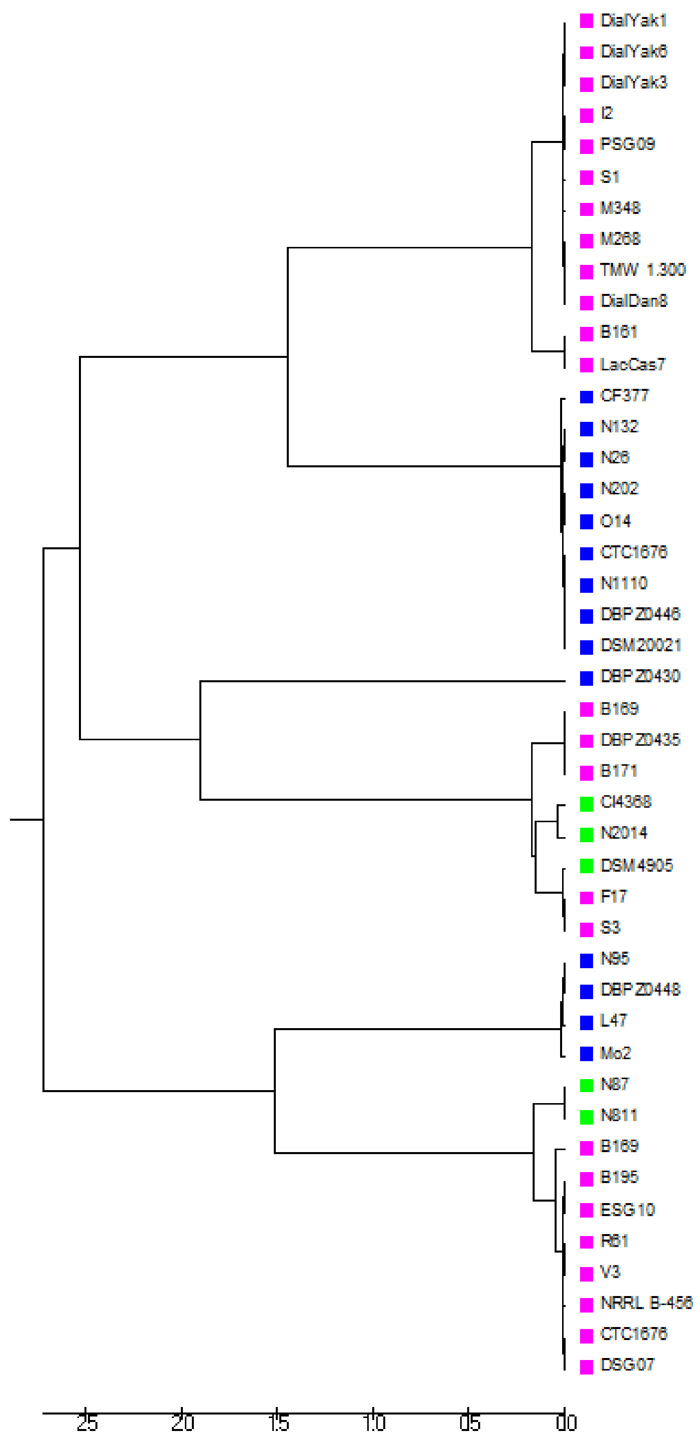
dnaK



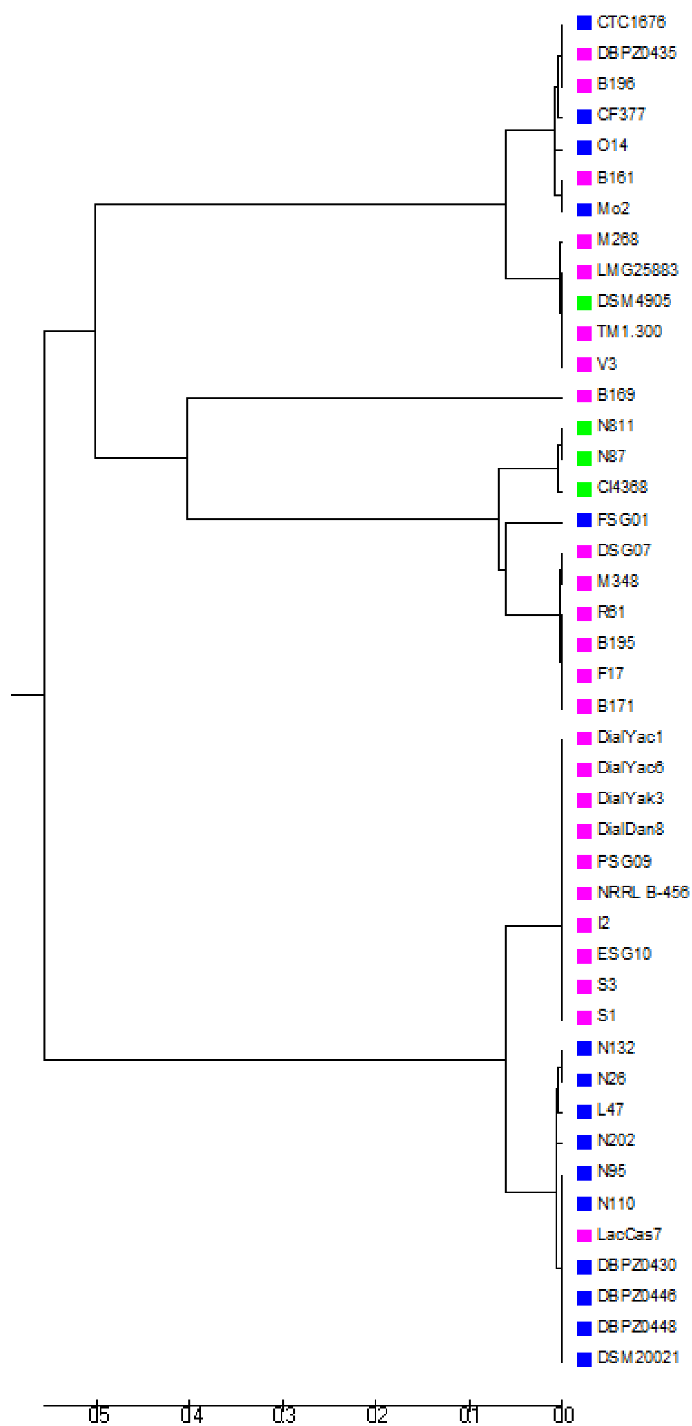
hrcA



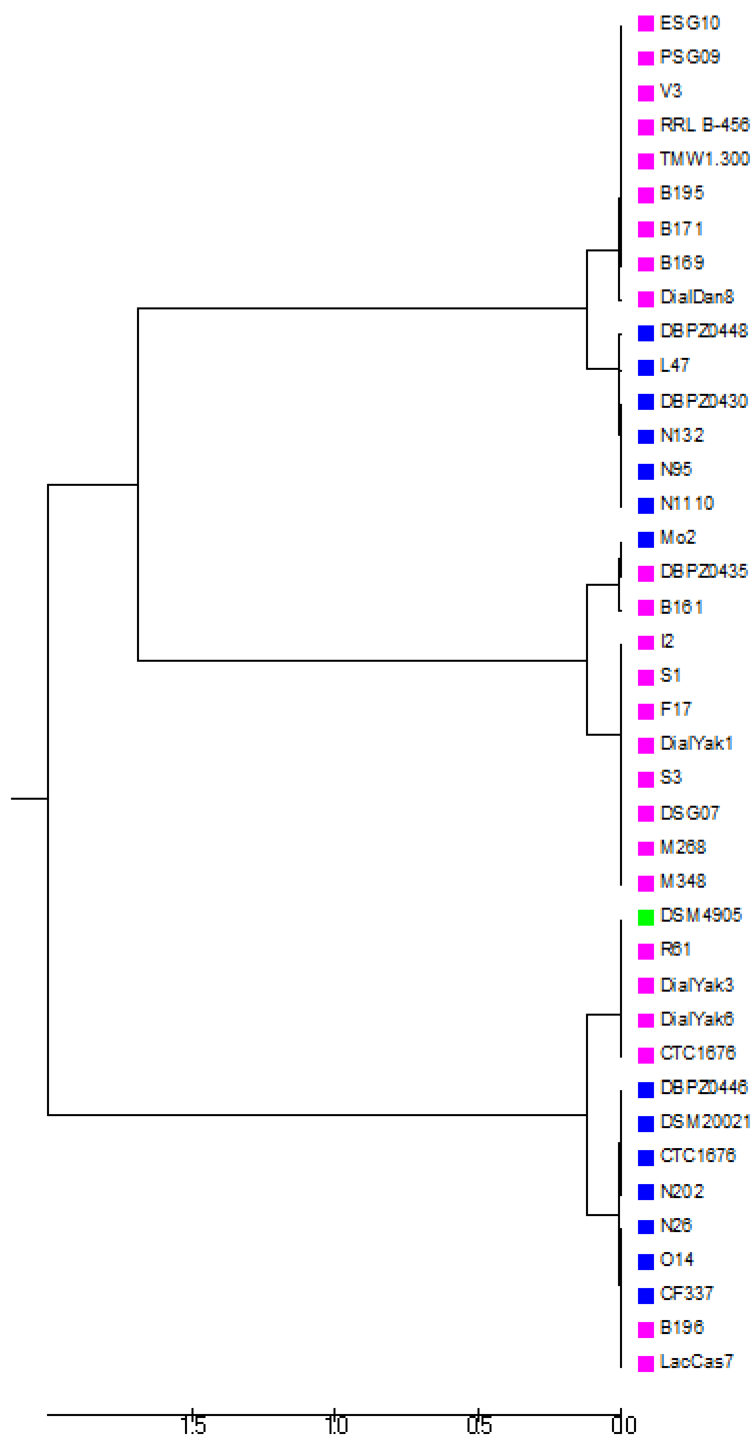
nox



npr

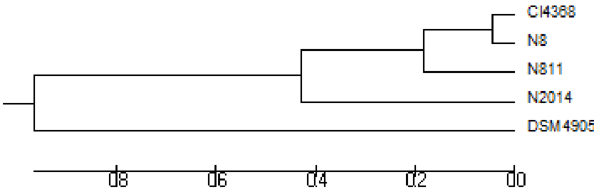


pox

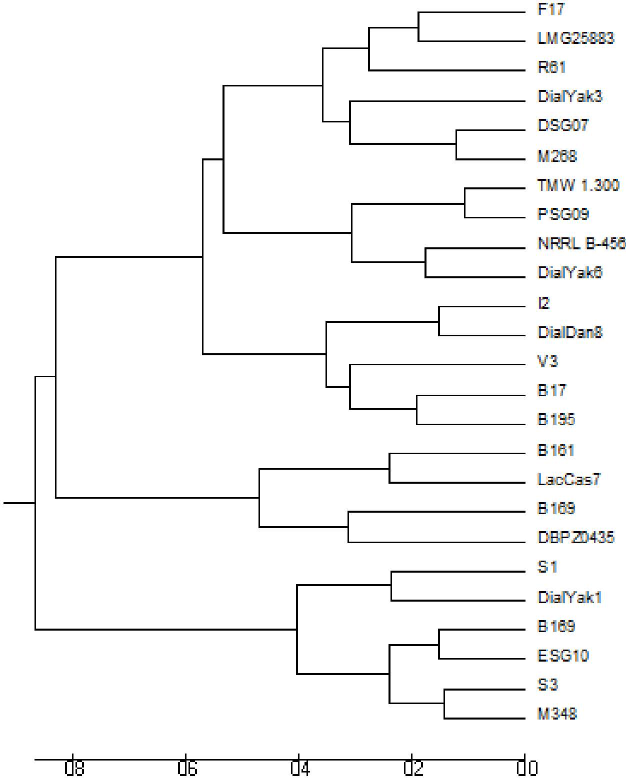


UPGMA trees obtained comparing the concatenated sequences of each species and of all the species.

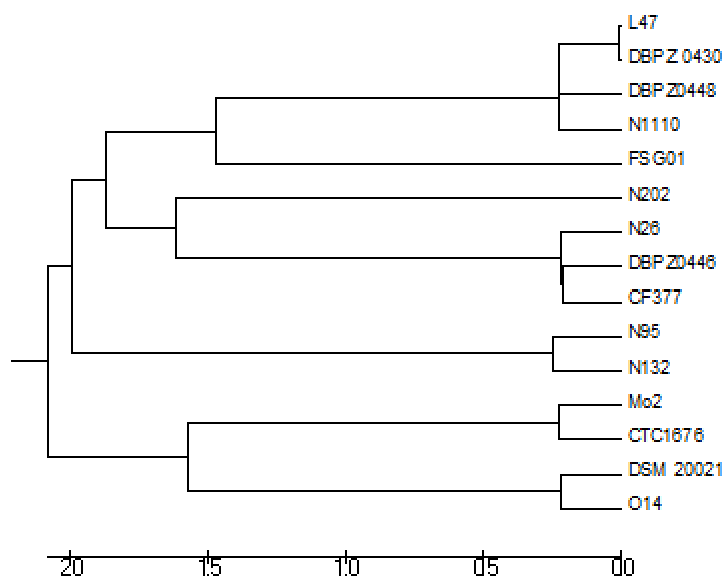
L. casei



L.paracasei



L. rhamnosus



L. casei group