Università degli Studi di Palermo

AREA RICERCA E TRASFERIMENTO TECNOLOGICO
SETTORE DOTTORATI E CONTRATTI PER LA RICERCA
U. O. DOTTORATI DI RICERCA

Course in Biodiversity in Agriculture and Forestry (BAF)

Dipartimento di Scienze Agrarie, Alimentari e Forestali (SAAF)

AGR/16 MICROBIOLOGIA AGRARIA

Role of lactic acid bacteria associated to wooden equipment used in traditional dairy production

IL DOTTORE

Gabriele Busetta

IL COORDINATORE

Riccardo Lo Bianco

Firmato digitalmente da: Riccardo Lo Bianco
Organizzazione: UNIVERSITA' DEGLI STUDI DI PALERMO/80023730825
Data: 03/02/2025 17:22:26

IL TUTOR

Raimondo Gaglio

Firmato digitalmente da: Raimondo Gaglio
Organizzazione: UNIVERSITA' DEGLI STUDI DI PALERMO/80023730825
Data: 31/01/2025 10:57:41

CO TUTOR

Luca Settanni

Firmato digitalmente da: Luca Settanni
Organizzazione: UNIVERSITA' DEGLI STUDI DI PALERMO/80023730825
Data: 31/01/2025 10:31:05

CICLO XXXVII

ANNO CONSEGUIMENTO TITOLO 2025

Table of contents

Abstract

Traditional cheese production plays a key role in preserving the culinary heritage of many regions around the word. It significantly contributes to the development and sustainability of rural areas. These cheeses are deeply connected to their regions of origin, still made using milk from animals of indigenous breeds and traditional methods and wooden equipment. The use of wooden tools promotes the formation of microbial biofilms, primarily composed of indigenous microorganisms, especially lactic acid bacteria (LAB). These microorganisms are crucial for developing the complex sensory characteristics that reflect the traditional and regional cheese heritage. Although wood's porous nature has sparked considerable debate, there is no scientific evidence linking it directly to foo-borne diseases. In Italy, the use of wooden equipment in cheese production is permitted under Regulation (EC) no. 2074/2005, which provides an exemption from Regulation (EC) no 852/2004 for foods with traditional characteristics related to the materials used in equipment manufacturing, packaging, and wrapping. In practice, microbial biofilms on wooden equipment represent one of the most vital reservoirs of dairy biodiversity. These biofilms not only influence the development of the LAB but also inhibit food-borne pathogens.

The primary objective of the research was to investigate, characterize, and enhance various traditional cheeses produced with wooden equipment and to study the role of LAB during the cheesemaking process. To achieve this, the first step involved characterizing the wooden vats used in producing two pasta filata cheeses typical of southern Italy and their influence on sensory, chemical, and organoleptic characteristics. The second step focused on characterizing the wooden shelves used for ripening pressed cheese and the inhibitory

potential of LAB against food-borne pathogens. Moreover, a third phase was initiated to further improve traditional cheeses by employing a technological strategy to reduce the cooking time during the production of a typical Sicilian cheese.

Numerous research articles have consistently shown that the wooden equipment is hygienically safe and serves as a reservoir of microbial biodiversity, which enhances the final quality, safety, and flavour profile of traditional dairy products. Ongoing research is focused on characterizing LAB isolated from this wooden equipment, exploring their antimicrobial properties, and testing their potential application on wooden prototypes. These prototypes will then be used on ripening boards in cheese factories during the ripening processes. Broadly, this research highlights on how traditional cheeses contribute to the culinary heritage of specific regions and play a crucial role in sustainability and economic viability.

Sommario

Le produzioni di formaggi tradizionali giocano un ruolo fondamentale nel salvaguardare il patrimonio culinario di molte regioni nel mondo. Esse contribuiscono fortemente allo sviluppo e alla sostenibilità delle aree rurali. Questi formaggi sono molto legati ai territori d'origine e ancora oggi sono ottenuti con latte di razze autoctone, trasformato attraverso processi tradizionali e facendo uso di attrezzature in legno. L'uso delle attrezzature in legno favorisce la formazione di biofilm microbici solitamente composti da microrganismi indigeni, prevalentemente batteri lattici, che giocano un ruolo determinante nello sviluppo di tratti sensoriali complessi che riflettono il patrimonio regionale tipico e tradizionale dei formaggi. A causa della sua natura porosa, l'utilizzo del legno è oggetto di numerose controversie, ma non vi è alcuna prova scientifica che attesti la diretta correlazione tra l'impiegno del legno e le malattie di origine alimentare. Ad oggi, in Italia l'impiego delle attrezzature in legno in caseificazione è possibile grazie al regolamento (CE) n. 2074/2005 che deroga l'applicazione del regolamento (CE) n. 852/2004 per gli alimenti con caratteristiche tradizionali "in relazione al tipo di materiale utilizzato per la fabbricazione delle attrezzature impiegate per la preparazione, imballaggio e confezionamento". In realtà, la formazione dei biofilm microbici sulle attrezzature in legno rappresenta una delle fonti di biodiversità casearia più importanti. Infatti, i biofilm presenti sulle superifici delle attrezzature non solo influenzano lo sviluppo della comunità lattica, ma sono anche responsabili dell'inibizione di molti microrganismi patogeni di origine alimentare. Sulla base delle sopracitate motivazioni, l'obiettivo principale dell'attivita di ricerca condotta è stato quello di approfondire, caratterizzare e valorizzare diversi formaggi tradizionali prodotti con le attrezzature in legno e approfondire le conoscenze sul ruolo dei batteri

lattici durante i processi di caseificazione. Per raggiungere questo obiettivo, inizialmente si è provveduto a caratterizzare i tini di legno ("tine") utilizzati durante la produzione di due formaggi a pasta filata tipici del sud Italia e a valutare la loro influenza sulle caratteristiche sensoriali, chimiche e organolettiche dei prodotti trasformati. In un secondo momento, sono state caratterizzate le scaffalature in legno utilizzate per la stagionatura dei formaggi con particolare attenzione al potenziale inibitorio dei batteri lattici isolati nei confronti dei microrganismi patogeni di origine alimentare. Inoltre, in una terza fase e sempre nell'ottica di valorizzare i formaggi tradizionali, è stata applicata una strategia tecnologica per ridurre i tempi di cottura durante il processo di produzione di un formaggio tipico siciliano. Attraverso diversi articoli di ricerca, i risultati hanno costantemente dimostrato la sicurezza igienico-sanitaria delle attrezzature in legno e come queste agiscano da vero e proprio "serbatoio" di biodiversità microbica contribuendo alla qualità finale e alla sicurezza dei prodotti lattiero-caseari tradizionali. Attualmente, sono in corso lavori per la caratterizzazione dei batteri lattici che hanno mostrato proprietà antimicrobiche isolati da queste attrezzature in legno mediante applicazione su prototipi di scaffali per un futuro impiego nell'attivazione delle assi di stagionatura nei caseifici che esplicano il processo di stagionatura. In una prospettiva più ampia, questa ricerca ha fornito evidenze concrete sul ruolo che i formaggi tradizionali e tipici svolgono nel valorizzare il patrimonio culinario di alcune regioni e nel loro contributo reale alla sostenibilità e alla redditività economica.

Introduction

1. History of cheeses

The origins of cheese date back to ancient times, with the earliest references found in Mesopotamia. It was here that shepherds, while carrying milk in a goat's stomach, stumbled upon the formation of a cheese-like substance. This discovery led to a new food product derived from milk that could be stored for longer periods and more easily than milk itself (Kindstedt, 2012). It is believed that cheese originated in the "Fertile Crescent" between the Tigris and Euphrates rivers in present-day Iraq (Fig. 1), around 8000 years ago during the 'Agricultural Revolution' when certain plants and animals were domesticated.

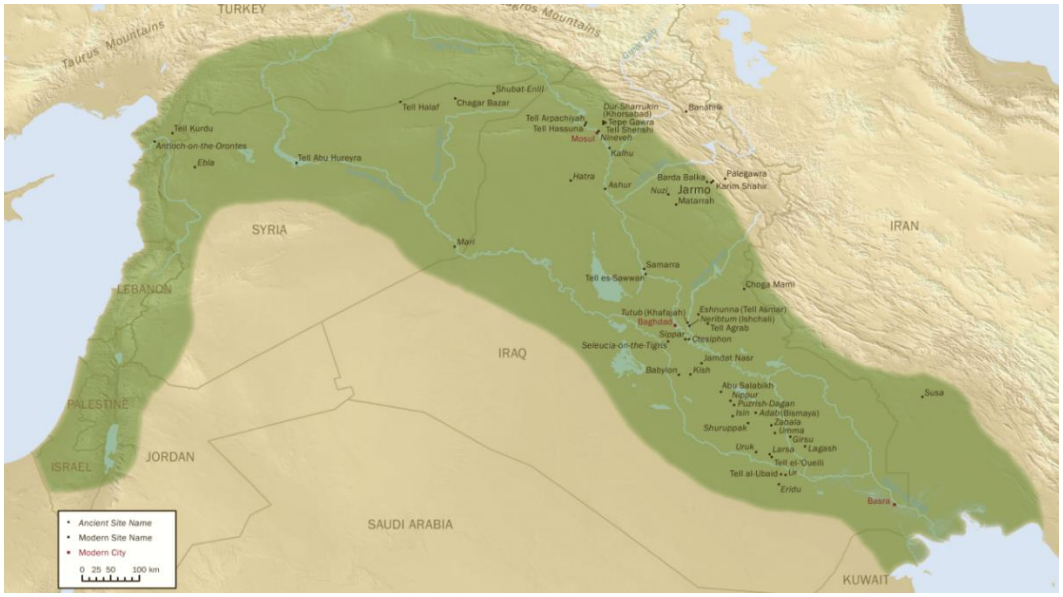

Fig. 1. Geographical map highlighting the "Fertile Crescent", (https://news.uchicago.edu/explainer/fertile-crescent-explained)

Goats and sheep were among the first animals to be domesticated due to their small size, sociable nature, and ease of breeding. These animals were ideal for providing meat, milk, hides, and wool (Fox et al., 2017). In contrast, cattle were much harder to domesticate; wild cattle were larger, more ferocious, and less suited to the arid environment of the

Middle East compared to goats and sheep. Initially, cattle were primarily used as work animals and only became a significant source of milk relatively recently. The nutritional value of milk from domesticated animals was quickly recognised, and milk and dairy products became essential components of the human diet.

Cheese production significantly contributed to the spread of civilisations across the Middle East, Egypt, Greece, and Rome. Several references to cheese appear in ancient texts. For instance, figures such as Job (1520 B.C.) and Samuel (1170–1017 B.C.) are associated with cheese, and ancient Egyptian tombs feature cheese-related depictions. Classical Greek literature by authors like Homer (1184 B.C.), Herodotus (484–408 B.C.), and Aristotele (384–322 B.C.) also mentions cheese. In Classical Rome, cheese manufacturing was well established and included in soldiers' rations. Roman writers such as Cato the Elder (234–149 B.C.), Varro (116–27 B.C.), Columella (4–70 A.C.), Pliny the Elder (23–79 A.C.), and Palladius (400–470 A.C.) described cheesemaking processes, quality attributes, and culinary uses. The Romans widely disseminated cheesemaking knowledge during the Empire, evidenced by the use of the word "caseus", the root for casein, the primary milk protein coagulated to make cheese. The Romans enhanced Greek cheesemaking techniques by introducing cow's milk, which was uncommon at the time, as cows were primarily used for labor rather than milk production. During the Roman era, Pliny praised the quality of Sicilian cheeses, particularly those produced in Agrigento from goat milk and those crafted in Messina and Syracuse, which were renowned for their excellence. The most comprehensive ancient description of cheesemaking is provided by Columella in his agricultural treatise, *De Re Rustica* (Fox et al., 2017). During Emperor Diocletian's reign (284–305 B.C.), a maximum price for cheese was established to ensure

its affordability for all citizens. The movements of Roman armies and administrators facilitated the spread of cheese throughout the known world at the time. After the fall of the Roman Empire, the widespread movement of populations across Europe, along with the Crusaders and pilgrims during the Middle Ages, furthered the dissemination of cheesemaking. However, the most significant contributors to the development of cheesemaking techniques and the evolution of cheese varieties during the Middle Ages were monasteries and feudal estates (Gobbetti et al., 2018b). Monasteries played a significant role in advancing food production, particularly in wine, beer, and cheese. Many current cheese varieties originated in monasteries, such as Wenslydale (Rievaulx Abbey, Yorkshire), Port du Salut or Saint Paulin (Monastery de Notre Dame du Port du Salut, Laval, France), Fromage de Tamie (Abbey of Tamie, Lac d'Annecy, France), Maroilles (Abbey Moroilles, Avesnes, France), Trappist (Maria Stern Monastery, Banja Luka, Bosnia), Bethlehem (Abbey of Regina Laudis, Connecticut, USA). The movement of monks between monastery likely contributed to the spread of cheese varieties and the development of new cheeses.

During the Renaissance period, cheesemakers introduced new and exciting varieties to complement the traditional cheeses handed down from Roman times and those developed in monasteries during the Middle Ages (Licitra et al., 2014). Only at the end of the 19th century in Italy large cheeses such as Grana began to be produced, driven by the growth of small and medium-sized farms. The history of cheesemaking is rich, reflected in the myriad techniques and methods used in its production. This evolution has led to the existence of approximately 400 types of cheese, with nearly 1000 distinct names. Italy's strong connection to its territory, tradition passed down throught generations, and

agricultural culture position it as a competor with France in terms of dairy heritage. Today, the dairy industry is one of the leading sectors in the European food industry, with Italy boasting the widest variety of cheese production. This sector's economic importance is substantial. According to the Codex Alimentarius, cheese is defined as a solid or semi-solid product, fresh or ripened, obtained by coagulating milk, skimmed milk, semi-skimmed milk, cream, or whey cream through the action of rennet or other coagulating agents (Corradini, 1995). This definition includes a wide range of cheeses that can be classified according to various criteria. According to Fox et al. (2004), cheese classification is based on:

- type of milk used (e.g., cow, sheep, buffalo, goat, etc.);
- production origin (e.g., Grana Padano, Parmigiano Reggiano, Pecorino Romano, etc.);
- textural characteristics (e.g., soft, semi-soft, hard, very hard cheeses);
- fat content (e.g., light, semi-fat, fat cheeses);
- technological characteristics (e.g., uncooked, semi-cooked, cooked, stretched curds);
- ripening period (e.g., fresh, short-ripened, ripened, long-ripened);
- coagulation type (e.g., acid or enzymatic).

2. Traditional technological processes

The production of artisanal cheese is characterized by several common features, including fragmented companies, significant variability in production processes, and limited supply, making these cheeses niche products typically sold in local markets. Farms involved in traditional cheese production often operate in marginal areas and produce

various cheese types closely linked to their regions of origin. Traditional cheeses are known for their intense and diverse flavours, with considerable variability even within the same variety. Each traditional cheese emerges from complex systems drawing on unique bio-organoleptic characteristics associated with various biodiversity factors, such as the environment, macro and microclimate, natural pastures, animal breeds, the use of raw milk and its natural microbiota, natural coagulants, cheesemaking technology, the unique role of the cheesemaker, and historical wooden equipment. These biodiversity factors collectively influence the quality of the final products (Licitra, 2010). Italy boasts a rich dairy heritage in traditional cheese production. According to the Ismea-Qualivita report (ISMEA, 2023), 56 traditional cheeses enjoy the status of Protected Designation of Origin (DOP) and Protected Geographical Indication (PGI), with 13 of these produced in Southern Italy. Different production technologies can classify these southern Italian cheeses into fresh and ripened stretched cheeses and hard or extra-hard cheeses.

2.1. Stretched cheese or Pasta filiata

Stretched cheese has its origins in southern Italy, where the basic conditions of milk production and transportation often resulted in milk arriving at processing sites already quite sour, leading to the plastic properties of the curd. Howewer other European countries such as Greece, Balkanian states, Turkey and Eastern Europe have also specialised in producing these cheeses. An early description of the technology behind these cheeses is provided by Ottolini (1787), who noted that the name “caciocavallo” comes from the practice of hanging the cheeses tied together from rafters to mature them. The term pasta filata, meaning ‘sput paste’ or stretched curd iin Italian, refers to the unique plasticization

and stretching process to all pasta-filata cheeses, giving this diverse group a shared identity (McMahon et al., 2017). Stretched cheeses are made throughout Italy, from the Alps to Sicily, using methods that range from the most modern to the highly artisanal.

Artisanal stretched cheese production typically follows the flow chart illustrated in Fig. 2. Initially, raw milk is poured into wooden vats for a few minutes. During this time, the milk is gently stirred with a traditional wooden stick, which will later be used for breaking the curd. This gentle agitation helps release LAB from the biofilms adhered to the wood surfaces. The milk is then coagulated using lamb's rennet paste to form a compact curd. After coagulation, the curd is allowed to acidify overnight until it reaches a pH of 5.3–5.1, which is essential for the stretching phase. The acidified curd is then cut and stretched with hot water (tiycally 80 °C) to achieve a soft and elastic texture.

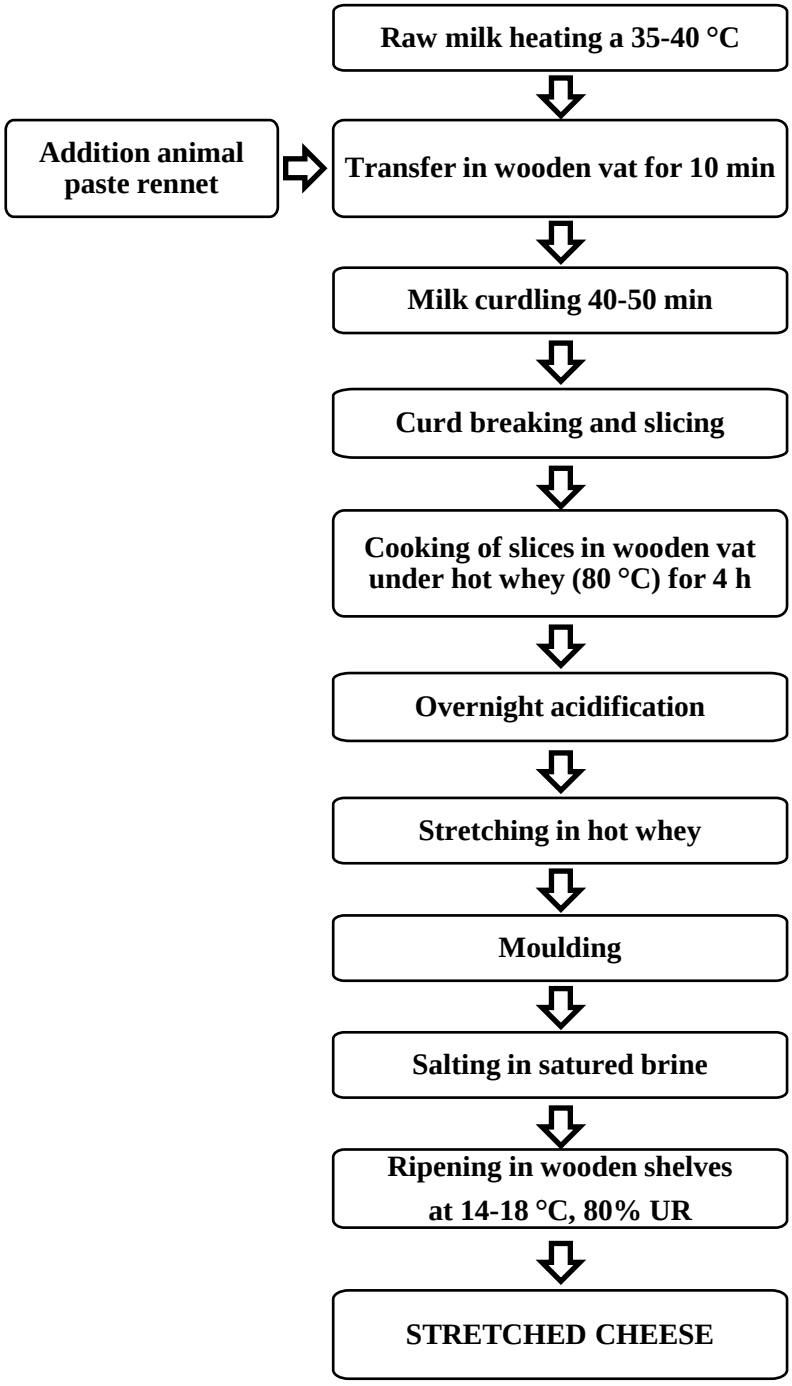

Fig. 2. Flowsheet of stretched cheese production.

The cheeses are ripened for several months at temperatures between14–18°C and a relative humidity of 80%. In southern Italy, traditional equipment is used to produce

stretched cheeses such as Provola dei Nebrodi, Caciocavallo Lucano, Caciocavallo Ragusano, and Vastedda del Belìce (Fig. 3).

Fig. 3.A. Traditional stretched cheeses in southern Italy; **B.** Traditional wooden equipment used for production.

2.2. Hard or extra-hard cheese

Hard or extra-hard cheeses are distinguished by the cooking of the curd and an extensive ripening period that can range from a few months to several years. In Italy, most traditional hard cheeses are produced using raw milk, with the curd cut into rice- or wheat-sized grains and cooked at high temperatures.

The production technology of most traditional hard cheeses from southern Italy, although varying greatly from one region to another, is generally represented by the flow chart in Fig. 4. The cheesemaking process begins with preheating the milk to 38–40 °C, then transferring it to a wooden vat where it is gently agitated for 5 min. This step is

crucial, as the wooden equipment facilitates the release of LAB, which are essential for acidification. The milk coagulation is enhanced by the use of lamb rennet paste.

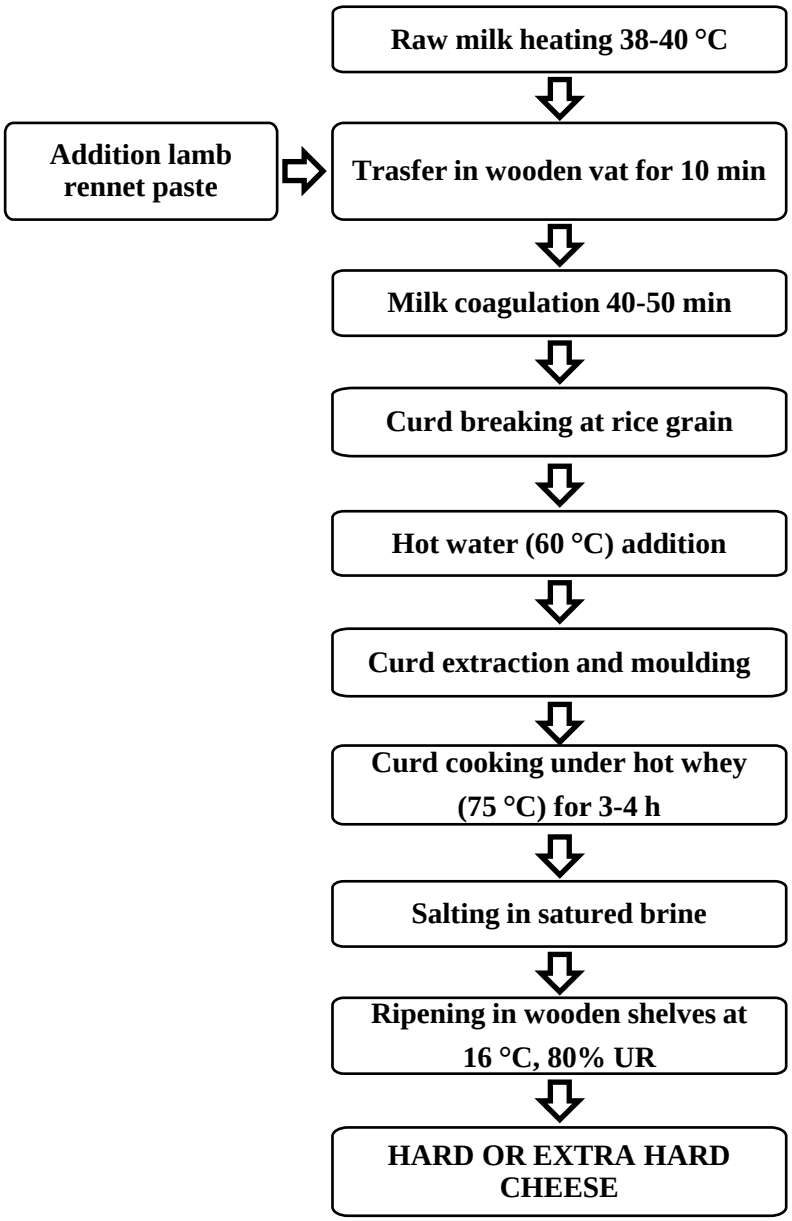

Fig. 4. Flowsheet of hard or extra hard cheese production.

The curd is broken with a wooden stick into rice grain-sized pieces, and hot water is added to facilitate syneresis. The curd is then hand-pressed to assist with whey drainage. It

is cooked for approximately 3–4 hours and covered with hot deproteinized whey at around 80°C. After 24 hours, the curd is salted in saturated brine or by manually dispersing the salt on cheese surface. Typically, traditional cheeses mature on wooden shelves at temperatures between 14 and 18 °C, with relative humidity levels of 75–80%, for a variable period depending on the type of cheese. In southern Italy, traditional hard cheeses include Pecorino Siciliano, Pecorino di Filiano, Piacentinu Ennese, Pecorino Crotonese, Canestrato Pugliese, and Pecorino Sardo (Fig. 5).

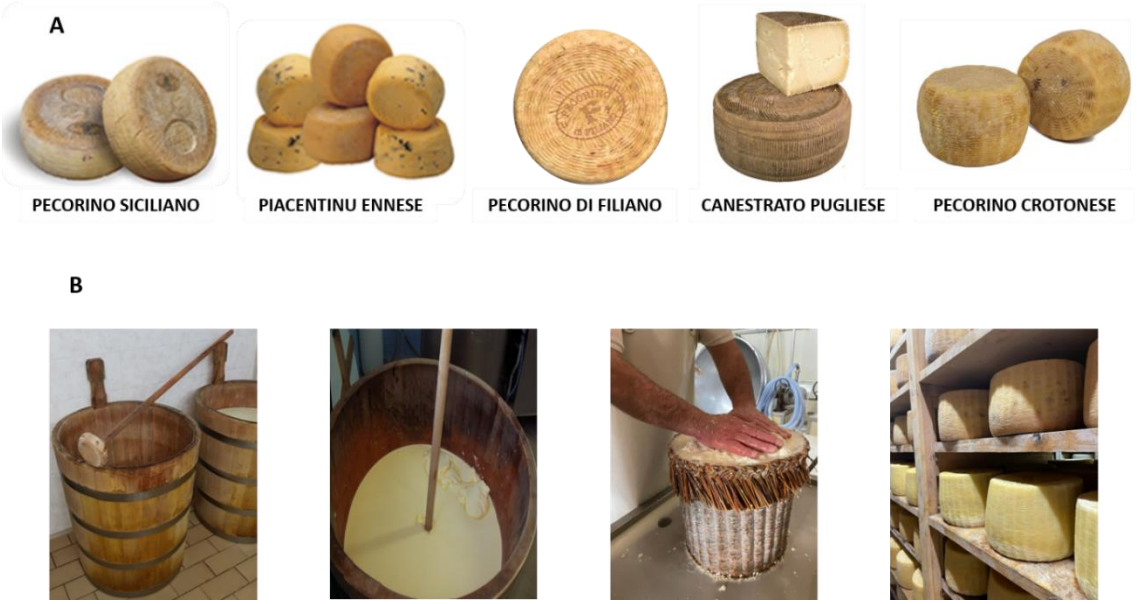

Fig. 5.A. Traditional pressed cheeses in Southern Italy; **B.** Traditional wooden equipment used for production

3. Microorganism involved in dairy production

A multitude of microorganisms play a role in transforming milk into cheese, initially operating as well-defined cellular entities and later as cytoplasmic enzymes released upon cell lysis. This lysis process is favoured by the stress conditions during cheesemaking, curd fermentation, and especially cheese ripening. As with any fermentation process, the

availability of sugars, nitrogen compounds, and mineral salts significantly impacts the development of microorganisms. Due to its complex composition, milk serves as a nutritionally rich medium that supports the growth of a diverse range of microorganisms (Franciosi et al., 2011). These microorganisms can be pathogenic, spoilage, or pro-technological. The latter group includes those responsible for curd acidification and transformation into mature cheese, as well as those enhancing the safety and stability of the final products. Besides LAB, which show superior adaptability in milk, other microorganisms like staphylococci, micrococci, coryneforms, propionibacteria, yeasts, and moulds also play pivotal roles in cheese production. These microorganisms contribute to gas production, surface colouring, softening, and, through their diverse enzymatic systems, the development of characteristic aromas (Parente and Cogan, 2004). Considering the microbiota involved in the production process, a further classification system for cheese has been proposed by Fox et al. (2004) (Fig. 6).

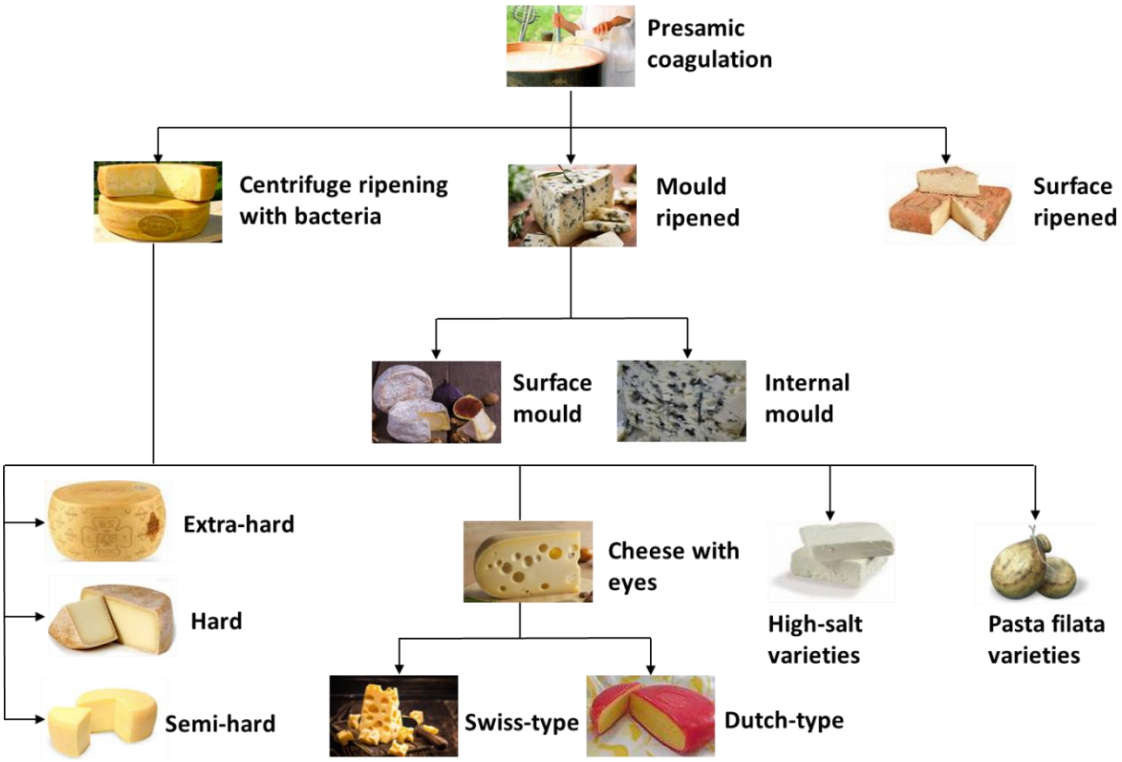

Fig. 6. Classification of the cheeses based on the microbiology of fermentation. Source: Fox et al., 2004.

4. Lactic acid bacteria

Certain bacteria play beneficial roles during fermentation, while others can lead to food spoilage or, worse, cause foodborne illnesses. The beneficial microorganisms, include those referred to as 'pro-technological', which have been traditionally used in the production of fermented foods. Among these, LAB are particularly significant; they can be naturally present in raw materials or added to foods as part of starter and/or protective cultures. LAB are widely used in the production of various fermented foods, including dairy, meat, vegetables, bakery products, and silage, contributing to their characteristics and stability in different ways (Leroy and De Vuyst, 2004). Depending on the product, other microorganisms such as micrococci, staphylococci, propionic acid bacteria,

coryneform bacteria, and certain yeasts may also play important roles (Parente and Cogan, 2004).

LAB form a highly diverse microbial group both morphologically and physiologically, playing vital roles in various fermentation processes. These bacteria primarily convert carbohydrates in food into lactic acid and enrich the flavour of various food products by breaking down proteins and lipids to generate compounds such as alcohols, aldehydes, acids, esters, and sulphur compounds. LAB are prokaryotic, heterotrophic, Gram-positive cocci, rods, or chains (Fig. 7). These non-sporulating, non-motile, anaerobic aerotolerant bacteria, which tolerate very small amounts of oxygen, lack catalase and nitrate reductase. Due to the absence of a respiratory chain, their metabolism is entirely fermentative, obtaining the necessary energy for anabolic functions through substrate-level phosphorylation (Kandler and Weiss, 1986). LABs are significant for their metabolic activity, utilizing sugars to produce organic acids and other beneficial compounds.

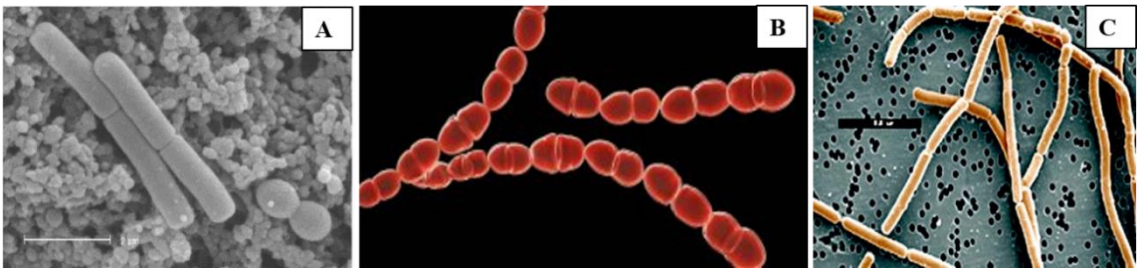

Fig. 7.A. Lactic acid bacteria cells focused on scanning electron micrograph; **B**, cocci chains; **C**, rod cells; (https://www.researchgate.net).

LAB might ferment glucose through two main pathways (Fig. 8), can grow at different temperatures, and are able to utilize carbohydrates other than glucose (e.g. carbohydrates pentose).

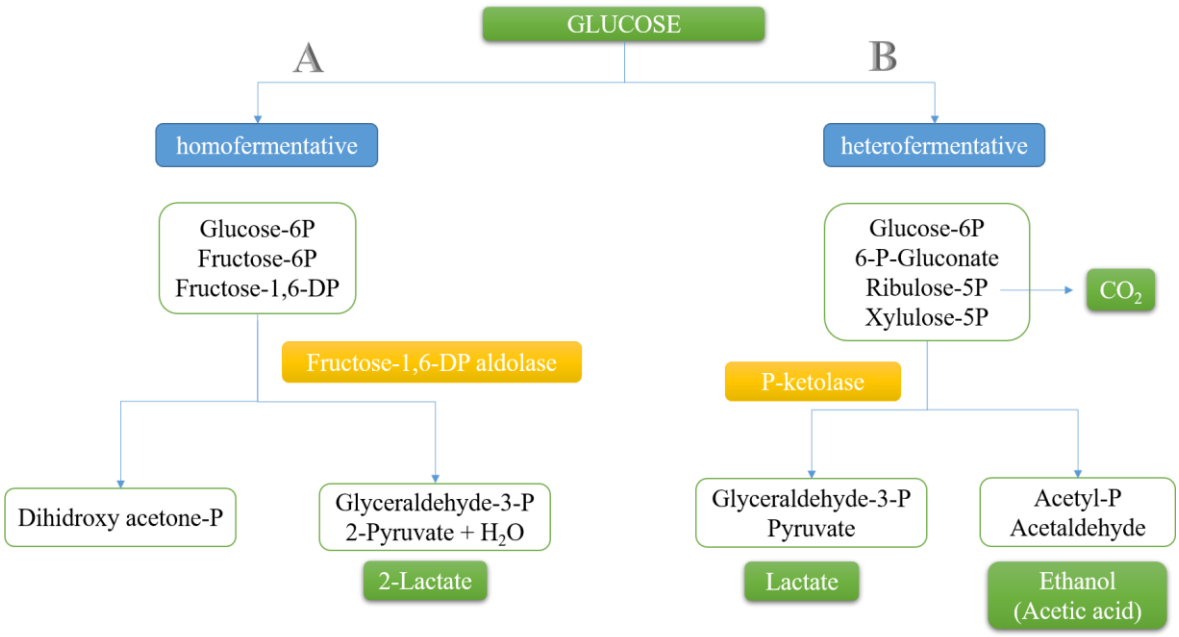

Fig. 8. Lactic acid fermentation. **A**, homolactic fermentation; **B**, heterolactic fermentation.

Homofermentative species primarily produce lactic acid from the fermentation of hexose carbohydrates via the Embden-Meyerhof (glycolysis) pathway and are unable to ferment pentose carbohydrates. In contrast, heterofermentative species generate not only lactic acid, but also ethanol/acetic acid, and carbon dioxide from hexoses through the 6-phosphogluconate/phosphoketolase (6PG/PK) pathway, though they do not produce CO_2 when fermenting pentose carbohydrates. Additionally, a third group of LAB, known as facultative heterofermentative, can utilize both metabolic pathways: glycolysis in the presence of hexoses and the 6PG/PK in the presence of pentoses.

In the dairy industry, particularly in cheese production, LAB are classified into starter LAB (SLAB) and non-starter LAB (NSLAB) (Settanni and Moschetti, 2010). SLAB are primarily tasked with acidifying the curd within a short timeframe (hours to days), and their role is relatively short. On the other hand, NSLAB have a prolonged function (months to years), crucial for the ripening process and the hygienic quality of the final product.

The key role of SLAB is to initiate lactic acid production, which promotes whey drainage from the curd, creates a hostile environment for undesired microorganisms (both spoilage and pathogenic), and contributes to a longer shelf life of the cheese (Salvadori Del Prato, 1998). Additionally, SLAB are valued for their resistance to bacteriophages and their autolysis capabilities (Parente and Cogan 2004). In most dairy production processes, SLABs are added to the milk. However, in artisanal production, biofilms on wooden equipment act as a natural starter, aiding in the cheesemaking process. During cheese production, various mesophilic species such as *Lactococcus lactis* and *Leuconostoc* spp., and thermophilic species such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Lactobacillus helveticus*, are crucial for lactic acid production from lactose (Settanni and Moschetti, 2010). On the other hand, NSLAB primarily consist of strains from the genus *Lactobacillus*. They include lactobacilli and pediococci, which can be obligate homofermenters, facultative heterofermenters, or obligate heterofermenters. Most non-starter lactobacilli are facultative heterofermentants, such as *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus*, *Lb. plantarum* and *Lb. curvatus*. The pediococci associated with cheese include *P. acidilactici* and *P. pentosaceus*. Some of these bacteria can utilize amino acids or certain glycoproteins from the fat globule membrane as a carbon source when sugar is absent. NSLAB are usually selected for their ability to enhance the organoleptic characteristics of the final product. During the ripening process, these bacteria degrade casein and peptides in cheese using their hydrolytic enzymes, releasing amino acids. While some of these compounds are directly aromatic (Fox and Wallace, 1997), most serve as precursors for developing aromatic compounds (α-ketothioacids, ammonia, amines, aldehydes, acids, and alcohols) that are pleasant and

typical of various cheeses, resulting from microbial enzyme-driven catabolism and modifications (Hemme et al., 1981). Additionally, these bacteria induce organoleptic and biochemical changes in the cheese during ripening, significantly influencing its texture and nutritional value. Furthermore, due to their capacity to produce bacteriocins, they play a pivotal role in preventing undesirable microorganisms (Gatti et al., 2014). The kinetics of SLAB and NSLAB usually follow the pattern shown in Fig. 9.

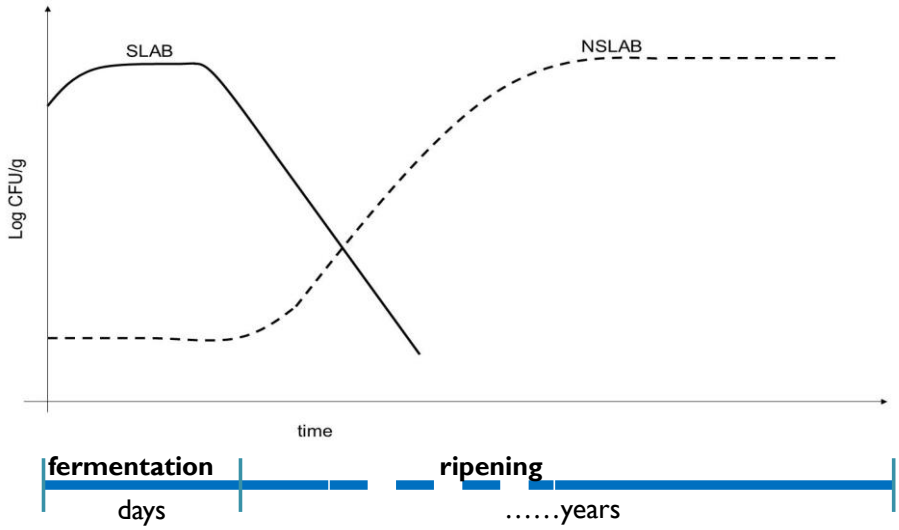

Fig. 9. Evolution dynamics of LAB in cheeses.

SLAB are introduced into the milk at a high density (approximately 10^6 and 10^7 CFU/g), but their numbers decrease during the initial stages of cheese production. In contrast, NSLAB are initially present in low concentrations in fresh curd and increase to 10^7 and 10^8 CFU/g, becoming the dominant microbiota in ripened cheese (Gobbetti et al., 2018a). Due to their widespread presence in foods and extensive use, they are naturally recognized as safe (GRAS) for human consumption by Food and Drug Administration (FDA) (Ibrahim et al., 2021; Kastner et al., 2006; Syrokou et al., 2022) and hold the 'qualified presumption of safety' (QPS) status granted by the European Food Safety

Authority (EFSA) (Barbosa et al., 2021). Nonetheless, EFSA has established guidelines (Koutsoumanis et al., 2020) for the analysis and study of whole genomes. Whole genome sequencing (WGS) enables the precise identification of a species and ensures its safety with regard to antibiotic resistance (Chokesajjawatee et al., 2020). These cultures represent a significant area of interest for the food industry, contributing positively to food production. Additionally, it is now well-established that bacteria can communicate with each other through a mechanism known as Quorum Sensing. This mechanism regulates gene expression in response to changes in cell-population density. Bacteria sensitive to Quorum Sensing produce and release chemical signal molecules, known as autoinducers, which increase in concentration as the bacterial population grows (Miller and Bassler 2001). This mechanism is foundational for regulating diverse bacterial actions, including the formation of microbial biofilms.

5. *Wood characteristics*

Wood is the primary raw material for manufacturing equipment and utensils in dairy factories for many traditional cheeses (Aviat et al., 2016). Unfortunately, because wood is biodegradable, few historical artifacts provide evidences of its past use (Lortal et al., 2014). Wood is a noble material, primarily composed of cellulose (40-50%), hemicellulose (10-30%), lignin (15-30%), and minerals. It is a heterogeneous material with pronounced anisotropy and porosity. The presence of low molecular weight volatile and non-volatile compounds influences its properties, including acidity, hygroscopicity, colour, odour, and mechanical strength, making wood a durable material (Licitra et al., 2017).

The physical properties of wood, such as mechanical strength, elasticity, and thermal conductivity, are closely related to its moisture content below the fibre saturation point (FSP), which is typically between 28% and 30%. Due to its irregular surface (crevices, cracks, etc.) and high porosity, wood is challenging to clean, particularly in light of the hygiene standards set by regulations across various regions. Today, the use of wood is permitted by numerous regulations.

6. Legislation regarding wood in contact with dairy products

Despite wood never being implicated in any food-borne disease outbreaks, the Codex Alimentarius does not endorse its use in contact with food, raising questions about its safety over the years (Codex Alimentarius, 1993). In Europe, the first legislation on food contact materials was directive 76/893/EEC of 23 November 1976 (EEC,1976), which did not mention wood. This changed with Directive 89/109/CEE of 21 December 1988 (EEC,1989), which marked wood's inaugural mention in this context. Similarly, Decision 96/536/EC of 29 July 1996 (EEC,1996) concerning milk-based products with traditional characteristics allowed the use of tools and equipment, regardless of their material, provided they were maintained in a satisfactory state of cleanliness and subjected to regular cleaning and disinfection. This was later superseded by Regulation 1935/2004 (Commission Regulation, 2004), listing wood among other materials for which specific measures may be adopted, while allowing Member States to maintain or adapt national decisions. Currently, dairy production in Italy can utilize wooden equipment under EC no. 2074/2005 (Commission Regulation, 2005), which permits derogations from EC no. 852/2004 for foods with traditional characteristics. In 2014, FDA issued an alert

concerning the microbiological risk associated with aging Italian and French cheeses on wooden shelves. The porous structure of wood can absorb and trap bacteria, increasing the risk of contamination of food products (Cutini, 2014). After substantial opposition from the US artisan cheese community regarding market disruption for unprocessed artisanal cheeses and considering the impact on imports of European and Canadian cheeses typically aged on wooden shelves (such as Parmigiano Reggiano, Grana Padano, Pecorino Romano, Asiago, Comté, Beaufort, Reblochon, Munster, and Cantal), the FDA clarified that it had not banned or prohibited the practice of using wooden shelves for artisanal cheeses (Licitra et al., 2017). Currently, there is no evidence linking any foodborne illness to the use of wood, provided appropriate hygienic standards are maintained during production, storage, and application (Sun and D'Amico 2024).

7. Wooden equipment used for cheesemaking

Wood has a long-standing history in the production of traditional cheeses, with various studies highlighting its beneficial role in bio-protection against pathogens. The study by Mariani et al. (2007) demonstrated the potential of wood in inhibiting the growth of *Listeria monocytogenes.* Recent studies by Sun and D'amico (2024) further indicated that even when *L. monocytogenes* and *Escherichia coli* were intentionally introduced into the milk, their levels did not increase. This suggests that competition from other microorganisms and their metabolites present in wooden equipment inhibits the growth of *L. monocytogenes* and *E. coli*. The use of wood in cheesemaking not only aids in bio-protection, but also serves as a reservoir for microbial biodiversity, enhancing the quality, safety, and final characteristics of the cheese.

In recent years, numerous studies conducted by Italian, French, and American research groups have emphasized the crucial role of wooden vats in cheese production (Busetta et al., 2023; Didienne et al., 2012; Licitra et al., 2007; Lortal et al., 2009; Scatassa et al., 2015; Sun and D'Amico, 2021). These authors have demonstrated the safety and efficacy of biofilms established on wooden vats, which act as a natural inoculum, predominantly composed of indigenous strains of *S. thermophilus* capable of rapidly fermenting lactose (Picon et al., 2017). Thus, the use of wooden vats is a primary source of beneficial microorganisms in traditional dairy production.

The use of wooden shelves during the ripening stages of traditional cheeses significantly impacts the quality and safety of the final product. They provide an optimal environment for developing desirable flavours and textures. Various studies (Busetta et al., 2024; Gaglio et al., 2022; Guzzon et al., 2017; Mariani et al., 2007; Mariani et al., 2011; Quijada et al., 2018; Settanni et al., 2021; Wadhawan et al., 2021; Wadhawan et al., 2023) have consistently negated the microbiological risk associated with wooden shelves, substantiating their suitability for the ripening process. Mariani et al. (2011) demonstrated that biofilm on wooden ripening shelves of French Reblochon de Savoie cheese exhibited a stable anti-listeria effect under experimental conditions. This highlights the potential of wood as a bioprotective agent against foodborne pathogens. Furthermore, LAB colonising the biofilms of wooden shelves act as a barrier to foodborne pathogens. Gaglio et al. (2022) demonstrated this inhibitory activity in their characterisation of 18 wooden shelves from Sicilian dairies. The use of wood thus represents a safe system without any microbiological risk, facilitating the production of high-quality products with excellent sensory qualities.

8. Microbiol biofilm

It is well documented that planktonic (single-cell) microbial growth is uncommon in nature. The term ‘biofilm’ was first introduced in 1978, and since then, numerous studies has shown that biofilm associated microorganisms exhibit different gene transcription patterns compared to their planktonic forms (Vu et al., 2009). A biofilm is an aggregate of bacterial cells, algae, fungi, and protozoa enclosed in a self-produced matrix mainly consisting of extracellular polymeric substances (EPS). The matrix makes up between 50-90% of the total organic matter present in a biofilm. It includes exopolysaccharides, proteins, and extracellular DNA, which are produced through cell lysis (Whitchurch et al., 2002). Water, which constitutes 97% of the biofilm (Table 1), plays a pivotal role in the hydrodynamic transport of nutrients within the biofilm structure (Vu et al., 2009; Mgomi et al., 2023).

Table 1. Composition of the biofilm matrix.

Substances	Percentage
Water	>97%
Bacterial cells	2-5%
Polysaccharides	1-2%
Proteins (from cell lysis)	<1-2%
DNA and RNA	<1-2%
Ions	bound or free

Fonte: http://dstunisannio.it/sites/default/files/2019-11/3.Biofilm%20microbici%20p2.pdf.

The primary function of a biofilm is to protect microorganisms in hostile environments and to serve as a nutrient trap. EPS play a crucial role in determining the structure of the biofilm. Their concentration varies depending on the microorganism and increases as the biofilm matures. The EPS contain open aqueous channels that extend to each microcolony, facilitating nutrient delivery and waste removal. The biofilm matrix can make bacteria 10 to 1000 times more resistant to antibiotics compared to their planktonic form (Post et al., 2007). This biofilm aggregate is of significant interest in the food industry, as it is a primary source of contamination during food production, particularly due to its presence on equipment, pipes, and surfaces. Contaminations can render food unfit for consumption due to spoilage bacteria like *Pseudomonas* or pathogenic bacteria such as *Escherichia coli*, *Bacillus*, and *Clostridium*, which pose health risks to consumers. Therefore, it is important to manage and prevent biofilm formation (which occurs only under certain conditions such as the availability of favourable nutrients, temperature, and pH) to avoid such contamination (Ghirardello, 2013).

Biofilm typically develop on inorganic surfaces or dead organic tissue, with bacterial adhesiveness being greatest on hydrophobic and non-polar surfaces (Bendinger et al., 1993). Various biofilm types can form on surfaces, consisting of either a single bacterial species or a consortium of multiple microbial species. The formation of a single-species

biofilm depends on the EPS produced, which acts as an inhibitor for other microorganisms, preventing the attachment of susceptible bacteria and allowing only resistant ones to colonise. Conversely, if the EPS do not act as inhibitors, aggregation can occur even between different species. The formation of biofilms is influenced by environmental factors, and the entire system is regulated by Quorum Sensing. The biofilm formation process includes several steps: surface conditioning; surface adhesion; growth and colonisation of the surface; biofilm formation; detachment and dispersion of biofilm (Fig. 10).

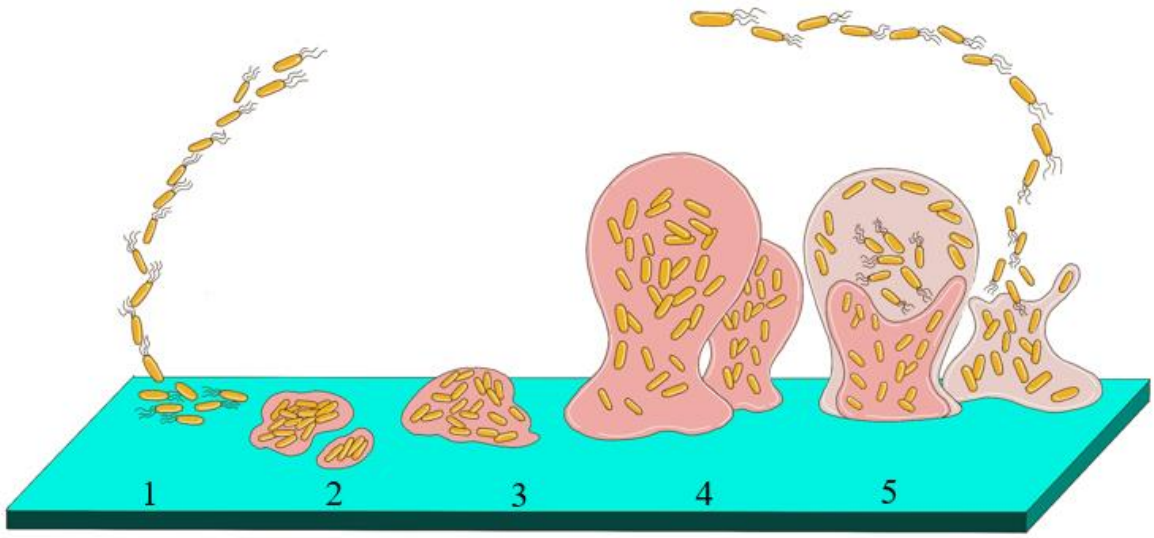

Fig. 10. Schematic cycle of biofilm development, divided into five phases.

8.1. Surface conditioning

When a solid material is submerged in a liquid, organic and inorganic molecules, along with bacteria, concentrate on the surface, forming a ‘conditioning film’. This leads to an accumulation of these molecules at the solid-liquid interface, resulting in a higher concentration of nutrients than in the fluid phase.

8.2. Surface adhesion

In the initial stages of development, bacteria exist in a free form and are sense environmental stimuli. They move towards nutrients, following the concentration gradient through chemotaxis. Bacterial cell surfaces are equipped with chemosensory systems that regulate movement and adhesion. Adhesion to a surface is influenced by the environment, substrate, and the bacteria themselves. The solid-liquid interface provides an ideal environment for microbial adhesion and growth. Factors such as pH, nutrient levels, cations (Na^+, $Ca2^+$, $Fe2^+$), and surface temperature can influence bacterial adhesion. Generally, biofilms adhere more rapidly to non-polar hydrophobic surfaces than to hydrophilic materials like glass or metal. Surface proteins called adhesins enables bacteria to bind to eukaryotic cell membranes or intercellular matrix proteins. Adhesion can be reversible or irreversible. It is reversible when bacteria initially adhere to surfaces or other microorganisms already attached. True intercellular adhesion makes the process irreversible.

8.3. Growth and colonisation of the surface

During the growth phase, bacteria produce polysaccharides, enabling them to anchor to the surface and facilitate colony growth. This process results in biofilm accumulation on the surface.

8.4. Biofilm formation

When nutrients are sufficiently available on the surface, cells replicate, forming microcolonies and initiating the heterogeneity typical of microbial biofilms. This phase achieves high cell density, triggering quorum sensing mechanisms. The synthesis of exopolysaccharides leads to forming a complex microbial biofilm structure, comprising

dense networks of channels that ensure nutrient distribution and waste removal, allowing bacteria in the deeper regions to survive.

8.5. Detachment and dispersion of biofilm

Once the biofilm reaches a specific size, it tends to break down mechanically, releasing floccules or cells that can revert to a planktonic condition, starting a new biofilm life cycle.

9. Properties of microbial biofilms

Microbial biofilms offer several advantages to bacteria, including protection from external chemical, physical, and biological agents, as well as the retention of water and nutrients (Ghirardello, 2013). These properties are of particular importance in the dairy industry, where biofilms can be leveraged for their metabolic activities. Numerous species of microorganisms, including Gram-positive and Gram-negative bacteria, yeasts, and moulds, have been identified in dairy environments. These microorganisms are present in raw milk and exhibit a different diversity and quantity of species compared to starter cultures or industrial cultures used in cheese production.

The variations in microbiota contribute to the diversity of raw milk cheeses, resulting in more intense flavour and odour than cheeses made from pasteurized milk. An abundance of native microbiota can lead to more complex flavour profiles and higher sensory evaluations in raw milk cheeses. Traditional raw milk cheeses also possess the ability to inhibit the growth of pathogenic microorganisms, primarily due to the presence of antimicrobial strains within the milk. LAB, for instance, can reduce fermentable sugars and lower the pH value by producing primary metabolites with antimicrobial activity. These

conditions inhibit the growth of pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. Additionally, the acidification of wooden surfaces and dense colonization of wooden equipment by bacteria can inhibit pathogens by limiting nutrient availability (Ghirardello, 2013).

References

Aviat, F., Gerhards, C., Rodriguez-Jerez, J.J., Michel, V., Bayon, I.L., Ismail, R., Federighi, M., 2016. Microbial safety of wood in contact with food: a review. Compr. Rev. Food Sci. Food Saf. 15 (3), 491–505.

Barbosa, J., Albano, H., Silva, B., Almeida, M.H., Nogueira, T., Teixeira, P., 2021. Characterization of a *Lactiplantibacillus plantarum* R23 isolated from Arugula by whole-genome sequencing and its bacteriocin production ability. Int. J. Environ. Research Public Health, 18 (11), 5515. https://doi.org/10.3390/ijerph18115515.

Bendinger, B., Rijnaarts, H.H., Altendorf, K., Zehnder, A.J., 1993. Physicochemical cell surface and adhesive properties of coryneform bacteria related to the presence and chain length of mycolic acids. Appl. Environ. Microbiol. 59 (11), 3973–3977. https://doi.org/10.1128/aem.59.11.3973-3977.1993.

Busetta, G., Gaglio, R., Mangione, G., Garofalo, G., Franciosi, E., Gannuscio, R., Caccamo, M., Todaro, M., Di Gerlando, R., Settanni, L., Licitra, G., 2023. Effect of commission implementing regulation (EU) 2020/1319 on the bacterial composition of PDO Provola dei Nebrodi cheese. Int. J. Food Microbiol. 394, 110188. https://doi.org/10.1016/j.ijfoodmicro.2023.110188.

Busetta, G., Garofalo, G., Claps, S., Sardina, M.T., Franciosi, E., Alfonzo, A., Francesca, N., Moschetti, G., Settanni, L., Gaglio, R., 2024. The wooden shelf surface and cheese rind mutually exchange microbiota during the traditional ripening process.Int. J. Food Microbiol. 409, 110478. https://doi.org/10.1016/j.ijfoodmicro.2023.110478.

Chokesajjawatee, N., Santiyanont, P., Chantarasakha, K., Kocharin, K., Thammarongtham, C., Lertampaiporn, S., Vorapreeda, T., Srisuk, T., Wongsurawat, T., Jenjaroenpun, P., Nookaew, I., Visessanguan, W., 2020. Safety assessment of a nham starter culture *Lactobacillus plantarum* BCC9546 via whole-genome analysis. Sci. Rep. 10, 10241. https://doi.org/10.1038/s41598-020-66857-2.

Codex Alimentarius Commission., 1993. Report of the Twenty-Sixth Session of the Codex Committee on Food Hygiene, Washington D.C., 1–5 March 1993. Codex Alimentarius Commission, Rome, Italy.

Corradini, C., 1995. Chimica e tecnologia del latte. (Eds.), Tecniche nuove, Milan, Italy.

Cutini, F., 2014. (Food: Divina (LN), the FDA Declares War on Our Chesees, AgenParl, Roma, Italy). Personal communication.

Didienne, R., Defargues, C., Callon, C., Meylheuc, T., Hulin, S., Montel, M.C., 2012. Characteristics of microbial biofilm on wooden vats (‘gerles’) in PDO Salers cheese. Int. J. Food Microbiol. 156 (2), 91–101.

Commission Regulation, 2004. CE Regulation 853 (Alleg. II, Sez. IX, Cap. I, Parag. III). https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32004R0853 Accessed 09.02.2023.

Commission Regulation, 2005. Commission regulation no 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union 338:1–26.

European Union. 1976. Council Directive 76/893/EEC of 23 November 1976 on the approximation of the laws of the Member States relating to materials and articles intended to come into contact with foodstuffs. Off J Eur Union L 340, pp 19–24.

European Union. 1989. Council Directive 89/109/EEC of 21 December 1988 on the approximation of the laws of the Member States relating to materials and articles intended to come into contact with foodstuffs. Off J Eur Union L 040, pp 38–44

European Union. 1996. Commission Decision 96/536/EC of 29 July 1996 establishing the list of milk-based products in respect of which Member States are authorized to grant individual or general derogations pursuant to Article 8 (2) of Directive 92/46/EEC and the nature of the derogations applicable to the manufacture of such products (Text with EEA relevance). Off J Eur Union L 230, pp 12–15.

Franciosi, E., Settanni, L., Cologna, N., Cavazza, A., Poznanski, E., 2011. Microbial analysis of raw cows' milk used for cheese-making: Influence of storage treatments on microbial composition and other technological traits. World J. Microbiol. Biotechnol, 27, 171–180. https://doi.org/10.1007/s11274-010-0443-2.

Fox, P.F., Guinee, T.P., Cogan, T.M., McSweeney, P.L.H., 2017. Pathogens in Cheese and Foodborne Illnesses. Fundamental of Cheese Science. In: Fox, P.F., Guinee, T.P., Cogan, T.M., McSweeney, P.L.H., (Eds.), Springer, New York, USA, pp. 681–713.

Fox, P.F., McSweeney, P.L.H., Cogan T.M., Guinee T.P., 2004. Cheese: Chemistry, Physics and Microbiology. (3rd Eds.), Elsevier Academic Press, London, UK.

Fox, P.F., Wallace, J.M., 1997. Formation of flavour compounds in cheese. Adv. Appl. Microbiol. 45, 17–85. https://doi.org/10.1016/S0065-2164(08)70261-2.

Gaglio, R., Busetta, G., Gannuscio, R., Settanni, L., Licitra, G., Todaro, M., 2022. A multivariate approach to study the bacterial diversity associated to the wooden shelves used for aging traditional Sicilian cheeses. Foods,11 (5), 774. https://doi.org/10.3390/foods11050774.

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. J. Dairy Sci. 97, 573– 591. https://doi.org/10.3168/jds.2013-7187.

Ghirardello, M., 2013. Redazione di una Procedura Operativa Standard per il "CDCBiofilm Reactor". Ph.D. thesis, unipd, Padova, Italy. http://tesi.cab.unipd.it/51086/1/Ghirardello_Mattia.pdf.

Gobbetti, M., Di Cagno, R., Calasso, M., Neviani, E., Fox, P.F., De Angelis, M., 2018a. Drivers that establish and assembly the lactic acid bacteria biota in cheeses. Trends Food Sci. Technol. 78, 244–254.

Gobbetti, M., Neviani, E., Fox, P., 2018b. The origins of cheesemaking. In The cheeses of Italy: Science and Technology. (1st Eds.), Springer International Publishing, Cham, Switzerland, pp. 1–12.

Guzzon, R., Carafa, I., Tuohy, K., Cervantes, G., Vernetti, L., Barmaz, A., Larcher, R., Franciosi, E., 2017. Exploring the microbiota of the red-brown defect in smear-ripened cheese by 454-pyrosequencing and its prevention using different cleaning systems. Food Microbiol. 62, 160–168. https://doi.org/10.1016/j.fm.2016.10.018.

Hemme, D.H., Schmal, V., Auclair, J.E., 1981. Effect of the addition of extracts of thermophilic lactobacilli on acid production by *Streptococcus thermophilus* in milk. J. Dairy Res. 51, 345–351. https://doi.org/10.1017/S0022029900021555.

Ibrahim, S.A., Ayivi, R.D., Zimmerman, T., Siddiqui, S.A., Altemimi, A.B., Fidan, H., Esatbeyoglu, T., Bakhshayesh, R.V., 2021. Lactic acid bacteria as antimicrobial agents: Food safety and microbial food spoilage prevention. Foods, 10 (12), 3131. https://doi.org/10.3390/foods10123131.

ISMEA, 2023. Rapporto sulle produzioni agroalimentari e vitivinicole italiane DOP, IGT e STG. Available online: http:// https://www.ismea.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/12678.

Kandler, O., Weiss, N., 1986. Genus Lactobacillus Beijerinck 1901, 212AL. In Sneath, P.H.A., Mair, N. S., Sharpe, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology. Williams & Wilkins, Baltimore, USA, pp. 1209–1234.

Kastner, S., Perreten, V., Bleuler, H., Hugenschmidt, G., Lacroix, C., Meile, L., 2006. Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. Syst. Appl. Microbiol. 29 (2), 145–155. https://doi.org/10.1016/j.syapm.2005.07.009.

Kindstedt, P.S., 2012. Cheese and Culture: A History of Cheese and Its Place in Western Civilization. (1st Eds.), Chelsea Green Publishing Company: Hartford, USA.

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., Cesare, A.D., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cocconcelli, P.S., Escámez, P.S.F., Maradona, M.P., Querol, A., Suarez, J.E., Sundh, I., Vlak, J., Barizzone, F., Correia, S., Herman, L., 2020. Scientific opinion on the update of the list of QPS

recommended biological agents intentionally added to food or feed as notified to EFSA (2017– 2019). EFSA J., 18, e05966. 38. https://doi.org/10.2903/j.efsa.2020.5966.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15, 67–78. https://doi.org/10.1016/j.tifs.2003.09.004.

Licitra, G., 2010. World wide traditional cheeses: Banned for business? Dairy Sci. Technol. 90(4), 357–374. https://doi.org/10.1051/dst/2010016.

Licitra, G., Caccamo, M., Valence, F., Lortal, S., 2017. Traditional wooden equipment used for cheesemaking and their effect on quality. Global Cheesemaking Technology: Cheese Quality and Characteristics, 157–172. https://doi.org/10.1002/9781119046165.ch0g

Licitra, G., Carpino, S., 2014. The microfloras and sensory profiles of selected protected designation of origin Italian cheeses. Microbiol. Spectr. 2(1), 10–1128. https://doi.org/10.1128/microbiolspec.cm-0007-2012

Licitra, G., Ogier, J.C., Parayre, S., Pediliggieri, C., Carnemolla, T.M., Falentin, H., Madec, M.N., Carpino, S., Lortal, S., 2007. Variability of bacterial biofilms of the "tina" wood vats used in the Ragusano cheese-making process. Appl. Environ. Microbiol. 73 (21), 6980–6987. https://doi.org/10.1128/AEM.00835-07.

Lortal, S., Di Blasi, A., Madec, M. N., Pediliggieri, C., Tuminello, L., Tanguy, G., Fauquant, J., Lecuona, Y., Campo, P., Carpino, S., Licitra, G., 2009. Tina wooden vat biofilm: a safe and highly efficient lactic acid bacteria delivering system in PDO Ragusano cheese making. Int. J. Food Microbiol. 132 (1), 1-8. https://doi.org/10.1016/j.ijfoodmicro.2009.02.026.

Lortal, S., Licitra, G., Valence, F., 2014. Wooden tools: reservoirs of microbial biodiversity in traditional cheesemaking. Microbiol. Spectr. 2 (1), 101–128. https://doi.org/10.1128/microbiolspec.cm-0008-2012.

Mariani, C., Briandet, R., Chamba, J.F., Notz, E., Carnet-Pantiez, A., Eyoug, R.N., Oulahal, N., 2007. Biofilm ecology of wooden shelves used in ripening the French raw milk smear cheese Reblochon de Savoie. J. Dairy Sci. 90 (4), 1653–1661.

Mariani, C., Oulahal, N., Chamba, J.F., Dubois-Brissonnet, F., Notz, E., Briandet, R., 2011. Inhibition of *Listeria monocytogenes* by resident biofilms present on wooden shelves used for cheese ripening. Food Control 22 (8), 1357–1362.

McMahon, D. J., Oberg, C. J., 2017. Pasta-Filata Cheeses. In McSweeney P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W. (4th Eds.), Cheese. Academic Press, San Diego, USA, pp. 1041–1068.

Mgomi, F.C., Yang, Y.R., Cheng, G., Yang, Z.Q., 2023. Lactic acid bacteria biofilms and their antimicrobial potential against pathogenic microorganisms. Biofilm, 5, 100118. https://doi.org/10.1016/j.bioflm.2023.100118.

Miller, M.B., Bassler, B.L., 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. 55(1), 165-199. https://doi.org/10.1146/annurev.micro.55.1.165. https://doi.org/10.1146/annurev.micro.55.1.165.

Ottolini, G., 1787. Prodromo dell'abate Gerolamo Ottolini, intorno alla maniera di migliorare la fabbrica de' formaggi. In Ottolini, G., (Eds.), Forgotten books, London, UK. pp. 123–148.

Parente, E., Cogan, T.M., 2004. Starter cultures: general aspects. In Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P. (Eds.), Cheese: chemistry, physics and microbiology. Elsevier, Amsterdam, Netherlands, pp. 123–148.

Picon, A., 2017. Cheese microbial ecology and safety. In Papademas, P., Bintis, T. (Eds.), Global cheesemaking technology: Cheese quality and characteristics. Wiley & Sons, New York, USA, 71–99. https://doi.org/10.1002/9781119046165.ch0d.

Post, J.C., Hiller, N.L., Nistico, L., Stoodley, P., Ehrlich, G.D., 2007. The role of biofilms in otolaryngologic infections: update 2007. Curr. Opin Otolaryngol. Head Neck Surg. 15 (5), 347–351. https://10.1097/MOO.0b013e3282b97327

Quijada, N.M., Dzieciol, M., Schmitz-Esser, S., Wagner, M., Selberherr, E., 2022. Metatranscriptomic analyses unravel dynamic changes in the microbial and metabolic transcriptional profiles in artisanal Austrian hard-cheeses during ripening. Front. Microbiol. 13, 813480. https://doi.org/10.3389/fmicb.2022.813480.

Salvadori del Prato., 1998. Trattato di Tecnologia Casearia, 1st (Eds.), Edagricole, Bologna, Italy.

Scatassa, M.L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., Di Grigoli, A., Moschetti, G., Settanni, L., 2015. Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol. 52, 31–41. https://doi.org/10.1016/j.fm.2015.06.008.

Settanni, L., Moschetti, G., 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiol. 27, 691–697. https://doi.org/10.1016/j.fm.2010.05.023.

Settanni, L., Puccio, V., Busetta, G., Licitra, G., Franciosi, E., Botta, L., Di Gerlando, R., Todaro, M., Gaglio, R., 2021. In-Depth Investigation of the Safety of Wooden Shelves Used for Traditional Cheese Ripening. Appl. Environ. Microbiol. 87, e01524-21. https://doi.org/10.1128/AEM.01524-2.

Sun, L., D'Amico, D.J., 2021. Composition, succession, and source tracking of microbial communities throughout the traditional production of a farmstead cheese. MSystems 6 (5), 101–128. https://doi.org/10.1128/msystems.00830-21.

Sun, L., D'Amico, D.J., 2024. Population dynamics and bidirectional transfer of *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* during cheese production in wooden vats. Food Microbiol. 120, 104483. https://doi.org/10.1016/j.fm.2024.104483.

Syrokou, M.K., Paramithiotis, S., Drosinos, E.H., Bosnea, L., Mataragas, M., 2022. A comparative genomic and safety assessment of six *Lactiplantibacillus plantarum* subsp. *argentoratensis* strains isolated from spontaneously fermented Greek wheat sourdoughs for potential biotechnological application. Int. J. Mol. Sci. 23 (5), 2487.

Vu, B., Chen. M., Crawford. R.J., Ivanova, P.E., 2009. Bacterial extracellular polysaccharides involved in biofilm formation. Molecules 14 (7) 2535–2554. https://doi.org/10.3390/molecules14072535.

Wadhawan, K., Steinberger, A.J., Rankin, S.A., Suen, G., Czuprynski, C.J., 2021. Characterizing the microbiota of wooden boards used for cheese ripening. JDS Communications, 2 (4), 171–176. https://doi.org/10.3168/jdsc.2020-0014.

Wadhawan, K., Steinberger, A., Rankin, S., Suen, G., Czuprynski, C., 2023. Inhibition of *Listeria monocytogenes* by broth cultures of surface microbiota of wooden boards used in cheese ripening. Appl. Sci. 13(10), 5872. https://doi.org/10.3390/app13105872.

Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C., Mattick, J.S., 2002. Extracellular DNA required for bacterial biofilm formation. Science 295 (5559), 1487–1487. https://10.1126/science.295.5559.1487.

Objective and organisation of the research project

In recent years, the demand for traditional dairy products has surged considerably. Modern consumers are increasingly seeking unique tastes and flavors rooted in specific cultural, historical, and geographical contexts. Traditional cheeses, in particular, hold a prominent place in this trend as they are cherished globally in their many varieties. These cheeses are often seen as small “works of art”, reflecting the distinctive and irreplaceable qualities of their regions of origin. Cheese symbolizes a unique connection between humanity and the land, blending artistry and biodiversity, especially when made from raw milk and using traditional wooden tools that impart rich enzymes during production.

During the three-year PhD program, the activities were carried out within the objectives of the project “Development of a synergy model aimed at qualifying and valorising the natural historical cheeses of southern Italy in the regions of Sicily, Sardinia, Basilicata, Calabria and Campania (Canestrum casei)” funded by AGER (Agri-food and reserach) - Foundations in network for agro-food research. The primary objective was to valorise the typical cheeses of southern Italy. In the first phase, research activities focused on the microbiological, chemical-physical and sensory characterization of two typical southern Italian cheeses: PDO Provola dei Nebrodi and PDO Caciocavallo Podolico. In a second phase, the wooden shelves used for ripening traditional cheeses were studied. In particular, these tools used for ripening three medium-aged Sicilian cheeses (PDO Pecorino Siciliano, PDO Piacentinu Ennese and Caciocavallo Palermitano) were investigated using a multivariate statistical approach to identify relationships between the bacterial diversity of the wooden shelves and the type of cheese. Additionally, the mutual microbial transfer between the rind and the wooden shelves used for ripening two Lucanian cheeses (PDO

Pecorino di Filiano and PGI Canestrato di Moliterno) was also evaluated. In a third phase, aiming to improve natural historical cheeses, replacing whey permeate with hot water was tested to accelerate the production of PDO Pecorino Siciliano by reducing the resting time before cooking. Our efforts culminated in several data-rich papers published in international journals, showcasing the collaborative work of my research group.

Part I

Microbial and quality characteristics of traditional stretched cheese

In this section, we focused on two traditional stretched cheeses produced with wooden equipment. Typically, traditional stretched curd cheeses are made in two phases: curd formation and acidification, followed by stretching the acidified curd into its final form. The acidification process is carried out by LAB. Since starter cultures are not deliberately added in traditional cheese production, acidification relies on LAB present in raw milk, on wooden equipment surfaces, and even in animal rennet. These LAB quickly convert milk lactose into lactic acid and thus as SLAB. NSLAB from the same sources are also transferred to the curd, contributing to ripening and giving the final cheeses the typical aromatic profile.

During the three years of PhD research, two traditional cheeses were produced and analyzed with wooden equipment such as PDO Provola dei Nebrodi and PDO Caciocavallo Podolico. To gain deeper insights into the microbial populations on the wooden equipment, the wooden vats used for cheese processing were also included in the study and their micobiological influence on the final cheese quality was evaluated. Furthermore, the final cheeses were studied for their chemical composition and sensory characteristics.

Chapter I

Effect of commission implementing regulation (EU) 2020/1319 on the bacterial composition of PDO Provola dei Nebrodi cheese

The present chapter has been published in

International Journal of Food Microbiology, 394

2023

Abstract

In this study, PDO Provola dei Nebrodi cheese was deeply characterized for its bacterial community and chemical composition. Four dairy factories (A – D) were monitored from milk to ripened cheese. Wooden vat biofilms were dominated by thermophilic rod LAB (4.6 – 6.5 log CFU/cm^2). Bulk milk showed consistent levels of total mesophilic microorganisms (TMM) (5.0 – 6.0 log CFU/mL) and, after curdling, a general increase was recorded. The identification of the dominant LAB in wooden vat biofilms and ripened cheeses showed that the majority of wooden vat LAB were lactococci and *Streptococcus thermophilus*, while cheese LAB mainly belonged to *Lacticaseibacillus paracasei* and *Enterococcus*. Illumina sequencing identified 22 taxonomic groups; streptococci, lactococci, lactobacilli and other LAB constituted the majority of the total relative abundance % of the wooden vat (69.01 – 97.58%) and cheese (81.57 – 99.87%) bacterial communities. Regarding chemical composition, the effect of dairy factories was significant only for protein content. Inside cheese color was lighter and yellower than surface. Differences in fatty acids regarded only myristic acid and total amount of monounsaturated fatty acids. The sensory evaluation indicated some differences among cheeses produced in the four dairies regarding color, homogeneity of structure, overall intensity, salty, spicy, and hardness. The integrated approach applied in this study showed that PDO Provola dei Nebrodi cheese characteristics are quite stable among the dairy factories analyzed and this has to be unavoidably imputed to the application of the same cheese making protocol among different dairies.

Keywords: antioxidant properties; cheese chemistry; Illumina; lactic acid bacteria; stretched cheese; wooden equipment.

Introduction

The Sicily is a southern Italian region where 33 traditional cheeses are produced, but only five of them enjoy a protected denomination of origin (PDO) status: Vastedda della valle del Belìce, Pecorino Siciliano, Piacentinu Ennese, Provola dei Nebrodi, and Ragusano. Among them, Provola dei Nebrodi cheese obtained the PDO recognition very recently, in light of Commission implementing regulation (EU) 2020/1319 of 22 September 2020, (G.U.R.I., 2020).

Provola dei Nebrodi PDO is a stretched cheese produced with raw whole cows' milk, coming from farms located in the Nebrodi area (Sicily), according to the product specifications described in the Production Regulations. The product shows a typical pear-shaped form tended to the oval, with a weight ranging from 1 to 10 kg and, depending on the aging duration, a straw yellow to intense yellow color. This cheese is produced in five different varieties (Busetta et al., 2021).

The entire cheese production process is characterized by the use of traditional wooden tools, namely "tina" (wooden vat), "ruotula" (wooden paddle used to break the curd), "piddiaturi" (wooden container), "manuvedda" (flattened wooden staff used for cutting) and mastredda (a large wooden open-topped board on which curd is traditionally left to acidify at room temperature for about 24 h) which, due to the porous structure of wood, facilitate absorption and trapping of the native dairy microbiota (Cruciata et al., 2019). In general, when no starter culture is added, curd acidification and maturation of traditional Sicilian cheeses depend exclusively on the adventitious microorganisms present in raw milk (Guarcello et al., 2016), in the animal rennet paste added for coagulation (Cruciata et al., 2014), onto the surface of the wooden equipment used during transformation (Di

Grigoli et al., 2015; Licitra et al., 2007), and in the dairy factory environment (Cruciata et al., 2019). In order to produce a given ripened cheese, the microbial community has to include lactic acid bacteria (LAB) because they perform the acidification of the curd (starter LAB) and contribute to the development of the typical sensory traits (non-starter LAB) (Grujović et al., 2022). Thus, the wooden equipment used to transform milk into traditional Sicilian cheeses are defining to transfer desired starter and non-starter LAB (Carpino et al., 2017; Di Grigoli et al., 2015). These bacteria produce extracellular polymeric substances (EPS) and form an aggregate known as "biofilm" (Azeredo et al., 2017).

The wooden vats used for milk curdling have been thoroughly characterized for their dairy LAB biofilms (Licitra et al., 2007; Lortal et al., 2009; Scatassa et al., 2015). Furthermore, those investigations did not reveal the presence of pathogenic bacteria. Regarding PDO Provola dei Nebrodi cheese production, so far, only "mastredda" has been microbiologically investigated and found to host several viable LAB belonging to *Enterococcus*, *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* genera (Busetta et al., 2021). In general, the whole production process of traditional Sicilian cheeses relies on wooden tools, from milking until ripening and even the wooden shelves used for ripening have been found to host harmless species belonging to cheese-surface-ripening groups, halophilic and moderately halophilic bacteria and LAB, confirming that all wooden equipment used to produce these cheeses are safe systems (Settanni et al., 2021).

In order to provide further insights on the microbiological significance of the wooden tools used to produce PDO Provola dei Nebrodi cheese, in this study the wooden vats used

during production were characterized by a combined culture-dependent and –independent approach and their influence was evaluated until ripened cheeses which were investigated for the total microbial diversity and for the dominant strains. The final cheeses were also investigated for their chemical composition as well as sensory traits.

Materials and methods

Cheese production and sample collection

Four dairy factories (A – D) located in the countryside of Randazzo and Maniace (Catania province), producing exclusively PDO Provola dei Nebrodi cheeses, were monitored during the entire production cycle from milking until the third month of ripening. Cheese production occurred in all four factories applying strictly the protocol established for the protection of this traditional product from raw cows' milk processed in wooden vats without the addition of starter cultures and curdled with animal rennet paste (G.U.R.I., 2020). The flowsheet of PDO Provola dei Nebrodi cheese is reported in Fig. 1. The characteristics of the wooden vats of the four factories were identical: made from douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] wood, activated contemporarily following the common protocol with hot water, coarse salt brushing and deproteinized whey contact (Cruciata et al., 2018) and used for three years. In each factory, cheese production was followed twice, the first was carried out in February and the second in March 2021. The wooden vat biofilms were sampled before milk contact following the brushing recovery method described by Didienne et al. (2012) using 100 cm^2 sterile plastic squares (Biogenetics s.r.l., Padua, Italy).

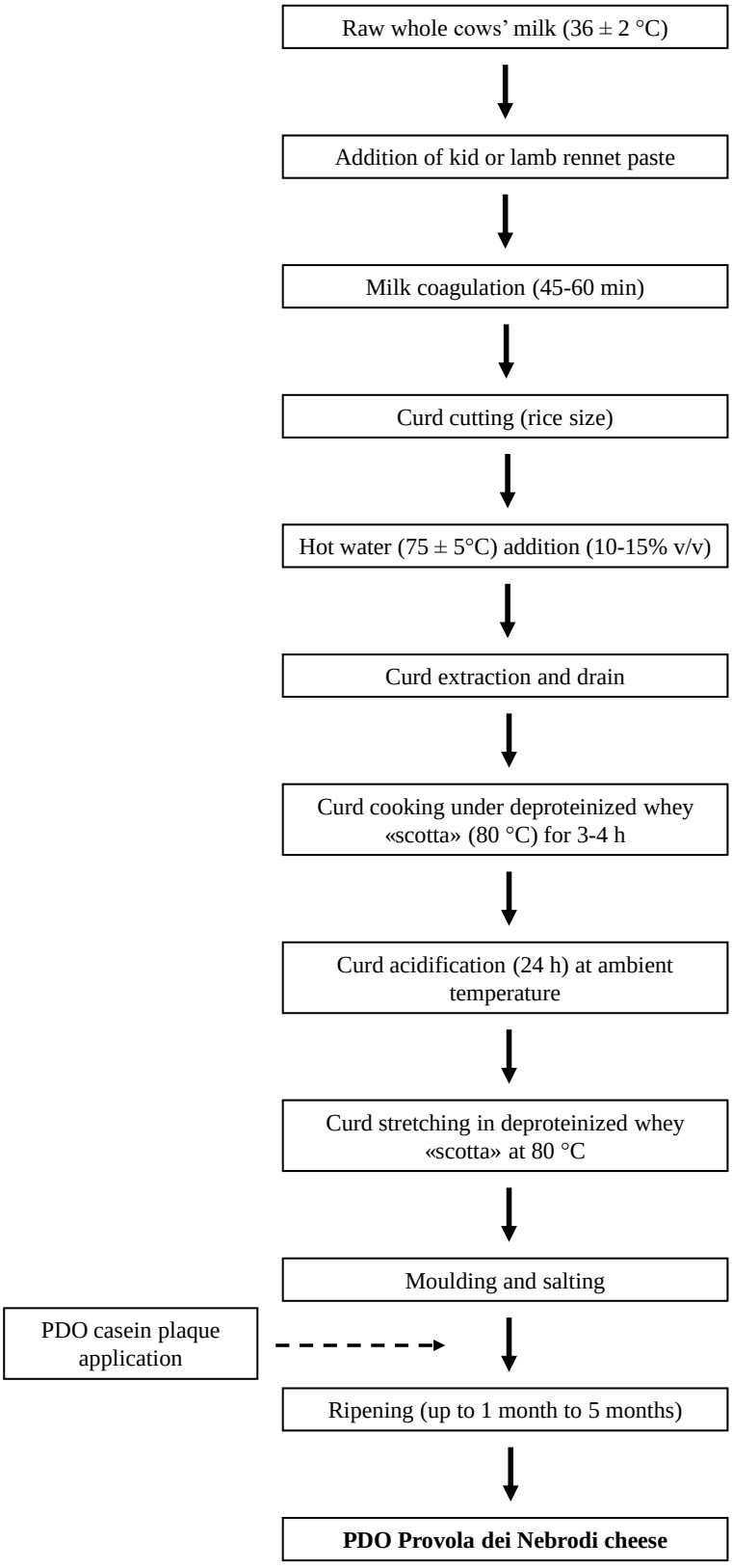

Fig.1. Flowsheet of PDO Provola dei Nebrodi cheese production.

In each factory, cheese production was followed twice, the first was carried out in February and the second in March 2021. The wooden vat biofilms were sampled before milk contact following the brushing recovery method described by Didienne et al. (2012) using 100 cm^2 sterile plastic squares (Biogenetics s.r.l., Padua, Italy). Delimited areas were brushed with a sterile toothbrush and microorganisms recovered by a sterile gauze as reported by Settanni et al. (2021). Bulk milk (500 L in factories A – C and 350 L in factory D) was sampled before being transferred into the wooden vat and after 5 min from transfer. During the contact with the vat surfaces, milk was kept under gentle manual agitation performed with the typical wooden stick used for curd breaking. Milk coagulation occurred with 30 g of lamb rennet paste for 100 L of milk, in order to obtain a firm coagulum in about 60 min. Curd acidification, molding and ripening were carried out as reported by PDO disciplinary for production (G.U.R.I., 2020).

The samples of wooden vat biofilms, bulk milk before and after wood contact, curd, acidified curd, stretched curd and 3-month ripened cheeses were transported under refrigeration in insulated boxes containing reusable ice packs to the laboratories of Agricultural Microbiology of University of Palermo.

Microbiological analysis and LAB isolation

All samples collected during PDO Provola dei Nebrodi cheese production, including vat biofilms, as well as 3-month ripened cheeses were subjected to the decimal serial dilution. In particular, the wooden vat biofilms were diluted in Ringer's solution; the first dilution of these samples was obtained from 1 mL of the cell suspensions deriving from toothbrush

and gauze. Milk samples (1 mL) were also diluted in Ringer's solution, while curd and cheese samples (15 g) were first homogenized in 2% (*w/v*) sodium citrate solution (135 mL) using a stomacher (Bag-Mixer 400, Interscience, Saint Nom, France) at the maximum speed for 2 min and then serially diluted as reported above in Ringer's solution.

Cell suspensions were plated on agar media to allow the development of total mesophilic microorganisms (TMM), total psychrotrophic microorganisms (TPM), members of the Enterobacteriaceae family, total coliforms, *Escherichia coli*, *Salmonella* spp., coagulase-positive staphylococci (CPS), *Listeria monocytogenes*, pseudomonads, thermophilic and mesophilic LAB cocci and rods, enterococci, yeasts, molds as reported by Gaglio et al. (2021a). LAB incubation occurred in anaerobiosis in hermetically sealed jars equipped with the AnaeroGen AN25 sachets. All media, supplements and the anaerobic gas generating sachets were purchased from Oxoid (Basingstoke, England). All microbiological counts were carried out in duplicates for all samples at each collection time.

Phenotypic and genetic characterization of LAB

Presumptive LAB colonies developed from cell suspensions of wooden vat biofilm and ripened cheese samples were isolated at the highest dilutions plated. Colonies (at least five) sharing the same morphological characteristics (width, thickness, shape, color and opaqueness, uniformity of structure and margin) were picked up from MRS, M17 and WBAM, considering all different morphologies, and transferred into the corresponding broth media. After growth at the optimal incubation conditions, the isolates were streaked repeatedly onto agar media until reaching the uniformity of

colonies. Purified bacteria were further phenotypically characterized following the procedure described by Gaglio et al. (2014).

All putative LAB were further processed by genetic tools. Overnight grown cultures were used to extract genomic DNAs by means of the DNA-SORB-B kit (Sacace Biotechnologies Srl, Como, Italy) applying manufacturer's guidelines. Strain differentiation was obtained through random amplification of polymorphic DNA (RAPD)-PCR analysis that is used as a fingerprinting for bacteria; to this purpose, each DNA was subjected to the amplification by single primers M13, AB111, and AB106 (Gaglio et al., 2017). The resulting polymorphic profiles were analyzed by GelCompar II software version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium). This program allows to generate a dendrogram to evaluate the similarity among the LAB isolates. The isolates sharing high similarity levels of their RAPD patterns were considered to be the same strain.

Furthermore, the different strains were subjected to the rRNA gene sequencing analysis using the 16S rRNA fragment as target sequence. The analysis was performed using the primers rD1/fD1 as described by Weisburg et al. (1991). The final sequences were loaded in the database available at http://www.ncbi.nlm.nih.gov (GenBank/EMBL/DDBJ) and http://eztaxon-e.ezbiocloud.net/ (EzTaxon-e). The doubtful identity of *Enterococcus* was resolved by the *sodA* gene-based multiplex PCR assay of Jackson et al. (2004).

Culture-independent analysis of total bacterial community

Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system (Illumina, USA) were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy) as described by Gaglio et al. (2020).

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative bacterial sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh and Standley, 2013; Price et al., 2009). For bacteria, taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database which had been previously trimmed to the V3 - V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by MiSeq Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. Number PRJNA893874.

Physicochemical characterization, antioxidant properties and oxidative stability of final cheeses

Cheese samples were freeze-dried and analyzed for dry matter (DM), fat, protein (N x 6.38), and ash content as reported by Bonanno et al. (2019). Soluble nitrogen (N) was determined on an aqueous filtrate using the Kjeldahl method (MAF, 1986), water activity was determined at 23 °C at the surface of each sample slice by using an activity-meter instrument (Rotronic Int., USA). Cheese samples were assessed for core and under ring color, measured in duplicate by a Minolta Chroma Meter CR300 (Minolta, Osaka, Japan) and for hardness by an Instron 5564 tester (Instron, Trezzano sul Naviglio, Milan, Italy) as reported by Gaglio et al. (2021b).

The antioxidant activity of PDO Provola dei Nebrodi cheeses followed in this study was evaluated by TEAC assay, according to Re et al. (1999) and Bonanno et al. (2019). The method applied is based on a decolorization assay that measures the radical scavenging ability of samples using the ABTS radical cation (ABTS•+), and Trolox as standard. A Trolox solution in phosphate-buffered saline, ranging from 0 to 2.5 mM, was used to develop a calibration curve (R^2 = 0.99), and the results were expressed as mmol Trolox/kg DM of cheese.

Regarding the oxidative stability of PDO Provola dei Nebrodi cheeses, the thiobarbituric acid–reactive substances (TBARS) as a measure of secondary lipid oxidation was evaluated according to the methods proposed by Tarladgis et al. (1960) and modified by Mele et al. (2011). To quantify TBARS, 1,1,3,3-tetramethoxypropane solutions at concentrations ranging from 0.016 to 0.165 µg/mL were used for the

calibration curve (R^2 = 0.99). Results were expressed as µg malonylaldehyde (MDA)/kg DM of cheese.

Cheese fatty acid profile determination

Fatty acids (FA) in lyophilized cheese samples (100 mg) were directly methylated in 1 mL hexane with 2 mL 0.5 M $NaOCH_3$ at 50 °C for 15 min, followed by 1 mL 5% (v/v) HCl in methanol at 50 °C for 15 min, based on the bimethylation procedure described by Lee and Tweed (2008). Fatty acid methyl esters (FAME) were recovered in 1.5 mL hexane. One microliter of each sample was injected by means of an autosampler, into an HP 6890 gas chromatography system equipped with a flame-ionisation detector (Agilent Technologies, Santa Clara, CA, USA). FAME from each sample were separated using a CP-Sil 88 capillary column (100 m long, 0.25 mm internal diameter, 0.25 µm film thickness) (Chrompack, Middelburg, The Netherlands). The injector temperature was kept at 255 °C and the detector temperature was kept at 250 °C, with hydrogen flow of 40 mL/min, air flow of 400 mL/min, and a constant helium flow of 45 mL/min. The initial oven temperature was held at 70 °C for 1 min, increased by 5 °C/min to 100 °C, held for 2 min, increased by 10 °C/min to 175 °C, held for 40 min, then finally increased by 5 °C/min to a final temperature of 225 °C held for 45 min. Helium, with a pressure of 158.6 kPa and a flow rate of 0.7 mL/min (linear velocity 14 cm/s), was used as carrier gas. A FAME hexane mix solution (Nu-Check-Prep, Elysian, MN, USA) was used to identify each FA. Individual standards (Larodan Fine Chemicals AB, Malmö, Sweden) were used to identify some branched FA, as C15:0 iso, C15:0 anteiso, C17:0 iso, and C17:0 anteiso.

To quantify total FA, C23:0 (Sigma-Aldrich, Milan, Italy) was used as internal standard (4 mg/g lyophilized cheese).

Sensory analysis

The sensory characteristics of PDO Provola dei Nebrodi cheese were evaluated after 3 months of ripening by a panel test carried out by 8 trained panelists following the ISO 13299:2016 protocol. The sensory analysis was performed for both cheese productions (February and March). Each panellist was asked to score a set of 14 descriptive attributes grouped into appearance, aroma, taste, and texture categories. All sensory attributes were evaluated on an increasing line-scale from 1 to 15 (from left to right) in steps of 0.1 and recorded by each panellist in individual computerized booths (UNI EN ISO 8589:2010). Data were gathered using the Compusense five V 4.6 software (Compusense, 2003).

Cheese samples, at room temperature at the time of testing, were portioned (approx. 1 cm of thickness) and presented on white plates to the panellists. An entire slice of each cheese was also shown to the panellists for the evaluation of appearance attributes. The samples were identified using random 3-digit codes.

Statistical analysis

Microbiological data were subjected to One-Way Variance Analysis (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft, New York, USA). Chemical, physical and fatty acid composition of PDO Provola dei Nebrodi cheese were analyzed with the following ANOVA linear model: $Y_{ik} = \mu + DF_i + \varepsilon_{ik}$ where

DF_i is the Dairy Factory (A.D), ε_{ik} is the error using the software SAS 9.1.2. Data on cheese sensory characteristics were analysed with the JMP 16 software (SAS Institute 2022) using a GLM mixed model to test the effect of the farm, while the variable panellist was considered as random effect. The Tukey's test was used for means comparisons. Statistical significance was attributed to p values of $p < 0.05$.

Results

Microbiological analysis by culture-dependent approach

The levels of the viable microbial groups investigated on wooden vat biofilms and all samples collected during cheese making and after ripening of PDO Provola dei Nebrodi cheeses are reported in Table 1.

Regarding wooden vat biofilms, their levels in terms of TMM ranged between 5.1 and 6.7 log CFU/cm^2 and LAB dominated the wooden vat microbial community of the four factories investigated. The wooden vats were actually characterized by different microbiological traits among the dairy factories; although *E. coli*, *Salmonella* spp., *Listeria monocytogenes* and CPS were not detected in any vat, WV-A was characterized by better hygienic conditions than the other three equipment used to collect milk, since members of Enterobacteriaceae, but also that of pseudomonads, enterococci and molds were below the detection limit.

Also, WV-C showed undetectable levels of pseudomonads. The levels of molds were particularly high (4.3 log CFU/cm^2) on WV-D surface. Within LAB community, a clear dominance of thermophilic cocci was registered in the vats WV-A and WV-C, while the vat WV-D showed a massive presence of mesophilic rods.

Bulk milk showed consistent levels of TMM (5.0 – 6.0 log CFU/mL) and, although no big differences were registered after contact with the wooden surfaces (5.2 – 6.6 log CFU/mL), the levels of LAB increased, especially for the thermophilic coccus group.

Table 1. Microbial loads of samples collected through experimental cheese production.

Sample	Media[b]														
	PCA-SkM 30 °C	PCA-SkM 7 °	VRBGA	VRBA	HEA (*E. coli*)	HEA (*Salmonella.* spp.)	BP (CPS)	LSAB	PAB	M17 44 °C	M17 30 °C	WBAM	MRS	KAA	YPD	PDA
WV-A	6.4 ± 0.3 b	1.3 ± 0.5 b	<1 d	0.8 ± 0.2 d	<1	<1	<1	<1	<1 c	6.5 ± 0.4 a	3.9 ± 0.0 c	3.6 ± 0.1 c	2.0 ± 0.0 c	<1 c	2.1 ± 0.1 d	<1 d
WV-B	6.7 ± 0.1 a	3.6 ± 0.3 a	3.1 ± 0.1 b	2.1 ± 0.1 b	<1	<1	<1	<1	3.6 ± 0.0 a	4.6 ± 0.2 b	4.7 ± 0.3 b	4.6 ± 0.1 b	4.6 ± 0.4 b	3.4 ± 0.2 a	3.6 ± 0.1 a	3.0 ± 0.0 b
WV-C	5.1 ± 0.1 c	3.3 ± 0.3 a	1.9 ± 0.1 c	1.7 ± 0.1 c	<1	<1	<1	<1	<1 c	6.7± 0.2 a	5.0 ± 0.3 b	5.7 ± 0.3 d	2.3 ± 0.5 c	1.4 ± 0.4 b	3.1 ± 0.2 b	1.6 ± 0.1 c
WV-D	5.9 ± 0.2 b	1.0 ± 0.1 b	4.1 ± 0.1 a	5.3 ± 0.0 a	<1	<1	<1	<1	2.2 ± 0.5 b	5.4 ± 0.4 b	6.1 ± 0.1 a	5.7 ± 0.6 a	7.1 ± 0.0 a	1.8 ± 0.2 b	2.7 ± 0.1 c	4.3± 0.1 a
SEM	0.19	0.36	0.46	0.51	-	-	-	-	0.47	0.27	0.24	0.54	0.62	0.37	0.17	0.48
p-value	<0.0001	<0.0001	<0.0001	<0.0001	-	-	-	-	<0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
BM-A	6.0 ± 0.2 a	3.8 ± 0.2 a	3.4 ± 0.3 a	2.3 ± 0.0 a	3.9 ± 0.0 a	<1	<1 b	<1	4.6 ± 0.4 ab	5.9 ± 0.1 a	5.6 ± 0.1 ab	4.5 ± 0.5 a	5.0 ± 0.2 b	3.8 ± 0.0	3.9 ± 0.2 a	2.4 ± 0.0 a
BM-B	5.7 ± 0.1 a	2.5 ± 0.1 c	1.7 ± 0.1 b	1.1 ± 0.1 c	1.8 ± 0.2 b	<1	2.0 ± 0.0 a	<1	3.3 ± 0.2 c	5.2 ± 0.1 b	5.9 ± 0.3 a	3.5 ± 0.2 b	6.0 ± 0.1 a	3.8 ± 0.1	2.7 ± 0.1 c	2.0 ± 0.0 b
BM-C	5.6 ± 0.2 a	3.5 ± 0.1 a	2.1 ± 0.2 b	3.2 ± 0.1 a	2.9 ± 0.4 ab	<1	<1 b	<1	4.2 ± 0.3 b	5.2 ± 0.2 b	5.2 ± 0.3 b	4.1 ± 0.0 ab	4.2 ± 0.1 c	3.9 ± 0.3	3.4 ± 0.2 b	1.8 ± 0.2 bc
BM-D	5.0 ± 0.2 b	3.0 ± 0.2 b	3.3 ± 0.2 a	1.5 ± 0.3 c	2.1 ± 0.6 ab	<	2.1 ± 0.1 a	<1	5.1 ± 0.1 a	4.0 ± 0.1 c	4.2 ± 0.1 c	4.0 ± 0.0 ab	3.6 ± 0.0 d	3.9 ± 0.0	2.8 ± 0.0 c	1.7 ± 0.0 c
SEM	0.12	0.15	0.23	0.25	0.26	-	0.31	-	0.21	0.21	0.20	0.13	0.27	0.04	0.15	0.08
p-value	0.001	<0.0001	<0.0001	<0.0001	0.008	-	<0.0001	-	0.0001	<0.0001	<0.0001	0.013	<0.0001	0.757	<0.0001	0.0001
MAC-A	5.9 ± 0.1 b	4.3 ± 0.0 b	3.5 ± 0.3 a	3.4 ± 0.1	3.8 ± 0.1 a	<1	<1	<1	3.5 ± 0.1 a	6.2 ± 0.0 a	5.5 ± 0.2 a	5.1 ± 0.2 a	5.0 ± 0.0 ab	3.3 ± 0.2	3.7 ± 0.1 a	2.8 ± 0.2 b
MAC-B	5.7 ± 0.1 b	3.5 ± 0.1 c	2.8 ± 0.1 b	2.5 ± 0.5	2.2 ± 0.7 b	<1	<1	<1	3.4 ± 0.1 a	5.4 ± 0.2 b	5.7 ± 0.3 a	5.1 ± 0.1 a	5.3 ± 0.2 a	3.3 ± 0.2	3.3 ± 0.0 b	3.6 ± 0.1 a
MAC-C	6.6 ± 0.2 a	5.0 ± 0.0 a	2.8 ± 0.3 b	3.4 ± 0.5	3.9 ± 0.1 a	<1	<1	<1	2.3 ± 0.1 b	6.4 ± 0.0 a	6.2 ± 0.4 a	5.0 ± 0.1 a	4.4 ± 0.5 b	3.3 ± 0.1	3.8 ± 0.0 a	2.1 ± 0.1 c
MAC-D	5.2 ± 0.1 c	3.9 ± 0.4 bc	2.4 ± 0.2 b	3.3 ± 0.2	3.2 ± 0.1 a	<1	<1	<1	2.7 ± 0.8 ab	4.8 ± 0.1 c	4.7 ± 0.0 b	3.9 ± 0.2 b	4.5 ± 0.2 b	3.1 ± 0.1	3.9 ± 0.2 a	3.9 ± 0.2 a
SEM	0.15	0.17	0.13	0.15	0.23	-	-	-	0.18	0.20	0.18	0.16	0.13	0.05	0.07	0.22
p-value	<0.0001	0.0001	0.003	0.048	0.004	-	-	-	0.020	<0.0001	0.001	<0.0001	0.015	0.370	0.001	<0.0001
C-A	7.9 ± 0.2 a	5.1 ± 0.0 ab	5.1 ± 0.1 a	4.1 ± 0.2 b	4.2 ± 0.2	<2	2.8 ± 0.2 b	<2	2.5 ± 0.3 c	8.2 ± 0.1 a	7.2 ± 0.3 a	4.8 ± 0.2 a	4.6 ± 0.1 c	5.5 ± 0.0 a	4.2 ± 0.0 b	2.5 ± 0.2 b
C-B	6.7 ± 0.1 b	4.1 ± 0.4 c	3.2 ± 0.3 c	2.3 ± 0.5 c	3.6 ± 0.1	<2	3.4 ± 0.2 a	<2	3.1 ± 0.1 bc	6.2 ± 0.1 b	6.2 ± 0.1 b	3.8 ± 0.2 b	4.7 ± 0.1 c	5.2 ± 0.2 b	3.2 ± 0.3 c	3.6 ± 0.0 a
C-C	8.0 ± 0.3 a	5.6 ± 0.0 a	4.5 ± 0.2 b	4.9 ± 0.0 a	4.6 ± 0.3	<2	<2 c	<2	5.1 ± 0.0 a	8.3 ± 0.3 a	7.5 ± 0.4 a	4.9 ± 0.3 a	6.2 ± 0.0 a	4.9 ± 0.0 c	4.8 ± 0.2 a	3.6 ± 0.1 a
C-D	7.1 ± 0.2 b	4.9 ± 0.1 b	4.3 ± 0.1 b	4.2 ± 0.0 ab	3.3 ± 0.1	<2	<2 c	<2	3.6 ± 0.4 b	6.1 ± 0.4 b	6.2 ± 0.3 b	4.2 ± 0.1 b	5.7 ± 0.1 b	3.0 ± 0.0 d	4.1 ± 0.2 b	2.8 ± 0.3 b
SEM	0.17	0.17	0.21	0.30	0.16	-	0.47	-	0.30	0.32	0.19	0.15	0.20	0.30	0.18	0.15
p-value	0.0001	0.0001	<0.0001	<0.0001	0.174	-	<0.0001	-	<0.0001	<0.0001	0.001	0.001	<0.0001	<0.0001	<0.0001	0.0001
AC-A	8.4 ± 0.0	2.7 ± 0.3 b	4.9 ± 0.0 a	5.0 ± 0.1	4.8 ± 0.7	<2	<2	<2	<2 b	9.0 ± 0.2	8.2 ± 0.3 ab	5.5 ± 0.1 b	5.2 ± 0.1 b	5.1 ± 0.2 b	5.3 ± 0.2 a	2.8 ± 0.2 b
AC-B	8.3 ± 0.3	2.8 ± 0.2 b	3.5 ± 0.0 c	4.1 ± 0.1	4.0 ± 0.1	<2	<2	<2	<2 b	9.1 ± 0.1	8.1 ± 0.1 b	4.7 ± 0.1 c	5.2 ± 0.2 b	5.9 ± 0.0 a	5.5 ± 0.0 a	<2 c
AC-C	8.3 ± 0.0	3.1 ± 0.5 b	4.3 ± 0.1 b	5.9 ± 1.5	4.7 ± 0.3	<2	<2	<2	2.8 ± 0.2 a	8.8 ± 0.4	8.7 ± 0.2 a	4.6 ± 0.1 c	5.1 ± 0.1 b	5.2 ± 0.1 b	4.6 ± 0.1 b	<2 c
AC-D	8.7 ± 0.4	4.0 ± 0.3 a	4.3 ± 0.1 b	5.7 ± 0.3	5.4 ± 0.1	<2	<2	<2	<2 b	8.5 ± 0.3	8.6 ± 0.1 ab	6.4 ± 0.1 a	6.9 ± 0.6 a	5.2 ± 0.0 b	5.2 ± 0.1 a	3.2 ± 0.2 a
SEM	0.08	0.18	0.15	0.28	0.15	-	-	-	0.37	0.10	0.09	0.22	0.24	0.10	0.11	0.46
p-value	0.240	0.006	<0.0001	0.075	0.077	-	-	-	<0.0001	0.109	0.013	<0.0001	0.0001	<0.0001	0.0001	<0.0001
SC-A	7.3 ± 0.1 b	<2	<1 c	<1 b	<2 b	<2	<2	<2	<2	8.5 ± 0.2 a	7.6 ± 0.4 b	5.9 ± 0.1 b	4.4 ± 0.5	3.6 ± 0.2 c	3.3 ± 0.2 c	<2b
SC-B	8.2 ± 0.2 a	<2	<1 c	<1 b	<2 b	<2	<2	<2	<2	7.8 ± 0.2 b	8.4 ± 0.3 a	4.5 ± 0.3 c	4.9 ± 0.3	5.2 ± 0.1 ab	5.0 ± 0.1 a	2.8 ± 0.2a
SC-C	7.5 ± 0.2 b	<2	2.7 ± 0.0 b	2.8 ± 0.2 a	2.2 ± 0.1 a	<2	<2	<2	<2	8.5 ± 0.1 a	8.2 ± 0.1 ab	3.8 ± 0.2 d	4.3 ± 0.5	5.0 ± 0.1 b	3.8 ± 0.1 b	<2b
SC-D	8.4 ± 0.1 a	<2	3.7 ± 0.2 a	2.8 ± 0.2 a	2.7 ± 0.2 a	<2	<2	<2	<2	7.6 ± 0.0 b	8.1 ± 0.0 ab	7.3 ± 0.2 a	5.1 ± 0.1	5.4 ± 0.0 a	3.3 ± 0.1 c	<2b
SEM	0.14	-	0.49	0.23	0.62	-	-	-	-	0.13	0.11	0.41	0.14	0.22	0.21	0.37
p-value	<0.0001	-	<0.0001	0.001	<0.0001	-	-	-	-	0.0001	0.026	<0.0001	0.096	<0.0001	<0.0001	<0.0001

Sample	Media[b]														
	PCA-SkM 30 °C	PCA-SkM 7 °	VRBGA	VRBA	HEA (*E. coli*)	HEA (*Salmonella.* spp.)	BP (CPS)	LSAB	PAB	M17 44 °C	M17 30 °C	WBAM	MRS	KAA	YPD	PDA
RC-A	8.0 ± 0.4 a	<2	<1	<1	<2	<2	<2	<2	<2	7.2 ± 0.1 ab	8.5 ± 0.1 a	7.3 ± 0.2 b	8.1 ± 0.4 a	4.3 ± 0.2 b	<2	<2
RC-B	8.1 ± 0.2 a	<2	<1	<1	<2	<2	<2	<2	<2	7.6 ± 0.7 ab	8.0 ± 0.0 ab	7.2 ± 0.3 b	7.8 ± 0.2 a	6.2 ± 0.4 a	<2	<2
RC-C	6.9 ± 0.3 b	<2	<1	<1	<2	<2	<2	<2	<2	6.8 ± 0.5 b	7.7 ± 0.5 b	6.4 ± 0.0 c	7.1 ± 0.1 b	5.8 ± 0.2 a	<2	<2
RC-D	7.9 ± 0.1 a	<2	<1	<1	<2	<2	<2	<2	<2	8.1 ± 0.0 a	8.2 ± 0.1 ab	8.2 ± 0.2 a	7.8 ± 0.1 a	4.4 ± 0.3 b	<2	<2
SEM	0.16	-	-	-	-	-	-	-	-	0.18	0.11	0.20	0.12	0.26	-	-
p-value	0.002	-	-	-	-	-	-	-	-	0.031	0.030	<0.0001	0.005	<0.0001	-	-

Results indicate mean values ± S.D. of four plate counts (carried out in duplicates for two independent productions). Units are log CFU/cm^2 for vat surfaces; log CFU/mL for milk samples; log CFU/g for curd, acidified curd, stretched curd and ripened cheeses. Data within a column followed by different letters are significantly different according to Tukey's test ($p < 0.05$). Abbreviations: PCA-SkM 30 °C, plate count agar added with skimmed milk incubated at 30 °C for total mesophilic microorganisms; PCA-SkM 7 °C, plate count agar added with skimmed milk incubated at 7 °C for total psychrotrophic microorganisms; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; VRBA, violet red bile agar for coliforms; HEA, hektoen enteric agar for *E. coli* (red colonies) and *Salmonella* spp. (black colonies); BP, baird-parker agar for CPS, coagulase-positive staphylococci; LSAB, *Listeria* selective agar base for *L. monocytogenes*; PAB, *Pseudomonas* agar base for pseudomonads; M17 44 °C, medium 17 agar incubated at 44 °C for thermophilic coccus LAB; M17 30 °C, medium 17 agar incubated at 30 °C for mesophilic coccus LAB; WBAM, whey-based agar medium for thermophilic rod LAB; MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; KAA, kanamycin aesculin azide agar for enterococci; YPD, yeast peptone dextrose agar for yeasts; PDA, potato dextrose agar for molds. WV, wooden vat; BM, bulk milk; MAC, milk after contact; C, curd; AC, acidified curd; SC, stretched curd; RP, ripened cheese; A–D, dairy factory A–dairy factory D; SEM, standard error of mean.

The milk after contact with the wooden vats was still characterized by the absence of *Salmonella* spp., *L. monocytogenes* and CPS, but the levels of members of Enterobacteriaceae, coliforms and *E. coli* were almost around 10^3 CFU/mL for all dairy factories.

After curdling, a general increase of the majority of microbial groups investigated was recorded. In particular, TMM reached 8.0 log CFU/g in factory C. This factory showed the highest increase of all LAB groups while *Salmonella* spp., *L. monocytogenes* and CPS were not registered. On the contrary, the curds of the factories A and B showed detectable levels of CPS (2.8 and 3.4 log CFU/g, respectively). After overnight acidification, plate counts increased showing levels above 8.0 log CFU/g of TMM for all four dairy factories. In all cases, the highest increase was registered in correspondence of thermophilic and mesophilic LAB cocci. In particular, thermophilic LAB cocci in acidified curds from factories A and B reached 9.0 and 9.1 log CFU/g, respectively. The acidification step decreased below the detection levels the group of pseudomonads in factories A, B and D, and that of mold in factories B and C. Furthermore, CPS compared during curdling in factories A and B were reduced until undetectable levels with the acidification.

The stretching operation exerted a sanitizing effect of the curds. In fact, even though very high levels of TMM and thermophilic and mesophilic LAB cocci were still enumerated in all factories, psychrotrophic microorganisms and pseudomonads, together with *Salmonella* spp., *Listeria monocytogenes* and CPS disappeared from the four stretched curds and, in case of samples from factories A and B it was also registered the decrease below detectability of *E. coli*, coliforms and members of

Enterobacteriaceae. Stretching also determined a reduction of molds until being undetectable from the curds A, C and D.

The characteristics of the ripened cheeses were quite comparable among the four dairy factories. The only groups detected were TMM and all LAB, while all other groups object of investigation were below the detection limit. However, within LAB community, enterococci ranged between 4.3 and 6.2 log CFU/g in ripened cheeses.

LAB differentiation and identification

The preliminary characterization of the 728 isolates for general LAB traits determined the recognition of 678 Gram-positive and catalase-negative bacteria which were distinguished into 559 cocci and 119 rods. Considering cell arrangement, growth in different conditions and use of different substrates, 14 phenotypic groups (Table S1) were obtained with the LAB isolated from wooden vats and final cheeses. Strain typing was performed exclusively on the isolates within each phenotypic group that developed from the highest dilutions of the cell suspensions of the wooden vat biofilms and ripened cheeses. The comparison of the polymorphic profiles indicated that the isolates from the wooden vat biofilms represented 14 strains (Fig. 2A), while those from ripened cheeses showed a similar biodiversity as being 18 strains (Fig. 2B). All strains were identified by 16S rRNA gene sequencing and were confirmed to belong to the group of LAB (Ac. No. OP714481 – OP714512). Furthermore, the application of the species-specific *sodA* gene based PCR confirmed that enterococci were *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* (results not shown).

Table S1. Phenotypic grouping of the LAB isolated from the wooden vat surfaces and ripened cheeses.

Characters	Clusters													
	I(n=11)	II (n=46)	III (n=37)	IV (n=16)	V (n=9)	VI (n=33)	VII (n=62)	VIII (n=38)	IX (n=142)	X (n=32)	XI (n=44)	XII (n=36)	XIII (n=116)	XIV (n=56)
Morphology	R	R	R	R	R	C	C	C	C	C	C	C	C	C
Cell disposition	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	t	lc	lc
Growth:														
15 °C	-	+	+	+	+	+	+	+	-	-	+	+	-	-
45 °C	+	-	+	+	-	-	-	-	+	+	+	+	+	+
pH 9.6	nd	nd	nd	nd	nd	+	+	-	+	+	-	-	-	-
6.5% NaCl	nd	nd	nd	nd	nd	-	-	+	+	+	+	+	-	-
Resistance to 60 °C	-	-	+	+	-	-	-	-	-	-	-	-	+	+
Hydrolysis of:														
arginine	-	-	-	-	-	+	-	-	+	+	-	-	+	-
aesculin	-	-	+	+	+	+	+	+	+	+	-	-	-	+
Acid production from:														
arabinose	-	-	-	+	+	-	-	+	-	+	-	+	+	-
ribose	-	-	-	+	+	+	+	+	+	+	-	+	+	-
xylose	-	-	-	+	+	-	-	+	+	+	+	+	+	-
fructose	+	+	+	+	+	+	+	+	+	+	-	+	+	+
galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	-	+	+	+	+	-	+	-	+	+	+	+	+
glycerol	-	-	-	+	+	+	+	+	+	+	-	+	+	+
CO_2 from glucose	-	-	+	-	-	-	-	+	-	-	+	-	-	-

Abbreviations: C, coccus; R, rods; lc, long chain; sc, short chain; t, tetrads; n.d., not determined.

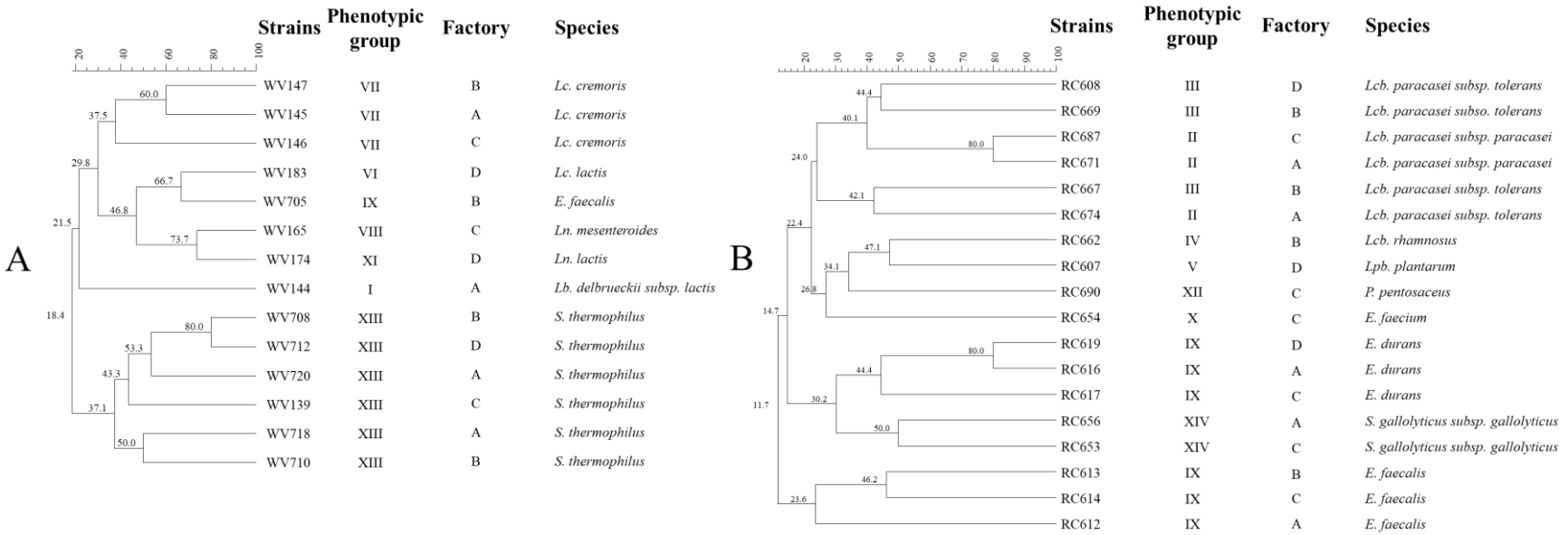

Fig.2. Dendrogram obtained from combined RAPD-PCR patterns of LAB strains from wooden vats (A) and 3-month ripened PDO Provola dei Nebrodi cheeses (B) generated with the primers M13, AB106 and AB111. Abbreviations: E., *Enterococcus*; Lb., *Lactobacillus*; Lc., *Lactococcus*; Lcb., *Lacticaseibacillus*; Lpb., *Lactiplantibacillus*; Ln., *Leuconostoc*; P., *Pediococcus*; S., *Streptococcus*.

58 .

The majority of LAB from the wooden vats were identified as lactococci (groups VI and VII) and *Streptococcus thermophilus* (group XIII), while those isolated from ripened cheeses were mainly allotted into *Lacticaseibacillus paracasei* (groups II and III) species and *Enterococcus* (groups IX and X) genus. In particular, the species *Enterococcus faecalis* was also found a member of the wooden vat biofilm community together with *Lactobacillus delbrueckii* (group I) and leuconostocs (groups VIII and XI), while the other species found in ripened cheeses were *Lactiplantibacillus plantarum* (group V), *Lacticaseibacillus rhamnosus* (group IV), *Pediococcus pentosaceus* (group XII) and *Streptococcus gallolyticus* subsp. *gallolyticus* (group XIV).

Characterization of cheese microbiota by Illumina analysis

The distribution of the relative abundances (%) of the bacterial OTUs identified by MiSeq Illumina within the wooden vat biofilms and 3-month ripened cheeses collected during PDO Provola dei Nebrodi cheese from four distinct factories are reported in Fig. 3. A total of 24 taxonomic groups, mainly identified at genus level, were detected. Although streptococci, lactococci, lactobacilli, leuconostocs, trichococci and other LAB constituted the majority of the total relative abundance % of the wooden vat (69.01 – 97.58%) and cheese (81.57 – 99.87%) bacterial communities, their proportions differed among cheese factories.

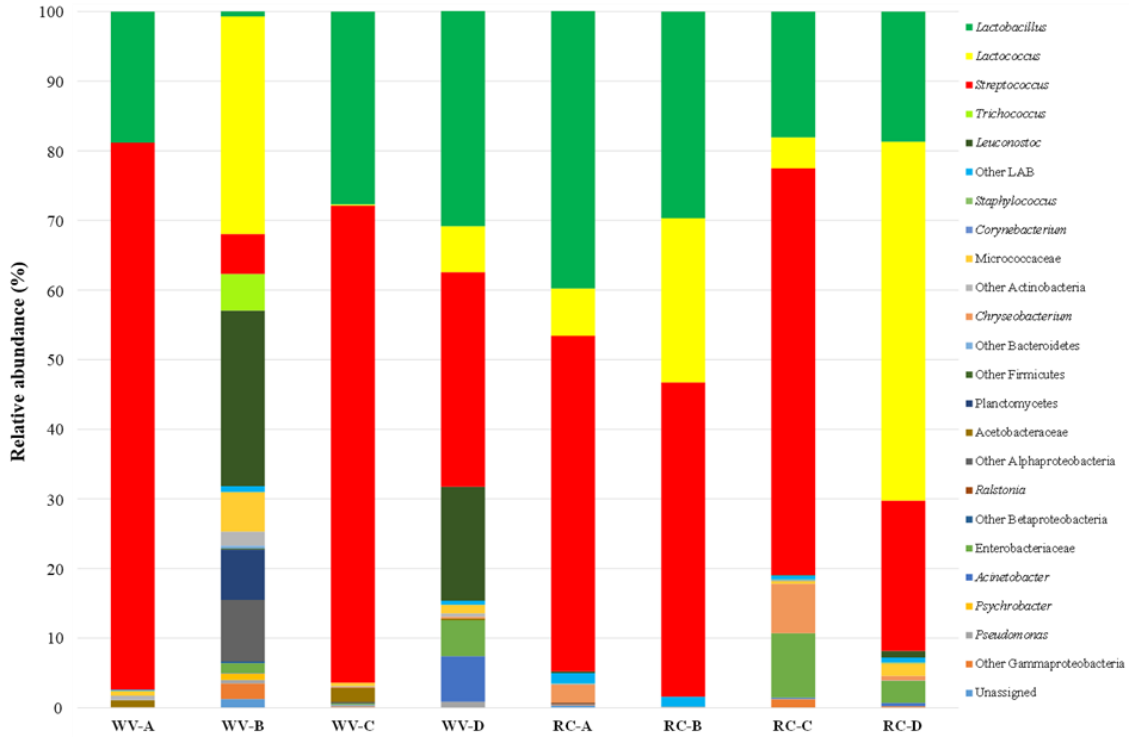

Fig. 3. Relative abundances (%) of bacterial taxa identified by MiSeq Illumina in wooden vat biofilms and ripened PDO Provola dei Nebrodi cheeses. Abbreviations: WV, wooden vat; RC, ripened cheese; A – D, factories A to D.

80

In particular, the wooden vats of the factories A and C were dominated by *Streptococcus* (78.57 and 68.53%, respectively) and *Lactobacillus* (18.84 and 27.70%, respectively), that of factory B by *Lactococcus* (31.26%), *Leuconostoc* (25.24%) and *Trichococcus* (5.26%), while the vat biofilm of the factory D by *Lactobacillus* (31.10%), *Streptococcus* (30.83%), *Leuconostoc* (16.36%) and *Lactococcus* (6.56%). Wooden vat biofilms also hosted low levels of Micrococcaceae, Enterobacteriaceae and other OTUs allotted, basically, into α- and γ-proteobacteria. Unlike wooden vat composition, ripened cheeses showed a more uniform situation in terms of relative abundance (%) of LAB. In general, the relative abundance (%) of lactobacilli detected in cheeses from factories A and B was higher than that observed for the corresponding wooden vats. Regarding lactococci, their relative abundance (%) registered in cheeses from factories A, C and D, was much higher than that of the wooden vat biofilms. Streptococci relative abundance (%) of the cheeses from factory B was 9-fold higher than that displayed by the wooden vat used for cheese making. Enterobacteriaceae were found only in cheese samples from the factories C and D, while *Chryseobacterium* in those from factories A, C and, at very low level, from factory D.

Physicochemical parameters of cheese samples

The physicochemical composition of PDO Provola dei Nebrodi cheese is reported in Table 2. Considering gross composition, dry matter (DM), fat and proteins were in the range 59.88 – 61.16%, 34.84 – 39.94 and 45.83 – 50.46 g/100 g DM, respectively. The effect of dairy factories was significant only for protein content. The differences for fat content were not significant probably due to the high variability, as evidenced by the high SEM value.

Table 2. Physicochemical analysis of PDO Provola dei Nebrodi cheeses.

Parameters	Samples				SEM	*p*-value
	RC-A	RC-B	RC-C	RC-D		
Dry matter (DM) (%)	60.63	59.88	61.08	61.16	0.745	0.639
Fat (g/100g DM)	39.94	34.84	37.00	36.65	1.430	0.233
Protein (g/100g DM)	45.83 d	50.46 a	49.04 b	47.92 c	0.551	0.017
Soluble N/total N (%)	10.59	14.06	13.95	13.10	1.390	0.377
Ash (g/100g DM)	7.55	8.47	8.10	8.51	0.496	0.558
NaCl (g/100g DM)	4.32	4.39	4.69	4.59	0.244	0.698
aw	0.970	0.960	0.968	0.957	0.006	0.449
Hardness (kg/cm²)	8.30	10.47	8.60	10.30	2.039	0.822
TEAC (mmol Trolox/kg DM)	12.29	18.40	17.93	18.06	3.205	0.541
TBARS, mg MDA/kg ss	0.44	0.87	0.69	0.38	0.141	0.186
Outside colour						
L*, lightness	59.55	59.24	59.15	62.67	1.427	0.365
a*, redness	-3.61	-4.48	-3.51	-4.20	-0.429	0.423
b*, yellowness	13.01	18.25	8.68	16.52	3.409	0.335
Chroma	13.54	18.80	9.38	17.04	3.370	0.338
Hue angle	-0.302	-0.255	-0.417	-0.255	0.060	0.318
Inside colour						
L*, lightness	82.87	78.56	74.30	79.43	2.892	0.346
a*, redness	-3.51	-4.25	-3.76	-4.06	0.400	0.617
b*, yellowness	18.18	23.62	13.70	23.68	3.645	0.296
Chroma	18.52	24.01	14.21	24.03	3.64	0.303
Hue angle	-0.196	-0.186	-0.278	-0.172	0.057	0.110

Abbreviations: RP, ripened cheese; A–D, dairy factory A–dairy factory D; SEM, standard error of mean. On the row, values with different letters are significant A, B, C: $p \leq 0.01$.

The effect of dairy factories was significant only for protein content. The differences for fat content were not significant probably due to the high variability, as evidenced by the high SEM value. N soluble/N total ratio ranged between 10 and 14%. Salt percentage was non influenced by dairies and ranged between 4.49 and 4.85 on DM, that correspond to 2.6-2.8% on fresh cheese. Cheese hardness was measured for minimum of 8.30 to a maximum of 10.47 kg/cm^2. Color parameters were determined outside and inside PDO Provola dei Nebrodi cheese samples. Although dairy factories did not affect the color of the cheeses, evident differences were found between the readings inside and onto the surface of Provola cheese. Inside Provola cheese color was lighter and yellower than

surface, while redness index did not change, consequently chroma detected at the inside of cheese was higher than cheese surface.

Antioxidant properties and fatty acid profile of cheeses

Cheese antioxidant properties evaluated on PDO Provola dei Nebrodi cheeses (Table 2) did not show differences among dairy farms. Similarly, also TBARS determination results were not statistically different among the four dairy factories investigated. Regarding cheese FA composition (Table 3), the effect of dairy factories resulted statistically significant only for a few FA detected, in particular, Myristic acid (C14) and the total amount of Monounsaturated FA (MUFA). Cheeses made in dairy factory C showed higher values of Myristic FA (p <0.05) and lower MUFA than cheeses processed in other dairy factories (p <0.05).

Table 3. Fatty acid composition (g/100 g FA) of PDO Provola dei Nebrodi cheeses.

Fatty Acids	Samples				SEM	*p*-value
	RC-A	RC-B	RC-C	RC-D		
C4	2.70	2.77	2.74	2.91	0.261	0.95
C6	2.27	2.36	2.42	2.30	0.134	0.86
C8	1.45	1.58	1.56	1.43	0.065	0.36
C10	3.15	3.62	3.53	3.01	0.230	0.31
C11	0.34	0.45	0.38	0.35	0.053	0.52
C12	3.54	4.13	3.89	3.29	0.355	0.44
C13	0.18	0.29	0.20	0.18	0.048	0.55
C14	11.59 b	12.10 ab	12.73 a	11.11 b	0.282	0.05
C15:iso	0.27	0.29	0.33	0.38	0.294	0.16
C14:1 t	0.55 b	0.66 ab	0.63 ab	0.75 a	0.033	0.05
C14:1 c	0.76	1.05	0.94	0.85	0.161	0.66
C15	1.06	1.33	1.15	1.47	0.145	0.29
C15:1 t	0.37	0.31	0.36	0.31	0.038	0.30
C16	30.16	26.88	31.97	28.89	1.487	0.24
C16:1	0.38	0.51	0.36	0.52	0.050	0.16
C17 anteiso	0.17	0.20	0.16	0.23	0.030	0.39
C16:1 t	1.70	1.85	1.92	1.71	0.187	0.80
C17	0.69	0.69	0.68	0.84	0.041	0.12
C17:1	0.23	0.22	0.21	0.25	0.026	0.75
C18	10.27	8.00	8.78	9.92	1.330	0.64
C18:1 t6	0.44 ABb	0.59 Aa	0.40 ABb	0.38 Bb	0.32	0.03
C18:1 t11 TVA	1.16	2.12	1.23	2.73	0.73	0.26
C18:1 t9	0.24	0.44	0.19	0.25	0.069	0.20
C18:1 c6 n12	0.45	0.99	0.45	0.63	0.169	0.21
C18:1 c9 n9	20.61	17.51	18.23	18.17	1.03	0.30
C18:1 c11	0.68 a	0.63 ac	0.53 bc	0.57 b	0.023	0.03
C18:2 t	0.12	0.44	0.10	0.19	0.112	0.26
C18:2 n6 LA	2.05	1.96	1.62	1.47	0.177	0.21
C20	0.18	0.13	0.17	0.20	0.026	0.27
C18:3 n6 GLA	0.14	0.12	0.12	0.18	0.013	0.08
C20:1 n11	0.33	0.72	0.25	0.99	0.246	0.26
C18:3 n3 ALA	0.42	1.30	0.46	1.03	0.314	0.27
C22	0.07	0.10	0.07	0.12	0.019	0.35
C20:3 n6 DGLA	0.09	0.09	0.08	0.07	0.013	0.64
C20:4 n6	0.13	0.12	0.14	0.10	0.007	0.16
Altri	1.06	2.45	1.00	0.07	0.469	0.21
SFA	68.10	64.93	70.76	66.68	1.448	0.16
MUFA	27.91 a	28.59 a	25.70 b	28.22 a	0.528	0.05
PUFA	2.94	4.03	2.53	3.05	0.551	0.37

Abbreviations: RP, ripened cheese; A–D, dairy factory A–dairy factory D; SEM, standard error of mean; TVA, trans vaccenic acid; LA, linoleic acid; GLA, gamma linoleic acid; ALA, alfa linolenic acid; DGLA, dihomo-γ-linolenic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. On the row, values with different letters are significant a, b, c: $p \leq 0.05$.

Sensory analysis

Results concerning the sensory analysis of the PDO Provola dei Nebrodi cheese are reported in Fig. 4. Among appearance attributes only color was affected by dairies effect (p <0.05) and cheese produced by B showed the highest score. Oiliness and homogeneity attributes were similar in all cheeses. Holes attributes ranged between 1.49-1.61. No significant differences were found in aroma attributes between the dairy factories, with pasture attribute ranging from 4.49 - 4.82. Most of the taste attributes were significantly different between the dairies. In particular B and D cheeses showed higher overall intensity, salty and spicy attributes compared with the others (p <0.0001, p <0.05 and p <0.01 respectively). Regarding the bitterness, no significant difference was found, and C cheese showed the highest score. As to texture, cheeses produced by A and C dairies tended to be less hard than B and D cheeses, with a significant difference (p<0.01). The chewiness and adhesiveness attributes did not show significant difference between the dairies, ranging from 10.52 to 11.15 and from 4.60 to 4.84 respectively.

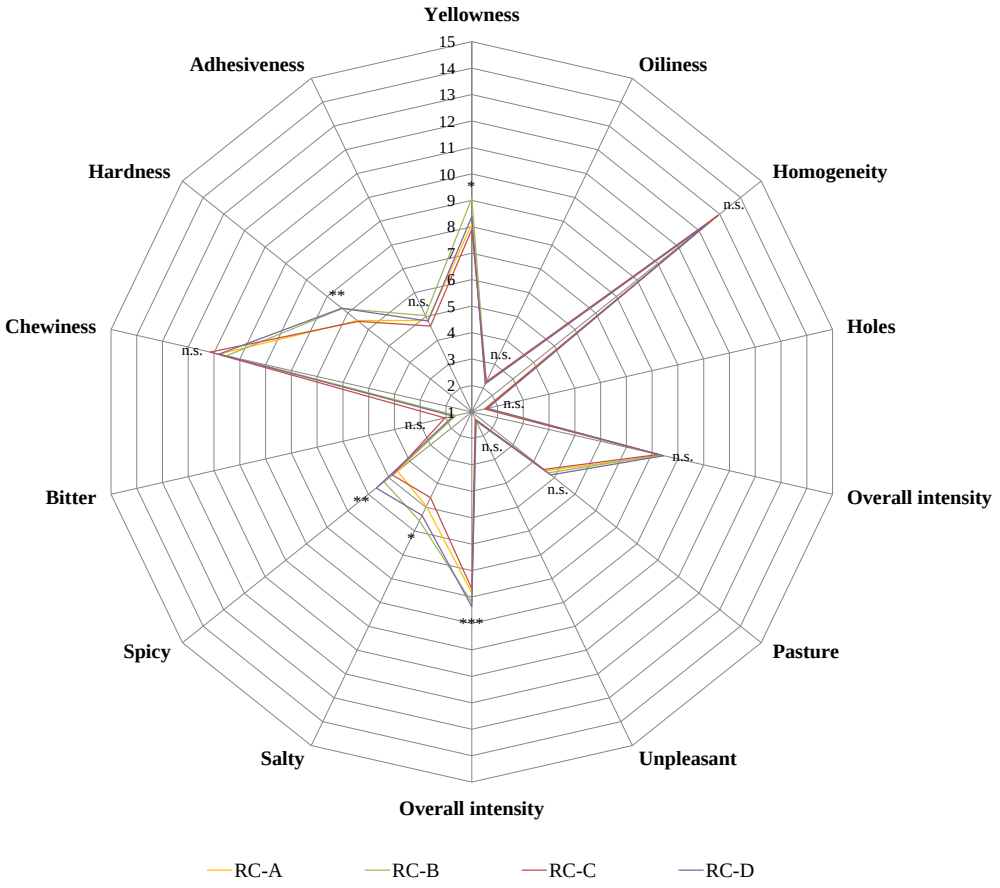

Fig. 4. Spider diagrams of descriptive sensory analysis of PDO Provola dei Nebrodi cheese. Abbreviations: RC, ripened cheese; A – D, factories A to D. *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant.

Discussions

The majority of traditional and the totality of PDO Sicilian cheeses are produced from raw milk kept into a wooden vat without the addition of starter cultures (Gaglio et al., 2021c). However, curd acidification and the development of the final cheese characteristics are ensured by the LAB biofilms present onto the wooden vat surface (Di Grigoli et al., 2015; Licitra et al., 2007; Scatassa et al., 2015; Settanni et al., 2012), but so far, these biofilms have not been characterized yet for the wooden vats used to process PDO Provola dei Nebrodi cheese. Since 2020, this cheese achieved the European quality status of “protected denomination of origin” (PDO) and the cheese

157 making protocols have been standardized among dairies. Thus, previous data available
158 on this cheese were influenced by different protocols and data need to be revised. The
159 four factories chosen for the present study were all characterized by the use of douglas
160 fir wooden vats, activated contemporarily three years before, to exclude variability of
161 data based on the different vat age.

162 The viable counts estimated on wooden vat biofilms differed among the four dairy
163 factories for almost all microbial groups investigated. These differences are imputable
164 to several factors, such as environmental conditions, efficacy of brushing during
165 cleaning and, especially, different bulk milks processed by each factory. *E. coli*,
166 *Salmonella* spp., *Listeria monocytogenes* and CPS, generally associated with poor
167 hygiene of dairy productions (Claeys et al., 2013), were never detected. The
168 attachment of these human pathogenic dairy bacteria has been proven to be hindered
169 by the LAB biofilms colonizing the inner vat surfaces (Cruciata et al., 2018). Microbes
170 from cheese whey are able to form stable biofilms on new wooden vats within a few
171 days from the first contact (Gaglio et al., 2016; Sun and D'Amico, 2022).

172 In view of the Commission Regulation 853 (2004) establishing that the maximum
173 level of total bacteria at 30°C is 100.000 per ml for raw cow's milk, the levels of
174 TMM in the raw milks processed in this study have to be considered high and only
175 factory D fully complied the European standard. However, this regulation refers to the
176 raw cow's milk intended for the manufacture of products by a process that does not
177 involve any heat treatment. In case of PDO Provola dei Nebrodi cheese, the production
178 process includes a heat treatment step because stretching is carried out at 80°C.
179 Furthermore, in our study, the dominant groups of the microbial community of the raw

milk from all factories were LAB. As matter of fact, after 3-month ripening, all potentially harmful microorganisms were below the detection limit and these results complies with the Commission Regulation 2073 (2005) on "microbiological criteria for foodstuff".

The isolates collected after plate counts were identified in order to identify the viable populations dominating the wooden vat biofilms and the final ripened cheeses. The species found (*Lactococcus lactis*, *Streptococcus thermophilus*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus*, *Enterococcus faecalis* and leuconostocs) were already detected in Provola dei Nebrodi cheeses not processed according to PDO protocol (Cronin et al., 2007; Randazzo et al., 2021) and other raw cow's milk stretched cheeses produced in Sicily (Licitra et al., 2007; Scatassa et al., 2015). As matter of fact, vat biofilms mainly hosted *S. thermophilus* and *Lc. lactis*, the most common starter LAB for cheese processing, while cheese isolated mainly belonged to the non starter LAB species *Lcb. pacaracasei* and enterococci (Settanni and Moschetti, 2010). These data showed a lack of correspondence between dominant cheese and vat LAB; the only species found at dominant level in both groups was *E. faecalis*, but the RAPD profile of the only wooden vat isolate WV705, associated to factory B, was not superimposable to that of the strain RC613 identified from ripened cheeses produced in the same factory. Indeed, the microbiological characterization of the open-topped table (mastredda) used to acidify Provola dei Nebrodi curd evidenced the presence of several dominating LAB in viable form (Busetta et al., 2021), indicating that, for this cheese, not only the wooden vat but also mastredda biofilms provide LAB for cheese fermentation. When

wooden surfaces represent the major source of LAB for the spontaneous fermentation no additional cultures are required (Sun and D'Amico, 2021).

The microbial community of the four wooden vat biofilms and the final cheeses was studied through a polyphasic phenotypic/genotypic culture-dependent approach combined with a next generation sequencing (NGS) technique. The characterization of isolates based on phenotypic tests followed by strain typing and 16S rRNA gene sequencing is a routine methodology applied to identify the dominant strains in dairy products (Franciosi et al., 2015; Rossetti et al., 2008), but also to evaluate their physiological and ecological functions (Rosselló-Mora and Amann, 2001). In particular, strain typing by RAPD-PCR analysis is a common technique to discriminate among LAB isolated from food matrices (Fusco et al., 2019). Regarding NGS approach, the study of total DNA from complex matrices became a routine activity to deeply describe microbial composition and evolution (Ercolini, 2013) and also to track the source of microbial communities in cheese facilities (Sun and D'Amico, 2021). In our study, MiSeq Illumina technology confirmed the dominance of streptococci, lactococci, lactobacilli and leuconostocs in the inner wooden vat surfaces, but also evidenced differences among cheese microbiota of the four dairy factories.

Considering the physicochemical composition of the cheeses gross parameters are in accordance with previous studies performed on Provola dei Nebrodi cheeses at 90 days of ripening (Condurso et al., 2006; Cronin et al., 2007). The differences encountered among the different productions depend on the high variability of dairy transformations. Indeed, even though PDO Provola dei Nebrodi cheese production

process is applied uniformly among the various dairy factories that adhere to PDO production regulations, cheese differences depend primarily on differences in bulk milk composition, but also on the craftsmanship of this cheese production, performed manually by the cheese makers. N soluble/N total ratio and salt percentage of PDO Provola dei Nebrodi cheese were similar to those registered for other stretched cheeses (Bonanno et al., 2013; Di Trana et al., 2022; Licitra et al., 2000), while hardness is higher than that displayed by other stretched cheeses at the same ripening period (Bonanno et al., 2013). Although color parameters at the rind of PDO Provola dei Nebrodi cheese is similar to those of other Sicilian stretched cheeses, the inside was yellower (Bonanno et al., 2013).

Several factors affect cheese antioxidant properties, mainly caseins, β-carotene, uric acid, vitamin E, phenols, whey protein, and folate (microbial influence) (Fardet and Rock, 2018). Moreover, TEAC value is influenced by the season of production, with higher values registered for summer cheeses (Revilla et al., 2016), and ripening time, since ripened cheeses show higher antioxidant activity than fresh cheeses (Gupta et al., 2009). The absence of statistical differences between PDO Provola dei Nebrodi cheeses produced in the four dairy factories followed in this study is not only due to the application of the same production protocol, but probably also to the very similar techniques of livestock applied. A comparison of data among various studies is difficult, due to the different methods used for cheese antioxidant assays (Di Trana et al., 2022). The level of TBARs recorded in this study is quite comparable or slightly lower than that observed for other Italian cheeses (Garofalo et al., 2021; Ponte et al., 2022). So far, PDO Provola dei Nebrodi cheese FA profile was only determined by

Condurso et al. (2006) who analyzed cheeses produced before the adoption of PDO production protocol. FA profile showed as saturated FA resulted between 65 and 70% of the total FA detected, confirming data displayed by other raw cow's milk stretched cheeses (Esposito et al., 2014; Maniaci et al., 2021; Pistoia et al., 2015). Our cheeses were produced during February and March when pasture availability is limited in Nebrodi area and the feeding of lactating cows includes hay and concentrate. Cows' diet might explain the high percentages of saturated FA in PDO Provola dei Nebrodi cheeses analyzed and also the higher percentage of myristic FA and the lower percentage of MUFA of the cheeses produced in dairy C due to the feeding of the lactating cows mainly with hay and concentrates (Chion et al., 2010; Coppa et al., 2011; Maniaci et al., 2021).

The different levels of pasture in the diet might also explain the different color perceived by the panelists who were asked to judge the sensory characteristics of PDO Provola dei Nebrodi cheeses of the four factories. Indeed, Carpino et al. (2004) demonstrated that yellowness was more pronounced when PDO Ragusano cheese was transformed from milk of cows consuming fresh native pasture. However, the results from sensory evaluation were in contrast with those measured with colorimeter that found no significant differences among factories probably because of adoption of a 15-points scale for the sensory evaluation and because this test was performed by trained personnel. The scores for holes are similar to those registered for other stretched cheeses reported in literature (Bonanno et al., 2013). This attribute is correlated with the microbiological quality of milk (Fox et al., 2004) and the low presence of holes is in accordance with the production regulation of PDO Provola dei Nebrodi cheese that

tolerate only a few holes with small dimensions. Pasture attribute of the final cheeses was scored at high levels and this is a trait of volatile compounds usually correlated with fresh pasture plants (Carpino et al., 2004). Bitterness was scored almost at the same level for the four dairy factories. Fallico et al. (2005) reported that this attribute might depend on a difference in cheese making technology or salting brine which consequently influence the microbial growth during aging. Sensory evaluation found some of the cheeses to be quite hard and, in general, the judges confirmed the hardness registered instrumentally. In general, sensory analysis showed a good sensory profile of PDO Provola dei Nebrodi cheeses analysed in this study, although it confirmed a high variability among farms characterized by traditional herd management and cheese making system.

In conclusion, the main microbiological parameters were almost comparable among the cheeses produced in different factories and also the physicochemical profiles and sensory attributes of the final cheeses were quite stable among dairy factories. This study has proven that the application of the same cheese making protocol among dairies producing PDO Provola dei Nebrodi cheese harmonized the microbial evolution and the final characteristics at ripening.

References

Azeredo, J., Azevedo, N.F., Briandet, R., Cerca, N., Coenye, T., Costa, A.R., Desvaux, M., Di Bonaventura, G., Hébraud, M., Jaglic, Z., Kačániová, M., Knøchel, S., Lourenço, A., Mergulhão, F., Meyer, R.L., Nychas, G., Simões, M., Tresse, O., Sternberg, C., 2017. Critical review on biofilm methods. Crit. Rev. Microbiol. 43, 313–351. https://doi.org/10.1080/1040841X.2016.1208146.

Bonanno, A., Di Grigoli, A., Vitale, F., Di Miceli, G., Todaro, M., Alabiso, M., Gargano, M.L., Venturella, G., Anike, F.N., Isikhuemhen, O.S., 2019. Effects of feeding diets supplemented with medicinal mushrooms myceliated grains on some production, health and oxidation traits of dairy ewes. Int. J. Med. Mushrooms 21, 89–103. 10.1615/IntJMedMushrooms.2018029327.

Bonanno, A., Tornambè, G., Bellina, V., De Pasquale, C., Mazza, F., Maniaci, G., Di Grigoli, A., 2013. Effect of farming system and cheese making technology on the physicochemical characteristics, fatty acid profile, and sensory properties of Caciocavallo Palermitano cheese. J. Dairy Sci. 96,710–724. http://dx.doi.org/10.3168/jds.2012-5973.

Busetta, G., Garofalo, G., Mangione, G., Botta, L., Franciosi, E., Di Gerlando, R., Todaro, M., Licitra, G., Scatassa, M.L., Gaglio, R., Settanni, L., 2021. Polyphasic Characterization of Microbiota of "Mastredda", a Traditional Wooden Tool Used during the Production of PDO Provola dei Nebrodi Cheese. Appl. Sci. 11, 8647. https://doi.org/10.3390/app11188647.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Campo, M.M., Nute, G.R., Hughes, S.I., Enser, M., Wood, J.D., Richardson, R.I., 2006. Flavour perception of oxidation in beef. Meat Sci. 72, 303-311. https://doi.org/10.1016/j.meatsci.2005.07.015.

Carpino, S., Mallia, S., La Terra, S., Melilli, C., Licitra, G., Acree, T.E., Barbano, D.M., Van Soest, P.J., 2004. Composition and aroma compounds of Ragusano cheese: Native pasture and total mixed rations. J. Dairy Sci. 87:816–830. http://dx.doi.org/10.3168/jds. S0022-0302(04)73226-9.

Carpino, S., Randazzo, C.L., Pino, A., Russo, N., Rapisarda, T., Belvedere, G., Caggia, C., 2017. Influence of PDO Ragusano cheese biofilm microbiota on flavour compounds formation. Food Microbiol. 61, 126–135. https://doi.org/10.1016/j.fm.2016.09.006.

Chion, A.R., Tabacco, E., Giaccone, D., Peiretti, P.G., Battelli, G., Borreani, G., 2010. Variation of fatty acid and terpene profiles in mountain milk and "Toma piemontese" cheese as affected by diet composition in different seasons. Food Chem. 121, 393–399. https://doi.org/10.3168/jds.2013-7292.

Claeys, W.L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K., De Zutter, L., Huyghebaert, A., Imberechts, H., Thiange, P., Vandenplas, Y., Herman, L., 2013. Raw or heated cow milk consumption: Review of risks and benefits. Food Control 31, 251–262. https://doi.org/10.1016/j.foodcont.2012.09.035.

Commission Regulation, 2004. CE Regulation 853 (Alleg. II, Sez. IX, Cap. I, Parag. III). https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32004R0853 Accessed 09.02.2023.

Commission Regulation, 2005. Commission regulation no 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off J Eur Union L 338:1–26.

Condurso, C., Ziino, M., Palmegiano, G.B., Romeo, V., Verzera, A., 2006. Free Fatty Acids In" Provola Dei Nebrodi", A Historical Sicilian Cheese. Ital. J. Food Sci. 18, 151–162.

Coppa, M., Ferlay, A., Monsallier, F., Verdier-Metz, I., Pradel, P., Didienne, R., Farruggia, A., Montel M.C., Martin, B. 2011. Milk fatty acid composition and cheese texture and appearance from cows fed hay or different grazing systems on upland pastures. J. Dairy Sci. 94, 1132–1145. https://doi.org/10.3168/jds.2010-3510.

Cronin, T., Ziino, M., Condurso, C., McSweeney, P.L.H., Mills, S., Ross, R. P., Stanton, C., 2007. A survey of the microbial and chemical composition of seven semi-ripened Provola dei Nebrodi Sicilian cheeses. J. Appl. Microbiol. 103, 1128–1139. https://doi.org/10.1111/j.1365-2672.2007.03364.x.

Cruciata, M., Gaglio, R., Scatassa, M.L., Sala, G., Cardamone, C., Palmeri, M., Moschetti, G., La Mantia, T., Settanni, L., 2018. Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production. Appl. Environ. Microbiol. 84, e02107-17. https://doi.org/10.1128/AEM.02107-17.

Cruciata, M., Gaglio, R., Todaro, M., Settanni, L., 2019. Ecology of Vastedda della valle del Belìce cheeses: a review and recent findings to stabilize the traditional production. Food Rev. Int. 35, 90–103. https://doi.org/10.1080/87559129.2018.1469142.

Cruciata, M., Sannino, C., Ercolini, D., Scatassa, M.L., De Filippis, F., Mancuso, I., La Storia, A., Moschetti, G., Settanni, L., 2014. Animal rennets as sources of dairy lactic acid bacteria. Appl. Environ. Microbiol. 80, 2050–2061. https://doi.org/10.1128/AEM.03837-13.

Di Grigoli, A., Francesca, N., Gaglio, R., Guarrasi, V., Moschetti, M., Scatassa, M.L., Settanni, L., Bonanno, A., 2015. The influence of the wooden equipment employed for cheese manufacture on the characteristics of a traditional stretched cheese during ripening. Food Microbiol. 46, 81–91. https://doi.org/10.1016/j.fm.2014.07.008.

Di Trana, A., Di Rosa, A.R., Addis, M., Fiori, M., Di Grigoli, A., Morittu, V.M., Spina, A.A., Claps, S., Chiofalo, V., Licitra, G., Todaro, M., 2022. The Quality of Five Natural, Historical Italian Cheeses Produced in Different Months: Gross Composition, Fat-Soluble Vitamins, Fatty Acids, Total Phenols, Antioxidant Capacity, and Health Index. Animals 12, 199. https://doi.org/10.3390/ani12020199.

Didienne, R., Defargues, C., Callon, C., Meylheuc, T., Hulin, S., Montel, M.C., 2012. Characteristics of microbial biofilm on wooden vats ('gerles') in PDO Salers cheese. Int. J. Food Microbiol. 156, 91–101.

Dolci, P., Ferrocino, I., Giordano, M., Pramotton, R., Vernetti-Prot, L., Zenato, S., Barmaz, A., 2020. Impact of Lactococcus lactis as starter culture on microbiota and metabolome profile of an Italian raw milk cheese. Int. Dairy J. 110, 104804. https://doi.org/10.1016/j.idairyj.2020.104804.

Ercolini, D., 2013. High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. Appl. Environ. Microbiol. 79, 3148–3155. https://doi.org/10.1128/AEM.00256-13.

Esposito, G., Masucci, F., Napolitano, F., Braghieri, A., Romano, R., Manzo, N., Di Francia, A., 2014. Fatty acid and sensory profiles of Caciocavallo cheese as affected by management system. J. Dairy Sci. 97, 1918–1928. https://doi.org/10.3168/jds.2013-7292.

Fallico, V., McSweeney, P.L.H., Horne, J., Pediliggieri, C., Hannon, J.A., Carpino, S., Licitra, G., 2005. Evaluation of bitterness in Ragusano cheese. J. Dairy Sci. 88, 1288–1300. https://doi.org/10.3168/jds.S0022-0302(05)72795-8.

Fardet, A., Rock, E., 2018. In vitro and in vivo antioxidant potential of milks, yoghurts, fermented milks and cheeses: A narrative review of evidence. Nutr. Res. Rev. 31, 52–70. https://doi.org/10.1017/S0954422417000191.

Fox, P.F., McSweeney, P.L.H., Cogan T.M., Guinee T.P., 2004. Cheese: Chemistry, Physics and Microbiology. 3rd ed. Elsevier Academic Press, London.

Franciosi, E., Carafa, I., Nardin, T., Schiavon, S., Poznanski, E., Cavazza, A., Larcher, R., Tuohy, K.M., 2015. Biodiversity and γ-aminobutyric acid production by lactic acid bacteria isolated from traditional alpine raw cow's milk cheeses. BioMed Res. Int. 625740. http://dx.doi.org/10.1155/2015/625740.

Fusco, V., Quero, G.M., Poltronieri, P., Morea, M., Baruzzi, F., 2019. Autochthonous and probiotic lactic acid bacteria employed for production of "advanced traditional cheeses". Foods 8, 412. https://doi.org/10.3390/foods8090412.

Gaglio, R., Barbaccia, P., Barbera, M., Restivo, I., Attanzio, A., Maniaci, G., Di Grigoli, A., Francesca, N., Tesoriere, L., Bonanno, A., Moschetti, G., Settanni, L. (2021b). The use of winery by-products to enhance the functional aspects of the fresh ovine "primosale" cheese. Foods 10, 461. https://doi.org/10.3390/foods10020461.

Gaglio, R., Cruciata, M., Di Gerlando, R., Scatassa, M. L., Cardamone, C., Mancuso, I., Sardina, M.T., Moschetti, G., Portolano, B., Settanni, L., 2016. Microbial activation of wooden vats used for traditional cheese production and evolution of neoformed biofilms. Appl. Environ. Microbiol. 82, 585–595. https://doi.org/10.1128/AEM.02868-15.

Gaglio, R., Francesca, N., Di Gerlando, R., Cruciata, M., Guarcello, R., Portolano, B., Moschetti, G., Settanni, L., 2014. Identification, typing and investigation of the dairy characteristics of lactic acid bacteria isolated from "Vastedda della valle del Belìce" cheeses. Dairy Sci. Technol. 94, 157–180. https://doi.org/10.1007/s13594-013-0150-5.

Gaglio, R., Francesca, N., Di Gerlando, R., Mahony, J., De Martino, S., Stucchi, C., Moschetti, G., Settanni, L., 2017. Enteric bacteria of food ice and their survival in alcoholic beverages and soft drinks. Food Microbiol. 67, 17–22. https://doi.org/10.1016/j.fm.2017.04.020.

Gaglio, R., Franciosi, E., Todaro, A., Guarcello, R., Alfeo, V., Randazzo, C. L., Settanni, L., Todaro, M., 2020. Addition of selected starter/non-starter lactic acid bacterial inoculums to stabilise PDO Pecorino Siciliano cheese production. Food Res. Int. 136, 109335. https://doi.org/10.1016/j.foodres.2020.109335.

Gaglio, R., Restivo, I., Barbera, M., Barbaccia, P., Ponte, M., Tesoriere, L., Bonanno, A., Attanzio, A., Di Grigoli, A., Francesca, N., Moschetti, G., Settanni, L., 2021a. Effect on the antioxidant, lipoperoxyl radical scavenger capacity, nutritional, sensory and microbiological traits of an ovine stretched cheese produced with grape pomace powder addition. Antioxidants 10, 306. https://doi.org/10.3390/antiox10020306.

Gaglio, R., Todaro, M., Settanni, L., 2021c. Improvement of raw milk cheese hygiene through the selection of starter and non-starter lactic acid bacteria: The successful case of PDO pecorino siciliano cheese. Int. J. Environ. Res. Public Health. 18, 1834. https://doi.org/10.3390/ijerph18041834.

Garofalo, G., Busetta, G., Maniaci, G., Sardina, M.T., Portolano, B., Badalamenti, N., Maggio, A., Bruno, M., Gaglio, R., Settanni, L., 2021. Development of "Quadrello di Ovino", a Novel Fresh Ewe's Cheese. Foods 11, 25. https://doi.org/10.3390/foods11010025.

Gazzetta Ufficiale della Repubblica Italiana (G.U.R.I.), 2020. Provvedimento 23 settembre 2020. Iscrizione della denominazione "Provola dei Nebrodi" DOP nel registro europeo delle denominazioni di origine protette e delle indicazioni geografiche protette. Serie Generale, n. 243. pp. 32–37. https://www.gazzettaufficiale.it/eli/id/2020/10/01/20A05246/sg. (accessed 17 June 2021).

Grujović, M.Ž., Mladenović, K.G., Semedo-Lemsaddek, T., Laranjo, M., Stefanović, O.D., Kocić-Tanackov, S.D., 2022. Advantages and disadvantages of non-starter lactic acid bacteria from traditional fermented foods: Potential use as starters or probiotics. Compr. Rev. Food Sci. Food Saf. 2, 1537–1567. https://doi.org/10.1111/1541-4337.12897.

Guarcello, R., Carpino, S., Gaglio, R., Pino, A., Rapisarda, T., Caggia, C., Marino, G., Randazzo, C.L., Settanni, L., Todaro, M., 2016. Large Factory-Scale Application of Selected Autochthonous Lactic Acid Bacteria for PDO Pecorino Siciliano Cheese Production. Food Microbiol. 59, 66–75. https://doi.org/10.1016/j.fm.2016.05.011.

Gupta, A., Mann, B., Kumar, R., Sangwan, R.B., 2009. Antioxidant activity of Cheddar cheeses at different stages of ripening. Int. J. Dairy Technol. 62, 339–347. https://doi.org/10.1111/j.1471-0307.2009.00509.x.

ISO 13299:2016. Sensory Analysis—Methodology—General Guidance for Establishing a Sensory Profile. https://www.iso.org/standard/58042.html (accessed on 25 October 2021).

Jackson, C.R., Fedorka-Cray, P.J., Barrett, J.B., 2004. Use of a genus- and species- specific multiplex PCR for identification of enterococci. J. Clin. Microbiol. 42:3558–3565. https://doi.org/10.1128/JCM.42.8.3558-3565.2004.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010.

Lee, M.R.F., Tweed, J.K.S., 2008. Isomerisation of cis-9 trans-11 conjugated linoleic acid (CLA) to trans-9 trans-11 CLA during acidic methylation can be avoided by a rapid base catalysed methylation of milk fat. J. Dairy Res. 75, 354–356. https://doi.org/10.1017/S0022029908003518.

Licitra, G., Campo, P., Manenti, M., Portelli, G., Scuderi, S., Carpino, S., Barbano, D.M., 2000. Composition of Ragusano cheese during aging. J. Dairy Sci. 83, 404–411. https://doi.org/10.3168/jds.S0022-0302(00)74896-X.

Licitra, G., Ogier, J.C., Parayre, S., Pediliggieri, C., Carnemolla, T.M., Falentin, H., Madec, M.N., Carpino, S., Lortal, S., 2007. Variability of the bacterial biofilms of the "tina" wood vat used in the Ragusano cheese making process. Appl. Environ. Microbiol. 73, 6980–6987. https://doi.org/10.1128/AEM.00835-07.

Licitra, G., Portelli, G., Campo, P., Longombardo, G., Farina, G., Carpino, S., Barbano, D.M., 1998. Technology to produce Ragusano cheese: a survey. J. Dairy Sci. 81, 3343–3349. https://doi.org/10.3168/jds.S0022-0302(98)75900-4.

Lortal, S., Di Blasi, A., Madec, M.N., Pediliggieri, C., Tuminello, L., Tangury, G., Fauquant, J., Lecuona, Y., Campo, P., Carpino, S., Licitra, G., 2009. Tina wooden vat biofilm. A safe and highly efficient lactic acid bacteria delivering system in PDO Ragusano cheese making. Int. J. Food Microbiol. 132, 1–8. https://doi.org/10.1016/j.ijfoodmicro.2009.02.026.

MAF, (Ministero dell'Agricoltura e delle Foreste), 1986. Decreto ministeriale 21/4/1986. Approvazione dei metodi ufficiali di analisi per i formaggi (Italian official methods of cheeses analysis), No. 8, Page 15. Supplemento ordinario alla Gazzetta Ufficiale della Repubblica Italiana (GURI), n. 229 del 2/10/1986.

Maniaci, G., Di Grigoli, A., Bonanno, A., Giosuè, C., Ilardi, V., Alabiso, M., 2021. Fatty acids as biomarkers of the production season of Caciocavallo Palermitano cheese. Animals 11, 2675. https://doi.org/10.3390/ani11092675.

Mele, M., Contarini, G., Cercaci, L., Serra, A., Buccioni, A., Povolo, M., Conte, G., Funaro, A., Banni, S., Lercker, G., Secchiari, P., 2011. Enrichment of pecorino cheese with conjugated linoleic acid by feeding dairy ewes with extruded linseed: Effect on fatty acid and triglycerides composition and on oxidative stability. Int. Dairy J. 21, 365–372. https://doi.org/10.1016/j.idairyj.2010.12.015.

Pistoia, A., Casarosa, L., Poli, P., Mani, D., Ferruzzi, G., 2015. Quality aspects of milk and Caciocavallo cheese in Podolica cattle. Large Anim. Rev., 21, 63–66.

Ponte, M., Maniaci, G., Di Grigoli, A., Gannuscio, R., Ashkezary, M.R., Addis, M., Pipi, M., Alabiso, M., Todaro, M., Bonanno, A., 2022. Feeding dairy ewes with fresh or dehydrated sulla (*Sulla coronarium* L.) forage. 2. Effects on cheese enrichment in bioactive molecules. Animals 12, 2462. https://doi.org/10.3390/ani12182462.

Price, M.N., Dehal, P.S., Arkin, A.P., 2009. FastTree: computing large minimum evolution, trees with profiles instead of a distance matrix. Mol. Biol. Evol. 26, 1641–1650. https://doi.org/10.1093/molbev/msp077.

Randazzo, C.L., Liotta, L., Angelis, M.D., Celano, G., Russo, N., Hoorde, K.V., Chiofalo, V., Pino, A., Caggia, C., 2021. Adjunct culture of non-starter lactic acid bacteria for the production of Provola Dei Nebrodi PDO cheese: In vitro screening and pilot-scale cheese-making. Microorganisms 9, 179. https://doi.org/10.3390/microorganisms9010179.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free. Radic. Biol. Med. 26, 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3.

Revilla, I., Gonzalez-Martin, M.I., Vivar-Quintana, A.M., Blanco-Lopez, M.A., Lobos-Ortega, I.A., Hernandez-Hierro, J.M., 2016. Antioxidant capacity of different cheeses: Affecting factors and prediction by near infrared spectroscopy. J. Dairy Sci. 99, 5074–5082. https://doi.org/10.3168/jds.2015-10564.

Rosselló-Mora, R., Amann, R., 2001. The species concept for prokaryotes. FEMS Microbiol. Rev. 25, 39–67. https://doi.org/10.1111/j.1574-6976.2001.tb00571.x.

Rossetti, L., Fornasari, M. E., Gatti, M., Lazzi, C., Neviani, E., Giraffa, G., 2008. Grana Padano cheese whey starters: microbial composition and strain distribution. Int. J. Food Microbiol. 127, 168–171. https://doi.org/10.1016/j.ijfoodmicro.2008.06.005.

Scatassa, M.L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., Di Grigoli, A., Moschetti, G., Settanni, L., 2015. Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol. 52, 31–41. https://doi.org/10.1016/j.fm.2015.06.008.

Settanni, L., Di Grigoli, A., Tornambé, G., Bellina, V., Francesca, N., Moschetti, G., Bonanno, A., 2012. Persistence of wild Streptococcus thermophilus strains on wooden vat and during the manufacture of a traditional Caciocavallo type cheese. Int. J. Food Microbiol. 155, 73–81. https://doi.org/10.1016/j.ijfoodmicro.2012.01.022.

Settanni, L., Moschetti, G., 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiol. 27, 691–697. https://doi.org/10.1016/j.fm.2010.05.023

Settanni, L., Puccio, V., Busetta, G., Licitra, G., Franciosi, E., Botta, L., Di Gerlando, R., Todaro, M., Gaglio, R., 2021. In-Depth Investigation of the Safety of Wooden Shelves Used for Traditional Cheese Ripening. Appl. Environ. Microbiol. 87, e01524-21. https://doi.org/10.1128/AEM.01524-2.

Smid, E.J., Erkus, O., Spus, M., Wolkers-Rooijackers, J., Alexeeva, S., Kleerebezem, M., 2014. Functional implications of the microbial community structure of undefined mesophilic starter cultures. Microb. Cell Factories 13, 1–9. https://doi.org/10.1186/1475-2859-13-S1-S2.

Sun, L., D'Amico, D.J., 2022. Characterization of microbial community assembly on new wooden vats for use in cheese production. Food Microbiol. 104154. https://doi.org/10.1016/j.fm.2022.104154.

Sun, L., D'Amico, D.J., 2021. Composition, Succession, and Source Tracking of Microbial Communities throughout the Traditional Production of a Farmstead Cheese. Msystems 6, e00830-21. https://doi.org/10.1128/mSystems.00830-21.

Tarladgis, B.G., Watts, B.M., Younathan, M.T., Dugan, L., 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. J. Am. Oil Chem. Soc. 37, 44–48. https://doi.org/10.1007/BF02630824.

Weisburg, W., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991.

Chapter II

Metagenomic, microbiological, chemical and sensory profiling of Caciocavallo Podolico Lucano cheese

The present chapter has been published in

Food Research International, 169

2023

Abstract

In this study, Caciocavallo Podolico Lucano (CPL) cheese was deeply characterized for its bacterial community, chemical composition and sensory aspects. The entire cheese making process (from milk collection to ripened cheese) was performed by strictly applying the traditional protocol for CPL production in four dairy factories (A – D) representative of the production area. The vat made of wood represents the main transformation tool for CPL cheese production and the biofilms hosted onto the internal surfaces of all vats analyzed in this study were dominated by lactic acid bacteria. Total mesophilic microorganisms present in bulk milk (4.7 – 5.0 log CFU/ml) increased consistently after contact with the wooden vat surfaces (5.4 – 6.4 log CFU/ml). The application of Illumina sequencing technology identified barely 18 taxonomic groups among processed samples; streptococci and lactobacilli constituted the major groups of the wooden vat biofilms [94.74 – 99.70 % of relative abundance (RA)], while lactobacilli dominated almost entirely (94.19 – 100 % of total RA) the bacterial community of ripened cheeses. Except coagulase positive staphylococci, undesirable bacteria were undetectable. Among chemical parameters, significant variations were registered for unsaturated, monounsaturated, polyunsaturated fatty acids and antioxidant properties (significantly lower for CPL cheeses produced in factory B). The cheeses from factories A, C and D were characterized by a higher lactic acid and persistence smell attributes than factory B. This work indicated that the strict application of CPL cheese making protocol harmonized the main microbiological, physicochemical and sensory parameters of the final cheeses produced in the four factories investigated.

Keywords: lactic acid bacteria; next generation sequencing; stretched cheese; typicality; volatile compounds; wooden vat.

Introduction

Caciocavallo Podolico cheese is made from raw cows' milk in four regions (Apulia, Basilicata, Calabria and Campania) of southern Italy. This cheese is produced applying the characteristic stretching technology including the acidification of the curd and the subsequent scalding and molding to the final pear shape (Licitra & Carpino, 2014). Alike other traditional southern Italian stretched cheeses, such as PDO Ragusano and Caciocavallo Palermitano (Licitra et al., 2007; Settanni et al., 2012), Caciocavallo Podolico is a semihard cheese produced with wooden tools without the inoculation of starter cultures. The cheeses under Caciocavallo Podolico (CP) denomination are all produced from the milk of Podolica breed cows, but are further differentiated based on their production area: "CP Dauno" is produced in Apulia region; "CP Lucano" (CPL) in Basilicata region; "CP of Calabria" in Calabria region; and "CP of Campania" in Campania region. Depending on inoculants, rennet, curd acidification, stretching conditions, salting, ripening conditions and duration CP cheeses processed in distant areas are particularly different (Uzun et al., 2020). In addition, the transformation traditions of a given production territory provide uniqueness to the final products. CPL cheese is included in the list of Traditional Agri-Food Products (TAP) by the Italian Ministry of Agriculture, Food Sovereignty and Forestry (G.U.R.I., 2021). This stretched cheese is obtained from the transformation of the raw milk of autochthonous Podolica breed cows reared at pasture year-round without any supplementation and it is round shaped with a soft, creamy white

consistency, sweet taste and delicate flavour. The minimum ripening time is three months and assumes a firmer structure and a darker yellow color with time; aging can be prolonged until three years (Claps, 2001).

The production of raw milk cheeses processed with wooden tools without starter addition in Sicily region relies on the indigenous microorganisms present in raw milk (Guarcello et al., 2016), in the animal rennet paste added for coagulation (Cruciata et al., 2014), onto the surface of the wooden equipment used during transformation (Di Grigoli et al., 2015; Licitra et al., 2007) as well as on the environmental contaminants of the dairy factory (Cruciata et al., 2019), and a similar trend is expected also for CPL cheese. Among the microorganisms responsible for the biochemical events occurring during cheese making and ripening, lactic acid bacteria (LAB) are of paramount importance. Specifically, dairy LAB necessary to produce a given ripened cheese belong to two groups: starter LAB defining for curd acidification; and, non-starter LAB that drive the ripening process (Barbaccia et al., 2020) and determine the typical sensory traits of the final cheese (Guarrasi et al., 2017).

Several works evidenced the key role played by bacterial biofilms associated to the wooden equipment during cheese production. In particular, the wooden vats used for milk coagulation have been thoroughly characterized and several works revealed the presence of dairy LAB (Licitra et al., 2007; Lortal et al., 2009; Scatassa et al., 2015; Sun & D'Amico, 2021). The species identified are both starter and non-starter LAB (Carpino et al., 2017; Di Grigoli et al., 2015). These bacteria are adsorbed and trapped onto the surface of the wooden vats because wood is a porous structure (Cruciata et al., 2019) and can easily form the typical aggregates of biofilms thanks to their extracellular polysaccharides (EPS) (Vert

et al., 2012), but so far, no information is available on the bacterial biofilms of the wooden vats used to process CPL cheese.

In order to provide in-depth insights on the microbial populations characterizing CPL cheese production, in this study the wooden vats used for cheese making and the final cheeses, produced in four dairy factories applying the traditional cheese making protocol, were investigated for total microbial diversity, chemical composition, and sensory traits.

Materials and methods

Cheese production and sample collection

Four dairy factories (A – D) producing CPL cheese, located in the cities of Castelgrande and Muro Lucano (Potenza province, southern Italy), were monitored during the entire transformation process from milking until 4-month ripening. Cheese making was performed following the traditional production protocol established with TAP disciplinary for protection of CPL cheese using raw cows' milk processed in wooden vats without the addition of starter cultures and curdled with animal rennet paste (B.U.R., 1999). The flowsheet of CPL cheese production is reported in Fig. 1. The wooden vats (WV.CPL-A – WV.CPL-D) were all made of ash-leaved maple (*Acer negundo* L.) wood and used for one year in factories A – C and four years in factory D. In each factory, cheese production was followed twice at 15-d interval, both during May 2021. The wooden vat biofilms were sampled before milk contact applying the brushing recovery method described by Didienne et al. (2012) using 100 cm^2 sterile plastic squares (Biogenetics s.r.l., Padua, Italy). Bulk milk (500 L) was sampled before and after filling in the wooden vat.

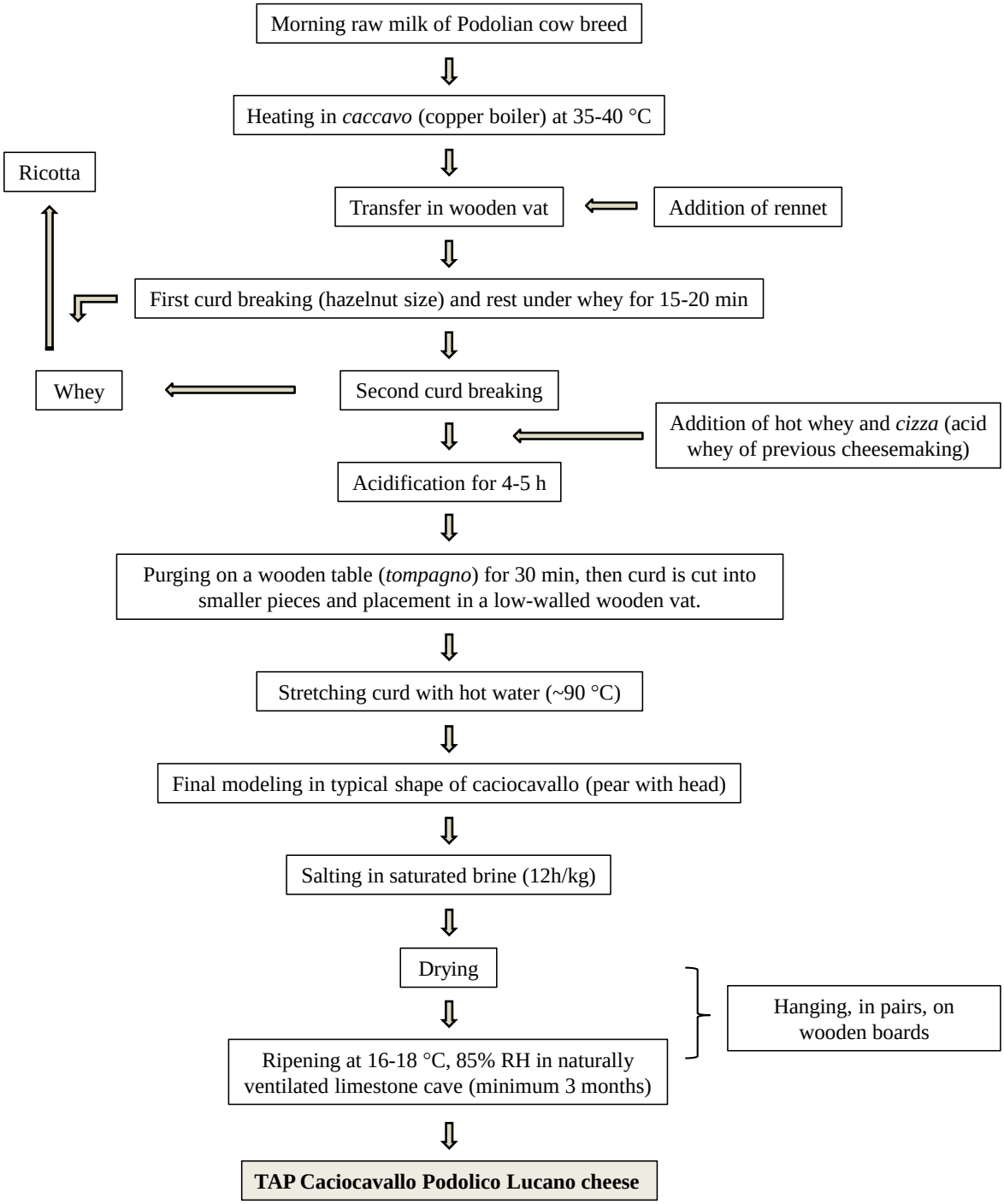

Fig. 1. Flowsheet of Traditional Agri-Food Products (TAP) Caciocavallo Podolico Lucano cheese production.

610 During the contact with the vat surfaces, milk was kept for 5 min under gentle manual
611 agitation performed with the typical wooden stick used for curd breaking. Milk coagulation
612 occurred with 30 g of lamb rennet paste (Camoscio® CSO 95/75, DSM Food Specialties,

Segrate, Italy) per 100 L of milk and the resulting milk coagulum was disrupted manually. The resulting curd was then left to acidify until a pH (5.2 – 5.4) necessary for the stretching phase that lasted about 10-15 min. The acidified curd was finally molded. Salting was performed just after shaping in saturated brine. Ripening occurred for four months at 16 – 18 °C and 85 % relative humidity in naturally ventilated limestone caves.

Samples of wooden vat biofilms, bulk milk before and after wood contact, curd, acidified curd, stretched curd and 4-month ripened cheeses were transported to the laboratories of Agricultural Microbiology of University of Palermo under refrigeration using insulated boxes containing reusable ice packs.

Microbiological analyses

All samples collected during CPL cheese production, including vat biofilms taken before milk transfer, as well as 4-month ripened cheeses were subjected to the decimal serial dilution. Wooden vat biofilms (cells released from toothbrush and gauze) and milk samples were directly diluted in Ringer's solution, while curd and cheese samples (15 g) were first homogenized in 2 % (*w/v*) sodium citrate solution (135 ml) by means of a stomacher (Bag-Mixer 400, Interscience, Saint Nom, France) at the maximum speed for 2 min and then serially diluted as reported above in Ringer's solution.

Cell suspensions were plated on agar media to allow the development of: total mesophilic microorganisms (TMM) spread on plate count agar (PCA) supplemented with 1 g/L skimmed milk and incubated for 72 h at 30 °C; total psychrotrophic microorganisms (TPM) spread on PCA plus skimmed milk, incubated for 7 d at 7 °C; members of the Enterobacteriaceae family poured in violet red bile glucose agar (VRGBA), incubated for

24 h at 37 °C; coliforms poured in violet red bile agar (VRBA), incubated for 24 h at 37 °C; *Escherichia coli* and *Salmonella* spp. spread on Hektoen enteric agar (HEA), incubated for 24 h at 37 °C; coagulase-positive staphylococci (CPS) spread on Baird-Parker (BP) agar supplemented with rabbit plasma fibrinogen (RPF), incubated for 48 h at 37 °C; *Listeria monocytogenes* spread on *Listeria* selective agar base (LSAB) added with SR0140E supplement, incubated for 48 h at 37 °C; pseudomonads spread on *Pseudomonas* agar base (PAB) supplemented with cephaloridine sodium fusidate cetrimide (CFC), incubated for 48 h at 25 °C; thermophilic and mesophilic coccus LAB poured in M17 agar, incubated for 48 h at 44 °C and 30 °C, respectively; thermophilic rod LAB poured in whey-based agar medium (WBAM) prepared as described by Settanni et al. (2012) and incubated for 48 h at 44 °C; mesophilic rod LAB poured in de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 M), incubated for 48 h at 30 °C; enterococci spread on kanamycin esculin azide (KAA) agar, incubated for 24 h at 37 °C; yeasts spread on yeast peptone dextrose (YPD), incubated for 48 h at 28 °C; molds spread on potato dextrose agar (PDA), incubated for 7 d at 25 °C. Growth of fungi on M17, WBAM and MRS was prevented by cycloheximide (10 mg/ml) addition, while YPD and PDA were supplemented with chloramphenicol (0.1 mg/ml) to inhibit the growth of bacteria. LAB incubation occurred in anaerobiosis in hermetically sealed jars equipped with the AnaeroGen AN25 sachets. All media, supplements and the anaerobic gas generating sachets were purchased from Oxoid (Milan, Italy). All microbiological counts were carried out in duplicates for all samples at each collection time.

Culture-independent analysis of total bacterial community

MiSeq library preparation and Illumina sequencing

Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing by Illumina MiSeq system (Illumina, USA) were performed at the Sequencing Platform of Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy). Briefly, 464-nucleotide sequences from bacterial 16S rRNA gene V3-V4 region (Baker et al., 2003; Claesson et al., 2010), corresponding to *Escherichia coli* positions 341 to 805, were amplified from each sample. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of the smaller amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to the manufacturer's instructions; subsequently, DNA concentrations of the amplicons were determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios.

Illumina data analysis and sequences identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric

sequences were identified and removed via the consensus method in DADA2. Representative bacterial sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh & Standley, 2013; Price et al., 2009). Bacterial taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99 % Operational Taxonomic Units (OTUs) database which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by MiSeq Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. Number PRJNA924124.

Chemical analyses of cheeses

Proximate composition of cheeses (moisture, protein, fat, lactose, ash and NaCl) and fatty acid profile as Saturated Fatty Acids, (SFA), Unsaturated Fatty Acids (UFA), Monounsaturated Fatty Acids (MUFA) and Polyunsaturated Fatty Acids (PUFA) were analyzed in duplicate with the FoodScan-TM2 using mid-infrared spectroscopy (MIRS) prediction models developed and commercialized by FOSS (FOSS Analytical, Italy).

Cheese antioxidant properties

Samples of milk before and after wood contact, curd and cheeses for antioxidant property determinations were stored at – 80 °C until analyses. Extracts of milk, curd and cheeses were performed according to the methods of Rashidinejad et al. (2013) with some modifications. Briefly, 8.5 ml of milk and 0.5 g of curd or cheese were suspended in 25 ml

of a 95 % methanol aqueous solution supplemented 1 % HCl and homogenized at 12,000 rpm using an Ultra-Turrax homogenizer (T 25 D, IKA WERKE, Staufen, Germany). The suspension from each sample was kept at 40 °C for 30 min in a water bath and vortexed for 30 s every 10 min. The mixture was cooled and filtered with cheese cloth and the residues were washed with 1 ml of the same solution. The filtrate was centrifuged at 7000 × g for 10 min at 4 °C, and the supernatant was kept at −80 °C until analyses. All samples were analyzed in duplicate for their antioxidant properties, measuring total phenolic content (TPC), ferric reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC) as described by Di Trana et al. (2022b).

Volatile organic compound emission

Headspace solid phase microextraction SPME (DVB/CAR/PDMS, 50 mm, Supelco) fiber was used to extract the volatile organic compounds (VOC) from cheeses. VOC profile was determined using a gas chromatograph (Agilent 6890) equipped with a mass spectrometry (MS) detector (Agilent 5975 c) and a DB-624 capillary column (Agilent Technologies, 60m, 0.25mm, 1.40µm). Cheese samples (5 g) were chopped, placed in a 25 ml glass vial and exposed to SPME-fiber under continuous stirring at 60 °C for 15 min. SPME fiber thermal desorption was performed through a splitless GC injector at 250 °C for 1 min. Chromatographic separation was achieved with helium carrier gas at 1 ml/min and an oven temperature program with a 5 min isotherm at 40 °C followed by a linear temperature increase of 5 °C min up to 200 °C, where it was held for 2 min. VOC analysis was performed in MS full scan mode applying interface temperature at 230 and acquisition mass range between 40 – 400 amu. Each VOC component was identified by MS

comparison with spectral data (NIST library). The relative proportions of the identified components were shown as percentages obtained by normalizing the area of the GC-MS peaks with the total area of the significant peaks. Three replicates were performed for each sample.

Sensory analysis

Sensory analysis of the CPL cheeses was performed to grasp the differences among the cheeses produced in the four factories. In individual cubicles at the CREA-ZA, the labelled cheese samples (1 cm per side) were presented to nine judges, following the ISO 8589 (2007) indications, in a random order on a white paper plate. The judges evaluated appearance attributes on a whole slice of each cheese. All sensory attributes were evaluated on a graduated scale from 0 (extremely low) to 8 (extremely high).

Statistical analysis

Data of microbiological, chemical and volatile organic compounds were analysed using the statistical software package Systat 13 (SYSTAT, 2009). All data were tested for the distribution of the variables with the Shapiro–Wilk test and analysed with ANOVA procedure. The model included the factory (4 levels = A, B, C and D) as fixed factor and means were compared by Tukey's test. For the sensory data analysis, smell, taste, structure and acceptability, the panellist effect was also introduced into the statistical model. Least square means were reported, and differences were considered significant at $P < 0.05$.

In addition, an explorative multivariate analysis was performed to investigate the relationship among cheeses. A hierarchical cluster analysis (HCA) (joining, tree clustering)

was carried out for grouping the cheeses according to their dissimilarity, measured by Euclidean distances, whereas cluster aggregation was based on the Ward's method (Martorana et al., 2015). In particular, the relationships between sensory attributes of the cheeses produced in the four factories and microbiological-chemical-VOCs data were evaluated. Graphic construction was achieved by using STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA).

Results and Discussions

Viable levels of microorganisms

The levels of the viable microbial groups of wooden vat biofilms and all samples collected during cheese making, as well as, ripened CPL cheeses are reported in Table 1. Wooden vat biofilms are generally developed after the contact with cheese whey (Gaglio et al., 2016a; Sun & D'Amico, 2023). The vats used to process CPL cheese hosted levels of TMM in the range 4.2 – 5.3 log CFU/cm^2. Only sample WV.CPL-C showed detectable levels of TPM (5.4 log CFU/cm^2). Except mesophilic LAB cocci of WV.CPL-A biofilm (1.9 log CFU/cm^2), LAB onto the wooden vat surfaces were generally in the range 10^3 – 10^4 CFU/cm^2.

Table 1. Microbial loads[a] of samples collected through Caciocavallo Podolico Lucano cheese production.

Sample	Growth media															
	PCA-SkM 30 °C	PCA-SkM 7 °	VRBGA	VRBA	HEA-E	HEA-S	BP (CPS)	LSAB	PAB	M17 30 °C	M17 44 °C	MRS	WBAM	KAA	YPD	PDA
WV.CPL-A	4.3 ± 0.2 c	<1 b	<1	<1	<1	<1	<1	<1	<1	1.9 ± 0.0 c	3.6 ± 0.4	2.7 ± 0.1 b	3.1 ± 0.1 b	<1	<1 c	<1 b
WV.CPL-B	4.2 ± 0.1 c	<1 b	<1	<1	<1	<1	<1	<1	<1	3.4 ± 0.1 b	3.5 ± 0.1	2.8 ± 0.0 b	2.9 ± 0.1 b	<1	1.7 ± 0.0 b	1.8 ± 0.2 a
WV.CPL-C	5.3 ± 0.2 a	5.4 ± 0.1 a	<1	<1	<1	<1	<1	<1	<1	4.4 ± 0.1 a	3.8 ± 0.4	3.3 ± 0.1 a	3.5 ± 0.2 a	<1	2.2 ± 0.1 a	<1 b
WV.CPL-D	4.8 ± 0.1 b	<1 b	<1	<1	<1	<1	<1	<1	<1	3.4 ± 0.3 b	3.4 ± 0.0	3.4 ± 0.0 a	3.7 ± 0.1 a	<1	1.9 ± 0.3 ab	1.9 ± 0.3 a
p-value	0.001	0.001	n.e	n.e	n.e	n.e	n.e	n.e	n.e	0.001	0.454	0.001	0.001	n.e.	0.001	0.001
BM.CPL-A	4.9 ± 0.0	2.0 ± 0.1 b	<1 b	2.3 ± 0.1 a	<1	<1	2.0 ± 0.0 c	<1	<1	5.0 ± 0.0 b	4.9 ± 0.0 a	3.0 ± 0.1 b	<1	3.0 ± 0.0 c	2.6 ± 0.0 b	<1 b
BM.CPL-B	4.7 ± 0.0	<1 c	<1 b	<1 d	<1	<1	2.8 ± 0.0 a	<1	<1	4.1 ± 0.0 c	3.7 ± 0.0 b	<1 c	<1	3.2 ± 0.1 b	1.3 ± 0.1 d	<1 b
BM.CPL-C	5.0 ± 0.8	3.9 ± 0.1 a	1.4 ± 0.1 a	1.9 ± 0.1 b	<1	<1	2.7 ± 0.0 a	<1	<1	5.4 ± 0.1 a	4.9 ± 0.0 a	<1 c	<1	<1 d	3.0 ± 0.0 a	0.8 ± 0.2 a
BM.CPL-D	4.7 ± 0.1	<1 c	1.3 ± 0.2 a	1.1 ± 0.1 c	<1	<1	2.2 ± 0.1 b	<1	<1	3.9 ± 0.1 d	3.8 ± 0.1 b	3.8 ± 0.1 a	<1	4.1 ± 0.0 a	2.0 ± 0.0 c	<1 b
p-value	0.707	0.001	0.001	0.001	n.e.	n.e.	0.001	n.e.	n.e.	0.001	0.001	0.001	n.e.	0.001	0.001	0.001
MAC.CPL-A	5.6 ± 0.2 ab	3.8 ± 0.2 a	2.3 ± 0.0 a	2.3 ± 0.2 a	<1	<1	2.0 ± 0.1 b	<1	<2 b	6.2 ± 0.0 a	6.4 ± 0.0 a	4.2 ± 0.0 c	5.3 ± 0.0 a	3.1 ± 0.1 a	3.3 ± 0.2 a	2.6 ± 0.3 a
MAC.CPL-B	5.4 ± 0.1 bc	3.7 ± 0.1 ab	<1 c	<1 c	<1	<1	2.3 ± 0.3 ab	<1	<2 b	5.3 ± 0.0 c	5.9 ± 0.1 b	4.5 ± 0.1 b	5.1 ± 0.1 b	1.7 ± 0.1 b	2.7 ± 0.0 b	2.8 ± 0.1 a
MAC.CPL-C	6.4 ± 0.7 a	3.7 ± 0.1 ab	2.2 ± 0.2 a	2.5 ± 0.0 a	<1	<1	2.5 ± 0.0 a	<1	<2 b	6.1 ± 0.0 b	5.7 ± 0.2 b	5.4 ± 0.0 a	4.9 ± 0.0 c	1.7 ± 0.0 b	3.3 ± 0.2 a	2.3 ± 0.2 ab
MAC.CPL-D	5.9 ± 0.0 ab	3.4 ± 0.1 b	1.1 ± 0.1 b	1.9 ± 0.1 b	<1	<1	2.1 ± 0.0 ab	<1	1.9 ± 0.1 a	5.4 ± 0.1 c	5.7 ± 0.1 b	4.5 ± 0.0 b	4.8 ± 0.0 c	1.8 ± 0.1 b	2.2 ± 0.1 c	2.1 ± 0.1 b
p-value	0.03	0.05	0.001	0.001	n.e.	n.e.	0.019	n.e.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.01
C.CPL-A	6.1 ± 0.0 c	3.1 ± 0.2 b	4.1 ± 0.1 a	3.7 ± 0.1 a	<2 b	<2	5.0 ± 0.0 d	<2	<2 b	7.3 ± 0.3 a	7.3 ± 0.1 a	5.4 ± 0.1 c	5.8 ± 0.1 b	4.3 ± 0.2 b	3.7 ± 0.1 c	3.2 ± 0.0 b
C.CPL-B	6.6 ± 0.2 b	2.1 ± 0.1 c	<1 d	<1 d	<2 b	<2	5.7 ± 0.0 b	<2	<2 b	7.1 ± 0.1 a	7.1 ± 0.2 a	5.7 ± 0.0 b	6.2 ± 0.0 a	3.9 ± 0.1 c	4.2 ± 0.1 b	3.7 ± 0.2 a
C.CPL-C	6.8 ± 0.1 ab	4.7 ± 0.6 a	3.2 ± 0.1 b	3.1± 0.0 b	<2 b	<2	5.2 ± 0.1 c	<2	<2 b	6.5 ± 0.1 b	6.6 ± 0.1 b	5.5 ± 0.0 bc	5.7 ± 0.2 b	<2 d	4.6 ± 0.1 a	3.3 ± 0.2 b
C.CPL-D	7.1 ± 0.0 a	5.1 ± 0.1 a	2.0 ± 0.0 c	2.7 ± 0.1 c	2.1 ± 0.1 a	<2	6.3 ± 0.0 a	<2	3.1±0.1 a	7.3 ± 0.2 a	7.1 ± 0.2 a	6.7 ± 0.1 a	6.5 ± 0.2 a	5.1 ± 0.0 a	4.1 ± 0.1 b	3.1 ± 0.1 b
p-value	0.001	0.001	0.001	0.001	0.001	n.e.	0.001	n.e.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
AC.CPL-A	5.7 ± 0.1 c	<2 b	<1 c	<1 c	<2 b	<2	4.5 ± 0.1 b	<2	<2 b	8.4 ± 0.0 b	8.3 ± 0.1 b	7.0 ± 0.0 a	6.9 ± 0.0 a	4.1 ± 0.0 b	3.2 ± 0.2 c	<2 c
AC.CPL-B	7.1 ± 0.0 a	<2 b	<1 c	<1 c	<2 b	<2	5.6 ± 0.2 a	<2	<2 b	8.4 ± 0.0 b	8.5 ± 0.0 b	7.1 ± 0.2 a	6.7 ± 0.0 b	4.0 ± 0.2 b	3.6 ± 0.1 b	3.0 ± 0.1 a
AC.CPL-C	7.1 ± 0.0 a	3.1 ± 0.1 a	2.7 ± 0.1 b	3.2 ± 0.1 a	<2 b	<2	5.5 ± 0.3 a	<2	<2 b	8.9 ± 0.1 a	8.9 ± 0.1 a	6.0 ± 0.1 c	5.9 ± 0.1 c	3.2 ± 0.2 c	4.1 ± 0.0 a	<2 c

Sample	Growth media															
	PCA-SkM 30 °C	PCA-SkM 7 °	VRBGA	VRBA	HEA-E	HEA-S	BP (CPS)	LSAB	PAB	M17 30 °C	M17 44 °C	MRS	WBAM	KAA	YPD	PDA
AC.CPL-D	6.8 ± 0.2 b	<2 b	4.7 ± 0.2 a	2.5 ± 0.1 b	2.2 ± 0.1 a	<2	6.0 ± 0.1 a	<2	2.7 ± 0.3 a	7.9 ± 0.0 c	7.6 ± 0.1 c	6.6 ± 0.2 b	6.5 ± 0.1 b	4.7 ± 0.1 a	4.1 ± 0.0 a	2.7 ± 0.1 b
p-value	0.001	0.001	0.001	0.001	0.001	n.e.	0.001	n.e.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
SC.CPL-A	5.5 ± 0.1 c	<2 b	<1	<1	<2	<2	3.3 ± 0.2 b	<2	<2	7.3 ± 0.2 c	7.8 ± 0.1 b	6.1 ± 0.1 c	6.5 ± 0.2 b	3.8 ± 0.1 b	<2	<2
SC.CPL-B	6.3 ± 0.2 b	<2 b	<1	<1	<2	<2	4.1 ± 0.0 a	<2	<2	7.5 ± 0.0 bc	7.8 ± 0.1 b	7.3 ± 0.2 a	7.2 ± 0.0 a	3.9 ± 0.0 b	<2	<2
SC.CPL-C	6.5 ± 0.1 b	<2 b	<1	<1	<2	<2	3.5 ± 0.2 b	<2	<2	7.8 ± 0.0 b	7.9 ± 0.1 b	6.1 ± 0.1 c	6.4 ± 0.1 b	3.2 ± 0.2 c	<2	<2
SC.CPL-D	8.6 ± 0.1 a	2.7 ± 0.1 a	<1	<1	<2	<2	4.1 ± 0.0 a	<2	<2	8.5 ± 0.1 a	8.7 ± 0.1 a	6.8 ± 0.1 b	7.2 ± 0.0 a	5.1 ± 0.0 a	<2	<2
p-value	0.001	0.001	n.e.	n.e.	n.e.	n.e.	0.001	n.e.	n.e.	0.001	0.001	0.001	0.001	0.001	n.e.	n.e.
RC.CPL-A	7.4 ± 0.2 bc	<2	<1	<1	<2	<2	<2	<2	<2	6.9 ± 0.0 b	7.1 ± 0.1 b	7.2 ± 0.1 b	6.9 ± 0.1 b	3.5 ± 0.1 c	<2	<2
RC.CPL-B	8.0 ± 0.1 a	<2	<1	<1	<2	<2	<2	<2	<2	7.2 ± 0.2 b	6.5 ± 0.1 c	7.7 ± 0.0 a	7.5 ± 0.1 a	2.9 ± 0.3 d	<2	<2
RC.CPL-C	7.2 ± 0.2 c	<2	<1	<1	<2	<2	<2	<2	<2	6.5 ± 0.2 c	6.5 ± 0.1 c	6.7 ± 0.0 c	6.2 ± 0.3 c	5.2 ± 0.1 b	<2	<2
RC.CPL-D	7.7 ± 0.1 ab	<2	<1	<1	<2	<2	<2	<2	<2	7.6 ± 0.1 a	7.4 ± 0.0 a	7.7 ± 0.0 a	7.3 ± 0.2 ab	5.8 ± 0.1 a	<2	<2
p-value	0.001	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	0.001	0.001	0.001	0.001	0.001	n.e.	n.e.

Loads are reported as log CFU/cm^2 for vat surfaces, log CFU/ml for milk samples, and log CFU/g for curds and cheeses. Results indicate mean values ± S.D. of four plate counts (carried out in duplicates for two independent productions). A, B, C, D capital letters and a, b, small letters in column within the same sample indicate significant differences for $p < 0.001$, $0.01 < p < 0.05$, respectively. Abbreviations: PCA-SkM 30 °C, plate count agar added with skimmed milk incubated at 30 °C for detection of total mesophilic microorganisms; PCA-SkM 7 °C, plate count agar added with skimmed milk incubated at 7 °C for detection of total psychrotrophic microorganisms; VRBGA, violet red bile glucose agar for detection of *Enterobacteriaceae*; VRBA, violet red bile agar for detection of total coliforms; HEA-E, hektoen enteric agar for detection of *E. coli* (red colonies); HEA-S, hektoen enteric agar for detection of *Salmonella* spp. (black colonies); BP, baird-parker agar for detection of coagulase-positive staphylococci; LSAB, *Listeria* selective agar base for detection of *L. monocytogenes*; PAB, *Pseudomonas* agar base for detection of pseudomonads; M17 30 °C, medium 17 agar incubated at 30 °C for detection of mesophilic coccus LAB; M17 44 °C, medium 17 agar incubated at 44 °C for detection of detection of thermophilic coccus LAB; MRS, de Man-Rogosa-Sharpe agar for detection of mesophilic rod LAB; WBAM, whey-based agar medium for detection of thermophilic rod LAB; KAA, kanamycin aesculinazide agar for detection of enterococci; YPD, yeast peptone dextrose agar for detection of yeasts; PDA, potato dextrose agar for detection of molds; WV, wooden vat; BM, bulk milk; MAC, milk after contact with wooden vat surface; C, curd; AC, acidified curd; SC, stretched curd; RP, ripened cheese; CPL, Caciocavallo Podolico Lucano; A – D, factories A – D; n.e., not evaluated.

The highest LAB levels were registered for mesophilic LAB cocci of WV.CPL-C biofilm counted at 4.4 log CFU/cm^2, explaining the high TPM levels associated to this wooden vat biofilm. High levels of TPM are often detected in wooden vat biofilms used to process PDO traditional Sicilian cheeses (Gaglio et al., 2016a; Settanni et al., 2013), and this finding is imputable to the psychrotrophic behaviour of LAB (Pothakos et al., 2012). Except WV.CPL-C, LAB counted at the highest levels in the other vats were generally thermophilic (cocci in WV.CPL-A and WV.CPL-B or rods in WV.CPL-D). The bacterial levels registered on the wooden vats used for CPL cheese production are similar to those generally reported for the wooden vats used for curdling raw cow's milk for the production of Caciocavallo Palermitano (Scatassa et al., 2015), PDO Ragusano (Licitra et al., 2007) and PDO Salers cheeses (Didienne et al., 2012).

All undesired groups represented by members of Enterobacteriaceae family, in particular, coliforms, and specifically *E. coli* and *Salmonella* spp., as well as CPS, *L. monocytogenes* and pseudomonads, generally associated with poor hygiene of dairy productions (Claeys et al., 2013), were below the detection limit in all wooden vat biofilms. These samples showed also undetectable levels of enterococci. Yeasts were uncountable in sample WV.CPL-A and counted at very low levels in the other samples, while molds were detected at low levels only in samples WV.CPL-B and WV.CPL-D (1.8 and 1.9 log CFU/cm^2, respectively). Except smear-ripened cheeses, yeasts and molds cause spoilage in other cheese typologies (Geronikou et al., 2022; Izzo et al., 2022).

Bulk milk before transformation hosted almost 10^5 CFU/ml of TMM and increased until 5.4 – 6.4 log CFU/ml after contact with the wooden vat surfaces. The increase of TMM levels of milk is an expected phenomenon after rest in wooden vats (Didienne et al., 2012).

Half of the factories (A and D) showed detectable levels of LAB rods in bulk milk, but after contact with the wooden surfaces this group increased until almost 5.0 log CFU/ml in all factories. No big differences were found for the levels of CPS, Enterobacteriaceae, yeasts and molds before and after wooden surface contact, while the average level of enterococci decreased. Regarding pseudomonads, these bacteria were not detected in wooden vat biofilms or in bulk milk, but the levels registered in milk after interaction with WV.CPL-D were 1.9 log CFU/ml. The contact with wood did not affect the detectability of *Salmonella* spp., *L. monocytogenes* and *E. coli*. Cruciata et al. (2018) demonstrated that the attachment of these human pathogens to the inner vat surfaces is hindered by the LAB biofilms.

A general increase of almost all microbial groups investigated was observed after curdling. On average, this increase was about 1 log cycle and, except *E. coli* for factory D (2.1 log CFU/g), the groups undetectable in milk were still below the detection limit in curds. The acidification of the curds, occurred during almost 4-5 h, determined the decrease of molds in all factories, and for factories A and C they decreased below the detection level. The acidification process did not kill members of Enterobacteriaceae family, coliforms and *E. coli* and well as pseudomonads, but inhibited their growth. All LAB groups (including enterococci) increased their levels, but those dominating the acidification process were mesophilic and thermophilic LAB cocci (on average, 8.4 and 8.3 log CFU/g, respectively). This behavior was expected considering the results registered during Caciocavallo Palermitano cheese production (Settanni et al., 2012).

The stretching operation, typical of Caciocavallo cheese making, exerted a definite sanitizing effect on the curds of all factories. Curd stretching is performed after

824 acidification and consists on the scalding of the curd at approximately 85 – 95 °C to allow
825 molding (Licitra et al., 2017). The thermal shock applied with stretching determined a
826 further reduction (until below the detection levels) of almost all undesired microbial groups
827 still present in curds after acidification. However, CPS, derived from bulk milk, were still
828 detected in stretched curds and their levels were quite consistent (3.3 – 4.1 log CFU/g).
829 These results are not surprising; de Andrade et al. (2022) reported that the treatment at
830 95 °C for a few minutes is not enough to inactivate completely CPS. However, after 4-
831 month ripening, also CPS levels decreased below the detection limit and these results
832 comply with the Commission Regulation 2073/2005 on "microbiological criteria for
833 foodstuff" (Commission Regulation, 2005), highlighting the high hygienic standards of
834 ripened CPL cheeses. Among the pro-technological groups, even though all LAB levels
835 slightly decreased, once again both mesophilic and thermophilic LAB cocci groups
836 dominated the microbial community. Only enterococci were not affected by the thermal
837 treatment applied during stretching. The thermal resistance of enterococci at the
838 temperatures applied during milk pasteurization is known (Barbaccia et al., 2022; García-
839 González et al., 2022).
840 Ripened CPL cheese microbiological characteristics were highly similar among the four
841 dairy factories. Basically, among the 16 microbial groups investigated by plate counts only
842 TMM and all LAB groups, including enterococci, were detected. Regarding enterococci
843 the cheeses produced in factories A and B (3.5 and 2.9 log CFU/g, respectively) showed
844 levels lower than those produced in factories C and D (5.2 and 5.8 log CFU/g,
845 respectively). These bacteria play several positive roles during cheese fermentation; in
846 particular, they are involved in the development of the organoleptic characteristics and,

847 generally, contribute consistently to the typicality of the ripened cheeses (Foulquié Moreno
848 et al., 2006). Concerning safety aspects, enterococci represent a risk for consumers when
849 they show multidrug resistance and/or virulence traits (Gaglio et al., 2016b). However, the
850 Commission Regulation 1441/2007 of 5 December 2007 does not set limits for their
851 presence in cheeses (Commission Regulation, 2007). No big differences were found
852 between LAB cocci and LAB rods; in particular, rod groups increased with ripening
853 following the common LAB evolution during cheese production (Samelis & Kakouri,
854 2022).
855
856 *Analysis of microbiota by Illumina analysis*
857 Microbiotas associated with the wooden vats and ripened CPL cheeses were deeply
858 studied by a next generation sequencing (NGS) approach that represents a routine
859 investigation to provide a deep description of the microbial composition and evolution of
860 complex environments (Jagadeesan et al., 2019). The relative abundances (%) of the
861 bacterial OTUs resulting from MiSeq Illumina analysis of the biofilms associated with the
862 wooden vats used to process CPL cheeses and the final 4-month ripened cheeses are
863 distributed according to the bar plot of Fig. 2. The bacterial diversity displayed by both
864 wooden biofilm and cheese biotas is quite limited; barely 18 taxonomic groups, mainly at
865 family and genus levels, were identified. RA % of all samples analyzed was mostly
866 represented by LAB: 94.74 – 99.70 % in wooden vat biofilms; 94.19 – 100 % in ripened
867 cheeses. LAB proportions among the four dairy factories varied and these differences can
868 be imputed to the environmental conditions, efficacy of brushing during cleaning and,
869 especially, different bulk milks processed by each factory (Seale et al., 2015).

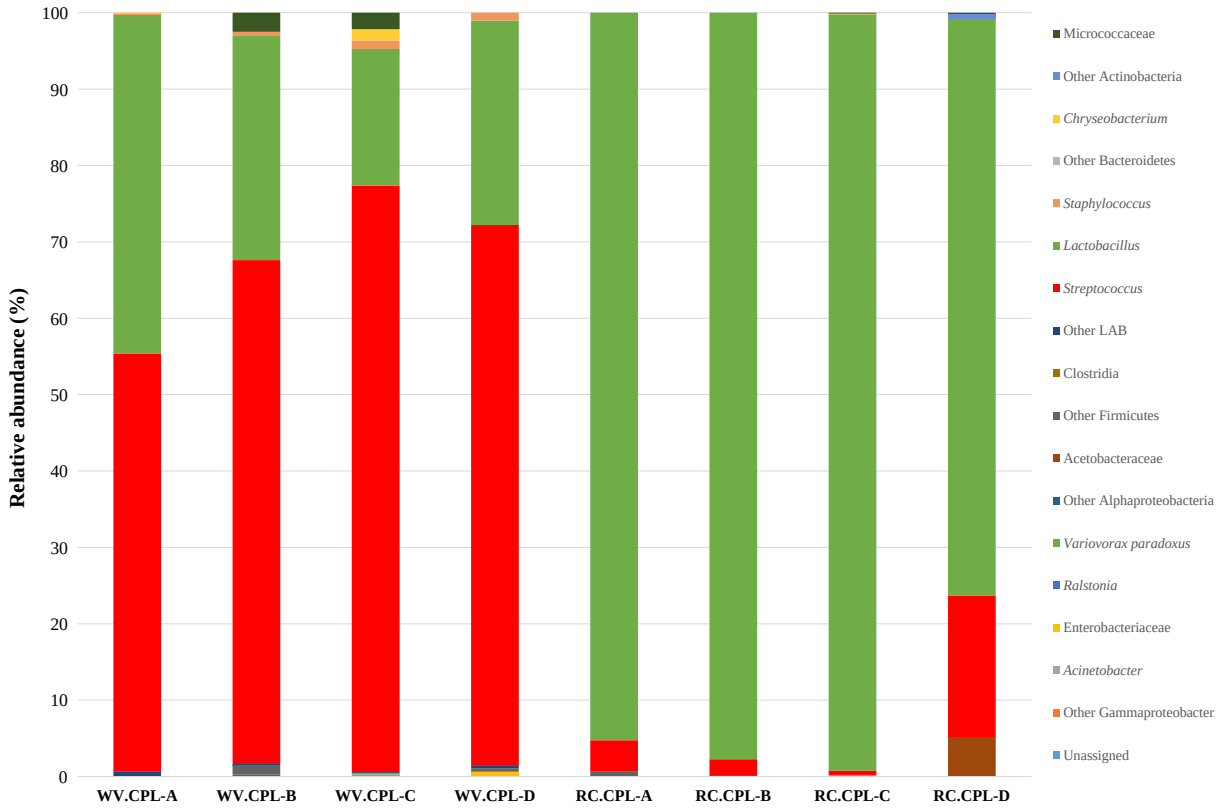

Fig. 2. Relative abundances (%) of of the taxonomic groups identified by MiSeq Illumina in wooden vat biofilms and ripened Caciocavallo Podolico Lucano (CPL) cheeses. Abbreviations: WV, wooden vat; RC, ripened cheese; A – D, factories A to D.

870

871

The vast majority of LAB were classified as lactobacilli and streptococci; in particular, *Lactobacillus* species dominated ripened cheeses ranging from 75.56 % in RC.CPL-D and even up to 98.99 % in RC.CPL-C. However, following the reclassification of Zheng et al. (2020), the group of lactobacilli includes several genera in addition to *Lactobacillus*. Wooden vat biofilms showed a major presence of *Streptococcus* species, whose RA % ranged between 54.70 and 76.76 %. These results follow a general trend observed for the wooden vats used to process other stretched cheeses in South Italy (Lortal et al., 2009; Scatassa et al., 2015). The consistent presence of lactobacilli explains the high increase in numbers registered for this group in milk after contact with the wooden vat for all factories.

Ripened cheeses hosted negligible levels (< 1 % of total bacterial diversity) of Micrococcaceae and other Actinobacteria, Firmicutes other than LAB, *Variovorax*, and Ralstonia. The presence of Acetobacteriaceae was only detected in RP.CPL-D and accounted for 5.06 %. All taxonomic groups detected in cheeses were also detected within wooden vat biofilms and, in addition, low percentages of *Chryseobacterium*, *Staphylococcus*, Enterobacteriaceae and *Acinetobacter* were also identified, especially from the wooden vat used in factory C. The presence of these bacteria is quite common on the surfaces of the wooden vats used to process milk into cheese (Sun & D'Amico, 2021).

Although culture-independent methods are being used as the sole tools to characterize complex matrix microbiota (Marino et al., 2019), these relatively new technologies are still evolving and not yet standardized (Zapka et al., 2017). Furthermore, culture-independent methods alone might not provide all information about microbiota behavior, like viability of dominant strains. For this reason, culture-based assessments are still particularly useful

to study cheese microbiotas. Thus, in the present study, both culture-dependent and –independent approaches were combined and provided important information in terms of dominance of desired dairy LAB and absence of potentially harmful *E. coli* and CPS as well as pathogenic species, such as *L. monocytogenes* and *Salmonella* spp. both in wooden vats and ripened CPL cheeses. These results confirmed that wooden tools might definitely contribute to the safety of the final traditional cheeses made from raw milk (Cruciata et al., 2019).

Chemical composition and fatty acid profile of cheese sample

Gross chemical composition and fatty acid profile of CPL cheese determined by FOSS are reported in Table 2. This methodology was utilized to predict dairy chemical composition and fatty acid content in several studies (Soyeurt et al. 2008; De Marchi et al. 2014; Penasa et al. 2015; Gottardo et al., 2017). The factory affected all parameters of chemical composition ($P < 0.01$) and, except SFA, fatty acid profile ($P < 0.001$). The cheeses produced in factory C showed higher protein and fat contents than the rest of cheeses. These macromolecules are both considered indicators of nutrition quality and energy availability. Protein fraction, mainly casein, represents an excellent source of essential amino acids and bioactive peptides exerting beneficial effect on the human body (Zimecki et al., 2007). The absence of lactose in CPL cheeses of factory C provides these products with added value, since lactose in hard and long-term ripened cheeses is generally absent or present at very low concentrations and can be consumed by individuals suffering primary lactose intolerance (Silanikove et al., 2019). Fat fraction of cheeses produced in factory C was characterized by the highest PUFA and MUFA contents that are considered

of health value (Dewhurst et al., 2006); increasing the level of PUFA in the consumer diet is an important nutritional recommendation are to (Department of Health, 1994).

Table 2. Gross chemical composition (g/100 g of cheese) and fatty acids profile (mg/100 g fat) of Caciocavallo Podolico Lucano cheeses.

Parameters	Samples				SEM	*p* value
	RC.CPL-A	RC.CPL-B	RC.CPL-C	RC.CPL-D		
Moisture	33.62 c	33.99 b	30.85 d	35.11 a	0.065	0.001
Protein	34.56 c	33.92 b	36.96 a	34.40 c	0.105	0.001
Fat	26.36 c	26.31 c	27.94 a	25.16 b	0.097	0.001
Lactose	0.69 a	1.30 a	0 b	0 b	0.167	0.001
Ash	4.77 b	4.47 b	5.81 a	5.87 a	0.076	0.001
NaCl	1.98 a	1.82 ac	1.53 bc	1.89 a	0.071	0.011
SFA	13.60	13.25	13.83	13.56	0.191	0.281
UFA	5.62 c	5.15 d	6.37 a	6.07 b	0.035	0.001
MUFA	5.08 b	4.85 c	5.44 a	5.43 a	0.040	0.001
PUFA	0.97 c	0.85 d	1.29 a	1.15 b	0.024	0.001

Results indicate the mean values of determinations carried out in duplicate for each of the two independent productions. A, B capital letters indicate significant differences for $p < 0.01$. Abbreviations: RC, ripened cheese; CPL, Caciocavallo Podolico Lucano; A – D, factories A – D; SEM = standard error of mean; SFA = Saturated fatty acids; UFA = Unsaturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Chemical composition and fatty acid profile of CPL cheese highlighted the uniqueness of this traditional cheese. These results showed a great variability due to the numerous factors characterizing the dairy factories, such as quality and quantity of forage available for grazing, pasture biodiversity, soil type, geographical position and climate. Furthermore, the artisanal techniques applied during cheese making and ripening play a relevant role in definition of chemical and fatty acid profiles. The average values of protein, fat, lactose, ash and salt of the CPL cheeses, ripened for four months, produced in the four factories were close to those reported by Di Trana et al., (2022a) for the CPL ripened 6 months and made at early spring. Regarding fatty acid profile, PUFA content of the cheeses manufactured in this study confirm that grazing is closely related to higher PUFA intake (Chilliard et al., 2007). The extensive rearing system of Podolica cattle is a peculiar characteristics of this traditional cheese production system; consequently, the differences in PUFA among factories are related to the type of plants ingested and their development

939 stage (Chilliard et al., 2007). In Alpine cheeses produced in summer and winter, Danieli et
940 al., (2022) reported a factory effect in MUFA and PUFA contents.
941
942 *Antioxidant properties of cheeses*
943 Results concerning the effect of dairy factory on cheese antioxidant properties are
944 reported in Table 3. In bulk milk before and after wood contact, TPC and TEAC were
945 affected by factory factors ($P < 0.05$ and $P < 0.001$, respectively), while no significant
946 differences were recorded for FRAP assay. Concerning curd, no effect of factory was
947 observed on any quantified parameters. On the contrary, production factory affected TPC,
948 FRAP and TEAC values of CPL cheeses ripened for four months ($P < 0.008$ and $P < 0.01$,
949 respectively).
950 Phenolic compounds are substances produced by several plants that are beneficial to the
951 human health (Di Lorenzo et al., 2021). The presence of these compounds originating from
952 forage species (De Feo et al., 2006; Di Trana et al., 2015), natural pastures (Chavez-Servin
953 et al., 2018; Hilario et al., 2010) and aromatic plants (Branciari et al., 2015; Garcia et al.,
954 2014) has been observed in milk and dairy products. Therefore, a strong relationship
955 between a given breeding environment (plant biodiversity and plant availability) and the
956 dairy products processed from milk of cows reared in that area is established. Furthermore,
957 the bioavailability of phenolic compounds is connected to the physiology of ruminant
958 digestion and to the degree of polymerization of polyphenols (Bravo, 1998), which act on
959 the degradation and absorption of these compounds and/or their metabolites (Di Lorenzo et
960 al., 2021; Tufarelli et al., 2017). This complex mechanism underlies the degree of transfer

961 of these bioactive molecules from the animal diet to milk and, finally, cheese. Thus, each
962 factory characterized by its own pasture determines the uniqueness of the final cheeses.

963 **Table 3.** Antioxidant capacity of samples collected through Caciocavallo Podolico Lucao cheese productions

Parameters	Factories	Samples			
		BM.CPL	MAC.CPL	C.CPL	RC.CPL
TPC (g GAE/kg)	A	0.212 b	0.154 b	2.795	3.714 c
	B	0.236 a	0.203 a	2.791	4.043 ab
	C	0.235 a	0.149 b	2.946	4.219 a
	D	0.200 b	0.202 a	2.656	4.007 b
	SEM	0.005	0.013	0.051	0.049
	p value	0.011	0.030	0.069	0.008
FRAP ($mmolFeSO_4/kg$)	A	0.588	0.565	1.692	3.354 a
	B	0.590	0.504	1.237	2.471 c
	C	0.688	0.577	1.618	2.918 b
	D	0.620	0.659	1.565	3.218 ab
	SEM	0.029	0.037	0.139	0.089
	p value	0.194	0.063	0.244	0.008
TEAC (mmolTrolox/kg)	A	5.648 A	2.740 c	18.535	47.563 b
	B	3.007 B	3.572 a	18.760	39.701 c
	C	3.282 B	3.079 b	16.813	57.995 a
	D	2.902 B	3.402 a	23.401	51.964 ab
	SEM	0.155	0.102	2.154	1.910
	p value	0.001	0.004	0.301	0.011

964 Results indicate the mean values of the determinations carried out in duplicate for each of the two independent productions. A, B capital
965 letters and a, b, c small letters in column within the same sample indicate significant differences for $p < 0.001$, $0.01 < p < 0.05$,
966 respectively. Abbreviations: BM, bulk milk; MAC, milk after contact with wooden vat surface; C, curd; RP, ripened cheese; CPL,
967 Caciocavallo Podolico Lucano; A – D, factories A – D; TPC = total phenolic content; GAE = gallic acid equivalent; FRAP = ferric
968 reducing ability power; TEAC = Trolox equivalent antioxidant capacity; SEM = standard error of mean.
969

970 In this study, the factory as a whole affected TPC of milk before and after contact with
971 the wooden vat and CPL cheese ripened for four months. The highest TPCs were observed
972 in factories B and C both for bulk milk and for CPL cheeses, but not for bulk milk after
973 contact with the wooden vat, because the samples from factory C showed the lowest TPC
974 value (Table 3). The effect of the feeding system, implemented in various factories, was
975 highlighted on the TPC content in the milk of cows reared on intensive rotational grazing,
976 semi-intensive conventional grazing and conventional grazing (Kuhnen, et al., 2014).

977 TPC values registered in this study are not always comparable with those from cheeses
978 obtained with the same production technique and ripening. Even though TPC values
979 measured for CPL cheeses were in the same range of those recorded for Caciocavallo
980 Palermitano cheese (3.52 – 4.65 g GAE/kg) (Di Trana et al., 2022b), they were higher than
981 those reported for stretched cheese Casizolu del Montiferru (2.98 – 3.65 g GAE/kg).
982 The antioxidant capacity of dairy products is mainly related to caseins, β-carotene, uric
983 acid, vitamin E, phenols, whey protein and microbiological profile (Fardet & Rock, 2018;
984 Khan et al. 2019), and it can hinge on cheese making procedure (Lucas et al., 2006), type
985 of coagulant (Pattorn et al., 2013), conditions and duration of ripening (Gupta et al., 2009).
986 In the present study, FRAP assay and TEAC (ABTS assay) were used to determine the
987 antioxidant capacity of CPL cheese. FRAP assay did not indicate great specificity for
988 antioxidant compound content in milk and curd of different factories (Table 3); no
989 difference was found among factories for this parameter. In CPL cheeses 4-month ripened,
990 the antioxidant capacity evaluated by FRAP test showed the highest values in cheeses from
991 factories A and D, and this result is in agreement with what was observed by Danieli et al.
992 (2022) who highlighted the factory effect on Alpine cheeses produced in winter.
993 Comparing CPL cheeses to other traditional southern Italian cheeses, FRAP values were
994 higher than those of 6-month ripened Casizolu del Montiferru cheese (1.69 – 2.08 mmol
995 $FeSO_4$/kg) and 2-month ripened Caciocavallo Palermitano cheese (1.84 – 2.00 mmol
996 $FeSO_4$/kg) (Di Trana et al., 2022b).
997 The bulk milk before wood contact of factory A showed the highest TEAC, while among
998 bulk milks after wood contact, the highest TEAC values were detected in factories B and
999 D. A similar trend was registered for TPC values. Regarding curd samples, no significance

was observed for the factory factor. In addition, differences were found for TEAC when comparing the factories; CPL cheeses produced in factory C showed TEAC values 32 %, 18 % and 10 % higher than those registered for the cheeses produced in factories B, A and D, respectively (Table 3). The factory effect on the TEAC seems reasonably explained by the cows' feeding regimes adopted in spring-summer and by plant biodiversity and plant availability in the different area of grazing. The highest values of TPC and TEAC observed in CPL cheeses produced in factory C reflect the positive correlation reported by Přikryl et al., (2018) between TPC and antioxidant capacity of cheese. TEAC values in CPL cheeses (Table 3) are close to those reported for Caciocavallo Palermitano cheese (46.8 – 52.4 mmol/kg) but much higher than those characterizing Casizolu del Montiferru cheese (10.3 – 18.9 mmol/kg) (Di Trana et al., 2022b).

Cheese VOC profiles

VOC profiles generated from cheese samples were identified by SPME-GC–MS; results are reported in Table 4. VOC belonging to fatty acids, alcohols, esters, aldehydes and ketones were detected in all cheese samples. For all factories, the main VOC class found in cheese samples was free fatty acids (FFA), specifically hexanoic and butanoic acids showed the highest values in all cheeses. This finding is not surprising; indeed, hexanoic and butanoic acids are generally recognized in cow's milk cheeses (Wolf et al 2011; Sulejmani et al., 2020). Short-chain FFA may generally result from lipolysis of milk fats due the action of the lamb rennet used for curdling and, partly, also to the activity of raw milk lipoprotein lipase (McSweeney & Sousa, 2000). Even acetic acid was found in comparable proportions among the cheeses from the four dairy factories; this compound

can be originated by the carbohydrate (primarily lactose) catabolism of LAB under anaerobic conditions (Rehman et al., 2000).

Table 4. Volatile organic compounds emitted from Caciocavallo Podolico Lucano cheeses

Chemical compounds	Samples				*p*-value
	RC.CPL-A	RC.CPL-B	RC.CPL-C	RC.CPL-D	
Acids					
Acetic acid	13.78 ± 2.62	14.51 ± 3.01	15.31 ± 2.30	12.25 ± 1.96	0.523
Butanoic acid	32.15 ± 4.82	35.11 ± 5.69	26.87 ± 4.03	30.63 ± 5.21	0.305
Pentanoic acid	0.34 ± 0.07 A	n.d. B	0.28 ± 0.05 A	0.29 ± 0.06 A	0.001
Exanoinc acid	42.06 ± 6.73	30.77 ± 5.91	40.26 ± 6.04	37.77 ± 6.42	0.214
Octanoic Acid	5.82 ± 0.81 a	3.00 ± 0.57 b	5.70 ± 1.09 a	5.48 ± 1.05 a	0.015
Nonanoic Acid	0.64 ± 0.13 B	2.11 ± 0.4 A	0.25 ± 0.05 B	0.25 ± 0.04 B	0.001
Esters					
Butanoic acid ethyl ester	0.27 ± 0.06 B	1.67 ± 0.31 A	0.75 ± 0.11 B	2.01 ± 0.44 A	0.001
Hexanoic acid ethyl ester	2.37 ± 0.44 C	6.54 ± 1.16 AB	5.17 ± 0.98 B	8.80 ± 1.38 A	0.001
Heptanoic acid, 2 methyl-2-butyl ester	n.d. B	0.53 ± 0.08 A	n.d. B	n.d. B	0.001
octanoic acid ethyl ester	0.24 ± 0.05 C	0.50 ± 0.09 BC	0.60 ± 0.12 AB	0.86 ± 0.16 A	0.001
Alcohol					
1 butanol 3 methyl (isoamyl alcohol)	1.08 ± 0.21 a	1.34 ± 0.2 a	1.08 ± 0.17 a	0.62 ± 0.1 b	0.007
1 hexanol/ 1 pentanol 4 methyl	0.53 ± 0.1 B	0.54 ± 0.09 B	2.22 ± 0.37 A	0.40 ± 0.07 B	0.001
2 heptanol	0.21 ± 0.03 B	1.20 ± 0.24 A	0.42 ± 0.07 B	0.33 ± 0.06 B	0.001
1 octanol	n.d. B	n.d. B	0.32 ± 0.05 A	n.d. B	0.001
Ketons					
2 Pentanone	0.22 ± 0.05 A	0.10 ± 0.02 B	0.07 ± 0.01 B	0.03 ± 0.01 B	0.001
2 Heptanone	0.17 ± 0.09 B	0.50 ± 0.02 A	0.13 ± 0.02 B	0.07 ± 0.02 B	0.001
2 Nonanone	n.d. C	1.25 ± 0.17 A	0.44 ± 0.07 B	n.d. C	0.001
Aldeydes					
2-Octenal	0.06 ± 0.01 C	0.21 ± 0.04 A	0.08 ± 0.01 BC	0.14 ± 0.02 B	0.001
Nonanal	0.06 ± 0.01 B	0.12 ± 0.02 A	0.04 ± 0.01 B	0.06 ± 0.01 B	0.001

Results indicate the mean percentage values of three measurements and are expressed as relative peak areas (peak area of each compound/total area of the significant peaks in all samples) x l00. A, B, C capital letters and a, b small letters on the row indicate significant differences for $p < 0.001$, $0.01 < p < 0.05$, respectively.
Abbreviations: RC, ripened cheese; CPL, Caciocavallo Podolico Lucano; A – D, factories A – D; n.d., not detectable.

After FFA, esters represented the second major class of compounds found in the volatile fraction of cheese samples. Within this class, ethyl hexanoate showed the highest area value, followed by ethyl butanoate, ethyl octanoate and ethyl decanoate. These compounds have floral and fruity notes and may contribute to cheese aroma by minimizing the sharpness of fatty acids and the bitterness of amines (Pinho et al., 2003). Similar ester profiles were also observed in other cheeses such as “Provola dei Nebrodi” (Ziino et al.,

2005). Aldehydes, alcohols and ketones are the most poorly represented classes in all cheeses. Among the four cheese productions, statistical analysis showed slight variations.

In particular, pentanoic acid was not detected in cheeses from factory B, while nonanoic acid concentration was higher than that of the other cheeses. Among esters, hexanoic acid ethyl ester in cheese from factory A was detected at low concentrations, while the highest were registered for production D. Heptanoic acid, 2 methyl-2-butyl ester was identified only for the cheeses processed in factory B. Regarding alcohols, isoamyl alcohol was less abundant in production D, but comparable values were displayed by the other cheeses.

Similarly, 1-hexanol was most abundant in cheese from factory C while comparable levels were registered in the other cheeses. Among ketones, production A differed for the highest content of 2-pentanone, while production B showed the highest 2-heptanone content. Finally, 2-nonanone was detected exclusively in cheeses from factories B and C. Generally, the aromatic profiles of the cheeses did not greatly differ among the four productions, especially with regard to the volatile compounds present at low levels. The production process may affect the content of esters that are mainly produced by enzymatic or chemical reaction of fatty acids with primary alcohols during ripening (Engels et al., 1997), whereas the presence of aldehydes, alcohols and ketones is directly influenced by the enzymatic activities of microorganisms (Muñoz et al., 2003).

Sensorial analysis

CPL is a high quality cheese with an intense and complex flavour. Fig. 3 reports the Kiviat graphic representation of the sensory characteristics evaluated on the CPL cheeses produced in four factories.

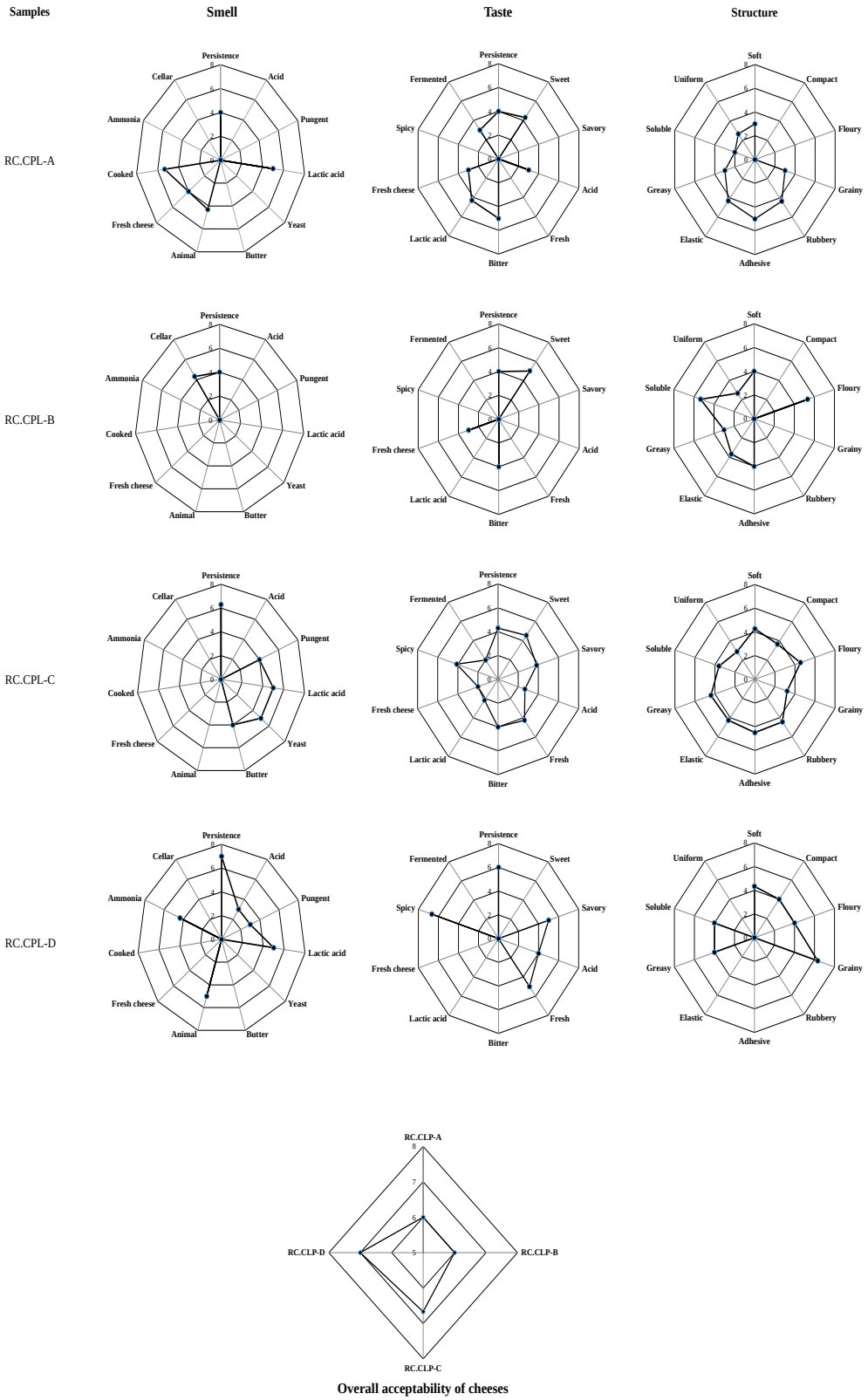

Fig. 3. Kiviat diagrams corresponding to the descriptive sensory analysis of Caciocavallo Podolico Lucano (CPL) cheeses. Abbreviations:RC, ripened cheese; A – D, factories A to D.

Farm management, animal diet (Carpino et al., 2004), cheesemaking technology, chemical and microbiological characteristics of raw milk (Martin et al., 2005) influence cheese sensory properties. As the factories were located in the same territory but on different altitude, the sensory attributes could present a variability linked to the different floristic composition of pasture. Except for persistence, all smell sensory descriptors of the cheeses were affected by factory. Acid smell attribute, typical of lactic and citric acids, was evident only in cheeses produced in factory D.

The pungent aroma is a sharp smelling or irritating with a physically penetrating sensation in the nasal cavity (Murray & Delahunty, 2000). Curioni and Bosset (2002) reported that the odour pungent descriptor in cheeses could be linked to the following volatile compounds: propanoic acid, ethanoic acid when pungent is vinegar-like, 3-methylbutanal and sulfur compounds products from Strecker degradation, in particular 3-methylthiopropanal which imparts a pungent acrid odour.

In our work, VOCs responsible for pungent aroma were not detected, but this attribute characterized the cheeses from factories C and D. Fermented aroma could be associated with a long-ripened cheese (Santillo et al., 2012) and except for the cheeses from factory B, was revealed in all cheeses. Butter odour has been associated with δ-dodecalactone and decanoic acid in Grana Padano, with 2-methylpropanoic acid in Gruyère cheese and with 2,3-butanedione (diacetyl) in Camembert, Cheddar and Emmental (Curioni & Bosset, 2002).

Furthermore, butter smell linked to 3-hydroxy-2-butanone was detected in Ragusano cheese obtained from cows fed on pasture, and not from cows that received a total mixed ration (Carpino et al, 2004). In our study, none of these VOCs was detected and all cows

1082 were fed on pasture, but the cheese produced in factory C were scored positive for butter
1083 smell. Animal odour was reported in bovine and water buffalo Mozzarella due to the
1084 presence of aldehydes nonanal, in Roncal cheese connected to tetradecanoic acid and in
1085 water buffalo Mozzarella linked to ketons 2-heptanone (Curioni & Bosset, 2002). In our
1086 study, animal smell was perceived in cheeses from factories A and D but does not seem to
1087 be related to nonanal and 2-heptanone emitted at the highest levels from the cheeses
1088 produced in factory B which resulted negative for this attribute. Fresh cheese smell
1089 attribute was linked to the presence of ethyl-3-methylbutanoate, and identified in
1090 Mozzarella bovine (Curioni & Bosset, 2002). Cooked smell, related with S-methional
1091 compounds was found in Grana Padano and Cheddar (Curioni & Bosset, 2002).

1092 In our study however, this was only evident in cheese produced in factory A and was
1093 not correlated with the sulphur compounds. Except for persistence, all taste sensory
1094 descriptors of cheeses were affected by factory. Sweet taste, typical of sucrose,
1095 characterized all cheeses except that produced in factory D. Furthermore, all cheeses
1096 except cheese D were characterized by bitter taste, typically associated to caffeine and
1097 quinine (Murray & Delahunty, 2000); this may related to the different cheesemaking
1098 technologies or brine (Fallico et al. 2005). The spicy or piquant note, irritating and
1099 aggressive taste perceived in the mouth or throat (Esposito et al., 2014), was evident in
1100 cheeses from factories C and D. Furthermore, this sensation is also a consequence of an
1101 intense lipolysis generating higher FFA (Santillo et al., 2012). Regarding CPL cheese
1102 structure, the products from factories A and C showed the greatest number of attributes
1103 (soft, grainy, rubbery, adhesive, elastic and greasy). Rubbery, adhesive and elastic were
1104 significantly affected by the factory. From a structural point of view, the mechanical

behavior of cheese is the result of complex interactions established between various components such as the resistance to deformation of the casein network (Lawrence et al., 1983), humidity, minerals and fat (Masi & Addeo, 1986). The elasticity of some stretched curd cheeses is positively related to fat and moisture content and negatively to mineral content (Masi & Addeo, 1986).

In fact, CPL cheeses produced in factory C with higher rubbery, adhesive and elastic attributes were characterized by higher protein, fat and lower salt and moisture contents, while the cheeses produced in factory A showed a lower protein and fat but a higher salt and humidity content and exhibited slightly lower scores for rubbery and elastic attributes. Elasticity was related to ripening and cheesemaking technology factors and, for Caciocavallo Palermitano it was correlated to the compressive stress (Bonanno et al., 2013). A relationship between rubbery attribute and the percentage of salt in brine has been detected in Caciocavallo dei Monti Dauni produced in different factories (Santillo et al., 2012). Buffa et al. (2001) and (Bonanno et al., 2013) proposed that cheese microbiota modifies the structure of cheese. The soft attribute has been related to cheese FA profile (Martin et al., 2005; Esposito et al., 2014) which, in turn, is closely connected to the nature and amount of fresh forage intake (Chilliard et al., 2001).

The cheeses produced in factories C and D, a higher UFA content associated with a slightly higher softness score was observed. In all factories, Podolica breed cows used pasture characterized by plant biodiversity and availability with an impact on cheese UFA content. In general, a good sensory profile was found in TAP CPL cheeses analyzed in this study. However, the variability among factories, considered as a peculiarity of all TAP cheeses, was confirmed. However, although sensory evaluation indicated some differences

among cheeses, sensory evaluation highlighted that the overall acceptability did not differ significantly ($p>0.05$) among factories. Similar results were observed by Claps (2001), who evaluated the sensory profile and acceptability of CPL cheeses produced on mountain and hilly pastures.

Data correlation

With the aim of investigating the relationships between cheese sensory attributes and data from microbiological, chemical and VOC analyses, a HCA was performed. This analysis allows to differentiate the samples examined (cheeses) in accordance with their mutual dissimilarity and relationship and join together in a dendrogram from the closest one, i.e., the most similar, to the furthest apart, which is the most different (Guarcello et al., 2016). The three resulting clusters are reported in Fig. 4. This figure clearly shows levels of dissimilarity at around 8 % among the cheeses for all relationships evaluated. In detail, regarding the relationships among sensory attributes and microbiological-VOCs (Fig. 4 A and C) data, the cheeses from the factories A and B formed a single cluster and they were clearly separated from those produced in factory C and factory D. The HCA performed with sensory attributes and chemical data formed two main clusters (Fig. 4 B) one for the cheeses from the factories A and B and one for those produced in factory C and factory D.

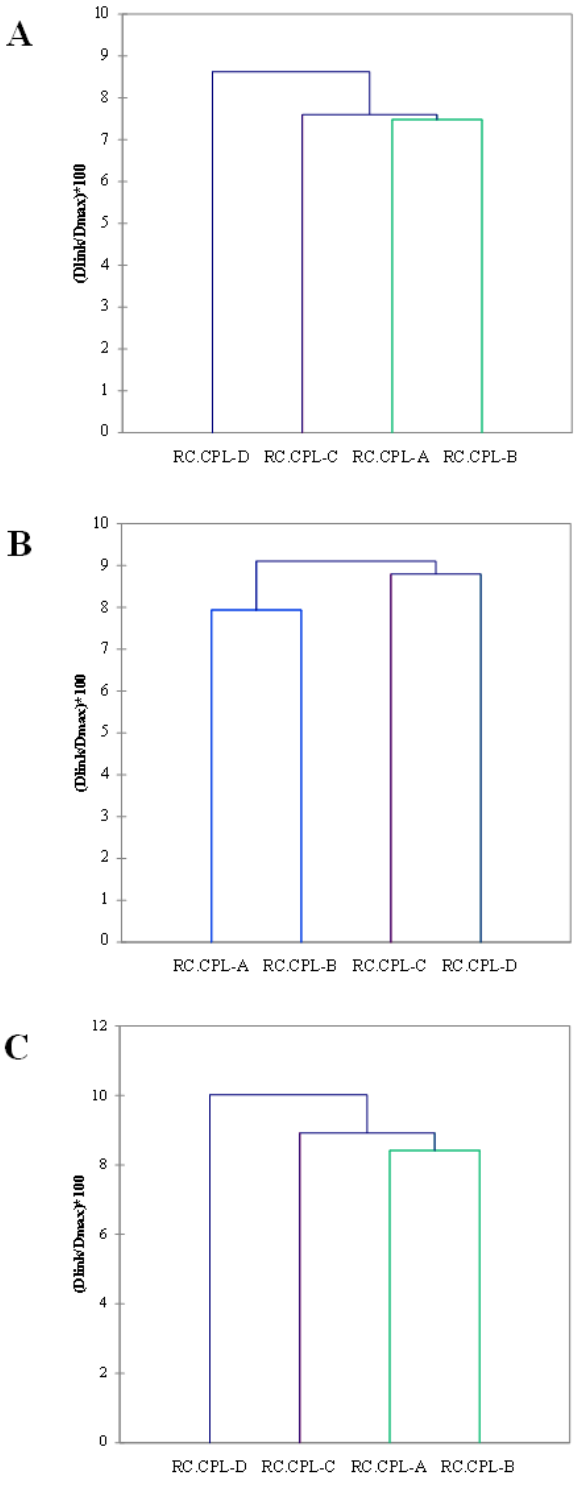

Fig. 4. Dendrograms resulting from HCA based on values of: A, sensory attributes and microbiological data; B, sensory attributes and chemical data; C, sensory attributes and VOCs data. The dissimilarity among cheeses was measured by Euclidean distance, while cluster aggregation was achieved by single linkage. Abbreviations: RC, ripened cheese; CPL, Caciocavallo Podolico Lucano; A – D, factories A – D.

Conclusions

CPL cheese is produced from raw milk without the addition of starter cultures. The use of the traditional wooden tools provides typicality to the final products. This work demonstrated that the transformation conditions determined the development and dominance of dairy LAB over undesired raw milk microbiota, even though the role of enterococci needs further investigation. The differences in terms of microbial levels and species composition revealed among the dairy factories object of the present study highlighted the strong link of the final cheeses with the production environment. Basically, the main microbiological parameters were highly comparable among the cheeses produced in different factories and also the physicochemical profiles, antioxidant properties and sensory attributes of CPL cheeses were quite stable among dairy factories. This study demonstrated that the slavish application of the traditional CPL cheese making protocol among dairies is the preferred strategy to harmonize the microbial evolution and to maintain almost constant the final characteristics of CPL cheese.

References

B.U.R. (Bollettino Ufficiale della Regione Basilicata). 1999. Legge regionale del 4 gennaio 1999, N.1. pp. 1–36.

Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domainspecific 16S primers. J. of Microbiol. Methods 55, 541–555. https://doi.org/10 .1016/j.mimet.2003.08.009.

Barbaccia, P., Busetta, G., Barbera, M., Alfonzo, A., Garofalo, G., Francesca, N., Moscarelli, A., Moschetti, G., Settanni, L., Gaglio, R., 2022. Effect of grape pomace from red cultivar Nero d'Avola on the microbiological, physicochemical, phenolic profile and sensory aspects of ovine Vastedda-like stretched cheese. J. Appl. Microbiol. 133, 130–144. https://doi.org/10.1111/jam.15354.

Barbaccia, P., Francesca, N., Gerlando, R.D., Busetta, G., Moschetti, G., Gaglio, R., Settanni, L., 2020. Biodiversity and dairy traits of indigenous milk lactic acid bacteria grown in presence of the main grape polyphenols. FEMS Microbiol. Lett. 367, fnaa066. https://doi.org/10.1093/femsle/fnaa066.

Bonanno, A., Tornambè, G., Bellina, V., De Pasquale, C., Mazza, F., Maniaci, G., Di Grigoli, A., 2013. Effect of farming system and cheesemaking technology on the physicochemical characteristics, fatty acid profile, and sensory properties of Caciocavallo Palermitano cheese. J. Dairy Sci. 96, 710-724. https://doi.org/10.3168/jds.2012-5973.

Branciari, R., Ranucci, D., Trabalza-Marinucci, M., Codini, M., Orru, M., Ortenzi, R., Valiani, A., 2015. Evaluation of the antioxidant properties and oxidative stability of Pecorino cheese made from the raw milk of ewes fed *Rosmarinus officinalis* L. leaves. Int. J. Food Microbiol. 50, 558–565. https://doi.org/10.1111/ijfs.12712.

Bravo, L., 1998. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. 56, 317–333. https://doi.org/10.1111/j.1753-4887.1998.tb01670.x.

Buffa, M.N., Trujillo, A.J., Pavia, M., Guamis, B., 2001. Changes in textural, microstructural, and colour characteristics during ripening of cheeses made from raw, pasteurized or high-pressure-treated goats' milk. Int. Dairy J. 11, 927-934. http://dx.doi.org/10.1016/S0958-6946(01)00141-8.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Carpino, S., Mallia, S., La Terra, S., Melilli, C., Licitra, G., Acree, T.E., Barbano, D.M., Van Soest, P.J., 2004. Composition and aroma compounds of Ragusano cheese: Native pasture and total mixed rations. J. Dairy Sci. 87:816–830. http://dx.doi.org/10.3168/jds. S0022-0302(04)73226-9.

Carpino, S., Randazzo, C.L., Pino, A., Russo, N., Rapisarda, T., Belvedere, G., Caggia, C., 2017. Influence of PDO Ragusano cheese biofilm microbiota on flavour compounds formation. Food Microbiol. 61, 126–135. https://doi.org/10.1016/j.fm.2016.09.006.

Chavez-Servin, J.L., Andrade-Montemayor, H.M., Vazquez, C.V., Barreyro, A.A., Garcia-Gasca, T., Martinez, R.A.F., Ramirez, A.M.O., de la Torre-Carbot. K., 2018. Effects of feeding system, heat treatment and season on phenolic compounds and antioxidant capacity in goat milk, whey and cheese. Small Rumin Res. 16, 54–58. https://doi.org/10.1016/j.smallrumres.2018.01.011.

Chilliard, Y., Ferlay, A., Doreau, M., 2001. Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. Livest. Prod. Sci. 70 (1-2), 31–48. https://doi.org/10.1016/S0301-6226(01)00196-8.

Chilliard, Y., Glasser, F., Ferlay, A., Bernard, L., Rouel, J., Doreau, M., 2007. Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. Eur. J. Lipid Sci. Technol. 109 (8):828–855. https://doi.org/10.1002/ejlt.200700080.

Claesson, M.J., Wang, Q., O'Sullivan, O., Greene-Diniz, R., Cole, J.R., Ross, R.P., O'Toole, P.W., 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res. 38, e200. https://doi.org/10.1093/nar/gkq873.

Claeys, W.L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K., De Zutter, L., Huyghebaert, A., Imberechts, H., Thiange, P., Vandenplas, Y., Herman, L., 2013. Raw or heated cow milk consumption: Review of risks and benefits. Food Control 31, 251–262. https://doi.org/10.1016/j.foodcont.2012.09.035.

Claps, S., 2001. Il caciocavallo podolico e la manteca. Un grande formaggio del SUD. ANFOSC-ONLUS.

Commission Regulation (EC) N° 1441/2007 of 5 December 2007 amending Regulation no 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union L 338/1; 2005. Off J Eur Union L 322/12; 2007.

Commission Regulation (EC) N° 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off J Eur Union L 338/1; 2005.

Cruciata, M., Gaglio, R., Scatassa, M.L., Sala, G., Cardamone, C., Palmeri, M., Moschetti, G., La Mantia, T., Settanni, L., 2018. Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production. Appl. Environ Microbiol. 84, e02107-17. https://doi.org/10.1128/AEM.02107-17.

Cruciata, M., Gaglio, R., Todaro, M., Settanni, L., 2019. Ecology of Vastedda della valle del Belìce cheeses: a review and recent findings to stabilize the traditional production. Food Rev. Int. 35, 90–103. https://doi.org/10.1080/87559129.2018.1469142.

Cruciata, M., Sannino, C., Ercolini, D., Scatassa, M.L., De Filippis, F., Mancuso, I., La Storia, A., Moschetti, G., Settanni, L., 2014. Animal rennets as sources of dairy lactic acid bacteria. Appl. Environ. Microbiol. 80, 2050–2061. https://doi.org/10.1128/AEM.03837-13.

Curioni, P.M.G., Bosset, J.O., 2002. Key odorants in various cheese types as determined by gas chromatography-olfactometry. Int. Dairy J. 12, 959–984. https://doi.org/10.1016/S0958-6946(02)00124-3.

Danieli, P.P., Lopez, A., Bellagamba, F., Vetturini, T., Bernabucci, U., Ronchi, B., Moretti, V.M., Basiricò, L., 2022. Effects of season and management on fatty acid profile, ACE-inhibitory activity and anti-oxidant properties of Italian Alpine cheeses. Ital. J. Anim. Sci. 21, 1021–1033. https://doi.org/10.1080/1828051X.2022.2086492.

De Andrade, N.M., De Carvalho, A.M., Saleh, M.M., Fonseca, A.B.M., De Mesquita, E.D.F.M., Duarte, M.C.K.H., Calixto, F.A.A. Do Nascimento, E.R., 2022. Hygiene conditions of mussels Perna perna captured in Niteroi, RJ, Brazil: thermal intervention and microbiological evaluation. Food Sci. Technol. 42. https://doi.org/10.1590/fst.107421.

De Feo, V., Quaranta, E., Fedele, V., Claps, S., Rubino, R., Pizza, C., 2006. Flavonoids and terpenoids in goats milk in relation to forage intake. Ital. J. Food Sci. 18, 85–92.

De Marchi, M., Toffanin, V., Cassandro, M., Penasa, M., 2014. Invited review: mid-infrared spectroscopy as phenotyping tool for milk traits. J. Dairy Sci. 97, 1171–1186. https://doi.org/10.3168/jds.2013-6799.

Department of Health. Nutritional Aspects of Cardiovascular Disease; Report on Health and Social Subjects; HMSO, (Her Majesty's Stationery Office): London, United Kingdom, 1994; Volume 46, pp. 37–46.

Dewhurst, R.J., Shingfield, K.J., Lee, M.R., Scollan, N.D., 2006. Increasing the concentrations of beneficial polyunsaturated fatty acids in milk produced by dairy cows in high-forage systems. Anim. Feed Sci. Technol. 131, 168–206. https://doi.org/10.1016/j.anifeedsci.2006.04.016.

Di Grigoli, A., Francesca, N., Gaglio, R., Guarrasi, V., Moschetti, M., Scatassa, M.L., Settanni, L., Bonanno, A., 2015. The influence of the wooden equipment employed for cheese manufacture on the characteristics of a traditional stretched cheese during ripening. Food Microbiol. 46, 81–91. https://doi.org/10.1016/j.fm.2014.07.008.

Di Lorenzo, C., Colombo, F., Biella, S., Stockley, C., Restani, P., 2021. Polyphenols and human health: The role of bioavailability. Nutrients 13, 273–302. https://doi.org/10.3390/nu13010273.

Di Trana, A., Bonanno, A., Cecchini, S., Giorgio, D., Di Grigoli, A., Claps. S., 2015. Effects of Sulla forage (*Sulla coronarium* L.) on the oxidative status and milk polyphenol content in goats. J. Dairy Sci. 98, 37–46. https://doi.org/10.3168/jds.2014-8414.

Di Trana, A., Claps, S., Quaranta, G., Salvia, R., Lovallo, C., 2022a. I formaggi della Basilicata. In G. Licitra (Eds) Casei - L'arte casearia mediterranea. pp. 239-287.

Di Trana, A., Di Rosa, A.R., Addis, M., Fiori, M., Di Grigoli, A., Morittu, V.M., Todaro, M., 2022b. The Quality of Five Natural, Historical Italian Cheeses Produced in Different Months: Gross Composition, Fat-Soluble Vitamins, Fatty Acids, Total Phenols, Antioxidant Capacity, and Health Index. Animals 12 (2), 199. https://doi.org/10.3390/ani12020199.

Didienne, R., Defargues, C., Callon, C., Meylheuc, T., Hulin, S., Montel, M.C., 2012. Characteristics of microbial biofilm on wooden vats ('gerles') in PDO Salers cheese. Int. J. Food Microbiol. 156, 91–101. https://doi.org/10.1016/j.ijfoodmicro.2012.03.007.

Engels, W.J.M., Dekker, R., De Jong, C., Neeter, R., Visser, S., 1997. A comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese. Int. Dairy J. 7(4), 255–263. https://doi.org/10.1016/S0958-6946(97)00003-4.

Esposito, G., Masucci, F., Napolitano, F., Braghieri, A., Romano, R., Manzo, N., Di Francia, A., 2014. Fatty acid and sensory profiles of Caciocavallo cheese as affected by management system. J. Dairy Sci. 97(4), 1918-1928. https://doi.org/10.3168/jds.2013-7292.

Fallico, V., McSweeney, P.L.H., Horne, J., Pediliggieri, C., Hannon, J.A., Carpino, S., Licitra, G., 2005. Evaluation of bitterness in Ragusano cheese. J. Dairy Sci. 88(4), 1288–1300. https://doi.org/10.3168/jds.S0022-0302(05)72795-8.

Fardet, A., Rock, E., 2018. In vitro and in vivo antioxidant potential of milks, yoghurts, fermented milks and cheeses: A narrative review of evidence. Nutr. Res. Rev. 31, 52–70. https://doi.org/10.1017/S0954422417000191.

Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L., 2006. The role and application of enterococci in food and health. Int. J. Food Microbiol. 106, 1–24. https://doi.org/10.1016/j.ijfoodmicro.2005.06.026.

Gaglio, R., Couto, N., Marques, C., Lopes, M.D.F.S., Moschetti, G., Pomba, C., Settanni, L., 2016b. Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses. Int. J. Food Microbiol. 236, 107–114. https://doi.org/10.1016/j.ijfoodmicro.2016.07.020.

Gaglio, R., Cruciata, M., Di Gerlando, R., Scatassa, M.L., Cardamone, C., Mancuso, I., Sardina, M.T., Moschetti, G., Portolano, B., Settanni, L., 2016a. Microbial activation of wooden vats used for traditional cheese production and evolution of neoformed biofilms. Appl. Environ. Microbiol. 82, 585–595. https://doi.org/10.1128/AEM.02868-15.

Garcia, V., Rovira, S., Boutoial, K., Lopez. M.B., 2014. Improvements in goat milk quality: A review. Small Ruminant Res. 121, 51–57. https://doi.org/10.1016/j.smallrumres.2013.12.034.

García-González, I., Corona-Cervantes, K., Hernández-Quiroz, F., Villalobos-Flores, L.E., Galván-Rodríguez, F., Romano, M.C., Miranda-Brito, C., Piña-Escobedo, A., Borquez-Arreortúa, F.G., Rangel-Calvillo, M.N., García-Mena, J., 2022. The Influence of Holder Pasteurization on the Diversity of the Human Milk Bacterial Microbiota Using High-Throughput DNA Sequencing. J. Hum. Lact. 38, 118–130. https://doi.org/10.1177/08903344211011946.

Geronikou, A., Larsen, N., Lillevang, S.K., Jespersen, L., 2022. Occurrence and Identification of Yeasts in Production of White-Brined Cheese. Microorganisms 10, 1079. https://doi.org/10.3390/microorganisms10061079.

Gottardo, P., Penasa, M., Righi, F., Lopez-Villalobos, N., Cassandro, M., De Marchi, M., 2017. Fatty acid composition of milk from Holstein-Friesian, Brown Swiss, Simmental and Alpine Grey cows predicted by mid-infrared spectroscopy. Ital. J. Anim. Sci. 16, 380–389. https://doi.org/10.1080/1828051X.2017.1298411.

Guarcello, R., Carpino, S., Gaglio, R., Pino, A., Rapisarda, T., Caggia, C., Marino, G., Randazzo, C.L., Settanni, L., Todaro, M., 2016. A Large Factory-Scale Application of Selected Autochthonous Lactic Acid Bacteria for PDO Pecorino Siciliano Cheese Production. Food Microbiol. 59, 66–75. https://doi.org/10.1016/j.fm.2016.05.011.

Guarrasi, V., Sannino, C., Moschetti, M., Bonanno, A., Di Grigoli, A., Settanni, L., 2017. The individual contribution of starter and non starter lactic acid bacteria to the volatile organic compound composition of Caciocavallo Palermitano cheese. Int. J. Food Microbiol. 259, 35–42. https://doi.org/10.1016/j.ijfoodmicro.2017.07.022.

Gupta, A., Mann, B., Kumar, R., Sangwan, R.B., 2009. Antioxidant activity of Cheddar cheeses at different stages of ripening. Int. J. Dairy Technol. 62, 339–347. https://doi.org/10.1111/j.1471-0307.2009.00509.x.

G.U.R.I. Gazzetta Ufficiale della Repubblica Italiana. 2021. Ministero delle Politiche Agricole Alimentari e Forestali Decreto ministeriale. Aggiornamento dell'elenco nazionale dei prodotti agroalimentari tradizionali ai sensi dell'articolo 12, comma 1, della legge 12 dicembre 2016, n. 238. (21A01168). No. 48, 15 Febbraio 2021.

Hilario, M.C., Puga, C.D., Ocana, A.N., Romo, F.P.G., 2010 Antioxidant activity, bioactive polyphenols in Mexican goats' milk cheeses on summer grazing. J. Dairy Res. 77, 20–26. https://doi.org/10.1017/S0022029909990161.

Holland, R., 2004. Esterases of lactic acid bacteria and cheese flavour. IDF symposium on cheese: ripening, characterization & technology. Prague, Czech Republic.

ISO 8589-2007. Sensory Analysis—General Guidance for the Design of Test Rooms.

Izzo, L., Mikušová, P., Lombardi, S., Sulyok, M., Ritieni, A., 2022. Analysis of Mycotoxin and Secondary Metabolites in Commercial and Traditional Slovak Cheese Samples. Toxins 14, 134. https://doi.org/10.3390/toxins14020134.

Jagadeesan, B., Gerner-Smidt, P., Allard, M.W., Leuillet, S., Winkler, A., Xiao, Y., Chaffron, S., Van Der Vossen, J., Tang, S., Katase, M., McClure, P., Kimura, B., Chai, L.C., Chapman, J., Grant, K., 2019. The use of next generation sequencing for improving food safety: Translation into practice. Food Microbiol. 79, 96–115. https://doi.org/10.1016/j.fm.2018.11.005.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010.

Khan, I.T., Bule, M., Rahman Ullah, M.N., Asif, S., Niaz, K., 2019. The antioxidant components of milk and their role in processing, ripening, and storage: Functional food. Vet. World 12, 12. https://doi10.14202/vetworld.2019.12-33.

Kuhnen, S., Moacyr, J.R., Mayer, J.K., Navarro, B.B., Trevisan, R., Honorato, L.A., Pinheiro Machado Filho, L.C., 2014. Phenolic content and ferric reducing antioxidant power of cow's milk produced in different pasture-based production systems in southern Brazil. J Sci. Food Agric. 94, 3110–3117. https://doi.org/10.1002/jsfa.6654.

Lawrence, R.C., Gilles, J., Creamer, L.K., 1983. The relationship between cheese texture and flavour. New Zealand J. Dairy Sci. Technol. 18, 175–190.

Licitra, G., Carpino, S., 2014. The microfloras and sensory profiles of selected protected designation of origin Italian cheeses. Microbiol. Spectr. 2, 1–12. https://doi.org/10.1128/microbiolspec.CM-0007-2012.

Licitra, G., Ogier, J.C., Parayre, S., Pediliggieri, C., Carnemolla, T.M., Falentin, H., Madec, M.N., Carpino, S., Lortal, S., 2007. Variability of the bacterial biofilms of the "tina" wood vat used in the Ragusano cheese making process. Appl. Environ Microbiol. 73, 6980–6987. https://doi.org/10.1128/AEM.00835-07.

Licitra, G., Radulovic, Z., Miocinovic, J., Uzunsoy, İ., Özer, B., Bintsis, T., Alichanidis, E., Herian, K., Jelen, P., 2017. Pasta-Filata Cheeses. In Papademas, P., Bintsis, T. (Eds), Global cheesemaking technology: cheese quality and characteristics. Wiley-IEEE Press, New York, pp.368–391. https://10.1002/9781119046165.ch8.

Lortal, S., Di Blasi, A., Madec, M.N., Pediliggieri, C., Tuminello, L., Tangury, G., Fauquant, J., Lecuona, Y., Campo, P., Carpino, S., Licitra, G.,2009. Tina wooden vat biofilm. A safe and highly efficient lactic acid bacteria delivering system in PDO Ragusano cheese making. Int. J. Food Microbiol. 132, 1–8. https://doi.org/10.1016/j.ijfoodmicro.2009.02.026.

Lucas, A., Rock, E., Chamba, J.F., Verdier-Metz, I., Brachet, P., Coulon, J.B., 2006. Respective effects of milk composition and the cheese-making process on cheese compositional variability in components of nutritional interest. Lait 86, 21–41. https://doi.org/10.1051/lait:2005042.

Marino, M., de Wittenau, G.D., Saccà, E., Cattonaro, F., Spadotto, A., Innocente, N., Radovic, S., Piasentier, E., Marroni, F., 2019. Metagenomic profiles of different types of Italian high-moisture Mozzarella cheese. Food Microbiol. 79, 123–131. https://doi.org/10.1016/j.fm.2018.12.007.

Martin, B., Verdier-Metz, I., Buchin, S., Hurtaud, C., Coulon, J.B., 2005. How do the nature of forages and pasture diversity influence the sensory quality of dairy livestock products? Animal Sci. 81, 205–212. https://doi.org/10.1079/ASC50800205.

Martorana, A., Alfonzo, A., Settanni, L., Corona, O., La Croce, F., Caruso, T., Moschetti, G., Francesca, N., 2015. An innovative method to produce green table olives based on "pied de cuve" technology. Food Microbiol. 50, 126–140. https://doi.org/10.1016/j.fm.2015.03.008.

Masi, P., Addeo, F., 1986. An examination of some mechanical properties of a group of Italian cheeses and their relation to structure and conditions of manufacture. J. Food Eng. 5, 217–229. https://doi.org/10.1016/0260-8774(86)90026-9.

McSweeney, P.L., Sousa, M.J., 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review., Le Lait 80, 293–324. https://doi.org/10.1051/lait:2000127.

Muñoz, N., Ortigosa, M., Torre, P., Izco, J.M., 2003. Free amino acids and volatile compounds in an ewe's milk cheese as affected by seasonal and cheese-making plant variations. Food Chem. 83, 329–338. https://doi.org/10.1016/S0308-8146(03)00133-X.

Murray, J.M., Delahunty, C.M., 2000. Mapping consumer preference for the sensory and packaging attributes of Cheddar cheese. Food Qual. Prefer. 11, 419–435. http://dx.doi.org/10.1016/S0950-3293(00)00017-3.

Pattorn, S., Hongsprabhas, P., 2013. Effect of coagulants on antioxidant capacity of milk protein curds and their tryptic hydrolysates. J. Food Biochem. 37, 203–211. https://doi.org/10.1111/j.1745-4514.2011.00625.x.

Penasa, M., Tiezzi, F., Gottardo, P., Cassandro, M., De Marchi, M., 2015. Genetics of milk fatty acid groups predicted during routine data recording in Holstein dairy cattle. Livest. Sci. 173, 9–13. https://doi.org/10.1016/j.livsci.2014.12.014.

Pinho, O., Pérès, C., Ferreira, I.M.P.L.V.O., 2003. Solid-phase microextraction of volatile compounds in "Terrincho" ewe cheese: comparison of different fibers. J. Chromatogr. A 1011, 1–9. https://doi.org/10.1016/S0021-9673(03)01066-5.

Pothakos, V., Samapundo, S., Devlieghere, F., 2012. Total mesophilic counts underestimate in many cases the contamination levels of psychrotrophic lactic acid bacteria (LAB) in chilled-stored food products at the end of their shelf-life. Food Microbiol. 32, 437–443. https://doi.org/10.1016/j.fm.2012.07.011.

Price, M.N., Dehal, P.S., Arkin, A.P., 2009. FastTree: computing large minimum evolution, trees with profiles instead of a distance matrix. Mol. Biol. Evol. 26, 1641–1650. https://doi.org/10.1093/molbev/msp077.

Přikryl, J., Hájek, T., Švecová, B., Salek, R.N., Černíková, M., Červenka, L., Buňka, F., 2018. Antioxidant properties and textural characteristics of processed cheese spreads enriched with rutin or quercetin: The effect of processing conditions. LWT - Food Sci. and Technol. 87, 266–271. https://doi.org/10.1016/j.lwt.2017.08.093.

Rashidinejad, A.E., Birch, J., Sun-Waterhouse, D., Everett, D.W., 2013. Effects of catechin on the phenolic content and antioxidant properties of low-fat cheese. Int. J. Food Sci. Technol. 48, 2448–2455. https://doi.org/10.1111/ijfs.12234.

Rehman, S.U., Banks, J.M., Brechany, E.Y., Muir, D.D., McSweeney, P.L.H., Fox, P.F., 2000. Influence of ripening temperature on the volatiles profile and flavour of Cheddar cheese made from raw or pasteurised milk. Int. Dairy J. 10, 55–65. https://doi.org/10.1016/S0958-6946(00)00019-4.

Samelis, J., Kakouri, A., 2022. Microbiological Characterization of Greek Galotyri Cheese PDO Products Relative to Whether They Are Marketed Fresh or Ripened. Fermentation, 8, 492. https://doi.org/10.3390/fermentation8100492.

Santillo, A., Caroprese, M., Ruggieri, D., Marino, R., Sevi, A., Albenzio, M., 2012. Consumer acceptance and sensory evaluation of Monti Dauni Meridionali Caciocavallo cheese. J. Dairy Sci. 95, 4203–4208. https://doi.org/10.3168/jds.2011-4777.

Scatassa, M.L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., Di Grigoli, A., Moschetti, G., Settanni, L., 2015. Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol. 52, 31–41. https://doi.org/10.1016/j.fm.2015.06.008.

Seale, B., Bremer, P., Flint, S., Brooks, J., Palmer, J., 2015. Overview of the problems resulting from biofilm contamination in the dairy industry. In Teh, K.H., Flint, S., Brooks, J., Knight, G. (Eds.), Biofilms in the dairy industry. Wiley Online Library. New York, pp. 49–64. https://doi.org/10.1002/9781118876282.ch4.

Settanni, L., Di Grigoli, A., Tornambé, G., Bellina, V., Francesca, N., Moschetti, G., Bonanno, A., 2012. Persistence of wild *Streptococcus thermophilus* strains on wooden vat and during the manufacture of a Caciocavallo type cheese. Int. J. Food Microbiol. 155, 73–81. https://doi.org/10.1016/j.ijfoodmicro.2012.01.022.

Settanni, L., Gaglio, R., Guarcello, R., Francesca, N., Carpino, S., Sannino, C., Todaro, M., 2013. Selected lactic acid bacteria as a hurdle to the microbial spoilage of cheese: Application on a traditional raw ewes' milk cheese. Int. Dairy J. 32, 126–132. http://dx.doi.org/10.1016/j.idairyj.2013.04.010.

Silanikove, N., Leitner, G., Merin, U., Prosser, C.G., 2010. Recent advances in exploiting goat's milk: quality, safety and production aspects. Small Rumin. Res. 89, 110–124. https://doi.org/10.1016/j.smallrumres.2009.12.033.

Soyeurt, H., Dardenne, P., Dehareng, F., Bastin, C., Gengler, N., 2008. Genetic parameters of saturated and monounsaturated fatty acid content and the ratio of saturated to unsaturated fatty acids in bovine milk. J. Dairy Sci. 91, 3611–3626. https://doi.org/10.3168/jds.2007-0971.

Sulejmani, E., Hayaloglu, A.A., 2020. Influence of starter culture on nitrogen fraction and volatile compounds in Beaten cow's milk cheese. J. Food Process. Preserv. 44, e14689. https://doi.org/10.1111/jfpp.14689.

Sun, L., D'Amico, D.J., 2023. Characterization of microbial community assembly on new wooden vats for use in cheese production. Food Microbiol. 109, 104154. https://doi.org/10.1016/j.fm.2022.104154.

Sun, L., D'Amico, D.J., 2021. Composition, Succession, and Source Tracking of Microbial Communities throughout the Traditional Production of a Farmstead Cheese. Msystems, 6, e00830-21. https://doi.org/10.1128/mSystems.00830-21.

SYSTAT. (2009). Statistics; Version 13 Edition. Evanston, IL: SYSTAT, Inc. 750 pp.

Thierry, A., Collins, Y.F., Mukdsi, M.C.A., McSweeney, P.L.H., Wilkinson, M.G., Spinnler, H.E., 2017. Lipolysis and metabolism of fatty acids in cheese. In McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W. (Eds.), Cheese: Chemistry, Physics and Microbiology, Academic Press, New York, pp. 423–444. https://doi.org/10.1016/B978-0-12-417012-4.00017-X.

Tufarelli, V., Casalino, E., D'Alessandro, A.G., Laudadio, V., 2017. Dietary phenolic compounds: Biochemistry, metabolism and significance in animal and human health. Curr. Drug Metab. 18, 905–913. https://doi.org/10.2174/1389200218666170925124004.

Uzun, P., Serrapica, F., Masucci, F., Assunta, B.C.M., Yildiz, H., Grasso, F., Di Francia, A., 2020. Diversity of traditional Caciocavallo cheeses produced in Italy. Int. J. Dairy Technol. 73, 234–243. https://doi.org/10.1111/1471-0307.12640.

Vert, M., Doi, Y., Hellwich, K.H., Hess, M., Hodge, P., Kubisa, P., Rinaudo, M., Schué, F., 2012. Terminology for biorelated polymers and applications (IUPAC recommendations 2012). Pure Appl. Chem. 84, 377–410. https://doi.org/10.1351/PAC-REC-10-12-04.

Wolf, I.V., Perotti, M.C., Zalazar, C.A., 2011. Composition and volatile profiles of commercial Argentinean blue cheeses. J. Sci. Food Agric. 91, 385–393. https://doi.org/10.1002/jsfa.4198.

Zapka, C., Leff, J., Henley, J., Tittl, J., De Nardo, E., Butler, M., Griggs, R., Fierer, N., Edmonds-Wilson, S., 2017. Comparison of standard culture-based method to culture-independent method for evaluation of hygiene effects on the hand microbiome. MBio, 8, e00093-17. https://doi.org/10.1128/mBio.00093-17.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. Int. J. Syst. Evol. Microbiol. 70, 2782–2858. https://10.1099/ijsem.0.004107.

Ziino, M., Condurso, C., Romeo, V., Giuffrida, D., Verzera, A., 2005. Characterization of "Provola dei Nebrodi", a typical Sicilian cheese, by volatiles analysis using SPME-GC/MS. Int. Dairy J. 15, 585–593. https://doi.org/10.1016/j.idairyj.2004.07.024.

Zimecki, M., Kruzel, M.L., 2007. Milk-derived proteins and peptides of potential therapeutic and nutritive value. J. Exp. Ther. Oncol. 6, 89–106.

Part II

Microbiol ecology of wooden shelves used for ripening of traditional cheese

The second section of the thesis presents the microbiological characterization of microbial biofilms on wooden shelves used for ripening typical cheeses. These biofilms, composed on SLAB and NSLAB, adhere to the porous and polymeric structure of the wood. is material that allows bacteria to adhere typical of these living systems. While lactic biofilms on wooden vats have been shown to defend against food pathogens, recent EU hygiene directives promote easily sanitized equipment, risking the standardization of cheeses with commercial starters. The traditional use of wood is crucial for the unique sensory profile of the finished products. Most studies have focused on the microbiological aspects of the biofilms on wooden vats used for milk coagulation, while very little is known about those on wooden boards used for cheese ripening and their hygienic impact. A study characterized the microbial diversity of wooden shelves used for ripening three Sicilian cheeses (PDO Pecorino Siciliano, PDO Piacentinu Ennese, and Caciocavallo Palermitano) using a multivariate statistical approach. Another study analyzed the bacterial and fungal communities on wooden shelves and the rind of two hard-pressed Basilicata cheeses (PDO Pecorino di Filiano and PGI Canestrato di Moliterno).

Chapter III

A Multivariate approach to study the bacterial diversity associated to the wooden shelves used for aging traditional sicilian cheeses


PECORINO SICILIANO


PIACENTINU ENNESE


CACIOCAVALLO PALERMITANO

The present chapter has been published in

Foods, *11*, *774*

2022

Abstract

The present study was carried to correlate the microbial diversity of the biofilms developed on the wooden boards used for aging traditional Sicilian cheeses with cheese typology. To this end, the microbial diversity of the shelves in contact with the cheeses PDO Pecorino Siciliano, PDO Piacentinu Ennese, and TAP Caciocavallo Palermitano, during ripening, was evaluated by a multivariate statistical approach. The shelf biofilms of this study were previously analyzed for their microbial composition, but no correlation between biodiversity and cheese type was investigated. Canonical discriminant analysis confirmed a cheese typology effect on the microbial loads of the wooden shelves investigated. Regarding the plate count data, the centroids of different cheeses were statistically distant from one another. This analysis also showed a good graphic separation of data regarding bacterial order operational taxonomy units (OTUs). Thus, the microbiological differences imputed to the cheese typologies were not affected by the environmental conditions of the facilities. Furthermore, wooden shelf lactic acid bacteria (LAB) were investigated for their ability to inhibit the main dairy pathogens. Although inhibitors were mainly enterococci, *P. pentosaceus* WS287 and *W. paramesenteroides* WS581 showed the highest inhibition activity, indicating their possible application to control the undesired bacteria in situ.

Keywords: cheese microbiology; cheese ripening; lactic acid bacteria; MiSeq Illumina; statistical analysis; traditional cheeses; wooden shelves.

Introduction

Wood has represented the main material for the manufacture of dairy equipment in Europe for centuries (Bruni 1859). In Sicily (southern Italy), the cheese making process remained almost unvaried over time for several cheese productions (Settanni and Moschetti, 2014), most of them enjoy quality status such as protected denomination of origin (PDO) or other recognitions by the European Union (Cruciata et al., 2019; Gaglio et al., 2021). Although there is no specific contraindication regarding the use of wood to process foods as in European Regulation (EC) no. 1935/2004 (Commission Regulation, 2004) the direct contact of food matrices with wood is still controversial in the majority of EU Countries. In Italy, dairy production can be carried out with wooden equipment thanks to regulation EC no. 2074/2005, which derogates from the EC no. 852/2004 for foods with traditional characteristics (Commission Regulation, 2005).

The controversial aspects of the use of wood in food production is due to its porous structure, which facilitate bacterial trapping. Once bacteria are adsorbed onto the wood surface, they might develop biofilms, becoming resident communities and, consequently, contaminating food products (Aviat et al., 2016). In the case of dairy products, specifically cheeses, several works have demonstrated how microbial biofilms formed on the surface of wooden vats used for milk processing are responsible for curd acidification, cheese ripening, and even the inhibition of undesired bacteria attachment and development thanks to the massive presence of lactic acid bacteria (LAB) (Cruciata et al., 2018; Didienne et al., 2012; Gaglio et al., 2016b; Licitra et al., 2007; Lortal et al., 2009; Scatassa et al., 2015; Settanni et al., 2012).

Recently, another wood equipment, the open-topped table (namely "mastredda") used for curd acidification during Sicilian stretched cheese production, was analyzed for the composition of LAB biofilms, evidencing the presence of *Enterococcus*, *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus, and Streptococcus* (Busetta et al., 2021).

Nowadays, there is renewed interest towards the safety aspects of wood in contact with cheese during ripening. In fact, the US Food and Drug Administration (FDA) expressed some concerns about cheeses ripened on wooden planks for the possible transfer of pathogenic bacteria, such as *Listeria monocytogenes* (Cutini, 2014). This aspect is often highlighted by detractors of traditional productions, without an in-depth consideration of the barrier effect of the resident microorganisms of a given wood biofilm against foodborne pathogens. Although limited research is available on this topic, some evidence has been provided. Mariani et al. (2007, 2011) characterized the microbial populations of the wooden shelves used to ripen French smear cheese, PDO Reblochon de Savoie, reporting the massive presence of micrococci-corynebacteria, yeasts, and molds, as well as LAB, *Staphylococcus*, and *Pseudomonas*, and evaluated the bio-protective action of the wooden shelf biofilms, specifically against *L. monocytogenes*. Regarding the microbial characterization, Guzzon et al. (2017) studied the biofilms of the wooden shelves used for the ripening of another smear cheese, the Italian PDO Fontina, and evidenced a cause–effect relationships between the dominant Actinobacteria populations and a red-brown pigmentation defect. The microbiota of wooden boards used for cheese ripening has been also recently considered in the US by Wadhawan et al. (2021), who detected Actinobacteria, Firmicutes, and Proteobacteria at dominant levels, and highlighted the

consistent presence of halophilic bacteria. The massive presence of halophilic and moderately halophilic bacteria was also revealed among biofilms of the wooden shelves used for aging traditional Sicilian cheeses, while the presence of the main dairy pathogens, such as coagulase-positive staphylococci (CSP), *Salmonella* spp., and *L. monocytogenes*, was not found (Settanni et al., 2021).

Considering that only a few studies have been carried out on the microbial diversity of the wooden boards used for cheese aging, the main limitations in this field are the low amount of data regarding cell densities and the taxon composition/distribution of the biofilms among traditional cheese productions, and the complete lack of information about the inhibitory properties of these biofilms towards *Salmonella* spp., *Escherichia coli*, and CPS.

This study is part of a project aimed to characterize the production processes of natural and historic cheeses of Southern Italy, specifically PDO Pecorino Siciliano, PDO Piacentinu Ennese, and traditional agri-food product (TAP) Caciocavallo Palermitano. PDO Pecorino Siciliano cheese is a hard cheese produced with raw milk of autochthonous Sicilian sheep breeds Valle del Belìce, Comisana, and Pinzirita, reared under an extensive system (Todaro et al., 2021). PDO Piacentinu Ennese cheese is a semi-hard product of a restricted area (Enna province) in central Sicily. In addition, this cheese is made from raw milk of the sheep breeds Valle del Belìce, Comisana, and Pinzirita, reared under an extensive system, but unlike PDO Pecorino Siciliano cheese, Sicilian saffron and black pepper are added during the manufacture (Fallico et al., 2006). TAP Caciocavallo Palermitano is a hard cheese produced within the Palermo province. It is a stretched cheese

obtained from raw bovine milk of Cinisara, Pezzata Rossa, and Bruna breeds and half-breeds (Bonanno et al., 2013).

The objectives of the present research were to analyze the results of culture-dependent and to perform independent microbiological investigations of the wooden shelves used for ripening the three medium-aged cheeses reported above, using a multivariate statistical approach to find relationships between wooden shelf bacterial diversity and cheese typology, as well as to characterize the inhibitory potential of wooden board LAB against *L. monocytogenes*, *Salmonella* spp., *Escherichia coli*, and CPS.

Materials and methods

Collection of wooden shelf biofilms and microbiological investigation

The microbial biofilms associated to the wooden shelves used to ripen PDO Pecorino Siciliano cheese, PDO Piacentinu Ennese cheese and TAP Caciocavallo Palermitano cheese were collected within the provinces of Agrigento, Enna, Palermo and Trapani (central and western Sicily, Italy). During cheese ripening, 18 shelves were sampled from 18 dairy facilities. The contact of the boards with the cheese rind lasted 1–2 months for the PDO Piacentinu Ennese cheese, and 4–5 months for the other two cheeses. A square surface (100 cm^2) of each board was subjected to a nondestructive microbial collection through brushing. Shelf biofilms were finally collected with a sterile gauze and were transferred into a Durham bottle containing 100 mL of Ringer's solution (Sigma-Aldrich, Milan, Italy). All wooden shelves were microbiologically investigated by culture-dependent and -independent methods, as reported by Settanni et al. (2021). Briefly, several microbial groups were detected, as follows: total mesophilic microorganisms (TMM) on

plate count agar (PCA) incubated at 30 °C for 72 h; members of the Enterobacteriaceae family on violet red bile glucose agar (VRBGA) incubated at 37 °C for 24 h; total coliforms (TC) on violet red bile agar (VRBA) incubated at 37 °C for 24 h; Escherichia coli on Hektoen enteric agar (HEA) incubated at 37 °C for 24 h; pseudomonads on *Pseudomonas* agar base (PAB) and incubated at 25 °C for 48 h; mesophilic and thermophilic LAB cocci on Medium 17 (M17) agar incubated at 30 and 44 °C, respectively, for 48 h; mesophilic and thermophilic LAB rods on de Man–Rogosa–Sharpe (MRS) agar incubated at 30 and 44 °C, respectively, for 48 h; enterococci on Kanamycin Aesculin Azide (KAA) agar incubated at 37 °C for 24 h; yeasts on dichloran Rose Bengal chloramphenicol (DRBC) agar incubated at 28 °C for 48 h; and molds on potato dextrose agar (PDA) incubated at 25 °C for 7 days. Regarding the culture-independent approach applied, DNAs from biofilms were extracted using the Power Food Microbial DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA), purified by PowerClean DNA Cleanup kit (Mo Bio Laboratories, Inc.) and quantified through Nanodrop 8800 fluorospectrometer (Thermo Scientific, Wilmington, NC, USA). A 464-nucleotide sequence of the V3-V4 region of the 16S rRNA gene (*E. coli* positions 341 to 805) was amplified from the total DNA of each sample and was paired-end sequenced by the Illumina MiSeq system. Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp, accessed on 25 March 2021) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). The sequences were then filtered, trimmed, denoised, and merged using DADA2 (87). The chimeric sequences were removed via the consensus method in DADA2. Taxonomic and compositional analyses were carried on using the plugins feature-classifier

(https://github.com/qiime2/q2-feature-classifier, accessed on 25 March 2021). A pretrained naive Bayes classifier based on the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database was applied to the paired-end sequence reads to generate taxonomy tables. In the present work, all data were reported per cheese typology.

Antagonistic activity of LAB

The antibacterial activity of the wooden shelf LAB isolated and identified by Settanni et al. (2021) was evaluated against the four main dairy pathogens: *Escherichia coli*, *L. monocytogenes*, *Salmonella* Enteritidis, and *Staphylococcus aureus*. The indicator (sensitive) bacteria used in this study were provided by the American Type Culture Collection: *E. coli* ATCC25922, *L. monocytogenes* ATCC 19114, *S.* Enteritidis ATCC13076, and *St. aureus* ATCC33862. All of the indicators were reactivated in Brain Heart Infusion (BHI) broth (Oxoid) at 37 °C for 24 h before testing the antagonistic activity of the LAB. The inhibitory assay was conducted as reported by Corsetti et al. (2008) using the well diffusion assay. The assays were performed in duplicate.

Statistical Analyses

The microbial loads of the wooden shelf biofilms to logarithmic transformation were subjected in order to normalize the distribution, while for the data of the culture-independent microbiological analysis, which presented a Poisson distribution with a large number of zeros, a square root transformation applied to y + 0.5, was carried out (Dey and Pandit, 2020). Box and whisker plots of microbial loads (log CFU/cm^2) of TMM, Enterococci, and LAB biofilms developed on the wooden shelves used to ripen traditional

Sicilian cheeses were carried out. On the transformed data, a dual statistical approach was applied—univariate and multivariate analyses. An ANOVA model was used to test the effect of the type of cheese (PDO Pecorino Siciliano, PDO Piacentinu Ennese, and TAP Caciocavallo Palermitano) on the dependent variables, while the multivariate analysis was carried out with a Canonical discriminant analysis (GLM and CANDISC procedures of SAS 9.1.2 software, 2004). The degree of dissimilarity among cheeses' wooden shelves was measured using squared Mahalanobis distances (MD), and the reliability of the canonical discriminant model was finally assessed by cross-validation, where the statistical tests used were Wilks Lambda, Pillai, Hotelling−Lawley, and Roy maximum root.

Results

Microbial loads of wooden shelf biofilms

The results of the plate count of the 12 main microbial groups associated with dairy productions are reported in Table 1.

Table 1. Microbial loads (LSM ± s.e.) of the biofilms developed on the wooden shelves used to ripen traditional Sicilian cheeses.

Bacterial Counts	Cheeses			*p* value
	TAP Caciocavallo palermitano	PDO Piacentinu Ennese	PDO Pecorino Siciliano	
TMM	5.09 ± 0.26 b	4.97 ± 0.28 b	5.84 ± 0.24 a	<0.036
Enterobacteriaceae	0 ± 0 B	0.61 ± 0.27 B	1.79 ± 0.23 A	<0.001
Total coliforms	0 ± 0 B	0.46 ± 0.26 AB	1.08 ± 0.22 A	<0.005
E. coli	0 ± 0 Bb	0.34 ± 0.28 ABb	1.17 ± 0.24 Aa	<0.004
Pseudomonas	0.94 ± 0.40 B	3.96 ± 0.44 A	3.21 ± 0.37 A	<0.001
Enterococci	1.95 ± 0.17 B	3.14 ± 0.19 A	2.11 ± 0.16 B	<0.001
Mesophilic LAB rods	3.40 ± 0.31 B	3.32 ± 0.34 B	4.64 ± 0.29 A	<0.004
Thermophilic LAB rods	1.79 ± 0.28 A	3.09 ± 0.30 B	1.29 ± 0.26 A	<0.001
Mesophilic LAB cocci	4.78 ± 0.35	5.31 ± 0.39	5.60 ± 0.32	<0.234
Thermophilic LAB cocci	2.45 ± 0.30	3.19 ± 0.33	2.57 ± 0.28	<0.213
Yeasts	3.84 ± 0.28 Aa	5.07 ± 0.31 Bb	4.75 ± 0.26 ABb	<0.010
Molds	2.34 ± 0.35 AB	1.47 ± 0.39 B	3.12 ± 0.33 A	<0.007

Units are log CFU/cm^2. LSM, least-square method; s.e., standard error; TMM, total mesophilic microorganisms; *E.*, *Escherichia*; LAB, lactic acid bacteria. In the rows, different capital letters are significant for $p < 0.01$; different letters are significant for $p < 0.05$.

Statistically significant differences among wooden shelves were found for the levels of almost all microbial groups investigated, with the exception of mesophilic and thermophilic LAB cocci. The PDO Pecorino Siciliano cheese wooden shelves showed the highest loads of TMM, members of Enterobacteriaceae family, total coliforms, and *E. coli*. The wooden boards used for TAP Caciocavallo Palermitano cheese ripening displayed the lowest loads of pseudomonads, while those used for PDO Piacentinu Ennese cheese showed the highest loads of enterococci. Regarding the other LAB groups, significant differences were registered among rod populations. To this purpose, the wooden shelves used for PDO Pecorino Siciliano cheese ripening displayed the highest loads for mesophilic species and the lowest for the thermophilic ones.

Yeasts were present at consistent levels on the wooden boards analysed. In particular, yeast loads of ovine milk cheeses (PDO Pecorino Siciliano and PDO Piacentinu Ennese cheeses) were significantly higher than those of the. TAP Caciocavallo Palermitano cheeses processed from cow's milk. The most abundant levels of molds were detected for the wooden shelves used for the PDO Pecorino Siciliano cheese, but the differences with those used for the TAP Caciocavallo Palermitano cheeses were not statistically significant.

The distribution of LAB, enterococci, and TMM loads is reported as box and whisker plots in Fig. 1. The TMM variability observed for the PDO Pecorino Siciliano wooden shelves was lower than that registered for the boards used for the other two cheeses (Fig. 1a). On the contrary, the TMM of the boards used for the Pecorino Siciliano cheese showed the highest levels. The enterococci distribution of PDO Piacentinu Ennese wooden shelves (Fig. 1b) showed the highest levels. The distribution of the levels of mesophilic LAB rods (Fig. 1c) showed the highest variability in the PDO Pecorino Siciliano wooden

shelves. In the case of thermophilic LAB rods (Fig. 1d), the distribution was quite wide for the very low levels (below detection limit) revealed on the wooden shelves used to ripen TAP Caciocavallo Palermitano and PDO Pecorino Siciliano cheeses.

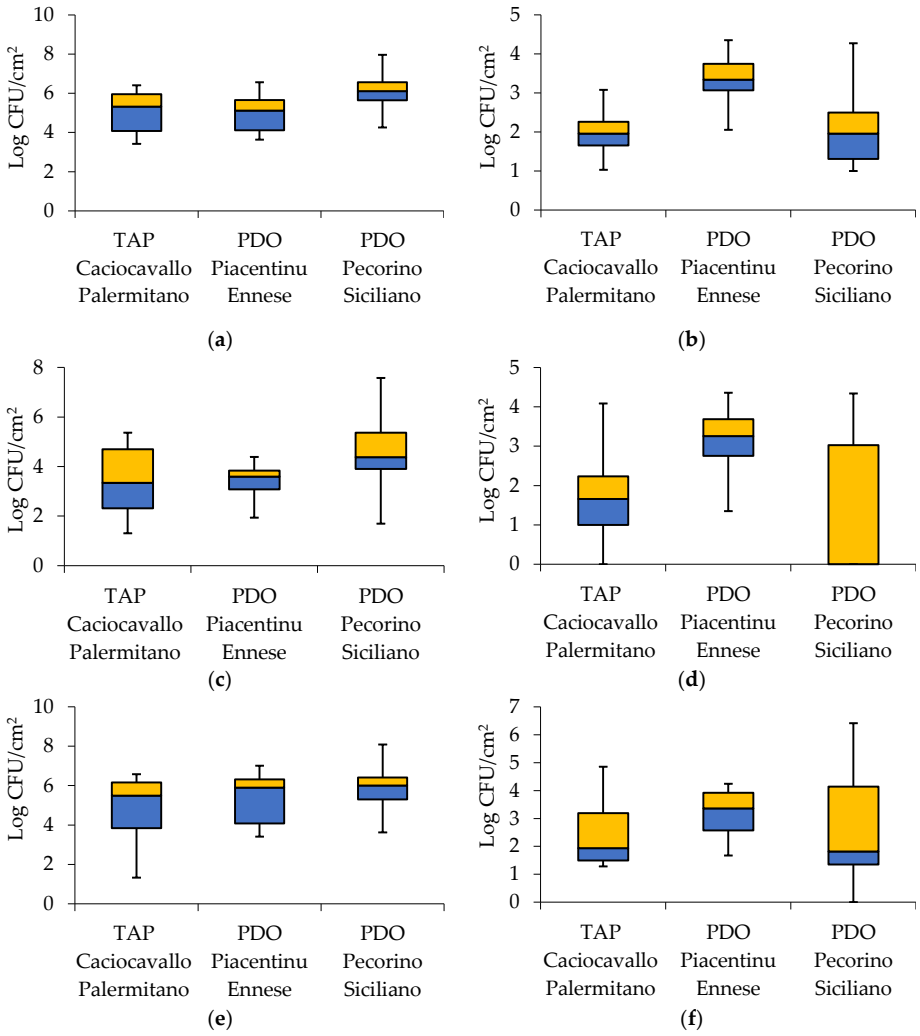

Fig. 1. Box and whisker plots of microbial loads of biofilms developed on the wooden shelves used to ripen traditional Sicilian cheeses: (a) total mesophilic microorganisms (TMM), (b) enterococci, (c) mesophilic LAB rods, (d) thermophilic LAB rods, (e) mesophilic LAB cocci, (f) thermophilic LAB cocci.

The mesophilic LAB cocci distribution (Fig. 1e) was highly similar among the wooden shelves analyzed. Regarding termophilic LAB cocci (Fig. 1f), the widest distribution was observed for the PDO Pecorino Siciliano wooden shelves.

1702 The multivariate approach confirmed the discrimination among the results described
1703 above. Statistical tests (Wilks Lambda, Pillai, Hotelling−Lawley, and Roy maximum root)
1704 on canonical discriminant analysis (Fig. 2) confirmed the effect of cheese typology on the
1705 microbial loads of the wooden shelves used for ripening.

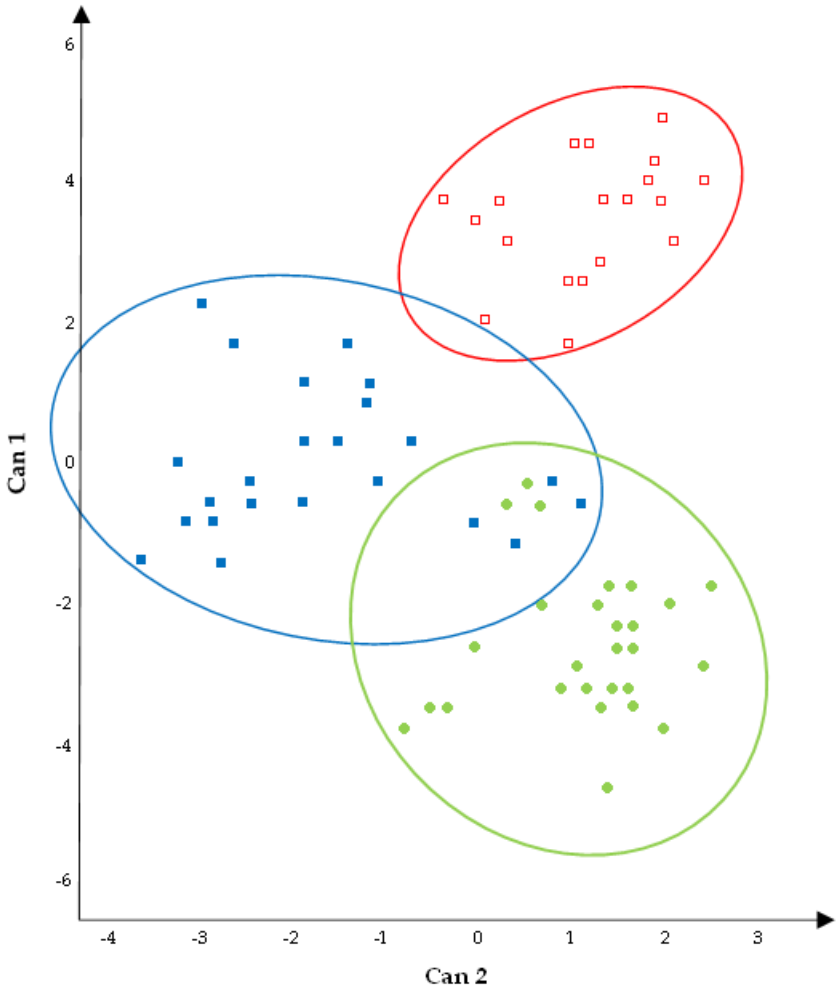

Fig. 2. Plot of Canonical 1 × Canonical 2 – variable: microbial loads of wooden shelves' biofilm. ▪, wooden shelves used for the ripening of TAP Caciocavallo Palermitano cheese; ●, wooden shelves used for the ripening of PDO Pecorino Siciliano cheese; ▫, wooden shelves used for the ripening of Piacentinu Ennese cheese. Centroids coordinates: ▪ (0.04; −1.90); ● (−2.5; 0.93); ▫ (3.46; 0.97).

1706 The MD between the three centroids of Fig. 2 was statistically significant. The longest
1707 distance was registered between the wooden shelves of the PDO Pecorino Siciliano cheese
1708 area and those of the PDO Piacentinu Ennese cheese (MD = 29.04; $p < 0.001$). On the

contrary, the shortest distance was measured between the centroid area of PDO Pecorino Siciliano wooden shelves and that of TAP Caciocavallo Palermitano wooden shelves (MD = 13.10; $p < 0.001$), for which a slight overlap of some points was also observed. The distances observed among the three centroids were mainly due to the first canonical variable (vertical axis of Fig. 1). In particular, thermophilic LAB rods were positively correlated, while mesophilic LAB rods were negatively correlated considering this canonical variable (Table 2).

Table 2. Standardized canonical discriminant function coefficients.

Variables	1st Canonical Variable	2nd Canonical Variable
TMM	−0.512	−1.141
Enterobacteriaceae	−1.843	0.503
Total coliforms	0.210	0.489
E. coli	0.388	−0.405
Pseudomonads	0.753	1.283
Enterococci	0.802	0.907
Mesophilic LAB rods	−2.042	0.192
Thermophilic LAB rods	1.390	−1.720
Mesophilic LAB cocci	1.091	−0.214
Thermophilic LAB cocci	−0.270	0.514
Yeasts	0.451	1.116
Molds	−0.282	0.017
Variance explained (%)	76.2	23.8

The LAB rod levels mostly influenced the separation of wooden shelves per cheese typology. This separation was also clear with regards to the second canonical variable, especially concerning the differences between the wooden shelves used for ripening ovine cheeses and those used for TAP Caciocavallo Palermitano cheeses. With the second canonical variable, thermophilic LAB rod counts were negatively correlated, while the loads of the pseudomonads were positively correlated.

Culture-independent microbiology analysis

The taxonomy classification allowed for identifying 14 phyla, 32 classes, 52 orders, 93 families, and 137 genera from the wooden shelf biofilms (Settanni et al., 2021). In the present study, the operational taxonomy units (OTUs) with a relative abundance >0.1%, were grouped per order (Table 3) and were processed by multivariate statistical analysis. In order to elaborate the relative abundance values, a square root transformation ($Y = \sqrt{((x) + 0.5)}$) was applied to normalize the data presenting a Poisson distribution.

Table 3. Relative abundance (%) of operational taxonomy units (LSM ± s.e.).

Orders	Cheeses			*p* value
	TAP Caciocavallo palermitano	PDO Piacentinu Ennese	PDO Pecorino Siciliano	
Actinomycetales	26.85 ± 0.60	19.04 ± 0.82	34.78 ± 0.44	<0.609
Flavobacteriales	0.11 ± 0.46 b	6.84 ± 0.45 a	0.29 ± 0.46 b	<0.001
Sphingobacteriales	0.87 ± 0.47	0.06 ± 0.47	0.05 ± 0.48	<0.137
Bacillales	43.72 ± 0.09 a	16.39 ± 0.01 b	26.23 ± 0.15 ab	<0.050
Lactobacillales	6.10 ± 0.09	4.17 ± 0.22	9.05 ± 0.02	<0.698
Clostridiales	0.62 ± 0.47	0.56 ± 0.47	0.29 ± 0.82	<0.696
Rhizobiales	3.58 ± 0.22	0.00 ± 0.16	0.08 ± 0.26	<0.171
Alteromonadales	0.00 ± 0.38	2.85 ± 0.36	0.73 ± 0.40	<0.124
Enterobacteriales	0.02 ± 0.46	0.75 ± 0.45	0.27 ± 0.47	<0.420
Oceanospirillales	1.90 ± 0.04	10.46 ± 0.05	4.98 ± 0.10	<0.249
Pseudomonadales	0.08 ± 0.27	1.96 ± 0.22	1.69 ± 0.30	<0.453
Salinisphaerales	0.02 ± 0.21	2.67 ± 0.15	0.87 ± 0.25	<0.439
Rhodobacterales	0.08 ± 0.48	0.64 ± 0.47	0.08 ± 0.48	<0.296

LSM, least-square method; s.e., standard error. In the rows, different capital letters are significant at $p < 0.01$; different letters are significant at $p < 0.05$.

The bacterial orders present at the highest relative abundances were Actinomycetales (19.04–34.78%) and Bacillales (16.39–43.72%), followed by Lactobacillales (4.17–9.05%). The effect of the cheese typology on the bacterial composition of the wooden shelves was statistically significant for Flavobacteriales ($p < 0.001$) and Bacillales ($p < 0.05$). In particular, the wooden shelves of the PDO Piacentinu Ennese cheese showed a consistently higher presence of Flavobacteriales (6.84%) than those of the TAP Caciocavallo Palermitano cheese (0.11%) and PDO Pecorino Siciliano cheese (0.29%).

Regarding the Bacillales order, the TAP Caciocavallo Palermitano cheese wooden shelves displayed the highest relative abundance and the differences found for the other wooden shelves used in the ripening of the other two cheeses (both from ovine milk) were not statistically significant. No statistical differences were found among cheese wooden shelves regarding the other orders identified, probably due to low sample size and/or to a high variability of the relative abundance of the OTUs. The OTUs' relative abundances were subjected to the multivariate statistical analysis. Not all of the results of the statistical tests obtained with the CANDISC procedure were significant: the Wilks Lambda and Hotelling−Lawley tests showed a significance for $p < 0.05$, while the Roy maximum root test was significant for $p < 0.01$. The canonical 1 x canonical 2 plot (Fig. 3) showed a good graphic separation between the three areas, with a clear remark between the wooden shelves used to ripen the PDO Piacentinu Ennese cheeses and those used for the ripening of the other two cheeses. The graphical observation was also confirmed by MD, which was only statistically significant between the PDO Piacentinu Ennese cheese versus Caciocavallo Palermitano PAT cheese wooden shelves (MD = 594; $p < 0.01$), and between the PDO Piacentinu Ennese cheese versus Pecorino Siciliano PDO cheese wooden shelves (MD = 386; $p < 0.05$).

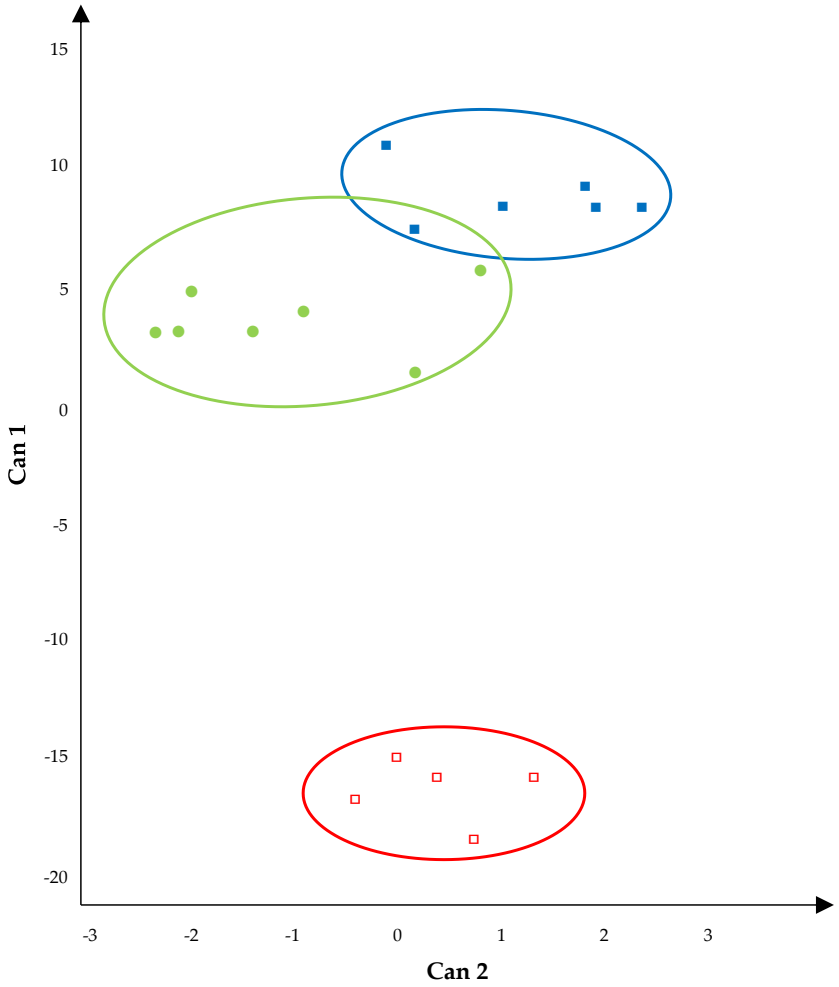

Fig. 3. Plot of Canonical 1 × Canonical 2—variable: relative abundance of OTUs detected on wooden shelves biofilm. Symbols: ▪, wooden shelves used for the ripening of TAP Caciocavallo Palermitano cheese; ●, wooden shelves used for the ripening of PDO Pecorino Siciliano cheese; ▫, wooden shelves used for the ripening of Piacentinu Ennese cheese. Centroids coordinates: ▪ (8.61; −1.07); ● (−15.74; 0.31); ▫ (3.85; −1.14).

The clear separation between the wooden shelves used for the Piacentinu Ennese cheese ripening and all of the other wooden shelves can be observed on the ordinate axis, which corresponds to first canonical variable, which explain the 99% total variability. With regards to the first canonical variable, the Rhizobiales, Bacillales, Actinomycetales, and Lactobacillales orders were positively correlated, while with Flavobacteriales, Sphingobacteriales, Alteromonadales, and Salinisphaerales the orders were negatively correlated (Table 4).

Table 4. Standardized canonical discriminant function coefficients.

Variables	1st Canonical Variable	2nd Canonical Variable
Actinomycetales	3.459	1.071
Flavobacteriales	−6.860	0.759
Sphingobacteriales	−3.574	1.601
Bacillales	3.895	1.753
Lactobacillales	2.788	0.757
Clostridiales	1.818	−1.384
Rhizobiales	5.211	−0.016
Alteromonadales	−3.253	1.885
Enterobacteriales	−0.328	−2.622
Oceanospirillales	4.884	−0.349
Pseudomonadales	−0.275	−0.322
Salinisphaerales	−3.170	−0.082
Rhodobacterales	−0.142	3.242
Variance explained (%)	99.0	1.0

Inhibitory activity of LAB

Among the dominant LAB, 75 strains were identified and allotted into the species *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus casseliflavus*, *Enterococcus gallinarum*, *Enterococcus italicus*, *Enterococcus lactis*, *Enterococcus thailandicus*, *Enterococcus viikkiensis*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus delbrueckii*, *Weissella hellenica*, and *Weissella paramesenteroides* (Settanni et al., 2021). In the present work, all LAB strains were tested for their inhibitory properties against the four main dairy pathogens. Thirty strains were able to inhibit at least one indicator strain (Fig. 4). In general, the most sensitive bacterium was *S.* Enteritidis ATCC13076, while the most resistant one *St. aureus* ATCC33862. Several enterococci were able to inhibit the undesired bacteria, but the highest inhibitory activity was registered for *P. pentosaceus* WS287 and *W. paramesenteroides* WS581 in terms of number of indicators inhibited and with of the clear areas.

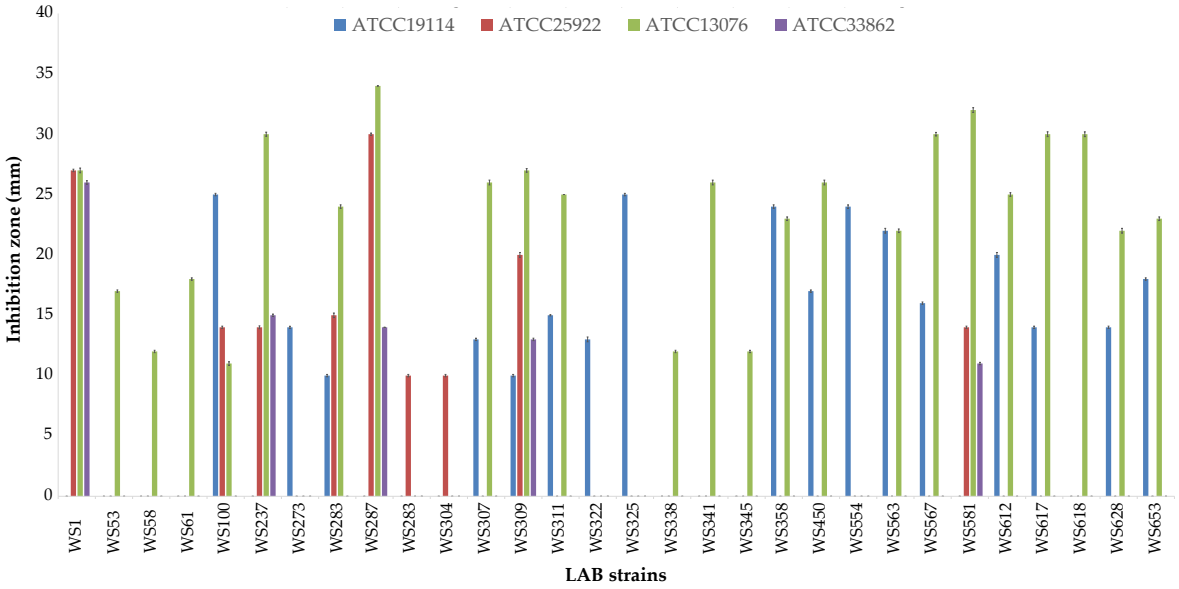

Fig. 4. Inhibitory activity of LAB isolated from wooden shelf biofilms. Strain codes belong to the following species: *En. faecalis* (WS1), *En. faecium* WS237, WS273, WS283, WS311, WS322, WS325, WS338, WS341, WS345, WS358, WS554, WS563, WS612, and WS653) *En. durans* (WS304), *En. lactis* (WS288), *En. viikkiensis* (WS58), *Lc. lactis* (WS61), *Ln. mesenteroides* (WS53, WS307, WS567, WS581, WS618, and WS628), *P. pentosaceus* (WS287, WS309, and WS450), *Lb. delbrueckii* (WS100), *W. hellenica* (WS617), and *W. paramesenteroides* (WS581).

Discussions

The use of wooden shelves in Sicily is mandatory for the ripening of several traditional semi-hard and hard cheeses, including PDO Pecorino Siciliano, PDO Piacentinu Ennese, and TAP Caciocavallo Palermitano (Settanni et al., 2021). These three cheeses are strongly linked to the production area. This implicates that the production environment exerts a defining role on the final cheese characteristics (Cruciata et al, 2019; Gaglio et al., 2021; Settanni et al., 2021). Although traditional cheeses produced in the Sicily region are not inoculated with starter cultures, the acidification and ripening processes rely on the indigenous LAB of raw materials or wooden vat biofilms (Di Grigoli et al., 2015; Pino et al., 2019; Settanni et al., 2013). This strongly indicates that the dairy environment of a given facility might influence the microbial evolution during cheese transformation. Furthermore, the microorganisms (especially bacteria) inside the cheese are characterized

by a different spatial distribution due to the physicochemical characteristics of the different layers (Gobbetti et al., 2018; Monfredini et al., 2012). Although the complex network of interactions between biotic (microbial interactions) and abiotic (pH, water activity, redox potential, and chemical composition) factors within cheese that determine the microbial dynamics is poorly understood (Montel et al., 2014), the microorganisms located just under the rind have to be particularly resistant to high salt concentrations and low water activity. The rind of stretched and pressed cheeses is basically considered to be a barrier, playing no active role during cheese ripening (Galanakis, 2019). However, Settanni et al. (2012) supposed that the wooden boards used for the ripening of PDO Pecorino Siciliano, PDO Piacentinu Ennese, and TAP Caciocavallo Palermitano were able to transfer the bacteria responsible for the centripetal maturation. Thus, the present study was undertaken to evaluate the relationships among wooden shelf bacterial diversity and cheese typology, applying a multivariate statistical approach.

First of all, the microbial loads of the wooden shelf biofilms were subjected to a canonical discriminant analysis, which confirmed a cheese typology effect on the microbial loads of the 18 wooden shelves investigated. The three graphic areas identified in the plot of can 1 x can 2 were quite distant, especially thanks to the levels of the rod LAB. Basically, the longest statistical distances were measured between the wooden shelves used for ripening ovine cheeses (PDO Pecorino Siciliano and PDO Piacentinu Ennese) and those used for the bovine TAP Caciocavallo Palermitano cheese. Canonical correlation analysis is generally applied in the dairy environment to discriminate among sources of contamination of *Pseudomonas* (Leriche et al., 2004), to investigate on the growth interaction between *St. aureus* and *Lactococcus lactis* subsp. *cremoris* in inoculated milk

(Nicolaou et al., 2011), and to correlate cheese volatile organic compounds with starter and non-starter LAB (Guarrasu et al., 2017), indicating a wide field of application of this statistical approach in dairy science. Strictly regarding discrimination about cheeses, Manca et al. (Manca et al., 2001) demonstrated that a multivariate treatment of casein and amino acid data differentiated numerous samples of Pecorino Sardo, Pecorino Siciliano, and Pecorino Pugliese cheeses according to place of origin (Sardinia, Sicilia, and Apulia regions, respectively). Based on our results, a similar discrimination could be tempted exclusively with microbiological data, given that the biofilm levels could provide differentiation among cheese production. For this purpose, the levels of TMM found on the wooden boards used in Sicily for the three cheeses of the present investigation were lower than those detected on the shelves used to ripen Reblochon de Savoie cheese in France (Mariani et al 2007) or Fontina cheese in northern Italy (Guzzon et al., 2017)—both smear cheeses—but higher than those reported for the shelves used for ripening Serro and Canastra cheeses in Brazil (Galinari et al, 2014). These data confirm that the levels of biofilms on the wooden shelves were strictly related to the cheese typology.

The same statistical approach was used to correlate bacterial OTUs at the order level and for cheese typology. The most relevant results showed that the wooden shelves used for PDO Piacentinu Ennese cheese ripening were characterized by the highest presence of Flavobacteriales, while those used for TAP Caciocavallo Palermitano cheese ripening were characterized by the highest relative abundance of Bacillales. Generally, Flavobacteriales are identified as cheese biomarkers during raw milk cheddar cheese production (Choi et al., 2020). Biomarkers are bacterial communities that are significantly and relatively highly abundant in two or more samples, that are useful to explain conditions of the sample source

(Segata et al., 2011). Bacillales are reported to dominate in raw milk during the warm seasons (spring and summer months) (Porcellato et al., 2018), and all of the TAP Caciocavallo Palermitano cheeses in contact with the wooden board analyzed in this study were produced during the summer season. Discriminant canonical analysis once again showed a good graphic separation among the three areas obtained from the data of the three cheeses analyzed, confirming the results obtained from the univariate analysis. The multivariate analysis of the metataxonomic and metafingerprinting data were successfully discriminated between dominant and subdominant taxa for the PDO Grana Padano cheese and generical hard cheeses (Zago et al., 2021), cheeses whose appearance is highly similar to that of the PDO Grana Padano cheese. Hence, our study represents a useful suggestion to also use the microbiota of the ripening tools to trace the typicality of PDO cheeses, and provides an additional measure to face their counterfeiting.

Generally, under the conditions of a given dairy facility, environmental contamination by LAB might be observed during cheese production (Calasso et al., 2016). So far, there is no evidence on what happens to the wooden shelf biofilms during cheese ripening; actually, knowledge about the microbial communities associated with cheese ripening wooden boards is limited (Wadhawan et al., 2021). In our opinion, there is a quite urgent need to fill this gap, especially after the so-called “cheese apocalypse” reported by Forbes in 2014, based on the FDA decree to answer the request from the New York State Department of Agriculture regarding the acceptability of wooden surfaces for cheese aging. The agency responded that “the use of wooden shelves, rough or otherwise, for cheese ripening does not conform to current good practices” (https://archive.kpcc.org/news/2014/06/13/44696/when-the-cheese-hits-the-ban-fda-

backtracks-on-sme/) (accessed on 24 February 2022), and issued an alert on the potential presence of pathogenic bacteria transferred by Italian and French cheeses ripened on wooden shelves (Cutini, 2014). In the present study, considering that the wooden shelves were sampled from several facilities characterized by their own environments, data from multivariate statistical elaboration highlighted how the microbial differences evaluated both in terms of viable levels and OTU classification among the board biofilms were mainly imputable to the cheese type, rather than the environmental factors of the facilities analyzed. The absence of pathogenic species after culture-dependent and -independent microbiological investigations assumed an inhibiting role of the microbial populations of wooden board biofilms. A previous investigation demonstrated a certain potential of the wooden shelf LAB to inhibit *L. monocytogenes* (Mariani et al., 2007). Based on this consideration, all strains isolated and identified by Settanni et al. (2021) from the wooden shelf biofilms characterized from PDO Pecorino Siciliano, PDO Piacentinu Ennese, and TAP Caciocavallo Palermitano cheeses were investigated for their ability to inhibit the growth of the main dairy pathogens, namely: *E. coli*, *L. monocytogenes*, *S. Enteritidis*, and *St. aureus*. The results clearly showed a definite activity against *S. Enteritidis* ATCC13076, while the most resistant strain was *St. aureus* ATCC33862. However, the most interesting result was that a huge number of wooden shelf LABs were able to inhibit at least one of the undesired bacteria. In general, it is known that LABs isolated from fermented foods, where adaptation to an environment rich in nutritional sources plays a major role for their persistence, are characterized by a very low ability to produce inhibitory substances, and that their raw materials host higher percentages of positive strains and a higher inhibitory activity (Galinari et al., 2014; Manca et al., 2001). This

work provides further evidence to that of Mariani et al. (2011) on the inhibitory spectrum of the LAB biofilms of wooden shelves used to ripen cheeses. Furthermore, it is worth noting that several enterococci were inhibitors of the indicator strains, but the highest activity was found for *P. pentosaceus* WS287 and *W. paramesenteroides* WS581. These results are particularly interesting because non-*Enterococcus* LAB have a wider application in dairy transformations than enterococci due to the innate presence of antibiotic resistance in several strains of the latter group (Gaglio et al., 2016a).

Conclusions

The dual statistical approach (multivariate and univariate analysis) applied in this study clearly showed that the microbial levels and the bacterial composition of the biofilms of the wooden shelves used to ripen traditional cheeses are influenced by the cheese typology. This is relevant information retrieved from the levels of the 12 microbial groups analyzed and the bacterial classification based on OTUs' attribution, in particular in consideration of the fact that all 18 facilities where wooden shelf biofilms were collected are characterized by quite unique environmental conditions. Furthermore, the biofilms associated with the wooden shelves investigated exerted an inhibitory activity against the main dairy pathogens, suggesting a barrier effect of LAB. In order to better and deeply investigate the development and composition of biofilms on the wooden shelves used to ripen traditional Sicilian cheeses, the effect of wood type, board age, and salting technology will be analyzed in future works. At present, works are being prepared to evaluate the in situ inhibitory activity of the selected strains showing the best performances in terms of

indicator inhibition on virgin boards subjected to the pressure of artificially inoculated pathogenic strains.

References

Aviat, F., Gerhards, C., Rodriguez-Jerez, J.J., Michel, V., Bayon, I.L., Ismail, R., Federighi, M., 2016. Microbial safety of wood in contact with food: a review. Compr. Rev. Food Sci. Food Saf. 15 (3), 491–505. https://doi.org/10.1111/1541-4337.12199.

Bonanno, A., Tornambè, G., Bellina, V., De Pasquale, C., Mazza, F., Maniaci, G., Di Grigoli, A., 2013. Effect of farming system and cheese making technology on the physicochemical characteristics, fatty acid profile, and sensory properties of Caciocavallo Palermitano cheese. J. Dairy Sci. 96, 710–724. http://dx.doi.org/10.3168/jds.2012-5973.

Bruni, A., 1859. Enciclopedia Agraria. Marghieri and Pellerano: Naples, Italy.

Busetta, G., Garofalo, G., Mangione, G., Botta, L., Franciosi, E., Di Gerlando, R., Todaro, M., Licitra, G., Scatassa, M.L., Gaglio, R., Settanni, L., 2021. Polyphasic Characterization of Microbiota of "Mastredda", a Traditional Wooden Tool Used during the Production of PDO Provola dei Nebrodi Cheese. Appl. Sci. 11, 8647. https://doi.org/10.3390/app11188647.

Calasso, M., Ercolini, D., Mancini, L., Stellato, G., Minervini, F., Di Cagno, R., De Angelis, M. Gobbetti, M., 2016. Relationships among house, rind and core microbiotas during manufacture of traditional Italian cheeses at the same dairy plant. Food Microbiol. 54, 115–126. https://doi.org/10.1016/j.fm.2015.10.008.

Choi, J., In Lee, S., Rackerby, B., Frojen, R., Goddik, L., Ha, S.D., Park, S.H., 2020. Assessment of overall microbial community shift during Cheddar cheese production from raw milk to aging. Appl. Microbiol. Biotechnol. 104, 6249–6260. https://doi.org/10.1007/s00253-020-10651-7.

Commission Regulation (EC) No 1935/2004 of the European Parliament an of the Council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC. Off. J. Eur. Union 338:4–17.

Commission Regulation (EC) No 2074/2005 Laying down implementing measures for certain products under regulation (EC) No. 853/2004 of the European Parliament and of the Council and for the organisation of official controls under regulation (EC) No. 854/2004 of the European Parliament and of the Council and regulation (EC) No. 882/2004 of the European Parliament and of the Council, derogating from regulation (EC) No. 852/2004 of the European Parliament and of the Council and amending regulations (EC) No. 853/2004 and (EC) No. 854/2004. Off J Eur Union 338:27–59.

Corsetti, A., Settanni, L., Braga, T.M., De Fatima Silva Lopes, M., Suzzi, G., 2008. An investigation on the bacteriocinogenic potential of lactic acid bacteria associated with wheat (*Triticum durum*) kernels and non-conventional flours. LWT-Food Sci. Technol. 41, 1173–1182. https://doi.org/10.1016/j.lwt.2007.07.022.

Cruciata, M., Gaglio, R., Scatassa, M.L., Sala, G., Cardamone, C., Palmeri, M., Moschetti, G., La Mantia, T., Settanni, L., 2018. Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production. Appl. Environ. Microbiol. 84, e02107-17. https://doi.org/10.1128/AEM.02107-17.

Cruciata, M., Gaglio, R., Todaro, M., Settanni, L., 2019. Ecology of Vastedda della valle del Belìce cheeses: a review and recent findings to stabilize the traditional production. Food Rev. Int. 35, 90–103. https://doi.org/10.1080/87559129.2018.1469142.

Cutini, F., 2014. (Food: Divina (LN), the FDA Declares War on Our Chesees, AgenParl, Roma, Italy). Personal communication.

Dey, P., Pandit, P., 2020. Relevance of data transformation techniques in weed science. J. Res. Weed Sci. 3, 81–89. https:// doi: 10.26655/jrweedsci.2020.1.8

Di Grigoli, A., Francesca, N., Gaglio, R., Guarrasi, V., Moschetti, M., Scatassa, M.L., Settanni, L., Bonanno, A., 2015. The influence of the wooden equipment employed for cheese manufacture on the

characteristics of a traditional stretched cheese during ripening. Food Microbiol. 46, 81–91. https://doi.org/10.1016/j.fm.2014.07.008.

Didienne, R., Defargues, C., Callon, C., Meylheuc, T., Hulin, S., Montel, M.C., 2012. Characteristics of microbial biofilm on wooden vats ('gerles') in PDO Salers cheese. Int. J. Food Microbiol. 156, 91–101.

Fallico, V., Tuminello, L., Pediliggieri, C., Horne, J., Carpino, S., Licitra, G., 2006. Proteolysis and microstructure of Piacentinu Ennese cheese made using different farm technologies. Int. J. Dairy Sci. 89, 37–48. https://doi.org/10.3168/jds.S0022-0302(06)72067-7.

Gaglio, R., Couto, N., Marques, C., Lopes, M.D.F.S., Moschetti, G., Pomba, C., Settanni, L., 2016a. Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses. Int. J. Food Microbiol. 236, 107–114. https://doi.org/10.1016/j.ijfoodmicro.2016.07.020.

Gaglio, R., Cruciata, M., Di Gerlando, R., Scatassa, M. L., Cardamone, C., Mancuso, I., Sardina, M.T., Moschetti, G., Portolano, B., Settanni, L., 2016b. Microbial activation of wooden vats used for traditional cheese production and evolution of neoformed biofilms. Appl. Environ. Microbiol. 82, 585–595. https://doi.org/10.1128/AEM.02868-15.

Gaglio, R., Todaro, M., Settanni, L., 2021. Improvement of raw milk cheese hygiene through the selection of starter and non-starter lactic acid bacteria: The successful case of PDO pecorino siciliano cheese. Int. J. Environ. Res. Public Health. 18, 1834. https://doi.org/10.3390/ijerph18041834.

Galanakis, C., 2019. Survival of Probiotics in Functional Foods during Shelf Life. In: Dinkci, N., Akdeniz, V., Akalin, A.S. (Eds.), Food Quality and Shelf Life, Academic Press, London, pp. 201-233. https://doi.org/10.1016/B978-0-12-817190-5.00006-9.

Galinari, É., Nóbrega, J.E.D., Andrade, N.J.D., Ferreira, C.L.D.L F., 2014. Microbiological aspects of the biofilm on wooden utensils used to make a Brazilian artisanal cheese. Braz. J. Microbiol. 45, 713–720. https://doi:10.1590/s1517-83822014000200047.

Gobbetti, M., Di Cagno, R., Calasso, M., Neviani, E., Fox, P.F., De Angelis, M., 2018. Drivers that establish and assembly the lactic acid bacteria biota in cheeses. Trends Food Sci. Technol. 78, 244–254. https://doi.org/10.1016/j.tifs.2018.06.010.

Guarrasi, V., Sannino, C., Moschetti, M., Bonanno, A., Di Grigoli, A., Settanni, L., 2017. The individual contribution of starter and non-starter lactic acid bacteria to the volatile organic compound composition of Caciocavallo Palermitano cheese. Int. J. Food Microbiol. 259, 35–42. https://doi.org/10.1016/j.ijfoodmicro.2017.07.022.

Guzzon, R., Carafa, I., Tuohy, K., Cervantes, G., Vernetti, L., Barmaz, A., Larcher, R., Franciosi, E., 2017. Exploring the microbiota of the red-brown defect in smear-ripened cheese by 454-pyrosequencing and its prevention using different cleaning systems. Food Microbiol. 62, 160–168. https://doi.org/10.1016/j.fm.2016.10.018.

Leriche, F., Bordessoules, A., Fayolle, K., Karoui, R., Laval, K., Leblanc, L., Dufour, E., 2004. Alteration of raw-milk cheese by P*seudomonas* spp.: Monitoring the sources of contamination using fluorescence spectroscopy and metabolic profiling. J. Microbiol. Methods 59, 33–41. https://doi.org/10.1016/j.mimet.2004.05.009.

Licitra, G., Ogier, J.C., Parayre, S., Pediliggieri, C., Carnemolla, T.M., Falentin, H., Madec, M.N., Carpino, S., Lortal, S., 2007. Variability of the bacterial biofilms of the "tina" wood vat used in the Ragusano cheese making process. Appl. Environ. Microbiol. 73, 6980–6987. https://doi.org/10.1128/AEM.00835-07.

Lortal, S., Di Blasi, A., Madec, M.N., Pediliggieri, C., Tuminello, L., Tangury, G., Fauquant, J., Lecuona, Y., Campo, P., Carpino, S., Licitra, G., 2009. Tina wooden vat biofilm. A safe and highly efficient lactic acid bacteria delivering system in PDO Ragusano cheese making. Int. J. Food Microbiol. 132, 1–8. https://doi.org/10.1016/j.ijfoodmicro.2009.02.026.

Manca, G., Camin, F., Coloru, G.C., Del Caro, A., Depentori, D., Franco, M.A., Versini, G., 2001. Characterization of the geographical origin of Pecorino Sardo cheese by casein stable isotope (13C/12C and 15N/14N) ratios and free amino acid ratios. J. Agric. Food Chem. 49, 1404–1409.

Mariani, C., Briandet, R., Chamba, J.F., Notz, E., Carnet-Pantiez, A., Eyoug, R.N., Oulahal, N., 2007. Biofilm ecology of wooden shelves used in ripening the French raw milk smear cheese Reblochon de Savoie. J. Dairy Sci. 90, 1653–1661. https://doi.org/10.3168/jds.2006-190.

Mariani, C., Oulahal, N., Chamba, J.F., Dubois-Brissonnet, F., Notz, E., Briandet, R., 2011. Inhibition of *Listeria monocytogenes* by resident biofilms present on wooden shelves used for cheese ripening. Food Control 22 (8), 1357–1362. https://doi.org/10.1016/j.foodcont.2011.02.012.

Monfredini, L., Settanni, L., Poznanski, E., Cavazza, A., Franciosi, E., 2012. The spatial distribution of bacteria in Grana-cheese during ripening. Syst. Appl. Microbiol. 35, 54–63. https://doi.org/10.1016/j.syapm.2011.07.002.

Montel, M.C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D., Desmasures, N., Berthier, F., 2014. Traditional cheeses: Rich and diverse microbiota with associated benefits. Int. J. Food Microbiol. 177, 136–154. https://doi.org/10.1016/j.ijfoodmicro.2014.02.019.

Nicolaou, N., Xu, Y., Goodacre, R., 2011. Fourier transform infrared and Raman spectroscopies for the rapid detection, enumeration, and growth interaction of the bacteria *Staphylococcus aureus* and *Lactococcus lactis* ssp. *cremoris* in milk. Anal. Chem. 83, 5681–5687. https://doi.org/10.1021/ac2008256.

Pino, A., Russo, N., Van Hoorde, K., De Angelis, M., Sferrazzo, G., Randazzo, C.L., Caggia, C., 2019. Piacentinu Ennese PDO Cheese as reservoir of promising probiotic bacteria. Microorganisms 7, 254. https://doi.org/10.3390/microorganisms7080254.

Porcellato, D., Aspholm, M., Skeie, S.B., Monshaugen, M., Brendehaug, J., Mellegård, H., 2018. Microbial diversity of consumption milk during processing and storage. Int. J. Food Microbiol. 266, 21–30. https://doi.org/10.1016/j.ijfoodmicro.2017.11.004.

Scatassa, M.L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., Di Grigoli, A., Moschetti, G., Settanni, L., 2015. Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol. 52, 31–41. https://doi.org/10.1016/j.fm.2015.06.008.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12, R60.

Settanni, L., Busetta, G., Puccio, V., Licitra, G., Franciosi, E., Botta, L., Di Gerlando, R., Todaro, M., Gaglio, R., 2021. In-Depth Investigation of the Safety of Wooden Shelves Used for Traditional Cheese Ripening. Appl. Environ. Microbiol. 87, e01524-21. https://doi.org/10.1128/AEM.01524-2.

Settanni, L., Di Grigoli, A., Tornambé, G., Bellina, V., Francesca, N., Moschetti, G., Bonanno, A., 2012. Persistence of wild *Streptococcus thermophilus* strains on wooden vat and during the manufacture of a traditional Caciocavallo type cheese. Int. J. Food Microbiol. 155, 73–81. https://doi.org/10.1016/j.ijfoodmicro.2012.01.022.

Settanni, L., Gaglio, R., Guarcello, R., Francesca, N., Carpino, S., Sannino, C., Todaro, M., 2013. Selected lactic acid bacteria as a hurdle to the microbial spoilage of cheese: Application on a traditional raw ewes' milk cheese. Int. Dairy J. 32, 126–132.

Settanni, L., Moschetti, G., 2014. New trends in technology and identity of traditional dairy and fermented meat production processes: preservation of typicality and hygiene. Trends Food Sci. Technol. 37 (1), 51–58. https://doi.org/10.1016/j.tifs.2014.02.006.

Todaro, M., Lo Presti, V., Macaluso, A., Alleri, M., Licitra, G., Chiofalo, V., 2021. Alkaline Phosphatase Survey in Pecorino Siciliano PDO Cheese. Foods 10, 1648.

Wadhawan, K., Steinberger, A.J., Rankin, S.A., Suen, G., Czuprynski, C.J., 2021. Characterizing the microbiota of wooden boards used for cheese ripening. JDS Commun. 2, 171–176.

Zago, M., Rossetti, L., Bardelli, T., Carminati, D., Nazzicari, N., Giraffa, G., (2021). Bacterial community of Grana Padano PDO cheese and generical hard cheeses: DNA metabarcoding and DNA metafingerprinting analysis to assess similarities and differences. Foods 10, 1826.

Chapter IV

The wooden shelf surface and cheese rind mutually exchange microbiota during the traditional ripening process

PECORINO DI FILIANO

CANESTRATO DI MOLITERNO

This work is published in

International Journal of Food Microbiology 409,

2024

Abstract

The rind acts as a protective barrier for internally-bacterial ripened cheeses. Unlike surface-inoculated smear cheeses, centripetal maturation is not assumed to occur in these cheeses. This research was aimed to evaluate the microbial diversity of the wooden shelves used for the ripening of Protected Denomination of Origin (PDO) Pecorino di Filiano and Protected Geographical Indication (PGI) Canestrato di Moliterno cheeses. The microorganisms associated with the rind of these cheeses were also investigated. Both wooden shelf surfaces and cheese rinds were sampled by brushing method to collect their biofilms. Wooden shelves showed levels of total mesophilic microorganisms (TMM) between 5.6 and 7.2 log CFU/cm^2, while cheese rinds between 6.1 and 7.8 log CFU/cm^2. The major dairy pathogens (*Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus*) were never detected, while mesophilic and thermophilic bacteria dominated the surfaces of all wooden shelves and cheese rinds. LAB community was represented by *Enterococcus* spp., *Leuconostoc* spp., and *Marinilactibacillus* spp. Among yeasts, *Debaryomyces* spp., *Candida* spp., were identified, while *Aspergillus* spp., and *Penicillium* spp., dominated the community of filamentous fungi. MiSeq Illumina analysis identified 15 phyla, 13 classes, 28 orders, 54 families, and 56 genera among bacteria. *Staphylococcus* spp. was identified from all wooden surfaces, with a maximum abundance of 71 %. *Brevibacterium*, *Corynebacterium* and halophilic bacteria were detected in almost all samples. Regarding fungi, wooden shelves mainly hosted *Aspergillus*, *Penicillium* and *Debaryomyces hansenii*, while cheese rinds especially *Penicillium* and *D. hansenii*. Alpha diversity confirmed a strict correlation between the microbiota of wooden shelves and that of cheese rinds for the majority of factories. This

study confirmed that the wooden shelves used for cheese ripening are microbiologically active and represent safe systems. Furthermore, the results of this work clarified the transfer flow between wooden shelves and PDO Pecorino di Filiano and PGI Canestrato di Moliterno cheese surfaces: smear-active microorganisms are mainly transferred from wooden shelves to cheese rind, which potentially contribute to the development of the final organoleptic characteristics; meanwhile, cheeses transfer LAB that are potentially involved in defining the safety aspects of the shelves.

Keywords: Cheese ripening; Microbial transfer; MiSeq Illumina; Lactic acid bacteria; Traditional cheeses; Wooden shelves

Introduction

Cheese production is a very ancient daily activity in Italy (Gobbetti and Neviani, 2018). This old cheese-making tradition has favored the production of several kinds of cheese, strongly linked to their respective territories of origin and, even today, produced using traditional processes; several traditional and typical southern Italian cheeses are still produced with tools made of wood (Busetta et al., 2023a; Busetta et al., 2023b; Settanni and Moschetti, 2014). For several traditional cheese productions, the ripening process occurs onto wooden shelves (Settanni et al., 2021; Wadhawan et al., 2021).

Thanks to its durability, low cost, and local availability, wood has been one of the most commonly used materials for dairy tools for centuries (Aviat et al., 2016). In the last years, the safety of wooden tools used in cheese making has been discussed at the European level, because the porous structure of the wooden surfaces makes their sanitization very difficult.

2115 However, according to several studies, wooden tools do not pose any hygiene issues and
2116 do not represent any microbiological risks for the human health (Busetta et al., 2023a;
2117 Cruciata et al., 2018; Licitra et al., 2007; Lortal et al., 2014; Scatassa et al., 2015; Sun and
2118 D'Amico, 2023a; Sun and D'amico, 2023b). In light of the European Regulation (EC) no.
2119 2074/2005, which derogates from EC no. 852/2004 for food with traditional characteristics
2120 (Commission Regulation 2005), wood is currently used in cheese making in France and
2121 Italy. In these countries, wood is in contact with the product throughout the entire
2122 transformation process until the final ripening stage. It is worth noting that after the US
2123 Food and Drugs Administration advice against Italian and French cheeses ripened on
2124 wooden shelves, there has been an American re-discovery of traditional cheeses ripened on
2125 wooden shelves in the very last years (Sun and D'Amico, 2021; Sun and D'amico, 2023a;
2126 Sun and D'amico, 2023b; Wadhawan et al., 2021).

2127 The use of wood during the ripening phase encourages the development of the rind and
2128 enhances the organoleptic qualities and typical features of cheeses (Richard, 1997).
2129 Mariani et al. (2007) demonstrated the suitability of wooden shelves for cheese ripening
2130 and reported that the microbial community of the shelves in contact with French
2131 Reblochon de Savoie smear cheeses is composed of micrococci-corynebacteria, yeasts, and
2132 molds, as well as LAB, *Staphylococcus*, and *Pseudomonas*. The authors monitored the
2133 microbial composition over time stating that it is quite stable and very similar to that of the
2134 cheese surface. Another work of the same research group (Mariani et al., 2011) verified the
2135 anti-*Listeria* activity of the resident biofilms present onto the wooden shelves used for
2136 cheese ripening, supporting the hypothesis that the wooden shelves regulate the
2137 development of microflora during ripening, and represent an essential source for the

development of the microbial ecosystem expected on the cheese rind. Besides the protection effects against undesired pathogenic and/or spoilage microorganisms, Irlinger and Mounier (2009) demonstrated also the role of rind microbiota in the development of the sensory characteristics. Settanni et al. (2021) analysed the microbial composition of the wooden shelves applied for ripening traditional cheeses produced in Sicily (southern Italy) focusing on the bacterial community, but there is still very little information on the microbiological traits and function of wooden shelves used to ripen traditional cheeses made in Italy. Information on the microbial composition of the wooden shelves and their transfer to cheese rind are necessary to better qualify traditional cheeses made with raw milk. These data are of considerable value also in view of territory valorization and, especially, to arrest land abandonment phenomenon of rural areas (Fontefrancesco et al., 2023).

Based on the above considerations, the present work aimed to deeply investigate and characterize bacterial and fungal communities associated with wooden shelves and rind of two typical hard cheeses of Basilicata region (southern continental Italy), Protected Denomination of Origin (PDO) Pecorino di Filiano and Protected Geographical Indication (PGI) Canestrato di Moliterno cheeses, through a culture-dependent and independent approach. Microbial biofilms from wooden shelves and cheese rinds were characterized using MiSeq Illumina technology and subjected to plate counts to detect the levels of the main dairy wanted and undesired microorganisms. Furthermore, bacteria, yeasts, and molds from wooden shelves and cheese rinds were genotypically characterized.

Materials and methods

Collection of wooden shelf and cheese rind biofilms

Biofilms formed onto the surface of the wooden shelves used to ripen traditional cheeses and those developed onto the rind of PDO Pecorino di Filiano and PGI Canestrato di Moliterno cheeses were collected from six dairies (A – F) of Basilicata region, all located in the inland of Potenza province. In particular, the dairy factories A and B produced PGI Canestrato di Moliterno cheeses, while factories C – F PDO Pecorino di Filiano cheeses. In each dairy factory investigated, samples were collected from the cheese rinds (CR-A – CR-F) and the wooden shelves (WS-A – WS-F) at the cheese rind/wooden shelf contact interface; the contact between shelves and cheese rinds lasted 3 – 4 months for PGI Canestrato di Moliterno cheeses and 1 – 2 months for PDO Pecorino di Filiano cheeses. The characteristics of the wooden shelves used for cheese ripening are reported in Table 1.

Table 1. Characteristics of the wooden shelves used for cheese ripening.

Wooden shelf[a]	Cheese typology	City	Age (years)	Wood type[b]	Cleaning procedure
WS-A	PGI Canestrato di Moliterno	Moliterno	2	chestnut	surface scraping – water pushing broom
WS-B	PGI Canestrato di Moliterno	Moliterno	8	silver fir	24 h sun exposure – surface scraping – pressure washer
WS-C	PDO Pecorino di Filiano	Filiano	5	stone pine	pressure washer – 8 h sun exposure
WS-D	PDO Pecorino di Filiano	Filiano	15	silver fir	water pushing broom
WS-E	PDO Pecorino di Filiano	Filiano	2	silver fir	water pushing broom
WS-F	PDO Pecorino di Filiano	Filiano	10	silver fir	10% NaOH pushing broom – pressure washer

[a] WS-A to -F, wooden shelves from factories A to F.
[b] Tree species: chestnut, *Castanea sativa* Miller; silver fir, *Abies alba* L.; stone pine, *Pinus pinea* L.

Biofilm collection from both cheese rinds and wooden shelves occurred by a non-destructive method through 100 cm^2 area delimiters (Area Space 100, VWR International PBI s.r.l., Milan, Italy). The method described by Settanni et al. (2021) was applied for sampling the surfaces of the wooden shelves and was also adapted to sample cheese rinds (Fig. 1).

Fig. 1. Biofilm collection from wooden shelves surfaces (A) and cheese rind surfaces (B) with a sterile toothbrush

Cheese rind was sampled from a given cheese from both top and bottom dishes, because cheeses were turned upside down weekly. Briefly, the delimited area was brushed with a sterile toothbrush and, then, a sterile gauze, previously wet in Ringer's solution (Sigma-Aldrich, Milan, Italy) was put on the brushed area to collect the microorganisms. The toothbrush was energetically shaken and washed in 100 ml of Ringer's solution contained into a 200 mL-volume Anicrin liquid container (Anicrin, Scorzé, Italy) and the contaminated gauze was transferred in this container. All containers with the biofilms from the wooden shelves or cheese rinds were kept under refrigeration by means of a portable fridge with reusable ice packs and transported to the Laboratory of Agricultural Microbiology of the University of Palermo (Italy). Both wooden shelves and cheese rinds were sampled in triplicate from each dairy factory: three 100 cm^2 areas from three distinct wooden shelves as well as top and bottom dishes from three cheeses (one per wooden shelf) were sampled.

Microbiological analyses

All samples were subjected to decimal serial dilution in Ringer's solution. In particular, the first dilution of these samples was obtained from 1 mL of the vigorously manually shaken cell suspension of toothbrush and gauze from each wooden shelf or cheese rind added with 9 mL Ringer's solution. All other dilutions were performed at 1:10 ratio. All cell suspensions were plated on agar media to allow the development of different microbial groups: total mesophilic microorganisms (TMM) on plate count agar (PCA) supplemented with 1 g/L skimmed milk (SM), typically used for dairy samples, under aerobic incubation at 30 °C for 72 h; total psychrotrophic microorganisms (TPM) on SM-PCA under aerobic incubation at 7 °C for 7 d; thermophilic and mesophilic LAB cocci on M17 agar under anaerobic incubation, occurred into hermetically sealed jars containing the AnaeroGen AN25 sachets (Oxoid), at 44 and 30 °C, respectively, for 48 h; thermophilic and mesophilic LAB rods on de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 M), under anaerobic incubation at 44 and 30 °C, respectively, for 48 h; enterococci on kanamycin esculin azide (KAA) agar under aerobic incubation at 37 °C for 24 h; coagulase-positive staphylococci (CPS) on Baird-Parker (BP) agar supplemented with rabbit plasma fibrinogen under aerobic incubation at 37 °C for 48 h; members of *Enterobacteriaceae* family on violet red bile glucose agar (VRGBA) under microaerobic incubation, obtained by pouring a top VRBGA layer onto the surface of the bottom VRBGA layer inoculated by pour plate method, at 37 °C for 24 h; total coliforms on violet red bile agar (VRBA) under microaerobic incubation at 37 °C for 24 h; pseudomonads on *Pseudomonas* agar base (PAB) supplemented with cephaloridine sodium fusidate cetrimide under aerobic incubation at 25 °C for 48 h; yeasts on yeast peptone dextrose (YPD) under

aerobic incubation at 28 °C for 48 h; molds on potato dextrose agar (PDA) supplemented with 0.1 g/L chloramphenicol to avoid bacterial growth under aerobic incubation at 25 °C for 7 d; *Escherichia coli* and *Salmonella* spp. on Hektoen enteric agar (HEA) under aerobic incubation at 37 °C for 24 h; *Listeria monocytogenes* on *Listeria* selective agar base (LSAB) added with SR0140E supplement under aerobic incubation at 37 °C for 48 h. All media and supplements were purchased from Oxoid (Milan, Italy). Plate counts were performed in duplicate.

Isolation and identification of LAB

Presumptive LAB were randomly picked up from the highest dilutions plated on agar media. At least five colonies characterized by the same shape, size, width, elevation, thickness, uniformity, color and surface opacity were picked up from MRS and M17 agar plates. The colonies were then transferred into the corresponding broth media. After overnight growth at the optimal incubation conditions, all isolates were purified by streaking technique until obtaining colonies with identical appearance. All presumptive LAB were preliminarily tested for their general characteristics: Gram type was determined after exposure to a 3 % (w/v) KOH solution (Gregersen, 1978); catalase test was carried out with 3 % (v/v) H_2O_2 (Koneman, 1997). Presumptive LAB cultures were stocked into 1.5 mL vials containing 20 % (v/v) glycerol at – 80 °C. As described by Barbaccia et al. (2021), all pure cultures were subjected to a grouping based on cell morphology and arrangement of cells. Furthermore, all isolates were tested for growth at 15 and 45°C, heat resistance (60°C for 30 min), hydrolysis of arginine and aesculin, acid production from carbohydrates, and CO_2 production from glucose as described by Gaglio et al. (2014).

Presumptive LAB cocci were also analyzed for their growth at pH 9.2 and in the presence of NaCl (6.5 g/liter) because enterococci are positive to these tests.

All pure cultures were then subjected to DNA extraction using a DNA-SORB-B kit (Sacace Biotechnologies Srl, Como, Italy) following the protocol provided by the manufacturer. The differentiation of the isolates was obtained through random amplification of polymorphic DNA (RAPD)-PCR analysis, that is commonly used to fingerprint bacteria; to this purpose, bacterial DNAs were individually amplified by single primers M13, AB111, and AB106 (Gaglio et al., 2017). The resulting polymorphic profiles were analyzed by GelCompare II software version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium). This program allows to generate a dendrogram to evaluate the similarity among PCR pattern products. The isolates sharing a very high similarity are considered to represent the same strain. All different strains were identified by sequencing the 16S rRNA gene and the sequences were compared with those available in GenBank/EMBL/DDBJ and EzTaxon database (http://eztaxon-e.ezbiocloud.net/ taxonomy).

Isolation and identification of fungi

Yeasts

At least 5 colonies with the same morphology were also isolated from YPD to analyze the composition of unicellular fungi. Fungal colonies were purified to homogeneity by streaking onto Malt Extract Agar (MEA). This operation was repeated for all different yeast colony morphologies. Yeast isolates were then subjected to genetic characterization. First discrimination of the yeasts was performed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and

ITS2) and the 5.8 S rRNA gene. DNA amplification occurred with the primer pair ITS1/ITS4 as described by Esteve-Zarzoso et al. (1999). The generated amplicons were digested with the endonucleases *Cfo*I, *Hae*III and *Hinf*I (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h. ITS amplicons as well as their restriction fragments were run on 1.5 % (w/v) agarose gel in 1× TBE (89 mM Tris–borate, 2 mM EDTA, pH 8) buffer.

The isolates were further processed by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary RFLP identification. The identities of the sequences were determined by BLASTN search (http://www.ncbi. nlm.nih.gov).

Filamentous fungi

Fungi were collected from PDA and transferred to malt extract agar (MEA) (Oxoid, Milan, Italy). Petri dishes were incubated at 25 °C and observed every 24 h until a consistent development of mycelium was observed (1-2 weeks). All different fungal colonies were picked up from agar plates and purified after consecutive sub-culturing steps onto MEA. After incubation, fungi were subjected to morphological characterization including color (on both sides of the plate), diffusible pigments, exudates, texture, growth zones, aerial and submerged hyphae, growth rate, and topography (White et al., 1990). According to the standard protocol described by O'Donnell et al. (1999), the genomic DNA was extracted from single-spore cultures. Fungi were analyzed by RLFP of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. DNA fragments were amplified with the primer pair ITS1F (Gardes and Bruns 1993)/ITS4 (White et al., 1990a) and the resulting amplicons were treated with the endonucleases *Cfo*I

and *Hae*III as above reported. The isolates were finally processed by sequencing the 5.8S-ITS rRNA region and the sequences were identified by BLASTN search.

Culture-indipendent analysis of total bacterial community

DNA Extraction, MiSeq library preparation and Illumina sequencing

Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system (Illumina, USA) were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy). Briefly, for each sample, a 464-nucleotide sequence of the V3-V4 region (Baker et al., 2003; Claesson et al., 2010), of the 16S rRNA gene (*E. coli* positions 341 to 805) and ITS3/ITS4 specific for the ITS2 fungi region (Mbareche et al., 2021) were amplified for bacteria and yeasts, respectively. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of the smaller amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to the manufacturer's instructions; subsequently, DNA concentrations of the amplicons were determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios.

Illumina data analysis and sequences identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative bacterial sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh and Standley, 2013; Price et al., 2009). For bacteria, taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99 % Operational Taxonomic Units (OTUs) database which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. For fungi, sequences were classified to the species-level using a 97 or 99 % threshold dynamic classifier created using UNITE software version 8.0 (Kõljalg et al., 2013; UNITE Community, 2019). The data generated by MiSeq Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. No. PRJNA996329.

Statistical analysis

Data on plate count were statistically analyzed by one-way variance analysis (ANOVA) using the XLStat software version 2020.3.1 for Microsoft Excel (Addinsoft, New York, NY, USA) and growth medium was the variable included in the model. The Tukey's test

for $p < 0.05$ was applied to determine the difference between means. Regarding Miseq Illumina data: Alpha-diversity was performed with observed OTUSs number, Evenness and Shannon diversity index; Beta-diversity was calculated using weighted Unifrac distance matrix and Jaccard distance matrix for bacteria and fungi respectively. Both alpha-diversity and beta-diversity were constructed for all samples using the plug-in in QIIME2 package. The similarity matrices were visualized using TREX webserver for visualization of phylogenetic Tree (http://www.trex.uqam.ca/index.php?action=trex, Boc et al., 2012).

Results

Microbiological analysis by culture-dependent approach

The levels of the microbial groups constituting the biofilms of the wooden shelves and cheese rinds sampled in this study are shown in Table 2. Wooden shelves hosted TMM in the range 5.6 – 7.2 log CFU/cm^2 with the lowest values showed by sample WS-D and the highest by WS-A. Except samples WS-E showing TPM (6.7 log CFU/cm^2) at higher levels than TMM, in general psychrotrophic microorganisms were counted at lower numbers than mesophilic ones. The presence of LAB was revealed in all samples wooden shelf biofilms. However, the four groups investigated (mesophilic and thermophilic rods and cocci) were not always detected altogether. Although LAB cocci were detected at consistent levels (3.9 – 6.4 and 2.8 – 5.3 log CFU/cm^2 for mesophilic and thermophilic, respectively) in all wooden shelves, only sample WS-C showed detectable levels of mesophilic rods (3.45 log CFU/cm^2).

Table 2. Microbial loads in wooden shelves and cheese rinds.

Sample	Bacterial count														
	LSAB	KAA	HEA	BP	VRBA	VRBGA	PCA 30°C	PCA 7°C	MRS 30°C	MRS 44°C	M17 30°C	M17 44°C	PAB	YPD	PDA
WS-A	<1	4.3 ± 0.5 ab	<1	<1	0	0	7.2 ± 0.1 a	6.1 ± 0.1 b	0 b	0 d	5.7 ± 0.2 b	3.6 ± 0.1 c	<1 c	5.8 ± 0.2 ab	5.2 ± 0.1 a
WS-B	<1	4.7 ± 0.2 a	<1	<1	0	0	7.2 ± 0.1 a	6.0 ± 0.2 b	0 b	0 d	5.6 ± 0.2 b	3.8 ± 0.2 c	<1 c	5.6 ± 0.2 b	5.4 ± 0.1 a
WS-C	<1	3.2 ± 0.4 bc	<1	<1	0	0	6.4 ± 0.1 b	4.1 ± 0.1 d	3.45 ± 0.2 a	2.6 ± 0.1 b	4.6 ± 0.2 c	4.3 ± 0.2 b	<1 c	4.2 ± 0.1 c	4.1 ± 0.2 b
WS-D	<1	3.1 ± 0.4 c	<1	<1	0	0	5.6 ± 0.2 c	3.2 ± 0.1 e	0 b	3.4 ± 0.2 a	3.9 ± 0.1 d	5.3 ± 0.2 a	2.9 ± 0.2 a	4.1 ± 0.1 c	4.5 ± 0.1 b
WS-E	<1	4.8 ± 0.6 a	<1	<1	0	0	6.4 ± 0.1 b	6.7 ± 0.1 a	0 b	3.4 ± 0.2 a	6.4 ± 0.1 a	4.3 ± 0.2 b	2.0 ± 0.1 b	6.1 ± 0.3 a	5.0 ± 0.1 a
WS-F	<1	3.8 ± 0.2 abc	<1	<1	0	0	6.7 ± 0.3 b	5.6 ± 0.2 c	0 b	2.1 ± 0.2 c	5.5 ± 0.1 b	2.8 ± 0.1 d	2.7 ± 0.2 a	6.2 ± 0.1 a	5.1 ± 0.2 a
p-value	n.e.	<0.001	n.e.	n.e.	n.e.	n.e.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CR-A	<1	2.0 ± 0.3 c	<1	<1	0	0	7.8 ± 0.1 a	6.2 ± 0.1 ab	5.1 ± 0.1 a	2.2 ± 0.1 d	7.2 ± 0.1 a	5.3 ± 0.1 b	3.7 ± 0.1 a	6.7 ± 0.2 a	2.5 ± 0.1 d
CR-B	<1	3.5 ± 0.1 a	<1	<1	0	0	7.2 ± 0.3 b	6.5 ± 0.1 a	0 b	3.2 ± 0.1 b	7.0 ± 0.1 ab	5.7 ± 0.1 a	3.5 ± 0.1 a	6.5 ± 0.1 a	4.2 ± 0.1 c
CR-C	<1	<1 d	<1	<1	0	0	6.8 ± 0.1 bc	5.9 ± 0.1 b	0 b	0 e	0 d	0 f	3.5 ± 0.1 a	5.1 ± 0.2 c	7.2 ± 0.1 a
CR-D	<1	2.4 ± 0.2 bc	<1	<1	0	0	6.1 ± 0.1 d	5.9 ± 0.2 b	0 b	2.8 ± 0.1 c	6.3 ± 0.2 c	2.9 ± 0.1 e	3.4 ± 0.2 a	6.4 ± 0.1 ab	4.7 ± 0.1 b
CR-E	<1	3.6 ± 0.3 a	<1	<1	0	0	6.7 ± 0.1 c	6.2 ± 0.1 ab	0 b	4.1 ± 0.1 a	6.8 ± 0.1 b	4.9 ± 0.1 c	3.4 ± 0.2 a	6.0 ± 0.1 b	4.3 ± 0.2 c
CR-F	<1	2.8 ± 0.1 b	<1	<1	0	0	7.2 ± 0.1 b	5.3 ± 0.1 c	0 b	3.3 ± 0.2 b	6.1 ± 0.1 c	4.3 ± 0.1 d	3.6 ± 0.1 a	5.3 ± 0.1 c	4.5 ± 0.1 bc
p-value	n.e.	<0.0001	n.e.	n.e.	n.e.	n.e.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.137	<0.0001	<0.0001

Results indicate mean values ± S.D. of four plate counts (carried out in duplicates for two independent samplings). Units are log CFU/cm^2 for wooden shelves and cheese rinds. Data within a column followed by different letters are significantly different according to Tukey's test ($p < 0.05$).
Abbreviations: LSAB, *Listeria* selective agar base for *L. monocytogenes*; KAA, kanamycin aesculinazide agar for enterococci; HEA, hektoen enteric agar for *E. coli* (red colonies) and *Salmonella* spp. (black colonies); BP, baird-parker agar for CPS, coagulase-positive staphylococci; VRBA, violet red bile agar for coliforms; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; PCA-SkM 30 °C, plate count agar added with skimmed milk incubated at 30 °C for total mesophilic microorganisms; PCA-SkM 7 °C, plate count agar added with skimmed milk incubated at 7 °C for total psychrotrophic microorganisms; MRS 30°C, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; MRS 44 °C, de Man-Rogosa-Sharpe agar medium for thermophilic rod LAB; M17 30 °C, medium 17 agar incubated at 30 °C for mesophilic coccus LAB; M17 44 °C, medium 17 agar incubated at 44 °C for thermophilic coccus LAB; PAB, *Pseudomonas* agar base for pseudomonads; YPD, yeast peptone dextrose agar for yeasts; PDA, potato dextrose agar for molds; WS, wooden shelves; CR, cheese rind; A–F, dairy factory; dairy factory A to B produce PGI Canestrato di Moliterno, and dairy factory C to F produce PDO Pecorino di Filiano.

Except WS-A and WS-B that did not host thermophilic rod LAB, the other wooden shelves showed different cell densities (2.6 – 3.4 log CFU/cm^2) within this group. Enterococci were detected in all wooden shelf associated biofilms and their levels were 0.8 – 1.7 log cycles lower than those registered on M17 incubated at 30°C. Filamentous and unicellular fungi were also investigated. Yeasts were consistently found on the examined wooden shelves. In particular, yeast loads of the wooden shelves WS-C and WS-D (4.1 and 4.2 log CFU/cm^2, respectively) were significantly lower than those developed on the other wooden shelf surfaces (on average, 5.9 log CFU/cm^2). The wooden shelves used for ripening PGI Canestrato di Moliterno cheeses (WS-A and WS-B) were characterized by the highest levels of molds (5.2 – 5.4 log CFU/cm^2), significantly different from the number of colonies (4.1 – 5.1 log CFU/cm^2) displayed by the wooden shelves used for ripening PDO Pecorino di Filiano cheeses (WS-C – WS-F). Members of Enterobacteriaceae and coliforms were not found, *E. coli*, *L. monocytogenes*, *Salmonella* spp. and coagulase-positive staphylococci were below their detection limit (1 log CFU/cm^2) in all wooden shelf biofilms collected, while pseudomonads were found only in biofilms collected from samples WS-D – WS-F (2.0 – 2.9 log CFU/cm^2).

TMM of the cheese rind biofilms ranged between 6.1 and 7.8 log CFU/cm^2 and lower values were registered for TPM. The rind of the PDO Pecorino di Filiano cheeses showed the lowest loads of pseudomonads (5.4 – 5.6 log CFU/cm^2), whereas PGI Canestrato di Moliterno cheese rinds showed the highest loads of TMM (7.5 log CFU/cm^2, on average) and yeasts (6.6 log CFU/cm^2, on average). The cheese rinds of PGI Canestrato di Moliterno cheeses showed also the highest loads for mesophilic and thermophilic LAB cocci. Only sample CR-A hosted detectable levels of mesophilic LAB rods and the levels

registered were quite consistent (5.1 log CFU/cm^2), while CR-C sample was characterized by undetectable levels of all LAB groups investigated; in sample CR-C not even enterococci were detected, even though they ranged between 2.0 and 3.6 log CFU/cm^2 in the other cheese rind samples. However, cheese rind from Factory C was characterized by very high levels of molds (7.2 log CFU/cm^2). Like wooden shelves, none of the cheese rind samples investigated showed detectable levels of members of Enterobacteriaceae, coliforms, *Salmonella* spp., *L. monocytog*enes, *E. coli* and coagulase-positive staphylococci. The levels of pseudomonads (3.4 – 3.7 log CFU/ cm^2) were similar among cheese rinds.

LAB differentiation and identification

Four hundred and eighty colonies of presumptive LAB were isolated from the biofilms of wooden shelves and cheese rinds. Only 442 isolates were still considered putative LAB as being Gram-positive and catalase-negative. After cell morphology determination by microscopic inspection, the vast majority of these isolates (n = 383) were coccus shaped, while barely 59 isolates were rods. The microscopic inspection also allowed to determine cell arrangement. Morphological data were then combined with the behavior of the cultures in different conditions and four phenotypic groups were obtained (Table 3). LAB cocci constituted three groups (I, II, and III); all of them were arranged in short-chain. Among these, only members of Group III showed an obligate heterofermentative metabolism, as being able to produce CO_2 from glucose. LAB rods were all members of Group IV characterized by a heterofermentative behavior.

Table 3. Phenotypic grouping of LAB isolated from wooden shelves and cheese rinds.

Characters	Clusters				
	I (n=329)	II (n=28)	III (n=26)	IV (n=59)	V (n=9)
Morphology	C	C	C	R	
Cell disposition	sc	sc	sc	sc	
Growth:					
15 °C	-	-	+	+	
45 °C	+	+	-	-	
pH 9.6	+	+	-	nd	
6.5% NaCl	+	+	+	nd	
Resistance to 60 °C	-	-	-	+	
Hydrolysis of:					
arginine	+	+	-	-	
aesculin	+	+	+	+	
Acid production from:					
arabinose	+	-	+	+	
ribose	+	+	+	+	
xylose	+	+	+	+	
fructose	+	+	+	+	
galactose	+	+	+	+	
lactose	+	+	+	+	
sucrose	+	-	+	+	
glycerol	+	+	+	+	
CO_2 from glucose	-	-	+	+	

[a]Abbreviations: C, coccus; R, rods; sc, short chain; n.d., not determined.

Approximately 25 % of the isolates within each phenotypic group was subjected to randomly amplified polymorphic DNA (RAPD) analysis to perform a LAB typing. RAPD patterns were used to construct two different dendrograms, one for the isolates of wooden shelf origin (Fig.2) and another one for the isolates collected from cheese rinds. This approach identified 29 distinct RAPD profiles, 20 for cheese rind LAB and nine for wooden shelf LAB. All 29 LAB characterized by diverse RAPD patterns were considered different strains and were then processed by 16S rRNA gene sequencing for species allotting. These 29 strains were confirmed to be representative of the LAB group (Ac. No OR337840-OR337840) and belonged to three genera: *Enterococcus, Leuconostoc* and *Marinilactibacillus.* LAB identified from the biofilms of wooden shelves were exclusively

Enterococcus faecium (Group I). This species was predominant also in cheese rinds. However, cheese rinds hosted other enterococci (Group II), *Leuconostoc mesenteroides* (Group III) and *Marinilactibacillus psychrotolerans* (Group IV).

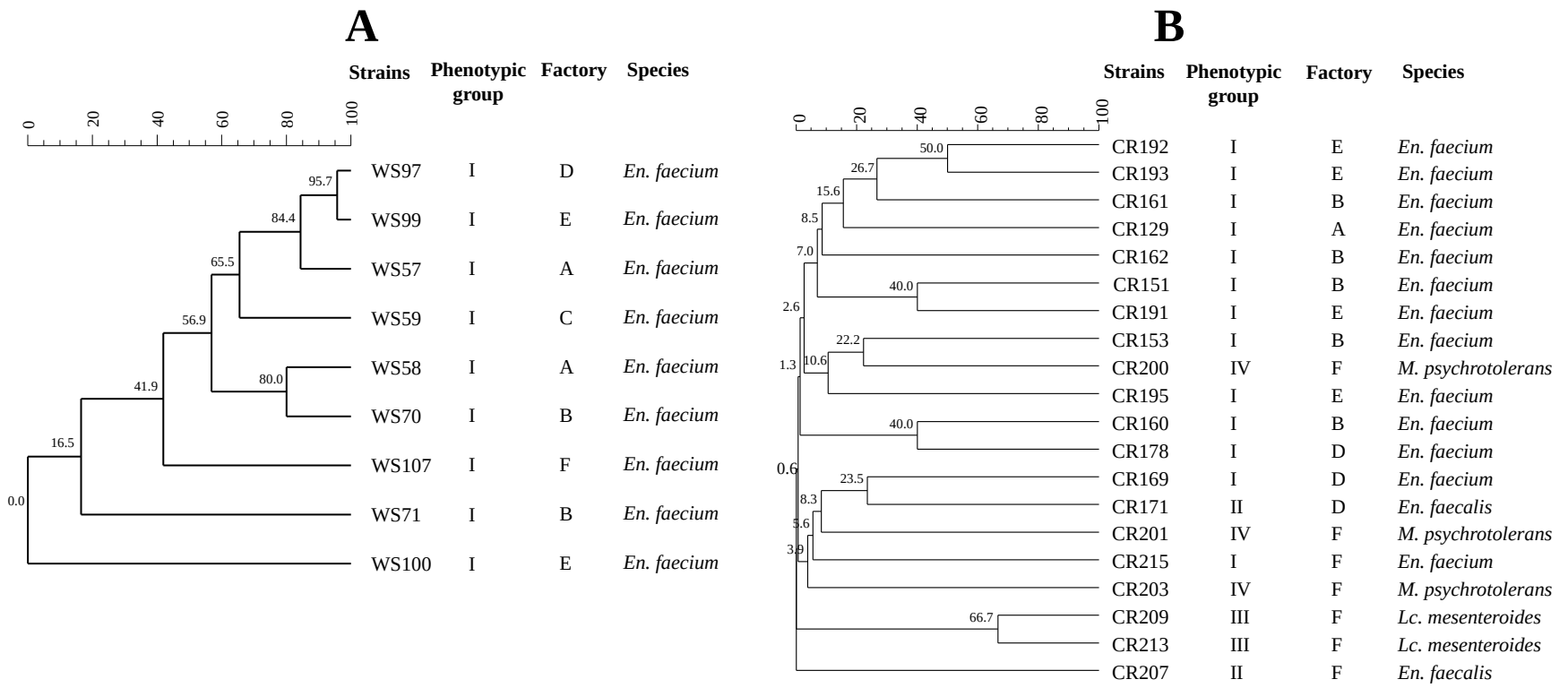

Fig.2. Dendrogram obtained from combined RAPD-PCR patterns of LAB strains from wooden shelves (A) and cheese rind (B) from two different cheeses (PGI Canestrato di Moliterno; PDO Pecorino di Filiano) generated with the primers M13, AB106 and AB111. Abbreviations: *En.*, *Enterococcus*; *M.*, *Marinilactibacillus*; *Lc.*, *Leuconostoc*.

2429

Identification and distribution of fungi

A total of 120 yeast colonies were isolated from the wooden shelves and the cheese rinds analyzed. Microscopic analysis did not succeed in differentiating yeasts. Thus, all isolates were subjected to molecular identification. The results of RFLP of the 5.8S-ITS region recognized two yeast groups (Table 4): *Candida parapsilosis* (Group I) and *Debaryomyces hansenii* (Group II). The genotypic identification of yeasts was completed by pairwise alignment of D1/D2 region of the 26S rRNA gene that confirmed species allotting.

Table 4. Molecular identification of yeast species.

Species	Restriction profile	5.8S-ITS PCR	Size of restriction fragment			No. of isolates[a]	Accession number[d]
			*Cfo*I	*Hae*III	*Hinf*I		
Candida parapsilosis	I	540	300+235	400+110	290+255	13 (2)	OR337917-OR337918
Debaryomyces hansenii	II	507-554	300+300+300	420+150+90	320+320	107 (16)	OR337919-OR337934

[a] number of isolates per each yeast species.
[b] accession number of D1/D2 region of the 26S rRna gene of isolates deposited into Genbank database

The same wooden shelves and cheese rinds allowed to isolate 170 filamentous fungi. The molds were divided into 10 groups after microscopic inspection (Table 5). The results of the RLFP for the 5.8S-ITS region using the endonucleases *Cfo*I and *Hae*III confirmed microscopic grouping. All isolates were also processed by sequencing of the 5.8S-ITS rRNA gene that unequivocally identified the following species: *Aspergillus* spp., *Aspergillus creber*, *Aspergillus versicolor*, *Penicillium* spp., *Penicillium chrysogenum*, *Penicillium commune*, *Penicillium crustosum*, *Penicillium solitum*, *Penicillium verrucosum*, and *Talaromyces rugulosus*. The species most frequently isolated were *Penicillium chrysogenum*, *Penicillium commune*, *Aspergillus creber*, and *Aspergillus versicolor*. In terms of mold diversity, the most diverse samples were wooden shelf from Factory F and cheese rinds from Factories A and D.

Table 5. Molecular identification of filamentous fungi.

Specie (% identity[a])	Group	5.8S-ITS PCR	Size of restriction fragments		Accession number	Source of isolation	No. of isolates
			*Cfo*I	*Hae*III			
Aspergillus creber (99 %)	I	746 - 747	264 + 247 + 177 + 58	401 + 164 + 70	OR342688-OR342689	WS-D, WS-E	2
Aspergillus spp. (99 %)	II		247 + 177 + 90	358 + 97 + 70	OR342690	WS-E	1
Aspergillus versicolor (100 %)	III	520	247 + 177 + 90	349 + 97 + 70	OR342691-OR342692	WS-F, CR-B	2
Penicillium chrysogenum (99 %)	IV	470 - 557	170 + 92	255 + 97 + 68	OR342693-OR342700	WS-A, WS-F, CR-A, CR-C, CR-E	8
Penicillium commune (99 %)	V	532 - 554	180 + 89 + 84	257 + 95 + 69	OR342701-OR342702	WS-C, CR-D	2
Penicillium crustosum (99 %)	VI	616	179 + 149 + 109	255 + 155 + 69+ 64	OR342703	WS-B	1
Penicillium solitum (100 %)	VII	562	180 + 89 + 77	258 + 95 + 68	OR342704	WS-C	1
Penicillium spp. (100 %)	VIII		180 + 88 + 78	257 + 94 + 68	OR342705	WS-D	1
Penicillium verrucosum (99 %)	IX	556	179 + 112 + 88	257 + 118 + 67	OR342706	CR-D	1
Talaromyces rugulosus (99 %)	X	506	166+ 144 + 111 + 60	399+ 92	OR342707	WS-F	1

[a]based on an NCBI database BlastN search of the 5.8S-ITS1 rRNA gene sequences

Comparison of polymorphic profiles of bacteria and fungi isolated from wooden shelves and cheese rinds

The possible transfer of microorganisms from the wooden shelves to cheese rinds and *vice versa* was estimated through comparison of the RAPD profiles of the strains identified from both sources of isolation. Regarding LAB, the nine polymorphic profiles characterizing the nine strains from wooden shelves were compared to the 20 profiles of cheese rind strains and only two strains shared the same RAPD patterns (WS58 and WS97 from the wooden shelves with CR129 and CR169 of cheese rind origin, respectively).

RAPD-PCR analysis was also applied on yeast strains (Fig. 3) and the comparison of the resulting patterns confirmed that several *D. hansenii* strains from wooden shelves and cheese rinds shared the same polymorphic profile.

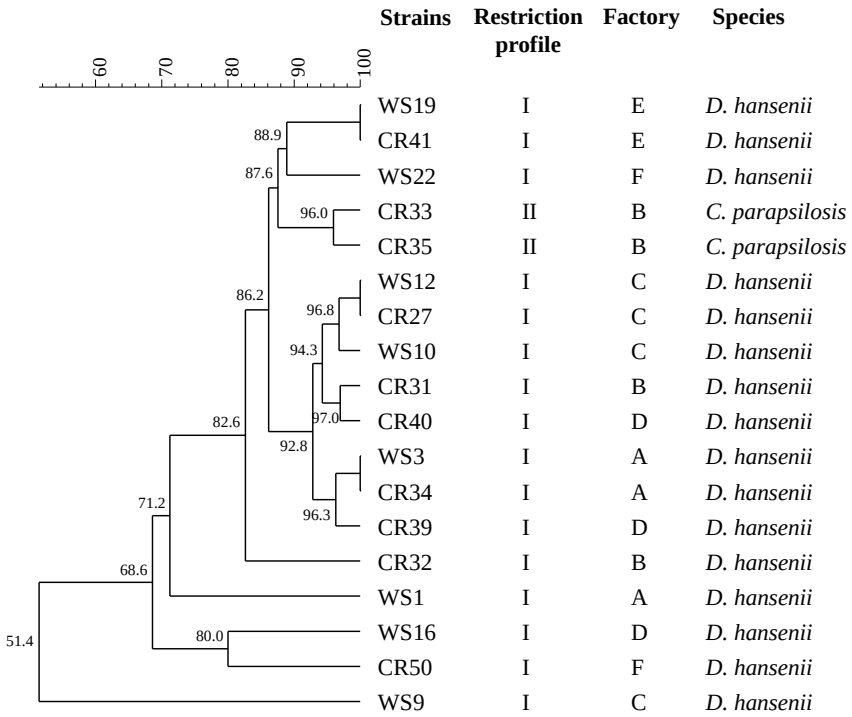

Fig. 3. Dendrogram obtained from combined RAPD-PCR profiles of yeast strains from wooden shelves (A) and cheese rind (B) from two different cheeses (PGI Canestrato di Moliterno; PDO Pecorino di Filiano) generated with the primers M13. Abbreviations: C., *Candida*; D., *Debaryomyces*.

As an example, the wooden shelf strain WS3 from factory A shares the same RAPD profile with the strain CR34 detected on the cheese rind analyzed in this factory.
In case of molds, RAPD analysis did not succeed in finding homology between the strains isolated from wooden shelves and cheese rinds (data not shown).

Characterization of cheese microbiota by Illumina analysis

DNA-based Illumina technology was successfully applied to deeply investigate on the composition of total bacterial and fungal communities of the samples object of this study. MiSeq Illumina analysis identified 15 phyla, 13 classes, 28 orders, 54 families, and 56 genera within bacteria. The sequences were grouped into operational taxonomic units (OTUs). The relative abundances (RA) % of the OTUs identified from both wooden shelves and cheese rinds are reported in Fig. 4. As suggested by Logares et al. (2014) the abundant communities considered were those with an individual RA $\geq$ 0.1 %. All wooden shelves were characterized by the presence of staphylococci. RA of bacterial group ranged from 3.01 to 74.21 %. In particular, the wooden shelves WS-A – WS-D showed an absolute dominance of staphylococci, with the species *Staphylococcus equorum* being mostly represented among this group (27.71 – 46.88 %). These four samples displayed also a massive presence of unspeciated staphylococci (14.70 – 42.86 %) and a minor presence of *Staphylococcus sciuri*. The wooden shelves analyzed hosted also consistent RA % of *Brevibacterium*, until 25.04 % in WS-B, and *Corynebacterium*, which was absent in WS-E biofilm, but accounted for 70.99 % in WS-F. Except for 1.28 % in WS-C sample, Micrococcaceae were present in wooden shelf biofilms at a RA <1 %.

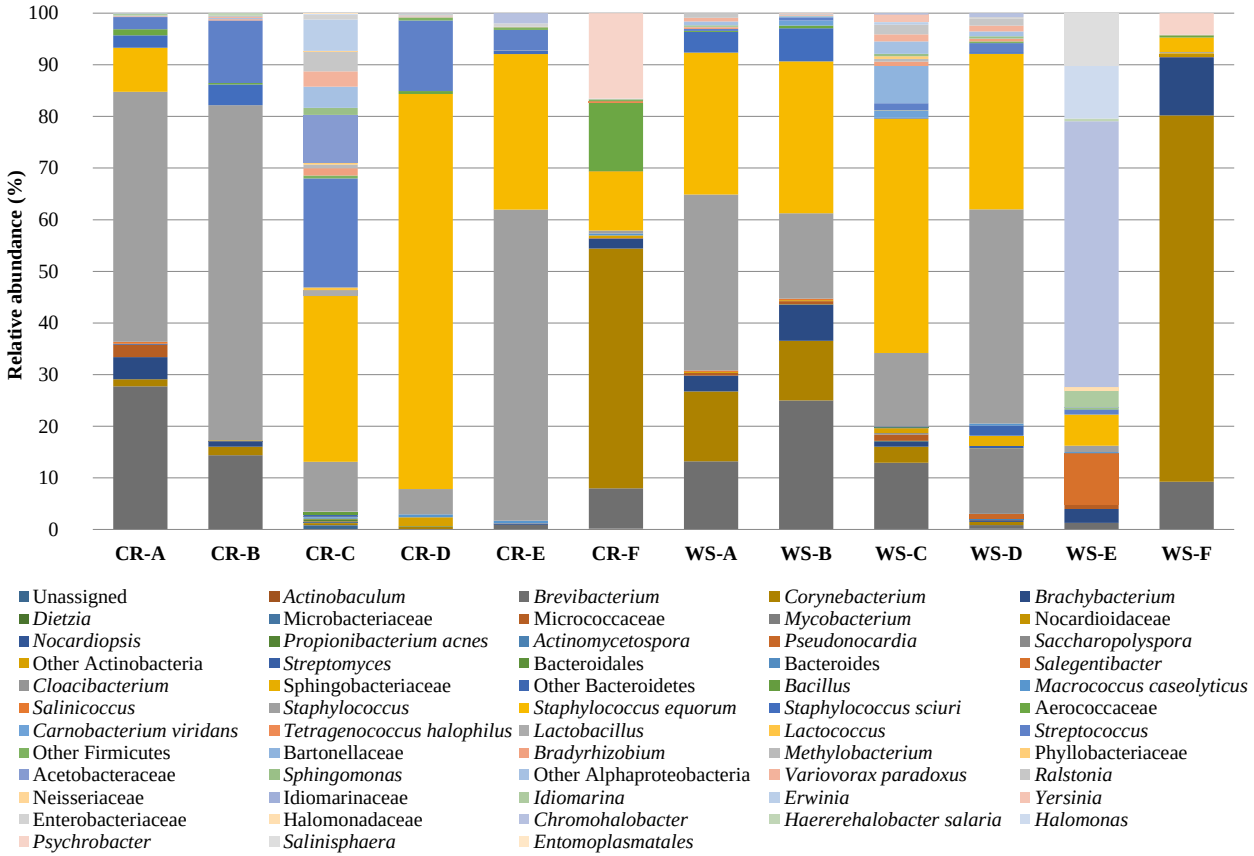

Fig. 4. Relative abundances (%) of bacterial genera identified by MiSeq Illumina in wooden shelves and cheese rind from two different cheeses. Abbreviations: WS, wooden shelves; RC, rind cheese; A – B, factories A to B produced PGI Canestrato di Moliterno; C – F, factories C to F produced PDO Pecorino di Filiano.

Among Actinobacteria, *Brachybacterium* was identified in all wooden shelves, even though the RA was barely 0.34 % in WS-D. LAB populations were poorly present on the wooden shelves and were only represented by *Streptococcus* genus (0.41 – 1.79 %) in five out of six biofilms analyzed. Halophilic and/or halotolerant bacteria were found at consistent levels only in WS-E sample. In this biofilm *Chromohalobacter* accounted for a very high RA (51.55 %), but *Halomonas* and *Salinisphaera* were detected at relevant levels (10.23 and 10.19 %, respectively). The bacterial community of the cheese rinds object of investigation showed a high biodiversity, but also these samples analyzed were greatly dominated by staphylococci (12.18 – 91.43 %). In particular, *S. equorum* represented 78.00 % of total biodiversity of cheese rind from Factory D. Samples CR-A, CR-B and CR-E displayed unspeciated *Staphylococcus* at 48.43, 64.93 and 60.53 %, respectively. *Brevibacterium* was not detected in CR-C and CR-D, but ranged from 0.91 until 27.74 % in the other samples. *Corynebacterium*, absent in CR-C, was detected at low levels in CR-A – CR-D samples (0.38 – 1.65 %) and accounted for 46.78 % of total biodiversity in CR-F. Micrococcaceae and *Brachybacterium* were basically present in CR-A and at a low RA % in a few other samples. Except *Lactobacillus* and *Lactococcus* (1.13 and 0.57 %, respectively) in CR-C, LAB community of cheese rinds of the factories A – E was mainly represented by *Streptococcus* genus (2.35 – 21.57). Regarding halophilic/halotolerant bacteria, apart 1.99 % for *Chromohalobacter* in CR-E and 16.68 % for *Psychrobacter* in CR-F, the presence of other genera within this group were not worth of noting on cheese rinds.

Regarding fungal microbiota, Fig. 5 shows RA % and distribution of the groups identified within wooden shelves and cheese rinds of the six factories object of study.

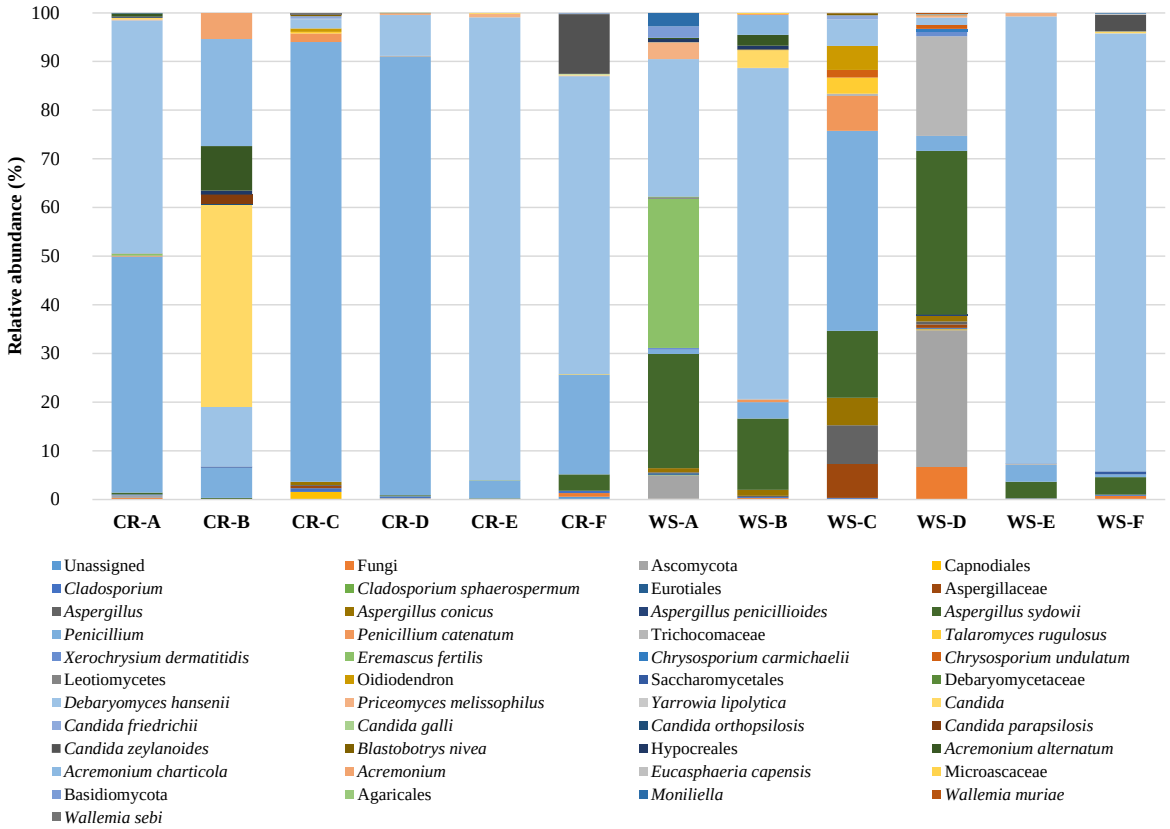

Fig. 5. Relative abundances (%) of fungi genera identified by MiSEq Illumina in wooden shelves and cheese rind from two different cheeses. Abbreviations: WS, wooden shelves; RC, rind cheese; A – B, factories A to B produced PGI Canestrato di Moliterno; C – F, factories C to F produced PDO Pecorino di Filiano.

2510

Illumina technology identified 3 phyla, 12 classes, 18 orders, 32 families, and 35 genera within fungi. All wooden shelves mainly hosted *Aspergillus*, *Penicillium* and *D. hansenii*. Among aspergilli, the species detected at the highest RA % (3.60 – 33.54 %) was *Aspergillus sydowii*. The genus *Penicillium* covered 41.07 % of total fungal OTUs in sample WS-C. *Debaryomyces hansenii* found on the wooden shelves used to ripen PDO Pecorino di Filiano of the factories E and F accounted for very high RA % (91.91 and 89.94 %, respectively). *Eremascus fertilis* was detected only in WS-A, but its presence was particularly consistent (30.61 %). WS-A samples also showed the presence of *Priceomyces melissophilus*, *Basidiomycota* and *Moniliella*. *Candida* and *Acremonium*, in particular *Acremonium charticola* and *Acremonium alternatum* characterized sample WS-B. *Candida zeylanoides* (3.41 %) was only detected on WS-F surface. WS-A and WS-D were the only two wooden shelves to host Ascomycota (4.83 – 28.01, respectively).

Cheese rind fungal community diversity was almost similar to that registered on the wooden shelves, even though the main group identified were *Penicillium* and *D. hansenii*. *Penicillium* ranged from 3.62 % in sample CR-E to 90.34 % in sample CR-C; on the contrary, *D. hansenii* from 1.89 % in sample CR-C to 95.07 in sample CR-E. *Candida* at a consistent RA (41.47 %), *Acremonium* (36.47 %), particularly with the species *A. charticola* (22.01 %) and *C. parapsilosis*, characterized cheese rind of Factory B. Capnodiales (1.55 %), *Cladosporium* (0.78 %), Aspergillaceae, *Penicillium catenatum* (1.76 %), *Candida friedrichii* (0.54 %) and *Blastobotrys nivea* (0.36 %) were also detected in sample CR-C. Low RA % of *Cladopsorium* were also found in CR-A, CR-D and CR-F. Regarding *A. sydowii*, found at consistent RA % onto the wooden shelves, it was not detected in sample CR-C and registered at low levels in the other cheese rind samples,

2534 except CR-F characterized by 3.34 % for this species. Alpha diversity analysis resulting
2535 from OTUs data of bacteria and fungi are graphically presented in Fig. 6.

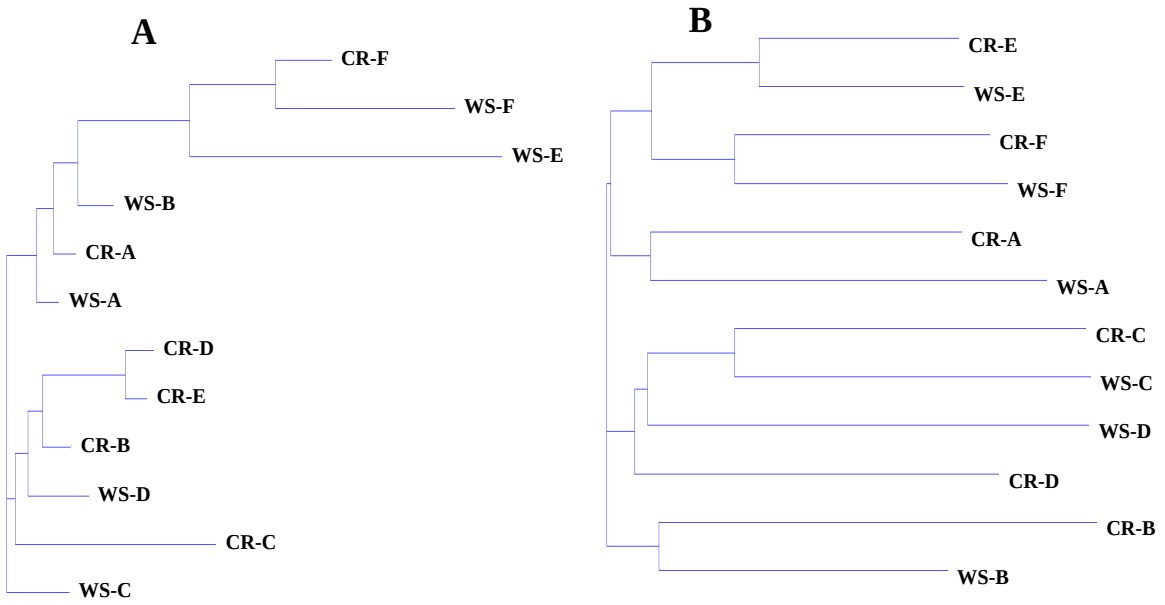

Fig. 6. Alpha diversity distribution: A, bacteria; B, fungi. Abbreviations: WS, wooden shelves; RC, rind cheese; A – B, factories A to B produced PGI Canestrato di Moliterno; C – F, factories C to F produced PDO Pecorino di Filiano.

2536 The weighted unifrac dist matrix for bacteria (data not shown) generated a tree (Fig.
2537 6A) that showed a few correlations among cheese crusts and wooden shelves. In particular,
2538 two mega-clusters were obtained: the upper cluster included both cheese rinds and wooden
2539 shelves of Factories A and F, while the downer cluster, showed the presence of cheese
2540 rinds CR-D and CR-C and the corresponding wooden shelf samples WS-D and WS-C.
2541 Regarding fungi, the Fig. 6B displays a higher level of similarity calculated by Jaccard's
2542 distance matrix (data not shown). As a matter of fact, fungal diversity of cheese rinds
2543 mirrored strongly that of the wooden shelves for all factories except Factory D. For the
2544 Factories A, B, C, E and F, each sub-cluster included the wooden shelf and the
2545 corresponding cheese rind.

Discussions

The present study was mainly aimed to characterize the microbial diversity of the wooden shelves of PDO Pecorino di Filiano and PGI Canestrato di Moliterno cheeses. Unlike previous works focusing exclusively of the bacterial communities, this work also took into account yeasts and filamentous fungi. Fungi are key elements of cheese ripening environment; indeed, they play important roles in shaping the communities of microbes in aging facilities. In view of a better understanding of the influence of these ripening tools on the microbiota of cheese rinds during ripening, the crusts of the cheeses were also object of the microbiological investigation performed in this study. A culture-dependent approach was applied to estimate the levels of viable microorganisms present on wooden shelves and cheese rinds.

The levels of TMM characterizing the six wooden shelves under investigation are comparable to those found for the aging of French Reblochon cheese (Mariani et al., 2007). In particular, TMM data registered for three of the four wooden shelves used for ripening Pecorino di Filiano cheeses are highly similar to those reported by Guzzon et al. (2017) who explored the microbiota of the red-brown defect in smear-ripened cheeses, starting from the wooden shelves. In the present study, LAB levels of the wooden shelves are comparable to those reported by Settanni et al. (2021), who found thermophilic lactococci in the range of 1.3 to 6.0 log CFU/cm^2 for the wooden shelves used for aging some typical Sicilian cheeses. However, mesophilic lactobacilli were found at lower levels than those found by Galinari et al. (2014) with regards to the wooden shelves used for the ripening of Brazilian cheeses. The levels of *Pseudomonas* estimated for the six wooden shelves of the present study are similar to those reported by Mariani et al. (2007).

The levels of TMM of the cheese rinds were very similar to those registered for Herve cheese, a soft cheese with a washed rind produced in Belgium (Delcenserie et al., 2014). Almost all cheese rinds showed high levels of LAB, specifically mesophilic cocci. These levels were superimposable to those of TMM, indicating that this group dominated the bacterial community of the cheese rinds. The dominance of cocci over rods was previously reported in Danish cheese surfaces (Gori et al., 2013). Molds and yeasts developed on all cheese rinds. PDO Pecorino di Filiano is known to host yeasts, in particular *D. hansenii*, on its crust (Capece and Romano, 2009). Although yeasts are in some cases responsible for cheese spoilage (Fleet, 1990), on the other hand, they can contribute to the centripetal ripening of cheeses by degrading lactate and causing rind deacidification. According to Parente et al. (2022), the increase in pH in the crust during ripening could facilitate growth and survival of pathogenic microorganisms such as *Salmonella* spp., *L. monocytogenes*, *E. coli*, and CPS. However, these microorganisms, generally associated with the criteria of poor hygiene and safety of dairy production, were never detected on the rinds of PDO Pecorino di Filiano and PGI Canestrato di Moliterno cheeses. Plate count method gave a general overview of the viable microbial groups present on both wooden shelves and cheese rinds of these two typical cheeses.

To provide a comprehensive overview on the populations inhabiting wooden shelves and cheese rinds and to retrieve also the level of interaction among them, LAB, yeasts, and molds were isolated, differentiated and identified. With regards to the LAB community, three out of the four viable groups detected were cocci and a total of 29 strains, belonging to three genera (*Enterococcus*, *Leuconostoc* and *Marinilactibacillus*), were identified. The great majority of the LAB species detected on wooden shelves and cheese rinds are

typically associated with dairy environments, such as raw milk (Franciosi et al., 2011), cheeses (Di Grigoli et al., 2015; Gaglio et al., 2014), wooden vats (Scatassa et al., 2015) and animal rennets (Cruciata et al., 2014), but the LAB communities associated with the samples analyzed from the two Lucanian cheese productions were dominated by enterococci. Regarding wooden shelves, these results are not surprising and confirmed what reported for traditional cheeses produced in Sicily (Settanni et al., 2021). The comparison of the RAPD profiles of the enterococci from wooden shelves with those from cheese rinds indicated that the surfaces of the ripening shelves and cheese crusts might exchange these bacteria. This phenomenon can be highly positive because enterococci contribute to the development of the typical flavor during cheese ripening (Giraffa, 2003). Also *M. psychrotolerans*, a halophilic and alkalophilic LAB species of marine origin, is strongly associated with the dairy environment, but is specifically isolated from the rinds of soft and semi-hard cheeses and derives from the sea salt added to the brines used for salting the cheeses (Vermote et al., 2018). This marine species plays an important role in cheese ripening (Delcenserie et al.,2014; Ishikawa et al., 2007; Ishikawa et al., 2003; Suzuki et al., 2021) and has been detected also in French and German cheeses (Feurer et al., 2004, Maoz et al., 2003).

After applying RLFP of 5.8S-ITS rRNA region and its sequencing, yeast isolates were differentiated into two groups represented by *Debaryomyces* and *Candida*. The results unequivocally revealed that *D. hansenii* dominated in both PDO Pecorino di Filiano and PGI Canestrato di Moliterno cheese productions, because they were detected on the wooden shelves as well as on the cheese rind surfaces. Low pH, high salt content and low water activity characterize the environment of this yeast species and, for this reason, it has

2615 been isolated from artisanal cheeses (Cosentino et al., 2001; Fadda et al., 2004), including
2616 PDO Pecorino di Filiano (Capece and Romano, 2009). Although *C. parapsilosis* is widely
2617 considered as an opportunistic human pathogen capable of causing invasive candidiasis
2618 (Banjara et al., 2015, Lima et al., 2019; Trofa et al., 2008), its presence on cheese rinds has
2619 never been linked to human outbreaks. Furthermore, Geronikou et al. (2022) confirmed its
2620 positive impact on the organoleptic characteristics of cheeses. Concerning molds,
2621 *Penicillum* and *Aspergillus* were most frequently found in both samples object of study
2622 (wooden shelves and cheese rinds). Several species detected in this study, such as *P.*
2623 *crustosum*, *P. solitum*, and *P. verrucosum* were previously reported as environmental
2624 contaminants of cheese and dairy productions (Kure et al., 2004, Serra et al., 2003). In
2625 general, conidia of molds are airborne transmittable and they can easily dominate cheese
2626 ripening environments (De Santi et al., 2010; Decontardi et al., 2017).

2627 Next generation sequence analysis showed that all samples were characterized by the
2628 presence of *S. equorum*. Bacteria of this species are generally found in cheese rinds and
2629 this is due to their high salt tolerance (Jeong et al., 2014; Jeong et al., 2017). The presence
2630 of *S. equorum* onto the surface of wooden shelves used for cheese ripening is also very
2631 common (Settanni et al., 2021; Wadhawan et al., 2021) and plays an important role in the
2632 inhibition of *L. monocytogenes* (Wadhawan et al., 2023). The presence of
2633 *Corynebacterium* in the biofilms collected from wooden shelves and cheese rinds is not
2634 negative, since these bacteria are commonly found onto the surface of cheeses
2635 (Bockelmann et al., 2005; Fontana et al., 2010; Rea et al., 2007) and are considered
2636 important for the ripening of smear cheeses (Brennan et al., 2004; Guzzon et al., 2017;
2637 Mariani et al., 2007). The presence of *Corynebacterium*, *Brevibacterium*, and

Brachybacterium is not surprising on the samples analyzed in this study, since they are part of the bacterial community of smear cheese rind (Schornsteiner et al., 2014). The presence of halophilic genera such as *Chromohalobacter*, *Halomonas* and *Salinisphaera* on wooden shelves could be related to the sea salt used for preparing the brines (Vermote et al., 2018). LAB community of the cheese rinds was mainly dominated by *Streptococcus*, confirming previous results showed for cheese crusts (Sant'Anna et al., 2019). Even though at very low percentages, LAB presence onto the wooden shelves is imputable to cheese rinds. These low percentages were also found by Settanni et al., (2021) who studied 18 wooden shelves for the bacterial communities.

Illumina technology was also applied to study yeast and mold microbiota of wooden shelves and cheese rinds. This approach confirmed what found with the culture-dependent tools: *D. hanseni* dominated cheese rinds of both cheeses object of investigation, providing further insights to the results showed by Capece and Romano (2009) of PDO Pecorino di Filiano cheese; *Aspergillus* and *Penicillum*, generally characterizing cheese aging processes (Moubasher et al., 2018), were mainly associated to PDO Pecorino di Filiano and PGI Canestrato di Moliterno cheeses.

Conclusions

The combined (culture-dependent and -independent) approach applied in this study clearly showed that the bacterial and fungal groups identified from the wooden shelves used for ripening PDO Pecorino di Filiano and PGI Canestrato di Moliterno cheeses are those typically associated with smear cheese rinds. The microbiotas found onto the rinds of both cheeses were confirmed to be those characterizing smear cheeses. Even though PDO

Pecorino di Filiano and PGI Canestrato di Moliterno cheeses are not inoculated with smear microorganisms, the contact with the wooden shelves determines a surface ripening. Thus, the rind of the cheeses in contact with these shelves take advantages from the wooden biofilms and are actively involved in generating a protective barrier against the major dairy pathogens. Some bacteria, specifically enterococci, were mostly isolated from cheese rinds, but also found on the wooden shelves, suggesting a mutual microbial transfer between wooden shelves and cheese rinds. In light of the results of the present study, PDO Pecorino di Filiano and PGI Canestrato di Moliterno cheeses are no more considered cheeses with an exclusive internal bacterial ripening, but have to be inserted in the smear cheese typology since bacterial and yeast microbiota of the rinds clearly indicated the occurrence of a centripetal maturation.

References

Aviat, F., Gerhards, C., Rodriguez-Jerez, J.J., Michel, V., Bayon, I.L., Ismail, R., Federighi, M., 2016. Microbial safety of wood in contact with food: a review. Compr. Rev. Food Sci. Food Saf. 15 (3), 491–505. https://doi.org/10.1111/1541-4337.12199.

Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods. 55, 541–555. https://doi.org/10 .1016/j.mimet.2003.08.009.

Banjara, N., Suhr, M.J., Hallen-Adams, H.E., 2015. Diversity of yeast and mold species from a variety of cheese types. Curr. Microbiol. 70, 792–800. https://doi.org/10.1007/s00284-015-0790-1.

Barbaccia, P., Busetta, G., Matraxia, M., Sutera, A.M., Craparo, V., Moschetti, G., Francesca, N., Settanni, L., Gaglio, R., 2021. Monitoring Commercial Starter Culture Development in Presence of Red Grape Pomace Powder to Produce Polyphenol-Enriched Fresh Ovine Cheeses at Industrial Scale Level. Ferment. 7, 35. https://doi.org/10.3390/fermentation7010035.

Boc, A., Diallo, Alpha B., Makarenkov, V., 2012. T-REX: a web server for inferring, validating and visualizing phylogenetic trees and networks, Nucleic Acids Research, 40 (W1), W573–W579. https://doi.org/10.1093/nar/gks485.

Bockelmann, W., Willems, K.P., Neve, H., Heller, K.H., 2005. Cultures for the ripening of smear cheeses. Int. Dairy J. 15, 719–73. https://doi.org/10.1016/j.idairyj.2004.08.022.

Brennan, N.M., Cogan, T.M., Loessner, M., Scherer, S., 2004. Bacterial surface-ripened cheeses. In: Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P. (Eds.), Cheese: chemistry, physics and microbiology. Elsevier, New York, pp.199-225.

Busetta, G., Gaglio, R., Mangione, G., Garofalo, G., Franciosi, E., Gannuscio, R., Caccamo, M., Todaro, M., Di Gerlando, R., Settanni, L., Licitra, G., 2023a. Effect of commission implementing regulation (EU) 2020/1319 on the bacterial composition of PDO Provola dei Nebrodi cheese. Int. J. Food Microbiol. 394, 110188. https://doi.org/10.1016/j.ijfoodmicro.2023.110188.

Busetta, G., Garofalo, G., Barbera, M., Di Trana, A., Claps, S., Lovallo, C., Franciosi, E., Gaglio, R, Settanni, L., 2023b. Metagenomic, microbiological, chemical and sensory profiling of Caciocavallo Podolico Lucano cheese. Food Res. Int. 169, 112926. https://doi.org/10.1016/j.foodres.2023.112926.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Capece, A., Romano, P., 2009. "Pecorino di Filiano" cheese as a selective habitat for the yeast species, *Debaryomyces hansenii*. Int. J. Food Microbiol., 132 (2-3), 180–184. https://doi.org/10.1016/j.ijfoodmicro.2009.04.007.

Claesson, M.J, Wang, Q., O'Sullivan, O., Greene-Diniz, R., Cole, J.R., Ross, R.P., O'Toole, P.W., 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res. 38, e200. https://doi.org/10.1093/nar/gkq873.

Commission Regulation (EC) No 2074/2005 Laying down implementing measures for certain products under regulation (EC) No. 853/2004 of the European Parliament and of the Council and for the organisation of official controls under regulation (EC) No. 854/2004 of the European Parliament and of the Council and regulation (EC) No. 882/2004 of the European Parliament and of the Council, derogating from regulation (EC) No. 852/2004 of the European Parliament and of the Council and amending regulations (EC) No. 853/2004 and (EC) No. 854/2004. Off J Eur Union 338:27–59.

Cosentino, S., Fadda, M.E., Deplano, M., Mulargia, A.F., Palmas, F., 2001. Yeasts associated with Sardinian ewe's dairy products. Int. J. Food Microbiol. 69 (1-2), 53–58. https://doi.org/10.1016/S0168-1605(01)00572-4.

Cruciata, M., Gaglio, R., Scatassa, M.L., Sala, G., Cardamone, C., Palmeri, M., Moschetti, G., La Mantia, T., Settanni, L., 2018. Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production. Appl. Environ. Microbiol. 84, e02107-17. https://doi.org/10.1128/AEM.02107-17.

Cruciata, M., Sannino, C., Ercolini, D., Scatassa, M.L., De Filippis, F., Mancuso, I., La Storia, A., Moschetti, G., Settanni, L., 2014. Animal rennets as sources of dairy lactic acid bacteria. Appl. Environ. Microbiol. 80, 2050–2061. https://doi.org/10.1128/AEM.03837-13.

De Santi, M., Sisti, M., Barbieri, E., Piccoli, G., Brandi, G., Stocchi, V., 2010. A combined morphologic and molecular approach for characterizing fungal microflora from a traditional Italian cheese (Fossa cheese). Int. Dairy J. 20 (7), 465–471. https://doi.org/10.1016/j.idairyj.2010.02.004.

Decontardi, S., Mauro, A., Lima, N., Battilani, P., 2017. Survey of Penicillia associated with Italian grana cheese. Int. J. Food Microbiol. 246, 25–31. https://doi.org/10.1016/j.ijfoodmicro.2017.01.019.

Delcenserie, V., Taminiau, B., Delhalle, L., Nezer, C., Doyen, P., Crevecoeur, S., Roussey, D., Korsak, N., Daube, G., 2014. Microbiota characterization of a Belgian protected designation of origin cheese, Herve cheese, using metagenomic analysis. J. Dairy Sci. 97 (10), 6046–6056.

Di Grigoli, A., Francesca, N., Gaglio, R., Guarrasi, V., Moschetti, M., Scatassa, M.L., Settanni, L., Bonanno, A., 2015. The influence of the wooden equipment employed for cheese manufacture on the characteristics of a traditional stretched cheese during ripening. Food Microbiol. 46, 81–91. https://doi.org/10.1016/j.fm.2014.07.008.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8 S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Evol. 49 (1), 329–337. https://doi.org/10.1099/00207713-49-1-329.

Fadda, M.E., Mossa, V., Pisano, M.B., Deplano, M., Cosentino, S., 2004. Occurrence and characterization of yeasts isolated from artisanal Fiore Sardo cheese. Int. J. Food Microbiol. 95 (1), 51–59. https://doi.org/10.1016/j.ijfoodmicro.2004.02.001.

Feurer, C., Irlinger, F., Spinnler, H.E., Glaser, P., Vallaeys, T., 2004. Assessment of the rind microbial diversity in a farmhouse-produced vs a pasteurized industrially produced soft red-smear cheese using both cultivation and rDNA-based methods. J. Appl. Microbiol. 97 (3), 546–556. https://doi.org/10.1111/j.1365-2672.2004.02333.x.

Fleet, G.H., 1990. Yeasts in dairy products. J. appl. bacteriol. 68, 199–211.

Fontana, C., Cappa, F., Rebecchi, A., Cocconcelli, P.S., 2010. Surface microbiota analysis of taleggio, gorgonzola, casera, scimudin and formaggio di fossa Italian cheeses. Int. J. Food Microbiol. 138 (3), 205–211. https://doi.org/10.1016/j.ijfoodmicro.2010.01.017.

Fontefrancesco, M.F., Zocchi, D.M., Pieroni, A., 2023. The Intersections between Food and Cultural Landscape: Insights from Three Mountain Case Studies. Land, 12, 676. https://doi.org/10.3390/land12030676.

Franciosi, E., Settanni, L., Cologna, N., Cavazza, A., Poznanski, E., 2011. Microbial analysis of raw cows' milk used for cheese-making: Influence of storage treatments on microbial composition and other technological traits. World J. Microbiol. Biotechnol., 27, 171–180. https://doi.org/10.1007/s11274-010-0443-2.

Gaglio, R., Francesca, N., Di Gerlando, R., Cruciata, M., Guarcello, R., Portolano, B., Moschetti, G., Settanni, L., 2014. Identification, typing and investigation of the dairy characteristics of lactic acid bacteria isolated from "Vastedda della valle del Belìce" cheeses. Dairy Sci. Technol. 94, 157–180. https://doi.org/10.1007/s13594-013-0150-5.

Gaglio, R., Francesca, N., Di Gerlando, R., Mahony, J., De Martino, S., Stucchi, C., Moschetti, G., Settanni, L., 2017. Enteric bacteria of food ice and their survival in alcoholic beverages and soft drinks. Food Microbiol. 67, 17–22. https://doi.org/10.1016/j.fm.2017.04.020.

Galinari, É., Nóbrega, J.E.D., Andrade, N.J.D., Ferreira, C.L.D.L F., 2014. Microbiological aspects of the biofilm on wooden utensils used to make a Brazilian artisanal cheese. Braz. J. Microbiol. 45, 713–720. https://doi:10.1590/s1517-83822014000200047.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2 (2), 113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x.

Geronikou, A., Larsen, N., Lillevang, S.K., Jespersen, L., 2022. Occurrence and Identification of Yeasts in Production of White-Brined Cheese. Microorganisms 10 (6), 1079. https://doi.org/10.3390/microorganisms10061079.

Giraffa, G., 2003. Functionality of enterococci in dairy products. Int. J. Food Microbiol. 88 (2-3), 215–222. https://doi.org/10.1016/S0168-1605(03)00183-1.

Gobbetti, M., Neviani, E., Fox, P., 2018. The cheeses of Italy, Science and technology, first ed. Springer, Switzerland.

Gori, K., Ryssel, M., Arneborg, N., Jespersen, L., 2013. Isolation and identification of the microbiota of Danish farmhouse and industrially produced surface-ripened cheeses. Microb. Ecol. 65, 602–615. https://doi.org/10.1007/s00248-012-0138-3.

Gregersen, T., 1978. Rapid method for distinction of Gram-negative from Gram-positive bacteria. Eur. J. Appl. Microbiol. Biotechnol. 5, 123–127. https://doi.org/10.1007/BF00498806.

Guzzon, R., Carafa, I., Tuohy, K., Cervantes, G., Vernetti, L., Barmaz, A., Larcher, R., Franciosi, E., 2017. Exploring the microbiota of the red-brown defect in smear-ripened cheese by 454-pyrosequencing and its prevention using different cleaning systems. Food Microbiol. 62, 160–168. https://doi.org/10.1016/j.fm.2016.10.018.

Irlinger, F., Mounier, J., 2009. Microbial interactions in cheese: implications for cheese quality and safety. Curr. Opin. Biotechnol. 20 (2), 142–148. https://doi.org/10.1016/j.copbio.2009.02.016.

Ishikawa, M., Kodama, K., Yasuda, H., Okamoto-Kainuma, A., Koizumi, K., Yamasato, K., 2007. Presence of halophilic and alkaliphilic lactic acid bacteria in various cheeses. Lett. Appl. Microbiol. 44 (3), 308–313. https://doi.org/10.1111/j.1472-765X.2006.02073.x.

Ishikawa, M., Nakajima, K., Yanagi, M., Yamamoto, Y., Yamasato, K., 2003. *Marinilactibacillus psychrotolerans* gen. nov., sp. nov., a halophilic and alkaliphilic marine lactic acid bacterium isolated from marine organisms in temperate and subtropical areas of Japan. Int. J. Syst. Evol. Microbiol.53 (3), 711–720. https://doi.org/10.1099/ijs.0.02446-0.

Jeong, D.W., Han, S., Lee, J.H., 2014. Safety and technological characterization of *Staphylococcus equorum* isolates from jeotgal, a Korean high-salt-fermented seafood, for starter development. Int. J. Food Microbiol. 188, 108–115. https://doi.org/10.1016/j.ijfoodmicro.2014.07.022.

Jeong, D.W., Heo, S., Ryu, S., Blom, J., Lee, J.H., 2017. Genomic insights into the virulence and salt tolerance of *Staphylococcus equorum*. Sci. Rep. 7 (1), 1–11. https://doi.org/10.1038/s41598-017-05918-5.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010.

Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., Bates, S.T., Bruns, T.D., Bengtsson-Palme, J., Callaghan, T.M., Douglas, B., Drenkhan, T., Eberhardt, U., Dueñas, M.,

Grebenc, T., Griffith, G.W., Hartmann, M., Kirk, P.M., Kohout, P., Larsson, E., Lindahl, B.D., Lücking, R., Martín, M.P., Matheny, P.B., Nguyen, N.H., Niskanen, T., Oja, J., Peay, K.G., Peintner, U., Peterson, M., Põldmaa, K., Saag, L., Saar, I., Schüßler, A., Scott, J.A., Senés, C., Smith, M.E., Suija, A., Taylor, D.L., Telleria, M.T., Weiss, M. and Larsson, K.-H., 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol. Ecol. 22, 5271–5277. https://doi.org/10.1111/mec.12481.

Koneman, E.W., Allen, S.D., Janda, W.M., Schreckenberger, P.C., Winn, W.C., 1997. The nonfermentative gram-negative bacilli. In Koneman, E.W., Allen, S.D., Janda, W.M., Schreckkenberger, P.C., Winn, W.C. (Eds.), Color Atlas and Textbook of Diagnostic Microbiology, seventh ed. Lippincott, Philadelphia, pp. 253–320.

Kure, C.F., Wasteson, Y., Brendehaug, J., Skaar, I., 2001. Mould contaminants on Jarlsberg and Norvegia cheese blocks from four factories. Int. J. Food Microbiol. 70 (1-2), 21–27. https://doi.org/10.1016/S0168-1605(01)00520-7.

Licitra, G., Ogier, J.C., Parayre, S., Pediliggieri, C., Carnemolla, T.M., Falentin, H., Madec, M.N., Carpino, S., Lortal, S., 2007. Variability of the bacterial biofilms of the "tina" wood vat used in the Ragusano cheese making process. Appl. Environ. Microbiol. 73, 6980–6987. https://doi.org/10.1128/AEM.00835-07.

Lima, G.B.L., Rosa, C.A., Johann, S., Vieira, M.D.L.A., Gomes, F.D.C.O., 2019. Yeasts isolated from tropical fruit ice creams: diversity, antifungal susceptibility and adherence to buccal epithelial cells. Brazilian J. Food Technol. 22. https://doi:10.1590/1981-6723.19718.

Logares, R., Audic, S., Bass, D., Bittner, L., Boutte, C., Christen, R., Claverle, J.M., Decelle, J., Dolan, J.R., Dunthorn, M., Edvardsen, B., Gobet, A., Kooistra, W.H.C.F., Mahé, F., Not, F., Ogata, H., Pawlowsky, J., Pernice, M.C., Romac, S., Shalchian-Tabrizi, K., Simon, N., Stoeck, T., Santini, S., Siano, R., Wincker, P., Zingone, A., Richiards, T.A., de Vergas, C., Massana, R., 2014. Patterns of rare and abundant marine microbial eukaryotes. Curr. Biol. 24 (8), 813–821. https://doi.org/10.1016/j.cub.2014.02.050.

Lortal, S., Licitra, G., Valence, F., 2014. Wooden tools: reservoirs of microbial biodiversity in traditional cheesemaking. Microbiol. Spectr. 2 (1), 2–1. https://doi.org/10.1128/microbiolspec.cm-0008-2012.

Maoz, A., Mayr, R., Scherer, S., 2003. Temporal stability and biodiversity of two complex antilisterial cheese-ripening microbial consortia. Appl. Environ. Microbiol. 69 (7), 4012–4018. https://doi.org/10.1128/AEM.69.7.4012-4018.2003.

Mariani, C., Briandet, R., Chamba, J.F., Notz, E., Carnet-Pantiez, A., Eyoug, R.N., Oulahal, N., 2007. Biofilm ecology of wooden shelves used in ripening the French raw milk smear cheese Reblochon de Savoie. J. Dairy Sci. 90, 1653–1661. https://doi.org/10.3168/jds.2006-190.

Mariani, C., Oulahal, N., Chamba, J.F., Dubois-Brissonnet, F., Notz, E., Briandet, R., 2011. Inhibition of *Listeria monocytogenes* by resident biofilms present on wooden shelves used for cheese ripening. Food Control 22 (8), 1357–1362. https://doi.org/10.1016/j.foodcont.2011.02.012.

Mbareche, H., Veillette, M., Bilodeau, G.J., 2021. In Silico Study Suggesting the Bias of Primers Choice in the Molecular Identification of Fungal Aerosols. J. Fungi. 7 (2), 99. https://doi.org/10.3390/jof7020099.

Moubasher, A.A., Abdel-Sater, M.A., Soliman, Z.S.M., 2018. Yeasts and filamentous fungi associated with some dairy products in Egypt. J. Mycol. Med. 28 (1), 76–86. https://doi.org/10.1016/j.mycmed.2017.12.003.

O'Donnell, K., Gherbawy, Y., Schweigkofler, W., Adler, A., Prillinger, H., 1999. Phylogenetic analyses of DNA sequence and RAPD data compared in *Fusarium oxysporum* and related species from maize. J. Phytopathol. 147 (7-8), 445–452. https://doi.org/10.1111/j.1439-0434.1999.tb03849.x.

Parente, E., Zotta, T., Ricciardi, A., 2022. A review of methods for the inference and experimental confirmation of microbial association networks in cheese. Int. J. Food Microbiol. 109618. https://doi.org/10.1016/j.ijfoodmicro.2022.109618.

Price, M.N., Dehal, P.S., Arkin, A.P., 2009. FastTree: computing large minimum evolution,trees with profiles instead of a distance matrix. Mol. Biol. Evol. 26, 1641–1650. https://doi.org/10.1093/molbev/msp077.

Rea, M.C., Görges, S., Gelsomino, R., Brennan, N.M., Mounier, J., Vancanneyt, M., Scherer, S., Swings, J., Cogan, T.M., 2007. Stability of the biodiversity of the surface consortia of Gubbeen, a red-smear cheese. J. Dairy Sci. 90 (5), 2200–2210. https://doi.org/10.3168/jds.2006-377.

Richard, J., 1997. Utilisation du bois comme matériau au contact des produits laitiers. Comptes rendus de l'Académie d'agriculture de France, 83 (5), 27–34.

Sant'Anna, F.M., Wetzels, S.U., Cicco, S.H.S., Figueiredo, R.C., Sales, G.A., Figueiredo, N.C., Nunes, C.A., Schmitz-Esser, S., Mann, E., Wagner, M., Souza, M.R., 2019. Microbial shifts in Minas artisanal cheeses from the Serra do Salitre region of Minas Gerais, Brazil throughout ripening time. Food Microbiol. 82, 349–362. https://doi.org/10.1016/j.fm.2019.02.016.

Scatassa, M.L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., Di Grigoli, A., Moschetti, G., Settanni, L., 2015. Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol. 52, 31–41. https://doi.org/10.1016/j.fm.2015.06.008.

Schornsteiner, E., Mann, E., Bereuter, O., Wagner, M., Schmitz-Esser, S., 2014. Cultivation-independent analysis of microbial communities on Austrian raw milk hard cheese rinds. Int. J. Food Microbiol, 180, 88–97. https://doi.org/10.1016/j.ijfoodmicro.2014.04.010.

Serra, R., Abrunhosa, L., Kozakiewicz, Z., Venâncio, A., Lima, N., 2003. Use of ozone to reduce molds in a cheese ripening room. J. Food Prot. 66 (12), 2355–2358. https://doi.org/10.4315/0362-028X-66.12.2355.

Settanni, L., Busetta, G., Puccio, V., Licitra, G., Franciosi, E., Botta, L., Di Gerlando, R., Todaro, M., Gaglio, R., 2021. In-Depth Investigation of the Safety of Wooden Shelves Used for Traditional Cheese Ripening. Appl. Environ. Microbiol. 87, e01524-21. https://doi.org/10.1128/AEM.01524-2.

Settanni, L., Moschetti, G., 2014. New trends in technology and identity of traditional dairy and fermented meat production processes: preservation of typicality and hygiene. Trends Food Sci. Technol. 37 (1), 51–58. https://doi.org/10.1016/j.tifs.2014.02.006.

Sun, L., D'Amico, D.J., 2021. Composition, Succession, and Source Tracking of Microbial Communities throughout the Traditional Production of a Farmstead Cheese. Msystems 6 (5), e00830-21. https://doi.org/10.1128/mSystems.00830-21. https://doi.org/10.1128/msystems.00830-21.

Sun, L., D'Amico, D.J., 2023. Characterization of microbial community assembly on new wooden vats for use in cheese production. Food Microbiol. 109, 104154.https://doi.org/10.1016/j.fm.2022.104154.

Sun, L., D'Amico, D.J., 2023. The impact of environmental conditions and milk type on microbial communities of wooden vats and cheeses produced therein. Food Microbiol. 104319. https://doi.org/10.1016/j.fm.2023.104319.

Suzuki, T., Matsutani, M., Matsuyama, M., Unno, R., Matsushita, H., Sugiyama, M., Yasamoto, K., Koizumi, Y., Ishikawa, M., 2021. Growth and metabolic properties of halophilic and alkaliphilic lactic acid bacterial strains of *Marinilactibacillus psychrotolerans* isolated from surface-ripened soft cheese. Int. Dairy J. 112, 104840. https://doi.org/10.1016/j.idairyj.2020.104840.

Trofa, D., Gácser, A., Nosanchuk, J.D., 2008. *Candida parapsilosis*, an emerging fungal pathogen. Clin. Microbiol. Rev. 21 (4), 606–625. https://doi.org/10.1128/cmr.00013-08.

UNITE Community, 2019. UNITE QIIME release for Fungi. 10.15156/BIO/786334.

Vermote, L., Verce, M., De Vuyst, L., Weckx, S., 2018. Amplicon and shotgun metagenomic sequencing indicates that microbial ecosystems present in cheese brines reflect environmental inoculation during the cheese production process. Int. Dairy J, 87, 44–53. https://doi.org/10.1016/j.idairyj.2018.07.010.

Wadhawan, K., Steinberger, A., Rankin, S., Suen, G., Czuprynski, C., 2023. Inhibition of *Listeria monocytogenes* by Broth Cultures of Surface Microbiota of Wooden Boards Used in Cheese Ripening. Appl. Sci. 13 (10), 5872. https://doi.org/10.3390/app13105872.

Wadhawan, K., Steinberger, A.J., Rankin, S.A., Suen, G., Czuprynski, C.J., 2021. Characterizing the microbiota of wooden boards used for cheese ripening. JDS communications, 2 (4), 171–176. https://doi.org/10.3168/jdsc.2020-0014

White, T.J., Bruns, T., Lee, S.J.W.T., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18 (1), 315–322.

White, T.J., Bruns, T., Lee, S.J.W.T., Taylor, J., 1990a. PCR protocols: a guide to methods and applications. In: Innis M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., (Eds) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press, San Diego, pp 315–322.

Part III

Tecnological strategy to improve cheese quality

In this session, a technological strategy to reduce cooking time in the production process of PDO Pecorino Siciliano cheese was applied. The production guidelines for PDO Pecorino Siciliano mandate the use of raw ewe's milk, traditional wooden equipment, and a maturation period of at least four months, without the addition of starter culture. The microbiota involved in cheesemaking and ripening is autochthonous, originating from the milk or the processing environment, especially the wooden vat.

Given the variability in microbiology of cheeses produced with raw milk and no starter culture, each production step, including ripening, can alter the ratios of microbial populations, although indigenous LAB are well-adapted to the environment, technology, and cheese type. In this regard, a study to evaluate the effects of cooking in hot water compared to traditional cooking under deproteinized whey and to gather scientific evidence to support modifications to the current production process was conducted. The final cheeses were assessed for their microbiological, chemical, and sensory composition. This innovation helped preserve the traditional process while reducing production times for PDO Pecorino Siciliano.

Chapter V

Reduction of PDO Pecorino Siciliano cheese making duration: Microbial dynamics and quality attributes deriving from replacing whey permeate with hot water during cooking

This work is published in

International Journal of Food Microbiology 410,

2024

Abstract

This work was carried out with the aim to reduce the transformation duration of Protected Designation of Origin (PDO) Pecorino Siciliano cheese. To this purpose, the cooking in hot water (experimental production, EXP) was compared to the traditional cheese cooking under whey permeate (control production, CTR). The microbiological composition of under rind (UR) and core (Co) section of CTR and EXP cheeses was determined by a combined culture-dependent and -independent approach. Total mesophilic microorganisms and lactic acid bacteria (LAB) present in raw ewes' milk (5.0 log CFU/mL) increased during cheese making and reached values of about 8.0 log CFU/g in both sections (UR and Co) of 5-month ripened cheeses of both productions (CTR and EXP) monitored. The identification of the viable LAB populations in ripened cheeses showed that *Enterococcus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Limosilactobacillus* and *Streptococcus* dominated UR and Co sections of all cheeses. MiSeq Illumina analysis demonstrated that LAB populations (lactobacilli, lactococci and streptococci) dominated the bacterial community of cheeses at 95.63–98.41 % of relative abundance. The two different cooking operations did not influence the physicochemical characteristics of PDO Pecorino Siciliano cheeses. Sensory evaluation performed by artificial senses analysis and trained panelists confirmed that the modification of PDO Pecorino Siciliano cheese production protocol did not significantly affect product characteristics and overall acceptance. Thus, data of this work confirmed that cooking under hot water allowed to reduce transformation duration and safeguard typicality of PDO Pecorino Siciliano cheese.

Keywords: Artificial senses; Illumina technology; Lactic acid bacteria; Physicochemical composition; Sensory traits; Traditional cheese.

Introduction

Pecorino Siciliano is a traditional semi-hard Italian raw ewes' milk cheese that gained the Protected Designation of Origin (PDO) status by the European Community (Commission Regulation 1107, 1996). PDO Pecorino Siciliano cheese is produced throughout Sicily only in small enterprises gathered into a protection consortium applying a century-old production protocol (GUCE C 170 EUR-Lex - 52020XC0518(03)). Recently, the production and consumption of Pecorino cheese type is on the increase (Centorotola et al., 2021), and this phenomenon is strictly linked to the re-discovery of natural and historical cheeses by the postmodern consumer (Braghieri et al., 2014). The use of wooden tools, lamb rennet paste, raw ewes' milk and the absence of lactic acid bacteria (LAB) starter cultures added characterize typical PDO Pecorino Siciliano cheese making (Gaglio et al., 2021). As a matter of fact, the production of this cheese relies on the presence of indigenous LAB present in raw materials, equipment and transformation environment (Ruta et al., 2023).

Microbiota naturally associated with the production of PDO Pecorino Siciliano cheese include starter LAB (SLAB), able to generate a high amount of lactic acid during the very first steps of cheese productions through the fermentation of lactose, and non starter LAB (NSLAB), involved in the development of the typical sensory traits of cheeses (Gaglio et al., 2021). Even though some undesirable microorganisms have been found in PDO Pecorino Siciliano cheese during ripening (Todaro et al., 2011), typical dairy pathogens

associated with the microbiological food safety criteria (Commission Regulation 2073, 2005), such as *Listeria monocytogenes* and *Salmonella* spp., have never been detected along the entire production process (Guarcello et al., 2016; Settanni et al., 2013).

Traditionally, PDO Pecorino Siciliano cheese is molded into rattan baskets which are kept under hot whey permeate at about 75 °C for 3–4 h; this production step is known as the cooking phase. This phase is intimately related to the production of Ricotta cheese obtained by thermal precipitation of whey proteins (Mangione et al., 2023). The whey permeates resulting from Ricotta cheese production, namely "scotta" (Settanni et al., 2020), is used to perform PDO Pecorino Siciliano cheese cooking. Thus, PDO Pecorino Siciliano cheese production depends on the availability of scotta which takes approximately two hours. During this time interval, Pecorino Siciliano pressed curd in the rattan baskets remains at room temperature and undesired microorganisms might negatively influence the quality of the final product.

The cooking phase represents a crucial step in cheese-making; it affects physicochemical and sensory aspects of semi-hard cooked cheeses (Güler et al., 2021). In particular, cheese cooking influences the syneresis of the curd, protein, fat and volatile organic compound losses, yield, color, texture and microstructure (Hayaloglu et al., 2008; Lucey and Kelly, 1994). Up to date, the studies available in literature on the use of whey permeate for cheese cooking only focused on the effects of the temperature applied on physicochemical and sensory traits of semi-hard cooked cheeses (Hayaloglu and Brechany, 2007; Hayaloglu et al., 2010; Sulejmani et al., 2014). To our knowledge, no studies have been conducted on the evaluation of the microbiological, chemical, textural and sensory characteristics of cheeses cooked under hot water.

In this study, the replacement of whey permeate with hot water was tested in order to accelerate PDO Pecorino Siciliano cheese production by reducing the resting time before cooking. This study is part of a research project aimed to valorize the natural historic cheeses and was performed to provide in-depth insights on the effect of the different cooking procedures on PDO Pecorino Siciliano cheese characteristics. The final cheeses were subjected to the evaluation of microbiological and chemical composition as well as sensory traits by artificial sense and panel evaluation.

Materials and methods

Cheese production

Cheese making trials were performed at an artisanal dairy factory, located in Santa Margherita di Belìce (Agrigento, Italy), belonging to the Consortium for the protection of PDO Pecorino Siciliano cheese. Raw ewe's milk of the autochthonous Sicilian sheep breed "Valle del Belìce" was transformed applying the traditional "PDO Pecorino Siciliano" cheese technology (Fig. 1). The experimental plan included two different trials: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water. Briefly, bulk milk (200 L), heated at 38 °C was transferred into a 12 years old Douglas wooden vat and was kept under gentle manual agitation for 5 min before addition of lamb rennet paste (36 g, Rennet Regional Consortium, Poggioreale, Italy). Curdling occurred in approximately 40–50 min. The unbroken coagulum was added with hot water (20 L) at 74 °C to facilitate syneresis and was cut with a wooden paddle called "rotula" until small rice-size grains (3–7 mm diameter) were reached.

3032

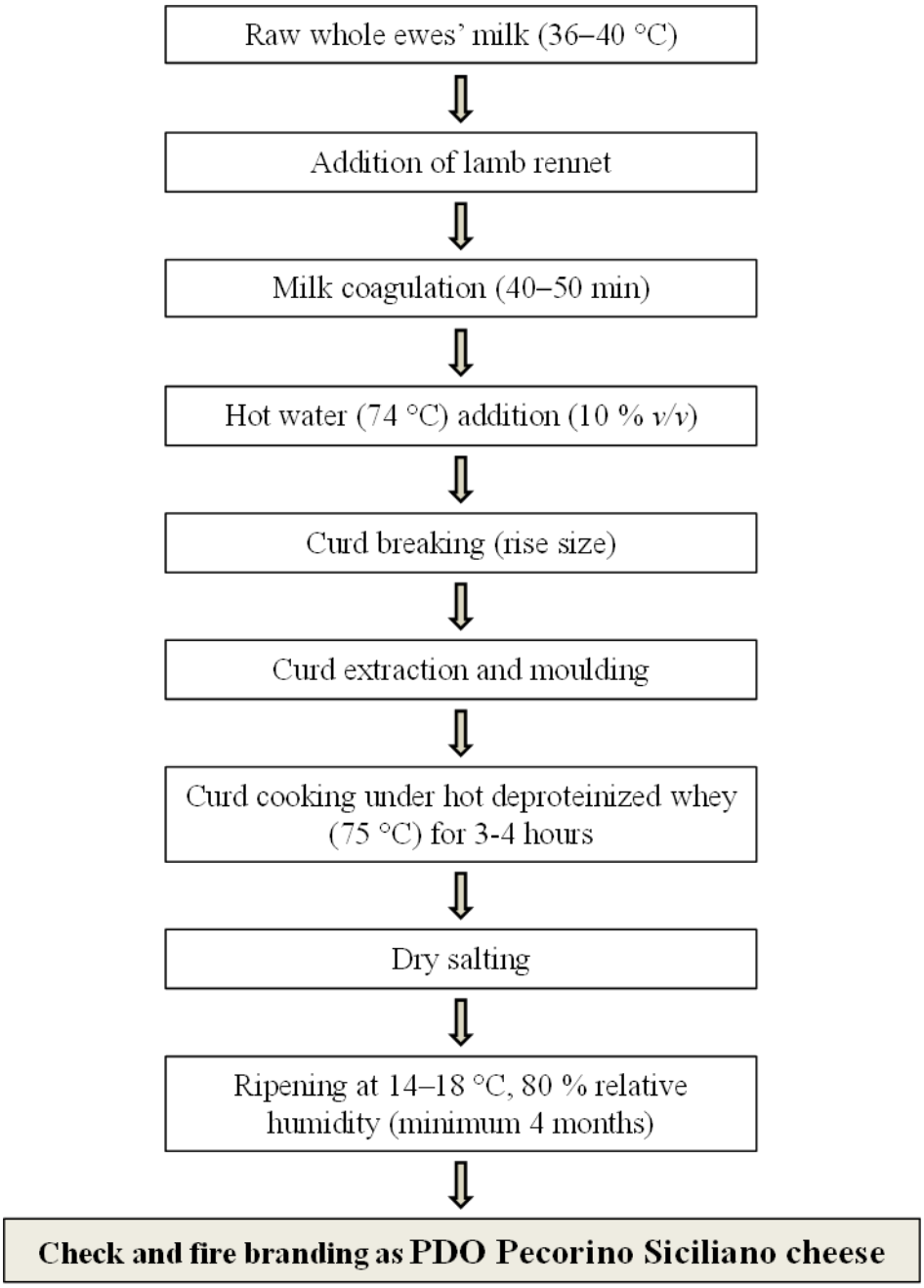

Fig.1. Flowsheet of PDO Pecorino Siciliano cheese production.

3033 After whey draining, curd was hand pressed into 7 kg rattan baskets, forming a total of
3034 four cheeses. Two cheeses representing EXP trial were immediately cooked under hot
3035 water at 72 °C for 3 h, while two cheeses of CTR trial remained at room temperature for 2

h, miming the average time generally occurring to obtain whey permeate from Ricotta cheese production. Also for CTR trial cheeses the cooking phase occurred at 72 °C for 3 h. Twenty-four hours after cooking, all cheeses were salted in saturated brine for 24 h, and ripened for 5 months in a storage chamber at 16 °C and 85 % of relative humidity (RH). Cheese productions was carried out in triplicate (one production per month) during February, March and April 2020 which represent the months with the higher green forage availability and traditionally suited for high quality production of PDO Pecorino Siciliano cheese.

Sample collection, temperature and pH monitoring

Control and experimental cheese making trials were thoroughly monitored; samples were collected from bulk milk before being transferred into the wooden vat and after 5 min from the transfer, curds just after curdling, cheese soon after cooking, and 5-month ripened cheeses. In order to analyze the entire cheese profile, the under rind (UR) section, located at 3 cm from the upper face, and core (Co), taken at a distance of 11 cm from both faces of each cheese, were collected. The wooden vat surface before milk contact was sampled following the brushing method described by Didienne et al. (2012). The monitoring of pH during the production processes (from raw ewe's milk to final cheeses) was performed with a portable pH-meter (waterproof pHTestr 30, Eutech Instruments, Nijkerk, The Nederlands). The temperature of cheeses during the cooking process was registered with two Thermo Button 22 T 8 K data loggers (VWR International Srl, Milano, Italy) inserted at molding in cheese Co and UR sections.

Microbiological analysis

One milliliter of liquid (wooden vat biofilms and milk) samples was directly serially decimally diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy) (Health Canada, 2015), while 25 g of solid (curd and cheese) samples were first homogenized in 225 mL of sodium citrate (2 % *w/v*) solution by a stomacher (Solís et al., 2009) and then serially diluted in Ringer's solution.

Appropriate dilutions from each sample were plated on agar media to allow the development of: total mesophilic microorganisms (TMM) spread on Skim Milk Agar (SMA) and incubated for 72 h at 30 °C; thermophilic and mesophilic coccus LAB poured in Medium 17 (M17) agar containing 5 g/L of lactose, incubated for 48 h at 44 °C and 30 °C, respectively; thermophilic rod LAB poured in whey-based agar medium (WBAM) prepared as described by Settanni et al. (2012) and incubated for 48 h at 44 °C; mesophilic rod LAB poured in de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 M), incubated for 48 h at 30 °C; total coliforms poured in violet red bile agar (VRBA), incubated for 24 h at 37 °C. In addition, all samples were also analyzed for the presence of the main pathogenic microorganisms: coagulase-positive staphylococci (CPS) applying the UNI EN ISO 6888-2 (ISO 2021); *Escherichia coli* the ISO 4832 (ISO 2006); sulfite-reducing anaerobes the ISO 15213 (ISO 2003); *Listeria monocytogenes* and *Salmonella* spp. by the Enzyme Linked Fluorescent Assay (ELFA) method as reported by Cruciata et al. (2018). All media and supplements were purchased from Biokar Diagnostics (Allonne, French), except SMA provided by Microbiol Diagnostici (Uta, Italy). All microbiological counts were carried out in duplicates.

LAB isolation, differentiation and identification

Presumptive LAB colonies developed from the highest cell suspensions of CTR and EXP ripened cheese samples were isolated and purified by successive sub-culturing following the approach reported by Busetta et al. (2023a). Pure cultures were tested for the preliminary LAB characteristics: Gram-positive and catalase-negative. Gram type was determined after treatment with 3 % (*w/v*) KOH (Gregersen, 1978), while catalase test was carried out by 3 % (*v/v*) H_2O_2 contact (Koneman et al., 1997). All pure cultures preliminary belonging to the LAB group were microscopically investigated to evaluate cell morphology and arrangement (Barbaccia et al., 2021). Physiological and biochemical characteristics were analyzed following the procedure described by Gaglio et al. (2014). All cultures with a coccus shape were tested for growth at pH 9.2 and with 6.5 g/L NaCl to identify enterococci able to grow in both conditions.

All LAB were subjected to genomic DNA extraction and strain differentiation following the approach reported by Gaglio et al. (2017). Briefly, crude cell extracts were analyzed by randomly amplified polymorphic DNA (RAPD)-PCR and the resulting polymorphic profiles were elaborated by the program GelCompar II version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium). The isolates showing different RAPD patterns were subjected to 16S rRNA gene sequencing following the procedures applied by Weisburg et al. (1991). The resulting DNA fragments of about 1600 bp were purified by the ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced at BMR Genomics (Padova, Italy). The unequivocal identities of the

sequences were determined by comparison with those available in two distinct databases, NCBI and EZ-Taxon (Gaglio et al., 2016).

DNA extraction, MiSeq library preparation, Illumina sequencing, data analysis and identification of sequences

Total genomic DNA was extracted with the QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. DNAs were quantified by the Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies Wilmintog, DE, USA). Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system were carried out at Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy) sequencing platform. The methodology was reported in detail by Gaglio et al. (2020). Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology, Qiime2, version 2020.11 (Bolyen et al., 2019). The sequences were quality-filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh and Standley, 2013). Taxonomic and compositional analysis were carried by using the plugins feature classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained, accessed Naive Bayes classifier based on the Greengenes gg_13_5_otus.tar.tgz Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/?prefix=downloads/greengenes_database/gg_13_5/)

, which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to the generate taxonomy tables. Data generated by Illumina sequencing were uploaded in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA997145.

Physicochemical analysis

Curd and cheese samples were analysed for dry matter (DM), fat, protein (N x 6.38), and ash content according to the International Dairy Federation (IDF) standards [4A (IDF, 1982), 5B (IDF, 1986), 25 (IDF, 1964a), and 27 (IDF, 1964b), respectively]. Cheese samples soluble nitrogen (N) was determined on an aqueous filtrate using the Kjeldahl method (MAF, 1986). Water activity was determined at 23 °C at the surface of each sample slice by using an activity-meter instrument (Rotronic Int., USA). Cheese samples were assessed for Co and UR color, measured in duplicate by a Minolta Chroma Meter CR300 (Minolta, Osaka, Japan) using the illuminant C; results are expressed as lightness (L*, from 0 = black, to 100 = white), redness (a*, from red = +a, to green = −a), and yellowness (b*, from yellow = +b, to blue = −b), according to the CIE L* a* b* system. Cheese hardness was evaluated with an Instron 5564 tester (Instron, Trezzano sul Naviglio, Milan, Italy) measuring the maximum resistance to compression (compressive stress, N/mm^2) of samples (2 cm × 2 cm × 2 cm) kept at room temperature (22 °C).

Electronic nose and tongue

Odor and taste profiles of CTR and EXP cheeses were evaluated using an E-nose (FOX 4000, Alpha M.O.S., Toulouse, France) with 18 MOS sensors and a potentiometric E-

tongue (αAstree, Alpha M.O.S., Toulouse, France) with 7 chemical sensors. The electronic senses were developed to mimic the function of the human senses and, in particular, the electronic nose and electronic tongue to perceive smells and tastes, respectively. This system consists of an array of non-specific, slightly selective electrochemical sensors with high stability and cross-selectivity towards volatile compounds or groups of substances present in complex liquid systems (Di Rosa and Leone, 2018). The array of sensors is combined with an appropriate pattern recognition system that can interpret complex signals from those sensors producing the fingerprint of the product as human senses produce in brain. Each sample was tested five times with the electronic nose and 10 times with the electronic tongue.

Sensory analyses

Five-month ripened CTR and EXP cheeses were also evaluated for their sensory traits by a panel of judges. All cheeses were judged by 12 assessor members including six men and six women (aged between 21–65 years old) familiar with the sensory analysis of cheeses. The analysis was carried out in single chambers and the panelists were specifically trained following the ISO 8589 (ISO 2007) indications. The cheeses were acclimated at about 20 °C for 1 h, cut into cubes (3 cm × 3 cm × 3 cm) and then coded and served in a random order. Sixteen descriptive attributes grouped into aspect, aroma, taste, and texture categories were judged and scored using a line scale from 1 to 9 (cm) as reported by Gaglio et al. (2019a).

Statistical analyses

Microbiological, chemical and physical data of cheeses were statistically analysed with the following ANOVA linear model: $Y_{ijk} = \mu + (\text{cooking x cheese})_{ij} + \varepsilon_{ijk}$ where cooking is the liquid utilized to cook the cheeses (CTR, EXP); cheese is the fixed factor cheese type (cheese soon after cooking, PDO Pecorino Siciliano); Chemical and physical cheese data were analyzed with the following ANOVA linear model: $Y_{ijk} = \mu + (\text{cooking x sampling})_{ij} + \varepsilon_{ijk}$ where cooking is the different liquid utilized to cook the cheeses (CTR, EXP); sampling is the zone of cheese analysed (UR, Co). Sensorial parameters were analysed with the following ANOVA linear model: $Y_{ijk} = \mu + \text{Panellist}_i + \text{cooking}_j + \varepsilon_{ijk}$ were Panellist_i is the fixed factor "expert judges" (1..12) and cooking is the liquid utilized to cook the cheeses (CTR, EXP); least square means were reported as spider graph. The Student "t" test was used for means comparisons at $p < 0.05$ and $p < 0.01$ significance level, while the statistical software used was SAS 9.1.2 (Procedure General Linear Model procedure). Results from E-nose and E-tongue sensors were subjected to exploratory Principal Component Analysis (PCA) and expressed based upon the Discrimination Index (DI).

Results and Discussions

Monitoring of temperatures during cheese making

Table 1 shows the temperatures of the curd detected during the entire cheese-making process. During molding, the temperature of the curds was highly similar, independently on the cooking system (CTR vs EXP) and cheese section (UR vs Co). After moulding,

EXP cheeses exhibited higher temperatures than CTR cheeses at the beginning of the cooking operation ($p < 0.01$). This observation is undoubtedly the result of the resting of CTR cheeses before being covered by the whey permeate residual from Ricotta cheese production.

Table 1. Temperatures detected during experimental cheese productions.

Items	Cooking (C)	Sampling (S)		SEM	*p* value "C x S"
		Co	UR		
Moulding	EXP	38.6	38.2	0.49	0.327
	CTR	37.6	37.4		
Start of cooking	EXP	38.5	37.3_{X}	0.57	0.008
	CTR	37.1^{A}	$34.7^{B}{}_{Y}$		
End of cooking	EXP	43.6^{a}	40.9^{b}	0.97	0.029
	CTR	41.3^{a}	38.9^{b}		
After 30 min from end of cooking	EXP	44.9^{A}	41.3^{B}	0.80	0.008
	CTR	43.0^{a}	40.4^{b}		
After 120 min from end of cooking	EXP	45.3^{a}	37.3^{b}	1.80	0.126
	CTR	42.6	39.3		
Brine	EXP	17.5	16.5	1.03	0.884
	CTR	17.4	16.9		

On the row, values with different superscript letters are significant A, B: $p \leq 0.01$; a, b: $p \leq 0.05$. On the column, values with different underscript letters are significant X, Y: $p \leq 0.01$. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; SEM = standard error of mean.

During cooking the temperatures increased for both trials, but, after 3 h, the differences between CTR and EXP cheeses were still significant ($p < 0.05$). After 30 and 120 min from the end of cooking, the temperatures were not statistically significant between the two production trials. Regarding cheese sections, core temperature was characterized by almost 2 °C higher than the under rind. The slow increase of core temperature is due to the slow heat penetration into the solid matrix of the cheese depending on its low thermal conductivity that delays heat transfer. The low thermal conductivity of dairy products depends on their composition; it is directly related to water content and inversely to fat and protein content (Tavman and Tavman, 1999).

Evolution of microbial populations during cheese making

The results of the plate counts carried out throughout cheese production from wooden vat surface to CTR and EXP cheeses after 5-month of ripening are reported in Table 2. The specific search for *L. monocytogenes* and *Salmonella* spp., responsible for food-borne outbreaks (Nguyen et al., 2016), did not show any growth from either of the samples analyzed (for this reason, these results are not included in Table 2). The surfaces of the wooden vat, hosted levels of TMM, mesophilic rod and coccus, as well as thermophilic rod and coccus LAB of about 10^6 CFU/cm^2. High levels of these microorganisms are often detected in wooden equipment used to process PDO traditional Sicilian cheeses (Busetta et al., 2021; Gaglio et al., 2016; Settanni et al., 2021), and this is imputable to the ability of LAB to colonize the inner surfaces of the wooden vats used to process these cheeses (Cruciata et al., 2018). Within the undesired bacteria, CPS were undetectable (<1 log CFU/mL), while total coliforms and *E. coli* were around 10^2 CFU/cm^2. Similar levels of these bacteria have been previously observed onto the surfaces of the wooden vats used for the production of Caciocavallo Palermitano and PDO Vastedda della Valle del Belìce (Scatassa et al., 2015). Bulk milk hosted TMM at almost 10^5 CFU/mL and it was dominated by mesophilic LAB cocci. An increase of about 1 log cycle was registered for TMM and all LAB groups, after contact with the wooden vat. No differences were found for the levels of total coliforms, *E. coli* and CPS before and after contact with the wooden vat surface. A similar trend was previously reported by Didienne et al. (2012). After

3236 curdling, an almost 1 log increase was registered for the densities of the majority of the
3237 microbial groups investigated.

Table 2. Microbial evolution during experimental cheese productions.

Samples	Growth media							
	SMA	M17 30 °C	M17 44 °C	MRS	WBAM	VRBA	BP	TBX
Wooden vat surface	6.49 ± 0.31	6.42 ± 0.19	6.21 ± 0.22	5.88 ± 0.56	5.49 ± 0.48	1.87 ± 0.40	<1	1.69 ± 0.35
Bulk milk	4.91± 0.35	4.94 ± 0.42	3.89 ± 0.18	3.99 ± 0.52	1.94 ± 0.59	2.85 ± 0.59	2.26 ± 0.18	1.38 ± 0.37
Bulk milk in wooden vat	5.87 ± 0.25	5.80 ± 0.48	4.54 ± 0.18	4.40 ± 0.52	3.38 ± 0.37	3.09 ± 0.34	2.64 ± 0.24	1.64 ± 0.37
Curd	6.61 ± 0.15	6.30 ± 0.27	5.68 ± 0.19	5.68 ± 0.29	4.08 ± 0.24	3.76 ± 0.26	3.58 ± 0.16	3.24 ± 0.18
Cheese after cooking "UR"								
CTR	7.95 ± 0.21	8.20 ± 0.34	7.79 ± 0.28	7.57 ± 0.32	7.31 ± 0.53	4.41 ± 0.27	4.71 ± 0.24	4.25 ± 0.24
EXP	7.75 ± 0.17	7.88 ± 0.30	7.75 ± 0.50	7.22 ± 0.24	7.56 ± 0.57	4.29 ± 0.31	4.66 ± 0.28	3.99 ± 0.12
p value	0.269	0.289	0.910	0.215	0.608	0.640	0.826	0.169
Cheese after cooking "Co"								
CTR	8.13 ± 0.34	8.20 ± 0.34	7.79 ± 0.28	7.57 ± 0.32	7.31 ± 0.53	4.41 ± 0.27 b	4.71 ± 0.24	4.25 ± 0.24 b
EXP	8.21 ± 0.22	8.13 ± 0.36	8.08 ± 0.24	7.71 ± 0.32	7.75 ± 0.36	5.09 ± 0.27 a	5.34 ± 0.45	4.81 ± 0.24 a
p value	0.749	0.819	0.245	0.620	0.300	0.037	0.099	0.046
Ripened cheese "UR"								
CTR	7.83 ± 0.21	8.00 ± 0.40	7.87 ± 0.33	7.51 ± 0.17	7.30 ± 0.33	<2	<2	<2
EXP	7.98 ± 0.31	7.95 ± 0.33	8.19 ±0.29	7.88 ± 0.33	7.28 ± 0.35	<2	<2	<2
p value	0.795	0.857	0.276	0.159	0.946	n.e.	n.e.	n.e.
Ripened cheese "Co"								
CTR	7.91 ± 0.23	7.73 ± 0.22	8.09 ± 0.37	7.79 ± 0.32	7.53 ± 0.35	<2	<2	<2
EXP	7.74 ± 0.21	7.79 ± 0.30	7.87 ± 0.39	7.93 ± 0.43	7.70 ± 0.30	<2	<2	<2
p value	0.398	0.794	0.518	0.674	0.654	n.e.	n.e.	n.e.

Loads are reported as log CFU/cm^2 for vat surface, log CFU/mL for milk samples, and log CFU/g for curd. Results indicate the mean values ± standard deviation (S.D.) of six plate counts (carried out in duplicate for three independent productions). Data within a row followed by different letters are significantly different according to Tukey's test. Abbreviations: SMA, Skim Milk Agar for detection of total mesophilic microorganisms; M17 30 °C, medium 17 agar incubated at 30 °C for detection of mesophilic coccus LAB; M17 44 °C, medium 17 agar incubated at 44 °C for detection of detection of thermophilic coccus LAB; MRS, de Man-Rogosa-Sharpe agar for detection of mesophilic rod LAB; WBAM, whey-based agar medium for detection of thermophilic rod LAB; VRBA, violet red bile agar for detection of total coliforms; BP, baird-parker agar for detection of coagulase-positive staphylococci; TBX, Tryptone Bile X-Gluc agar for detection of *E. coli*; CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; UR, under rind; Co, core; n.e., not evaluated.

3245 This increase is an expected phenomenon after whey draining (Settanni et al., 2013). As
3246 reported in Table 2, no significant differences ($p > 0.05$) were found for the levels of all
3247 microbial groups object of investigation between the different portions (UR and Co) of
3248 both EXP and CTR cheese soon after cooking and even after 5-month of ripening.
3249 Regardless of the cooking treatment applied (water or whey permeate) and the section
3250 analyzed, all cheeses soon after production showed values of TMM and all LAB groups of
3251 about 10^8 CFU/g. These levels remained almost constant in both CTR and EXP cheeses
3252 after 5-month of ripening as previously reported for this type of cheese by different authors
3253 (De Pasquale et al., 2016; Guarcello et al., 2016; Randazzo et al., 2006). In general, higher
3254 differences are registered in terms of LAB populations characterizing the different sections
3255 of high-volume cheeses, such as Trentingrana cheese, during ripening (Monfredini et al.,
3256 2012). Concerning safety aspects, after 5-month ripening, all potentially harmful
3257 microorganisms (total coliforms, *E. coli* and CPS) were below the detection limits and
3258 were in compliance with the Commission Regulation 2073 (2005) on microbiological
3259 criteria for foodstuffs (Commission Regulation 2073, 2005).
3260
3261 *Phenotypic differentiation and genotypic identification of LAB*
3262 Four hundred and forty-two colonies of LAB (Gram-positive and catalase-negative)
3263 distinguished into 220 cocci and 222 rods were isolated from the 5-month ripened PDO
3264 Pecorino Siciliano cheeses processed in this study by cooking in water or whey permeate.
3265 Considering morphological, physiological and biochemical features, the 442
3266 presumptive LAB cultures were separated into eight groups (Table 3).
3267

Table 3. Phenotypic grouping of LAB isolated from ripened cheeses.

Characters	Clusters							
	I (n=143)	II (n=61)	III (n=16)	IV (n=157)	V (n=10)	VI (n=14)	VII (n=9)	VIII(n=32)
Morphology	C	C	C	R	R	R	R	R
Cell disposition	lc	sc	sc	sc	sc	sc	sc	sc
Growth:								
15 °C	-	+	+	+	-	+	-	+
45 °C	+	+	+	-	+	+	+	+
pH 9.6	-	+	+	n.d.	n.d.	n.d.	n.d.	n.d.
6.5 % NaCl	-	+	+	n.d.	n.d.	n.d.	n.d.	n.d.
Resistance to 60 °C	-	-	-	-	-	-	-	+
Hydrolysis of:								
Arginine	-	+	+	-	-	-	+	+
Aesculin	-	+	+	+	-	-	-	+
Acid production from:								
Arabinose	-	+	+	-	-	+	+	+
Ribose	-	+	+	+	+	+	+	+
Xylose	-	+	+	-	-	+	+	+
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Lactose	+	+	+	-	+	+	+	+
Sucrose	+	+	-	+	+	+	+	+
Glycerol	+	+	+	-	-	+	+	+
CO_2 from glucose	-	-	-	-	-	-	+	-

Abbreviations: C, coccus; R, rods; lc, long chain; sc, short chain; n.d., not determined.

The community of coccus isolates included three groups with cells organized in long chains (group I) and short chains (groups II and III). LAB rods constituted five groups (IV to VIII), all forming short chains of cells. Among these, only one group (VII) showed a hetero-fermentative metabolism. Two hundred and nine presumptive LAB cultures (approximately 50 % of the isolates from eight phenotypic groups) were selected from both sections (UR and Co) of CTR and EXP cheeses and processed by RAPD analysis. This PCR-based technique is commonly applied for the intra- and inter-specific differentiation of dairy LAB (Rossetti and Giraffa, 2005). The dendrogram reported in Fig. 2 shows the presence of 29 distinct strains representing the viable populations dominating all sections of the final cheeses.

The identification by 16S rRNA gene sequencing indicated that all 29 strains belonged to the LAB group and they were allotted into nine species: *Enterococcus durans* (Ac. No. OR226616-OR226617); *Enterococcus faecalis* (Ac. No. OR226618-OR226619); *Enterococcus lactis* (Ac. No. OR226620); *Lacticaseibacillus paracasei* (Ac. No. OR226621-OR226631); *Lacticaseibacillus rhamnosus* (Ac. No. OR226632); *Lactiplantibacillus pentosus* (Ac. No. OR226633); *Levilactobacillus brevis* (Ac. No. OR226635- R226635); *Limosilactobacillus fermentum* (Ac. No. OR226636); *Streptococcus gallolyticus* subsp. *macedonicus* (Ac. No. OR226637-OR226644). These species are part of the typical NSLAB cultures involved in traditional cheese productions (Settanni and Moschetti 2010).

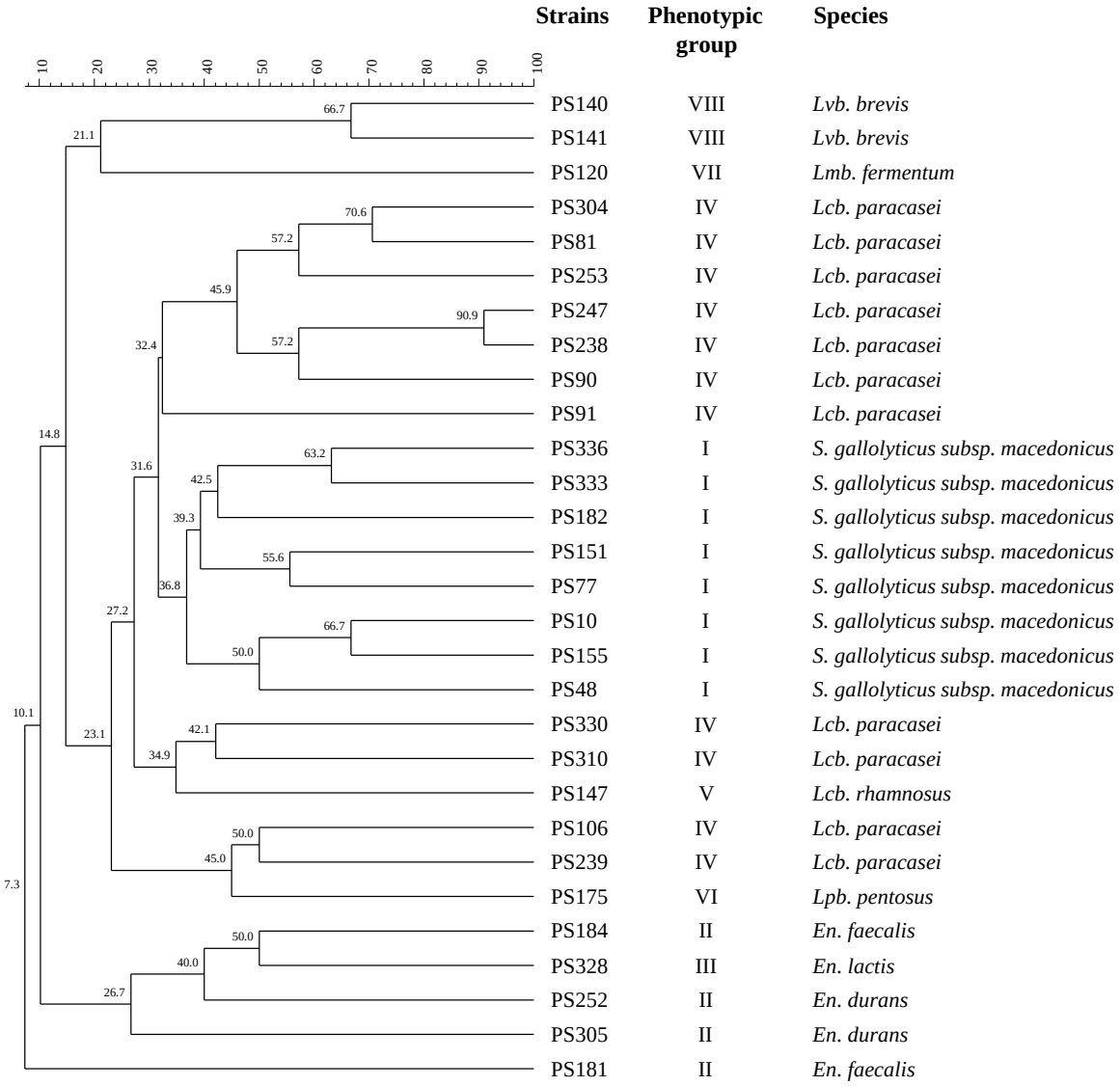

Fig. 2. Dendrogram obtained from combined RAPD-PCR patterns of LAB strains isolated from 5-month ripened cheeses. Abbreviations: *En.*, *Enterococcus*; *Lcb.*, *Lacticaseibacillus*; *Lactiplantibacillus*; *Lvb.*, *Levilactobacillus*; *Lmb.*, *Limosilactobacillus*; *Lpb.*, *S.*, *Streptococcus*.

In particular, enterococci are generally isolated from PDO Pecorino Siciliano cheeses (Randazzo et al., 2006; Vernile et al., 2008; Todaro et al., 2011) and, thanks to their biochemical traits, such as lipolytic activity and citrate utilization, are considered important for the development of typical organoleptic traits that the cheese acquire with ripening (Foulquié Moreno et al., 2006). *Lacticaseibacillus paracasei* and *Lcb. rhamnosus* isolated

from PDO Pecorino Siciliano cheeses have been object of selection for NSLAB addition due to their probiotic features (Caggia et al., 2015). Regarding *Streptococcus* species detected in this study, *S. gallolyticus* subsp. *macedonicus* is of dairy origin and has been used as NSLAB in PDO Pecorino Siciliano cheese production (Guarcello et al., 2016; Gaglio et al., 2020; Settanni et al., 2013).

Culture-independent microbiological investigation

The microbiota associated with the different sections (UR and Co) of CTR and EXP cheeses after 5-month of ripening was also studied by DNA-based Illumina technology. This approach has been widely applied to provide a deep description of the entire bacterial composition of processed foods (Jagadeesan et al., 2019; Settanni et al., 2020). Fig. 3 reports the bar plot of the distribution of the relative abundance (RA) % of the bacterial operational taxonomy units (OTUs) identified by MiSeq Illumina. The figure includes only the OTUs with a RA > 0.1 %, which is the minimum level generally fixed as the threshold for abundant communities (Logares et al., 2014). The taxonomy classification allowed the identification of 10 groups, the majority of which at family and genus level. LAB were detected in all samples at consistent RA % (95.63–98.41), and were classified as lactobacilli, lactococci and streptococci. The major group among cheeses, both in UR and Co section, was *Streptococcus* that ranged between 59.28 % (in UR section of CTR cheeses) and 71.31 % (in Co section of EXP cheeses) of the bacterial RA. Generally, *Streptococcus* is component of the starter LAB group (Settanni and Moschetti, 2014), but the high RA % registered in 5-month ripened cheeses could derive from the residual DNAs

3317 of no more viable cells (Gaglio et al., 2019b) or from the DNAs of *St. gallolyticus* subsp.
3318 *macedonicus* isolated at high cell densities in all sections of cheeses.

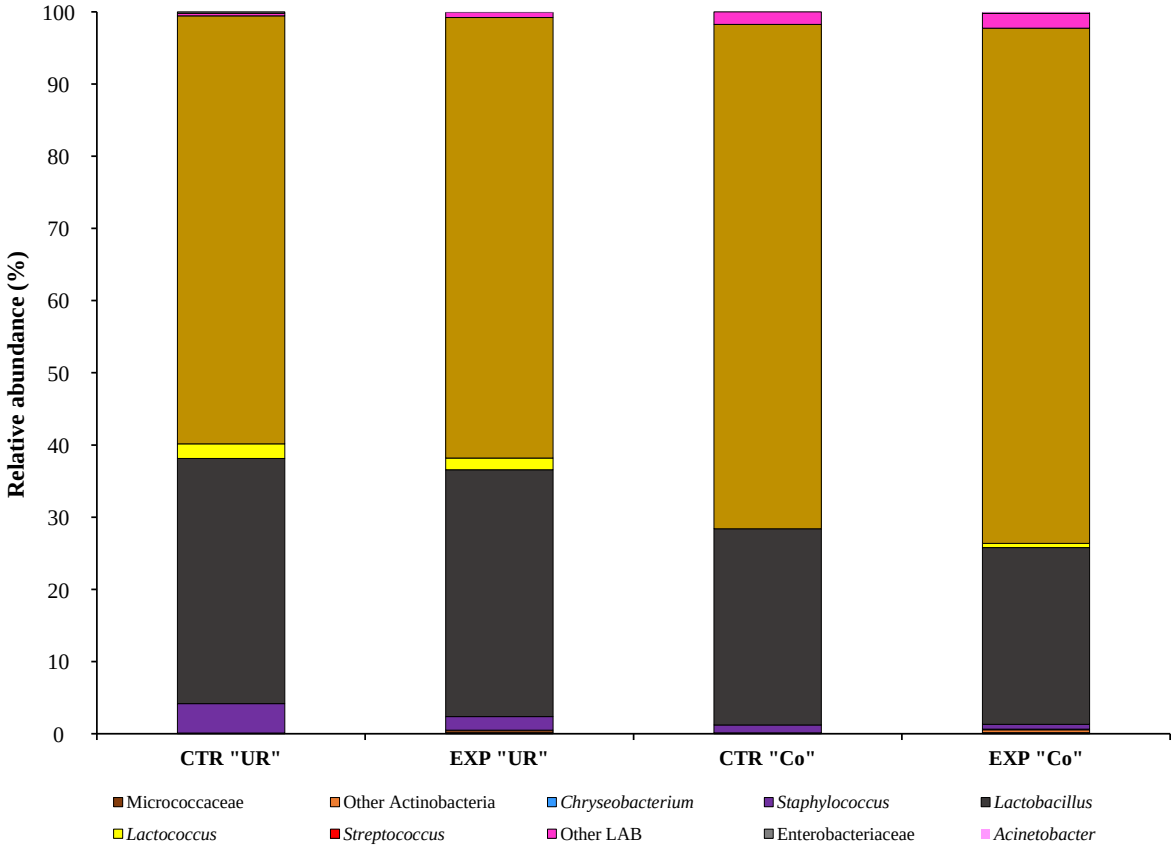

Fig. 3. Relative abundances (%) of the bacterial operational taxonomy units (OTUs) identified by MiSeq Illumina. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; UR, under rind; Co, core.

3319 However, the high percentage of these microorganisms found in the Co of both CTR
3320 and EXP cheeses is probably due to the temperatures higher to 40 °C kept in this section
3321 for at least 2 h from end of cooking, which represent the optimal growth temperatures of
3322 the species belonging to *Streptococcus* genus associated to dairy products (Gobbetti and
3323 Calasso, 2014). *Lactobacillus* was the second group most abundant in all cheese samples
3324 analyzed (27.15–33.97 % of RA). This group includes several genera in addition to
3325 *Lactobacillus*, as a consequence of the reclassification by Zheng et al. (2020). Regarding

Lactococcus, low percentages of OTUs ranged between <0.1 % and 2.03 % were identified in the UR section of both cheese productions. The presence of lactobacilli, lactococci and streptococci at the same percentage of RA revealed in this study was previously reported for PDO Pecorino Siciliano cheese (Gaglio et al., 2020), Gran Ovino cheese (Gaglio et al., 2019a) and Grana Padano cheese (Bassi et al., 2015).

All samples object of investigation were characterized by the presence of *Staphylococcus* at very low RA % (<4 %). The presence of these bacteria at low levels is imputable to high hygiene conditions of the raw milk used for the cheese productions (Giammanco et al., 2011). Micrococcaceae, other Actinobacteria, *Chryseobacterium*, Enterobacteriaceae and *Acinetobacter* were present in all cheeses at negligible levels (<1 % of total bacterial diversity). These bacteria are commonly presents at low percentages in ripened raw milk cheeses (Busetta et al., 2023a; Busetta et al., 2023b; Gaglio et al., 2020). These results confirmed those obtained by culture-dependent approach that highlighted the dominance of LAB in both sections (UR and Co) of CTR and EXP cheeses.

Physicochemical analyses of cheeses

Gross composition of the cheeses produced in this study is reported in Table 4. The different cooking procedures did not affect the chemical composition of the final cheeses. In particular, 5-month ripened CTR and EXP cheeses were characterized by an average DM, fat, protein, and ash content of 61.56, 43.19, 48.30, and 7.06 %, respectively. These results are comparable to those registered for PDO Pecorino Siciliano cheeses produced at a large-scale level in dairy factories characterized by different pedoclimatic conditions, sheep breed, and pasture (Guarcello et al., 2016).

Table 4. Chemical analysis of curds and cheeses.

Items	Curd just after curdling	Cooking (C)	Cheese (Ch)		*p* value "C x Ch"
			Cheese soon after cooking	Ripened cheeses	
Dry matter (DM) (%)	47.02 ± 1.69	EXP	54.07 ± 0.50 ^{A}	60.72 ± 0.70 ^{B}	0.001
		CTR	55.52 ± 0.50 ^{A}	62.39 ± 0.70 ^{B}	
Fat (g/100g DM)	41.99 ± 0.79	EXP	40.94 ± 0.68	42.78 ± 0.97	0.147
		CTR	41.49 ± 0.68	43.60 ± 0.97	
Protein (g/100g DM)	45.76 ± 0.73	EXP	47.08 ± 0.50	48.14 ± 0.70	0.408
		CTR	47.70 ± 0.50	48.46 ± 0.70	
Ash (g/100g DM)	5.50 ± 0.12	EXP	5.91 ± 0.11^{A}	7.09 ± 0.15^{B}	0.001
		CTR	5.83 ± 0.11^{A}	7.02 ± 0.15^{B}	

Results indicate least square means ± s.d. On the row, values with different superscript letters are significant A, B: $p \leq 0.01$; a, b: $p \leq 0.05$. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water.

The increase of DM and ash registered during ripening is mainly due to dehydration and to the increase of chloride content over time (Caridi et al., 2003). The results of the physicochemical analysis performed on the different sections (UR and Co) of CTR and EXP cheeses are reported in Table 5.

Table 5. Physicochemical parameters of different section of cheeses.

Items	Cooking (C)	Sampling (S)		SEM	*p* value "C x S"
		Co	UR		
a_w, activity water	EXP	0.99	0.98	0.01	0.292
	CTR	0.98	0.98		
pH	EXP	5.76	5.56	0.06	0.183
	CTR	5.65	5.60		
Soluble N/total N (%)	EXP	23.72	23.72	0.49	0.991
	CTR	23.88	23.88		
Hardness (kg/cm²)	EXP	0.47	0.60	0.13	0.538
	CTR	0.52	0.73		
L*, lightness	EXP	76.87 ^{a}	68.88 ^{b}	2.25	0.008
	CTR	77.69 ^{A}	64.39 ^{B}		
a*, redness	EXP	-3.49 $_{x}$	-3.57	0.21	0.174
	CTR	-4.16 $_{y}$	-3.81		
b*, yellowness	EXP	14.60	12.45	1.26	0.152
	CTR	15.63	11.45		

Results indicate the mean values of six determination (carried out in duplicate for three independent productions). On the row, values with different superscript letters are significant A, B: $p \leq 0.01$; a, b: $p \leq 0.05$. On the column, values with different underscript letters are significant x, y: $p \leq 0.05$. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; UR, under rind; Co, core; SEM = standard error of mean.

No differences ($p > 0.05$) were found among the sections (UR and Co) of CTR and EXP cheeses for N soluble/N total ratio (on average, 23.88 and 23,72 %, respectively). Values of this ratio between 12 to 24 % indicate that the cheeses are characterized by high amounts of bioactive peptides (Rizzello et al., 2005). Regarding colorimetric index, significant differences ($p = 0.008$) were found only for the redness (a*) value. This parameter showed a lower value in the Co of CTR cheeses. However, similar colorimetric parameters, on the whole, were previously registered for 5-month ripened PDO Pecorino Siciliano cheeses (Todaro et al., 2011) and, in general, in Pecorino cheese typology (Bennato et al., 2023; Grasso et al., 2023).

Electronic nose and tongue response

Fig. 4 shows the separate PCA plot for E-nose and E-tongue. Results indicate that the modification of traditional protocol of production of PDO Pecorino Siciliano cheese did not affect the organoleptic attributes in terms of volatile profile and taste properties. The DI is -9 % for E-nose and -12 % for E-tongue and the first two planes (PC1 and PC2) represent 99.3 % and 95.4 % of the total variance between CTR and EXP cheeses, respectively for E-nose and E-tongue. Overall, the results showed a great potential of artificial senses to find even the most subtle differences. In fact, within the CTR and EXP cheeses, the E-senses underline the variability due to the different quality of the milk over the months and the sensory attributes of the cheeses are generally correlated with the sensory quality of milk that is the result of different components related to feed and chemical compounds.

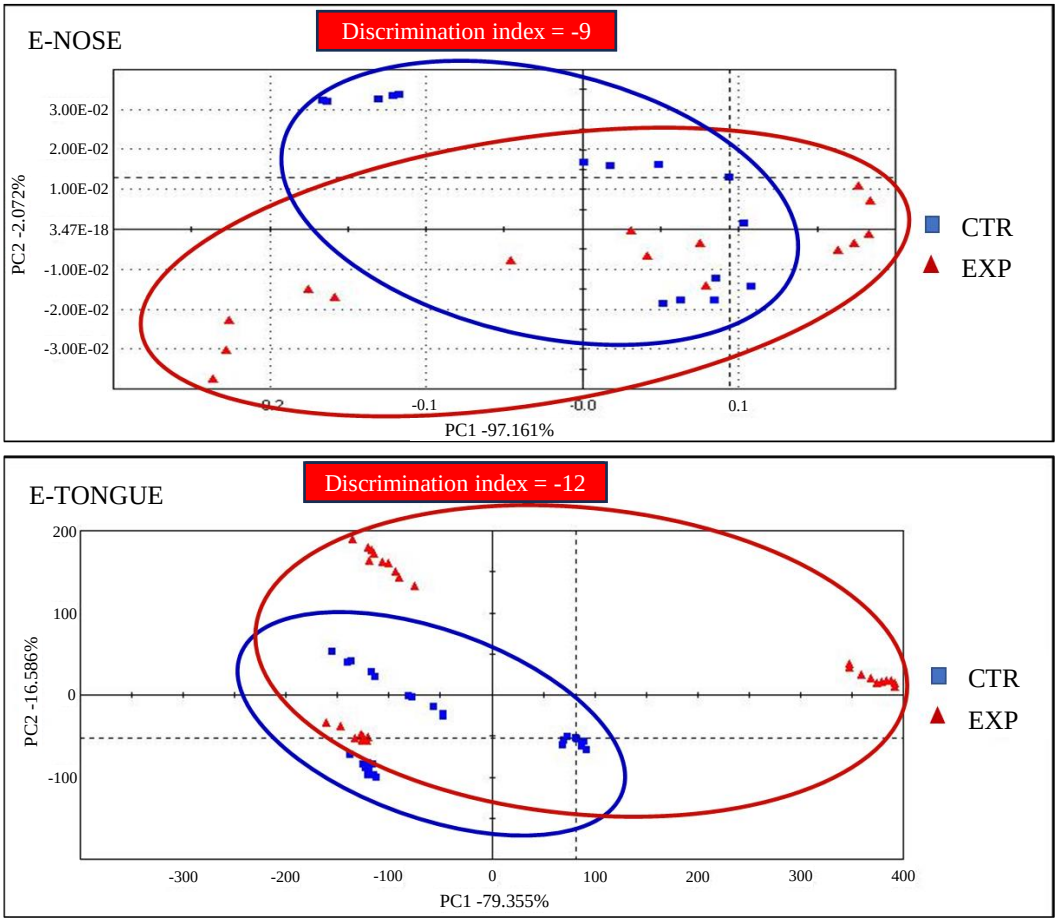

Fig. 4. Principal component analysis plot resulting from e-nose and e-tongue. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water.

Moreover, the diet can influence the sensory perception of milk and dairy products (Chiofalo et al., 2020; Liotta et al., 2019) as well as other potential factors play a role at farm level (Kilcawley et al., 2018) or during the production phase (Di Rosa et al., 2018). The different response of the E-nose and E-tongue sensors could be explained by the distribution and abundance of volatile compounds of pasture as a consequence of the different plant species (Mariaca et al., 1997), and seasonal climatic changes (Rajeswara et al., 1996). The sensors that responded mostly to the volatile profile of cheese in the various months were TA/2, P30/1 and P30/2 with a strong affinity for ethanol, hydrocarbons,

ketones and hydrogen sulfide (Lo Presti et al., 2023). These findings are in agreement with the observations of some authors (Bendall and Olney, 2001; Coppa et al., 2011) which found two ketones (hept-cis-4-enal and 2,3-octanedione) as important volatile aromatic components in milk obtained from different pastures, suggesting that this was due to the oxidation of linoleic and linolenic acids mostly represented in pasture milk samples. These results confirmed that the different protocols applied for cheese cooking did not influence the sensory profile of PDO Pecorino Siciliano cheeses.

Sensory evaluation of cheeses

Fig. 5 reports the radar chart of the sensory attributes evaluated on CTR and EXP ripened cheeses during taste sessions. This analysis is mandatory before marketing of a new food product since the application of a different technology of production might influence the consumers' acceptability (Fiorentini et al., 2020). In this study, the modification of traditional protocol of production of PDO Pecorino Siciliano cheese through cooking in water did not particularly affect the sensory attributes of final products. Except for the intensity of odor and unpleasant smell, all other sensory attributes object of evaluation did not differ among CTR and EXP ripened cheeses. The absence of big differences among CTR and EXP for the main sensory attributes evaluated is undoubtedly due to the use of the same raw ewes' milk for both productions. In fact, the sensory attributes of cheeses are generally correlated with the microbiological quality of milk (Fox et al., 2004), the animal diet and farm management (Kilcawley et al., 2018).

The cheese cooking under hot water influenced negatively intensity of odor and unpleasant smell.

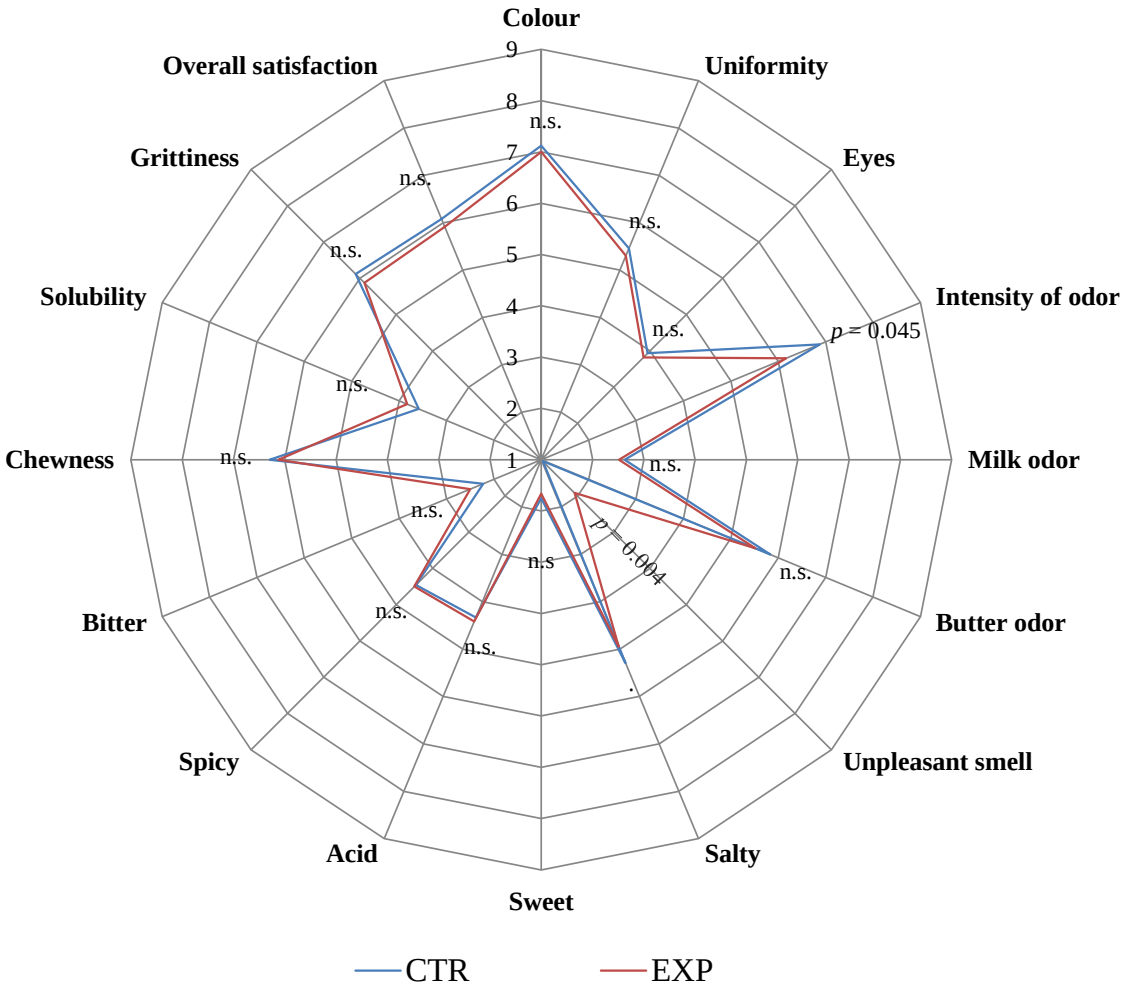

Fig. 5. Spider graph of descriptive sensory analysis of cheeses. Abbreviations: CTR, control production that included cheese cooking under hot whey permeate; EXP, experimental production obtained by cheese cooking under hot water; n.s., not significant.

These results are not surprising, since whey permeate used for the cooking of CRT cheeses is rich in aromatic compounds (Saglam et al., 2019), which can affect the odor intensity of the final products. However, the scores registered in this research are similar to those reported in literature for PDO Pecorino Siciliano cheeses (Gaglio et al., 2020; Guarcello et al., 2016; Settanni et al, 2013). Overall satisfaction, intended as an overall rating determined on the basis of all sensory attributes with their scores (Qasem et al., 2017), did not differ significantly ($p > 0.05$) among CTR and EXP cheeses. These results

confirmed that the modification of the production protocol of PDO Pecorino Siciliano cheeses did not affect the product characteristics and overall acceptance.

Conclusions

This study provides an analysis of the microbiological, chemical, textural and sensory characteristic of PDO Pecorino Siciliano cheese cooked under hot water. This technology did not affect the growth, survival and LAB species evolution in the different PDO Pecorino Siciliano cheese sections (UR and Co). Experimental PDO Pecorino Siciliano cheese was characterized by the same ash, dry matter, fat and protein content in comparison to CTR productions. Sensory traits evaluated on the basis of human and artificial senses were comparable among CTR and EXP cheeses. The results of this study clearly highlighted that the cheese cooking under hot water represent a useful innovation to reduce the transformation duration of PDO Pecorino Siciliano cheese without compromising the typical sensory profile of this traditional cheese.

References

Barbaccia, P., Busetta, G., Matraxia, M., Sutera, A.M., Craparo, V., Moschetti, G., Francesca, N., Settanni, L., Gaglio, R., 2021. Monitoring commercial starter culture development in presence of red grape pomace powder to produce polyphenol-enriched fresh ovine cheeses at industrial scale level. Fermentation 7 (1), 35. https://doi.org/10.3390/fermentation7010035.

Bassi, D., Puglisi, E., Cocconcelli, P.S., 2015. Understanding the bacterial communities of hard cheese with blowing defect. Food Microbiol. 52, 106–118. https://doi.org/10.1016/j.fm.2015.07.004.

Bendall, J.G., Olney, S.D., 2001. Hept-cis-4-enal: Analysis and flavour contribution to fresh milk. Int. Dairy J. 11, 855–864. https://doi.org/10.1016/S0958-6946(01)00136-4.

Bennato, F., Ianni, A., Bellocci, M., Grotta, L., Sacchetti, G., Martino, G., 2023. Influence of dietary grape pomace supplementation on chemical and sensory properties of ewes' cheese. Int. Dairy J. 143, 105671. https://doi.org/10.1016/j.idairyj.2023.105671.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37 (8), 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Braghieri, A., Girolami, A., Riviezzi, A.M., Piazzolla, N., Napolitano, F., 2014. Liking of traditional cheese and consumer willingness to pay. Ital. J. Anim. Sci. 13 (1), 3029. https://doi.org/10.4081/ijas.2014.3029.

Busetta, G., Gaglio, R., Mangione, G., Garofalo, G., Franciosi, E., Gannuscio, R., Caccamo, M., Todaro, M., Di Gerlando, R., Settanni, L., Licitra, G., 2023a. Effect of commission implementing regulation (EU) 2020/1319 on the bacterial composition of PDO Provola dei Nebrodi cheese. Int. J. Food Microbiol. 394, 110188. https://doi.org/10.1016/j.ijfoodmicro.2023.110188.

Busetta, G., Garofalo, G., Barbera, M., Di Trana, A., Claps, S., Lovallo, C., Franciosi, E., Gaglio, R., Settanni, L., 2023b. Metagenomic, microbiological, chemical and sensory profiling of Caciocavallo Podolico Lucano cheese. Food Res. Int. 169, 112926. https://doi.org/10.1016/j.foodres.2023.112926.

Busetta, G., Garofalo, G., Mangione, G., Botta, L., Franciosi, E., Di Gerlando, R., Todaro, M., Licitra, G., Scatassa, M. L., Gaglio, R., Settanni, L., 2021. Polyphasic characterization of microbiota of "mastredda", a traditional wooden tool used during the production of PDO Provola dei Nebrodi cheese. Appl. Sci. 11 (18), 8647. https://doi.org/10.3390/app11188647.

Caggia, C., De Angelis, M., Pitino, I., Pino, A., Randazzo, C.L., 2015. 'Probiotic features of *Lactobacillus* strains isolated from Ragusano and Pecorino Siciliano cheeses. Food Microbiol. 50, 109–17. https://doi.org/10.1016/j.fm.2015.03.010.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13 (7), 581–583. https://doi.org/10.1038/nmeth.3869.

Caridi, A., Micari, P., Caparra, P., Cufari, A., Sarullo, V., 2003. Ripening and seasonal changes in microbial groups and in physico-chemical properties of the ewes' cheese Pecorino del Poro. Int. Dairy J. 13 (2-3), 191–200. https://doi.org/10.1016/S0958-6946(02)00157-7.

Centorotola, G., Sperandii, A.F., Tucci, P., D'Alterio, N., Ricci, L., Goffredo, E., Mancini, M.E., Iannetti, L., Salini, R., Migliorati, G., Pomilio, F., Valiani, A., Ortenzi, R., Lanni, L., 2021. Survival rate of *Escherichia coli* O157 in artificially contaminated raw and thermized ewe milk in different Pecorino cheese production processes. Int. J. Food Microbiol. 347, 109175. https://doi.org/10.1016/j.ijfoodmicro.2021.109175.

Chiofalo, B., Di Rosa, A.R., Lo Presti, V., Chiofalo, V., Liotta, L., 2020. Effect of supplementation of herd diet with olive cake on the composition profile of milk and on the composition, quality and sensory profile of cheeses made therefrom. Animals 10 (6), 977. https://doi.org/10.3390/ani10060977.

Commission Regulation, 1996. Commission regulation no 1107/1996 of 12 June 1996 on the registration of geographical indications and designations of origin under the procedure laid down. Off. J. Eur. Union L148/6.

Commission Regulation, 2005. Commission regulation no 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union 338, 1–26.

Coppa, M., Martin, B., Pradel, P., Leotta, B., Priolo, A., Vasta, V., 2011. Effect of a hay-based diet or different upland grazing systems on milk volatile compounds. J. Agric. Food Chem. 59, 4947–4954. https://doi.org/10.1021/jf2005782.

Cruciata, M., Gaglio, R., Scatassa, M.L., Sala, G., Cardamone, C., Palmeri, M., Moschetti, G., La Mantia, T., Settanni, L., 2018. 'Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production. Appl. Environ. Microbiol. 84 (4), https://doi.org/10.1128/AEM.02107-17.

De Pasquale, I., Di Cagno, R., Buchin, S., De Angelis, M., Gobbetti, M., 2016. Spatial distribution of the metabolically active microbiota within Italian PDO ewes' milk cheeses. PLOS One 11 (4), https://doi.org/10.1371/journal.pone.0153213.

Di Rosa, A.R., Leone, F. 2018. Application of electronic nose systems on animal source food – An overview. In: Albastaki, Y.A., Albalooshi, F. (Eds.), Electronic Nose Technologies and Advances in Machine Olfaction. IGI Global, pp. 151–174.

Di Rosa, A.R., Leone, F., Bressan, F., Battaglia, G., Veccia, T., Chiofalo, V., 2018. Radio frequency heating of milk–effects on quality, safety, and shelf life assessed using artificial senses and chemometric tools. Electronics 7 (12), 402. https://doi.org/10.3390/electronics7120402.

Didienne, R., Defargues, C., Callon, C., Hulin, S., Montel, M.C., 2012. Characteristics of microbial biofilm on wooden vats ("gerles") in PDO Salers cheese. Int. J. Food Microbiol. 156 (2) 91–101. https://doi.org/10.1016/j.ijfoodmicro.2012.03.007.

Fiorentini, M., Kinchla, A.J., Nolden, A.A., 2020. Role of sensory evaluation in consumer acceptance of plant-based meat analogs and meat extenders: A scoping review. Foods 9 (9), 1334. https://doi.org/10.3390/foods9091334.

Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L., 2006. The role and application of enterococci in food and health. Int. J. Food Microbiol. 106, 1–24. https://doi.org/10.1016/j.ijfoodmicro.2005.06.026.

Fox, P.F., McSweeney, P.L., Cogan, T.M., Guinee, T.P. 2004. Cheese: Chemistry, physics and microbiology, third ed. Elsevier.

Gaglio, R., Cruciata, M., Di Gerlando, R., Scatassa, M.L., Cardamone, C., Mancuso, I., Sardina, M.T., Moschetti, G., Portolano, B., Settanni, L., 2016. Microbial activation of wooden vats used for traditional cheese production and evolution of the neo-formed biofilms. Appl. Environ. Microbiol. 82 (2), 585–595. https://doi.org/10.1128/AEM.02868-15.

Gaglio, R., Cruciata, M., Scatassa, M.L., Tolone, M., Mancuso, I., Cardamone, C., Corona, O., Todaro, M., Settanni, L., 2019b. Influence of the early bacterial biofilms developed on vats made with seven wood types on PDO Vastedda della valle del Belìce cheese characteristics. Int. J. Food Microbiol. 291, 91–103. https://doi.org/10.1016/j.ijfoodmicro.2018.11.017.

Gaglio, R., Francesca, N., Di Gerlando, R., Cruciata, M., Guarcello, R., Portolano, B., Settanni, L., 2014. Identification, typing and investigation of the dairy characteristics of lactic acid bacteria isolated from "Vastedda della valle del Belìce" cheeses. Dairy Sci. Technol. 94 (2), 157–180. https://doi.org/10.1007/s13594-013-0150-5.

Gaglio, R., Francesca, N., Di Gerlando, R., Mahony, J., De Martino, S., Stucchi, C., Moschetti, G., & Settanni, L., 2017. Enteric bacteria of food ice and their survival in alcoholic beverages and soft drinks. Food Microbiol. 67, 17–22. https://doi.org/10.1016/j.fm.2017.04.020.

Gaglio, R., Franciosi, E., Todaro, A., Guarcello, R., Alfeo, V., Randazzo, C.L., Todaro, M., 2020. Addition of selected starter/non-starter lactic acid bacterial inoculums to stabilise PDO Pecorino Siciliano cheese production. Food Res. Int. 136, 109335. https://doi.org/10.1016/j.foodres.2020.109335.

Gaglio, R., Todaro, M., Scatassa, M.L., Franciosi, E., Corona, O., Mancuso, I., Settanni, L., 2019a. Transformation of raw ewes' milk applying "Grana" type pressed cheese technology: Development of extra-hard "Gran Ovino" cheese. Int. J. Food Microbiol. 307, 108277. https://doi.org/10.1016/j.ijfoodmicro.2019.108277.

Gaglio, R., Todaro, M., Settanni, L., 2021. Improvement of raw milk cheese hygiene through the selection of starter and non-starter lactic acid bacteria: The successful case of PDO Pecorino Siciliano cheese. Int. J. Environ. Res. Public Health. 18 (4), 1834. https://doi.org/10.3390/ijerph18041834.

Giammanco, G.M., Pepe, A., Aleo, A., D'Agostino, V., Milone, S., Mammina, C., 2011. Microbiological quality of Pecorino Siciliano" primosale" cheese on retail sale in the street markets of Palermo, Italy. New Microbiol. 34 (2), 179–185.

Gobbetti, M., Calasso, M., 2014. *Streptococcus*: introduction. In: Batt, C.A., Tortello, M.L. (Eds.), Encyclopedia of Food Microbiology. Elsevier, pp. 535–553.

Grasso, C., Eramo, V., Lembo, M., Forniti, R., Carboni, C., Botondi, R., 2023. Effects of gaseous ozone treatment on the mite pest control and qualitative properties during ripening storage of pecorino cheese. J. Sci. Food Agric. (4), 2124–2133. https://doi.org/10.1002/jsfa.12400.

Gregersen, T. (1978). Rapid method for distinction of gram-negative from gram-positive bacteria. Appl. Microbiol. Biotechnol. 5, 123–127. https://doi.org/10.1007/BF00498806.

Guarcello, R., Carpino, S., Raimondo, G., Pino, A., Rapisarda, T., Caggia, C., Marino, G., Randazzo, C., Settanni L., Todaro, M., 2016. A large factory-scale application of selected autochthonous lactic acid bacteria for PDO Pecorino Siciliano cheese production. Food Microbiol. 59, 66–75. https://doi.org/10.1016/j.fm.2016.05.011.

Güler, Z., Türkmen, D., Dursun, A., 2021. Different scalding duration in hot whey influencing chemical, biochemical, physical, microstructural, textural and sensorial characteristics of brine-type cheese. LWT-Food Sci. Technol. 145, 111312. https://doi.org/10.1016/j.lwt.2021.11131.

Hayaloglu, A.A., Brechany, E.Y., 2007. Influence of milk pasteurization and scalding temperature on the volatile compounds of Malatya, a farmhouse Halloumi-type cheese. Lait, 87, 39–57. https://doi.org/10.1051/lait:2006025.

Hayaloglu, A.A., Deegan, K.C., McSweeney, P.L.H., 2010. Effect of milk pasteurization and curd scalding temperature on proteolysis in Malatya, a Halloumi-type cheese. Dairy Sci. Technol. 90 (1), 99–109. https://doi.org/10.1051/dst/2009052.

Hayaloglu, A.A., Ozer, B.H., Fox, P.F., 2008. Cheeses of Turkey: 2. Varieties ripened under brine. Dairy Sci. Technol. 88 (2), 225–244. https://doi.org/10.1051/dst:2007014.

Health Canada, 2015. MFHPB-18 determination of the aerobic colony count in foods. https://www.canada.ca/en/health-canada/services/food-nutrition/research-programs-analytical-methods/analytical-methods/compendium-methods/methods-microbiological-analysis-foods-compendium-analytical-methods.html. Retrieved: 1 July 2023.

IDF, 1964a. Determination of the Protein Content of Processed Cheese Products. In: International Standard FILI-IDF. No. 25. International Dairy Federation, Brussels, Belgium.

IDF, 1964b. Determination of the Ash Content of Processed Cheese Products. In: International Standard FILI-IDF. No 27. International Dairy Federation, Brussels, Belgium.

IDF, 1982. Cheese and Processed Cheese Product. Determination of the Total Solids Content. In: International Standard FILI-IDF Standard 4A. International Dairy Federation, Brussels, Belgium.

IDF, 1986. Cheese and Processed Cheese Product. Determination of Fat Content-Gravimetric Method. In: International Standard FILI-IDF Standard 5B. International Dairy Federation, Brussels, Belgium.

ISO 15213, 2003. Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions.

ISO 4832, 2006. Microbiology of food and animal feeding stuffs-Horizontal method for the enumeration of coliforms - Colony-count echnique.

ISO 8589, 2007. Sensory Analysis-General Guidance for the Design of Test Rooms.

ISO 6888–2, 2021. Microbiologia della catena alimentare-Metodo orizzontale per la conta di stafilococchi coagulasi-positivi (*Staphylococcus aureus* e altre specie) — Parte 2: Metodo che utilizza il terreno agar al plasma di coniglio e al fibrinogeno.

Jagadeesan, B., Gerner-Smidt, P., Allard, M.W., Leuillet, S., Winkler, A., Xiao, Y., Chaffron, S., Van Der Vossen, J., Tang, S., Katase, M., McClure, P., Kimura, B., Chai, C.L., Chapman, J., Grant, K., 2019. The use of next generation sequencing for improving food safety: Translation into practice. Food Microbiol. 79, 96–115. https://doi.org/10.1016/j.fm.2018.11.005.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30 (4), 772–780. https://doi.org/10.1093/molbev/mst010.

Kilcawley, K.N., Faulkner, H., Clarke, H.J., O'Sullivan, M.G., Kerry, J.P., 2018. Factors influencing the flavour of bovine milk and cheese from grass based versus non-grass based milk production systems. Foods 7 (3), 37. https://doi.org/10.3390/foods7030037.

Koneman, E.W., Allen, S.D., Janda, W.M., Schreckenberger, P.C., Winn, W.C., 1997. The nonfermentative gram-negative bacilli. In Koneman, E.W., Allen, S.D., Janda, W.M., Schreckkenberger, P.C., Winn, W.C. (Eds.), Color Atlas and Textbook of Diagnostic Microbiology, seventh ed. Lippincott, Philadelphia, pp. 253–320.

Liotta, L., Randazzo, C.L., Russo, N., Zumbo, A., Di Rosa, A.R., Caggia, C., Chiofalo, V., 2019. Effect of molasses and dried orange pulp as sheep dietary supplementation on physico-chemical, microbiological and fatty acid profile of comisana ewe's milk and cheese. Front. Nutr. 6, 1. https://doi.org/10.3389/fnut.2019.00001.

Lo Presti, V., Tudisco, R., Di Rosa, A.R., Musco, N., Iommelli, P., Infascelli, F., Grossi, M., Ferrara, M., Chiofalo, B., Lombardi, P., Oteri, M., 2023. Influence of season on milk fatty acid profile and sensory characteristics of grazing goats in a Mediterranean environment: a sustainable agro-food system. Anim. Prod. Sci. 63 (7), 689–703. https://doi.org/10.1071/AN21538.

Logares, R., Audic, S., Bass, D., Bittner, L., Boutte, C., Christen, R., Claverie, J.M., Decelle, J., Dolan, J.R., Dunthorn, M., Edvardsen, B., Gobet, A., Kooistra, W.H.C.F., Mahé, F., Not, F., Ogata, H., Pawlowski, J., Pernice, M.C., Romac, S., Massana, R., 2014. Patterns of rare and abundant marine microbial eukaryotes. Curr. Biol. 24, 813–821. https://doi.org/10.1016/j.cub.2014.02.050.

Lucey, J., Kelly, J., 1994. Cheese yield. Int. J. Dairy Technol. 47 (1), 1–14. https://doi.org/10.1111/j.1471-0307.1994.tb01264.x.

MAF (Ministero dell'Agricoltura e delle Foreste), 1986. Decreto ministeriale 21/4/1986. Approvazione dei metodi ufficiali di analisi per i formaggi (Italian official methods of cheeses analysis), No. 8, Page 15. Supplemento ordinario alla Gazzetta Ufficiale della Repubblica Italiana (GURI), n. 229 del 2/10/1986.

Mangione, G., Caccamo, M., Natalello, A., Licitra, G., 2023. Graduate Student Literature Review: History, technologies of production, and characteristics of ricotta cheese. J. Dairy Sci. 106 (6), 3807–3826. https://doi.org/10.3168/jds.2022-22460.

Mariaca, R., Berger, T.R., Imhof, M., Jeangros, B., Bosset, J.O., 1997. Occurrence of volatile mono-and sesquiterpenoids in highland and lowland plant species as possible precursors for flavor compounds in milk and dairy productsJ. Agric. Food Chem. 45 (11), 4423–4434. https://doi.org/10.1021/jf970216t.

Monfredini, L., Settanni, L., Poznanski, E., Cavazza, A., Franciosi, E., 2012. The spatial distribution of bacteria in Grana-cheese during ripening. Syst. Appl. Microbiol. 35 (1), 54–63. https://doi.org/10.1016/j.syapm.2011.07.002.

Nguyen, T.T., Van Giau, V., & Vo, T.K., 2016. Multiplex PCR for simultaneous identification of *E. coli* O157: H7, *Salmonella* spp. and *L. monocytogenes* in food. 3 Biotech 6, 1–9. https://doi.org/10.1007/s13205-016-0523-6.

Qasem, A.A.A., Alamri, M.S., Mohamed, A.A., Hussain, S., Mahmood, K., Ibraheem, M.A., 2017. Soluble

fiber-fortified sponge cakes: formulation, quality and sensory evaluation. J. Food Meas. Charact. 11 (3), 1516–1522. https://doi.org/10.1007/s11694-017-9530-5.

Rajeswara Rao, B.R., Kaul, P.N., Mallavarapu, G.R., Ramesh, S., 1996. Effect of seasonal climatic changes on biomass yield and terpenoid composition of rose-scented geranium (*Pelargonium species*). Biochem. Syst. Ecol. 24, 627–635. https://doi.org/10.1016/S0305-1978(96)00071-3.

Randazzo, C.L., Vaughan, E.E., Caggia, C., 2006. Artisanal and experimental Pecorino Siciliano cheese: microbial dynamics during manufacture assessed by culturing and PCR–DGGE analyses. Int. J. Dairy Technol. 109 (1-2), 1–8. https://doi.org/10.1016/j.ijfoodmicro.2005.11.002.

Rizzello, C.G., Losito, I., Gobbetti, M., Carbonara, T., De Bari, M.D., Zambonin, P.G., 2005. Antibacterial activities of peptides from the water-soluble extracts of Italian cheese varieties. J. Dairy Sci. 88 (7), 2348–2360. https://doi.org/10.3168/jds.S0022-0302(05)72913-1.

Rossetti, L., Giraffa, G., 2005. Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. J. Microbiol. Methods 63 (2), 135–144. https://doi.org/10.1016/j.mimet.2005.03.001.

Ruta, S., Murray, M., Kampff, Z., McDonnell, B., Lugli, G.A., Ventura, M., Todaro, M., Settanni, L., Van Sinderen, D., Mahony, J., 2023. Microbial Ecology of Pecorino Siciliano PDO Cheese Production Systems. Fermentation, 9 (7), 620. https://doi.org/10.3390/fermentation9070620.

Saglam, H., Sarioglu, T., Karahan, A.G., Oner, Z., 2019. An investigation for the development of whey-based probiotic beverages. Rom. Biotechnol. Lett. 24 (6), 1097–1106. doi: 10.25083/rbl/24.6/1097.1106.

Scatassa, M.L., Cardamone, C., Miraglia, V., Lazzara, F., Fiorenza, G., Macaluso, G., Arcuri, L., Settanni, L. Mancuso, I., 2015. Characterisation of the microflora contaminating the wooden vats used for traditional Sicilian cheese production. Ital. J. Food Saf. 4 (1), 4509. doi: 10.4081/ijfs.2015.4509.

Settanni, L., Barbaccia, P., Bonanno, A., Ponte, M., Di Gerlando, R., Franciosi, E., Di Grigoli, A., Gaglio, R., 2020. Evolution of indigenous starter microorganisms and physicochemical parameters in spontaneously fermented beef, horse, wild boar and pork salamis produced under controlled conditions. Food Microbiol. 87, 103385. https://doi.org/10.1016/j.fm.2019.103385.

Settanni, L., Busetta, G., Puccio, V., Licitra, G., Franciosi, E., Botta, L., Di Gerlando, R., Todaro, M., & Gaglio, R., 2021. In-depth investigation of the safety of wooden shelves used for traditional cheese Ripening. Appl. Environ. Microbiol. 87 (23). https://doi.org/10.1128/AEM.01524-21.

Settanni, L., Cruciata, M., Guarcello, R., Francesca, N., Moschetti, G., La Carrubba, V., Gaglio, R., 2020. Valorisation of dairy wastes through kefir grain production. Waste Biomass Valorization, 11, 3979–3985. https://doi.org/10.1007/s12649-019-00718-6.

Settanni, L., Di Grigoli, A., Tornambé, G., Bellina, V., Francesca, N., Moschetti, G., Bonanno, A., 2012. Persistence of wild Streptococcus thermophilus strains on wooden vat and during the manufacture of a traditional Caciocavallo type cheese. Int. J. Food Microbiol. 155 (1-2), 73–81. https://doi.org/10.1016/j.ijfoodmicro.2012.01.022.

Settanni, L., Gaglio, R., Guarcello, R., Francesca, N., Carpino, S., Sannino, C., Todaro, M., 2013. Selected lactic acid bacteria as a hurdle to the microbial spoilage of cheese: application on a traditional raw ewes' milk cheese'. Int. Dairy J. 32 (2), 126–132. https://doi.org/10.1016/j.idairyj.2013.04.010.

Settanni, L., Moschetti, G., 2014. New trends in technology and identity of traditional dairy and fermented meat production processes: preservation of typicality and hygiene. Trends Food Sci. Technol. 37 (1), 51–58. https://doi.org/10.1016/j.tifs.2014.02.006.

Settanni, L., Moschetti, G., 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiol. 27, 691–697. https://doi.org/10.1016/j.fm.2010.05.023.

Solís, L., Sánchez, E., García, S., Heredia, N., 2008. Traditional methods for detection of foodborne pathogens. In: Heredia, N., Wesley, I., Garcia, S. (Eds.), Microbiologically Safe Foods. Wiley, New York, pp. 525–545.

Sulejmani, E., Rafajlovska, V., Güneser, O., Karagül-Yüceer, Y., Hayaloglu, A.A., 2014. Volatile compounds and proteolysis in traditional Beaten (Bieno sirenje) ewe's milk cheese. Int. J. Dairy Technol. 67, 584–593. https://doi.org/10.1111/1471-0307.12159.

Tavman, I.H., Tavman, S., 1999. Measurement of thermal conductivity of dairy products. J. Food Eng. 41, 109–114. http://dx.doi.org/10.1016/s0260-8774(99)00079-5.

Todaro, M., Francesca, N., Reale, S., Moschetti, G., Vitale, F., Settanni, L., 2011. Effect of different salting technologies on the chemical and microbiological characteristics of PDO Pecorino Siciliano cheese. Eur. Food Res. Technol. 233 (6), 931–940. https://doi.org/10.1007/s00217-011-1593-7.

Vernile, A., Giammanco, G., Spano, G., Beresford, T.P., Fox, P.F., Massa, S., 2008. Genotypic characterization of lactic acid bacteria isolated from traditional Pecorino Siciliano cheese. Dairy Sci. Technol. 88 (6), 619–629. https://doi.org/10.1051/dst:2008009.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173 (2), 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M., Harris, H.M., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. Int. J. Syst. Evol. 70 (4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107.

Ending notes

Traditional cheeses from around the world should not be viewed merely as food, but as reflections of the history, culture, and lifestyle of their producer communities. These cheeses are deeply connected to their regions of origin and uniquely embody the interaction between human resources, community culture, and nature, significantly contributing to development, sustainability, and economic viability. However, cheeses made with raw milk and wooden equipment have sparked international debate about food safety.

The main objective of this PhD program was to enhance historical cheeses typical of southern Italy and to thoroughly understand the role of LAB associated with the wooden equipment used in artisanal production. Our findings revealed that LAB in the microbial biofilms on wooden equipment act as reservoir of microbial biodiversity, improving the quality, authenticity, safety, and flavour profile of artisanal cheeses. These protechnological bacteria on wooden equipment surfaces play a crucial role in curd acidification and cheese ripening. Additionally, the use of wood was shown to provide biodefense against major food pathogens, indicating that wood is a suitable material for cheese-making and maturing processes. Ongoing work aims to apply selected bacteria from dairy productions to evaluate their antimicrobial activity against foodborne pathogens, stabilizing artisanal cheeses made with raw milk and wooden equipment.

Papers published in the three-year PhD course

Publications in international journals:

1. Barbaccia, P., Francesca, N., Gerlando, R. D., **Busetta, G.**, Moschetti, G., Gaglio, R., Settanni, L. (2020). Biodiversity and dairy traits of indigenous milk lactic acid bacteria grown in presence of the main grape polyphenols. FEMS Microbiology Letters, 367(8), fnaa066. https://doi.org/10.1093/femsle/fnaa066.
2. Barbaccia, P., **Busetta, G.**, Matraxia, M., Sutera, A. M., Craparo, V., Moschetti, G., Francesca, N., Settanni, L., Gaglio, R. (2021). Monitoring commercial starter culture development in presence of red grape pomace powder to produce polyphenol-enriched fresh ovine cheeses at industrial scale level. Fermentation, 7(1), 35. https://doi.org/10.3390/fermentation7010035.
3. **Busetta, G.**, Garofalo, G., Mangione, G., Botta, L., Franciosi, E., Di Gerlando, R., Todaro, M., Licitra, G., Scatassa, M.L., Gaglio, R., Settanni, L. (2021). Polyphasic characterization of microbiota of "Mastredda", a traditional wooden tool used during the production of PDO Provola dei Nebrodi cheese. Applied Sciences, 11, 8647. https://doi.org/10.3390/app11188647.
4. Gaglio, R., Barbera, M., Tesoriere, L., Osimani, A., **Busetta, G.**, Matraxia, M., Attanzio, A., Restivo, I., Aquilanti, L., Settanni, L. (2021). Sourdough "ciabatta" bread enriched with powdered insects: Physicochemical, microbiological, and simulated intestinal digesta functional properties. Innovative Food Science & Emerging Technologies, 72, 102755. https://doi.org/10.1016/j.ifset.2021.102755.
5. Settanni, L., **Busetta, G.**, Puccio, V., Licitra, G., Franciosi, E., Botta, L., Di Gerlando, R., Todaro, M., and Gaglio, R. (2021). In-depth investigation of the safety of wooden shelves used for traditional cheese Ripening. Applied and Environmental Microbiology, 87(23), e01524-21. https://doi.org/10.1128/AEM.01524-21.
6. Passafiume, R., Gugliuzza, G., Gaglio, R., **Busetta, G.**, Tinebra, I., Sortino, G., & Farina, V. (2021). Aloe-based edible coating to maintain quality of fresh-cut italian pears (Pyrus communis L.) during cold storage. Horticulturae, 7(12), 581. https://doi.org/10.3390/horticulturae7120581.
7. Garofalo, G., **Busetta, G.**, Maniaci, G., Sardina, M.T., Portolano, B., Badalamenti, N., Maggio, A., Bruno, M., Gaglio, R., Settanni, L. (2022). Development of "Quadrello di Ovino", a novel fresh ewe's cheese. Foods, 11, 25. https://doi.org/10.3390/foods11010025.
8. Tinebra, I., Scuderi, D., **Busetta, G.**, Palazzolo, E., Settanni, L., Farina, V. (2022). Modified atmosphere packaging and low temperature storage extend marketability of cherimoya (*Annona cherimola* Mill.). Italus Hortus, 29, 115-137. https://doi: 10.26353/j.itahort/2022.1.115137.
9. Gaglio, R., **Busetta, G.**, Gannuscio, R., Settanni, L., Licitra, G., Todaro, M. (2022). A multivariate approach to study the bacterial diversity associated to the wooden shelves used for aging traditional Sicilian cheeses. Foods, 11(5), 774. https://doi.org/10.3390/foods11050774.
10. Barbaccia, P., **Busetta, G.**, Barbera, M., Alfonzo, A., Garofalo, G., Francesca, N., Moscarelli, A., Moschetti, G., Settanni, L., Gaglio, R. (2022). Effect of grape pomace from red cultivar

'Nero d'Avola' on the microbiological, physicochemical, phenolic profile and sensory aspects of ovine Vastedda-like stretched cheese. Journal of Applied Microbiology, 133, 130-144. https://doi.org/10.1111/jam.15354.

11. Mannino, G., Serio, G., Gaglio, R., **Busetta, G.**, La Rosa, L., Lauria, A., Settanni, L., Gentile, C. (2022). Phytochemical profile and antioxidant, antiproliferative, and antimicrobial properties of *Rubus idaeus* seed powder. Foods, 11(17), 2605.
12. **Busetta, G.**, Ponte, M., Barbera, M., Alfonzo, A., Ioppolo, A., Maniaci, G., Guarcello, R., Francesca, N., Palazzolo, E., Bonanno, A., Moschetti, G., Settanni, L., Gaglio, R. (2022). Influence of citrus essential oils on the microbiological, physicochemical and antioxidant properties of Primosale cheese. Antioxidants, 11(10), 2004.
13. **Busetta, G.**, Gaglio, R., Mangione, G., Garofalo, G., Franciosi, E., Gannuscio, R., Caccamo, M., Todaro, M., Di Gerlando, R., Settanni, L., Licitra, G. (2023). Effect of commission implementing regulation (EU) 2020/1319 on the bacterial composition of PDO Provola dei Nebrodi cheese. International Journal of Food Microbiology, 394, 110188. https://doi.org/10.1016/j.ijfoodmicro.2023.110188.
14. **Busetta, G.**, Garofalo, G., Barbera, M., Di Trana, A., Claps, S., Lovallo, C., Franciosi, E., Gaglio, R., Settanni, L. (2023). Metagenomic, microbiological, chemical and sensory profiling of Caciocavallo Podolico Lucano cheese. Food Research International, 169, 112926. https://doi.org/10.1016/j.foodres.2023.112926.
15. Maniaci, G., Ponte, M., Giosuè, C., Gannuscio, R., Pipi, M., Gaglio, R., **Busetta, G.**, Di Grigoli, A., Bonanno, A., Alabiso, M. (2024). Cladodes of *Opuntia ficus-indica* (L.) as a source of bioactive compounds in dairy products. Journal of Dairy Science, 107(4), 1887-1902. https://doi.org/10.3168/jds.2023-23847.
16. Garofalo, G., Ponte, M., **Busetta, G.**, Tolone, M., Bonanno, A., Portolano, B., Gaglio, R., Erten, H., Sardina, M.T., Settanni, L. (2023). A Thorough Investigation of the Microbiological, Physicochemical, and Sensory Properties of Ewe's Yoghurt Fermented by a Selected Multi-Strain Starter Culture. Foods, 12, 3454. https://doi.org/10.3390/foods12183454.
17. **Busetta, G.**, Garofalo, G., Claps, S., Sardina, M.T., Franciosi, E., Alfonzo, A., Francesca, N., Moschetti, G., Settanni, L., Gaglio, R. (2024). The wooden shelf surface and cheese rind mutually exchange microbiota during the traditional ripening process. International Journal of Food Microbiology, 409, 110478. https://doi.org/10.1016/j.ijfoodmicro.2023.110478.
18. Todaro, M., Garofalo, G., **Busetta, G.**, Gannuscio, R., Di Rosa, A.R., Scatassa, M.L., Cardamone, C., Mancuso, I., Franciosi, E., Rando, F., Agnolucci, M., Chiofalo, V., Gaglio, R., Settanni, L. (2024). Reduction of PDO Pecorino Siciliano cheese making duration: Microbial dynamics and quality attributes deriving from replacing whey permeate with hot water during cooking. International Journal of Food Microbiology, 410, 110481. https://doi.org/10.1016/j.ijfoodmicro.2023.110481.
19. Viola, E., Garofalo, G., **Busetta, G.**, Supper, M., Alfonzo, A., Tolone, M., Francesca, N., Moschetti, G., Sottile, F., Gaglio, R., Settanni, L. (2024). Selection of lactic acid bacteria from home-made sourdoughs for resistance to the main almond skin polyphenols. Journal of Agriculture and Food Research, 15, 100951. https://doi.org/10.1016/j.jafr.2023.100951.
20. Garofalo, G., Gaglio, R., **Busetta, G.**, Ponte, M., Barbera, M., Riggio, S., Piazzese, D., Bonanno, A., Erten, H., Sardina, M.T., Settanni, L. (2024). Addition of fruit purees to enhance

quality characteristics of sheep yogurt with selected strains. Journal of Agriculture and Food Research, 16, 101153. https://doi.org/10.1016/j.jafr.2024.101153.

21. Garofalo, G., Ponte, M., **Busetta, G.**, Barbera, M., Tinebra, I., Piazzese, D., Franciosi, E., Di Grigoli, A., Farina, V., Bonanno, A., Gaglio, R., Settanni, L. (2024). Microbial dynamics and quality characteristics of spontaneously fermented salamis produced by replacing pork fat with avocado pulp. Food Microbiology, 122, 104536. https://doi.org/10.1016/j.fm.2024.104536
22. Garofalo, G., Taspinar, T., **Busetta, G.**, Mastrangelo, S., Portolano, B., Sardina, M. T., Gaglio, R., Erten, H., Settanni, L. (2024). Description of Ewiss cheese, a new ewe's milk cheese processed by Swiss cheese manufacturing techniques: microbiological, physicochemical and sensory aspects. Journal of Dairy Science, 6614-6628. https://doi.org/10.3168/jds.2024-24711.
23. Gaglio, R., Garofalo, G., Taspinar, T., **Busetta, G.**, Barbera, M., Sutera, A. M., Alfonzo, A., Sardina, M. T., Erten, H., Settanni, L. (2024). Description of "Ovino Belmontese", a new semisoft sheep's milk cheese processed using "Italico" cheese technology. Food Bioscience, 104450.

Publications in national journals:

1. Garofalo, G., **Busetta, G.**, Alfonzo, A., Francesca, N., Moschetti, G., Settanni, L., Gaglio, R. (2022). Effect of red wine soaking on the microbiological profile, total phenolic content and sensory aspects of an ovine pressed cheese. Scienza e Tecnica Lattiero-Casearia, 72(2), 56–62. https://doi.org/10.36138/STLC.02.2022.02.
2. **Busetta, G.**, Guarcello, R., Ioppolo, A., Sciurba, L., Palazzolo, E., Settanni, L., Gaglio, R. (2023). Anti-*Listeria* activity of citrus essential oils in a fresh ovine cheese. Scienza e Tecnica Lattiero-Casearia, 73(3), 50-55. https://dx.doi.org/10.36138/STLC.03.2023.01.

International symposium submissions:

1. **Busetta, G.**, Garofalo, G., Elena, F., Clamps, S., Francesca, N., Moschetti, G., Gaglio, R., Settanni, L. Wooden shelves: an ancient tool for sustainable cheese ripening in future. 7th International Conference on Microbial Diversity – "Agrifood microbiota as a tool for a sustainable future". Parma, 26-29 Settembre 2023.

National symposium submissions:

1. Liguori, G., Greco, G., **Busetta, G.**, Cannatella, R., Gaglio, R., Inglese, P. (2021). Valutazione dell'applicazione di edible coating sulla qualità dei frutti di fragola durante la frigoconservazione. XIII Giornate Scientifiche SOI. Catania, 22-23 giugno 2021. 54.
2. Garofalo, G., Taspinar, T., **Busetta, G.**, Mastrangelo, S., Portolano, B., Sardina, M.T., Erten, H., Gaglio, R., Settanni, L. Description of Ewiss cheese, a new ewe's milk cheese processed by Swiss cheese manufacturing techniques: microbiological, physicochemical and sensory aspects. Aissa under 40 2024 V convegno Giugno 26-27, 2024 – Firenze, Italia
3. Ponte, M., Maniaci, G., Pipi, M., Gannuscio, R., **Busetta, G.**, Gaglio, R., Settanni, L., Alabiso, M., Todaro, M., Bonanno, A., Di Grigoli, A. Dehydration preserves the ability of tanniferous

sulla forage (Sulla coronaria (L.) Medik.) to improve nutritional, microbial and sensory traits of sheep cheese. 25th Aspa Congress. Monopoli, 13-16 Giugno 2023

Oral communications in national conferences:

1. **Busetta, G.**, Garofalo, G., Franciosi E., Claps S., Francesca, N., Moschetti, G., Gaglio, R., Settanni, L. Wooden shelves: an ancient tool for sustainable cheese ripening in future. Microbial Diversity 2023 7th International Conference on Microbial Diversity Agrifood microbiota as a tool for a sustainable future September 26-29, 2023 – Parma, Italy.
2. **Busetta, G.** Microbial Characterization of wooden equipment used in southern Italy for the production of traditional cheese. 28th Workshop on the Developments in the Italian PhD research on Food Science, Tecnology and Biotecnology September 18-20, 2024 – Catania, Italy.