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New findings on the adaptation of *Lactobacillus casei* group to cheese environment

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"The sea where once living beings were immersed,
is now enclosed within their bodies."

Time and the Hunter, Italo Calvino

"Il mare in cui un tempo gli esseri viventi erano
immersi, ora è racchiuso entro i loro corpi."

Ti con 0, Italo Calvino

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Abstract

Cheese represents a very complex environment, where the components initially present in milk are modified by technological processing, which lead to substantial changes in the biochemical properties of the initial matrix, and directs the development of the microbiota.

Lactobacillus casei group is a species of technological interest, particularly in Italian long ripened cooked hard cheese, where it becomes a prevalent species of the microbiota involved in the maturation of the curd until the end of ripening. The aim of this thesis was to study the adaptation of *Lb. casei* group to cheese environment, using a comprehensive approach involving the use of *in vitro* and *in situ* model systems, as well as confirmation of the results in real cheese.

Two systems potentially involved in adaptation of *Lb. casei* group to cheese environment, are *spxB* gene, which encodes for a pyruvate oxidase (POX), and a plasmid-encoded toxin-antitoxin system, involved in plasmid maintenance.

POX catalyses the oxidation of pyruvate to acetyl-phosphate, which is then converted to acetate with the production of ATP from an acetate kinase (ACK), potentially representing an alternative metabolic pathway for bacterial growth during cheese manufacturing.

spxB gene was found to be widespread in dairy isolates of *Lb. casei* group, and its sequence heterogeneity provides differentiation among isolates through high-resolution melting technique. Combining the taxonomic potential of *spxB* gene and high-throughput sequencing allowed to describe the population dynamics of *Lb. casei* group in ripening cheese up to the sequence-type level.

Furthermore, activation of *spxB* was also measured in response to various oxygen concentrations in an expressly designed miniaturised cheese model, as well as bacterial growth and metabolite production. All the considered approaches have underlined the relevance of *spxB* gene in adaptation to cheese environment, as well as its suitability to be used to identify the species within *Lb. casei* group.

The toxin antitoxin (TA) system detected in dairy *Lb. rhamnosus* consists of a plasmid-located two component system, coding for a stable toxin, and an unstable antitoxin, and failure in transmitting the plasmid to newborn

cells causes its post-segregational killing. A novel type I toxin-antitoxin system from dairy *Lb. rhamnosus* is described for the first time, and an in-depth bioinformatics analysis reveals the wide distribution of this system on plasmids harboured by *Lb. casei* group. Transcriptional analysis showed that TA system is induced in cheese-based media, and the transcript can be detected in cheese as well, suggesting its importance in adaptation to this environment. The role of TA systems is relevant for plasmid stability and maintenance, but also in dormancy and apoptosis of the cell, making it one of the most versatile global regulatory system in bacteria.

The projects presented highlight the importance of using a combined approach, integrating the results of experiments performed in model systems and real cheese, to describe the drivers of bacterial adaptation during cheese manufacturing.

Sommario

Il formaggio è un ambiente molto complesso, in cui i component inizialmente presenti nel latte sono stati modificati dai trattamenti tecnologici, portando a dei cambiamenti sostanziali nelle proprietà biochimiche della matrice iniziale, influenzando lo sviluppo del microbiota.

Le specie *Lactobacillus rhamnosus*, *Lb. casei* e *Lb. paracasei*, che insieme formano il gruppo *Lb. casei*, sono di grande interesse tecnologico, particolarmente nei formaggi italiani a pasta dura e lunga stagionatura, diventando le specie prevalenti del microbiota coinvolte nella maturazione della cagliata fino alla fine della stagionatura.

L'obiettivo di questa tesi è stato quello di studiare l'adattamento del gruppo *Lb. casei* alle condizioni ambientali del formaggio, utilizzando un approccio completo che ha visto l'impiego di sistemi modello *in vitro* ed *in situ*, affiancati alla conferma dei risultati sperimentali nel formaggio reale.

Due sistemi potenzialmente coinvolti nell'adattamento del gruppo Lb. casei al formaggio sono il gene *spxB*, che codifica per una piruvato ossidasi (POX), e un sistema tossina-antitossina (TA) localizzato su un plasmide, e coinvolto nel mantenimento dello stesso.

L'enzima POX catalizza l'ossidazione del piruvato ad acetil-fosfato, che viene a sua volta convertito ad acetato con la produzione di una molecola di ATP da parte dell'enzima acetato chinasi (ACK), che lo rende un potenziale metabolismo alternativo per consentire la crescita batterica durante la lavorazione del formaggio.

Il gene *spxB* è risultato ampiamente diffuso in ceppi del gruppo *Lb. casei* isolati da prodotti caseari, e l'eterogeneità di questa sequenza ha permesso la loro discriminazione attraverso la tecnica dell'high resolution melting (HRM). Combinando le potenzialità tassonomiche del gene *spxB* e la tecnica dell'high-throughput sequencing (HTS) è stato possibile descrivere le dinamiche di popolazione del gruppo *Lb. casei*, rivelando i biotipi che si susseguono durante la stagionatura.

Inoltre, l'attivazione del gene *spxB*, così come la crescita batterica e la produzione di vari metaboliti sono stati misurati in risposta a diverse

concentrazioni di ossigeno, in un formaggio miniaturizzato appositamente realizzato.

Gli approcci utilizzati hanno sottolineato l'importanza del gene *spxB* nell'adattamento al substrato del formaggio, così come la possibilità di utilizzare questa sequenza per distinguere le specie all'interno del gruppo *Lb. casei*.

Il sistema tossina-antitossina (TA) scoperto in *Lb. rhamnosus* isolati da formaggio consiste di un meccanismo a due componenti localizzato su plasmide, codificante per una tossina stabile ed un'antitossina instabile. La mancata trasmissione del plasmide ad una cellula figlia ne causa la morte, che viene così definita post-segregazionale. Il nuovo sistema TA di tipo I è stato descritto per la prima volta in questo studio, ed un'approfondita analisi bioinformatica ha rivelato che la distribuzione di questo sistema su plasmidi del gruppo *Lb. casei* è molto ampia. L'analisi trascrizionale ha mostrato che il sistema TA viene espresso in seguito a coltivazione delle cellule batteriche in un terreno a base di formaggio, e il trascritto è stato identificato anche nel formaggio stesso, suggerendo la sua importanza nell'adattamento all'ambiente. Il ruolo del sistema TA, infatti, non è limitato alla propagazione e stabilizzazione del plasmide, ma anche in processi di dormienza ed apoptosi della cellula, rendendolo uno dei sistemi di regolazione batterica più versatile.

I progetti presentati nell'ambito di questa tesi sottolineano l'importanza di utilizzare un approccio combinato, integrando i risultati sperimentali ottenuti in diversi sistemi modello e nel formaggio stesso, per descrivere i fattori chiavi dell'adattamento batterico durante la lavorazione e la maturazione del formaggio.

Chapter I

General introduction and aim of the thesis

Introduction

The history of cheese-making has most probably originated in Middle Eastern countries about 10.000 years ago, and made its long way until our age, and still sees an increase in the consumption trends (International Dairy Federation, 2016 data, http://www.fil-idf.org).

The key for success of this fermented product might have been determined by the prolonged conservation that lactic bacteria development conferred to the fresh milk, transforming lactose into lactic acid, which prevented the growth of spoilage bacteria.

While the first fermented dairy products might have originated from natural contamination of the milk by lactic acid bacteria, forming acid-coagulated products, the use of rennet, as well as the introduction of further technological steps such as cooking, salting or pressing of the curds has led to the development of hundreds of cheese varieties.

Furthermore, control of the fermentation process has been achieved by the use of fermentation starter cultures, such as defined or undefined starters, or natural whey culture, the latter consisting of the whey produced during cheese-making, stored in conditions that favour the development of the bacteria therein, and used to inoculate the milk in the following day.

The different technological processes used in cheese-making define some fundamental traits of the fermenting curd such as firmness, water activity, salt concentration, and impose a selective pressure on the lactic acid bacteria developing in the curd, leading to the growth of species that can cope with the varying environmental parameters.

These species are responsible of fundamental changes that lead from the milk to the final cheese and, besides fermenting the lactose with subsequent lactate accumulation and curd acidification, are involved in the production of the characteristic flavours of typical cheeses (Smid and Kleerebezem, 2014).

The cheese environment

Cheese represents a very complex environment, where the components initially present in milk are modified by processing, such as thermal

treatment, renneting, pressing, salting and maturation, which lead to substantial changes in the biochemical properties of the initial matrix, and directs the development of the microbiota.

From milk to curd

Freshly collected milk is a highly nutritious substrate, which can support the growth of a rich microbiota. Bacteria can reach milk from various sources present in the milking farm environment, and can either positively affect dairy fermentations (e.g. *Lactococcus*, *Lactobacillus*, *Streptococcus*, etc.), or hinder the fermentation process (*Pseudomonas*, *Clostridium*, *Bacillus*, etc.) (Quigley et al., 2013).

The microbiota of raw milk consists of psychrotolerant bacteria, mainly belonging to the genus *Pseudomonas*, as well as other species from the family of Lactobacillaceae, such as the genus *Lactobacillus* or *Lactococcus* (Ercolini et al., 2009; Vacheyrou et al., 2011).

After collection, milk can be subjected to pasteurisation or other thermal treatment, which lead to the inactivation of most non-spore forming bacteria, or can be used raw for cheese manufacturing, as is the case for various typical cheese products, including Italian cooked, long ripened, hard cheeses such as Grana Padano (GP) or Parmigiano Reggiano (PR) as described from Gatti et al. (2014) (Fig. 1). The manufacturing of these cheeses requires a spontaneous creaming of the raw milk, with a resting period of 6 to 12 hours, which changes the composition of the milk microbiota, due to the interaction of the bacteria with the milk fat globule and to the growth rate of the bacteria. The first phenomenon has to do with bacterial adhesion to the fat globule, which might lead to its entrapment within the floating cream, that has a higher bacterial count of the raw milk and of the resulting partially skimmed milk (Franciosi et al., 2011). Bacterial growth rate is greatly influenced from the temperature at which this creaming step is performed, which can range between 8° and 20°C, and greatly affects the development of psychrotrophic and pathogen species (Carminati et al., 2008).

Despite a resident microbiota is present in the raw milk, addition of a bacterial starter triggers the fermentation, and plays a key role in the whole cheese manufacturing process. The use of starter cultures is necessary for fast acidification of the milk used for cheese production, and the rate of this acidification process is of paramount importance, since

lowering of the pH not only hampers the growth of spoilage bacteria, but also enhances the expulsion of the whey from cheese curd and prepares the environment that will influence microbial growth and activity throughout the ripening period (Bottari et al., 2010; Stanley, 1998).

Figure 1 Flow sheet for Parmigiano Reggiano (PR) and Grana Padano (GP) cheese-making. *Only for PR cheese; **only for GP cheese. (Reprinted from: Invited Review: Microbial evolution in raw-milk, long ripened cheeses produced using undefined natural whey starters, Gatti et al., 2013, with permission from Elsevier).

Nowadays commercial starters are available, prepared from specialised companies and provided either as defined or undefined starters, the former composed of one or more strains with known characteristics, and the latter having an undefined composition of lactic acid bacteria (LAB) (Smid and Kleerebezem, 2014).

In the case of Italian long ripened hard cooked cheeses, such as the aforementioned GP and PR, the starter is obtained from the previous day whey, using a back-slopping procedure. Briefly, the whey remaining from the cheese-making process is incubated overnight under thermophilic conditions, and delivers a bacterial population mainly composed of thermophilic LAB strains, characterized from an assortment of species and, particularly, of strains (Gatti et al., 2014) .

The dominant species found in GP and PR natural whey starter (NWS) are *Lactobacillus helveticus* and *Lb. delbrueckii* sp. *lactis*, two thermophilic and homofermentative LAB, together with low amounts of *Lb. fermentum* and *St. thermophilus*. Furthermore, mesophilic species such as the facultative heterofermentative *Lb. paracasei*, *Lb. rhamnosus* and other species belonging to the genus *Lactabacillus* could be detected (De Filippis et al., 2014; Neviani et al., 2013; Pogačić et al., 2013).

Among the species introduced in the milk by NWS, the dominating thermophilic strains are involved in lactic acid production and acidification of cheese curd, while the mesophilic bacteria do not contribute to acid production, and become relevant later, during ripening.

In the case of Italian cooked hard cheeses, after inoculation of the cheese milk with NWS, addition of the rennet and cutting of the curd coagulum, the curd is fast cooked to a temperature that ranges from 53°C to 56°C, and then the curd grains are left to rest under hot whey for about 40-70 minutes, before extraction and molding of the cheese curd, favouring whey syneresis from curd grains.

The curd cooking temperature, room temperature, and size of the wheel are the main variables affecting the rate of the curd cooling and the heat-load gradient occurring within the wheel. Consequently, these factors affect the heat stress exerted on the LAB, other bacteria, and enzymes from the milk, rennet, and microorganisms. Molding lasts for approximately 2 to 3 d, during which the curd is placed in a plastic mold

on the first day , while on the following day(s), a finely pierced steel mold is used (Gatti et al.,2014).

About 12 to 24 hours after curd extraction, there is a consistent increase in bacterial counts, especially thermophilic LAB. At the same time lactose is depleted and pH drops, due to the accumulation of lactic acid (Santarelli et al., 2013b). During this step, the thermal stress exerts a selection over the thermophilic LAB introduced with the NWS, causing a decrease of the diversity indexes of the curd, compared with those calculated for the complex raw milk microbiota (Santarelli et al., 2013a).

After molding, the cheese wheels are submerged in saturated brine for a time ranging from 18 to 25 days, which leads to a reduction in the viable counts of starter LAB, leading to a continuous decrease of *Lb. helveticus* and *Lb. delbrueckii* sp. *lactis* until the second month of ripening (Alessandria et al., 2016; Santarelli et al., 2013a). Autolysis of starter LAB is a well-studied event, that leads to the release in the cheese matrix of cytoplasmic enzymes, such as proteases and esterases, which contribute to development of cheese flavour, as well as proteins, oligopeptides, lipids, fats, and fatty acids (Lortal and Chapot-Chartier, 2005; Smid and Kleerebezem, 2014).

Cheese ripening

The environment of cheese, at this early ripening point, presents a low water activity (a_w), low pH, and lactose is absent, but these hostile conditions are tolerated by non starter LAB, which were introduced in the fermentation at low concentration, deriving both from the raw milk and from NWS. Potential substrates for non starter LAB growth are represented by milk components, such as small peptides or amino acids, citrate, lactate, and free fatty acids (Fox et al., 1993), as well as the above mentioned cellular components released from starter LAB autolysis.

Growth of non starter LAB in ageing cheeses is often correlated with the development of positive traits of the cheese, particularly to the catabolism of peptides and free aminoacids, which leads to the synthesis of aroma compounds. Only rarely the development of this bacterial population is associated with detrimental effect on the final characteristics of cheese (Settanni and Moschetti, 2010).

The environment present around the first 2-4 months of ripening presents the highest diversity throughout the manufacturing of GP cheese

(Alessandria et al., 2016; Santarelli et al., 2013a), probably due to the competition between bacteria strains to adapt and succeed in occupying the niche previously dominated from starter LAB, leading to the activation of peculiar adaptation strategies.

In the case of PR and GP, the main non starter LAB species found during ripening are *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*, three closely related species that are frequently described as *Lb. casei* group. Despite their taxonomy is still debated, the three species present a wide biodiversity at the strain-level, as reported by the occurrence of different biotypes during ripening, and their genotypic diversity (Bove et al., 2011; Pogačić et al., 2013).

After 6 months of ripening, and until a ripening age of 24-30 months, which is the case of premium quality PR cheese, bacterial counts decrease steadily, together with cultivability, that presents a large variability depending on the culture media used for viable cell counting (Neviani et al., 2009). Metabolically active cells of *Lb. casei* group can be found until 12 mo. of ripening, and accounts for most of the microbiota, while other minor species, together with members of the starter population, are present in a transcriptionally active status until late ripening stage, despite their role over the development of the cheese characteristics are still not clear (Ruggirello et al., 2016; Chapter 3 of this thesis).

General features of *Lb. casei* group

Lactobacillus casei group includes the three closely related species *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*, which find many applications in food and health related products. These species are very interesting and relevant for genetic and physiological studies because they have a wide ecological distribution (human host, vegetables, meat, dairy products) and may have a role as probiotics (Toh et al., 2013).

The most interesting technological traits of *Lb. casei* group reside in their ability to contribute to improving the quality of some dairy product, and to the probiotic properties of some strains of this group.

The role of *Lb. casei* group in cheese-making has already been anticipated in the first paragraph, at least for the making of GP and PR, and the development of bacteria from these species during ripening highly impact cheese proteolysis, release of free amminoacids (FAA), and formation of

flavour compounds with paramount effect in the development of the characteristic cheese aroma (Settanni and Moschetti, 2010).

The probiotic properties of *Lb. rhamnosus* are widely documented, in particular it has an acknowledged role in the treatment of acute infectious diarrhoea in children, in the prevention of rotavirus nosocomial infection in children and as adjuvant in therapies for *Helicobacter pylori* eradication; it is also effective in the prevention of antibiotic-associated diarrhoea and in reduction of irritable bowel syndrome symptoms (Guarino et al., 2009; Aureli et al., 2011). The presence of proteinaceous pili encoded by the *spaCBA* gene cluster allows this species to colonize the GIT, thanks to its mucus-binding properties, and this gene cluster is present in other strains belonging to *Lb. casei* group (Kankainen et al., 2009; Toh et al., 2013).

Combination of these technological traits led to the development of cheeses and dairy products with added probiotic value, and the obtained cheeses showed comparable attributes respect to the control cheeses, while preserving the viability of the probiotic strain, opening some interesting scenario for the production of "functional" dairy products (Burns et al., 2012; Cichosz et al., 2014).

The three species forming *Lb. casei* group have been in a debate with respect to their classification and, despite technological advancement in sequencing technology and an increasing amount of publicly available data, the taxonomy of these species is still unclear.

To date (December 2016), *Lb. casei* group consists of 3 species and 7 subspecies, of which 5 belonging to the species *Lb. casei* and 2 to *Lb. paracasei*, as reported in Table 1, according to the "List of Prokaryotic names with Standing in Nomenclature" (Parte, 2014; http://www.bacterio.net). Recently, some studies have dealt with the taxonomic classification of the type strains of this group, especially concerning the classification of type strains ATCC393 and ATCC334, whose attribution to either *Lb. casei* or *Lb. paracasei* with multiple genotypic and phenotypic approaches lead to controversial (Acedo-Felix, 2003; Felis et al., 2001; Judicial Commission of the International Committee on Systematics of Bacteria, 2008). The hurdle in identification of species belonging to *Lb. casei* group is due to the dual nature of their genomic complexity: from one side, we have that comparative analysis of

genome sequences has revealed extensive occurrence of gene loss, duplication, and acquisition (Felis and Dellaglio, 2007). On the other hand, other studies report an atypical stability of variable sequences frequently used for fine typing of homogeneous groups, which fails to distinguish among *Lb. casei* and *Lb. paracasei* (Diancourt et al., 2007).

Table 1 Species and subspecies currently recognised as part of *Lb. casei* group.

Species	Subspecies	Reference
Lactobacillus casei		Orla-Jensen 1916, Hansen and Lessel 1971 *
	Lactobacillus casei subsp. *alactosus*	Mills and Lessel 1973 *
	Lactobacillus casei subsp. *casei*	Orla-Jensen 1916, Abo-Elnaga and Kandler 1965 *
	Lactobacillus casei subsp. *pseudoplantarum*	Abo-Elnaga and Kandler 1965 *
	Lactobacillus casei subsp. *rhamnosus*	Hansen 1968 *
	Lactobacillus casei subsp. *tolerans*	Abo-Elnaga and Kandler 1965 *
Lactobacillus paracasei		Collins et al. 1989, sp. nov.
	Lactobacillus paracasei subsp. *paracasei*	Collins et al. 1989, subsp. nov.
	Lactobacillus paracasei subsp. *tolerans*	Abo-Elnaga and Kandler 1965, Collins et al. 1989, comb. nov.
Lactobacillus rhamnosus		Hansen 1968, Collins et al. 1989, comb. nov.

* Approved Lists 1980

The distinction between these three species, though difficult, is important to understand the evolutionary relationship between the strains and to allow recognition and monitoring of their genetic characters. Furthermore, because of their industrial importance, a tool for accurate

taxonomic identification of these microorganisms is essential to generate accurate labels for food products and probiotics (Koirala et al., 2015).

Niche adaptation of Lb. casei group

While the increasing availability of genomic information allows to predict the bacterial functionality in complex environments, the mere presence of certain genes does not always reflect their functionality. The physiological and technological properties of strains mainly depend on expressed features in a given set of conditions.

Lb. casei, Lb. paracasei and *Lb. rhamnosus* are able to grow in different environments, and their tolerance to different types of stress is at the basis of their ability to colonize various environments, ranging from gastrointestinal tract (GIT) to dairy products, fermented vegetables and meat product (Papadimitriou et al., 2016).

As described in the previous paragraphs, the environment of maturing cheese can be characterized by growth-limiting conditions and only the species that can adapt to these conditions are able to thrive in such environments, as is the case of *Lb. casei* group.

The genomic features of dairy isolates of this group suggest that genetic information that are redundant in this environment, such as genomic islands coding for sugars utilization, can be loss during replications. On the other hand, other bacteria present in the niche, as well as plasmids or phages, might provide a source of exogenous DNA, which might diversify the strain response in a complex environment such as cheese.

Metabolic characteristics

Carbon metabolism

The term "central carbon metabolism" (CCM) describes the integration of pathways of transport and oxidation of main carbon sources inside the cell. In most bacteria, the main pathways of the CCM are those of the phosphotransferase system (PTS), glycolysis, gluconeogenesis, pentose phosphate (PP) pathway, and the tricarboxylic acid cycle (TCA) with the glyoxylate bypass. As a whole, the system has a complex structure and it is regulated by complex networks of reactions (Papagianni, 2012).

The metabolism performed by lactobacilli in milk is the fermentation of lactose by three pathways: homolactic fermentation, heterolactic fermentation and heterofermentative pathways.

Homolactic fermentation leads to the production of lactate as the major end product, through the enzymatic conversion performed by lactate dehydrogenase (LDH), and is responsible of the fast acidification of the curd mass. The pathway that leads to lactate starts with glycolysis, with the Emben-Meyerhoff Pathway (EMP), that converts glucose into pyruvate, and pyruvate is subsequently converted into lactate from lactate dehydrogenase (LDH, Fig.2).

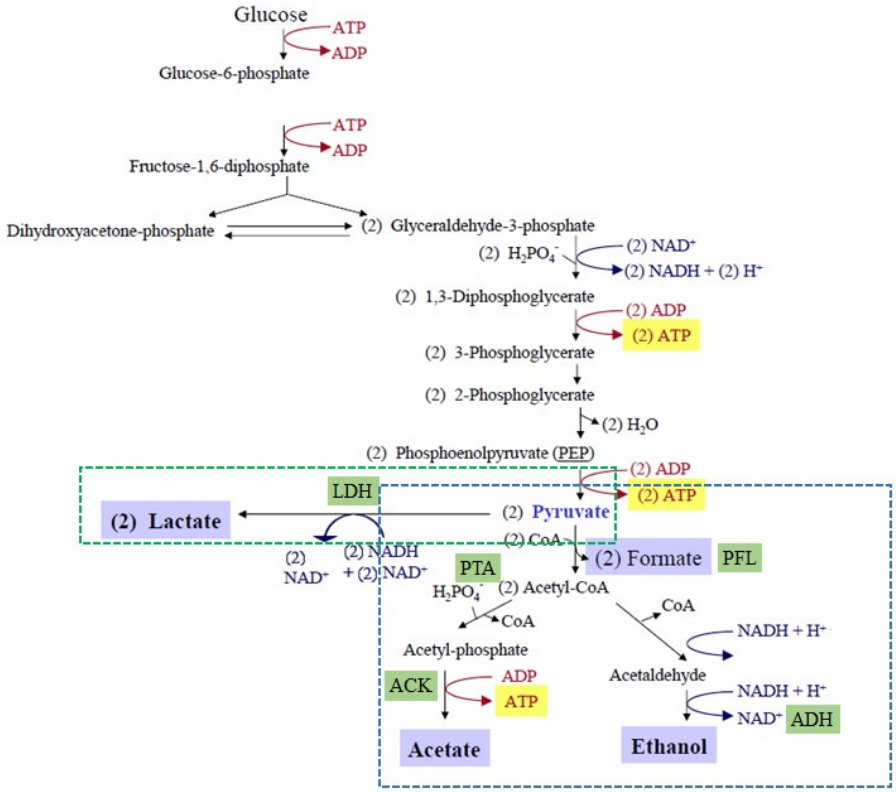

Figure 2 Homo- and heterolactic fermentation of glucose. In homolactic fermentation the pyruvate produced from EMP pathway is converted to lactate from LDH (dashed green box). During heterolatic fermentation part of the pyruvate can be rerouted towards the synthesis of acetate or ethanol (dashed blue box). (Images adapted with permission from original material of Eddy J. Smid). LDH: lactate dehydrogenase, PTA: phosphate acetyltransferase, ACK: acetate kinase, PFL: pyruvate formate lyase; ADH: alcohol dehydrogenase.

Bacteria belonging to *Lb. casei* group are also capable to perform heterolactic fermentation of lactose, which leads to the synthesis of lactate, together with acetate, ethanol, formate and CO_2 as final products (Fig.2). The pyruvate produced from EMP pathway, besides conversion

via the LDH, can be rerouted to pyruvate formate lyase (PFL), leading to the synthesis of formate and acetyl-CoA. Acetyl CoA can be subsequently converted to acetate by phosphate acetyl transferase (PTA) and acetate kinase (ACK), producing one extra molecule of ATP, or to ethanol, synthetized by alcohol dehydrogenase (ADH).

Finally, an heterofermentative pathway exists, that relies on the activation of the pentose phosphate pathway, and the phosphoketolase (PK) enzyme, with formation of glyceraldehyde-3-phosphate, which is then rerouted through the EMP pathway, and acetyl phosphate (Fig. 3). Acetyl phosphate can subsequently be converted into ethanol, through alcohol dehydrogenase (ADH), or to acetate by acetate kinase (ACK), with substrate-level phosphorylation of ADP to ATP.

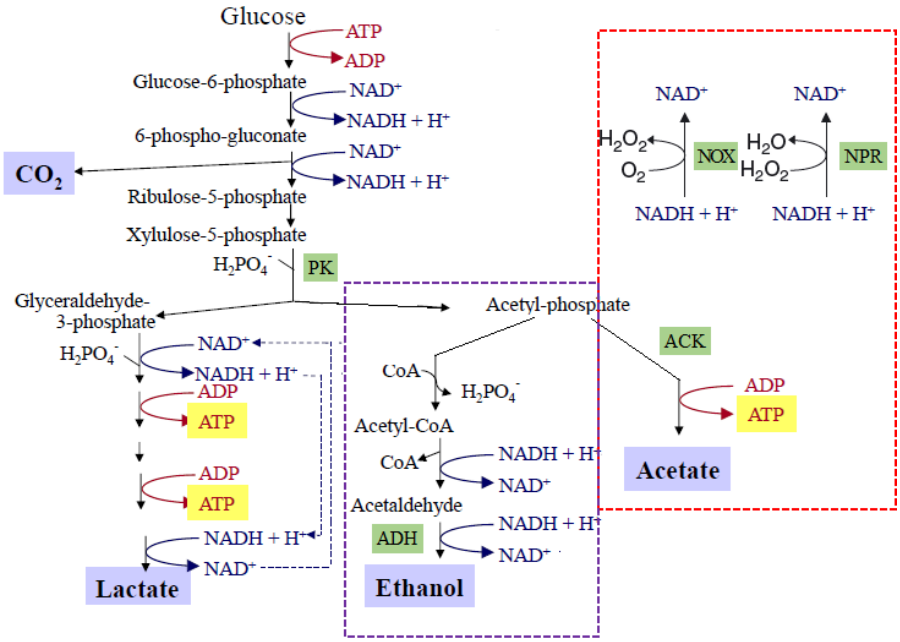

Figure 3 Pentose phosphate and heterofermentative pathways. Glucose is metabolized through the pentose-phosphate pathway, PK produces glyceraldehyde 3-phosphate, that is converted to lactate, and acetyl phosphate that can be converted to ethanol during growth in anaerobic conditions (dashed violet box), or to acetate during growth in aerobic conditions (dashed red box). In aerobic conditions NOX and NPR are responsible for regeneration of NAD+. (Figure adapted with permission from original material of Eddy J. Smid). PK: phosphoketolase, ADH: alcohol dehydrogenase; ACK: acetate kinase, NOX: NADH oxidase; NPR: NADH peroxidase.

The latter pathways are alternative, and mainly regulated by redox balance of the growing bacteria, indeed, the major end product of pentose phosphate pathway is ethanol under anaerobic conditions, since this pathway leads to the oxidation of cofactor NADH, with regeneration of NAD+, required for continuation of the glycolytic activity. On the other hand, when growth conditions are aerobic, thus the NADH/NAD+ ratio is lower, acetyl phosphate is converted to acetate, with ATP production, and the task of regenerating NAD from NADH is carried out by NADH oxidase (NOX), or NADH peroxidase (NPR), with the production of hydrogen peroxide (Fig.3).

The bacteria belonging to *Lb*. *casei* group have the metabolic potential to perform all the above mentioned pathways and are classified as facultative heterofermentative lactobacilli. Furthermore, some recent studies have pointed out, through transcriptomic approaches, that cultivation of *Lb*. *rhamnosus* in a cheese-mimicking broth leads to the upregulation of *spxB* gene encoding for pyruvate oxidase (POX), an enzyme that catalyses the oxidation of pyruvate to acetyl-phosphate, which can subsequently be converted into acetate by ACK with ATP production (Fig. 4).

POX pathway is involved in the aerobic metabolism of LAB, and in *Lb*. *plantarum* its activation leads to an increased synthesis of acetate in the early stationary phase of aerobic growth (Goffin et al., 2006; Lorquet et al., 2004; Quatravaux et al., 2006). The activation of *spxB* gene from *Lb*. *rhamnosus* was recently assessed *in situ* during ripening of Italian cooked hard cheese (Savo Sardaro et al., 2016; chapters 2 and 3 of this thesis), although the cheese environment is generally reported as anaerobic.

Nevertheless, diffusion of oxygen in cheese matrix is hard to assess, particularly considering the limitations imposed from a cheese which has an average weight of 30 kg (Mucchetti and Neviani, 2006). For this reason, chapter 4 reports a study performed to assess the correlation between oxygenation conditions and microbial metabolism in a miniaturized model cheese.

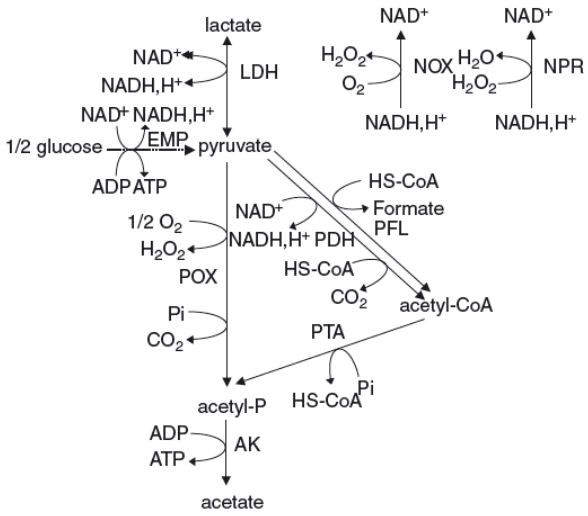

Figure 4 Lactate and acetate production pathways in *Lactobacillus plantarum*. EMP, Embden–Meyerhof Parnas pathway; LDH, lactate dehydrogenase; POX, pyruvate oxidase; PDH, pyruvate dehydrogenase; PFL, pyruvate formate lyase; PTA, phosphotransacetylase; AK, acetate kinase; NOX, NADH oxidase, NPR, NADH peroxidase (Reprinted from: Quatravaux et al., 2006, with permission from John Wiley and Sons).

Proteolysis and amino acid catabolism

Milk, as well as the deriving curd, is a protein rich environment, due to the presence of caseins, and probably for this reason LAB isolated from such environments have incomplete metabolisms for amino acids synthesis, but possess well developed systems for protein and peptides degradation (Fernández and Zúñiga, 2006).

However, proteolysis in Italian cheeses has other actors, besides LAB, such as enzymes from the coagulant, and the enzymes present in raw milk (McSweeney and Sousa, 2000). The initial step for protein degradation in lactobacilli is extracellular, and is performed by a cell-envelope proteinase (CEP, Fig.5) (Savijoki et al., 2006). In LAB five different CEPs were identified, and *Lb. rhamnosus* and *Lb paracasei* encode for PrtR and PrtP proteinases, respectively (Pastar et al., 2003).

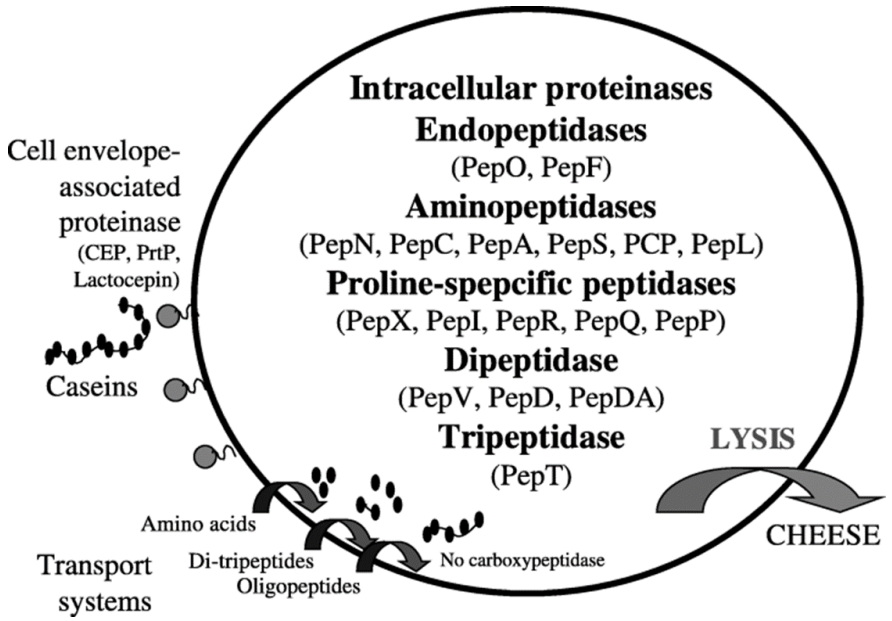

Figure 5 Summary of the proteolytic system of *Lactococcus*. The proteolytic systems of other lactic acid bacteria are generally similar (Reprinted from: Biochemistry of cheese ripening, McSweeney 2004, with permission from Springer).

CEPs degrade casein into oligopeptides, which are subsequently transported into the cells where they are subjected to further degradation into shorter peptides and amino acids by the action of a number of peptidases with distinct but partly overlapping specificities (Fernández and Zúñiga, 2006).

After breaking down of the casein molecules into smaller peptides, the oligopeptides are mainly transported into the cell by the Opp system, an oligopeptide ATP-binding cassette (ABC) transporter, while other systems are capable to transport di-tripeptides, such as the ABC transporter Dpp, or the ion-linked transporter DtpT, which have been extensively reviewed from Doeven et al. (2005). Most of the studies have concerned, so far, the species *Lactococcus lactis* and few data are available about peptide transport system in *Lb. casei* group.

After intake, the di-, tri- and oligopeptides are subject to the action of several intracellular oligopeptidase (Fig.5), whit different modes of action, such as endopeptidases (PepO, PePF), general aminopeptidases (PepN,

PepC, PepP), X-prolyl dipeptidyl aminopeptidase (PepX), tripeptidase (PepT), prolidase (PepQ), prolinase (PepR), proline iminopeptidase (PepI), and dipeptidases D and V (PepD, PepV) (Savijoki et al., 2006).

The final products of these enzymatic degradations are free amino acids (FAA), whose concentration generally increase when non starter LAB are used as adjunct cultures (Settanni and Moschetti, 2010).

Amino acids are further converted in a variety of compounds, such as aldehydes, alcohols, acids, esters and sulphur compounds, that have a relevant role in aroma formation during ripening (Smid and Kleerebezem, 2014). The formation of these aroma compounds can proceed either through transamination, or elimination. The latter, mainly observed for methionine, leads to the formation of major sulphur aroma compounds. The transamination pathway is generally initiated by an aminotransferase, and is the main pathway for degradation of all amino acids by LAB (Yvon and Rijnen 2001), leading to the formation of the α-ketoacids of the corresponding amino acids (Fig.6). Particularly, bacterial aminotransferases are further classified, on the basis of their substrate specificity, into branched chain-, aromatic- and aspartate-aminotransferases (BcAT, ArAT, and AspAT), despite experimental evidences suggest that these enzymes have a broader substrate specificity (Liu et al., 2008). Interestingly, the latter authors identified six aminotransferase genes in *Lb. casei* genome. The transaminase reaction is generally coupled with deamination of glutamate to α-ketoglutarate, catalysed by glutamate dehydrogenase (GDH), whose sequence was not found in the genome of *Lb. casei*.

The α-ketoacids represent a key intermediate in the synthesis of other flavour compounds, and are involved in a series of subsequent transformations. The first transformation involves a ketoacid decarboxylase (KdcA) which catalyses the decarboxylation of the ketoacid to the corresponding aldehyde, despite among LAB this enzymatic activity was found only in few *Lc. lactis* strains, and putative genes encoding for this function were not identified in lactobacilli (Liu et al., 2008; Smit and Smit, 2005).

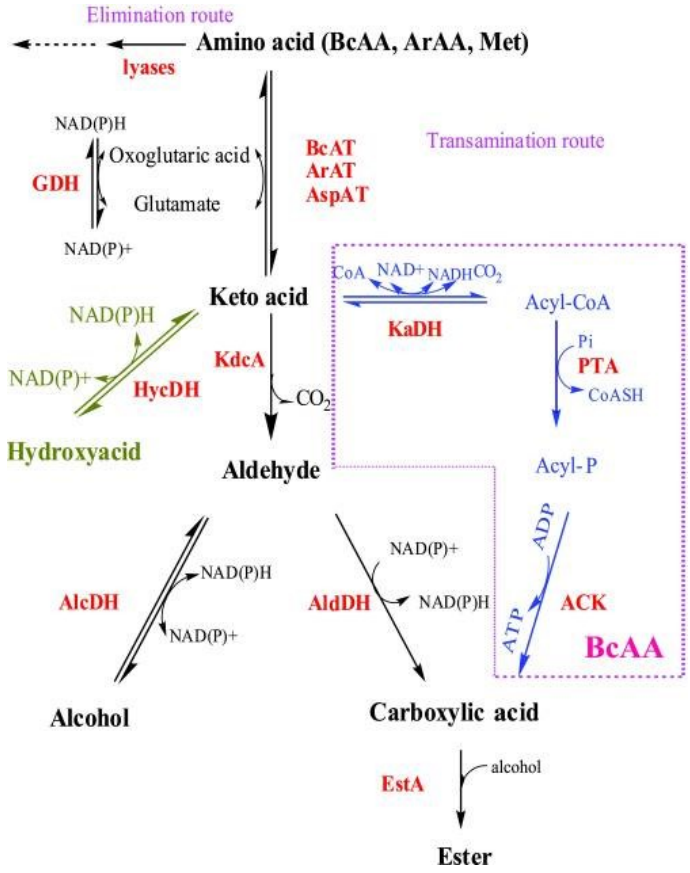

Figure 6 Generic amino acid (branched-chain amino acids, aromatic amino acids, and methionine) degradation pathway initiated by transamination in LAB. The enzymes are BcAT, ArAT, AspAT, GDH, HycDH, KdcA, AlcDH, AldDH, and EstA (esterase A). Enzymes specific for branched-chain amino acids degradation (dotted box): KaDH, α-keto acid dehydrogenase complex; PTA, phosphotransacylase; and ACK, acyl kinase. (Reprinted from Liu et al., 2008, with permission from American Society for Microbiology)

Other pathways for conversion of α-ketoacids include oxidative decarboxylation to carboxylic acids, via ketoacid dehydrogenase complex, or reduction to hydroxy acids by hydroxyl acid dehydrogenase, and the existence of alternative routes for the formation of organic acids might explain the lack of the aldehyde-forming activity.

When aldehydes are synthesised, they can be converted either into alcohols, via the reaction catalysed by alcohol dehydrogenase (AlcDH), or to carboxylic acids, from the aldehyde dehydrogenase enzyme (AldDH). The carboxylic enzymes can then be esterified by esterase (EstA) to the

corresponding (thio)esters, and these compounds represent important contributors of the final cheese aroma (Smit et al., 2005).

Lipolytic activity

Cheese lipids can be oxidised or hydrolysed by the LAB of the microbiota, however, oxidation is probably limited because of the low redox potential in cheeses. Lipases in PR and GP cheese originate from milk, starter and non-starter LAB. Lipolytic enzymes are hydrolases active on the ester linkage between a fatty acid and the glycerol core of the triacylglyceride, producing FFA, and mono- and diacylglycerides. Lipolytic enzymes may be classified as esterases or lipases, according to the length of the hydrolysed acyl ester chain, the physico-chemical nature of the substrate and the enzymatic kinetics (Collins et al., 2003).

LAB possess intracellular esterolytic/ lipolytic enzymes, although lipolytic activity is weak in comparison with some other cheese-related microorganisms. However, because of their presence in cheese at high numbers over an extended ripening period, LAB might contribute to the levels of FFA found in cheese (Collins et al., 2003).

Intensive lipolysis is undesirable in most cheese varieties because of the development of rancidity. However, low concentration of short- and intermediate-chain fatty acids contribute considerably to the flavour of some cheese. Short-chain fatty acids are substrate of other catabolic reactions such as esterification with alcohols or sulphydrilic compounds, or ß-oxidation.

When fatty acids are esterified with alcohols, ethanol is the most common substrate available for this reaction, since it might derive from heterofermentative pathway. This reaction, in LAB, is performed by esterases, which catalyse not only hydrolysis of milk fat glycerides to release FFAs, but also synthesis of esters via a transferase reaction. The esterases of LAB prefer di- and monoglycerides for both hydrolysis and ester synthesis and are, in fact, alcohol acyltransferases that use both water (hydrolysis) and alcohol (alcoholysis) as acyl acceptors. When glycerides are esterified with thiols, thioesters are the final products (Collins et al., 2004; Holland et al., 2005).

FFA released by lipases can be oxidised to ß-ketoacids and then decarboxylated to alkan-2-ones. Alkan-2- ones may be reduced to the corresponding secondary alcohols (alkan-2-ols), a step which is reversible

under aerobic conditions (McSweeney and Sousa, 2000), despite this pathway is not relevant for LAB.

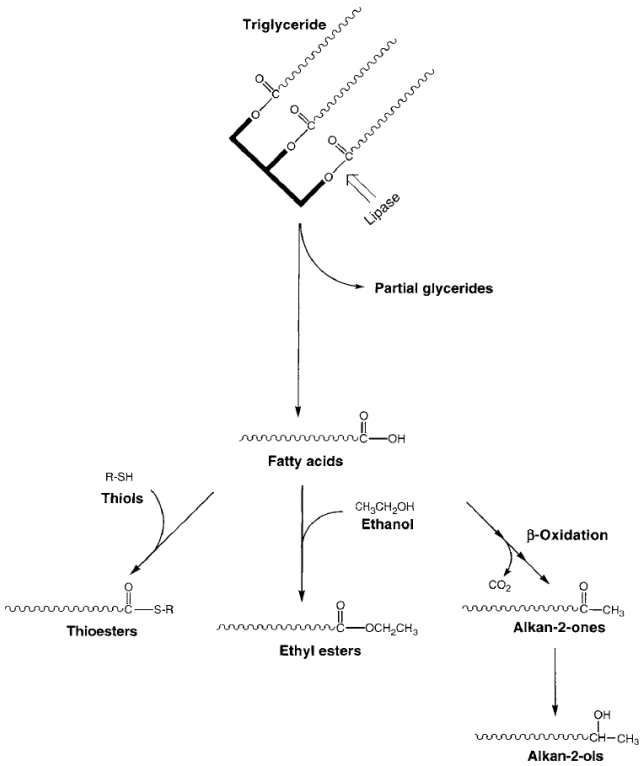

Figure 7 Pathways for the formation of fatty acids. (Adapted from Collins et al., 2004)

Genome plasticity and adaptation

The adaptation of LAB to growth in specific niches can be considered as a "domestication" of wild, pre-existing strains to food environment, driven by the human forcing of the fermentation process, leading to changes in growth rate, yield, and fitness of the originating strains (Mora et al, 2013). Experimental evolution performed to assess the adaptation of a plant-derived *Lc. lactis* strain to milk has shown that this adaptation is quite fast, "only" 1000 generations are enough to see the first changes. Modification in physiological parameters is accompanied by a fast response of the regulatory mechanisms, which readily adapt the transcription of necessary genes, but also by a rapid genomic response, with the appearance of point mutations, and loss of a mobile elements (Bachmann et al., 2009).

Recently, the availability of an increasing number of sequenced genomes highlighted the heterogeneity of *Lb. casei* group, which share a core genome of at least 1682 conserved genes, as well as clear signatures of niche adaptation, which is particularly relevant for dairy isolated strain that, for instance, present a loss of the genetic clusters involved in sugar utilisation (Broadbent et al., 2012; Toh et al., 2013).

Another work defines the existence of a "variome", or a flexible gene pool, estimated to comprise at least 2400 orthologous genes in *Lb. paracasei* (Smokvina et al., 2013), which comprises gene families correlated with phages, plasmids, transposon/conjugative elements, sugar utilization cassettes and other functions. Characterization of the *Lb. casei* genome suggested that it could have had access to genetic material present in its ecological niche, and its genomic composition has been influenced by ecological co-localization with other bacterial species, especially lactobacilli.

Plasmids are semi-autonomously replicating extrachromosomal DNA entities, which are normally dispensable for bacterial growth, and can confer traits that impart important niche-specific features (Kelly et al., 2010). Plasmid-associated phenotypic traits may include industrially important survival strategies, metabolic capabilities, virulence factors and antibiotic resistances (Ainsworth et al., 2014).

To date, there are 41 deposited plasmid sequences for bacteria belonging to *Lb. casei* group, and their size ranges from 1.3 Kb up to over 73 Kb for a *Lb. paracasei* strain, containing 91 genes and encoding 82 proteins (Table 2; https://www.ncbi.nlm.nih.gov).

Plasmid inheritance is due to the presence of gene cassettes that can be grouped into three classes: centromere like systems, site-specific recombination systems, and cassettes that cause post-segregational killing, that is the killing of newborn, plasmid-free cells (Gerdes et al., 2000). The latter, also called toxin-antitoxin (TA) system, consists of a two-component system, coding for a stable toxin, and an unstable antitoxin; the nature of these two interacting components, defines five different types of TA systems (Schuster and Bertram, 2013).

TA system could be located on plasmid and/or chromosome and their different location suggests a different physiological role. When TA system is carried on plasmid it acts as mechanism for plasmid maintenance (post

segregational killing) leading to cell death; conversely, when it is present on chromosome, it could kill the cell or induce dormancy often by inhibition of ATP synthesis.

In a recent study, Lazzi et al. (2014) has identified a set of genes involved in response to a cheese-mimicking environment, among which a transcript

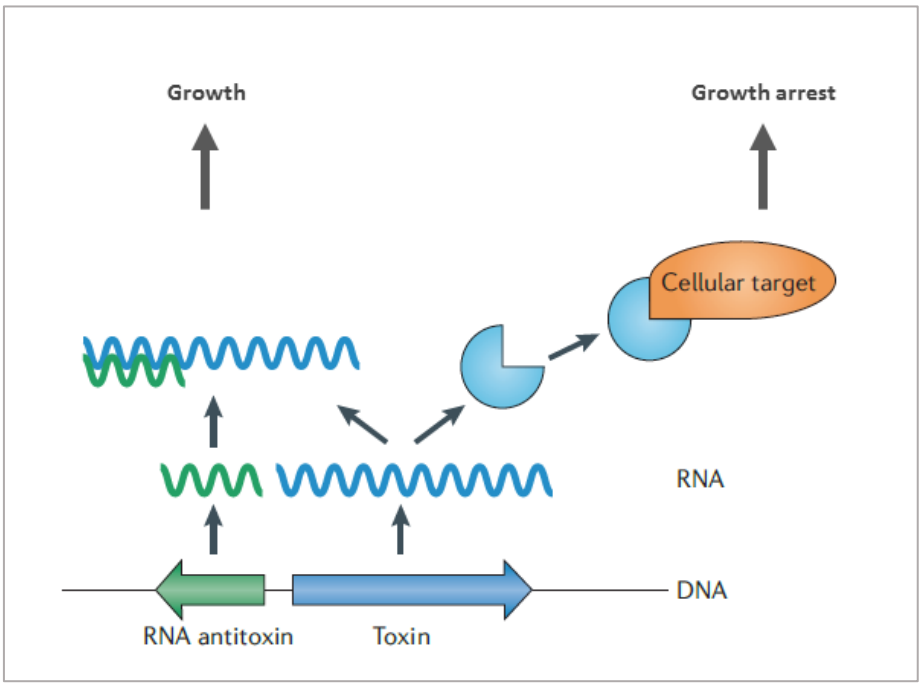

Figure 8 Model for the regulation of type I toxin–antitoxin (TA) systems. The toxin and RNA antitoxin (antisense RNA) genes are transcribed separately. RNA antitoxin binds to the toxin-encoding mRNA to form a duplex, inhibiting translation of the toxin-encoding mRNA. (Adapted from Yamagouchi et al., 2011)

related to *Lb. casei* plasmids. In particular, Chapter 5 reports for the first time a plasmid located type I TA system in a food-isolate (Fig., 8; Chapter 5). Overexpression of this TA system is observed in cheese-mimicking conditions, compared to growth of *Lb. rhamnosus* in a rich medium, as well as in ripening cheese, confirming the observation that plasmid-encoded features might be relevant for adaptation to environmental stress.

Table 2 Characteristics of sequenced plasmids from Lb. casei group, as reported in NCBI website.

Organism name	Plasmid Name	Accession number	Size (Kb)	GC%	Protein	Gene	Pseudogene
***Lb. rhamnosus* HN001**	pLR001	NC_011223.1	8.8	41.9	9	11	2
***Lb. rhamnosus* HN001**	pLR002	NC_011225.1	31.5	42.5	36	37	1
***Lb. rhamnosus* Lc 705**	pLC1	NC_013200.1	64.5	43.5	53	61	8
Lb. casei	pYIT356	NC_006257.1	4.9	43.4	1	1	-
Lb. casei	pSMA23	NC_010242.1	3.5	38.2	2	2	-
Lb. casei	pRCEID3.2	NC_014619.1	3.3	44.2	5	5	-
Lb. casei	pRCEID13.9	NC_014620.1	13.9	40.4	13	13	-
Lb. casei	pRCEID7.6	NC_016975.1	7.6	40.4	9	9	-
Lb. casei	pRCEID2.9	NC_017466.1	3.0	43.2	3	3	-
Lb. casei	pMC11	NC_025132.1	11.3	43.9	8	8	-
***Lb. casei* BD-II**	pBD-II	NC_017476.1	57.4	43.7	64	65	1
***Lb. casei* LC2W**	pLC2W	NC_017475.1	38.4	42.6	42	43	1
***Lb. casei* LcA**	pACT	NZ_CM001862.1	59.6	43.7	68	69	1
***Lb. casei* LcY**	pYAK	NZ_CM002348.1	59.6	43.7	68	69	1
***Lb. casei* LOCK919**	pLOCK919	NC_021722.1	29.8	43.9	28	31	3
***Lb. casei* str. Zhang**	plca36	NC_011352.1	36.5	40.1	42	42	-

***Lb. casei* subsp. *casei* ATCC 393**	pLBCZ-1	NZ_AP012545.1	26.7	44.1	25	32	7
***Lb. casei* subsp. *casei* ATCC 393**	pLBCZ-2	NZ_AP012546.1	1.3	43.3	1	2	1
***Lb. casei* W56**	pW56	NC_020057.1	56.3	43.7	61	63	2
Lb. paracasei	unnamed1	NZ_CP012188.1	73.3	42.9	82	91	9
Lb. paracasei	unnamed2	NZ_CP012189.1	47.7	41.6	50	55	5
Lb. paracasei	unnamed3	NZ_CP012190.1	50.4	43.0	48	62	14
Lb. paracasei	unnamed4	NZ_CP012191.1	31.5	42.7	30	37	7
Lb. paracasei	pMA3	NC_010913.1	5.1	39.5	4	4	-
Lb. paracasei	pSJ2-8	NC_012222.2	14.4	42.2	15	15	-
Lb. paracasei	pCD01	NC_013543.1	19.9	39.5	23	23	-
Lb. paracasei	pCD02	NC_013544.1	8.6	39.5	9	9	-
Lb. paracasei	pTXW	NC_013952.1	3.2	42.9	4	4	-
Lb. paracasei	pWCZ	NC_019669.1	3.1	42.6	3	3	-
Lb. paracasei	pLP5401	NC_021572.1	9.8	43.9	14	14	-
Lb. paracasei	pLP5402	NC_021573.1	6.7	39.0	10	10	-
Lb. paracasei	pLP5403	NC_021574.1	1.8	37.7	5	5	-
***Lb. paracasei* ATCC 334**	1	NC_008502.1	29.1	42.2	20	20	-
***Lb. paracasei* N1115**	unnamed_1	NZ_CP007123.1	3.8	38.7	3	5	2
***Lb. paracasei* N1115**	unnamed_2	NZ_CP007124.1	55.1	43.6	60	64	4

***Lb. paracasei* N1115**		unnamed_3	NZ_CP007125.1	58.5	44.0	62	66	4
***Lb. paracasei* N1115**		unnamed_4	NZ_CP007126.1	8.8	38.5	5	5	-
***Lb. paracasei* subsp. *paracasei* 8700:2**		1	NC_022114.1	24.2	40.5	29	29	-
***Lb. paracasei* subsp. *paracasei* 8700:2**		2	NC_022123.1	62.1	44.0	61	66	5
***Lb. paracasei* subsp. *paracasei* JCM 8130**		pLBPC-1	NZ_AP012542.1	11.2	42.5	12	12	-
***Lb. paracasei* subsp. *paracasei* JCM 8130**		pLBPC-2	NZ_AP012543.1	10.8	40.2	9	9	-

The role of TA systems is not limited to plasmid stability and maintenance, for instance these systems are involved in the generation of persistent cells, leading to multidrug resistance (Yamaguchi et al., 2011), reduce the energy requirements and allow the cells to better cope with stress (Gerdes et al., 2000) and prevention of the replication and diffusion of phages (Samson et al., 2013).

So far, few TA systems located on plasmids of LAB were identified, thus more studies are needed for a better characterization and to elucidate causes and effects of their activation.

Strategies to study bacterial adaptation

The complexity of cheese and of the physical, biochemical and microbiological conditions that interact and change during the manufacturing process, makes necessary some assumptions and simplification in order to study the physiological response of the microorganism to such environment.

Most of the studies about LAB physiology are performed on pure cultures, using either rich, undefined, or chemically defined media, in order to control the effect of every component on the growth and survival of the bacteria.

The study of microbial physiology and adaptation in response to the cheese environment requires a polyphasic approach, that makes use of model systems, allowing to isolate the stress components and to generate unambiguous answers. This objective is obtained by *in vitro* model systems, such as cheese-mimicking media, which allow to culture pure strains in a condition that provides the same substrates present in cheese.

Among these models are the cheddar-based CCE (Budinich et al., 2011) and the PR-based CB (Bove et al., 2011). The latter cheese-based media was used by Bove et al. (2012) to assess the physiological response of cheese isolates of *Lb. rhamnosus* by use of proteomics, revealing different profiles in comparison with bacteria grown in MRS media. Similarly, Lazzi et al. (2014) observed a great impact of CB cultivation on the transcriptomic profile of two *Lb. rhamnosus* and, in combination with an untargeted cDNA-AFLP approach, allowed to identify some peculiar metabolism that are relevant for growth and survival of *Lb. casei* group dairy strains. Nevertheless, the *in vitro* model system, compared to cheese

condition, has the drawback that bacterial growth in liquid media is generally planktonic, while growth in cheese is characterized by the formation of immobilised micro- or macro-colonies, leading to different metabolic response depending on the feature of the colony (Jeanson et al., 2015; Le Boucher et al., 2016). To this purpose, *in situ* model systems can be used, like miniaturized cheeses, that allow to study bacterial response without isolating the cells from the cheese matrix.

Model cheeses have been realised from different authors, and applied to a wide variety of cheeses (Bachmann et al., 2009; Erkus et al., 2013; Ruggirello et al., 2016). Regarding cooked, hard cheeses, which represent the target product of this thesis, only two models are available (Mucchetti et al., 2002; Vèlez et al., 2015), but in both the cases the proposed cheese-making protocols do not allow high-throughput manufacturing, which is desirable for systematic screening of technological parameters and microbial strains. Thus, one of the project of this thesis consisted in the realization of a high throughput model for cooked hard cheese (Chapter 4), and its application to study the *in situ* response of *Lb. rhamnosus* to varying oxygen concentration.

The major purpose of these models is to provide an easily manageable tool to perform a broad range of screening, despite they cannot entirely reproduce the manufacturing conditions found in real cheese, particularly in the case of miniaturization, since size affects some physical parameters relevant for bacterial growth (temperature gradients, a_w, oxygen diffusion, brine permeation, etc.). Thus, the research conducted on model systems cannot leave out of consideration the studies performed in real food products, using *in situ* approach, for instance, directly in cheese matrix.

Currently, the study of microbial adaptation to cheese is made simpler from advancing technologies that allow monitoring of the bacterial dynamics, as well as of gene and protein expression and metabolite production. As an example, the study of microbial dynamics in fermented foods has gained great advantage from the development of high throughput sequencing, that allows to depict the bacterial communities involved in the food manufacturing, and their development during ripening, overcoming the limitations represented by classical culture-dependent approach, that might give an incomplete picture of the microbiota composition (De Filippis et al., 2016).

In chapter three of this thesis, a metagenetic approach allowed to describe the metabolically active population during ripening of GP cheese and the evolution of *Lb. casei* group, targeting a metabolic gene with taxonomic potential. Furthermore, changes in the relative expression of the same metabolic gene could be assessed *in situ*, confirming the data obtained in CB with a previous study (Savo Sardaro et al., 2016, Chapter 2).

This corroborates the importance of using a combined approach, integrating the results of different model systems, to describe the drivers of bacterial adaptation during cheese manufacturing and elucidate the mechanism of growth in food conditions.

Aim of the thesis

The aim of this thesis was to study the adaptation of *Lb. casei* group to cheese environment, using a comprehensive approach involving the use of *in vitro* and *in situ* model systems, as well as *in situ* confirmation of the results in real cheese.

This thesis aimed to describe two systems potentially involved in adaptation of *Lb. casei* group to cheese environment, in particular the activation of *spxB* gene and the expression of a plasmid-encoded toxin-antitoxin system.

spxB gene encodes for a pyruvate oxidase (POX), and is widely present in the genomes of dairy *Lb. casei* group strains. POX catalyses the oxidation of pyruvate to acetyl-phosphate, which is then converted to acetate with the production of ATP from an acetate kinase (ACK), potentially representing an alternative metabolic pathway for bacterial growth during cheese manufacturing.

The toxin antitoxin (TA) system detected in dairy *Lb. rhamnosus* consists of a plasmid-located two component system, coding for a stable toxin, and an unstable antitoxin, involved in plasmid inheritance mechanism. The role of TA systems is not limited to plasmid stability and maintenance, but also in dormancy and apoptosis of the host cell, making it one of the most versatile global regulatory system in bacteria.

The second chapter of this thesis describes the distribution of *spxB* gene in dairy isolates of *Lb. casei* group, and how its sequence heterogeneity provides differentiation among isolates through high-resolution melting technique, allowing taxonomic identification of wild strains. Furthermore, it allows identification of *Lb. casei* group in cheese matrix and monitoring of these species during manufacturing.

Chapter three describes the microbial evolution of metabolically active microbiota during ripening of GP by pyrosequencing of 16S rRNA and of *spxB*. This approach further expands the taxonomic potential of this metabolic gene, allowing to distinguish strains belonging to *Lb. casei* group up to sequence type level, and to follow their dynamics in relation to ripening age.

The project described in chapter four deals with the set-up of a model mill cheese system for manufacturing of Italian-like cooked hard cheese. The

protocol for manufacturing is described, as well as the critical technological parameters that might affect bacterial development in real cheese. The model is then applied to investigate how different oxygen concentrations might influence lactic acid bacteria growth dynamics, and the aroma profile of the milli-cheese.

Finally, a novel type I toxin-antitoxin system from dairy *Lb. rhamnosus* is described for the first time in chapter five, and an in-depth bioinformatics analysis reveals the wide distribution of this system on plasmids harboured by *Lb. casei* group. Transcriptional analysis showed that this TA system is induced in CB media, and the transcript can be detected also in cheese, suggesting a role in adaptation to cheese environment.

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Chapter 2

The *spxB* gene as a target to identify *Lactobacillus casei* group species in cheese

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Abstract

This study focused on the *spxB* gene, which encodes for pyruvate oxidase. The presence of *spxB* in the genome and its transcription could be a way to produce energy and allow bacterial growth during carbohydrate starvation. In addition, the activity of pyruvate oxidase, which produces hydrogen peroxide, could be a mechanism for interspecies competition. Because this gene seems to provide advantages for the encoding species for adaptation in complex ecosystems, we studied *spxB* in a large set of cheese isolates belonging to the *Lb. casei* group. Through this study, we demonstrated that this gene is widely found in the genomes of members of the *Lb. casei* group and shows variability useful for taxonomic studies. In particular, the HRM analysis method allowed for a specific discrimination between *Lb. rhamnosus*, *Lb. paracasei* and *Lb. casei*. Regarding the coding region, the *spxB* functionality in cheese was shown for the first time by real-time PCR, and by exploiting the heterogeneity between the *Lb. casei* group species, we identified the bacterial communities encoding the *spxB* gene in this ecosystem. This study allowed for monitoring of the active bacterial community involved in different stages of ripening by following the POX pathway.

Introduction

Lactic acid bacteria (LAB) constitute a group of Gram-positive and strictly fermentative bacteria that produce lactic acid as the major end product from carbohydrates. The *Lactobacillus genus*, which currently contains 204 species (http://www.bacterio.net/lactobacillus.html), is the largest group in the family *Lactobacillaceae*. Within this genus, the facultative heterofermentative species *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus,* which are closely phylogenetically and phenotypically related, are regarded together as the *Lactobacillus casei* group (Felis and Dellaglio, 2007). Despite the controversial nature of their classification and nomenclature (Acedo-Felix, 2003; Diancourt et al., 2007; Felis et al., 2001; Judicial Commission Of The International Committee On Systematics Of Prokaryotes, 2008; Toh et al., 2013), these species can be isolated from different environments, including plants, and in particular, they play a key role in different food products, including meat and cheese. Regarding dairy products, the presence of bacteria belonging to the *Lb. casei* group is due to their natural occurrence in milk, together with their ability of growing in curd and cheese. Indeed, they represent the major microbiota group during ripening, due to their metabolic activities that allow them to tolerate the environment of the cheese (Peterson and Marshall, 1990; Settanni and Moschetti, 2010). Recent studies performed on *Lb. rhamnosus* growing in a cheese model system (Bove et al., 2012; Lazzi et al., 2014) revealed a redirection of bacterial metabolism toward acetate, an important flavor compound. In particular, Lazzi et al. (2014) observed an upregulation of the *spxB* gene, which encodes for pyruvate oxidase (POX), an enzyme that catalyzes the oxidation of pyruvate to acetyl phosphate, which is then converted to acetate in the subsequent reaction, leading to ATP production. Although *spxB* is present in the genome of different *Lactobacilli* (Lazzi et al., 2014), this metabolic pathway has been deepen studied only in *Lb. plantarum* (Goffin et al., 2006; Lorquet et al., 2004; Zotta et al., 2012). A more in-depth knowledge regarding the presence of genes encoding for the POX pathway in bacterial genomes and their transcription is required, as it could indicate a way to produce energy in different habitats and permit bacterial growth in specific nutritional conditions. Furthermore, the activity of POX, due to hydrogen peroxide production, could be a mechanism involved in interspecies competition, as different authors have

described for the *Streptococcus* community that inhabits the oral cavity (Zheng et al., 2011; Zhu et al., 2014). Complete genome sequencing of different *Lb. rhamnosus* and *Lb. paracasei* show the occurrence of multiple copies of genes encoding putative POXs, and it is known that gene duplication is an important evolutionary force that provides an organism with an opportunity to evolve new functions. It is well known how different bacterial species grow in succession during cheese ripening, and as reviewed by Gatti et al. (2014), this is especially extensive for long-ripened cheeses. The presence of different strains that mark specific moments in cheese ripening or that can develop differently throughout the ripening process are linked to the strains' abilities to adapt to specific environmental and technological conditions and could influence the features of cheese. All of these findings stress the importance of following the dynamics of technologically relevant bacteria during ripening: culture-independent methods, based on bacterial DNA or, even better, on RNA that identify active members of the microbial population, represent a significant tool to monitor species. The sequence of 16S rRNA has been extensively used to identify bacterial species and to fingerprint complex communities by different methods. Recently, certain LAB studies that were all based on 16S rRNA reported the use of High Resolution Melting (HRM), a post-PCR method that can be used to study sequence variation due to a single nucleotide polymorphism leading to specific melting temperatures and specific melting profiles (Patel, 2009; Reed et al., 2007). Porcellato et al. (2012) proposed the first application of this technique to characterize isolates from Norwegian cheese; subsequently, Iacumin et al. (2014) extended the method to a wider collection of bacteria belonging to the *Lb. casei* group, proving its effectiveness in comparison with other molecular approaches. Recently, the *groEL* gene, which encodes a heat shock protein, has been used as a marker for the genetic typing of the *Lb. casei* group species (Koirala et al., 2015), confirming that taxonomic assignment deserves attention from the scientific community and that the HRM technique is a promising technique.

Given the potential role of *spxB* in bacterial growth during cheese ripening, the aim of this study was to propose this gene as a possible target of the metabolically active microflora for the first time. For this purpose, the *spxB* gene was examined within the *Lb. casei* group species: 74 isolates from Parmigiano Reggiano cheese at different ripening stages were screened for the presence and the sequence heterogeneity of this gene. In

addition, HRM and transcription analysis of *spxB* in cheese was investigated to target the metabolically active microflora directly in the cheese matrix.

Materials and methods

Strains and culture conditions

Seventy-four strains belonging to the species *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus,* isolated from Parmigiano Reggiano (Table 1A) and previously identified by Ward and Timmins (1999), were used in this study. All of these strains have been deposited in the bioresource collection of the Department of Food Science (University of Parma). The twelve reference strains used for the PCR specificity study were reported in Table 1B. All bacterial strains were maintained as frozen stocks (-80 °C) in MRS (*Lactobacillus*) or M17 (*Streptococcus*, *Lactococcus* and *Enterococcus*) broth (Oxoid, Milan, Italy) supplemented with 15% glycerol (w/v). Before use, the cultures were propagated twice with a 2% (v/v) inoculum into the appropriate media and incubated for 18 h at 37 °C anaerobically (AnaeroGen, Oxoid).

Nucleic acid preparation

Genomic DNA was extracted from bacterial cultures using the DNeasy Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The DNA concentration was measured using an Eppendorf BioPhotometer Plus instrument and checked by agarose gel electrophoresis. The genomic DNA was diluted to 20 ng μL^{-1} for PCR. RNA was extracted by TRIzol (Invitrogen, Milano, Italy) from 6- and 24-month-old Parmigiano Reggiano cheeses and their respective raw milk sources. After the extraction, cheese samples were prepared following the protocol of Monnet et al. (2008) with a few modifications. Briefly, 150 mg of grated cheese was placed in a 2 ml screw cap tube containing 500 µl of zirconia/glass beads (diameter, 0.1 mm; BioSpec Products, Bartlesville, OK), followed by the immediate addition of 1.5 ml of TRIzol. The tubes were processed with a Mini-BeadBeater 8 (BioSpec Products, Bartlesville, OK) by three 60 s mixing sequences at maximum speed, interspersed with 60 s pauses at room temperature. Homogenized cheese samples and raw milk were processed following the TRIzol manufacturer's instruction.

Table 1 Bacterial strains included in the study. (A) Strains belonging to the bioresource collection of the Food Science Department (University of Parma) used for the determination of the presence and sequence heterogeneity of *spx*B gene. (B) Reference strains used for the PCR specificity evaluation. (C) Strains used for degenerate primer design and the neighbor-joining tree.

(A)

Species	Strain*	Origin
Lactobacillus rhamnosus	**2075**, **2077**, 2101, **2233**	Parmigiano Reggiano 2 months
	1019	Parmigiano Reggiano 4 months
	2114, 2118, 2132, **2140**, 2407, 2465, 2466, 2471	Parmigiano Reggiano 6 months
	2177, **2178**, **2190**	Parmigiano Reggiano 9 months
	1200, **2352**, **2360**, **2362**, **2411**	Parmigiano Reggiano 12 months
	1473	Parmigiano Reggiano 20 months
Lactobacillus paracasei	**2203**	Milk
	2247, **2461**, **2462**	Parmigiano Reggiano 2 months
	2092, **2093**, **2115**, **2122**, 2124, **2125**, **2126**, 2127, **2133**, 2145, 2150, 2152, **2155**, 2167, 2186, **2187**, **2188**, **2189**, **2333**, **2468**, **2469**,	Parmigiano Reggiano 6 months
	2349, **2367**, **2369**, **2416**	Parmigiano Reggiano 13 months
Lactobacillus casei	**2046**, **2057**, **2240**, 2243, **2246**, **2306**, **2464**	Parmigiano Reggiano 2 months

	2094, **2107**, **2116**, 2120, 2121, 2136, 2138, 2142, 2413	Parmigiano Reggiano 6 months
	2154	Parmigiano Reggiano 9 months
	2334, 2348, 2356, 2410	Parmigiano Reggiano 12 months

* Bold character indicates strains submitted for sequencing

(B)

Species	**Strain**	**Origin**
Lactobacillus casei	ATCC 334	Emmental cheese
Lactobacillus fermentum	LMG 6902^T	Fermented beets
Lactobacillus plantarum* subsp. *plantarum	LMG 6907^T	Pickled cabbage
Lactococcus lactis* subsp. *lactis	LMG 6890^T	Unknown
Lactococcus lactis* subsp. *cremoris	LMG 6897^T	Cheese starter
Lactobacillus helveticus	LMG 6413^T	Swiss Emmental cheese
Streptococcus thermophilus	LMG 6896^T	Pasteurized milk
Streptococcus gallolyticus* subsp. *macedonicus	LMG 18490	Kasseri cheese
Lactobacillus delbrueckii* subsp. *lactis	LMG 7942^T	Emmental cheese
Lactobacillus delbrueckii* subsp. *bulgaricus	LMG 6901^T	Bulgarian yoghurt
Enterococcus faecium	LMG 11423^T	Unknown
Lactobacillus rhamnosus GG	ATCC 53103	Human feces

(C)

Species	Strain	Origin	Accession number
Lactobacillus rhamnosus	GG (ATCC 53103)	Human feces	FM179322
	LOCK900	Human feces	CP005484
	LOCK908	Human feces	CP005485
	ATCC 8530	Human gastrointestinal tract	CP003094
	Lc 705	Human gastrointestinal tract	FM179323
Lactobacillus paracasei	JCM 8130[T]	Dairy	AP012541
	8700:2	Human gastrointestinal tract	CP002391
Lactobacillus casei	ATCC 334	Emmental cheese	CP000423
	LC2W	Chinese dairy product	CP002616
	BL23	Dairy	FM177140
	LOCK919	Human feces	CP005486
	str. Zhang	Koumiss	CP001084
	W56	Human	NC_018641
	BD-II	Koumiss	CP002618
	str.12A	Corn silage	CP006690.1

[T] Type strain

After extraction, RNA was quantified using a spectrophotometer (Jasco, Japan) and checked by agarose gel electrophoresis.

RNA was reverse transcribed into cDNA with Quantiscript Reverse Transcriptase (QuantiTect Reverse Transcription Kit, Qiagen, Milan, Italy) using random hexamer primers according to the manufacturer's instructions.

In silico analysis of spxB gene

The partially transcribed RNA sequence of the *spxB* gene of *Lb. rhamnosus*, accession number AB896775 (Lazzi et al., 2014), was used to find homologous pyruvate oxidase sequences in the NCBI database. Fourteen sequences from different species (Table 1C) were aligned and used to design primers for *spxB* gene region amplification within the *Lb. casei* group strains. A set of degenerate primers was designed (Table 2): the forward primer *poxPromFw* was located in the promoter region of the gene, and the reverse primer *poxPromRv* was in the coding region, which yielded an amplification product of 260 bp.

PCR amplification of the target gene and nucleotide sequencing

The partial fragments of the *spxB* genes of the *L. casei* group strains were amplified and sequenced in 44 out of 74 strains originating from milk and cheese at different stages of ripening (13 *Lb. rhamnosus*, 10 *Lb. casei* and 21 *Lb. paracasei*) (Table 1A) using the primers *poxPromFw* and *poxPromRv* (Table 2). PCR reactions were composed of 7 µL of sterile MilliQ water, 10 µL of 2X PCR GoTaq Master Mix (Promega), 1 µL of forward primer (10 mM), 1 µL of reverse primer (10 mM) and 1 µL of template DNA (20 ng/µL). The following thermal cycling conditions were used: initial strand denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The resulting amplicons were purified using the QIAquick PCR purification Kit (Qiagen), sequenced by MACROGEN Europe (Amsterdam, The Netherlands) and aligned using DNAman software (vers. 4.15, Lynnon Biosoft Company). This software was used to generate a neighbor-joining (NJ) dendrogram where relative branch length was indicated. The bootstrap values were based on 1,000 replications.

PCR primer specificity for Lb. casei group strains

The alignment of the 44 *spxB* partial sequences obtained from the *Lb. casei* group selected strains allowed for the design of a new experimental primer combination. This second combination, composed of the forward primer (*poxcDNAFw*) located in the coding region of the gene and the reverse primer (*poxProm Rv*) (Table 2) previously used in the sequencing PCR, yielded an amplification product of 150 bp. The oligonucleotide primer pair specific for the *Lb. casei* group (*poxcDNAFw- poxPromRv),* located in the transcribed region of the gene *spxB*, was evaluated for PCR specificity. Twelve reference strains belonging to species other than the *Lb. casei* group, reported in Table 1B, were used to test the primer pair for its specificity. The following amplification conditions were used: initial strand denaturation at 94 °C for 2 min, followed by 36 cycles of 94 °C for 30 s, 63 °C for 30 s and 72 °C for 30 s, and a final extension step at 72 °C for 7 min. The PCR products were visualized on a 2% agarose gel, stained with SYBR Safe Dye (Invitrogen).

Development of High-Resolution Melting (HRM) analysis

The initial transcribed region within the *spxB* gene was selected for HRM analysis by using the primers *poxcDNAFw* and *poxPromRv* for the amplification reaction. PCR was performed in a final volume of 10 µl containing 1X MeltDoctor™ HRM Master Mix (Life Technologies), 0.5 µM of primers and 20 ng of genomic DNA of the *Lb. casei* group strains. HRM was performed in a 7900HT Fast Real-Time PCR System thermal cycler (Life Technologies, Carlsbad, USA) under the following amplification conditions: 95 °C for 10 min, 50 cycles at 95 °C for 15 s and 60 °C for 1 min. Melting analysis was carried out as follows: (i) denaturation at 95 °C for 10 s, (ii) annealing at 60 °C for 1 min and (iii) HRM curve generation at 95 °C for 15 s and annealing at 60 °C for 15 s. The ramp rate of the HRM step was 1% of the rate of the annealing step. The amplification and dissociation curves were analyzed using the SDS Software v 2.3 (Life Technologies), and post-PCR analysis of the melt curves was carried out using the HRM Software v 2.0.1 (Life Technologies). The aligned melt curves and the difference plots were obtained for the *Lb. casei, Lb. paracasei* and *Lb. rhamnosus* strains using the fluorescence value obtained from each strain and a confidence level of 90% (Andersson *et al.*

2009). All 74 strains of the *Lb. casei* group were tested in triplicate; positive controls and non-template controls were included in each run.

Table 2 Primer sequences and characteristics of the amplicons used in this study.

Primer name	Sequence (5'-3')	Length (bp)	Amplicon size (bp)	Temperature of annealing (°C)
poxPromFw	TGAAAGGGyTTGCATTGTTAT	21	260 [a]	58
poxcDNAFw	CAGACGCAATGATCAAGGTG	20	150 [b]	60
poxPromRv	AATGCGCCyACTTCTTCATG	20		60
GAPDH*Fw*	GTTGGTACCATGACCACCGT	20	122	60
GAPDH*Rv*	GTGCTGTGAGGAATCGTGTT	20		60

[a] Refers to the primer pair *poxPromFw – poxPromRv*

[b] Refers to the primer pair *poxcDNAFw – poxPromRv*

Relative quantification by real-time RT-PCR and melting profiles analysis

Relative quantification was performed using an EP Gradient S Mastercycler® (Eppendorf, Hamburg, Germany) with the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, California, USA). The 20 µl PCR reaction included 1 µl of cDNA, 0.5 µl of reverse primer, 0.5 µl of sense primer and 10 µl of SYBR green. The reactions were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence signal was acquired at 60 °C. Melting curve analysis (60- 95 °C with a heating rate of 0.1 °C per second and a continuous fluorescence measurement) was carried out. After the reaction, the Ct data were determined using default threshold settings, and the mean Ct was determined from the triplicate PCRs. The *poxcDNAFw* and *poxPromRv* primers were used for the relative quantification of *spxB* using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene (Table 2). GAPDH primers were specifically designed for the amplification of the *Lb. casei* group. The fold change in

spxB from 6- and 24- month-old Parmigiano Reggiano cheeses were obtained with respect to the raw milk calibrator sample using the $\Delta\Delta C_T$ method (Livak *et al.* 2001). The real-time PCR amplification efficiencies (E) in the exponential phase were calculated according to the equation: $E = 10^{(-1/slope)}$. The results showed that the amplification efficiencies were 91% for *spxB* and 92% for GAPDH, with a difference of less than 5% between these genes.

Results and discussion

Microorganisms belonging to the *Lactobacillus* genus can metabolize pyruvate into acetate via pyruvate dehydrogenase (PDH), pyruvate formate lyase (PFL) or pyruvate oxidase (POX) pathways (Quatravaux et al., 2006). In this last pathway, the POX enzyme, encoded by the *spxB* gene, catalyzes pyruvate oxidation leading to the production of hydrogen peroxide and acetyl phosphate, followed by acetate and ATP generation via acetate kinase (ACK). Recently, the presence of this pathway has been described in *Lb. rhamnosus*, *Lb. casei* and *Lb. paracasei* (Zotta et al., 2014). Lazzi et al. (2014) predicted the monocistronic transcription and showed that this gene is overexpressed when *Lb. rhamnosus* grows in a cheese-like medium. Similarly, Mortera et al. (2013) suggested that the growth of *Lb. casei* in a substrate lacking sugars and rich in citrate principally leads to the activation of this metabolic pathway, instead of those involving PFL or PDH.

Because this gene seems to provide advantages for growth and adaptation to complex ecosystems for the encoding species, we decided to study the *spxB* gene both in wild cheese isolates and directly in the cheese matrix. Given that the microflora of long-ripened cheeses is mainly constituted by the species belonging to the *Lb. casei* group (*Lb. casei, Lb. paracasei* and *Lb. rhamnosus*), as a starting point we investigated whether a wide number of isolates retrieved from these cheeses contained the *spxB* gene.

First, a BLAST search was performed using the partially expressed sequence of *spxB* (pyruvate oxidase) present in Genbank with the accession number AB896775 (Lazzi et al. 2014).

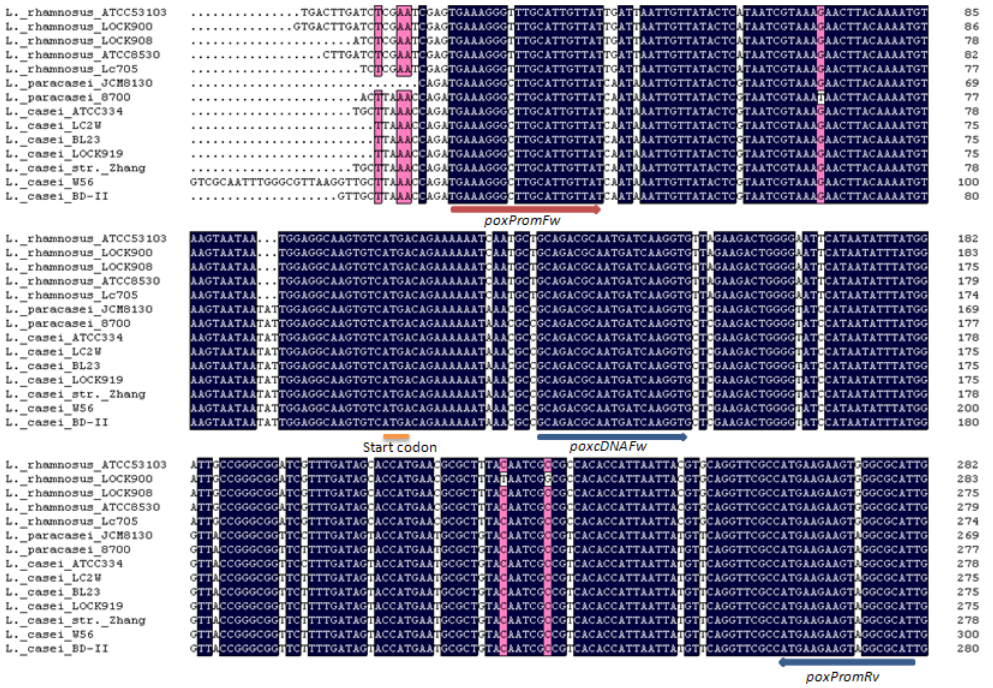

Figure 1 Nucleotide sequence alignment of the *spxB* gene and the promoter regions from fourteen *Lb. casei* group strains. In silico analysis was used for the primers design: the arrows show the primer positions, and the line shows the start codon of the gene. In the alignments, the black color designates 100% similarity, whereas the violet color designates a similarity equal or higher than 75% among the *Lb. casei* group.

Fourteen full-length DNA sequences related to the *Lb. casei* group (Table 1C) were found with homology values higher than 82%. These sequences were used to design one set of degenerate primers (*poxPromFw - poxPromRv)* able to amplify a fragment of the *spxB* genomic sequence starting from the promoter region of the gene at approximately -90 bp and spanning 170 bp into the coding region (Fig. 1), producing a fragment of 260 bp. Despite the many conserved portions, degenerate primers were needed to address sequence variations between the species. In addition, the conserved sequences were not long enough to design suitable primer pairs (at least 18-20 base pairs) without degenerate bases.

This primer combination was then used to test for the presence of the *spxB* gene sequence in all of the 74 bacterial strains selected for this study from the bioresource collection of the Department of Food Science (University of Parma). A positive amplification was observed in all of the strains tested (data not shown). The presence of the *spxB* gene in a wide set of

Lactobacilli has never been reported to date, therefore 44 amplicons (13 from *Lb. rhamnosus*, 10 from *Lb. casei* and 21 from *Lb. paracasei*) were sequenced to confirm the presence of the *spxB* gene in the *Lb. casei* group. The sequences were compared, which showed an overall sequence homology of 84.96%. The neighbor-joining dendrogram of the *Lb. casei* group strains based of their *spxB* gene sequences, depicted in Fig. 2, highlights the genetic variability and the phylogenetic relationships present in this bacterial group. Four main clusters could be distinguished. The cluster I group is composed of the reference strains of the *Lb. casei* species *(Lb. casei* 12A and *Lb. casei* str. Zhang), including the *Lb. casei* strains collection and some strains previously identified by Ward and Timmins (1999) as *Lb. paracasei*. Because the reference strains (*Lb. casei* 12A and *Lb. casei* str. Zhang) used in the phylogenetic analysis were never be considered to be controversial, we can assume that the cluster I group *Lb. casei* strains and that the classification using the method described by Ward & Timmins (1999) is not suitable above all to discriminate among the *Lb. casei* and *Lb. paracasei* strains collected from the same ecological niche.

The cluster II group is composed of the *Lb. paracasei* strains, the *Lb. paracasei* type strain JCM 8130 and *Lb. casei* ATCC334. As reported by different authors (Judicial Commission Of The International Committee On Systematics Of Prokaryotes 2008; Koirala *et al.,*2015; Iacumin *et al.* 2015), ATCC334, despite being annotated in GenBank as *Lb. casei*, has been reclassified as *Lb. paracasei*. The cluster III group is composed of all *Lb. rhamno*sus strains, including strains LOCK908 and LOCK900.The wild strains, LC2154 and LP2462, which were previously identified as *Lb. casei* and *Lb. paracasei*, were now reclassified as *Lb. rhamnosus*, similar to what was observed with cluster I. Interestingly, the cluster IV group is composed of three *Lb. casei* strains that show a higher level of genetic similarity with the sequences of *Lb. rhamnosus* (95.69%) than with the closer members of *Lb. casei* (cluster I) (91.56%). Overall, these results showed that the sequence variability of the *spxB* partial gene among *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* could be used for their discrimination.

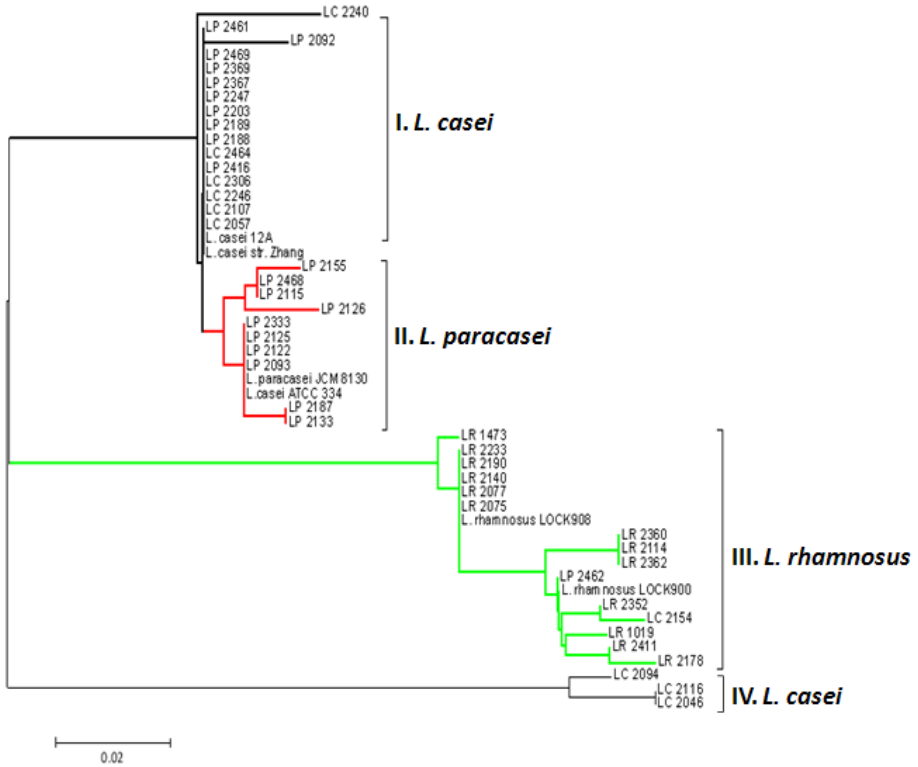

Figure 2 Neighbor-joining dendrogram of the aligned *spxB* gene sequences from the 44 *Lactobacillus casei* group strains and the 6 reference strains listed in Table 1A-1C.

Given that the functionality of the bacterial community is driven at the RNA level, and because the *spxB* gene could have a functional role for microorganisms growing in cheese, we proposed to use this gene to identify the *Lb. casei* community present in cheese by using the HRM analysis. This approach follows the indications of certain authors for using non-16S rRNA genomic amplicons, such as the l*acS* gene, for a culture-independent identification of types within species in food (De Filippis et al., 2014; Ercolini et al., 2005). For this purpose, the primer pair *poxcDNAFw-poxPromRv* (Table 2, Figure 1), which gave an amplification product of 150 bp in the internal transcribed region of the gene, was used for HRM analysis of the 74 *Lb. casei* strains and also for the subsequent real-time PCR in the cheese matrix.

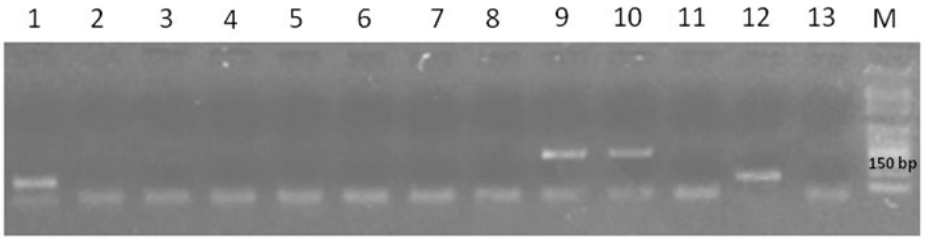

Figure 3 Gel electrophoresis of the *spxB* amplicons (expected size 150 bp) for PCR specificity study performed on DNA extracted from the type strains listed: 1) *Lactobacillus casei* ATCC334, 2) *Lactobacillus fermentum* (LMG6902), 3) *Lactobacillus plantarum* subsp. *plantarum* (LMG6907), 4) *Lactococcus lactis subsp. lactis* (LMG6890), 5) *Lactococcus lactis* subsp. *cremoris* (LMG6897), 6) *Lactobacillus helveticus* (LMG6413), 7) *Streptococcus thermophilus* (LMG6896), 8) *Streptococcus gallolyticus* subsp. *macedonicus* (LMG18490), 9) *Lactobacillus delbrueckii* subsp. *lactis* (LMG7942), 10) *Lactobacillus delbrueckii* subsp. *bulgaricus* (LMG6901), 11) *Enterococcus faecium* (LMG11423), 12) *Lactobacillus rhamnosus* GG, 13) Negative control, M) Molecular marker 1 Kb plus (Life Technologies).

To expand the application of this primer set to the amplification of complex cDNA samples derived from cheese, the specificity of the new primer pair was tested with end-point PCRs. The template DNA for the specificity assay was derived from cultures of well-characterized references strains representing some of the major bacterial species found in cheese (Table 1B). The agarose gel showed a single amplification product of 150 bp corresponding only to the *Lb. casei* group species (Fig. 3, lanes 1 and 12). *Lb. delbrueckii* subsp. *bulgaricus* (LMG6901) and *Lb. delbrueckii* subsp. *lactis* (LMG7942) (lanes 9 and 10, respectively) showed a non-specific amplification product of 500 bp that, having a different melting temperature, did not affect the HRM analysis. A preliminary primer optimization step was then performed in the real-time PCR experiment using three strains to identify the optimal primer concentrations to ensure the highest PCR efficiency and the absence of dimers. These requirements were met using 0.3 µM of the primers (both 5′ and 3′) in a 20 µl reaction mix (data not shown).

After the primer specificity and the optimization step, an HRM analysis on the 74 *Lb. casei* group strains was performed. The melting curves

obtained were then analyzed with the commercial HRM software v 2.01 (Life Technologies) to verify the possibility of distinguishing the species in the *Lb. casei* group. The aligned melt curves (Fig. 4A) and the difference plots (Fig. 4B) obtained from all of the strains allowed them to be grouped into four variants with a confidence higher than 90%. Variant 1 represented strains belonging to *Lb. rhamnosus*, with an average Tm of 80.5 ± 0.5 °C, while variant 2, with an average Tm of 78.5 ± 0.1 °C, was linked to strains that are listed in the cluster I group (*Lb. casei*) (Fig. 2). Variants 3 - 4 referred to *Lb. paracasei* strains, which have an average Tm of 79.2 ± 0.1 °C and 78.8 ± 0.1 °C, respectively (Fig. 4A and B). An optimal confidence was not obtained for the three strains present in cluster IV (Fig. 2), for which further studies are required to elucidate their taxonomical position.

Overall, the HRM data were in agreement with the analysis of the *spxB* sequences, and compared with the neighbor-joining dendrogram, two different variants (3-4) in the *Lb. paracasei* species were found. This is consistent with data from Koirala et al. (2015), who suggest the presence of two different subspecies, *paracasei* and *tolerans,* in the *Lb. paracasei* species based on their HRM results.

However, the methods proposed by Koirala et al. (2015) and Iacumin et al. (2015) to identify species belonging to the *Lb. casei* group by HRM focused on strains coming from *different* ecological niches with different selective pressures. In contrast, in this work, the strains analyzed were isolated from the same source (i.e., dairy samples from Parmigiano Reggiano cheese-making), and a greater genetic similarity among the strains due to the same cheese-making parameters that induce the same selective pressures could make discrimination more difficult.

The heterogeneity observed in the transcribed region of *spxB,* a functional gene that we found to be conserved in a wide number of strains, makes it suitable to directly identify the species involved in the ripening of cheese.

For this purpose, raw milk samples and 6- and 24-month-old Parmigiano Reggiano cheeses were subjected to RNA extraction and cDNA reverse transcription. The cDNA obtained were used in real-time PCR amplification and HRM analysis.

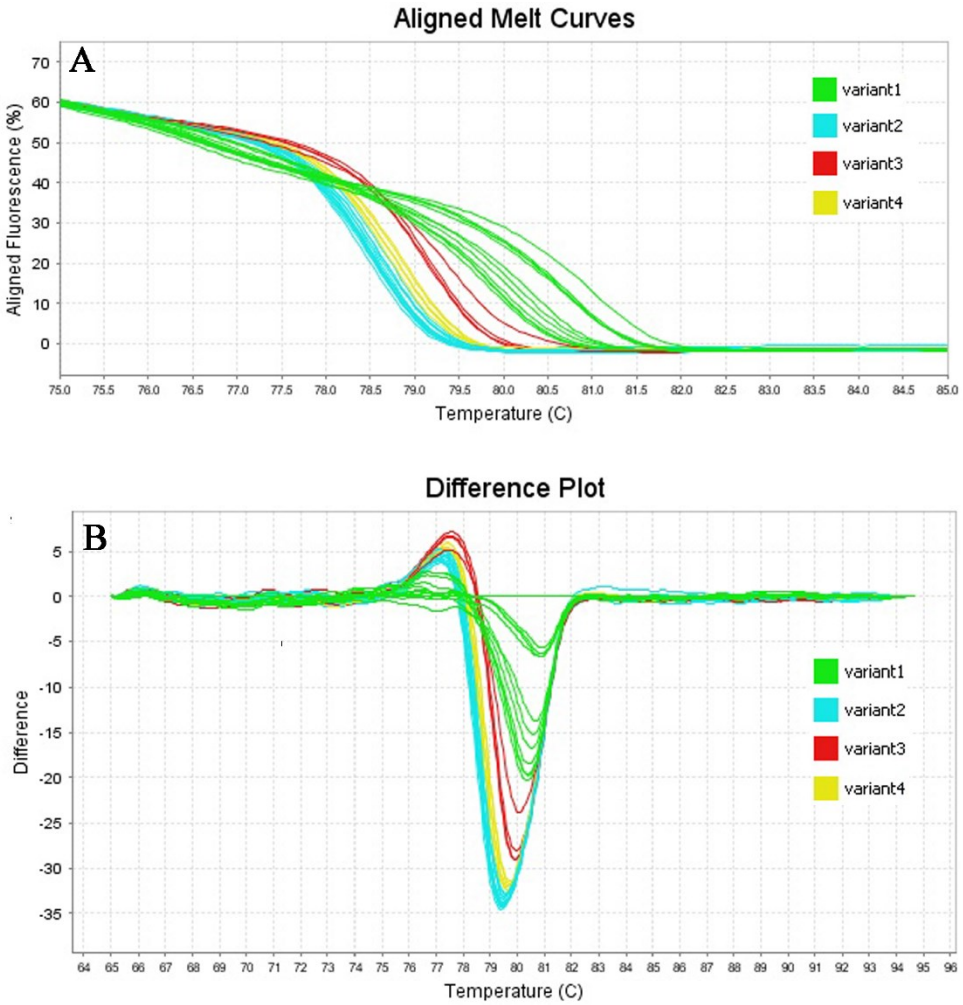

Figure 4 High resolution melting analysis of the 150bp *spxB* DNA fragments amplified by real-time PCR from the 74 *Lb. casei* group strains (Table 1). The aligned melt curves (A) and the difference plots (B) reported the four variants identified. Variant 1 (green) indicates *Lb. rhamnosus* strains; variant 2 (blue) indicates *Lb. casei*; variants 3 and 4 (red and yellow) indicate *Lb. paracasei* strains.

According to the melting temperatures, it was possible to identify the metabolically active community encoding this gene. On the basis of the melting profiles of the obtained fragments, we can assume that the *spxB* gene in the 6-month-old Parmigiano Reggiano was primarily related to *Lb. paracasei*, with a Tm of 79.1 °C (variant 3) (Fig. 5A), whereas the 24-month-old Parmigiano Reggiano showed the presence of a melting curve related to *Lb. rhamnosus*, with a Tm of 80.1 °C (variant 1) (Fig. 5B). In

addition, the relative expression analysis in Fig. 5C showed that the presence of the expressed *spxB* gene in 6-month-old Parmigiano Reggiano was four-fold higher than in the raw milk samples and the 24-month-old Parmigiano Reggiano (Fig. 5D). To our knowledge, this is the first evidence of the expression of the *spxB* gene in cheese and its functionality in this ecosystem.

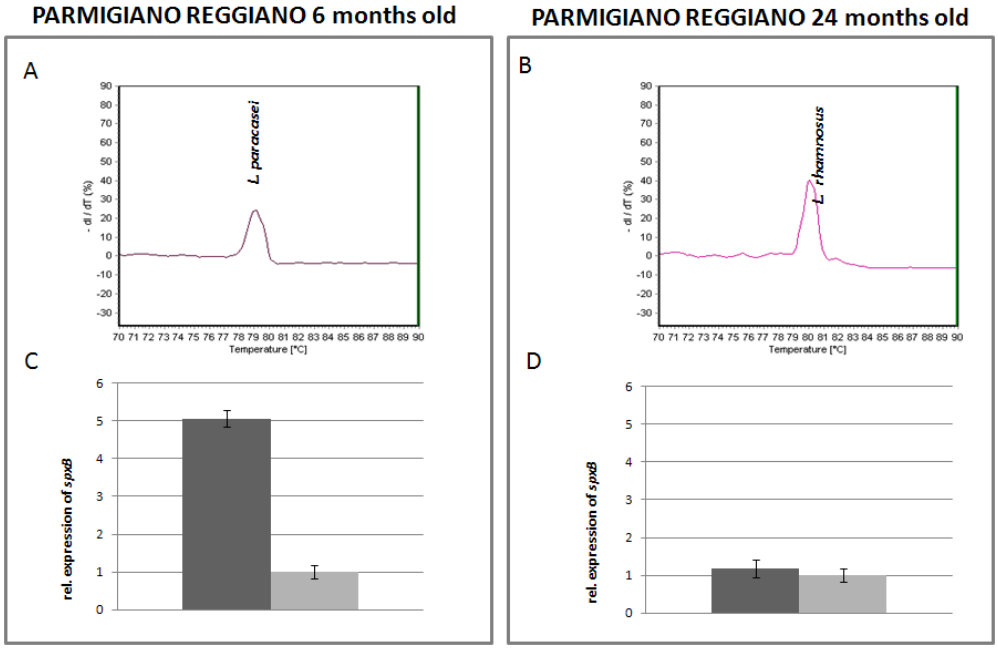

Figure 5 Melting profiles of cDNA-derived PCR amplicons from 6- (A) and 24- (B) month-old Parmigiano Reggiano cheese. Relative quantification of the *spxB* expression in 6- (C) and 24- (D) month-old Parmigiano Reggiano cheese (dark) and in their raw milk starting samples (grey).

Conclusions

Through this study, we demonstrated that *spxB* is a metabolic gene of ecological interest. Indeed, not only it is widely shared in the genome of *Lb. casei* group species, favoring its suggested potential role in the adaptation of these species to different ecological niches, but it also shows an appreciable sequence variability.

Furthermore, whereas previously this was only a hypothesis (Lazzi *et al.* 2014), the *spxB* functionality in cheese was shown for the first time by relative quantification by real-time RT-PCR, and by exploiting the

heterogeneity between the *Lb. casei* group species, we identified by HRM the bacterial communities encoding the *spxB* gene in this ecosystem. As this study confirms the expression of *spxB* in long-ripened cheese, further studies will aim to learn more about the specific function of the gene in microbial growth during ripening and to monitor the functionality of the bacterial community involved in different stages of ripening.

Acknowledgments

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Chapter 3

Metabolic gene-targeted monitoring of non starter lactic acid bacteria during cheese ripening

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Abstract

Long ripened cheeses, such as Grana Padano (GP), a Protected Designation of Origin (PDO) Italian cheese, still harbour a viable microbiota at long ripening ages, mainly composed of non starter lactic acid bacteria (LAB), which contribute to the final characteristics of cheese.

The non starter LAB species *Lactobacillus rhamnosus*, *Lb. casei* and *Lb. paracasei* are frequently found in GP, and form a closely related taxonomic group (*Lb. casei* group), making it difficult to distinguish the three species through 16S rRNA sequencing. *SpxB*, a metabolic gene coding for pyruvate oxidase in *Lb. casei* group, was recently used to distinguish the species within this bacterial group, both in pure cultures and in cheese, where it could provide an alternative energy source through the conversion of pyruvate to acetate.

The aim of this work was to study the evolution of the metabolically active microbiota during different stages of GP ripening, targeting 16S rRNA to describe the whole microbiota composition, and *spxB* gene to monitor the biodiversity within the *Lb. casei* group. Furthermore, activation of pyruvate oxidase pathway was measured directly in cheese by reverse transcription real time PCR (RT –qPCR).

The results showed that *Lb. casei* group dominates throughout the ripening and high-throughput sequencing of *spxB* allowed to identify 4 clusters inside the *Lb. casei* group. The dynamics of the sequence types forming the clusters were followed during ripening. Pyruvate oxidase pathway was expressed in cheese, showing a decreasing trend over ripening time. This work highlights how the composition of the microbiota in the early manufacturing stages influences the microbial dynamics throughout ripening, and how targeting of a metabolic gene can provide an insight into the activity of strains relevant for dairy products.

Introduction

Cheese is a biochemically dynamic product and significant microbial and biochemical changes occur during its ripening. A ripening period of 24 months or longer is common for different Italian and European cheeses. Despite the long ripening time, these cheeses harbour a viable microbiota at the end of ripening, mainly consisting of non starter lactic acid bacteria (LAB). Non starter LAB can arise from raw milk and dairy environment (Montel et al., 2014), they do not contribute to acid production during cheese-making, but they can strongly influence the biochemistry of cheese maturation, contributing to the final sensorial properties (Beresford et al., 2001; De Filippis et al., 2016a; Settanni and Moschetti, 2010).

The evolution of the microbiota during GP manufacturing has been extensively studied (Alessandria et al., 2016; De Filippis et al., 2014; Neviani et al., 2013; Santarelli et al., 2013). Among non starter LAB, *Lactobacillus rhamnosus*, *Lb. casei* and *Lb. paracasei*, commonly referred to as *Lb. casei* group, were frequently found in ripened cheeses and they usually become the dominant bacterial population during ripening of extra-hard, cooked Italian Protected Designation of Origin (PDO) cheese, such as Grana Padano (GP) (Gatti et al., 2014). The development of non starter microbiota during ripening has been attributed to their ability to use nutrient sources available in lactose-free ripened cheese, where milk components modified by technological treatments (rennet addition and curd cooking), starter LAB metabolites and cell lysis products may become a substrate for their growth. Recently, the knowledge about *Lb. rhamnosus* growth in cheese has been deepened through proteomics (Bove et al., 2012) and transcriptomic (Lazzi et al., 2014). The growth of this microorganism in a cheese-like medium (Neviani et al., 2009) causes a metabolic shift towards acetate production. In particular, an upregulation of the *spxB* gene, coding for pyruvate oxidase (POX) was observed (Lazzi et al., 2014). POX catalyses the oxidation of piruvate to acetyl-phosphate, which is then converted to acetate with the production of ATP from an acetate kinase (ACK). The increased expression of these enzymes suggests a potential role of this pathway in non starter LAB growth during cheese ripening.

Furthermore, a recent study has proposed the use of *spxB* coding sequence as a target to distinguish the species belonging to *Lb. casei* group (Savo

Sardaro et al., 2016), since their identification is often controversial due to their close phylogenetic relationships (Felis and Dellaglio, 2007). Culture-independent approaches based on high-throughput sequencing (HTS) allows an in-depth study of the microbial diversity, potentially revealing also subdominant microbial species (De Filippis et al., 2016b; Ercolini, 2013). Nevertheless, 16S rRNA sequencing does not allow the discrimination within *Lb. casei* group, due to the high similarity in 16S sequence. The sequencing-based monitoring of the microbiota beyond the species level was recently proposed, selecting key genes with significant intra-species heterogeneity such as the *lacZ* and *serB* genes of *Streptococcus thermophilus* (De Filippis et al., 2014; Ricciardi et al., 2016).

In the current study, HTS was used to study the evolution of the metabolically active microbiota during different stages of GP ripening, targeting 16S rRNA to describe the whole microbiota composition, and *spxB* gene to monitor the biodiversity within *Lb. casei* group. Furthermore, activation of POX-ACK pathway was measured directly in cheese by reverse transcription real time PCR (RT –qPCR).

Materials and Methods

Sampling

PDO Grana Padano cheese was manufactured with raw cows' milk, using natural whey starter, according to the usual manufacturing technique (EU regulation OJ L 160, 18.6.2011, p. 65–70), at Caseificio San Vitale (Brescia, Italy). Cheese manufacturing was performed with two different batches of milk, leading to the production of two cheese-making, hereafter referred to as CM1 (cheese-making 1) and CM2 (cheese-making 2). Cheese samples were collected after 2, 6 and 12 months of ripening, immediately grated, and stored at –80 °C.

RNA extraction and cDNA synthesis

RNA was extracted from Grana Padano cheese by modifying a protocol described in a previous work (Monnet et al., 2008). In brief, 150 mg of grated cheese were placed in a 2 ml screw cap tube containing 500 µl of zirconia/glass beads (diameter, 0.1 mm; BioSpec Products, Bartlesville, USA) and immediately added with 1.5 ml of TRIzol (Invitrogen, Milano, Italy). The tubes were processed with a Mini-BeadBeater 8 (BioSpec Products, Bartlesville, USA) by using three 60-s mixing sequences at

maximum speed, spaced out with 60-s pauses at room temperature. The homogenized sample was then processed following the TRIzol manufacturer's instruction.

RNA was quantified spectrophotometrically, and the integrity was evaluated by denaturing agarose gel electrophoresis. Reverse transcription was performed with QuantiTect Reverse Transcription Kit (Qiagen, Milan, Italy) using random hexamer primers and according to the manufacturer instructions.

16S rRNA gene amplicon library preparation and sequencing

The microbial diversity was studied by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene using primers and PCR conditions previously described (Ercolini et al., 2012). 454-adaptors were included in the forward primer followed by a 10 bp sample-specific Multiplex Identifier (MID). PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy), quantified using the QuantiFluorTM (Promega, Milano, Italy) and an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) by using the GS Junior + emPCR and Sequencing kits (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions.

SpxB gene amplicon library preparation and sequencing

In order to prepare amplicon libraries for *Lb. casei* group *spxB* gene sequencing, the 454 Universal Tailed Amplicon protocol was used with a double PCR step (454 Sequencing System – Guidelines for Amplicon Experimental Design). Only 5 libraries were successfully prepared from the 6 samples. A sequence of 684 bp of the coding region of *spxB* was amplified using the primers Pox_FW 5'-AGACGCAATGATCAAGGTG**Y**T and Pox_RV 5'- GTGATGATCGG**R**ATATGCGTT. The universal primers M13f 5'-TGTAAAACGACGGCCAGT and M13r 5'-CAGGAAACAGCTATGAC were included at 5' and 3' ends of the POX primers (Daigle et al., 2011). Each PCR mixture (final volume, 50 µl) contained 100 ng of template cDNA, 0.1 µmol/L of each primer, 0.50 mmol of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, 5 µl of 10X PCR buffer and 2.5 U of native Taq polymerase (Invitrogen, Milano, Italy). The following PCR conditions were used: 95°C for 3 min, followed by 40 cycles at 94°C for 45s, 57°C for 45 s, 72°C for 1 min. A final extension was carried

out at 72°C for 5 min. Twenty ng of the amplicon were used as template in a second PCR step, where primers M13f and M13r were used, with the addition of 454-adaptors and a 10 bp sample-specific Multiplex Identifier (MID). The PCR mixture was prepared as above described and the PCR conditions were modified as follows: 94°C for 5 min, 30 cycles at 94°C for 1 min, 50°C for 1 min, 72 °C for 2 min, and a final extention of 72°C for 7 min, as previously described (De Filippis et al., 2014). PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy) and then quantified using the QuantiFluorTM (Promega, Milano, Italy). An equimolar pool of amplicons was prepared and it was used for pyrosequencing as described above.

Detection of spxB expression from cheeses

cDNA was also used for qPCR in a Mastercycler ® ep realplex S instrument (Eppendorf). In brief, 1 µl of of cDNA template was used in each reaction, using Power SYBR® Green PCR Master Mix (Applied Biosystems, Milano, Italy) and 5 µM of each primer, according to the target to detect, in 15 µl final volume. The primers used were different from the ones used in HTS library preparation: a universal primer pair was used to detect a 130 bp fragment of 16S rRNA gene (Denman and McSweeney, 2006), and *Lb. casei* group specific primers were used to detect *spxB* gene (Savo Sardaro et al., 2016). A third primer pair was designed, targeting a 227 bp fragment of *ackA* gene, and tested for specificity against members of the *Lb. casei* group (data not shown). Details about the primer pairs used for RT qPCR can be found in Table 1. The following cycler temperatures were set for all the primer pairs: 2 min at 50°C, 10 min at 95°C, 40 repeats at 95°C for 15 s, and 60°C for 1 min, and finally a melting step with an initial step of 95°C for 15 s, followed by a ramp from 60°C to 90°C, with fluorescence detection in function of temperature increase for the comparison of melting curve profile. Samples were assayed in triplicate, and no-template control were included for each primer pair. Amplification efficiency for each primer pair was calculated from the slope of standard curves generated with two-fold serial dilutions of the same cDNA sample, as $E= 10^{(-1/slope)}$.

No significant variations in 16S rRNA abundance was observed in samples from CM1 and CM2 (the amount of cDNA in each reaction was standardized), thus it was used as reference gene in relative quantification. Relative expression of the target genes was determined

using the $\Delta\Delta C_t$ method, after Pfaffl correction (Pfaffl, 2001). Statistical analysis was performed with SPSS v.23, using ANOVA followed by Dunnett's test.

Table 1 Primer pairs used for RT-qPCR. Degenerated bases are indicated in bold, the code is standard IUPAC.

Gene	Primer pair	Sequence (5' --> 3')	Amplicon(bp)	Correlation(R^2)	PCR eff.
spxB	poxcDNAfw	CAGACGCAATGATCAAGGTG	151	-0.993	1.82
	poxPromRv	AATGCGCC**Y**ACTTCTTCATG			
ackA	AckAfw	GCAGAAGGCGC**R**ACTGA**Y**ATCCT	183	-0.99	1.84
	AckArv	GCGATGRCC**R**ATGCCGGTAATTTCA			
16S	TBAfw	CGGCAACGAGCGCAACCC	130	-0.999	2.04
	TBArv	CCATTGTAGCACGTGTGTAGCC			

Bioinformatics and data analysis

16S rRNA data were analysed by using QIIME 1.8.0 software (Caporaso et al., 2010). After the quality filtering, raw reads were excluded from the analysis if they had an average quality score lower than 25, if they were shorter than 300 bp and if there were ambiguous base calls. OTU defined by a 99% of similarity were clustered by using the UCLUST method (Edgar, 2010), clusters with less than 10 sequences were removed and taxonomic assignment of representative sequences was achieved by using the Greengenes 16S rRNA gene database (McDonald et al., 2012). In order to improve taxonomic assignment within *Lactobacillus* genus, sequences belonging to OTUs identified as *Lactobacillus* were double-checked using the BLAST (BLASTn) search program (http://www.ncbi.nlm.nih.gov/blast/).

For the *spxB* gene reads, the analysis pipeline carried out was the following: demultiplexed sequences that passed the quality filters (sequences shorter than 600 bp, with mean qual score <25, with forward or reverse primer mismatches or ambiguous base calls were discarded)

were clustered at 100% of similarity using the UCLUST method (Edgar, 2010). Clusters with less than 10 sequences were discarded and the longest sequence of each cluster was picked as representative. The representative sequences were aligned to the *spxB* sequences belonging to *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* sequences retrieved from databases (Table 2) by using MEGA 7.0.20 software (Kumar et al., 2016), manually checked in order to confirm mutations detected and corrected. After alignment, a phylogenetic tree was built using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. Those clusters represented by sequences characterized by less than 99.5% of similarity were merged in order to achieve the relative abundance of each *spxB* sequence types.

Multiple sequence alignment of the translated *spxB* primary sequence were performed on MEGA 7.0.20, and compared with publicly available data sets using CDD database from NCBI. Heatplot was built in R environment (http://www.r-project.org), by using the *heatplot* function in *made4* package.

Table 2 List of type strains used for phylogenetic analysis of the *spxB* gene.

Species	Strain	Origin	Accession number
Lactobacillus rhamnosus	ATCC 53103	Human faeces	FM179322
	LOCK900	Human faeces	CP005484
	LOCK908	Human faeces	CP005485
	ATCC8530	Human gastrointestinal tract	CP003094
	Lc 705	Human gastrointestinal tract	FM179323
Lactobacillus paracasei	N1115	Chinese fermented milk	CP007122
	ATCC 334	Emmental cheese	CP000423
	8700:2	Human gastrointestinal tract	CP002391
Lactobacillus casei	ATCC 393	Dairy product	AP012544

LC2W	Chinese dairy product	CP002616
BL23	Dairy	FM177140
LOCK919	Human faeces	CP005486
Zhang	Koumiss	CP001084
W56	Human	NC_018641
BD-II	Koumiss	CP002618
12A	Corn silage	CP006690

Nucleotide sequence accession number

16S rRNA and *spxB* sequence data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (accession number SRP063447).

Results

16S rRNA gene pyrosequencing

The metabolically active microbiota of two different Grana Padano cheese-making was studied through pyrosequencing of 16S rRNA over a ripening time ranging from 2 to 12 months. A total of 45,783 16S rRNA reads were obtained after the quality filtering, with an average of 7,630 sequences/sample and an average length of 516 bp. The calculated Good's estimated sample coverage (ESC), the ChaoI and Shannon indices are reported in Table S1, together with the number of reads obtained for each sample after quality filtering and the number of OTUs. Rarefaction analysis showed a satisfactory coverage, with ESC close to or above 90% for all the samples. This result is in agreement with the reported alpha metrics, such as Chao1 index, which suggests that the samples present a high number of singletons, despite the low value of Shannon index indicates that the communities are dominated by few species (Table S1).

As expected, *Firmicutes* largely prevailed in all the samples, while the phylum *Actinobacteria* and *Proteobacteria* were on average 0.12% and 0.07% of the microbial population, respectively. The bacterial classes observed in the samples were: *Actinobacteria*, *Bacilli*, *Clostridia*, *Alphaproteobacteria* and *Gammaproteobacteria*, as shown on the leftmost bar of the heatplot (Fig.1).

Eighty OTUs were identified, and their abundance and distribution among the samples neatly distinguished samples belonging to CM1 and to CM2 (Fig. 1). For instance, CM2 presented a distinctive contamination of *Clostridiaceae*, with an initial level of 0.7%, rising up to 3.6 % after 12 months of ripening, while *Clostridiaceae* reached 1.1% in CM1 after 12 months of ripening.

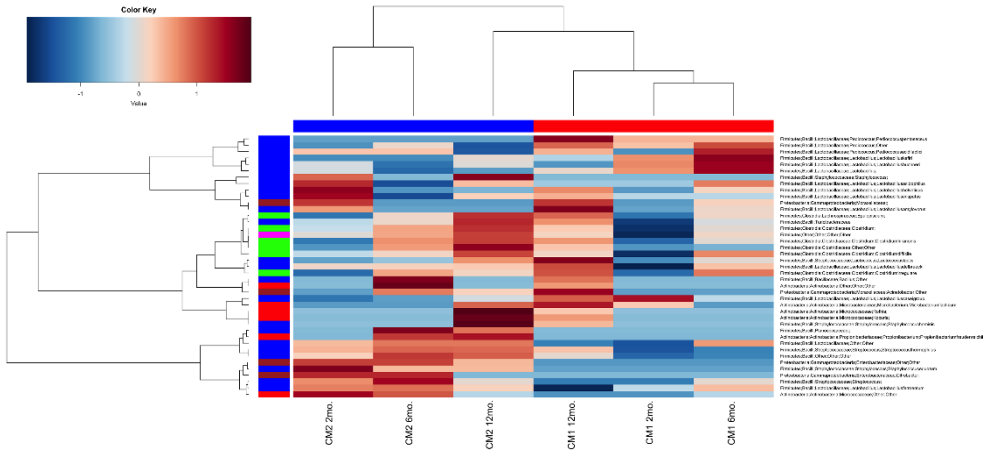

Figure 1 Hierarchical average-linkage clustering of the samples based on the Pearson's correlation coefficient of the abundance of bacterial OTUs present in at least 20% of samples. The colour scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance. Column bar is coloured according to the cheese-makings (red: first; blue: second); row bar is coloured according the bacterial class (red: Actinobacteria; blue: Bacilli; green: Clostridia; brown: Gammaproteobacteria; magenta: other).

Among the 80 OTUs, only 6 showed a relative abundance higher than 1% in both cheese-makings (Fig. 2) and *Lb. casei* group represented on average 51% of the bacterial population, showing fluctuation connected to the ripening age of the cheese. The metabolically active microbiota included *Lb. fermentum* (23% on average) *Lb. helveticus* (6%), *Lb. delbrueckii* (5%), *S. thermophilus* (5%) and *Lb. buchneri* (2%). All of the above mentioned species were significantly different between the two cheese-making ($p<0.05$). Moreover, the two most abundant groups, *Lb. casei* and *Lb. fermentum* groups, showed a strong negative correlation (Spearman's correlation coefficient = - 0.886, $p< 0.01$), which is particularly evident in samples belonging to CM2.

Lb. casei group abundance in CM2 was 37% in GP at 2 months, and showed an increasing trend during ripening (fig. 2, 40% at 6 mo., 49% at 12 mo.). In this cheese-making, higher amount of metabolically active *Lb. fermentum* was also found (34% at 2 mo.), and it still represented 23% of the population in GP from CM2 at 12 mo. Conversely, the metabolically active *Lb. casei* group in CM1 represented 70% of the microbiota at 2 mo. of ripening, while decreasing thereafter (Fig. 2, 47% at 6 mo. and 63% at 12 mo.). In this cheese-making *Lb. fermentum* initial presence was far lower, and never rose over 25% of the total population, decreasing to about 6%.

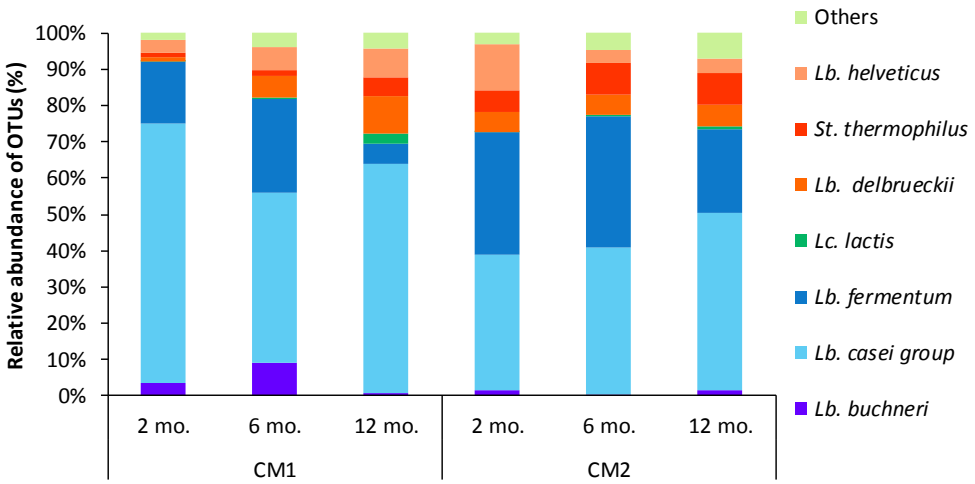

Figure 2 Incidence of OTUs based on 16S rRNA gene pyrosequencing at different ripening times. The bar graph describes the relative abundance of each species for CM1and CM2 at different sampling times (2, 6 and 12 months). OTUs with abundance lower than 1% in all the samples were included in the category "Others".

Twenty sub-dominant OTUs were observed with an abundance higher than 0.1% in both the cheese-makings. Subdominant species belonging to the family of *Lactobacillacee*, such as *Lb. acidophilus* and *Pediococcus acidilactici*, were identified during ripening. Unexpectedly, within *Streptococcacee* family, *Lactococcus lactis* was found in all the samples: with a low abundance in 2 mo. old cheese (0.07% in CM1, and 0.09% in CM2), but increased in 12 mo. old cheese (2% in CM1 and 0.7 % in CM2).

Finally, the genus *Kocuria*, belonging to the *Micrococcaceae* family, was present at 12 mo. of ripening, with a level of 0.01% in CM1 and 0.04% in CM2. It is interesting to notice that this genus has only been detected with culture-independent techniques in cheese (Gatti et al., 2008), probably due to its difficult cultivability.

SpxB gene pyrosequencing

Lb. casei group represented the prevalent metabolically active group during ripening. Nevertheless, 16S rRNA sequencing did not allow to distinguish between the closely related *Lb. casei*, *Lb. rhamnosus* and *Lb. paracasei*. Therefore, *spxB*-targeted pyrosequencing was implemented. A total of 17,551 reads were obtained after quality filtering, with an average number of 3,510 reads/sample and an average length of 691 bp.

One hundred forty-five *spxB* sequence types were aligned with previously published sequences (Table1) and a phylogenetic tree was built (data not shown). Using a similarity cut-off of 99.5%, 75 *spxB* sequence types were identified, whose phylogenetic relationships were reconstructed by a UPGMA tree (Fig. 3).

Multiple sequence alignment of a 642 bp fragment of *spxB* coding sequence from 16 database deposited sequences (Table 1) identified 137 parsimony informative sites in this portion of the sequence, or nucleotide positions which showed substitutions in at least two sites (data not shown)

When the 75 *spxB* sequence types obtained by pyrosequencing were introduced into the alignment, the parsimony informative sites rose to 204. Among these substitutions, 37 were shared only with *Lb. casei* sp. *casei* ATCC393. This strain was recently renamed from *Lb. paracasei* to *Lb. casei* sp. casei, after a genome-wide comparison with other strains belonging to *Lb. casei* group (Toh et al., 2013) and, remarkably, it was also isolated from cheese environment.

When nucleotide sequences were translated to amino acidic sequences, the informative sites dropped to 38, highlighting that most of the nucleotide substitutions led to silent mutation, with no effect on the primary sequence of the protein. Furthermore, there was only one position consistently shared with the primary sequence of *Lb. casei* sp. *casei* ATCC393 (see alignment in Figure S2).

All the substitutions regarded coil or loop sites of the secondary protein structure, which should not affect either the functionality, nor the capability to form dimers and tetramers in the quaternary structure, as was confirmed with a search in the CDD database (Conserved Domain Database, https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, see alignment in Figure S3).

The tree based on the 75 *spxB* sequence types allowed to group the sequences into four clusters, respectively named R1, R2, PC1 and PC2 depending on the species of the type strains that clustered together with the sequences (Fig. 3).

Two of the clusters showed higher similarity to *spxB* sequences of *Lb. rhamnosus* (R1-1 to 31 and R2-1 to 16), a third cluster grouped together with *Lb. casei* sp. *casei* ATCC393 (PC1-1 to 16), and the last group included sequence types of both *Lb. casei* and *Lb. paracasei* (PC2-1 to 12), one sequence was removed from the UPGMA tree) (Fig. 3). The distribution of these clusters clearly showed that sequences belonging to PC-2 dominated in both the cheese-making, and that the diversity in sequence types increased during the ripening time (Fig. 4A).

Among the 76 sequence types, thirty-one reached an abundance higher than 1% in at least one sample, but only 9 were present at > 5% abundance (Fig. 4B). Only one sequence type belonging to the *Lb. casei*/*paracasei* group (PC2-5) was present in all the samples. It was present at low levels in CM1 at the beginning of ripening (1.4%), and it increased during ripening (58% at 2 mo. and 45% at 12 mo.), while it showed an opposite trend in CM2 (33% at 2 mo., and 1% at 6 mo.).

Notably, each sample appeared to be dominated by one or few sequence types, and their abundance dynamically changed during ripening. In CM1, PC2-9 dominated at 2 mo. (87.8%), then PC2-5 increased at 6 mo., and still prevailed at 12 mo., despite a rise of R1-1. In CM2, 2 mo. sample was dominated by R1-9 and PC2-5 (31 and 33%, respectively), while PC2-11 was the prevalent type at 6 mo. of ripening, although R1-9 (25.9%) was still present.

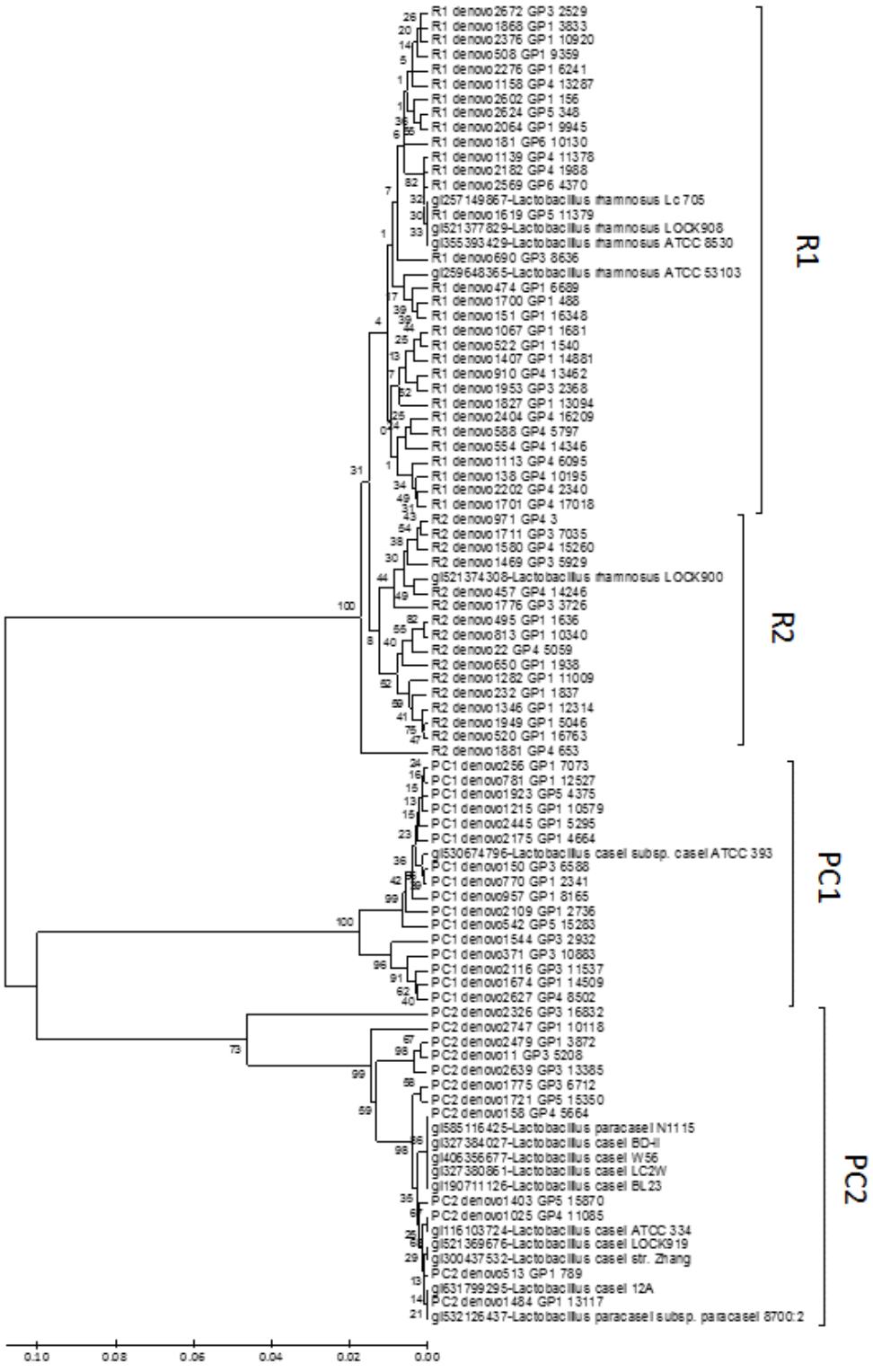
R1 denovo2672 GP3 2529
R1 denovo1868 GP1 3833
R1 denovo2376 GP1 10920
R1 denovo508 GP1 9359
R1 denovo2276 GP1 6241
R1 denovo1158 GP4 13287
R1 denovo2602 GP1 156
R1 denovo2624 GP5 348
R1 denovo2064 GP1 9945
R1 denovo181 GP6 10130
R1 denovo1139 GP4 11378
R1 denovo2182 GP4 1988
R1 denovo2569 GP6 4370
gi|257149867-Lactobacillus rhamnosus Lc 705
R1 denovo1619 GP5 11379
gi|521377829-Lactobacillus rhamnosus LOCK908
gi|355393429-Lactobacillus rhamnosus ATCC 8530
R1 denovo690 GP3 8636
gi|259648365-Lactobacillus rhamnosus ATCC 53103
R1 denovo474 GP1 6689
R1 denovo1700 GP1 488
R1 denovo151 GP1 16348
R1 denovo1067 GP1 1681
R1 denovo522 GP1 1540
R1 denovo1407 GP1 14881
R1 denovo910 GP4 13462
R1 denovo1953 GP3 2368
R1 denovo1827 GP1 13094
R1 denovo2404 GP4 16209
R1 denovo588 GP4 5797
R1 denovo554 GP4 14346
R1 denovo1113 GP4 6095
R1 denovo138 GP4 10195
R1 denovo2202 GP4 2340
R1 denovo1701 GP4 17018
R2 denovo971 GP4 3
R2 denovo1711 GP3 7035
R2 denovo1580 GP4 15260
R2 denovo1469 GP3 5929
gi|521374308-Lactobacillus rhamnosus LOCK900
R2 denovo457 GP4 14246
R2 denovo1776 GP3 3726
R2 denovo495 GP1 1636
R2 denovo813 GP1 10340
R2 denovo22 GP4 5059
R2 denovo650 GP1 1938
R2 denovo1282 GP1 11009
R2 denovo232 GP1 1837
R2 denovo1346 GP1 12314
R2 denovo1949 GP1 5046
R2 denovo520 GP1 16763
R2 denovo1881 GP4 653
PC1 denovo256 GP1 7073
PC1 denovo781 GP1 12527
PC1 denovo1923 GP5 4375
PC1 denovo1215 GP1 10579
PC1 denovo2445 GP1 5295
PC1 denovo2175 GP1 4664
gi|530674796-Lactobacillus casei subsp. casei ATCC 393
PC1 denovo150 GP3 6568
PC1 denovo770 GP1 2341
PC1 denovo957 GP1 8165
PC1 denovo2109 GP1 2736
PC1 denovo542 GP5 15283
PC1 denovo1544 GP3 2932
PC1 denovo371 GP3 10883
PC1 denovo2116 GP3 11537
PC1 denovo1674 GP1 14509
PC1 denovo2627 GP4 8502
PC2 denovo2326 GP3 16832
PC2 denovo2747 GP1 10118
PC2 denovo2479 GP1 3872
PC2 denovo11 GP3 5208
PC2 denovo2639 GP3 13385
PC2 denovo1775 GP3 6712
PC2 denovo1721 GP5 15350
PC2 denovo158 GP4 5664
gi|565116425-Lactobacillus paracasei N1115
gi|327384027-Lactobacillus casei BD-II
gi|406356677-Lactobacillus casei W56
gi|327380861-Lactobacillus casei LC2W
gi|190711126-Lactobacillus casei BL23
PC2 denovo1403 GP5 15870
PC2 denovo1025 GP4 11085
gi|116103724-Lactobacillus casei ATCC 334
gi|521369676-Lactobacillus casei LOCK919
gi|300437532-Lactobacillus casei str. Zhang
PC2 denovo513 GP1 789
gi|631799295-Lactobacillus casei 12A
PC2 denovo1484 GP1 13117
gi|532126437-Lactobacillus paracasei subsp. paracasei 8700:2
R1
R2
PC1
PC2
0.10
0.08
0.06
0.04
0.02
0.00

Figure 3 (on the left) Phylogenetic analysis of *spxB* gene. Partial sequences were obtained either from HTS (75 sequences, this study) or from public databases (16 sequences). The evolutionary history was inferred using the UPGMA method. The clusters identified correspond to *Lb. rhamnosus* 1 and 2 (LR1 and LR2), and *Lb. paracasei/casei* 1 and 2 (PC1 and PC2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated.

RT-qPCR of spxB gene in cheese

Relative expression ratios of *spxB* gene in cheese are presented in Figure 5, together with the data for *ackA* gene, a gene involved in the POX pathway, responsible for ATP synthesis at the end of the metabolic pathway.

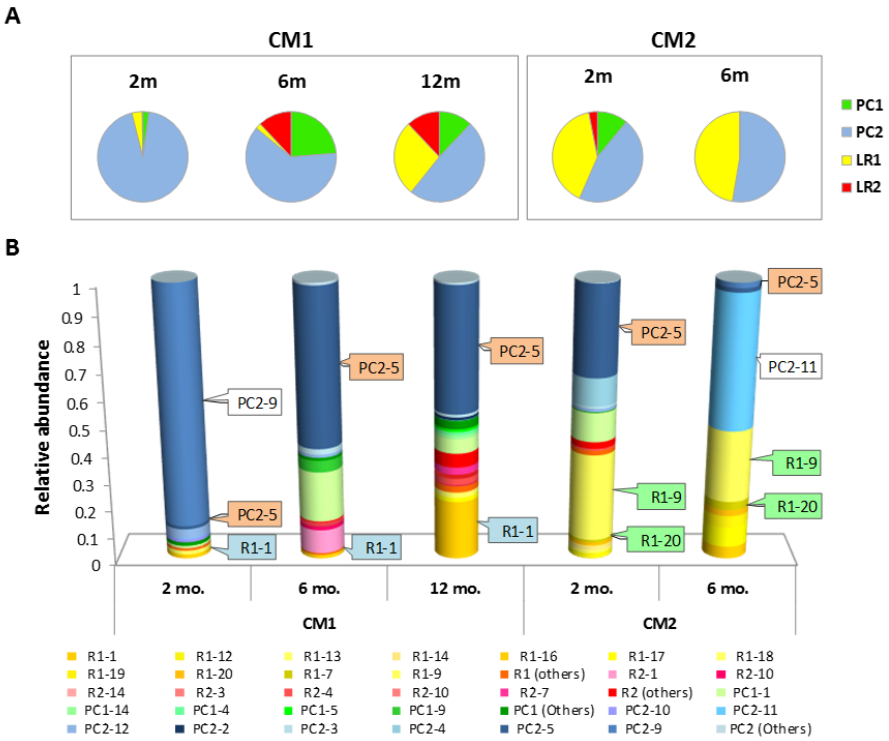

Figure 4 *spxB* sequence types distribution in cheese samples. **A** Distribution of the four *Lb. casei* group clusters in the cheese samples; **B** Relative abundance of *spxB* sequence types in cheese samples. Sequence types with relative abundance lower than 1% in all the samples were grouped in the "others" category of the corresponding cluster.

The expression was normalized to the sample with the lowest *spxB* expression, which in both the cheese-makings was represented by the 12 mo. old cheese sample, using 16S rRNA as housekeeping gene. Both the genes are expressed during the whole ripening time, with a decreasing expression level during ripening.

SpxB and *ackA* genes were overexpressed in 2 mo. old samples of CM1 ($p<0.001$), of about 7-fold each, while at 6 months of ripening only *spxB* was 3.5-folds overexpressed ($p<0.001$). On the contrary, *ackA* had an expression level comparable to the control condition (12 mo. old samples). CM2 presented a similar trend, where *spxB* was 8-folds and *ackA* 2.7 folds overexpressed at 2 mo. ($p< 0.001$), while at 6 months only *spxB* was overexpressed ($p<0.001$), compared to the 12 mo. old sample.

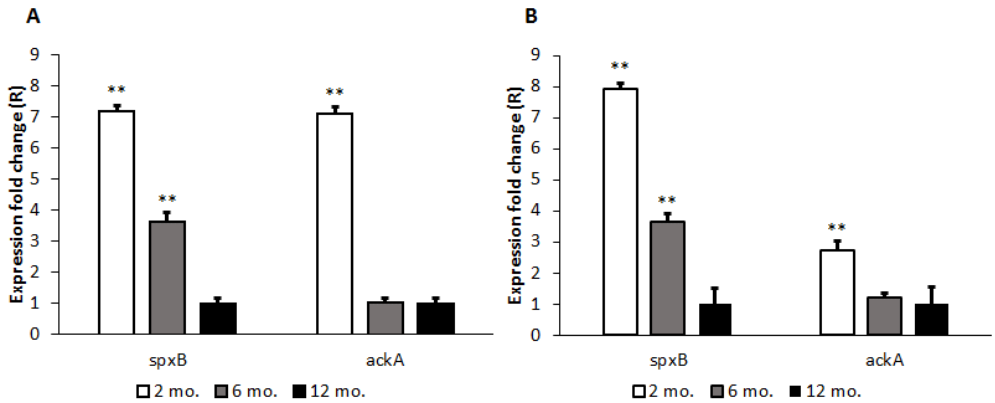

Figure 5 *spxB* and *ackA* relative gene expression in cheese, measured for CM1 (**A**) and CM2 (**B**), error bars represent the standard deviation of expression ratio, asterisks represent significantly different data (**= $p \leq 0.001$).

The main difference between the two cheese-makings was observed at the beginning of the ripening: *ackA* relative expression in CM2 was lower than *spxB* in the sample at 2 mo. The imbalance in the expression ratios between the two genes would require further studies to define the physiological role of the enzymes involved in POX-ACK pathway.

Discussion

This study aims to give a deeper insight into the overall bacterial evolution during ripening of Grana Padano cheese and, furthermore, to

evaluate the efficacy of *spxB* gene as target in sequencing-based monitoring of *Lb. casei* group.

HTS was recently used to investigate the microbial dynamics of different cheeses and dairy processes (Alessandria et al., 2016; Dolci et al., 2014; Guidone et al., 2016; Parente et al., 2016) and proved to be a powerful tool to investigate population dynamics (De Filippis et al., 2016b; Ercolini, 2013), or food spoilage processes (Bassi et al., 2015; Stellato et al., 2015).

In this study, GP samples belonging to two independent cheese-making were collected from 2 to 12 months of ripening.

Eighty OTUs were identified by 16S rRNA sequencing, in good agreement with previous results (De Filippis, 2014). The distribution of these OTUs drives the clustering of samples belonging to the two cheese-making, that differed for the abundance of species belonging to the family of *Lactobacillaceae*, except for *S. thermophilus* ($P<0.05$). These differences may arise from the different batches of milk used for the production of CM1 and CM2, leading to diversified microbial balances in the first ripening steps. At the first sampling point, with 2 months ripening, we can see that the active population composition is quite dissimilar: CM2 presented a higher amount of *Lb. fermentum* (over 30%), while CM1 was dominated by *Lb. casei* group (over 70%). The presence of *Lb. fermentum* in natural whey starter (NWS) for the production of long ripened, hard cooked cheeses was previously reported, especially in GP (Bottari et al., 2013; De Filippis et al., 2014, Alessandria et al., 2016). In this study, we highlight that *Lb. fermentum* is able to survive throughout the ripening, and a higher concentration in the first ripening steps affects the amount that is metabolically active at a more advanced stage of ripening.

Moreover, *Lb. fermentum* and *Lb. casei* group might be competing for the same substrates, since there is a strong negative correlation between these two species (Spearman's correlation coefficient = - 0.886, $p< 0.01$), more evident in samples belonging to CM2.

CM2 also shows a distinctive contamination of Clostridia, responsible for butyric contamination and late blowing (Bassi, 2014), despite the cheeses used in this study did not develop any defect. Relative abundance of Clostridia increases during CM1 ripening as well, but to a lower extent. Moreover, CM2 also shows lower abundance of *Lb. casei* during ripening, highlighting the importance of initial contamination coming from raw

milk, NWS or environment to determine the presence of spoilage microorganisms in the final cheese product (Stellato et al, 2015).

Interestingly, other species commonly indicated as starter lactic acid bacteria, namely *Lb. helveticus*, *Lb. delbrueckii*, *S. thermophilus, Lc. lactis* and, to a minor extent, *Lb. buchneri*, associated to the NWS, were found to be metabolically active until 12 months of ripening. Previous studies already reported that whole cells of *Lb. delbrueckii* spp. and *Lb. helveticus* could be present until 24 months of ripening (Gatti et al., 2008). cDNA sequencing allows to prove that those cells are likely to be metabolically active, even if their role on the overall ripening process is still unknown.

Despite providing many useful information about the population dynamics, 16S rRNA does not allow to distinguish the species within *Lb. casei* group, namely *Lb. rhamnosus*, *Lb. casei* and *Lb. paracasei*, due to their high phylogenetic relatedness (G. Felis & Dellaglio, 2007). Recently, strain monitoring of the starter species *St. thermophilus* in cheese was performed by HTS of metabolic genes (De Filippis et al., 2014; Ricciardi et al., 2016). Savo Sardaro et al. (2016) previously observed that *spxB* gene, coding for a pyruvate oxidase (POX) involved in the pyruvate utilization pathway, has the potential to distinguish among the species within *Lb. casei* group.

The activation of *spxB* metabolism by *Lb. rhamnosus* was observed for the first time in a cheese-mimicking media (Lazzi C., et al. 2014), and its expression was confirmed in Parmigiano Reggiano, another long ripened Italian cheese (Savo Sardaro et al., 2016). *SpxB* gene was chosen in this work as a molecular target for library preparation and pyrosequencing, to study the transcriptionally active fraction of *Lb. casei* group in GP cheese, and its dynamics during ripening.

Phylogenetic analysis of the 75 *spxB* sequence types identified through pyrosequencing, together with the 16 database-retrieved sequences allowed to retrace the evolutionary relationships of this gene in *Lb. casei* group, leading to the identification of 4 clusters, two grouping with sequences from *Lb. rhamnosus* type strains (R1 and R2), a third group clustering with *Lb. casei* sp. *casei* ATCC 393, and a fourth group clustering with sequences belonging to various isolates of *Lb. casei* and *Lb. paracasei*. Due to the debated phylogeny of the latter two species (Acedo-Felix, 2003; Diancourt et al., 2007; Felis et al., 2001; Judicial Commission

of the International Committee on Systematics of Bacteria, 2008), we decided to define the two groups as *Lb. paracasei-casei* 1 and 2, abbreviated to PC1 and PC2, respectively. Sequences attributes PC1 forms a defined cluster with high similarity with *spxB* from *Lb. casei* ATCC 393. A genome-wide study has shown that his strain is distinct from other strains previously described as *Lb. paracasei* (Toh et al., 2013), and this difference is evident also in the phylogenetic reconstruction provided from *spxB* gene.

The evolution of *spxB* sequence types during ripening shows that the more abundant clusters at the beginning of ripening prevail throughout cheese ageing. Furthermore, some sequence types persist throughout the ripening, while other dominate only at specific sampling points. Interestingly, PC2-5 is the only sequence type found in both the cheese-making, and in both of them it accounts for over 30% of *Lb. casei* group sequence types in the first stages of ripening. On the contrary, the only sequence type that reaches a significant relative abundance in the 12 mo. old cheese is PC2-9, suggesting that persistence and adaptation to stress condition might be strain specific.

Indeed, a previous study has demonstrated that *Lb. rhamnosus* strains isolated from an Italian dairy product presented a wide genotypic variability (Bove et al., 2011), as well as a diverse distribution in the different phases of cheese ripening.

While pyrosequencing allows to describe fluctuations in the composition of the bacterial population, the study of expression of metabolic pathways still requires the use of complementary techniques, such as RT qPCR, that was performed on cheese cDNA to investigate POX-ACK pathway activation during ripening. Indeed, pyruvate oxidase expression is higher after 2 and 6 mo. of ripening, in both cheese-making, while the acetate kinase shows different expression levels between CM1 and CM2, but decreases as well during ripening. The activation of POX-*ACKA* pathway leads to the production of ATP, while the conversion of pyruvate to acetate seems to be involved in pH homeostasis (Quatravaux et al., 2006). The overexpression of acetate kinase from *Lb. rhamnosus* when growing under cheese-mimicking conditions was already observed in a proteomic study (Bove et al., 2012), but this is the first time that expression of this gene is reported in cheese. The role of pyruvate oxidase metabolism on bacterial growth was firstly investigated in *Lb. plantarum* (Goffin et al., 2006;

Lorquet et al., 2004), and proved to be activated in the early stationary phase, after glucose depletion. Furthermore, other authors suggested that members of *Lb. casei* group could perform aerobic growth, and that this feature seems to be strain specific (Brooijmans et al., 2009; Zotta et al., 2014). Aerobic growth is generally regarded as a positive technological trait, since it is correlated with higher cell counts and prolonged survival (Pedersen et al., 2012), despite the activation of POX in *Lb. plantarum* was shown to be temperature and oxygen dependent (Zotta et al., 2013). Both the parameters could induce *spxB* activation in GP ripening, which is performed at low temperatures (15-22 °C, Gatti et al., 2014), assuming that oxygen might be introduced in cheese matrix during the curd cutting step. Indeed, overexpression of *spxB* in the first stages of ripening, and its decrease over time, is probably due to the progressive oxygen depletion. A similar trend in the expression of *spxB* was observed in PR cheese by Savo Sardaro et al. (2016), suggesting that POX activity might have a role in the metabolism of *Lb. casei* group, at least in hard cooked cheeses. The observation that respiration competence might positively affect some technological traits of bacteria belonging to the *Lb. casei* group was previously reported (Zotta et al., 2014), and the knowledge of specific sequences connected with respiration metabolism might allow the selection and monitoring of strains throughout the fermentation, gaining a deeper control of the cheese manufacturing process and improving its quality.

Supplementary material

Supplementary figures and tables are listed in Appendix I.

Acknowledgements

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Chapter 4

Effect of oxygen concentration on *Lactobacillus rhamnosus* metabolism in a model

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Manuscript in preparation

Abstract

Lactobacillus rhamnosus is a species of technological interest, particularly for Italian long ripened cooked hard cheese, where this species becomes prevalent during ripening, affecting the development of typical characteristics of the cheese. Ripening curd is a complex environment, where lactose is depleted and growing bacteria need to rely on alternative substrates for energy production, which are still poorly understood. Furthermore, cheese undergoes physiochemical variations during maturing, which direct the activation of bacterial metabolic pathways.

Lb. rhamnosus is capable to activate, in cheese-mimicking condition, a gene encoding for pyruvate oxidase (POX) which might provide an alternative metabolic pathway for ATP production, and expression of the same gene was demonstrated in cheese as well. In this work, a miniaturized model for the manufacturing of Italian cooked hard cheeses was developed, and used to evaluate the response of *Lb. rhamnosus* during ripening under different oxygen concentrations. The different oxygenation conditions taken into exam influenced the bacterial growth dynamics, the production of metabolites, and the volatile organic compounds profile of the miniaturized cheeses.

Despite the model can provide only an approximation of the real cheese system, it represents a valuable tool to assess bacterial response to various technological treatments encountered during manufacturing.

Introduction

Lactobacillus rhamnosus is a species of technological interest, particularly in Italian long ripened cooked hard cheese, where it becomes a prevalent species of the microbiota involved in the maturation of the curd until the end of ripening. The microbiota of Italian cooked hard cheeses is influenced by the bacterial species coming from natural whey starter, still produced using a back-slopping procedure, dominated by the thermophilic species *Lactobacillus helveticus* and *Lactobacillus delbrueckii* sp. *lactis*. Due to the use of raw milk, a secondary microflora is also introduced during the cheese-making, which mainly consists of *Lb. rhamnosus* and the closely related species of *Lb. casei* group (Gatti et al., 2014). Despite *Lb. rhamnosus* metabolism influences texture, flavor and aroma of the final product (Settanni and Moschetti, 2010), the metabolic activities of this species in cheese are still poorly understood.

The capability of *Lb. rhamnosus* to grow in the lactose-free maturing curd relies on a wide metabolic potential to use alternative substrates, available in the cheese environment, for energy production and cell replication.

Potential substrates for microbial growth in cheese environment are represented by small peptides or amino acids, citrate, lactate, and free fatty acids (Fox et al., 1993). Additionally, sugars, phospholipids, nucleic acids and peptides released in the cheese matrix after lysis of the starter lactic acid bacteria (LAB) can be catabolized from non starter microbiota (Budinich et al., 2011). These substrates not only allow energy production for the growing bacteria, but their metabolic conversion leads to the formation of aroma compounds, which determine the flavor of fermented dairy products (Smid and Kleerebezem, 2014; Smit and Smit, 2005).

Besides the metabolic potential of the bacteria involved in fermentation, several physicochemical parameters such as fermentation temperature, pH, redox potential and salinity affect the activation of certain metabolic pathways from bacteria involved in the cheese-making process (Beresford et al., 2001). Among these parameters, redox potential is known to affect flavor development of fermented products, and a negative redox potential is generally correlated with the development of a balanced flavour (Aubert et al., 2002). The development of a negative redox potential in cheeses is generally regarded as an establishment of anaerobic conditions, which

should be required to trigger flavor-forming reactions (Urbach, 1997). Only few data exist about how oxygen diffusion in the cheese matrix influences cheese quality (Thomas, 1987), though several studies indicate the existence of various gradients (pH, salt, redox potential, oxygen) between cheese rind and core, which have various impact on microbial development and metabolite formation (Colas et al., 2007; De Filippis et al., 2016).

Recent work by Lazzi et al. (2014) has shown that cultivation of *Lb. rhamnosus* in a cheese-mimicking broth leads to the upregulation of *spxB* gene encoding for pyruvate oxidase (POX), an enzyme that catalyzes the oxidation of pyruvate to acetyl-phosphate, with subsequent ATP production.

POX pathway is involved in the aerobic metabolism of LAB, and in *Lb. plantarum* its activation leads to an increased synthesis of acetate in the early stationary phase of aerobic growth (Goffin et al., 2006; Lorquet et al., 2004). The activation of *spxB* gene from *Lb. rhamnosus* was recently assessed during ripening of Italian cooked hard cheese (Savo Sardaro et al., 2016), although the cheese environment is generally reported as anaerobic. Nevertheless, diffusion of oxygen in cheese matrix is hard to assess, particularly considering the limitations imposed from a cheese which has an average weight of 30 kg (Mucchetti and Neviani, 2006).

Miniaturization of cheese manufacturing process has successfully been applied to a wide variety of cheeses, and used to test several physical and microbiological parameters involved in cheese manufacturing (Le Boucher et al, 2015; Ruggirello et al., 2016; Vèlez et al., 2015; Shakeel-Ur Rheman et al., 1998), but only two model systems allow high-throughput manufacturing, which is desirable for systematic screening of technological parameters or microbial strains (Bachmann et al., 2009; Erkus et al., 2013). Nevertheless, the latter model cannot be used to simulate the conditions encountered from bacteria in Italian cooked hard cheeses, without tailoring the model-cheese manufacturing to the parameters used in the real cheese-making process.

Since oxygen concentration might affect not only the growth of the bacteria, but also the activation of metabolic pathways involved in cheese flavor development, we decided to investigate how different oxygen concentrations might influence growth dynamics and the aroma profile of

the cheese by setting up a milli-cheese model system for the production of lab-scale cheese mimicking Italian cooked hard cheeses.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study were *Lb. delbrueckii* sp. *lactis* 1617 (LD), *Lb. helveticus* 5 (LH) and *Lb. rhamnosus* 1019 (LR). All of these strains have been deposited in the bioresource collection of the Department of Food Science (University of Parma). All bacterial strains were maintained as frozen stocks (−80 °C) in MRS broth (Oxoid, Milan, Italy) supplemented with 15% glycerol (w/v). Before use, the cultures were propagated twice with a 2% (v/v) inoculum into MRS media (Oxoid, Milan) and incubated for 18 h at the appropriate temperature conditions (42°C for LD and LH, and 37°C for LR) in microaerophilic conditions (Anoxomat, MART Microbiology B.V.).

Lab-scale cheese manufacturing and sampling

A milligram-scale model system for the production of long ripened hard cheeses was developed adapting previously published protocols for the production of Gouda-like cheese (Bachmann et al., 2009; Erkus et al., 2013). Briefly, a mixture of pasteurized full fat and half fat bovine milk was used, in a 50:50 ratio, in order to reach a final fat content of 2.5%. The bacterial coltures were propagated twice in MRS, washed twice in PBS buffer, and resuspended in PBS buffer (pH 7.4) in order to obtain a final cell concentration of 10^9 cfu/ml for *Lb. helveticus* and *Lb. delbrueckii*, respectively, and a concentration of 10^6 cfu/ml for *Lb. rhamnosus*. Subsequently, 125 ml of milk were inoculated with 3% of starter culture and 3% w/v of rennet (Lebstremsel vloeibaar, Brouwmarkt, Almere, The Netherlands) was added, and 5 ml of the inoculated milk were distributed in each well of a 24 deep-well plate. The inoculated milk was heated to 32°C for 15 minutes, and subsequently the coagulum was vigorously cut for 5 minutes using a custom-made stainless steel device. The curd was subsequently cooked for 7 minutes at a temperature of 53°C, and left to rest under the hot whey for 30 minutes. The deep-well plate was then centrifuged for 10 minutes at 4600 rpm at 40°C, and the whey carefully removed. The moulding step was performed for 1h at a temperature of 50°C in a water bath, then the temperature was lowered until 30°C and

the sealed plate was kept in the water bath for 18h. At the end of the moulding step the milli-cheeses were brined by addition of 50 µl of 15% (w/v) sterile sodium chloride solution. The plates were sealed with a gas permeable seal (Thermo Scientific) and ripened at 12°C for two weeks. Different oxygen conditions were set up as aerobic (A, 21% oxygen concentration, environmental conditions), microaerophilic (B, 6% oxygen concentration) and anaerobic (C, 0.16% oxygen concentration, with MART® Catalyst), by means of Anoxomat SW80 instrument (MART Microbiology B.V.). The cheeses were manufactured in duplicate, and sampled before brining (24h), after one week (w1) and two weeks (w2) of ripening. Each milli-cheese was assigned an unambiguous code, the codes were shuffled with Excel, and randomized sampling was applied.

PMA treatment and DNA extraction

Prior to DNA extraction, cheese samples were homogenized and treated with propidium monoazide (PMA) dye (Biotium Inc., Hayward,California). Briefly, each cheese was grinded with mortar and pestle, the homogenate was resuspended in trisodium citrate 2% (w/v) in a 1:10 ratio, and incubated in a water bath for 30 min at 50°C. Subsequently, the solution was washed 3 times by centrifugation at 10.000 rpm for 10 min in trisodium citrate 2%. The cell suspension was centrifuged and the pellet resuspended in 1 ml of trisodium citrate 2% and treated with 2.5 µl 20 mM PMA dye (Biotium Inc., Hayward,California), followed by incubation for 5 min at room temperature. Afterwards, the samples were exposed to the PMA-Lite™ LED photolysis device (Biotium Inc., Hayward,California) for 15 minutes. The cells were recovered by centrifugation for 10 min at 10.000rpm, and the extraction was performed from the bacterial pellet as specified in the DNeasy Tissue and Culture DNA Isolation Kit (QIAGEN, Hilden, Germany) user kit, following the Gram+ protocol specified from the manufacturer. Each condition was assayed in triplicate.

RNA extraction and cDNA synthesis

RNA was extracted from milli-cheeses by modifying a protocol described in a previous work (Monnet et al. 2008). At the moment of sampling milli-cheeses were snap frozen in liquid nitrogen immediately after extraction from the deep-well plate, and store at -80°C until usage. Subsequently 150 mg of frozen cheese were placed in a 2 ml screw cap tube containing 500

µl of zirconia/glass beads (diameter, 0.1 mm; BioSpec Products, Bartlesville, OK) and immediately added with 1 ml of TRIzol (Invitrogen, Milano, Italy). The tubes were processed with a Mini-BeadBeater 8 (BioSpec Products, Bartlesville, OK) by using three 60-s mixing sequences at maximum speed, spaced out with 5 minutes pauses in ice. The homogenized sample was then processed following the TRIzol manufacturer's instruction. RNA quantification was carried out using NanoDrop 1000 (Thermo Fisher Scientific B.V., The Netherlands) and the integrity was evaluated by agarose gel electrophoresis. Contaminating genomic DNA was removed by treatment with TURBO DNA-free™ Kit (Thermo Fisher Scientific) following manufacturer's instructions, and RNA was reverse transcribed with SuperScript® III Reverse Transcriptase (Thermo Fisher Scientific) using random hexamer primers, following the protocol provided with the kit.

Quantitative Real-time PCR

Real time experiments were performed using CFX96 instrument (Bio-Rad) with the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, California, USA). DNA extracted from milli-cheeses was used for absolute quantification of the bacterial species during ripening; cDNA obtained from the reverse transcription of RNA extracted from milli-cheeses was used to assess relative expression of *Lb. rhamnosus* genes.

The PCR reaction was set up in the same way for both relative and absolute quantification, in a final volume of 20 µl, with the following components: 2 µl of DNA (or cDNA), 0.5 µl of each primer (10 µM, Sigma Aldrich), 10 µl of Power SYBR 2X mastermix, 7 µl of PCR-grade water. The reactions were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence signal was acquired at 60 °C. Melting curve analysis (60–95 °C with a heating rate of 0.1 °C per second and a continuous fluorescence measurement) was carried out. After the reaction, the Ct data were determined using default threshold settings, and the mean Ct was determined from three technical repeats. The primer used for amplification, designed for being specific towards only one of the species under exam, and are listed in table 1.

Table 1 Sequences of the primers used in this work. **LH**: *Lb. helveticus*, **LD**: *Lb. delbrueckii* sp. *lactis*; **LR**: *Lb. rhamnosus*

Target	Primers	Sequence (5'-3')	Amplicon (bp)	PCR Efficiency	References	Use
LH	LHpheSF	TTGATGGTGAAGACTTGCTTAGAA	50	2.01	1	qPCR
	LHpheSR	TCTGGCTTGGTCACCTGAAG				
LD	LLpheSF	ACGTTGACGCTGACCACC	50	2.06	1	qPCR
	LLpheSR	GGCTTGAACTGGTGAAGTCTG				
LR	PoxcDNAFW	CAGACGCAATGATCAAGGTG	151	1.97	This study.	qPCR/RT-qPCR
	PoxPromRV	AATGCGCCYACTTCTTCATG				
LR	PtaFW	CGGCCTGGGGTTCATTTAGT	49	1.99	This study.	RT-qPCR
	PtaRV	GACCGCGTTGCATGATGAAA				
LD, LH, LR	TBAfw	CGGCAACGAGCGCAACCC	130	2.04	2	RT-qPCR
	TBArv	CCATTGTAGCACGTGTGTAGCC				

High pressure liquid chromatography (HPLC) determination of sugars and organic acids

Cheese samples were pretreated by homogenization in PBS buffer (pH 7.5) in a 1:10 ratio, and the homogenized samples were deproteinized using Carrez reagents. Organic acids present in the homogenate were analysed using an Ultimate 3000 (Dionex) instrument, equipped with an Aminex HPX-87H 300x7.8mm column (Biorad) with pre-column. 5mM H2SO4 was used as eluent at a flow rate of 0.6 ml/min at 40˚C. Detection was achieved by refractive index (Shodex RI 101). Statistical analysis was performed on calculated concentrations of the organic compounds, using SPSS v.20.

Headspace gas chromatography–mass spectrometry analysis (HS-GC–MS) of volatile organic compounds

Volatile organic compounds (VOCs) produced during cheese ripening were analysed as follows: samples for each oxygenation conditions (0.16%,6% or 21%) were taken in triplicate from each of the biological replicate plates after 1 week or 2 weeks of ripening, put into a GC–MS vial and stored at −20 °C until GC–MS analysis was performed. VOCs were extracted and detected by headspace solid-phase micro-extraction GC–MS, using a HS-SPME fibre (carboxen/polydimethylsiloxane; CAR/PDMS; Supelco, USA) (Gamero et al., 2013b). Samples were pre-incubated at 60 °C for 2 min without agitation, followed by exposure of the fibre to the sample headspace for 5 min at 60 °C. Volatile compounds were injected into the GC column by desorption of the fibre for 10 min. A Finnigan Trace GC Ultra (Thermo Fisher Scientific, USA) equipped with a Stabilwax®–DA-Crossband®–Carbowax® polyethylene glycol column (30 m long × 0.32 mm i.d., 1 μm internal thickness; Restek, Bellefonte, PA, USA) was used for GC–MS analysis. The injection device was a TriPlusTM autosampler (Thermo Fisher Scientific) in PTV splitless mode (5 min) at 250 °C. Helium was used as the carrier gas at a constant flow of 10 ml/min. The GC oven was initially at 40 °C for 2 min, then was raised to 250 °C at 10 °C/min, and then kept at 250 °C for 5 min. The total run time was 28 min. Mass spectral data were collected over a range of m/z 33–250 in full scan mode; scan time, 0.5 s. HS-GC–MS data were analysed as described from van Rijswijck et al. (2015), and statistical analysis were performed on log2 transformed peak areas, using SPSS v.20.

Results

Development of the small scale cheese model

The protocol for high throughput manufacturing of Italian cooked hard cheese employs a 24-deep-well plate, which becomes an array of cheese vats. The cheese-making protocol developed in this study was adapted from the Gouda cheese model system described previously (Erkus et al., 2013), in order to adapt it to cooked hard cheeses manufacturing process, which differs in the passages described in figure 1 and detailed below. The milk used for the production of cooked hard cheeses is generally a mixture of partially skimmed milk obtained from spontaneous overnight milk

creaming and whole milk from morning milking (Gatti et al., 2014). The milk used for real cheese manufacturing is raw, but to set up the model system a mixture of pasteurised full and partially skimmed milk was used, in order to have a fat tenure of approximately 2.5%, differently from Gouda cheese, which has a fat tenure of around 3.5%.

The starter for the milli-cheese consisted of wild strains of *Lb. helveticus* and *Lb. delbrueckii* sp. *lactis*, isolated from natural whey starter (NWS) used for the production of cooked hard cheeses, while a wild *Lb. rhamnosus* isolated from 4 months old cheese was added as a representative strain of non starter LAB. As a comparison, Gouda model cheeses were produced using commercial starter cultures (Erkus et al., 2016) and for this cheese the main species involved in fermentation is *Lactococcus lactis* spp.

Another difference between cheese manufacturing processes is the cutting step, which in the Gouda model system consists of short (20s) cutting intervals followed by resting periods (3min) repeated for 5 minutes, while in the cooked hard cheese model system the best approximation was found to be a continuous and vigorous cutting for 3 minutes, leading to the formation of small curd grains.

After cutting of the curd, the two cheese manufacturing processes differ considerably, because in the case of Gouda, the curd is washed before complete whey removal, while Italian cheese is cooked at a temperature of 53°C for 7 minutes, and left resting under hot whey (50°C) for 30 minutes before whey removal. Extraction of the curd was simulated by centrifugation of the plate, and removal of the whey.

Finally, both cheeses undergo an overnight moulding step at 30°C before being brined. Submerged brining could not be performed for the experimental cheeses, therefore a defined amount of brine was added to each milli-cheese vat, and penetration of the brine into the curd was obtained with centrifugation.

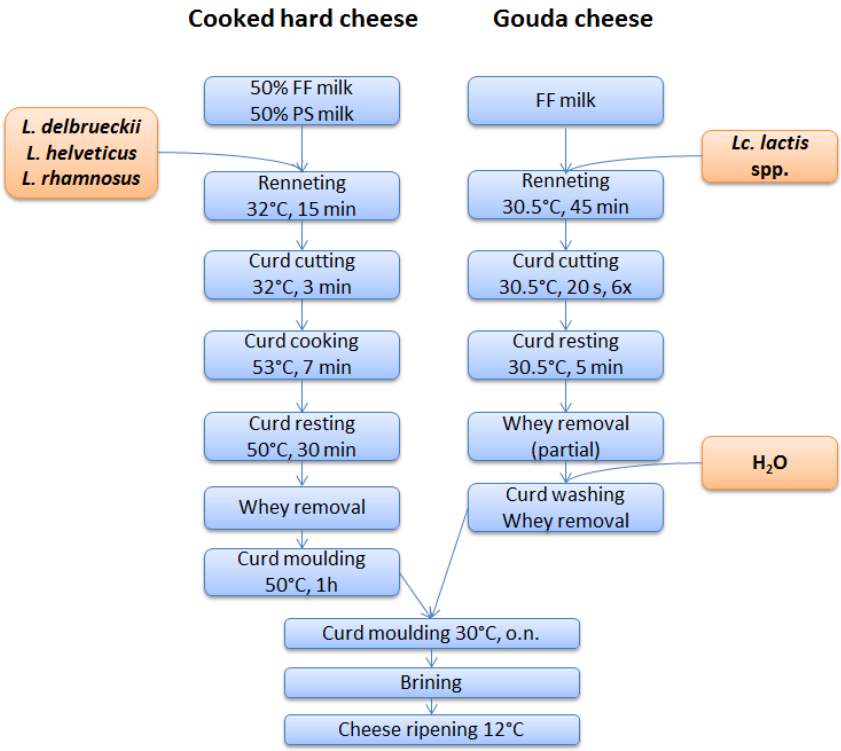

Figure 1 A flow-chart comparison between cooked hard cheese and Gouda cheese model manufacture protocol.

Bacterial population dynamics

The dynamics of the three bacterial species used for cheese manufacturing were assessed through a culture-independent, qPCR based approach, and plate counts on MRS.

While plating does not allow for discrimination of the three bacterial species involved in the fermentation process, qPCR performed with primer pairs specifically targeting *Lb. helveticus*, *Lb. delbrueckii* or *Lb. rhamnosus* has permitted to precisely monitor and quantify the bacterial composition after one and two weeks of ripening. Furthermore, the treatment of cheese homogenates with PMA followed by photolysis, prior to DNA extraction, allows to selectively inactivate the DNA from dead and injured cells, due to permeation of the dye through the cell wall, so that in the subsequent DNA extraction and PCR quantification only viable cells are taken into account.

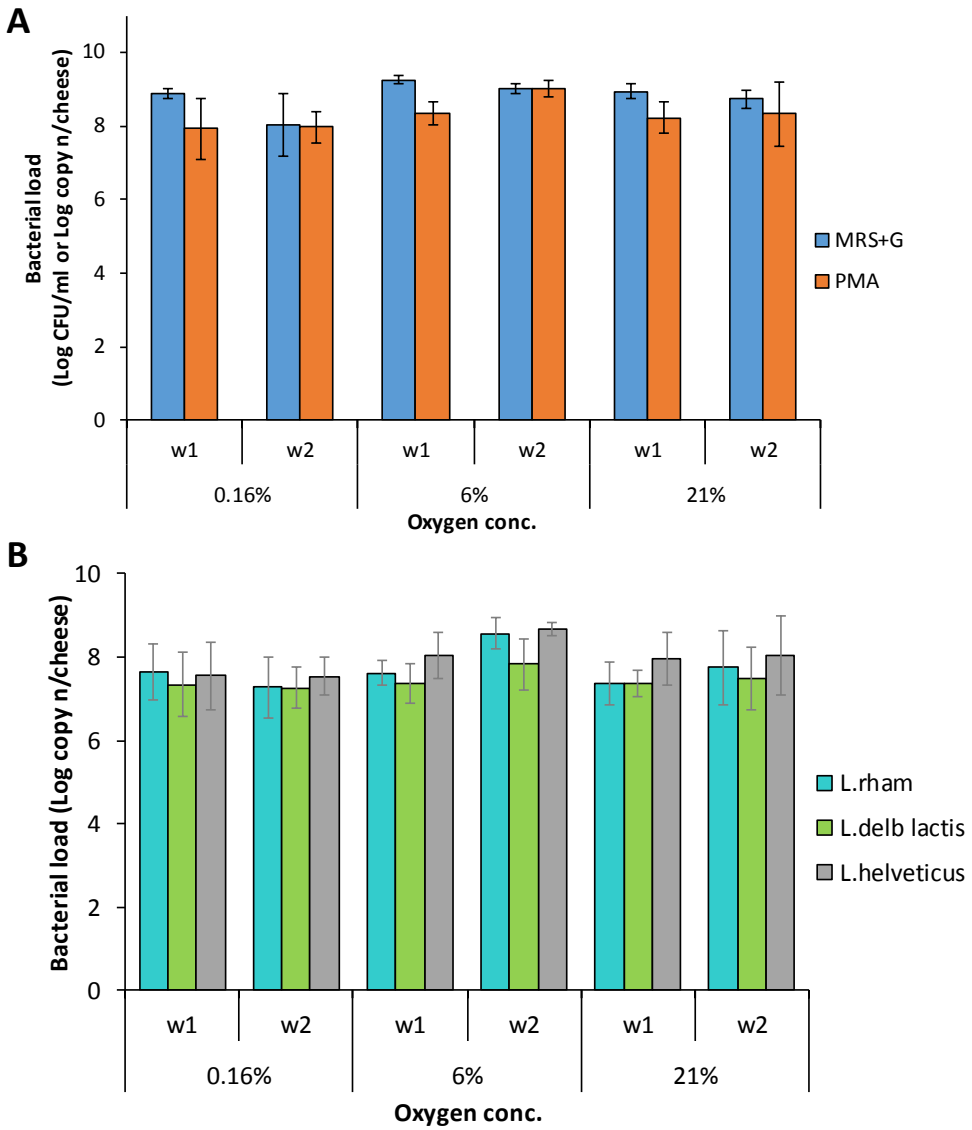

Figure 2 Bacterial population dynamics. **A** Comparison of the bacterial counts obtained with plate counting and PMA-qPCR. **B** Bacterial species dynamics obtained with PMA-qPCR.

The comparison between total viable count (TVC) from plating and cumulative counts obtained with qPCR (fig. 2a) shows a good agreement between the two techniques, though statistical testing shows a different result for samples from 1 week old milli-cheeses ($p<0.05$), while after two

weeks of ripening total counts show no statistically significant difference ($p>0.41$).

The results presented highlight that in both oxygenation conditions there is an increase in the viable population during the first week of ripening, with TVC reaching 9 log CFU/g, with an average increase of 2 log CFU/g. Only under anaerobiosis the bacterial count starts to decrease during the second week of ripening.

qPCR data, presented in panel b of figure 2, represent the mean of 3 milli-cheeses for each of the two biological replicates. In detail, aerobic condition shows a predominance of *Lb. helveticus* over the other two species, with a concentration of 8 log CFU/gr; microaerophilic conditions appear to stimulate the growth of *Lb. rhamnosus* and *Lb. helveticus* up to 8.5 log CFU/gr ($p<0.05$), while under anaerobic conditions the three species are stable around 7 log CFU/gr during the two weeks of ripening (Fig. 2b).

Relative gene expression

The metabolic activity of *Lb. rhamnosus* was evaluated through real time PCR targeting the transcripts of genes *pta* and *spxB*, encoding phosphate acetyltransferase and pyruvate oxidase respectively, both involved in the synthesis of acetate from pyruvate, while 16S was chosen as a housekeeping gene. Before proceeding to relative quantification experiments, the primer pairs specificity towards *Lb. rhamnosus* was tested against genomic DNA of *Lb. rhamnosus*, *Lb. helveticus* and *Lb. delbrueckii*, along with a mixture of the DNAs, in order to exclude the possibility of cross amplification that could lead to wrong interpretation of threshold cycle (c_q) in an experimental setting based on SYBR green chemistry (data not shown). Due to the similar amplification efficiencies reported for the primer pairs, $\Delta\Delta c_t$ method was used for the evaluation of the expression ratio without applying further corrections. The results presented in fig. 3 show the expression ratio for the genes *pta* and *spxB* after two weeks of ripening, normalised against anaerobiosis, showing that *pta* activity is overexpressed under oxygenation conditions, with a ratio of about 3. Overexpression is particularly evident for *spxB* gene, which has a ratio of about 25 in aerobic conditions and of about 5 under microaerophilic conditions, in comparison with anaerobiosis, that was

chosen as a control, where *spxB* transcript was barely detectable compared to the housekeeping gene (Δc_t>15).

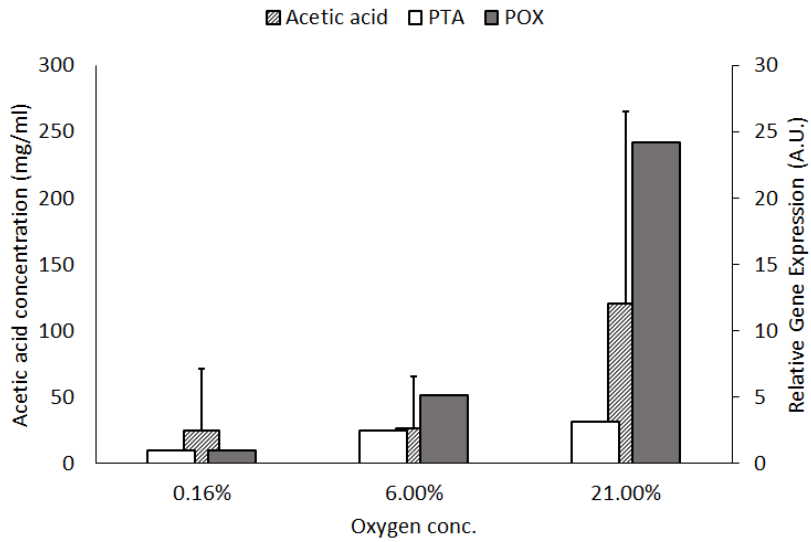

Figure 3 Relative gene expression of POX and PTA after two weeks of ripening at the selected oxygen concentration, compared with the measured acetic acid concentration, measured with GC-MS. Anaerobic (0.16%) condition is chosen as a control.

Liquid chromatography

The use of HPLC allows quantification of the organic acids produced during ripening, as well as other compounds present during fermentation. The compounds identified with HPLC are lactose, citric acid, pyruvate, succinic acid lactic acid and ethanol.

Lactose decreases from week 1 to week 2 in all samples, with concomitant increase of lactic acid. The compounds that significantly vary are pyruvate, which increases in aerobic condition after two weeks of ripening ($p<0.05$) and ethanol, which is present only in aerobic and microaerophilic conditions (Fig.4). No significant correlation was found between oxygenation conditions and organic acids synthesis.

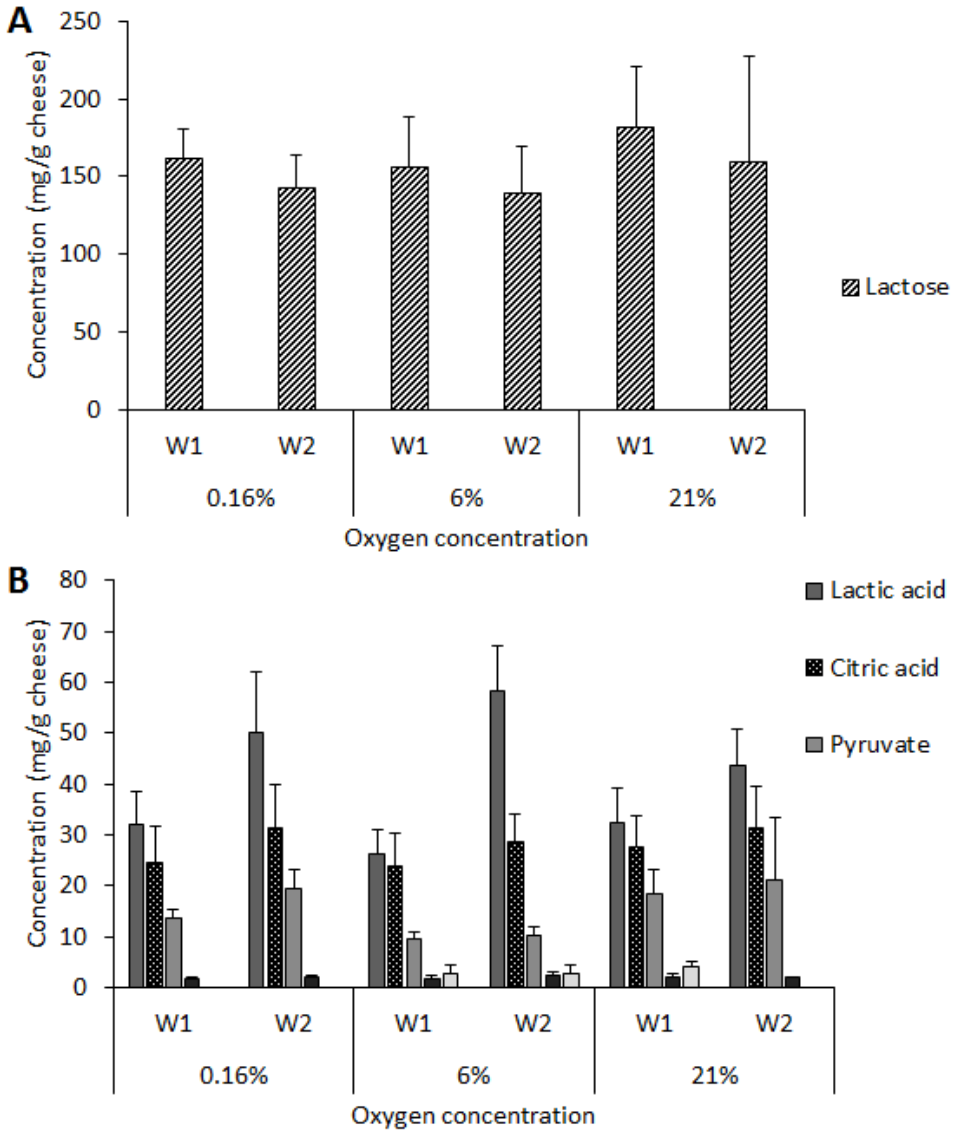

Figure 4 Primary metabolites identified through HPLC from milli-cheeses incubated under three oxygen concentration. **A** Lactose concentration at different steps of fermentation. **B** Organic acids profile during fermentation.

Volatile organic compounds

In order to assess the effect of the three different oxygenation conditions on the milli-cheese volatile organic compounds profile, 36 samples were analysed with HS-GC-MS analysis. 15 VOCs were identified, and are listed in table 2, belonging to the chemical classes of ketones, esters and

acids. Comparing the relative abundance of each classes of compounds in the volatile profile of the milli-cheeses, it can be easily seen that ketones, in this case acetoin and diacetyl, represent the most abundant compounds in aerobic and microaerophilic cheeses, while their abundance is lower in one week old anaerobic cheese, and further decreases with further ripening. Conversely, under anaerobic conditions, there is a great increase during ripening of the acids fraction, in this case mainly represented by short and medium chain fatty acids. Esters, despite less abundant, seem to slightly increase in all the oxygenation conditions, especially under microaerophilic ones.

The volatile compounds that significantly vary ($p<0.05$) in the conditions chosen in these study are diacetyl, 2 –propenoic acid 2 methyl methyl ester and acetoin. In order to highlight further correlations between oxygenation conditions and production of volatile organic compounds a principal component analysis (PCA) was conducted. Figure 5a reports the PCA loadings plot on the first 2 factors describing the percentage of the variance explained from the volatile fraction of the cheeses in the experimental settings. Component 1 accounts for 19.6% of the variability and component 2 for 14.8%. Despite the explained variability is not high, Fig. 5b shows how anaerobically incubated milli-cheeses have a greater prevalence of compounds that contribute to factor one with a negative sign, especially short and medium chain fatty acids (butanoic acid, 3 methyl- pentanoic acid, hexanoic acid, octanoic acid) and methyl hexanoate. Conversely, cheeses that were incubated with higher oxygen concentration are characterized by volatile compounds that contribute to factor 1 with a positive sign, such as the aforementioned acetoin and diacetyl. Indeed, there is a positive correlation between increased oxygen concentration and synthesis of diacetyl (Pearson's correlation coefficient, $p<0.05$), acetoin and 2 propenoic acid 2 methyl methyl ester ($p<0.01$), while the only compounds whose production shows a negative correlation with increased oxygen incubation is 3-methyl pentanoic acid ($p<0.05$).

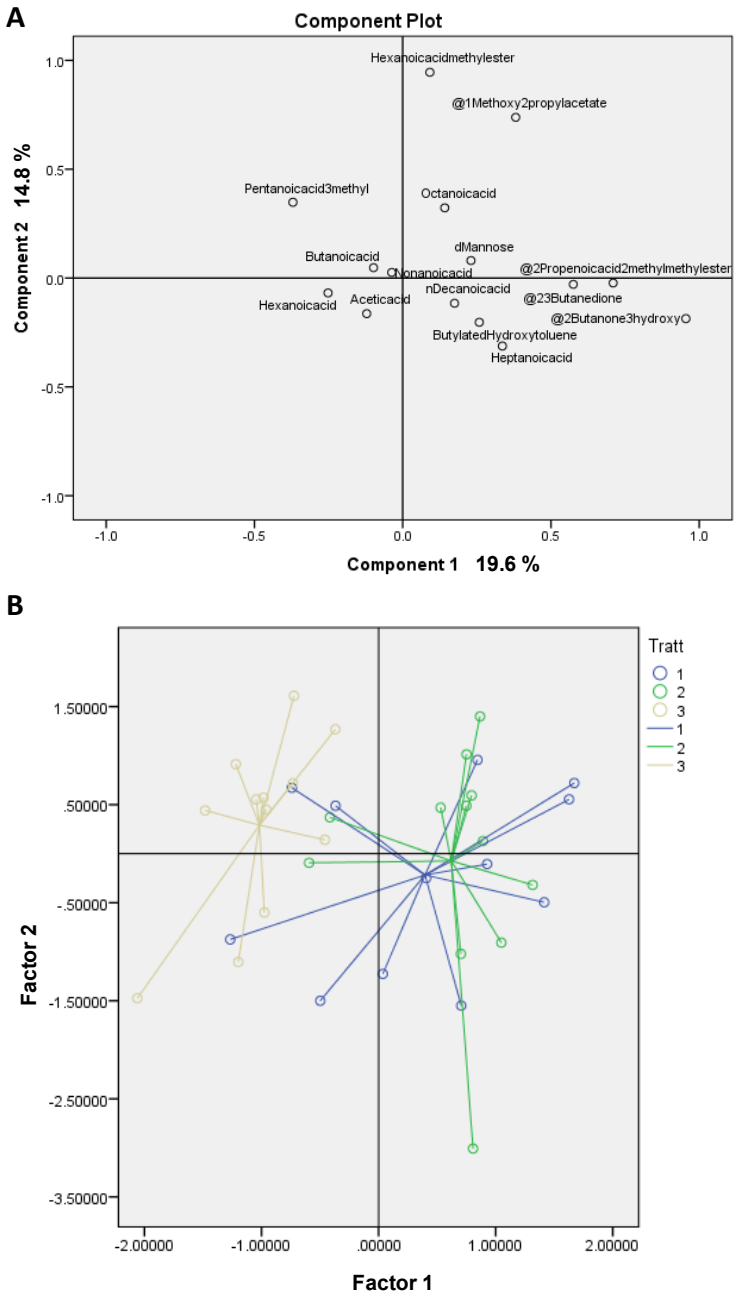

Figure 5 Principal component analysis of VOCs in milli-cheeses. **A** Variables component plot for the two extracted components. **B** Score plot of the samples used in this study, legend corresponds to oxygenation condition, where A=21%, B=6%, C=0.16%. Lines represent distance from the centroids for samples incubated under the same conditions.

Table 2 Volatile organic compounds identified in this study. Peak areas are expressed as arbitrary units (AU=peak area/10^3), N.I.***** = not identified

Compounds	**Oxygenation conditions**					
	21%		**6%**		**0.16%**	
	Mean	SD	Mean	SD	Mean	SD
Acids						
Acetic acid	2293.2	856.9	2056.7	1072.1	2618.1	832.6
Butanoic acid	316.4	92.3	336.7	122.1	415.6	142.4
Pentanoic acid, 3-methyl-	17.9	24.5	40.3	37.2	37.3	16.5
Hexanoic acid	380.1	168.9	415.7	183.9	475.2	66.3
Octanoic acid	173.3	58.4	180.2	77.3	171.8	46.4
Nonanoic acid	1.3	0.4	1.0	0.8	0.7	0.6
n-Decanoic acid	4.3	11.7	10.1	29.7	1.2	0.7
Heptanoic acid	0.3	0.2	0.3	0.3	0.3	0.3
Σ of acids	3186.8		3040.9		3720.2	
Esters						
2-Propenoic acid, 2-methyl-, methyl ester	83.8	32.3	260.8	51.0	6.1	3.5
Hexanoic acid, methyl ester	12.0	13.7	25.9	29.7	27.8	27.7
1-Methoxy-2-propyl acetate	55.9	85.9	65.6	144.2	33.2	71.0
Σ of Esters	151.7		352.3		67.1	
Ketones						
Diacetyl	6818.2	1704.5	5847.7	1525.3	1654.8	1500.4
Acetoin	4169.0	6014.3	2151.0	3386.5	16.2	19.8
Σ of ketones	10987.2		7998.7		1671.1	
Other						
Butylated Hydroxy Toluene	0.1	0.1	0.2	0.2	0.1	0.1
N.I. *	145.0	114.9	110.6	78.1	125.7	97.0
Σ of others	145.1		110.8		125.8	

Discussion

In this work, a new model system for the production of small scale cooked hard cheese is proposed, and used to study the metabolic response of *Lb. rhamnosus* to varying oxygen concentrations. While many model systems have been used to study several variety of cheeses which are relevant for the food industry (Ruggirello et al. 2016; Erkus et al., 2013, Bachmann et al, 2009; Shakeel-Ur-Rheman et al., 1998), in the case of cooked hard cheeses only two models are available (Mucchetti et al., 2005, Vèlez et al., 2015), but in both the cases the proposed cheese-making protocols do not allow high-throughput manufacturing.

The protocol presented in this study employs a 24-deep-well plate, which becomes an array of cheese vats. A similar high-throughput cheese-making protocol was proposed recently (Erkus, 2013), but was adapted to the cheese-making protocol for Italian cooked hard cheeses, where different milk composition, starter culture, the presence of a cooking step and the absence of a curd washing step greatly influences the development of the microbial populations and of the final cheese characteristics (Gatti et al., 2014).

In particular, the model cheese was used to test the effect of incubation with an oxygen concentration of 21%, 6% and 0.16%, respectively defined as aerobic, microaerophilic and anaerobic, on microbial dynamics and metabolite compounds produced at the early stages of ripening.

Few studies have dealt with the effect of varying oxygen concentration on cheese quality, aiming to assess acetate formation in Cheddar cheese wrapped in films with different oxygen- permeability (Thomas, 1987), or to study surface and core redox potential in cheese (Colas et al., 2007).

The artificial starter culture produced for the manufacturing of milli-cheeses comprises three autochthonous *Lactobacillus* species, isolated from different stages of cheese manufacturing, to simulate the complex microbiota involved in the fermentation of Italian cooked hard cheeses.

For the enumeration of the three species during cheese manufacturing a culture-independent approach was used, relying on PMA-qPCR analysis of the total DNA extracted from cheese samples (Erkus, 2016), since plate count could not distinguish between the species used in this study.

PMA-qPCR method proved to be a valid tool to assess viable population in the model system, and was validated by comparison with the total viable counts (TVC) obtained through plating on MRS. Despite some differences were found between plate counts and cumulative cell counts obtained with qPCR, the culture independent approach is sensitive and well-suited for the purpose of selective enumeration of the three *Lactobacillus* species.

The results show that after one week of ripening, the total population size is reached, and slight variations occur during the second week of ripening, except for anaerobiosis, that shows a significant reduction in the bacterial loads compared to the oxygenated conditions ($p<0.05$). On the other hand, microaerophilic incubation appears to stimulate the growth of *Lb. helveticus* and *Lb. rhamnosus* ($p<0.05$) during the second week of ripening, suggesting that oxygen sensing mechanism might be species-specific.

Bacterial growth is accompanied, in all the experimental conditions, by consumption of residual lactose, and a concomitant increase of lactic acid, but also from the production of acetic acid and ethanol, deriving from heterofermentative pathways. *Lb. helveticus* and *Lb. delbrueckii* sp. *lactis* are obligate homofermentative, while *Lb. rhamnosus*, like other non starter LAB, is a facultative heterofermentative species (Gatti et al., 2014).

Acetate is an important metabolite, it is involved in cheese flavour, and bacterial synthesis through the acetate kinase pathway leads to substrate-level phosphorylation, producing an additional ATP. A metabolic pathway for acetate production in *Lb. rhamnosus* involves the expression of pyruvate-coding *spxB* gene, and its activation in cheese has already been investigated (Savo Sardaro et al., 2016).

Results obtained with the milli-cheese show that an increased oxygen concentration leads to overexpression of this gene, and to an increased acetate synthesis, despite the presence of residual lactose. *SpxB* gene is under control of carbon catabolite protein A (ccpA; Lorquet et al., 2004), but some studies have shown that in *Lb. plantarum* catabolite repression is not activated from lactose (Sedewitz et al., 1984). Further studies should be performed to assess the extent of acetate production and *spxB* activation from *Lb. rhamnosus* in lactose-containing media. Acetate was detected in anaerobic milli-cheeses as well, though in lower

concentrations, in agreement with the reduced expression of *spxB* and *pta* genes found in this condition. However, acetate synthesis in anaerobic conditions might involve the activation of pyruvate formate lyase, an enzyme active only under strict anaerobic conditions (Hugenholtz et al., 1993). No traces of formate were however detected.

Under aerobic and microaerophilic conditions citrate is consumed and pyruvate is formed during fermentation, as well as the above mentioned acetic acid and small amounts of ethanol. Heterofermentative bacteria can produce ethanol from acetyl-phosphate from the phosphoketolase pathway, but also from pyruvate, through the action of alcohol dehydrogenase. The absence of detection of ethanol in anaerobiosis might be due to the lower counts for *Lb. rhamnosus* in this condition, thus the produced ethanol might be below the detection limit of our chromatographic method, or to the fact that, under anaerobic conditions, the major end product of bacterial fermentation is lactic acid (Pedersen et al., 2008).

Among the volatile organic compounds distinctive of the profile of cheeses in aerobic and microaerophilic conditions, we have an increased synthesis of acetoin and diacetyl. Under oxygenic conditions, the production of lactic acid is reduced and the glycolytic flux is redirected toward production of acetate, ethanol, acetoin, diacetyl, and CO2 (Papadimitriou et al., 2016). This effect is well documented in several studies regarding the metabolic shifts of LAB as a response to a reduced NADH/NAD ratio under varying oxygenation conditions, and is likely to be due to an increased redox potential.

Other compounds detected by GC-MS reveals the synthesis of metabolites that might derive from amino acid degradation and fatty acid catabolism pathways.

Catabolism of amino acids is strongly correlated to the synthesis of flavour compounds in cheese (Smit and Smit, 2005) and lactic acid bacteria possess a wide enzymatic potential for their synthesis (Liu et al., 2008). The only compound that could be associated with amino acids catabolism is 3 methyl pentanoic acid, which might derive from Leucine degradation pathway. The compounds methyl methacrylate, 1methoxy 2 propyl acetate and 2 propenoic acid 2 methyl methyl ester could not be linked to

any specific pathway from any of the species involved in the fermentation, despite they might likely derive from amino acid degradation pathways.

Among the volatile compounds identified in this study, a relevant amount consists of short- and medium-chain fatty acids. Lipolysis occurs to a significant level in Italian long ripened cheeses, and this activity is mainly attributed to lipases that might originate from raw milk, from rennet paste, or from the microbiota development throughout the ripening (Collins et al., 2004). Production of these compounds in the milli-cheeses can be attributed only to the lipolytic activity of the three LAB used for fermentation, since milk pasteurisation leads to inactivation of milk lipases (Driessen, 1989), and the enzyme used for the renneting step consisted of purified enzyme (chymosin).

The available literature about LAB esterases/lipases reports that most of the lipolytic activity of these bacteria is intracellular, and bacterial lysis causes their release in the cheese matrix; this is relevant also for Italian cooked hard cheeses, where extensive starter LAB lysis could be correlated with an increased production of free fatty acids (Lazzi et al., 2016).

In this study, the fatty acids identified range from butanoic acid to n-decanoic acid, showing that the substrate specificity of LAB esterase is not limited to short-chained glyceride substrates, as frequently reported (Holland et al., 2005).

Anaerobic milli-cheeses presented an extensive amount of fatty acids and, despite no significant differences could be observed, the relative amounts of octanoic, hexanoic and butanoic acids were the highest, compared with the other oxygenation conditions. Since anaerobiosis is the only condition where a small decrease in the viable population counts occurs, lysis of the cells might be contributing to the release of enzymes in the cheese, and consequently to an increased lipolysis.

Together with fatty acids, esters are important contributors of the fruity note of Italian cheeses (Barbieri et al., 1994), but the only fatty acid ester that could be identified was methyl hexanoate, which was prevalently synthesised in anaerobiosis, while other esters corresponding to the fatty acids detected could not be identified, probably due to the lack of ethanol synthesis in this condition.

Conclusions

The strain of *Lb. rhamnosus* employed in this study responded to the aerobic and microaerophilic oxygenation conditions in cheese environment by activating heterofermentative pathway, which led to an increased synthesis of acetate and ethanol, and also of acetoin and diacetyl.

Even if miniaturisation of the model represents an approximation of the real cheese system, it gained an insight in microbial metabolisms responses to different oxygenation conditions in a real cheese environment. This is a valuable addition since often these are assessed in controlled fermentation experiments performed in liquid media.

Acknowledgements

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Chapter 5

Lactobacillus plasmid toxin: a type I toxin-antitoxin system from dairy *Lb. rhamnosus*

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Abstract

Plasmids harbor useful traits in bacteria among which genes encoding toxin-antitoxin (TA) systems for plasmid maintenance. To date, in the *Lactobacillus* genus, TA systems have been found only in plasmids from *Lb. plantarum*, *Lb. salivarius Lb. curvatus* and *Lb. casei*, but none of these systems belong to the type I.

Here we identify, for the first time in *Lb. rhamnosus* isolated from cheese, a type I TA system located on plasmid DNA with a structural organization resembling the Fst TA system which comprises two small convergently RNAs, with the role of toxin and antitoxin. The 29 amino acid peptide with the role of toxin, named *Lactobacillus* plasmid toxin (Lpt), has shown the ability to inhibit *E. coli* growth and resulted upregulated in conditions mimicking cheese ripening and is detectable also directly in cheese as shown by transcription analysis. We also demonstrate, by an in-depth bioinformatic analysis, that this system is widely distributed on plasmids harbored by *Lactobacillus*, in particular within *Lb. casei* group. The present study highlights the wide distribution of this type I TA system in *Lactobacillus* plasmids and suggests for it a relevant role for the growth in stress conditions typical of cheese environment.

Introduction

Lactobacillus rhamnosus is a non-starter lactic acid bacterium that plays a significant role during cheese ripening, leading to the formation of flavor. In long-ripened cheeses it persists throughout the whole ripening time due to its capacity to adapt itself to the environmental conditions. The metabolic response of *Lb. rhamnosus* to different ecosystems, particularly in cheese dairy environment, is still poorly understood.

Fermented foods, like cheese, represent very complex environment for bacterial life in which the nutrients are almost exhausted and waste products are very abundant. Moreover, many intrinsic factors affect survival, growth and biochemical activities of the cheese microbiota, like moisture content, salt concentration, pH of the curd as well as availability of oxygen (Fleet, 1999). Several studies have been carried out to understand the bacterial metabolic response in these adverse conditions, and in particular to identify which biochemical pathways are activated to permit survival and growth in cheese habitat (Bove et al., 2012). In a recent study (Lazzi et al., 2014) a set of genes of *Lb. rhamnosus* related to the growth ability in a cheese-like medium (CB) was identified by cDNA-amplified fragment length polymorphism (cDNA-AFLP), one of the most robust and sensitive transcriptomic technologies for gene expression analyses. Interestingly, an upregulation of a transcript related to a *Lb. casei* plasmid was reported. It is known that plasmids often carry genes that might be essential for survival under hostile conditions, such those encoding enzymes involved in secondary metabolic pathways, conferring adaptive advantages to the host strains (Wegrzyin and Wegrzyn, 2002). To date, more than 20 species of *Lactobacillus* containing plasmids have been identified and over than 400 lactic acid bacteria (LAB) plasmids have been isolated and studied (Cui et al., 2015), but only three plasmids isolated from *Lb. rhamnosus* have been sequenced and are available in DNA database (pLR001, GenBank accession number CP001155; pLR002 GenBank accession number CP001156; pLC1 GenBank accession number FM179324).

Plasmids encode important traits in LAB, like resistance to phages or antibiotics, lactose catabolism, and production of proteolytic enzymes or bacteriocins (Cui et al., 2015). It is known that plasmids may harbor also genes encoding toxin-antitoxin (TA) systems for plasmid maintenance

(Gerdes et al., 1986); in the genus *Lactobacillus* these systems have been found only in plasmids from *Lb. plantarum* (Sorvig et al., 2005; Heis et al., 2015), *Lb. salivarius* (Fang et al., 2008), *Lb. curvatus* (Weaver et al., 2009) and from *Lb. casei* (Zhang et al., 2008).

The plasmid inheritance is due to the presence of gene cassettes that could be grouped into three classes: (i) centromere-like systems that actively secure ordered segregation of replicons prior to cell division, (ii) site-specific recombination systems that actively resolve tandem plasmid multimers into monomers, and (iii) cassettes that mediate killing of newborn, plasmid-free cells resulting from failure of the first two systems to secure plasmid maintenance (Gerdes and Jensen, 2000).

This latter phenomenon by which plasmid-encoded TA systems ensure plasmid stability is called plasmid addiction or post-segregational killing (Brzozowska and Zielenkiewicz, 2013). TA loci are a two-component system that code for a stable "toxin", able to kill the cells or confer growth stasis, and for an unstable "antitoxin" (Gerdes and Wagner, 2007). To date at least five different types of TA systems that differ markedly in their genetic architectures and in their activity regulation have been identified (Schuster and Bertram, 2013).

Among these, in type I and type III, the antitoxin is a RNA molecule with different mode of action. In type I, an antitoxin RNA is able to repress toxin peptide expression by interacting with toxin mRNA, preventing its translation or targeting it for degradation. In type III, antitoxin RNA directly binds to the toxin peptide sequestering it. In type II, IV and V both toxin and antitoxin are proteins and the toxin is neutralized by protein-protein interaction or by different mechanisms (Schuster and Bertram, 2013). The antagonist regulator that neutralizes the toxin, which could be an antisense RNA or a protein, is unstable. If a plasmid-free variant is produced, owing to a replication error or to other defect in plasmid maintenance, a rapid depletion of these unstable regulators occurs in newborn plasmid-free cells. In these conditions the stable toxin inherited from the mother cell which is no longer neutralized by the antitoxin, causes the death of the plasmid-free cell (Gerdes et al., 2005). Recently, homologous systems have been found in bacteria chromosomes as a part of integrated mobile elements (Wen and Fozo, 2014). Conversely, newly identified loci on bacterial chromosomes do not show homology to

mobile genetic elements (Wen and Fozo, 2014), suggesting that TA systems could have different biological function.

Most of the characterized TA systems, located both in chromosome and plasmids, belong to type II. Studies focused on type I or III TA systems are more limited probably because their identification is more difficult due to the small size of the hydrophobic toxin and to the hard prediction of small RNAs (Wozniak and Waldor, 2009; Fozo et al., 2010). Different molecular mechanisms of type I and type III systems have been described to date (Brielle et al., 2016) mainly in *Bacillus subtilis* and *Escherichia Coli* (Wen and Fozo, 2014), but more studies are needed to elucidate causes and effects of the activation of these systems.

In this work we identified a new toxin peptide (*Lactobacillus* plasmid peptide, Lpt) belonging to type I toxin-antitoxin systems in plasmids from two different strains of. *Lb. rhamnosus* isolated from cheese. This system is upregulated when the *Lb. rhamnosus* is grown in a cheese-like medium strongly indicating a role in cell stress response. Using qRT PCR, we also demonstrated the Lpt toxin expression in long ripened cheese; this is the first time that an RNA belonging to a TA system was detected directly in food. Moreover, an in-depth bioinformatic analysis permitted us to identify homologous systems widely distributed on plasmids harbored by *Lactobacillus casei* group (*Lb. rhamnosus*, *Lb. casei* and *Lb. paracasei*) and *Lb. brevis*, highlighting a relevant function for this TA system in *Lactobacillus* genus.

Methods

Bacterial strains, media and culture conditions

Lb. rhamnosus PR1019 and PR1473 were isolated from Parmigiano Reggiano cheese (PR) at 4 and 20 months of ripening, respectively (Neviani et al., 2009) and identified by 16S rDNA gene sequencing (Bove et al., 2011) and species-specific PCR (Ward and Timmins, 1999). Both strains were cultivated in MRS broth (Oxoid) or Cheese Broth (CB) at 30°C, under anaerobiosis, for 24 or 48 h, respectively. CB, a culture medium that mimics raw- milk long-ripened cheese, was prepared according to the protocol described in (Bove et al., 2011b).

cDNA-AFLP

Total RNA was isolated from *Lb. rhamnosus* PR1473 and PR 1019 grown in MRS and CB medium at the top of logarithmic phase by using the RNeasy Protect Bacteria Mini Kit (QIAGEN). cDNA synthesis and cDNA-AFLP analysis were carried out as described in Bove et al. (2011b). Briefly, after a step of mRNA enrichment and polyadenylation of RNA transcripts, cDNA was synthesized by reverse transcription (RT) using a biotinylated oligo (dT). A first digestion was carried out by employing *Eco*RI, biotinylated fragments were separated by using streptavidine-coated Dynabeads and subsequently further digested by using *Mse*I. cDNA fragments were ligated to the specific adaptors (Table S1) and amplified by using the non-selective *Eco*RI and *Mse*I primers (Table S1). Subsequently, a selective amplification were performed with a *Eco*RI primer labeled with an infrared dye (IRDye™ 700 phosphoramidite), and an unlabeled *Mse*I primer (Table S1). Three primer combinations were used to selectively amplify the expressed genes: 5'DY-*Eco*RI-AC/*Mse*I-AT, 5'DY-*Eco*RI-AT/*Mse*I-AC and 5'DY-*Eco*RI-AT/ *Mse*I-AT (Table S1). cDNA-AFLP fragments were separate on polyacrylamide gel and visualized by Odissey (LI-COR Biosciences).

Transcript-derived fragments overexpressed in CB culture were cut from polyacrylamide gel as reported by Vuylsteke et al. (2007), resuspended in water and subsequently re-amplified using the same selective primers lacking the infrared dye (Table S1). The experiments were carried out in triplicate. Amplified products were cloned in pGem vector (Promega) and recombinant plasmids were sequenced on both strands.

In silico analysis

Nucleotide database was searched with the BLASTn program (Altschul et al., 1990) by using the overexpressed cDNA sequence identified by AFLP or the complete Lpt TA system region as a query. The detection of protein homology was performed with the help of HHPRED program (http://toolkit.tuebingen.mpg.de/hhpred). The identification of putative promoter sequences were carried out by using BPROM available at www.softberry.com, the prediction of terminator sequences was carried out with the help of the web server "Arnold finding terminators" available at http://rna.igmors.u-psud.fr. RNA secondary structures were predicted by using RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Sequence alignments were constructed by Clustal

Omega (Sievers et al., 2011) and rendered by GeneDoc or Espript3 (http://espript.ibcp.fr; Gouet et al., 1999) programs.

Plasmid isolation, DNA sequencing and 5'/3' RACE-PCR

Plasmid DNA was extracted from the two strains grown both in MRS and CB medium and the predicted complete sequence including all the regulatory regions was amplified. Briefly, plasmid DNA was extracted by using Plasmid DNA Extraction Mini Kit (Fisher Molecular Biology) and amplified by standard PCR employing primers fst plus and fst minus (Table S1) designed on the basis of plasmid pLBPC-2 sequence (GenBank accession number AP01254). The amplification products were sequenced on both strands by using the same primers.

Enriched and polyadenylated mRNA from PR1019 and PR1473 strains grown in CB obtained as previously described in cDNA-AFLP section, was used in RACE-PCR experiments in order to identify the full length sequence of toxin encoding RNA. In 3' RACE-PCR the synthesis of the first cDNA strand was carried out by using the oligo-dT-adaptor primer (anchored polyT, Table S1) and the SuperScript II reverse transcriptase (Invitrogen). Touch-down PCR was then used to amplify the 3' end of cDNA molecule using a forward gene specific primer 3 μM (3RACEplus, Table S1), and a reverse primer corresponding to the adaptor sequence 1 μM (3-5RACE-PCR primer, Table S1). 5' RACE-PCR was carried out using SMART™ mRNA Amplification Kit (Clontech, USA) following manufacturer's instructions with some modifications. Briefly, the synthesis of the first cDNA strand was performed by using the oligo-dT-adaptor primer (anchored polyT, Table S1) and the SuperScript II reverse transcriptase (Invitrogen) in the presence of the oligo-dG-adaptor primer (anchored polyG , Table S1). cDNA molecules obtained were then amplified by using forward and reverse primers corresponding to adaptor sequences (5RACE PCR primer and 3-5RACE-PCR primer, Table S1). Finally, the specific cDNA sequence was amplified by using touch-down PCR, a forward primer corresponding to the adaptor sequence 1 μM (5RACE PCR primer, Table S1) and a reverse gene specific primer 3 μM (5RACEminus). The amplification products were then cloned into pGEM vector (Promega) and recombinant plasmids were sequenced on both strands.

Expression of toxic peptide in E. coli

The toxin DNA sequence, from the start codon to the transcription terminator, were PCR amplified by using plasmid DNA extracted from *Lb. rhamnosus* 1473 as a template and two sequence specific primers, a *Nde*I-tailed upstream primer (lpt plus, Table S1) and a BamHI-tailed downstream primer (lpt plus, Table S1). The amplification product cloned into pGEM vector (Promega) was digested with *Bam*HI and *Nde*I enzymes and subcloned into the expression vector pSRKKm (Khan et al., 2008). The resulted recombinant plasmid (pSRKKm-lpt) was electroporated into *E.coli* DH10bT1R competent cells. Growth assays were performed for *E.coli* DH10bT1R transformed with pSRKkm or with pSRKKm-lpt on plates containing LB medium added of glucose or lactose.

Quantitative reverse transcription PCR

Quantitative real-time PCR was carried out on cDNA obtained from RNA of *Lb. rhamnosus* grown in different conditions or from RNA extracted directly from cheese. Total RNA was extracted from *Lb. rhamnosus* PR1473 and PR 1019 grown on MRS and CB at the top of logarithmic phase by using the RNeasy Protect Bacteria Mini Kit (QIAGEN).The same procedure was used to isolated total RNA from two samples of cheese (Parmigiano Reggiano) ripened 6 and 12 months. cDNA was generated from total RNA using the QuantiTect Reverse Transcription Kit (Qiagen) using random hexamers primers, according to the manufacturer's protocol.

qRT PCR was carried out using a QuantStudio® 3 (Thermo Fisher Scientific) using the Power SYBR Green PCR Master Mix (Applied Biosystems). The 20 µl PCR reaction included 0.1 µl of cDNA, 0.5 µl of specific primers targeting Lpt cDNA (Table S1) and 10 µl of Master Mix.

The reactions were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After the reaction, the Ct data were determined using default threshold settings, and the mean Ct was determined from three replicates of PCR experiments. Calculations were performed using 16S rRNA (gene/16S) as internal standard. The $2-\Delta\Delta CT$ method was used to determine the relative gene expression (Livak and Schmittgen, 2001). The real-time PCR amplification efficiencies (E) in the exponential phase were calculated according to the equation: E = 10(-1/slope). The amplification efficiencies were 103.62% for Lpt and 99.79%

for 16S rRNA, with a difference of less than 5% between the two genes. Statistical analysis were performed by IBM SPSS v.23, using Student's t-test.

Results

Identification of a RNA toxin-antitoxin system in Lb. rhamnosus

The transcriptomic profile of *Lb. rhamnosus* PR1473 grown in MRS and CB media evaluated by using cDNA-AFLP with different primer sets is shown in Figure 1A. By comparing these results with those previously observed for the strain PR1019[Lazzi et al., 2014] (Fig. 1B), a common sequence overexpressed in CB medium in both strains was identified. This sequence corresponds to one differentially amplified fragment in PR1473 and to five differentially amplified fragments in PR1019 (Fig. 1A and B).

By searching DNA database by using BLASTn program and the overexpressed transcript from the strain PR1473 as a query, we were able to retrieve two plasmid sequences from *Lb. casei* group strains. In particular, high identities were found with the plasmid pLBPC-2 from *Lb. paracase*i subsp. *paracasei* JCM8130 (100%) and with the plasmid pNCD0151 from *Lb.casei* (99%). However, the homologous sequences are both annotated in the database records as non coding DNA regions.

By searching the protein database with the help of BLASTx program (Altschul et al., 1990) no significant similarity was found. On the contrary, the detection of protein homology by using HHPRED program (Soding et al., 2005) led us to identify the faecalis plasmid stabilization toxin (Fst) belonging to the type I TA systems (Weaver et al., 2009 Fozo et al., 2010), which has been identified for the first time on plasmid pAD1 from *Enterococcus faecalis* (Greenfield et al., 2000; Greenfield et al., 2001). We then proceeded analyzing a larger region of the plasmid pLBPC-2 with the aim of verifying the presence of the complete regulatory region characterizing the Fst toxin-antitoxin mechanism. By using bionformatic tools we were able to identify two small convergently transcribed RNAs, named RNAI and RNAII (Fig. 2A and B) containing a direct repeat sequence (DR in Fig. 2A), as previously described for Fst system (Weaver et al., 2009) .

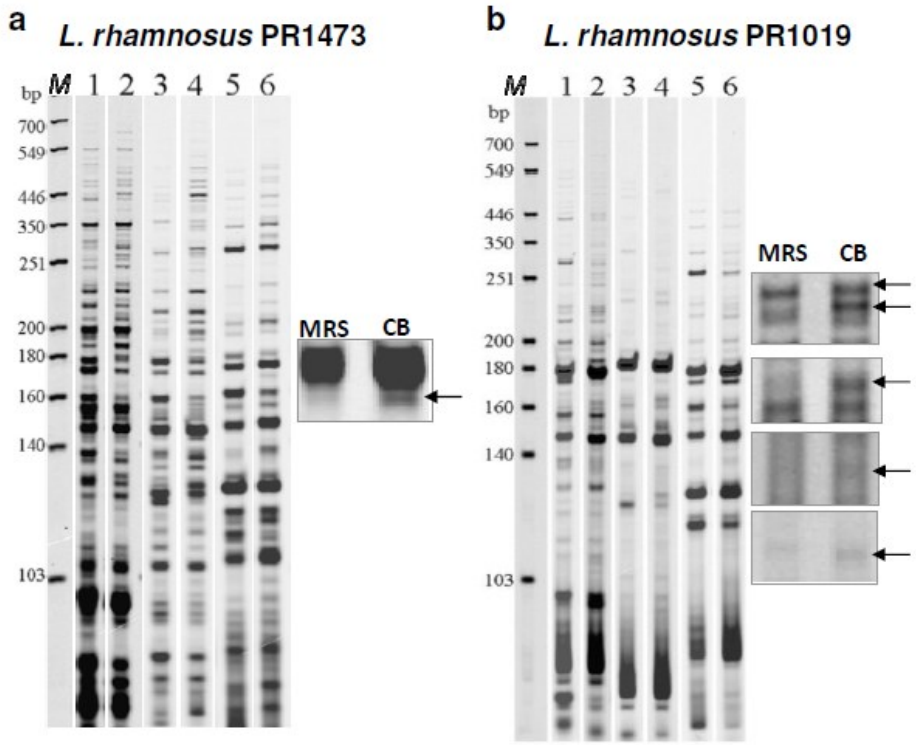

Figure 1 Electrophoretic patterns of cDNA-AFLP experiments conducted on *Lb. rhamnosus* strains. cDNA-AFLP analysis conducted for the strain PR1473 (a) and PR1019[3] (b) of *Lb. rhamnosus* grown in MRS and CB broth. In a and b: M, marker IRDye700 sizing standard; lanes 1, 3 and 5, cDNA-AFLP fingerprinting of *Lb. rhamnosus* strains cultured in MRS using EcoRI-AC/*MseI*-AT (lane 1), EcoRI-AT/*MseI*-AC (lane 3) and EcoRI-AT/*MseI*-AT (lane 5) primer combinations; lanes 2, 4 and 6, cDNA-AFLP fingerprinting of *Lb. rhamnosus* strains cultured in CB using EcoRI-AC/*MseI*-AT (lane 2), EcoRI-AT/*MseI*-AC (lane 4) and EcoRI-AT/ *MseI*-AT (lane 6) primer combinations. The overexpressed amplified fragments corresponding to the plasmid sequence are enlarged in the boxes for both strains.

Table 1 Comparison between Lpt TA system locus of *Lb. rhamnosus* PR1473 and the homologous regions identified in plasmid of *Lactobacillus* species.

Plasmid	Organism	Identity	GeneBank	Sequence range
pLBPC-2	*Lb. paracasei* subsp. *paracasei* JCM 8130	96%	AP012543	1598-1974
pNCD0151	*Lb.casei*	96%	Z50861	3644-4019
pLR001	*Lb. rhamnosus* HN001	74%	CP001156	3317-3693
pCD01 (sequence a)	*Lb. paracasei* subsp. *paracasei* NFBC338	73%	AY662330	3749-4122
pLP5401	*Lb. paracasei*	71%	KC812101	3715-4091
pRCEID7.6	*Lb. casei* TISTR1341	71%	JN793951	1051-1425
pMC11	*Lb. casei* MCJ	70%	KF986324	10797-10421
pMA3	*Lb. paracasei* MA3	68%	EU255257	1614-1238
pCD01 (sequence b)	*Lb. paracasei* subsp. *paracasei* NFBC338	68%	AY662330	9015-9390
pLP5402	*Lb. paracasei*	67%	KC812102	5330-4955
pLB925A03	*Lb. brevis*	62%	AB370336	8351-8724
pSJ2-8	*Lb. paracasei* subsp. *paracasei*	62%	FM246455	10850-10479
pLBPC-1	*Lb. paracasei* subsp. *paracasei* JCM 8130	60%	AP012542	883-1261

The plasmid sequences overexpressed in both strains of *Lb. rhamnosus* identified by cDNA-AFLP experiments, were found on RNAI. This molecule encodes an open reading frame for a 29 amino acids peptide with the putative role of toxin (Fig. 2B) that we named *Lactobacillus* plasmid toxin (Lpt). Moreover, a prediction of the secondary structures of RNAI and II (Fig. 2C) has shown peculiar folds which share specific features with the structures previously proposed for toxin and antitoxin RNAs of Fst TA system (Greenfield et al., 2000; Greenfield et al., 2001; Greenfield et al., 2001). In this system, it has been previously demonstrated that the upstream helix (UH) located at the 5' end is essential to ensure the required stability of the molecule (Shokeen et al., 2009), while the next stem loop region (SL), including the RBS sequence, is implicated in an intramolecular mechanism of translation repression (Shokeen et al., 2008). Similarly, in the structural model of the Lpt encoding RNA identified in this study (Fig. 2C), it is possible to found at the 5' end the UH followed by the SL region including most of the RBS sequence. Moreover, the DR sequence is mainly located in a single strand region in the antitoxin RNA II, while on RNAI, this region, which includes also the start codon, is located in a region characterized by a low pairing stability. It is therefore possible to postulated, as reported in a similar way for Fst system (Greenfield et al., 2001; Shokeen et al., 2008), that the toxin synthesis is controlled by two different mechanism of translation inhibition: the intramolecular regulation involving SL region which is able to sequester the RBS sequence, and the intermolecular mechanism mediated by the interaction between DR sequences located on RNAI and RNAII.

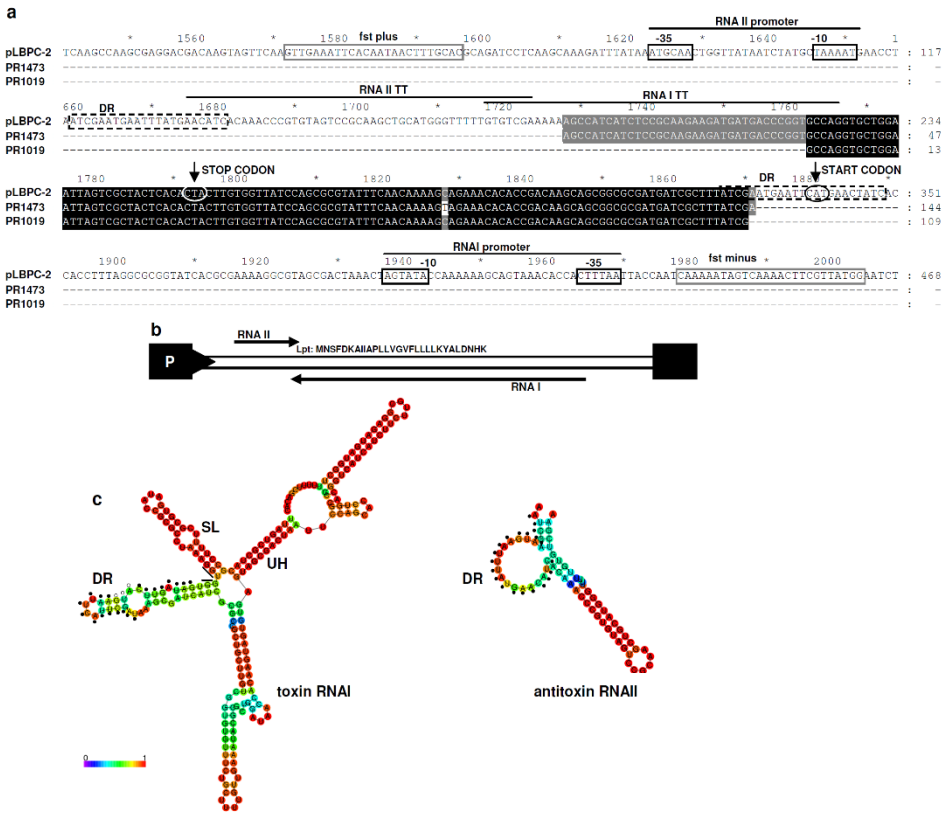

Figure 2 Structure of the identified toxin-antitoxin system locus. **a** alignment between the plasmid sequences overexpressed in *Lb. rhamnosus* PR1473 and PR1019 identified by cDNA-AFLP and the homologous sequence on the plasmid pLBPC-2 from *Lb. paracasei* (GenBank accession number AP012543 range 2009-1541). The predicted structure of the identified TA locus is shown: RNA I and RNAII promoters and TT are indicated with black lines; the direct repeat regions (DR) are shown in dashed black boxes; the start and the stop codons of the sequence encoding for the toxin peptide are identified by black arrows and black and white circles, respectively. gray boxes indicate the sequence employed to design the primers required to amplify the complete TA system region. **b** TA system structure scheme showing the two convergently transcribed RNA I and RNAII indicated by the arrows preceded by the promoter regions indicated by the letter P. The shaded box corresponds to the toxin coding region, and the peptide sequence is also reported. **c** secondary structures of RNAI and II molecules predicted by using RNAfold WebServer (for details see methods section). DR, direct repeat region; UH, upstream helix; SL stem-loop.

Characterization of Lpt system in Lb. rhamnosus

The region containing the TA system locus was PCR-amplified from plasmid DNA extracted from *Lb. rhamnosus* PR1473 and *Lb. rhamnosus* PR1019 grown in MRS or CB medium by using primers designed on the basis of pLBPC-2 sequence (Fig. 3, Table S1). The results were positive for all the conditions analyzed confirming the plasmid location of the type I toxin-antitoxin system and indicating that the plasmid carrying the TA system is not lost as a result of growth in these different conditions (Fig. S1).

The DNA regions amplified from the two different strains have been sequenced and compared to the plasmid pLBPC-2 deposited in GenBank database (Fig. 3A). The sequence from PR1473 strain shares a 97% identity with DNA from PR1019 strain and from pLBPC-2, while the sequence from PR1019 strain shares an identity of 99% with pLBPC-2. The region coding for Lpt peptide is thus identical in all the three compared sequences with the only exception of a conservative single nucleotide substitution in PR1019 strain (Fig. 3A). In order to experimentally verify the length of the in silico predicted coding transcript RNAI, RACE experiments were carried out starting from total enriched RNA extracted from PR1473 and PR1019 strains grown in CB medium. 3' RACE identified the end of RNA I transcript 8 nucleotides upstream of the predicted transcription termination site (Fig. 3a and b; Fig.S2a); otherwise, 5' RACE identified the start of RNA I transcript 27 nucleotides downstream of the predicted transcription starting site (Fig. 3a and c; Fig. S2b). The experimentally identified RNAI molecule is thus shorter than the predicted molecule and possesses a transcription starting site located at a non conventional distance from the promoter sequence; this result could suggest that the RNAI molecule has been processed after synthesis by a specific cleavage at its 5' end. The processing of toxin RNA before it could be translated has been previously proposed for Fst system as a possible intramolecular mechanism of translation regulation, but the processed molecule has never been identified (Weaver, 2012). To verify the activity of the predicted toxin, the corresponding cDNA was then cloned in the expression vector pSRKKm, and the recombinant plasmid was used to transform *E.coli* DH10bT1R.

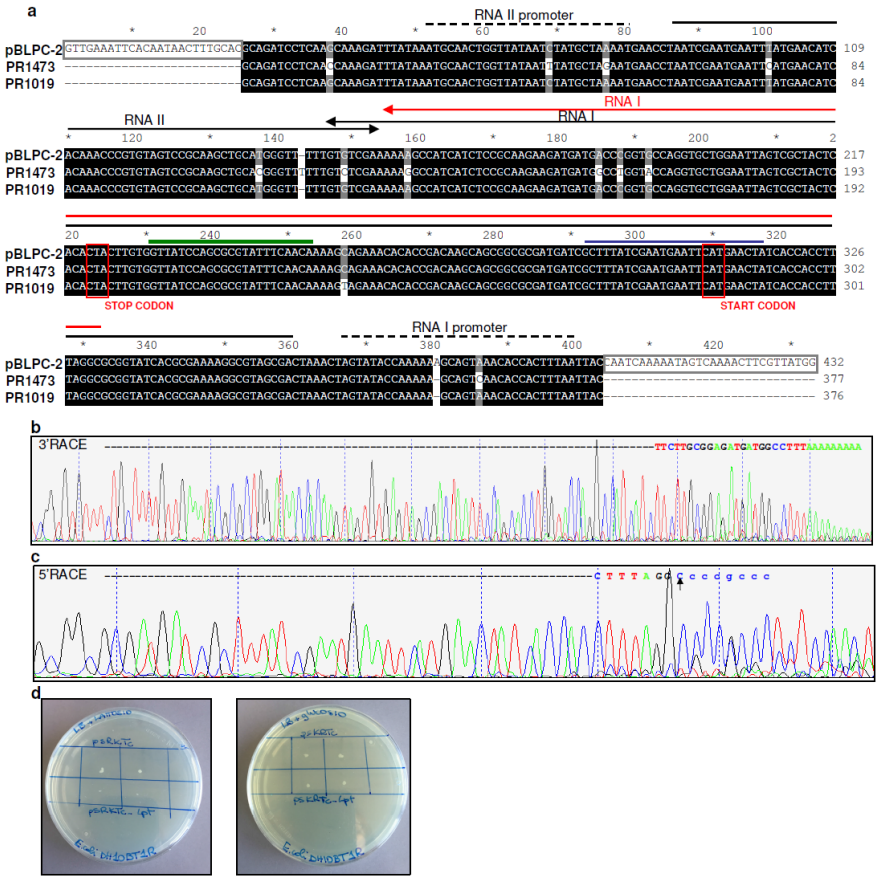

Figure 3 Characterization of TA system regions identified on *Lb. rhamnosus* strains. **a** alignment between the sequences amplified from *Lb. rhamnosus* PR1473 and 1019 and the plasmid pLBPC-2 from *Lb. paracasei*. Black arrows: predicted RNA I and RNAII molecules; red line: experimentally determined RNA I molecule. Black dashed lines: predicted promoter regions. Red boxes: start and stop codons of the toxin coding region on RNAI. Blue and green lines: sequence-specific primers employed in 3' RACE and 5' RACE experiments, respectively. **b** sequencing of the amplified fragment obtained by 3' RACE experiments corresponding to the 3' end of RNAI molecule. **c** sequencing of the amplified fragment obtained by 5' RACE experiments. Capital letters: 5' end of RNAI molecule, lowercase letters: primer sequence. **d** growth assays of transformed *E. coli* DH10bT1R on LB medium added of lactose (left) or of glucose (right). For every plate two different colonies of *E. coli* DH10bT1R transformed with the empty vector pSRKKm (top) or with the recombinant pSRKKm-lpt vector (bottom) are shown.

By comparing transformed cells cultured on plates in the presence or in the absence of lactose, it was possible to observe a noticeable difference in cell growth; in particular lactose, inducing the expression of the peptide, leads to a strong inhibition of cell growth. This result demonstrates not only that the predicted peptide has a toxic effect but also that it could be active in Gram- bacteria.

Detection of Lpt expression in cheese mimicking environment and cheese samples

To confirm the reliability of cDNA-AFLP results, a qRT PCR was carried out to evaluate the expression of Lpt in *Lb. rhamnosus* PR1473 and PR1019 grown in MRS or CB medium (Fig. 4a). For both strains, the quantity of Lpt encoding RNA resulted significantly different in the two growth conditions considered. In particular, upregulation occurred when cells were grown in CB medium in comparison with the growth in MRS medium, showing a transcription ratio of about 1.9 and 1.3 for PR1019 and PR1473, respectively.

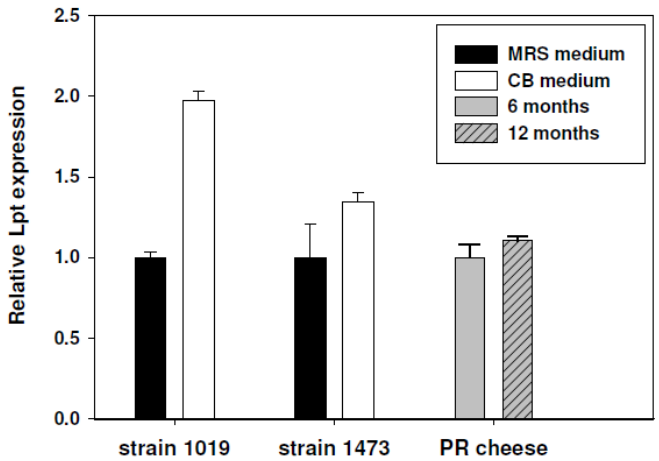

Figure 4

Detection by RT qPCR of Lpt expression for *Lb. rhamnosus* PR 1019 and PR 1473 strains grown in CB (black bars) compared to growth in MRS (white bars). Significant differences for both strains were determined by Student's t-test ($p < 0.05$ for 1473 strains and $p < 0.001$ for 1019 strain); Lpt expression detected in 12 month ripened PR cheese (dashed gray) and 6 month ripened PR cheese (gray). Experiments were conducted in triplicate, mean values with error bars indicative of standard deviations are reported.

In order to verify the expression of Lpt directly in cheese, qRT PCR was carried out on RNA extracted from two sample of Parmigiano Reggiano, at 6 and 12 months of ripening, in which *Lb. rhamnosus* represents the predominant species (Gatti et al., 2014). As shown in Fig. 4b the expression of Lpt was confirmed also *in situ*, with slight variations between the two samples.

Distribution of the Lpt peptide in bacteria

In order to assess the prevalence of this type I TA system in the bacterial world, a screening of nucleotide sequences from bacteria was performed by using BLASTn program and the entire TA locus identified in *Lb. rhamnosus* PR1473 as a query. Interestingly, all the sequences which showed significant identities are located on plasmids carried by bacteria belonging to *Lactobacillus* genus. In particular, we were able to identify 6 plasmids isolated from *Lb. paracasei*, 3 isolated from *Lb. casei*, 2 from *Lb. brevis* and 1 from *Lb. rhamnosus* (Table 1 and Fig. 5).

The comparison of the homologous plasmid regions containing the entire TA system (Fig. 5) have shown sequence identities ranging from 60% to 96% with Lpt TA locus. The highest identities were found with the same plasmids retrieved by searching the database using cDNA-AFLP fragments: pLBPC-2 and pNCD0151 from *Lb. paracasei* and *Lb. casei*, respectively. Interestingly, the plasmid pCD01 is characterized by the presence of two TA systems (sequence a and b in Table 1) both homologous to Lpt TA system located in distinct DNA regions. This system appears therefore to be specific for plasmids widespread among *Lactobacillus*, and in particular within *Lb. casei* group.

By analyzing all the identified plasmid sequences homologous to the Lpt TA system as a whole, we could verify that promoter and transcription termination regions, previously predicted for both RNA molecules (RNAI and II, Fig. 2a), are well conserved (Fig. 5). Moreover, it is possible to note that the single direct repeat region identified on pLBPC-2 (Fig. 2a) is actually present in all the compared plasmids, and it is characterized by two highly conserved regions separated by a short plasmid-specific non-conserved sequence (Fig. 5). Interestingly, the region corresponding to the predicted 5'end of RNAI molecule, comprising the UH and SL secondary structures likely responsible of functional roles, is highly conserved in all the plasmids (Fig. 5). As regards the translation signals, the start site on

RNAI, represented by an ATG or GTG codon preceded by the ribosome binding site, results highly conserved in all the analyzed sequences; stop codon is thus conserved in 12 sequences of 14, conversely in pSJ2-8 and pLBPC-1 it is located three codons downstream (Fig. 5).

Furthermore, in the plasmid pNCD0151, a single nucleotide deletion generates a frameshift leading to a five amino acid longer peptide (Fig. 5 and 6). Lpt and its homologues toxin peptides reported in Fig. 6, contain a number of amino acid residues ranging from 29 to 34 and share different sequence identities (from 38 to 100%). The comparison of the toxin sequences allows to distinguish the plasmids in six different groups (Fig. 6), each characterized by a different toxin peptide; nevertheless, all peptides share highly conserved amino acids in specific positions (Fig. 6), suggesting a structural or functional role for these residues.

Discussion

In this study we identified, for the first time in *Lactobacillus* species the presence of a TA system belonging to the type I located on plasmid DNA, whose toxic peptide was named *Lactobacillus* plasmid toxin (Lpt).

Initially, an overexpressed RNA was identified by transcriptomic analysis conducted on two strains of *Lb. rhamnosus* grown in a cheese like condition. Despite this sequence showed a significant similarity to plasmid sequences from *Lb. casei* group, its identification was complex. The nucleotide regions with high identities from the plasmids pLBPC-2 of *Lb. paracasei* and pNCD0151 of *Lb. casei*, are indeed annotated in the databases as non coding sequences; moreover, by using BLAST programs it was not possible to identify homologous proteins. These results are therefore consistent with data present in the literature; articles concerning toxin-antitoxin systems are effectively increasing, but only limited results are related to type I systems. For instance, 1196 results can be found querying the ISI web database by using the keywords “toxin antitoxin system”, but only 148 research papers were retrieved when the query is oriented specifically to type I systems. This is certainly due to the features of these systems, such as short length and hydrophobicity of the toxin, and challenge in the bioinformatic prediction of RNA molecules.

Recently a computational approach based on PSI-BLAST and TBLASTN has been used to discover a great number of type I TA systems encoded in

bacterial genomes (Fozo et al., 2010). With this study, known and new type I TA systems were identified in different bacteria lineage, but none of these referred to plasmid DNA of *Lactobacillus*.

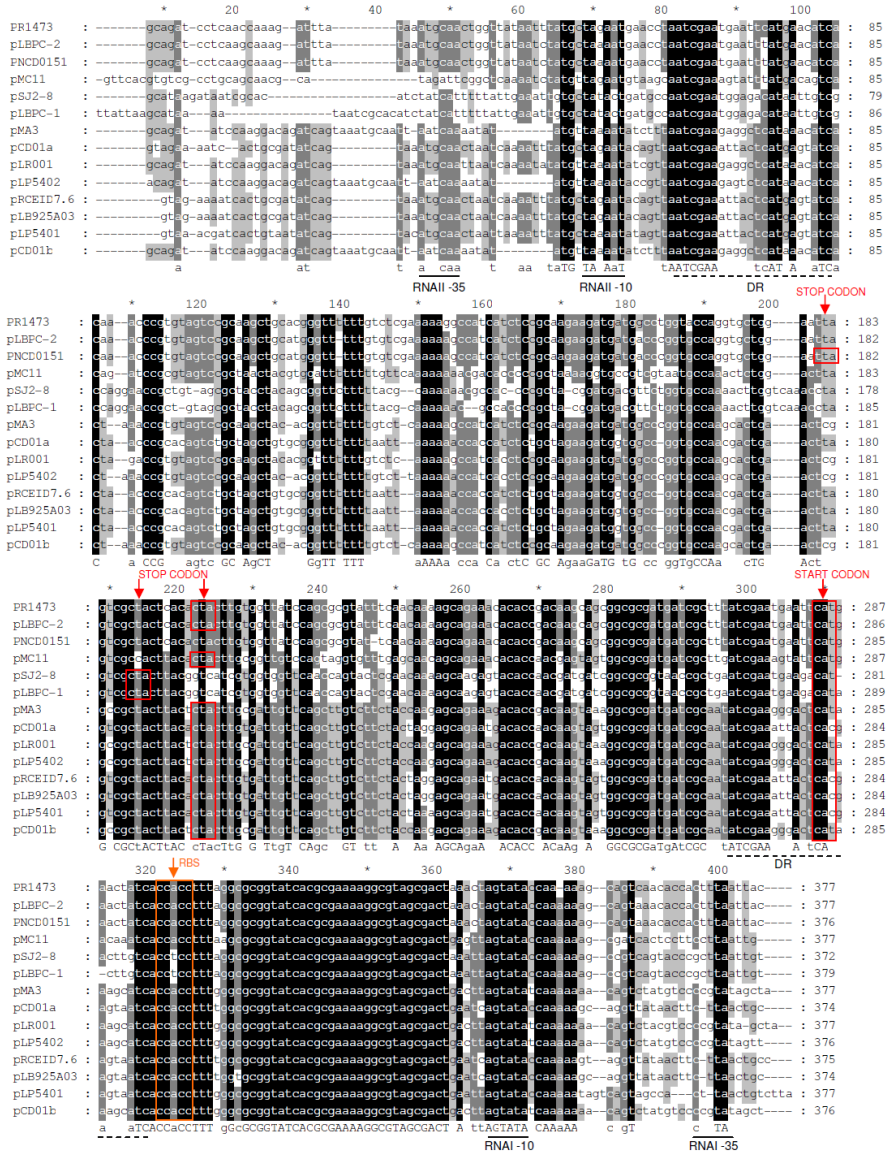

Figure 5 Alignment of Lpt TA system from *Lb. rhamnosus* PR1473 with homologous plasmid sequences identified in *Lactobacillus* species. RNA I and RNAII promoters are indicated with black lines; the direct repeat region (DR) is marked with the dashed black line. Start and stop codons on RNAI and RBS are boxed in red and orange, respectively. Accession numbers and specific sequence ranges of the aligned TA loci are reported in Table 1

Thanks to the searches carried out by using HHPRED, considered a more sensitive tool (Soding et al., 2005), it was possible to associate the overexpressed sequence to the Fst protein family. Fst is the toxin peptide of the type I TA system identified on the plasmid pAD1 from *Enterococcus faecalis* whose organization comprises two small, convergently transcribed RNAs, designated as RNAI and RNAII, which have the role of toxin and antitoxin, respectively (Greenfield et al., 2000). Since we found the same structural organization in *Lb. rhamnosus* TA system, we can postulate a common mechanism of regulation in which the region of RNAI including the toxin start codon is sequestered by the antitoxin (RNAII) preventing toxin translation. Regardless of this RNA-RNA interaction, an important role in the toxin production is assumed to be also played by the secondary structure of the 5' end of RNAI molecule. It has been indeed demonstrated for Fst system that an helix (UH) and a stem-loop (SL) region control the stability of the molecule and its translation, respectively (Shokeen et al., 2009; Shokeen et al., 2008). Interestingly, by employing RACE experiments (Fig. 3a, b and c), we were able to identify an RNA I molecule shorter than that in-silico predicted, suggesting that RNA I is processed after synthesis. Despite the processing of RNA I molecule has been previously postulated for Fst system as a mechanism able to activate the translation of RNAI with the synthesis of the toxic peptide (Weaver, 2012), the processed molecule has never been isolated before. The finding of a processed Lpt encoding RNA strongly supported the role of its 5' end in the regulation of translation process leading to toxin synthesis. Moreover, experiments conducted in *E. coli* have shown a strong inhibition of cell growth when the predicted toxin is expressed indicating that this peptide can have a toxic effect also on Gram- bacteria.

Interestingly, in this work we also find out that Lpt and its homologous peptides are widespread among *Lactobacillus* genus and in particular in *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*. Notably, the distribution of this type I TA system is restricted to plasmid DNA; when the entire locus of Lpt system was used as a query to search nucleotide database no significant homology was indeed observed with chromosomes. It is interesting to note that also in the multiple approach study conducted by (Fozo et al., 2010), which highlighted a wide distribution of the type I TA modules on plasmid and chromosome DNA and identified novel toxin families, the Lpt toxin and its homologous peptides were not reported. By

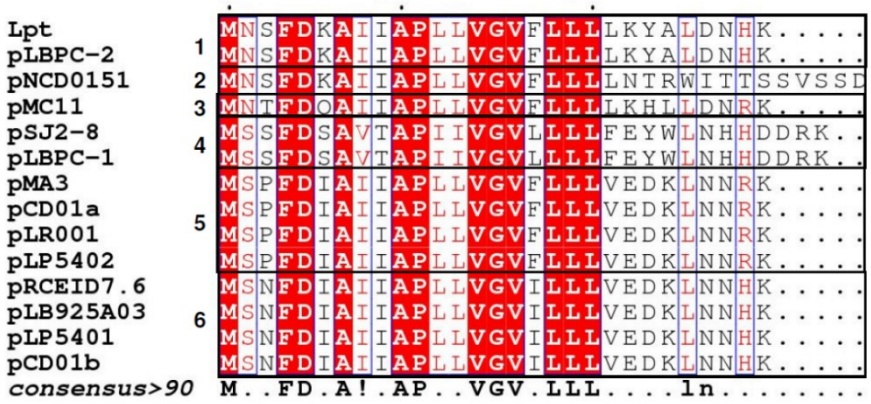

Figure 6 Alignment of peptides homologous to Lpt identified in Lactobacillus genus. Numbered boxes show the six different peptides identified.

considering the Lpt homologous peptides encoded by different plasmids it is possible to observe a distribution which seems to be strain-dependent instead of specie-dependent probably reflecting a possible horizontal gene transfer by conjugation. It is interesting to note that another study argued the employment of TAS, specifically type II TA encoded by the chromosome of *Lb. rhamnosus,* as a marker for strains diversity (Klimina et al., 2013). Species belonging to *Lb. casei* group, closely genetically related, were generally used in commercial and traditional fermented foods. In particular, in dairy products their presence is due to their natural occurrence in milk and to their ability of growing in curd and cheese becoming dominant species in ripening cheese (Gatti et al., 2014). The ripening represents a key moment for growth and survival of bacteria because different factors (salt, pH, low moisture, low temperature and nutrient starvation) cause environmental changes to which bacteria respond with stress-induced reactions. The ability to survive in hostile environment has been yet reported for species of *Lb. casei* group thanks to different mechanisms of adaptation (Lazzi et al., 2014; Zotta et al., 2014; Rossi et al., 2016). The presence of plasmids can contribute to stress tolerance, since they could encode for important traits conferring adaptive advantages (Cui et al., 2015).

Cui et al. (2015) have recently demonstrated that the strain *Lb. rhamnosus* 1019 activates genes involved in alternative metabolic pathways when grown on cheese medium. In our work we applied the

same experimental approach for a different strain of *Lb. rhamnosus* (1473) to identify the presence of a common behavior that could be linked to adaptation on cheese. By analyzing the overexpressed transcripts shared by both the strains we were able to identify Lpt TA system. The employed approach by using cDNA-AFLP does not require any prior sequence knowledge allowing the analysis of both known and unknown genes including those lowly expressed (Vuylsteke et al., 2007). We can thus speculate that thanks to this approach we manage to identify the new toxin Lpt, demonstrating not only its presence in *Lb. rhamnosus* but also its upregulation in conditions mimicking cheese ripening.

Despite the presence in the literature of relevant researches concerning the physiological role of TA systems, particularly conducted on pathogen microorganisms (Tiwari et al., 2015; Bukowski et al., 2012), TA system regulation has never been reported in food or in condition mimicking food environment. In this work, we initially found the upregulation of toxin encoding RNA in *Lb. rhamnosus* grown in a medium formulated with 20-month ripened cheese by cDNA AFLP (Fig. 1a and b) and qRT PCR (Fig. 4a) experiments. Moreover, we investigated the presence of toxin RNAI directly in two different samples of cheese (Parmigiano Reggiano) ripened 6 and 12 months, finding out similar and significant amounts of RNAI (Fig 4b).

The putative role of TA systems is generally referred to plasmid stability and maintenance but also other biological functions have been proposed. In particular, TA systems are considered elements to cope with stress, to guard against DNA loss and to protect against phage invasion (Van Melderen, 2010); moreover Bukowski et al. (2012) define the TA system as one of the most versatile global regulatory system in bacteria. The expression of Lpt in cheese suggests that in this conditions, cells could take an advantage from the presence of the plasmid, possibly providing genes which permit to survive to stress conditions. Future experiments to explain the functional significance of Lpt in the *Lactobacillus* stress adaptation will focus to the identification of the factors trigger the overexpression and to the investigation of the toxin mechanism of action on bacteria cells.

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Supplementary material

Supplementary figures and tables are listed in Appendix II.

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Chapter 6

Summary and conclusions

Summary and conclusions

The work presented in this thesis has focused on the adaptation of *Lb. casei* group to cheese environment. The research question has been examined through different approaches, using either model systems mimicking the conditions encountered from the bacteria during cheese-making, or the commercial cheese itself.

A previous study performed on dairy isolates of *Lb. rhamnosus* had highlighted how adaptation to cultivation in a cheese-based (CB) media affected the transcriptomic profile of the bacteria, compared to growth in MRS (Lazzi et al., 2014). The use of an untargeted cDNA-AFLP approach allowed to identify some transcripts, whose role in adaptation to growth in cheese condition was not extensively addressed before.

The newly identified transcripts discussed within this thesis are *spxB* gene, coding for pyruvate oxidase (POX) in *Lb. casei* group, and Lpt toxin, coded by a toxin-antitoxin (TA) system located on plasmid, the first to be identified in this bacterial group.

Pyruvate oxidase is an enzyme that leads to the conversion of pyruvate to acetyl-phosphate, which can subsequently be converted to acetate by acetate kinase, with simultaneous substrate-level phosphorylation of ADP into ATP. Therefore, activation of this pathway in cheese might provide supplementary energy for the bacteria.

The first step to understand whether it might have a general role in adaptation to the cheese environment was to assess its presence in strains of *Lb. casei* group. A screening was performed over 74 strains isolated from PR or GP with different ripening ages, and previously identified as belonging to this bacterial group. All the tested strains resulted positive for the presence of *spxB*, and sequencing of the amplified gene fragments revealed a wide variability of this sequence between isolates. The dissociation curves profiles of the amplified sequences were analysed through high-resolution melting (HRM), a post-PCR analysis that can be used to study sequence variation and polymorphic sites, identifying specific melting temperatures and melting profiles of the different amplicons (Patel, 2009; Reed et al., 2007).

The melting curves obtained allowed to differentiate isolates belonging to *Lb. rhamnosus* by *Lb. casei/paracasei* isolates. *spxB* activation was also

assessed in cheese, for the first time, by relative quantification with RT-qPCR, and led to discriminate the species activating this pathway at different ripening times.

The sequence variability of *spxB* gene allowed to monitor population dynamics of *Lb. casei* group during ripening of two different cheese-makings. Several works have studied the microbiota of cheeses by high throughput sequencing of 16S rRNA gene, that provides identification of the species involved in the manufacturing, and allows to estimate their relative abundance. In few studies, RNA can be preferred to prepare 16S RNA libraries, since it allows obtaining a structure of the microbiota that is potentially ascribable to the viable populations (De Filippis et al., 2016). Nevertheless, 16S rRNA does not provide discrimination among the species included in *Lb. casei* group, due to the close taxonomic relatedness. To correctly describe species that show a high similarity for their ribosomal genes, some studies have proposed the use of key genes with significant intra-species heterogeneity, such as the *lacZ* and *serB* genes of *Streptococcus thermophilus*, for monitoring beyond the species level (De Filippis et al., 2014; Ricciardi et al., 2016).

The composition of *Lb. casei* group metabolically active population was obtained by pyrosequencing of *spxB* transcript, at different stages of ripening. The evolution of *spxB* sequence types during ripening shows that the more abundant clusters at the beginning of ripening prevail throughout cheese ageing. Furthermore, some sequence types persist during all the ripening, while other dominate only at specific sampling points.

The enzymatic reaction catalyzed by POX enzyme uses oxygen as the final electron acceptor and, despite its activation could be detected *in situ* in real cheese, detection of different oxygen presence in full-sized cheese wheels is not trivial, and only few studies have dealt with oxygen permeation or measurement of related physico-chemical parameters in real-size cheeses (Thomas 1987, Colas et al., 2007).

Miniaturization of cheese manufacturing was used to overcome this limitation, in fact this process has successfully been applied to a wide variety of cheeses, and used to test several physical and microbiological parameters involved in different cheese manufacturing, including cooked hard cheeses (Bachmann et al., 2009; Erkus et al., 2013; Le Boucher et al.,

2016; Mucchetti et al., 2002; Ruggirello et al., 2016; Vélez et al., 2015). The protocol for the production of small scale cooked hard cheese developed in this thesis has the advantage of being high-throughput. This model was used to assess the metabolic response of *Lb. rhamnosus* to varying oxygen concentrations. The results have shown that the different conditions affected both the growth dynamics and the synthesis of organic acids and volatile organic compounds.

All the experimental data collected within the projects of this Thesis allowed to describe the distribution of *spxB* in dairy isolates of *Lb. casei* group and to study some of the factors leading to overexpression of this gene. Screening of dairy isolates revealed that the presence of this gene is widespread and, despite the strains were collected in the same niche, revealed an appreciable sequence variability (Chapter 2). But since the mere presence of a sequence in a certain species or environment does not always reflect its functionality, all the projects described have employed a transcript-based approach, allowing to detect variations in relative gene expression in the various conditions tested.

The results from growth in an *in vitro* model system have first shown the relevance of *spxB* activation during growth of *Lb. rhamnosus* in cheese like conditions (Lazzi et al., 2014). Model cheeses allowed to impose different oxygenation conditions during the fermentation, and results indicate that expression of this gene significantly decreases with lower O_2 concentration, despite being weakly expressed also in anaerobic conditions (Chapter 4). Finally, the results on *spxB* gene expression in cheese show that this gene is transcribed *in situ* in the real food product, and its activity decreases over ripening time (Chapter3).

In light of the results obtained we confirm that *spxB* functionality has a role in growth and survival of strains of *Lb. casei* group in cheese. Activation of this gene in early stages of cheese ripening might be induced by the presence of residual oxygen in the matrix, coupled with decreased lactose concentration. In these conditions, rerouting of intracellular pyruvate to POX pathway leads to an increased energy production, providing an adaptation strategy to the changing environment. As oxygen is progressively consumed, the relevance of this metabolism decreases, as is confirmed by the reduced transcription level of this gene.

The other adaptation strategy investigated in this thesis regarded a toxic peptide coded by a dairy isolate of *Lb. rhamnosus*. The system identified in the work described in Chapter 5 belongs to a plasmid-located type I TA system, the first to be described for the species *Lb. rhamnosus*.

The role of TA system is to ensure plasmid inheritance during cell replication, and failure to transmit the plasmid results in post-segregational killing of the newborn plasmid-free cells (Gerdes, 2000). It consists of a two-component system, coding for a stable toxin, and an unstable antitoxin, that prevents translation of the toxin-coding sequence.

The study of the plasmid-encoded TA system of *Lb. rhamnosus* was conducted with a two phase approach: evaluation of the expression of this system *in vitro*, using the already discussed CB, and detection of the transcript coding for Lpt toxin *in situ* in cheese (Chapter 5).

Lpt and its homologous peptides are widespread among *Lactobacillus* genus and in particular in *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*. Notably, the distribution of this type I TA system is restricted to plasmid DNA, and is probably the reason why this class of TA systems is only rarely described. Furthermore, the Lpt homologous peptides encoded by different plasmids show a distribution which seems to be strain-dependent instead of species-dependent, probably reflecting a possible horizontal gene transfer by conjugation.

The experiments conducted in *E. coli*, described in this thesis, show that when the Lpt toxin is expressed a strong inhibition of cell growth occurs, indicating how this peptide has a broad spectrum of action, including Gram - bacteria.

Quantification of the relative gene expression through RT-qPCR has shown an overexpression of the Lpt coding sequence in CB, compared to growth in MRS, confirming the results obtained with cDNA-AFLP. Also, the expression of Lpt in cheese suggests that the plasmid might provide the cells with functionalities that allow the adaptation of the bacteria to the environment.

The newly identified plasmid might encode important traits like resistance to phages or antibiotics, lactose catabolism, or production of proteolytic enzymes or bacteriocins, as reported for other LAB. Furthermore, TA systems are not limited to plasmid stability and maintenance, but they are involved in global regulation of the bacterial

cell in response to stress, and might lead to cell dormancy, reducing the energy requirement of the strain, and preventing the replication and diffusion of phages (Gerdes et al., 2005; Samson et al., 2013).

Transcriptional activity of *lpt* gene in response to CB cultivation and in food matrix indicates that the functionalities encoded in the plasmids or the toxin-antitoxin system itself are relevant for adaptation to cheese environment. Future projects will aim to a deep comprehension of how and to which extent technological parameters can influence TA system activation. Whether it only affects plasmid maintenance, or if it also triggers a wide cascade of signals involved in stress response, this system is an interesting target in terms of basic and applied research.

The works presented in this thesis have demonstrated the importance of a polyphasic approach to dissect the response of *Lb. casei* group to cheese environment. Indeed, cheese represent a biochemically complex environment, that challenges bacterial adaptive capacities with chemical, physical and microbiological changes.

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Appendix

Appendix I.

Supplementary table S1 Number of reads for each sample, and corresponding alpha diversity metrics . The values are reported as average of obtained with 10 rarefactions, +- standard deviation.

Cheese making	Ripening mo.	Reads	Shannon	obs.species	ESC	Chao1
CM1	2	7515	5.2 ± 0.02	760.1 ± 6.47	0.92	1738.4 ± 72.5
	6	8301	6.8 ± 0.02	1187.7 ± 10.6	0.87	3041.6 ± 201.6
	12	7959	6.0 ± 0.01	880.7 ± 11.2	0.91	2064.0 ± 127
CM2	2	6567	6.5 ± 0.01	1143.2 ± 4.5	0.87	3064.2 ± 69.4
	6	6235	6.0 ± 0.00	1018.6 ± 0.7	0.89	2447.5 ± 6.0
	12	9194	5.8 ± 0.03	992.3 ± 11.5	0.89	2728.2 ± 110.8

Supplementary figure S1 Multiple sequence alignment of a 642 bp fragment of *spxB* gene retrieved from 16 publicly available genome sequences (Table 1) and 4 representative sequences obtained in this study. Legend: N parsimony informative sites, · identical nucleotide, - missing data. The complete names of the strains/sequences are:

1) R1denovo813
2) R2denovo1158
3) PC1denovo781
4) PC2denovo1400
5) *Lb. casei*12A
6) *Lb. paracasei*N1115
7) *Lb. paracasei*8700:2
8) *Lb. casei*ATCC393
9) *Lb. rhamnosus*LOCK908
10) *Lb. rhamnosus*LOCK900
11) *Lb. casei*LOCK919
12) *Lb. casei*W56
13) *Lb. rhamnosus*ATCC8530
14) *Lb. casei* BD-II
15) *Lb. casei* LC2W
16) *Lb. casei* str.Zhang
17) *Lb. rhamnosus* ATCC53103
18) *Lb. rhamnosus* Lc705
19) *Lb. casei* BL23
20) *Lb. casei* ATCC334

```
                          *       20        *       40        *
1)  R1denovo81   : ..........A..T.............A..G........A..G..... :  50
2)  R2denovo11   : ..........A..T.............A..G........A..G..... :  50
3)  PC1denovo7   : ..........C..T.....C..........G..T..T.......... :  50
4)  PC2denovo1   : .................................................. :  50
5)  Lb. casei12A : .................................................. :  50
6)  Lb. paracase : .................................................. :  50
7)  Lb. paracase : .................................................. :  50
8)  Lb. caseiATC : ..........C..T.....C..........G..T..T.......... :  50
9)  Lb. rhamnosu : ..........A..T.............A..G........A..G..... :  50
10) Lb. rhamnosu : ..........A..T.............A..G........A..G..... :  50
11) Lb. caseiLOC : .................................................. :  50
12) Lb. caseiW56 : .................................................. :  50
13) Lb. rhamnosu : ..........A..T.............A..G........A..G..... :  50
14) Lb. caseiBD- : .................................................. :  50
15) Lb. caseiLC2 : .................................................. :  50
16) Lb. caseistr : .................................................. :  50
17) Lb. rhamnosu : ..........A..T.............A..G........A..G..... :  50
18) Lb. rhamnosu : ..........A..T.............A..G........A..G..... :  50
19) Lb. caseiBL2 : .................................................. :  50
20) Lb. caseiATC : ...........................................C..... :  50
                  GAAGACTGGGG AT CATAAtATTTATGG TT CCgGGcGG TC TTTGA
```

```
                      60        *       80        *       100
R1denovo81   : ...C........C.....T..T.....G..C..............C..G. : 100
R2denovo11   : ...C........C.....T..T.....G..C..............C..G. : 100
PC1denovo7   : ...C..G........A..T..T.....G........G..C..C....... : 100
PC2denovo1   : .................................................. : 100
Lb. casei12A : .................................................. : 100
Lb. paracase : .................................................. : 100
Lb. paracase : .................................................. : 100
Lb. caseiATC : ...C..G........A..T..T.....G........G..C..C....... : 100
Lb. rhamnosu : ...C........C.....T...........C..............C..G. : 100
Lb. rhamnosu : ...C........C.....T..T.....G..C..............C..G. : 100
Lb. caseiLOC : .................................................. : 100
Lb. caseiW56 : .................................................. : 100
Lb. rhamnosu : ...C........C.....T...........C..............C..G. : 100
Lb. caseiBD- : .................................................. : 100
Lb. caseiLC2 : .................................................. : 100
Lb. caseistr : .................................................. : 100
Lb. rhamnosu : ...C........C.....T...........C..............C..G. : 100
Lb. rhamnosu : ...C........C.....T...........C..............C..G. : 100
Lb. caseiBL2 : .................................................. : 100
Lb. caseiATC : .................................................. : 100
               TAG ACcATGAA GCgCT TA AATCG CG CACACcATtAAtTA GT C
```

```
                    *       120         *       140         *
R1denovo81   : ...............G..G.............G..T..C.....A... : 150
R2denovo11   : ...............G..G.............G..T..C.....A... : 150
PC1denovo7   : .......G..........C..T..G..A..A..A..T..........A : 150
PC2denovo1   : ................................................. : 150
Lb. casei12A : ................................................. : 150
Lb. paracase : ................................................. : 150
Lb. paracase : ................................................. : 150
Lb. caseiATC : .......G..........C..T..G..A..C..A..C..........A : 150
Lb. rhamnosu : ...................G.............G..T..C.....A... : 150
Lb. rhamnosu : ...................G.............G..T..C.....A... : 150
Lb. caseiLOC : ................................................. : 150
Lb. caseiW56 : ................................................. : 150
Lb. rhamnosu : ...................G.............G..T..C.....A... : 150
Lb. caseiBD- : ................................................. : 150
Lb. caseiLC2 : ................................................. : 150
Lb. caseistr : ................................................. : 150
Lb. rhamnosu : ...................G.............G..T..C.....A... : 150
Lb. rhamnosu : ...................G.............G..T..C.....A... : 150
Lb. caseiBL2 : ................................................. : 150
Lb. caseiATC : ................................................. : 150
               AGGTTCGcCATGAAGAaGT GGcGCaTTgGCtGC GC GG GAAGC AAg


                   160         *       180         *       200
R1denovo81   : ........A..C..T.....C........G..A..A..........C.. : 200
R2denovo11   : ........A..C..T.....C........G..A..A..........C.. : 200
PC1denovo7   : .....T..C..C..T..C..A..............T..G.....G..A.. : 200
PC2denovo1   : ......................A........C..........C..C.. : 200
Lb. casei12A : ................................................. : 200
Lb. paracase : ................................................. : 200
Lb. paracase : ................................................. : 200
Lb. caseiATC : .....T..C..C..T..C..A..............T..G.....G..A.. : 200
Lb. rhamnosu : ........A..C..T.....C........G..A..A..........C.. : 200
Lb. rhamnosu : ........A..C..T..C..C........G..A..A..........C.. : 200
Lb. caseiLOC : ................................................. : 200
Lb. caseiW56 : ................................................. : 200
Lb. rhamnosu : ........A..C..T.....C........G..A..A..........C.. : 200
Lb. caseiBD- : ................................................. : 200
Lb. caseiLC2 : ................................................. : 200
Lb. caseistr : ................................................. : 200
Lb. rhamnosu : ........A..C..T.....C........G..A..A..........C.. : 200
Lb. rhamnosu : ........A..C..T.....C........G..A..A..........C.. : 200
Lb. caseiBL2 : ................................................. : 200
Lb. caseiATC : ................................................. : 200
               GTAACgGG CG AT GGgGC ACgTTTGG Tc gc GGtCCTGGtGc GT
```

```
                    *       220         *       240         *
R1denovo81   : ......GT.A..C..T..A.....T..G.TA..C...C....A..G..A. : 250
R2denovo11   : ......GT.A..C..T..A.....T..G..A..C...C....A..G..A. : 250
PC1denovo7   : A.....G.................T..T..A..C...C.C.....G.... : 250
PC2denovo1   : G.............................................. : 250
Lb. casei12A : .............................................. : 250
Lb. paracase : .............................................. : 250
Lb. paracase : .............................................. : 250
Lb. caseiATC : A.....G.................T..T..A..C...C.......G.... : 250
Lb. rhamnosu : ......GT.A..C..T..A.....T..G..A..C...C....A..G..A. : 250
Lb. rhamnosu : ......GT.A..C..T..A.....T..G..A..C...C....A..G..A. : 250
Lb. caseiLOC : .............................................. : 250
Lb. caseiW56 : .............................................. : 250
Lb. rhamnosu : ......GT.A..C..T..A.....T..G..A..C...C....A..G..A. : 250
Lb. caseiBD- : .............................................. : 250
Lb. caseiLC2 : .............................................. : 250
Lb. caseistr : .............................................. : 250
Lb. rhamnosu : ......GT.A..C..T..A.....T..G..A..C...C....A..G..A. : 250
Lb. rhamnosu : ......GT.A..C..T..A.....T..G..A..C...C....A..G..A. : 250
Lb. caseiBL2 : .............................................. : 250
Lb. caseiATC : .............................................. : 250
               tCATCT  T AA GG TT TATGA GC Ca TA GAT AtGT CC GT T
```

```
                   260         *       280         *       300
R1denovo81   : .A..........C..G..G..G.....A........T.....C.....T : 300
R2denovo11   : .A..........C..G..G..G.....A........T.....C.....T : 300
PC1denovo7   : ....T.....C.....G..G..G...................C.T.... : 300
PC2denovo1   : .............................................. : 300
Lb. casei12A : .............................................. : 300
Lb. paracase : .............................................. : 300
Lb. paracase : .............................................. : 300
Lb. caseiATC : ....T.....C.....G..G..G...................C.T.... : 300
Lb. rhamnosu : .A..........C..G..G..G.....A........T.....C.....T : 300
Lb. rhamnosu : .A..........C..G..G..G.....A........T.....C.....T : 300
Lb. caseiLOC : .............................................. : 300
Lb. caseiW56 : .............................................. : 300
Lb. rhamnosu : .A..........C..G..G..G.....A........T.....C.....T : 300
Lb. caseiBD- : .............................................. : 300
Lb. caseiLC2 : .............................................. : 300
Lb. caseistr : .............................................. : 300
Lb. rhamnosu : .A..........C..G..G..G.....A........T.....C.....T : 300
Lb. rhamnosu : .A..........C..G..G..G.....A........T.....C.....T : 300
Lb. caseiBL2 : .............................................. : 300
Lb. caseiATC : .............................................. : 300
               T GCaTTGGTaGG CA GT CC ACTGC GCcATGAA ACCAA TaTTT
```

```
                    *       320         *       340         *
R1denovo81    : ..G..........A.....G.....C..C........G..T..T..T.. : 350
R2denovo11    : ..G........T..A.....G.....C..C.....A..G..T..T..T.. : 350
PC1denovo7    : ..G........T.....T..G........C.....T..G..T.....T.. : 350
PC2denovo1    : ..................................................... : 350
Lb. casei12A  : ..................................................... : 350
Lb. paracase  : ..................................................... : 350
Lb. paracase  : ..................................................... : 350
Lb. caseiATC  : ..G........T.....T..G........C.....T..G..T.....T.. : 350
Lb. rhamnosu  : ..G........T..A.....G.....C..C.....A..G..T..T..T.. : 350
Lb. rhamnosu  : ..G........T..A.....G.....C..C........G..T..T..T.. : 350
Lb. caseiLOC  : ..................................................... : 350
Lb. caseiW56  : ..................................................... : 350
Lb. rhamnosu  : ..G........T..A.....G.....C..C.....A..G..T..T..T.. : 350
Lb. caseiBD-  : ..................................................... : 350
Lb. caseiLC2  : ..................................................... : 350
Lb. caseistr  : ..................................................... : 350
Lb. rhamnosu  : ..G........T..A.....G.....C..C........G..T..T..T.. : 350
Lb. rhamnosu  : ..G........T..A.....G.....C..C.....A..G..T..T..T.. : 350
Lb. caseiBL2  : ..................................................... : 350
Lb. caseiATC  : ..................................................... : 350
                CA GAAATGAA GA AAcCC ATGTT GC GATGT TC GT TA AA CG


                     360         *       380         *       400
R1denovo81    : G..T........G....CA........A..C.....C..T..A..C..C. : 400
R2denovo11    : G..T........G....CA........G..C.....C..T..A..C..C. : 400
PC1denovo7    : G.....G.....G....CT..G.....A...........T..A..C.... : 400
PC2denovo1    : ..................................................... : 400
Lb. casei12A  : ..................................................... : 400
Lb. paracase  : ..................................................... : 400
Lb. paracase  : ..................................................... : 400
Lb. caseiATC  : G.....G.....G....CT..G.....A...........T..A..C.... : 400
Lb. rhamnosu  : G..T........G....CA........G..C.....C..T..A..C..C. : 400
Lb. rhamnosu  : G..T........G....CA........A..C.....C..T..A..C..C. : 400
Lb. caseiLOC  : ..................................................... : 400
Lb. caseiW56  : ..................................................... : 400
Lb. rhamnosu  : G..T........G....CA........G..C.....C..T..A..C..C. : 400
Lb. caseiBD-  : ..................................................... : 400
Lb. caseiLC2  : ..................................................... : 400
Lb. caseistr  : ..................................................... : 400
Lb. rhamnosu  : G..T........G....CA........A..C.....C..T..A..C..C. : 400
Lb. rhamnosu  : G..T........G....CA........G..C.....C..T..A..C..C. : 400
Lb. caseiBL2  : ..................................................... : 400
Lb. caseiATC  : ..................................................... : 400
                AC GCcATGAC GCTG  CAaTTGCC CA GTTGT GA GC AT C
```

```
                    *       420         *       440         *
R1denovo81   : ....A..G......T..C.A..C.....................C...... : 450
R2denovo11   : .C..A.........T..C.A..C.....T.............T...... : 450
PC1denovo7   : .......G..C......C.G..T..G..A..G.....G.....T..A... : 450
PC2denovo1   : ....................................T............ : 450
Lb. casei12A : ....................................T............ : 450
Lb. paracase : ....................................A............ : 450
Lb. paracase : ....................................T............ : 450
Lb. caseiATC : .......G..C......C.G..T..G..A..G.....G.....T..A... : 450
Lb. rhamnosu : .C..A.........T..C.A..C.....T.............T...... : 450
Lb. rhamnosu : ....A..G......T..C.A..C.....................T...... : 450
Lb. caseiLOC : ....................................T............ : 450
Lb. caseiW56 : ....................................A............ : 450
Lb. rhamnosu : .C..A.........T..C.A..C.....T.............T...... : 450
Lb. caseiBD- : ....................................A............ : 450
Lb. caseiLC2 : ....................................A............ : 450
Lb. caseistr : ....................................T............ : 450
Lb. rhamnosu : ....A..G......T..C.A..C.....T.............T...... : 450
Lb. rhamnosu : .C..A.........T..C.A..C.....T.............T...... : 450
Lb. caseiBL2 : ....................................A............ : 450
Lb. caseiATC : ....................................T............ : 450
               GtCA GC TAtAAG AT A GG GTtGC GTtGTGAC ATTCC AAgGAT


                   460         *       480         *       500
R1denovo81   : C.C......CAGG..........T........T.....G........T.. : 500
R2denovo11   : C.C......CAGG.A..C.....T........T.....G..T.....T.. : 500
PC1denovo7   : C.T......GAA..A.....C...........T..G...........C.. : 500
PC2denovo1   : ................................................... : 500
Lb. casei12A : ................................................... : 500
Lb. paracase : ................................................... : 500
Lb. paracase : ................................................... : 500
Lb. caseiATC : C.T......GAA..A.....C...........T..G...........C.. : 500
Lb. rhamnosu : C.C......CAGG.A..C.....T........T.....G..T.....T.. : 500
Lb. rhamnosu : C.C......CAGG..........T........T.....G........T.. : 500
Lb. caseiLOC : ................................................... : 500
Lb. caseiW56 : ................................................... : 500
Lb. rhamnosu : C.C......CAGG.A..C.....T........T.....G..T.....T.. : 500
Lb. caseiBD- : ................................................... : 500
Lb. caseiLC2 : ................................................... : 500
Lb. caseistr : ................................................... : 500
Lb. rhamnosu : C.C......CAGG..........T........T.....G........T.. : 500
Lb. rhamnosu : C.C......CAGG.A..C.....T........T.....G..T.....T.. : 500
Lb. caseiBL2 : ................................................... : 500
Lb. caseiATC : ................................................... : 500
               T GGTTGG    A ATtGAtGA AATTATGT TCaTC GCcAATCT TA
```

```
                    *       520         *       540         *
R1denovo81   : T..A.T....T.A..A.....A..G..T..C..G..A.-.GA.AATC... : 549
R2denovo11   : T..A......T.A........A..G..T..C..G..A.-.G..AA.C... : 549
PC1denovo7   : ..............A.....G..G..T.....A....-....AA.G..C : 549
PC2denovo1   : ..........................................-.......... : 549
Lb. casei12A : ..........................................-.......... : 549
Lb. paracase : ..............................A.......-.......... : 549
Lb. paracase : ..........................................-.......... : 549
Lb. caseiATC : ...A...........A.....G..G..T.....A....-....AA.G..C : 549
Lb. rhamnosu : T..A......T.A........A..G..T.....G..A.-.G..AA.C... : 549
Lb. rhamnosu : T..A......T.A........A..G..T..C..G..A.-.G..AA.C... : 549
Lb. caseiLOC : ......................C.................-.......... : 549
Lb. caseiW56 : ..............................A.......-.......... : 549
Lb. rhamnosu : T..A......T.A........A..G..T.....G..A.-.G..AA.C... : 549
Lb. caseiBD- : ..............................A.......-.......... : 549
Lb. caseiLC2 : ..............................A.......-.......... : 549
Lb. caseistr : ......................C.................-.......... : 549
Lb. rhamnosu : T..A......T.A........A..G..T..C..G..A.-.G..AA.C... : 549
Lb. rhamnosu : T..A......T.A........A..G..T.....G..A.-.G..AA.C... : 549
Lb. caseiBL2 : ..............................A.......-.......... : 549
Lb. caseiATC : ..........................................-.......... : 549
               CA AaGCCG T TTgCCTGA CC GA CC GA CA G T gC  c GCt


                      560         *       580         *       600
R1denovo81   : ...AGC..T.......G...C..AT....G..TC.A..C.....T...-. : 598
R2denovo11   : ...AGC..T..A..G.....C........G...C.A..C.....T...-. : 598
PC1denovo7   : ...AGC...A.C....G...C........G..T..A..C.....T...-. : 598
PC2denovo1   : ................................................... : 599
Lb. casei12A : ..............................................-. : 598
Lb. paracase : A.............................................-. : 598
Lb. paracase : ..............................................-. : 598
Lb. caseiATC : ...AGC...A.C....G...C........G..T..A..C.....T...-. : 598
Lb. rhamnosu : ...AGC..T..A..G.....C..A.....G...C.A..C.....T...-. : 598
Lb. rhamnosu : ...AGC..T..A..G.....C........G...C.A..C.....T...-. : 598
Lb. caseiLOC : ..............................................-. : 598
Lb. caseiW56 : ..............................................-. : 598
Lb. rhamnosu : ...AGC..T..A..G.....C..A.....G...C.A..C.....T...-. : 598
Lb. caseiBD- : ..............................................-. : 598
Lb. caseiLC2 : ..............................................-. : 598
Lb. caseistr : ..............................................-. : 598
Lb. rhamnosu : ...AGC..T..A..G.....C..A.....G..TC.A..C.....T...-. : 598
Lb. rhamnosu : ...AGC..T..A..G.....C..A.....G...C.A..C.....T...-. : 598
Lb. caseiBL2 : ..............................................-. : 598
Lb. caseiATC : ..............................................-. : 598
               tGG   AT tT AA GaTGC AA aAGCC ATc T TA GTTGG AAT G
```

```
                    *       620         *       640
R1denovo81    : .T.-....T..A-..T..-...T..A.-....T..A...TC.GA....- : 642
R2denovo11    : .T.-.A..T..C-..T..-...T.TA.-....T..A...TC.GA....- : 642
PC1denovo7    : ...-....T.-.-..C..G......A.-.T..T......TC.GA....- : 642
PC2denovo1    : ...-......-.-......-.......-..........-.......... : 642
Lb. casei12A : ...-.........-......-.......-..........-.......... : 642
Lb. paracase : ...-.........-......-.......-..........-.......... : 642
Lb. paracase : ...-.........-......-.......-..........-.......... : 642
Lb. caseiATC : ...-....T...-..C..G-.......-.T..T......TC.GA....- : 642
Lb. rhamnosu : .T.-....T..A-..T..-...T..A.-....T..A...TC.GA....- : 642
Lb. rhamnosu : .T.-....T..A-..T..-...T..A.-....T..A...TC.GA....- : 642
Lb. caseiLOC : ...-.........-......-.......-..........-.......... : 642
Lb. caseiW56 : ...-.........-......-.......-..........-.......... : 642
Lb. rhamnosu : .T.-....T..A-..T..-...T..A.-....T..A...TC.GA....- : 642
Lb. caseiBD- : ...-.........-......-.......-..........-.......... : 642
Lb. caseiLC2 : ...-.........-......-.......-..........-.......... : 642
Lb. caseistr : ...-.........-......-.......-..........-.......... : 642
Lb. rhamnosu : .T.-....T..A-..T..-...T..A.-....T..A...TCAGA....- : 642
Lb. rhamnosu : .T.-....T..A-..T..-...T..A.-....T..A...TC.GA....- : 642
Lb. caseiBL2 : ...-.........-......-.......-..........-.......... : 642
Lb. caseiATC : ...-.........-......-.......-..........-.......... : 642
               G CgCG Gg  GC CG  GA A TcAT GC TT   c  AAAA
```

Supplementary figure S2 Multiple sequence alignment of the translated *spxB* gene retrieved from 16 publicly available genome sequences (Table 1) and 4 representative sequences obtained in this study (translation frame=+1). The polymorphic site shared with *Lb. casei* ATCC 393 is highlighted in red (see text for more details). Legend: N parsimony informative sites, · identical nucleotide, - missing data. The complete names of the strains/sequences are:

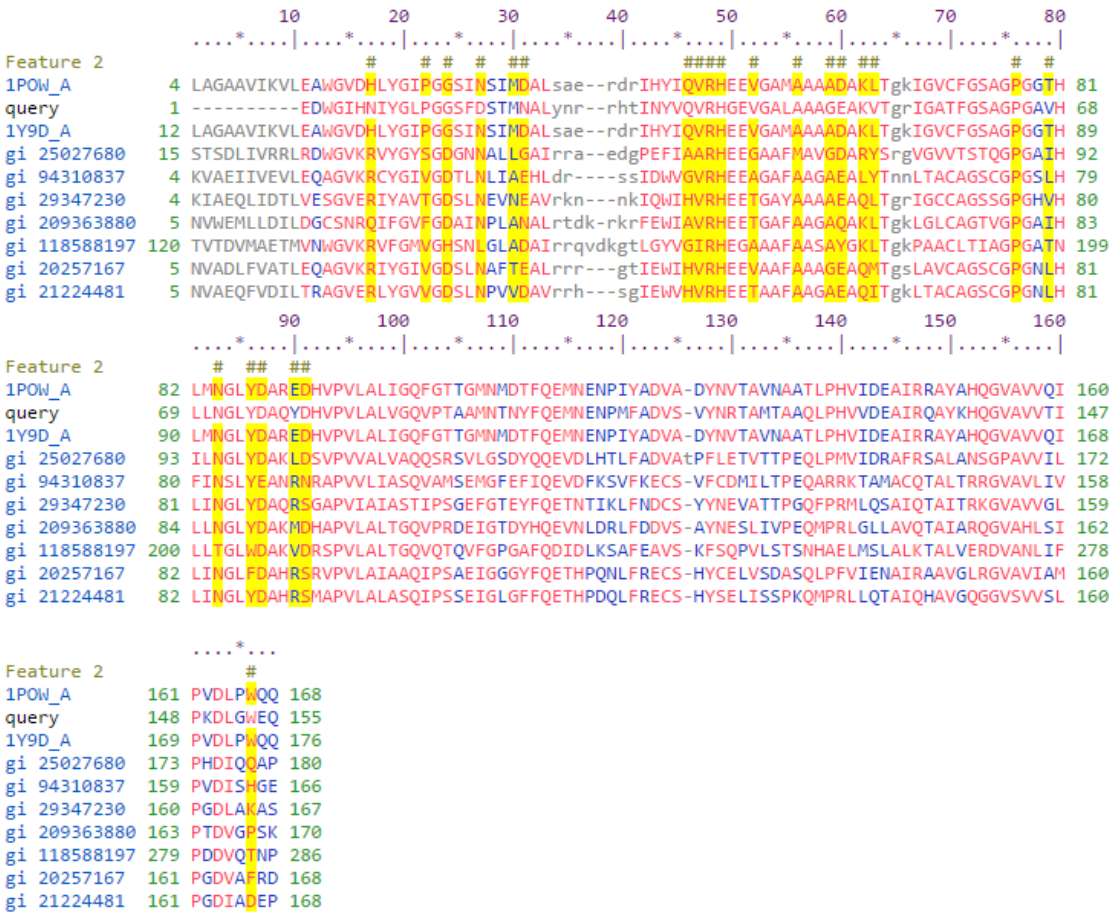
1) R1denovo813
2) R2denovo1158
3) PC1denovo781
4) PC2denovo1400
5) *Lb. casei*12A
6) *Lb. paracasei*N1115
7) *Lb. paracasei*8700:2
8) *Lb. casei*ATCC393
9) *Lb. rhamnosus*LOCK908
10) *Lb. rhamnosus*LOCK900
11) *Lb. casei*LOCK919
12) *Lb. casei*W56
13) *Lb. rhamnosus*ATCC8530
14) *Lb. casei* BD-II
15) *Lb. casei* LC2W
16) *Lb. casei* str.Zhang
17) *Lb. rhamnosus* ATCC53103
18) *Lb. rhamnosus* Lc705
19) *Lb. casei* BL23
20) *Lb. casei* ATCC334

```
                  *        20         *        40         *
    1)  R1denovo81 : ..................................................... :  50
    2)  R2denovo11 : ..................................................... :  50
    3)  PC1denovo7 : ..................................................... :  50
    4)  PC2denovo1 : ..................................................... :  50
    5)  Lb. casei12A : ................................................... :  50
    6)  Lb. paracase : ................................................... :  50
    7)  Lb. paracase : ................................................... :  50
    8)  Lb. caseiATC : ................................................... :  50
    9)  Lb. rhamnosu : ................................................... :  50
    10) Lb. rhamnosu : ................................................... :  50
    11) Lb. caseiLOC : ................................................... :  50
    12) Lb. caseiW56 : ................................................... :  50
    13) Lb. rhamnosu : ................................................... :  50
    14) Lb. caseiBD- : ................................................... :  50
    15) Lb. caseiLC2 : ................................................... :  50
    16) Lb. caseistr : ................................................... :  50
    17) Lb. rhamnosu : ................................................... :  50
    18) Lb. rhamnosu : ................................................... :  50
    19) Lb. caseiBL2 : ................................................... :  50
    20) Lb. caseiATC : ................................................... :  50
                      EDWGIHNIYGLPGGSFDSTMNALYNRRHTINYVQVRHEEVGALAAAGEAK

                     60         *         80         *        100
R1denovo81   : ..............................H..................... : 100
R2denovo11   : ..............................H..................... : 100
PC1denovo7   : ..............................H..................... : 100
PC2denovo1   : ..................................................... : 100
Lb. casei12A : ..................................................... : 100
Lb. paracase : ..................................................... : 100
Lb. paracase : ..................................................... : 100
Lb. caseiATC : ..............................H..................... : 100
Lb. rhamnosu : ..............................H..................... : 100
Lb. rhamnosu : ..............................H..................... : 100
Lb. caseiLOC : ..................................................... : 100
Lb. caseiW56 : ..................................................... : 100
Lb. rhamnosu : ..............................H..................... : 100
Lb. caseiBD- : ..................................................... : 100
Lb. caseiLC2 : ..................................................... : 100
Lb. caseistr : ..................................................... : 100
Lb. rhamnosu : ..............................H..................... : 100
Lb. rhamnosu : ..............................H..................... : 100
Lb. caseiBL2 : ..................................................... : 100
Lb. caseiATC : ..................................................... : 100
                VTGRIGATFGsaGPGaVHLLNGLYDAqYD VPVLALVGQVPTAAMNTN5F
```

```
                    *       120         *       140         *
R1denovo81    : ......................A..............YQ..........  : 150
R2denovo11    : ......................A..............YQ..........  : 150
PC1denovo7    : ......................A..............Q...........  : 150
PC2denovo1    : ..................................................  : 150
Lb. casei12A  : ..................................................  : 150
Lb. paracase  : ..................................................  : 150
Lb. paracase  : ..................................................  : 150
Lb. caseiATC  : ......................A..............Q...........  : 150
Lb. rhamnosu  : ......................A..............YQ..........  : 150
Lb. rhamnosu  : ......................A..............YQ..........  : 150
Lb. caseiLOC  : ..................................................  : 150
Lb. caseiW56  : ..................................................  : 150
Lb. rhamnosu  : ......................A..............YQ..........  : 150
Lb. caseiBD-  : ..................................................  : 150
Lb. caseiLC2  : ..................................................  : 150
Lb. caseistr  : ..................................................  : 150
Lb. rhamnosu  : ......................A..............YQ..........  : 150
Lb. rhamnosu  : ......................A..............YQ..........  : 150
Lb. caseiBL2  : ..................................................  : 150
Lb. caseiATC  : ..................................................  : 150
                QEMNENPMFADVSVYNRTAMTA QLPHVVDEAIRQAYK  GVAVVTIPKD

                      160         *       180         *       200
R1denovo81    : ...Q...................E...E.-DNR.EHF....I..S.R.--  : 197
R2denovo11    : ...Q...................E...E.-GNR.EHFKG.....P.R.--  : 197
PC1denovo7    : ...E...................E...E.-.NG.EHH.......F.R.--  : 197
PC2denovo1    : ..............................-.....................  : 199
Lb. casei12A  : ..............................-.....................-  : 198
Lb. paracase  : ..............................-...-.................-  : 197
Lb. paracase  : ..............................-.....................-  : 198
Lb. caseiATC  : ...E...................E...E.-.NG.EHH.......F.R.--  : 197
Lb. rhamnosu  : ...Q...................E...E.-GNR.EHFKG..K..P.R.--  : 197
Lb. rhamnosu  : ...Q...................E...E.-GNR.EHFKG.....P.R.--  : 197
Lb. caseiLOC  : ..............................-.....................-  : 198
Lb. caseiW56  : ..............................-.....................-  : 198
Lb. rhamnosu  : ...Q...................E...E.-GNR.EHFKG..K..P.R.--  : 197
Lb. caseiBD-  : ..............................-.....................-  : 198
Lb. caseiLC2  : ..............................-.....................-  : 198
Lb. caseistr  : ..............................-.....................-  : 198
Lb. rhamnosu  : ...Q...................E...E.-GNR.EHFKG..K..S.R.--  : 197
Lb. rhamnosu  : ...Q...................E...E.-GNR.EHFKG..K..P.R.--  : 197
Lb. caseiBL2  : ..............................-.....................-  : 198
Lb. caseiATC  : ..............................-.....................-  : 198
                LGW 2IDDNYVSSANLYQkPLLP PDP Q    l     C2 A1 6 W
```

```
                     *
R1denovo81    : V-..-S-M.-...SE. : 209
R2denovo11    : V-..-S-M--...SE. : 208
PC1denovo7    : .-.--P...-...SE. : 209
PC2denovo1    : .-.--.-..-..-... : 209
Lb. casei12A  : .-..-.-..-..-... : 209
Lb. paracase  : .-..-.-..-..-... : 208
Lb. paracase  : .-..-.-..-..-... : 209
Lb. caseiATC  : .-..-P-..-...SE. : 209
Lb. rhamnosu  : V-..-S-M.-...SE. : 209
Lb. rhamnosu  : V-..-S-M.-...SE. : 209
Lb. caseiLOC  : .-..-.-..-..-... : 209
Lb. caseiW56  : .-..-.-..-..-... : 209
Lb. rhamnosu  : V-..-S-M.-...SE. : 209
Lb. caseiBD-  : .-..-.-..-..-... : 209
Lb. caseiLC2  : .-..-.-..-..-... : 209
Lb. caseistr  : .-..-.-..-..-... : 209
Lb. rhamnosu  : V-..-S-M.-...SE. : 209
Lb. rhamnosu  : V-..-S-M.-...SE. : 209
Lb. caseiBL2  : .-..-.-..-..-... : 209
Lb. caseiATC  : .-..-.-..-..-... : 209
                Rg      IA   K
```

Supplementary figure S3: Multiple sequence alignment of the conserved domains of one *spxB* sequence obtained in this study, and the 10 most dissimilar representative sequences of the family TPP_PYR_POX (cd07039). Query sequence is denovo811, the most similar sequences retrieved in CDD database belongs to *Lb. plantarum* (1POW_A, 1Y9D_A). Upper case aa: conserved positions, Lower case aa:unaligned aa, dashes: gaps, hash marks (#): conserved feature residues, red to blue color scale represents high to low conservation. (For more information see: https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd_help.shtml#ConservedFeatures)

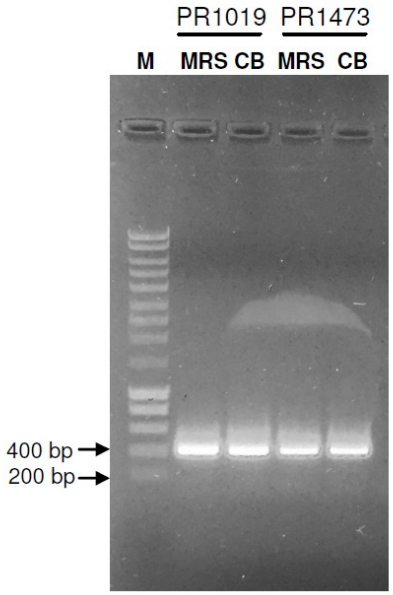

10 20 30 40 50 60 70 80
Feature 2
1POW_A 4 LAGAAVIKVLEAWGVDHLYGIPGGSINSIMDALsae--rdrIHYIQVRHEEVGAMAAAADAKLTgkIGVCFGSAGPGGTH 81
query 1 ----------EDWGIHNIYGLPGGSFDSTMNALynr--rhtINYVQVRHGEVGALAAAGEAKVTgrIGATFGSAGPGAVH 68
1Y9D_A 12 LAGAAVIKVLEAWGVDHLYGIPGGSINSIMDALsae--rdrIHYIQVRHEEVGAMAAAADAKLTgkIGVCFGSAGPGGTH 89
gi 25027680 15 STSDLIVRRLRDWGVKRVYGYSGDGNNALLGAIrra--edgPEFIAARHEEGAAFMAVGDARYSrgVGVVTSTQGPGAIH 92
gi 94310837 4 KVAEIIVEVLEQAGVKRCYGIVGDTLNLIAEHLdr----ssIDWVGVRHEEAGAFAAGAEALYTnnLTACAGSCGPGSLH 79
gi 29347230 4 KIAEQLIDTLVESGVERIYAVTGDSLNEVNEAVrkn---nkIQWIHVRHEETGAYAAAAEAQLTgrIGCCAGSSGPGHVH 80
gi 209363880 5 NVWEMLLDILDGCSNRQIFGVFGDAINPLANALrtdk-rkrFEWIAVRHEETGAFAAGAQAKLTgkLGLCAGTVGPGAIH 83
gi 118588197 120 TVTDVMAETMVNWGVKRVFGMVGHSNLGLADAIrrqvdkgtLGYVGIRHEGAAAFAASAYGKLTgkPAACLTIAGPGATN 199
gi 20257167 5 NVADLFVATLEQAGVKRIYGIVGDSLNAFTEALrrr---gtIEWIHVRHEEVAAFAAAGEAQMTgsLAVCAGSCGPGNLH 81
gi 21224481 5 NVAEQFVDILTRAGVERLYGVVGDSLNPVVDAVrrh---sgIEWVHVRHEETAAFAAGAEAQITgkLTACAGSCGPGNLH 81
90 100 110 120 130 140 150 160
Feature 2
1POW_A 82 LMNGLYDAREDHVPVLALIGQFGTTGMNMDTFQEMNENPIYADVA-DYNVTAVNAATLPHVIDEAIRRAYAHQGVAVVQI 160
query 69 LLNGLYDAQYDHVPVLALVGQVPTAAMNTNYFQEMNENPMFADVS-VYNRTAMTAAQLPHVVDEAIRQAYKHQGVAVVTI 147
1Y9D_A 90 LMNGLYDAREDHVPVLALIGQFGTTGMNMDTFQEMNENPIYADVA-DYNVTAVNAATLPHVIDEAIRRAYAHQGVAVVQI 168
gi 25027680 93 ILNGLYDAKLDSVPVVALVAQQSRSVLGSDYQQEVDLHTLFADVAtPFLETVTTPEQLPMVIDRAFRSALANSGPAVVIL 172
gi 94310837 80 FINSLYEANRNRAPVVLIASQVAMSEMGFEFIQEVDFKSVFKECS-VFCDMILTPEQARRKTAMACQTALTRRGVAVLIV 158
gi 29347230 81 LINGLYDAQRSGAPVIAIASTIPSGEFGTEYFQETNTIKLFNDCS-YYNEVATTPGQFPRMLQSAIQTAITRKGVAVVGL 159
gi 209363880 84 LLNGLYDAKMDHAPVLALTGQVPRDEIGTDYHQEVNLDRLFDDVS-AYNESLIVPEQMPRLGLLAVQTAIARQGVAHLSI 162
gi 118588197 200 LLTGLWDAKVDRSPVLALTGQVQTQVFGPGAFQDIDLKSAFEAVS-KFSQPVLSTSNHAELMSLALKTALVERDVANLIF 278
gi 20257167 82 LINGLFDAHRSRVPVLAIAAQIPSAEIGGGYFQETHPQNLFRECS-HYCELVSDASQLPFVIENAIRAAVGLRGVAVIAM 160
gi 21224481 82 LINGLYDAHRSMAPVLALASQIPSSEIGLGFFQETHPDQLFRECS-HYSELISSPKQMPRLLQTAIQHAVGQGGVSVVSL 160
Feature 2
1POW_A 161 PVDLPWQQ 168
query 148 PKDLGWEQ 155
1Y9D_A 169 PVDLPWQQ 176
gi 25027680 173 PHDIQQAP 180
gi 94310837 159 PVDISHGE 166
gi 29347230 160 PGDLAKAS 167
gi 209363880 163 PTDVGPSK 170
gi 118588197 279 PDDVQTNP 286
gi 20257167 161 PGDVAFRD 168
gi 21224481 161 PGDIADEP 168

Appendix II.

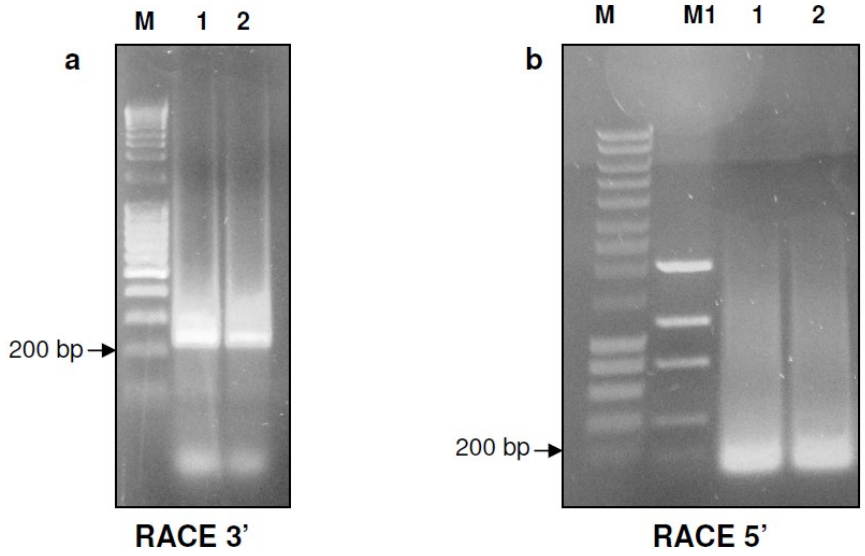
Supplementary Table 1 List of oligonucleotides used in this study.

Name	Sequence (5'-3')	Application
EcoRI	GACTGCGTACCAATTC	non-selective primer (cDNA-AFLP)
MseI	GATGAGTCCTGAGTAA	non-selective primer (cDNA-AFLP)
5'DY-EcoRI-AT	GACTGCGTACCAATTCAT	selective labelled primer
5'DY-EcoRI-AC	GACTGCGTACCAATTCAC	selective labelled primer
MseI-AT	GATGAGTCCTGAGTAAAT	selective primer (cDNA-AFLP)
MseI-AC	GATGAGTCCTGAGTAAAC	selective primer (cDNA-AFLP)
EcoRI-plus	CTCGTAGACTGCGTACC	adaptor (CDNA-AFLP)
EcoRI-minus	AATTGGTACGCAGTCTAC	adaptor (CDNA-AFLP)
MseI-plus	GACGATGAGTCCTGAG	adaptor (CDNA-AFLP)
MseI-minus	TACTCAGGACTCAT	adaptor (CDNA-AFLP)
fst-plus	GTTGAAATTCACAATAACTTTGCAC	primer (PCR and sequencing)
fst-minus	CCATAACGAAGTTTTGACTATTTTTG	primer (PCR and sequencing)
anchored polyT	ACAACTTTGTACAAGAAAGTTGGGTACT(30)VN	oligo-dT-adaptor primer (3' and 5'RACE)
3RACE-plus	AGTTCATGAATTCATTCGATAAAGC	sequence-specific primer (3' RACE and qRT PCR)
5RACE-minus	GTTATCCAGAGAGTATTTCAACA	sequence-specific primer (5' RACE and qRT PCR)
anchored polyG	GGACAACTTTGTACAAAAAAGTTGGAGAGGGCGGG	oligo-dG-adaptor primer (5' RACE)
5RACE PCR	TCGTCGGGGACAACTTTGTACAAAA	primer corresponding to adaptor sequence (5' RACE)
3-5RACE-PCR	GGCGGCCGCACAACTTTGTACAAGAAAGTTGGGT	primer corresponding to adaptor sequence (3' RACE and 5' RACE)
lpt-plus	CATATGAATTCATTCGATAAAGCGA	Primer for Lpt cDNA cloning
lpt-minus	GGATCCAAGCCATCATCTCCG	Primer for Lpt cDNA cloning

Supplementary Figure 1 PCR-amplification of Lpt TA system locus from *Lb. rhamnosus* strains. Agarose gel electrophoresis of the amplification products obtained by standard PCR employing plasmid DNA extracted from *Lb. rhamnosus* PR1473 and 1019 grown in MRS or CB medium. M: marker SMART ladder (Biosense).



Supplementary Figure 2 Agarose gel electrophoresis of the amplification products of RACE3' experiments (a. lanes 1 and 2) and RACE 5' experiments (b, lanes1 and 2). M: marker SMART ladder (Biosense); M1: marker Low DNA mass ladder (Invitrogen).



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The task of enclosing three years of work in a doctoral thesis is always difficult, because in addition to data, tables, figures and all the rest, the hardest part is to compress in a few lines all the experiences and human wealth accumulated in the meanwhile.

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About the author

Alessia Levante was born on 11th May 1989 in Palermo, a province of the beautiful island of Sicily, in Italy. Raised by the seaside, in 2007 was accepted to the degree programme in Biotechnology at the University of Parma, and after completion of the bachelor prosecuted her studies in the same university with the master degree programme in Industrial Biotechnologies. During her last year she moved to Max Planck Institut fur Chemische Energiekonversion where under the supervision of Prof. Wolfgang Gartner and Prof. Cristiano Viappiani she realised the project for her master thesis. The findings of that experience are collected in the master thesis "Building of a photoswitchable 1393GAF3 – RsbRA protein construct for FRET studies in bacterial cells ". Shortly after graduating, Alessia passed the selection for the Doctoral School in Food Sciences at University of Parma, under the supervision of Prof. Camilla Lazzi and Prof. Erasmo Neviani. During the three years of her PhD she dealt with various aspects concerning the adaptation of *Lactobacillus casei* group to cheese environment, developing a personal interest towards molecular tools for microbial dynamics investigation, bioinformatics and metabolomics. The results achieved in the three years of the PhD are described in this thesis.