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CICLO XXXV

Non-starter Lactic Acid Bacteria characterization for their control in cheese making

Coordinatore:
Chiar.ma Prof.ssa Chiara Dall'Asta

Tutore:
Chiar.ma Prof.ssa Monica Gatti

Dottorando: Luca Bettera

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Preface

This thesis has been submitted to meet the requirements to obtain a PhD degree at the PhD Course in Food Science, Department of Food and Drug, University of Parma. The PhD scholarship was funded by the italian Ministry of University and Research. I spent a period as Visiting PhD hosted by Agroscope (Bern, Switzerland).

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Luca Bettera, January 2023.

"La bocò l'è mìo stracò, se la sent mìo dè acò"

Detto popolare Bresciano

"The mouth is not tired if it does not taste like cow"

Popular quote from Brescia

Abstract (English)

Cheese making date his practice over millennia and evolved nowadays being part of a sector representing one of the biggest business of the food industry. In the wide cheese variety, raw milk cheeses have particular economic relevance for many European countries. These products owe their popularity to a typic organoleptic profile whose formation is recognized to be strongly influenced by the raw milk microbiota. Non-starter lactic bacteria (NSLAB) represent the part of this microbiota which has shown the capability to better adapt in the cheese environment and their activity has been positively correlated with cheese flavor formation. Although much is known about the cheese microbiota and from which NSLAB species it is composed, further research has been highlighted as necessary to better understand the origin, development dynamics along the production chain, and metabolic traits at the strain level of NSLAB.

The object of the present thesis was to do a step forward over what is known about the NSLAB, studying their diversity at the strain level through the characterization of their genotypic, phenotypic and metabolic traits. The knowledge of this information is at fundamental for the control of the interested NSLAB within the complex raw milk microbiota and as consequence govern their effect on the cheese's final characteristics.

More than 120 NSLAB strains have been isolated from 20 dairies and screened in order to be qualified as potentially applicable as adjunct cultures. The possibility of positively influencing the cheese characteristic controlling the raw milk microbiota was also investigated by developing a natural adjunct milk culture. Finally, an investigation on hard cheeses produced from centrifugated raw milk gave new insight into the description and consequent prevention of structural defects possibly correlated with the NSLAB abundance unbalancing within the cheese microbiota.

The results achieved lay the foundation to a better exploit of the raw milk microbiota, contributing to the possibility of predicting more precisely the final cheese characteristics. Cheese making trials will be necessary to test the effectiveness of the adjunct strains screened in influencing the cheese aroma. In order to obtain the same goal in the context of an artisanal dairy, more research is needed to optimize the natural adjunct milk culture developed, testing different treatments that could more specifically select desired NSLAB present in raw milk.

Riassunto (Italiano)

La produzione di formaggio risale a millenni fa e si è evoluta fino a diventare parte di un settore che oggi rappresenta una delle maggiori attività dell'industria alimentare. Nell'ampia varietà di formaggi presenti sul mercato, quelli prodotti da latte crudo hanno una particolare rilevanza economica per molti paesi europei. Questi prodotti devono la loro popolarità a un profilo organolettico tipico, la cui formazione è riconosciuta essere fortemente influenzata dal microbiota del latte crudo. I batteri lattici non starter (NSLAB, dall'inglese *non-starter lactic acid bacteria*) rappresentano la parte di questo microbiota che ha dimostrato di meglio adattarsi nel formaggio stagionato per lunghi periodi, e la loro attività è stata correlata alla formazione del sapore e degli aromi tipici del formaggio. Sebbene la ricerca in passato abbia contribuito ad una maggiore conoscenza del microbiota del formaggio e delle specie di NSLAB che lo compongono, è stata evidenziata la necessità di ulteriori ricerche per comprendere meglio l'origine, le dinamiche di sviluppo lungo la catena produttiva e le caratteristiche metaboliche a livello di ceppo dei NSLAB.

L'obiettivo della presente tesi è stato quello di fare un passo avanti rispetto a quanto noto sui NSLAB, studiando la loro diversità a livello di ceppo attraverso la caratterizzazione dei loro tratti genotipici, fenotipici e metabolici. La conoscenza di queste informazioni è fondamentale per il controllo dei NSLAB di interesse caseario presenti all'interno del complesso microbiota del latte crudo e, di conseguenza, per governare il loro effetto sulle caratteristiche finali del formaggio.

Più di 120 ceppi NSLAB sono stati isolati da 20 caseifici e testati per essere qualificati come potenziali colture aggiuntive aromatizzanti. La possibilità di influenzare positivamente le caratteristiche del formaggio controllando il microbiota del latte crudo è stata studiata anche attraverso lo sviluppo di una coltura naturale aggiuntiva in latte. Infine, un'indagine su formaggi a pasta dura prodotti da latte crudo centrifugato ha fornito nuovi spunti per la descrizione e la conseguente prevenzione di difetti strutturali potenzialmente correlati allo squilibrio dell'abbondanza di NSLAB all'interno del microbiota del formaggio.

I risultati ottenuti pongono le basi per un miglior controllo del microbiota del latte crudo, contribuendo alla possibilità di prevedere con maggiore precisione le caratteristiche finali del formaggio. Saranno necessarie prove di caseificazione per testare l'efficacia dei ceppi selezionati nell'influenzare l'aroma del formaggio. Per ottenere lo stesso obiettivo nel contesto di un caseificio tradizionale, sono necessarie ulteriori ricerche per ottimizzare la coltura naturale aggiuntiva in latte sviluppata, testando diversi trattamenti che potrebbero selezionare in modo più specifico i NSLAB desiderati presenti nel latte crudo.

General list of abbreviations

PDO = protected designation of origin

LAB = lactic acid bacteria

SLAB = starter lactic acid bacteria

NSLAB = non-starter lactic acid bacteria

NWC = natural whey culture

CFU = colony forming unit

NGS = Next-generation sequencing

OTU = Operation taxonomic units

MRS = de Man-Rogosa-Sharpe

PCA = plate count agar (microbial growth medium)

PCA = principal component analysis (data analysis technique)

AFLP = Amplified Fragment Length Polymorphisms

Contents

1 Introduction

1.1 Thesis outline

The present thesis addressed the study of a microbial group of dairy interest named non-starter lactic acid bacteria (NSLAB). The characterization of NSLAB geno-pheno-type and dynamics in raw milk and cheese ecosystems are fundamental for their potential control at the farm and cheese making level. This thesis has been subdivided into the steps undertaken to achieve this goal.

This first chapter introduces the state of the art of the topic, briefly addressing the relevance of the raw milk cheese production and the basis of cheese microbiology. Finally, the specific aim of the thesis is described.

The second chapter reviewed NSLAB abundance in the complex raw milk microbiota, also analyzing their absolute quantification. Clarifying these aspects by reviewing the scientific literature available was considered the priority at the beginning of this PhD project since the number of bacterial cells is known to be one of the most important factors in determining microbial activity in food.

In the third chapter, a research about phenotyping of NSLAB isolated from raw milk is presented. The strategy applied lays the foundation for the exploitation of metabolic features of specific strains qualified as adjunct cultures.

The fourth chapter presents the investigation of enriching the cheese with NSLAB using artisanal methods, i.e. a natural adjunct milk culture reproducible with backslopping, a tool that could represent for the artisanal cheese makers an alternative to the modern approach of developing selected strain cultures that require not always available specifics know-how and laboratory facilities.

The fifth chapter addresses the investigation of microbial and physico-chemical characteristics of a raw milk hard cheese produced from centrifugated milk that presented structural defects, highlighting the importance of the control of the raw milk microbiota also to reduce the occurrence of defects.

Overall conclusion and perspectives are discussed in the final chapter.

1.2 Raw milk cheese

Cheese making is an ancient method of preserving milk properties born from the empirical know-how of herdsmen (Mucchetti and Neviani 2022) and is commonly believed to date back some 8000 years ago (McSweeney et al. 2017). In particular, in Italy, cheese manufacture has a very long history, that strongly developed at the time of the Roman Empire (Gobbetti et al. 2018).

Nowadays the dairy sector represents one of the biggest businesses of the food industry (fil-idf.org/). It is estimated that there are more than 1000 cheese varieties, obtained using different types of milk (the most common are bovine, buffalo, ewe and goat) and applying different technologies. In **Figure 1**, a schematization of the principal cheese varieties is reported. The technology applied will regulate the microbial activity; the cheese is in fact, with few exceptions, a fermented food which relies on microorganism for its manufacture. The microbial component can

be added with the use of starters, or it can arise from contamination in the farm and dairy environments. In the case of cheeses produced with heat treated milk (pasteurized or thermized), this latter microbial component is strongly reduced, both in terms of quantity and species richness, while in the case of cheese produced with raw milk it is preserved. This increased microbial diversity can change the characteristics of the cheese due to the presence of other metabolic pathways. On the one hand, this can lead to authenticity and added value such as possible benefits for the consumer deriving from the production of bioactive peptides (Egger et al. 2021), but on the other hand, it can also lead to the occurrence of deficiencies and/or also represent a pathogenic risk for the consumer. The present thesis addressed aspects related to the quality of the specific variety of raw milk cheeses; aspects regarding hygiene and consumer health risk were not studied and will not be discussed.

The production of raw milk cheese is a common practice in Europe, that reflects the historic tradition of cheesemaking of the continent (Montel et al. 2014). This is tradition is recognized as an added value for the cheese products, so much so that many of them are regulated by specific laws by branding them with geographical indications such as Protected designation of origin (PDO), Protected geographical indication (PGI) and Traditional speciality guaranteed (TSG) (Vandecandelaere et al. 2010; EP and Council of EU 2012). The production of raw milk cheese is particularly strong in Italy, France, Spain and Switzerland. As shown in **Table 1,** in 2021 in Italy were manufactured more than 500.000 tons of PDO/PGI cheese where the raw milk is either mandatory or allowed in mix with thermized/pasteurized milk for the production. **Table 2** shows the number of cheese varieties produced using raw milk in different European countries. It is generally accepted that cheese made from raw milk develops a more intense flavor than that made from pasteurized milk and this has been correlated with the raw milk microbiota activity (Bachmann et al. 2011). The raw milk microbiota is complex, harboring more than 40 phyla (Quigley et al. 2013; Eugenio Parente, Ricciardi, and Zotta 2020). However, only a small part generally called non-starter lactic acid bacteria (NSLAB) withstand the cheese making stresses and few of them resist in the cheese environment when long ripening occurs (Bottari et al. 2018). The recognition of the lactic acid bacteria (LAB) pro-technological feature has prompted their domestication and use in the form of microbial cultures.

1.3 Microbial cultures

Although the production of cheese with the autochthonous raw milk microbiota as the sole microbial source is still practised by small artisanal dairies, in the last century the cheese industry has developed the production of a variety of starter and secondary microbial cultures. Primary starters, or just starters, are involved mainly in the production of lactic acid from lactose, which occurs early in cheese production, while secondary (or adjunct) microorganisms have features important for cheese ripening (e.g., eye formation, rind modification, flavor enhancement).

Microbial cultures can be artisanally prepared with the backslopping technique where an old batch of a fermented product is used to inoculate a new one, characterizing the natural culture in a undefined microbiota. Natural whey cultures (NWC) and natural milk cultures (NMC) are used for the production of several cheeses, for example Grana Padano PDO and Parmigiano Reggiano PDO

(Gatti et al. 2014), Mozzarella TSG, Caciocavallo Silano PDO (Ercolini et al. 2008), Berner Alp- und Hobelkäse PDO and Le Gruyère PDO.

Alternatively, commercial cultures are available. The ability to isolate, purify and characterize bacterial strains opened the way to the industrial production of mixed- or even defined- strain cultures. (E. Parente, Cogan, and Powell 2017; Powell, Broome, and Limsowtin 2022). These cultures found their application in improving the sensory properties of cheese also because the improvement in the hygiene of milk and the need for standardization and acceleration of ripening have broadly resulted in cheeses with less complex flavor cheese (McSweeney et al. 2017). Cases of biodiversity loss of LAB in the dairy chain has been already reported by Morandi et al. (2019). The authors monitored over two years the reduction of microbial diversity of NWC used for Grana Padano PDO (Trentingrana) production, correlating this with changings in the cheese volatile organic compounds profile, concluding that the recognition of the microbial depletion drivers should be necessary to preserve the cheese quality and prevent the loss of typical cheese traits.

The fact that some cheeses have become blander in taste raised awareness of the decisive role played by the raw milk microbiota and this increased the demand for secondary starters and the curiosity of researchers to solve the cheese quality sustainability challenge.

1.4 Aim of the thesis

The aim of this thesis was to do a step forward over what is known about the non-starter lactic acid bacteria, studying their diversity through the characterization of their genotypic, phenotypic and metabolic traits. These analyses were applied in a polyphasic approach consisting of classical microbiology methods for the culturing and isolation of the microorganisms, culture-independent techniques for taxonomic and genomic evaluations, metabolic assays as phenotypic methods, and chemical and physico-chemical evaluations on the dairy samples.

To reach this goal this PhD carried on four research lines. The first reviewed the literature intending to clarify which and how many lactic acid bacteria are present in the raw milk used for cheese production. The second isolated and screened NSLAB from raw milk with the object to qualify adjunct strains for cheese making through the study of their phenotypic traits. The third investigated the development of a natural adjunct milk culture with the aim to standardize the enrichment in cheese making of the raw milk microbiota. The fourth aimed to analyze some physico-chemical and microbiological characteristics of hard cheese produced from centrifugated raw milk and clarify the effect on cheese quality when the NSLAB component is altered.

1.5 Tables

Table 1. Italian PDO/PGI cheeses of which the use of raw milk is compulsory or allowed: volume production updated to the year 2021. Data retrieved from CLAL.it (www.clal.it).

n.	Cheese production (tons) (2021)	PDO Cheese	Milk	Milk type	Natural starter	Curd cooking (°C)	Long ripened
1	203.290	Grana Padano	Raw	Cow	X	56	X
2	163.647	Parmigiano Reggiano	Raw	Cow	X	54-56	X
3	53.766	Mozzarella di Bufala Campana	Raw/Thermized/Pasteurized	Buffalo	X	-	-

4	34.282	Pecorino Romano	Raw/Thermized	Ewe	X	45-48	-
5	21.969	Asiago	Raw/Thermized/Pasteurized	Cow	X	45-49	X
6	8.674	Taleggio	Raw/Pasteurized	Cow	X	-	-
7	7.419	Provolone Valpadana	Raw/Thermized/Pasteurized	Cow	X	-	-
8	6.255	Montasio	Raw	Cow	X	42-48	X
9	4.000	Fontina	Raw	Cow	X	-	X
10	3.373	Pecorino Toscano	Raw/Pasteurized	Ewe	X	-	-
11	2.791	Quartirolo Lombardo	Raw/Pasteurized	Cow	X	35-44	-
12	1.980	Piave	Raw/Pasteurized	Cow	X	44-47	X
13	1.832	Pecorino Sardo	Raw/Thermized/Pasteurized	Ewe	X	43	-
14	1.583	Valtellina Casera	Raw	Cow	-	40-45	X
15-52	7.490	Other PDO/PGI	-	-	-	-	-
Tot.	522.351						

Table 2. Number of cheese varieties around Europe where the raw milk is mandatory or allowed for the production. Data updated to the year 2021 and retrieved from eAmbrosia.eu (ec.europa.eu) and formaggiosvizzero.ch (www.formaggiosvizzero.ch).

Country	PDO/PGI raw-milk cheeses (n)
France	40
Spain	16
Switzerland	11
Portugal	10
United Kingdon	7
Austria	6
Slovakia	6
Greece	4
Slovenia	3
Denmark	2
Germany	2
Poland	2
Romania	2
Croatia	1
Netherland	1

1.6 Figures

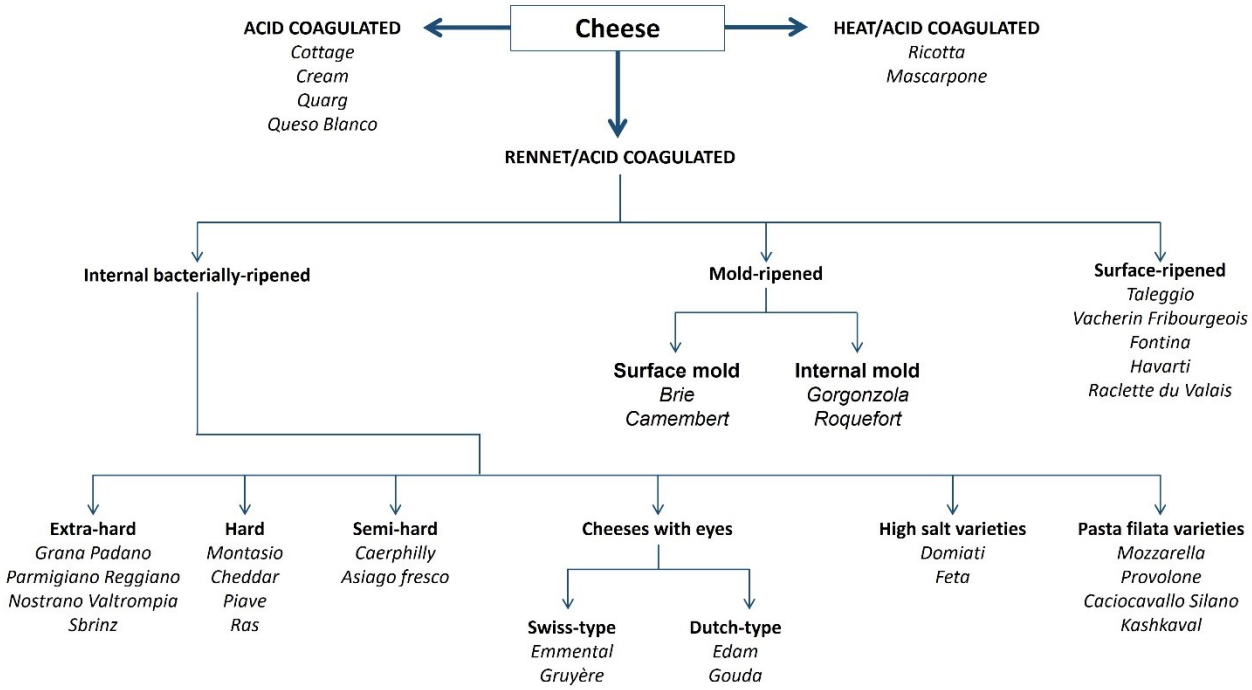

Figure 1. Schematization of cheese varieties. Super-families classify cheeses based on the method of coagulation; a further subdivision is based on the principal ripening agent and/or characteristic technology and microbiology. Adapted by Fox et al.(2017).

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2 LAB quantification in raw milk

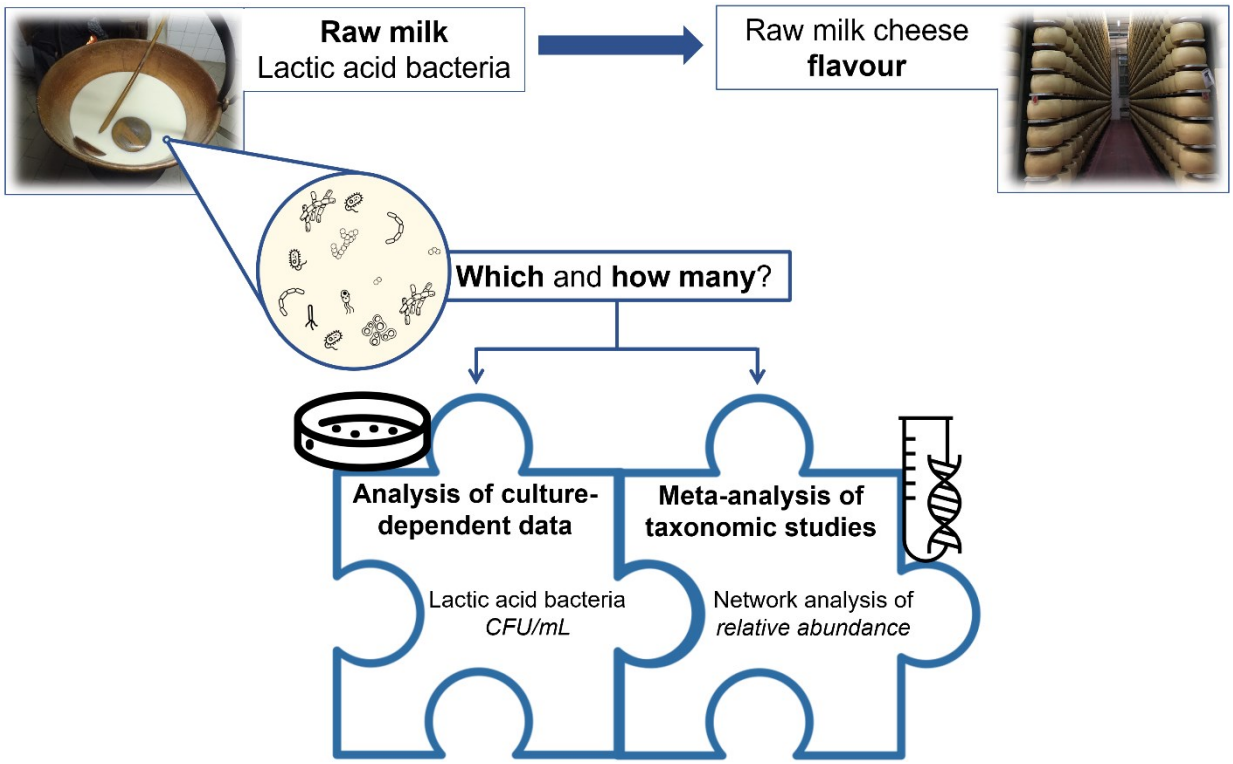
Lactic acid bacteria in cow raw milk for cheese production: which and how many?

Luca Bettera[1], Alessia Levante[1], Elena bancalari[1], Benedetta Bottari[1], Monica Gatti[1*]

[1]Department of Food and Drug, University of Parma, Parco Area delle Scienze 49/A, Parma 43124, Italy.

* Corresponding author

Keywords: Raw milk cheese, Raw milk microbiota, Cheese microbiota, Cheese ripening, LAB, NSLAB, Lacticaseibacillus.



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Abstract

Lactic Acid Bacteria (LAB) exert a fundamental activity in cheese production, as starter LAB in curd acidification, or non-starter LAB (NSLAB) during ripening, in particular in flavor formation. NSLAB originate from the farm and dairy environment, becoming natural contaminants of raw milk where they are present in very low concentrations. Afterward, throughout the different cheesemaking processes, they withstand chemical and physical stresses becoming dominant in ripened cheeses. However, despite a great body of knowledge is available in the literature about NSLAB effect on cheese ripening, the investigations regarding their presence and abundance in raw milk are still poor.

With the aim to answer the initial question: "which and how many LAB are present in cow raw milk used for cheese production?", this review has been divided in two main parts. The first one gives an overview of LAB presence in the complex microbiota of raw milk through the meta-analysis of recent taxonomic studies. In the second part, we present a collection of data about LAB quantification in raw milk by culture-dependent analysis, retrieved through a systematic review.

Essentially, the revision of data obtained by plate counts on selective agar media showed an average higher concentration of coccoid LAB than lactobacilli, which was found to be consistent with meta-taxonomic analysis.

The advantages of the impedometric technique applied to the quantification of LAB in raw milk were also briefly discussed with a focus on the statistical significance of the obtainable data.

Furthermore, this approach was also found to be more accurate in highlighting that microorganisms other than LAB are the major component of raw milk. Nevertheless, the variability of the results observed in the studies based on the same counting methodology, highlights that different sampling methods, as well as the "history" of milk before analysis, are variables of great importance that need to be considered in raw milk analysis.

2.1 Introduction

Microbiological and biochemical changes in the curd are crucial factors for the production of raw-milk, ripened cheeses. The microbial ecology of this cheese variety consists of a complex interaction between starter lactic acid bacteria (SLAB, usually deliberately added for curd acidification) and non-starter LAB (NSLAB, adventitious milk contaminants from farm and dairy environments) from milking to ripening (Blaya et al., 2018; Gatti et al., 2014).

NSLAB frequently recovered from cheese are facultative heterofermentative lactobacilli (Beresford et al., 2001). *Lacticaseibacillus* [formerly *Lactobacillus casei* group, (Zheng et al., 2020)] is one of the most prevalent genera found in hard cooked, long-ripened cheeses. It includes the species *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus rhamnosus*, which are of particular interest because of their proven role in cheese flavor formation during ripening (Bottari et al., 2018), as well as their potential health benefits through the consumption of fermented foods (De Filippis et al., 2020; Hill et al., 2018). Their trend from low abundance in raw milk, to dominance in ripened cheese, involves adaptation to chemical and physical stresses throughout the cheesemaking and ripening processes (i.e. heat-related, acidic, osmotic, and oxidative stresses),

besides their ability to grow using energy sources other than lactose (Gatti et al., 2014). These are the physiological criteria used for the delineation of the name of the new genus *Lacticaseibacillus* from *caseus* (cheese) (Zheng et al., 2020).

The mechanisms responsible for NSLAB survival to stresses encountered during cheesemaking might be attributed to various strategies of adaptation, such as the capability to utilize different energy sources (Lazzi et al., 2014; Papadimitriou et al., 2016) or the activation of strategies that can increase their tolerance to the food manufacturing process, such as the recently described toxin-antitoxin systems (Levante et al., 2021, 2019). Although NSLAB have been extensively studied for their role in cheese ripening (Bottari et al., 2020; De Dea Lindner et al., 2008; Levante et al., 2017; Sgarbi et al., 2014, 2013; Solieri et al., 2012), and reviews are available on this topic (Beresford et al., 2001; Bottari et al., 2018; Gobbetti et al., 2015; Montel et al., 2014; Settanni and Moschetti, 2010), the source of their origin and their relative abundance in raw milk are not well clarified yet.

Further research is necessary to demonstrate whether live microorganisms are present in milk inside a healthy mammary gland (Oikonomou et al., 2020), although milk endogenous bacterial contamination via enteromammary pathway has been hypothesized (Addis et al., 2016). However, it is known that microbial colonization occurs from different sources throughout the route from farm to cheese factory (Gobbetti et al., 2018) and raw milk represents an ideal environment for the growth of many microorganisms (Quigley et al., 2013). To shed light on which and how many of these biotypes will be part of the ripened cheese microbiota, it is of primary importance to know their initial abundance in raw milk, since the number of bacterial cells is known to be one of the most important factors in determining microbial activity in food (Fleet, 1999; Giraffa, 2004). It is expected that in the next future quantitative methods such as flow cytometry will help understanding how microbial loads of certain species in raw milk may affect the microbiome of resulting cheeses (Porcellato et al., 2021; Skeie et al., 2019), as it was also shown for other complex microbiomes (Vandeputte et al., 2017).

Although many studies deal with the description of the complex dairy ecosystem focusing on LAB in raw milk, often reporting isolation, identification, and relative quantification of the most abundant species, they rarely provide absolute quantification. With the aim to answer the initial question: “which and how many lactic acid bacteria are present in cow raw milk used for cheese production?”, this review analyses and comments on the data of the studies available in the literature addressing LAB quantification in raw milk used for the production of different cow's milk cheeses.

The different analytical techniques used for LAB evaluation in milk can be divided into two methodological approaches: I) conventional culture-dependent methods which analyze microorganisms after their growth in liquid or solid media; II) more recent culture-independent methods which directly detect microbial nucleic acids, avoiding the culturing step. A prior careful consideration of numerous factors (e.g. taxonomic resolution, workload) is necessary to define the appropriate methodology for a certain purpose (Temmerman et al., 2004). These different approaches are complementary, thus their combination in a polyphasic approach is suggested to set up more complete and accurate studies (Agrimonti et al., 2019; Delbès et al., 2007; Ercolini et al.,

2001) that better describe the dynamic evolution of microbial communities in food ecosystems, in particular in the complex transformation of raw milk to ripened cheese.

In both methods, the qualitative information (i.e. microorganisms taxonomic identification) can be addressed by common molecular analysis (such as PCR- and sequencing-based methods). On the other hand, the quantitative results differ: most of the culture-independent studies on LAB in raw milk report the relative abundance (%) of prevalent taxa; culture-dependent studies mainly based on plate counts, report instead results as Colony Forming Units (CFU/mL).

For this reason, data collected from the studies applying different approaches have been discussed separately in the following two chapters: the first one aims at giving an overview of LAB's abundance within the complex microbiota of cow raw milk through the review of recent taxonomic studies; the second includes a collection of data about quantification of LAB by culture-dependent analysis in cow raw milk.

2.2 Culture-independent quantification

Culture-independent techniques available for milk and cheese microbiota analysis are constantly evolving and have been extensively reviewed elsewhere (Addis et al., 2016; Levante et al., 2020; Ndoye et al., 2011; Neviani et al., 2013; Quigley et al., 2011; Randazzo et al., 2009; Tilocca et al., 2020). Common outlines of these reviews are: I) the usefulness of the culture-independent approach in describing complex ecosystems such as raw milk and cheese, and the ability to overcome culture-dependent limits (e.g. ability to distinguish between dead or viable cells by a combination of DNA- and RNA-based approaches), although a polyphasic approach is recommended; II) -omics approaches are expected to greatly contribute in shedding light on microbial ecology dynamics.

Methods for absolute quantification at the species level have been implemented, such as Fluorescence in-situ Hybridization [FISH (Bottari et al., 2006)], quantitative PCR [qPCR (Agrimonti et al., 2019)], and Total Bacterial Count computed with the percentage of taxon after 16S rRNA Amplicon Sequencing (Porcellato et al., 2021; Props et al., 2017; Skeie et al., 2019). However, to the authors' knowledge, no studies applied these methods for the absolute quantification of the *Lacticaseibacillus* group in cow raw milk used for cheese production, except for Masoud and colleagues who quantified *Lacticaseibacillus rhamnosus* in raw milk used for Danish cheese production (Masoud et al., 2012).

Different studies are instead available in the literature about the characterization of raw milk microbiota by amplicon-based high-throughput sequencing (HTS) analysis. This method is successful to describe changes in microbiota related to seasonality, geographical origin, and the microbiota evolution at different steps of cheese making, but it suffers from bias because results are usually reported as relative abundance of taxa which are not converted to quantitative values (Skeie et al., 2019), and identification of bacteria beyond the genus level is often not possible (Claesson et al., 2010; Dreier et al., 2022). On the other hand, a fundamental advantage of these studies is the possibility to use the generated raw sequences to perform meta-analysis.

A very useful tool to conduct the meta-analysis is FoodMicrobionet [FMBN (De Filippis et al., 2018; Parente et al., 2019, 2016)], a collection of datasets created by 16S rRNA gene amplicon HTS studies of food bacterial communities. This tool was already used to widely review the microbiota

of dairy milk, discussing comparisons between the pasture and feed, farm environments, teat skin, teat milk (from different species, also affected by diseases), bulk tank milk and finally also HTST (high-temperature short time) milk (Parente et al., 2020).

In the present review, the updated version of FMBN 4.1.2 (Parente et al., 2022), integrated with the taxonomy reclassification for the genus *Lactobacillus* (Zheng et al., 2020) and further recent studies on the raw milk microbiota, has been used. Among the studies available in the database on cow raw milk, those analyzing cow whole raw milk used for cheese production were selected (Calasso et al., 2016; Cremonesi et al., 2020; De Filippis et al., 2016; De Pasquale et al., 2014; Dolci et al., 2014; Falardeau et al., 2019; Giello et al., 2017; Kamimura et al., 2020; Nikoloudaki et al., 2021) (**Supplementary table 1, 2**). This dataset included reads from 250 raw milk samples, classified in 1594 total taxa belonging to 45 different phyla (*Chloroplast*, *Eukaryota*, *Mithocondria*, and unidentified OTUs at the domain level were removed). Nodes and edges tables (.gml file generated by FMBN Shiny app) were then imported into Gephi software [v 0.9.2 (Bastian et al., 2009)] for bipartite network analysis, i.e. network with nodes belonging to OTUs and samples.

Nodes and edge statistics were calculated in Gephi: the *degree* is the number of connections to a certain node, meaning that for a sample, it represents the number of present OTUs, *vice versa* for an OTU, it represents the number of milk samples where it was found; the *weight* is a value assigned to an edge, corresponding to the OTU relative abundance in the sample; the *weighted degree* is the sum of *weights* for a node (**Supplementary table 3**). Nodes and taxa label size are proportional to their *weighted degree*, hence the bigger they are, the more the OTU is abundant in raw milk. Only nodes with a *weighted degree* > 10 were considered for the network (**Figure 1**). ForceAtlas2 algorithm (Jacomy et al., 2014) was applied for the layout: as a consequence, taxa are closer to samples where they are present with a greater abundance (thus, taxa to the periphery are less abundant); also, taxa close to each other were present more frequently in the same samples.

After the filtering step, 149 taxa were retained (**Supplementary table 3**), which belong to 10 different phyla. Part of the OTUs were classified only at the kingdom level (*Bacteria,* NA dark grey node). Among the most abundant genera, the psychrotrophic bacteria are present in significant proportions: these include *Pseudomonas* (23.85% of the tot *weighted degree* in the network), *Chryseobacterium* (6.56%), and *Acinetobacter* (6.01%). It is reasonable to assume that their dominance could be due to the refrigerating temperature usually applied for raw milk storage which decreases bacterial diversity (Raats et al., 2011).

According to these data, LAB relevant for cheese production are present as dominant taxa such as *Lactococcus* (7.24%), or subdominant such as *Streptococcus* (3.6%), *Lacticaseibacillus* (2.42%), *Lactobacillus* (2.35%), *Leuconostoc* (1.15%), and *Enterococcus* (0.41%). Despite their relatively low abundance in the raw milk ecosystem, these subdominant taxa can develop in subsequent cheese manufacturing. An example is found in long ripened cheeses, where the *Lacticaseibacillus* genus is known to become dominant throughout the ripening of raw milk, hard cooked, long-ripened cheeses (Bottari et al., 2018).

The low abundance of some subdominant species makes their isolation from raw milk difficult, limiting thus a potential targeted use. A strategy to overcome this limit could be the enrichment of the autochthonous raw milk microbiota through its spontaneous fermentation. Following this

approach, Bancalari and colleagues carried out a spontaneous fermentation of Parmigiano Reggiano raw milk samples to isolate LAB strains potentially usable as adjunctive aromatic starters (Bancalari et al., 2017), while Galli and colleagues incubated the raw milk for 24 and 48h, at 30 and 40°C, with the purpose to isolate additional LAB with potentially high GABA (γ-aminobutyric acid) producing capabilities (Galli et al., 2022).

2.3 Culture-dependent quantification

2.3.1 A systematic review of plate counts on agar media of LAB in raw milk

2.3.1.1 Search strategy

Articles were identified by searching in Scopus (https://www.scopus.com/) on the 10th of October 2022, using the following key words: TITLE-ABS-KEY ((({raw milk}) OR ({raw-milk}) OR ({bulk milk}) OR ({tank milk}) OR ({vat milk}) OR ({milk microbiota}) OR ({milk microflora}) OR ({Raw cows' milk}) OR ({Raw milk/cheese})) AND (({lactic acid bacteria}) OR (lab) OR (lactobacilli) OR (lactobacillus) OR (lacticaseibacillus) OR (nslab)) AND (cheese) AND NOT (({human milk}) OR (camel) OR (goat) OR (ewe) OR (sheep) OR (deer) OR (donkey))). After a titles/abstract screening, articles were included in the review on the base of the following criteria about the milk analyzed: 1) it must be raw [as defined by (EC, 2004)]; 2) it must derive from cow; 3) it must be used for cheese production; 4) it must be analyzed for LAB Colony Forming Unit. In the **Figure 1**, the four-phase PRISMA flow diagram (Page et al., 2021) schematizing the studies search process is reported.

2.3.1.2 Data elaboration

According to the definition of raw milk (EC, 2004), we considered only data from the analysis of milk samples which were not heat treated or undergone any treatment that has an equivalent effect. LAB quantification data (CFU/mL) evaluated by plate counts on elective or selective agar media after incubation at different temperatures were extracted and transformed in $\log_{10}$ scale when necessary. Quantification in broth media using the Most Probable Number (MPN) was rarely used (Cremonesi et al., 2020; Mucchetti et al., 2009). Results of standard total bacterial count through Plate Count Agar (PCA) (EC, 2006; ISO 4833-2, 2013) have been also extracted when available. Data were grouped based on the LAB group analyzed, i.e. by growth media and incubation temperature used. Only the two largest, more robust datasets underwent a statistical analysis for the evaluation of a significant difference among the two LAB groups: I) Lactobacilli, cultivated on Man-Rogosa-Sharpe (MRS) at 30-32°C (n = 82); II) Coccoid LAB, cultivated on M17 at 30-32°C (n = 70). The two datasets (supplementary data 1) were checked for their normal distribution through the *Shapiro-Wilk* test; the homogeneity of variance was assessed with a *F*-test; finally, a *t*-test ($\alpha = 0.05$) was applied. The data elaboration and statistical analysis were done in the R environment (R Core Team, 2022) using the packages "stats" and "tidyverse" (Wickham et al., 2019).

2.3.1.3 Lactic Acid Bacteria concentration in raw milk for cheese production

Out of the 453 records retrieved from the systematic search (**Figure 1**), the first country in terms of publication number in this field is Italy (n = 102), followed by France (57), and Spain (48) (Scopus stats, data not shown). Together with Ireland, Switzerland, and Portugal, they represent the 59% of the articles retrieved by this search. This is evidence of the high research interest of the European

Institutions in the dairy sector, reflecting the historic tradition of cheesemaking of the continent (Montel et al., 2014). The rest of the records were from Brazil, Turkey, Mexico and the United States of America.

Raw milk cheeses are produced worldwide. However, different cow raw milk cheeses analyzed in the studies retrieved through the literature search, were excluded from this review because the authors did not evaluated the LAB quantification in the raw milk by plate counts. Some examples are: Cotija [Mexico (Escobar-Zepeda et al., 2016)], Tulum [Turkey (Gezginc et al., 2022)], Fontina [Italy (Giannino et al., 2009)], Kraški and Tolminc [Slovenia (Trmčić et al., 2011)], Zlatar [Serbia (Terzic-Vidojevic et al., 2007)], Sir iz Mišine [Croatia (Vrdoljak et al., 2022)], Bitto [Italy (Colombo et al., 2009)], São Jorge [Portugal (Kongo et al., 2009)], and Poro de Tabasco [Mexico (De la Rosa-Alcaraz et al., 2020)].

Table 1 reports the list of the 64 studies available in the literature that quantified LAB in cow raw milk used for the production of 42 types of cheese. Mean values of log CFU/mL have been grouped by media type and incubation temperature to define the core LAB raw milk microbiota (**Figure 3**). Thus, the observed variability is likely explained by the fact that other factors take part in the modulation of LAB load in raw milk, such as sampling procedures, milk pre-treatments (e.g. skimming), and seasonality. Furthermore, although the studies considered eligible in this review have in common the use of the same growth media and incubation temperature, they may have slight differences in the analysis procedure, such as plate incubation time, degree of aerobic/anaerobic conditions, use of selective additives (**Supplementary data 2**).

The total number of values found in literature about LAB quantification in raw milk used for Grana Padano cheese is 103, the highest among the values found for the other cheeses addressed in this review. This indicates a high research interest in this cheese type. The raw milk used for Grana Padano PDO (Protected designation of origin) cheese (including Trentingrana) was analyzed by sampling whole evening raw milk and skimmed morning raw milk, thus testing the effect of overnight creaming (Franciosi et al., 2011a; Santarelli et al., 2013a). With a similar sampling procedure, also the microbial composition of morning whole raw milk and vat raw milk was evaluated (Monfredini et al., 2012), investigating also different storage temperatures (Franciosi et al., 2012, 2011b). More recently, the effect of the use of different machine cleaning detergents on the raw milk microbiota was investigated by sampling bulk tank raw milk at the farm and skimmed raw milk (Cremonesi et al., 2020), whereas Bava and colleagues analyzed bulk tank raw milk sampled at farms which had different management practices (Bava et al., 2021). In total, 6 studies quantified the LAB in raw milk used for Parmigiano Reggiano (**Table 1**), which allowed the collection of 28 values, the highest data number after Grana Padano and Traditional Mountain Cheese from Trentino alpine area (103 data in 7 studies and 70 data in 4 studies, respectively). Raw milk used for Parmigiano Reggiano PDO is also skimmed by overnight creaming, and different studies analyzed evening whole and morning skimmed raw milk (Coloretti et al., 2016; CRPA, 2011). Franceschi and colleagues sampled bulk raw milk from tanks directly at the farm after storage at two different temperatures (Franceschi et al., 2021). Other studies analyzed vat raw milk, obtained by mixing whole evening raw milk and morning skimmed raw milk (Coppola et al., 2000; CRPA, 2011; Gatti et al., 2008; Neviani et al., 2009).

The vat raw milk has also been studied in several other types of cheese: Provolone del Monaco (Aponte et al., 2008), Castelmagno (Dolci et al., 2008), Saint-Nectaire (Delbès et al., 2007), Swiss-type (Beuvier et al., 1997), Traditional Mountain cheese (Carafa et al., 2019, 2016), Cheddar (Gelsomino et al., 2001), Arzúa (Centeno et al., 1994), Cantal (De Freitas et al., 2007) and Fior di Latte di Agerola (Coppola et al., 2006). In the case of Caciotta, Caciocavallo Pugliese, and Emmental de Savoie the raw milk was sampled from tanks at the cheese factory (Calasso et al., 2016; Sohier et al., 2012), while for other cheeses directly at the farm, sometimes evaluating the effect of seasonality (Arenas et al., 2004; Castro et al., 2016; Desmasures et al., 1997; Franciosi et al., 2009; Luiz et al., 2017; Mallet et al., 2012), breeding practices (Gagnon et al., 2020; Giello et al., 2017), or transhumance period (Carafa et al., 2020).

Many studies, instead, did not report detailed information about the raw milk samples analyzed (Agarwal et al., 2006; Aquilanti et al., 2011; Carraro et al., 2011; Çetinkaya and Ece Soyutemiz, 2006; Denis et al., 2001; García Fontán et al., 2001; McSweeney et al., 1993; Randazzo et al., 2002; Rehman et al., 2000b, 2000a; Rodriguez Medina et al., 1995; Yunita and Dodd, 2018).

The main growth media used in the 64 reviewed studies were de Man-Rogosa-Sharpe (MRS) (De Man et al., 1960) elective for lactobacilli (55 studies), M17 (Terzaghi and Sandine, 1975) elective for coccoid LAB (40 studies), and PCA (31 studies) for total bacterial count. The results of 5 growth temperature ranges are shown in **Figure 3**: 7°C for psychrotrophs; 22-25, 30-32 and 37°C for mesophiles; 42-45°C for thermophiles.

PCA medium was modified for coccoid LAB count (PCA-BCP, supplementary data 2), to analyze raw milk used to produce Camembert de Normandie [2.91 log CFU/mL raw milk in winter period; 2.84 log CFU/mL in spring/summer period (Desmasures et al., 1997)] and cheese of Basse Normandie area [1.91 log CFU/mL raw milk in winter period; 1.87 log CFU/mL in spring period (Mallet et al., 2012)]; for this reason, these values were not included in **Figure 2**. Since the average of total bacterial counts at 30-32 °C (4.74 ± 1.26 log CFU/mL) is similar in comparison with LAB counts, especially for M17 counts, this may indicate that LAB represent the majority of mesophiles among culturable bacteria in raw milk. The slightly higher average concentration of total psychrotrophs in comparison with total mesophiles at 22-25°C is likely caused by the low number of data found in the literature for this temperature range (n=19 and n=6, respectively), in addition to the high values of 6.53 and 6.93 log CFU/mL found respectively in the raw milk used for the Spanish cheeses Leòn (Rodriguez Medina et al., 1995) and Genestoso (Arenas et al., 2004) (the latest was indeed considered an outlier).

Focusing on the LAB counts, cocci were found in very high concentrations at 37°C (5.88 log CFU/mL on average). However, this value is influenced by the high counts of raw milk used to produce artisanal cheeses from Sicily [7.9 log CFU/mL (Randazzo et al., 2002)], from Brasil [6.53 and 6.86 log CFU/mL in raw milk sampled in dry and rainy season respectively (Castro et al., 2016)], and from Turkey [6.64 log CFU/mL (Çetinkaya and Ece Soyutemiz, 2006)]. The raw milk used for artisanal cheese Caciotta di Montefeltro (Aquilanti et al., 2011) was also found to have a particular high value of mesophiles (22-25°C) coccoid LAB of 7.61 log CFU/mL. The same artisanal productions, together with raw milk used for artisanal Rugova cheese from Kosovo (Ajazi et al., 2018), Kashar from Turkey (Çetinkaya and Ece Soyutemiz, 2006), and Minas from Brazil

(Castro et al., 2016), showed the highest count of lactobacilli at the same incubation temperature of 37°C, expanding the value range of LAB found in MRS (1st quartile = 3.69 log CFU/mL; 3rd quartile = 6.29 log CFU/mL), which had an average of 5.05 log CFU/mL (**Figure 3**). The concentration of lactobacilli able to grow at this temperature in raw milk used for Cheddar cheese was found to be 2.28 log CFU/mL (McSweeney et al., 1993), even if the authors performed the cultivation on a different growth medium named *Lactobacillus* Selection Agar (LBS, Baltimore Biological Laboratories, Rockville, MD, USA; Becton Dickinson Microbiology Systems, USA). The same medium incubated at 30°C resulted in a lactobacilli concentration of 1.94 log CFU/mL in the raw milk used for the same cheese type (Gelsomino et al., 2001; Hickey et al., 2007)

More common and thus more useful for comparison was the incubation of MRS and M17 at 30-32°C, performed in 33 (n=82) and 26 (n=70) studies respectively, and representing the raw milk used for 24 of the 42 types of cheese analyzed in the review. The average concentration of lactobacilli (3.69 ± 1.21 log CFU/mL) was found significantly lower ($p < 0.001$) than coccoid LAB (4.62 ± 1.07 log CFU/mL). This result is consistent with what we found in the meta-analysis of HTS studies on the raw milk microbiota, where *Lactococcus* and *Streptococcus* genera were present in greater abundance compared to *Lacticaseibacillus* and *Lactobacillus* genera.

At the same growth temperature, total acidifying mesophiles had similar counts in raw milk used for cheese in Savoie-Haute Savoie using Elliker medium (Michel et al., 2001). Few works instead quantified coccoid LAB at 22-25°C, thus the higher concentration found in comparison with counts at 30-32°C may not be relevant.

The thermophiles population was counted in raw milk used for Swiss-type (Beuvier et al., 1997), Fior di Latte di Agerola (Coppola et al., 2006), Caciotta di Montefeltro (Aquilanti et al., 2011), Piedmont hard cheese (Bautista-Gallego et al., 2014), Caciocavallo di Castelfranco (Giello et al., 2017), Caciocavallo Palermitano (Settanni et al., 2012), and PDO cheeses such as Saint-Nectair (Delbès et al., 2007), Montasio (Carraro et al., 2011; Marino et al., 2008), Provolone del Monaco (Aponte et al., 2008), Salers (Callon et al., 2004), Comté (Bouton et al., 1998) and Parmigiano Reggiano (Coppola et al., 2000; Gatti et al., 2008). Thermophilic streptococci were particularly high in raw milk used for Fior di Latte di Agerola (6.7 log CFU/mL on average), causing a wider data distribution in comparison with thermophilic lactobacilli, searched in the same cheeses, except for Saint-Nectaire, which accounted an average of 3.06 log CFU/mL.

With the intention to discriminate LAB present in raw milk that better adapt to the biochemical transformation of the substrate during cheesemaking, some authors have developed specific media: Whey Agar Medium (WAM) (Gatti et al., 2003), Curd Agar Medium (CURDAM) (Lazzi et al., 2007), Cheese Agar Medium (CAM) (Neviani et al., 2009); Heterofermentative Isolini Agar (Isolini et al., 1990) and Arginine Bromocresol Meat Extract Vancomycin (ABEV) (Sohier et al., 2012) where used instead to discriminate facultative and obligate heterofermentative lactobacilli, which are known to differently adapt and grow in cheese during ripening; finally, Cheese Ripening Bacteria Media (CRBM) (Denis et al., 2001) was proposed to selectively count LAB growing on the cheese surface. Low concentration of thermophiles was found in raw milk used for Parmigiano Reggiano (Gatti et al 2008, Neviani et al 2009) and Grana Padano (Franciosi et al., 2011b, 2012; Monfredini et al., 2012) when cultivated on WAM. The raw milk used for the same cheeses showed

a slightly higher concentration of LAB able to grow in curd and cheese media (Gatti et al., 2008; Neviani et al., 2009; Santarelli et al., 2013a). Heterofermentative lactobacilli were mainly analyzed at 37°C using Isolini agar and ABEV, resulting in a mean concentration of 2.82 ± 0.98 log CFU/mL. Although these specific media were used by few studies for the quantification of LAB in raw milk, they have the potential to be applied for the selective recovery of subdominant LAB species often difficult to isolate (Neviani et al., 2009).

2.3.2 Other culture-dependent methods

An alternative method that enables the quantification of LAB in milk, based on their duplicative capacity, is the impedometric analysis. Similarly to plate count, this is a culture-dependent method, but differently, it is not based on the quantification of formed colony but on the detection and estimation of LAB metabolites during their growth.

Impedometric analysis measures the resistance that an alternating current finds when passing through a conductive culture medium, where microorganisms are developing (Bancalari et al., 2016; Yang and Bashir, 2008). When this method is applied to the study of LAB growth in milk, the system can quantify the presence of ions resulting from the degradation of lactose into lactic acid, between two electrodes (Lanzanova et al., 1993; Mucchetti et al., 1994).

Using an instrument such as the BacTrac 4300®, two impedance components can be revealed and measured over time. The first component is the overall relative change in conductivity compared to an initially recorded value (M%) and the second is the variation of the ionic double layer near the surface of the two electrodes (E%) (Bancalari et al., 2019; Futschik and Pfutzner, 1995). Both measures are recorded every 10 min, and incubation time and temperature can be set depending on the chosen conditions.

By plotting these measures against time, conductimetric or impedometric curves can be obtained. If these curves are then fitted with the Gompertz equation, three different parameters can be obtained: Lag, Rate and Yend (Bancalari et al., 2016). Particularly, the first parameter, Lag, is measured in hours and is defined as the time that the LAB cells need to adapt to the analysis conditions, before starting to duplicate. This value is inversely correlated with the amount of LAB cells: the smaller the time, the higher the number of LAB cells (Bancalari et al., 2016).

However, even if both impedance analysis and plate counts depend on the physiological state of the cells and their concentration, the Lag value cannot be converted into CFU. In fact, the Lag value is a more complex concept that depends on several factors such as the LAB genus, the species, biotype and, definitely, the physiological state of LAB cells at the beginning of the analysis. As compared to plate count, this method allows to collect more information about the behaviours of LAB strains, and even small differences can be highlighted within the same species (Bancalari et al., 2016).

Taking into consideration all these aspects, the impedometric method was recently used to quantify the mesophilic LAB in raw milk used to produce Grana Padano by measuring M% every 10 minutes for 48 consecutive hours at 25°C (D'Incecco et al., 2020). The obtained results allow underlining differences between samples in Lag values, ranging from 15 to 20 hours, that were attributed to the different initial concentrations of LAB cells (D'Incecco et al., 2020).

One of the most evident advantages of this technique applied to the analysis of raw milk is the possibility of increasing the throughput, by analysing many samples and variables at the same time and easily having more than two repetitions of each sample, which makes subsequent statistical data analysis and interpretation more reliable, especially if compared to plate counts.

2.4 Conclusions and perspectives

The route of some LAB bearing positive features for cheese production, begins when they reach the raw milk. With the intention of defining which and how many LAB are present in cow raw milk used for cheese production, this review compared and discussed the results of the available studies that addressed this topic through culture-dependent and -independent methods.

The critical review of the literature highlighted an inconsistency in the definition of "raw milk", a term that can be referred to milk sampled at the farm rather than from the cheese vat after skimming. Together with the sampling procedures, other factors such as different farming systems, milk pre-treatments, and seasonality, take part in the modulation of the raw milk microbiota.

The meta-analysis conducted with the use of FMBN was very effective to highlight the complexity of the microbiota of cow raw milk used for cheese production which is composed of over 45 phyla and showed how the genera belonging to LAB are not the most abundant in this microbial ecosystem. It also indicated how LAB belonging to *Lactobacillus* and *Lacticaseibacillus* genera are present in lower relative abundance in comparison with *Lactococcus* and *Streptococcus*.

This result is consistent when compared with LAB concentration evaluated by means of plate counts on agar media: lactobacilli were in fact found on average in lower concentration than coccoid LAB in the raw milk used for the production of 24 different cheese types.

This allows once more to conclude that both culture-dependent and -independent techniques complementarily describe the raw milk microbiota, and the combination of the two approaches restores a complete picture of which and how many LAB are present in the raw milk.

A better knowledge of both the amount and the species of LAB present in the raw milk could be useful in the perspective of monitoring and maintaining the biodiversity of the microbiome of this complex substrate through the managing of parameters at the farm and cheese making level and finally predict the expected outcomes in the resulting raw milk cheeses.

2.5 Author Contributions

LB and MG conceptualized the study; LB performed the investigation, data curation and visualization, and wrote the original draft; MG supervised the study; AL and MG contributed to the methodology of data elaboration, and acquired the fundings: EB and MG cured the section 3.2 of the manuscript. BB critically revised the paper. All authors contributed to manuscript revision, read, and approved the submitted version.

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2.8 Tables

Table 1. List of cheeses whose raw milk was analyzed for LAB concentration through culture-dependent techniques. PDO = Protected Designation of Origin; PGI = Protected Geographical Indication (EP and Council of EU, 2012); PAT = Prodotti Agroalimentari Tradizionali (Mipaaf, 2022).

n.	Cheese	Country	Origin certification	Reference
1	**Arzúa**	Spain	-	(Centeno et al., 1994)
2	**Bergkäse**	Austria	-	(Eliskases-Lechner et al., 1999)
3	**Caciocavallo di Castelfranco**	Italy	PAT	(Giello et al., 2017)
4	**Caciocalvallo Palermitano**	Italy	PAT	(Settanni et al., 2012)
5	**Caciocavallo Pugliese**	Italy	-	(Calasso et al., 2016)
6	**Caciotta**	Italy	-	(Calasso et al., 2016)
7	**Caciotta Montefeltro**	Italy	-	(Aquilanti et al., 2011)
8	**Camembert de Normandie**	France	PDO	(Desmasures et al., 1997) (Henri-Dubernet et al., 2008)
9	**Cantal**	France	PDO	(De Freitas et al., 2007)
10	**Casín**	Spain	PDO	(Alegría et al., 2009)
11	**Casizolu**	Italy	PAT, Slow Food Presidia	(Mangia et al., 2016)
12	**Castelmagno**	Italy	PDO	(Dolci et al., 2008)
13	**Cheddar**	Ireland, USA	-	(McSweeney et al., 1993) (Rehman et al., 2000a) (Rehman et al., 2000b) (Gelsomino et al., 2001) (Agarwal et al., 2006) (Hickey et al., 2007)
14	**Cheese Basse-Normandie area**	France	-	(Denis et al., 2001) (Mallet et al., 2012)
15	**Cheese Quebec area**	Canada	-	(Gagnon et al., 2020)
16	**Cheese Savoie-Haute Savoie area**	France	-	(Michel et al., 2001)
17	**Chihuahua**	Mexico	-	(Béjar-Lio et al., 2020)
18	**Comté**	France	PDO	(Bouton et al., 1998)
19	**Dil pasta-filata**	Turkey	-	(Irkin, 2010)

20	**Emmental de Savoie**	France	PGI	(Sohier et al., 2012)
21	**Fior di Latte di Agerola**	Italy	-	(Coppola et al., 2006)
22	**Genestoso**	Spain	-	(Arenas et al., 2004)
23	**Grana Padano (including Trentingrana)**	Italy	PDO	(Santarelli et al., 2013b) (Bava et al., 2021) (Franciosi et al., 2011a) (Franciosi et al., 2011b) (Franciosi et al., 2012) (Monfredini et al., 2012) (Cremonesi et al., 2020)
24	**Kashar**	Turkey	-	(Çetinkaya and Ece Soyutemiz, 2006)
25	**León**	Spain	-	(Rodriguez Medina et al., 1995)
26	**Minas**	Brasil	-	(Castro et al., 2016) (Luiz et al., 2017)
27	**Montasio**	Italy	PDO	(Carraro et al., 2011) (Marino et al., 2008)
28	**Nite pasta-filata**	Slovakia	-	(Medved'ová et al., 2020)
29	**Nostrano di Primiero**	Italy	PAT	(Poznanski et al., 2004)
30	**Pannerone**	Italy	PAT	(Mucchetti et al., 2009)
31	**Parmigiano Reggiano**	Italy	PDO	(Coppola et al., 2000) (Gatti et al., 2008) (Neviani et al., 2009) (Bortolazzo et al., 2010) (Coloretti et al., 2016) (Franceschi et al., 2021)
32	**Piedmont hard cheese**	Italy	-	(Bautista-Gallego et al., 2014)
33	**Provolone del Monaco**	Italy	PDO	(Aponte et al., 2008)
34	**Ragusano**	Italy	PDO	(Randazzo et al., 2002)
35	**Saint-Nectaire**	France	PDO	(Delbès et al., 2007)
36	**Salers**	France	PDO	(Callon et al., 2004)
37	**San Simón**	Spain	PDO	(García Fontán et al., 2001)
38	**Swiss-type**	France	-	(Beuvier et al., 1997)
39	**Toma tipo Piemonte**	Italy	-	(Astegiano et al., 2014)
40	**Traditional Mountain Cheese Trentino alpine area**	Italy	-	(Carafa et al., 2016) (Carafa et al., 2019) (Carafa et al., 2020) (Franciosi et al., 2009)

41	**Traditional Rugova**	Kosovo	-	(Ajazi et al., 2018)
42	**UK blue-veined**	United Kingdom	-	(Yunita and Dodd, 2018)

2.9 Figures

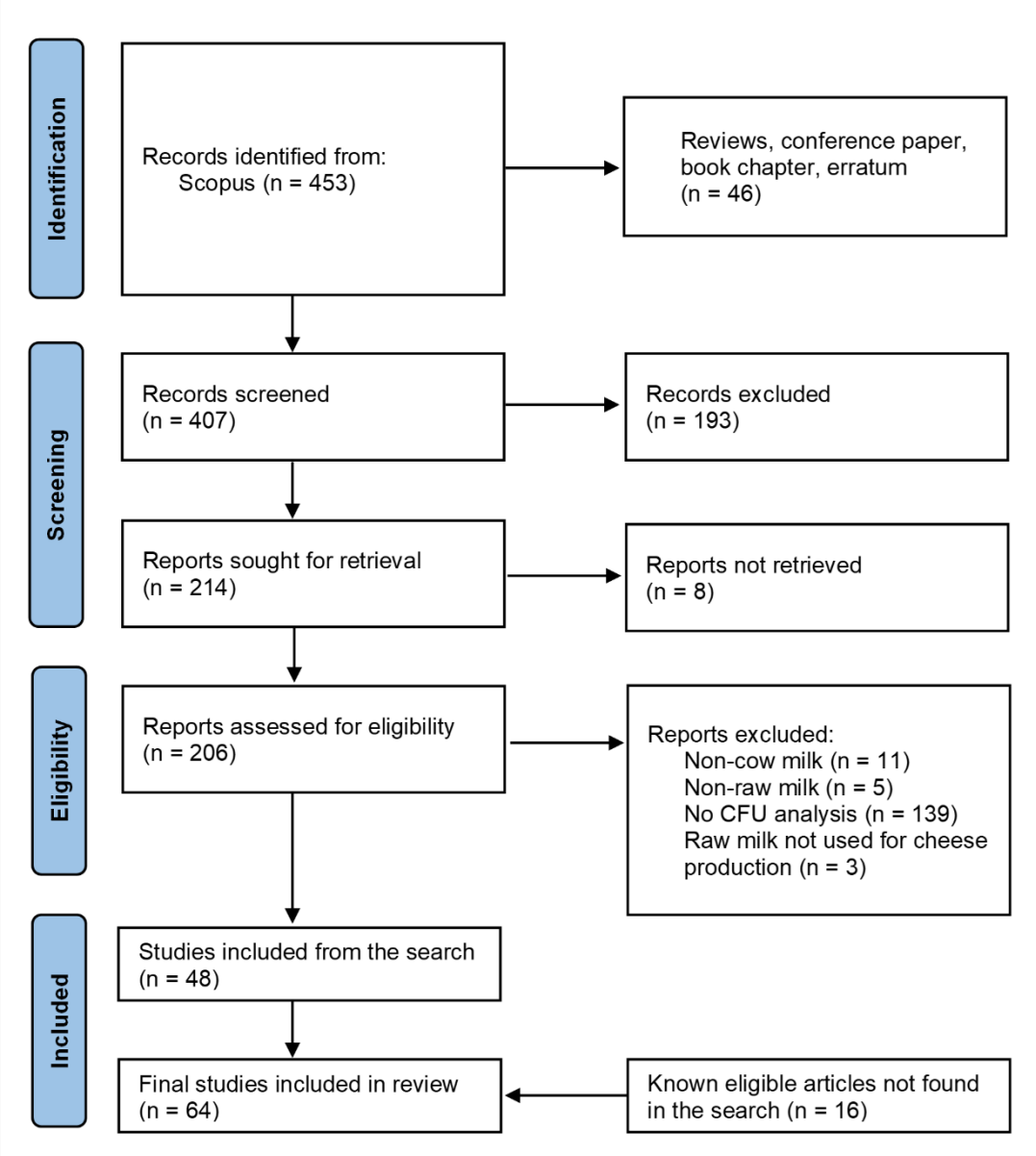

Figure 1. Flow diagram of the studies search for the systematic review of plate counts on agar media of Lactic Acid Bacteria in raw milk. CFU = Colony forming unit.

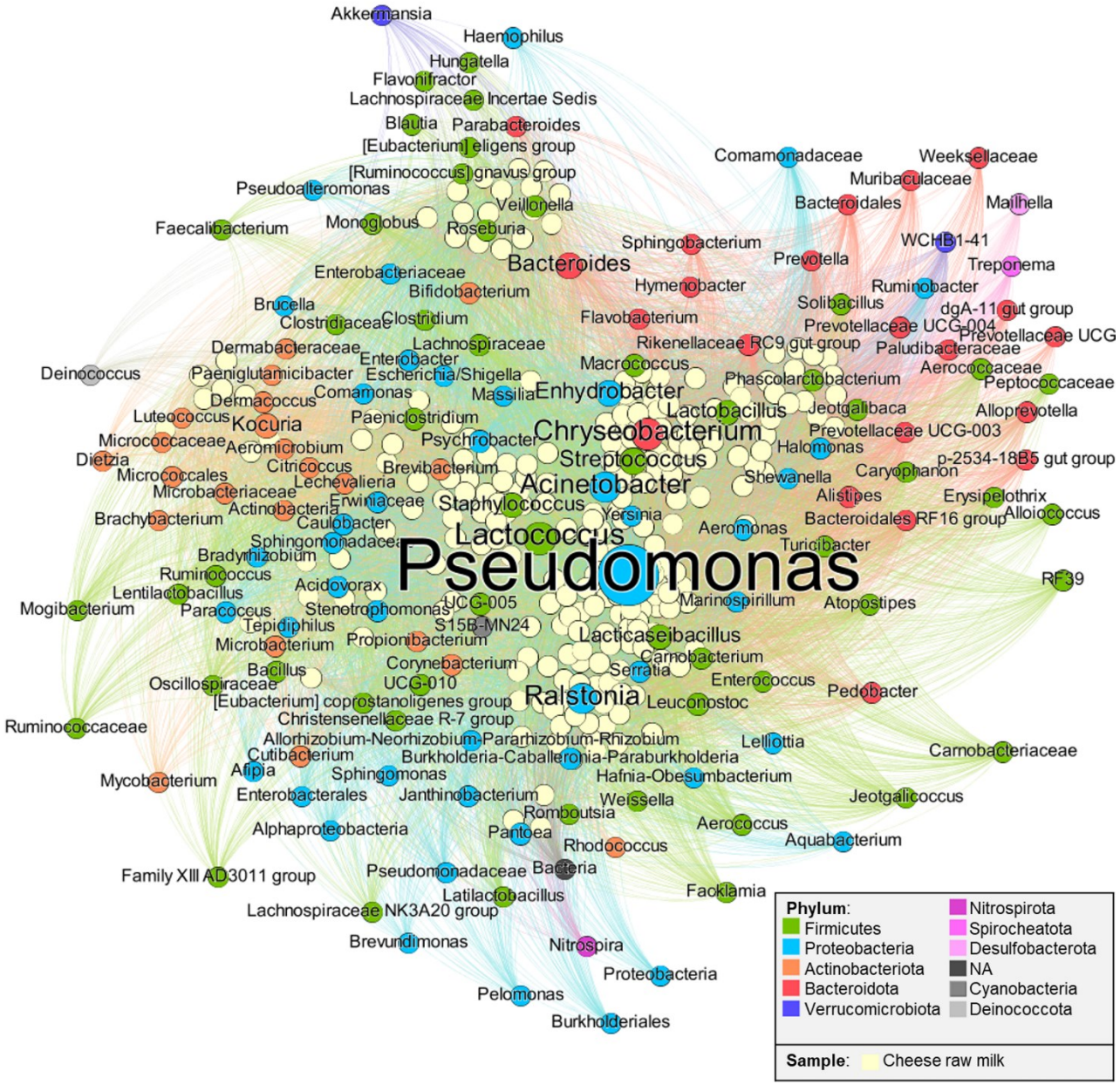

Figure 2. Bipartite network. Nodes represent cheese raw milk samples and Operational Taxonomic units (OTUs), linked to each other by edges. OTUs' label and node size are directly proportional to their relative abundance in all the raw milk samples. Data were extracted from studies listed in supplementary table 2.

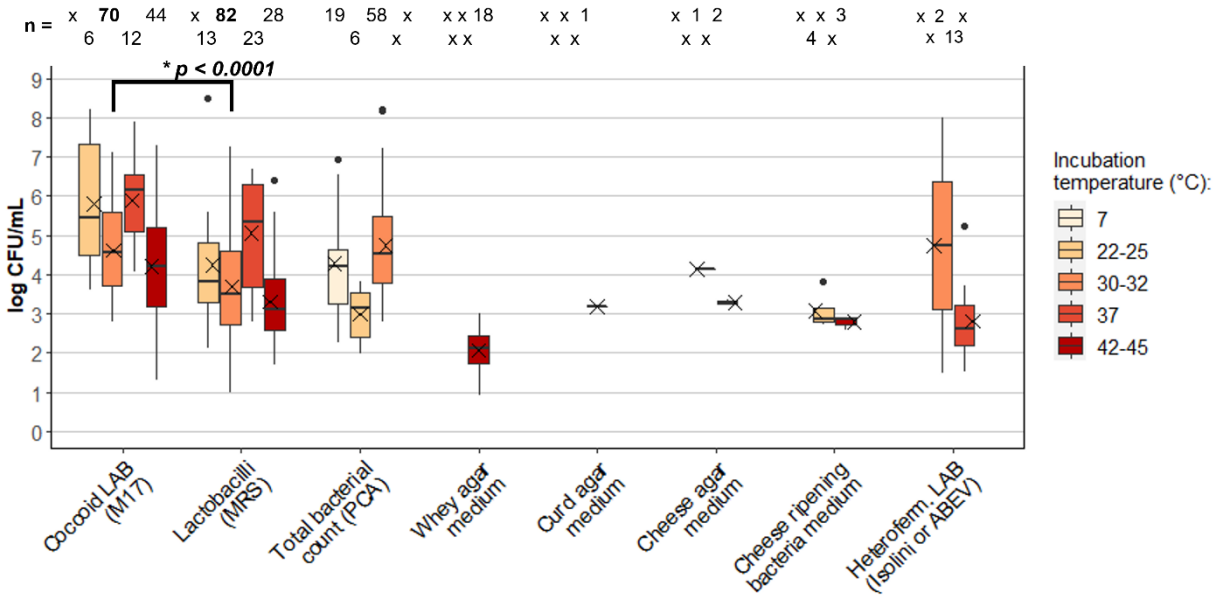

Figure 3. Box plot of Lactic Acid Bacteria (LAB) concentration (log CFU/mL) in cheese raw milk: comparison between different growth media and incubation temperatures. Extremes of the bars represent the max. and min. values; the box lines indicate the 1st quartile, the median, and the 3rd quartile; the mean is indicated by the X within the box; isolated data points are outliers. The number of data (n) is reported at the top of each box (x = missing data). Data were extracted from studies listed in **Table 1**. The p-value (*p) resulting from the t-test applied to Coccoid LAB and Lactobacilli results incubated at 30-32°C is reported (datasets are reported in **Supplementary data 1**). MRS = Man-Rogosa-Sharpe; PCA = plate count agar; ABEV = Arginine Bromocresol Meat Extract Vancomycin.

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2.11 Supplementary material

Supplementary table 1. Selection in the "Filter" section of FMBN version 4.1.2-Shiny App (De Filippis et al., 2018; Parente et al., 2019, 2016) to obtain the taxonomic studies object of the meta-analysis.

Field	Filter for:
Select one or more food codes	*A02LY*
sample type	*sample*
issues	*from -1 to 0*
nature	*raw*
process	*none*
spoilage	*unspoiled*

Supplementary table 2. List of taxonomic studies that analyzed cow raw milk samples used for cheese production through 16S rRNA gene amplicon high-throughput sequencing. FMBN = FoodMicrobionet (De Filippis et al., 2018; Parente et al., 2019, 2016); PDO = Protected Designation of Origin (EP and Council of EU, 2012)

FMNB study ID	Sample type	Raw-milk cheese	Reference
ST7	Cow milk, whole	Caciocavallo Pugliese	(De Pasquale et al., 2014)
ST8	Cow milk, whole	Fontina PDO	(Dolci et al., 2014)
ST10	Cow milk, whole	Caciocavallo Silano PDO	(De Filippis et al., 2016)
ST22	Cow milk, whole	Caciotta	(Calasso et al., 2016)
		Caciocavallo Pugliese	
ST44	Cow milk, whole	Caciocavallo di Castelfranco	(Giello et al., 2017)
ST74	Cow milk, whole	Cheddar	(Falardeau et al., 2019)
		Gruyere	
		Jarlsberg	
ST107	Cow milk, whole	Serra da Canastra	(Kamimura et al., 2020)
ST149	Cow milk, whole	Grana Padano "Trentingrana" PDO	(Cremonesi et al., 2020)
ST178	Cow milk, whole	Cheese South Tyrol area	(Nikoloudaki et al., 2021)

Supplementary table 3. List of the 149 most abundant (weighted degree > 10) Operational Taxonomic Units identified in whole cow raw milk samples analysed in the studies of supplementary table 2, and visualized in the bipartite network (figure 2).

n	Operational Taxonomic Unit	Taxa level	Degree	Weighted degree	Weighted degree %
1	*Pseudomonas*	genus	246	5622.05	23.85
2	*Lactococcus*	genus	224	1706.73	7.24
3	*Chryseobacterium*	genus	172	1545.24	6.56
4	*Acinetobacter*	genus	241	1416.53	6.01
5	*Ralstonia*	genus	107	1341.35	5.69
6	*Streptococcus*	genus	211	847.62	3.60
7	*Bacteroides*	genus	163	826.56	3.51
8	*Enhydrobacter*	genus	161	776.76	3.30
9	*Kocuria*	genus	110	577.73	2.45
10	*Lacticaseibacillus*	genus	178	570.47	2.42
11	*Lactobacillus*	genus	157	554.82	2.35
12	*Staphylococcus*	genus	226	542.62	2.30
13	*Leuconostoc*	genus	167	272.20	1.15
14	*Psychrobacter*	genus	159	263.85	1.12
15	*Macrococcus*	genus	93	245.55	1.04
16	*Burkholderia-Caballeronia-Paraburkholderia*	genus	79	219.70	0.93
17	*Veillonella*	genus	77	206.53	0.88
18	*Pantoea*	genus	29	204.16	0.87
19	*Paeniclostridium*	genus	101	202.47	0.86
20	*UCG-005*	genus	149	198.00	0.84
21	*Phascolarctobacterium*	genus	124	176.82	0.75
22	*Roseburia*	genus	38	152.24	0.65
23	*Bifidobacterium*	genus	81	149.63	0.63
24	*Rikenellaceae RC9 gut group*	genus	156	149.58	0.63
25	*Acidovorax*	genus	78	142.66	0.61
26	*Carnobacterium*	genus	50	134.31	0.57

27	*Serratia*	genus	59	129.68	0.55
28	*Lachnospiraceae*	family	137	128.56	0.55
29	*Alistipes*	genus	162	126.20	0.54
30	*Escherichia/Shigella*	genus	95	122.43	0.52
31	*Paracoccus*	genus	89	122.18	0.52
32	*Corynebacterium*	genus	135	114.86	0.49
33	*Jeotgalibaca*	genus	116	113.30	0.48
34	*Stenotrophomonas*	genus	152	104.87	0.44
35	*Halomonas*	genus	93	101.43	0.43
36	*Enterococcus*	genus	157	96.30	0.41
37	*UCG-010*	family	126	84.97	0.36
38	*Brevibacterium*	genus	82	82.44	0.35
39	*Flavobacterium*	genus	156	81.05	0.34
40	*Sphingomonadaceae*	family	83	77.13	0.33
41	*Caulobacter*	genus	86	74.77	0.32
42	*Shewanella*	genus	68	74.04	0.31
43	*Clostridium*	genus	88	72.60	0.31
44	*Propionibacterium*	genus	33	63.90	0.27
45	*Turicibacter*	genus	143	61.47	0.26
46	*Micrococcaceae*	family	50	59.02	0.25
47	*Christensenellaceae R-7 group*	genus	120	59.00	0.25
48	*Parabacteroides*	genus	57	58.25	0.25
49	*Atopostipes*	genus	152	56.27	0.24
50	*Prevotellaceae UCG-003*	genus	126	55.03	0.23
51	*Bacteria*	kingdom	126	53.23	0.23
52	*Bacteroidales RF16 group*	family	142	52.19	0.22
53	*Lechevalieria*	genus	43	52.07	0.22
54	*Prevotellaceae UCG-004*	genus	107	51.15	0.22
55	*Micrococcales*	class	66	50.97	0.22

56	*[Eubacterium] eligens group*	genus	47	48.92	0.21
57	*Weissella*	genus	46	48.66	0.21
58	*[Eubacterium] coprostanoligenes group*	family	106	47.87	0.20
59	*Bacillus*	genus	70	47.11	0.20
60	*Enterobacteriaceae*	family	93	46.57	0.20
61	*Prevotella*	genus	107	45.84	0.19
62	*[Ruminococcus] gnavus group*	genus	23	45.68	0.19
63	*Dermacoccus*	genus	46	45.00	0.19
64	*Actinobacteria*	class	65	44.80	0.19
65	*Enterobacter*	genus	48	41.76	0.18
66	*Aerococcus*	genus	142	39.26	0.17
67	*Solibacillus*	genus	65	38.43	0.16
68	*Cutibacterium*	genus	103	36.18	0.15
69	*Janthinobacterium*	genus	44	36.15	0.15
70	*Bacteroidales*	class	129	35.95	0.15
71	*Clostridiaceae*	family	65	35.90	0.15
72	*Monoglobus*	genus	76	34.66	0.15
73	*Ruminococcus*	genus	91	32.72	0.14
74	*Tepidiphilus*	genus	16	32.64	0.14
75	*Romboutsia*	genus	40	29.99	0.13
76	*Marinospirillum*	genus	77	29.40	0.12
77	*Caryophanon*	genus	59	29.11	0.12
78	*Microbacterium*	genus	62	28.85	0.12
79	*Sphingobacterium*	genus	92	28.70	0.12
80	*Latilactobacillus*	genus	89	28.69	0.12
81	*Massilia*	genus	52	28.66	0.12
82	*Bradyrhizobium*	genus	63	27.94	0.12
83	*Alphaproteobacteria*	class	76	27.94	0.12
84	*Allorhizobium-Neorhizobium-*	genus	37	27.22	0.12

	Pararhizobium-Rhizobium				
85	*Blautia*	genus	41	26.77	0.11
86	*Paludibacteraceae*	family	71	25.89	0.11
87	*Microbacteriaceae*	family	59	25.87	0.11
88	*Aerococcaceae*	family	75	23.80	0.10
89	*Hafnia-Obesumbacterium*	genus	30	23.67	0.10
90	*Luteococcus*	genus	41	23.53	0.10
91	*Dietzia*	genus	58	23.33	0.10
92	*Ruminobacter*	genus	57	23.09	0.10
93	*WCHB1-41*	class	85	23.04	0.10
94	*p-2534-18B5 gut group*	family	89	23.03	0.10
95	*Comamonas*	genus	51	22.98	0.10
96	*Deinococcus*	genus	52	22.81	0.10
97	*Alloprevotella*	genus	93	22.49	0.10
98	*Oscillospiraceae*	family	80	22.30	0.09
99	*Facklamia*	genus	102	22.19	0.09
100	*Aeromonas*	genus	22	21.70	0.09
101	*Yersinia*	genus	39	21.51	0.09
102	*Comamonadaceae*	family	99	21.30	0.09
103	*Lentilactobacillus*	genus	76	21.08	0.09
104	*Muribaculaceae*	family	89	21.06	0.09
105	*Jeotgalicoccus*	genus	130	20.53	0.09
106	*Brucella*	genus	50	19.32	0.08
107	*Erysipelothrix*	genus	68	18.81	0.08
108	*Pedobacter*	genus	87	18.72	0.08
109	*dgA-11 gut group*	genus	72	18.53	0.08
110	*S15B-MN24*	class	6	18.35	0.08
111	*Paeniglutamicibacter*	genus	32	17.03	0.07
112	*Aeromicrobium*	genus	23	16.73	0.07
113	*Brachybacterium*	genus	30	16.11	0.07
114	*Treponema*	genus	66	15.86	0.07

115	*Pseudomonadaceae*	family	64	15.78	0.07
116	*Lelliottia*	genus	34	15.64	0.07
117	*Sphingomonas*	genus	38	15.58	0.07
118	*RF39*	class	94	15.30	0.06
119	*Alloiococcus*	genus	62	15.19	0.06
120	*Mailhella*	genus	79	14.98	0.06
121	*Nitrospira*	genus	57	13.96	0.06
122	*Afipia*	genus	43	13.80	0.06
123	*Lachnospiraceae NK3A20 group*	genus	53	13.27	0.06
124	*Dermabacteraceae*	family	22	13.23	0.06
125	*Haemophilus*	genus	41	13.12	0.06
126	*Mogibacterium*	genus	77	13.09	0.06
127	*Peptococcaceae*	family	54	12.87	0.05
128	*Hymenobacter*	genus	32	12.84	0.05
129	*Lachnospiraceae Incertae Sedis*	genus	20	12.70	0.05
130	Flavonifractor	genus	21	12.56	0.05
131	*Enterobacterales*	class	49	12.43	0.05
132	*Erwiniaceae*	family	15	12.37	0.05
133	*Carnobacteriaceae*	family	113	12.20	0.05
134	*Faecalibacterium*	genus	52	11.80	0.05
135	*Pseudoalteromonas*	genus	41	11.46	0.05
136	*Family XIII AD3011 group*	genus	71	11.37	0.05
137	*Citricoccus*	genus	18	11.21	0.05
138	*Ruminococcaceae*	family	77	11.00	0.05
139	*Mycobacterium*	genus	49	10.87	0.05
140	*Pelomonas*	genus	60	10.74	0.05
141	*Burkholderiales*	classe	64	10.62	0.05
142	*Brevundimonas*	genus	47	10.50	0.04
143	*Akkermansia*	genus	54	10.40	0.04
144	*Hungatella*	genus	17	10.21	0.04
145	*Rhodococcus*	genus	23	10.20	0.04

146	*Prevotellaceae UCG-001*	genus	46	10.17	0.04
147	*Aquabacterium*	genus	55	10.15	0.04
148	*Weeksellaceae*	family	53	10.13	0.04
149	*Proteobacteria*	phylum	51	10.03	0.04

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Futher supplementary material is available online at:

https://www.frontiersin.org/articless/10.3389/fmicb.2022.1092224/full#supplementary-material.

Data Sheet 1.xlsx

Supplementary data 1: dataset of lactobacilli (n = 82) and coccoid LAB (n = 70) concentration (log CFU/mL) in raw milk used for cheese production analyzed using MRS and M17 growth media, respectively, incubated at 30-32°C.

Supplementary data 2: growth media, supplements, and incubation conditions [temperature (°C), time (hours), and atmosphere modification] used in the studies that analyzed raw milk with culture-dependent techniques.

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3 Raw milk *Lacticaseibacillus* subsp. geno-phenotyping

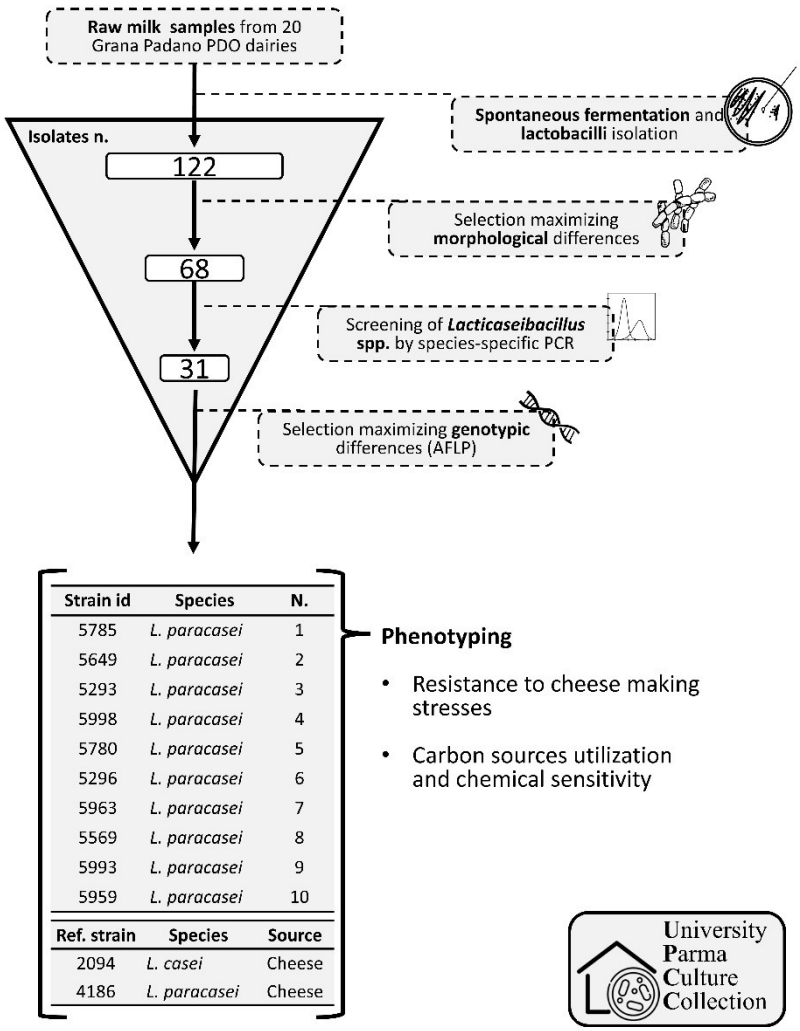
***Lacticaseibacillus* strains isolated from raw milk: screening strategy for their qualification as adjunct culture in cheese making**

Luca Bettera[a1*], Alessia Levante[1a], Elena Bancalari[a], Benedetta Bottari[a], Martina Cirlini[a], Monica Gatti[a]

[a] Department of Food and Drug, University of Parma, Parma, Italy

[1] These authors contributed equally to the work

* Corresponding author



Abstract

Microbiological and biochemical changes of the curd are crucial factors to produce raw-milk, long-ripened cheeses. The microbial ecology fundamentals of this cheese variety consist of a complex interaction between starter lactic acid bacteria (LAB) and non-starter LAB (NSLAB). The need to standardize cheesemaking and accelerate ripening has driven the use of several NSLAB as adjuncts or attenuated adjuncts secondary starters. From this perspective, this work focused on the isolation and characterization of NSLAB from raw cow's milk from 20 dairies that produce Grana Padano, the PDO cheese most exported in the world. Although the aromatic properties of the strains are paramount, other phenotypic traits need to be considered, such as the capability to endure the technological parameters encountered during cheese making. One hundred and twenty-two strains have been isolated using the strategy of low temperature spontaneous fermentation. Sixty-eight belonging to *Lacticaseibacillus casei* group strains were genotypically characterized and their metabolic activity and chemical sensitivity by phenotypic microarray have been carried out. The impedance technique was used to evaluate 31 strains' growth performance in milk in combination with the application of thermal stress, salt, and lysozyme. Finally, the production of volatile compounds after their growth in milk was assessed, using solid-phase microextraction gas chromatography-mass spectrometry. The complex characterization of 10 strains have been discussed to reach the elements necessary to choose which of them can be the best for potential use as adjunct strains in the production of raw milk, long-ripened hard cheeses. The two best strains were 5959_Lbparacasei and 5296_Lbparacasei even if resistance to fusidic acid for the first and to vancomycin for the latter have to be correctly measured before their employment. Other strains with interesting aromatic capabilities but lower heat resistance were 5293_Lbparacasei, 5649_Lbparacasei and 5780_Lbparacasei, which could be good candidates as adjunct strains for uncooked cheese production.

3.1 Introduction

Microbiological and biochemical changes of the curd are crucial factors to produce raw-milk, long-ripened cheeses. Manufacturing of traditional cheeses that fall within this category, such as Grana Padano PDO [GP, protected designation of origin; (EP and Council of EU 2012)], rely on a defined set of technological operations, as well as a complex microbiota that plays a key role in defining the quality of the final cheese product.

The microbial ecology distinguishing this cheese variety is largely influenced by the technological processes that are applied during cheesemaking, so much so that two different microbial populations develop successively: firstly, the starter lactic acid bacteria (SLAB), deliberately added mainly for curd acidification, followed by non-starter LAB (NSLAB), adventitious milk contaminants correlated to cheese flavor formation during ripening (Gatti et al. 2014; Giraffa 2021).

The NSLAB moiety of GP cheese is mainly composed of facultatively heterofermentative lactobacilli, belonging to the *Lacticaseibacillus* genus, which was recently updated regarding its taxonomy by Zheng et al. (2020). This taxonomic clade includes the species *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus*, which were previously defined as the *Lb. casei* group, due to their pylogenetic relatedness (Toh et al. 2013).

These strains enter the cheesemaking process from raw cow's milk, where they are not the prevalent genus (Bettera et al. 2023), but possess adaptation capabilities that allow them to survive the initial steps of the manufacturing process, making *Lb. casei* group the dominant microbiota of GP cheese during the subsequent ripening process (Bottari et al., 2018; Bottari et al., 2020). Indeed, during the early GP cheese manufacturing, technological parameters (cooking temperature, pH decrease, salt exposure, lysozyme addition) will ensure the quality of the produced cheese, and prevent the development of spoilage and pathogen bacteria, throughout the subsequent long ripening process (Gatti et al., 2014; Giraffa 2021).

Therefore, NSLABs need to possess strategies to adapt to the varying environment of the ripening cheese, and strains belonging to the *Lb. casei* group could benefit from the ability to ferment different carbohydrates (Buron-Moles et al., 2019; Lazzi et al., 2013), thus allowing them to develop in the cheese matrix after SLAB have depleted lactose. Another aspect that could improve their fitness in the cheese environment is the capability of entering a dormancy state (Levante et al., 2021), where environmental conditions do not hamper microbial viability, turning into a metabolically active and dividing state when the stress factors are released, after the brining step, as shown by various studies that focused on the dynamics of GP cheese microbiota during ripening (Levante et al. 2017; Lazzi et al. 2016; Alessandria et al. 2016).

The development of NSLAB in long-ripened cheeses is associated with the formation of the peculiar organoleptic properties of the final product and for this reason strains belonging to the *Lb. casei* group have been proposed as adjunctive cultures for the dairy industry (Gobbetti et al. 2015). Indeed, several studies have proposed the characterization of NSLAB belonging to the *Lb. casei* group of cheese origin, aiming to characterize the capability of these strains to produce volatile compounds in milk (Levante et al. 2020) or in cheese-mimicking substrates (Cuffia et al. 2020), for the informed selection of strains to be used as aromatic adjunct starter for cheesemaking.

Another approach is to focus on *Lb. casei* isolates that originate from raw cow's milk used for cheese manufacturing, as reported by Bancalari et al. (2017), who used impedance analysis to measure isolates' technological performance and studied the volatile profile to identify potential adjunct strains for the dairy industry.

Although the aromatic properties of the selected strain(s) are paramount, other phenotypic traits need to be considered, such as the capability to endure the technological parameters encountered during cheesemaking. In this perspective, this work focused on the isolation and characterization of NSLAB from raw cow's milk from 20 dairies that produce GP cheese. The strains have been first genotypically characterized, followed by a screening of their metabolic activity and chemical sensitivity by phenotypic microarray. The impedance technique was used to evaluate the strains' growth performance in milk in combination with the application of thermal stress, salt, and lysozyme. Finally, the production of volatile compounds after their growth in milk was assessed, using solid-phase microextraction (SPME) gas chromatography-mass spectrometry (GC-MS).

This multifactorial approach allows to highlight the genotypic and phenotypic biodiversity of strains from the raw milk microbial community, while collecting data on their different suitability for dairy applications by evaluating adaptation and aromatic properties.

3.2 Material and methods

3.2.1 Raw milk sampling

Raw milk for Grana Padano DOP (Consorzio Tutela Grana Padano 1996)(GP) production was taken from 20 dairies dislocated in the entire PDO area. More precisely, vat raw milk (pH = 6.71 ± 0.03; temperature = 16.07 ± 6.46°C), consisting of a mix of partially skimmed milk by overnight spontaneous creaming and morning whole milk in a 50:50 v/v ratio; was delivered to the laboratory in refrigerated conditions.

3.2.2 Strain isolation and screening

3.2.2.1 Raw milk spontaneous fermentation

Raw milk samples (100 mL) were immediately incubated anaerobically at 8°C for 60 days in order to perform a spontaneous fermentation and select the subdominant NSALB strains, as proposed by (Bancalari et al. 2017). At the end of the incubating period, a simple odor evaluation was performed by the operators to assess the intensity of the following aroma descriptors on a scale from 0 (absent) to 5 (strong): expired milk (off-flavor), acidic (yogurt-like), and cheesy.

3.2.2.2 Bacteria culturing and isolation

The spontaneous fermented raw milk was analyzed for the lactobacilli viable count; furthermore, the pH was measured using a pHmeter Beckman Instrument mod F350 (Furlenton, CA, USA) and glass electrode (Hamilton, Bonaduz, Switzerland)

Samples (1mL) were ten-fold serially diluted in sterile Ringer's solution (Oxoid, Ltd., Basingstoke, United Kingdom), and plated (dilution ranging from 10^{-2} to 10^{-7}) on acidified de Man, Rogosa and Sharpe agar (De Man, Rogosa, and Sharpe 1960) (MRSa; pH=5,4 reached by acetic acid addition; Oxoid). Plates were incubated at 37°C for 48h in anaerobic conditions with a gas pack (Fisher Scientific, Rodano, Italy) in MRS broth. The lactobacilli concentration was calculated as colony forming units per mL (CFU/mL).

A number between 5−7 colonies for each sample were selected maximizing their macroscopic diversity (i.e. size, thickness, shape, color; data not shown) using Nikon Eclipse Si microscope (Nikon Europe, Firenze, Italy). The number was further reduced by screening the most morphologically different isolates based on descriptors of cell length (short, medium, long) and organization (single rod, diplo- strepto-bacilli; data not shown). Isolates were purified by streaking repeatedly on MRS agar, then grown in MRS broth and stored at -80°C with the addition of 30% v/v of glycerol (solution 50% v/v glycerol-distilled H_20).

Three strains belonging to the University of Parma Culture Collection (UPCC) were used in this study as reference, i.e., strains *Lb. rhamnosus* 1473, *Lb. casei* 2094 and *Lb. paracasei* 4186, isolated from ripened cheeses and characterized at the genome level (Levante et al., 2021)

3.2.2.3 DNA extraction and strain identification

Bacterial genomic DNA was extracted using the DNeasy Kit (Qiagen, Milan, Italy) following the manufacturer's instructions.

The strains belonging to the *Lacticaseibacillus casei* group (*Lb. casei* gr.) were identified by doing an endpoint species-specific PCR targeting the *spxB* gene. The primer used were *poxcDNAFw* 5'-CAGACGCAATGATCAAGGTG-3' and *poxPromRv* 5'-AATGCGCC**Y**ACTTCTTCATG-3' as proposed by (Levante et al. 2017; Savo Sardaro et al. 2016). Each PCR mixture contained 1 µL of each primer at concentration [10 µM], 10 µL of 2X GoTaq Master Mix (Promega, Madison, WI, USA), 7 µL of sterile MilliQ water, and 1 µL of DNA (final volume, 20 µl). The following amplification conditions were used: initial strand denaturation at 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The PCR results were checked with a 1% agarose electrophoresis gel run. Discrimination among the *Lacticaseibacillus casei*/*paracasei* and *rhamnosus* species of the isolates was performed according to the melting temperature curve analysis method, a post-PCR method described by Savo Sararo et al. (2016), however, all the newly isolated strains belonged to the *Lb. paracasei* species, as confirmed by corresponding melting peak temperatures (**Supplementary. Figure 1**).

The strains which not belonged to the *Lacticaseibacillus* genus were identified by the amplification and sequencing of the 16S rRNA gene. The primers used were 46Fw 5'-GC**Y**TAACACATGCAAGTCGA-3' and 536Rv 5'-GTATTACCGCGGCTGCTGG-3' (Marchesi et al. 1998). Each PCR mixture contained 1 µL of each primer at concentration [10 µM], 10 µL of 2X GoTaq Master Mix (Promega, Madison, WI, USA), 7 µL of sterile MilliQ water, and 1 µL of DNA (final volume, 20 µl). PCR products were checked on 1.5 % w/v agarose gel in 1×TAE buffer at 90 V/cm for 20 min and visualized on a GelDoc Go gel imaging system (BioRad, Milan, Italy). The amplified sequences were compared with reference sequences using the Basic Local Alignment Search Tool (BLAST®, https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the 16S ribosomal RNA sequences database.

3.2.2.4 Strain genotypic characterization

The isolates belonging to the *Lacticaseibacillus* genus were further screened in order to select the most genotypically different. The Amplified Fragment Length Polymorphisms (AFLP) technique was applied following the protocol improved by (Bertani et al. 2019).

Briefly: after a restriction-ligation step, the digested-ligated DNA underwent a pre-amplification using the pre-selective primers *Eco*RI-0 5'- GACTGCGTACCAATTC and *Mse*I-0 5'-GATGAGTCCTGAGTAA; finally, a selective PCR using the primers *Eco*RI-A 5'-GACTGCGTACCAATTCA and *Mse*I-A 5'-GATGAGTCCTGAGTAAA was done. The selective primers were previously checked for their efficiency using the tool *In silico* AFLP-PCR amplification (Bikandi et al. 2004).

First, a restriction-ligation (R/L) step was performed, and the reactions were composed of10 µl of 250 ng/µl BSA (New England Biolabs, Ipswich, MA, USA), 5 µl of 1× T4 DNA ligase buffer with 1 µl of 1 mM ATP (New England Biolabs, Ipswich, MA, USA), 1 µl of 50 pmol/µl MseI adaptor, 1 µl of 5 pmol/µl EcoRI adaptor, 1 µl of 10 mM ATP (Invitrogen S.R.L., Milano, Italy), 0,5 µl of 10 U/µl MseI (New England Biolabs, Ipswich, MA, USA), 0,25 µl of 20 U/µl EcoRI (New England Biolabs, Ipswich, MA, USA), 0,1 µl of 200U/µl of T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and 15 µl of template DNA (500 ng), volume was adjusted to 50 µl with milliQ water.

The reaction was incubated at 37°C for 4 hours. After the R/L step, the digested-ligated DNA underwent two amplification reactions that were executed after incubation. The first pre-selective PCR included 10 µl GoTaq® Master Mix 2X (Promega, Madison, WI, USA), 0,6 µl of 10 µM EcoRI-0 Primer (5'- GACTGCGTACCAATTC-3'), 0,6 µl of 10 µM MseI-0 Primer (5'-GATGAGTCCTGAGTAA-3') and 2 µl of digested-ligated DNA, until a final volume of 20 µl. Amplified samples were diluted 1:10 in TE (10 mM_TRIS HCl at pH=8, 0.1 mM EDTA at pH=8). The selective amplification reaction was composed of 10 µl GoTaq® Master Mix 2X (Promega, Madison, WI, USA), 0,6 µl of 10 µM EcoRI-A Primer (5'- GACTGCGTACCAATTCA-3'), 0,6 µl of 10 µM MseI-A Primer (5'-GATGAGTCCTGAGTAAA-3') and 2 µl of diluted DNA, until a final volume of 20 µl. All PCRs were performed in a GeneAmp® PCR System 2700 (Applied Biosystem, Foster City, CA, USA) by following the thermocycler program of Bertani et al. (2019). Amplifications were followed by electrophoresis on a 1.2% agarose gel in 1×TAE buffer at 90 V/cm for 20 min and visualized as previously described for 16S gene PCR products, to confirm successful PCR reactions. AFLP profiles were compared through Bionumerics version 8.1 (Applied Maths NV, Sint-Martens-Latem, Belgium) by setting GeneScan-500 [LIZ] as size standard to normalize peaks. Bands with a length ranging between 50 and 500 bp were considered. AFLP profiles were compared by Jaccard similarity coefficient and clustered according to Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The correlation similarity cut-off was set at 40% according to the similarity coefficient obtained for biologically independent replicates of type strains used as reference, i.e. *Lb. rhamnosus* 1473, *Lb. casei* 2094 and *Lb. paracasei* 4186.

3.2.3 Strain phenotypic characterization

3.2.3.1 Evaluation of the strain resistance to cheesemaking stresses

The 12 strains selected from the screening (10 isolates and 2 reference, **Figure 1**) were evaluated for their ability to grow in UHT partially skimmed milk (UPSM). Frozen broth cultures were revitalized by inoculation in 6 mL of MRS broth (3% v/v) and incubating overnight anaerobically at 37°C. This propagation was repeated three times. The viable count of each strain after growing in MRS broth (t = 20 h) was calculated by plating on MRSa agar. Cultures were then propagated twice in UPSM (2% v/v) at the same conditions; viable counts on MRSa agar after growing in UPSM (t = 20 h) were also calculated (**Table 1**). Each culture was then tenfold diluted and inoculated in 6 mL of UPSM (2% v/v) in six different conditions: i) UPSM; ii) UPSM + NaCl (1% w/v); iii) UPSM + NaCl (2,5% w/v); iv) UPSM + NaCl (5% w/v); v) UPSM + Lisozime (200 mg/L); vi) the culture was heat treated at 54°C for 1h and before the inoculation in UPSM. The inoculated sample was analyzed by means of impedometric analysis, following the methodology described by (Bancalari et al. 2016). Briefly, a BacTrac 4300® Microbiological Analyzer system (SY-LAB, Neupurkersdorf, Austria) was used to evaluate the impedometric curves. The M% values over time (48 h at 37 °C) curves were interpolated with Gompertz model using DMFit v3.5 (Baranyi and Roberts 1994). The strain's growth kinetic parameters extracted were: lag (h), adaptation time to the substrate before the growth; rate (-), acidification speed; and yEnd (M%), maximum acidification capacity. If the model was not applicable because of absence of growth, the values used were lag = 48 h, rate = 0, and yEnd = 0. The analysis was done in triplicate.

3.2.3.2 Evaluation of metabolic capabilities

Carbon source utilization and chemical sensitivity

The Biolog GEN III MicroPlates (BIOLOG Inc.©, USA) were used to study the carbon sources utilization and chemical sensitivity of screened strains. All tested compounds are prefilled and dried into the microplate together with Tetrazolium Violet redox dye used as colorimetrical indicator of microbial metabolic activity.

For the preparation of the inoculum, pure cultures were grown on solid MRS agar media, and single colonies were picked from the plate and suspended in 12 ml of inoculation fluid IF-A (BIOLOG Inc.©, USA) until reaching the recommended cell density for LAB using a cotton-tipped swab, until reaching 90-98% of transmittance (T) on the BIOLOG turbidimeter. Cell suspension was gently inoculated into the 96-wells GEN III microplate avoiding cross-contamination among substrates, by using a multichannel pipette. The microplate was incubated at the optimal strain's temperature to allow the phenotypic fingerprint to form. Metabolic activity and chemicals sensitivity of strains was detected on BIOLOG MicroReader Station (BIOLOG Inc.©, USA) by absorbance reading at a wavelength of 590nm, before incubation and after 72 h. Measurement of the reduced Tetrazolium Violet dye (purple colored), indirectly represents the increased metabolic activity in wells where cells used the substrate (Acin-Albiac et al. 2020).

Having single end-point measurements, the phenotypic raw data were elaborated calculating the Average Well Color Development (AWCD) as reported in literature (Acin-Albiac et al. 2020; Dubey, Malla, and Kumar 2022). To reduce the noise levels, all absorbance values of the carbon source utilization (GEN III microplate columns 1-9) were referenced against the negative control well (A1), while the absorbance values of the chemical sensitivity (GEN III microplate columns 10-12) were subtracted from half of the positive control well (A10), and subsequently all divided by the respective AWCD obtaining the Absorbance Ratios parameter (**Table 2**). Negative values were set to 0. Normalized data were used for statistical analysis in the R environment (R Core Team 2022), using the UPGMA clustering method of the "stats" package. Results of metabolic and sensitivity assay were plotted in two different heatmaps using "heatmaps" package (Kolde 2019).

Volatile compounds

The 10 LAB strains were tested for their ability to produce volatile compounds in fermented milk following the method proposed by (Bancalari et al. 2017) with slight modifications. Briefly, 5 mL of UHT milk was inoculated with each strain and incubated anaerobically for 4 days at 37 ◦C. The analysis was done in duplicate. For each considered sample, the fermentation step was conducted directly in 20 mL glass vials.

The samples were then analyzed by Head Space Solid Phase Microextraction technique coupled with Gas Chromatography-Mass Spectrometry analyses (HS-SPME/GC-MS). Before analysis, 5 µL of Toluene (100 mg/L, Sigma, St. Louis, MO, USA) were added into the sealed vials to be used as an internal standard. Volatile compounds were extracted at 40° C for 20 min, after an equilibration time of 20 min at the same temperature and adsorbed on a SPME fibre functionalized with a divinylbenzene-carboxen-polydimethylsiloxane coating (Supelco Inc., Bellefonte, PA, USA).

All the analyses were conducted on a TRACE 1300 gas chromatograph (Thermo Fisher Scientific Inc.) coupled to a ISQ mass spectrometer (Thermo Fisher Scientific Inc.) equipped with an electronic impact source. Analytes were separated on a Supelcowax 10 capillary column (Supelco, Bellefonte, PA, USA; 30 m ×0.25 mm× 0.25 µm), applying a temperature ramp as follows: at starting oven temperature was set at 50°C and maintained for 3 min, then it was increased at 130°C (5°C/min), and then at 220°C (15°C), after that, the temperature was maintained at 220°C for 10 min. The detection was performed setting the mass spectrometer in full scan mode, registering the spectra in the mass range of 40-500 m/z.

Peak identification was performed both by comparison of the experimental mass spectra with those reported in the NIST14 instrument library, as by the linear retention indexes (LRIs) calculation, based on the analysis of a C_8–C_{20} alkane standard solution (Sigma–Aldrich, Milan, Italy) performed in the same instrumental conditions applied for sample characterization. A semi-quantitative approach was performed by comparison of the relative peak areas of the identified volatiles to the peak area of Toluene. When a compound was not produced by the strain, the value = 0 mg/L was assigned.

3.2.4 Statistical analysis

To evaluate the significance of the factors "strain" and "analysis condition" in influencing the impedometric parameters during the resistance to cheesemaking stress test, the one-way ANOVA model was applied ($\alpha = 0.05$) using PROC GLM statement of SAS® OnDemand for Academics (©2022 SAS Institute Inc, Cary, NC, USA). A post hoc LSMEANS test with *t* adjustment was applied to check the difference between the samples.

Impedometric and gas-chromatography results were subjected to principal component analysis (PCA) using the packages "FactoMineR" (Le, Josse, and Husson 2009) and "factoextra" for the results plotting (Kassambra and Mundt 2020). Furthermore, the Pearson coefficient for variables correlations was calculated and plotted respectively using the packages "stats" and "corrplot" (Taiyun and Simko 2021). Both the PCA and the Pearson correlation were done in the R environment.

3.3 Results

3.3.1 Screening of the isolates

A schematization of the isolates' screening strategy is reported in **Figure 1.** At the end of the incubation period (60 d at 8 °C anaerobically), all the raw milk samples developed a cheesy note, and in many of them (8 out of 20) this was perceived as a strong flavor. The acidic flavor was present in almost all the samples, while only two of them developed an unpleasant expired milk off-flavor. The spontaneous low temperature fermented raw milks were rich in lactobacilli, having a viable count on average of 7.23 log CFU/mL (range 8.55−5.76) and an average pH = 4.52 (range 4.69−4.31) (**Table 2**).

A total of 122 lactobacilli were isolated from all the samples. The 68 most morphologically different lactobacilli were selected for molecular identification (**Figure 2**). The specie-specific PCR revealed that 31 of these isolates belonged to the *Lb. casei* group. Analyzing the melting

temperature curves, the strains were discriminated as belonging to the *Lb. casei/paracasei* species (**Supplementary Figure 1**). The remaining isolates belonged to the species *Lentilactobacillus parabuchneri* (n = 8), *Lentilactobacillus otakiensis* (n = 7), *Leuconostoc lactis* (n = 5), *Leuconostoc pseudomesenteroides* (n = 4), *Leuconostoc mesenteroides* (n = 3), *Lentilactobacillus hilgardii* (n = 3), *Lentilactobacillus diolivorans* (n = 3), *Pediococcus acidilactici* (n = 1), *Lentilactobacillus buchneri* (n = 1) and *Lactiplantibacillus plantarum* (n = 1).

The 31 strains belonging to the *Lb. casei* group, together with reference strains, were subjected to AFLP analysis. Samples were clustered with the UPGMA method on AFLP profiles' Jaccard similarity coefficient. Based on the 41 % similarity cut-off set, the strains were grouped in 9 main clusters. All the isolates were clustered with a similar AFLP profile with the reference strain *Lacticaseibacillus paracasei* 4186 (4186_Lbparacasei). Ten strains with high genotypic diversity were selected for the phenotypic analysis, in addition to the refence strains *Lacticaseibacillus casei* 2094 (2094_Lbcasei) and 4186_Lbparacasei.

3.3.2 Phenotypic tests

3.3.2.1 Resistance to cheesemaking stresses

The strains were tested for their ability to grow in milk in optimal conditions (no stress applied) and under the simulated main stresses encountered during GP cheese making, i.e. presence of lysozyme, heating at the curd cooking temperature of 54 °C, and high NaCl concentration. Three parameters were used to describe the strain's growth kinetic: i) lag, measured in hours and representing the adaptation time of the strain before starting to grow; ii) rate, the slope of the kinetic curve indicating the acidification speed; iii) yEnd, the maximum M% value measured and representing the strain's acidification capacity. Rate and yEnd are strongly positively correlated, and both are inversely correlated with the lag (**Figure 2**). The results are reported in the **Figures 3, 4** and **5**, together with the pH values reached in inoculated milk after the incubating period (**Figure 6**).

The strains 2094_Lbcasei (reference) and 5963_Lbparacasei were the faster to adapt and start growing in milk in the absence of any stress, having lag values respectively of 6.4 ± 1 h (mean ± SD) and 5.3 ± 0.4 h, which is significantly lower in comparison with the lag parameter of other strains. The heating treatment was the stress that most affected the time needed from the strains to start growing, except for strains 4186_Lbparacasei (reference) and 5296_Lbparacasei that well resisted this stress. 5993_Lbparacasei was not able to grow after the heating treatment, furthermore, it had a lag parameter significantly higher in all the conditions tested. The influence of the stresses on the acidification speed and capacity was strain dependent. Excluding the reference strains, 5785_Lbparacasei was the isolate with the highest rate and yEnd, in all the growth conditions. In general, the rate and yEnd values were more negatively impacted by the salt addition, although strain 5959_Lbparacasei and 5993_Lbparacasei were sensitive also to the heating treatment. The pH values had a trend inversely correlated with rate and yEnd values, but they were less influenced by the different treatments among the strains.

3.3.2.2 Aromatic compounds production

The results of the volatile compound concentration produced by the strains after growing in milk are reported in **Table 4**. The Kovats calculated index and the reference for the compound

identification are reported in **Supplementary Table 1**. 5649_Lbparacasei was the isolate producing more ketones, in particular high amount of Acetoin, and together with 5293_Lbparacasei and 5296_Lbparacasei they were the only producing 2,3-Butanedione (diacetyl, who has sweet, creamy, buttery odor). The acid compounds were produced in higher concentrations from 5780_Lbparacasei and 5785_ Lbparacasei.

The strains' similarity based on their ability to produce aromatic compounds and growth in milk was assessed by reducing the dimensionality of both chemical (n = 11) and impedometric (n = 4) features by principal component analysis (PCA). The ordination biplot is shown in **Figure 8**; the features' % contribution to the explained variance is reported in the **Supplementary Figure 2**. The most diverse strains were: 5649_Lbparacasei, 5785_Lbparacasei, and 5293_Lbparacasei, which are respectively characterized by high acetone and 2,3-Butanedione production, high rate and n-Decanoic acid production, as well as a low rate but high dimethyl sulfone and hexanoic acid production. The strain 5959_Lbparacasei showed similar features to 5785_Lbparacasei, while all the other strains were less diverse among each other as demonstrated by their proximity in the biplot.

3.3.2.3 Carbon source and sensitivity assays

The strains were analyzed for their ability to use different carbon sources as growth substrate, as well as their resistance to different chemicals. The results were used to cluster them based on their similar response and are reported separately for the two assays (**Figures 9** and **10**). The strain 5993_Lbparacasei showed a singular profile, being able to grow very well using D-fructose and N-Acetyl-D-Glucosamine. The strain 5293_Lbparacasei was also very different mainly because of his ability to metabolize D-Salicin and D-Cellobiose. No one of our isolates was able to grow using citric acid, while 5780_Lbparacasei, 5649_Lbparacasei and 5959_Lbparacasei were the only ones able to metabolize acetic acid. Regarding the chemical sensitivity, the strains 5296_Lbparacasei and 5293_Lbparacasei were particularly resistant to vancomycin, while 5649_Lbparacasei, 5785_Lbparacasei, 5780_Lbparacasei and 5959_Lbparacasei to fusidic acid.

3.4 Discussion

When produced with raw milk, the microbial ecosystem of traditional long-ripened cheeses such as Grana Padano PDO harbors a non-starter microbiota that is correlated with a peculiar cheese flavor formation. The dominant bacteria of this ecosystem are the NSLAB, in particular the species belonging to the *Lacticaseibacillus casei* group, which contribute with their metabolic pathways to the cheese proteo-lipo-lysis as well as citrate degradation, so as to be proposed as adjunctive cultures for the cheese industry. In this study, we assessed some important phenotypic features (growth kinetic in milk; resistance to high temperature, salt and lysozyme; aroma production) of *Lb. casei* group strains isolated from raw milk, in order to evaluate their potential use as adjunct strains in the production of raw milk, long-ripened hard cheeses.

3.4.1 Strains' resistance to cheesemaking stresses

The isolation and screening process allowed the selection of ten *Lb. paracasei* strains morphologically and genotypically diverse based on microscope and AFLP profiles clustering evaluations. All the strains were able to grow in milk showing kinetic parameters comparable with

those of other strains of the same species tested in the same conditions (Bancalari et al. 2017). Strains 5785_Lbparacasei and 5963_Lbparacasei were characterized by a significantly shorter lag, meaning earlier adaptation in milk and as consequence possible competition in lactose metabolism with SLAB (Blaya, Barzideh, and LaPointe 2018). This may interfere with the curd acidification process and compromise the cheese making. When the heat stress was applied (54 °C for 1 h) 5993_Lbparacasei was not able to grow in the incubation condition tested, indicating a possible lower expression of heat-shock proteins (HSPs) and excluding this strain as adjunct in curd cooked cheese production. The inability of *Lb. paracasei* strains to grow at thermophilic temperatures (45 °C) was also reported by Fitzsimons et al. (1999), who phenotypically characterized NSLAB isolated from mature Irish Cheddar cheeses. Strains 5959_Lbparacasei, 5998_Lbparacasei and 5296_Lbparacasei showed good recovery to the heat stress, resistance to salt and lysozyme; furthermore, they showed not excessively short lag and at the same time a good acidification capacity and rate, making them good candidates as adjunct strain.

3.4.2 Strains' metabolic capabilities

Different studies have highlighted the genotypic diversity that is often found in isolates from the same niche, especially in the case of dairy products (Bove et al. 2011; Solieri, Bianchi, and Giudici 2012). Genetic variability at the genus level is relevant in *Lacticaseibacillus*, as reported in various studies (Toh et al. 2013; Zheng et al. 2020) and to some extent, this variability has been recognised also during the different stages of GP ripening (Levante et al., 2017). However, there is a general awareness that similar phenotypes displayed by strains do not always correspond to similar or even closely related genotypes (Temmerman, Huys, and Swings 2004). According to the results presented in this study, the genetic variability does not seem to be reflected in the capability of the strains to metabolise the different substrates of the Biolog GEN III plate. Most of the strains tested showed similar phenotypic profiles sharing the use of different carbon sources such as simple sugars (α-D-glucose, α-D-galactose, D-fructose, etc.) but also D-Gluconic acid and D-salicin (**Figure 9**). As expected, our strains were not able to ferment L-Rhamnose since this distinguish the species *Lb. casei/paracasei* to *Lb. rhamnosus* within the *Lb. casei* group (Minervini and Calasso 2022). Although the isolated tested were not able to use L-arginine as growing substrate, indicating the possible absence of arginine deaminase (ADI) pathway which is important for the adaptation in the cheese environment (Zúñiga et al. 1998), strains 5293_Lbparacasei, 5296_Lbparacasei and 5649_Lbparacasei were able to produce 2,3-Butanedione (diacetyl), a ketone compound that is related to the pleasant sweet, creamy and buttery notes, and as consequence of interest for the aroma formation of dairy products (Barbieri et al. 1994)

The obtained results showed a widespread resistance to fusidic acid, an antibiotic increasingly used for the treatment of skin infections due methicillin-resistant *S. aureus* for skin treatment (Hajikhani et al. 2021). On the other hand, the resistance to vancomycin, the last resort for the treatment of severe infections caused by Gram-positive bacteria such as *Enterococcus* species, *Staphylococcus aureus*, and *Clostridium difficile* (Stogios and Savchenko 2020) was limited. Anyway, the use of GEN III was useful for a preliminary evaluation and this resistance should be evaluated and measured through more accurate and official antimicrobial susceptibility testing methods such as agar diffusion method (Gajic et al. 2022) or also by mean of impedometric analysis (Bancalari et al.

2020). Usually, the evaluation of antibiotic resistance is required for the LAB to use as starter (Zarzecka, Zadernowska, and Chajęcka-Wierzchowska 2020). However, in the case of ripened cheeses, the vitality of this type of bacterial cells is destined to drastically decrease in a few months, contrary to the number of NSLAB cells which increase during cheese ripening and then decrease again after very long ripening times. This implies that NSLAB can be present and viable in large amounts when the cheese is consumed, for this reason, it is important to correctly assess their antibiotic resistance before suggesting their technological application (Nunziata et al. 2022; European Food Safety Authority (EFSA) 2021).

3.4.3 Adjunct culture implementation

At the end of the complex characterization, we can summarize the elements needed to choose the strains with the best potential to be used as adjunct strains in the production of raw milk, long-ripened hard cheeses.

Strains 5959_Lbparacasei, 5998_Lbparacasei and 5296_Lbparacasei showed good recovery to the heat stress, resistance to salt and lysozyme; furthermore, they showed no excessively short lag and at the same time a good acidification capacity and rate. On the other hand, 5293_Lbparacasei, 5296_Lbparacasei and 5649_Lbparacasei, but also 5780_Lbparacasei and 5959_Lbparacasei to a lesser extent, are more involved in keton compounds production.

Thus, the two best strains should be 5959_Lbparacasei and 5296_Lbparacasei even if resistance to fusidic acid for the first and to vancomycin for the latter has to be correctly measured before their employment.

Otherwise, other strains with interesting aromatic capabilities, such as 5293_Lbparacasei, 5649_Lbparacasei and 5780_Lbparacasei, could be good candidates as adjunct strains for uncooked cheese production.

Finally, the results obtained in this study can be also useful to choose strain, such 5993_Lbparacasei able to grow very well using D-fructose, to use as starter in the fermentation of plant-based food matrices (Harper et al. 2022), or 5293_Lbparacasei, able to metabolize D-Salicin and D-Cellobiose, for fermentation of food by-product (Hadj Saadoun et al. 2021).

3.5 Acknowledgments

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3.6 Tables

Table 1. Strains viable count (log CFU/mL) after growing 20 h at 37 °C (anaerobic condition) in MRS broth and UHT partially skimmed milk (UPSM).

Strain id	MRS	UPSM
2094_Lbcasei_C	9.15	9.77
4186_Lbparacasei_C	9.23	8.19
5293_Lbparacasei	8.62	7.96

5296_Lbparacasei	8.91	8.78
5569_Lbparacasei	8.96	8.55
5649_Lbparacasei	9.28	8.93
5780_Lbparacasei	9.38	8.81
5785_Lbparacasei	9.03	8.52
5959_Lbparacasei	9.09	8.46
5963_Lbparacasei	9.37	8.83
5993_Lbparacasei	9.22	7.98
5998_Lbparacasei	8.12	8.64

Table 2 Data calculation to obtain values for statistical analysis and metabolic functional diversity indices (Acin-Albiac et al., 2020).

Estimation	**Formula**	**C source utilization**	**Chemical sensitivity**
Average Well Color Development (AWCD)	$AWCD = \sum_{i=1}^{n} \frac{(ODi - R)}{n}$	*ODi* = raw OD value of each well from 1 to 9 column *R* = OD value of negative control well *n* = number of substrates analized (71)	*ODi* = raw OD value of each well from 10 to 12 columns *R* = half of the OD value of positive control well *n* = number of substrates analized (23)
Absorbance Ratios	$R_{si} = \frac{(OD_i - R)}{AWCD}$		

Table 3. Features of raw milk samples after the spontaneous fermentation (incubation for 60 d at 8°C anaerobically). Intensity scale of odor descriptors: 0 = absent, 5 = strong.

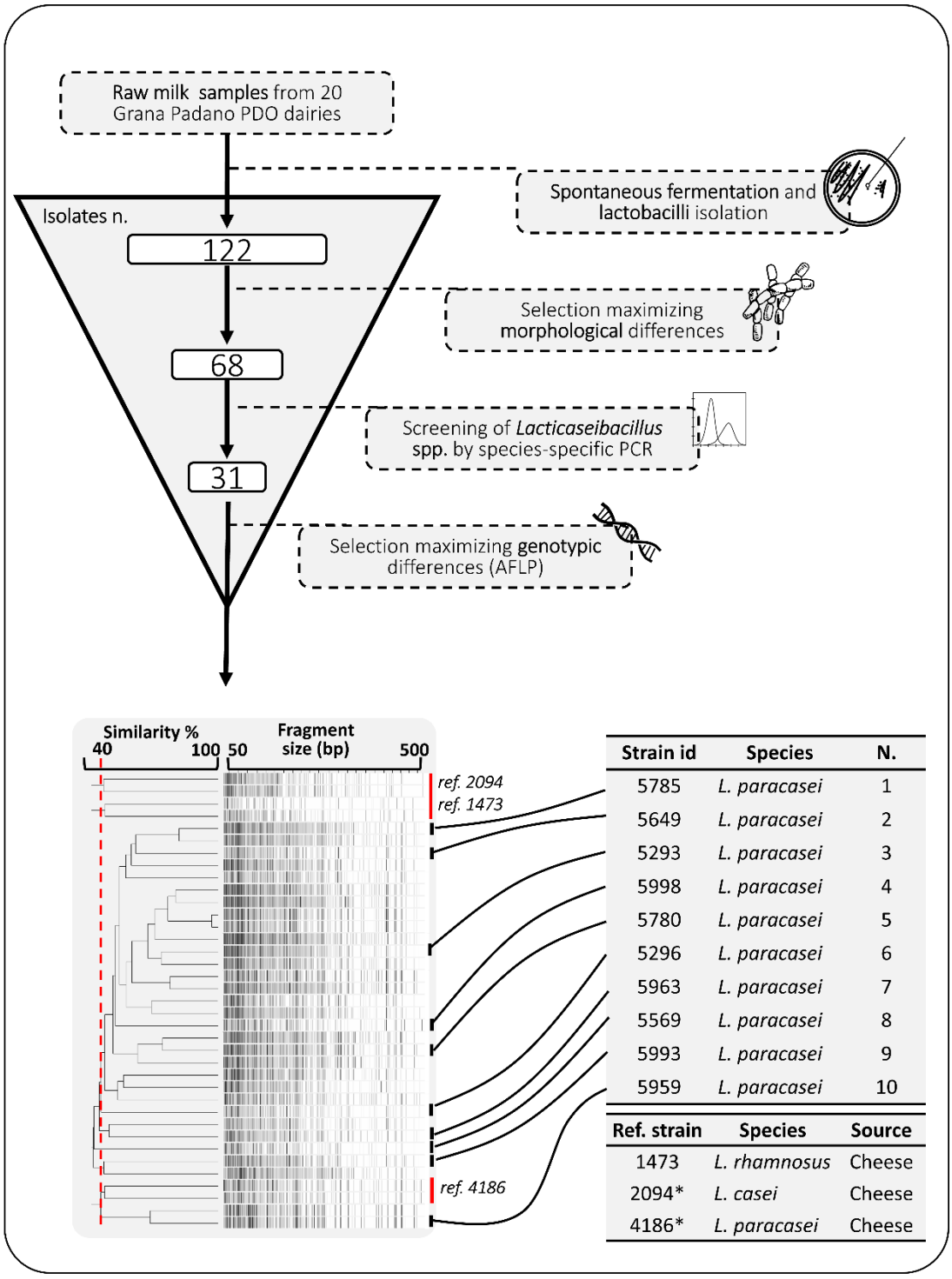
Provence of milk sampling	**Lacobacilli (log CFU/mL)**	**pH**	**Odor evaluation**			
			Expired milk	Acidic (yoghurt-like)	Cheesy	Notes
Bergamo	7.39	4.40	0	4	3	High gas formation, fizzy
Brescia	5.90	4.60	0	4	2	Slight fruity notes
Brescia	7.08	4.52	2	2	3	-
Brescia	7.58	4.59	0	2	5	-
Cremona	6.34	4.53	2	2	2	-
Cremona	7.67	4.60	0	2	5	-
Cuneo	6.62	4.50	0	2	3	-

Lodi	7.70	4.50	0	2	5	-
Lodi	7.95	4.69	0	3	2	High gas formation, fizzy
Mantova	7.75	4.60	0	2	3	-
Mantova	7.59	4.50	0	0	5	-
Padova	6.94	4.40	0	4	2	High gas formation, fizzy
Pavia	6.82	4.50	0	5	1	Slight fruity notes
Piacenza	7.39	4.50	0	2	5	-
Piacenza	5.76	4.56	0	2	4	-
Trento	7.95	4.45	0	3	5	-
Verona	6.53	4.50	0	3	3	-
Verona	7.53	4.50	0	3	4	-
Vicenza	8.55	4.59	0	4	5	-
Vicenza	7.46	4.31	0	1	5	-

Table 4. Volatile compounds produced by the isolated strains (refer to **Figure 1** for the strain species). Data (mg/L) are expressed as mean ± standard deviation (n = 2). Kovats indexes are reported in the **Supplementary Table 1**. (a) Odor types were retrieved from TGSC (www.thegoodscentscompany.com/allodor.html)

Compound class	Aromatic compound	Odor type[(a)]	Strain ID											
			2094_C	**4186_C**	**5293**	**5296**	**5569**	**5649**	**5780**	**5785**	**5959**	**5963**	**5993**	**5998**
Acids	Acetic acid	sharp pungent sour vinegar	0.215 ± 0	0	0	0	0	0	0	0	0	0	0	0
	Hexanoic acid	sour fatty sweet cheese	0.049 ± 0.034	0.003 ± 0	0.007 ± 0.001	0	0	0	0	0	0	0	0	0
	n-Decanoic acid	sour, creamy, buttery, fatty	0	0.005 ± 0	0	0.002 ± 0.001	0.003 ± 0	0	0	0.011 ± 0.005	0.004 ± 0.001	0.004 ± 0.001	0.003 ± 0	0.003 ± 0.001
	Octanoic acid	fatty waxy rancid oily vegetable cheesy	0.021 ± 0.014	0.007 ± 0	0.005 ± 0	0.005 ± 0.002	0.006 ± 0.001	0	0.026 ± 0.005	0.017 ± 0.007	0.007 ± 0.002	0.007 ± 0.001	0.006 ± 0	0.006 ± 0.002
Acids sum			0.285	0.015	0.012	0.007	0.009	0	0.026	0.028	0.011	0.011	0.009	0.009
Alcohols	2-Furanmethanol	alcoholic chemical musty sweet caramel bread coffee	0	0.004 ± 0.001	0	0	0	0	0	0	0	0	0	0
	Ethanol	strong alcoholic ethereal medical	0.003 ± 0.002	0.002 ± 0	0.001 ± 0	0	0.001 ± 0	0.006 ± 0.001	0.01 ± 0.001	0.003 ± 0	0.001 ± 0	0.001 ± 0.001	0.001 ± 0	0
Alcohols sum			0.003	0.006	0.001	0	0.001	0.006	0.01	0.003	0.001	0.001	0.001	0
Ketons	2,3-Butanedione	Sweet, creamy, buttery, pungent caramellic nuance	0	0	0.001 ± 0	0.001 ± 0	0	0.002 ± 0	0	0	0	0	0	0
	2-Heptanone	Cheese, fruity, ketonic, green banana, with a creamy nuance	0	0.002 ± 0	0	0	0	0	0	0	0	0	0	0
	Acetoin	sweet buttery creamy dairy milky fatty	0.114 ± 0.073	0.015 ± 0.001	0	0	0	0.037 ± 0.003	0	0	0.014 ± 0	0	0	0
	Acetone	solvent ethereal apple pear	0	0	0	0	0	0.011 ± 0.002	0.001 ± 0	0	0	0	0	0
	Dimethyl sulfone	sulfurous burnt	0.011 ± 0.01	0.001 ± 0	0.001 ± 0	0	0	0	0	0	0	0	0	0
Ketons sum			0.125	0.019	0.003	0.001	0	0.049	0.001	0	0.014	0	0	0
Others	2,4-Dimethyl-1-heptene	absent	0	0.005 ± 0	0	0	0	0	0	0	0	0	0	0.002 ± 0.001
	3-Buten-1-ol, 3-methyl-	sweet fruity	0	0	0	0	0.001 ± 0	0	0	0	0	0	0	0
	alfa-Pinene	Woody, piney and turpentine-like, a fresh herbal lift	0	0	0.001 ± 0	0.001 ± 0	0.001 ± 0	0	0	0.004 ± 0.001	0.002 ± 0.001	0.003 ± 0.001	0	0.003 ± 0.001
Others sum			0	0.005	0.001	0.001	0.002	0	0	0.004	0.002	0.003	0	0.005

3.7 Figures



Strain id	Species	N.
5785	*L. paracasei*	1
5649	*L. paracasei*	2
5293	*L. paracasei*	3
5998	*L. paracasei*	4
5780	*L. paracasei*	5
5296	*L. paracasei*	6
5963	*L. paracasei*	7
5569	*L. paracasei*	8
5993	*L. paracasei*	9
5959	*L. paracasei*	10

Ref. strain	Species	Source
1473	*L. rhamnosus*	Cheese
2094*	*L. casei*	Cheese
4186*	*L. paracasei*	Cheese

Figure 1. Screening process to select 10 strains to be phenotypically characterized. AFLP = Amplified Fragment Length Polymorphisms. * = reference strains selected as controls for the phenotypic study.

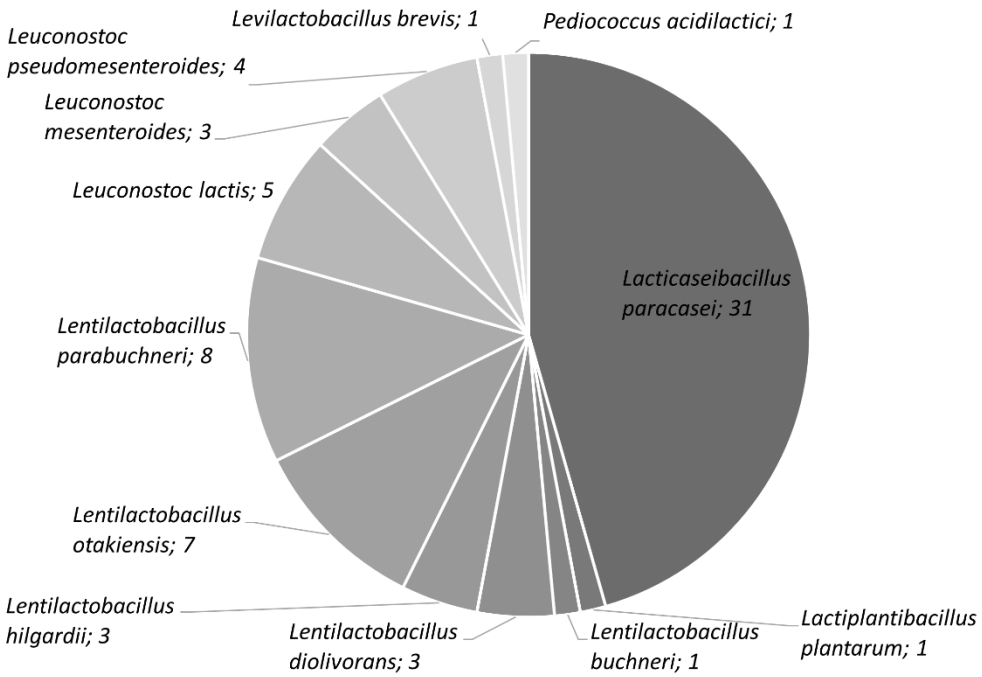


Figure 2. Strains isolated from spontaneous fermented raw milk selected for species identification. *Lacticaseibacillus paracasei* were identified via specie-specific PCR and AFLP clustering, while the remaining strains via 16S rRNA sequencing. The number of isolates is reported beside the species (tot. number = 68).

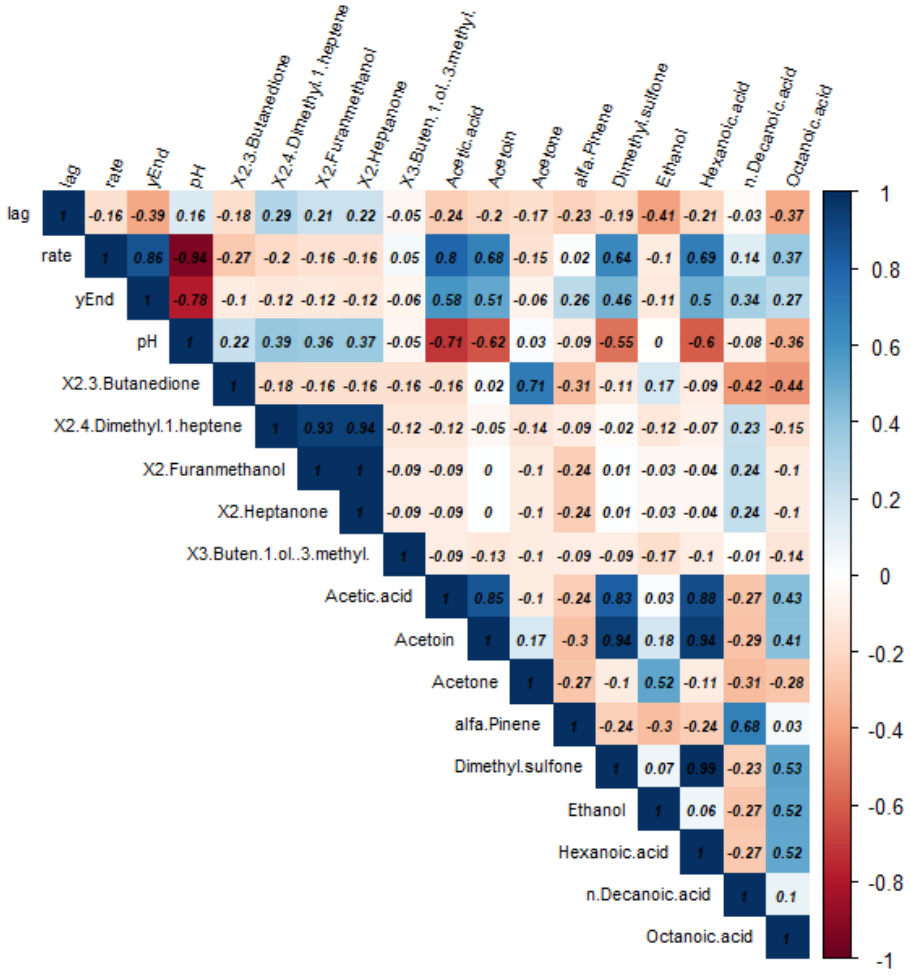


Figure 3. Pearson correlation between aromatic and impedometric features of the strains after their growth in milk.

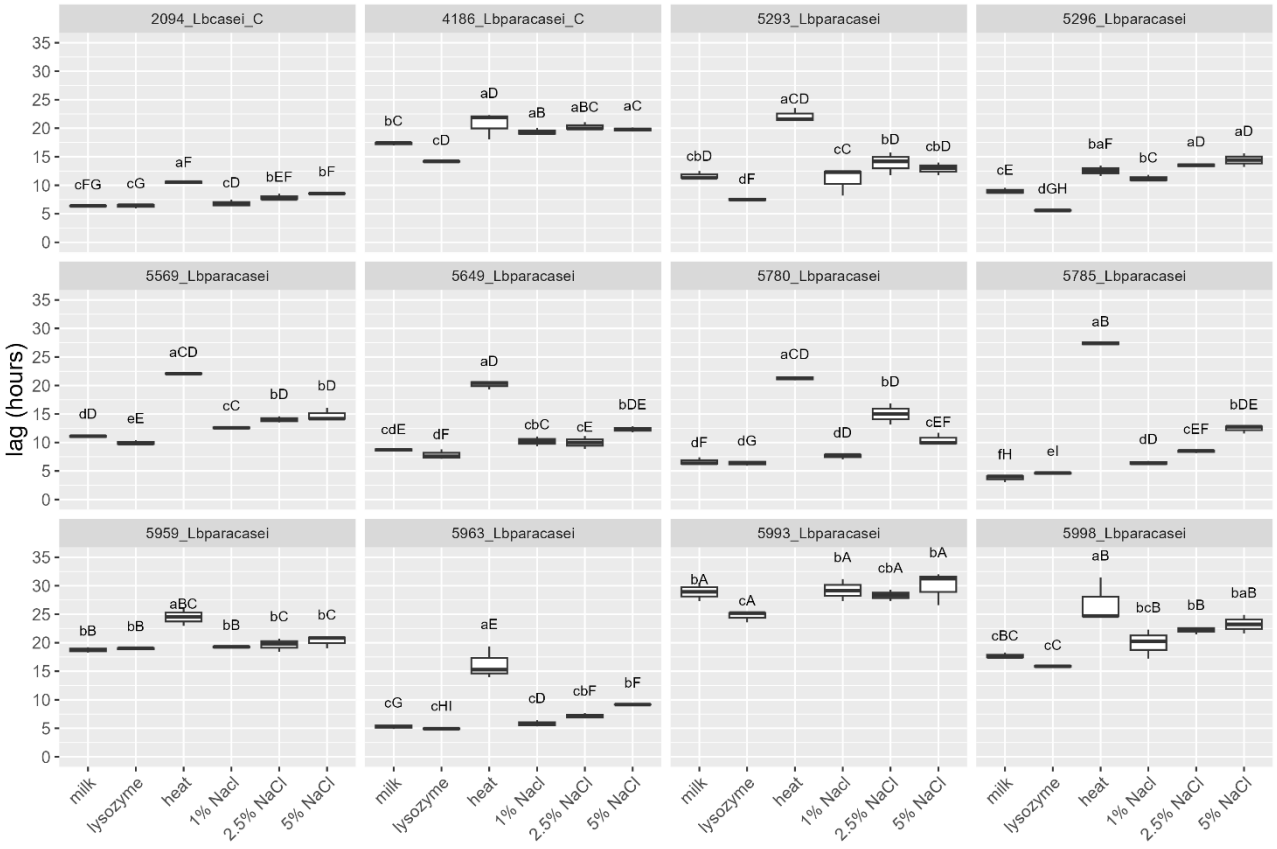


Figure 4. Lag values of the strains after their growth (48h at 37 °C) in milk in different conditions: absence of stress (milk), in presence of 200mg/L of lysozyme (lysozyme), after heat treatment of 54 °C for 1 h (heat) and in presence of different % (w/v) of NaCl. Growth conditions within the same strain with different lowercase letters are significantly different ($p < 0.05$); strains within the same growing condition with different uppercase letters are significantly different.

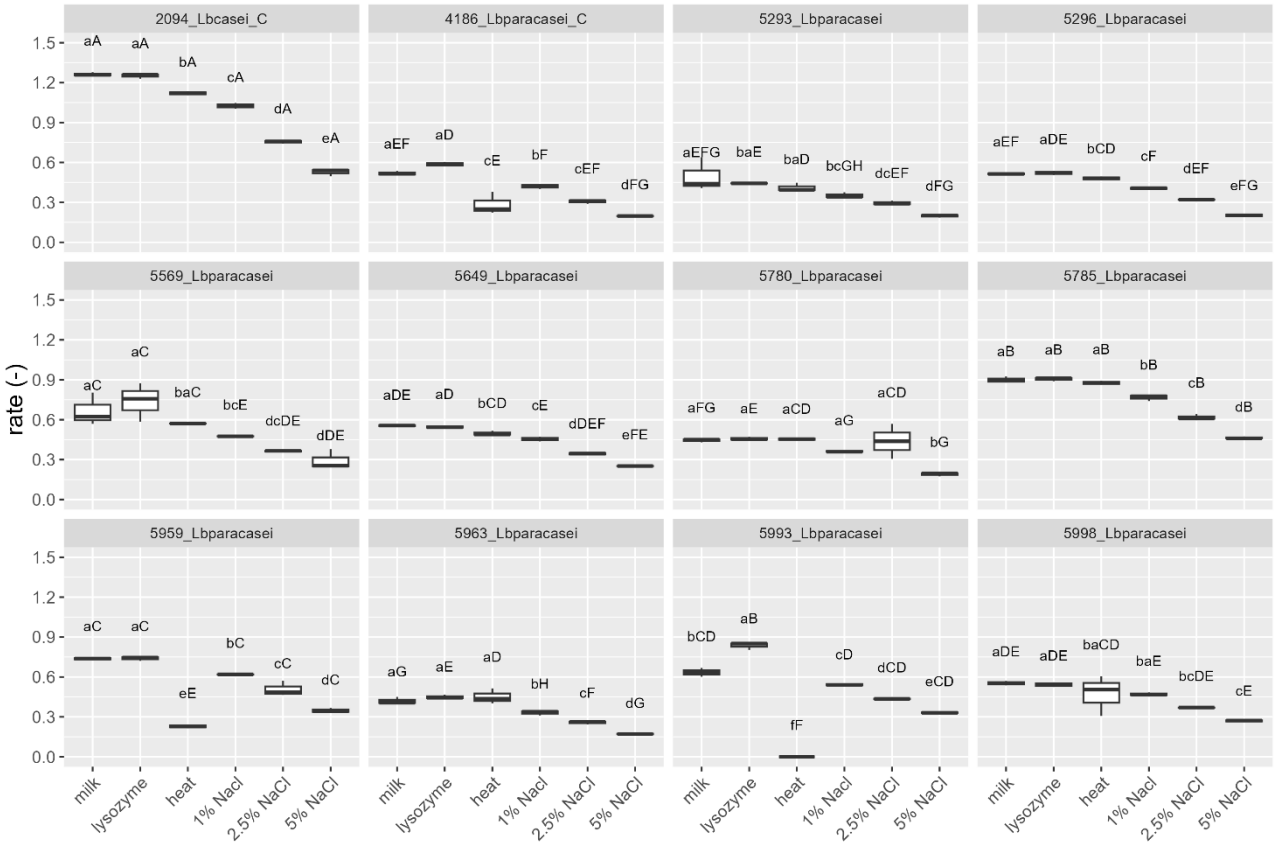


Figure 5. Rate values of the strains after their growth (48h at 37 °C) in milk in different conditions: absence of stress (milk), in presence of 200mg/L of lysozyme (lysozyme), after heat treatment of 54 °C for 1 h (heat) and in presence of different % (w/v) of NaCl. Growth conditions within the same strain with different lowercase letters are significantly different ($p < 0.05$); strains within the same growing condition with different uppercase letters are significantly different.

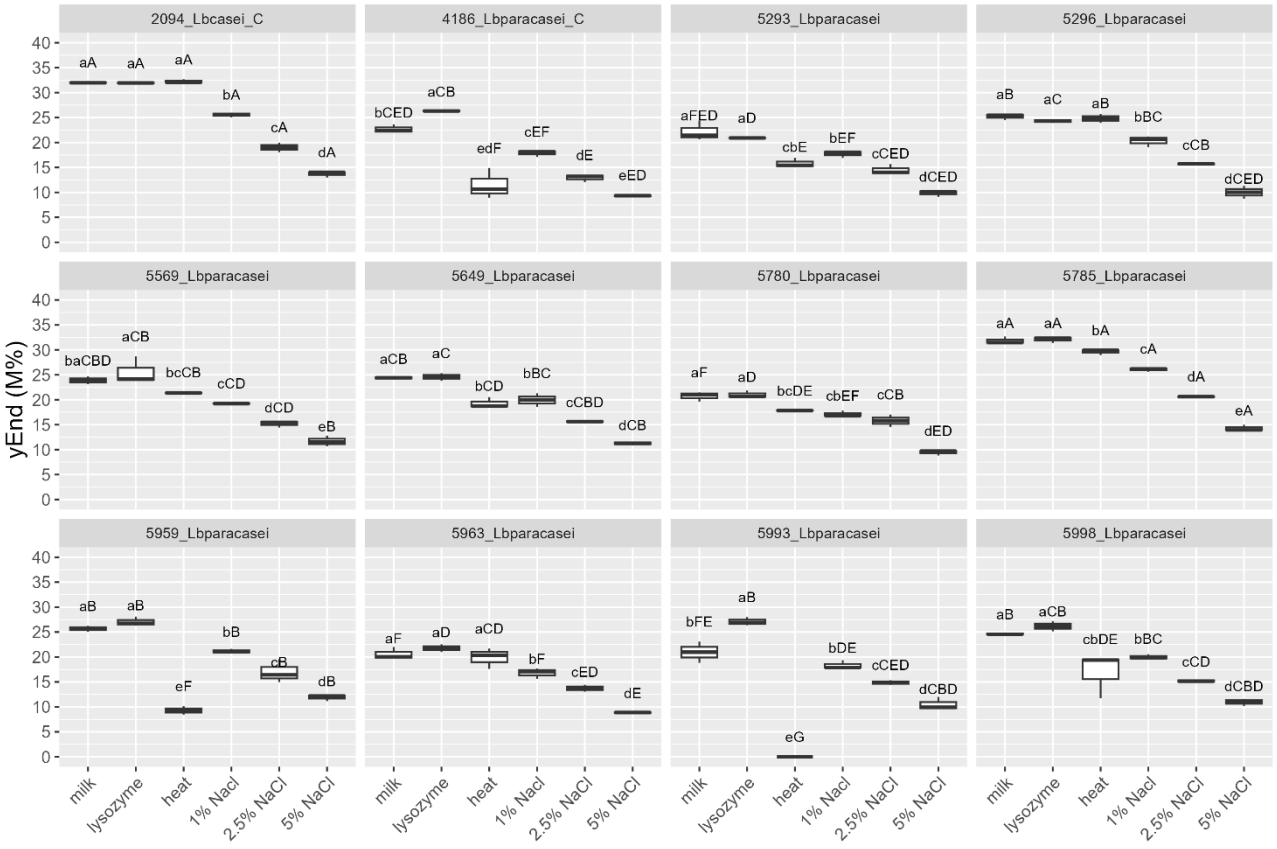


Figure 6. yEnd values of the strains after their growth (48h at 37 °C) in milk in different conditions: absence of stress (milk), in presence of 200mg/L of lysozyme (lysozyme), after heat treatment of 54 °C for 1 h (heat) and in presence of different % (w/v) of NaCl. Growth conditions within the same strain with different lowercase letters are significantly different ($p < 0.05$); strains within the same growing condition with different uppercase letters are significantly different.

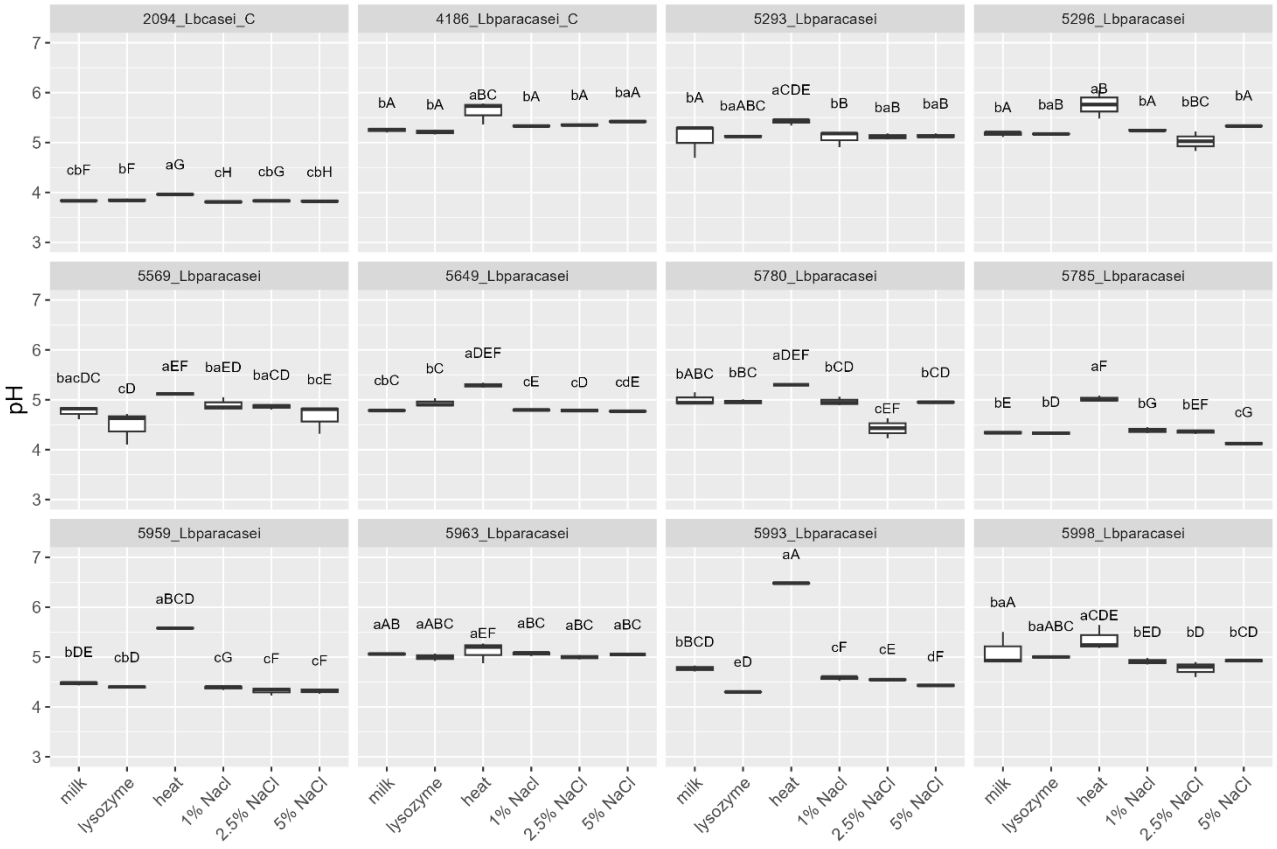


Figure 7. pH values of the milk after the strains' growth (48h at 37 °C) in different conditions: absence of stress (milk), in presence of 200mg/L of lysozyme (lysozyme), after heat treatment of 54 °C for 1 h (heat) and in presence of different % (w/v) of NaCl. Growth conditions within the same strain with different lowercase letters are significantly different ($p < 0.05$); strains within the same growing condition with different uppercase letters are significantly different.

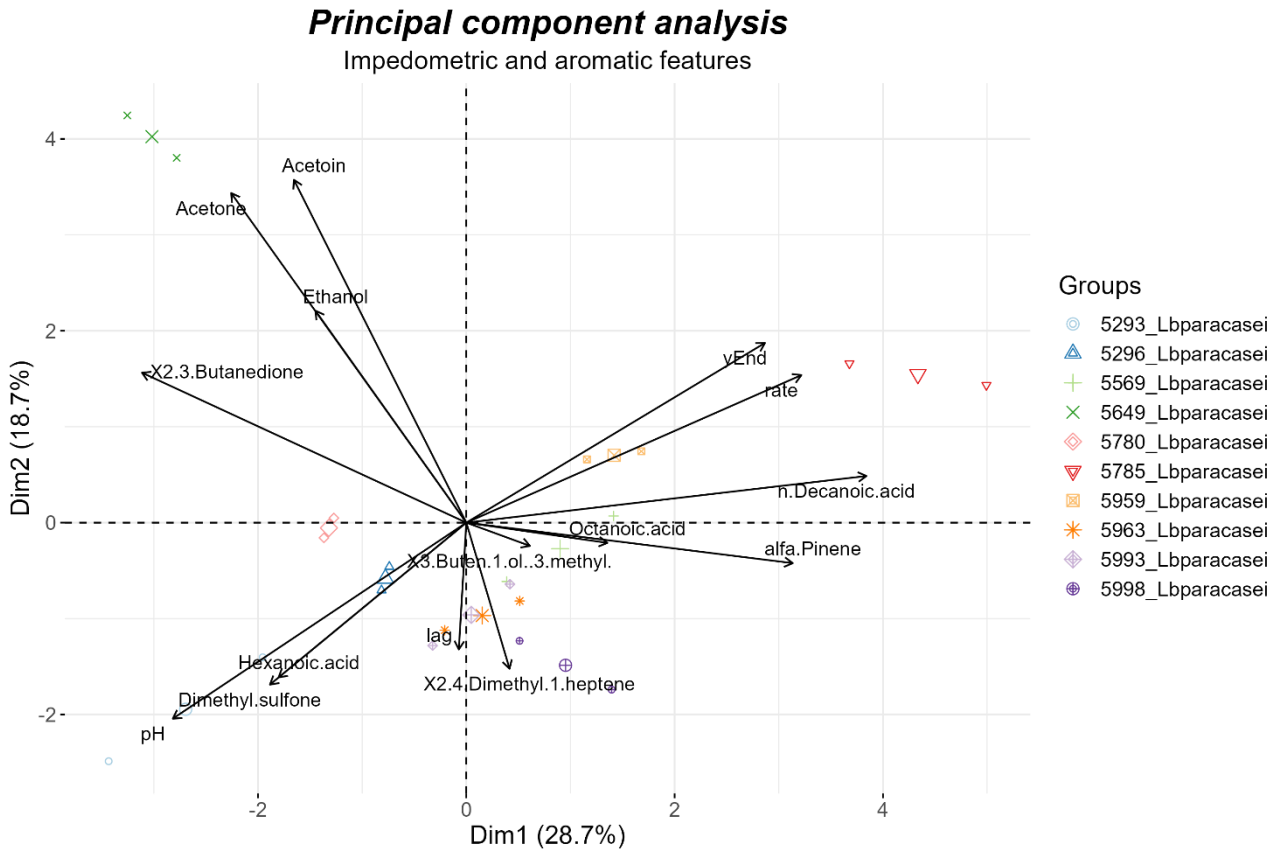


Figure 8. Principal component analysis of impedometric (n = 4) and aromatic (n = 10) features of strains after their growth in milk.

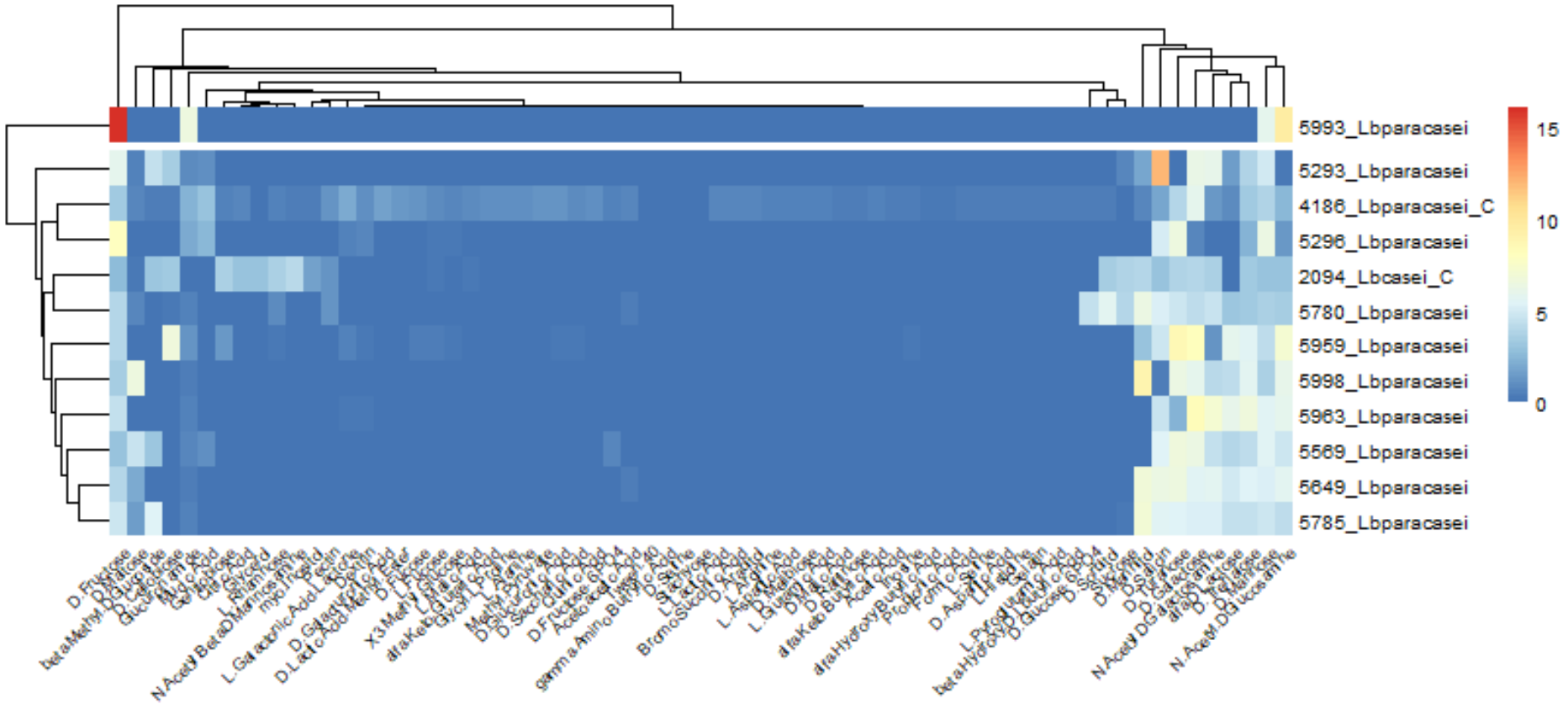


Figure 9. Strains clustering (vertical axis) based on their ability to growth on different carbon sources (horizontal axis). The growth rate increases from blue (no growth) to red.

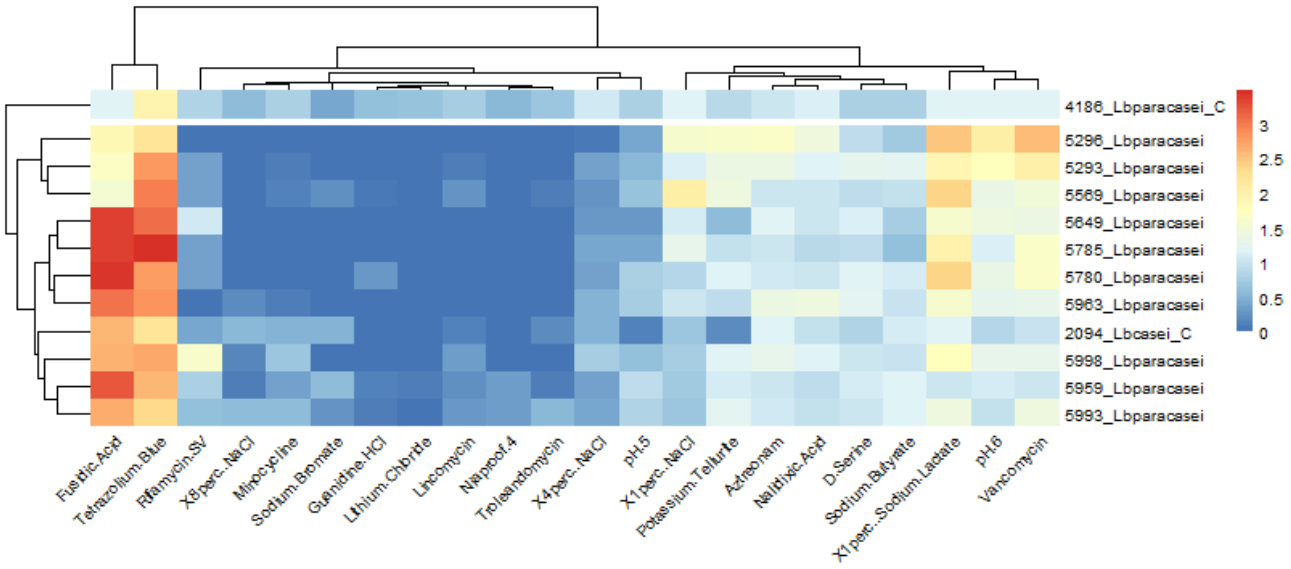


Figure 10. Strains clustering (vertical axis) based on their ability to resist different chemical inhibitors (horizontal axis). The growth rate increases from blue (no growth) to red.

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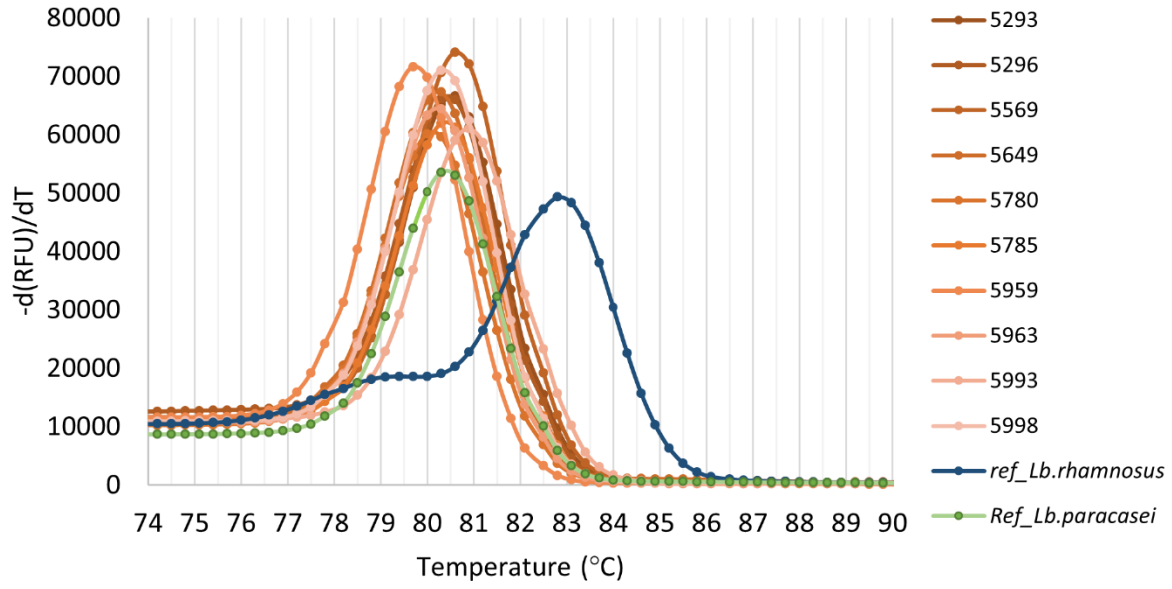
3.9 Supplementary Material

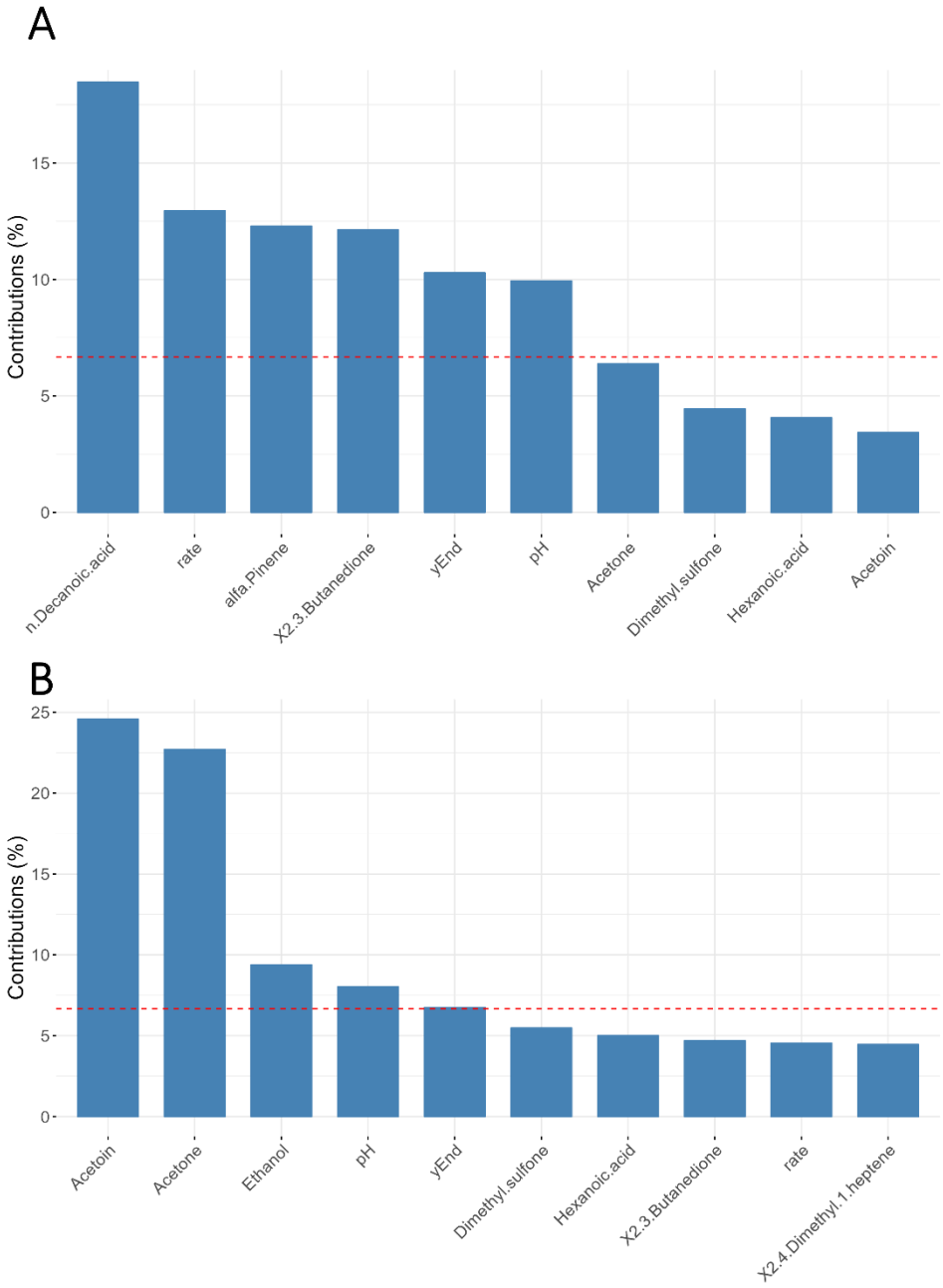
Supplementary Table 1. Identification of the compound produced by the strains as measured by means of SPME GC-MS. (a) Retrieved from the NIST database (www.nist.gov/)

Compound	CAS number	Retention time (min)	Kovats index (calculated)	Kovats index (literature)[(a)]	Reference Kovats index literature[(a)]
Acetone	67-64-1	1.53	864.47	847	(Waggott and Davies 1984)
2,4-Dimethyl-1-heptene	19549-87-2	1.72	889.47	885	(Muresan et al. 2000)
Ethanol	64-17-5	2.05	932.89	931	(Bonastre and Grenier 1968)
2,3-Butanedione	431-03-8	2.41	980.26	980	(Katsumi Umano et al. 1986)
alfa-Pinene	80-56-8	2.76	1011.43	1011	(K. Umano and Shibamoto 1988)

Toluene	108-88-3	3.1	1030.86	1030	(Egazaryants and Maximov 1998)
2-Heptanone	110-43-0	5.85	1167.25	1169	(Katumi Umano, Hagi, and Shibamoto 2002)
3-Buten-1-ol, 3-methyl-	763-32-6	7.57	1238.19	1237	(Yuhong 2007)
Acetoin	513-86-0	8.56	1277.17	1277	(T. H. Kim et al. 2002)
Acetic acid	64-19-7	14.7	1521.01	1498	(Soria, Martínez-Castro, and Sanz 2008)
2-Furanmethanol	98-00-0	17.86	1655.9	1656	(J. H. Kim et al. 2004)
Dimethyl sulfone	67-71-0	21.83	1904.26	1906	(Chung et al. 2002)
Hexanoic acid	142-62-1	22.01	1923.4	1889	(Peng 2000)
Octanoic acid	124-07-2	23.82	2115.96	2106	(Peng 2000)
n-Decanoic acid	334-48-5	25.23	2265.96	2266	(Welke et al. 2012)



Supplementary Figure 1. Discrimination among the *Lacticaseibacillus casei/paracasei* and *Lacticaseibacillus rhamnosus* species of the isolates according to the melting temperature curve analysis. The reference strains are indicated with their numerical ID in red scale lines; references strains are in green (*Lacticaseibacillus paracasei*) and blue (*Lacticaseibacillus rhamnosus*) line.



Supplementary Figure 2. Ten most contributing (%) variables in defining the strains' differentiation on the first (A) and the second (B) principal component. See **Figure 8** in the article for the principal component analysis plot.

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4 Natural adjunct milk culture

Selective enrichment of the raw milk microbiota in cheese production: concept of a natural adjunct milk culture

Luca Bettera[1,2*], Matthias Dreier [2], Remo S. Schmidt [2], Monica Gatti[1], Hélène Berthoud[2], Hans-Peter Bachmann[2]

[1] *Department of Food and Drug, University of Parma, Parma, Italy*

[2] *Agroscope, Bern, Switzerland*

* Correspondence:
luca.bettera@unipr.it

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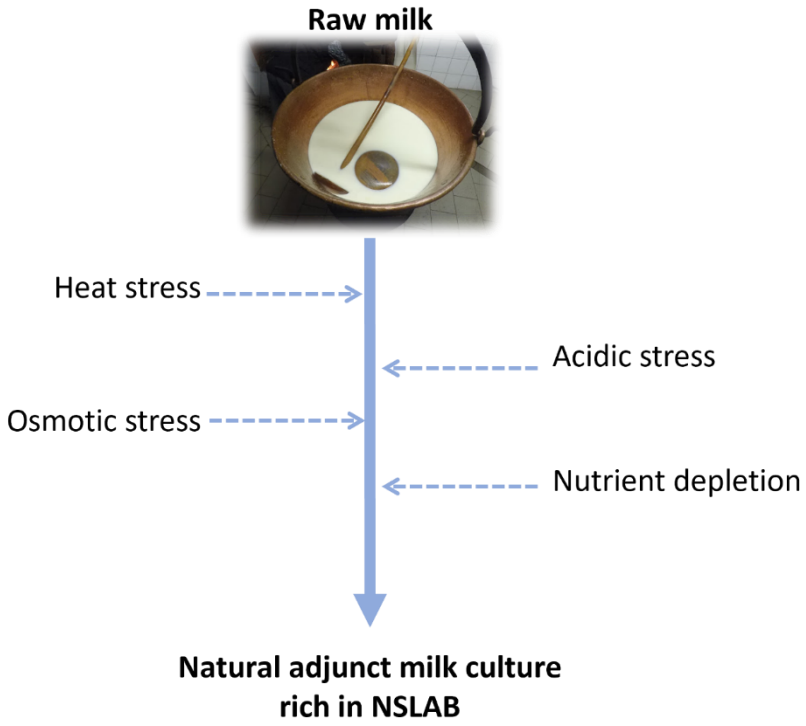

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Abstract

In cheese production, microorganisms are usually added at the beginning of the process as primary starters to drive curd acidification, while secondary microorganisms, with other pro-technological features important for cheese ripening, are added as selected cultures. This research aimed to investigate the possibilities of influencing and selecting the raw milk microbiota using artisanal traditional methods, providing a simple method to produce a natural supplementary culture. We investigated the production of an enriched raw milk whey culture (eRWC), a natural adjunct microbial culture produced from mixing an enriched raw milk (eRM) with a natural whey culture (NWC). The raw milk was enriched by spontaneous fermentation for 21 days at 10 °C. Three milk enrichment protocols were tested: heat treatment before incubation, heat treatment plus salt addition, and no treatment. The eRMs were then co-fermented with NWC (ratio of 1:10) at 38 °C for 6h (young eRWC) and 22h (old eRWC). Microbial diversity during cultures' preparation was evaluated through the determination of colony forming units on selective growth media, and next-generation sequencing (16S rRNA gene amplicon sequencing). The enrichment step increased the Streptococci and Lactobacilli but reduced microbial richness and diversity of the eRMs. Although the lactic acid bacteria viable count was not significantly different between the eRWCs, they harbored higher microbial richness and diversity than NWC. Natural adjunct cultures were then tested in cheese making trials, following the microbial development, and assessing the chemical quality of the 120 days ripened cheeses. The use of eRWCs slowed the curd acidification in the first hours of cheese making but the pH 24 h after production settled to equal values for all the cheeses. Although the use of diverse eRWCs contributed to having a richer and more diverse microbiota in the early stages of cheese making, their effect decreased over time during ripening, showing an inferior effect to the raw milk microbiota. Even if more research is needed, the optimization of such a tool could be an alternative to the practice of isolating, geno-pheno-typing, and formulating mixed-defined-strain adjunct cultures that require knowledge and facilities not always available for artisanal cheese makers.

4.1 Introduction

Microorganisms are most often intentionally applied in cheese making. They are added at the beginning of the process as primary starters, where they drive curd acidification by metabolizing milk lactose into lactic acid. Further, secondary microorganisms have features important for cheese ripening (e.g., eye formation, rind modification, flavor enhancement). Additionally, in cheese varieties made from raw milk, an autochthonous non-starter microbiota of milk origin is present in the ripened cheeses. These microbes, mostly lactic acid bacteria (LAB), contribute to the formation of the cheese flavor and texture. This increased microbial diversity can change the characteristics of the cheese due to the presence of other metabolic pathways. On the one hand, this can lead to authenticity and added value, but on the other hand, it can also lead to off-flavors and/or texture deficiencies. Reproducibility of consistent flavor and quality when using raw milk is difficult because the composition of the microbiota in the milk varies. The growth dynamics of strains from raw milk during production and maturation are difficult to predict.

Artisanal natural cultures represent a practical tool for cheesemakers to influence the fermentation process while keeping a high biodiversity linked to the terroir of production. These cultures can be

maintained in-house by backslopping (i.e., use of an old batch of a fermented product to inoculate a new one). Natural cultures have an undefined strain composition, although the application of selective pressure (heat treatment, incubation temperature, low pH) favors the dominance of desired LAB (Parente et al., 2017; Powell et al., 2022). These cultures are produced either from milk or whey (Parente, 2006), and they are required by the standards of identity of several traditional protected designation of origin [PDO; (EP and Council of EU, 2012)] cheese types because a strict relationship is believed to exist between their use, the cheese quality and the territory of production.

Natural milk cultures (NMC) are used in the production of several traditional cheeses, such as Argentinian cheese (Reinheimer et al., 1997), Montasio PDO (Marino et al., 2003, 2008; Carraro et al., 2011), Asiago PDO (Gobbetti et al., 2018), and Mozzarella TSG [Traditional Speciality Guaranteed; (EP and Council of EU, 2012)]. These cultures are produced starting from raw milk, which is thermized (60–65 °C, 10–30 min), and incubated at 42–45 °C until a titratable acidity of 0.4–0.6 % lactic acid is reached (Parente, 2006). Dominating species of NMC are *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus helveticus*, but other thermophilic and mesophilic LAB (e.g. *Streptococcus gallolyticus* subsp. *macedonicus*, enterococci) may be present as co-dominant or sub-dominant species (Zotta et al., 2022).

Natural whey cultures (NWC) are produced by incubating whey drained from the cheese (commonly referred as “sweet” whey) in conditions that favour the selection of desirable LAB. The incubating temperature varies according to the cheese variety, and can be controlled or uncontrolled, i.e. spontaneously decreasing from the initial temperature to room temperature. NWC are widely used for the production of Italian cheese varieties, including Mozzarella di Bufala Campana (De Filippis et al., 2014), Caciocavallo Silano (Ercolini et al., 2008), Nostrano Valtrompia, Provolone, (Gobbetti et al., 2018), but also French [e.g., Comté PDO, Rocamadour PDO (Demarigny et al., 2006), Picodon PDO (Yann and Pauline, 2014)] and Swiss varieties (L’Etivaz PDO, Berner Alp- und Hobelkäse PDO, Le Gruyère PDO). The characteristics of NWC used for the production of Grana Padano and Parmigiano Reggiano have been reviewed by (Gatti et al., 2014). The amount of culture added to the vat milk varies between 2.7–3.5 % depending on the value of the titratable acidity determined on the production day. The main drivers for the selection of wanted LAB are the high incubation temperature, the low pH at the end of incubation and the backslopping process itself, as favorable LAB are present at the beginning of the inoculation. These conditions lead to the dominance of the aciduric and thermophilic LAB that reach a concentration ranging from 7.7 to 9.9 log CFU/mL. Dominant LAB species of Grana Padano and Parmigiano Reggiano NWC are *L. helveticus, L. delbrueckii* subsp. *lactis*, and less frequently *Limosilactobacillus fermentum* and *S. thermophilus* (Gatti et al., 2014). The NWC used for Gruyère cheese making is also composed of thermophilic LAB, since the initial whey is incubated at 38 °C for 20 h after having reached the curd cooking temperature of 54–59 °C and a further thermization to about 60−63 °C (Moser et al., 2018).

However, to the authors’ knowledge, natural cultures are exclusively used as primary cultures. Commercial secondary cultures with non-starter lactic acid bacteria (NSLAB) strains (also called adjunct cultures) are already available on the market, but this may not be a suitable solution to ensure broad species diversity (Gobbetti et al., 2015). Moreover, these mixed- or defined-strain

cultures are usually maintained and cultivated in the laboratory and cheese makers may not be able to maintain them using traditional methods (Powell et al., 2022).

In this work, we wanted to investigate the possibilities of influencing and selecting the microbiota of raw milk using artisanal traditional methods, with the aim of providing a simple method for the production of a natural supplementary culture for artisanal cheese makers. We investigated the production of an enriched raw milk whey culture (eRWC), a natural adjunct microbial culture produced from co-fermenting an enriched raw milk (eRM) with a natural whey culture (NWC). The eRWC was produced in triplicate testing different protocols and analyzed for the viable microbial profile with classical microbiology, and for its microbiota through next-generation sequencing (NGS). Afterwards, the eRWC were tested in two trials as adjunct cultures for the production of Vacherin Fribourgeois PDO, evaluating the microbial evolution and the cheese chemical features up to 120 days of ripening.

4.2 Materials and Methods

4.2.1 Natural cultures production

The raw milk and the sweet whey (deriving from Gruyère cheese production) were provided by the Agricultural Institute in Grangeneuve (Switzerland) within the framework of the Center of Excellence for Raw Milk Products. The samples were delivered to the laboratory for analysis on the same morning in refrigerated condition.

The eRWCs production process is schematized in **Figure 1**. Briefly, the raw milk (RM) was enriched by spontaneous fermentation for 21 days at 10 °C. Three milk enrichment protocols were tested: heat treatment (1h at 54 °C) before incubation (eRM.H), heat treatment plus salt addition (1h at 54 °C, 5 % w/v NaCl; eRM.HS), and no treatment (eRM). After mixing 1 part of eRM with 9 of NWC, the cultures were further incubated at 38 °C for 6h and 22h to obtain the so-called "young" (y) and "old" (o) cultures, respectively, resulting in a total of 6 final cultures: 1) eRWC.y; 2) eRWC.o; 3) eRWC.H.y; 4) eRWC.H.o; 5) eRWC.HS.y; 6) eRWC.HS.o. A simple odor evaluation was done by the operators to detect off-flavors (expired milk) and aroma descriptors of fresh (milk), acidic-fresh (yoghurt), and cheese (**Table 1**).

The pH-value of the samples was measured using a pH electrode (Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany).

The titratable acidity was determined by titration of 10 ml of sample with NaOH (0.25 N) using phenolphthalein (2 %) as indicator. Results were expressed in Soxhlet-Henkel degrees (SH°) (Deutscher Normenausschuss, 1970).

4.2.2 Microbiological analysis

For the microbiological analysis, 10 g of cheese core samples were homogenized in 90 mL of peptone-buffered saline water using a Stomacher (Masticator, IUL Instruments, Königswinter, Germany).

Homogenized cheese and the liquid samples were diluted ten-fold in peptone-buffered saline water for the viable counts of different microbial groups. **Table 2** reports the agar growth media used and their incubation condition. Each sample was plated in duplicate; results are expressed as $\log_{10}$ of

colony forming units (CFU) per mL (log CFU/mL). For plates without colonies due to the detection limit or excessive sample dilution, microbial counts were either expressed as zero (i.e. absent in 1 mL) or just below the lowest dilution analyzed (e.g. absent in the 10^1 dilution = 9 CFU/mL).

The count of *Clostridium* spores was performed by the most probable number technique (MPN), using the BY liquid growth medium (Bryant and Burkey, 1956). The sample (1 mL) of sample was mixed with 10 mL of medium, and then heat treated in a water bath at 80 °C for 10 minutes to kill the cells in the vegetative form. The glass tubes containing a glass bell as gas formation indicator, were incubated at 37 °C for 7 days. The MPN/mL was calculated according to the McGrady tables for three replicates. The following controls were used: negative control using sterile milk, and two positive controls using sterile milk inoculated with *Clostridium tyrobutyricum* FAM22553 (5 MPN/mL) and *Clostridium sporogenes* FAM1752 (5 MPN/mL) (Agroscope Microbial Collection).

The cheese making samples (i.e. vat milk at day 0, cheese at day 1, cheese at day 60, and cheese at day 120) were analyzed by an external laboratory for the absence of listeria, salmonella, coagulase positive staphylococci and *Escherichia coli*.

4.2.3 Next-generation sequencing (NGS)

The DNA extraction of 120 d ripened cheese core, and subsequent 16S rRNA gene amplicon sequencing and analysis were performed following (Dreier et al., 2022).

For the DNA extraction of liquid samples, a pellet was obtained by centrifuging 1 mL of sample at 16.000 × g for 10 min and discarding the supernatant. Then, 600 µL of guanidinium chloride 8 M were added and centrifugated at 16.000 × g for 10 min (this step was avoided for RM samples). After another supernatant removal, 1 mL of guanidium chloride 4 M were added and centrifugated for 5 minutes at 8.000 × g. After another supernatant removal, 400 µL of G2 buffer solution (EZ1 DNA Tissue kit, Qiagen, Hilden, Germany) were added and the whole sample was transferred to a 0.5 ml skirted tubes containing 100 mg 0.1 mm low binding zirconium beads (OPS Diagnostics, Lebanon, NJ, USA) and shaken for 1 min in a bead ruptor (Omni International Inc., Kennesaw, GA, USA). The tube was centrifugated at 16.000 × g for 10 min; after that, 200 µL of the supernatant were transferred to a tube with 10 µL of proteinase K (Qiagen) and incubated for 1h at 56 °C. Cell lysates were then processed by the BioRobot® EZ1 workstation (Qiagen,).

The 16S rRNA gene amplicon sequencing and data analysis were performed as described by (Dreier et al., 2022). Briefly, PCR of the V1–V2 16S rRNA gene region was performed in 50 µl reactions using 4 µl of DNA, 0.1 µM primer NGS_ABCxF27 (5′- CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG |Barcode X| AG AGT TTG ATC MTG GCT CAG − 3′) and 0.1 mM primer NGS_trP1_355 (5′- CCT CTC TAT GGG CAG TCG GTG ATG CWG CCT CCC GTA GGA GT − 3′), and 45 µl Platinum™ PCR SuperFiTM PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The amplification was carried out as follows: 98 °C for 30 s, followed by 18-35 cycles of 98 °C for 10 s, 55 °C for 20 s, and 72 °C for 30 s, and a final elongation 72 °C for 5 min. Sequencing was carried out on an IonTorrent Ion GeneStudio™ S5 System instrument (Thermo Fisher Scientific).

The raw sequences were primer trimmed and quality filtered in DADA2 (Callahan et al., 2016). Amplicon sequence variances (ASVs) were obtained in DADA2 with the parameter POOL =

"pseudo". Taxonomic annotation was performed using DAIRYdb v1.2.4 (Meola et al., 2019) with IDTAXA (Murali et al., 2018). Biostatistical analyses were done using the PHYLOSEQ package (McMurdie and Holmes, 2013) in R v4.2.2 (R Core Team, 2022).

4.2.4 Cheese production and quality analysis

Eight cheeses were produced following the Vacherin Fribourgeois PDO technology (**Figure 2**). The trials were replicated in two consequent days in the pilot plant facility at Agroscope (Liebefeld, Switzerland). Two controls, one with raw milk and the other with thermized milk, were produced using only the starter culture. A starter culture commonly used in Vacherin Fribourgeois PDO production (*Lactococcus lactis* and *Leuconostoc mesenteroides*; Liebefeld Kulturen AG) was also added at the proportion of 0.12 % to the vat milk. The natural adjunct cultures were added at the proportion of 0.5 % to the vat milk. The pH-value of the cheeses was determined using a pH electrode.

4.2.4.1 Volatile carboxylic acids

Volatile carboxylic acids were analyzed in the 120 d ripened cheeses as described by (Fröhlich-Wyder et al., 2013). After esterification with ethanol, the gas chromatography analysis was conducted using a Hewlett Packard HP 6890 gas chromatograph (Agilent Technologies, Basel, Switzerland) equipped with a Hewlett Packard Ultra 2 cross linked phenyl methyl silicone fused silica capillary column (50 m, 0.32 mm, 0.52 mm) and a flame ionization detector (FID).

4.2.4.2 Biogenic amines

Biogenic amines were analyzed in the 120 d ripened cheeses as described by (Ascone et al., 2017) using a UPLC system (UltiMate 3000 RS; Thermo Fisher Scientific) equipped with a C18 column (Accucore C18: 2.6 mm, 150 _ 4.6 mm; Thermo Fisher Scientific). All measurements were carried out in duplicate.

4.2.4.3 Free amino acids and di- and tripeptides

Total free amino acids and di- and tripeptides were analyzed in the 120 d ripened cheeses with the ophthaldialdehyde (OPA) method (Egger et al., 2019). Briefly, the samples were diluted ten-fold, prior to precipitation with perchloric acid (0.5 mol/L), and then derivatized with OPA in the presence of 2-mercapto-ethansulfonic acid. The produced 1-alkylthio-2-alkylisoindol compound was measured at 340 nm. To calculate the results, a standard curve based on glutamic acid was used.

4.2.4.4 Proteolysis

The extent of proteolysis in the 120 d ripened cheese was measured by analyzing the following compounds: total nitrogen (TN), water-soluble nitrogen (WSN) and non-protein nitrogen (NPN) according to Kjeldahl (Collomb et al., 1990).

4.2.4.5 Moisture and fat content

Cheese samples at 1 d and 120 d of ripening were analysed for moisture, dry matter (IDF, 1982), fat (IDF, 1987), and fat in dry matter (FDM) using common standard methods.

4.2.4.6 *Lactic acid, citric acid, and L-leucine aminopeptidase*

Cheese samples at 1 d and 120 d of ripening were analysed for lactic acid and citric acid concentration and L-leucine aminopeptidase (LAP) activity. For the determination of lactate, 1.25 g of cheese were homogenized in 50 mL of water using an OmniPrep Multi-Sample Homogenizer (Omni International, Kennesaw, USA). For the determination of citrate, 5 g of cheese were used. The homogenates were then incubated at 2 °C for 20 min. Particles and fat were removed by filtration. The concentration of D- and L-lactate, and citrate in the filtrates was determined using commercial enzymatic assay kits (R-Biopharm AG, Murten, Switzerland). L-leucine-aminopeptidase (LAP) activity was determined using a colorimetric assay with L-leucine-4-nitroanilide as substrate. For the assays, 60 µl cheese filtrate (1.25 g cheese sample homogenized in 50 ml water and filtered) and 250 µl of L-leucine-4-nitroanilide (final concentration: 0.995 mmol/L) in phosphate buffer containing 2 mmol/L Mg^{2+} (pH=7.4) were mixed in a microtiter plate. Enzyme activity was calculated based on the micromolar extinction coefficient of 4-nitroaniline measured by a SpectraMax ABS plus plate reader (Molecular Devices) after two hours of incubation using the SoftMax Pro software (Molecular Devices).

4.2.5 Statistical analysis

A statistical analysis of the microbiological results was conducted to evaluate the influence of the enrichment step in changing the microbial composition. RM, eRM, NWC, and eRWC were available in triplicate. All the results reported in the text are expressed as mean ± standard deviation. RM and eRM results were compared with a Student's t-test ($\alpha = 0.05$) when the variances were homogeneous (F-test; $\alpha = 0.05$); a Welch's t-test ($\alpha = 0.05$) was instead applied when the variances were heterogeneous. RM.H, eRM.H, and eRM.HS were instead analysed with an ANOVA model ($\alpha = 0.05$). In this case, the homogeneity of variances was previously tested with Bartlett's test ($\alpha = 0.05$); if the ANOVA showed significant differences, a multiple comparisons Tukey's HSD test was applied. If the ANOVA assumptions were not meet, non-parametric Kruscal-Wallis ($\alpha = 0.05$) and Dunn's post hoc tests were applied. The same statistical evaluation was done to test the significance of the difference between the three eRMs, and between eRWCs and NWC. The statistical analysis was done using the packages "stats", "agricolae" (de Mendiburu, 2021) and "FSA" (Derek et al., 2022) in the R environment (R Core Team, 2022).

Microbiological features (n = 14) of vat milk and cheese after 60 days, and both chemical (varying n, range 7−29) and microbiological (n = 14) features of cheeses after 1 and 120 days were subjected to Principal Component Analysis (PCA). The analysis was done in the R environment using the packages "FactoMineR" (Le et al., 2009) and "factoextra" for the results plotting (Kassambra and Mundt, 2020).

4.3 Results

4.3.1 Culture production

The enriched raw milk (eRMs) underwent phase separation at the end of the 21 day long incubation period. As the samples were left unagitated during incubation, fat accumulated at the top due to spontaneous creaming, while a protein coagulum deposited at the bottom. For eRM.HS, however, we did not observe protein coagulation. All the final eRWCs reached a pH below 4.5 (**Table 1**). The

extended incubation to produce the "old" cultures led to further acidification, with pH values reaching a range of 3.8–3.9.

The raw milk treatment influenced the odor of the eRMs and of the final cultures ready for addition to vat milk at the end of their respective incubation periods. For the eRMs, the outcomes differed for the three setups: two out of three replicates of eRM developed a cheese flavor; two out of three replicates of eRM.H developed an acidic-fresh yoghurt-like flavor; while all the three eRM.HS replicates kept a flavor similar to fresh milk (**Table 1**). The final eRWC cultures had an acidic-fresh yoghurt flavor like the NWC, with two exceptions, where off-flavors were detected.

4.3.1.1 Microbiological analysis

The RM, eRM, NWC and eRWC samples were analyzed for the viable count of the microbial groups listed in **Table 2**. All the results are presented in the boxplots in **Figure 3.** The hygienic quality of the RM was satisfactory, having an average value of total aerobic mesophilic (TAM) within European and Swiss legal criteria (EC, 2004; EDI, 2017), equal to 4.9 ± 0.9 log CFU/mL (mean ± SD; **Figure 3A**). When the RM was heat treated, all the bacterial groups decreased in concentration (TAM = 2.1 ± 0.2 log CFU/mL).

The enrichment step significantly increased all the microbial group concentrations in all the eRMs produced, except for facultative heterofermentative (FH) lactobacilli and yeast and molds, which were not significantly different between RM.H and eRM.HS (**Figure 3A**). The eRM showed the highest LAB concentrations (9.1 ± 0.2 log CFU/mL for both lactobacilli and streptococci), while the heat treatment and the NaCl addition inhibited the LAB growth. Streptococci concentration was not significantly different between eRM.H and eRM.HS, indicating that the NaCl addition did not influence their growth during the enrichment (**Figure 3A**). The NaCl addition was instead effective in reducing enterobacteriaceae growth in eRM.HS. Staphylococci were not inhibited either by heat or salt.

The final eRWCs and the NWC showed more variable results in different microbial groups (wider data distribution of the boxes, **Figure 3B**). As expected, viable streptococci decreased in the old cultures (eRWC.o, streptococci = 6.5 ± 0.2 log CFU/mL; mean ± SD), whereas the young cultures contained higher bacterial counts, with the highest count of lactobacilli in eRWC.y at 7.9 ± 0.2 log CFU/mL. Significant differences were found in the concentration of enterococci (eRWC.H.o vs. NWC), yeast and molds (eRWC.H.o, eRWC.o vs. NWC), staphylococci (eRWC.HS.o, NWC vs. eRWC.H.y), and enterobacteriaceae (eRWC.H.y, eRWC.o vs. NWC).

No clostridia were detected in any sample.

4.3.1.2 NGS analysis

After the raw sequences trimming, a total of 557 ASVs were identified in the RM, eRM, NWC and eRWC samples, assigned to 274 species. **Figure 4A** shows the alpha diversity of all the samples, measured with different indices (Thukral, 2017). The Chao1 and Shannon indices were used to define the microbial richness and diversity, respectively. The RM showed the highest microbial richness and diversity, although with clear differences between replicates. On the other hand, NWC was the sample that showed the least variability between the three replicates, but it had low values of Chao1 and Shannon. Heat treatment and salting of the eRM led to the smallest decrease in

microbial richness and diversity. This effect was less evident in the eRWCs, which all showed a similar species richness independent of the eRM treatment prior to their production. The further incubation to produce old eRWCs increased the microbial diversity on average.

Non-metric multidimensional scaling of Bray–Curtis dissimilarity was used to study the between-sample diversity (**Figure 4B**). RM, eRM.HS, and eRM.H samples showed a distinct microbial composition. On the other hand, eRM, NWC, and the eRWCs were all close to each other in the ordination plot, meaning that they had a similar microbiota. eRWC.H and eRWC are separated along the secondary axis of the graph, indicating that the type of eRM used for their production influenced their microbial profile. There was no clear clusterization of the eRWCs based on the "young" and "old" treatment.

Figures 5A and **5B** show the dominant and subdominant species present in the NWC and the final eRWCs. Four species, namely S. thermophilus, Lactococcus lactis, L. helveticus, and L. delbrueckii, dominated the cultures, having an average relative abundance above one per cent. Two replicates of NWC were primarily composed of *S. thermophilus*, at 99.3 % and 93.2 % abundance, respectively. Surprisingly, the third replicate contained only a small proportion of *S. thermophilus* (0.31 %), whereas the main constituent was *L. lactis* at 99 %, a species not commonly found in NWCs (De Filippis et al., 2014). This influenced the microbial composition of the final cultures: All the eRWC replicates .2 and .3, derived from the NWCs rich in *S. thermophilus*, showed a higher concentration of *S. thermophilus* than (when present) *L. lactis*, while the eRWC replicates .4 always had a concentration of *L. lactis* > 92 %. Except for the replicates .4, all the "old" cultures were characterized by the presence and high abundance of *L. delbrueckii* and *L. helveticus*. Among the low-abundant species (average relative abundance < 1 %), *Streptococcus salivarus* and species belonging to the *Pseudomonaceae* family were the most present and abundant.

4.3.2 Cheese production

Cheese cross sections after 120 days of ripening are shown in **Supplementary Figure 1**.

The use of eRWCs slowed the acidification process during the cheese making, as shown by the higher pH values in the curd after 6h of production (**Figure 2B**). After 24h, this difference disappeared, and all the cheeses reached a pH in the target range of 4.95–5.01.

4.3.2.1 Microbiological and chemical analysis

We assessed the overall similarity of analyzed samples by reducing the dimensionality of all measured parameters (n = 43), including both chemical (varying n, range 7–29) and microbiological (n = 14) features by principal component analysis (PCA). Ordination plots are shown in **Figure 6.** The control cheeses produced from thermized milk resulted in very different chemical and microbiological features; for this reason, in order to better evaluate the effect of the adjunct cultures on cheese properties, we excluded their results from the PCA. Constant features between the samples were removed because they did not contribute to the explanation of the samples' similarity.

For the vat milk and the 60 d cheese, only microbiological features were analysed. The first two principal components explained 59 % of the variance in vat milk samples (**Figure 6A**). When produced with the same adjunct culture, milk samples shared a similar microbial profile as shown by their proximity in the ordination plot. Three clusters formed. By coloring the results by culture

type, i.e. young and old (**Supplementary Figure 2B**), it was possible to see how the microbial profile of the vat milk was influenced by this factor. The control and the vat milk with eRWC.HS.o showed a similar microbial profile. After 60 days, the variance explained by the first two principal components, but only based on the microbiological analysis, was lower (55.8 %, **Figure 6C**). The contribution of each variable on the first two principal components is reported in **Supplementary Figure 3**.

In addition to the microbiological features, different chemical features were analyzed for the cheeses one day (Supplementary Figure 4C) and 120 days (Supplementary Figure 5C) after production. The one day ripened cheeses showed two distinct clusters separated along the first component (**Figure 6B**), which explained 39.5 % of the total variance. The factor influencing these differences was the production day (**Supplementary Figure 3A**), which mainly caused changes in the dry matter, dry loss, and pH of the cheese (**Supplementary Figure 3B**). Even if the effect was less pronounced, the use of different cultures influenced the 1 d cheese properties. This is more evident looking at the clustering of the samples on the second principal component in **Figure 6B**, which explained 26.9 % of the variance. Being clustered at the bottom of the plot, the control cheeses showed different microbiological and chemical characteristics from the cheese produced with adjunct culture. In particular, the samples produced with eRWC.H.o were the most different from the control. The features mainly driving this distinction were the amount of D-Lactic acid, citric acid, and FH lactobacilli (**Supplementary Figure 4C**). At the end of ripening, after 120 days, the NaCl content, together with the volatile profile and the concentration of some biogenic ammines, were the features that most contributed to the samples' diversity (**Supplementary Figure 5C**). As shown in **Supplementary Figure 5A**, cheese samples are divided on the first component (32.6 % of the total variance explained) grouping in function of the production day. Also, the adjunct culture used for cheese production influenced the cheese properties: in **Figure 6D**, pairs of samples are clearly separated along the second component mainly because of their content in LAP, NPN, formic acid, acetic acid, and D-lactic acid (**Supplementary Figure 5C**).

4.3.2.2 NGS analysis

The total number of ASVs found in all the cheese samples ripened at 120 d was 62, which were assigned to 22 species. **Figure 7A** shows the alpha diversity of the cheeses. Samples are grouped based on the type of adjunct culture used for cheese production. Looking at the Chao1 and Shannon indices of microbial richness and diversity, respectively, it is possible to see how the control cheese produced without adjunct cultures had the greatest variability between the two production replicates, while the use of eRWCs limited this variability. The cheese with the highest species richness and diversity were those produced with eRWC.H.y and eRWC.H.o, respectively. The cheese produced with eRWC.y showed lower Chao1 and Shannon values, more similar to the control cheese.

Figure 7B shows the difference in the microbiota between the ripened cheese based on Bray–Curtis dissimilarity. Cheeses produced with eRWC.H.y and eRWC.H.o are close to each other, meaning that they had a similar microbiota and were less influenced by the y and o incubation. This was different for cheese produced with eRWC and eRWC.HS. Overall, the samples did not show a distinct microbiota influenced by the different cultures. On the other hand, the cheeses microbial

profiles were more influenced by the production day, i.e. by the different raw milk used for cheese making (**Supplementary Figure 5A**).

Figures 8A and **8B** show the dominant and subdominant species found in the ripened cheeses. *S. thermophilus*, *L. lactis*, and *Leuconostoc mesenteroides* were the only three species present with a relative abundance above one per cent on average. *S. thermophilus* was always present in the cheeses produced with the adjunct culture, but not in the control. Among the subdominant species, *L. delbrueckii* was present with higher relative abundance in cheese produced with "old" eRWCs, while in the other cheeses it was classified as *Other species* having a maximum relative abundance < 0.6 %. The cheeses produced with eRWC.H.y and eRWC.H.o had a particular presence of *Enterococcus faecalis*, with a relative abundance of 5.89 and 5.51 % respectively.

4.4 Discussion

As a fermented food, cheese relies on microorganisms for its production. Microorganisms are often added in the form of cultures during the cheese making mainly to drive the acidification (starter cultures) or the ripening process (starter and adjunct cultures). While starter cultures are produced both at the laboratory or industrial (mixed defined-strain cultures), and artisanal (natural undefined strain culture) level, adjunct cultures with NSLAB are only commercialized as mixed defined-strain cultures. NSLAB are naturally present in raw milk, however, although some species may have very low abundance, since LAB are just a subdominant part of the raw milk microbiota (Bettera et al., 2023). This makes their selection and isolation difficult. Strategies to overcome this limit could be the use of selective/elective growth media (Coeuret et al., 2003; Neviani et al., 2009) and/or the spontaneous fermentation of raw milk with the application of selective conditions, as already applied for the isolation of potentially probiotic yeasts and LAB (Galli et al., 2022a, 2022b).

4.4.1 Natural adjunct culture microbiota

In this study, we performed a spontaneous fermentation of raw milk with the aim of producing a natural adjunct culture rich in NSLAB. The strategy was to apply the conditions that are known to promote the selection of NSLAB of our interest and inhibit the rest of the microbiota, as it happens in raw milk, cooked, long-ripened cheese varieties (Gatti et al., 2014; Bottari et al., 2018). Low values of pH, curd cooking, NaCl, low temperature, lack of lactose and microbial competition are factors that make the cheese environment hostile during ripening. NSLAB are subjected to these stress conditions, and their adaptation responses are efficient (Gobbetti et al., 2015).

We applied these conditions to raw milk to produce the eRWCs (**Figure 1**). A similar approach was used by Bancalari and colleagues, who subjected Parmigiano Reggiano raw milk samples to spontaneous fermentation for 4 months at 8 °C in order to isolate potential aroma-producing *Lacticaseibacillus* strains (Bancalari et al., 2017).

The raw milk enrichment step increased the LAB concentration (**Figure 3A**) and decreased the overall microbial richness and diversity (**Figure 4A**), although differently between eRM, eRM.H and eRM.HS (**Figure 4C**). eRM in fact selected an aciduric microbiota due to its lower pH (**Table 1**) with values closer to a *lattoinnesto* (Parente et al., 2017), while eRM.H and eRM.HS were most likely composed of heat- and salt-resistant microorganisms.

We then mixed 10 mL of eRMs in 90 mL of NWC and incubated for 6 and 22 h to apply further acid stress. The LAB viable count was not significantly different between the eRWCs, ranging from 6.5–7.9 log CFU/mL which is comparable to LAB concentrations found in natural milk cultures (Parente et al., 2017) or natural whey cultures (Gatti et al., 2014). However, the eRWCs harbored higher microbial richness and diversity than NWC (**Figure 4A**), especially in the old cultures where lower pH selected *L. helveticus* and the more acid-resistant *L. delbrueckii* (De Angelis and Gobbetti, 2004).

The applied pre-treatments allowed to have adjunct cultures with diverse microbiota, which were tested in cheese making trials.

4.4.2 Influence of eRWCs on cheese features

The addition of 0.5 % eRWC was enough to influence the vat milk microbial profile (**Figure 6a**). Old and young eRWC samples clearly differed from each other and the control mainly because of their streptococci and yeast and mould concentrations (**Supplementary Figure 2B**). The use of eRWCs slowed curd acidification in the first hours of cheese making (**Figure 2B**). Although the acidifying performances are known to be strains-dependent (Bancalari et al., 2016), this was surprising since one of the common drawbacks of adjunct cultures is the curd over-acidification due to lactose fermentation in addition to primary starters, which is the reason why adjunct strains are usually attenuated (Gobbetti et al., 2015). Competition for nutrients between SLAB and NSLAB could be a possible explanation supported by the higher D-lactate detected in cheese with adjunct culture (samples distribution on the second dimension, Supplementary Figure 3B), which is known to be produced from NSLAB by fermentation of residual lactose or by isomerization of L-lactate (McSweeney et al., 2017; Blaya et al., 2018). However, the pH after 24 h settled to equal values for all the cheeses.

The cheese features after one day were only partially influenced by the microbiota of the eRWCs. The different amounts of D-lactic acid, citric acid, and FH lactobacilli separated the experimental cheeses along the secondary axis of the PCA (**Figure 6B**). In particular, the cheese produced with eRWC.H.o, the culture with the highest concentration of *L. helveticus* (**Figure 5A**), was the most distant from the control. On the other hand, the cheese chemical and microbiological features were mainly influenced by the production day, therefore most likely by the raw milk characteristics (**Supplementary Figure 4A**). The same was noticed after 120 d of ripening. Although the use of diverse eRWCs contributed in some cases to producing cheese with richer and more diverse microbiota, the final cheese features were more influenced by the production day (**Supplementary Figure 5A**). Variability in cheese characteristics arising from the diverse raw milk microbiota evolution during ripening is known to occur, even if the same technology is applied and starter cultures are used to drive the fermentation. Such an example was reported for Grana Padano PDO (Lazzi et al., 2016) and Cantal PDO (Frétin et al., 2018) cheeses. This result could also be caused by the cheese variety analyzed in this study. Vacherin Fribourgeois PDO is in fact a surface smear-ripened cheese. Although the richer rind microbiota of this cheese variety is known to be different from the core (Dugat-Bony et al., 2016), the surface microbiota strongly affects the final cheese characteristic. This could have masked the effect of the adjunct culture used in our trials. Perspective for future studies could be the control of the entire cheese microbiota including the rind.

Furthermore, testing the eRWCs with different cheese varieties (i.e. different technological stress) could reveal different dynamics in the microbiota evolution and possibly a stronger effect of the adjunct culture on the ripened cheese.

This study provides new insights into the possibility to enrich the raw milk microbiota for the production of cheese. The applied protocols allowed us to test the effect of different stresses on the microbial populations present in raw milk, leading to the production of natural adjunct cultures harboring diverse microbiota. This microbial diversity influenced the early stages of cheese making, but its effect decreased over time during ripening, showing an inferior effect to the raw milk microbiota. More research is needed to optimize the culture production, testing different treatments that could more specifically select desired NSLAB present in raw milk. The optimization of such a tool could be an alternative to the practice of isolating, geno-pheno-typing, and formulating mixed-defined-strain adjunct cultures that require knowledge and facilities not always available for artisanal cheese makers.

4.5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.6 Author Contributions

LB, MD, RS and HPB contributed to conception and design of the study; LB, MD and HBM performed the experiments; LB and MD performed the data curation, elaboration and statistical analysis; LB wrote the first draft of the manuscript; MG, RS and HPB acquired the fundings. All authors contributed to manuscript revision, read, and approved the submitted version.

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4.9 Tables

Table 1. Acidity and odor evaluation of RM, RH.Heated, eRMs, NWC, sweet whey (SW), and eRWCs. Refer to **Figure 1** for samples' abbreviation. (✓ = perceived; × = not perceived; - = not evaluated).

Sample (n = 3)	Acidity		Odor evaluation											
	pH	°SH/10mL	Off-flavors (expired milk)			Fresh (milk)			Acidic-fresh (yoghurt)			Cheese		
			Replicates											
			1	2	3	1	2	3	1	2	3	1	2	3
RM	6.6 ± 0	7.3 ± 0.6	-	-	-	-	-	-	-	-	-	-	-	-
RM.H	6.6 ± 0	7.5 ± 0.5	-	-	-	-	-	-	-	-	-	-	-	-

eRM	4.7 ± 0.6	32.7 ± 7.1	✓	×	×	×	×	×	×	×	×	×	✓	✓
eRM.H	6.2 ± 0.2	13 ± 2	✓	×	×	×	×	×	×	✓	✓	×	×	✓
eRM.HS	6.4 ± 0.1	10.5 ± 0.9	×	×	×	✓	✓	✓	×	×	×	×	×	×
eRWC.y	4.2 ± 0.1	21 ± 2	✓	×	×	×	×	×	×	✓	✓	×	×	×
eRWC.H.y	4.2 ± 0.2	21 ± 3.5	×	×	×	×	×	×	✓	✓	✓	×	×	×
eRWC.HS.y	4.2 ± 0.2	21.7 ± 4	×	×	×	×	×	×	✓	✓	✓	×	×	×
eRWC.o	3.9 ± 0.4	32.7 ± 11.5	✓	×	×	×	×	×	×	✓	✓	×	×	×
eRWC.H.o	3.9 ± 0.4	33 ± 12.1	×	×	×	×	×	×	✓	✓	✓	✓	×	×
eRWC.HS.o	3.8 ± 0.4	32.3 ± 10.3	×	×	×	×	×	×	✓	✓	✓	×	×	×
SW	6.5 ± 0.1	5.3 ± 0.6	-	-	-	-	-	-	-	-	-	-	-	-
NWC	4.6 ± 0.5	16.7 ± 3.2	×	×	×	×	×	×	✓	✓	✓	×	×	×

Table 2. Microbial groups analyzed for viable counts and methods used.

Microbial group	Incubation conditions	Atmosphere conditions	Growth medium	Supplier
Total aerobic mesophilic (TAM)	30 °C for 2 days	aerobic	Standard Methods Agar with Casein	-BD (Franklin Lakes, NJ, U.S.) -Merck (Casein peptone) (Darmstadt, Germany)
Streptococci	37 °C for 2 days	aerobic	M 17 agar + D(+) Glucose (Terzaghi and Sandine, 1975)	Merck
Lactobacilli	30 °C for 2 days	anaerobic	MRS-lactose agar (5M lactic acid) (De Man et al., 1960)	-Biolife (Milan, Italy) -Oxoid (Agar No 2.) (Waltham, MA, U.S.)
Facultative heterofermentative lactobacilli (FHL)	30 °C for 3-4 days	anaerobic	FH agar (Isolini et al., 1990)	-Biolife
Propionibacteria	30 °C for 7 days	anaerobic	Lactate agar	-Merck (Lactate solution, casein peptone) -BBL (Yeast extract) -Oxoid (Agar No 2)
Enterococci	37 °C for 2 days	aerobic	Kanamycin Esculin Azide agar (Mossel et al., 1978)	Merck
Yeasts and molds	30 °C for 3-4 days	aerobic	Phytone-Yeast-Extract Agar	BBL
Staphylococci	37 °C for 2 days	aerobic	Mannitol salt phenol-red agar	Biolife
Enterobacteriaceae	37 °C for 2 days	aerobic	MacConkey agar	Oxoid

4.10 Figures legend

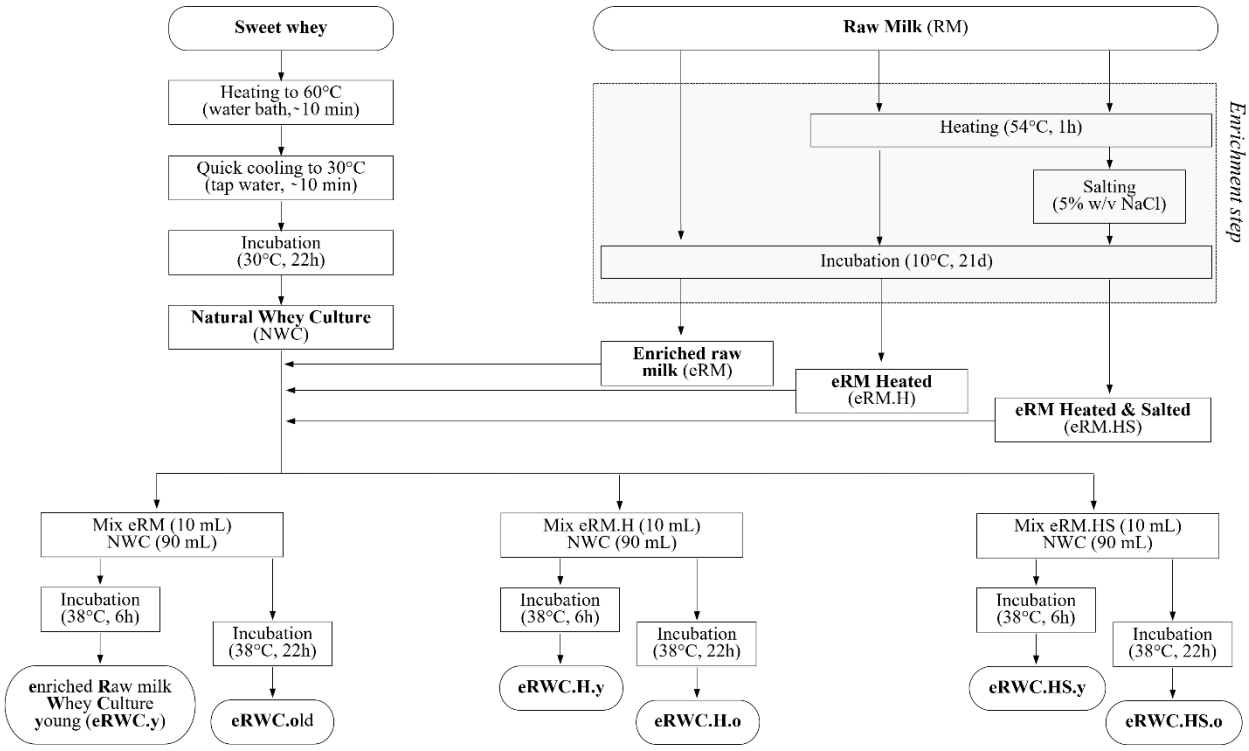

Figure 1. Natural adjunct culture production flow chart. Reference for the samples' abbreviation.

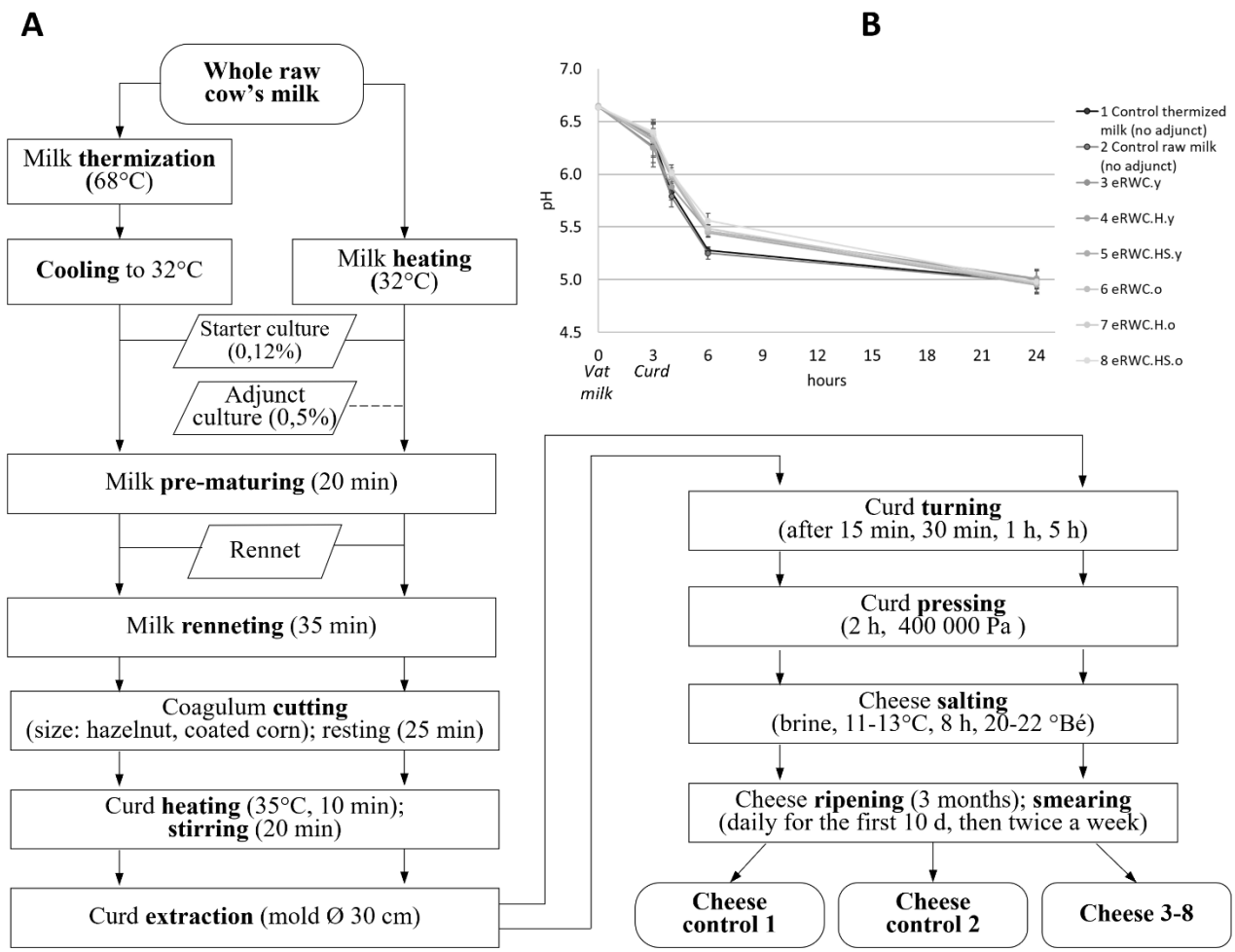

Figure 2. A) Vacherin Fribourgeois PDO cheese making flow chart. **B**) Acidification curve during the cheese making; the adjunct culture added in each trial is reported in the legend. Refer to **Figure 1** for samples' abbreviation.

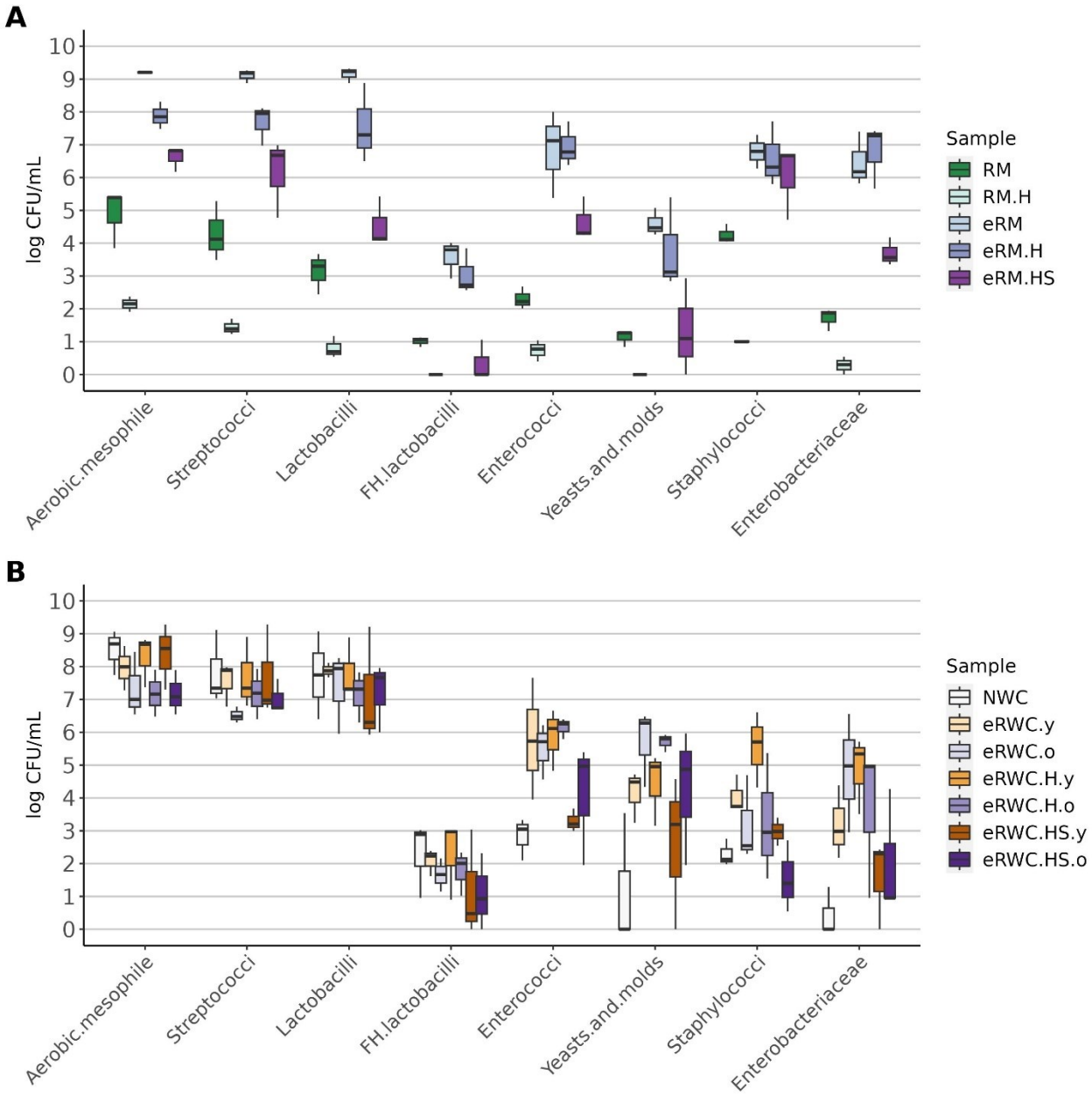

Figure 3. Viable microbial counts. **A**) RM, RH.Heated and eRMs; **B**) NWC and eRWCs. Refer to Figure 1 for samples' abbreviation.

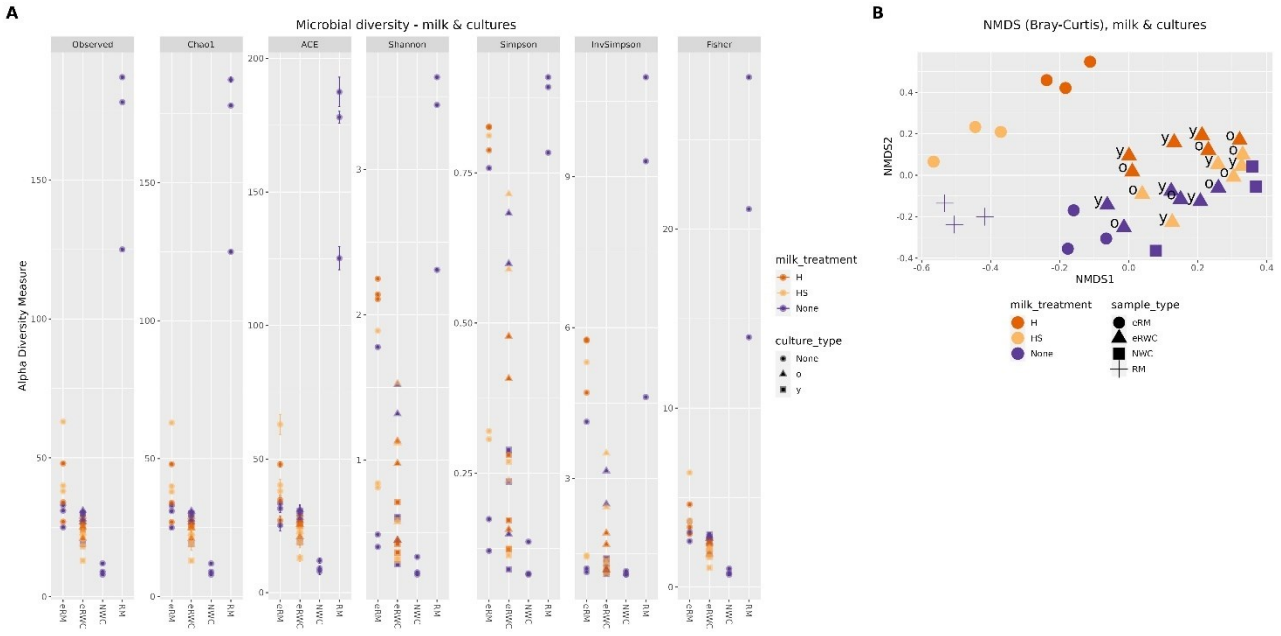

Figure 4. NGS results of RM, eRMs, NWC, and eRWCs. **A**) Alpha diversity measures; **B**) Ordination plot of non-metric multidimension scaling on Bray-Curtis dissimilarities; "y" = young, "o" = old. Refer to **Figure 1** for samples' abbreviation.

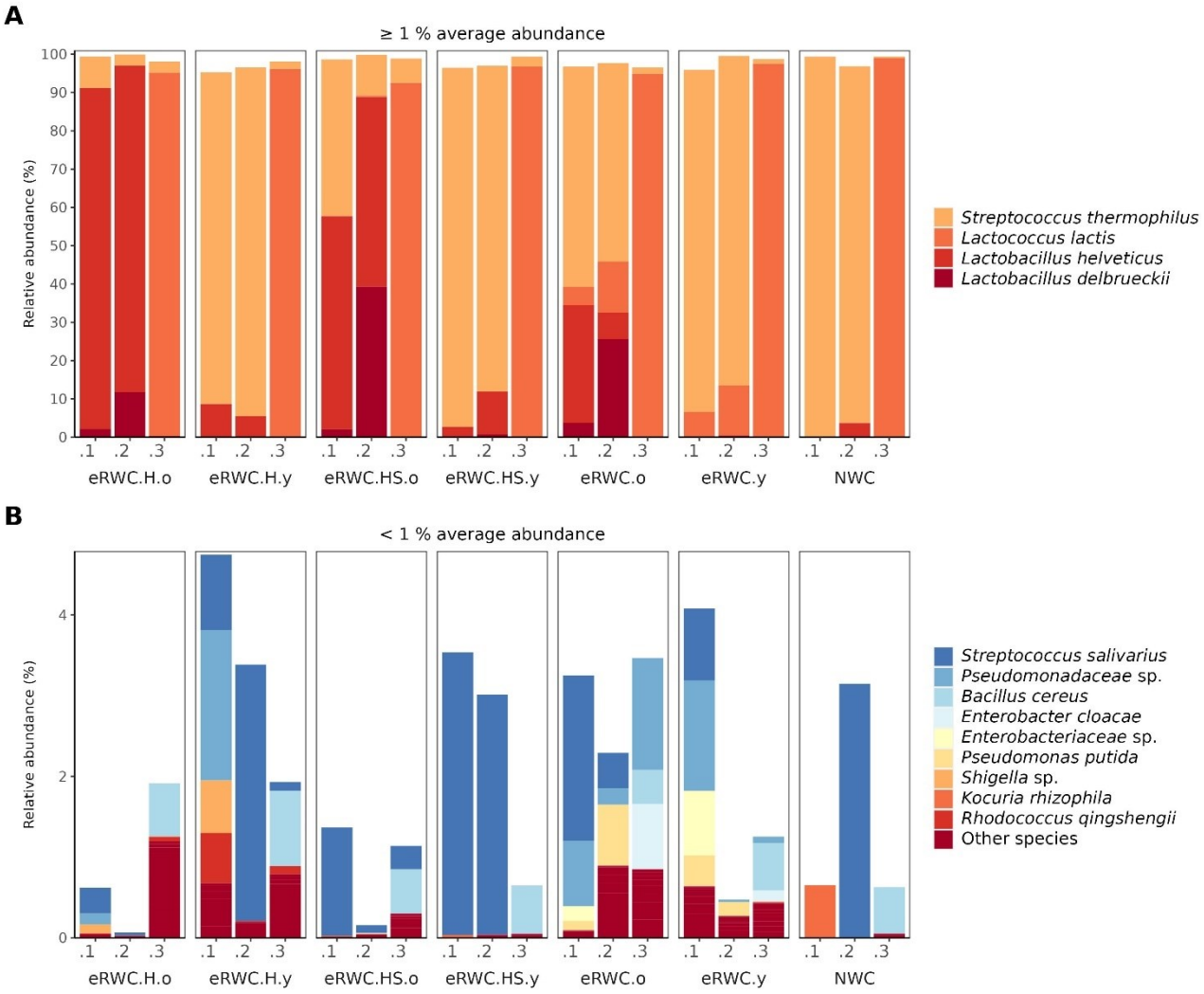

Figure 5. NGS results of NWC, and eRWCs. **A**) Stacked bar plot of most abundant species; **B**) stacked bar plot of least abundant species. Refer to **Figure 1** for samples' abbreviation; the three replicates of samples are reported.

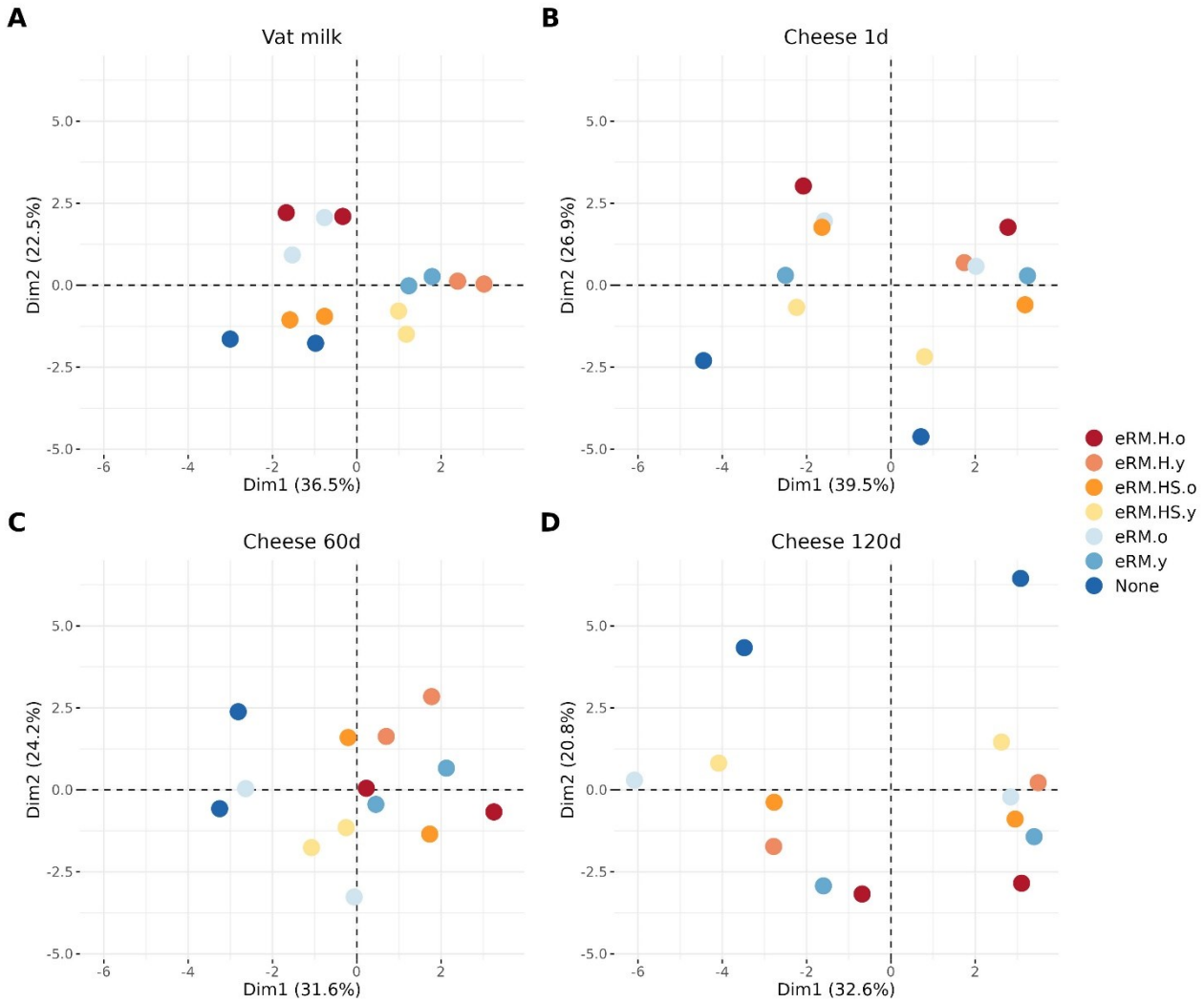

Figure 6. Ordination plots of principal component analysis: **A**) vat milk, microbial features (n = 14); **B**) 1 d cheese, microbial (n = 14) and chemical features (n = 7); **C**) 60 d cheese microbial features (n = 14); **D**) 120 d cheese, microbial (n = 14) and chemical features (n = 29). Refer to Figure 1 for samples' abbreviation.

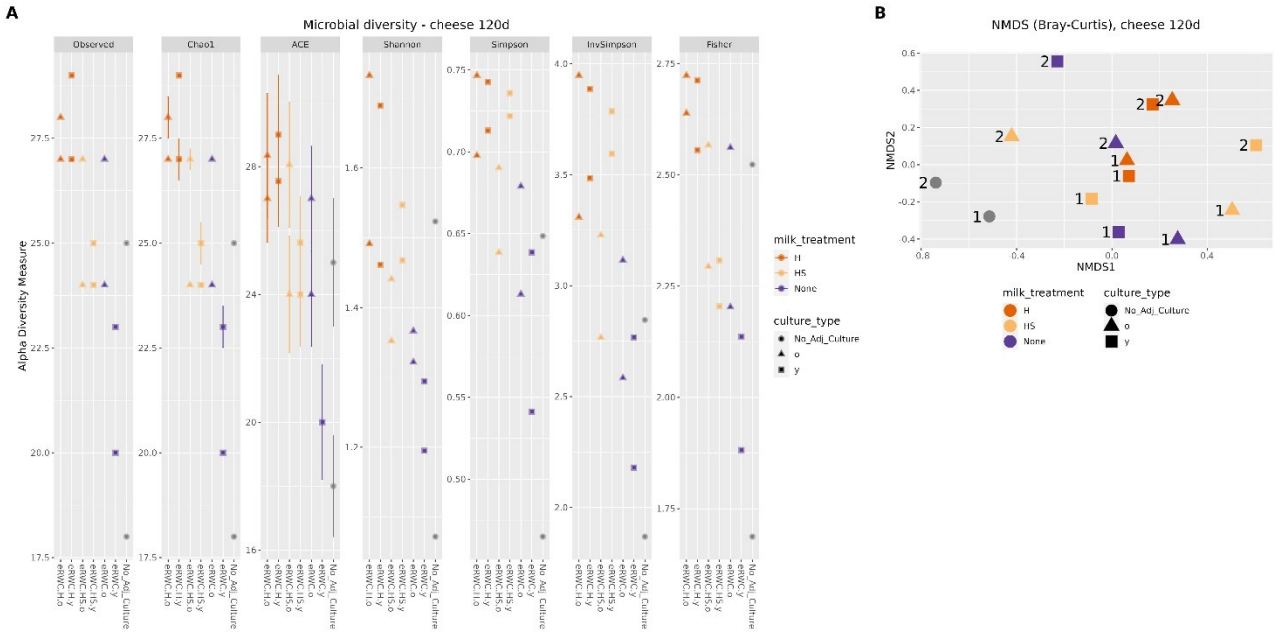

Figure 7. NGS results of 120 d cheese. **A**) Alpha diversity measures; **B**) Ordination plot of non-metric multidimension scaling on Bray-Curtis dissimilarities; "1" = first production day, "2" = second production day. Refer to **Figure 1** for samples' abbreviation.

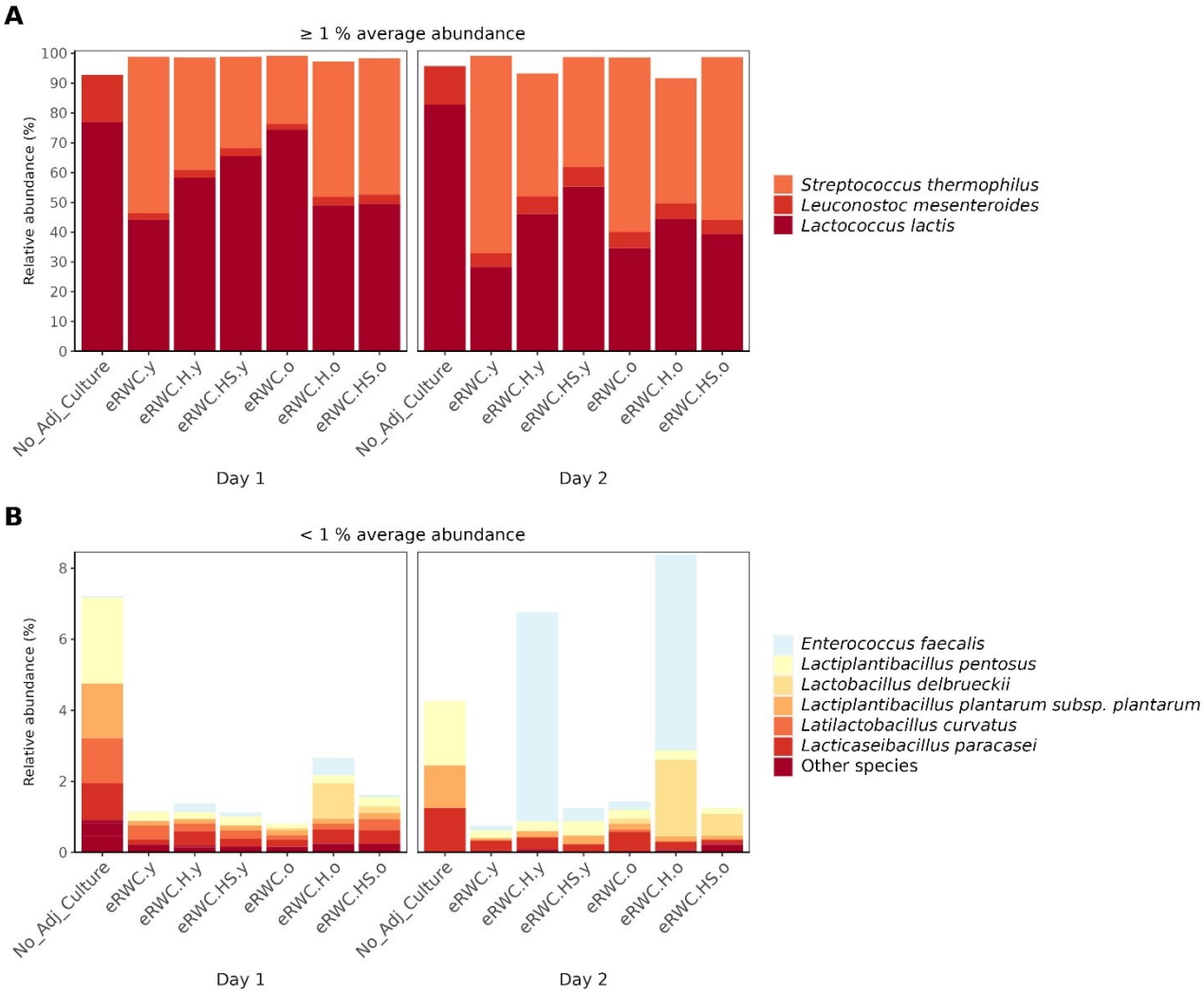

Figure 8. NGS results of 120 d cheese. **A**) Stacked bar plot of most abundant species; **B**) Stacked bar plot of least abundant species. Refer to **Figure 1** for samples' abbreviation; the two replicates (day 1 and day 2) of samples are reported.

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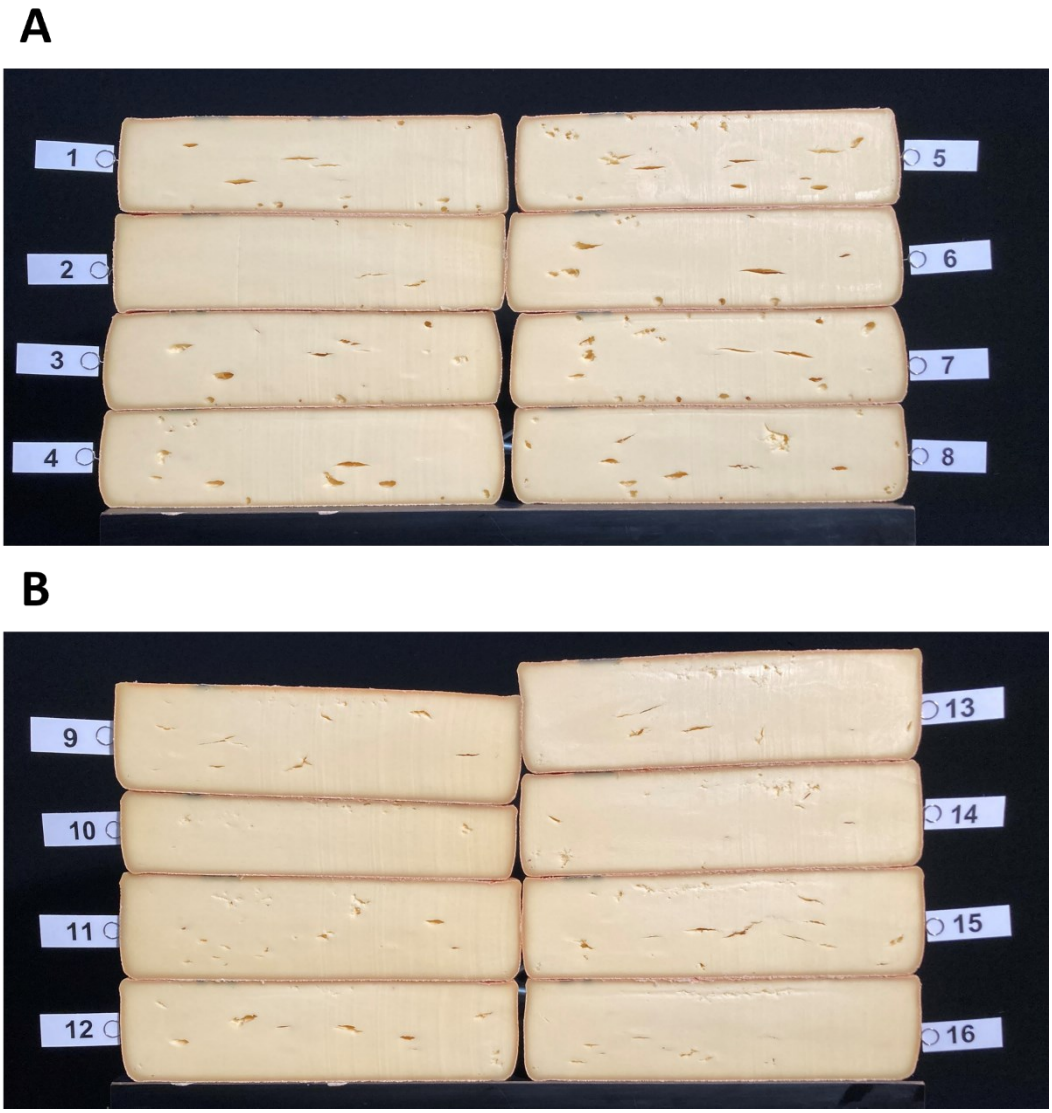
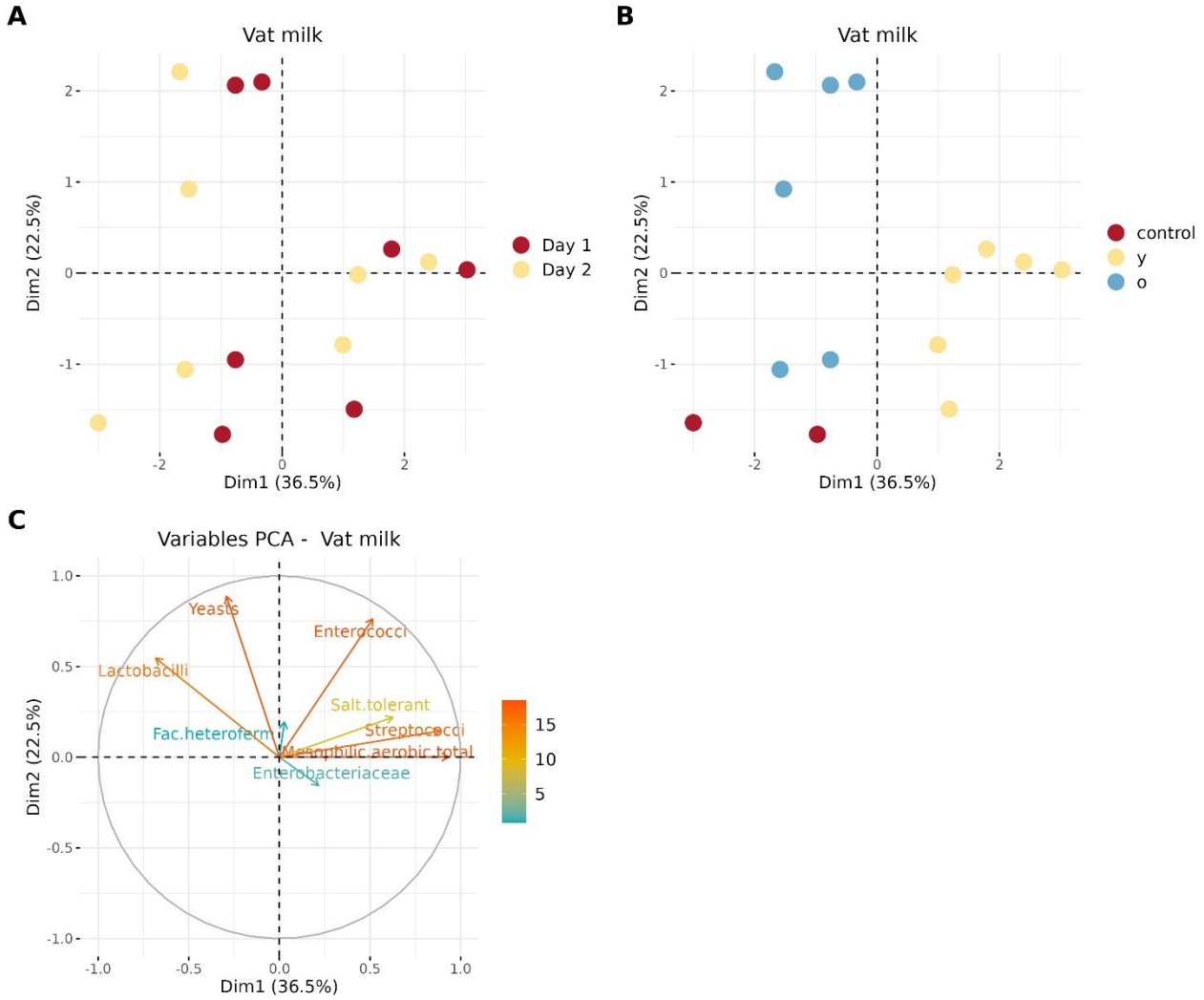
4.12 Supplementary Material



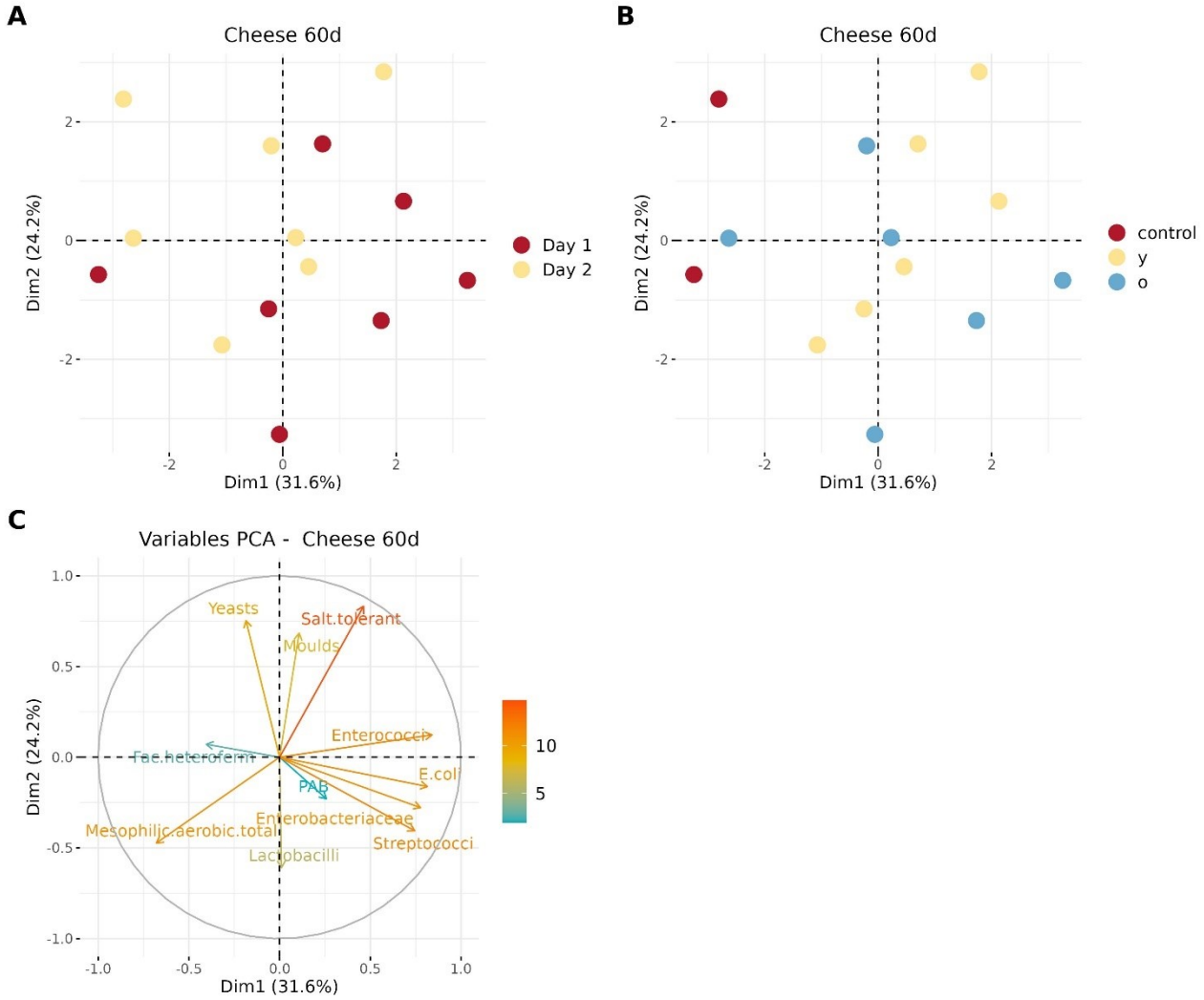
Supplementary Figure 1. Cheese cross sections after 120 days of ripening. A) First replicate; B) Second replicate. 1 and 9 = control, raw milk; 2 and 10 = control, thermized milk; 3 and 11 =

eRWC.y; 4 and 12 = eRWC.H.y; 5 and 13 = eRWC.HS.y; 6 and 14 = eRWC.o; 7 and 15 = eRWC.H.o; 8 and 16 = eRWC.HS.o. Refer to **Figure 1** in the article for samples' abbreviation.



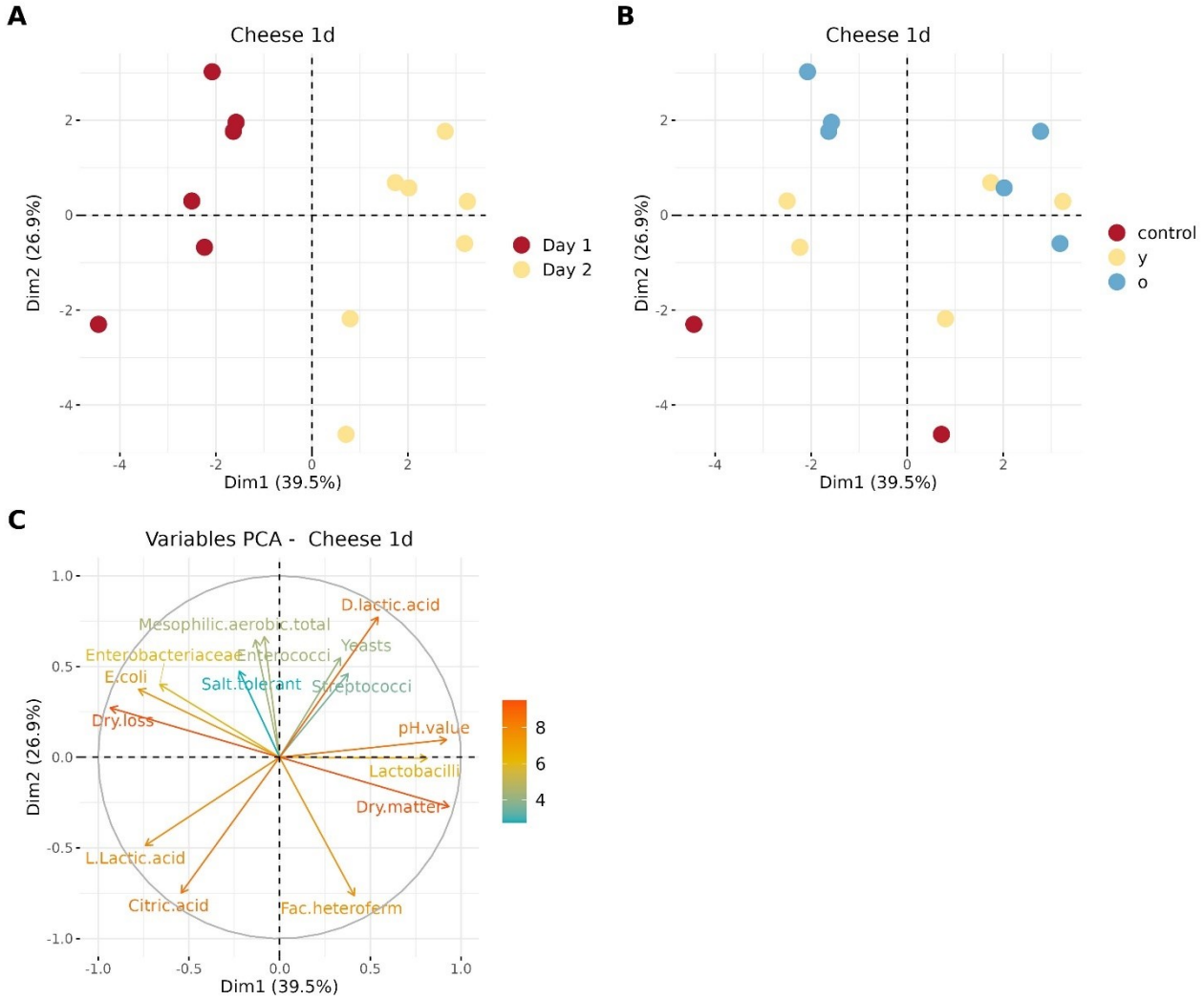
Supplementary Figure 2. Principal component analysis of vat milk microbial features (n = 14). A) Ordination plot grouping by eRWC production days 1 and 2; B) Ordination plot grouping by eRWC

treatment young (y) and old (o) (control = no use of adjunct culture); C) Variables contribution plot. Refer to **Figure 1** in the article for samples' abbreviation.



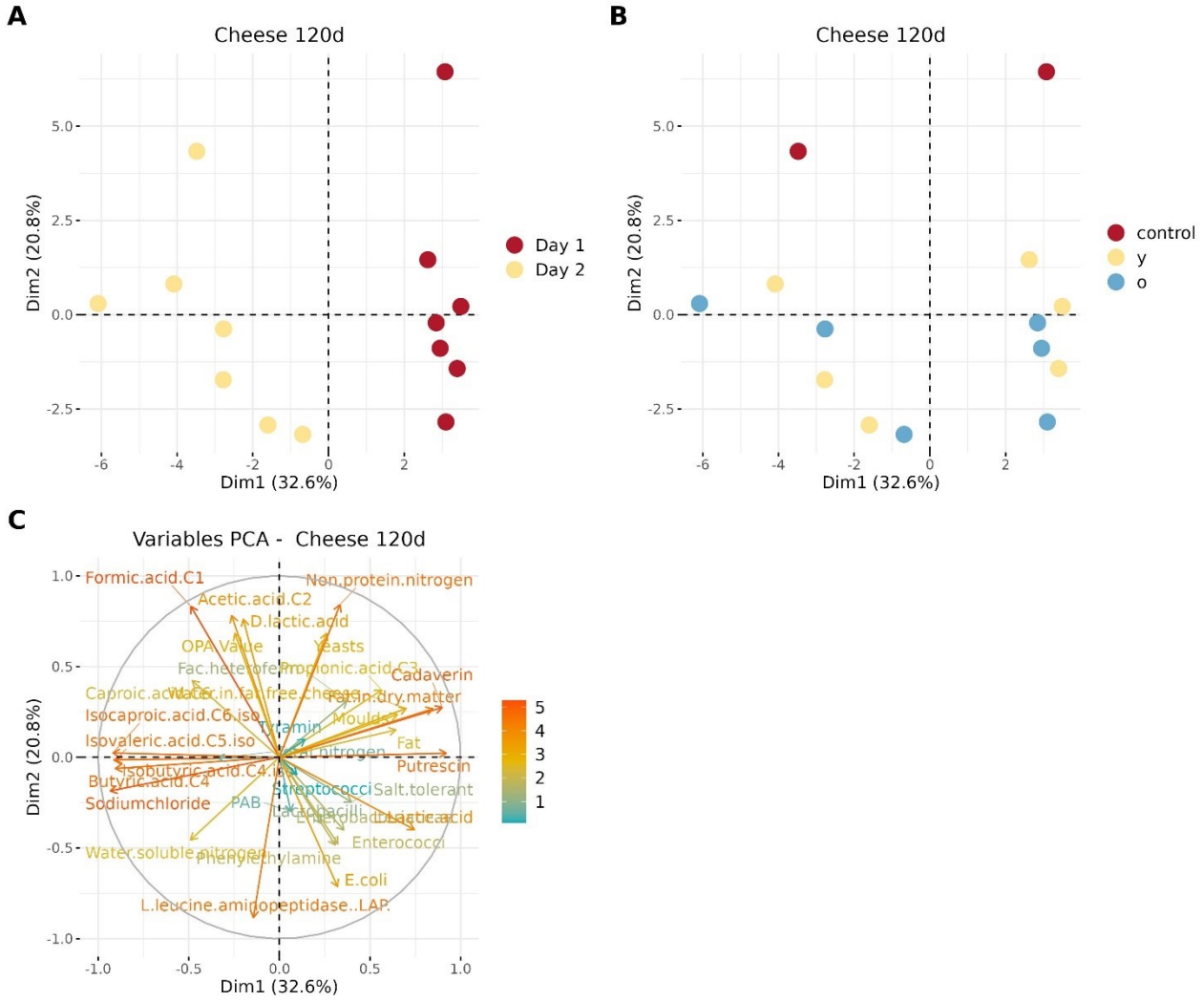
Supplementary Figure 3. Principal component analysis of 60 d ripened cheese microbial features (n = 14). A) Ordination plot grouping by eRWC production days 1 and 2; B) Ordination plot

grouping by eRWC treatment young (y) and old (o) (control = no use of adjunct culture); C) Variables contribution plot. Refer to **Figure 1** in the article for samples' abbreviation.



Supplementary Figure 4. Principal component analysis of 1 d ripened cheese microbial (n = 14) and chemical (n = 7) features. A) Ordination plot grouping by eRWC production days 1 and 2; B)

Ordination plot grouping by eRWC treatment young (y) and old (o) (control = no use of adjunct culture); C) Variables contribution plot. Refer to **Figure 1** in the article for samples' abbreviation.



Supplementary Figure 5. Principal component analysis of 120 d ripened cheese microbial (n = 14) and chemical (n = 29) features. A) Ordination plot grouping by eRWC production days 1 and 2; B) Ordination plot grouping by eRWC treatment young (y) and old (o) (control = no use of adjunct culture); C) Variables contribution plot. Refer to **Figure 1** in the article for samples' abbreviation.

4.13 Data availability statement

The datasets analyzed for this study can be found in the eRWC repository, https://doi.org/10.5281/zenodo.7729272. The NGS datasets generated and analyzed during the current study are available in the Sequence Read Archive (SRA) under the BioProject: PRJNA937653.

5 Structural defects in extra hard raw milk cheese

Investigating structural defects in extra hard cheese produced from high-speed centrifugation of milk

Luca Bettera[a1], Marcello Alinovi [a1], Elena Bancalari [a]*, Eleonora Carini[ac]; Paolo D'Incecco[b], Luisa Pellegrino[b], Monica Gatti[ac]

[a] Department of Food and Drug, University of Parma, Parma, Italy

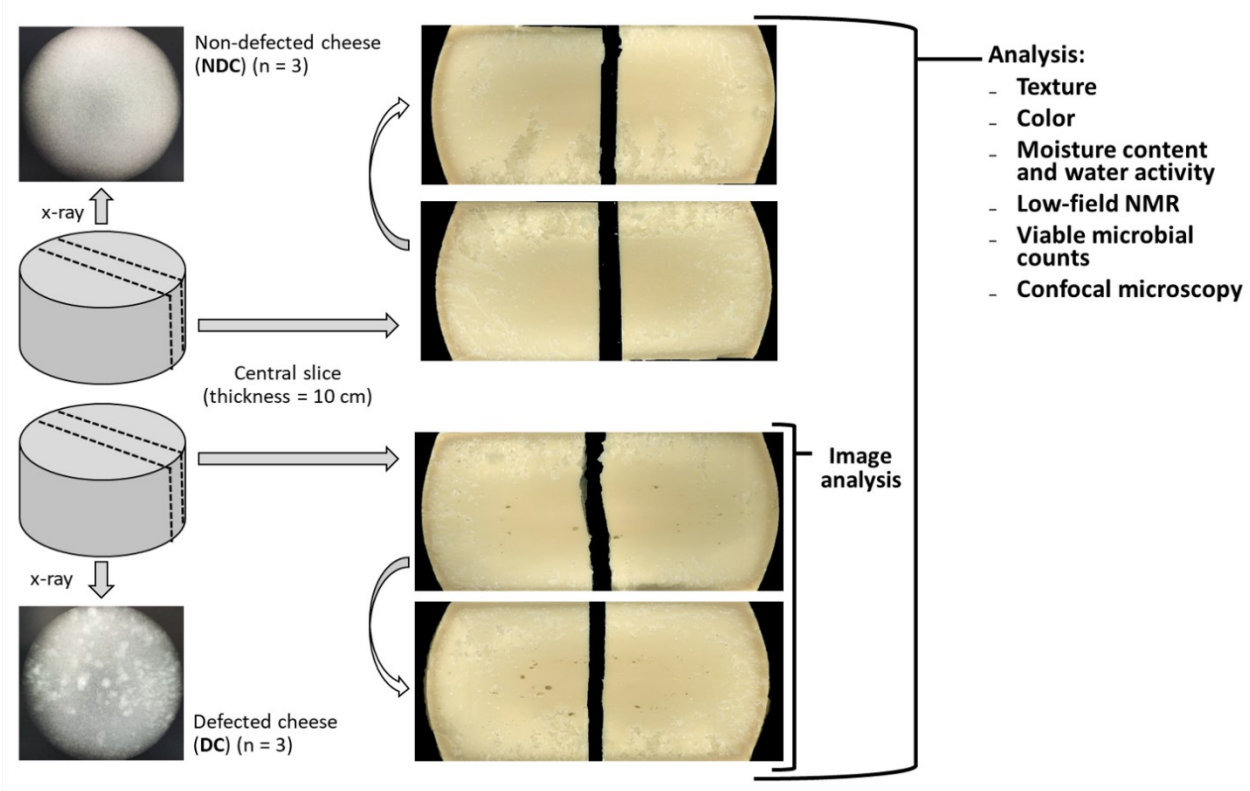
[b] Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy

[c] SITEIA.PARMA Interdepartmental Centre, University of Parma, Parma, Italy

[1] These authors contributed equally to the work

* Corresponding author

Keywords: hard cheese, cheese defects, cheese ripening, centrifugation, raw milk



Abstract

The present study investigated some physico-chemical and microbiological properties of 20 months ripened hard cheeses produced from low temperature high-speed centrifuged raw milk that developed a structural defect of eyes/slits opening in the paste. These cheeses were compared with controls that did not develop the defect. Color, texture, moisture, water activity, molecular structure, microstructure, extend of proteolysis of cheeses and microorganisms viable in all samples have been evaluated in triplicate and the significant differences between defected and not defected cheeses have been critically discussed.

At the microstructural level this caused fat coalescence and unevenly organized protein with small cracks in proximity to the openings. The different fat organization was correlated to a different transverse relaxation time of population protons at higher ms.

Textural and colour features were not different from the control cheese produced with the same technology but without defects, and the values were comparable with those of other long ripened hard cheeses found in literature. On the other hand, the defected cheese showed higher moisture %, lower lactobacilli and total mesophilic bacteria concentration, but the microbial origin of the defect remains an unlikely hypothesis that deserves to be further investigated.

5.1 Introduction

Visual appearance and texture are important factors to determine the quality and the consumer acceptance of cheeses (Clydesdale, 1993; Nestle et al., 1998; Spence, 2019). The occurrence of openings in the cheese structure can have physico-chemical and/or microbiological origin but also milk treatment as well as manufacturing may have a role. The presence of openings represents a typical feature of certain cheese types, such as the holes in Roquefort caused by unpressed curd, or the so called “eyes” in the Dutch and Swiss type cheeses formed as consequence of gas production by bacteria (Fox et al., 2017). The size, number, shape, and distribution of eyes are extremely important quality parameters, especially in Swiss-type cheese, where the eye is mainly the result of propionic acid fermentation involving the conversion of lactate into propionate, acetate, and CO2 during warm room storage (Guggisberg et al., 2015). On the other hand, holes or eyes represent a defect in blind cheeses such as Italian hard cheeses where the texture is compact and homogeneous without any kind of opening thanks to a strong cohesion among curd granules ensured by the vat cooking phase as well as the absence of late fermentations during ripening.

Early and late blowing are the most common structural defects of microbial origin in cheese and can represent an important economic cost for the dairies. While the first occurs within a few days after production and it is caused by coliform bacteria (Tabla et al., 2022), late gas formation arises after few months and it is a consequence of lactate and/or citrate fermentation. Late blowing is a frequent problem of hard and semi-hard ripened cheese, that generally also causes unpleasant flavour. It is mainly caused by Clostridium tyrobutyrricum, but other Clostridium species such as C. sporogenes, C. beijerinckii and C. butyricum were also found to be responsible for the problem (Julien et al., 2008; Vissers et al., 2007). The main sources of contamination are thought to be silage and unhygienic animal bedding (Langó & Heinonen-Tanski, 1995). The occurrence of this defect results in a downgrading of the final cheese with a consequent economic loss for the producers. Different

methods have been proposed to prevent the late blowing defects, such as addition of nitrate or lysozyme (Ávila et al., 2014), addition of protective cultures containing lactic acid bacteria strains biologically active against gram-positive bacteria (Gómez-Torres et al., 2014; Martínez-Cuesta et al., 2001; Silva et al., 2018), and centrifugation (also referred as "bactofugation") or microfiltration of milk (Elwell & Barbano, 2006).

In a previous work, (D'Incecco, Bancalari, et al., 2020) demonstrated how low-temperature high-speed (LTHS) centrifugation effectively reduced the clostridia spores in raw milk used for hard cheese production. Despite of this, some of the cheeses produced with the same LTHS centrifuged milk presented openings at the end of the ripening period. Although the structural defect does not necessarily strongly affect the taste and flavour of the ripened product, the structural appearance of the cheese does not comply with the consumers' expectation of hard cheese types commonly characterized by a compact structure. As consequence, in function of their characteristics, these defected cheeses can follow a different utilization, such as for grated, processed, or powdered cheese production. For this reason, it is of great interest deepen the knowledge on the characteristics of this kind of defected cheeses.

The aim of this study was to analyze some physico-chemical and microbiological characteristics of hard cheese produced with centrifuged milk that presented a paste structural defect at the end of the ripening (20 months). These cheeses were compared with controls that did not develop the defect. Furthermore, this research provided insight to suggest hypotheses in the explanation of the origin of the described cheese defect.

5.2 Material and methods

5.2.1 Cheese production and sampling

The raw bulk milk was centrifuged as described by (D'Incecco, Bancalari, et al., 2020) in the configuration 1. Briefly, after the fat separation by natural creaming at 8–12 °C for ~10 h, partly skimmed milk (fat 2.2 g/100 mL) was submitted to single centrifugation adopting one-phase centrifuge CSI-23001-772 Westfalia (Germany) operating at 39 °C and flow rate 21,000 L/ h. After centrifugation, milk was held in a degassing tank at ~13 °C for 4 h and then transferred to the vat. Hard cheeses were produced using only this low temperature high-speed (LTHS) centrifuged raw milk following the methodology described by (D'Incecco et al., 2018). After 20 months of ripening, three cheeses, produced in three different days, with structural defects (henceforth shorten DC, defected cheese) verified by x-ray were selected for the analysis. Three additional cheeses, produced in the same three days but in different vats, without the defect (henceforth shorten NDC, non-defected cheese) were analyzed as control. A vertical section slice from the center of the cheese wheel (thickness = 10 cm) was sampled and transported to the laboratory for the analysis under vacuum and refrigerated condition.

5.2.2 Image analysis

The image analysis was carried out only for the DC samples. Images of the two faces of the sample slice were acquired using a Hewlett Packard Scanjet 8200 scanner (Palo Alto, CA, USA) with a resolution of 600 dpi (corresponding to 236 pixels cm−1) and saved in TIFF format. A black

background was used to enhance the contrast of acquired images. Images were processed as described by (Bettera et al., 2020).

To evaluate the openings size distribution, minimum, maximum, mean, and the 25th, 50th and 75th percentiles (D25, D50 and D75) values of the opening area (mm2) are reported. The cheese porosity (%) was also calculated according to the following Equation (1):

$$Porosity\ \% = \frac{\sum openings\ area\ (mm^2)}{cheese\ section\ area\ (mm^2)} \times 100 \quad (1)$$

Based on the eccentricity (e) parameter calculated by the image analysis software, openings were classified as eyes ($e < 0.9$) or cracks/slits ($e \geq 0.9$).

5.2.3 Cheese physico-chemical analysis

5.2.3.1 Texture analysis

The analysis of the DC and NDC samples paste texture was performed by means of a TA.XTplus Texture Analyzer (Stable Micro Systems, Godalming, UK) equipped with a 30 kg load cell and a 3 mm diameter stainless steel cylindrical probe (SMS P/3, Stable Micro Systems) according to a previously described penetration test (Alinovi et al., 2019). Young's modulus (MPa), stress (MPa) and strain (-) at fracture were derived from true strain (ε) and true stress (δ) parameters, as calculated according to (Hort et al., 1997; Hort & Le Grys, 2001) (Equations (2) and (3)):

$$\varepsilon = ln\ (h_0/h_0 - \Delta h) \quad (2)$$

$$\delta(t) = F(t)/A(t) \quad (3)$$

where h0 is the original height (m), Δh represents the change in height of the sample (m), F(t) is the force at time (t), and A(t) is the surface area at time (t).

Colorimetric characteristics

The DC and NDC samples color was measured using a Minolta Colorimeter (CM 2600d, Minolta Co., Osaka, Japan) equipped with a standard illuminant D65. CIELAB color space was considered, and the parameters L* (lightness, black = 0, white = 100), a* (redness > 0, greenness < 0), b* (yellowness > 0, blue < 0) were determined. Nine replicated measurements were performed through the whole sample surface in order to have a representative value of the cheese paste.

5.2.3.2 Moisture content and water activity

Moisture content of DC and NDC samples was measured by oven-drying samples at 102 ◦C (AOAC, 1990) until a constant weight was reached. Data are expressed as moisture % (w/w).

Water activity (aw) of cheese samples was measured at 25 °C using an AquaLab Water Activity Meter Series 3TE with internal temperature control (Decagon Devices, Inc., Pullman, WA, USA). Before the analyses, the instrument was calibrated with saturated salt solutions and distilled water in the aw range of 0.846–1.000.

5.2.4 Low resolution 1H NMR

NMR analyses of DC and NDC samples were performed at 25.0 ± 0.1 °C using a low resolution 1H NMR spectrometer (the Minispec, Bruker, Massachusetts, USA, frequency: 20 MHz, magnetic field strength: 0.47 T). Cheese paste was sampled using a cork borer and transferred into NMR tubes

(outer diameter of 10 mm) that were filled up to 10 mm height; to avoid moisture loss during the analysis, the tube was sealed with laboratory film. 1H T2 spin–spin relaxation curves were measured with a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence by performing twelve scans for each replication, with a RD of 3.5 s (> 5 1H T1), an interpulse spacing (τ) of 80 μs and 30,000 data points. 1H T2 relaxation curves were analyzed as quasi-continuous distributions of relaxation times using UPENWin software (Alma Mater Studiorum, Bologna, Italy) and by applying multiexponential models using Sigmaplot, v.10 (Systat Software Inc., USA) as previously reported (Alinovi et al. 2022; Alinovi et al., 2020).

Each sample was analyzed in sextuplicate.

5.2.5 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) was applied to study the microstructure of DC and NDC. Sampling of DC and NDC portions was carried out as previously performed by D'Incecco et al., (2020b) with some modifications. Three sections of cheese of around 2 mm × 2 mm × 1 mm were cut using a razor blade from each cheese slice at 5-cm depth from the rind in NDC while also a sampling close to the defect was performed for DC. Cheese sections were stained within embryo dishes (Electron Microscopy Sciences, Hatfield, PA, USA) using Nile Red (Sigma Aldrich, St Louis, MO, USA) to visualize fat and Fast Green FCF (Sigma Aldrich) to visualize protein. Just prior to staining, both stock solutions of Nile Red (1 mg/mL in dimethyl sulfoxide) and Fast Green (1 mg/mL in Millipore MilliQ purified water) were tenfold diluted in water. Samples were analyzed using an inverted confocal laser scanning microscope A1+ (Nikon, Minato, Japan). Nile red was excited at 488 nm and emission was collected at 520-590 nm. Fast Green was excited at 638 nm and emission was collected at 660-740 nm.

5.2.6 Capillary zone electrophoresis (CZE)

The extent of DC and NDC samples proteolysis was investigated through capillary zone electrophoresis (CZE) that allows the separation of intact casein fraction as well as major peptides adopting the conditions described by D'Incecco et al. (2020). Briefly, samples were prepared by dissolving 1 g of grated cheese in 10 mL of DDT buffer (pH 8.6) at room temperature for 4 hours. Solubilized samples were further diluted 1:5 with the same buffer and then filtered using 0.22 μm membrane filter (Millipore) before analysis. The separation was carried out at 45°C using a Beckman P/ACE System MDQplus, equipped with a 50 cm fused silica column (DB-WAX 126-7012, Agilent Technologies, Milan, Italy). Detection was carried out at 214 nm and the corrected peak areas, calculated as peak area/migration time, were used to calculate ratios between peptide fragments and parent casein fractions (D'Incecco et al., 2020).

5.2.7 Microbiological analysis

To evaluate the viable microorganisms in the DC and NDC samples, different cultural media were used. Each DC and NDC sample were grated, and 10 g were suspended in 90 mL of 20 gL-1 trisodium citrate (pH 7.5) (Sigma–Aldrich, St. Louis, USA) and homogenized for 2 min in a blender at 230 rpm (Seward, London, United Kingdom). Decimal dilutions were made in quarter-strength Ringer solution (Oxoid, Basingstoke, United Kingdom). Samples were plate cultured in duplicate for the count of different microbial groups: mesophilic lactobacilli, on acidified MRS agar (Oxoid)

(pH = 5.4, reached by acetic acid addition) and incubated in anaerobic condition with a gas pack (Fisher Scientific, Rodano, Italy) at 30°C for 48 h; the total mesophilic count, on Milk Plate Agar (Oxoid) in aerobic condition (at 30 °C for 48 h; yeasts and molds, on Yeast extract dextrose chloramphenicol agar (YEDC, Oxoid) in aerobic condition at 30 °C for 5 d; propionibacteria (PAB), according to (Carini & Casadei, 1970) using P2 agar (containing peptone 5 g; beef extract, 3 g; yeast extract, 5 g; sodium lactate, 1 g; agar 15 g L−1) in anaerobic condition at 30 °C for 7 d; coliforms, on Violet Red Bile Agar (Oxoid) in aerobic condition at 37°C for 24 h. For the enumeration of sporeforming bacteria, the homogenized samples were heated to 80 °C for 10 min prior being cultured. The growth media used was tryptone soy agar (TSA, Merck KGaA, Darmstadt, Germany), incubated at 30°C for 72h: for aerobic sporeforming bacteria, the sample was plated on the surface, while for the anaerobic ones it was mixed with the liquified TSA and then solidified.

5.2.8 Statistical analysis

The three DC and the three NDC samples were analyzed in triplicates. Results are reported in the article as mean ± standard deviation. To test significant differences between samples (α=0.05), a Welch's t-test when DC and NDC results showed a heterogeneous variance to the F-test; when the variance was instead homogeneous, a Student's t-test was applied. Statistical evaluations were done using the "stats" package in the R environment (R Core Team, 2022).

5.3 Results and discussion

5.3.1 Cheese openings

The results of the image analysis conducted to characterize the cheese structure defect are reported in **Table 1**. The openings caused a porosity of 0.26 ± 0.10 % in the DC samples. The largest holes found measured on average 36.45 ± 1.10 mm2, while the smallest 0.39 ± 0.03 mm2. The mean size of the opening was 3.48 ± 1.83 mm2, while the D50 was equal to 1.31 ± 0.25 mm2, indicating that the opening size follow a right-skewed distribution. The 74.86 % of the openings were oval-/round-shaped and as consequence identified as eyes, while the remaining were cracks/slits. The cheese porosity (0.26 ± 0.10%) was in general lower than the data reported by Bettera et al., (2020), who characterized raw milk, PDO [protected designation of origin; (EP & Council of EU, 2012)] hard cheeses made with artisanal cheesemaking practices (Nostrano Valtrompia PDO, with a porosity ranging between 0.0% and 10.6%, with a mean of 2.0 ± 2.5%), and Innocente & Corradini, 1998 who evaluated Montasio PDO cheese (porosity ranging between 3.1% and 18.3%). On the contrary, similar porosity values were encountered in Pecorino cheese (~0.2%) (Rinaldi et al., 2010).

5.3.2 Cheese physico-chemical characteristics

5.3.2.1 Cheese color and texture

No significant differences were detected in the texture and the color between the DC and NDC (**Figure 1A, B**). The lightness (L*) of the two cheese types showed an average value ≈75.5, while the a* and b* parameters values ≈1.5 and ≈17.5 respectively. The results were consistent with other long-ripened hard cheeses such as Parmigiano Reggiano PDO (Romani et al., 2002; V. Sherveglieri et al., 2017), Montasio (Aprea et al., 2016), Nostrano Valtrompia PDO (Bettera et al., 2020), and Asiago PDO (Cozzi et al., 2009; Marchesini et al., 2009). Also the texture parameters of stress and

strain at fracture were comparable with hard cheeses ripened for 18 (Noël et al., 1996) or 16 months (Bettera et al., 2020), although our cheeses were harder showing higher values of Young's modulus equal to 7.87 ± 1.72 MPa and 7.55 ± 0.28 MPa respectively for NDC and DC.

5.3.2.2 Cheese moisture and water activity

The values of moisture (%) and water activity of DC and NDC are reported in **Figure 1C**. While the water activity was similar between the two samples with an average value ≈ 0.091, the moisture content showed higher values in DC compared to NDC (32.19 ± 0.28 % and 31.57 ± 0.3 %, respectively), although this difference did not result significant at the statistical test, despite being at the limit of significance ($p = 0.059$). Both the moisture and the water activity values were consistent with those found for other hard cheeses with a similar ripening time (D'Incecco, Limbo, et al., 2020; Pellegrino et al., 1997).

5.3.3 Cheese microstructure

Microstructure of cheeses was investigated by confocal laser scanning microscopy (CLSM). Differences were observed between DC and NDC at the expense of both fat and protein components. Fat was mostly organized as irregular globular-shaped areas of partially coalesced fat globules within a continuous protein network in NDC. This organization is in accordance with previous observations of cheese microstructure in long ripened hard cheese (D'Incecco, Limbo, et al., 2020). Differently, fat fully coalesced in DC and fat matrix showed to be damaged and very irregular, often as free fat in the proximity of the defect, especially at the inner surface of the eye (**Figure 2**). Protein appeared unevenly organized with small cracks, few microns in size, that were frequently filled by free fat. The formation of cracks and slits in cheeses with inelastic structure is reported to be direct consequence of high overpressure of CO2 that leads to a spontaneous formation of cracks in weak zone of the cheese (Guggisberg et al., 2015). The microstructure of DC in cheese areas not affected by defects were comparable to that of NDC.

5.3.4 Cheese proteolysis

The extent of primary proteolysis was evaluated by CZE separating intact caseins as well as fragments originating from their proteolysis operated by chymosin or indigenous milk enzymes. The ratios between peak areas of major fragments and the relative parent caseins were calculated. Specifically, the ratios αs1-I/αs1, αs(f1-23)/αs(1+0), ɣ/β and αs1-PL/αs1-CN were considered to monitor the proteolysis (**Table 2**). No significant differences were found between ratios in DC and NDC suggesting an equivalent enzymatic activity in both cheeses. Specifically, the αs1-I and αs(f1-23) fragments result from the activity of rennet chymosin, because it cleaves, besides k-casein, also the αs1-CN at Phe23-Phe24 bond, splitting the protein chain into two fragments, i.e., αs-CN f(1-23) and f(24-199), also called αs1-I-CN. A progressive decrease of αs1-I/αs1-CN ratio is expected during ripening as effect of faster degradation of αs1-I-CN fragments than αs1-CN (D'Incecco, Limbo, et al., 2020). Differently, β-casein is known to be primary degraded into ɣ-caseins by plasmin, especially in cooked cheeses (Vélez et al., 2015). These peptides are instead quite stable and thus the ɣ/β casein ratio increases during ripening (McSweeney, 2004). The activity of plasmin is also exerted towards the αs1-casein. However, even the αs1-PL/αs1-CN ratio did not show differences between cheeses. It is known that proteolysis extent is very important for the development of a define texture and flavour in ripened cheese. In fact, different proteolysis

pathways may stimulate growth of some microbial population instead of others, according to a different availability of small peptides and free amino acids used a growth substrate (Fröhlich-Wyder et al., 2002). However, this cannot be the case due to the equivalent casein degradation observed in DC respect to NDC.

5.3.5 Low resolution 1H NMR analyses

The results of low resolution 1H T2 NMR analyses (**Table 3**) showed the presence of four 1H populations. As it can be observed from a representative **Figure 3**, the T2 relaxation distribution showed quite resolved proton populations. This observation was consistent with previous studies that performed low field 1H T2 NMR analyses on long ripened, Italian hard cheeses (Bordoni et al., 2011; De Angelis Curtis et al., 2000).

In particular, population A (T2 ~1 ms) has been previously attributed to protons of water molecules tightly bound to the macromolecules or to the protons belonging to the most rigid casein portions (inter-micellar water fraction), population B (T2 ~10 ms) to water in exchange with the casein surface (intra-micellar water fraction) or to the protons belonging to the most flexible regions of casein (Bordoni et al., 2011; De Angelis Curtis et al., 2000). Bordoni et al. (2011) attributed populations C (T2 ~50 ms) and D (T2 ~170 ms) to the solid and liquid lipid fractions, respectively. However, it is difficult to support the attribution of pop. C to solid fat, as the value of T2 for proton in the solid fat phase (that can be expected of the order of μs) cannot be observed on the timescale of CPMG experiments (Chen et al., 2020; Song, 2009; Vermeir et al., 2019). De Angelis Curtis et al. (2000) attributed these two proton populations to the saturated (pop. C) and unsaturated (pop. D) lipid fractions; Mulas et al., (2016), who studied Grana Padano PDO cheese by performing MRI analyses, attributed pop. C both to a protons fraction of water molecules exhibiting less interaction within the protein matrix and to a fraction of liquid fat.

Significant differences ($P<0.05$) between NDC and DC samples were observed in the case of T2D and T2B: NDC showed shorter transverse relaxation times of pop. B and pop. D compared to DC samples. In particular, the different organization of the fat domain observed with CLSM analyses can be related to the different transverse relaxation time of pop. D (~170 ms for NDC vs. ~180 ms for DC), as an increase of T2D indicate a greater conformational freedom of the fat protons, that can be the consequence of a coarser and more irregular distribution of fat globules in the matrix of DC rather than NDC (Noronha et al., 2008) and of the formation of free fat in the proximity of the defects. The lower T2B reported in the case of NDC compared to DC may be also indicative of a different structural organization of the protein matrix, characterized by higher capacity of reducing the molecular mobility of the interstitial water fraction.

5.3.6 Microbiological analysis

The concentration of viable microorganisms found in the 20 months ripened cheeses are reported in **Figure 4**. Except for yeasts/molds, the DC had a more variable microbiological profile between the replicates as shown by the wider boxplots. The DC and NDC had a similar concentration of yeasts/molds and PAB; the latter was the microbial group that showed the highest variability between the three replicates. Differences were instead noticed in the lactobacilli and total mesophilic counts. The latter was significantly higher in the NDC (log CFU/mL = 4.86 ± 0.14, against the average value of 3.87 ± 0.41 of DC). NDC showed also a higher concentration of

lactobacilli, with a p-value = 0.063 slightly above the significance level (α = 0.05). D'Incecco, Bancalari, et al., (2020) discussed the influence of the LTHS centrifuagtion in modify the LAB composition in milk. In particular, they observed a selective effect of the centrifugation in reducing rod-shaped LAB, indicating the possibility to selectively unbalance the LAB species abundance. This may have led to a different interaction between PAB and LAB resulting in the stimulation of the propionic pathways, as it was demonstrated to occur during the cheese ripening by Fröhlich-Wyder et al., (2002). In fact, these authors found that the absence of facultatively heterofermentative lactobacilli have made PAB able to produce excessive CO2 with consequent formation of larger eyes. This finding could explain our results where the DC with eyes were associated with lower counts of lactobacilli. Facultatively heterofermentative non-starter LAB such as Lacticaseibacillus casei and Lacticaseibacillus rhamnosus are in fact know to slow down propionic acid fermentation, while thermophilic starter species such as Lactobacillus helveticus may stimulate late fermentation representing a risk of structural defects of eyes in the ripened cheese, or slits in case of less elastic paste of our hard cheeses. The interaction between lactic acid bacteria and PAB is known to be the key to improve and control the structural quality of hard cheeses, including the control of CO2 produced (Karjean et al., 2000). In our experimental research conducted with three replicates of three different cheesemaking on three different days, in the 20 months cheeses the concentration of PAB was highly variable. In particular, in one of the three samples the PAB concentration was much lower than the more constant average value of the NDCs (**Figure 4**). Although the significance of the difference cannot confirm what was previously observed, this hypothesis remains open as a possible cause of the defect (Fröhlich-Wyder et al., 2002). Thus, the microbiological origin of the defect remains an unlike hypothesis that deserves to be further investigated by following the process throughout the long cheese ripening time and taking cheese samples at different moment to allow the evaluation of the vital microbial cells also through the level of gene expression of different metabolites.

The formation of structural defects in the form of eyes or openings can also have a non-microbiological origin. Guggisberg et al., (2015) showed that the formation of eye defects in cheese is particularly difficult to control especially when centrifuged or microfiltered milk is used. Auer et al., (2021) and O'Sullivan et al., (2016) also mentioned that the entrapment of air during molding of the curd could be responsible for the formation of eye defects. In the case of DC we can also speculate that excessive air entrapment took place during LTHS centrifugation of milk followed by insufficient degassing phase.

5.4 Conclusions

Long ripened hard cheeses are characterized by a compact paste structure. For many factors related to the production technology, the cheese can develop structural defects of physico-chemical and/or microbial origin. Improper structure appearance leads to degradation of cheese, even if the taste is not necessarily affected. Late blowing is a known common defect of hard cheeses that can be avoided using milk centrifugation. Despite the use of low temperature high-speed centrifugation, we experienced the production of cheese with a structural defect apparently not directly and solely of microbial origin.

The present study investigated some physico-chemical and microbiological properties of 20 months ripened hard cheeses produced from bactofugated raw milk that developed a structural defect of eyes/slits opening in the paste. At the microstructural level this caused fat coalescence and unevenly organized protein with small cracks in proximity of the openings. The different fat organization was correlated to a different transverse relaxation time of population protons at higher ms.

Textural and color features were not different from the control cheese produced with the same technology but without defects, and the values were comparable with those of other long ripened hard cheeses found in literature. The defected cheese showed higher moisture % and lower lactobacilli and total mesophilic bacteria concentration, but the microbial origin of the defect remains an unlikely hypothesis that deserves to be further investigated. These results not only offer new insight in the description and consequent prevention of hard cheese defects, but demonstrate that different cheese properties were not altered by the defect, providing in this way useful information about the possible use of the defected cheeses.

5.5 Tables

Table 1. Size frequency distribution percentiles (D25, D50, D75), mean, maximum and minimum size of the defected cheese openings measured with image analysis.

Parameter		**Defected cheese (n=3)**	
		Mean	St. dev.
Opening area (mm^2)	Min.	0.39	0.03
	D25	0.71	0.13
	D50	1.31	0.25
	D75	3.48	1.83
	Max.	36.45	12.37
	Mean	3.92	1.10
Porosity (%)		0.26	0.10

Table 2. Casein fraction ratios in defected (DC) and non-defected (NDC) cheeses at 20 months of ripening.

Cheese	**αs1-I/αs1**	**αs(f1-23)I/αs$_{(1+0)}$**	**ɣ/β**	**αs1-PL/αs1**
NDC	0.38 ± 0.04	0.19 ± 0.05	4.38 ± 1.45	0.68 ± 0.20
DC	0.29 ± 0.12	0.13 ± 0.01	3.55 ± 0.58	0.56 ± 0.06
Sign.	n.s.	n.s.	n.s.	n.s.

The results are expressed as the mean ± standard deviation. N.s. = not significant ($p>0.05$)

Table 3. Relative abundance (%) and 1H T2 relaxation time (ms) of the four proton populations (population A, B, C, D) measured by low resolution NMR relaxometry for non-defected cheeses (NDC) and defected cheeses (DC).

Cheese	pop. A (%)	pop. B (%)	pop. C (%)	pop. D (%)	T_{2A} (ms)	T_{2B} (ms)	T_{2C} (ms)	T_{2D} (ms)
NDC	5.44 ± <0.01	55.06 ± 0.08	23.58 ± 0.05	15.92 ± 0.04	1.55 ± 0.12	10.78 ± 0.05	51.10 ± 1.37	170.79 ± 5.68
DC	5.32 ± 0.08	54.55 ± 1.01	23.71 ± 0.79	16.42 ± 0.48	1.62 ± 0.19	11.13 ± 0.19	54.07 ± 2.16	180.54 ± 1.79
Sign. (*p<0.05)	n.s. (p=0.065)	n.s.	n.s.	n.s.	n.s.	*	n.s.	*

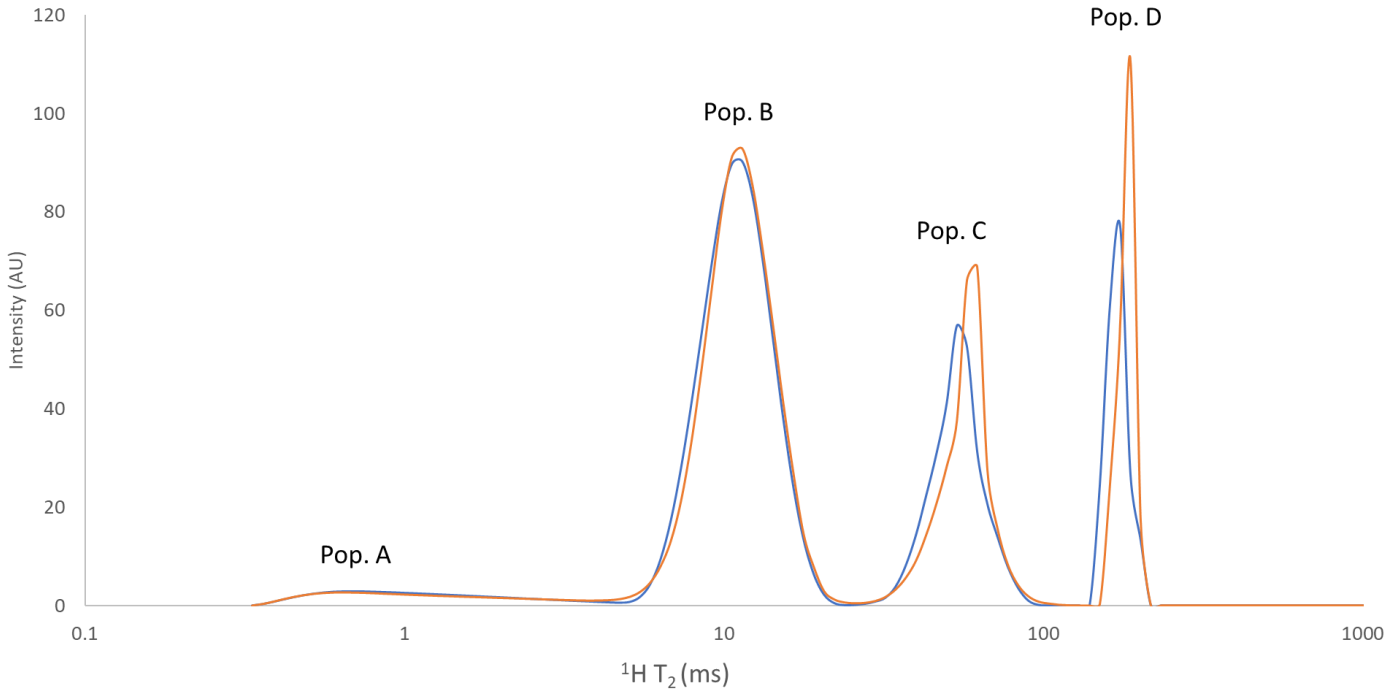


Figure 3. Representative 1H T2 relaxation distribution of measured by CPMG sequence of DC (orange line) and NDC (blue line) samples.

5.6 Figures

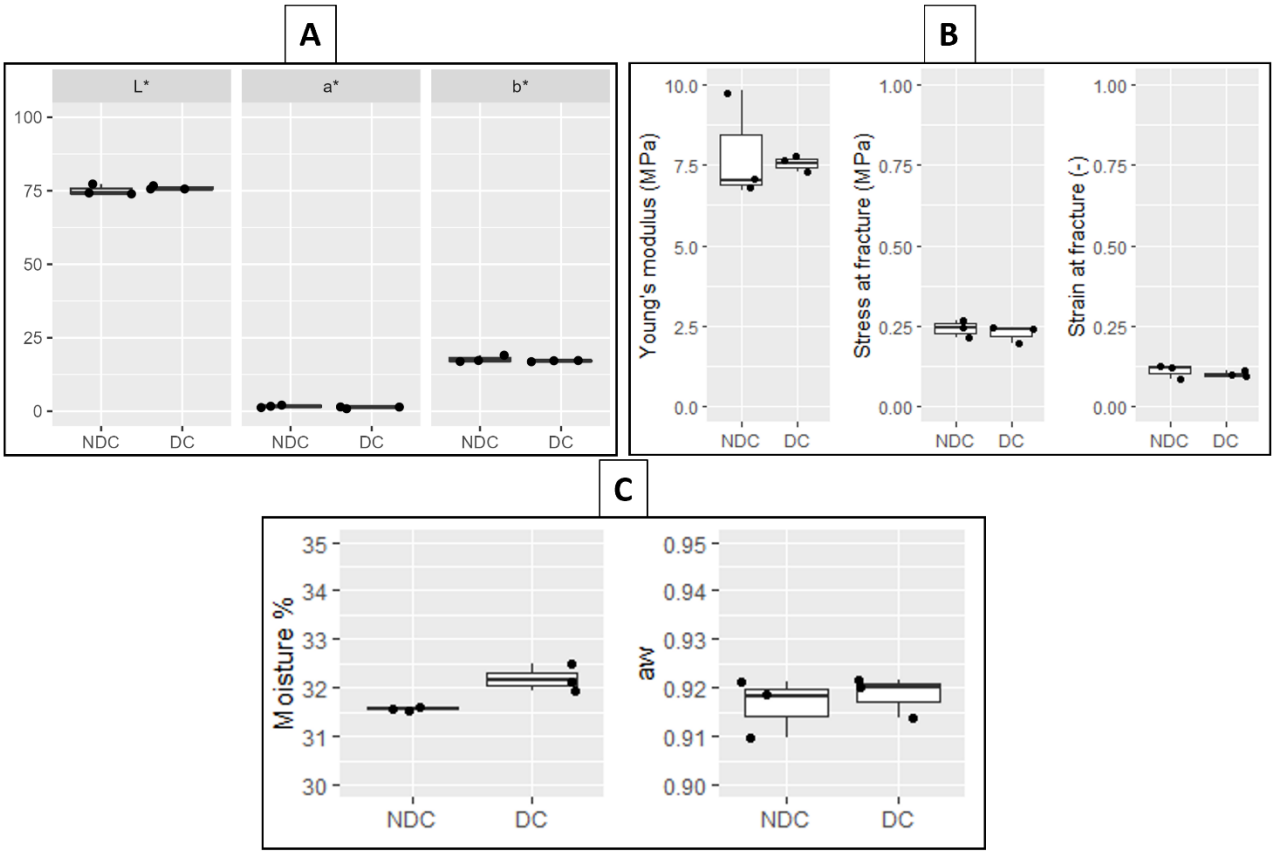


Figure 1. Physico-chemical characteristic of 20 months ripened non-defected cheese (NDC) and defected cheese (DC): color (panel A), texture (panel B), moisture (%) and water activity (a_w) (panel C).

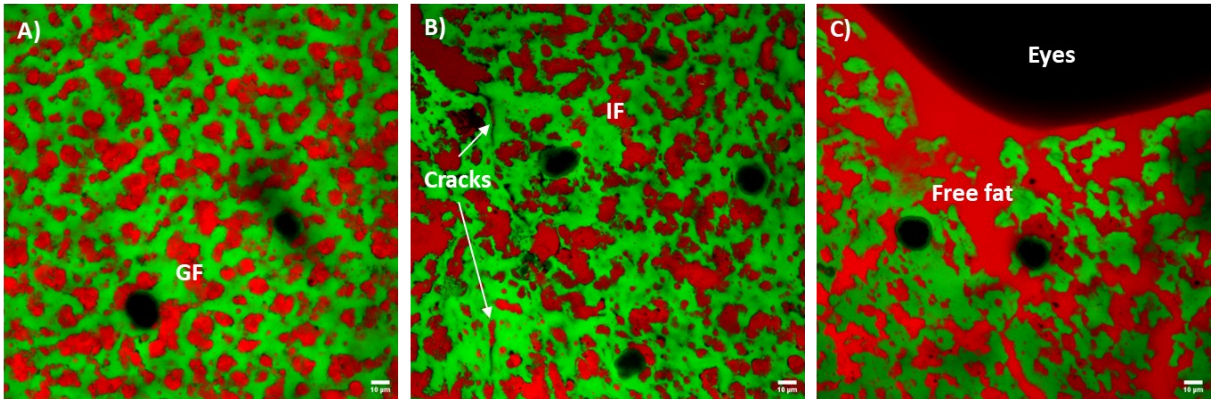


Figure 2. Confocal laser scanning microscopy of non-defected (A) and defected (B, C) cheeses. Fat was organized as globular fat (GF) in non-defected cheese while irregular fat pools (IR) were visible in defected cheese. Cracks of the protein matrix were observed in defected cheese only.

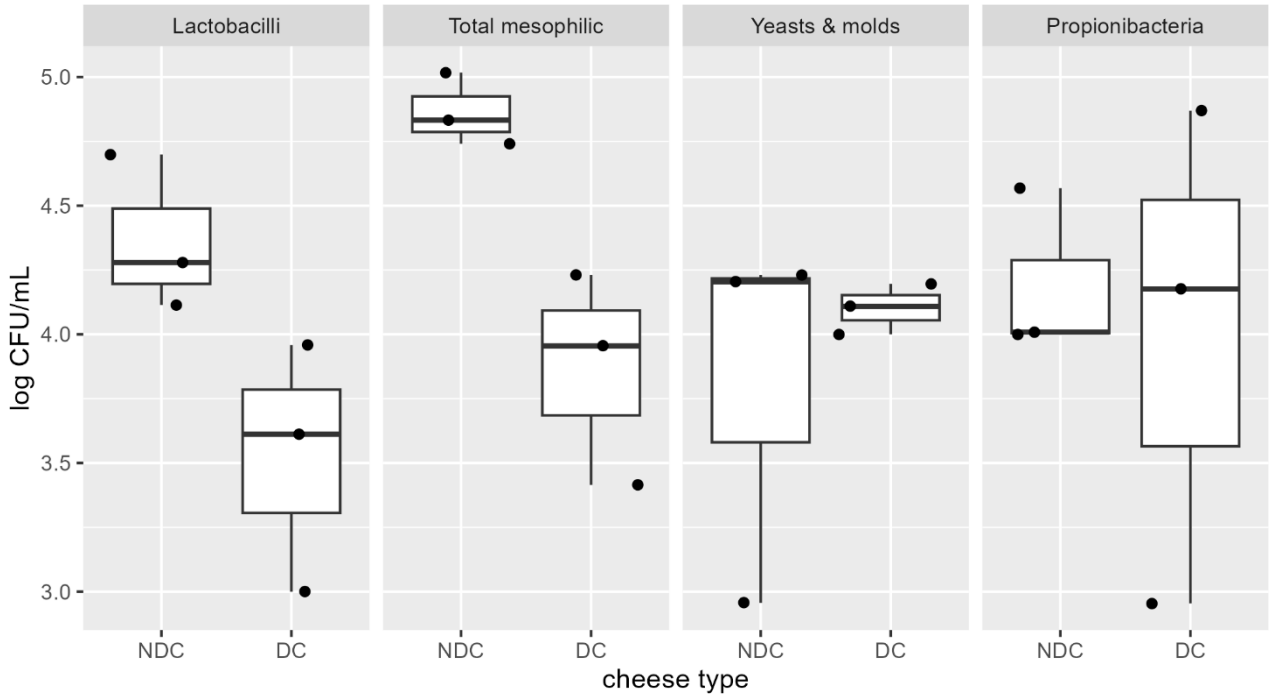


Figure 4. Concentration (log CFU/mL) of the microbial population found in 20 months ripened non-defected cheese (NDC) and defected cheese (DC).

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6 Conclusions and perspectives

The object of the present thesis was to do a step forward over what is known about the non-starter lactic acid bacteria (NSLAB), studying their abundance in raw milk, development dynamics in ripened cheese, and genotypic, phenotypic and metabolic traits at the strain level. The knowledge of this information is fundamental for the control of the interested NSLAB present in the complex raw milk microbiota and as consequence govern their effect on the cheese's final characteristics.

The meta-analysis conducted during the first part of this PhD project was very effective to highlight the complexity of the microbiota of cow raw milk used for cheese production which is composed of over 45 phyla and showed how the genera belonging to LAB are not the most abundant in this microbial ecosystem. It also indicated how LAB belonging to *Lactobacillus* and *Lacticaseibacillus* genera are present in lower relative abundance in comparison with *Lactococcus* and *Streptococcus*. This result is consistent when compared with LAB concentration evaluated by means of plate counts on agar media: lactobacilli were in fact found on average in lower concentration than coccoid LAB in the raw milk used for the production of 24 different cheese types.

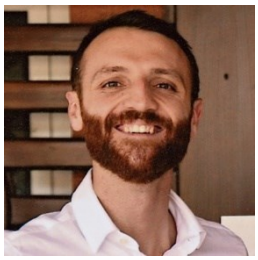
Based on the currently available knowledge about the low abundance of NSLAB in raw milk, the experimental part of this thesis followed an approach aimed at favoring NSLAB growth in raw milk in order to study their traits and exploit them in the form of selected strains and natural adjunct culture. The results achieved lay the foundation for a better exploitation of the raw milk microbiota, contributing to the possibility of predicting more precisely the final cheese characteristics.

More than 120 NSLAB strains have been isolated from the raw milk of 20 different dairies. A screening process selected the best strains potentially applicable as adjunct cultures in cooked hard cheese production, i.e. characterized by good resistance to salt and lysozyme, good recovery to heat stress and ability to produce volatile compounds typical of cheese aroma. Cheese making trials will be necessary to test the effectiveness of the adjunct strains screened in influencing the cheese aroma and monitor the absence of interference with the starter LAB acidification activity.

The possibility of positively influencing the cheese characteristic controlling the raw milk microbiota was also investigated by developing a natural adjunct milk culture. Our results showed that the applied raw milk enrichment protocols were able to increase the concentration of autochthonous LAB. This allowed the production of natural adjunct cultures harboring diverse microbiota. Chemical and microbiological results suggested that this microbial diversity influenced the early stages of cheese making, but its effect decreased over time during ripening, showing an inferior effect than that of raw milk microbiota. More research is needed to optimize the culture production, including testing different treatments that could more specifically select desired NSLAB present in raw milk and validating the reproducibility of the method by monitoring the microbial diversity during the backslopping process. The optimization of such a tool could be an alternative to the practice of isolating, geno-pheno-typing, and formulating mixed-defined-strain adjunct cultures that require knowledge and facilities not always available for artisanal cheese makers.

Finally, the investigation on hard cheeses produced from centrifugated raw milk gave new insight into the description and consequent prevention of structural defects possibly correlated with the NSLAB abundance unbalancing within the cheese microbiota.

7 Short Educational CV



7.1 Education

2008 – 2013: Professional agricultural high school diploma (98/100), “Giardino” V. Dandolo, Località Giardino, 25030 Orzivecchi, BS, Italy

2013 – 2016: BSc Food Science and Technology (104/110), Department of Food and Drug, University of Parma, Viale delle scienze, 49/a , 43124 Parma , Italy

2016 – 2018: MSc Food Science and Technology, (110/110), Department of Food and Drug, University of Parma, Viale delle scienze, 49/a ,43124, Italy

Jan – Jun 2019: Post graduate researcher, University of Copenhagen, Rolighedsvej 26, DK-1958 Frederiksberg, Denmark

Jan – Jun 2022: Visiting PhD student, Agroscope, Schwarzenburgstrasse 161, 3003 Bern, Switzerland

7.2 List of publications

Bettera, L., Alinovi, M., Mondinelli, R. and Mucchetti, G., 2020. Ripening of nostrano valtrompia PDO cheese in different storage conditions: Influence on chemical, physical and sensory properties. Foods, 9(8), p.1101. https://doi.org/10.3390/foods9081101

da Silva, D.F., **Bettera, L**., Ipsen, R. and Hougaard, A.B., 2021. Cheese powders as emulsifier in mayonnaise. LWT, 151, p.112188. https://doi.org/10.1016/j.lwt.2021.112188

Bettera, L., Levante, A., Bancalari, E., Bottari, B., and Gatti, M. (2023). Lactic acid bacteria in cow raw milk for cheese production: Which and how many? Front. Microbiol. 13, 1092224. doi:10.3389/fmicb.2022.1092224.

7.3 List of pubblication under review

Bettera, L.*, Dreier, M., Schmidt, R. S., Gatti, M., Berthoud-dit-Gallon Marchand, H. and Bachmann, H. Selective enrichment of the raw milk microbiota in cheese production: concept of a natural adjunct milk culture. Submitted to *Frontiers in Microbiology*.

* Publication presented as corresponding author

7.4 List of presentations

7.4.1 Oral presentations

Bettera, L. Non-starter Lactic Acid Bacteria: origin and characterization for a potential targeted use in cheesemaking. September 2022, 26th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Asti, Italy.

Flash talk: Bettera, L. Selective enrichment of the raw milk microbiota in cheese production: concept of a natural adjunct milk culture. October 2022, 3rd Agroscope PhDs-PostDocs Symposium 2022, Changins, Switzerland

7.4.2 Poster presentations (see annex)

1) e-Poster: Bettera, L., Bancalari, E., Monica, S., Neviani, E., Gatti M. and Bottari B. The interplay between farming system and raw milk quality: development of new payment parameters of milk in the Parmigiano Reggiano cheese area. June 2021, IDF International Cheese Science and Technology Symposium, University of Laval, Québec, Canada. Virtual Event.
2) Video e-Poster: Bettera, L. and Gatti, M. Lactic acid bacteria in raw milk for long-ripened cheeses: origin and characterization for a potential targeted use. September 2021. First Virtual (XXV) WORKSHOP on "The Developments In The Italian PhD Research On Food Science Technology And Biotechnology", Palermo, Italy. Virtual event.
3) Video e-Poster: Bettera, L., Levante, A., Bancalari, E., Bertani, G., Neviani, E. and Gatti M. Geno- and phenotypic characterization of *Lacticaseibacillus* strains isolated from raw milk for cheese production. December 2021. MD2021, Advances in Microbial Diversity. Virtual event.

7.5 Didactic activities

7.5.1 Didactic laboratories

Assistance to the Food Microbiology Laboratories. Bachelor and Master course of "Food Science and Technology" (10 hours). Academic year 2019-2020.

Assistance to the Food Microbiology Laboratories. Bachelor and Master course of "Food Science and Technology" (10 hours). Academic year 2020-2021.

7.5.2 Correlator of student's thesis

Giulia Mariotti: "Raw milk lactic acid bacteria involved in long ripened cheeses production: a critical study of data found in the literature". Master Thesis, course of "Food Science and Technology", academic year 2019 – 2020, University of Parma, Parma, Italy.

Alessio Campagnolo: "Interactive map of italian raw milk cheeses: an important heritage still to be enhanced". Bachelor thesis, course of "Gastronomic Science", academic year 2020 – 2021, University of Parma, Parma, Italy.

Miriana Bello: "Raw milk cheeses: an italian heritage to be collected and preserved". Bachelor thesis, course of "Gastronomic Science", academic year 2020 – 2021, University of Parma, Parma, Italy.

Sara Grassi: “The alps of Val Leventina: past and present of places, people and dairy traditions”. Bachelor thesis, course of “Gastronomic Science”, academic year 2020 – 2021, University of Parma, Parma, Italy.

Gabriele Caroli: “Autochthonous Lactic Acid Bacteria in raw milk used for Parmigiano-Reggiano cheese: quantification through culture-dependent and -independent techniques”. Master thesis, course of “Food Science and Technology”, academic year 2021 – 2022, University of Parma, Parma, Italy.

7.5.3 Tutoring

Students’ International Mobility tutor. Academic year 2019 – 2020. According to Regulation L. 341 del 19/11/1990 and D.lgs. N. 68 del 29 marzo 2012.Tutor. Internationalization Office, University of Parma, Parma, Italy.

7.5.4 Other

Social media manager. Co-founder of the PhD Doctoral School Twitter account @FoodPhDUnipr.

7.6 ANNEX

7.6.1 Poster 1

Abstract

Parmigiano Reggiano (PR) is one of the most famous Italian Protected Designation of Origin cheese. It is definable as raw-milk, cooked, long-ripened cheese. The raw milk quality links the product to its territory representing the basis of its final, unique characteristics. For this reason, the control of factors related to farming practices is of great importance since they directly impact the dairy milk quality influencing the cheese properties. Upon arrival to the cheese factory, milk is currently paid according to quality parameters, among which the microbial load, independently from the species present. Besides total microbial count, the biodiversity and the presence of specific lactic acid bacteria (LAB) should represent an added value for milk quality; however, they are not considered.

Through the project Farm4PR, granted by MIPAAF, Italian Ministry of Agriculture, Forestry and Tourism, we aimed at classifying farms in the PR area based on their environmental and breeding practices, and to correlate these with milk microbial profiles and cheesemaking parameters, to develop a novel milk payment system. Particular significance will be given to updating the definition of milk microbiological quality, considered so far as total bacterial count, evaluating LAB's presence by impedometric analysis and Real-Time qPCR. This approach could define a new parameter to give importance to the autochthonous raw milk microbiota relevant for PR production.

The correlation between farming variables and milk microbiological and cheesemaking characteristics will make possible to select the best drivers of milk quality. This will allow the definition and prediction of the most significant parameters to be considered in the milk payment system. The main expected outcomes are the valorization of farming systems that contribute the most to the production of high-quality milk, and the improvement of PR production chain by controlling the factors related to milk production.

UNIVERSITÀ DI PARMA

IDF INTERNATIONAL Cheese Science and Technology Symposium — June 7 to 11, 2021 — 100% virtual edition

The interplay between farming system and raw milk quality: development of new payment parameters of milk in the Parmigiano Reggiano cheese area

Luca Bettera*, Elena Bancalari, Saverio Monica, Erasmo Neviani, Monica Gatti and Benedetta Bottari
Department of Food and Drug, University of Parma, Italy
*Correspondence: luca.bettera@unipr.it

Introduction

Parmigiano Reggiano is one of the most famous Italian Protected Designation of Origin (PDO) raw milk cheese

The quality of **raw milk** links the product to its **territory** representing the basis of its final and unique characteristics

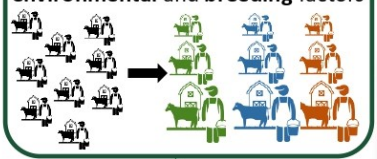
Farming practices directly impact the milk quality, especially the presence and abundance of **Lactic Acid Bacteria (LAB)**

Milk is currently paid according to **quality parameters**, among which total microbial load, independently from the species present

How to give importance to high-quality raw milk?

Material and methods

Farms **clustering** based on **environmental** and **breeding** factors



Raw milk analysis:

- **Impedometric** and **Real-Time qPCR** for the quantification of **autochthonous LAB**
- **Chemical** and **coagulation** properties by FT-IR and mechanical test

Expected results

The correlation between farming variables and milk microbial and cheesemaking characteristics will determine the **best drivers of milk quality**

Development of a **novel raw milk payment system**

Recognize the added value of producing **high-quality** raw milk

mipaaf Ministero delle politiche agricole alimentari e forestali

The project is granted by MIPAAF, Italian Ministry of Agriculture, Forestry and Tourism (grants for research projects under the Dairy Investment Fund, MD Sept.25th 2018 n. 27443)

7.6.2 Poster 2

Lactic acid bacteria in raw milk for long-ripened cheeses: origin and characterization for a potential targeted use

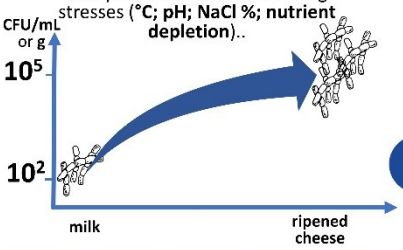
PhD student Luca Bettera (luca.bettera@unipr.it)
Department of Food and Drug, University of Parma, Parma, Italy
Tutor: Prof.ssa Monica Gatti

Introduction

Non Starter Lactic Acid Bacteria (NSLAB- *Lacticaseibacillus* spp.) originate from **raw milk** where they are in low concentration...

Source at farm level?

..they withstand cheese making stresses (**°C; pH; NaCl %; nutrient depletion**)..



..and during **ripening**, dominate the cheese environment contributing to **flavor** formation [1].

Geno- phenotypic characteristics?

Material and methods

Raw milk from 128 farms

Growth kinetics[2] of autochthonous NSLAB (Impedometric analysis at 25°C and 45°C)

Quantification *Lacticaseibacillus* spp. (Real-Time qPCR)

[3]

Gene	Primer pair	Sequence (5' → 3')
spxB	poxcDNAfw	CAGACGCAATGATCAAGGTG
	poxPromRv	AATGCGCCYACTTCTTCATG

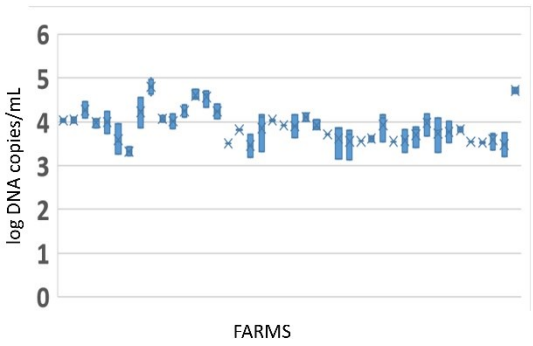
Raw milk spontaneous fermentation

Isolation of **lactobacilli** on MRS

Specie-specific PCR and **Amplified Fragment Length Polymorphism (AFLP)**[4]

Results

- PCR efficiency = 0,99
- Standard curve R^2 = 0,99



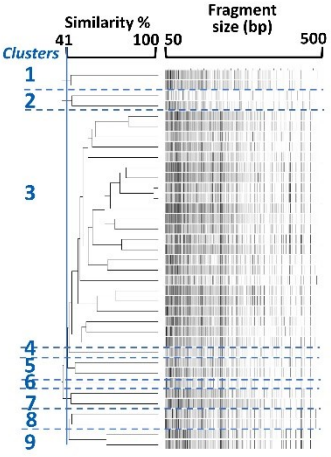
Total of 122 isolates

Specie-specific PCR

Screening of 31 *Lacticaseibacillus* spp.

AFLP

Selection of **10 strains** for phenotypic analysis



Conclusions and perspectives

- *Lacticaseibacillus* spp. have been quantified in raw milk → The correlation between milk microbial profile and farming condition will determine the **best drivers of raw milk quality**
- The screening of isolates allowed to select the most genotypically divers *Lacticaseibacillus* spp. strains → Selected strains will be tested for their thermal, pH, NaCl% resistance, beside for their flavor formation in model systems

References

(1) Gatti, M., Bottari, B., Lazzi, C., Neviani, E., Mucchetti, G., 2014. Invited review: Microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. Journal of Dairy Science 97, 573–591.
(2) Bancalari, E., Bernini, V., Bottari, B., Neviani, E., 2016. Application of Impedance Microbiology for Evaluating Potential Acidifying Performances of Starter Lactic Acid Bacteria to Employ in Milk Transformation 7, 1–11.
(3) Savo Sardaro, M.L., Levante, A., Bernini, V., Gatti, M., Neviani, E., Lazzi, C., 2016. The spxB gene as a target to identify Lactobacillus casei group species in cheese. Food Microbiology 59, 57–65.
(4) Bertani, G., Savo Sardaro, M.L., Neviani, E., Lazzi, C., 2019. AFLP protocol comparison for microbial diversity fingerprinting. Journal of Applied Genetics 60, 217–223.

First Virtual Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology
Palermo 14/15 Settembre

7.6.3 Poster 3

Abstract

Microbiological and biochemical changes to curd are crucial factors for the production of raw-milk, long-ripened cheeses. The microbial ecology fundamentals of this cheese variety consist of a complex interaction between starter lactic acid bacteria (SLAB, deliberately added manly for curd acidification) and non-starter LAB (NSLAB, adventitious milk contaminants correlated to cheese flavor formation during ripening). Nevertheless, the need to standardize cheesemaking and accelerate ripening has driven the use of several NSLAB, often called as adjuncts or attenuated adjuncts secondary starters. Their proper targeted use, however, relies on the understanding of geno- and phenotypic characteristics at strain level.

This study aimed to characterize NSLAB isolated from raw milk used for cheese production sampled from 20 different cheese factories. From a total of 122 strains isolated, 31 were screened as *Lacticaseibacillus* spp. through specie-specific PCR, and 10 strains from different Amplified Fragment Length Polymorphism (AFLP) clusters intended to cover the diversity of the species were selected. Strains were phenotypically characterized on the basis of their thermal, pH, NaCl% resistance, and ability to grow on different substrates by means of impedometric and Biolog MicroPlates (Biolog, Inc.) analysis.

By the investigation of geno- and phenotypic traits of NSLAB isolated from raw milk, this study will allow the selection and targeted use of strains taking into account the complexity and dynamicity of the ecosystem that contribute to the cheese core microbiota formation.

MD21, December 14-15, 2021

Geno- and phenotypic characterization of *Lacticaseibacillus* strains isolated from raw milk for cheese production

Luca Bettera*, Alessia Levante, Elena Bancalari, Gaia Bertani, Erasmo Neviani, Monica Gatti
Department of Food and Drug, University of Parma
Parco Area delle Scienze 49/a, 43124, Parma, Italy
*correspondence author: luca.bettera@unipr.it

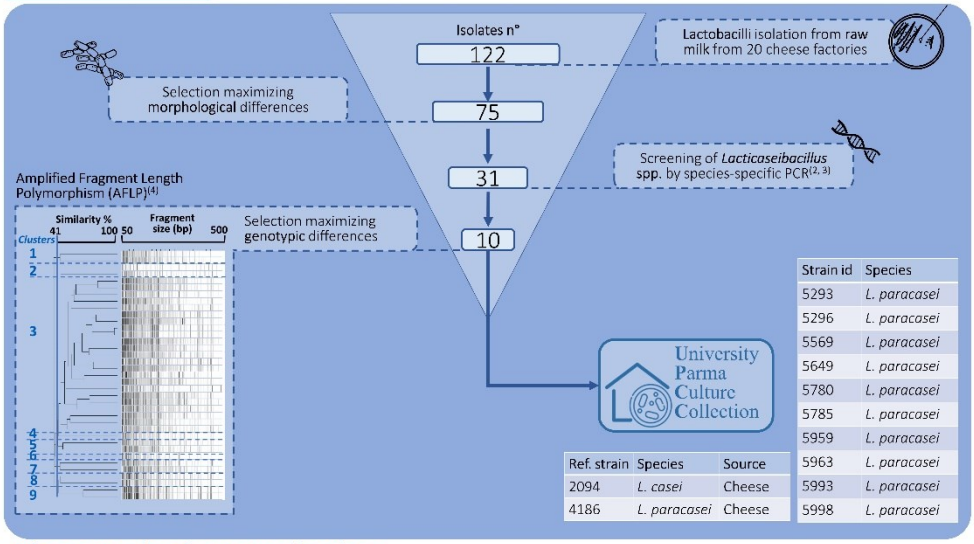
Introduction

Microbiological and biochemical changes occurring in the curd are crucial factors for the production of **raw-milk, long-ripened cheeses**. The microbial ecology fundamentals of this cheese variety relies on a complex interaction between starter lactic acid bacteria (SLAB, deliberately added manly for curd acidification) and **non-starter LAB** (NSLAB, adventitious milk contaminants correlated to **cheese flavor** formation during ripening)[1].

Nevertheless, the need to standardize cheesemaking and accelerate ripening has driven the use of several NSLAB, often called as adjuncts or attenuated adjuncts secondary starters. Their proper targeted use, however, relies on the understanding of **geno-** and **phenotypic characteristics at strain level**.

This study aimed to characterize ***Lacticaseibacillus* spp.** strains isolated from raw milk. GEN III microplates (Biolog, Inc.) were used to test strains' ability to grow on different substrates and resist under chemical stresses, while **impedometric analysis** were applied to follow strains' **growth kinetic** in milk and under **osmotic, thermal,** and **lysozyme stresses**.

Strain selection

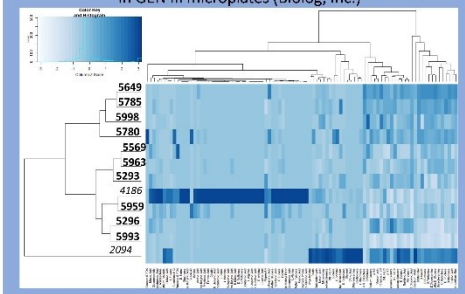


Ref. strain	Species	Source
2094	*L. casei*	Cheese
4186	*L. paracasei*	Cheese

Strain id	Species
5293	*L. paracasei*
5296	*L. paracasei*
5569	*L. paracasei*
5649	*L. paracasei*
5780	*L. paracasei*
5785	*L. paracasei*
5959	*L. paracasei*
5963	*L. paracasei*
5993	*L. paracasei*
5998	*L. paracasei*

Phenotypic characterization

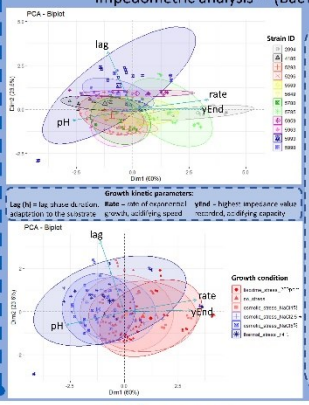
Metabolic assay, growth ability (48h, OD_{590}) in GEN III microplates (Biolog, Inc.)



-Despite they belong to the same species, isolates showed **different ability to use carbon and nitrogen source**, as well as **different resistance to chemical stresses**

-In particular, strains 5296, 5993, 5293, 5569 and 5959 showed less ability to use **alfa-D-Lactose** and **D-Galactose** as growth substrates

Growth kinetic (60h, 37°C) in partially skimmed milk- impedometric analysis [5] (BacTrac 4300®)



-Principal Component Analysis highlights **differences in strains' growth kinetics**

-Ref. strain 2094 separates mainly for its high acidifying capacity

-Strain 5993 were not able to grow after thermal stress

-Thermal stress and 5% NaCl concentration in milk were the conditions that most affect (reduce) strains' growth

-Lower NaCl concentration and lysozyme presence didn't affect the growth in comparison with no-stress condition

Conclusions

- The present work allowed the isolation of more than one hundred of **lactic acid bacteria** strains with potential technological applications in **food industry**
- The screening process allowed the selection of the 10 most genotypically diverse ***Lacticaseibacillus* spp.** strains
- According to their phenotypic characteristics, some strains may not perform well as adjunct culture for cooked cheese production (low resistance to thermal stress), while others seem to be promising thanks to their relative low acidifying capacity (important to avoid growth overlapping with SLAB)
- Strains with the best phenotypic profile will be first tested for their **aroma compounds** production, and then applied in cheesemaking as **adjunct culture**

References

(1) Gatti, M., Bottari, B., Lazzi, C., Neviani, E., Mucchetti, G., 2014. Invited review: Microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. Journal of Dairy Science 97, 573–591.
(2) Savo Sardaro, M.L., Levante, A., Bernini, V., Gatti, M., Neviani, E., Lazzi, C., 2016. The spxB gene as a target to identify *Lactobacillus casei* group species in cheese. Food Microbiology 59, 57–65.
(3) Levante, A., F. De Filippis, A. La Storia, M. Gatti, E. Neviani, D. Ercolini, and C. Lazzi. 2017. Metabolic gene-targeted monitoring of non-starter lactic acid bacteria during cheese ripening. International Journal of Food Microbiology 257:276–284
(4) Bertani, G., Savo Sardaro, M.L., Neviani, E., Lazzi, C., 2019. AFLP protocol comparison for microbial diversity fingerprinting. Journal of Applied Genetics 60, 217–223.
(5) Bancalari, E., Bernini, V., Bottari, B., Neviani, E., 2016. Application of Impedance Microbiology for Evaluating Potential Acidifying Performances of Starter Lactic Acid Bacteria to Employ in Milk Transformation 7, 1–11.