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Application of *ad hoc* transformation protocols and selected lactic acid bacteria for the innovation in the ewes' milk dairy production sector

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Abstract

Innovation plays a pivotal role across various economic sectors, and dairy products hold significant sway in the food market. In Italy, age-old tradition of making cheese from ewe's milk has gained renewed prominence. Ewe's milk cheeses now serve not only as a primary economic pursuit but also as vital contributors to the tourism industry. They help showcase local products, preserve cultural heritage, and celebrate natural landscapes. Consequently, cheese production has emerged as a critical driver for rural development, diversification, and innovation. Sicily, a Euro-Mediterranean region, actively promotes the transformation of this milk into different cheese varieties. However, the small ruminant dairy sector faces organisational hurdles. Many dairies focus on the breeding and niche-market cheese production, relying on traditional techniques and equipment. Despite these challenges, sectoral innovation remains essential for the economic survival of small dairy farms.

Given these considerations, within the context of a three-year PhD program, a decision was reached to advance the ovine dairy industry. The primary objective was to expand the range of Sicilian dairy offerings by introducing novel cheeses with significant nutritional and sensory value for consumers. Achieving this involved incorporating polyphenolic compounds, which have long been recognized for their beneficial antioxidant properties in human body, as well as ingredients like essential oils that act as natural preservatives due to their antimicrobial effects. This strategic approach not only addressed the growing consumer demand for transparency regarding raw material origin, quality, and safety, but also paved the way for innovative protocols. These protocols were specifically designed for producing fresh cheeses from pasteurized ewe's milk, ensuring compliance with rigorous hygienic and microbiological standards. Additionally, they extended to the

creation of fermented dairy products such as yogurt and ripened cheeses, using raw and pasteurized milk. Utilizing carefully selected microbial starters, a Whole Genome Sequencing Analysis (WGSA) was conducted to characterize their genetic patterns comprehensively. This approach allowed for rigorous quality and safety control. Across multiple research papers, the results consistently demonstrated the hygienic and sanitary safety of the final dairy products. Additionally, there was notable improvement in nutritional attributes, including fatty acids and organic compounds. Judges, evaluating these products on a sensory level, expressed overall satisfaction.

Zooming out, this research sheds light on how product innovation within the primary sector can impact rural development and local economies, especially in marginal areas. For instance, through tourism, cheese serves as an embodiment of gastronomic culture and cultural identity. Modern consumers increasingly appreciate the authenticity and genuine connection to the world that cheese represents.

Sommario

L'innovazione riveste un ruolo fondamentale in vari settori economici e i prodotti lattiero-caseari esercitano un'influenza significativa sul mercato alimentare. In Italia la secolare tradizione di produrre formaggi di pecora ha guadagnato nuova rilevanza. I formaggi ovini rappresentano non solo un'attività economica primaria, ma fanno anche da volano per l'industria del turismo. Essi contribuiscono a valorizzare i prodotti locali, preservare il patrimonio culturale e apprezzare i paesaggi naturali. Di conseguenza, la produzione di formaggi si è affermata come un motore critico per lo lo sviluppo, la diversificazione e l'innovazione. In Sicilia, una regione euro-mediterranea, si promuove attivamente la trasformazione di questo latte in diverse varietà di formaggio. Tuttavia, il settore lattiero-caseario dei piccoli ruminanti affronta ostacoli organizzativi. Molte aziende casearie si concentrano sulla riproduzione e sulla produzione di formaggi di nicchia, affidandosi a tecniche e attrezzature tradizionali. Nonostante queste sfide, l'innovazione settoriale rimane essenziale per la sopravvivenza economica delle aziende casearie.

Tenendo conto di queste considerazioni, all'interno del contesto di un programma di dottorato triennale, si è giunti a una decisione per promuovere l'industria lattiero-casearia ovina. L'obiettivo principale era ampliare la gamma prodotti caseari siciliani introducendo formaggi innovativi ad elevato valore nutrizionale e organolettico per i consumatori. Per raggiungere questo obiettivo, si è proceduto con l'aggiunta di sostanze polifenoliche, da tempo riconosciute per le proprietà antiossidanti benefiche per il corpo umano, nonché di ingredienti quali oli essenziali, che agiscono come conservanti naturali grazie ai loro effetti antimicrobici. Questo approccio strategico non solo ha affrontato la crescente domanda dei consumatori riguardo alla trasparenza sull'origine delle materie prime sulla qualità e sulla

sicurezza, ma ha anche aperto la strada a protocolli innovativi. Tali protocolli sono stati appositamente progettati per la produzione di formaggi freschi a base di latte ovino pastorizzato, garantendo il rispetto di rigorosi standard igienici e microbiologici. Inoltre, si sono estesi alla creazione di prodotti lattiero-caseari fermentati, come lo yogurt e i formaggi stagionati, utilizzando sia il latte crudo che quello pastorizzato. Sfruttando starter microbici attentamente selezionati, è stata condotta un'Analisi del Sequenziamento del Genoma Completo (WGSA) per caratterizzarne in modo completo i modelli genetici. Questo approccio ha permesso un rigoroso controllo di qualità e sicurezza. Attraverso diversi articoli di ricerca, i risultati hanno costantemente dimostrato la sicurezza igienico e sanitaria dei prodotti finiti. Inoltre, si è registrato un notevole miglioramento delle caratteristiche nutrizionali, tra cui gli acidi grassi e i composti organici. Gli assaggiatori, valutando questi prodotti a livello sensoriale, hanno espresso un apprezzamento generale.

Allargando la prospettiva, questa ricerca getta luce su come l'innovazione dei prodotti, nel settore primario possa influenzare lo sviluppo rurale e le economie locali, specialmente nelle aree marginali. Ad esempio, attraverso il turismo, il formaggio rappresenta un forte elemento della cultura culinaria e dell'identità culturale. I consumatori moderni apprezzano sempre più l'autenticità e il legame genuino con il mondo che il formaggio rappresenta.

Introduction

1. Cheese, history overview and production technology

For millennia, milk and dairy products have been an integral part of the human diet, playing a crucial role in nourishing and supporting the development of human populations worldwide.

Food fermentations were adopted by humans due to fortuitous events, leading to changes in the biochemical properties of raw materials and subsequent repetition of processes that allowed for empirical reproduction [1]. Evidence indicates that the first rudimentary cheeses were discovered after transporting milk in containers made from sheep stomachs. Enzymatic residues within the stomach, involved in casein digestion, led to the initial presamic coagulation during milk storage and transport. Humans became aware of a new food product derived from milk, which, unlike milk itself, could be stored longer and more easily [2].

Food reflects human society, and the cheesemaking process is believed to date back to the “Fertile Crescent” between the Tigris and Euphrates rivers in Iraq, approximately 8000 years ago, during the “agricultural revolution” when animals were domesticated. Among the earliest domesticated animals were goats and sheep, which were small, social, and easily herded [3]. These animals served as source of meat, milk, hides, and wool. Cattle, on the other hand, were more challenging to domesticate. Wild cattle were larger and more aggressive than modern cattle and were less suited to the arid Middle East compared to goats and sheep. Initially, cows were primarily used as work animals, and it was only recently that they were recognized as a milk source (Figure 1) [4]. However, the nutritional

value of milk from domesticated animals was soon acknowledged, and milk and its derivatives became vital components of the human diet.

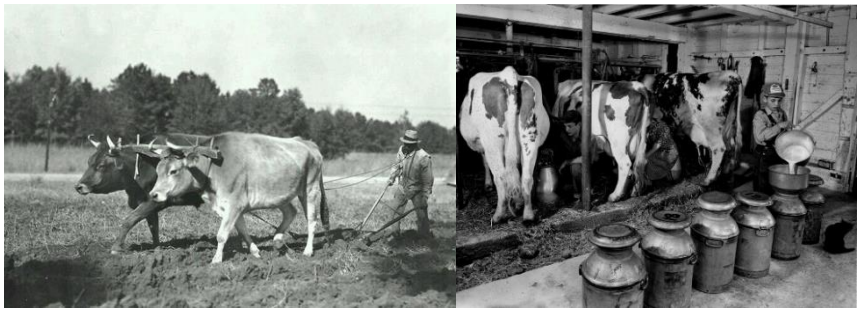

Figure 1. Cattle taming, (https://www.gravelroots.net/history).

Cheesemaking has played a significant role in the spread of civilisation across the Middle East, Egypt, Greece and Rome. References to cheese abound are found in various historical texts and contexts: in the Old Testament, figures like Giobbe (1520 B.C.) and Samuele (1170–1017 B.C.) are mentioned in connection with cheese; Ancient Egyptian tombs feature depictions related to cheese; classical Greek literature by authors such as Homer (1184 B.C.), Herodotus (484–408 B.C.) and Aristotele (384–322 B.C.) also alludes to cheese.

Classical Rome firmly established cheese production, and it even formed part of the rations for Roman soldiers. Numerous Roman writers, including Catone the Elder (234–149 B.C.), Varrone (116–27 B.C.), Columella (4–70 A.D.), Plinio the Elder (23-79 A.D.), and Palladius (400-470 A.D.), provided insight into cheese production, quality characteristics, and culinary uses. Notably, Columella offered a detailed report on cheese production in his agricultural treatise, *De Re Rustica* [5].

The extensive adoption of cheese was facilitated by mass migrations of people across Europe following the decline of the Roman Empire, as well as by the crusaders and pilgrims during the Middle Ages [6]. However, the monasteries and fiefdoms played

pivotal roles in advancing cheese technology and shaping the diversity of cheese varieties during this period [7].

Cheesemaking boasts a rich history, evident in the diverse range of techniques employed in its production. This historical evolution has led to the existence of about 400 types of cheeses with nearly 1000 distinct names. Remarkably, cheeses stand out as the most versatile category of dairy products. Despite sharing common raw materials, such as cow's, sheep's, goat's, or buffalo's milk, lactic acid bacteria (LAB), coagulant, and NaCl, a vast array of cheeses can be produced (Figure 2). Indeed, the saying holds true: “there is a cheese for every taste preference and a taste preference for every cheese” [8]. The high nutritional value and pleasing sensory attributes of cheese contribute to its widespread consumption [9].

In the present day, the dairy industry stands as a cornerstone of the European agri-food sector, and Italy ranks among the countries with the most extensive and diverse cheese production made from cow’s, sheep’s, goat’s and buffalo’s milk. This not only underscores the economic and industrial significance of this sector, but also emphasizes the ubiquitous consumption of dairy products and their impact on consumers' health and quality of life.

Despite the wide variety of cheese products, each distinguished by specific sensory and textural characteristics, the Codex Alimentarius broadly defines "cheese" as encompassing fresh, cured, semi-cured, solid, and semi-solid product resulting from the coagulation of milk. This milk can be whole, skimmed, or semi-skimmed, and the coagulation process may also involve other products such as cream, buttermilk, and whey, either individually or in combination. Although this definition appears simplistic, it effectively encompasses a

diverse range of cheeses, each distinct in its own right and amenable to various classification criteria [10].

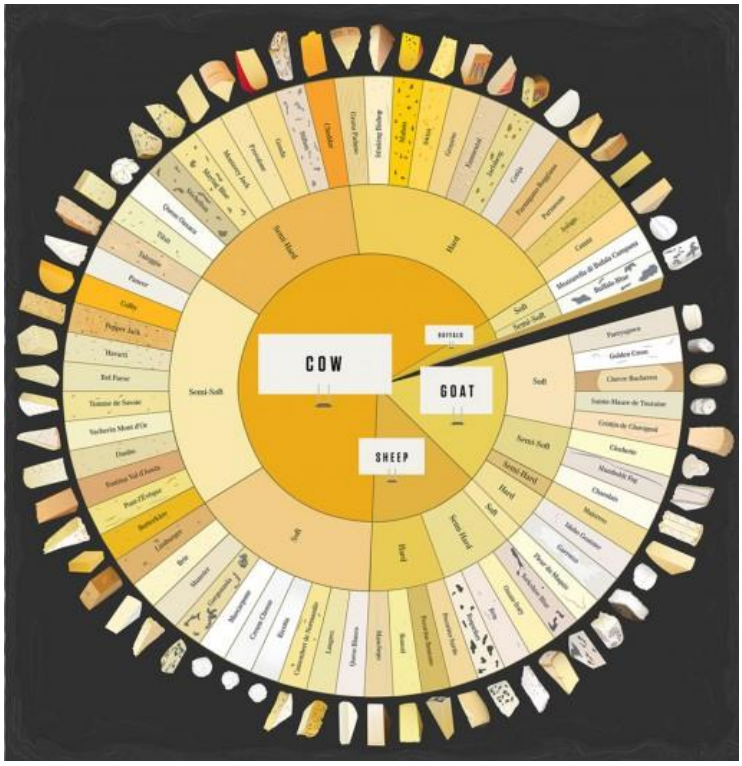

Figure 2. Variety of cheeses obtainable from the same raw materials, (https://www.fastcompany.com).

Cheese production spans the globe, yielding numerous varieties with distinct flavors, aromas, and textures, all following a general process. The first phase of cheese making involves the acidification of milk; fermenting agents, notably LAB, and rennet enzymes initiate this phase. Milk transition from a liquid state to a semi-solid one (Figure 3) due to destabilization in the primary milk protein structure.

Caseins in cows, goat, and sheep milk come in five subtypes: α-, β-, αs1-, αs2-, and κ-casein. κ-casein, crucial for preventing casein micelles from sticking together due to ionic charge repulsion, is packaged in stable micelles [11]. Acidification destabilizes κ-casein, leading to micelle collision and curd formation. Curd comprises casein, calcium, and other divalent minerals tightly associated with milk fat. The second step is to break the coagulum

and separate curd from whey. Whey contains water, lactose and truly soluble proteins like α-lactalbumin, β-lactoglobulin, and serum albumin. It also includes some enzymes and protein fractions (about one-third of total milk protein) and monovalent ions (mainly K^+ and Na^+). During the final step, the curd is moulded into shape, salted, and ripened based on the desired cheese product [12]. This intricate process results in a rich tapestry of cheeses, each with its own story and character.

Figure 3. Coagulation of milk.

Dairy products, including milk, fall into the category of nutrient-rich foods. They offer a substantial amount of nutrients and play a crucial role in maintaining health across all stages of life [13]. As consumer demand for dairy products continues to rise, the global dairy market is expected to expand further in the year ahead. Addressing this challenge requires collaboration between academia and industry [14].

2. Milk and the principal milk species

Milk, produced by female mammals, is a glandular secretion. It appears as a rich white emulsion, primarily composed of water and containing essential components such as fat, protein, lactose, minerals, enzymes, cells, hormones, immunoglobulins, and vitamins. With an almost neutral pH and high water activity, milk's composition varies significantly across species [15]. Even within the same species, differences arise due to various natural factors, including the animal's breed, lactation period, number of lactations, seasonal

variations, diet, and geographical location. Milk serves as a highly nutritious food, particularly crucial for meeting the energy requirements of newborns [16].

The primary species involved in dairy farming include cows, goats, sheep, and buffalo. Among these, cattle are the dominant species in dairy production (Figure 4). Globally, cow's milk constitutes approximately 85% of total milk production, accounting for at least 80% in all regions [17]. The success of bovine milk in the market can be attributed to its relatively stable composition the year, with minimal variation between seasons due to year-round breeding. In contrast, sheep and goat milk, produced mainly by seasonal rearing, exhibit more pronounced changes in composition towards the end of lactation. These changes include increased fat, protein, solids, and minerals, along with a decrease in lactose content [3]. Moreover, a large-scale industrialisation of this supply chain is limited due to the low production yield of around 50 kg per year [18].

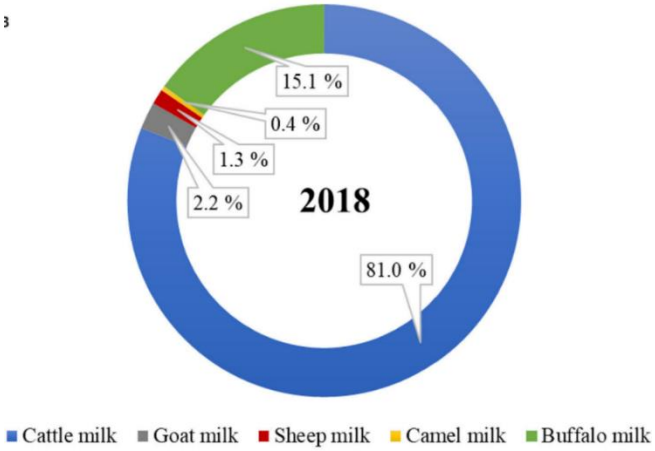

Figure 4. Percentage of bovine and non-bovine milk produced globally in 2018. (Source: FAOstat, March 2020).

Despite cow's milk being the primary source of global milk production, dairy products derived from small ruminants' milk are gaining popularity among researchers and industry professionals due to their unique taste and nutritional properties [19]. The distinct protein

and fat content differences between ewe's milk and cow's milk contribute to the varying technological and sensory characteristics of cheese.

Ewe's milk boasts higher levels of protein and fat compared to cow's milk [20]. Additionally, the fat globules in sheep's milk are smaller, and the membrane is more susceptible to oxidation. These lipids contain a greater proportion of short-chain fatty acids, including caproic, caprylic, and capric acids, which contribute to the distinctive flavor of sheep's milk cheeses [21]. Moreover, faster lipolysis in sheep's milk plays a significant role in taste development. Several parameters further differentiate ovine milk from bovine milk; sheep's milk has a different pH, casein micelles are larger, and contains varying quantities of calcium per unit weight of casein. Furthermore, differences in mineral content affect coagulation time, coagulation speed, curd firmness, and the amount of rennet required during cheese production [18]. Table 1 gives a schematic overview of the main chemical differences that exist between cow's and sheep's milk.

Table 1. General composition (g 100 mL^{-1}) of milk from different mammalian species.

Ruminants	**Properties**					
	Total solids	Protein	Fat	Lactose	Ash	Oligosaccharides
Cattle	11.8–13.0	3.0–3.9	3.3–5.4	0.7–0.8	4.4–5.6	0.003–0.006
Sheep	18.1–20.0	4.5–7.0	5.0–9.0	4.1–5.9	0.8–1.0	0.002–0.004

Understanding the composition and physico-chemical characteristics of ewe's milk is crucial for sustainable development in the dairy industry and successful commercialization of its products. Notably, sheep breeding systems for milk production often thrive in economically and physically disadvantaged areas, significantly affecting the agricultural economy [13]. These systems frequently employ low-input practices, which offer a broader range of ecosystem services compared to intensive farming methods. The rearing of dairy sheep exemplifies this trend. Furthermore, sheep farming contributes to social benefits

such as safeguarding livelihoods in rural areas, providing employment, and preventing depopulation [22,23].

Rearing sheep offers several advantages compared to large ruminants. Sheep have a natural inclination for grazing, allowing them to feed on weeds and shrubs. Their dietary flexibility contributes to their adaptability in various environments. Due to their small size, sheep require less space then larger ruminants. This compactness minimizes soil damage and compaction, making them suitable for diverse landscapes [24]. Sheep are easier to manage and work with. Their manageable size simplifies tasks such as feeding, health monitoring, and transportation. Sheep are more affordable to purchase and maintain compared to larger livestock. Their lower input costs make them an attractive option for farmers. In many countries, especially in the Mediterranean and Middle East regions, sheep farming plays a crucial role in the national economy [25]. Interestingly, France, Italy, Spain, and Greece specialize in this sector [26]. These regions experience marked seasonal fluctuations in pasture composition and productivity.

Southern Italian regions focus extensively on ewe herds, amphasizing lamb production and milk transformation into various cheese varieties tied to local traditions, collectively known as “Pecorino” [27]. About 30% of Italian ewe's milk contributes to the production of PDO Pecorino cheese, a leading ewe's milk cheese in Italy and a prominent player in the international market [28]. Unfortunetely, the majority of ovine specialities remain farmstead cheeses with limited economic impact, primarily consumed locally. To ensure the survival of these farming systems, promoting a diverse range of products from small ruminants is essential. Encouraging not only farmers but the entire sheep dairy product chain will contribute to sustainable growth and market responsiveness.

3. Lactic acid bacteria and technological applications

LAB form a heterogeneous group of bacteria that play vital roles in various fermentation processes. These remarkable microbes primarily convert food carbohydrates into lactic acid, a central product of fermentation, but their influence extends beyond lactic acid production; LAB contribute to the rich flavors of different food products by breaking down proteins and lipids, generating a range of compounds, including alcohols, aldehydes, acids, esters, and sulfur compounds.

LAB are Gram-positive, non-spore forming bacteria, existing as rods, cocci, or chains (Figure 5). Their versatility shines in the realm of fermented foods, where they act as starter cultures [29]. They also function as adjunt cultures in cheese production, enhancing flavor, texture, and nutritional value of cheeses. Additionally, some LAB act as bioprotective cultures, producing bacteriocins and antifungal compounds to safeguard some foods [30]. Certain LAB can also play a "probiotic" role, because, benefiting gut health by promoting a balanced microbial environment in the host [31].

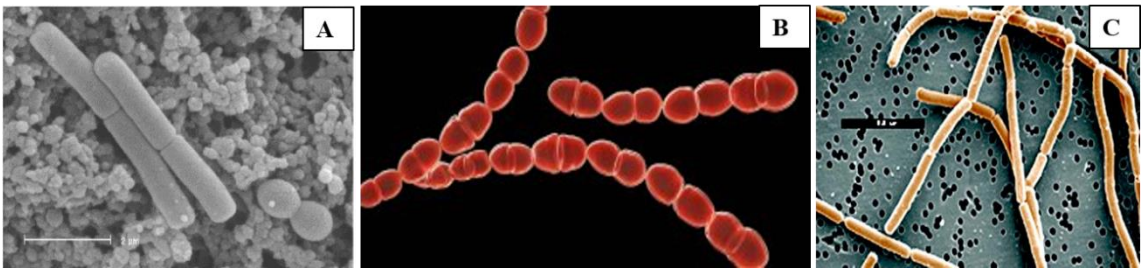

Figure 5. Lactic acid bacteria cells focused on scanning electron micrograph (A); cocci chains (B); rod cells (C), (https://www.researchgate.net).

LAB importance derives from their metabolic activity, fueled by available sugars, that results in the production of organic acids and other beneficial compounds. Due to their widespread presence in foods and extensive use, they are naturally recognized as safe (GRAS) for human consumption [32].

LAB exert a fundamental activity in cheese production. These microorganisms are defined “starters” when they main role is to “initiate” (start) the production of lactic acid. They deliberately added to the milk, except in certain traditional productions, which are based on exploiting the biofilm present in the wooden equipment used in the cheesemaking process. In addition to their acidification capacity as well as fermentation rate, the importance of lactic starters is also linked to other characteristics such as their resistance to bacteriophages, their attitude to autolysis [33]. The major species involved include *Lactococcus lactis*, *Leuconostoc species* among mesophilic species and *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* among thermophilic species. Their main function is undoubtedly to produce lactic acid from lactose during cheese production [32].

Others LAB are also intentionally added during cheesemaking as “adjunct cultures”, meaning selected strains added in order to fulfil different purposes and they have no function in acid production. Indeed, their main role is to induce organoleptic and biochemical changes into or onto the cheese during ripening, significantly contribute to the flavor, texture, nutritional value and microbial safety of fermented foods thanks to their ability to produce bacteriocins (biopreservation), this LAB are indicated as non-starter LAB (NSLAB) [34]. The group of NSLABs includes lactobacilli including *Companilactobacillus farciminis* among the obligatory homofermentative species; *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, *Lactiplantibacillus pentosus*, *Latilactobacillus curvatus* and *Lacticaseibacillus rhamnosus* among the heterofermentative species. Also commonly encountered are *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus durans*, *Enterococcus faecalis*,

Enterococcus faecium and also leuconostocs with the same species acting as starter cultures [35].

Trends of SLAB and NSLAB in raw milk cheese follow a general dynamic (Figure 6). SLAB are inoculated into the milk in high numbers (approximately 10^6 and 10^7 cfu/g) and regularly decrease in the first hours of cheese production. In contrast, NSLAB occur at low concentrations in fresh curd and increase by about four to five orders of magnitude dominating the microbiota of mature cheese [36].

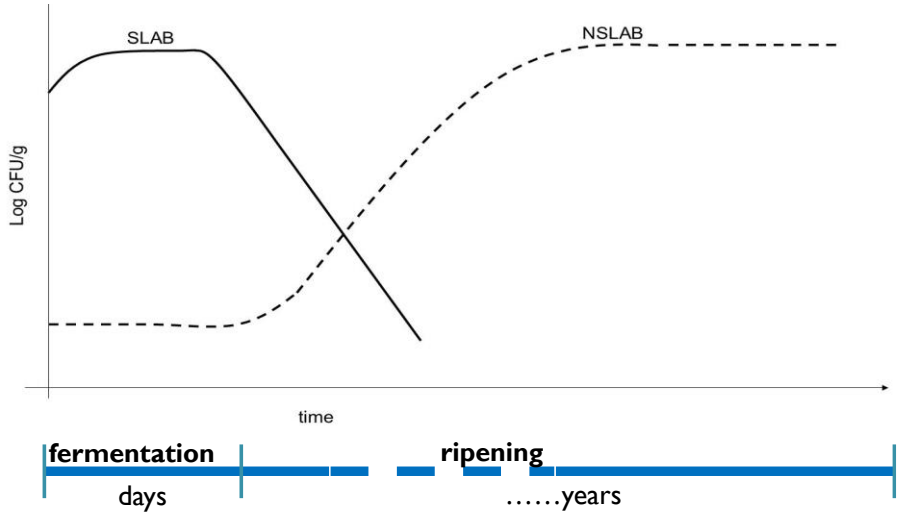

Figura 6. Evolution dynamics of LAB in cheeses.

Although LAB have long been employed in food production, recent attention to food safety has prompted EFSA guidelines [37] requiring the study of whole genomes. Whole Genome Sequencing (WGS) has emerged as a powerful tool for assessing microbial strains, enabling precise identification and safety evaluation [38]. Advances in genetics, molecular biology, physiology, and LAB biochemistry have expanded our understanding of these bacteria. As a result, the food industry now produces safe, nutritious products with different flavors and potential health benefits. These innovations cater to consumer

demands while maintaining the essence of traditional foods. In addition, the use of selected LAB strains with well-defined metabolic properties enhances overall production quality.

4. Microbiology quality of cheese

The microbiological quality and safety of cheese start with milk. While milk is rich in nutrients and serves as a complete diet for young mammals, it also provides an ideal environment for the growth of both spoilage and pathogenic microorganisms [39], contamination can occur through two routes: by endogenous transfer, via direct transfer of blood or infection from the udder itself; by exogenous transfer during or after the milking process. Microorganisms from the farm environment, animal feed, milking equipment, udder hygiene conditions, and even operator's hands can contaminate the milk. Some of these microorganisms can penetrate the teat canal and cause infection, ultimately being excreted in the milk [40]. Furthermore, microorganisms adhering to the surfaces of milking and processing equipment, due to inadequate cleaning and sanitisation, can also contribute to milk contamination.

Throughout the cheesemaking process – from production to consumption – cheese is continuously exposed to varying environmental conditions. Consequently, there is a significant risk of microbiological contamination and the growth of pathogenic and spoilage microorganisms. These factors can compromise product quality and render the cheese unsuitable for consumption [41]. Cheese spoilage is attributed to bacteria, yeasts, and fungi. However, not all cheese types are equally susceptible to the same microorganisms [42]. Some of these spoilage microorganisms enzymatically alter milk components, leading to negative changes in the cheese sensory attributes. Among pathogens, certain bacteria pose risks in cheese: *Staphylococcus aureus* and *Listeria*

monocytogenes (Gram-positive bacteria); *Escherichia coli* O157:H7 and *Salmonella* spp. (Gram-negative bacteria). These bacteria are associated to foodborne illnesses and are commonly found in cheese [43]. Given this, cheesemakers, must prioritize contamination control. High microbiological quality milk is essential for achieving optimal cheese yield, quality, and safety.

In the last decades, there has been a growing interest in developing food safety measures to ensure the production of safe and high-quality food products. One such systematic tool widely used in the food industry is the Hazard Analysis Critical Control Point (HACCP) system. It is defined by Regulation 852/2004 on the hygiene of foodstuffs in the European Union [44]. This regulation emphasizes the responsibility of food operators for product safety. Basically, the HACCP system focuses on identifying, assessing, and controlling hazards, with a strong emphasis on preventing identified risks. To prevent or minimise bacterial contamination, including the occurrence or growth of pathogens, several control measures are planned. These include: good production practices ensuring hygienic practices during production; sanitisation and hygiene measures for proper handling of raw materials; environmental control, managing the food industry environment to prevent foodborne outbreaks. The microbial control at the farm level ensures that the safety of dairy products begins at farm; several hurdles are set up to minimise contamination. To this purpose, maintaining animal health and welfare allows to produce high quality milk and minimise contamination [45]. Somatic cell count (SCC) parameter is an indicator of animal health. Lower SCC values correlate with better animal health. The accepted upper limit varies by country [46]. However, European Union sets a maximum limit of 400,000 cells/mL. Coliform bacteria are indicators of udder preparation

quality or hygienic handling of milking machines. For milk intended for pasteurization, coliform counts should be less than 100 CFU/mL. If the milk will be consumed raw coliform counts should be less than 10 CFU/mL. In summary, the HACCP system, combined with diligent practices at the farm level, contributes to safe and wholesome dairy products.

The microbial ecology of cheese is intricates, and pathogenic microorganisms often find their way into cheese through the use of raw milk. The safety of raw milk remains a highly debated topic. Advocates of pasteurization argue for managing pathogenic risks by applying thermal heat to reduce microbial loads in equipment and milk. They also emphasize standardizing production by inoculating selected strains into milk [47]. In contrast, proponents of raw-milk cheeses emphasize the importance of maintaining a high taxonomic diversity within indigenous cheese microbial communities. They argue that diverse microbial activities, combined with distinct cheese production methods, lead to unique characteristics, including low pathogen risk and varied taste traits. Raw milk plays a crucial role in many traditional cheeses, connecting the product to its geographical origin [48].

While raw milk products tend to exhibit more robust flavor profiles than pasteurized milk products, recent health concerns have prompted discussions about requiring milk pasteurization for cheese production [49]. Nevertheless, continuous microbiological monitoring and strict hygiene practices throughout the dairy supply chain are essential to ensure milk and cheese safety [50].

5. *Ewe's cheese consumption*

Cheese, a widely consumed long-term dairy product, enjoys popularity worldwide. However, its consumption varies significantly across countries in terms of annual intake. Europe and North America are the primary hubs for global cheese production (Figure 7), and this trend is expected to continue due to dietary westernisation, improved accessibility, and robust marketing efforts [51]. These trends apply universally to all types of cheeses. Interestingly, there are no specific statistics available for ewe's milk cheese consumption. However, based on FAOSTAT data from 2018 (since more recent data are not available), Greece stands out with a daily per capita consumption of 36 grams of ewe's milk cheese [52]. In contrast, other countries typically consume only 0–5 g per capita per day. Cow's milk cheeses exhibit a wider range, with values ranging from 0 to 96 g/capita.

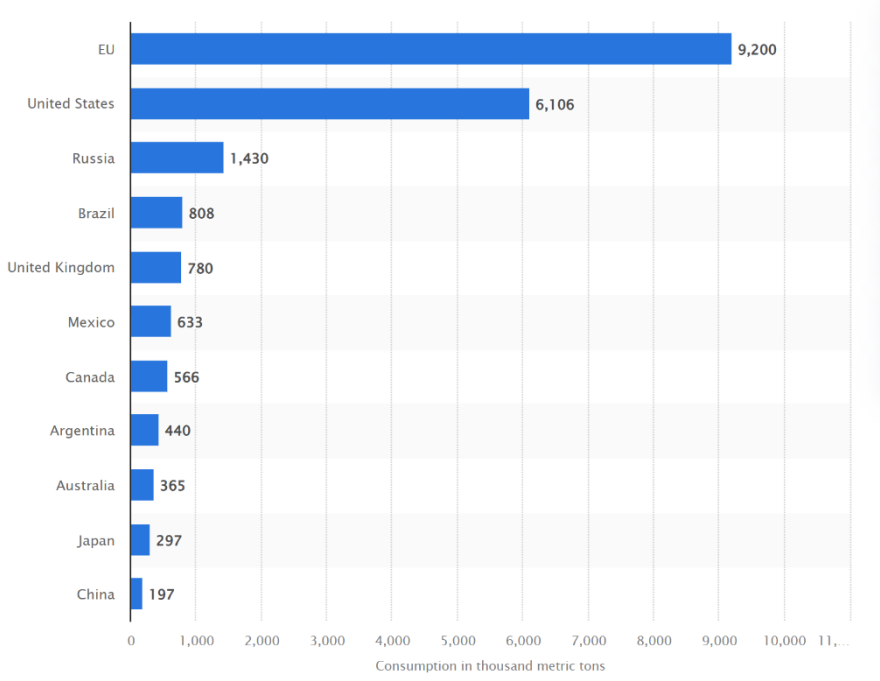

Figure 7. Annual consumption of cheese worldwide in 2023, by selected country, (https://www.statista.com/statistics/868231/global-annual-consumption-of-cheese-by-country).

In today's health-conscious landscape, especially in developed nations, there is a growing demand for high-quality, healthy foods [53]. Cheeses made from ewe's milk are

well-positioned to meet this demand due to their rich content of functional and physiologically active compounds. These include vitamins, minerals, fatty acids, terpenes, sialic acid, orotic acid, and L-carnitine, primarily derived from milk. Additionally, the fermentation and aging processes further enhance these cheeses by introducing bioactive peptides, γ-aminobutyric acid (GABA), and biogenic amines [54]. Furthermore, the Department of Health advocates for dairy product consumption as an integral part of a healthy diet. Research indicates that cheese contributes approximately 9.2% of the total calcium intake and serves as a valuable source of vitamin K2, which plays a protective role against vascular calcification [55,56]. Other studies highlight the potential benefits of consuming ewe's cheese in preventing various health conditions, including hypertension, obesity, and cancer [57].

In a market predominantly dominated by cow's milk cheese products, safeguarding ewe's milk cheese has gained newfound significance. Ewe's milk cheeses possess a distinct taste and flavor profile, setting them apart from their cow's milk counterparts [58]. Moreover, it must be recognized that ewe's milk cheeses often fall into the category of traditional, artisanal cheeses. Many of these cheeses hold legal protection through certificates of authenticity, enhancing their appeal to consumers (Figure 8). This connection to their place of origin, coupled with the historical and cultural heritage associated with these cheeses, imbues them with a unique quality. Their production typically occurs in nonindustrial environment, characterised by small-scale production and minimal mechanisation [59].

Figure 8. Niche sheep products, (https://www.vivasicilia.com/formaggi-siciliani).

6. *Sheep supply chain in Sicily*

Sicily, a region of Southern Italy, actively promotes the utilization of ewe's milk by crafting various kinds of cheese. According to data provided by BDN-Anagrafe Zootecnica, Sicily ranks as the second Italian region in terms of the number of ewes bred specifically for milk production, boasting approximately 699,000 head (Figure 9) [60]. The dairy sheep sector significantly contributes to the region economy, particularly in marginal rural areas where challenging environmental conditions limit alternative economic activities. Simultaneously, this sector plays a vital role in preserving local cultural heritage linked to shepherd traditions, mountain communities, and distinct cuisine [61].

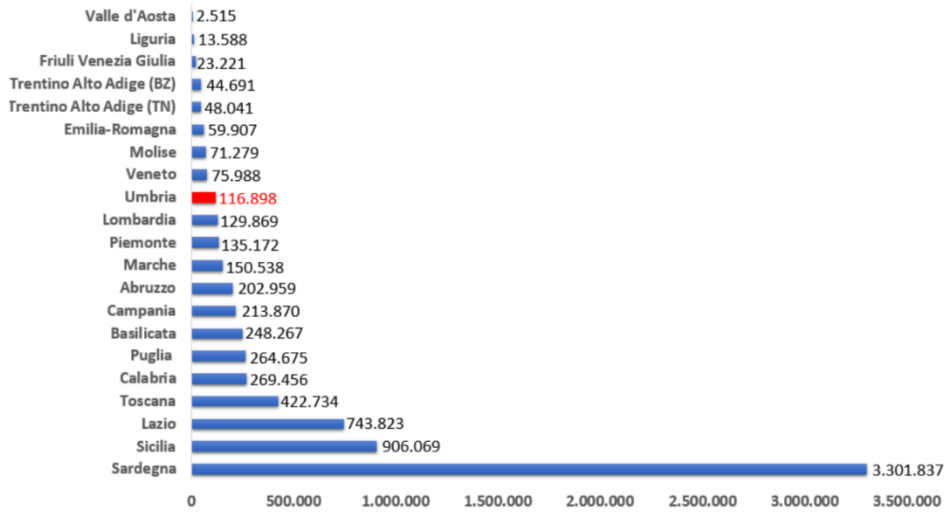

Figure 9. Italian sheep herd consistency distributed per region (IZS source in the year 2017).

Presently, Sicily hosts only four native sheep breeds: Barbaresca, Comisana, Pinzirita, and Valle del Belíce. Despite enduring harsh climates and semi-arid environments, these breeds remain essential for producing high-quality milk and typical dairy products [62]. Their significance extends beyond mere production, they serve as a valuable genetic reservoir, crucial for adapting to environmental shifts and responding to disease evolution [60].

The regional dairy sector in Sicily boasts an essential feature, an extraordinary range of products derived from ewe's milk. These include renowned cheeses such as Pecorino Siciliano, Pecorino “Primosale”, Piacentinu ennese, Vastedda della Valle del Belíce, and Maiorchino. These cheeses have flourished and gained prominence within specific regional areas, environmental setting, and social-economic contexts. Their historical and cultural significance is widely acknowledged, and modern consumer perceive them as authentic and genuine [63]. Despite this recognition, competing in today’s market dynamics remains challenging. Therefore, it becomes crucial to valorize these distinctive and niche products while simultaneously rejuvenating the sector. Doing so not only supports the preservation of local breeds, but contributes to maintaining biodiversity and preventing the abandonment of increasingly widespread lands.

7. Tradition and innovation in the ovine dairy supply chain

Ewe’s cheese holds a prominent place in Sicily cuisine, where it graces tables during both lunch and dinner. The art of cheese-making thrives, resulting in a diverse array of products, many deeply tied to their specific regional origins. Dairy farming plays a pivotal role in mountainous and marginal lands, contributing to the production of typical and traditional food items. The concept of safeguarding and preserving this rich food diversity,

particularly cheese, aims to honor the unique heritage of specific regions while maintaining product authenticity [64].

In economically advanced countries, consumers' perception of food quality has evolved. Quality is now considered as a multidimensional concept, with one critical dimension being the association of a food product with specific geographical areas [65]. Consequently, traditional dairy products are often regarded as high-quality due to the methods employed in their production and the natural environment in which the milk is sourced.

The European Commission' 2006 definition of "traditional" emphasizes proven usage within the community market over a generational time span—typically at least 25 years [66]. Traditional food are products crafted through centuries-old process that continue to be appreciated today. When such processes occur within specific geographical boundaries, the resulting product is deemed "typical". It is important to note that a typical product is also traditional, though not necessarily the inverse [67].

The concept of typicality arises from several interconnected factors tied to geographical origin, social customs, and cultural traditions within the production area. In the context of typicality and product identification, the use of locally sourced raw materials significantly influences consumers' perception of quality (Figure 10) [68]. However, these products cannot remain static in an ever-evolving ecological, technical, and social environment. Surprisingly, innovation has always been part of this sector. Without innovation, many traditional dairy cheese-making practices would fade away. It is precisely through innovation that typical cheeses have endured. Importantly, today's tradition builds upon past innovations, and current innovations may well become tomorrow's tradition.

Figure 10. Examples of wooden equipment used to make traditional cheeses.

Traditional cheese producers face the challenge of enhancing safety, healthiness, and convenience through innovative approaches. These adaptations allow them to thrive and expand in the fiercely competitive global market. Innovations extend beyond the product itself, they also encompass packaging, labelling, branding, and additional certifications, all guided by a specific production disciplinary [69,70].

Meeting demands from distant markets often requires introducing various forms of innovations. Bishop [71], emphasized that "innovation is the key to the future growth of the cheese market and will continue to be the focus of research and technological development". However, balancing tradition and innovation prove challenging, especially when consumers resist changes that impact the authentic essence of traditional foods. Therefore, it becomes essential to educate consumers about these abstract concepts (tradition and innovation). Generally, innovations that offer tangible and relevant benefits find acceptance among consumers [69]. For companies in the food industry, this understanding serves as a valuable tool to differentiate themselves from competitors, meet consumer expectations, and rejuvenate their image [72].

Beyond basic nutrition, food now serves a broader purpose; not only to provide nutrients and satiate hunger, but also to aid in preventing nutrition-related diseases and enhance physical and mental well-being. Consequently, there is a growing need to develop foods with specific nutritional functions, addressing gaps left by intolerances, strict diets,

or personal preferences. According to the OECD and FAO, the dairy supply chain will remain one of the fastest-growing agricultural sub-sectors in the coming decade, with cheese continuing to be one of the most popular and widely consumed products [73].

8. Functional food and food safety

In recent years, the food industry has witnessed significant innovations primarily centered around scientific and technical advancements in food processing and the introduction of novel foods. These innovations serve as crucial tools for companies to distinguish themselves from competitors and meet consumer expectations [74]. In particular, consumer expectations regarding food production have undergone substantial changes. People now increasingly recognize the direct impact of food on their health, a relationship well-established through research [75]. Consequently, there is a growing emphasis on "functional food". Researchers unanimously agree that functional foods constitute one of the most intriguing areas of research and innovation within the food industry [76].

Functional foods are foods that, when consumed in sufficient amounts over the long-term, enhance overall bodily health, reduce the risk of specific diseases (e.g. cholesterol-related conditions), and even serve as therapeutic aids for certain illnesses [77]. To achieve these benefits, various food products have been enriched with natural compounds. Examples include antioxidant additives, specific phenolic compounds, natural plant extracts (e.g. grape or green tea extract, cranberry powder, etc.), as well as natural substances and preservatives (e.g. extract of pomegranate peel, essential oil of *Thymus vulgaris*). These enrichments have been proposed for a range of products, including dairy beverages, yogurt, milk powder, and processed cheese [78–81]. Furthermore, probiotic

bacteria, such as lactobacilli and *Bifidobacterium* strains, have been harnessed in several studies. When present in sufficient levels (10^6–10^7 CFU/g), these probiotics offer health benefits to the host [82,83].

Cheeses and dairy products are widely utilized due to their advantageous properties, including a solid and consistent texture, optimal pH, and buffering capacity. These features allow for their incorporation in various ways, enhancing microbiological safety, sensory attributes, and nutritional value. Consequently, dairy products play a significant role in the functional foods sector, representing over 40% of this market [84].

The development of functional foods is a long-term trend with substantial market potential. Research-generated information supports consumer decisions, investments, and government regulations. As a result, these products emerge as a sustainable global trend, continually witnessing the launch of new offerings. The competitive landscape in this sector is intensifying [85].

Beyond health-enhancing foods, food safety remains a critical concern in the industry. Ensuring safer food production ranks among the top priorities alongside combating food spoilage. Food producers, regulatory bodies, researchers, and consumers all share concerns about the occurrence of food-borne diseases [86].

Over the years, food additives known as GRAS have been commonly used in foods. However, with consumers increasingly mindful of their well-being, synthetic preservatives are gradually giving way to natural alternatives [87]. These alternatives include natural antioxidants and antimicrobials derived from animals such as lysozyme and lactoferrin, botanicals such as polyphenols and essential oils, and microorganisms with bacteriocins being notable examples [88].

A growing body of evidence indicates the significant rise in the use of natural antimicrobials in food, particularly essential oils (EOs). EOs exhibit the ability to reduce or eliminate pathogenic bacteria, thereby enhancing overall food quality [89,90]. Additionally, EOs have been reported to enhance the nutritional potential of lactic acid-containing formulations [91,92]. EOs are volatile hydrophobic liquids extracted from plants (Figure 11). They primarily consist of aromatic and volatile compounds found naturally in various plant parts, including seeds, flowers, skin, stem, bark, and whole plants [93]. These secondary metabolites are important for the defence mechanism of plants. Some EOs also exhibit antimicrobial effects, including components from oregano, cloves, cinnamon, citral, garlic, coriander, rosemary, parsley, lemongrass, sage and vanillin [94].

Figure 11. Several types of essential oils, (https://www.cuisineaz.com/articles/comment-et-pourquoi-utiliser-les-huiles-essentielles-en-cuisine-672.aspx).

In Europe, the use of EOs and extracts in food is legally regulated by the European Commission. However, a common limitation of EO applications in food products lies in their impact on sensory attributes. Their strong odor and intense taste can alter the organoleptic characteristics of products, sometimes exceeding consumers' acceptable level [95].

9. *Circular economy, use of by-products*

In every production cycle, including food processing, waste inevitably occurs. Agricultural waste and food by-products can have adverse environmental consequences if not properly managed. These materials are highly susceptible to microbial spoilage, which limits their further utilization [96]. For producers, waste and by-products management represents a cost. Expenses related to drying, storage, and transportion of by-products pose economic limitions. Consequently, these materials are often repurposed as feed or fertiliser [97]. However, the challenge of by-product disposal is compounded by legal restrictions. Specific guidelines must be followed to prevent negative effects. On a European policy level, Directive 2008/98/EC governs waste management. Nationally, Italy's "Environmental Consolidation Text" defines by-product as substances resulting from production processes not primarily intended for their creation. Their subsequent lawful use is essential [98].

Interest in managing food by-products has surged, leading to their utilization as functional food ingredients. These by-products are rich in protein, dietary fibre, and beneficial bioactive compounds [99]. Thus, efficient, practical, and environmental-friendly use of these materials becomes increasingly crucial [100]. After recovery, food by-products find new life in food processing, particularly in fortifying various products. Their health benefits have made them popular choices for food fortification, positioning them as valuable raw materials and ingredients for food production. Some products like soybean meal and rice bran have shown promising results by providing phytonutrients and enhancing the nutritional value of tortillas [101,102]. Apple peel powder used as a partial replacement for wheat flour in muffin baking contributes to both flavor and health [103].

Grape pomace rich in antioxidant polyphenol compounds has been added to bread [104], breadsticks [105], semi-hard cheeses [106] and also pasta [107]. In all studies, grape pomace contributed positively to the dietary fiber and total phenolic content of the products. The antioxidant properties of products were significantly increased and the products were readily accepted by consumers at a certain level of pomace addition [108].

In the Mediterranean region, local agricultural by-products (residues of grapes, olives, tomatoes, citrus pulp, and myrtle) are extensively studied for their impact on the diets of small ruminants. However, the winemaking sector significantly contributes to Sicily's agri-food production and offers abundant agro-industrial by-products, including grape pomace (Figure 12). This surplus presents an exciting opportunity for creating innovative cheeses. The drive to revitalize the sheep industry has led to the development of new fresh dairy products. This dual objective aims at both sustainable waste management of winery by-products and functional dairy production [64]. Recent literature highlights a growing demand for health-conscious cheeses within the dairy industry. Notably, grape pomace has been investigated as an antioxidant rich dietary fibre, enhancing the nutritional value of yogurt and ice cream [109].

Figure 12. Grape pomace, a by-product of the wine supply chain, (https://www.grappamarolo.it/storie/le-vinacce-materia-prima-nobile).

Increasing awareness of climate change and environmental concerns has prompted people to reevaluate their eating habits, aiming for more sustainable food systems. Recent shifts in consumer preferences emphasize productivity, sustainability, and safety while preserving the unique qualities of food product. Notably, there is growing attention to the principles of the circular economy, particularly within the European Union. Consumers now seek food without synthetic additives, valuing naturalness and high quality [110]. In this context, utilizing by-products becomes essential, addressing global worries about limited natural resources. The FAO advocates the 3R principles (Reduce, Reuse and Recycle) across all productive sectors for sustainable development.

However, incorporating new ingredients, despite their health benefits, requires careful consideration. Sensory variations may occur, impacting consumer acceptance, especially when fortifying or enriching familiar foods. Familiarity with a product influences how deviations from expected sensory properties are perceived [111].

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Objective and organisation of the research project

The dairy sector is a multifaceted component of the agri-food industry, characterised by intense competition. It encompasses diverse production processes, ranging from fresh and UHT milk to butter and various types of cheese (both fresh and aged, traditional or industrial). Additionally, there is a wide array of evolving by-products, making it a supply chain significantly impacted by ongoing segmentation and product innovation. Cheeses occupies a unique position in the market, catering to both established adult consumers and health-conscious young individuals. Within the dairy sector, cheeses contribute approximately 20% of the turnover generated from fresh product sales.

In this context, a PhD initiative emerged with two key objectives for dairy businesses: innovation and quality assurance. Regarding innovation, the aim is to expand the product portfolio while honoring Sicilian tradition and authenticity, offering consumers novel dairy products with high health, nutritional, and sensory value. Furthermore, to address the growing consumer demand for transparency regarding the origin, quality, and safety of raw materials and finished dairy products.

Specifically, the focus was on using ewe's milk from the Valle del Belíce breed to produce innovative dairy items. This initiative aimed to bridge the gap between research and the dairy industry by developing cutting-edge ewe's milk cheeses, establishing production standards, and activating relevant production lines.

Over the course of a 3-year PhD program, we successfully produced ewe's milk cheeses by processing both raw and pasteurised milk. Our goal was to create a diverse range of cheese types, including ripened cheese (similar to “Swiss” cheese), fresh cheese (akin to “Crescenza” cheese), and fermented milks such as yogurt. The productions included the

use of lactic acid bacteria isolated from the Sicilian ovine dairy environment and selected for their exceptional dairy performance. The resulting cheeses retained a strong connection to their geographical origin. In the pursuit of innovation, fresh ewe's cheese infused with varying concentrations of oregano essential oil were produced. This not only extended the shelf life of the cheese, but also enhanced its nutritional value and sensory attributes. Additionally, sustainability by fortifying fresh ewe's cheese with grape pomace powder, a by-product from the wine industry, was explored. This initiative aligned with the principles of a circular economy, emphasizing waste reduction and reuse. Importantly, this fortified cheese is a staple in Sicilian diets.

Throughout the process, all raw materials, intermediate products, and final products were evaluated for microbiological safety. The new ewe's cheeses were studied from a physicochemical point of view, analyzing their fatty compsotision and volatile organic compounds. Finally, sensory analyses were carried out under the guidance of experienced judges, providing insights into the acceptability of these novel products.

Our research efforts culminated in several data-rich papers published in international journals, a testament to the collaborative work of my research group.

Part I

Novel fresh ewe's milk cheeses

In this section, the focus shifts to fresh ewe's milk cheeses. Fresh cheeses, exemplified by varieties like Crescenza, are ready for consumption immediately after the production process concludes. These cheeses primarily consist of starter LAB, typically present at levels of 10^8–10^9 CFU/g. Crafted from pasteurized milk, they undergo no ripening period and have a short shelf life when stored at refrigerated temperatures. Their defining characteristics include high moisture content, and a sweet flavor with milky aroma.

Fresh cheeses find favor among young consumers. Several factors contribute to their popularity: their soft, easy-to-swallow consistency, mild taste, and freshness. Furthermore, diet-conscious consumer perceives them as a wholesome choice. In addition, attractive and convenient retail packaging enhances their appeal.

During the three years of the PhD research, various fresh ewe's milk cheeses were developed. Specifically, ewe's milk was pasteurized and used for the production of a Crescenza-type cheese; cheesemaking was carried out with commercial freeze-dried starters to develop a production protocol. This cheese, traditionally made with cow's milk, now had an innovative twist. By incorporating different percentages of oregano EOs, a natural antimicrobial preservative, flavor and safety were enhanced using *Lactococcus lactis* strains as starter cultures. Finally, leveraging by-products from winemaking (rich in polyphenolic compounds), fresh ovine cheeses with added nutritional value were also produced. These endeavors aligned with our commitment to innovation and sustainability.

Chapter I

Development of "Quadrello di Ovino", a novel fresh ewe's cheese

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Abstract

This work was performed to produce a new soft ewe's milk cheese, namely "Quadrello di ovino" (QdO) cheese, to enlarge ewe's dairy product portfolio of South Italy, barely limited to Pecorino cheese typology. Cheese making was performed applying the technology for "Crescenza" cheese typology with some modifications. In particular, pasteurized ewes' milk was inoculated with two commercial starter formulations (SF1 and SF2) of *Streptococcus thermophilus* to obtain two different productions (QdO-P1 and QdO-P2, respectively). Plate counts demonstrated the ability

of both starter formulations to drive the fermentation process, since *S. thermophilus* counts reached 10^9 CFU/g in both productions. Generally, the two starter formulations did not affect the chemical composition of QdO cheeses that contained, on average, 64.08% dry matter of which approximately 54.99% were fats and 36.39% proteins. Among chemical parameters, significant differences were registered for secondary lipid oxidation state (significantly lower for QdO-P2), fatty acids and volatile organic compounds (VOCs). However, the differences registered among cheese VOCs from were not perceived by the panelists who recognized both cheese productions highly similar, although QdO-P2 cheeses were mostly appreciated by the judges. This study allowed to produce a novel fresh ovine cheese with specific chemical and sensorial characteristics well appreciated by consumers.

Keywords: ewe's milk, fresh cheese, lactic acid bacteria, physicochemical parameters, starter cultures.

Introduction

The great diversity of cheeses registered worldwide depends on several factors, mainly type of milk, technology applied, starter and secondary cultures inoculated and conditions of ripening [1]. Strictly considering the microbiological aspects, all cheeses are subjected to an internal bacterial transformation, mainly operated by lactic acid bacteria (LAB). Dairy LAB act in distinct phases of cheese production: those involved in the acidification of the curd are known are starter LAB, while the species responsible for the biochemical changes occurring during ripening are indicated as non-starter LAB [2]. Furthermore, during ripening a different spatial distribution of LAB can be found among cheese profile, from under rind to core (central part) [3].

Italy and France are probably the countries with the highest world's cheese diversity. In particular, Italy is the world's country with the highest number of food products that enjoy a recognition of quality status. Among these, 56 different cheeses are indicated as protected designation of origin (PDO), protected geographical indication (PGI) or traditional specialty guaranteed (TSG) products [4]. These recognitions are very important for the valorisation of dairy products, especially cheeses that acquired a bad reputation in terms of healthy properties due to their general high fat content [5]. Among cheese products, those mostly affected by this negative image are those processed from ewe's milk that is particularly rich in fats [6].

Besides traditional cheeses, new types of cheeses are being produced for several reasons such as improving the functional properties, to face allergies, to encounter the request of vegetarians, to respond to new lifestyles, to increase economic revenues and also for religious motivations [7–9]. In this framework, the milk mostly used for novel

cheese productions is cow's milk. Up to date, innovation in cheese production included the addition of fruit and vegetable by-products to obtain functional cheeses [10,11], the adjunct of natural food colorants to produce more attractive colored products [12], the inclusion of cereals as prebiotics to enhance the development of probiotic bacteria at intestinal level [13], even the incorporation of ripened cheese in novel fresh cheeses to valorize cheese surpluses [14]. However, there is also the need to valorize some milk productions generally addressed to a few specific productions.

Regarding the economic implications of developing new cheeses, in southern Italy there is a strong need to valorize ewe's milk productions. The two main Italian islands, Sicily and Sardinia, represent the regions where sheep milk is produced at consistent levels [15]. In order to contribute to the rural development and to impact positively on the local regional economy, Sicilian research institutes and cheese producers are collaborating to offer new dairy products with the aim of limiting the land abandonment phenomenon, particularly common in the internal hilly areas [16]. Generally, Italian hard cheeses processed from ewe's milk are referred to as "Pecorino" cheeses [17]. This cheese typology is often characterized by a strong aromatic flavour [18]. Due to the characteristic aroma, ripened Pecorino cheeses are not appreciated by several people, especially those who are sensitive to strong smells. "Pecorino" is actually an adjective that comes from "pecora", the Italian translation of ewe, but in common thinking, all cheeses processed from ewe's milk are identified as cheeses with an aggressive smell, even those that do not undergo a long ripening period. Thus, the major challenge to innovate ewe's milk cheese production is the manufacture of new products welcomed by consumers. Another dairy segment for

which the production of new products is urgent is buffalo's milk transformation. To this purpose, buffalo's milk has been used to process Stracchino cheese, a soft cheese belonging to Crescenza typology [19], that was well appreciated by judges [20].

The main objective of this work was to produce a new soft ewe's milk cheese applying the technology of Crescenza cheese typology by means of commercial thermophilic LAB. The final cheeses were characterized for their microbiological and physicochemical traits, volatile organic compounds (VOCs) profile as well as sensory features.

Materials and Methods

Milk and starter culture

Raw ewe's whole milk was obtained from several farms and transported daily by a temperature-controlled road tanker to the dairy factory "Il Cacio Siciliano" located in Belmonte Mezzagno (Italy). Bulk milk (500 L) was kept refrigerated (4–6 °C) under stirring until cheese making. Pasteurization occurred at 71 °C for 10 s in a PS15351 COMAT pasteurizer (CO.MAT. s.r.l., Bellizzi, Italy). After heat treatment, the milk was cooled at 38 °C to perform starter inoculation. The characteristics of pasteurized milk (average data of the bulks used in this study) were: pH 6.60 ± 0.03; somatic cell count 3.12 ± 0.13 Log, fat 5.86 ± 0.25%; protein 4.99 ± 0.15%; casein 3.87 ± 0.31%; and lactose 3.81 ± 0.35%.

Two different freeze-dried starter formulations CR/A (SF1) and LYOBAC-D CRM (SF2) were purchased from microMilk s.r.l. (Cremosano, Italy) and Alce International s.r.l. (Quistello, Italy), respectively. Both commercial starter cultures were prepared

from defined strains of *S. thermophilus* (SF1: strains CR/A1 and CR/A2; SF2: strains CRM4 and CRM6). Freeze-dried cells (5 Units) were re-activated for 10 min in 2 L of pasteurized ewe's milk, kept under manual agitation, to prepare starter inoculums.

"Quadrello di Ovino" cheese making

The novel cheese object of study, "Quadrello di ovino" (QdO) cheese, owes its name to the little square shape given to this sheep milk cheese. QdO production (Figure 1) was performed by modification of the classical flowsheet of "Crescenza" cheese making. The two distinct productions carried out with SF1 and SF2 were indicated as QdO-P1 and QdO-P2.

Bulk milk was transferred to a stainless-steel vat and, after starter preparation inoculation, subjected to a gentle agitation (20 rpm) for 5 min, then left to rest for 40 min. The milk was re-heated by direct steam injection to bring the temperature back to 38 °C and added with 60 mL of Naturen Premium 225 liquid rennet (Chr. Hansen's Laboratory, Parma, Italy) characterized by a strength of 1:22,500. After about 20 min, the coagulum was first subjected to a coarse cut performed by a stainless-steel rod and, after additional 50 min, the cut was concluded with a cheese harp containing vertical and horizontal wires to obtain cubes of about 3 cm x 3 cm x 3 cm. The curd was transferred into cuboid moulds (10 cm x 10 cm x 7 cm) and stewed under steam at 45 °C for 30 min, turned upside down stewed for other 30 min and then left acidifying until pH 5.4. The cheeses were transferred into a cold chamber [6 °C, relative humidity (RH) > 90%] for 24 h before being immersed into a saturated brine for 15 min and

then stored at 6 °C, RH > 90% covered by plastic sheets for 4 d. Finally, the cheeses were packed into plastic boxes and sealed with a transparent film.

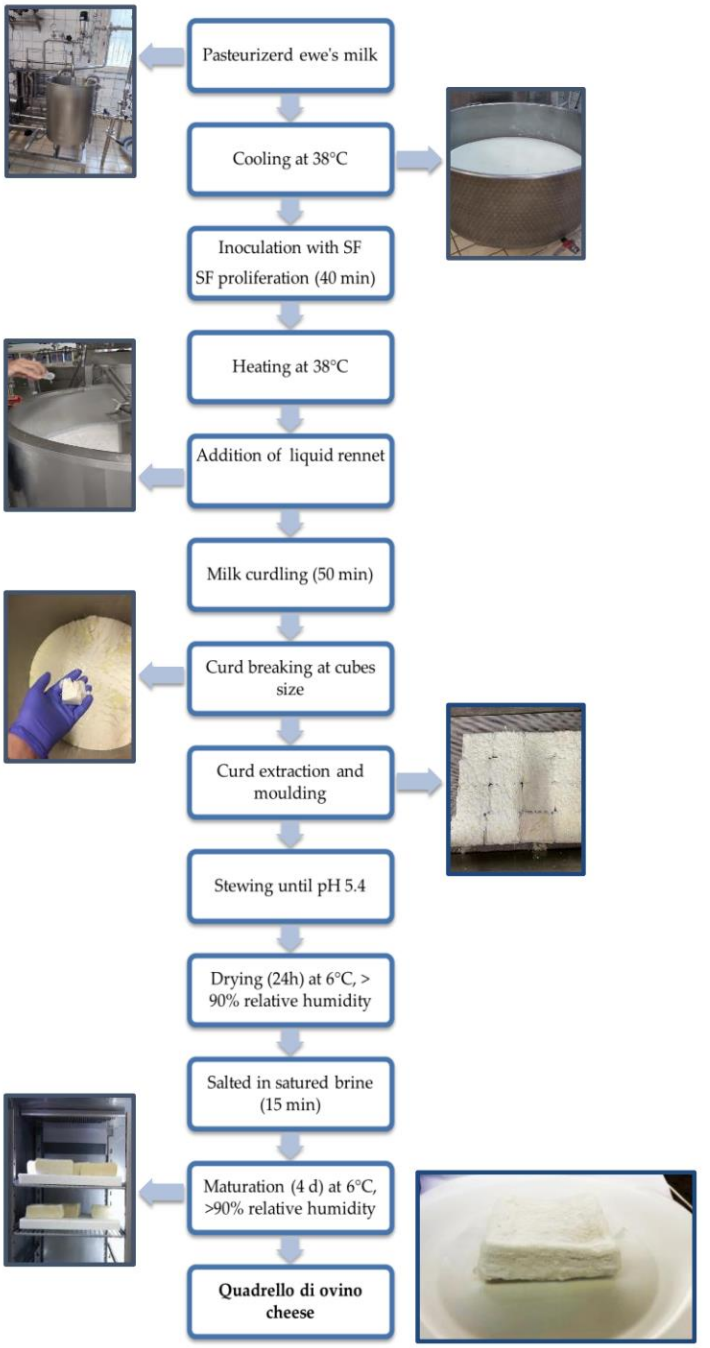

Figure 1. Flow diagram set up for this study to produce "Quadrello di ovino" cheese. Abbreviations: SF, starter formulations.

The day after, cheese production was performed with the second starter inoculum and both productions were repeated three times in three consecutive months. Samples of pasteurized milk, inoculated milk, curd and cheese, were collected from each cheese making and subjected to several analyses.

Microbiological analyses

Milk samples (1 mL) were serially diluted (1:10) in Ringer's solution (Sigma-Aldrich, Milan, Italy). Curd and cheese samples (15 g) were homogenized with 2% (w/v) sodium citrate solution (135 mL) by means of the stomacher Bag-Mixer 400 (Interscience, Saint Nom, France) for 2 min at the maximum speed (blending power-4). Curd and cheese homogenates were then subjected to the decimal serial dilution as reported above in Ringer's solution.

Cell suspensions of pasteurized milk samples were analysed by plate count for the enumeration of the total mesophilic microorganisms (TMM), LAB, *Pseudomonas* spp., coagulase positive staphylococci (CPS), members of Enterobacteriaceae family, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* spp. TMM were grown on Skim milk agar ISO 6610 incubated aerobically at 30 °C for 72 h. de Man–Rogosa–Sharpe (MRS) agar and Medium 17 (M17) were used for the growth of rod- and coccus-shaped LAB, respectively, after 48 h incubation at 30 °C for mesophilic groups and at 44 °C for thermophilic groups. *Pseudomonas* species were investigated on *Pseudomonas* agar base (PAB) supplemented with Cephaloridine-Fucidin-Cetrimide (CFC), incubated aerobically at 25 °C for 48 h. Members of Enterobacteriaceae family were detected on violet red bile glucose agar (VRBGA), after incubation at 37 °C for

24 h. *L. monocytogenes* were cultivated on *Listeria* selective agar base (LSAB) added with SR0140E supplement, incubated at 37 °C for 48 h. Hektoen enteric agar (HEA), incubated at 37 °C for 24 h, was specifically used for enteric Gram-negative bacteria (*Salmonella* spp. and *E. coli*), while Baird Parker (BP) agar with rabbit plasma fibrinogen supplement, incubated in the same conditions, for CPS.

Cell suspensions of inoculated milk, curds and cheeses were analysed for TMM and thermophilic coccus-shaped LAB on M17 agar as reported above. All media and chemicals were purchased from Oxoid (Milan, Italy). Plates counts were performed in triplicate.

Physicochemical analyses

Physical determination

Color of internal surfaces of the cheeses was assessed by a Minolta Chroma Meter CR300 (Minolta, Osaka, Japan) using the illuminant C; measurements of 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) were performed according to the CIE L* a* b* system [21].

Cheese hardness was evaluated by the maximum resistance to compression (compressive stress, N/mm^2) of samples (2 cm x 2 cm x 2 cm) kept at room temperature (22 °C). This parameter was measured as index of cheese hardness with an Instron 5564 tester (Instron Corp., Trezzano sul Naviglio, Milan, Italy).

Content of basic ingredients and antioxidant activity

Cheese samples were freeze-dried by a SCANVAC Coolsafe 55–9 (Labogene Aps, Lynge Denmark) and analysed for content of basic ingredients as reported by Bonanno et al. [22]. Briefly, dry matter (DM) was determined after drying in an oven at 105 °C until constant weight, fat content through the extraction of fatty substances with petroleum ether and diethyl, after acid hydrolysis, protein content using the Kjeldahl method (N 6.38), ash content through calcitation at 550 °C, and NaCl content through the precipitation of chlorides with the addition of silver nitrate and subsequent titration with ammonium sulphocyanide. The determination of soluble nitrogen was carried out by treatment with a sodium citrate solution and subsequent precipitation of the proteins at pH 4.6 and was determined on aqueous filtrate using the Kjeldahl method, the proteolysis index (PI) was calculated as the percentage ratio between NPN and total nitrogen (TN) [23].

The oxidation status of fat was assessed in the freeze-dried cheese samples by determining the peroxide value (POV, mEq O_2/kg fat) as an index of primary lipid oxidation [24], in screw-cap Pyrex culture tube, 20 mg of fat are dissolved with 9.8 mL of a chloroform-methanol solution (70:30) and added with 50 µL of iron chloride and 50 µL of ammonium thiocyanate, after 5 min of incubation it is read the absorbance at 500 nm using an Hach DR3900 spectrophotometer (Hach Company, Ames, Iowa, USA). The products of secondary lipid oxidation were determined as thiobarbituric acid reactive substances (TBARS), expressed as µg malonylaldehyde (MDA)/kg DM, as reported by Bonanno et al. [22]. In brief, phosphate buffer aqueous solution (pH 7.0) (8 mL) was added to cheese (2 g) in a 25-mL Sovirel tube, and the mixture was homogenized using an Art-Miccra D-8 high-speed homogenizer

(Moderne Labortechnik, Heitersheim, Germany). A 30% (v/v) trichloroacetic acid aqueous solution (2 mL) was then added and the sample was mixed in a vortex mixer for a few seconds, then filtered through Whatman No. 1 filter paper. A 0.02 M aqueous solution (5 mL) of thiobarbituric acid was added to 5 mL filtrate. The solution was placed in a hot water bath (90 °C) for 20 min then refrigerated. The absorbance of the supernatant was read at 530 nm using a Hach DR3900 spectrophotometer (Hach Company), after centrifugation at 4500 rpm for 5 min. TBARS were quantitatively determined, using 1,1,3,3-tetramethoxypropane solutions at concentrations ranging from 0.016 to 0.165 µg/mL for the calibration curve (R^2 = 0.99). Physicochemical determinations were performed in triplicate.

Determination of cheese fatty acids

Fatty acids (FAs) were directly methylated in screw-cap Pyrex culture tube on lyophilized samples (500 mg) with the addition of 0.7 mL of 10 N KOH in water, 5.3 mL of MeOH and 0.58 mL of 24 N H_2SO_4 in water [25]. Methyl ester of C23:0 (Sigma-Aldrich, Milan, Italy) was used as internal standard (0.5 mg/g freeze-dried sample) for the FA quantification. Fatty acid methyl esters (FAME) were recovered in hexane (1.5 mL). Each sample (1 µL) was injected by autosampler into an Agilent 7000C GC system, fitted with a fused silica DB-5MS capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) (Santa Clara, CA, USA) coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 270 °C. Solvent Delay: 0 min. Helium was the carrier gas (1 mL/min). The oven temperature was held at 70 °C for 1

min, increased of 5 °C/min to 100 °C, held for 2 min, increased of 10 °C/min to 175 °C, held for 40 min and finally increased at 5 °C/min to 225 °C, held for 45 min. The identification of each FAs was performed as described by Alabiso et al. [26]: the individual FAs were identified by comparing their retention times with those of a standard FAs (FAME mix C4-C24, CRM47885 Supelco, and tricosanoic acid conjugated methyl ester, Sigma-Aldrich). The health-promoting index (HPI) was calculated as reported by Ashkezary et al. [27]: (n-3 PUFA + n-6 PUFA + MUFA)/(C12:0 + (4 × C14:0) + C16:0).

Volatile organic compound determination

The method proposed by Tunick and co-workers [28] was followed for the determination of the volatile compounds of cheeses. All cheese samples were stored in the freezer at 15 °C and subsequently defrosted at 20 °C in a fridge. Five grams of each individual sample were grated and placed into a 20 mL vial (75.5 x 22.5 mm) (Supelco, Bellefonte, PA, USA). Each sample was added with an internal standard solution (0.1 mL). The internal standard solution was ethyl benzoate (Sigma Aldrich, St. Louis, MO, USA) at 5 ppm. The most suitable internal standard was chosen to quantify each analyte, depending on the best linearity and precision parameters found. A fiber assembly was evaluated and used: 50/30 µm divinylbenzene (DVB)/carbowax (CAR)/polydimethylsiloxane (PDMS) (Supelco®, Bellefonte, PA, USA). The samples were equilibrated at 40 °C for 30 min. The SPME fiber was exposed to the cheeses for 30 min in the headspace of the sample kept at 40 °C. The flavor compounds were desorbed for 10 min from the fiber to the column through a splitless injector at 250 °C.

Before use, fiber was conditioned and cleaned at 270 °C for 30 min, following instructions from Supelco®. All samples were prepared in triplicates in standard 20 mL-volume headspace vials.

Quantification of volatile compounds was performed using an Agilent 7000C GC system, fitted with a fused silica DB-5MS capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) (Santa Clara, CA, USA) coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 270 °C. Solvent delay: 0 min. Helium was the carrier gas (1 mL/min). The oven temperature was initially kept at 40 °C for 5 min. Then gradually increased to 250 °C at 2 °C/min rate. Held for 15 min and finally raised to 270 °C at 10 °C/min. Volatile compounds were injected at 250 °C automatically with the splitless mode. The individual peaks were analysed using the GC-MSolution package, Version 2.72. Identification of compounds was carried out using Adams [29], NIST 11, Wiley 9 and FFNSC 2 mass spectral database.

Sensory evaluations

Cheese samples were also evaluated for their sensory characteristics by a panel of 15 assessor members (nine men and six women, from 25 to 54 years old). All panelists were trained at the Department of Agricultural, Food and Forest Sciences—University of Palermo following the ISO 8589 [30] indications. The panelists were asked to score 17 descriptive attributes regarding aspect (color and structure uniformity), smell (intensity of odor, odor of butter, odor of milk, and unpleasant odor), taste (salty, sweet, acid, bitter, and spicy), consistency (chewiness, solubility, and grittiness

following mastication) and overall acceptability. The sensory evaluation of QdO cheeses were compared to that of commercial cow's Crescenza cheese (CCCh) purchased from a retail store. Each attribute was scored using an ordinal sensory scale of intensity from 1 (low) to 9 (high) as reported by Tidona et al. [31].

Statistical analyses

Microbiological, physicochemical and sensory evaluation data were subjected to one- way variance analysis (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft, NY, USA). The Duncan procedure and Tukey's test was applied for pairwise comparison. Statistical significance was attributed to *p* values of $p < 0.05$ and are marked with different letters.

Results

Microbiological evolution during cheese productions

The levels of the different microbial groups investigated in pasteurized milk used for QdO-P1 and QdO-P2 are reported in Figure 2. No statistically significant differences ($p > 0.05$) were observed among both pasteurized milks for the levels of TMM, mesophilic and thermophilic rod- and coccus-shaped LAB. Thermophilic coccus and mesophilic rod LAB were found at the same level (10^3 CFU/mL) of TMM, while thermophilic rod and mesophilic coccus LAB were one log cycle lower for both matrices. The results of *Pseudomonas*, members of Enterobacteriaceae family, *L. monocytogenes*, *Salmonella* spp., *E. coli* and CPS are not reported in Figure 2 because their levels were below the detection limit for both milks.

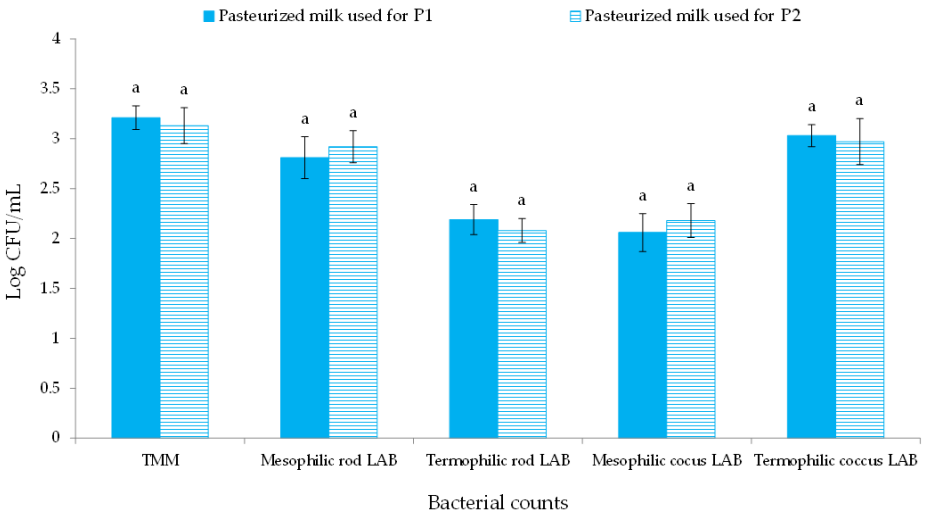

Figure 2. Microbial loads (Log CFU/mL) of pasteurized milk samples. Results indicate mean values and standard deviation of six determinations (carried out in duplicate for three independent productions). Abbreviations: P1, production performed with starter culture SF1 (starter formulation CR/A); P2, production performed with starter culture SF2 (starter formulation LYOBAC-D CRM); TMM, total mesophilic microorganisms; LAB, lactic acid bacteria.

The bulk milks were then inoculated with the commercial *S. thermophilus* starter cultures and the levels of fermenting microorganisms in inoculated milks, curds and cheeses are reported in Table 1.

Table 1. Growth of commercial starter LAB during Quadrello di ovino cheese productions.

Samples	**Bacterial Counts**	
	TMM	*S. thermophilus*
Inoculated milk		
P1	6.84 ± 0.21[a]	7.09 ± 0.12[a]
P2	7.03 ± 0.13[a]	7.26 ± 0.16[a]
Curd		
P1	7.77 ± 0.23[a]	7.98 ± 0.18[a]
P2	7.92 ± 0.20[a]	8.02 ± 0.22[a]
Cheese		
QdO-P1	8.82 ± 0.11[a]	8.99 ± 0.29[a]
QdO-P2	8.73 ± 0.14[a]	8.89 ± 0.11[a]

Units are log CFU/mL for liquid samples and log CFU/g for solid samples. Results indicate mean values ± S.D. of nine plate counts (carried out in triplicate for three independent productions). Data within a column followed by the same letter are not significantly different according to Duncan test. Abbreviation: TMM, total mesophilic microorganisms; *S.*, *Streptococcus*; P1, production inoculated with the SF1; P2, production inoculated with the SF2; QdO, Quadrello di ovino cheese.

According to Duncan test, no statistically significant differences ($p > 0.05$) were found for the levels of TMM and *S. thermophilus* in all samples analysed. In particular, the level of these microorganisms for the P1 and P2 productions were almost perfectly superimposable. After inoculation with each commercial starter culture, P1 and P2 milks showed about 10^7 CFU/mL of TMM and *S. thermophilus*. After coagulation, these microorganisms were counted at about 10^8 CFU/g in curds for both productions showing an increase of about 1 Log cycle. Both QdO-P1and QdO-P2 cheeses reached LAB values of about 9 Log CFU/g.

Physicochemical characterisation of cheeses

Cheese color and hardness

Physical measurements (colorimetric parameters and hardness) of the final cheeses are reported in Table 2. The three colour parameters (L*, a*, and b*), as well as the hardness of the cheeses were significantly influenced ($p < 0.05$) by the two starter formulations.

Table 2. Physical parameters of Quadrello di ovino cheeses.

Parameters	Samples	
	QdO-P1	QdO-P2
Lightness (L*)	88.32 ± 0.30[a]	86.30 ± 0.63[b]
Redness (a*)	-3.81 ± 0.28[a]	-4.76 ± 0.24[b]
Yellowness (b*)	15.18 ± 0.69[b]	16.88 ± 0.08[a]
Hardness, N/mm^2	0.18 ± 0.02[b]	0.26 ± 0.03[a]

Results indicate mean values ± S.D. of nine determinations (carried out in triplicate for three independent productions). Data within a line followed by the same letter are not significantly different according to Duncan test. Abbreviation: QdO-P1, Quadrello di ovino cheese produced with starter culture SF1 (starter formulation CR/A); QdO-P2, Quadrello di ovino cheese produced with starter culture SF2 (starter formulation LYOBAC-D CRM).

Basic chemical composition and antioxidant capacity of cheese

The final cheeses were characterized (Table 3) by an average DM of 64.08%, mainly represented by fats (54.99%) and proteins (36.39%). Although no statistically significant differences ($p > 0.05$) were observed between QdO-P1 and QdO-P2 cheeses regarding the basic compositional analysis, TBARS values registered for QdO-P1 cheese were higher than those recorded for QdO-P2 cheese.

Table 3. Compositional characteristics of Quadrello di ovino cheeses.

Parameters	Samples	
	QdO-P1	QdO-P2
Dry matter (DM), %	63.17 ± 1.47[a]	64.99 ± 0.13[a]
Ash, %	5.57 ± 0.08[a]	5.53 ± 0.05[a]
Protein, %	35.52 ± 0.89[a]	37.26 ± 0.91[a]
Fat, %	54.99 ± 1.01[a]	54.98 ± 0.88[a]
NaCl, %	1.68 ± 0.08[a]	1.54 ± 0.01[a]
PI, %	1.02 ± 0.04[a]	1.01 ± 0.08[a]
POV, meq peroxide/kg fat	0.42 ± 0.01[a]	0.44 ± 0.03[a]
TBARs, MDA mg/kg DM	42.71 ± 0.74[a]	36.69 ± 0.37[b]

Results indicate mean values ± S.D. of nine determinations (carried out in triplicate for three independent productions). Data within a line followed by the same letter are not significantly different according to Duncan test. Abbreviation: QdO-P1, Quadrello di ovino cheese produced with starter culture SF1 (starter formulation CR/A); QdO-P2, Quadrello di ovino cheese produced with starter culture SF2 (starter formulation LYOBAC-D CRM); PI, proteolysis index; POV, peroxide value; TBARs, thiobarbituric acid-reactive substances; MDA, malonylaldehyde.

Cheese Fatty Acids Composition

FAs composition of the experimental cheeses is shown in Table 4. Saturated fatty acids (SFAs) were consistently present in QdO cheeses (66.6 and 63.8 g/100 g for QdO-P1 and QdO-P2, respectively); among these, palmitic acid (C16:0; 24.9 and 25.7 for QdO-P1 and QdO-P2, respectively) showed a higher concentration followed by stearic acid (C18:0; 9.81 and 10.5 for QdO-P1 and QdO-P2, respectively) and myristic acid (C14:0; 11.0 and 10.6 for QdO-P1 and QdO-P2, respectively).

Table 4. Fatty acid profile (g/100 g FA) of Quadrello di ovino cheeses.

Parameters	Samples	
	QdO-P1	QdO-P2
Total FAs, % DM	48.38 ± 0.05[a]	49.80 ± 1.24[a]
Butanoic acid (C4)	1.67 ± 0.04[a]	1.45 ± 0.00[b]
Caproic acid (C6)	1.88 ± 0.02[a]	1.44 ± 0.03[b]
Caprylic acid (C8)	2.31 ± 0.01[a]	1.71 ± 0.03[b]
Capric acid (C10:0)	7.25 ±0.02[a]	5.50 ± 0.06[b]
Caproleic acid (C10:1Δ^{c9})	0.28 ± 0.00[a]	0.22 ± 0.00[b]
Lauric acid (C12:0)	4.11 ± 0.02[a]	3.43 ± 0.02[b]
Dodecenoic acid (C12:1Δ^{c5})	0.13 ± 0.00[a]	0.11 ± 0.00[b]
Myristic acid (C14:0)	11.01 ± 0.05[a]	10.67 ± 0.10[b]
Myristoleic acid (C14:1Δ^{c9})	0.23 ± 0.00[a]	0.23 ± 0.00[a]
Pentadecanoic acid (C15:0)	1.89 ± 0.01[a]	1.77 ± 0.02[b]
Pentadecenoic acid (C15:1Δ^{c14})	0.51 ± 0.00[a]	0.48 ± 0.00[b]
Palmitic acid (C16:0)	24.9 84 ± 0.05[b]	25.73 ± 0.09[a]
Palmitoleic acid (C16:1Δ^{c9})	0.23 ± 0.00[a]	0.22 ± 0.00[a]
Heptadecanoic acid (C17:0)	1.47 ± 0.00[a]	1.46 ± 0.01[a]
Heptadecenoic acid (C17:1Δ^{c9})	0.29 ± 0.00[a]	0.24 ± 0.00[b]
Stearic acid (C18:0)	9.81 ± 0.09[b]	10.5 ± 0.04[a]
Oleic acid (C18:1Δ^{c9})	17.11 ± 0.04[b]	20.14 ± 0.07[a]
cis-Vaccenic acid C18:1Δ^{c11}	0.64 ± 0.00[a]	0.59 ± 0.01[b]
trans-Vaccenic acid (C18:1Δ^{t11})	3.68 ± 0.02[b]	3.91 ± 0.04[a]
Octadecenoic acid (C18:1Δ^{c13})	3.28 ± 0.02[b]	3.82 ± 0.02[a]
Octadecadienoic acid (C18:2$\Delta^{t9,c12}$)	0.88 ± 0.01[a]	0.62 ± 0.01[b]
Linoleic acid (C18:2$\Delta^{c9,c12}$)	1.91 ± 0.01[a]	1.86 ± 0.01[b]
Rumenic acid (C18:2$\Delta^{c9,t11}$)	1.20 ± 0.03[a]	1.30 ± 0.04[a]
Octadecadienoic acid (C18:2$\Delta^{t10,c12}$)	0.46 ± 0.00[a]	0.39 ± 0.01[b]
α-linolenic acid (C18:3$\Delta^{c9,c12,c15}$)	1.54 ± 0.00[a]	1.15 ± 0.00[b]
γ-linolenic acid (C18:3$\Delta^{c6,c9,c12}$)	0.34 ± 0.00[b]	0.37 ± 0.00[a]
Icosanoic acid (C20:0)	0.10 ± 0.01[a]	0.09 ± 0.01[a]
Gondoic acid (C20:1Δ^{c11})	0.07 ± 0.00[b]	0.09 ± 0.00[a]
Eicosadienoic acid (C20:2$\Delta^{c11,c14}$)	0.10 ± 0.00[a]	0.08 ± 0.00[b]
Eicosatrienoic acid (C20:3$\Delta^{c11,c14,c17}$)	0.02 ± 0.00[a]	0.01 ± 0.00[a]
Dihomo-γ-linolenic acid (C20:3$\Delta^{c8,c11,c14}$)	0.02 ± 0.00[a]	0.02 ± 0.00[a]
Arachidonic acid (C20:4$\Delta^{c5,c8,c11,c14}$)	0.11 ± 0.01[a]	0.11 ± 0.00[a]
Eicosapentaenoic acid (C20:5$\Delta^{c5,c8,c11,c14,c17}$)	0.15 ± 0.02[a]	0.15 ± 0.00[a]
Docosanoic acid (C22:0)	0.12 ± 0.00[a]	0.13 ± 0.00[a]
Docosadienoic acid (C22:2$\Delta^{c13,c16}$)	0.15 ± 0.00[a]	0.15 ± 0.00[a]
Docosadienoic acid (C22:4$\Delta^{c8,c11,c14,c17}$)	0.03 ± 0.00[a]	0.04 ± 0.00[a]
Docosapentaenoic acid (C22:5$\Delta^{c7,c10,c13,c16,c19}$)	0.12 ± 0.00[a]	0.10 ± 0.00[b]
Cervonic acid (C22:6$\Delta^{c4,c7,c10,c13,c16,c19}$)	0.05 ± 0.00[a]	0.03 ± 0.00[b]
Tetracosanoic acid (C24:0)	0.02 ± 0.01[a]	0.02 ± 0.00[a]

SFA	66.59 ± 0.04[a]	63.82 ± 0.09[b]
MUFA	26.51 ± 0.03[b]	29.92 ± 0.10[a]
PUFA	6.94 ± 0.07[a]	6.25 ± 0.01[b]
n6	2.53 ± 0.00[a]	2.49 ± 0.01[b]
n3	1.86 ± 0.03[a]	1.45 ± 0.01[b]
n6/n3	1.36 ± 0.02[b]	1.71 ± 0.00[a]
HPI	0.42 ± 0.00[b]	0.47 ± 0.00[a]

Results indicate mean values ± S.D. of nine determinations (carried out in triplicate for three independent productions). Data within a line followed by the same letter are not significantly different according to Duncan test. Abbreviation: QdO-P1, Quadrello di ovino cheese produced with starter culture SF1 (starter formulation CR/A); QdO-P2, Quadrello di ovino cheese produced with starter culture SF2 (starter formulation LYOBAC-D CRM); FA = fatty acid; DM = dry matter; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; HPI = health promoting index.

Among MUFA, oleic acid (OA, C18:1Δ^{c9}) was the most representative as fatty acid (17.1 and 20.1 for QdO-P1 and QdO-P2, respectively). All cheeses were characterized by a high content of trans-vaccenic acid (VA, C18:1Δ^{t11}, 3.68 and 3.91 for QdO-P1 and QdO-P2, respectively) while the cis-vaccenic acid (C18:1Δ^{c11}) showed a much lower concentration, barely 0.64 and 0.59 for QdO-P1 and QdO-P2, respectively. Regarding PUFA, linoleic acid (LA, C18:2$\Delta^{c9,c12}$) was the most representative FAs (1.91 and 1.86 for QdO-P1 and QdO-P2, respectively). The use of different *S. thermophilus* strains determined significant differences in FAs composition of QdO cheeses. Considering the short-chain (SCFA; C4:0–C8:0) and medium-chain (MCFA; C10:0–C15:0) FAs, QdO-P1 cheeses showed significantly higher concentrations than QdO-P2 cheeses, except for myristoleic acid (C14:1Δ^{c9}) which showed no significant differences. Significant differences were also found between long-chain FAs (LCFA; C16:0–C24:0). In particular, palmitic, stearic, oleic, trans-vaccenic, γ-linolenic, eicosenoic acid and the other C18:1 isomers, were significantly higher in QdO-P2 cheeses than in QdO-P1 cheeses. An opposite trend was registered for heptadecenoic (C17:1), cis-vaccenic (C18:1Δ^{c11}), linoleic, α-linolenic (ALA, C18:3$\Delta^{c9,c12,c15}$), eicosadienoic acid (C20:2$\Delta^{c11,c14}$), and the other C18:2 isomers, that were significantly

higher in P1. The other identified LCFAs did not show significant differences among cheese productions.

Individual fatty acid determined differences in SFA (p = 0.001), PUFA (p = 0.005), n6 PUFA (p = 0.013) and n3 PUFA (p = 0.002) which were higher in QdO-P1 rather than in QdO- P2 cheeses, while MUFA (p < 0.001) were at the highest concentrations in QdO-P2 cheeses. The health indices investigated were also influenced by the type of inoculum used: both n6/n3 ratio and HPI were higher in QdO-P2 cheeses (p = 0.002 and p = 0.004, respectively).

Chemical composition of volatile organic compounds

The analysis of VOCs of the two QdO cheeses was carried out by SPME-GC/MS and the results are reported in Table 5. Relevant qualitative and quantitative differences were evaluated between the productions carried out with the two SFs.

Twenty-one compounds of different chemical classes (alcohols, ethers, aldehydes, ketones, carboxylic acids, esters, amines, and monoterpenes) were identified among cheeses. Ketones represented the major VOCs class of QdO-P1 cheeses (41.21 ± 0.96 ppm), followed by esters (19.32 ± 0.56 ppm) and ethereal compounds (9.03 ± 0.21 ppm). Among ketones, acetoin was the most abundant compound (31.33 ± 0.73 ppm). In descending order of quantity, the second is phenethyl hexanoate (17.25 ± 0.40 ppm). QdO-P2 cheeses showed a higher presence of short-chain carboxylic acids (acetic acid and butanoic acid) (21.25 ± 0.49 ppm), straight-chain esters (ethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate) (25.52 ± 0.60 ppm), and a lower concentration of ketones (1.49 ± 0.04 ppm) than QdO-P1 cheeses.

Aldehydes were negligible in QdO-P2 cheeses, but were detected at very low concentrations (benzaldehyde, 0.33 ± 0.01 ppm) in QdO-P1 cheeses. Significant differences were found among QdO-P1 and QdO-P2 cheeses for the production of ethyl ether (9.03 ± 0.21 and 10.33 ± 0.24 ppm for, respectively) and ethyl benzene, 1,3-dimethyl benzene and 1-butenyl benzene. Among terpenes sylvestrene was found in QdO-P2 cheeses. Among amines only dimethylamine (4.94 ± 0.39 ppm) was detected in QdO-P1 cheeses.

Table 5. Abundance of VOCs emitted from Quadrello di ovino cheeses.

Compounds[a] (Common names)	**Samples**		**Identification**
	QdO-P1	QdO-P2	
Σ Alcohols	4.30 ± 0.34[a]	1.49 ± 0.03[b]	
3-Methyl-1-butanol	4.30 ± 0.34[a]	1.49 ± 0.03[b]	b
Σ Ethers	9.03 ± 0.21[b]	10.33 ± 0.24[a]	
Ethyl ether	9.03 ± 0.21[b]	10.33 ± 0.24[a]	b,c
Σ Aldehydes	0.33 ± 0.01[a]	n.d.[b]	
Benzaldehyde	0.33 ± 0.01[a]	n.d.[b]	b,c
Σ Ketones	41.21 ± 0.96[a]	1.49 ± 0.04[b]	
2-Pentanone	5.93 ± 0.14[a]	n.d.[b]	b
Butan-2-one-3-hydroxy (Acetoin)	31.33 ± 0.73[a]	n.d.[b]	b
2-Hexanone	1.22 ± 0.03[a]	n.d.[b]	b
2-Heptanone	n.d.[b]	1.09 ± 0.03[a]	b
2-Nonanone	2.73 ± 0.06[a]	0.40 ± 0.01[b]	b
Σ Carboxylic Acids	1.29 ± 0.10[b]	21.25 ± 0.49[a]	
Acetic acid	1.29 ± 0.10[b]	15.35 ± 0.35[a]	b,c
Butanoic acid	n.d.[b]	5.90 ± 0.14[a]	b
Σ Esters	19.32 ± 0.56[b]	25.52 ± 0.60[a]	
Ethyl acetate	n.d.[b]	8.43 ± 0.20[a]	b,c
Ethyl butanoate	n.d.[b]	9.83 ± 0.23[a]	b
Phenethyl hexanoate (Phenethyl caproate)	17.25 ± 0.40[a]	n.d.[b]	b
Ethyl hexanoate (Ethyl caproate)	0.23 ± 0.02[b]	3.40 ± 0.08[a]	b
Ethyl octanoate (Ethyl caprylate)	1.14 ± 0.09[b]	2.33 ± 0.05[a]	b,c
Ethyl decanoate (Ethyl caprate)	0.70 ± 0.05[b]	1.53 ± 0.04[a]	b
Σ Amines	4.94 ± 0.39[a]	n.d.	
Dimethylamine	4.94 ± 0.39[a]	n.d.	b
Σ Monoterpenes	n.d.[b]	0.20 ± 0.00[a]	
Sylvestrene	n.d.[b]	0.20 ± 0.00[a]	b
Σ Others	1.11 ± 0.03[b]	1.45 ± 0.04[a]	

Ethyl benzene	0.28 ± 0.01[b]	0.67 ± 0.02[a]	b
1,3-Dimethyl benzene	0.83 ± 0.02[a]	0.39 ± 0.01[b]	b
1-Butenyl benzene	n.d.[b]	0.39 ± 0.01[a]	b

Results are expressed in parts per million (ppm). [a] Compounds are tabulated according to the organic class and to retention time; b comparison with mass spectrum libraries; c co-elution with authentic sample. Data within a line followed by the same letter are not significantly different according to Duncan test. Abbreviation: QdO-P1, Quadrello di ovino cheese produced with starter culture SF1 (starter formulation CR/A); QdO-P2, Quadrello di ovino cheese produced with starter culture SF2 (starter formulation LYOBAC-D CRM); n.d., not detectable.

Sensory aspects of cheeses

The sensory characteristics of QdO cheeses were evaluated by judges of different age and gender (Figure 3). In particular, the sensory characteristics of both QdO cheeses were compared to those of commercial Crescenza cheese (CCCh) taken as reference cheeses. According to the Tukey's test, no statistically significant differences ($p > 0.05$) were found for uniformity, odor of milk, odor of butter, salty, acid, bitter and unpleased odor and aroma, while different levels of significance emerged for all other attributes evaluated. QdO-P1 and QdO-P2 cheeses were scored at similar level by the panelists for color, intensity of odor, sweet, spicy and taste persistency. All these attributes reached the highest in QdO. Solubility of CCCh cheese was comparable to that of QdO-P2 cheese. CCCh and QdO-P2 cheeses were not statistically different also for overall assessment, intended as the degree of overall satisfaction.

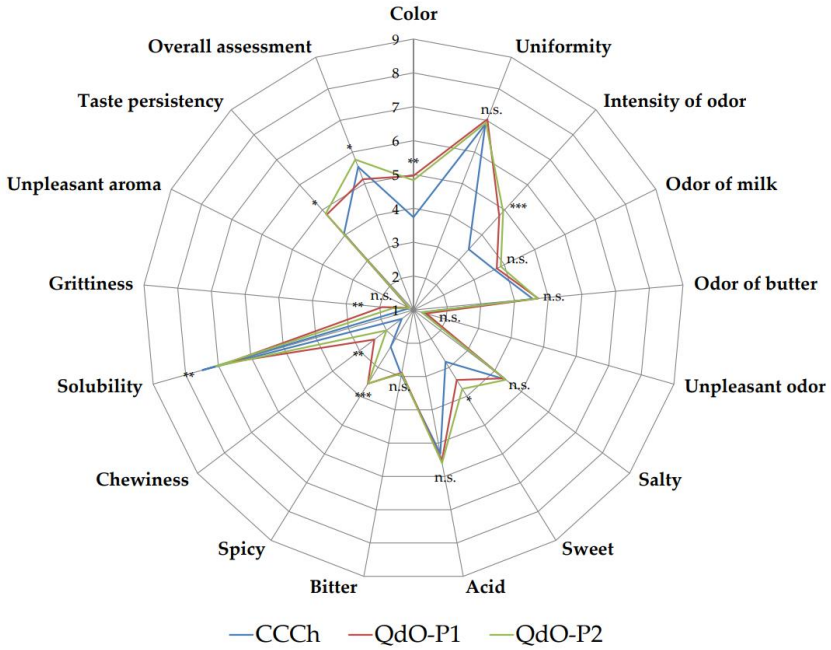

Figure 3. Spider diagrams of descriptive sensory analysis of cheeses. Abbreviations: CCCh, commercial crescenza cheese; QdO-P1, Quadrello di ovino cheese produced with starter culture SF1 (starter formulation CR/A); QdO-P2, Quadrello di ovino cheese produced with starter culture SF2 (starter formulation LYOBAC-D CRM). *p* value: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s., not significant.

Discussion

Sicily is a southern Italian region where cheese making is a very ancient daily activity [32]. Up to date, five out of 33 traditional cheeses produced in Sicily enjoy a PDO status: Vastedda della valle del Belìce, Pecorino Siciliano, Piacentinu Ennese, Provola dei Nebrodi, and Ragusano. Among these, only ewe's milk Vastedda della valle del Belìce cheese can be considered a fresh product [33]. Except this particularly rare example, in Italy ewe's milk is generally transformed to produce hard or semi-hard cheeses, especially in the south part of the country [17].

The main hypothesis of this work was that a new typology of cheese could be produced from ewe's milk and that this fresh cheese could be well appreciated by

consumers. With this in mind, QdO cheese was processed with commercial culture preparations of *Streptococcus thermophilus*, typical thermophilic dairy LAB [34], used as starters for Crescenza cheese production [31,35,36]. Among fresh cheese, Crescenza is an Italian product particularly appreciated by consumers [37]. It is a soft rindless cheese with a very short ripening time, processed from pasteurized cow's milk in the northern Italy, mainly in Lombardia region [38].

Regarding the inoculums for curd acidification, two different commercial preparations were used in order to evaluate the influence of different strains on the final characteristics of QdO cheese. The metabolic activities of *Streptococcus* strains are particularly flexible and diverse [39] and metabolomics are being widely applied for food quality analyses [40]. Thus, the use of different starter strains might provide unique metabolomics for the final products. For this reason, in this work two commercial starter preparations were used.

The pasteurised bulk milks were microbiologically investigated, and the densities of LAB were almost at the same level of TMM. These results might be ascribable to the ability of the indigenous milk bacteria to survive during the pasteurization process [41,42], but also to a post-pasteurization contamination [43,44]. Furthermore, this phenomenon seems to be common in pasteurized ewe's milk [8,16,45]. All undesired bacteria (*Pseudomonas*, members of Enterobacteriaceae family, *L. monocytogenes*, *Salmonella* spp., *E. coli* and CPS) were undetectable. Barbaccia et al. [45] reported similar findings for pasteurized ewes' milk used for another fresh ewe's milk cheese production, Pecorino "Primosale". After inoculation, starter cultures were monitored during cheese making. They increased until 10^8 CFU/g in curds as a consequence of

whey draining [46]. Both QdO cheese productions showed LAB levels of about 9 Log CFU/g following the general trend commonly observed for ovine pressed cheeses [8,47].

The cheeses were analyzed for several quality parameters, first of all for their physical characteristics (color and hardness). Color represents an important quality index of soft cheeses, including Crescenza type, that strongly affects the acceptability by consumers [48]. The three color parameters lightness, redness and yellowness of QdO cheese were, on average 87.31, 4.29, and 16.03, respectively. These values are comparable to those of the only fresh ewe's milk cheese (Vastedda della valle del Belìce) produced in Sicily [49]. Regarding redness, the results of QdO cheeses are almost identical to that of Roncal cheese [50], while yellowness is quite lower than those characterizing several ripened cow's milk cheeses. The initial b* values of soft cow's milk Stracchino (synonymous of Crescenza) cheeses are in the range 11.5–12.0 [51]. Comi et al. [48] stated that an increase of yellowness in Stracchino cheese, impacts both cheese shelf life and acceptability. An increase of yellowness is commonly observed during storage of high moisture cheeses [52]. In this study, the hardness of QdO cheese, which is the force required to obtain a given deformation of the sample, was 0.22 N/mm^2 on average. This value is extremely lower than those estimated for "Primosale" cheese for which hardness was in the range 0.98–1.10 N/mm^2 [8]. For this reason, the texture of Crescenza type cheese is considered soft and somewhat viscous [53].

The basic chemical composition of QdO cheese showed an average content of fats and proteins of 35.24 and 23.32%, respectively, which are much higher than the levels

reported for Crescenza cheese generally characterized by 24% of fats and 14% of proteins [31,36]. These results were quite expected, due to the high content of dry matter, proteins and fats of ewe's milk [54]. Furthermore, the compositional analysis of ovine QdO cheeses showed data comparable to those measured for other ovine fresh cheeses such as pressed Primosale cheese and stretched Vastedda-like cheese [8,16]. QdO-P1 cheese showed a higher antioxidant activity, in terms of TBARS, than QdO-P2 cheese. The lower values registered for the latter cheese are presumably due to the higher content in polyphenols of the bulk milk [55] or to the high ability of SF2 starter bacteria to inhibit lipid oxidation [56,57].

Regarding the composition of FAs, both experimental cheeses showed profiles comparable to those detected for other Sicilian ewe's milk cheeses [49,58]. The two productions were characterized by different levels of SFA, MUFA and PUFA. Bacterial metabolism during cheese processing clearly contributed to the final cheese FAs profiles. The lipolytic activity of the two commercial strain preparations could be characterized by a different enzyme kinetics and act selectively against the FAs present in milk triglycerides, leading to a different concentration of free FAs (FFA) on which beta-oxidation occurs [59]. Furthermore, it could be possible that elongation reactions can lead to the re-elaboration of the FFA present. Barely a few studies evaluated the effect of starter cultures on cheese FAs composition, obtaining conflicting results: Gursoy et al. [60] reported an effect of different bacteria inoculums on the final FAs composition of white pickle cheese, while Branciari et al. [61] registered no effect on Pecorino cheese.

VOCs emitted from food products are decisive for the perception of aroma and taste [62]. Recently, the relationship between VOCs and sensory attributes has been successfully evaluated for Gorgonzola cheese [63].

QdO cheese was characterized by alcohols, ethers, aldehydes, ketones, carboxylic acids, esters, amines, and monoterpenes. The class of carboxylic acids is mostly present in cheeses [64]. In particular, a significant increase in ester and ketones can be induced by ruminants' diet [65]. Acetoin was the most abundant ketone detected in QdO cheese. This chemical derives from the metabolism of lactose and citrate and is a common compound found in many other cheeses made with sheep and goat milk; it is responsible for the smell of butter perceived by panelists. Phenethyl hexanoate, the only ester that does not derive from linear FAs, was the second most abundant compound of this novel cheese. Carboxylic acids can be formed through lipolysis, proteolysis and fermentation of lactose. The presence of these acids provides dairy products with their characteristic aroma. Among the main acids detected, butanoic acid is characterized by a rancid and unpleasant smell [64]. According to Bontinis et al. [66], esters are originated by esterification between SCFA and ethanol, derived from the catabolism of amino acids. The presence of ethyl esters (mainly ethyl acetate, ethyl butanoate and ethyl octanoate) is characteristic of ovine cheeses. Among these compounds, ethyl butanoate, ethyl hexanoate and ethyl octanoate derive from acids and possess the typical smell of caprine cheese. Aldehydes are generally transient compounds in cheeses as they break down into acids and alcohols [67] and were detected at very low levels in QdO cheeses. Among these, benzaldehyde has a typical almond aroma, and it has been detected in other works aimed to characterize cheese

flavors [68,69]. Among terpenes which derive from plants and are transferred to dairy products through animal feeding [69], only the monoterpene hydrocarbon sylvestrene was found in QdO-P2 cheeses. On the other hand, regarding amines, produced via decarboxylation of amino acids [70], only dimethylamine was detected in QdO-P1 cheeses. This amine is characterized by fruity, alcoholic, and varnish flavor notes [71]. Generally, the concentrations of amines in fresh cheeses are lower than those found in long ripened cheeses [72].

Before commercialization of novel food products, the evaluation of sensory traits plays an important role in the assessment of overall acceptability [73]. For this reason, QdO cheeses were subjected to a sensory panel evaluation and their sensory traits were compared to those of Crescenza cheese. In particular, the novel ewe's cheese was characterized by a higher level of color, intensity of odor, sweet, spicy and taste persistency than cow's Crescenza cheese, no matter which starter cultures was used. These results were quite predictable since ewe's milk determines higher taste complexity in the final transformation products than cow's milk [74].

Conclusions

This study provided an extended analysis on the microbiological, physicochemical, VOCs profile and sensory characteristics of a novel soft ewe's milk cheese produced at industrial scale level with two defined commercial *S. thermophilus* starters. The microbiological analysis demonstrated the suitability of *S. thermophilus* to be used as fermenting agent in QdO cheese production. Lowest secondary lipid oxidation was observed in QdO-P2 cheese and, although the differences of VOCs emitted from the

two QdO cheeses were not perceived by the panelists, they showed a higher appreciation for QdO-P2 cheese that, in terms of judges' preference was comparable to commercial Crescenza cheese. These results clearly highlighted the huge marketability perspectives of QdO cheese and provided evidence on the possibility to enlarge the dairy product portfolio of fresh ewe's cheeses produced in Sicily.

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Chapter II

Application of whole genome sequenced selected lactic acid bacteria to tailor the making of the “Quadrello di Ovino” fresh ewe's milk cheese to the production area

The present chapter reports a work in progress

Introduction

Most cheeses produced today worldwide rely on the inclusion of specific starter microorganisms. These "starters", initiate cheese fermentation by consuming lactose and converting it into lactic acid [1]. Commonly referred to as "primary starters", these microorganisms typically belong to the lactic acid bacteria (LAB) genera, including *Streptococcus*, *Lactococcus*, *Lactobacillus* and *Leuconostoc* [2]. However, in cheesemaking, alongside starter cultures, non-starter cultures play a significant role. These non-starter cultures contribute to the development of flavorful organoleptic and sensory characteristics. Additionally, some of these cultures serve as probiotics – living microorganism that offer health benefits when consumed in adequate amounts, as recognized by the FAO/WHO [3].

In the commercial realm, several strains have been successfully incorporated as probiotic cultures into diverse cheese types. These include lactobacilli, *Bifidobacterium* spp., and *Propionibacterium* ssp. [4–6]. Dairy products, within this context, serve as intriguing and effective vehicles for probiotic consumption, thereby fostering the creation of functional and health-promoting foods. Minervini et al. [7] explored this concept in their research on Fior di Latte cheese, while Buriti et al. [8] investigated Minas fresh cheese. These studies underscore the suitability of different cheese varieties as potential carriers of probiotics, likely influenced by factors such as texture, pH, oxygen levels, buffering capacity, and fat content [9]. Researchers continue to explore novel food preparations and functional ingredients with the aim of enhancing health and preventing or managing diseases [10].

When incorporating new LAB as starter, prioritizing food safety it is crucial. Although the use of starter cultures has a long history, recent research indicates that microorganisms within these cultures may acquire antibiotic resistance genes. This development raises concerns about the potential antibiotic resistance of bacteria associated with LAB [11,12]. It is worth noting that the antibiotic resistance observed in starter culture bacteria does not directly endanger consumers, as these bacteria are not pathogenic. However, they can serve as environmental reservoirs for antibiotic resistance determinants. Consequently, caution should be exercised when using bacteria with antibiotic resistance genes in food production. Furthermore, stress factors during food production and storage may influence the antibiotic resistance profile of these microorganisms [13]. While the development of antibiotic resistance in starter cultures is not a critical concern, it remains an important consideration that cannot be overlooked [14].

Food producers employing starter cultures during the food production process must prioritize consumer health and safety. It is essential to ensure that any introduced microorganisms do not pose a direct or indirect risk to consumers. In Europe, stringent regulations govern food products to maintain high standards of safety and quality. Remarkably, the application of a microbial starter involves intentionally adding substantial amounts of cells per gram of specific agents to food matrices (approximately 10^7–10^8 cells per gram of inoculated raw material). Ensuring the safety of these microbial starter agents is a primary consideration when selecting and evaluating suitable strains and species. These microorganisms used in food must be classified as GRAS (Generally Recognised as Safe). Specifically, they should lack pathogenicity factors, antibiotic resistance, or virulence genes. Simultaneously, they must contribute to technological aspects without

posing any risk to consumer health. This concept aligns with the EFSA 2020 guidelines, which mandate comprehensive genome investigations of microorganisms to mitigate potential risks to consumers. Parallel with the relevance to normative context, there is also, in the evaluation and management of risk and in technological enhancement of starter cultures in foods, a crucial aspect related to biological sciences. This concerns taxonomy and nomenclature, pivotal aspects that allow unequivocal communication about every living species [15]. To meet this need, EFSA proposed a rapid system for assessing the risk associated with the use of products containing microorganisms for which authorization is required before being commercially available, born the concept of Qualified Presumption of Safety (QPS). Briefly, it was proposed that the safety evaluation of a taxonomic group could be done on the basis of four pillars (taxonomic identification, analysis of the knowledge, evaluation of potential security concerns and intended use). If the taxonomic grouping raised no safety concerns, or if safety concerns existed but could be defined and excluded, the grouping could be awarded QPS status. Afterwards, any microorganism strain whose identity could be unequivocally established and assigned to a QPS cluster would be exempt from the need for further safety evaluations, apart from meeting the required qualifications. Microorganisms not deemed suitable for QPS status remain susceptible to a full safety evaluation [16].

Based on the above considerations, this research aimed to utilize specific strains of *Pediococcus acidilactici*. These strains were isolated from the Sicilian dairy environment and had demonstrated intriguing acidifying capabilities. Interestingly, these microorganisms are not commonly employed as primary starters in cheesemaking, making them an excellent subject for exploration. The primary goal was to investigate whether

these strains could impart unique sensory properties to the final cheese, thereby establishing a connection between the product and its geographical origin. Additionally, these bacteria might offer potential benefits through the release of compounds resulting from proteolytic and lipolytic action on proteins and fats. Before their use in cheesemaking, the *P. acidilactici* strains underwent whole genome analysis. Subsequently, these strains were employed as starters to produce the fresh ewe's milk Quadrello di Ovino cheese, which had been previously described by Garofalo et al. [17]. The resulting cheese underwent thorough assessment, covering microbiological, physicochemical characteristics, composition, and sensory aspects.

Materials and methods

Whole genome sequencing analysis of pediococci

Total genomic DNA of was extracted from *Pediococcus acidilactici* RCUNIPA2 e RCUNIPA5 from 1.8 mL of broth culture using the Wizard Genomic DNA Purification System kit (Promega Italia, Milan, Italy), following the manufacturer's instructions. The DNA quality was estimated by Qubit Fluorometer (Thermo Fischer Scientific, Waltham, MA, USA) and NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, US).

Library preparation and sequencing were performed by Centro Piattaforme Tecnologiche (CPT University of Verona, Italy). In detail, libraries were obtained using KAPA PCR-Free Kit (Roche Diagnostics spa, Monza, Italy) according to the manufacturer's instructions; Illumina MiSeqDX in paired-end 150 mode was employed as

platform sequencing. The raw reads were demultiplexed and adapters were masked by CPT tools.

Genome annotation was performed with RAST (Rapid Annotation using Subsystem Technology) (http://rast.nmpdr.org/rast.cgi) which was used to obtain the subsystem category distribution for annotation [18].

Taxonomic identification and genome-based safety assessment

The Average Nucleotide Identity (ANI) [19], was calculated using the ANI Calculator in EzBiocloud (https://www.ezbiocloud.net/tools/ani) while digital DNA-DNA hybridization (dDDH) [20] was determined using GGDC (Genome to Genome Distance Calculator 3.0) [21].

The annotated sequences of both strains were employed to query the Comprehensive Antibiotic Resistance Database (CARD, version 3.2.7; https://card.mcmaster.ca/) through the Resistance Gene Identifier tool (RGI, version 6.0.2) [22] selecting only “Perfect” and “Strict” hits and to query ResFinder 4.1 database (https://cge.food.dtu.dk/services/ResFinder/) [23] with 90% as percentage of identity and 60% as query coverage for acquired antimicrobial resistance genes.

Experimental plan and starter preparation

Drawing inspiration from the research conducted by Garofalo et al. [17], this study devised two distinct cheese production approaches. The control production (CP) involved incorporating freeze-dried commercial starter formulations CRBS7 purchased from Calza Clemente (Acquanegra Cremonese, Italy). The lyophilised starter was reactivated

following the manufacturer's instructions. Specifically, 5 units were reactivated in 2 L of pasteurized ewe's milk through manual agitation for 10 min, after which they were directly added to the pasterurized ewe's milk. The experimental production (EXP) was carried out by inoculating the two selected strains of *Pediococcus acidilactici* (RCUNIPA2 and RCUNIPA5), as previously described. These distinct approaches aimed to explore the impact on cheese quality and characteristics.

The two bacterial strains were revitalized in a suitable culture broth (M17) medium (Oxoid, Milan, Italy) and incubated at 37 °C for 24 h. Subsequent refreshments were performed to generate an adequate pellet volume for inoculation. Following this, two consecutive washes with Ringer's solution (0.9% v/v) were carried out using a Neya 16R centrifuge (Securlab SRL, Rome, Italy) at 7000 rpm for 2 min to remove any remnants of the broth medium. The milk starter culture (MSC) was then prepared by inoculating approximately 10^6 colony-forming units (CFU)/mL of the washed cells from both strains into whole UHT ewe's milk (Leeb Vital, Wartberg an der Krems, Austria) and then incubated at 37 °C for 24 h.

Cheesemaking and sample collection

Cheeses production followed the methodology outlined by Garofalo et al. [17] using ewe's milk sourced from various dairy farms. This milk is daily transported to the "Il Cacio Siciliano" factory (Sicily, Italy) via tanker trucks maintained at controlled temperatures. Subsequently, the milk underwent pasteurization in a COMAT PS15351 pasteurizer (CO.MAT. s.r.l., Bellizzi, Italy) for 15 s at 71 °C. After heat treatment, the milk was cooled to 38 °C and inoculated with the starter strains. The same protocol used to make

"Quadrello di Ovino" cheese [17] was then repeated. Both cheese productions occurred on the same day, with a subsequent repeatition one month later. Throughout the cheesemaking process, samples of raw ewe's milk (RM), pasteurized milk (PM), inoculated milk (IM), whey (W), and curds (C) were collected under aseptic conditions. All samples were transported under cooled conditions to the Agricultural Microbiology Laboratory at the University of Palermo. Finally, the cheeses (Ch) were sampled after 4 d of refrigerated storage.

Microbiological analyses

One mL of liquid samples (RM, PM, LIM, W) was aliquoted and subjected to ten-fold dilutions in Ringer's solution prepared at 0.9% (v/v) salt. Additionally, 15 g of solid samples (C and Ch) were first initially homogenized with 135 mL of an isotonic sodium citrate solution prepared at 0.2% (v/v) using a Bag-Mixer 400 paddle homogenizer (Interscience, Saint Nom, France). The homogenizer operated at maximum speed for 2 min. Subsequently, the solid samples underwent the dilution procedure in Ringer's solution, effectively reducing the initial density by an order of magnitude. Cell suspensions from RM and PM were subjected to plate counts to enumerate several microbial groups: total mesophilic microorganisms (TMM) on Plate Cout Agar (PCA), incubated at 30 °C for 72 h; pseudomonads on *Pseudomonas* Agar Base (PAB) supplemented with Cephaloridine–Fucidin–Cetrimide (CFC), incubated at 25 °C for 48 h; enterococci on Kanamycin Aesculin Azide (KAA) agar, incubated at 37 °C for 24 h; coagulase-positive staphylococci on Baird-Parker (BP) agar supplemented with rabbit plasma fibrinogen, incubated at 37 °C for 24 h. *Listeria monocytogenes* was cultured on *Listeria* Selective

Agar Base (LSAB) supplemented with SR0140E, incubated at 37 °C for 24 h; *Escherichia coli* and *Salmonella* spp. were grown on Hektoen enteric agar (HEA) at 37 °C for 24 h. Unicellular and filamentosus fungi were cultivated on Yeast Peptone Dextrose (YPD) supplemented with chloramphenicol (0.1 mg/mL) to inhibit bacterial growth. These cultures were incubated at 30 °C for 48 h. Potato Dextrose Agar (PDA) was used for fungal growth and incubated at 30 °C for 7 d. Additionally, lactic acid bacteria (LAB), total coliforms, and members of the Enterobacteriaceae family were included. Mesophilic LAB rods were cultured on de Man-Rogosa-Sharpe (MRS) agar acidified with 5 M lactic acid to pH 5.4, and incubated at 30 °C for 48 h. Thermophilic LAB rods were grown on WBM agar, following the preparation described by Settanni et al. [24], and incubated at 44 °C for 48 h. Mesophilic and thermophilic LAB cocci were cultured on M17 agar medium, with mesophilic LAB incubated at 30 °C for 48 h and thermophilic LAB at 44 °C for 48 h. Enumeration of members of the Enterobacteriaceae family and coliforms was performed on Violet Red Bile Glucose Agar (VRGBA) and Violet Red Bile Agar (VRBA), respectively, both incubated at 37 °C for 24 h.

The group of LAB was incubated under anaerobic conditions using anaerobic jars (AnaeroGen, Thermo Fisher Scientific, Waltham, MA, USA). The growth medium for LAB was supplemented with cycloheximide (10 mg/mL) to prevent fungal growth. Samples from IM, W, C and Ch were specifically analysed for TMM and mesophilic and termophilic cocci LAB as described above. All growth media and supplements were sourced from Oxoid (Milan, Italy). Microbiological counts were carried out in duplicate for all samples collected at any time.

Physical analysis

The interior surface of both control and experimental cheese productions was used to assess color parameters based on CIE system [25]. These parameters include: lightness (L*) which ranges from 0 (black) to 100 (white); redness (a*), varying from red (+a) to green (−a); and yellowness (b*), spanning from yellow (+b) to blue (−b). To determine the pH value, a portable pH meter (Hanna HI98161) was immersed in a homogenized cheese sample. Measurements for both pH and color were taken from three distinct areas, and the results were averaged. The hardness of cheeses resulting from both CP and EXP productions was evaluated using an Instron 5564 tester (Instron, Trezzano sul Naviglio, Milan, Italy). The maximum compressive strength (N/mm^2) was measured for samples previously cut into dimensions of 3 cm × 3 cm × 3 cm and kept at room temperature (22 °C).

Chemical Analysis

Contents of dry matter (DM), fat, ash, titrable acidity and sodium chloride were determined in accordance with AOAC guidelines [26–30]. Protein (N × 6.38) was established according to IDF standards [31].

Total free fatty acid (FFA) and free fatty acid profile of cheese samples was carried out according to the methodology of De Jong et al. [32].

Organic acid and sugar analysis were done as described by Manolaki et al. [33].

Cheese proteolysis

Total nitrogen (TN), water-soluble nitrogen (WSN), trichloroacetic acid soluble nitrogen (TCASN) and phosphotungstic acid soluble nitrogen (PTASN) contents of samples were calculated by the methods described by Bütikofer et al. [34]. Proteolytic maturation parameters were calculated from TN, WSN, TCASN, and PTASN values using the following equations:

$$Ripening\ extension\ index\ (REI) = \frac{WSN}{TN} \times 100$$

$$Ripening\ depth\ \ index\ (RDI) = \frac{TCASN}{TN} \times 100$$

$$Free\ amino\ acid\ index\ (FAAI) = \frac{PTASN}{TN} \times 100$$

In addition to PTASN values, the total free amino acid (FAA) content of cheese samples was quantified using the 2,4,6-trinitrobenzenesulphonic acid (TNBS) method. TNBS reacts with primary amines, producing a yellow color, and the absorbance of this color was measured at 420 nm [35]. Through this method, total amino acid content in cheeses can be better quantified because there are only free amino acid groups [36].

Volatile organic compounds analysis

Volatile organic compounds (VOC) extraction and analysis were carried out using solid phase dynamic microextraction (SPME) and GC/MS, according to the method described by Gioacchini et al. [37]. Chromatographic separation was done using a capillary column (60 m × 0.25 mm × 0.25 μm) (Agilent 7890B GC, 7010B MS triple quadrupole, MS Agilent Technologies, USA). SCAN mode (50-500 m/z) was used for compound identification. The peak areas were assumed to be the relative abundances of each volatile

compound. Each compound was identified by means of the NIST 14 library standards for comparison.

Sensory analysis

A total of 12 assessors (comprising five men and seven women, aged between 26 and 63 years) evaluated CP (control production) and EXP (experimental production) cheese samples for their sensory attributes. These assessors received specific training at the Department of Agricultural, Food, and Forestry Sciences of the University of Palermo, following the guidelines outlined in ISO 8589 [38]. Using tablets, the panelists assessed 17 descriptive attributes on a growing intensity score ranging from 1 to 9. These attributes covered various aspect (color and uniformity of the paste), odor (intensity of odor, butter odor, milk odor and unpleasant odor), taste (salty, sweet, sour, bitter, spicy, taste persistence and unpleasant aromas), and mouthfeel (chewability, solubility and grittiness after chewing) charateristics, and overall acceptability. The sensory evaluation of the cheeses was compared to that of commercial cow's Crescenza (C-C) purchased in a retail market.

Statistical analysis

Microbiology, physical and chemical data as well as VOCs were examined by one-way analysis of variance (ANOVA) with the software XLStat version 7.5.2 for Excel (Addinsoft, New York, NY, USA). Differences in means were determined by Tukey's test at $p \leq 0.05$.

Results and discussions

General features of Pediococcus acidilactici RCUNIPA2 and RCUNIPA5 genomes and genome-based safety assessment

The genome sequence of *P. acidilactici* RCUNIPA2 consisted of 24 scaffolds and a size of 2.1 Mb (scaffolds size ≥ 1000 bp), the GC content was 41.7%, whereas the N50 value was 162,631 bb, whereas the genome of *P. acidilactici* RCUNIPA5 consisted of 44 scaffolds with a size of 2.2 Mb (scaffolds size ≥ 1000 bp), a GC content of 41.8%, and an N50 value of 113,046 bp. A total of 1955 and 2150 genes were predicted for RCUNIPA2 and RCUNIPA5, respectively; most of the genes were related to Carbohydrates (306 and 281), followed by Protein metabolism (199 and 210), DNA metabolism (107 and 121) and Cell Wall and Capsule (107 and 127, respectively). The two genomes were analysed with both CARD and Resfinder and no genes related to antibiotic resistance traits were found.

Whole-genome analysis applied to *P. acidiliactici* species generally shows a high genetic diversity which is basically related to variable genomes, mobile elements, and hypothetical genes obtained through horizontal gene transfer. Li et al. [39] explained through comparative genomics the adaptation of *P. acidilactici* for host environment. Furthermore, different *P. acidilactici* strains can metabolize a variety of carbon sources, enhancing the adaptability of this species and survival in different environments.

Monitoring of microbiological levels in Quadrello di Ovino cheese

Figure 1 presents the results of ewes' milk plate count level before and after pasteurization. Initially, raw ewe's milk exhibited a TMM load of 5.86 Log CFU/mL, a value consistent with findings from Pisano et al. [40] in their study on Fiore Sardo cheese,

an ewe's milk cheese produced in Sardinia. TMM is commonly assessed to evaluate on-farm sanitation practices and the quality of milk and dairy products [41]. It serves as an indicator of bacterial contamination stemming from factors such as udder hygiene, milking tools, water quality, and inadequately sanitized milking equipment [42]. Following the sanitization treatment, the TMM load decreased by approximately 2 log cycles.

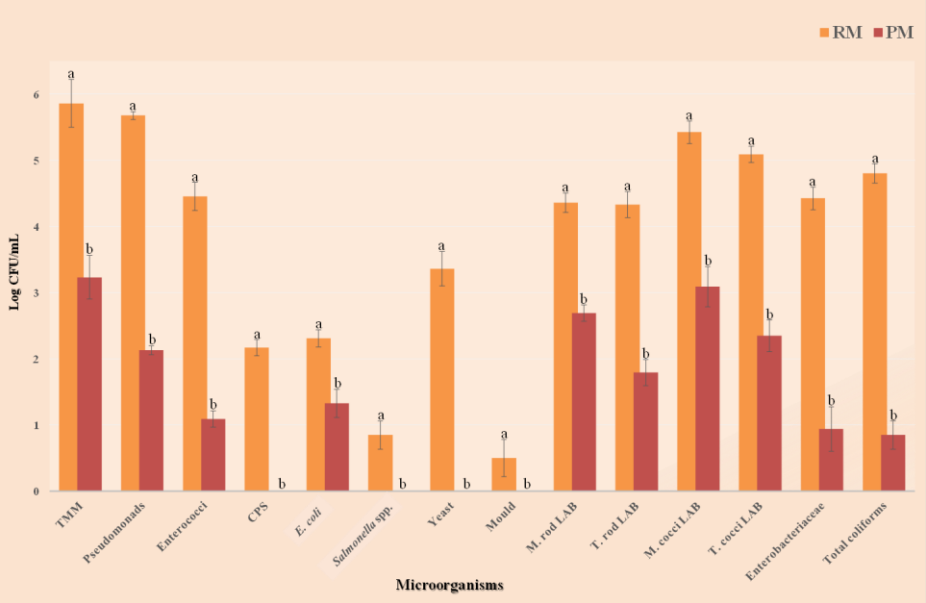

Figure 1. Microbial level of milk samples.

Among spoilage microorganisms, pseudomonads were found in higher cell densities in milk compared to total coliforms and Enterobacteriaceae members (5.67, 4.80 and 4.42 Log CFU/mL respectively). Raw ewe's milk, being one of the most complex and unpredictable food matrices, may pose a risk for the viability and proliferation of alterative or, in the worst cases, pathogenic microorganisms [43]. To address this concern, various thermal methods can be used to reduce the levels of these microorganisms. Among these methods, pasteurization treatment (using high temperature/short time) effectively eliminates the risk of viable pathogens organisms. However, a portion of protechnological

microorganisms and enzymes that contribute to the development of a cheese's distinctive sensory profile is also eliminated [44].

According to EU Regulation 853/2004 [45], goat's and ewe's milk with a total mesophilic count greater than 5×10^5 Log CFU/mL must undergo pasteurization [46]. Consequently, thermal sanitation significantly impacted the initial level of spoilage microorganisms. Specifically, pseudomonads decreased by approximately 3 log cycles, while total coliforms and members of the Enterobacteriaceae family decreased by approximately 4 log cycles. A similar trend was observed for pathogenic microorganisms (such as *E. coli*, *Salmonella* spp., and CPS) and unicellular and filamentous fungi. These were initially detected at low levels in raw milk but were not detected following pasteurization, consistent with findings reported by Fotou et al. [46]. Interestingly, *L. monocytogenes*, was never found even in raw milk samples.

Within LAB, mesophilic cocci were the most highly counted after sanitisation (3.09 Log CFU/mL), consistent wth the findings in the work of Garofalo et al. [47]. The separation of whey (Table 1) from the curd resulted in a concentration of the microbial load, causing an increase of approximately one Log cycle in the TMM within the two curd productions [48]. During fermentation of both productions, mesophilic coccus LAB remained dominant, with counts of 7.65 and 7.86 Log CFU/g in the CCP and CEXP samples, respectively. In contrast, whey exhibited a lower microbial load, approximately 4 Log CFU/mL in both productions. These results are not surprising, as the lowest cell density in whey samples are attributed to the curd coagulation process [49]. Enumeration of inoculated milk samples showed no significant differences in the detected LAB levels

between CP and EXP. However, a slightly higher count was observed in the control production.

After refrigerated storage for 4 days, the Quadrello di Ovino cheese was analyzed. The plate count results revealed statistically significant differences between the two productions. Specifically, the EXP production exhibited levels of approximately 10^8 CFU/g for mesophilic and thermophilic coccus LAB. This suggests that pediococci may play an interesting role as starters for fresh ovine cheeses. In contrast, the results of LAB in the CP production align with those reported in the work of Natrella et al. [50] for Canestrato Pugliese cheese.

Table 1. Microbial loads[a] of samples.

Samples	**Microorganisms[a]**		
	TMM	Mesophilic cocci LAB	Termophilic cocci LAB
IM CP	7.32	7.36	6.24
IM EXP	7.42	6.95	5.85
SEM	0.04	0.07	0.08
p value	0.556	0.058	0.166
C CP	7.88[b]	7.65	6.45
C EXP	8.51[a]	7.86	6.71
SEM	0.09	0.04	0.05
p value	0.008	0.106	0.125
W CP	5.55[b]	4.41	4.39
W EXP	6.64[a]	4.57	4.47
SEM	0.16	0.06	0.03
p value	0.004	0.485	0.425
Ch CP	7.16[b]	7.09[b]	6.95[b]
Ch EXP	8.74[a]	8.50[a]	8.46[a]
SEM	0.23	0.20	0.23
p value	0.003	0.001	0.017

Results indicate mean values of four plate counts (carried out in duplicates for two independent productions).
[a] Log CFU/mL for inoculated milk and whey samples; Log CFU/g for curd and cheeses.
[b] Abbreviations: TMM, total mesophilic microorganisms; LAB; lactic acid bacteria. IM, inoculated milk; CP, control production; EXP, experimental production; C, curd; W, whey; Ch, cheese. SEM, standard error of the mean.
Data within a row followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$).

Physical properties of Quadrello di Ovino cheese

Physical characteristics of the cheeses are summarized in Table 2. The values of pH differed between CP and EXP cheeses. Notably, the EXP cheese exhibited higher acid values than the CP cheese. Interestingly, there was no significant diversity between the cheese made with selected pediococci and the commercial crescenza purchased from a local supermarket. Previous studies, including those by Gobbetti and collaborators [51] and Burns et al. [52], have reported pH values around 5.2, which aligns with our findings. In contrast to pH values, the three color parameters lightness (L*), redness (a*), and yellowness (b*) were not affected by the two starter cultures. Furthermore, both CP and EXP cheeses showed no differences compared to the commercial Crescenza in terms of color. Color plays a significant role in consumer acceptance, especially for fresh cheeses like Crescenza and Stracchino. For instance, an intensification of yellow coloration can be negatively perceived by consumers [53]. Our results are comparable to those observed in other fresh ewe's cheeses, such as Vastedda della valle del Belíce cheese [54] and Primosale cheese [55].

Table 2. Physico-chemical composition of Quadrello di Ovino cheeses.

Parameters	**Samples**			***p* value**	**SEM**
	C-C	CP	EXP		
Color					
L*	92.19	88.79	92.28	0.280	0.73
a*	2.38	2.94	2.60	0.086	0.08
b*	13.43	14.92	12.49	0.490	0.58
pH	5.22^{a}	4.88^{b}	5.25^{a}	<0.0001	0.04
Hardness (N/mm^2)	0.035	0.043	0.067	0.078	0.00
DM (%)	43.16^{c}	45.49^{b}	49.07^{a}	<0.0001	0.66
Fat on DM (%)	60.24	62.65	62.16	0.587	0.68
Ash on DM (%)	2.07^{b}	2.11^{b}	3.34^{a}	<0.0001	0.16
Titrable acidity (%)	1.02^{c}	1.59^{a}	1.20^{b}	<0.0001	0.06

Salt (%)	1.46[b]	1.47[b]	2.39[a]	<0.0001	0.12
Protein	15.39	14.81	16.85	0.111	0.32

Results indicate mean values of three determinations carried out in duplicate for each of the two independent cheese-making.
Abbreviations: C-C, commercial Crescenza; CP, control production made with freeze-dried starter culture (CRBS7); EXP, experimental production made with selected strains (RCUNIPA2 and RCUNIPA5); DM, dry matter.
On the row: a, b, c = $p < 0.05$. SEM, standard error of the mean.

The hardness parameters of Crescenza-type cheeses made using the two different starters did not exhibit significant differences, and they were also comparable to the commercial counterpart. This finding is encouraging for promoting the use of indigenous starters. Notably, the low hardness values, averaging 0.05 N/mm², can be attributed to several factors. Cheese texture is primarily influenced by moisture, fat content, and the integrity of the protein matrix [56]. High moisture levels, combined with elevated fat content and reduced proteolytic activity, contribute to a softer cheese texture [57].

Cheese chemical content and free fatty acids composition

The chemical characteristics of the cheeses are summarized in Table 2. Notably, the fat and protein content remained unaffected by the type of starter culture used. However, significant differences were observed in terms of total dry matter and titratable acidity across all cheeses. Additionally, the EXP cheese exhibited higher levels of ash and salt content. It's worth noting that these values fall within the typical range encountered in other fresh ewe's cheeses [35, 58].

The total fatty acid amounts, along with the fatty acid profile, are detailed in Table 3. While no statistically significant differences were observed in the overall fatty acid content among the cheeses, variations were evident in their specific fatty acid profiles. Saturated fatty acids (SFAs), such as palmitic acid (C16:0) and myristic acid (C14:0), are naturally

abundant in ewe's milk [59]. The nutritional value of fat in ewe's milk is highly regarded due to the potential health benefits associated with fatty acids like conjugated linoleic acid [60]. Our study aligns with this, revealing palmitic and myristic acid levels of 26.31 and 10.37, respectively, in the CP cheese, and 25.99 and 9.42 in the EXP cheese. Additionally, CLA was present at similar levels in both CP and EXP productions (2.50 and 2.61, respectively). Short-chain fatty acids (SCFAs; C4:0-C10:0) significantly contribute to the final taste of cheese [61]. In our investigation, caproic, caprylic, and capric acid exhibited significantly higher concentrations in the CP cheese compared to the EXP cheese. A similar trend was observed for medium-chain fatty acids (lauric, myristic, and pentadecanoic acid), which were more abundant in the CP cheese. On the other hand, the EXP cheese displayed higher concentrations of stearic acid (14.35), linolenic acid (0.70), and oleic acid (26.80).

Table 3. Total free fatty acid amount (%) and fatty acid profile (%) of Quadrello di Ovino cheeses.

Parameters	Samples			*p* value	SEM
	C-C	CP	EXP		
FFA (% in DM)	1.49	2.68	1.83	0.123	0.19
Caproic acid (C6)	2.23^{a}	2.30^{a}	1.84^{b}	0.009	0.06
Caprylic acid (C8)	1.49^{b}	1.96^{a}	1.58^{b}	0.005	0.06
Capric acid (C10:0)	3.67^{c}	5.40^{a}	4.44^{b}	0.001	0.20
Lauric acid (C12:0)	4.39^{a}	3.46^{b}	2.94^{c}	<0.0001	0.16
Myristic acid (C14:0)	13.20^{a}	10.37^{b}	9.42^{c}	<0.0001	0.43
Pentadecanoic acid (C15:0)	1.43^{a}	1.24^{b}	1.18^{c}	<0.0001	0.03
Palmitic acid (C16:0)	35.68^{a}	26.31^{b}	25.99^{c}	<0.0001	1.19
Palmitoleic acid (C16:1)	1.79^{a}	1.49^{b}	1.48^{b}	<0.0001	0.04
Stearic acid (C18:0)	10.18^{c}	13.10^{b}	14.35^{a}	<0.0001	0.47
Oleic acid (C18:1 Δ^{c9})	21.36^{c}	25.07^{b}	26.80^{a}	<0.0001	0.61
Oleic acid (C18:1 Δ^{t9})	1.68^{c}	6.16^{b}	6.67^{a}	<0.0001	0.60
Linoleic acid (C18:2)	2.90^{a}	2.50^{b}	2.61^{b}	<0.0001	0.05
Linolenic acid (C18:3)	0.48^{c}	0.66^{b}	0.70^{a}	<0.0001	0.03

Results indicate mean values of three determinations carried out in duplicate for each of the two independent cheese-making.

Abbreviations: C-C, commercial Crescenza; CP, control production made with freeze-dried starter culture (CRBS7); EXP, experimental production made with selected strains (RCUNIPA2 and RCUNIPA5); FFA = free fatty acid.
On the row: a, b, c = $p < 0.05$. SEM, standard error of the mean.

Notably, oleic acid belongs to the group of medium-chain fatty acids (MUFAs). These MUFAs play a crucial role; they are directly absorbed and transported to the liver, where rapid metabolism enhances the thermogenic effect induced by the diet [62]. Interestingly, the fatty acid profile of the EXP cheese sample closely resembles that of other fresh cheeses [63].

In cheese production and ripening, three primary biochemical events play crucial roles: glycolysis, proteolysis, and lipolysis. These events directly or indirectly influence the chemical composition, sensory characteristics, and overall quality, including flavor and texture, of diary products [64]. During production, starter bacteria convert lactose into lactic acid [65]. For the fresh cheeses manufactured in our study, which have a limited maturation period of just a few days, lactic acid was the sole organic acid detected by HPLC. Its concentration was measured at 3147.92 and 2630.10 ppm in CP and EXP samples, respetively.

The concentrations of glucose, a hydrolysis product of lactose, was higher in the EXP samples (720.966 ppm) compared to the CP samples (243.880 ppm).

Cheese proteolysis

Proteolysis, a crucial biochemical process, significantly impacts the organoleptic qualities of cheese. It is influenced by residual enzymes from curd, milk proteinases, and proteolytic enzymes from both starter and non-starter bacteria [66]. The results of the proteolytic parameters of Quadrello di Ovino cheese samples are presented in Table 4. To

specify the degree of proteolysis, one straightforward approach is to examine the fractions of soluble nitrogen. Additionally, water-soluble nitrogen (WSN) provides insights into proteolysis. WSN quantification involves assessing coagulant and plasmin activity, as well as measuring small peptides and amino acids within WSN [36]. WSN, TCASN, and PTASN values were in the ranges 0.18–0.45%, 0.05–0.21% and 0.04–0.13, respectively. Based on these data, three indices were calculated: REI, ranged from 7.56 to 16.92%; RDI, ranged from 2.10 to 9.24%, and FAAI, ranged from 0.78 to 5.62%. Total free amino acid (FAA) content in the cheese samples ranged from 0.39 to 1.5 mg Leu/g cheese. These ranges, however, appear lower than those reported in the current literature. This discrepancy can be partly attributed to the nature of Crescenza, which is a fresh cheese with limited proteolytic activity. Its positively perceived lactic and slightly acidic taste may be linked to the survival of a high density of starter cells within the cheese matrix [67].

Table 4. Proteolytic parameters of Quadrello di Ovino cheeses.

Parameters	Samples			*p* value	SEM
	C-C	CP	EXP		
TN	2.41	2.32	2.64	0.107	0.05
WSN (%)	0.18^{c}	0.21^{b}	0.45^{a}	<0.0001	0.03
TCASN (%)	0.05^{c}	0.21^{a}	0.15^{b}	<0.0001	0.02
PTASN (%)	0.04^{c}	0.13^{a}	0.10^{b}	<0.0001	0.01
Total FAA	0.39^{c}	1.47^{a}	0.90^{b}	<0.0001	0.12
REI (%)	7.56^{c}	9.04^{b}	16.92^{a}	<0.0001	1.10
RDI (%)	2.10^{c}	9.24^{a}	5.57^{b}	<0.0001	0.78
FAAI (%)	0.78^{c}	5.62^{a}	3.64^{b}	<0.0001	0.53

Results indicate mean values of three determinations carried out in duplicate for each of the two independent cheese-making.

Abbreviations: C-C, commercial Crescenza; CP, control production made with freeze-dried starter culture (CRBS7); EXP, experimental production made with selected strains (RCUNIPA2 and RCUNIPA5); TN, total nitrogen; WSN, water-soluble nitrogen; TCASN, tricholoroacetic acid-soluble nitrogen; PTASN, phosphotungstic acid-soluble nitrogen; FAA, free amino acid (mg leucine g^{-1} cheese); REI, ripening extension index; RDI, ripening depth index; FAAI, free amino acid index.

On the row: a, b, c = $p < 0.05$. SEM, standard error of the mean.

VOC profile of cheeses

The analysis of volatile organic compounds (VOCs) in cheeses was conducted using SPME-GC/MS, and the results are presented in Figure 2. A total of 53 volatile compounds were identified, categorized into nine phytochemical clusters: acids, alkanes, alcohols, aldehydes, amines, ketones, esters, lactones, and terpenes. The overall content of VOCs varied significantly among the cheeses. Interestingly, five compounds were shared by all three cheeses; one acid (isovaleric acid), three alcohols (phenylethyl alcohol, [S,S]-2,3-butanediol and [R,S]-2,3-butanediol) and one ester (ethyl caproate). Phenylethyl alcohol, in particular, plays a key role in shaping the aromatic profile of ewe's milk cheeses. Its delicate rose-like scent adds to the sensory experience of these cheeses [68].

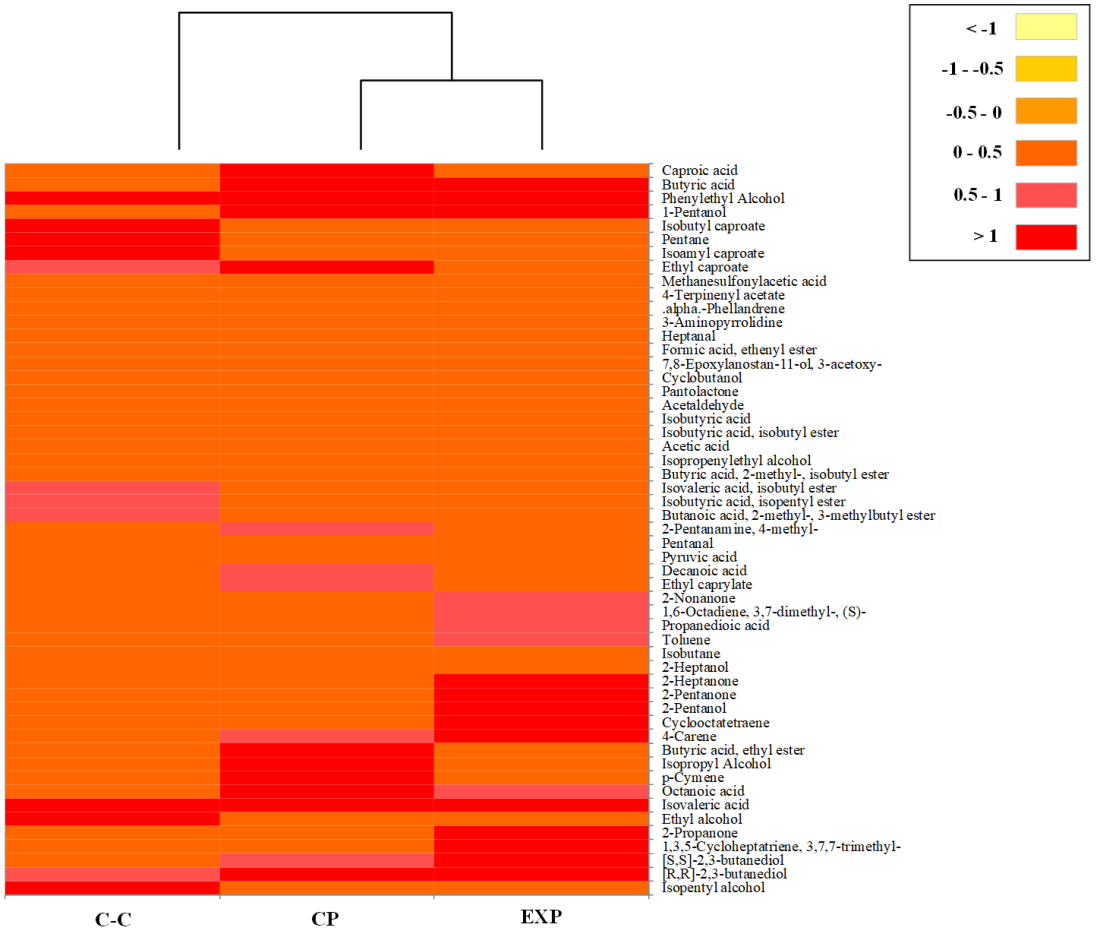

Figure 2. Heat mapof VOCs emitted from Quadrello di ovino cheeses.

In both commercial Crescenza cheese and EXP Quadrello ovino, alcohols emerged as the most abundant class of compounds. Numerous studies have highlighted that alcohols play a pivotal role in shaping the chemical landscape of certain ewe's milk cheeses, potentially influencing their flavor profiles [69, 70]. However, in CP Quadrello di Ovino cheese, a different trend emerged. Acids dominated the volatile organic compounds, with butyric acid and caproic acid occurring in higher proportions (36.57% and 19.21%, respectively). Butyric acid, notorious for its rancid cheese-like odor, significantly contributes to the flavor of cheeses such as Camembert, Cheddar, Grana Padano, Gruyère, Pecorino, Ragusano, and Roncal [71]. Interestingly, amines, aldehydes, and lactones were negligible in all three cheeses examined. On the other hand, alkanes and ketones were significantly present in the EXP cheese (10.22% and 13.26%, respectively), directly contributing to its flavor profile [72]. Esters, however, took center stage in the aromatic ensemble. They were predominantly detected in both commercial Crescenza and CP ovine cheese. Among these esters, ethyl caproate and isoamyl caproate stood out with the highest concentrations. Notably, ethyl caproate also holds the title of the most abundant ester in Pecorino Romano [73] and Canestrato Pugliese cheeses [74]. Esters, being a common volatile fraction in cheese, significantly contribute to the overall aroma. Their high volatility at ambient temperatures and low perception threshold make them essential players in the sensory experience [75].

In our study, the milk used for CP and EXP cheese production was ewe's milk, while commercial cheese was made from cow's milk. According to McSweeney et al. [76], the potential sources of variability in the volatile composition of our ovine cheese include rennet enzymes, indigenous dairy enzymes (particularly plasmin and non-starter bacteria)

and any organisms that survive milk pasteurization or contaminate pasteurized milk and curd during manufacturing. However, the compounds detected in ewe's cheeses align with the findings of other studies where fresh ewe's cheeses were examined [77,78].

Sensorial assesment of Quadrello di Ovino cheeses

The introduction of a new product on the market inevitably requires consumer approval. Consequently, a sensory analysis conducted by a panel of judges can yield valuable insights into the perception and acceptance of the product. In this study, sensory features of rated cheeses were visualized using a spider plot (Figure 3). The parameters related to unpleasant odor, texture homogeneity, saltiness, and sweetness exhibited the highest values for the experimental cheese (EXP). Interestingly, both cheeses made from pasteurized milk with a commercial starter (CP) and pasteurized milk with a selected starter (EXP) did not show statistically significant differences ($p > 0.05$) according to Tukey's test. This lack of difference can be attributed to the use of the same ewe's milk for both cheeses. Sensory attributes of cheeses are generally influenced by the microbiological quality of the milk, the animals' diet, and dairy management practices [79].

The judges rated the EXP cheese higher in terms of texture homogeneity, a result comparable to that reported for fresh ewe's milk cheeses in the work of Garofalo et al. [17]. Additionally, the stronger unpleasant odor detected in the EXP cheese is plausible, given the ongoing debate surrounding the flavor profiles of sheep and goat cheeses. Some consumers prefer neutral flavors associated with cow's milk cheeses, while others appreciate the distinct goat or sheep aroma. These characteristic flavors arise from complex combinations of volatile compounds [80]. Ultimately, the question about the real

familiarity of judges with goat and ewe flavors remains. Lastly, both cheeses received similar overall evaluations in terms of satisfaction.

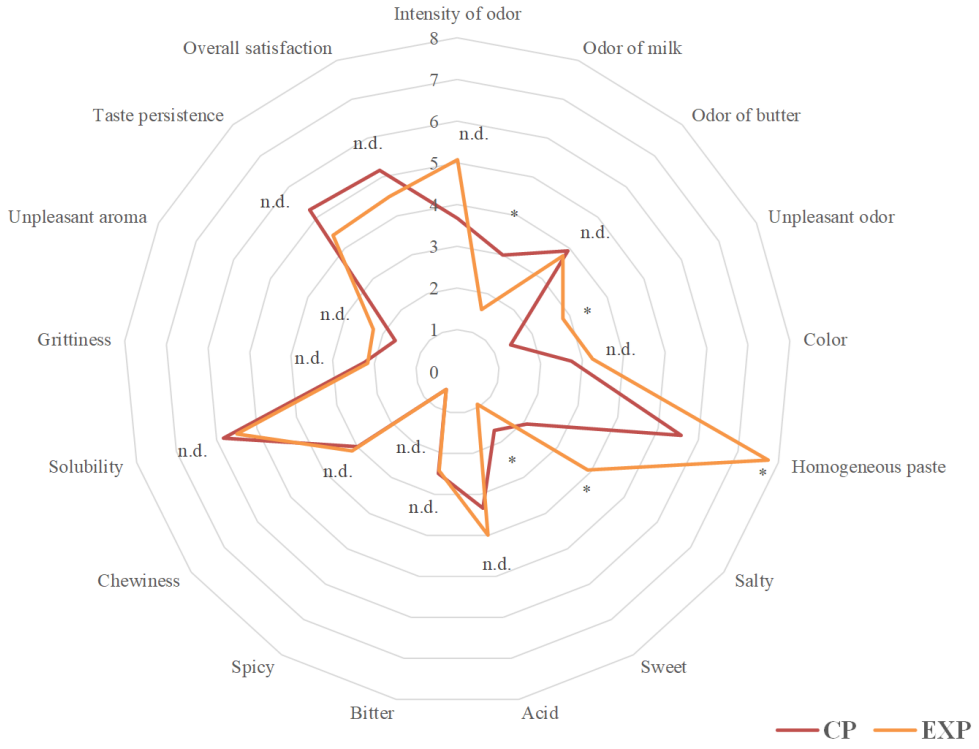

Figure 3. Radial plot of sensory analysis of Quadrello di Ovino cheeses

Conclusions

In this study, an in-depth analysis was conducted on the microbiological, physico-chemical, fatty acid, VOC, and sensory profile of Quadrello di Ovino, a fresh ewe's milk cheese produced at industrial scale. The cheese was made using selected starter bacteria isolated from Sicilian ewe's dairy production. Compliance with food regulations necessitates a thorough examination of the entire genome of new strains to ensure the absence of virulence and antibiotic resistance genes. The whole-genome sequencing (WGS) analysis revealed a non-pathogenicity and absence of genes involved in transferable antimicrobial resistance, virulence as well as in the formation of biogenic

amines, confirming the safety of the employed strains as suitable candidates as starters for cheesemaking. Their industrial application resulted in the production of Quadrello di Ovino cheeses that were microbiologically safe and highly appreciated in sensory terms by judges. Additionally, these cheeses exhibited a rich profile of fatty acids and volatile organic compounds.

These findings indicate a favorable market prospect for Quadrello di Ovino experimental cheese, offering an opportunity to expand the portfolio of fresh ewe's milk cheeses while maintaining their connection with the Sicilian territory.

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Chapter III

Description of "Ovino Belmontese", a new semisoft sheep's milk cheese processed using "Italico" cheese technology

The present chapter has been submitted for publication in

Food Bioscience

Abstract

The objective of this study was to create a new semisoft sheep's milk cheese called "Ovino Belmontese" cheese (OBCh) by applying the "Italico" cheese-making technology. The cheese production took place under industrial conditions, with the addition of a commercial starter formulation containing *Streptococcus thermophilus*. The microbiological, physicochemical, and sensory characteristics of OBCh were assessed and compared to those of a commercially available cow's Italico cheese (CICh). *Streptococcus thermophilus* dominated the microbial community during the cheese-making process, reaching levels of approximately 9.0 Log CFU/g in both OBCh and CICh. Among physical characteristics, no statistically significant difference ($p \geq 0.05$) was registered in terms of lightness, redness, yellowness, and hardness between the two cheeses. OBCh exhibited a twofold higher short-chain fatty acid content compared to CICh. Both cheeses displayed similar classes of volatile organic compounds, although their relative percentages differed. The application of Italico cheese technology to process sheep's milk did not negatively affect sensory attributes. This study highlighted that utilizing a cheese-making technology not commonly used for processing sheep's milk represents a promising strategy to diversify Sicilian dairy productions.

Keywords: sheep's milk; *Streptococcus thermophiles*, novel cheeses, physicochemical properties, fatty acids, sensory evaluation.

Introduction

Cheese, a culinary staple with a rich global history, has been produced for centuries [1]. Archaeological evidence, including cave paintings, traces cheese making back to the Palaeolithic era [2]. Over time, cheese production techniques have evolved due to various factors, including population growth, lifestyle changes, and the integration of cheese as a fundamental ingredient in the food service industry [3].

Sicily, strategically positioned in the Mediterranean Sea, has significantly influenced European cheese history [4]. Here, sheep farming prevails over cattle breeding due to the arid climate and rugged soil conditions [5]. Ovine breeding plays a crucial role in the regional economy [6]. Sicilian ewe's cheeses are intrinsically tied to their specific production areas and remain niche products due to their ancient and traditional methods [7]. Among these cheeses, Pecorino Siciliano, Piacentinu Ennese, and Vastedda della valle Belíce have earned the prestigious protected denomination of origin (PDO) status. While Vastedda della valle Belíce thanks to its stretching phase can be enjoyed soon after production [8], the other two cheeses, made from raw milk as well, require a minimum ripening period of four months [9]. During this maturation, the cheeses develop a robust and enduring aromatic profile, which may not be fully appreciated by all consumers, especially those with post-modern tastes [10]. To address this, the Sicilian sheep dairy industry is actively exploring innovative approaches. Developing ewe's milk products that can be marketed shortly after production while satisfying modern consumer preferences is a priority.

Traditionally, the production of typical cheeses has limited opportunities for innovation within the sheep's milk sector. However, diversifying dairy products remains a crucial

competitive strategy to adapt the ever-changing market dynamics [11]. Recently advancements have explored the application of Crescenza cheese technology, commonly used for cows' milk, to create a novel Sicilian ewes' cheese [12]. This innovative approach has yielded quality characteristics that resonate well with consumers. Beyond product diversification, this initiative also serves a broader purpose: revitalizing sheep breeding in rural marginal areas marked by significant land abandonment [13]. By embracing new cheese making techniques, Sicily aims to encourage sustainable sheep farming practices.

This research represents an initial endeavour to produce innovative ewe dairy products, drawing inspiration from the well-established and beloved Italico-cheese, a soft-rind, short-ripened cows' cheese [14].

Cheese making trials were performed on an industrial scale using commercial *Streptococcus thermophilus* starter cultures. The focus was on creating a new semisoft ewe's milk cheese "Ovino Belmontese" (OBCh), hailing from the homonymous municipality in Palermo province (Belmonte Mezzagno, Palermo, Italy), which was evaluated for its microbiological, physicochemical, and sensory characteristics. This research is part of a broader project aimed at promoting the value of Sicilian ewes' milk by developing innovative dairy products.

Materials and methods

Milk and milk starter culture preparation

The bulk milk used for cheese production came from several farms within Palermo province (Sicily, Italy). These farms raised sheep of the Valle del Belíce and Comisana breeds. Collected milk was transported in a refrigerated road tanker (4–6 °C) to the "Il

Caciocavallo" industrial dairy factory in Belmonte Mezzagno (Italy). The whole milk underwent pasteurization at 75 °C for 15 s using a Comat PS 15351 system (Bellizzi, Italy), previously sanitized with a UNIPLUS solution (Sydex S.p.A., Cercola, Italy). Freeze-dried cheese lactic acid bacteria (LAB) starter culture LYOBAC-D (Alce International s.r.l., Quistello, Italy) was employed to start the fermentation process. This starter culture consisted of various strains of *Streptococcus thermophilus*. Specifically, a package containing 5 units of freeze-dried starter preparation was reactivated in 2 L of pasteurized milk. After incubation at 44 °C for 50 min, this mixture became the Milk Starter Culture (MSC), the essential fermenting agent for cheese production.

Cheese production and sample collection

The production of Ovino Belmontese cheese (OBCh) followed the principles of the "Italico" semisoft cheese technology (Figure 1). Five hundred liters of pasteurized ewe's milk were transferred to a multi-purpose cheese vat (Comat mod. POL15P12, Bellizzi, Italy). The milk was cooled to 42 °C and then gently stirred for 10 min while inoculating it with the MSC. Coagulation was initiated by adding 225 mL of Astro Chymosin 200 liquid rennet (Calza Clemente s.r.l., Acquanegra Cremonese, Italy). After 20 min, the coagulum was manually crosscut using a stainless-steel rod, called "lira". An additional 20 min of mechanical agitation broke the curd into nut-size grains. Partial whey was drained, and the curd was promptly transferred into rectangular perforated plastic containers (20 cm × 13 cm × 11 cm) purchased from GR s.r.l. (Trapani, Italy). The curds underwent an initial 30 min steam stewing at 45 °C. They were then inverted in the molds and stewed for an additional 30 min. After 24 h of stewing, all cheeses were immersed in 18 °Bé brine for 20

min. The cheeses were then stored for 10 d at 6 °C and 90% relative humidity (RH) in a seasoning cabinet model 701 Glass (Everlasting s.r.l., Suzzara, Italy). Cheese production was performed in triplicate over three consecutive months (three independent experimental replicates). Samples were collected at various stages: pasteurized milk, freeze-dried starter preparation, inoculated milk with MSC, curd, and final cheese. All analyses performed on OBCh were compared to those of a commercial cow's Italico cheese (CICh) (Lactalis Galbani, Milan, Italy).

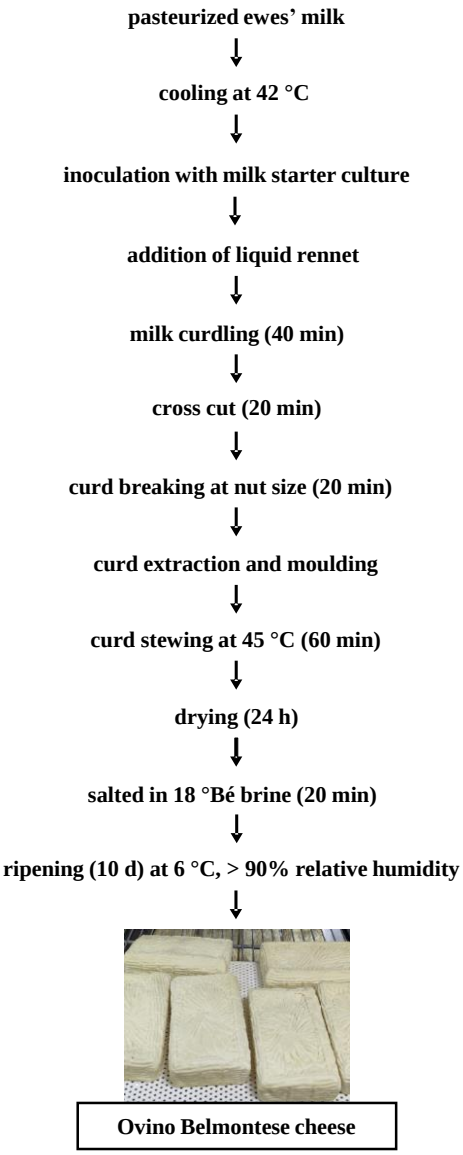

Figure 1. Flowsheet set up to produce "Ovino Belmontese" cheese.

Microbiological analyses of cheeses

All samples collected throughout the production chain of OBCh were subjected to the serial decimal dilution procedure [12]. Cell suspensions at decreasing cell densities were plated on: Plate Count Agar (PCA) incubated aerobically for 3 d at 30 °C for the enumeration of total mesophilic microorganisms (TMM); Sucrose Peptone Yeast pH 9.3 (SPY9.3) agar incubated anaerobically for 2 d at 42 °C for *S. thermophilus* [15]; Coliforms Chromogenic Medium (CHROM) agar incubated aerobically for 1 d at 37 °C for *Escherichia coli*; *Listeria* Selective Agar Base incubated aerobically for 1 d at 37 °C for *Listeria monocytogenes*; Baird Parker (BP) agar incubated aerobically for 2 d at 37 °C for coagulase-positive staphylococci (CPS); Xylose Lysine Deoxycholate (XLD) agar incubated aerobically incubated aerobically for 1 d at 37 °C for *Salmonella* spp.. All media, except for CHROM (provided by Condalab, Madrid, Spain) were purchased from Oxoid (Basingstoke, United Kingdom). Analyses were performed in duplicates for each replicate production.

Isolation, typing and identification of thermoduric milk LAB

All presumptive *S. thermophilus* developed on SPY9.3 inoculated with the cell suspensions of pasteurized milk were purified and subjected to Gram reaction and catalase activity tests [16]. Differentiation of the collected isolates was carried out using random amplification of polymorphic DNA (RAPD)-PCR analysis as described by Garofalo et al. [17]. Genotypic identification of the distinct strains was performed at the AGRIVET Centre (Palermo, Italy), following the approach reported by Gaglio et al. [18].

Monitoring of commercial starter culture

The dominance of commercial *S. thermophilus* starter culture over LAB resistant to pasteurization was carried out by RAPD-PCR analysis. Specifically, RAPD profiles obtained from bacteria isolated from SPY9.3 at the various stages of the OBCh production chain were compared with a pure culture of the *S. thermophilus* strain originating from the freeze-dried starter preparation.

Physicochemical analyses of cheeses

The colorimetric parameters of the cheese samples were determined using a tristimulus chromometer Minolta CR-400 (Minolta, Osaka, Japan), measuring the values of L* (lightness), a* (redness/greenness), and b* (yellowness/blueness), according to the Commission Internationale de l'Eclairage standard (CIE, 2019).

The pH was measured by immersing a portable Hanna HI98161 pH meter (Hanna Instruments, Woonsocket, USA) into homogenized cheese sample. Hardness analysis was carried out using a TA.XTplus Texture Analyser (Stable Micro Systems, Godalming, England). The cheeses were cut into cubes (3 cm × 3 cm × 3 cm) using a sharp knife and then compressed at a constant crosshead speed of 2 mm/s. The centesimal chemical composition of the samples was analyzed, and the dry matter (DM), protein, fat, and ash content were determined according to AOAC International methods [19–22]. Physicochemical determinations were performed in duplicate.

Determination of cheeses fatty acids

The fatty acid composition of the cheeses was analysed using Gas Chromatography-Mass Spectrometry (7890B GC - 7010B MS, Agilent). Grated cheese samples weighing 10 g underwent fatty acid esterification following the method outlined by De Jong and Badings [23] with modifications. Specifically, a 1 µL aliquot of the sample with a split ratio of 1:40 was injected into a GC-MS/MS system equipped with a flame ionization detector. Separation of the fatty acids was conducted using a capillary Agilent J&W DB-WAX column (60 m x 0.25 µm x 0.25 µm) with helium as the carrier gas flowing at a rate of 1 mL/min. The oven temperature program started at 50 °C for 1 min, then increased to 200 °C at a rate of 25 °C/min, held for 10 min, further increased to 230 °C at a rate of 3 °C/min, and maintained at this temperature for 26 min. The inlet temperature and detector were set to 250 °C and 300 °C, respectively. Identification of fatty acids was confirmed by comparing the retention times of sample peaks with those of reference standards (Supelco 37 Component FAME Mix, Sigma-Aldrich).

Analysis of volatile organic compounds emitted from cheeses

The volatile organic compounds of cheeses were determined using the headspace solid-phase microextraction method (HS-SPME) and analysed via Gas Chromatography (Agilent 7890B GC) coupled with mass spectrometry (7010B MS, Agilent). Initially, the samples were heated to 30 °C for 15 min, allowing the volatile compounds to be adsorbed onto a coated fiber (Carboxen TM/PDMS StableFlexTM) for 30 min. Subsequently, the samples

were desorbed for 5 min through a splitless GC injector and injected into a capillary column (60 m x 0.25 mm i.dx 0.25µm, J&W Scientific-Folsom, USA).

The column temperature was programmed to increase gradually from 40 °C to 90 °C at a rate of 3 °C per min, followed by maintaining an isothermal hold at 130 °C for 4 min with a ramp of 4 °C per min. Afterwards, the temperature was further raised to 240 °C at a rate of 5 °C per min and held for 8 min. Helium served as the carrier gas at a flow rate of 1 mL/min. The acquisition was conducted under scanning conditions within a mass range spanning from 40 to 600 m/z. The partition ratio was 1:10.

Identification of volatile compounds was accomplished using the NIST library, and the results were expressed as percentages of the peak area relative to the total area of significant peaks.

Sensory evaluation of cheeses

A group of 13 judges (comprising six women and seven men, aged between 27–62 years) assessed the sensory characteristics of OBCh and CICh cheeses. The evaluation followed EN ISO 22935–2:2023 guidelines [24]. These evaluators were chosen based on their familiarity with cheese consumption and were unaware of the experimental setup. The cheeses, cut into 2 cm cubes, were allowed to acclimate at room temperature for 1 h. They were then served in a random order on white plastic plates, each labeled with a unique digit code unrelated to the experimental batches. The sensory evaluation took place in individual chambers illuminated by white light. An iPad connected to the Smart Sensory Box software (Smart Sensory Solutions S.r.l., Sassari, Italy) facilitated the assessment. The judges evaluated 17 sensory attributes related to aspect, smell, taste, and consistency. Their

scores were recorded using a left-anchored hedonic scale ranging from 1 to 9 cm, as previously described by Garofalo et al. [12].

Statistical analyses

Microbiological, physicochemical, and sensory characteristics were analysed using One-Way Variance Analysis (ANOVA) and pairwise comparisons with Tukey's test at a significance level of $p \leq 0.05$. Heat map cluster analysis was used to identify the distribution of VOCs emitted from OBCh and CICh. All analyses were conducted using XLSTAT software version 2020.3.1 (Addinsoft, New York, USA) evaluating only the effect of cheese (OBCh and CICh).

Results and discussion

Evolution of microbiological parameters during cheese production

The results of the microbiological investigation carried out throughout the production chain of OBCh cheese, from ewes' milk to curd samples, are reported in Table 1.

Table 1. Microbial counts of freeze-dried starter culture, milk, and curd samples

Parameters	**Samples**				***p* value**
	DSC	PM	IM	C	
TMM	10.14 ± 0.26^{a}	3.34 ± 0.27^{d}	7.09 ± 0.22^{c}	7.97 ± 0.19^{b}	≤ 0.0001
S. thermophilus	10.36 ± 0.33^{a}	3.05 ± 0.31^{d}	6.91 ± 0.29^{c}	7.83 ± 0.21^{b}	≤ 0.0001
CPS	<2	<1	<1	<2	n.e.
E. coli	<2	<1	<1	<2	n.e.
L. monocytogenes	<2	<1	<1	<2	n.e.
Salmonella spp.	<2	<1	<1	<2	n.e.

Units are CFU/g for freeze-dried starter culture and curd samples; CFU/mL for milk samples. Results indicate mean values ± S.D. of six plate counts (carried out in duplicate for three independent productions). Abbreviations: DSC, dried starter culture; PM, pasteurized milk; IM, inoculated milk; C, curd; TMM, total mesophilic microorganisms; CPS, coagulase-positive staphylococci; *E.*, *Escherichia*; *L.*, *Listeria*; n.e., not evaluated. On the row: a, b, c, d = $p \leq 0.05$.

The targeted search for *E. coli*, CPS, *L. monocytogenes*, and *Salmonella* spp., which are relevant for monitoring food hygiene and safety standards [25], yielded no colonies in any of the analyzed samples. Notably, the commercial dried starter culture was predominantly composed of *S. thermophilus* (10.36 Log CFU/mL). In pasteurized milk, the presence of TMM and streptococci at approximately 3.0 Log CFU/mL was observed. This aligns with the typical microbial levels found in pasteurized ewes' milk used for cheese production [26,27]. The occurrence of TMM and LAB primarily results from the inability of the pasteurization process to completely inhibit the growth of thermoduric milk microbiota [28].

When analyzing milk inoculated with MSC showed an increase in *S. thermophilus* levels, reaching up to 6.91 Log CFU/mL, was observed. Blaiotta et al. [29] also documented a similar trend when studying bovine milk inoculated with the same starter culture used for producing Italico-type cheese. Following curdling, the cell densities of these microorganisms reached approximately 8.0 Log CFU/g. The observed increase in curd samples is an anticipated phenomenon attributed to whey drainage [30]. Interestingly, no statistically significant differences ($p \geq 0.05$) were detected in the levels of TMM and *S. thermophilus* between CICh and OBCh samples (Figure 2).

Both cheeses exhibited *S. thermophilus* levels of approximately 9.0 Log CFU/g, consistent with the patterns commonly observed in pressed ovine and bovine cheeses [31,32].

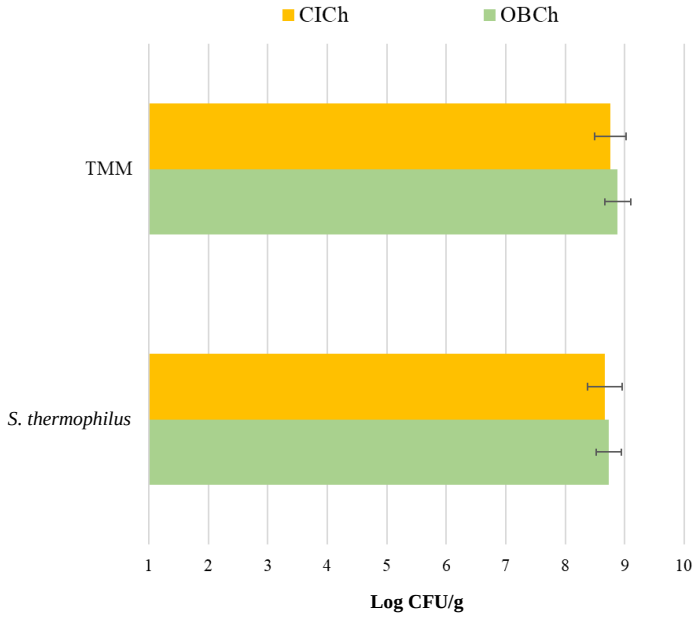

Figure 2. Microbiological loads of cheeses. Units are Log CFU/g. Results indicate mean values ± S.D. of six plate counts (carried out in duplicate for three independent productions). Abbreviations: CICh, commercial cow's Italico cheese; OBCh, ovino Belmontese cheese; TMM, total mesophilic microorganisms; *S.*, *Streptococcus*.

Identification of thermoduric milk LAB

After enumeration, all presumptive *S. thermophilus* isolates from pasteurized ewes' milk underwent strain typing using RAPD-PCR. The resulting analysis revealed that the LAB community isolated from pasteurized ewes' milk consisted of four distinct strains (Figure 3). Subsequent identification via 16S rRNA gene sequencing confirmed that all four strains indeed belonged to the LAB group and were specifically identified as *S. thermophilus*. This particular LAB species is characteristic of sheep milk microbiota [33] and falls within the group of thermophilic starter LAB (SLAB). These bacteria play a crucial role in the acidification process of the curd during cheese production [34]]. Despite their typical association with sheep milk, the presence of *S. thermophilus* in pasteurized

milk primarily stems from its remarkable ability to withstand the conventional heat pasteurization process [35].

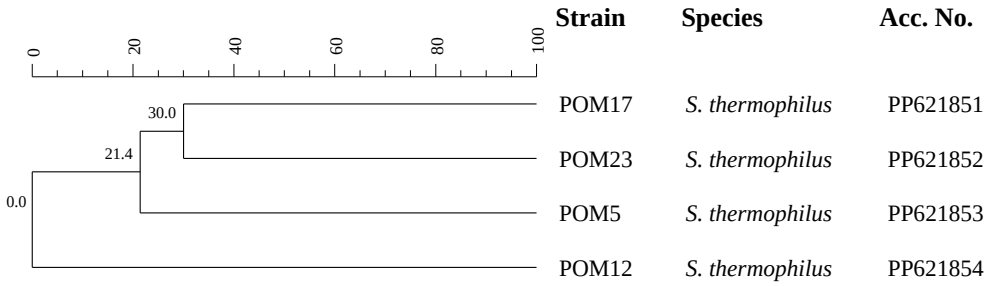

Figure 3. Dendrogram obtained from RAPD-PCR patterns of the thermoduric milk lactic acid bacteria strains identified. Abbreviations: *S.*, *Streptococcus*.

Dominance of S. thermophilus starter cultures

The prevalence of commercial starter cultures in relation to thermoduric milk LAB was monitored throughout the cheeses-making process. To achieve this, 107 isolates were collected and subjected to a comprehensive characterization using both microscopic inspection and RAPD-PCR analysis. This approach is commonly used to assess the dominance of added starter cultures in cheese productions [36]. Upon microscopic inspection, all isolates exhibited a characteristic arrangement: cells organized in long chains, a typical feature of streptococci [37]. Although specific results are not shown, the RAPD-PCR analysis conducted on isolates obtained from the commercial freeze-dried starter revealed the presence of three distinct *S. thermophilus* strains. The strategic use of multiple-strain combinations of LAB is of paramount importance in mitigating phage-related challenges [38]. Furthermore, a direct comparison of the polymorphic profiles of all LAB isolated along the OBCh production chain unequivocally demonstrated the dominance of the added *S. thermophilus* strains originating from freeze-dried commercial starter. These strains effectively outcompeted the thermoduric milk LAB.

Physicochemical characterization of cheeses

The physicochemical characteristics of CICh and OBCh are summarized in Table 2. Notably, no statistically significant differences ($p \geq 0.05$) were observed between the two cheeses regarding color parameters (L*, a*, and b*) and hardness. These physical attributes play a defining role in determining visual acceptability and influencing consumer purchase decisions, especially for fresh cheeses [39]. Our findings align with those reported by Mohamed et al. [40] in fresh cheeses made from both sheep's and cow's milk. While the pH values exhibited variation between CICh and OBCh, they remained within the typical range of 5.06 to 5.52, commonly observed for rennet-curd cheeses [41].

Table 2. Physicochemical analysis of cheeses

Parameters	**Samples**		***p* value**
	CICh	OBCh	
Color			
Lightness L*	87.76 ± 0.23	87.59 ± 0.35	0.637
Redness a*	-3.61 ± 0.41	-4.53 ± 0.35	0.106
Yellowness b*	16.71 ± 1.45	15.28 ± 0.68	0.317
Hardness (N)	0.41 ± 0.03	0.33 ± 0.02	0.059
pH	5.12 ± 0.02^{b}	5.21 ± 0.01^{a}	0.012
Dry matter (%)	57.37 ± 0.67^{a}	51.23 ± 0.68^{b}	0.003
Fat in DM (%)	59.70 ± 0.88	56.12 ± 1.43	0.065
Protein (%)	22.00 ± 0.38	21.61 ± 0.22	0.319
Ash (%)	3.53 ± 0.05^{a}	2.97 ± 0.07^{b}	0.003

Results indicate mean values ± S.D. of six determinations (carried out in duplicate for three independent productions). Abbreviations: CICh, commercial cow's Italico cheese; OBCh, ovino Belmontese cheese. On the row: a, b = $p \leq 0.05$.

Regarding the chemical composition of the cheeses, significant differences ($p \leq 0.05$) were evident only in terms of dry matter and ash content. In particular, CICh showed higher values than those of OBCh, which can be attributed to the different milk types used in cheese production [42]. Both cheeses shared an average fat content of 57.91% and a

protein content of 21.81%. These results are consistent with previous findings reported by Gobbetti et al. [43] for fresh cow's milk cheeses and by Garofalo et al. [12] for sheep's milk cheeses.

Fatty acid composition of cheeses

The fatty acid composition of cheeses is influenced by various factors, and distinct characteristics emerge between the two productions (Table 3). Specifically, during OBCh production, significantly higher average percentages of short-chain fatty acids (SCFA) (17.35%) and medium-chain fatty acids (MCFA) (20.32%) were observed, while the average percentage of long-chain fatty acids (LCFA) was lower (62.30%) compared to CICh (SCFA = 7.78%; MCFA = 18.71%; LCFA = 73.93%). Comparable trends were observed in similar productions [44,45]. Among the long-chain polyunsaturated fatty acids (PUFA), the isomer cis-9, trans-11 of linoleic acid (LA) (commonly known as rumenic acid) exhibited higher levels in OBCh production, corroborating existing literature from Contarini et al. [46], Cruz-Hernandez et al. [47], and Prandini et al. [48]. Notably, PUFA levels are not synthesized by ruminant tissues and strongly depend on animal feeding practices [49–51]. Interestingly, previous studies indicate that among cows, goats, and sheep, the highest LA concentration is found in ewe's milk, even when these ruminant species are fed similar forages [52,53]. This aspect holds significant health benefits, as rumenic acid is associated with anticarcinogenic, immunomodulatory, and anti-atherosclerotic properties [54,55]. Additionally, both productions prominently featured the long-chain monounsaturated fatty acid oleic acid (C18:1 cis9). The presence of this

compound is noteworthy due to its documented to possess anti-carcinogenic and anti-atherogenic properties, making it beneficial for inclusion in daily diets [56].

Table 3. Free fatty acid profile of cheeses

Parameters	Samples		*p* value
	CICh	OBCh	
Caproic acid (C6:0)	2.43 ± 0.12^{b}	3.37 ± 0.31^{a}	0.032
Caprylic acid (C8:0)	1.57 ± 0.23^{b}	3.51 ± 0.40^{a}	0.011
Capric acid (C10:0)	3.78 ± 0.14^{b}	10.47 ± 0.17^{a}	≤ 0.0001
Lauric acid (C12:0)	4.42 ± 0.31^{b}	5.89 ± 0.36^{a}	0.025
Myristic acid (C14:0)	12.91 ± 0.33	13.16 ± 0.36	0.542
Pentadecanoic acid (C15:0)	1.38 ± 0.22	1.27 ± 0.11	0.591
Palmitic acid (C16:0)	35.89 ± 0.27^{a}	26.22 ± 0.46^{b}	0.000
Palmitoleic acid (C16:1)	1.97 ± 0.17^{a}	1.38 ± 0.16^{b}	0.043
Stearic acid (C18:0)	9.75 ± 0.24	8.84 ± 0.33	0.058
Oleic acid (cis) (C18:1)	21.59 ± 0.12^{a}	15.43 ± 0.25^{b}	≤ 0.0001
Oleic acid (trans) (C18:1)	1.45 ± 0.24^{b}	5.41 ± 0.24^{a}	0.001
Linoleic acid (C18:2)	2.87 ± 0.09^{a}	3.02 ± 0.12^{a}	0.272
Linolenic acid (C18:3 n3)	0.41 ± 0.21^{b}	2.02 ± 0.10^{a}	0.003

Results indicate mean values ± S.D. of six determinations (carried out in duplicate for three independent productions). Abbreviations: CICh, commercial cow's Italico cheese; OBCh, ovino Belmontese cheese. On the row: a, b = $p \leq 0.05$.

In OBCh cheese, higher contents of short-chain fatty acids, such as caproic (C6:0), caprylic (C8:0), capric (C10:0), and lauric (C12:0) acids, were found compared to CICh, following classic fatty acid profiles of sheep's milk cheeses [57,58]. The increased presence of short-chain fatty acids not only improves the digestibility of the product but also contributes to the distinctive flavors found in cheeses from small ruminant animals. Additionally, these fatty acids can serve as indicators for identifying milk blends from various species [58].

Volatile organic compounds profile of cheeses

Results of the analysis for the volatile organic profile of OBCh and CICh are presented in Figure 4. These volatile organic compounds (VOCs) encompass a variety of chemical

classes, including acids, alcohols, esters, aldehydes, and ketones. Carboxylic acids constituted the primary class of VOC in both CICh (70.9%) and OBCh (39.1%). Alcohols followed in descending order, accounting for 16.7% in CICh and 29.9% in OBCh. Ketones contributed 6.3% in CICh and 20.8% in OBCh, aldehydes 4% in CICh and 10% in OBCh, while esters 1.9% in CICh and 0.2% in OBCh.

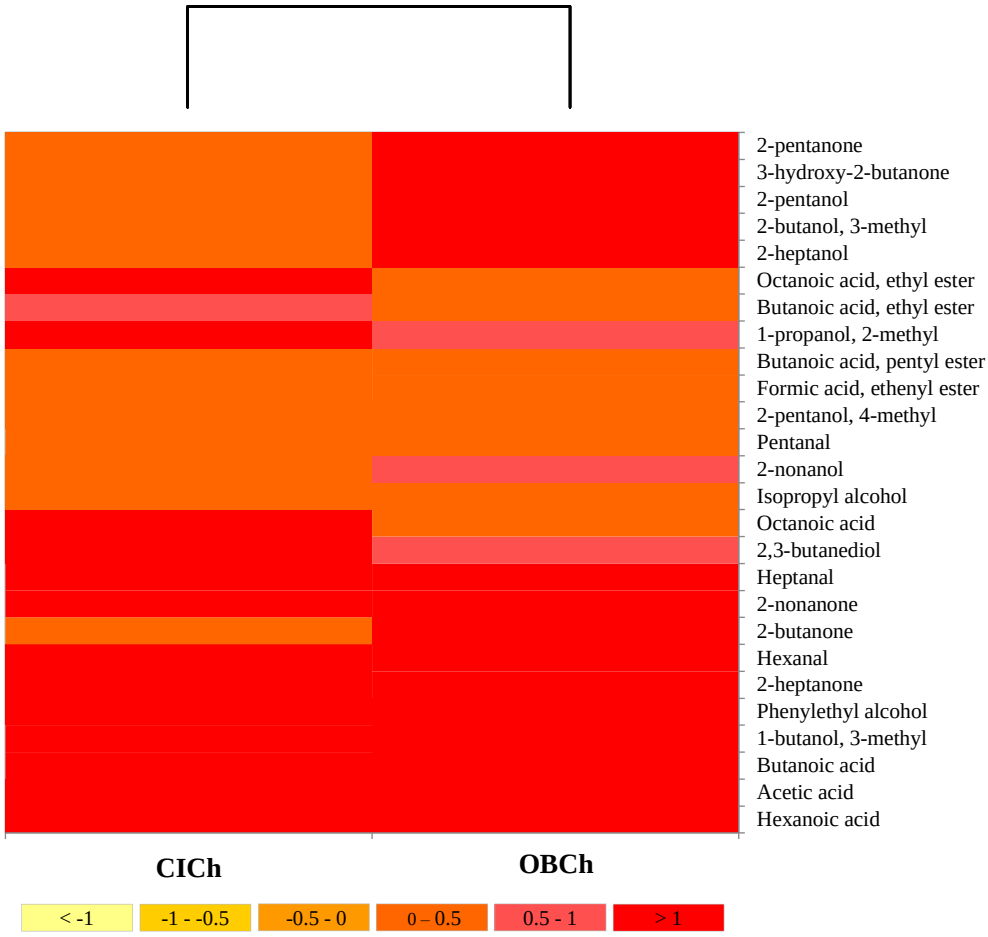

Figure 4. Distribution of volatile organic compounds among cheeses. The heat map plot depicts the relative concentration of each VOC. Abbreviations: CICh, commercial cow's Italico cheese; OBCh, ovino Belmontese cheese.

Among the acids, hexanoic, butyric, and acetic acids were prominent volatile compounds in CICh, and these same compounds were also detected in OBCh. Carboxylic acids significantly contribute to the overall flavor of cheese [59]. Specifically, hexanoic acid imparts a sour note, butanoic acid adds a cheesy flavor, and acetic acid contributes to

vinegar and acidic notes [10]. However, while acids are important in cheese aroma, they also serve as precursors for other compounds, including ketones, alcohols, aldehydes, and esters [60,61].

Ketones, commonly found in dairy products, originate from the β-oxidation of fatty acids [62]. These compounds possess a distinctive odor and are detectable at low levels [63]. Among the ketones, 2-butanone, 2-heptanone, and 2-nonanone were present in higher amounts (6.0%, 5.6%, and 4.6%, respectively, in the OBCh sample; and 0.3%, 3.2%, and 2.1% in the CICh sample). Similar findings have been observed in other PDO cheeses made from raw milk [64], suggesting that these ketones play a crucial role in the final aroma of these cheeses. In particular, 2-butanone imparts a buttery odor, while 2-heptanone exhibits an herbaceous odor [65]. Various methyl ketones, like nonanone, contribute fruity and floral notes, enhancing cheese flavor [64]. Despite the prevalence of carboxylic acids in all cheese samples, esters were poorly detected, likely due to the fresh nature of the investigated cheeses [66,67]. In OBCh, additional odor-active compounds such as alcohols (1-butanol-3-methyl) and aldehydes (hexenal and heptanal) were also identified. Overall, the volatile composition in OBCh aligns with the profile observed in cheeses produced from sheep's milk in various studies [27,32,68].

Sensory traits of cheeses

The spider plot depicted in Figure 5 illustrates the outcomes of the descriptive sensory evaluation conducted on OBCh and CICh. This evaluation is essential for assessing consumer satisfaction with new food products before their market launch [69]. While it is widely recognized that the sensory characteristics of dairy products are primarily

influenced by factors such as the type of milk used, animal diet [70], and raw milk characteristics [71], the comparison between OBCh and CICh did not reveal statistically significant differences ($p \geq 0.05$) for most of the evaluated attributes. However, some distinctions were observed: color, intensity of odor, spiciness, and taste persistency were higher for OBCh. These results are not surprising, since ovine milk imparts greater sensory complexity to the final products compared to cows' milk [72]. However, the scores registered in this study are similar to those reported by Blaiotta et al. [29] for bovine Italico cheese. Interestingly, unpleasant odors, a critical factor affecting consumers' acceptance of new products [73], were not detected in either of the evaluated cheeses. Overall, both OBCh and CICh received similar overall satisfaction scores, affirming that the transformation of sheep's milk using the Italico cheese technology does not adversely impact sensory characteristics.

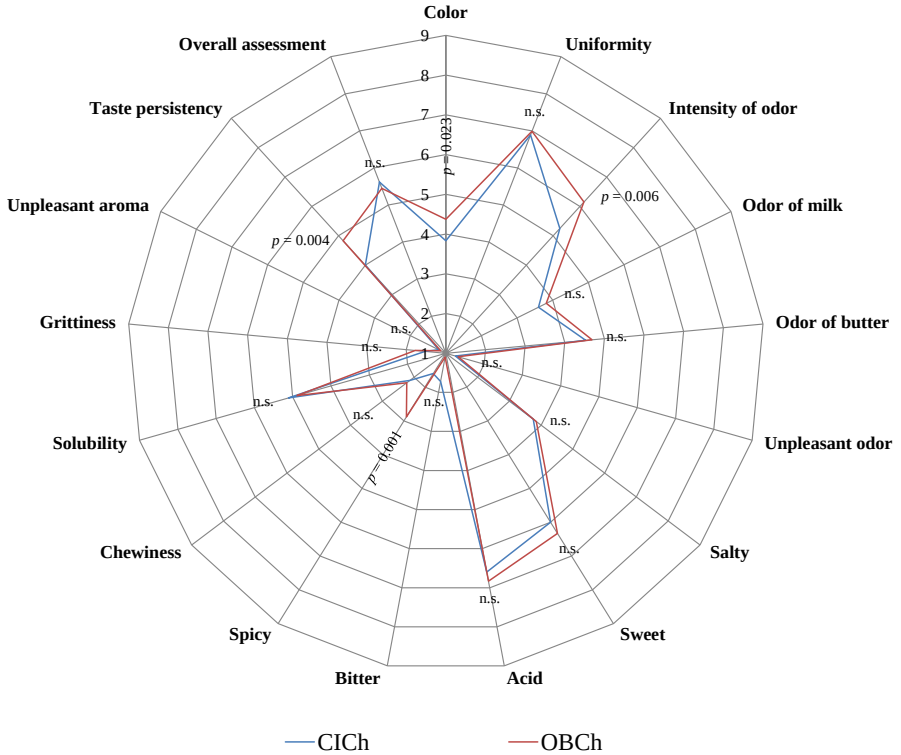

Figure 5. Spider chart of descriptive sensory evaluation of cheeses. Abbreviations: CICh, commercial cow's Italico cheese; OBCh, ovino Belmontese cheese; n.s., not significant.

Conclusion

In this comprehensive investigation, a novel Sicilian semisoft cheese made from sheep's milk underwent several analyses. The microbiological assessment confirmed the safety of the final cheeses and validated the use of a commercially available *S. thermophilus* formulation as a starter culture for OBCh production. Elevated levels of short-chain fatty acids were detected in OBCh, potentially enhancing product digestibility. OBCh exhibited higher values of the cis-9, trans-11 isomer of linoleic acid, known for its numerous health benefits. Despite varying proportions, both cheeses displayed comparable classes of volatile organic compounds, which did not significantly alter their aromatic profiles. Remarkably, the sensory analysis revealed that OBCh was on par with commercially available Italico cheese in terms of overall appreciation. This work has not only led to the creation of an unconventional dairy product in the Sicilian region but also holds promise for making sheep farming economically viable while preserving native breeds and mitigating land abandonment.

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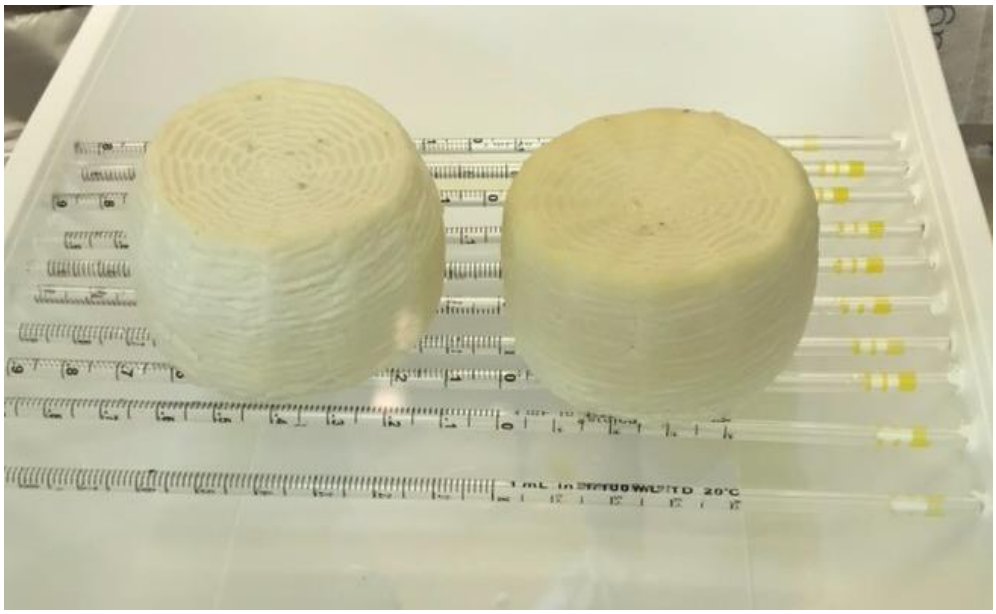
Chapter IV

Improvement of fresh ovine “Tuma” cheese quality characteristics by application of oregano essential oils



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Antioxidants

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Abstract

In the present work, oregano essential oils OEOs was applied to process the fresh ovine cheese "Tuma" obtained by pressed cheese technology. Cheese making trials were performed under industrial conditions, using ewe's pasteurized milk and two strains of *Lactococcus lactis* (NT1 and NT5) as fermenting agents. Two experimental cheese productions (ECP) were obtained by the addition of 100 (ECP100) and 200 (ECP200) µL/L of OEO to milk, while control cheese production (CCP) was OEOs free. Both *Lc. lactis* strains showed in vitro and in vivo ability to grow in presence of OEOs and to dominate over indigenous milk lactic acid bacteria (LAB) re-sistant to pasteurization. In presence of OEOs, the most abundant compound found in cheese was carvacrol, constituting more than 65% of the volatile fraction in both experimental productions. The addition of OEOs did not influence ash, fat and protein content, but increased of 43% the antioxidant capacity of experimental cheeses. ECP100 cheeses showed the best appreciation scores by the sensory panel. In order to investigate the ability OEOs to be used as natural preservative, a test of artificial contamination was carried out and the results showed a significantly reduction of the main dairy pathogens in OEOs added cheeses.

Keywords: oregano essential oil, lactic acid bacteria, novel fresh ovine cheese, physicochemical properties, antioxidant capacity, volatile organic compounds, sensory evaluation, dairy pathogenic bacteria.

Introduction

Cheese is an ancient, fermented food produced worldwide. Its distribution and consumption have increased over the years [1], and the cheese market is projected to reach the USD 112 billion mark by 2025 [2]. The interest of consumers toward this product is mainly due to the high content of proteins with biological value and high digestibility, minerals (e.g., calcium), vitamins, and fatty acids [3]. Such a complex nutritional composition of cheese enables the growth of the main microbial groups associated with foods, including spoilage and pathogenic species [4], endangering their stability and consumer safety [5]. Therefore, the application of chemical additives, in particular in fresh cheeses characterized by high pH and moisture, is necessary to extend their shelf life [6]. The use of chemical additives can be harmful to the consumer's health [7]; for this reason, the dairy sector is being more and more active in proposing natural alternatives to preserve and improve the quality and safety of cheeses [8]. For this purpose, naturally synthesized substances, especially plant essential oils (EOs), are considered important to produce cheeses with long shelf lives, high nutritional values, and sensory characteristics [9].

EOs are highly hydrophobic products extracted from various parts of plants, herbs, spices, and fruits [10] added to foods for flavoring and to improve their antioxidant properties [11]. Due to their harmlessness, many EOs are included in a list (21 Code of Federal Regulations part 182) of food substances that, when used for the purposes indicated and in accordance with good manufacturing practice, are classified as GRAS (Generally Recognized as Safe) by the U.S. Food and Drug Administration [12]. EOs play an important role in food microbial control. In fact, very recently, these natural preservatives were used in food applications for their antibacterial, antifungal, antiviral,

and antibiofilm formation properties [13]. Among the herbaceous aromatic plants used for the extraction of EOs, oregano assumes a role of particular interest; it is one of the most cultivated species in the Mediterranean area [14], exhibiting high antioxidant and health-promoting activities. These characteristics depend on its high content in phenolic compounds, which are mainly carvacrol and thymol [15,16].

Dairy products contain low concentrations of phenolic compounds [17]; thus, the addition of cheeses with plant EOs represents a novel approach to enhancing their functional properties [18]. The use of oregano essential oils (OEOs) for processing dairy foods is not new, but their application has been primarily performed with bovine milk in order to improve the microbiological and chemical properties of cheeses [19,20]. To our knowledge, no studies have been specifically performed on the evaluation of the effect of the addition of OEOs to fresh ovine cheeses.

This study is part of a research project aimed to enlarge the ewe's dairy product portfolio of South Italy through the use of essential oils extracted from aromatic plants of Mediterranean origin. The purpose of the present study was to evaluate, for the first time, the effect of the addition of OEOs on the microbiological, physicochemical, antioxidant, and sensory aspects of "Tuma" cheese, a Sicilian fresh-pressed cheese made from ewes' milk characterized by a typical cylindrical shape with a uniform structure and a white or ivory white color [21].

Cheese making trials were performed at an industrial scale using *Lactococcus lactis* starter cultures and using OEOs as natural antimicrobial substances. For this purpose, a scale-up approach was followed to test, firstly, the antibacterial activity of OEOs against the main dairy pathogenic bacteria in vitro; second, after efficacy evaluation, the same

components were applied in vivo. This study also aimed to evaluate the chemical composition of the OEOs, monitor starter lactic acid bacteria (LAB) during cheese production, and evaluate the physicochemical, antioxidant, volatile organic compounds, and sensory traits of the final cheeses.

Materials and Methods

Oregano essential oil extraction and gas chromatography analysis

The plants of *Origanum vulgare* ssp. *viridulum* x *Origanum vulgare* ssp. *hirtum* were provided by a farm located in Agrigento (37°27'28" N, 13°36'01" E). After harvesting, the plants were air dried and transferred into plastic bags at the laboratories of the Research Centre for Plant Protection and Certification (Bagheria, Italy). EOs were extracted from the leaves and flowers using a distiller in a current of steam of 12 L volume (Spring Extractor, Albrigi Luigi, Verona, Italy). After extraction, OEOs were transferred to 25 mL amber glass bottles with a screw cap (Laboindustria, Arzergrande, Italy) and stored at 4 °C.

Volatile Organic Components (VOC) of oregano essential oils were analysed by solid-phase microextraction (SPME) GC–MS after dilution with hexane (1:100). The SPME fiber (DVB/CAR/PDMS, 50 mm, Supelco, Bellefonte, PA, USA) was exposed to the diluted oils under stirring at 60 °C. After an extraction time of 5 min, fiber was inserted in a GC splitless injector, and volatile organic components were desorbed for 1 min at 250 °C. Chromatographic separation was performed using a DB-624 capillary column (Agilent Technologies, Santa Clara, CA, USA, 60 m, 0.25 mm, 1.40 µm). The oven temperature program was set with a 5 min isotherm at 40 °C followed by a linear temperature increase of 5 °C every minute up to 200 °C, where it was held for 2 min and the helium carrier gas

was set at 1 mL/min. The interface temperature was fitted at 230 °C and mass spectra were recorded in the range of m/z 40–400 amu under full-scan acquisition mode. Single volatile organic compounds were identified by comparing each MS spectra with the commercial library NIST05. Results were obtained from three replicates and reported as percentages relative to the significant peak.

Bacterial strains, milk starter culture preparation, and culture conditions

In order to evaluate the antibacterial properties of OEOs, four bacterial strains belonging to the American Type Culture Collection (ATCC) were used as indicators of microorganisms (sensitive to antimicrobial compounds). In particular, two gram-positive (*Listeria monocytogenes* ATCC19114 and *Staphylococcus aureus* ATCC33862) and two gram-negative (*Escherichia coli* ATCC25922 and *Salmonella* Enteritidis ATCC13076) bacteria were chosen as representatives of the main dairy bacterial pathogens. These bacteria were reactivated in Brain Heart Infusion (BHI) broth (Condalab, Madrid, Spain) and incubated at 37 °C for 24 h.

Milk starter cultures (MSC) were developed with two strains of *Lc. lactis* (NT1 and NT5) belonging to the culture collection of the Department of Agricultural, Food, and Forest Sciences (University of Palermo, Italy). These strains were previously isolated from LYOBAC-D NT freeze-dried starter preparation (Alce International s.r.l., Quistello, Italy) [22]. *Lc. lactis* strains were cultivated in M17 broth (Oxoid, Hampshire, UK) at 30 °C for 24 h and centrifuged at 10,000 g for 5 min. The cells were then washed twice in Ringer's solution (Oxoid) and re-suspended in the same solution. The washed cells of *Lc. lactis* were inoculated (1%, v/v) into ovine whole fat UHT milk (Leeb Vital, Wartberg an der

Krems, Austria) and incubated at 30 °C for 24 h [23]. MSC containing the multi-strain culture at about 10^9 CFU/mL, as verified by plate count, was then used for cheese production.

In vitro antibacterial activity of oregano essential oil

The antibacterial activity of the OEOs was tested in vitro against a cell density of approximately 10^7 CFU/mL of the four pathogenic bacterial strains (*E. coli*, *L. monocytogenes*, *S.* Enteritidis, and *St. aureus*) and the two LAB (*Lc. lactis*) in BHI or M17 soft agar (0.7% w/v) by the paper disc diffusion method, as indicated by Gaglio et al. [24]. Streptomycin at 10% (w/v) was used as a positive control, while sterile water was used as a negative control [25]. After incubation at 37 °C for 24 h, inhibitory activity was assessed and considered positive only if a clear area around the paper discs was present. The test was carried out in triplicate.

Once scored positive, the antibacterial activity against the four pathogenic bacteria was quantified as Minimum Inhibitory Concentration (MIC) following the methodology reported by Militello et al. [26]. Briefly, the OEOs were serially 2-fold diluted in acetone (Carlo Erba Reagents, Rodano, Italy), added to Brain Heart Infusion (BHI) broth (Condalab, Madrid, Spain), and tested by employing the strains at approximately 10^6 colony forming units (CFU)/mL. The test was carried out in triplicate.

Description of dairy plant

Cheese was produced on an industrial scale at the dairy factory Azienda Agricola Giuseppe Basile in Ventimiglia di Sicilia (Palermo, Italy). The dairy plant (Figure 1) used

for cheese production is a multi-purpose system with a working capacity of 200 L (Sfoggia & C. SAS, Montebelluna, Italy).

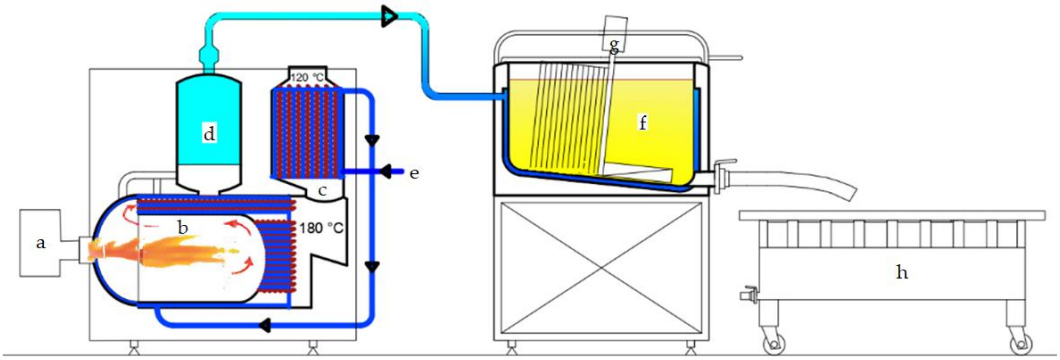


Figure 1. Scheme of dairy plant. (a) diesel burner; (b) furnace (first and second stage of smoke circuits); (c) third stage of smoke circuits; (d) steam accumulator with dispensing valve; (e) water inlet; (f) multipurpose coagulation vats; (g) gearmotor for mechanically cutting curd; (h) perforated steel table.

The plant is equipped with a high-efficiency wet-bottom condensing steam generator (Figure 1a–e) with flame reversal, and it is fueled by diesel (TFRE series, Manara Roberto S.r.l Company, Fontevivo, Italy). The generator is characterized by a steam production of 150 kg/h, a stamp pressure of 5 ÷ 12 bar, a potential yield of 90,000 kcal/h, and horizontal smoke tubes. There is a fully inspectable front cavity door with water recirculation from the generator. There are three passes of smoke (the first and second in the hearth, the third in the pipes) to increase efficiency. The feed water flows against the current, raising the temperature by over 40 °C and, consequently, lowering the flue gas temperature to 120 °C, thus obtaining an efficiency of 95%. It is completed with the automatic drain in electropneumatic stainless steel adjustable by PLC with functions also in manual mode. The tank used for milk pasteurization and coagulation (Figure 1f) is double-bottomed, steam heated, and equipped with a gearmotor for mechanically cutting curd (Figure 1g). It is connected to two probes, which transmit into an electrical panel the temperature of the milk, curd, and heating water. The bottom of the tank is slightly inclined and has a drain

valve, located in the lower part, through which it is possible to drain the curd and whey. A perforated steel table (Figure 1h) is used for curd molding.

Cheese production and sample collection

The strains *Lc. lactis* NT1 and NT5 were used to prepare a milk starter culture (MSC). Briefly, the washed cells of *Lc. lactis* were inoculated (1% v/v) into ovine whole fat UHT milk (Leeb Vital, Wartberg an der Krems, Austria) and incubated at 30 °C for 24 h [25]. An MSC containing the multi-strain culture at about 10^9 CFU/mL, as verified by plate count, was then used for cheese production.

Pasteurized ewe's milk (60 °C for 30 min) from crossbreeds between "Valle del Belíce" × "Sarda" sheep was used as the raw material. Cheese making trials were performed by applying Tuma pressed cheese technology (Figure 2).

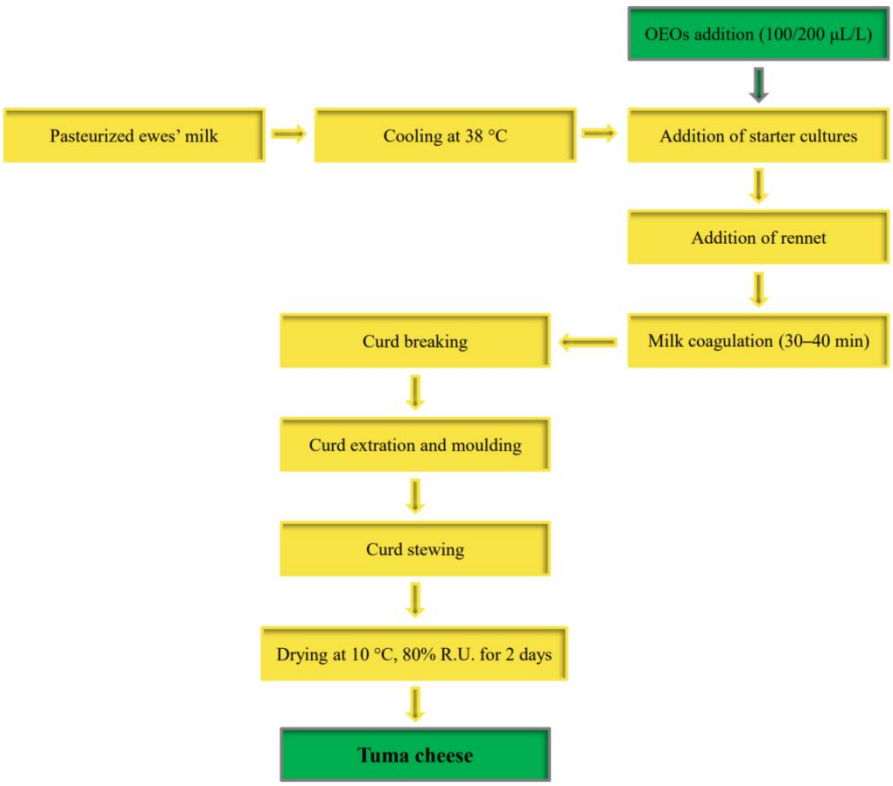


Figure 2. Flow diagram of Tuma cheese production. Abbreviations: OEOs, Oregano essential oil.

Three cheese products were obtained using MSC as a fermenting agent: CCP, a control cheese product prepared from pasteurized ewe's milk; ECP100, an experimental cheese product prepared from pasteurized ewe's milk enriched with 100 µL/L of OEOs; and ECP200, an experimental cheese product prepared from pasteurized ewe's milk enriched with 200 µL/L of OEOs. Each production was performed in a stainless steel vat previously sanitized with a DTH101 isopropyl solution (Trezzo sull'Adda, Italy) with 200 L of pasteurized whole ewe's milk. After cooling at 40 °C, the milk of the CCP and ECP trials was inoculated with the MSC at a concentration of approximately 10^7 CFU/mL. Before liquid rennet (Micromilk Srl, Cremosano, Italy) was added (60 mL), the milk used for the ECP productions was inoculated with 100 and 200 µL/L of OEO, respectively. After curdling, the curd was cut until attaining the dimension of small rice-size grains, which were hand pressed into 1 kg cylindrical perforated plastic molds, kept at 40 °C for 50 min (the so-called stewing step), and then dried for 2 days at 10 °C. All cheese productions were replicated for two consecutive weeks. Samples of raw milk, pasteurized milk, inoculated milk after the addition of MSC and OEOs, whey, curds, and cheeses after two days following production were collected for analyses.

Microbiological analyses

First, 1 mL of the liquid (milk and whey) samples was directly serially diluted in Ringer's solution (Oxoid), while 10 g of the solid (curd and cheese) samples was first homogenized in 90 mL of sodium citrate (2% w/v) solution in the Bag-Mixer 400 stomacher (Interscience, Saint Nom, France) at the maximum speed for 1 min and then serially diluted in Ringer's solution (Oxoid). Appropriate dilutions of raw milk and

pasteurized milk were plated on agar media to allow the development of: total mesophilic microorganisms (TMM) spread on Skim Milk Agar (SMA) (Microbiol Diagnostici, Cagliari, Italy) and incubated for 72 h at 30 °C; mesophilic lactic acid bacteria (LAB) cocci poured in Medium 17 (M17) agar (Oxoid) incubated for 48 h at 30 °C; mesophilic LAB rods poured in de Man-Rogosa-Sharpe (MRS) agar (Condalab), adjusted to pH 5.4 with 5 Mol lactic acid, incubated for 48 h at 30 °C; enterococci spread on kanamycin Esculin Azide (KAA) agar (Biotec, Grosseto, Italy) incubated for 24 h at 37 °C; *Pseudomonas* spp. spread on *Pseudomonas* Agar Base (PAB) (Condalab) incubated at 22 °C for 72 h; members of the Enterobacteriaceae family poured in Violet Red Bile Glucose Agar (VRBGA) (Biolife Italiana, Monza, Italy) incubated for 24 h at 37 °C; *E. coli* spread on Coliforms Chromogenic Medium (CHROM) agar (Condalab); *L. monocytogenes* spread on Agar *Listeria* to Ottaviani and Agosti (ALOA) added with ALOA enrichment-selective supplement (Biolife Italiana) incubated for 24 h at 37 °C; *Salmonella* spp. spread on Xylose Lysine Deoxycholate (XLD) agar (Liofilchem, Roseto degli Abruzzi, Italy) incubated for 24 h at 37 °C; and coagulase-positive staphylococci (CPS) spread on Baird-Parker (BP) agar added with enrichment-selective supplement (Oxoid) incubated for 48 h at 37 °C. All plate counts were incubated aerobically, except those used for growing LAB, which were incubated anaerobically using the AnaeroGen AN25 system (Oxoid).

The appropriate dilutions of inoculated milk, curd, and whey and cheese samples were analyzed for TMM and mesophilic coccus LAB exclusively on M17 agar (Biotec). Plate counts were carried out in triplicate.

Monitoring of starter cultures and identification of the thermoduric milk LAB

In order to monitor the dominance of *Lc. lactis* (NT1 and NT5) inoculated as a starter culture over thermoduric LAB, all presumptive LAB collected during cheese making were analysed by the randomly amplified polymorphic DNA (RAPD)-PCR technique, as reported by Gaglio et al. [27]. Briefly, the analysis was performed using the single primers M13 (5′-GAGGGTGGCGGTTCT-3′), AB111 (5′-GTAGACCCGT-3′), and AB106 (5′-TGCTCTGCCC-3′) in a 25 µL reaction volume. The PCR program applied to all primers comprised an initial template denaturation step for 2 min at 94 °C, followed by 40 cycles of denaturation for 1 min at 94 °C, annealing for 20 s at 40 °C, extension for 2 min at 72 °C, and a final extension at 72 °C for 10 min. Amplifications were performed by means of Swift™ MaxPro Thermal Cycler (Esco Technologies Inc., Oak Ridge, NJ, USA). The obtained polymorphic profiles of the axenic cultures of *Lc. lactis* strains were compared to those of the LAB colonies developed on agar media using the software Gelcompare II version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium). All different LAB strains isolated from pasteurized milk before MSC addition were subjected to 16S rRNA gene sequencing following the procedures applied by Weisburg et al. [28]. 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 available data in two distinct databases, as reported by [29].

Physicochemical analysis of tuma cheeses

The assessment of cheese color was performed in duplicate by a Minolta Chroma Meter CR-300 (Minolta, Osaka, Japan) measuring the values of lightness (L* = 0–100, from black to white), redness (a* = -a/+a, from green to red), and yellowness (b* = -b/+b, from blue to yellow), according to the CIE L*a*b* system [30]. The evaluation of maximum resistance to compression (compressive stress, N/mm^2) was performed by measuring the hardness of cheese samples (2 x 2 x 2 cm) maintained at 22 °C (room temperature) using an Instron 5564 tester (Instron, Trezzano sul Naviglio, Milan, Italy). The water activity (a_w) of different samples was measured with the HygroPalm portable water activity meter (Rotronic, Bassersdorf, Germany), according to ISO 21807 [31].

Lyophilized cheese samples were analysed for dry matter (DM), protein (N × 6.38), fat, and ash content in accordance with International Dairy Federation (IDF) standards [32–34]. Extracts of lyophilized cheese samples were prepared according to the method of Rashidine- jad et al. [35] with minor changes. Briefly, a milled cheese sample (0.5 g) was dissolved in methanol 95% aqueous solution (25 mL) added with 1% HCl. The suspension was mixed by vortex for 30 s and then maintained at 40 °C in an ultrasonic water bath (LBS1 Sonicator; Falc Instruments, Treviglio, Italy) for 30 min, during which time it was mixed for 5 s every 10 min. Then, the suspension was cooled, filtered with linen a cloth, centrifuged at 7000 rpm at 9 °C for 10 min, and kept at −18 °C until analysis.

Antioxidant capacity of tuma cheeses

Analyses for antioxidant properties of cheeses were performed in duplicate on extracted samples by TEAC (Trolox equivalent antioxidant capacity) assay.

The total antioxidant capacity (TEAC) in extracted cheese samples was measured by TEAC assay as Trolox equivalent according to a published procedure [33], as described by Bonanno et al. [36] (with some modifications). TEAC is a decolorization assay by which samples are evaluated for their radical scavenging ability using the ABTS radical cation (ABTS•+) and Trolox as standard [37]. To obtained the ABTS radical cation, equal volumes of a 14 mM ABTS aqueous solution and 4.9 mM potassium persulphate were mixed and incubated in the dark for 16 h at room temperature. To perform the assay, the ABTS radical cation solution was diluted in 5 mM phosphate buffered saline (PBS, pH 7.40) until obtaining an absorbance of 0.795 (±0.020) at 734 nm by the Hach DR/4000 U spectrophotometer. The mixture of 150 µL of PBS with 2850 µL of a diluted ABTS radical cation solution was placed in a cuvette, and its absorbance was recorded at 734 nm immediately and after incubation for 6 min at 30 °C. At the same way, 150 µL of extracted samples was mixed with 2850 µL in the same diluted solution of ABTS radical cation, and their absorbance was read at 734 nm after a 6 min incubation at 30 °C; using the read absorbance, the percentage decrease of the absorbance due to decolorization was calculated in comparison with the absorbance obtained with PBS. Solutions of Trolox in PBS (0–2.5 mM) were used to construct a calibration curve (R^2 = 0.99), with results expressed as mmol Trolox/kg DM.

The stability and susceptibility of cheese fat to oxidation were estimated by determining in duplicate the POV (peroxide value, mEq O_2/kg fat) expressing the primary lipid oxidation [38], and TBARs (thiobarbituric acid-reactive substances, µg of malonylaldehyde (MDA)/kg DM) as products of secondary lipid oxidation, as described by Tarladgis et al. [39] and slightly modified by Mele et al. [40].

Briefly, for TBARs analysis, 2 g of lyophilized cheese was mixed with 8 mL of aqueous solution of phosphate buffer (pH 7) and vortexed. Then, the sample was added to 2 mL of 30% (v/v) aqueous solution of trichloroacetic acid and vortexed for 5 s and filtered with Whatman filter paper No. 1. Then, 5 mL of filtrate was added with 0.02 M thiobarbituric acid aqueous solution and placed for 20 min in a hot water bath at 90 °C and then refrigerated. After centrifugation (4500 RPM for 5 min), the reading of the supernatant absorbance was performed at 530 nm by the Hach DR/4000 U spectrophotometer. Solutions of 1,1,3,3-tetramethoxypropane at concentrations between 0.016 and 0.165 µg/mL were read to construct the calibration curve (R^2 = 0.99).

Volatile organic compounds emitted from tuma cheeses

Volatile organic compounds (VOCs) of cheese samples were extracted by SPME and identified using the GC–MS technique. Two grams of both control and OEOs-enriched cheese samples were finely chopped and placed in a glass vial for the headspace solid-phase microextraction. Both extraction and desorption of the analytes from the SPME fiber were carried out by applying the same procedure reported above for the determination of the aromatic profile of oregano essential oil (Section 2.1). Furthermore, chromatographic and mass spectrometry instrumental conditions set up for the analysis of the OEOs' volatile profile were also applied to obtain the mass spectra of the volatile compounds emitted by the cheeses.

Sensory evaluation of tuma cheeses

The sensory properties of CCP and ECP cheeses were evaluated following the ISO [41] indications by a descriptive panel of 16 evaluators, including 7 women and 9 men aged between 22 and 64 years old. The evaluators were trained in order to recognize the specific attributes for Tuma cheese and were asked to score 22 descriptors grouped into aspect, aroma, taste, and texture categories, as reported by Ashkezary et al. [42]. All sensory attributes were evaluated using a hedonic scale from 1 to 9 (1 = extremely low; 9 = extremely high).

In vivo antibacterial effect of oregano essential oil

To thoroughly investigate the antibacterial activity of OEOs during the production of Tuma cheese, tests of artificial contamination were carried out. Three additional cheese making trials were performed under controlled laboratory conditions, including one control production without the addition of OEOs and experimental productions obtained by the addition of 100 and 200 µL/L of OEOs to milk. Before OEOs were added, the milk of all trials was inoculated with 10^7 CFU/mL of the starter cultures (*Lc. lactis* NT1 and NT5) and 10^4 CFU/mL of pathogenic bacteria *(E. coli* ATCC 25922, *L. monocytogenes* ATCC 19114, *S.* Enteritidis ATCC13076, and *St. aureus* ATCC 33862) to simulate a massive contamination. Each trial was carried out with 20 L of pasteurized whole ewe's milk following the protocol of production reported above (Section 2.4). Samples of pasteurized ewe's milk, inoculated milk after the addition of MSC, pathogenic bacteria and OEOs, curds, and cheeses after two days from ripening were subjected to microbiological analysis.

Statistical analyses

Microbiological and VOC data were subjected to One-Way Analysis of Variance (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft, New York, NY, USA). Differences between means were determined by Tukey's test at $p \leq 0.05$.

The physicochemical data were statistically analyzed using the MIXED procedure in SAS 9.2 software (SAS Institute Inc., Campus Drive Cary, NC, USA). In the mixed model, cheese production (3 levels: CCCP, ECCPO100, ECCPO200) represented the fixed factor, and cheese making (2 levels as replicates) was the random factor used as the error term. When the effect of production was significant ($p \leq 0.05$), the averages were compared using *p*-values adjusted according to multiple comparison Tukey–Kramer.

Results and Discussion

Chemical composition of oregano essential oil

Figure 3 reports the graphical representation of the volatile profile of OEOs. Globally, thirty-two volatile compounds belonging to six phytochemical groups (monoterpenes, monoterpenoids, sesquiterpenes, ethers, ketones, and alcohols) were found.

Most of the compounds identified belong to the group of monoterpenes; among these, the most abundant chemicals were p-cymene, γ-terpinene, and myrcene. However, the most abundant volatile compound detected was carvacrol (79.9 ± 3.5%), determining the monoterpenoids groups as the most abundant class of VOCs in OEOs analysed in this study. Carvacrol, p-cymene, and γ-terpinene are also the main compounds detected in other oregano plants grown in the Mediterranean area [43–45]. Although the volatile

composition of OEOs may be affected by many factors (environmental conditions, season of collection, age of plants, and geographical origin) [44,46,47], several studies have reported the possibility of classifying oregano chemotypes according to their essential oil composition in carvacrol type, thymol type, and both of them (carvacrol and thymol in almost equal amounts) [43,46,48]. According to Fleisher and Sneer's [48] classification, our results show that because of the higher content of carvacrol (~80%), the OEOs studied corresponded to the carvacrol oregano type, thus determining the smell and use of the condiment oregano.

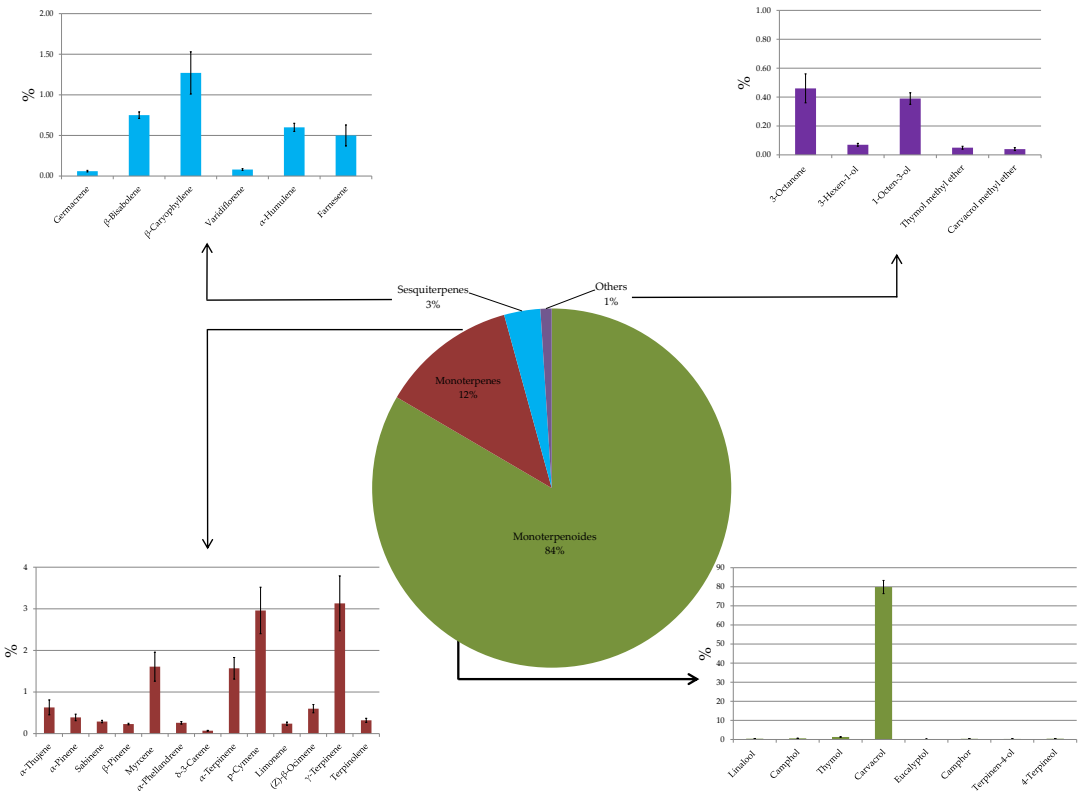


Figure 3. Volatile organic compounds emitted from oregano essential oil. Results indicate mean percentage values ± standard deviation (S.D.) of three measurements and are expressed as relative peak areas (peak area of each compound/total area of the significant peaks to all samples) x l00.

Besides characterizing OEOs' aroma, carvacrol exhibits a plethora of bioactivities, including antioxidative properties, inhibition of antibiotic-resistant bacteria, inhibition of microbial and fungal toxins, and anti-carcinogenic activity [49–52]. These aspects

highlight the potential of using OEOs belonging to the carvacrol oregano type as a multifunctional food ingredient.

Antibacterial activity of oregano essential oil

The antibacterial activity of OEOs against the four main dairy pathogens (*E. coli*, *L. monocytogenes*, *S.* Enteritidis, and *St. aureus*) responsible for foodborne diseases associated with cheese consumption [53] and the two LAB (*Lc. lactis*) commonly used as fermenting agents in dairy production [54] is shown in Table 1.

Table 1. Antibacterial activity of oregano essential oil.

Species	Strains	Inhibition (mm)	MIC (µL/mL)
Pro-technological			
Lc. lactis	NT1	-	n.d.
Lc. lactis	NT5	-	n.d.
Pathogenic			
E. coli	ATCC25922	42.5 ± 0.1	1.25
L. monocytogenes	ATCC19114	35.8 ± 0.2	2.50
S. Enteritidis	ATCC13076	42.0 ± 0.2	0.625
St. aureus	ATCC33862	38.2 ± 0.1	1.25

Results indicate the mean value of three independent assays. Abbreviations: MIC, minimum inhibitory concentration; *Lc.*, *Lactococcus*; *E.*, *Escherichia*; *L.*, *Listeria*; *S.*, *Salmonella*; *St.*, *Staphylococcus*; n.d., not determined. Symbols: -, no inhibition found.

The high sensitivity of human pathogens to EOs extracted from different varieties of oregano cultivated in Sicily is well known [55]. However, OEOs tested in this study showed very high antibacterial activity against all pathogenic strains with a diameter of the inhibition area around the paper disc in the range 35.8–42.5 mm. Considering the strong activity shown by OEOs, it was also characterized in terms of an MIC that represents the lowest concentration of an active compound able to inhibit microbial growth [56]. MIC confirmed the strong activity of OEOs tested in this study with values of 2.50 µL/mL against *L. monocytogenes*, 1.25 µL/mL against *S.* Enteritidis and *St. aureus*, and 0.625

µL/mL against *E. coli*. These results suggest that this natural product has great potential in food preservation to ensure for consumers a safe food supply. Interestingly, the development of the lactococci selected as starter cultures was not inhibited by OEOs, indicating their harmlessness against LAB, a basic condition for their application in cheese making. The resistance of LAB to the OEOs' components may be explained by the fact that bacterial susceptibility to antimicrobial agents is strain dependent [57].

Evolution of microbial populations during cheese making

The results of the plate counts carried out in ewes' milk before and after pasteurization are reported in Table 2.

Table 2. Microbial load of raw and pasteurized ewe's milk.

Microbial counts	**Samples**		**SEM**	***p*-value**
	RM	PM		
TMM	6.02[a]	3.24[b]	0.45	<0.0001
Coccus LAB	5.71[a]	2.91[b]	0.45	<0.0001
Rod LAB	4.27[a]	1.49[b]	0.44	<0.0001
Enterococci	2.87[a]	<1[b]	0.46	<0.0001
Entericacteriaceae	3.03[a]	<1[b]	0.48	<0.0001
E. coli	2.79[a]	<1[b]	0.44	<0.0001
CPS	2.42[a]	<1[b]	0.38	<0.0001
L. monocytogenes	<1	<1	n.e.	n.e.
Salmonella spp.	<1	<1	n.e.	n.e.
TMM	6.02[a]	3.24[b]	0.45	<0.0001

Units are Log CFU/mL. Results indicate the mean values of six plate counts (carried out in triplicate for two independent products). Data within a row followed by different letters are significantly different according to Tukey's test. Abbreviations: RM, raw milk; PM, pasteurized milk; SEM, standard error of the mean; TMM, total mesophilic microorganisms; LAB, lactic acid bacteria; *E.*, *Escherichia*; CPS, coagulase-positive staphylococci; *L.*, *Listeria*; n.e., not evaluated.

Raw ewes' milk hosted levels of TMM, mesophilic coccus, and rod LAB of 6.02, 5.71, and 4.27 Log CFU/mL, respectively. Similar results were previously reported by Guarcello et al. [58] and by Gaglio et al. [59] in raw milk used for the production of two traditional

Sicilian cheeses; specifically, PDO Pecorino Siciliano and PDO Vastedda della valle del Belíce. After the application of the pasteurization treatment, the levels of TMM, coccus, and rod LAB decreased by about three Log cycles, as previously observed by Barbaccia et al. [22,60] for raw and pasteurized ewe's milk used for ovine pressed and stretched cheese productions. These results confirmed the ability of LAB to survive during thermal pasteurization [61]. *E. coli* and CPS, analysed through microbiological process hygiene criteria [62], were found at low levels (2.79 and 2.42 Log CFU/mL, respectively) in raw ewes' milk and decreased below the detection limit in pasteurized milk. Regarding *L. monocytogenes* and *Salmonella* spp., which are associated with the microbiological food safety criteria [62], they were never detected in either of the matrices. The microbiological counts of inoculated milk after the addition of MSC and OEOs and whey, curd, and cheese samples included only TMM (Figure 4a) and *Lc. lactis* (Figure 4b) to evaluate the ability of the starter LAB to act as a fermenting agent in the presence of OEOs.

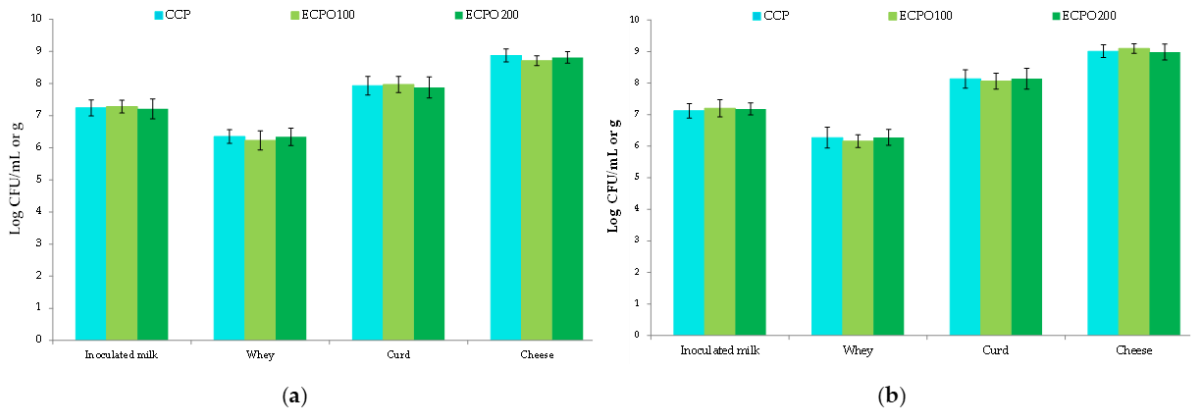


Figure 4. Growth of starter cultures during Tuma cheese productions. (a) total mesophilic microorganisms; (b) *Lactococcus lactis*. Units are Log CFU/mL for milk and whey samples; Log CFU/g for curd and cheese samples. Abbreviations: CCP, control cheese production inoculated with the Milk Starter Cultures (MSC); ECPO100, experimental cheese production inoculated with MSC + 100 µL/L of oregano essential oils (OEOs); ECPO200, experimental cheese production inoculated with MSC + 200 µL/L of OEOs.

According to Tukey's test, no statistically significant differences were found for the levels of TMM and *Lc. lactis* in all the samples analysed. In particular, these

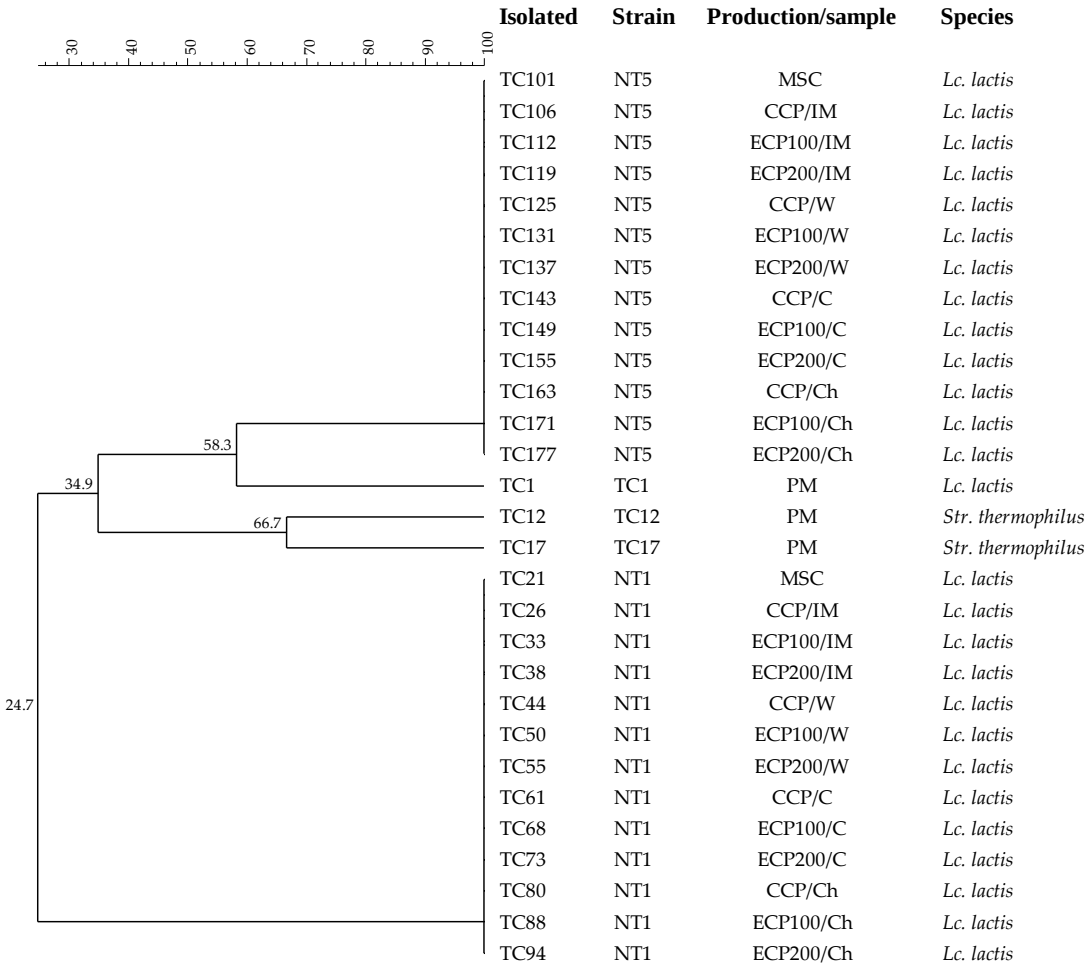
microorganisms were found in inoculated milk used for CCP and ECP productions at almost the same levels inoculated, confirming that *Lc. lactis* inoculums occurred at 10^7 CFU/mL. The analysis of whey at the time of curd separation showed a decrease of about one Log cycle for TMM and *Lc. lactis* that were present at levels of about 6 Log CFU/mL. Settanni et al. [63] observed a similar behavior by analysing cows' milk and whey samples during the production of Caciocavallo-type cheese. Control and experimental curds showed values of TMM and L*c. lactis* of about 10^8 CFU/g, and their levels reached values of about 9 Log CFU/g in the final cheeses, showing clearly that the addition of OEOs did not interfere with *Lc. lactis* development. Marcial et al. [64] observed a similar behavior in cows' milk inoculated with OEOs used for the production of Argentinean fresh bovine cheese.

Composition of thermoduric lab populations

One hundred and eighty-two colonies of LAB (gram-positive and catalase-negative) were isolated from all samples collected during cheese production, from pasteurized ewe's milk before MSC addition to processed cheeses. All isolates were subjected to RAPD analysis, a technique commonly used for strain typing and to monitor the starter LAB deliberately added [65]. The dendrogram reported in Figure 5 shows only 29 strains; this is because the rest of the isolates were characterized by polymorphic profiles already included in the dendrogram.

Two major RAPD clusters were identified. Each cluster included one of the *Lc. lactis* strains used for MSC preparation. In particular, *Lc. lactis* (NT1 and NT5) was detected in all samples collected from the control and experimental products. Three different strains

isolated from pasteurized ewe's milk were identified as *Lc. lactis* subsp. *lactis* (Ac. No. OQ675106) and *Str. thermophilus* (Ac. No. OQ675104-OQ675105). These species are part of the typical starter LAB cultures, which are able to generate a high amount of lactic acid during the very first steps of cheese production through the fermentation of lactose [66].



Isolated	Strain	Production/sample	Species
TC101	NT5	MSC	*Lc. lactis*
TC106	NT5	CCP/IM	*Lc. lactis*
TC112	NT5	ECP100/IM	*Lc. lactis*
TC119	NT5	ECP200/IM	*Lc. lactis*
TC125	NT5	CCP/W	*Lc. lactis*
TC131	NT5	ECP100/W	*Lc. lactis*
TC137	NT5	ECP200/W	*Lc. lactis*
TC143	NT5	CCP/C	*Lc. lactis*
TC149	NT5	ECP100/C	*Lc. lactis*
TC155	NT5	ECP200/C	*Lc. lactis*
TC163	NT5	CCP/Ch	*Lc. lactis*
TC171	NT5	ECP100/Ch	*Lc. lactis*
TC177	NT5	ECP200/Ch	*Lc. lactis*
TC1	TC1	PM	*Lc. lactis*
TC12	TC12	PM	*Str. thermophilus*
TC17	TC17	PM	*Str. thermophilus*
TC21	NT1	MSC	*Lc. lactis*
TC26	NT1	CCP/IM	*Lc. lactis*
TC33	NT1	ECP100/IM	*Lc. lactis*
TC38	NT1	ECP200/IM	*Lc. lactis*
TC44	NT1	CCP/W	*Lc. lactis*
TC50	NT1	ECP100/W	*Lc. lactis*
TC55	NT1	ECP200/W	*Lc. lactis*
TC61	NT1	CCP/C	*Lc. lactis*
TC68	NT1	ECP100/C	*Lc. lactis*
TC73	NT1	ECP200/C	*Lc. lactis*
TC80	NT1	CCP/Ch	*Lc. lactis*
TC88	NT1	ECP100/Ch	*Lc. lactis*
TC94	NT1	ECP200/Ch	*Lc. lactis*

Figure 5. Dendrogram obtained from combined RAPD-PCR patterns of LAB strains isolated from pasteurized ewe's milk to Tuma cheeses. Abbreviations: CCP, control cheese production inoculated with the Milk Starter Cultures (MSC); ECPO100, experimental cheese production inoculated with MSC + 100 µL/L of oregano essential oils (OEOs); ECPO200, experimental cheese production inoculated with MSC + 200 µL/L of OEOs; PM, pasteurized milk; IM, inoculated milk; W, whey, C, curd; Ch; cheese; *Lc.*, *Lactococcus*; *Str.*, *Streptococcus*.

Although they are commonly part of raw milk microbiota [67], their ability to survive the pasteurization process is well known [68,69]. However, raw milk LAB strains were not found at dominant levels in any of the samples analysed after the addition of MSC,

evidencing the ability of the starter cultures to dominate over the indigenous milk LAB that survived to the pasteurization treatment. These results confirmed those obtained by microbial counts that excluded any negative influence of OEO during the fermentation process.

Physicochemical characterisation of tuma cheeses

The physicochemical traits of the cheeses are reported in Table 3. The chemical composition of cheese in terms of ash, protein, and fat was not affected by the addition of OEO. Additionally, chemical components of the processed cheeses were in the ranges registered in other investigations [18,70,71].

Table 3. Physicochemical and antioxidant traits of Tuma cheeses.

	Samples			SEM	*p*-value
	CCP	ECPO100	ECPO200		
Cheese weight at 48 h, kg	2.93	3.52	3.61	2.244	0.2222
Cheese yield at 48 h, g/100 g	14.63	15.97	16.45	0.350	0.1206
Cheese yield at 48 h, g/100 g dry matter	8.65	9.02	9.34	0.208	0.1145
Dry matter (DM), %	59.16^{a}	56.52^{b}	56.79^{b}	0.608	<0.0001
Ash, % DM	6.54	6.81	6.53	0.414	0.8531
Protein, % DM	43.34	42.39	43.24	1.413	0.5178
Fat, % DM	45.92	45.65	42.20	4.846	0.8041
pH	5.52	5.23	5.21	0.260	0.6878
Water activity, a_w	0.971^{b}	0.979^{ab}	0.986^{a}	0.015	0.0267
Hardness, N/mm^2	0.451^{a}	0.300^{b}	0.347^{ab}	0.041	0.0137
Lightness L*	83.27^{b}	86.91^{a}	86.12^{a}	1.0988	0.0005
Redness a*	-3.551^{b}	-3.161^{a}	-3.078^{a}	0.5096	0.0014
Yellowness b*	12.96^{a}	12.48^{ab}	11.94^{b}	1.4333	0.0111
TEAC, mmol/kg DM	55.51^{b}	68.26^{b}	98.25^{a}	4.763	0.0005
POV, mEq O^2/kg fat	2.39^{b}	3.14^{a}	2.81^{ab}	0.148	0.0089
TBARs, mg MDA/kg DM	0.066^{c}	0.109^{b}	0.172^{a}	0.006	<0.0001

Results indicate mean values of determinations carried out in duplicate for each of the two independent cheese making processes. Abbreviations: CCP, control cheese product inoculated with the milk starter cultures (MSC); ECPO100, experimental cheese product inoculated with MSC + 100 µL/L of oregano essential oils (OEOs); ECPO200, experimental cheese product inoculated with MSC + 200 µL/L of OEOs; SEM, standard error of the mean. On the row: a, b = $p < 0.05$.

The lowest hardness, expressed as resistance to compression, was found in the experimental production cheeses (ECPO100 and ECPO200), which reflects the lower consistency of their paste, justified by their higher humidity and then by their lower DM percentage.

Statistically significant differences were obtained for all cheese color indexes. Indeed, experimental products at both OEO levels (ECPO100 and ECPO200) showed higher values of lightness (L*) and redness (a*) and a corresponding lower yellow index (b*) compared to CCP cheeses. These results were expected considering the dark color of the extract attributed to the chlorophyll content in oregano. Furthermore, Boroski et al. [72], who added OEOs to milk, found that the color varied more consistently at increasing OEO concentrations. However, the color indexes recorded in CCP cheeses were within the ranges observed for this type of cheese [18,36].

Antioxidant capacity of tuma cheeses

The results of the antioxidant capacity of the cheeses analysed are reported in Table 3. The antioxidant capacity of OEO-added cheeses, expressed as TEAC, was higher than that registered for CCP; in particular, the highest antioxidant capacity was observed for the cheeses produced with the addition of 200 µL/L of OEOs. This result confirmed the well-known antioxidant properties of oregano, mainly due to the presence of relevant contents in phenolic compounds, such as carvacrol and thymol [15,16]. For this reason, OEOs are commonly used in food production to prolong shelf life by preventing oxidation and to preserve health properties.

Cheese fat stability to oxidation is expressed by POV and TBARs, representing, respectively, the indexes of primary and secondary lipid oxidation. The inclusion of OEOs was able to increase the antioxidant capacity of the cheeses, while it was not able to avoid an early fat oxidation. Indeed, the POV level was lower in 48 h control cheeses, whereas TBARs increased with the OEOs' inclusion. Similar results were obtained by Busetta et al. [69], who produced cheeses with EOs from citrus fruits. Based on these results, further investigations are necessary to explore the antioxidant potential of OEOs in preserving the oxidative stability of cheese fat during a prolonged storage time.

Volatile organic compounds emitted from tuma cheeses

Table 4 shows the VOC profiles generated from cheese samples with and without OEOs' addition. Twenty-one compounds were detected in control cheeses: seven acids, five ketones, four aldehydes, and four alcohols.

The main class found in cheese samples was free fatty acids (FFA). Hexanoic acid showed the highest values, followed by butanoic and acetic acid. Butanoic and hexanoic acids may generally result from the lipolysis of milk fat due to the action of the lamb rennet used for curdling, and also, in part, due to the activity of raw milk lipoprotein lipase [73,74]. Acetic acid can originate from different processes, including the oxidation of lactose by lactic acid bacteria under anaerobic conditions and carbohydrate catabolism by lactic acid bacteria [75]. Other odor-active compounds, such as alcohols (1-butanol-3-methyl (isoamyl alcohol) and aldehydes (hexenal and heptanal), were also revealed. Globally, the volatile composition detected in control cheeses reflects the volatile profile of cheeses produced from sheep's milk, as observed in many studies [18,76]. SPME-GC–MS

analysis clearly showed the effect of OEOs' addition, as both experimental products (ECPO100 and ECPO200) were characterized by components characteristic of the control cheese, such as hexanoic, butyric, and acetic acids and hexenal, heptanal, and isoamyl alcohol. However, the major volatile fraction was constituted by the typical compounds detected in OEOs, including, first of all, carvacrol. Furthermore, compounds belonging to the monoterpene hydrocarbons class, such as γ-terpinene, myrcene, β-pinene, p-cymene, and α-terpinene, and oxygenated monoterpenes, such as linalool, thymol, and β-caryophyllene, were also detected. Our results showed that independently of the amount of OEOs added, generally, no significant differences were found for VOCs emitted from cheese, but the percentage of carvacrol significantly increased. A similar effect was observed by Busetta et al. [69] on the VOC profiles of cheeses processed with different concentrations of citrus EOs. These results revealed that a small addition of OEOs (100 μL/L of OEO) significantly influenced the cheese's flavor profile, demonstrating that OEO compounds are easily transferred to cheese.

Table 4. Volatile organic compounds emitted from Tuma cheeses.

Chemical Compounds	**Samples**			**SEM**	***p*-value**
	CCP	ECPO100	ECPO200		
Acids					
Acetic acid	6.93^{a}	1.27^{b}	1.09^{b}	0.97	<0.0001
Butanoic acid	8.05^{a}	2.65^{b}	2.35^{b}	0.96	<0.0001
Exanoinc acid	13.41^{a}	2.94^{b}	3.19^{b}	1.76	<0.0001
2-hydroxy-4-methyl-Pentanoic acid	6.51^{a}	1.98^{b}	1.07^{b}	0.86	<0.0001
Octanoic Acid	4.47^{a}	1.54^{b}	0.97^{b}	0.57	0.001
Nonanoic acid	1.83^{a}	0.30^{b}	0.09^{b}	0.29	<0.0001
Ketons					
2-pentanone	1.53^{a}	0.10^{b}	0.07^{b}	0.25	<0.0001
3-hydroxy-2-butanone,	4.97^{a}	0.80^{b}	1.19^{b}	0.70	0.001
2-heptanone	0.58^{a}	0.010^{b}	0.03^{b}	0.09	<0.0001
2,3 octanedione	1.97^{a}	0.66^{b}	0.17^{b}	0.28	0.000
3,5 octadien-2-one	0.39^{a}	n.d.^{b}	n.d.^{b}	0.07	<0.0001
Alcohol					

3-Methyl-1-butanol	13.65[a]	4.76[b]	3.36[b]	1.70	0.001
1 pentanol	0.81[a]	0.19[b]	0.12[b]	0.11	<0.0001
2-butanol	2.64[a]	0.39[b]	0.24[b]	0.40	<0.0001
Octan-1-ol	2.79[a]	0.30[b]	0.5[b]	0.40	<0.0001
Hydrocarbons					
Hexane-2-methyl	1.46[a]	0.10[b]	n.d.[c]	0.24	<0.0001
Heptane 2,4 dimenthyl	2.45[a]	0.90[b]	0.24[b]	0.34	<0.0001
Aldeyde					
4 heptenal	0.18	0.020	n.d.	0.04	0.169
Hexanal	13.03[a]	5.09[b]	4.34[b]	1.45	<0.0001
Heptanal	10.53[a]	3.98[b]	2.63[b]	1.29	0.001
Nonanal	1.82[a]	0.33[b]	0.09[b]	0.28	<0.0001
Monoterpenes					
α-Thujene	n.d.[b]	0.20[a]	n.d.[b]	0.03	<0.0001
α-Pinene	n.d.[b]	0.06[a]	0.04[a]	0.01	0.000
Sabinene	n.d.[c]	0.05[a]	0.01[b]	0.01	<0.0001
β-Pinene	n.d.[c]	0.10[a]	0.01[b]	0.02	<0.0001
Myrcene	n.d.[c]	0.90[a]	0.62[b]	0.13	<0.0001
α-Phellandrene	n.d.[b]	0.11[a]	0.08[a]	0.02	<0.0001
α-Terpinene	n.d.[b]	0.90[a]	0.81[a]	0.15	<0.0001
p-Cymene	n.d.[b]	1.02[a]	0.89[a]	0.16	<0.0001
Limonene	n.d.[b]	0.10[a]	0.11[a]	0.02	<0.0001
(Z)-β-Ocimene	n.d.[b]	n.d.[b]	0.21[a]	0.04	<0.0001
γ-Terpinene	n.d.[b]	1.07[a]	0.99[a]	0.18	<0.0001
Monoterpenoids					
Linalool	n.d.[b]	0.10[a]	0.10[a]	0.02	0.002
Thymol	n.d.[c]	0.30[a]	0.20[b]	0.04	<0.0001
Carvacrol	n.d.[c]	66.08[b]	73.09[a]	11.66	<0.0001
Camphor	n.d.[b]	n.d.[b]	0.10[a]	0.02	0.001
Terpinen-4-ol	n.d.[b]	0.10[a]	n.d.[b]	0.02	<0.0001
β-Bisabolene	n.d.[b]	n.d.[b]	0.20[a]	0.03	<0.0001
β-Caryophyllene	n.d.[c]	0.60[b]	0.80[a]	0.12	<0.0001

Results are reported as relative peak areas (peak area of each compound/total area of identified VOC) × l00 and indicate the mean values ± standard deviation (S.D.) of four measurements (carried out in triplicate for two independent productions). Different superscript letters on the row indicate statistically significant differences according to Tukey's test. The retention times and mean peak area values are reported in Table S1. Abbreviations: CCP, control cheese product inoculated with the milk starter cultures (MSC); ECPO100, experimental cheese product inoculated with MSC + 100 µL/L of oregano essential oils (OEOs); ECPO200, experimental cheese product inoculated with MSC + 200 µL/L of OEOs; n.d., not detectable.

Sensory aspects of cheeses

Figure 6 reports the spider plot of the sensory attributes evaluated on control and experimental cheeses after 2 d of ripening. This approach makes it feasible to predict the consumer's attitude toward a new food product before its production at an industrial level and its introduction into the marketplace [77]. In this study, the addition of OEOs did not particularly affect the sensory attributes of Tuma cheeses. Except for the intensity of odor, milk odor, and butter odor, all other sensory attributes that were the object of evaluation were not influenced by the addition of OEOs.

In particular, the addition of OEOs increased odor intensity but influenced negatively milk and butter odor. These differences increased with OEOs' concentration and followed the same trend commonly reported for cheeses produced with the addition of EOs [64,69]. With regard to overall satisfaction, intended as an overall rating of the cheeses expressed considering all attributes with their scores [78], cheeses produced with 100 µL/L of OEOs were particularly appreciated by the evaluators and reached a score higher than that registered for control cheeses. On the contrary, the overall satisfaction of Tuma cheeses enriched with 200 µL/L of OEO was lower than that of control products, confirming that the addition of high concentrations of EOs in processed foods can negatively alter their taste and aroma and, consequently, exceed acceptability for consumption [79].

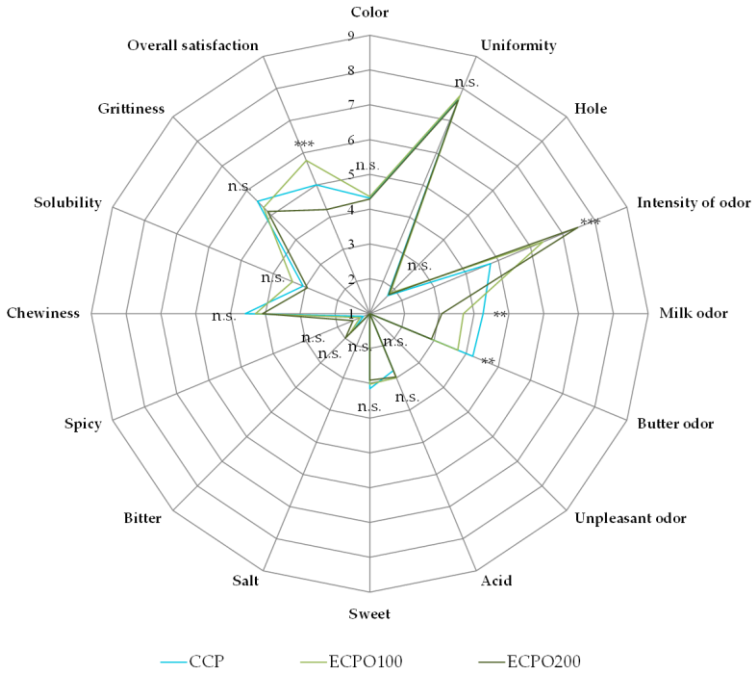


Figure 6. Spider diagrams of descriptive sensory analysis of Tuma cheeses. Abbreviations: Abbreviations: CCP, control cheese production inoculated with the Milk Starter Cultures (MSC); ECPO100, experimental cheese production inoculated with MSC + 100 µL/L of oregano essential oils (OEOs); ECPO200, experimental cheese production inoculated with MSC + 200 µL/L of OE-Os. $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant.

Artificial contamination test

The results of plate counts carried out for all samples collected during the production of Tuma cheeses from milk artificially contaminated with dairy pathogens are reported in Table 5.

Table 5. Microbial loads of samples collected during the artificial contamination test.

Samples	**Microbial counts**					
	TMM	*Lc. lactis*	*E. coli*	*L. monocytogenes*	*S.* Enteritidis	*St. aureus*
Pasteurized milk	2.71 ± 0.21	2.64 ± 0.26	<1	<1	<1	<1
Inoculated milk						
CCP	7.09	7.01	3.99	3.91	4.02	3.88
ECPO100	7.13	7.06	3.86	3.82	3.97	3.80
ECPO200	7.08	7.09	3.90	3.90	4.05	3.99
SEM	0.06	0.05	0.06	0.05	0.05	0.06
p value	0.972	0.914	0.829	0.865	0.928	0.673

Curd						
CCP	7.96	7.95	4.90	4.82	4.79	5.01
ECPO100	8.03	7.87	4.91	4.73	4.82	4.94
ECPO200	8.01	7.92	4.84	4.66	4.76	5.05
SEM	0.05	0.04	0.05	0.06	0.04	0.04
p value	0.926	0.870	0.929	0.782	0.942	0.817
Cheese						
CCP	8.85	8.67	5.71[a]	5.31[a]	5.09[a]	5.66[a]
ECPO100	8.96	8.84	2.99[b]	2.55[b]	3.01[b]	3.11[b]
ECPO200	8.90	8.70	2.74[b]	2.23[b]	2.93[b]	3.01[b]
SEM	0.05	0.06	0.34	0.35	0.25	0.31
p value	0.857	0.727	<0.0001	<0.0001	<0.0001	<0.0001

Units are Log CFU/mL for milk samples and Log CFU/g for curds and cheeses. Results indicate the mean values of six plate counts (carried out in triplicate for two independent productions). Data within a column followed by the same letter are not significantly different according to Tukey's test. Abbreviations: TMM, total mesophilic microorganisms; *Lc.*, *Lactococcus*; *E.*, *Escherichia*; *L.*, *Listeria*; S., *Salmonella*; *St.*, *Staphylococcus*; CCP, control cheese product inoculated with the milk starter cultures (MSC); ECPO100, experimental cheese product inoculated with MSC + 100 µL/L of oregano essential oils (OEOs); ECPO200, experimental cheese product inoculated with MSC + 200 µL/L of OEOs.

Pasteurized ewe's milk showed a TMM and coccus LAB count of approximately 3 Log CFU/mL, while dairy pathogenic bacteria were below the detection limit (<1 Log CFU/mL).

LAB and pathogens were found in inoculated milk used for control (CCP) and experimental products (ECP100 and ECP200) at 107 and 104 CFU/mL, respectively. As expected, both groups showed an increase of about one Log cycle after milk curdling. Cardamone et al. [80] observed a similar behavior in ewes' milk inoculated with the same four pathogenic strains during the production of PDO Pecorino Siciliano cheese. Significant differences ($p < 0.0001$) were found for the levels of all dairy pathogenic bacteria between the control and experimental cheeses. However, although these microorganisms were found both in ECP100 and ECP200 products, their levels were three Log cycles lower than those registered for control prods. This observation is probably due

to the limited contact time. Our results indicated a clear in vivo antibacterial activity of OEOs using Tuma cheese as a model cheese and confirmed previous observations of de Campos et al. [81]. These results confirmed those obtained by in vitro assay that showed very high antibacterial activity of OEOs against the main four dairy pathogenic bacteria, excluding any negative influence against LAB.

Conclusions

The addition of OEOs at 100 and 200 µL/L to milk did not affect the survival or growth of the two *Lc. lactis* starter cultures during cheese making. Experimental cheeses were characterized by the same ash, fat, and protein content in comparison to control products. OEOs' addition significantly increased the antioxidant activity of Tuma cheeses and impacted cheese VOC profiles with carvacrol, suggesting a high carryover of the volatile fraction of EOs from milk to final cheeses. Sensory traits were not negatively affected by the addition of OEO, but the highest values of overall satisfaction were evidenced for the Tuma cheeses enriched with 100 µL/L of OEOs. The addition of OEO was not able to completely inhibit the growth of the main dairy pathogens in the final cheeses, but it reduced significantly their development in OEO-added cheeses. The results of this study clearly highlighted the positive role of OEOs to enlarge the Sicilian ewes' milk-derived products portfolio, and may open new promising opportunities for the prevention of dairy pathogenic bacteria growth during cheese production.

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Chapter V

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

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Abstract

Aims: The purpose of this study was to functionalize an ovine stretched cheese belonging to "Vastedda" typology with red grape pomace powder (GPP) of Nero d'Avola cultivar and to characterize the microbiological, physicochemical, phenolic profile and sensory characteristics of the final cheeses.

Methods and Results: Before cheeses production, GPP was characterized for its microbiological profile, antibacterial activity and polyphenolic content. No colonies of bacteria and yeasts were detected in the GPP. GPP showed a large inhibition spectrum against spoilage and pathogenic bacteria. Three classes of polyphenolic compounds belonging to flavan-3-ols, flavonol and phenolic acids were identified. Two cheeses [0 and 1% (w w^{-1}) of GPP] were produced with pasteurized ewe's milk and commercial starter cultures. Plate counts and randomly amplified polymorphic DNA analysis demonstrated the ability of the starter strains to drive the fermentation process in the presence of GPP. GPP enrichment resulted in an increase of protein, phenolic compounds, sensory traits and reduced fat.

Conclusions: GPP addition to cheese represents an optimal strategy for the valorization of winemaking by-products and to obtain polyphenol-enriched cheese.

Significance and Impact of the Study: This study allowed to achieve an ovine cheese with specific physicochemical, nutraceutical and sensorial characteristics able to enlarge the functional dairy product portfolio.

Keywords: commercial starter culture, functional dairy product, grape pomace powder, ovine stretched cheese, physicochemical properties, polyphenolic profile.

Introduction

Alcoholic fermentation of grape is one of the world's economic most important transformation processes. Winemaking generates approximately 20%–25% volume of by-products, known as grape pomace, composed of a mix of grape skins and seeds [1]. This organic waste represents a serious environmental issue and also an economic loss for the winemaking industries in terms of pollution and exploitation of resources [2]. However, the increasing attention to the principles of the circular economy of wastes applied in the European Union [3] and the consumers request for food with no synthetic additives [4] has encouraged academic and industrial research institutes to experiment with alternative uses of grape pomace mass [5]. Based on its content in bioactive molecules [6], grape pomace in powder (GPP) form has been added to different foods of animal and vegetable origins poor in polyphenols and dietary fibre to enrich their functional value [7]. It is well documented that polyphenols exert several benefits on human health, including antioxidant and antimicrobial activity, and the prevention against chronic diseases [8].

Dairy products are rich in proteins, minerals, vitamins, short chain fatty acids [9], and contain also bioactive metabolites produced during bacterial fermentation [10,11]. However, in the last decade, increasing demand for functional cheeses has been observed and several studies have demonstrated the benefits of cheese enrichment with fruits and vegetables by-products [12]. The enrichment of dairy products with GPP is not completely new. The fortification with GPP in yogurt has been reported in recent review articles [13-15] highlighting their positive effect on the nutritional quality of

dairy products. Regarding ovine dairy products, although Gaglio et al. [16] and Gaglio, et al. [17] performed studies on the enrichment of pressed and stretched cheeses with GPP, they provided data on cheeses processed at pilot plant scale level using selected individual *Lactococcus lactis* strains. So far, there have been no studies in the literature on the determination of the phenolic profile of cheeses containing GPP and subjected to the stretching technology at the industrial level. In fact, considering the unique characteristics of different phenolic classes on biosynthesis, metabolism and health effects [18-20], the characterization of the phenolic profile of enriched foods is crucial to formulate new products with positive effects on consumers' health.

Hence, the purpose of the present research was to perform for the first time the production of an ovine stretched cheese enriched with GPP at the industrial level using a commercial starter culture. The specific objectives of the present study were to: (i) characterize the GPP for their microbiological characteristics, antibacterial activity and phenolic profile; (ii) monitor the ability of commercial starter LAB to drive the fermentation process in the presence of GPP; (iii) evaluate the phenolic profile of final cheeses;(iv) evaluate the sensory traits of the final cheeses.

Materials and Methods

Grape pomace collection and grape pomace powder production

Grape pomace is composed of a mix of grape skins and seeds from fermented red *Vitis vinifera* cv. Nero d'Avola was provided by the factory Cantine Europa (Petrosino, Trapani, Italy). Grape pomace (25 kg) was collected after 150 days of post-fermentation maceration during the winter 2021 and transferred into 25 sterile bags

(BagLightR 400, Interscience) containing 1 kg each. The 25 grape pomace aliquots were picked up randomly from different heaps and transported in a portable fridge to the Agricultural Microbiology laboratory (Department of Agricultural, Food and Forestry Science, University of Palermo, Italy) where they were dried in an oven Compact Combi (Electrolux) at 54 °C for 48 h and then ground with a Retsch centrifugal Mill ZM1 to obtain grape pomace powder (GPP) with a particle size of 250 μm [21].

Microbiological characterization of GPP

Plates counts

Fifteen grams of GPP were transferred into a sterile stomacher bag, added with 135 mL of Ringer's solution (Sigma Aldrich) and homogenized in the Bag-Mixer 400 stomacher (Interscience) for 2 min at the maximum speed. Homogenized GPP was then subjected to the decimal serial dilution in Ringer'ssolution (1:10) and the cell suspensions were used for the plate count of the main microbial groups belonging to the pro-technological, spoilage and pathogenic populations following the approach of Gaglio et al. [17]. Briefly, total mesophilic microorganisms (TMM) on Plate Count Agar (PCA); mesophilic and thermophilic LAB cocci on Medium 17 (M17) agar; mesophilic and thermophilic LAB rods on de Man–Rogosa–Sharpe (MRS) agar; enterococci on Kanamycin Esculin Azide (KEA) agar; members of Enterobacteriaceae on violet red bile glucose agar (VRBGA); coagulase-positive staphylococci (CPS) on Baird Parker (BP) supplemented with rabbit plasma fibrinogen (RPF); *Listeria monocytogenes* on *Listeria* selective agar base with SR0140E supplement; *Salmonella* spp. and *E. coli* on Hektoen Enteric Agar (HEA); yeasts

a on Yeast extract Peptone Dextrose (YPD) agar supplemented with 0.1 g/L chloramphenicol to avoid bacterial growth. All media and supplements were purchased from Oxoid Microbiology Products (Thermo-Scientific), except HEA provided by Microbiol Diagnostici. GPP was analysed in duplicate while plates counts were performed in triplicate.

Determination of antibacterial activity of GPP

GPP was suspended in sterile distilled water to the concentration of 200 mg mL^{-1}. Bacterial strains of food origin representative of the spoilage and pathogenic bacterial groups were used as indicators (sensitive to the inhibitory activity). Specifically, species within the genera *Brochotrix* and *Pseudomonas* were chosen among spoilage bacteria, while species within the genera *Acinetobacter*, *Bacillus*, *Escherichia*, *Listeria*, *Pseudomonas*, *Salmonella*, *Staphylococcus* and *Stenotrophomonas* among the agent associated with foodborne diseases. All strains belong to the culture collection of the Agricultural Microbiology Unit of the Department of Agricultural, Food and Forest Science – University of Palermo (Italy). All bacteria were sub-cultured in Brain Heart Infusion (BHI) broth (Condalab) incubated at 37 °C for 24 h.

GPP was tested for antibacterial activity applying the well diffusion assay [22]. Sterile water was used as a negative control, while streptomycin (10% w v^{-1}) as a positive control [23]. The inhibitory activity is considered positive if a definite clear area is detected around the wells. This test was performed in triplicate.

Minimum inhibitory concentration (MIC) was determined for the sensitive strains. MICs were determined by the broth microdilution method in 96-well microplates. GPP

suspension was serially diluted in BHI (1:1) and its concentration ranged between 100 and 3.125 mg mL^{-1}. Each well was inoculated with approximately 10^6 CFU per mL of the sensitive strain. The bacterial growth was followed by optical density (OD), measured using a ScanReady Microplate photometre P-800 (Life Real Biotechnology Co., Ltd) at 595 nm wavelength. BHI alone was used as a negative control, while BHI inoculated with each sensitive strain as a positive control. MIC was defined as the lowest concentration at which no growth was observed. This test was performed in triplicate.

Determination of phenolic profile of GPP

Two grams of GPP were added with 5 mL of extracting solution (20:80 water: methanol with 1% of formic acid). The mixture was shaken for 10 min, then centrifuged at 10,000 g at 4 °C for 10 min. The extract (2 mL) was filtered through a 0.2 µm membrane filter (Agilent Technologies) [24].

GPP polyphenol determination was carried out using a UPLC-MS/MS Micromass Quattro microTM (Waters), coupled with a Z-spray electrospray ionisation (ESI) source operating in negative mode. The sampled extract was injected into a reversed-phase column (BEH C18, 1.7 µm, 2.1 × 150 mm, Waters), which was maintained at 30 °C. Compounds were separated using gradient elution with 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B) at a flow rate of 0.3 mL min^{-1}. The injection volume was 20 µL. The gradient elution was performed as follows: solvent A 20%–35% from 0 to 10 min, 35%–50% from 10 to 20 min and 50%–100% from 20 to 25 min. All the ion source and ion optic parameters were optimized and they were finally set as follows: capillary voltage 2.5 kV; cone voltage

25 V; cone gas flow rate 70 L h^{-1}; desolvation temperature 350 °C and nebulizer pressure at 50 psi. Multiple reaction monitoring (MRM) parameters were optimized as summarized in Table 1. External calibration of commercial standards of polyphenol compounds was performed to determine the analytes concentration in samples. MassLynxTM Workstation (version 4.0, Waters) was used for data acquisition and processing.

Table 1. Optimized HPLC-ESI–MS/MS transition of phenolic compounds (in negative ion mode).

Phenolic compounds	**MRM transition [m z^{-1}]**
Epicatechin	289 > 109
Catechin	289 > 205
Quercetrin	447 > 299
Mirecetin	317 > 179
Quercetin	301 > 151
Vanilli acid	167 > 152
Siringic acid	197 > 123
Protocatechuic acid	153 > 109
Cumaric acid	163 > 119
Caffeic acid	179 > 135
Chlorogenic acid	353 > 191

Cheese production and sample collection

Pasteurized ewe's milk (72 °C for 15 s) from the indigenous Sicilian sheep breed "Valle del Belíce" and GPP were used as raw materials. Freeze-dried starter preparation (LYOBAC-D T, Alce International s.r.l.) composed of defined strains of *Streptococcus thermophilus* (MO097), as indicated by the producer company, was used as the fermenting agent. Cheesemaking was performed at an industrial level in the dairy factory "Biopek" located at Gibellina following the protocol of production for the stretched cheese "Vastedda" as reported by Gaglio et al. [17]. Two cheese trials were obtained with the starter culture: CTR, control cheese produced from pasteurized ewe's milk; EXP,

experimental cheese produced from pasteurized ewe's milk and 1% (w w^{-1} of curd) GPP, based on the previous studies by Barbaccia et al. [25], Gaglio et al. [16] and Gaglio et al. [17]. Each trial was carried out in a stainless steel vat containing 500 L of milk. Briefly, the curd was mechanically broken after coagulation until reaching small rice-sized grains. After whey draining, the control curds were immediately put into perforated containers, while the experimental curds were added with 1% (w/w) GPP, uniformly mixed, and then transferred into perforated containers. Both cheese trials were carried out in triplicate over three consecutive weeks. Samples of raw milk, pasteurized milk, freeze-dried starter preparation, inoculated milk with a starter culture, curds, acidified curds, cheeses just after production and after 15 days of refrigerated storage, were collected for analyses.

Microbiological analyses

Cell suspensions of milk samples were subjected to decimal serial dilutions in Ringer's solution, while freeze-dried starter preparation, curd and cheese samples (10 g) were first homogenized in 90 mL of sodium citrate (2% w v^{-1}) in a stomacher and then serially diluted.

As reported above for GPP, cell suspensions of raw milk and pasteurized milk were subjected to plate count for the enumeration of pro-technological, spoilage and pathogenic microorganisms.

Cell suspensions of freeze-dried starter preparation, inoculated milk, curds and cheeses were analysed for total mesophilic microorganisms (TMM) and thermophilic coccus LAB exclusively on M17 agar. Plates counts were performed in duplicate.

Monitoring of commercial starter culture and identification of the indigenous milk LAB resistant to the pasteurization process

The presence of the commercial starter culture added as a fermenting agent in the trials, and its dominance over LAB resistant to pasteurization was confirmed by randomly amplified polymorphic DNA (RAPD)-PCR technique as reported by Alfonzo et al. [27]. The polymorphic patterns of the LAB colonies developed on agar media were compared to those of the axenic cultures of *S. thermophilus* strains originating from commercial starter culture. Genotypic identification of the LAB strains isolated from pasteurized bulk milk before commercial starter culture addition was performed by amplification and sequencing of the 16S rRNA gene [27]. The sequences obtained were compared to those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) and EzTaxon-e (http://eztaxon-e.ezbiocloud.net/) databases.

Physicochemical analyses of cheeses

Moisture, fat, protein, saturated fatty acid, residual dry matter, salt content and the color parameters L* (luminosity), a* (redness) and b* (yellowness) were determined by FoodScan TM 2 Lab (Instrument number 91861723 - Foss Electric A/S). This instrument is based on near-infrared (NIR) technology [28]. All physicochemical measurements were carried out in triplicate.

Determination of phenolic profile of cheeses

The phenolic profile of both control and experimental cheese samples were detected by optimized QuEChERS extraction [29-32]. The QuEChERS procedure was as follows: 3 g

of homogenized sample was placed in a 50 mL falcon tube with 5 mL of water and 10 mL of MeOH (1% formic acid). Afterthat, the mixture was mechanically shaken for 2 min. QuEChERS extraction salts (4 g $MgSO_4$, 1 g NaCl) were added to the tube and it was shaken for another 2 min and centrifuged at 2291 g for 10 min. The supernatant (6 mL) was transferred into a 15-mL tube, kept at −20 °C for 3 h and then centrifuged (2291 g, 5 min, −4 °C). The supernatant was transferred into a 15-mL dSPE tube (PSA/C18/$MgSO_4$), stirred by vortex for 2 min and centrifuged (2291 g, 10 min). MeOH extract was transferred into a vial, evaporated under a gentle stream of N_2 and the final residue was re-dissolved with 500 µL of MeOH. Determination and quantification of polyphenols were performed as reported above for GPP.

Sensory evaluation

Control and experimental cheeses after 15 days of refrigerated storage were analysed for their sensory traits. A descriptive panel of 13 assessors composed of seven women and six men in the age range of 21–65 years old were specifically trained following the ISO 8589 [33] indications. The panellists were asked to evaluate 12 descriptors including intensity of odor and aroma, sweet, salt, bitter, acid, fibre, grainy, adhesiveness, hardness, humidity, and the overall assessment [34,35]. The analysis was performed following the guidelines of the ISO 13299 [36]. The assessors scored the level of each attribute with a mark on a seven-point hedonic scale (0 = extremely low; 7 = extremely high) as reported by Faccia et al. [37].

Statistical analyses

Microbiological, physicochemical and sensory evaluation data were subjected to one-way variance analysis (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft). The Duncan procedure and Tukey's test was applied for pairwise comparison. Statistical significance was attributed to *p* values of $p < 0.05$.

Results

Microbiological characterization of GPP

The specific search for pro-technological, spoilage and pathogenic microbial populations did not generate any colonies from the GPP, showing its hygienic suitability for food production.

The antibacterial activity of GPP against spoilage and pathogenic bacteria is shown in Table 2. Except for the strain *Brochotrix thermosphacta* SP10, all other indicator strains were inhibited by GPP, indicating a positive role of this by-product to limit the growth of undesired bacteria. The strongest inhibition determined by GPP, with a diameter of the inhibition area around the wells higher than 15 mm, was observed with all pseudomonadaceae (strains 4G764, 4G628 and 4G558), *Escherichia coli* PSL52, all *Listeria monocytogenes* strains (ATCC19114, 11B0 and 13B0), *Salmonella* Typhimurium 50432 and *Stenotrophomonas maltophilia* ICE272. Considering the strong activity shown by GPP, it was also characterized in terms of MIC against all of the most sensitive strains (Table 2). GPP showed a MIC of 25 mg mL^{-1} against all the *Pseudomonas* spp. and *L. monocytogenes strains*, but a lower inhibitory activity was exerted against *E. coli* PSL52, *S.* Typhimurium 50432 and *St. malthophilia* ICE272 for which MIC was 50 mg mL^{-1}.

Table 2. Antibacterial activity[a] of grape pomace powder.

Species	Strains	Source of isolation	Inhibition[a]	Antimicrobial MIC (mg ml^{-1})
Spoilage				
Brochotrix thermosphacta	SP10	Pork meat	-	n.d.
Pseudomonas endophytica	4G764	Ready to eat salad	17.8 ± 0.2	25
Pseudomonas fluorescens	4G628	Ready to eat salad	16.4 ± 0.1	25
Pseudomonas poae	4G558	Ready to eat salad	15.4 ± 0.2	25
Pathogenic				
Bacillus cereus	ICE170	Ice cubes	12.2 ± 0.1	n.d.
Escherichia coli	PSL52	DOP Pecorino Siciliano chesse	17.4 ± 0.2	50
Escherichia coli	ATCC25922	Clinical isolate	12.6 ± 0.1	n.d.
Enterobacter amnigenus	60A2	Freeze-dried lamb	12.3 ± 0.1	n.d.
Listeria monocytogenes	ATCC19114	Animal tissue	17.5 ± 0.1	25
Listeria monocytogenes	11BO	Meat factory	15.7 ± 0.1	25
Listeria monocytogenes	13BO	Gorgonzola cheese	15.1 ± 0.2	25
Pseudomonas aeruginosa	PSA68	Animal tissue	12.3 ± 0.1	n.d.
Salmonella Typhimurium	50432	Molluscs	15.2 ± 0.1	50
Salmonella Enteritidis	ATCC13076	Unknown	12.3 ± 0.2	n.d.
Staphylococcus aureus	ATCC33862	Unknown	12.2 ± 0.1	n.d.
Stenotrophomonas maltophilia	ICE272	Ice cubes	17.2 ± 0.1	50

Note: Results indicate the mean value of three independent assays. Abbreviations: MIC, minimum inhibitory concentration; n.d., not determined. [a]Antibacterial activity of grape pomace powder is indicated by the width of the inhibition zone (mm) around the well.

Phenolic profile of GPP

Figure 1 reports the graphic representation of the phenolic profile of GPP. Eleven components including 6 phenolic acids, 2 flavan-3-ols and 3 flavonols were quantified by the method UPLC-MS/MS developed in this study. In particular, high concentrations of catechin and epicatechin (1014.1 and 732.5 µg g^{-1}, respectively), among the flavanols/flavan-3-ols, quercetin and myricetin (530.5 and 402.2 µg g^{-1}, respectively), among the flavonols, syringic acid and caffeic acid (273.3 and 109.7 µg g^{-1}, respectively) and among phenolic acids were detected. The concentrations of the other phenolic compounds detected were lower than 100 µg g^{-1}. The total phenolic content of Nero

d'Avola GPP was 3310.8 μg g^{-1}.

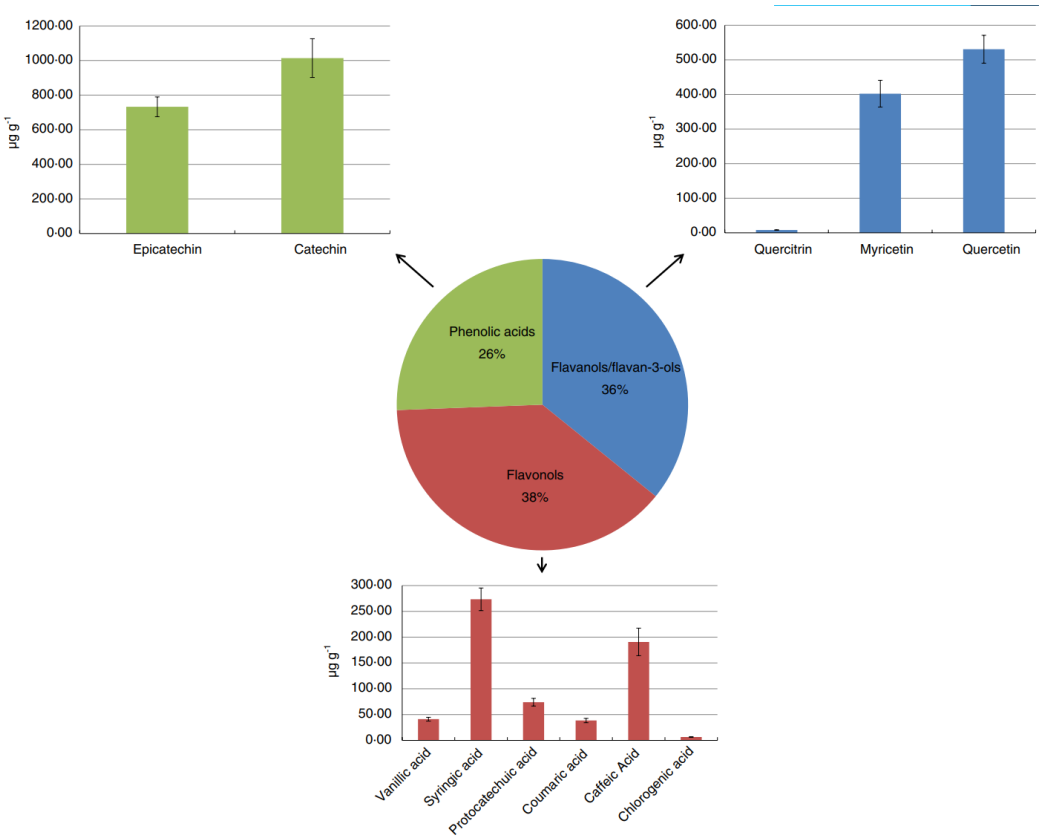

Figure 1. Polyphenolic compounds in GPP. Results as expressed in μg g^{-1} and indicate mean values ± SD of three determinations.

Microbiological evolution during cheese productions

The levels of the different microbial groups investigated in commercial starter culture and milk samples are reported in Table 3. The microbiological counts included only TMM and thermophilic coccus LAB from commercial starter culture and inoculated milk. The results of *L. monocytogenes* and *Salmonella* spp. are not reported because their levels were below the detection limit for both raw and pasteurized milk. Dried starter culture was dominated by thermophilic coccus LAB (>10^{10} CFU per gram). The levels of TMM in raw ewe's milk were found at 6.58 Log CFU per mL and decreased by about 3 Log cycles after pasteurization. After commercial starter culture addition, TMM increased by about 1 Log

cycle. Regarding LAB, raw ewe's milk hosted 10^6 CFU per mL of mesophilic rod and coccus LAB and 10^4 CFU per mL of thermophilic rod and coccus LAB. After pasteurization, mesophilic rod and coccus, as well asthermophilic rod and coccus LAB, decreased to 10^4 and 10^2 CFU per mL, respectively. After starter culture addition, thermophilic LAB cocci increased up to 7.42 CFU per mL. Enterococci were 2.52 Log CFU per mL in raw milk, but were below the detection level after pasteurization. The levels of undesired microbial groups, especially members of the Enterobacteriaceae family, CPS and *E. coli* in raw milk were 3.57, 2.40 and 2.24 Log CFU per mL, respectively, but they completely disappeared in pasteurized milk. Similar behavior was recorded for yeasts, which were at 2.35 Log CFU per mL in raw milk and were not found in pasteurized milk.

Table 3. Microbial load of commercial starter and milk samples.

Microbial counts	Samples				Statistical significance
	CSC	RM	PM	IM	
TMM	10.41 ± 0.21^a	6.58 ± 0.22^c	3.62 ± 0.13^d	7.36 ± 0.12^b	***
Mesophilic rod LAB	n.a.	5.93 ± 0.21^a	2.63 ± 0.14^b	n.a.	***
Thermophilic rod LAB	n.a.	3.85 ± 0.11^a	1.94 ± 0.20^b	n.a.	***
Mesophilic coccus LAB	n.a.	6.21 ± 0.20^a	2.51 ± 0.23^b	n.a.	***
Thermophilic coccus LAB	10.67 ± 0.24^a	4.03 ± 0.15^c	2.07 ± 0.13^d	7.02 ± 0.24^b	***
Enterococci	n.a.	2.52 ± 0.25^a	<2^b	n.a.	***
Entericacteriaceae	n.a.	3.57 ± 0.12^a	<1^b	n.a.	***
CPS	n.a.	2.40 ± 0.23^a	<2^b	n.a.	***
E. coli	n.a.	2.24 ± 0.16^a	<2^b	n.a.	***
Yeasts	n.a.	2.35 ± 0.17^a	<2^b	n.a.	***

Note: Units are CFU g^{-1} for dried starter culture; CFU mL^{-1} for milk samples. Results indicate mean values ± SD of six plate counts (carried out in duplicate for three independent productions). Data within a line followed by the same letter are not significantly different according to Tukey's test. *p* value: ***$p < 0.001$. Abbreviations: CPS, coagulase-positive staphylococci; CSC, commercial starter culture; *E.*, *Escherichia*; IM, inoculated milk; n.a., not analysed; PM, pasteurized milk; RM, raw milk; TMM, total mesophilic microorganisms.

Figure 2 reports the growth of the fermenting agent, added as a starter culture, evaluated on curds and cheeses samples. According to the Duncan test, no statistically significant differences ($p > 0.05$) were found for the levels of TMM and *S. thermophilus* in all samples

analysed. TMM and *S. thermophilus* were counted both in control and experimental curd at 10^8 CFU per gram. After acidification, control and experimental curds reached values of TMM and *S. thermophilus* of about 10^9 CFU per gram showing an increase in concentrations of these groups of about 1 Log cycle. Control and experimental cheeses just after production as well as after 15 day of refrigerated storage hosted levels of TMM and LAB a little lower than 9 Log CFU per gram.

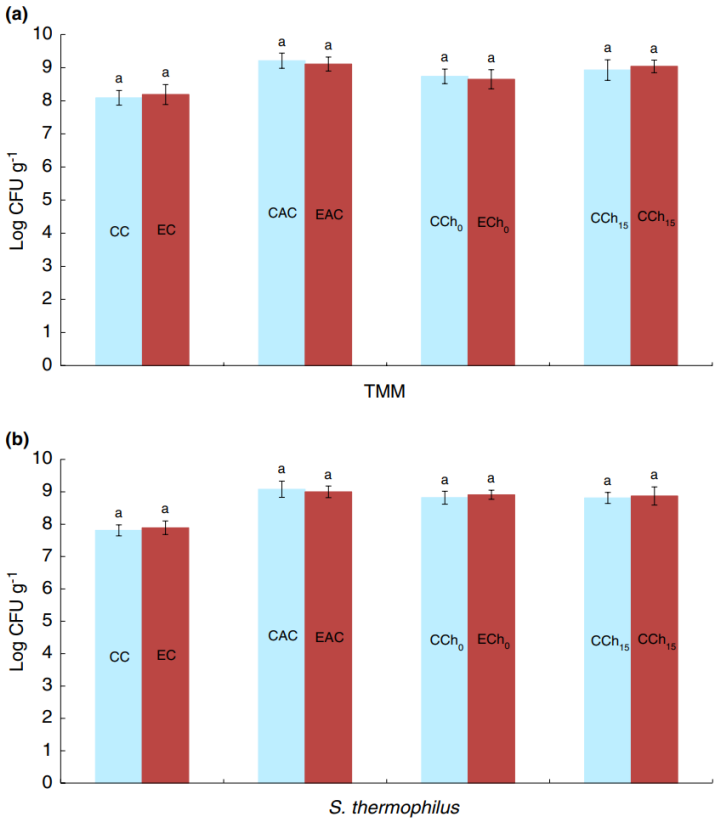

Figura 6. Growth of commercial starter LAB during cheese production (Log CFU per gram). (a) total mesophilic microorganisms (TMM); (b) *Streptococcus thermophilus*. Results indicate mean values ± SD of six determinations (carried out in duplicate for three independent productions). Histograms followed by the same letter are not significantly different according to the Duncan test between control and experimental samples for $p < 0.05$. CAC, control acidified curd; CC, control curd; $CCht_0$, control cheese soon after production; $CCht_{15}$, control cheese at 15 days of refrigerated storage; EAC, experimental acidified curd; EC, experimental curd; $ECht_0$, experimental cheese soon after production; $ECht_{15}$, experimental cheese at 15 days of refrigerated storage. Control production was inoculated with commercial starter culture, while experimental production with a commercial starter culture +1% of GPP.

Commercial starter culture recognition and identification of thermoduric indigenous milk LAB

After enumeration, 12 presumptive LAB (Gram-positive and catalase-negative) were isolated from commercial starter culture, 20 from pasteurized milk before commercial starter additionand 88 from inoculated milk with starter culture through to the final cheeses. All 120 isolates were subjected to strain typing by RAPD-PCR in order to distinguish the different strains and to monitor the added commercial starter LAB strain(s) by polymorphic profile comparison. Figure 3 shows only 14 isolates because they were isolated at least once in different samples. The other 106 isolates were not included in the dendrogram because they shared the same polymorphic profiles as those shown in Figure 3. The major RAPD cluster included all strains isolated from milk inoculated with the starter culture. These strains were detected even in cheeses (both CTR and EXP) after 15 days of refrigerated storage. In particular, the strain was coded as *S. thermophilus* MO097 in this study originated from commercial LAB starter culture, in all samples analysed. Four LAB strains (VEC13, VEC17, VEC20, and VEC31), isolated from pasteurized milk before commercial starter addition, were identified by 16S rRNA gene sequencing. This analysis indicated that the indigenous thermoduric milk LAB was represented by the species *Enterococcus faecalis* (Ac. No. MZ457323), *Leuconostoc mesenteroides* (Ac. No. MZ457324 - MZ457325) and *Leuconostoc pseudomesenteroides* (Ac. No. MZ45732).

As reported in Figure 3, indigenous milk LAB strains were not detected at dominating levels in milk after the addition of starter *S. thermophilus* strains. These results undoubtedly confirmed the ability of the commercial starter culture to dominate over the

LAB community resistant to pasteurization process in both control and experimental cheeses.

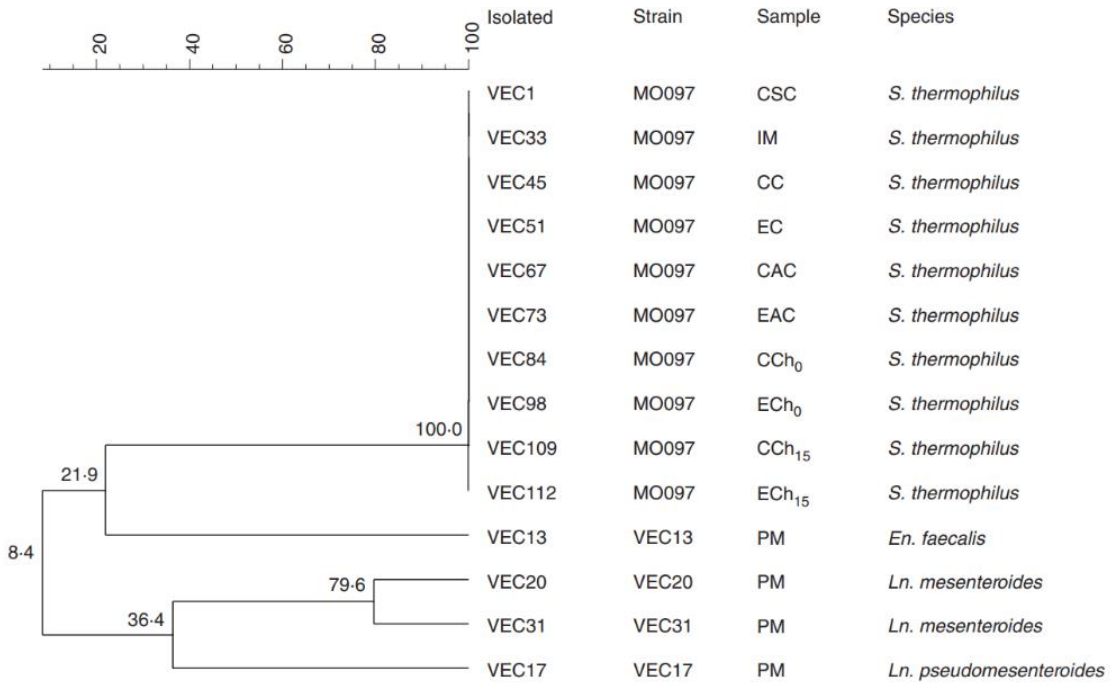

Figure3. Dendrogram obtained with combined RAPD-PCR patterns generated with three primers for LAB strains isolated during cheese productions. CAC, control acidified curd; CC, control curd; $CCht_0$, control cheese soon after production; $CCht_{15}$, control cheese at 15 days of refrigerated storage; CSC, commercial starter culture; *E.*, *Enterococcus*; EAC, experimental acidified curd; EC, experimental curd; $ECht_0$, experimental cheese soon after production; $ECht_{15}$, experimental cheese at 15 days of refrigerated storage; IM, inoculated milk; *Ln.*, *Leuconostoc*; *S.*, *Streptococcus*. Control production was inoculated with a commercial starter culture, while experimental production with a commercial starter culture +1% of GPP.

Physicochemical analyses of cheeses

The physicochemical parameters of control and experimental cheeses after 15 days of refrigerated storage are reported in Table 4. The addition of GPP did not determine significant variations ($p > 0.05$) for salt and saturated fatty acid percentages, while significant differences ($p < 0.001$) were observed for fat, moisture, protein and residual dry matter content. In particular, the enrichment of cheese with GPP induced a fat and moisture decrease, which was followed by a protein and residual dry matter increase. The evaluation

of the three color parameters showed that the color of cheeses was strongly affected by GPP addition. In particular, experimental cheeses showed the lowest values of lightness (L*) and yellowness (b*) and the highest values of redness (a*).

Table 4. Physicochemical traits of cheeses after 15 days of refrigerated storage.

Parameters	Samples		Statistical significance
	CCh_{15}	ECh_{15}	
Fat (%)	27.82 ± 0.08[a]	25.41 ± 0.06[b]	***
Moisture (%)	40.87 ± 0.13[b]	42.87 ± 0.00[a]	***
Salt (%)	1.54 ± 0.04[a]	1.47 ± 0.09[a]	n.s.
Protein (%)	25.28 ± 0.10[b]	29.08 ± 0.11[a]	***
Saturated fatty acid (%)	17.20 ± 0.14[a]	17.00 ± 0.01[a]	n.s.
Residual dry matter (%)	57.13 ± 0.03[b]	59.13 ± 0.13[a]	***
Lightness (L*)	59.25 ± 0.21[a]	43.75 ± 0.07[b]	***
Redness (a*)	−0.35 ± 0.07[b]	3.20 ± 0.10[a]	***
Yellowness (b*)	12.60 ± 0.11[a]	3.10 ± 0.05[b]	***

Note: Results indicate mean values ± SD of six determinations (carried out in duplicate for three independent productions). Data within a line followed by the same letter are not significantly different according to Duncan test. *p* value: ***$p < 0.001$. Abbreviations: CCh15, control cheese at 15 days of refrigerated storage; ECh15, experimental cheese at 15 days of refrigerated storage; n.s., not significant.

Phenolic profile of cheeses

The phenolic profile of control and experimental cheeses are reported in Table 5. According to the Duncan test, statistically significant differences ($p < 0.001$) were found among cheeses. All polyphenolic compounds detected in GPP were found in experimental cheeses, but not in control cheeses. In particular, each phenolic compound was detected in experimental cheese at about 1% of the concentration detected in GPP. Moreover, the addition of 1% (w w^{-1}) of GPP determined an increase of about 32 µg g^{-1} of total phenolic concentration.

Table 5. Polyphenolic compounds of GPP found in cheeses after 15 days of refrigerated storage.

Polyphenolic compounds	Samples		Statistical significance
	CCh_{15}	ECh_{15}	
Flavanols/flavan-3-ols			

Epicatechin	n.d.[b]	7.40 ± 1.55[a]	***
Catechin	n.d.[b]	10.72 ± 2.12[a]	***
Flavonols			
Quercitrin	n.d.[b]	0.06 ± 0.01[a]	***
Myricetin	n.d.[b]	3.80 ± 0.83[a]	***
Quercetin	n.d.[b]	4.22 ± 1.23[a]	***
Phenolic acids			
Vanillic acid	n.d.[b]	0.33 ± 0.07[a]	***
Syringic acid	n.d.[b]	2.32 ± 0.47[a]	***
Syringic acid	n.d.[b]	0.74 ± 0.17[a]	***
Coumaric acid	n.d.[b]	0.35 ± 0.07[a]	***
Caffeic acid	n.d.[b]	1.80 ± 0.33[a]	***
Chlorogenic acid	n.d.[b]	0.07 ± 0.02[a]	***

Note: Results as expressed in μg g^{-1} and indicate mean values ± SD of six determinations (carried out in duplicate for three independent productions). Data within a line followed by the same letter are not significantly different according to Duncan test. *p* value: ***$p < 0.001$. Abbreviations: CCh_{15}, control cheese at 15 days of refrigerated storage; ECh_{15}, experimental cheese at 15 days of refrigerated storage; n.d., not detected.

Sensory test

The results of the sensory evaluation performed by the panellists on the control and experimental cheeses after 15 days of refrigerated storage are reported in Figure 4. The spider graphic representation clearly shows that the addition of 1% (w w^{-1}) of GPP lead to the production of cheese with different sensory traits. No differences were found for salt and bitter, while high differences emerged for all other attributes evaluated. In particular, experimental cheeses were characterized by the highest odor and aroma intensity, acid perception, fibre, grainy, adhesiveness and humidity, but also showed the lowest sweetness and hardness. Finally, the highest score for the overall assessment, described as the degree of overall satisfaction, was shown by the experimental cheeses.

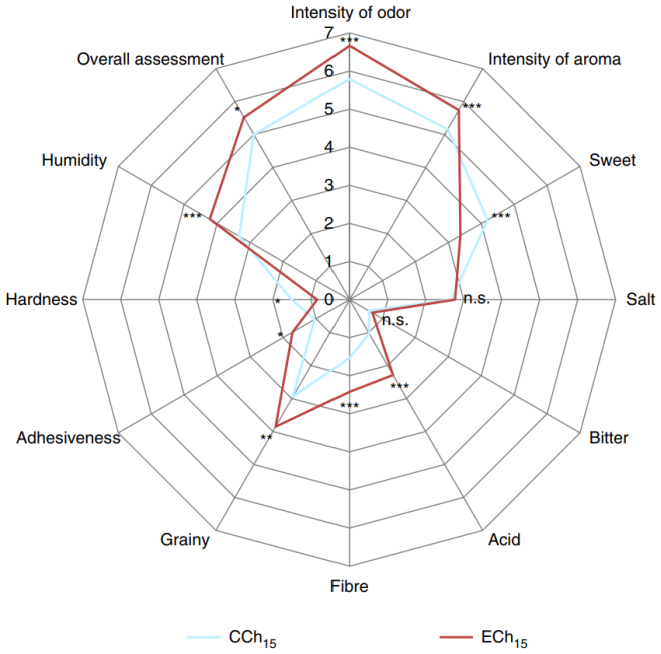

Figure 4. Spider diagrams corresponding to the descriptive sensory analysis of control and experimental cheeses. CCh_{15}, control cheese at 15 days of refrigerated storage; ECh_{15}, experimental cheese at 15 days of refrigerated storage. *p* value: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; n.s., not significant. Control production was inoculated with commercial starter culture, while experimental production with commercial starter culture +1% of GPP.

Discussion

Recently, natural antioxidants extracted from plant by-products have been considered by food producers in order to satisfy the increasing demand of consumers for healthy and functional foods [38]. In particular, regarding dairy products, the enrichment of cheeses with different non-conventional ingredients containing biologically active compounds and a balanced nutritional profile increased and this phenomenon is still on the increase [12]. The most interesting antioxidants present in fruits and vegetables are phenols [39]. For this reason, there is a growing interest in the bioactive phenolic compounds present in winemaking industry by-products [40].

The present study was undertaken to evaluate the effect of red grape pomace addition

on the microbiological, physicochemical, phenolic profile and sensory aspects of an ovine stretched cheese produced at industrial levels using commercial starter LAB culture.

Before cheese production, GPP from fermented red *Vitis vinifera* cv. Nero d'Avola was characterized for microbial load, antibacterial activity and phenolic profile. Bacteria and yeast were below the detection limits most likely due to the oven-drying treatment performed on grape pomace [5]. The absence of microorganisms at detectable levels in Nero d'Avola cultivar GPP was already shown by Gaglio et al. [16], Gaglio et al. [17] and Barbaccia et al. [25] for the vintage 2018, 2019 and 2020.

In view of the valorization of GPP as a natural alternative to traditional chemical additives, the antimicrobial activity of this powder was also evaluated. The inhibitory spectrum was determined against spoilage and pathogenic bacteria commonly associated with food items [41-45]. Only *Brochotrix thermosphacta*, commonly associated with meat spoilage [46], was insensi tive to the GPP assayed. However, a considerable inhibition activity was observed against *Pseudomonas* strains commonly involved in the spoilage of foods of animal origin [47] and several food-borne pathogens such as *E. coli*, *L. monocytogenes*, *S.* Typhimurium and *St. maltophilia* that represent a risk for consumers [45,48,49]. The above strains were also used as indicators to determine GPP MIC. GPP was found to have effective antimicrobial activity at low concentrations. A similar trend was previously observed for grape pomace of Emir and Kalecik karasi grape cultivars by Özkan et al. [50]. These results most likely depend on the phenolic composition of GPP [51-53].

The main phenolic components detected in the GGP sample analysed in this study belonged to three classes including flavan-3-ols (cathechin and epicatechin), flavonols

(myricetin, quercetin and quercitrin) and phenolic acids (syringic acid, vanillic acid, protocatechuic acid, cumaric acid, caffeic acid and chlorogenic acid). Flavan-3-ols were the most abundant phenols group in GPP samples, as also observed in grape pomace from different cultivars [54,55]. Regarding flavonols, high concentrations of quercetin and myricetin (>400 µg g^{-1}) were detected, while phenolic acids were in the range of 38.5–273.3 µg g^{-1}. Similar trends were previously detected by Antoniolliet al. [54] in red grape pomace from cv. Malbec. These phenolic compounds are considered the most important natural antioxidants gaining great importance as phytochemical compounds. Indeed, they are associated with several health benefits including cardiovascular disease, cancer, osteoporosis, diabetes and neurodegenerative diseases as well as oxidative stress [56]. In particular, flavan-3-ol and flavonol showed specific health protective effects such as antimicrobial activity against viruses, bacteria and fungi [19,57]. Also, the neuroprotective role of phenolic acids from food and by-products has been reported by many authors [58-60]. Our results highlighted the potential of GPP from the Nero d'Avola cultivar to be used as a natural additive for dairy products. To this purpose, a GPP by-product was applied at 1% (w w^{-1}) during fresh ovine cheese production.

Cheese trials were carried out in February 2021 applying "Vastedda" type stretched cheese technology. The microbiological parameters during control and experimental cheese productions were evaluated by plate count. The ewes' milk before pasteurization was characterized by a concentration of TMM higher than the limit of <500.000 CFU per mL fixed in Europe for raw ewes' milk [61]. Similar TMM levels are often detected in raw ewe's milk from Valle del Belíce breed [62,63]. High levels of TMM in raw milk might depend on the udder surface, the milking procedures, transport, and the storage condition

[64]. After pasteurization, the levels of TMM decreased by about 3 Log cycles, while LAB cocci and LAB rods by about 2 Log cycles. These results are not surprising, since a similar trend was previously observed for cow's milk by Gaglio et al. [65] and ewe's milk by Gaglio et al. [16] and Gaglio et al. [17]. These results might be ascribable to the initial cell densities of microorganisms in the raw milk [65], the temperatures applied [66] and the ability of the indigenous milk bacteria to survive during the pasteurization process [67]. *Salmonella* spp. and *L. monocytogenes* were never detected, while members of the Enterobacteriaceae family, coagulase- positive staphylococci (CPS) and *E. coli*, were detected at very low densities in raw milk and their levels were below the detection limit in pasteurized milk. These findings confirmed previous observations by Barbaccia et al. [25]. The analysis of milk after commercial starter culture addition, composed of defined strains of *S. thermophilus*, showed an increase in cell numbers for thermophilic coccus LAB that were present at levels higher than 7 Log CFU per mL. Settanni et al. [68] observed this behavior by analysing cows' milk inoculated with the same commercial starter culture used for the production of Caciocavallo-type cheese. After coagulation, an increase in the TMM and *S. thermophilus* levels was observed in both control and experimental curds due to whey draining as stated previously by Settanni et al. [63].

The microbial increase of 1 Log cycle for the levels of TMM and *S. thermophilus* in both control and experimental acidified curds followed the general trend commonly observed after curd acidification [69]. However, the levels of these microbial groups remained almost constant in control and experimental cheeses soon after production and after 15 days of refrigerated storage. This trend was previously reported by Gaglio et al. [17] for ovine-stretched cheese produced with GPP addition at pilot plant scale level and

selected individual *Lactococcus lactis* strains as the fermenting agents. The results of the microbial counts highlighted that the enrichment of curd with 1% (w w^{-1}) of GPP did not negatively influence the growth and survival of the commercial starter LAB used as a fermenting agent during ovine stretched cheese production.

RAPD analysis clearly showed the persistence of the added *S. thermophilus* strain, originating from commercial LAB starter culture, during control and experimental cheese productions [70]. A total of four different strains isolated from pasteurized milk were identified as *E. faecalis*, *Ln. mesenteroides* and *Ln. pseudomesenteroides* species. These species are part of the microbiota of ovine dairy products [71,72] and their presence in pasteurized milk was previously reported by Barbaccia et al. [25] and Gaglio et al. [17].

The physicochemical parameters (fat, moisture, salt, protein, saturated fatty acid, residual dry matter and colour) followed the same trends commonly reported for cheeses enriched with GPP [17,73].

The determination of phenolic content of the final cheeses confirmed the presence of all the polyphenols quantified in the GPP in the experimental cheese at a concentration of about 32 µg g^{-1} excluding their presence in control cheese.

Panellists evaluated control and experimental cheeses for their sensory traits and demonstrated several differences among the two cheeses. However, it is well known that the addition of GPP or other by-products exert a strong effect on the sensory traits of cheeses [12,21,74]. Experimental cheeses showed a higher complexity of odors (odor and aroma intensity). With regard to the overall assessment, GPP-enriched cheese was the most appreciated by the panellists, confirming that the addition of vegetable by-products to dairy products resulted in a favourable influence on their sensory traits [34,75] generating

innovative dairy products.

In conclusion, this study revealed that incorporation of GPP into ovine stretched cheese, at a concentration of 1% (w w^{-1}), did not affect the fermentation process carried out by commercial starter LAB. Moreover, this enrichment allowed to achieve an ovine cheese with specific physicochemical, functional and sensory characteristics. GPP-enriched cheese was characterized by higher protein content, higher polyphenols content and lower fat content. Thus, this dairy product appears appropriate to enable the balance of essential nutrients in the human body and also could be represent a promising resource in the novel foods market.

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Chapter VI

Effect of red wine soaking on the microbiological profile, total phenolic content and sensory aspects of an ovine pressed cheese

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Abstract

The aim of this study was to produce an ovine pressed cheese with high polyphenol content. To this purpose, fresh Primosale Pecorino cheese was soaked for seven days in Nero d'Avola wine (NDW) and was indicated as experimental cheese production (EP). The final cheese was characterized for its microbiological, chemical and sensory characteristics. Specifically, cheese making was performed with pasteurized ewes' milk and a commercial starter culture. Control cheese production (CP) did not include the soaking phase in NDW. Plate counts confirmed the dominance of the commercial starter until 10^9 CFU/g in both CP and EP cheeses. The soaking in NDW increased cheese total phenolic compounds (TPC) of 0.36 g GAE/kg. Sensory evaluation indicated that EP cheese was mostly appreciated by the panelists. This work indicated that soaking in NDW represents a promising strategy to produce a fresh ovine cheese with a high TPC content well appreciated by consumers.

Keywords: Nero d'Avola wine, commercial starter culture, ewe's milk, ovine cheese, total phenolic content.

Introduction

In recent years, the dairy sector is being more and more active in proposing novel products to enlarge milk derived product portfolio to encounter the increasing demand of consumers for novel healthy dairy foods with distinct organoleptic characteristics [1, 2]. Although dairy products are rich in proteins, minerals, vitamins and short chain fatty acids [3], they are basically poor in functional and bioactive compounds [4]. For

this reason, academic institutes and private industry research and development units are focusing on the use of natural resources in order to improve the organoleptic, hygienic and functional properties of cheeses [5, 6]. Hence some cheeses have been fortified with fruit and vegetables by-products [7] or covered by non-conventional products [8]. Among the last products, "Imbriago" cheese produced in north-eastern Italy is left under wine or grape pomace of different red and white cultivar for a while [9].

So far, this production process has only been applied to bovine and goat milk cheeses, e.g. "Ubriaco di Rabosa" cheese [8] and "Murcia al vino" cheese [10], respectively. Regarding ovine dairy products, Gaglio et al. [11] and Barbaccia et al. [12] performed studies on the enrichment of Pecorino "Primosale" cheeses with grape pomace powder (GPP) from fermented red *Vitis vinifera* cv. Nero d'Avola and evaluated their effect on nutritional, sensory and microbiological traits of the final cheeses, but no wine soaking as a step to enrich cheeses in phenolic compounds has been applied yet.

Pecorino "Primosale" is a traditional Sicilian fresh-pressed cheese made from ewes' milk [13], which is commonly consumed after a very short ripening time [14]. The purpose of the present research was to perform for the first time the production of "Primosale" cheese by soaking in Nero d'Avola wine (NDW) at pilot plant scale level using a commercial starter culture. The specific objectives of the present study were to: (i) monitor the commercial starter lactic acid bacteria (LAB) during cheese production; (ii) evaluate the total phenolic content of final cheeses; (iii) evaluate the sensory traits of the final cheeses.

Materials and methods

Raw material and starter culture

Red wine of Nero d'Avola cultivar was provided by the winery "Di Bella Vini S.r.l." located in San Giuseppe Jato (Palermo, Italy). The NDW was characterized by pH 3.43, total acidity 9.67 g/L (as tartaric acid), volatile acidity 0.27 g/L (as acetic acid), reducing sugars 0.25 g/L, ethanol 13.04% vol, glycerol 14.62 g/L, malic acid 0.02 g/L and lactic acid 1.99 g/L. Whole ewes' bulk milk used for cheese production came from sheeps of Valle del Belìce breed and was characterized as follows: pH 6.61, somatic cell count 4.01 Log, fat 6.15%, protein 5.51%, casein 4.11%, and lactose 4.36%. Commercial freeze-dried starter preparation LYOBAC-D NT included two defined strains of *Lactococcus lactis* as declared by the producer company Alce International s.r.l. (Quistello, Italy).

Cheese productions and sample collection

Cheese productions were performed under controlled conditions at a dairy pilot plant of the dairy factory "Biopek", located in Gibellina (Trapani, Italy). The production was performed applying "Primosale" type pressed cheese technology during February 2021. Twenty-five liters of pasteurized (72 °C for 15 s) whole ewe's milk was cooled at 38 °C and transferred into plastic vats previously sanitized with a PROMOX P900 solution (Leggiuno, Italy). The milk was inoculated with the commercial starter culture under slow agitation for 30 min and 7.5 mL of liquid rennet (Fromase® 220 TL, DSM Bright Science Brighter Living, Heerlen, Netherlands) was

added. After curdling, the coagulum was manually broken with a stainless-steel curd beater until reaching the dimension of rice grains. After whey draining, the curd was hand pressed into cylindrical, perforated plastic molds and subjected to cooking under hot deproteinized whey (75 °C) for 1 h. The cheeses were salted in saturated brine for 6 h, dried for 24 h at room temperature and ripened for 30 d at 13 °C and 80% of relative humidity. At the end of the process, two cheeses representing the experimental production (EP) were soaked for 7 d in NDW (Figure 1a) at 13–15 °C and then ripened for 20 d following the scheme reported by Innocente et al. [7]. All other cheeses represented control production (CP) (Figure 1b) and were ripened without wine immersion. Cheese productions were carried out in triplicate in three consecutive weeks. Samples of wine, raw milk, pasteurized milk, freeze-dried starter preparation, inoculated milk after addition of starter culture, curds, and final cheeses were collected for analyses.

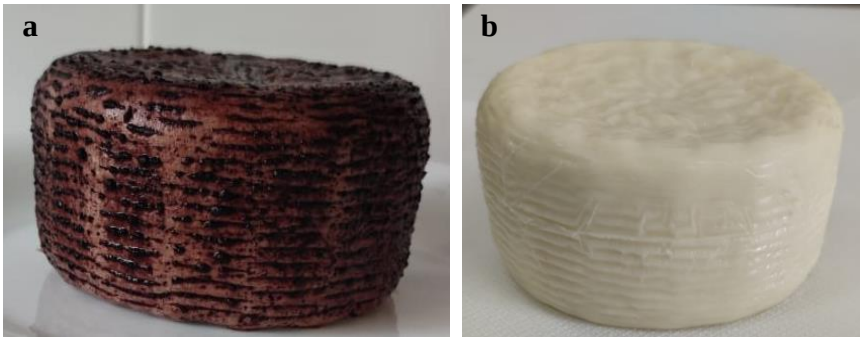

Figure 1. Final cheeses. a, experimental cheese; b, control cheese.

Microbiological analyses

Liquid (wine and milk) samples (1 mL) were directly serially diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy), while solid (freeze-dried starter preparation, curd and cheese) samples (10 g) were first homogenized in 90 mL of sodium citrate

[2% (w/v)] solution in the Bag-Mixer 400 stomacher (Interscience, Saint Nom, France) for 2 min at the maximum speed (blending power 4) and then serially diluted in Ringer's solution (Sigma-Aldrich).

Cell suspensions of wine, raw milk and pasteurized milk were subjected to plate count for the enumeration of protechnological, spoilage and pathogenic microorganisms following the approach of Cruciata et al. [15]. Cell suspensions of freeze-dried starter preparation, inoculated milk after addition of reactivated starter culture, curds and cheeses were analyzed for total mesophilic microorganisms (TMM) on plate count agar (PCA), incubated aerobically at 30 °C for 72 h and LAB exclusively on Media 17 (M17) agar, incubated aerobically for 48 h at 30 °C (16). PCA and M17 media were purchased from Biotec (Grosseto, Italy). Plates counts were performed in duplicate.

Total phenolic content

Total phenolic content (TPC) of wine and cheese samples was evaluated through the analyzer iCubio iMagic M9 (Shenzhen iCubio Biomedical Technology Co. Ltd. Shenzhen, China) as described by Barbaccia et al. [12]. The reagent Enzytec™ Polyphenols Cod. E2530 used for the determination was purchased from R-Biopharm AG (Darmstadt, Germany). The final concentrations of polyphenols were then reported as g of gallic acid equivalent (GAE)/L for wine or g GAE/ kg of product for cheeses. All measurements were performed in duplicate.

Sensory evaluation

Sensory evaluation of CP and EP cheeses was performed by a descriptive panel of 11 assessors (six women and five men aged between 23 and 62 years old). The judges were trained specifically following the ISO 8589 [17] indications and were asked to score 10 descriptors including intensity of odor and aroma, sweet, salt, bitter, acid, adhesiveness, hardness, humidity, and the overall assessment [8]. The assessors scored the level of each attribute with a mark on a 7-point hedonic scale (0 = extremely low; 7 = ex- tremely high).

Statistical analyses

Microbiological data were subjected to One-Way Variance Analysis (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft, New York, USA). The Duncan test was applied to evaluate the level of significance between control and experimental samples. $p < 0.05$ was considered significant.

Results

Microbiological evolution during cheese productions

The microbiological counts of NDW did not reveal the presence of any protechnological, spoilage and pathogenic microbial populations. The levels of the different microbial groups investigated in ewes' milk before and after pasteurization are reported in Figure 2. Statistical significant differences ($p < 0.0001$) were observed among raw and pasteurized milk for the levels of all microbial groups object of investigation. The results of *L. monocytogenes* and *Salmonella* spp. are not reported in Figure 2, because their levels were below the detection limit for both bulk milks

processed. Mesophilic coccus LAB were found at the same level (10^6 CFU/mL) of TMM, while mesophilic rod LAB were two Log cycles lower. After pasteurization, this microbial population was recorded at about 3 Log cycles lower. Enterococci were detected only in raw milk (2.25 Log CFU/mL). The levels of undesired microbial groups, such as members of Enterobacteriaceae family, CPS and *E. coli* in raw milk were in the range 10^2–10^3 CFU/mL and decreased below the detection limit in pasteurized milk.

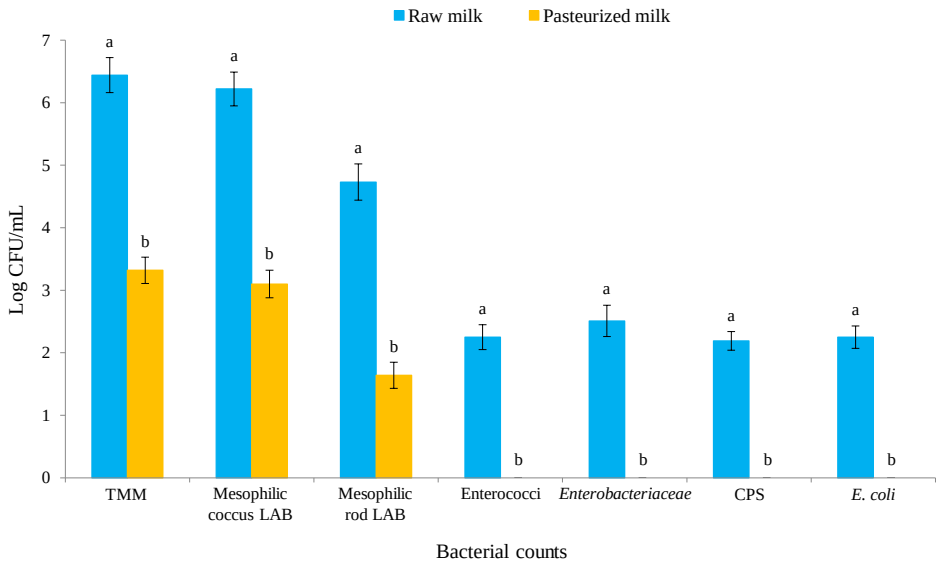

Figure 2. Microbial loads (Log CFU/mL) of raw and pasteurized milk samples. Abbreviations: TMM, total mesophilic microorganisms; CPS, coagulase-positive staphylococci; *E.*, *Escherichia*. Results indicate mean values and standard deviation of six determinations (carried out in duplicate for three independent productions). Different superscript letters indicate significant differences on microbial concentrations according to Duncan test between raw and pasteurized milk samples for $p < 0.05$.

The levels of TMM and mesophilic coccus LAB of all samples analysed are reported in Table 1. Commercial starter culture was dominated by mesophilic coccus LAB with levels of 10.64 Log CFU/g. Inoculated milk showed levels of mesophilic coccus LAB at about 10^7 CFU/mL. After curdling, TMM and mesophilic coccus LAB were counted at 10^8 CFU/g, while in the final CP and EP cheeses, their levels were above 9.0 Log CFU/g.

Table 1. Growth of commercial starter LAB during cheese production.

Samples	Bacterial counts	
	TMM	*L. lactis*
Dried starter culture	10.42 ± 0.31	10.64 ± 0.25
Inoculated milk	6.96 ± 0.24	7.15 ± 0.25
Curd	7.81 ± 0.21	8.01 ± 0.22
Cheese		
CP	9.21 ± 0.27[a]	9.04 ± 0.24[a]
EP	9.15 ± 0.29[a]	9.12 ± 0.21[a]
p value	0.806	0.686

Units are CFU/g. Results indicate mean values ± S.D. of six plate counts (carried out in duplicate for three independent productions). Data within a column followed by the same letter are not significantly different according to Duncan test. Abbreviations: TMM, total mesophilic microorganisms; CP, control production; EP, experimental production.

Total phenolic content

The results of TPC content are reported in Figure 3. NDW was characterized by a concentration of TPC of 2.3 g GAE/L. According to Duncan test, statistically significant differences ($p < 0.0001$) were found for TPC levels among cheeses. As expected, the amount of TPC detected in CP cheeses was lower than that registered for EP cheeses. In particular, the average TPC content of CP cheeses was 1.01 g GAE/kg while that of EP cheeses was 1.37 g GAE/kg.

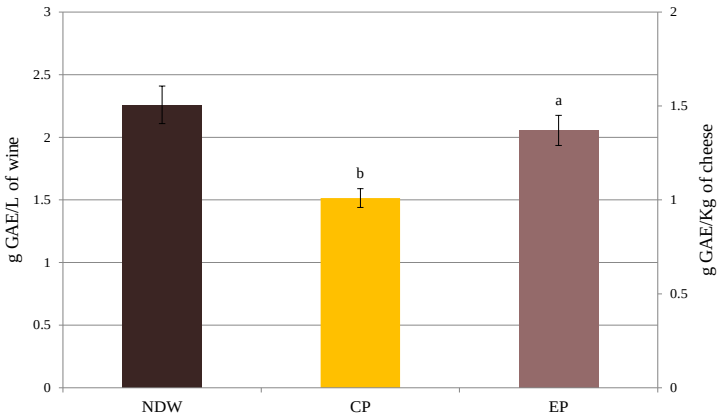

Figure 3. Total polyphenolic content (TPC) of NDW and cheese samples. Units are g GAE/L for NDW sample and g GAE/Kg for control and experimental cheese samples. Abbreviations: NDW, Nero d'Avola wine; CP, control production; EP, experimental production. Results indicate mean values and standard deviation of six determinations (carried out in duplicate for three independent productions).

Different superscript letters indicate significant differences on TPC according to Duncan test between control and experimental cheese samples for $p < 0.05$.

Sensory test

The results of the sensory evaluation performed by the panelists on CP and EP cheeses are reported in Figure 4. The spider graph clearly shows that the soaking step in NDW led to the production of Primosale cheeses characterized by a sensory profile particularly different from that generally recognized for traditional Primosale cheeses. Except for salt and bitter that were scored similarly, all other sensory traits evaluated were influenced by the soaking step. In particular, NDW soaking increased odor and aroma intensity, acid perception, adhesiveness and hardness, but influenced negatively sweet and humidity. The overall assessment clearly indicated that the cheeses soaked in NDW were particularly appreciated by the judges and reached a score higher than that registered for traditional Primosale cheese.

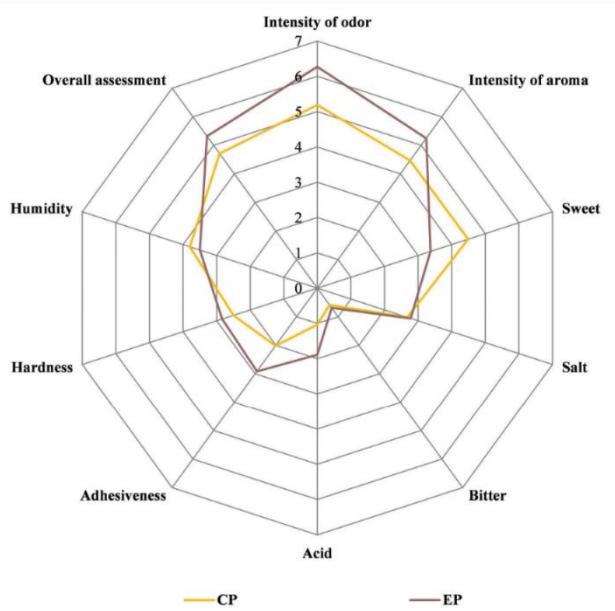

Figure 4. Spider diagrams corresponding to the descriptive sensory analysis of cheeses. Abbreviations: CP, control production; EP, experimental production.

Discussion

This study was carried out to evaluate the effect of wine soaking using red Nero d'Avola cultivar on the microbiological characteristics, total phenolic content and sensory aspects of the ovine pressed Primosale cheese. The experimental production was carried with a pilot plant using a commercial starter culture composed of lactococci as LAB. The wine used for cheese immersion did not evidence the presence of any of the microbial groups investigated confirming that the filtration process used before bottling was effective in removing microorganisms [18]. Ewes' milk before pasteurization hosted levels of TMM above the European limit of <500.000 CFU/mL for raw ewes' milk [9]. High levels of TMM are often detected in raw ewe's milk from Valle del Belìce breed [20, 21] and this is imputable to the microbial contamination of the udder surface, that occurring during the milking procedures or during transport or to the growth of indigenous microorganisms of milk thanks to the thermal conditions applied during storage [22]. After pasteurization, the levels of TMM, mesophilic coccus and rod LAB, decreased by about 3 log cycles, showing the ability of the indigenous milk bacteria to survive during the pasteurization process [23, 24]. A similar trend was previously observed for ewe's milk by Barbaccia et al. [12] and Gaglio et al. [11, 25]. The presence of the pathogenic bacteria *L. monocytogenes* and *Salmonella* spp. was never revealed while members of Enterobacteriaceae family, coagulase-positive staphylococci (CPS) and *E. coli*, were detected at very low cell densities in raw milk and their levels were below the detection limit in pasteurized milk. The same trend was previously observed by Barbaccia et al. [12].

The analysis of milk after commercial starter culture addition, composed of defined strains of *L. lactis*, showed levels of mesophilic coccus LAB above 7 Log CFU/mL. Barbaccia et al. [12] observed this behavior in ewes' milk inoculated with the same commercial starter culture used for the production of ovine pressed cheeses. After coagulation, an increase of the TMM and *L. lactis* levels was observed in curd due to whey draining as previously reported by Settanni et al. [20]. However, the levels of these microbial groups reached values above 9 Log CFU/g in CP and EP cheeses highlighting that the soaking phase in NDW did not alter the microbiological parameters of the final cheeses. A similar trend was previously reported by Gaglio et al. [11] for ovine pressed cheeses produced with GPP addition at pilot plant scale level and selected individual *L. lactis* strains used as fermenting agents in place of the commercial starter preparation. The amount of TPC detected in NDW in our study was 2.3 g GAE/L comparable with the values reported by Gervasi et al. [26] for the same wine typology. Regarding final cheeses, our results confirmed that the soaking phase in NDW determined an increase of 0.36 g GAE/kg of TPC in EP cheeses. Barbaccia et al. [12] reported similar findings with GPP addition.

The panelists evaluated all cheeses for their sensory attributes and evidenced several differences among CP and EP cheeses. Experimental cheeses showed a higher complexity of odor and aroma intensity. Similar results were observed by Gaglio et al. [11] and Barbaccia et al. [12] who tested red wine grape pomace of NDW to fortify ovine Primosale cheese. With regard to the overall assessment, the cheeses soaked in NDW were the most appreciated ones by the panelists.

Conclusions

This study provided, for the first time, an analysis of the microbiological, chemical and sensory characteristics of an ovine pressed cheese soaked in NDW. The results revealed that the soaking phase did not affect the microbiological parameters of the final cheeses and resulted in a general appreciation by the panelists. Moreover, soaking phase enriched Primosale cheese with TPC, increasing its antioxidant properties. Further studies are being prepared to better evaluate the real beneficial effect on human health through the determination of the antioxidant bioavailability of these dairy products.

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Part II

Development of ewe's yogurt

In this section, ewe's yogurt was produced and characterized for several aspects. FAO (1984) defined yogurt as "the coagulated milk product obtained by lactic acid fermentation through the action of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* from milk and milk products. The microrganisms in the final product must be viable and abundant". According to the *Codex Alimentarius*, yogurt should meet specific criteria: a minimum of 2.7% (m/m) milk proteins; a maximum of 15% milk fat; a minimum of 0.6% titratable acidity (expressed as % of lactic acid); and a minimum of 10^7 CFU/g of microorganisms (total microorganisms in the starter culture).

Yogurt consumption is associated with positive health patterns, improved diet quality, and favorable metabolic profiles. While yogurt preferences vary globally, it is increasingly recognized as a marker of a wholesome diet. Notably, it is more prevalent among healthier, leaner, well-educated individuals with higher socio-economic status, as well as being favoured by women. Today's yogurt market offers a diverse array of products, including low-fat yogurt, probiotic yogurt, yogurt mousse, frozen yogurt, drinking yogurt, and high-protein yogurt. Alongside traditional white yogurts, fruit-flavored yogurts are gaining popularity. The inclusion of fruit preparations, flavorings, purees, and extracts is perceived as healthy by consumers. These additions not only enhance color, but also contribute natural flavors, antioxidants, and dietary fibre.

This research journey began with an initial study focused on developing ewe's milk yogurt using carefully selected LAB. This new product underwent evaluation in terms of microbiological, physicochemical properties, and sensory attributes. Subsequently, a second study explored fortifying the same ewe's yoghurt with five different fruit purees.

Chapter VII

A thorough investigation of the microbiological, physicochemical, and sensory properties of ewe's yogurt fermented by a selected multi-strain starter culture

The present chapter has been published in

Foods

12, 3454

2023

Abstract

This work was carried out with the aim to investigate the microbiological, physicochemical, and sensory properties of an innovative yogurt produced from ewe's milk. Experimental yogurt productions were performed with a commercial freeze-dried starter preparation and a natural milk starter culture (NMSC) of *Streptococcus thermophilus* and *Lactobacillus delbrueckii*. The two yogurts did not differ for color parameters, showing an average value of lightness, redness, and yellowness of 94.99, –3.74, and 9.37, respectively. The yogurt produced using the NMSC as a fermenting agent was characterised by a significantly lower fat percentage and a higher antioxidant potential than commercial starters. Microbiological analysis confirmed the safety of the final product and a level of living lactic acid bacteria of 10^8 CFU/g. Sensory analysis revealed some differences among yogurts regarding unpleasant odor, homogeneity, and persistence in the mouth, but the yogurt processed with NMSC was more appreciated. Thus, the production of ewe's yogurt fermented by a selected multi-strain starter culture represents an interesting strategy to enlarge the functional ovine dairy product portfolio.

Keywords: ewe's yogurt, milk starter culture, lactic acid bacteria, antioxidant capacity, sensory traits.

Introduction

Yogurt is the most popular fermented milk product [1], and its unique properties are attributed to the symbiotic fermentation of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [2,3]. The nutritional value of this fermented milk product

determines its worldwide consumption [4]. Indeed, yogurt consumption can help people meet their nutritional needs for various nutrients, such as calcium, potassium, phosphorus, and vitamins B2 and B12 [5]. For this reason, yogurt is generally known to contribute to the overall quality of the diet [6] and provide health benefits [4]. In some Asian countries, yogurt is even used in folk medicine [7]. This product can also lower the risk of cardiovascular diseases and support bone health, especially for the elderly [8,9]. Moreover, yogurt-fermenting bacteria express functional lactase, helping the digestibility of yogurt compared to milk in intolerant subjects [10]. More than twenty years ago, Kailasapathy et al. [11] provided the first evidence of the positive impact of yogurt consumption on the gut microbiota, which is associated with a reduced risk of gastrointestinal disease. Thus, the FAO/WHO recommended daily yogurt intake due to its rich nutritional profile and its therapeutic and general benefits on the human body [12].

Yogurt is usually made from cow's milk. However, in several European and non-European countries, the milk from other species, such as sheep or goats, is processed into yogurt [2,13]. In countries where climatic and soil conditions are not favourable for cow farming, the use of different types of milk would be a suitable alternative to cow's milk to produce yogurt. These conditions characterise Sicily, a southern Italian island region where sheep breeding is more common than cattle breeding (https://www.istat.it/, accessed on 27 July 2023). Ewe's milk has several beneficial attributes over cow's milk, providing higher levels of proteins, lipids, minerals, and vitamins essential for human health [14]. Several studies suggested that high levels of protein and fat positively affect the production of fermented milk products [15–17], contributing to increased firmness, cohesiveness, and viscosity of the yogurt [18]. In particular, the viscosity of yogurt may be useful in

preserving probiotic cultures throughout the gastrointestinal tract. They offer added protection to probiotics in the stomach [19], as well as during the commercial storage period [20]. Furthermore, ewe's milk yogurt exhibits desirable flavor properties, such as the creamy-sour attribute appreciated by many consumers [21].

This work was carried out with the aim to develop an ewe's milk yogurt adapted to cow's milk yogurt technology. The goal was to expand the dairy Sicilian portfolio by targeting modern consumers who are increasingly interested in functional foods with higher nutritional values and health benefits. A natural milk starter culture (NMSC) was created using selected strains of *S. thermophilus* and *L. delbrueckii* isolated from typical Sicilian ewe's dairy products. The use of NMSC is considered important to better link the novel product with the Sicilian area and to avoid the taste homologation phenomena determined by commercial starter cultures [22]. The resulting yogurts were analysed for hygienic safety, physicochemical traits, and antioxidant capacity. Moreover, the yogurts were also assessed for their sensory properties to determine how appealing the product is to consumers.

Materials and Methods

Raw materials and starter cultures

Yogurt was prepared using bulk milk of the "Valle del Belíce" breed, an indigenous Sicilian sheep species. Raw milk was obtained from several dairy farms located in Agrigento province (Cammarata, Italy) and was then transformed at "Cooperativa Agricola Tumarrano" (Cammarata, Italy). The experimental plan of this study consisted of two different ewe's milk yogurt productions by applying the technology typically followed in

the production of cow's yogurt. The first production (EY-1) was performed using the commercial freeze-dried starter culture YODX091 (ALCE s.r.l., Quistello, MN, Italy), composed of *S. thermophilus* and *L. delbrueckii* spp. *bulgaricus* (one strain each), while a second production (EY-2) was carried out with the addition of a multi-strain NMSC developed in this study.

Preparation of natural milk starter culture

The NMSC used in this study included the following strains: *S. thermophilus* (PON244) and *L. delbrueckii* (WT601). These strains, selected for their high dairy performances such as acidification capacity, autolysis kinetics, diacetyl production, and antibacterial activity belonged to the collection of the laboratory of agricultural microbiology at the University of Palermo and were previously isolated from traditional PDO Sicilian cheeses [23,24]. Briefly, both strains were refreshed in their optimal growth media. *S. thermophilus* PON244 was reactivated in M17 medium [25], while *L. delbrueckii* WT601 was reactivated in MRS medium [26]. Both strains were incubated at 40 °C for 48 h. After growth, strains were subjected to two consecutive washes in Ringer's solution (Sigma-Aldrich, Milan, Italy). Each washing step was followed by centrifugation at 6000 g for 2 min using a Heraeus Biofuge Pico (Kendro Lab, Waltham, MA, USA) in order to remove any residue of growth medium. The washed cells were inoculated at 10^6 colony-forming units (CFU)/mL into ovine whole-fat UHT milk (Leeb Vital, Wartberg an der Krems, Austria) and incubated at 40 °C for 24 h. NMSC containing both *S. thermophilus* and *L. delbrueckii* at approximately 10^9 CFU/mL, as verified by plate count, was used for yogurt production.

Yogurt production and sample collection

Yogurts were prepared according to the production protocol indicated by Gaglio et al. [27]. Briefly, a volume of 100 L of raw milk was pasteurized at 72 °C for 15 s in a stainless-steel Comat PS 20351 pasteurizer (Bellizzi, SA, Italy) and cooled at 42 °C. The entire bulk milk was then transferred into an automatic Due Ci Inox s.r.l. yogurt maker (Guastalla, RE, Italy). After cooling, starter cultures were directly inoculated, and the milk was stirred for 10 min. Fermentation took place at 38 °C for 6 h and was stopped at pH 4.5 [28]. Finally, the yogurt was packaged into 120 mL plastic pots with an air-tight cap (FD Store s.r.l., Vignola, Italy) and stored at 4 °C for 3 d before distribution. Figure 1 provides an overview of the production protocol followed.

Both trials (EY-1 and EY-2) were repeated after two weeks (two independent replicates). Raw milk (RM), pasteurised milk (PM), inoculated milk (IM), and yogurt (Y) after 3 d of refrigerated (4 °C) storage were collected for analyses.

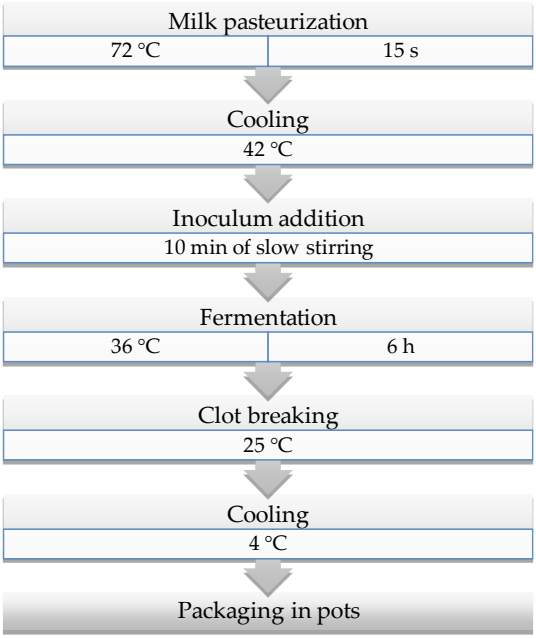

Figure 1. Flow chart of ewe's yogurt.

Microbiological analysis

All samples collected before and after yogurt production were microbiologically evaluated to enumerate total mesophilic microorganisms (TMM) by spreading on plate count agar (PCA), incubated aerobically at 30 °C for 72 h, and thermophilic rod and coccus-shaped lactic acid bacteria (LAB) after pouring on de Man-Rogosa-Sharpe (MRS) agar acidified to pH 5.4 with lactic acid (5 mol/L) and M17 agar, respectively, incubated anaerobically in anaerobic jars (AnaeroGen, Thermo Fisher Scientific, Waltham, MA, USA) at 42 °C for 48 h. Fungal growth was inhibited by adding chloramphenicol (1 mg/L) to both media. Raw and pasteurized milk samples were also analysed for the presence of the main four food-borne pathogens in compliance with Commission Regulation (EC) No 2073/2005 [29] on microbiological criteria for foodstuffs: *Escherichia coli* and coagulase-positive staphylococci (CPS) were analysed as process hygiene criteria applying ISO 16649- 1 [30] and ISO 6888-2 [31], respectively; *Salmonella* spp. and *Listeria monocytogenes* were analysed as food safety criteria by spreading on Hektoen enteric agar (HEA) and on *Listeria* selective agar base (LSAB) added with SR0140E supplement, respectively, both incubated at 37 °C for 24 h. All media and supplements were purchased from Oxoid.

The plate count method was applied after serial decimal dilutions. Aliquots of 1 mL of liquid samples (RM, PM, and IM) were diluted with 9 mL of sterile 0.9% (v/v) Ringer's solution, while 15 g of yogurt just after production and after 24 h of refrigerated storage were first homogenised in 135 mL of sodium citrate (2% v/v) solution in a stomacher (Bag Mixer 400; Interscience, Saint Nom, France) for 2 min at the highest speed and then serially diluted as described for liquid samples. Bacterial enumerations were carried out in

duplicate (technical repeats) for each trial. The results were expressed as CFU per mL (liquid samples) or gram (solid samples).

Physicochemical analysis

Milk samples before processing were analysed for pH, lactose, fat, protein, and urea by the infrared method (Combi-Foss 6000, Foss Electric, Hiller'd, Denmark). The color of milk and yogurts was evaluated in duplicate using a Minolta Chroma Meter CR-300 (Minolta, Osaka, Japan), which measures values of lightness (L* = 0/100, from black to white), redness (a* = -a/+a, from green to red) and yellowness (b* = -b/+b, blue to yellow), according to the CIE L*a*b* system [32]. The values of L*, a*, and b* were used for the calculation of chroma $[(a^{*2} + b^{*2})^{0.5}]$, measuring the color intensity or saturation, hue angle $[\tan^{-1}(b^*/a^*)]$, as a measure of color tone, and the whiteness index $[100 - ((100 - L^*)^2 + a^{*2} + b^*2)0.5]$, according to Vargas et al. [33]. Total color change (ΔE) after yogurt production was calculated as $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5}$, where ΔL*, Δa*, and Δb* are the differences of L*, a*, and b*, respectively, between milk and yogurt.

All yogurt samples were freeze-dried and analysed for centesimal chemical composition [dry matter (DM), protein (N 6.38), fat, and ash content] in accordance with FIL-IDF standards [34–36].

Oxidation products and antioxidant capacity

The extracts of lyophilised samples were prepared following the protocol described by Rashidinejad et al. [37] with minor modifications. Briefly, each lyophilised yogurt sample (0.5 g) was dissolved in a 95% aqueous methanol solution (25 mL) with 1% (v/v) HCl. The

suspension was mixed by vortex for about 30 s and then held at 40 °C in an ultrasonic water bath (LBS1 Sonicator; Falc Instruments, Treviglio, Italy) for 30 min, during which it was mixed by vortex for 5–10 s every 10 min. The resulting suspension was cooled, filtered with linen cloth, centrifuged at 7000 g at 9 °C for 10 min, and finally kept at −18 °C until analysis. Extracted samples were analysed in duplicate for antioxidant properties, measuring the antioxidant capacity and the indexes of primary and secondary lipid oxidation.

Total antioxidant capacity in extracted yogurt samples was assessed by the Trolox equivalent antioxidant capacity (TEAC) assay, according to a published protocol [37], as modified by Bonanno et al. [38]. TEAC is a discoloration assay aimed at evaluating the radical scavenging ability of the samples with the use of the [2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)] (ABTS) radical cation (ABTS•+) and Trolox as standards [39]. To obtain the ABTS radical cation, equal volumes of a 14 mM ABTS aqueous solution and 4.9 mM $K_2S_2O_8$ were mixed and placed in the dark for 16 h at room temperature. ABTS radical cation solution was diluted in 5 mM PBS solution (phosphate buffered saline, pH 7.40) until it reached an absorbance of 0.795 (±0.020) at 734 nm using a Hach DR/4000 U spectrophotometer (Hach Company, Ames, Iowa, USA). The absorbance reading of a mixture of 150 µL of PBS with 2850 µL of a diluted ABTS radical cation solution was recorded at 734 nm immediately (as white sample at 0 min) and after incubation at 30 °C for 6 min (as white sample at 6 min). Similarly, the absorbance of the 150 µL solution of each extracted sample mixed with 2850 µL of the dilute ABTS radical cation solution was read at 734 nm after 6 min of incubation at 30 °C. The absorbance values were used to determine the percentage decrease of the absorbance due to

decolorization, calculated by comparison with the absorbance obtained with PBS. Solutions of Trolox in PBS (0–2.5 mM) were used to construct the calibration curve (R^2 = 0.99), and the results are expressed in mmol Trolox/kg DM.

The oxidation of yogurt fat was estimated by determining in duplicate the peroxide value (POV, mEq O_2/kg fat), expressing the primary lipid oxidation [40], and the thiobarbituric acid reactive substances (TBARS, µg malonylaldehyde (MDA)/kg DM) as secondary lipid oxidation products, in line with the method described by Tarladgis et al. [41] and modified by Mele et al. [42]. Briefly, TBARS analysis was conducted on 2 g of lyophilised yogurt, which was mixed with an 8 mL aqueous solution of phosphate buffer at pH 7 by vortex. After that, 2 mL of a 30% (v/v) aqueous solution of trichloroacetic acid was added, and the resulting solution was vortexed for about 5 s and filtered with Whatman No. 1 filter paper. Five millilitres of the filtered solution were mixed with an equal volume of 0.02 M thiobarbituric acid aqueous solution, placed in a hot water bath at 90 °C for 20 min, and then refrigerated. After centrifugation at 4500 x g for 5 min, the absorbance of the supernatant at 530 nm was read spectrophotometrically. Solutions of 1,1,3,3-tetramethoxypropane at concentrations between 0.016 and 0.165 µg/mL were read to construct the calibration curve (R^2 = 0.99).

Sensory Evaluation

EY-1 and EY-2 yogurts were sensory evaluated by a panel of 19 judges composed of 10 males and 9 females aged between 25 and 62 years old. The evaluation of odors, tastes, and appearance was performed according to a list of eleven attributes: odor intensity and unpleasant odors (odor perception); sweet, acid, bitter, persistence, and off flavor (taste

sensation); and color, homogeneity, spontaneous syneresis, and viscosity (appearance and texture). The judges were also asked to give an overall evaluation in terms of satisfaction with the final product, considering the scores of all attributes [20,43,44]. To this purpose, the members of the descriptive panel used a sensory 9-rating scale as reported by Gaglio et al. [27], where 0 corresponds to the lowest score of the character and 9 to the highest value. Sensory assessment was performed in individual temperature-controlled cabs (20 °C). A commercial white cow's yogurt (CCY) (Muller, Fischach, Germania) purchased at a retail market was used as a control. Each yogurt (35 mL) was served at 7 °C using plastic cups coded with an alphanumeric random code. Water was used for rinsing between samples that were randomly presented as described by Akalın et al. [45].

Statistical Analyses

The data were analysed using XLStat software version 2020.3.1 for Excel software (Addinsoft, New York, NY, USA). A statistical analysis of bacterial counts was performed after logarithmic conversion of the data. Results are given as means standard deviation (SD). Differences between the means were determined by Tukey's multiple range post hoc test. p values of < 0.05 were deemed to be significant.

The effect of starter culture on the physicochemical traits of yogurt was evaluated statistically using the generalised linear model (GLM) procedure in SAS 9.2 software (SAS Institute Inc., Campus Drive, Cary, NC, USA).

Results and Discussion

Microbiological monitoring

The results of microbiological analyses of raw milk samples from EY-1 and EY-2 productions are shown in Figure 2.

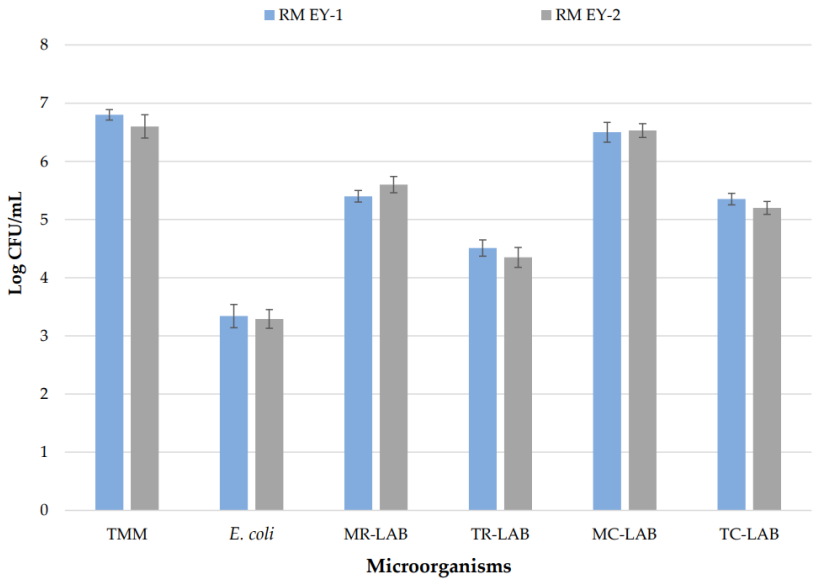

Figure 2. Microbiological loads of milk samples before pasteurization. Results indicate mean values and SD of four determinations (carried out in duplicate for two independent productions). Abbreviations: EY-1, production performed with freeze-dried commercial starter culture (starter formulation YODOX91); EY-2, production performed with a multi-strain milk starter culture consisting of *S. thermophilus* and *L. delbrueckii*; RM, raw milk; TMM, total mesophilic microorganisms; *E.*, *Escherichia*; LAB, lactic acid bacteria; MR, mesophilic rod; TR, thermophilic rod; MC, mesophilic cocci; TC, thermophilic cocci.

In both productions, RM samples show no statistically significant differences ($p > 0.05$) for the microbial groups investigated. These samples show levels of TMM at about 10^7 Log CFU/mL in EY-1 and EY-2. Similar levels were registered by Gaglio and co-workers [27], who analysed raw cow's milk before transformation into yogurt, while higher TMM levels have been detected in raw cow's milk in other studies [46,47]. The high TMM cell densities found in raw milk are not surprising since this food matrix, thanks to its high concentrations and variety of nutrients and its neutral pH, represents the ideal growth medium for a wide variety of microorganisms [48]. Raw milk microbiota is very

heterogeneous [49] and can be made up of pathogenic and spoilage microorganisms as well as bacteria of technological importance [50,51]. Regarding the specific search for undesired bacteria, plate counts did not detect *L. monocytogenes* or *Salmonella* spp. To date, the regulation (EC) No 2073/2005, which includes microbiological parameters [29], indicates the complete absence of these microorganisms for the human consumption of milk. Indeed, none of the samples show the presence of CPS.

The absence of staphylococci, potential contaminants in raw milk samples, is evidence of good hygiene practices applied during milking, conveyance, and storage [52], because bacterial cross-contamination of milk can arise as a result of many factors [53]. Nevertheless, plate counts revealed the presence of *E. coli* at levels of 3.34 and 3.29 CFU/mL in EY-1 and EY-2 production, respectively. Similar results were found by Caro et al. [54] on samples of raw ewe's milk used to make "Castellano" cheese. *E. coli* represents an indicator of faecal contamination [55], and such contamination during the milking process is quite common [56–58]. For this reason, a sanitization treatment becomes mandatory if the final product has a short maturation period (<2 months) [59].

LAB were dominant in raw milk samples; levels of mesophilic rod and cocci LABs were almost superimposable to those of TMM (log 5.4 and 6.5 CFU/mL, respectively, in EY-1; Log 5.6 and 6.53 CFU/mL, respectively, in EY-2). Similar results were previously registered by Samelis et al. [60] for raw milk used for Greek hard cheese production.

Thermophilic LAB were detected at levels lower than mesophilic LAB since log 10^5 CFU/mL were recorded for thermophilic cocci. Thermophilic rods were even lower (Log 10^4 CFU/mL) in both productions. Garofalo et al. [61] registered a similar trend for raw ewe's bulk milk collected in a different area (Palermo province) of Sicily. The microbial

ecology of raw milk consists of a complex interaction among indigenous LAB, which play different roles during cheese-making [62], and they are generally high in number [63].

Inoculated starter cultures drove the fermentation process during yogurt manufacture. Therefore, microbiological analysis included the detection of thermophilic LAB cocci and rods representing the starter cultures inoculated. Figure 3 shows the results of the microbial growth from pasteurized milk to final yogurts.

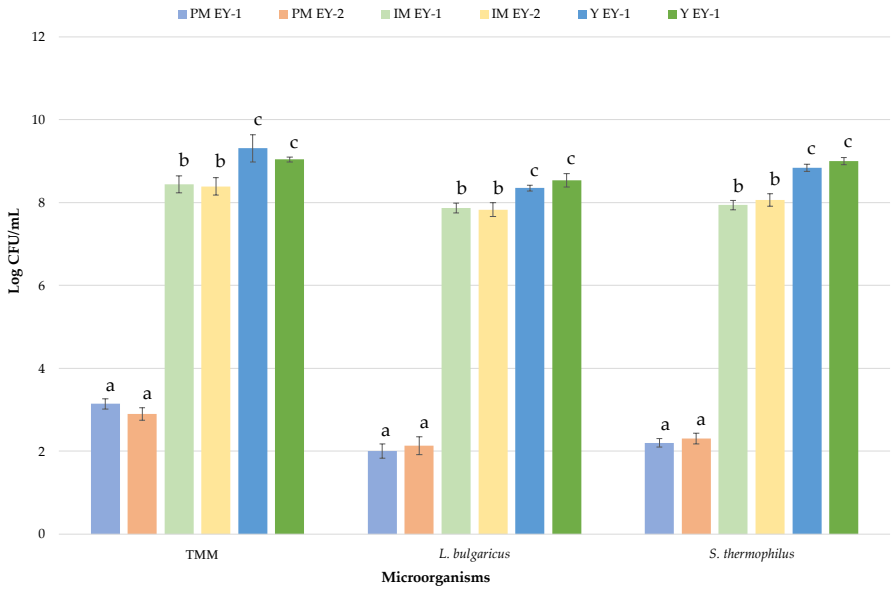

Figure 3. Growth of starter cultures during yogurt production. Units are log CFU/mL for liquid samples and log CFU/g for solid samples. Results indicate mean values ±SD of four plate counts (carried out in duplicate for two independent productions). a, b, c = $p < 0.05$. Abbreviations: TMM, total mesophilic microorganisms; *S.*, *Streptococcus*; *L.*, *Lactobacillus*; PM, pasteurized milk; IM, inoculated milk; Y, yogurt; EY-1, production of ewe's yogurt performed with freeze-dried commercial starter culture (starter formulation YODOX91); EY-2, production of ewe's yogurt performed with a multi-strain milk starter culture consisting of *S. thermophilus* and *L. delbrueckii*.

After pasteurization, the levels of TMM in milk were recorded at approximately three log cycles in comparison to RM samples. Pasteurization treatment is necessary to guarantee the hygiene and security of the final products [64]. Figure 3 clearly highlights a decrease of approximately three log cycles for TMM in PM samples. The main aim of thermal treatment (72 °C for 15 s) is to sanitise raw milk from any potential trace

contaminants it may have. A sudden and consistent drop was also registered for the levels of LAB and *E. coli*. In particular, *E. coli* was undetectable after the application of the thermal treatment. Although pasteurization causes a drastic reduction in microbial levels, this treatment does not determine the complete elimination of the indigenous microbiota [65]. This treatment leads to a reduction of at least 90% of the bacterial population, and usually only thermoduric bacteria survive [66].

Immediately after starter addition, the levels of *S. thermophilus* and *L. delbrueckii* in the yogurt were approximately Log 10^8 CFU/g in both productions. It is widely known that starter microorganisms should be added at levels of 10^7 CFU/g of viable bacteria since they have the primary task to produce lactic acid from lactose [67]. *S. thermophilus* was observed at slightly higher levels than *L. delbrueckii* in IM of both productions, with no statistically significant differences ($p > 0.05$). These results are in line with those argued in the study of Güler-Akin (2005) [68]. After fermentation, the final yogurts show count loads one Log cycle higher than inoculums (*S. thermophilus* and *L. delbrueckii* were detected at 8.84 and 8.35 CFU/g, respectively, in EY-1 and 9.00 and 8.54 in EY-2). Similar results can be found in the work of Shazly et al. [69], who produced probiotic yogurt from ewe's milk. Birollo et al. [70] stated that the high levels of viable LAB in fermented milks are correlated with consumers' health benefits.

Physicochemical traits of ewe's yogurt

Chemical composition and color parameters of milk used for the production of yogurts (Table 1) show some fluctuations, especially for protein, casein, urea, and yellowness, that can be attributed to the different feeding systems of the origin farms of milk [71].

However, milk parameters fully reflect those characterizing sheep milk from Valle del Belìce sheep [72].

Table 1. Composition of milk used for yogurt production.

Milk	First Yogurt Production	Second Yogurt Production
pH	6.07	6.31
Lactose, %	4.33	4.16
Fat, %	6.80	6.23
Protein, %	5.43	4.43
Casein, %	4.04	3.12
Urea, mg/dL	57.00	67.40
Colour		
Lightness L*	91.94	86.35
Redness a*	−3.63	−3.44
Yellowness b*	5.64	3.60
Chroma	6.70	4.97
Hue angle	−57.21	−46.30
Whiteness index	89.52	85.47

Regarding the physicochemical traits of the yogurts produced with the different types of starters (Table 2), the differences in protein content can be attributed to the different starting milk composition since the protein percentage changes from milk to yogurt occurred at similar levels (about +20%) for both production lines.

Compared to milk fat, yogurt fat shows a greater reduction with the use of NMSC than commercial starter, suggesting an effect of more intense bacterial activity in degrading fatty acids as an energy source [27].

None of the color parameters of yogurts (Table 2) were significantly affected by the starter culture, but the use of NMSC induced a significant increase in the total color change. This result can be explained by the larger change from milk to yogurt observed in the EY-2 yogurt than in the EY-1 yogurt for lightness and yellow index, even if these differences between yogurts did not emerge at a statistically significant level; these results

can be imputable to the microbial activity carried out by the bacteria constituting the selected starter culture.

Table 2. Physicochemical traits of ewe's yogurt.

	Samples		SEM	*p* value
	EY-1	EY-2		
Dry matter (DM), %	20.30	17.09	0.594	0.0620
Ash, % DM	3.77	4.58	0.149	0.0618
Protein, % DM	35.47	33.04	0.147	0.0072
Fat, % DM	35.07	34.62	0.051	0.0252
Protein change, %	20.27	20.73	0.536	0.6017
Fat change, %	−5.05	−10.03	0.139	0.0016
Color				
Lightness L*	95.13	94.85	0.521	0.7405
Redness a*	−3.89	−3.59	0.154	0.2964
Yellowness b*	10.25	8.49	0.596	0.1725
Total color change	5.65	9.84	0.397	0.0175
Chroma	10.96	9.22	0.607	0.1792
Hue angle	−69.21	−67.03	0.626	0.1332
Whiteness index	87.99	89.43	0.578	

Abbreviations: EY-1, production performed with freeze-dried commercial starter culture (starter formulation YODOX91); EY-2, production performed with a multi-strain milk starter culture consisting of *S. thermophilus* and *L. delbrueckii*. SEM, standard error of the mean.

Oxidation and antioxidant activity of ewe's yogurt

The antioxidant capacity is defined as the action of all the antioxidant molecules present in the food matrix [73]. Authors such as Jaster et al. [74] and Guz et al. [75] describe yogurt as a potential carrier of antioxidant compounds derived from its enrichment with a matrix containing bioactive molecules such as polyphenols. However, milk and its derivatives constitute a complex mixture of enzymatic systems, proteins, minerals, and vitamins, and all these substances together confer antioxidant capacity [76]. The antioxidant characteristics of milk and dairy products were extensively studied. Thus, studies on cow's milk and cow's yogurt have shown that the fermented version of milk products has a higher antioxidant capacity than milk [77]; this result can be linked to the

antioxidant properties of peptides produced from milk protein by the enzymatic activity of inoculated microorganisms [78]. In this regard, there is no study on non-fortified yogurt made from ewe's milk. The present study demonstrates that the application of selected bacteria can improve the antioxidant properties of ewe's yogurt, contributing to enhanced health benefits for consumers [79].

The antioxidant activity and the primary and secondary lipid oxidation values of the yogurts processed in this study are reported in Table 3.

Table 3. Oxidation products and antioxidant capacity of ewe's yogurt.

Samples	TEAC, mmol/kg DM	POV, mEq O2/kg fat	TBARS, mg MDA/kg DM
IM EY-1	0.63	0.16	0.092
IM EY-2	15.41	0.43	0.074
SEM	0.263	0.011	0.001
p-Value	0.0006	0.0036	0.0087

Abbreviations: EY-1, production performed with freeze-dried commercial starter culture (starter formulation YODOX91); EY-2, production performed with a multi-strain milk starter culture consisting of *S. thermophilus* and *L. delbrueckii*; TEAC, Trolox equivalent antioxidant capacity; POV, peroxide value; TBARS, thiobarbituric acid reactive substances; MDA, malonylaldehyde; SEM, standard error of mean.

The antioxidant activity of yogurts was evaluated by the TEAC assay in order to verify the effects of the use of selected strains. A better antioxidant capacity emerged in the EY-2 yogurt (15.41 mmol TE/kg DM) produced with the selected starters *S. thermophilus* PON244 and *L. delbrueckii* WT601; these microorganisms were able to exert a more intense proteolytic activity and release peptides with antioxidant properties. In addition, the antioxidant capacity could be related to several enzymatic and non-enzymatic compounds produced by lactobacilli; these enzymes are able to minimise reactive oxygen species generation and control the transition metal ions preventing the oxidation reaction [76,78,80]. Future studies on sheep yogurt should evaluate its antioxidant capacity during storage, since some studies have stated that this property decreases over time [81].

Moreover, since the antioxidant properties of this type of dairy product can derive from different events, more antioxidant assays are opportune.

Lipid oxidation was evaluated by POV and TBARS analyses. Foods with a low peroxide value are generally perceived as having high oxidative stability and a long shelf life [82]. However, the number of peroxides is often affected [83] and increases with storage time [84,85]. In this study, the level of primary lipid oxidation in yogurts produced using different starters was compared. Higher values were found for EY-2 yogurt (0.43 O_2/kg fat), presumably due to a greater microbial activity than that of EY-1, which led to an anticipated oxidation of the product. On the contrary, for secondary lipid oxidation, EY-2 yogurt shows the lowest value (0.074 MDA/kg DM), and this is attributable to its higher antioxidant capacity than EY-1. In the literature, no other study reports the values of primary and secondary lipid oxidation of ewe's yogurt; however, the values observed in this study were analogous to those reported for fresh sheep cheese [61].

Ewe's yogurt sensory evaluation

The panellists scored the three yogurt samples as objects of evaluation (EY-1, EY- 2, and CCY), and the results for all attributes are given in the form of a spider plot (Figure 4). It is well known that the sensory attributes of dairy products are strictly associated with the milk composition, starter cultures, heat treatment, and storage conditions [85]. For this reason, the sensory evaluation of a new dairy product is mandatory before its commercialization in the marketplace [86]. The scores of the assessors for odor intensity, color, sweetness, acidity, and bitterness were not statistically different ($p > 0.05$) among samples. These results are encouraging because they suggest that ovine milk yogurts meet

the basic requirements of consumers, which, according to Routray et al. [17], are color, odor, and flavor. These attributes represent the most significant sensory factors used for the promotional marketing of a new dairy product [88]. Statistically significant differences ($p < 0.0001$) observed for unpleasant odor, homogeneity, and persistence in the mouth may be attributable to the type of milk used. Indeed, sheep's milk is different from cow's milk in terms of gross composition and single constituents. For example, the lipid fraction contains a higher percentage of volatile, short-chain fatty acids, such as rancid flavor notes [89]. The fat content also serves as substrate for important flavor-generating reactions performed by microorganisms, but it also contributes to the body and texture of cheese, which explains the differences ($p < 0.01$) observed between spontaneous syneresis, viscosity, and off-flavor. Bonanno et al. [90] stated in their work that the differences observed are induced by the breed, the nature of the feed consumed, and even the time of milking (i.e., morning vs. evening). In addition, the use of sheep milk is well known to enrich the nutritional properties of the final product because of the high levels of total solids and nutrients [91].

Finally, the panel was also asked to express their preference between the samples, and even if no difference was noticed between all samples, EY-2 received a higher score (3.6). This means that LAB strains consistently impacted the organoleptic characteristics of the final yogurts.

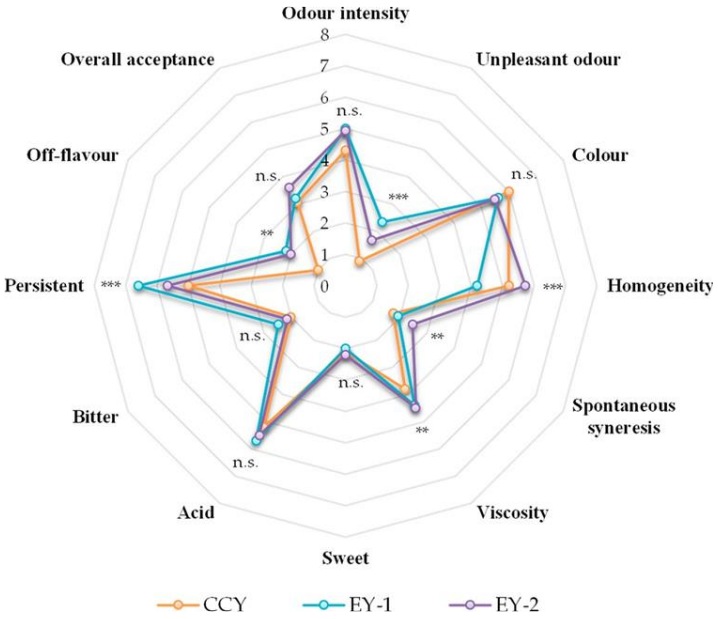

Figure 4. Spider diagram of sensory evaluation of yogurts. Abbreviations: CCY, commercial cow's yogurt; EY-1, production of ewe's yogurt performed with freeze-dried commercial starter culture (starter formulation YODOX91); EY-2, production of ewe's yogurt performed with a multi-strain milk starter culture consisting of *S. thermophilus* and *L. delbrueckii*; ** $p < 0.01$; *** $p < 0.001$; n.s., not significant ($p > 0.05$).

Conclusions

The results of this work show that selected starter cultures can be used to create new and safe products from ewe's milk, such as yogurt, with good antioxidant properties. The consumers also liked the sheep's milk yogurt, which is essential for its market success. This work explored a novel dairy product for Sicily that can benefit the sheep farmers by preserving the native breeds and biodiversity. In a future perspective, sheep yogurt can help face the increasing phenomenon of abandonment of inland areas affecting mountain provinces of the main island.

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Chapter VIII

Addition of fruit purees to enhance quality characteristics of sheep yogurt with selected strains

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Abstract

The aim of this research was to assess the effects of fruit purees of banana, kiwi, mango, red berry, and strawberry on the microbiological, physicochemical, antioxidant, and sensory properties of sheep yogurt. The fruit purees were characterized for their microbiological profile before yogurt production, and no spoilage or pathogenic microorganisms were detected in any of the purees analyzed. Yogurt productions were carried out under industrial conditions using pasteurized sheep's milk and selected starter cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. Five experimental yogurt productions (EYP) were made by adding 10% (w/w) of each fruit puree, while the control yogurt production (CYP) was puree-free. Plate counts revealed that levels of viable *Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* exceeded 8.0 Log CFU/g in all CYP and EYP samples after 3 d of refrigerated storage. The addition of fruit purees reduced fat percentage until to 7% and increased of antioxidant activity, especially with red berry puree. Except for banana, the addition of fruit purees resulted in a statistically significant increase ($p < 0.0001$) in the total terpene VOC profiles of EYP. Notably, the terpene content in mango-flavored yogurt was eightfold greater than that observed in the control trial. Sensory evaluation revealed a reduction in unpleasant odor and off-flavor, and an increase of about 50% in overall acceptance for all EYP in comparison to CYP. Therefore, adding fruit purees to sheep yogurt is a promising strategy for the valorization of Sicilian ewes' milk.

Keywords: sheep yogurt, selected starter culture, fruit purees, microbiological traits, physicochemical properties, sensory evaluation.

Introduction

Yogurt is a fermented milk product that has been consumed worldwide for centuries due to its nutritional and health benefits [1]. According to a report by Market Research [2] the global yogurt market was valued at USD 113.5 billion in 2022 and is expected to reach USD 163.8 billion by 2028. The consumption of yogurt has steadily increased over time [3]. The production of this dairy product relies on the beneficial symbiotic relationship (protocooperation) between *Lactobacillus delbruekii* subsp. *bulgaricus* and *Streptococcus thermophilus* [4,5]. Yogurt-fermenting bacteria and their metabolites can help modulate the gut microbiota and improve immune system function [6]. For this reason, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) recommend a daily intake of yogurt [7].

Yogurt is primarily made from cow's milk in the western world, but it can also be made from milk of other animals such sheep [8]. Sheep's milk boasts several functional properties that position it as a compelling alternative to cow's milk. Remarkably, it offers elevated levels of protein, fat, vitamins, and minerals [9]. The smaller size of fat globules in sheep's milk, compared to cow's milk, contributes to its enhanced digestibility [10]. Additionally, the abundance of conjugated linoleic acids (CLA) and bioactive peptides in sheep's milk plays a significant role in cancer prevention, cardiovascular health, and diabetes management [11]. Italy is one of the main sheep milk producer countries in the European Mediterranean area [12]. Sheep farming is mainly concentrated in the inner hilly areas of Sicily and Sardinia. These areas are known for their rugged terrain and are ideal for sheep farming [13]. The majority of ewes' milk produced in these regions is used to make "Pecorino" cheese, which requires a long ripening period and is known for its strong

and persistent aroma [14]. As a result, dairy producers have encouraged academic research institutes to develop new products that can be brought to market more quickly and satisfy the tastes of modern consumers. Among dairy products, yogurt can be marketed soon after production and does not require any ripening period. Currently, yogurt producers offer consumers an impressive variety of products, spanning diverse qualities and price ranges [15]. Among these, fruit-flavored yogurts reign supreme as the most popular choice [16]. In traditional bovine yogurt, fruit components such as fruit cubes, fruit flavors, fruit purees, and flavor extracts are commonly incorporated. The most favored fruit flavors in yogurt production include banana, berry, cherry, kiwi, lemon, lime, mango, passion fruit, peach, and strawberry [17–19]. These fruits are added to yogurt not only to enhance its flavor, but also to boost its functionality and health properties [20–22]. Regarding Sicilian sheep's yogurts, while Garofalo et al. [23] have provided data on their production using commercial and selected starter cultures, literature lacks studies on their fortification with fruit purees. In fact, adding fruit purees to sheep yogurt could mask the typical strong aromatic flavor attribute that some consumers may not appreciate [24], while also improving further its nutritional value [25]. It is widely recognized that daily consumption of fruits and vegetables can reduce or prevent the risk of non-communicable diseases [26]. The health benefits of fruit are mainly linked to their antioxidant components, particularly phenolic compounds and flavonoids [27].

The purpose of this study was to create new types of sheep yogurt by adding commercial fruit purees and selected *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* starter cultures for the valorization of Sicilian ewes' milk, with the goal of enhancing the value of Sicilian ewes' milk. The specific research objectives were: (i) to evaluate the

microbiological quality of the ewes' milk and fruit purees; (ii) to monitor the presence of *Lb. delbruekii* subsp. *bulgaricus* and *S. thermophilus* during yogurt productions; (iii) to assess the physicochemical properties, antioxidant activity, volatile organic compounds and sensory characteristics of the final yogurts.

Materials and methods

Raw material and natural milk starter culture preparation

To produce new types of sheep yogurt, five single commercial fruit purees were purchased from Les Vergers Boiron (Châteauneuf-sur-Isère, France). These purees consisted of 100% pulp from banana [with a total soluble solid (TSS) of 23 °Brix, protein content of 1.1%, and sugar content of 17.8%], kiwi (TSS 13 °Brix, protein 0.6%, sugars 7.2%), mango (TSS 19 °Brix, protein 0.8%, sugars 13.9%), red berry (TSS 10 °Brix, protein 0.5%, sugars 8.8%), and strawberry (TSS 8 °Brix, protein 0.5%, sugars 5.1%). These specific fruit flavors were chosen from those commonly used in commercial flavored yogurt production [17–19]. The raw ewes' milk from sheep of "Valle del Belíce" breed was provided by several artisanal dairy farms located within Agrigento province (Sicily, southern Italy). The strains *Lb. delbrueckii* subsp. *bulgaricus* WT601 and *S. thermophilus* PON244, which were previously selected for their dairy aptitudes [28,29] and ability to improve the antioxidant properties of white sheep yogurt [23], were used to prepare a Natural Milk Starter Culture (NMSC). Briefly, *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were grown in de Man-Rogosa-Sharpe (MRS) (Biotec, Grosseto, Italy) and Medium 17 (M17) (Oxoid, Hampshire, UK) broths, respectively, for 48 h at 44 °C. After growth, the cells of each strain were washed in Ringer's solution (Oxoid) and

individually inoculated at 1% (v/v) into UHT sheep milk (Leeb Vital, Wartberg an der Krems, Austria). These milks, after incubation at 44 °C for 24 h, represented the two NMSCs to be used as fermenting agents in yogurt production.

Yogurt production and sample collection

Yogurt production was carried out at a dairy pilot plant of the dairy factory "Cooperativa Agricola Tumarrano" located in Cammarata (Italy), during March 2022. The experimental design (Figure 1) included a control yogurt production (CYP) and five experimental yogurt productions (EYP), one for each fruit puree. Briefly, 40 L of ewe's milk underwent conventional heat pasteurization at 72 °C for 15 s using a P75 50/2 pasteurizer (Tecnolat S.p.a., Nocera Inferiore, Italy), which did not include high-pressure milk homogenization. After pasteurization, the milk was transferred into the yogurt machine (Due Ci Inox s.r.l., Guastalla, Italy) previously sanitized with an alkaline chlorinated solution (Mersolat, Cercola, Italy). The milk was cooled at 42 °C and added with 400 mL of each NMSC under gentle agitation. The fermentation process took place for 6 h at 40 °C under static conditions, and it was stopped at pH around 4.5 [30]. For the CYP, the mixture was initially stirred and then packaged into 120 mL round plastic pots sealed with hermetic pressure caps (FD Store s.r.l., Vignola, Italy). In contrast, the EYP were enriched with 10% (w/w) of each fruit puree, gently mixed for 2 min, and subsequently packaged.

All yogurts were kept under refrigerated condition (4 °C) for 3 d. Two independent yogurt productions were carried out at seven days of distance. Samples of fruit purees,

pasteurized milk, inoculated milk with NMSC, and yogurts after three days of storage were collected for analysis.

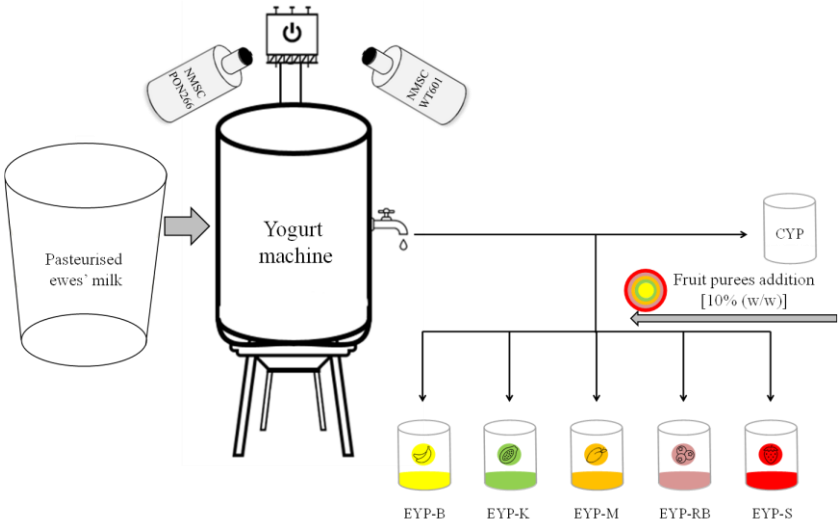

Figure 1. Experimental design of yogurt productions. Abbreviations: NMSC PON244, natural milk starter culture produced with the starter *S. thermophilus* PON244; NMSC WT601, natural milk starter culture produced with the starter *Lb. delbrueckii* subsp. *bulgaricus* WT601; CYP, control yogurt production; EYP-B, experimental yogurt production enriched with banana puree; EYP-K, experimental yogurt production enriched with kiwi puree; EYP-M, experimental yogurt production enriched with mango puree; EYP-RB, experimental yogurt production enriched with red berry puree; EYP-S, experimental yogurt production enriched with strawberry puree.

Microbiological analyses

All samples collected along the production chain of CYP and EYP were serially diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy). Cell suspensions of raw materials (fruit purees and pasteurized milk) were plated on: Skim Milk Agar (SMA) (Microbiol Diagnostici, Cagliari, Italy) incubated aerobically for 72 h at 30 °C for the enumeration of total mesophilic microorganisms (TMM); acidified MRS agar (Biotec) and M17 agar (Oxoid) incubated anaerobically for 48 h at 44 °C for lactobacilli and streptococci, respectively; Hektoen Enteric Agar (HEA) (Microbiol Diagnostici) incubated aerobically at 37 °C for 24 h for *Escherichia coli* and *Salmonella* spp.; Baird Parker (BP) agar (Oxoid)

incubated aerobically at 37 °C for 48 h for coagulase-positive staphylococci; *Listeria* Selective Agar Base (Oxoid) incubated aerobically at 37 °C for 24 h for *Listeria monocytogenes*. Inoculated milk and yogurt samples were analyzed only for TMM, *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. The presence of the last two microorganisms was confirmed following the ISO 7889 guidelines [31]. Briefly, cell suspensions from the inoculated milk and yogurt samples were plated on: acidified MRS agar (Biotec) incubated anaerobically for 72 h at 37 °C for *Lb. delbrueckii* subsp. *bulgaricus* and M17 agar (Oxoid) incubated aerobically for 48 h at 37 °C for *S. thermophilus*. After growth, the colonies were collected, purified, tested for Gram reaction and catalase activity, and microscopically investigated [32]. Analyses were performed in duplicate.

Physicochemical and antioxidant analyses

The yogurt samples were subjected to the determination of colorimetric parameters by a Minolta Chroma Meter CR-300 (Minolta, Osaka, Japan), measuring lightness (L* = 0/100, from black to white), redness (a* = -a/+a, from green to red) and yellowness (b* = -b/+b, from blue to yellow), according to the CIE L*a*b* system [33]; these values (L*, a*, and b*) were used to calculate chroma $[(a^{*2} + b^{*2})^{0.5}]$, expressing the color intensity or saturation, hue angle $[\tan^{-1}(b^*/a^*)]$, as a measure of color tone, and the whiteness index $[100-((100-L^*)^2 + a^{*2} + b^{*2})^{0.5}]$, according to Vargas et al. [34].

All samples of yogurts were analyzed for pH by HI 9025 pH meter (Hanna Instruments, Ann Arbor, MI, USA), titratable acidity by Soxhlet-Henkel method (°SH/50 mL) and total soluble solids (TSS) with an optical Brix refractometer (Manual Refractometer MHRB-40

ATC, Mueller Optronic, Erfurt, Germany). Successively, the samples were first frozen at –20 °C and then lyophilized to be analyzed for dry matter (DM), protein, fat and ash content, according to standard of International Dairy Federation [35–38].

From the lyophilized samples, extracts were prepared according to the method of Rashidinejad et al. [39], with minor modifications, for the subsequent analysis to determine the antioxidant capacity. Briefly, 0.5 g of each sample was suspended in 25 mL of methanol aqueous solution (95% v/v) containing 1% HCl. The mixture was shaken by vortex for 30 s. Then the suspension remained in an ultrasonic bath (LBS1 Sonicator; Falc Instruments, Treviglio, Italy) at 40 °C for 30 min, during which it was shaken by vortex every 10 min for a few seconds. As soon as cold, the suspension was filtered with linen cloth and centrifuged at 7,000 rpm/min for 10 min at 9 °C, then stored at –18 °C until analysis.

The yogurt extracts were analyzed in duplicate to determine the total polyphenols by the Folin-Ciocalteau colorimetric method, with gallic acid as standard, and the antioxidant capacity by TEAC assay using Trolox as standard, as described by Ponte et al. [40]; the respective results were expressed in gallic acid equivalent (GAE)/kg DM and mmol Trolox/kg DM. Regarding TEAC assay, the ABTS radical cation was obtained by reacting 14 mM ABTS aqueous solution with an equal volume of 4.9 mM persulfate of potassium and incubating the mixture in the dark for 16 h at 22 °C (room temperature). For the assay, the ABTS radical cation solution was diluted with 5 mM phosphate-buffered saline (PBS) at pH 7.4 to an absorbance of 0.795 (± 0.02) at 734 nm. The absorbance of a mixture of 75 µL of PBS with 1,425 µL of a diluted ABTS solution was read at 734 nm after incubation for 6 m at 30 °C. In the same way, 75 µL of each extracted sample were mixed with 1,425

µL diluted ABTS radical cation solution, and after incubation at 30 °C for 6 min the absorbance read at 734 nm was used to calculate the percentage decrease of the absorbance due to decolorization in comparison with the absorbance read with PBS. Trolox solutions in PBS, between 0 and 2.5 mM, were used to develop a calibration curve (R^2 = 0.99).

The oxidative stability of yogurt fat was evaluated by determining the peroxide values (POV, meq O_2 kg/fat), as a primary lipid oxidation index [41], and the thiobarbituric acid reactive substances [TBARS, mg malondialdehyde (MDA)/kg DM] as secondary lipid oxidation index, performed according to the method proposed by Tarladgis et al. [42] and modified by Mele et al. [43], as described by Ponte et al. [40].

Volatile organic compounds determination

The volatile organic compounds (VOCs) analysis was conducted using headspace solid-phase microextraction (SPME) coupled with the Gas Chromatography-Mass Spectrometry (GC-MS) technique. Five grams of CYP and EYP samples were exposed to an SPME fiber (DVB/CAR/PDMS 50 mm, Supelco) for 15 min at 60 °C with continuous stirring. Following adsorption, the SPME fiber was thermally desorbed through a splitless GC injector at 250 °C for 1 min. Chromatographic separation was achieved using a DB-624 capillary column (Agilent Technologies, 60 m, 0.25 mm, 1.40 µm) with a carrier gas (helium) at 1 mL/min. The oven temperature program was initiated with a 5-min isotherm at 40 °C, followed by a linear increase of 5 °C per min up to 200 °C, and the final temperature was held at 200 °C for 2 min. The acquisition was performed under scanning (SCAN) conditions with the interface temperature set at 230 °C, and the acquisition mass range spanning from 40 to 400. Identification of VOC compounds was carried out by

comparing the MS spectra with the NIST05 commercial library. The relative proportions of identified compounds were expressed as percentages, obtained by normalizing GC-MS peak areas with the total area of selected peaks. Three replicates of each sample were carried out.

Sensory evaluation

A descriptive panel of 15 judges (8 females and 7 males, aged between 27–63 years) evaluated the sensory traits of all yogurts. The evaluation was carried out in individual chambers using an iPad connected to the Smart Sensory Box software (Smart Sensory Solutions S.r.l., Sassari, Italy). The judges underwent training for evaluating yogurt attribute, following the guidelines outlined in ISO 8586 (ISO 8586:2023, 2023) indications [44]. Approximately 35 mL of each yogurt were placed in white plastic glasses and served in a random order. The panelists were asked to score the following sensory attributes: odor intensity, unpleasant odor, color, homogeneity, viscosity, sweet, acid, bitter, taste persistency and overall acceptance. The intensity each attribute was quantified on a line scale ranging from 0 (low quality) to 9 (high quality) cm.

Statistical analyses

One-Way Variance Analysis (ANOVA) was used to analyze data for plate counts and total terpene compounds. The generalized linear model (GLM) procedure with the yogurt productions (6 levels) as unique factor was used to analyze physicochemical traits. Tukey's test was applied for pairwise comparisons at a significance level of $p < 0.05$. Heat map cluster analysis was used to identify the distribution of VOCs emitted from yogurts. Two-

factor ANOVA, with panelists (p = 1 . . . 15) and yogurt (y = 1 . . . 6) as fixed factors, was applied to test the data on sensory evaluations. Statistical processing of plate counts, VOCs and sensory data, were carried out using XLStat software version 2020.3.1 for Excel (Addinsoft, New York, NY, USA), while the physicochemical data were analyzed using the SAS 9.2 software (SAS Institute Inc., Campus Drive Cary, NC, USA).

Results and discussion

Evolution of microbiological parameters during yogurt production

The microbiological evaluation of commercial fruit purees used in this study was performed to detect the presence of the main pathogenic and spoilage microorganisms. This assessment is mandatory before their use in food applications because, despite undergoing heat treatment, they are known to be vehicles for undesirable microorganisms [45]. The specific search for spoilage and pathogenic microorganisms did not reveal any colonies in any of the analyzed fruit purees (data not shown), indicating the hygienic suitability of these products for use in yogurt production. Figure 2 reports a graphic representation of the microbiological plate counts performed on raw and pasteurized ewes' milk. Raw milk hosted consistent levels of TMM and streptococci (6.0 Log CFU/mL), while lactobacilli were two log cycles lower. Raw ewes' milk used for traditional Sicilian cheese production commonly hosts consistent levels of these microorganisms [46]. The presence of high levels of TMM and LAB is mainly due to the milking practices and conservation conditions [47]. Within the main dairy pathogenic bacteria [48], *L. monocytogenes*, CPS, and *Salmonella* spp., were undetectable (< 1 Log CFU/mL), while *E. coli* was around 3.0 Log CFU/mL. *E. coli* is often detected in raw milk, and its presence is

usually attributed to fecal contamination [49]. Following pasteurization, ewes' milk only contained TMM, streptococci, and lactobacilli at levels of 2.94, 2.63, and 2.21 Log CFU/mL, respectively. Similar results were previously reported by Busetta et al. [50] and Barbaccia et al. [51] in pasteurized milk used to produce sheep pressed and stretched cheeses. These results are not surprising since the pasteurization process does not have the ability to inhibit the growth of the thermoduric indigenous milk microbiota [52].

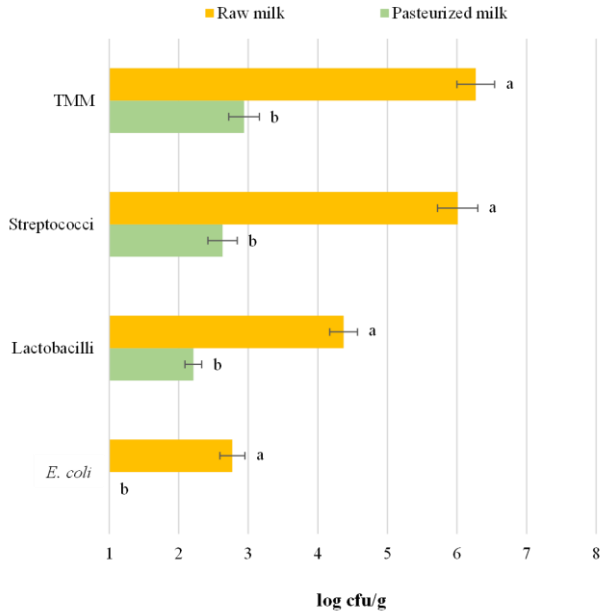

Figure 2. Microbiological loads of ewes' milk samples before and after pasteurization. Units are Log CFU/mL. Results indicate mean values ± S.D. of four plate counts (carried out in duplicate for two independent productions). Abbreviations: TMM, total mesophilic microorganisms; LAB, lactic acid bacteria; *E.*, *Escherichia*. a, b = $p < 0.05$.

The growth of the selected starter cultures during yogurt productions is reported in Table 1. The analysis of milk after NMSCs addition showed levels of TMM, *Lb. delbrueckii* subsp. *bulgaricus*, and *S. thermophilus* above 7.0 Log CFU/mL. It is widely recognized that starter cultures should be added at levels of 10^7 CFU/g of viable bacteria to promptly initiate the milk fermentation process [53]. No significant differences ($p > 0.05$) were found for the levels of TMM, *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*

among control and experimental yogurt samples. In particular, TMM and *S. thermophilus* reached values of about 10^9 cfu/mL, while *Lb. delbrueckii* subsp. *bulgaricus* reached levels one order of magnitude lower. Other authors have reported this trend in yogurt produced with ovine and bovine milk [54,55]. These results indicated that the addition of 10% (w/w) of each fruit puree did not have a negative impact on the survival of *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* used as fermenting agents in yogurt production.

Table 1. Levels of selected starter culture during yogurt production.

Samples	**Bacterial counts**		
	TMM	*Lb. delbrueckii* subsp. *bulgaricus*	*S. thermophilus*
Inoculated milk	7.37	7.09	7.23
CYP	9.21	8.15	8.93
EYP-B	9.33	8.02	8.97
EYP-K	9.01	7.93	8.85
EYP-M	9.25	8.09	8.94
EYP-RB	9.02	7.95	8.76
EYP-S	9.13	8.05	9.01
SEM	0.04	0.04	0.05
p value	0.310	0.777	0.652

Units are Log CFU/mL for inoculated milk samples and Log CFU/g for yogurt samples. Results indicate the mean values of four plate counts (carried out in duplicate for two independent productions). Abbreviations: TMM, total mesophilic microorganisms; *Lb.*, *Lactobacillus*; *S.*, *Streptococcus*; CYP, control yogurt production; EYP-B, experimental yogurt production enriched with banana puree; EYP-K, experimental yogurt production enriched with kiwi puree; EYP-M, experimental yogurt production enriched with mango puree; EYP-RB, experimental yogurt production enriched with red berry puree; EYP-S, experimental yogurt production enriched with strawberry puree; SEM, standard error of the mean.

Physicochemical and antioxidant characterization of yogurts

The physicochemical properties of control yogurt and the five experimental yogurt productions with fruit puree are indicated in Table 2. As expected, the addition of fruit puree had a visible effect on the color traits of yogurt, such as lightness, red index and yellow index (respectively L*, a*, b*) shown in Table 2. In the research of Ścibisz et al. [56], yogurt with blueberry puree compared to that with strawberry puree showed lower

lightness and yellow index values. By assimilating the blueberry puree to the red berry puree, this trial revealed similar results regarding the values of L* and b*, that are lower in yogurt with the addition of red berry puree in comparison with each other. Nevertheless, it is evident that the addition of fruit puree reduced the lightness values (L*) of all yogurts [57], especially those with the addition of red berry puree. Understandably, the same yogurt showed the significantly highest and positive red index (a*), which was followed by that of strawberry yogurt, while less marked but statistically significant differences were recorded among the other yogurts. The yellow index (b*) was much higher for yogurt with the addition of mango puree, which was visibly yellow. With regard to whiteness index, yogurt with banana and kiwi purees were not different than control yogurt. Instead, the yogurt with mango, red berry and strawberry purees showed lower levels of whiteness index due to the more intense color of puree. The trend of chroma and hue angle, being influenced by a* and b* values, reflects those of the original red and yellow indices.

As expected, the addition of fruit purees was responsible of a reduction of both pH and titratable acidity, more markedly with red berry puree. The Brix values of the six yogurt samples were significantly different from each other; this diversity is certainly due to the variable water content of the fruits used in the productions, as emerged by their respective TSS level. In each experimental production, the addition of fruits significantly decreased the protein and fat content of yogurt in comparison with the control yogurt. A similar result also emerged in Amal et al. [58] in the comparison between yogurts without and with the addition of fruit puree. Among yogurts with fruit puree, that with mango puree had a significantly higher protein content (31.68%), whereas, as emerged also in Concha-Meyer et al. [59], yogurts with strawberry puree and kiwi puree had almost similar protein levels,

along with that with banana puree and berries. Fat content of yogurts, on the other hand, ranged from 27.68% to 34.62%. The consistent decrease in the fat content of the yogurts containing fruits puree, is evidently due to the low fat content in fruit puree [58]. As regards the white yogurt without the addition of fruit puree (CYP), its chemical composition was almost similar to that of sheep yogurt produced in the Garofalo et al. [23].

The total polyphenols content did not increase with the addition of fruit purees in the yogurt, with the only exception when the red berry puree was used. On the contrary, the values of antioxidant capacity (TEAC) of sheep yogurt greatly improved when it was enriched with each of fruit purees, and more markedly with the red berry puree by which the highest level was reached. Accordingly, Razola-Díaz et al. [60] observed that fruits such as blueberries and blackberries (commonly called berries) and strawberries have higher antioxidant capacity than other fruits; indeed, in their work, determining the antioxidant capacity of several smoothies, the authors found the best antioxidant capacity values for samples that contained red fruits in their ingredients. The same trend was observed by Müller et al. [61] and Nowicka et al. [62] who in smoothies with higher amounts of red fruits obtained a higher antioxidant capacity than in those composed of mango, banana, apple, and pear. This greater antioxidant activity can be explained by the major presence of tannins and anthocyanins in the red fruits, in line with the highest polyphenols content found in the red berry yogurt. Instead, the increase of TEAC value recorded in the other yogurts was not supported by a concomitant increase of polyphenols in comparison with the CYP, suggesting that other components have exerted antioxidant power, such as terpenes and vitamins [27], or that the phenolic compounds from fruits

could have formed complexes with yogurt proteins, responsible for a reduction of the measured polyphenols amount [56].

Moreover, it can be noticed that the high antioxidant action of red berry was not able to protect the yogurts fat from oxidation, as emerged by the higher POV and TBARS values in comparison with the other yogurts. These results imply the possibility that the more intense red color could have interfered with the analytical determination. Nevertheless, in general both primary and secondary lipid oxidation recorded after 3 days from production were higher in yogurts with fruits than in CYP, although the increase was less consistent with mango, presumably due to its greatest richness in terpenes. In literature, there is no study that reports POV and TBARS values of sheep yogurt, therefore the higher values of lipid oxidation induced by the addition of fruit purees could be explained by a greater microbial activity, as hypothesized by Garofalo et al. [23]. However, on the basis of sensory evaluation (Table 3), the lipid oxidation apparently induced by the addition of fruits puree does not seem to have altered the yogurt taste or appearance.

Table 2. Physicochemical traits, antioxidant capacity and oxidation products of sheep yogurts.

	Samples						**SEM**	***p* value**
	CYP	EYP-B	EYP-K	EYP-M	EYP-RB	EYP-S		
Color								
Lightness L*	94.85^{a}	93.20^{a}	93.26^{a}	89.49^{b}	57.92^{d}	82.97^{c}	0.552	<0.0001
Redness a*	-3.59^{cd}	-2.76^{c}	-3.98^{d}	-3.24^{cd}	24.77^{a}	4.52^{b}	0.186	<0.0001
Yellowness b*	8.49^{b}	7.48^{b}	9.11^{b}	35.61^{a}	-3.01^{d}	3.79^{c}	0.723	<0.0001
Chroma	95.07^{a}	93.35^{a}	93.48^{a}	92.99^{a}	57.96^{c}	83.02^{b}	1.10	<0.0001
Hue angle (°)	-66.98^{c}	-69.67^{d}	-66.43^{c}	-84.74^{e}	-6.94^{b}	39.88^{a}	0.566	<0.0001
Whiteness index	89.37^{a}	89.44^{a}	87.98^{a}	62.72^{c}	51.08^{d}	81.97^{b}	0.774	<0.0001
Dry matter (DM), %	17.09^{d}	21.51^{b}	22.01^{a}	17.16^{d}	16.26^{e}	21.03^{c}	0.058	<0.0001
Ash, % DM	4.58^{a}	4.31^{b}	3.69^{c}	4.63^{a}	4.35^{b}	3.57^{d}	0.021	<0.0001
Protein, % DM	33.04^{a}	30.30^{c}	30.29^{c}	31.68^{b}	30.48^{c}	30.71^{c}	0.160	<0.0001
Fat, % DM	34.62^{a}	27.68^{d}	28.65^{c}	27.75^{d}	31.22^{b}	28.83^{c}	0.116	<0.0001
TEAC, mmol/kg DM	16.46^{d}	36.88^{c}	45.43^{bc}	45.60^{bc}	132.29^{a}	47.91^{b}	2.34	<0.0001
POV, mEq O_2/kg fat	0.432^{d}	1.19^{b}	0.964^{c}	0.952^{c}	1.32^{a}	0.269^{e}	0.008	<0.0001
TBARs, mg MDA/kg DM	0.07^{e}	0.14^{c}	0.12^{d}	0.11^{d}	1.75^{a}	0.19^{b}	0.003	<0.0001

Abbreviations: CYP, control yogurt production; EYP-B, experimental yogurt production enriched with banana puree; EYP-K, experimental yogurt production enriched with kiwi puree; EYP-M, experimental yogurt production enriched with mango puree; EYP-RB, experimental yogurt production enriched with red berry puree; EYP-S, experimental yogurt production enriched with strawberry puree; SEM, standard error of the mean; TSS, total soluble solid; GAE, gallic acid equivalent; TEAC, trolox equivalent antioxidant capacity; POV, peroxide value; TBARs, thiobarbituric acid–reactive substances; MDA, malonylaldehyde. On the row: a, b, c, d = $p < 0.05$.

Volatile organic compounds emitted from yogurts

The analysis of the volatile composition was conducted on both control and experimental yogurt productions to evaluate how the addition of fruit puree affects the volatile profile of yogurts in terms of sensory-active compounds. Figure 3 reports the volatile profiles generated by the yogurt samples analyzed. The control yogurt emitted a total of 17 VOCs, which consisted of 7 ketones, 3 aldehydes, 3 acids, 2 alcohols, 1 ester, and 1 terpene. Ketones are prevalent components of yogurt and their formation occurs through both the β-oxidation of saturated fatty acids, followed by the decarboxylation of β-ketoacids in the metabolic pathway [14,75,76]. Diacetyl, acetone, and acetoin were the main ketones emitted by the control yogurt. Diacetyl, a direct product of glucose and citrate catabolism [77,78], is the predominant significant flavor compound among these C4 chemicals. Both *S. thermophilus* and *L. bulgaricus* are capable of producing diacetyl. Acetoin, the reduced form of diacetyl, is generated by the enzyme diacetyl reductase acting on diacetyl. Acetoine plays a crucial role in reducing the sharpness of diacetyl and contributes to the pleasant, creamy flavor of yogurt. [79–82]. Additionally, acetone, originating from bacteria in milk and starter cultures, imparts a sweet fruity aroma and positively influences the flavor of yogurt [83]. Other ketones like 2-butanone, 2,3-Pentanedione, 3-Hydroxy-butanone, and 2-Heptanone were also detected. A similar ketone composition was also reported in goats' yogurt [84]. The concentration of carboxylic acids

was significantly higher than those of aldehydes, esters, and alcohols. Acetic acid was identified as the most abundant acid in CYP, followed by hexanoic acid and butanoic acid. Acetic acid results from the metabolism of lactose, citric and lactic acid [14] or derives from the catabolism of amino acids [85]. Hexanoic acid was generally found in goat's yogurt, reflecting the high amounts of short-chain fatty acids in goat's milk [86]. Overall, the carboxylic acids identified in this study, including hexanoic, butanoic, and acetic acids, contribute to the flavor of yogurt. Specifically, hexanoic acid is responsible for a sour note, butanoic acid imparts a cheesy flavor, and acetic acid is responsible for vinegar and acidic notes [14].

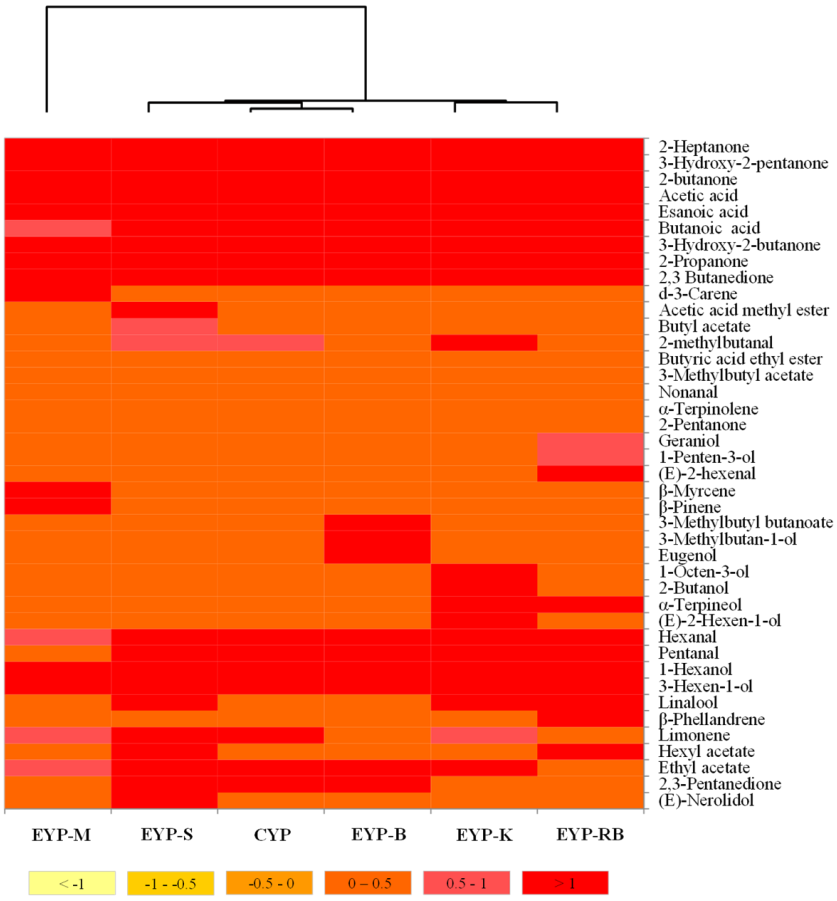

Figure 3. Distribution of volatile organic compounds among yogurts. The heat map plot depicts the relative concentration of each VOC. Abbreviations: CYP, control yogurt production; EYP-B, experimental yogurt production enriched with banana puree; EYP-K, experimental yogurt production enriched with kiwi puree; EYP-M, experimental yogurt production enriched with mango puree; EYP-RB, experimental yogurt

production enriched with red berry puree; EYP-S, experimental yogurt production enriched with strawberry puree. Specific sensory notes of volatile compounds identified in yogurt productions are reported in Table S1.

Compounds belonging to the classes of aldehydes (4-pentanal, hexanal, 2-methylbutanal), esters (ethyl acetate), alcohols (1-hexenol, 3-hexen-1-ol), and terpenes (limonene) have been identified in yogurt, but they constitute only a minor fraction of the total composition. Similar profiles have been reported in yogurt made from ovine milk [80,84]. Compared to the control yogurt, the addition of fruits resulted in a decrease in the content of ketones and acids in experimental yogurt production. On the other hand, the addition of fruit purees in experimental yogurt productions particularly affected sensory-active compounds, increasing the overall content of the terpene class (Figure 4) as observed in other studies on similar productions [25,87,88]. Specifically, the highest levels of terpene class were found in yogurt enriched with mango puree, followed in descending order by strawberry, red berry and kiwi.

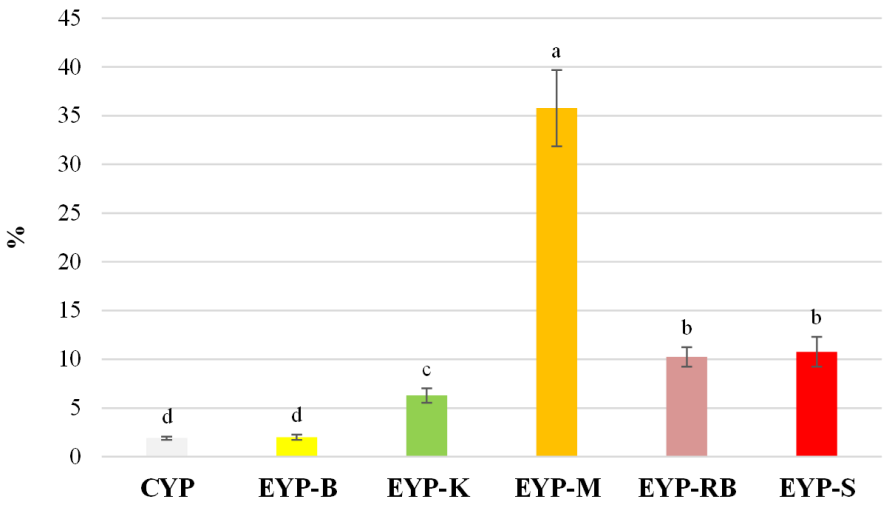

Figure 4. Distribution of total terpene compounds among yogurts. Results indicate mean percentage values ± S.D. of four measurements (carried out in duplicate for two independent productions) and are expressed as relative peak areas x l00. Abbreviations: CYP, control yogurt production; EYP-B, experimental yogurt production enriched with banana puree; EYP-K, experimental yogurt production enriched with kiwi puree; EYP-M, experimental yogurt production enriched with mango puree; EYP-RB, experimental yogurt

production enriched with red berry puree; EYP-S, experimental yogurt production enriched with strawberry puree. a, b, c, d = $p < 0.05$.

The consistent terpenes content registered in the yogurt with mango puree may contribute to the limited increase observed in its secondary lipid oxidation (Table 2). The absence of predominant VOCs in banana fruits [89–91] resulted in minimal aromatic influence on the final product, leading to no significant differences ($p > 0.05$) between banana-flavored yogurt and control production. Mango yogurt contained high levels of d-3-carene, which is characteristic of mango fruit and is the most abundant terpene found in the yogurt. Many studies reported values between 60 and 80% of the total VOCs [92,93]. The increase in terpenes in yogurt enriched with strawberry puree is mainly due to the detection of nerolidol and linalool. According to literature findings, compounds such linalool, β-phellandrene, and α-terpineol are responsible for the increase in terpenes in red berry flavored yogurt [94]. These compounds are reported as the predominant chemical class in red berries [95]. Similarly, the increase in terpene compounds in kiwi-enriched yogurt is attributed to the detection of linalool, geraniol, and α-terpineol, which are present in kiwi fruit [96–98].

The findings suggest that the aromatic profile is primarily influenced by the addition of fruit, as evidenced by the increase in terpenes and the detection of compounds absent in the control yogurt. However, despite the differences in physicochemical parameters observed between the control and experimental productions, such as the reduction in fat content due to the addition of fruits, it is not possible to determine if these variations influenced the final aromatic profile because the same production method was used for all samples. Therefore, further investigation is needed to evaluate the influence of production type (milk and starter cultures) on the final VOC profile.

In general, the presence of terpenes can positively impact consumer acceptability [99]. Terpenes significantly enhance aromatic perception [100], and numerous studies on fruits and vegetables have highlighted their role in enhancing flavor perception and consumer preference [101–105]. Furthermore, our results suggest that while there is an overall increase in terpenes, the specific terpene sensory-active compounds differ between the various production methods, which may influence sensory perceptions.

Sensory traits of yogurts

Both the control and experimental yogurts underwent sensory analysis to assess the impact of fruit puree addition on appearance, aroma, flavor, and texture attributes. This analysis is essential for evaluating consumer acceptance of new yogurts before their market launch [106]. Table 3 reports the results of the quantitative descriptive analysis of yogurts.

Table 3. Evaluation of the sensory traits of yogurts.

Attributes	**Trials**						**SEM**	***P* value**	
	CYP	EYP-B	EYP-K	EYP-M	EYP-RB	EYP-S		Panelists	Yogurt
Odor intensity	5.33^{c}	5.89^{b}	5.96^{b}	6.22^{ab}	6.43^{a}	6.67^{a}	0.07	0.824	< 0.0001
Unpleasant odor	2.22^{a}	1.09^{bc}	1.41^{b}	1.15^{bc}	1.04^{bc}	0.77^{c}	0.07	0.995	< 0.0001
Color	4.04^{d}	4.21^{d}	4.33^{d}	5.62^{c}	7.47^{a}	6.34^{b}	0.16	1.000	< 0.0001
Homogeneity	6.13^{a}	5.83^{ab}	5.61^{b}	5.80^{b}	5.65^{b}	5.72^{b}	0.04	0.911	0.0001
Viscosity	4.90^{b}	6.34^{a}	6.41^{a}	6.37^{a}	6.19^{a}	6.23^{a}	0.07	1.000	< 0.0001
Sweet	2.13^{e}	4.22^{a}	3.17^{cd}	2.80^{d}	3.25^{c}	3.73^{b}	0.09	0.997	< 0.0001
Acid	5.20^{a}	5.12^{a}	5.36^{a}	5.24^{a}	5.49^{a}	5.32^{a}	0.05	0.437	0.223
Bitter	1.99^{a}	1.55^{ab}	1.47^{b}	1.33^{b}	1.61^{ab}	1.37^{b}	0.05	0.745	0.001
Taste persistency	6.16^{a}	4.92^{b}	5.21^{b}	5.04^{b}	5.31^{b}	5.12^{b}	0.07	0.996	< 0.0001
Off-flavor	2.59^{a}	1.42^{bc}	1.34^{bc}	1.26^{c}	1.55^{bc}	1.66^{b}	0.06	0.993	< 0.0001
Overall acceptance	4.25^{b}	6.22^{a}	6.01^{a}	6.09^{a}	6.13^{a}	6.11^{a}	0.09	0.996	< 0.0001

Results indicate mean value. Abbreviations: CYP, control yogurt production; EYP-B, experimental yogurt production enriched with banana puree; EYP-K, experimental yogurt production enriched with kiwi puree; EYP-M, experimental yogurt production enriched with mango puree; EYP-RB, experimental yogurt production enriched with red berry puree; EYP-S, experimental yogurt production enriched with strawberry puree; SEM, standard error of the mean. On the row: a, b, c, d = $p < 0.05$.

Although it is well known that the aspect, aroma, taste, and texture of yogurt depend on the type and composition of milk, as well as the starter cultures used as fermenting agents [107], the addition of fruits and vegetables significantly impacts the sensory properties of this dairy product [108]. In this study, all sensory parameters except for acid perception attribute were scored differently among yogurts. Specifically, the addition of fruit purees, regardless of their taste, increased odor intensity, color, viscosity and sweetness, while reducing unpleasant odors, bitter, taste persistence and off-flavors. Sobti et al. [20] tested different commercial fruit purees to enrich camel yogurt and observed similar trends. In terms of color, the higher score given to the yogurt with mango puree is consistent with its intensely yellow index, whereas the scores for the red berry and strawberry puree correspond to their respective red color indices. The overall satisfaction, defined as the overall sensory acceptability of the food product evaluated [51], was higher for all yogurt productions enriched with fruit purees, regardless of their taste, compared to the control. Despite significant lipid oxidation in the experimental yogurts, there seems to be no negative impact on sensory properties.

Conclusion

This study delved into an extensive analysis of Sicilian sheep yogurts enriched with fruit purees at a concentration of 10% (w/w). These novel yogurts were manufactured at the pilot plant scale using selected starters of *Lactobacillus delbrueckii* subsp. *bulgaricus*. The addition of fruit purees did not affect hygiene and safety aspects of the final product. The two starter cultures (*Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) drove the acidification process. The addition fruit purees decreased led to a 7% reduction of fat

content across all experimental yogurts. Antioxidant properties were enhanced, especially with red berry puree. The terpene class significantly impacted the VOC profiles of all yogurts, except for banana yogurt. The addition of fruit purees resulted in a reduction of the typical strong aromatic flavor associated with sheep yogurt.

These results suggest that these new types of sheep yogurt may offer health benefits and provide dairy producers with products that cater to the tastes of the post-modern consumer. Further studies will validate this manufacturing method at an industrial level and explore the beneficial effects of these novel yogurts on human health through gastrointestinal digestion and radical scavenging activities over time.

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Part III

Novel ripened ewe's milk cheeses

In this section, the complex world of ripened ewe's cheese was approached.

Cheese is a complex ecosystem, teeming with a diverse group of microorganisms, collectively known as the cheese microbiota. These microorganisms originate from raw milk, starter cultures, and adjunct cultures. The interplay of milk proteins, carbohydrates, and fats within this ecosystem significantlyinfluences the sensory qualities of various cheese types. This transformation primarily occurs during a critical technological phase in cheese production known as “ripening”. Ripening is a pivotal step characterized by a sequence of biochemical and microbiological events. These processes play a key role in shaping the cheese's aroma and taste properties. Ewe's milk serves as the foundation for ripened cheeses. Furthermore, extended ripening periods allow for the use of raw milk, which imparts a wealth of organoleptic attributes to the final product, attributes that surpass those achievable with heat-treated milk.

Our research endeavors centered around studying a novel ewe’s cheese, following the well-established techniques used for Swiss-type cheese. Both raw and pasteurized milk were explored to create a distinctive cheese that embodies the essence of this remarkable dairy tradition.

Chapter IX

Description of Ewiss cheese, a new ewe's milk cheese processed by Swiss cheese manufacturing techniques: microbiological, physicochemical and sensory aspects

This work has been accepted for pubblication in

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Abstract

Typically, Swiss-type cheese is made from cow's milk. However, in the present work an attempt to expand the sheep supply chain and product offering in this field was made by developing a new type of cheese using Swiss-type cheese technology. The cheese was manufactured under industrial conditions, and fermentations were carried out using freeze-dried commercial starters that are traditionally used in the production of Swiss cheese. Two experimental "Ewiss cheese" (EC) products were produced using raw milk (RM-EC) and pasteurized milk (PM-EC), respectively. Fourteen microbial groups were investigated by plate counts from curd until ripened cheeses. According to microbiological analyses, no statistically significant differences were found between the two productions with respect to the group of lactic acid bacteria (LAB). The curds were mainly characterized by mesophilic LAB cocci (7.45 Log CFU/g in RM-EC and 7.33 Log CFU /g in PM-EC). However, at the end of the ripening period (9 mo), the cheeses exhibited a higher presence of mesophilic LAB rods. Undesired microbiological groups were found only in the curd of raw milk cheese in the range of 10^4–10^5 CFU/g, but reaching undetectable levels by plate count in the cheese at the end of ripening. RM-EC and PM-EC were characterized by 76% and 68% of dry matter, respectively. These cheeses contained 29.30% and 34.36% of protein, and 51.31% and 50.38% of fat, respectively. Textural analysis showed differences in terms of hardness, chewiness, and gumminess between the experimental cheeses and Swiss cheese sold on the market. These differences could be attributed to the higher protein content of ewe's milk. The main fatty acids in the cheeses were palmitic acid, myristic acid, oleic acid, and capric acid. Among the organic acids, RM-EC had higher concentrations of lactic acid, while PM-EC was higher in propionic acid. The ewe's

cheeses emitted forty-six volatile compounds, including acids, aldehydes, ketones, esters, alcohols, and other compounds. PM-EC was characterized by the main compounds of Swiss-type cheese: acetic acid, butyric acid, ethyl butyrate, ethyl caproate, propanoic acid, and tetramethylpyrazine. Sensory evaluation showed that the new dairy products were generally appreciated, and PM-EC was the most preferred by the judges. This research has enabled the development of new ewe's milk products, which could stimulate the valorisation of a sector that has been long neglected and still has a large margin of improvement.

Key words: ewe's cheese, novel dairy products, Swiss-type cheese, microbiological safety, sensory evaluation.

Introduction

The Italian agrifood industry is a key sector of the country's economy. Several local economies rely on the strategic production chains of foods and wines that have earned a recognition of quality status. Among these foods, dairy products, particularly cheeses, are of importance for milk producers, transformation industries, food traders, and distributors [1]. Over the past 20 years, cheese consumption has been on a steady rise [2].

Among cheeses, Swiss-type cheese is long-ripened, hard cheese known for its characteristic round, regular holes (eyes) and slightly sweet flavor. This cheese is consumed almost worldwide and is manufactured in almost all industrialised countries [3], usually from raw cow's milk. Cows' milk stands as the predominant milk type globally for cheese making, primarily due to its ability to yield high-quality final products. This

superiority arises from the specialized breeding practices within the cattle industry and the efficient milk processing techniques employed. These factors contribute to optimal yields and consistent quality. While there has been recent growth in the production of non-bovine milk varieties [4], cow's milk remains the dominant choice. However, in specific peripheral and mountainous regions, such as the Mediterranean areas, ewe breeding takes center stage in the local agricultural economy [5]. These regions prioritize ewe's milk for cheese production, emphasizing its importance in sustaining livelihoods and culinary traditions.

Currently, non-bovine milk (including goat and ewe milk) amounts to 133 million tons, representing more than 17% of total milk output worldwide [6]. Ewe's milk is not extensively utilized in comparison to cow or goat milk. While goat milk has gained prominence and offers various health benefits, ewe's milk remains relatively less utilized globally, resulting in a narrower range of derived products. However, its unique properties make it an interesting option for specific applications. The most famous ewe's cheeses produced in Europe are Roquefort in France, Pecorino in Italy, Feta in Greece, Manchego in Spain, and Bryndza in central and eastern Europe. In Italy, ovine milk is predominantly employed in the production of Pecorino cheese which comes in several varieties, made in various regions, particularly in the central and southern areas. However, the production of only typical cheeses does not allow for innovation in the ovine milk sector. Given the nutritional advantages of sheep milk over goat and cow milk [7], research efforts are essential to unlock the potential value of sheep milk by creating innovative products through ewe's milk processing. In recent years, there has been growing interest in revitalizing sheep breeding and promoting ovine milk production in rural areas [8]. Thus,

these areas, often characterized by challenging environmental conditions and dwindling agricultural activity, require the following targeted initiatives to sustain local economies and preserve cultural heritage: sheep breeding revival; ovine milk production; sustainable land use. The cheese industry's expansion is strongly influenced by consumers' demand for unusual (unique) cheeses [2]. Thus, the production of new cheeses, particularly those made from sheep's milk, could be instrumental in enhancing the value of marginal rural areas.

The objective of this study was to create a new type of ewe's cheese using Swiss cheese technology. To validate the new protocol, ewe's Swiss-type cheese was produced using both raw and pasteurized milk. The cheeses were evaluated for their microbiological traits and compared based on their physicochemical parameters, fatty acid profile, organic acid, volatile compounds and sensory acceptability.

Materials and methods

Raw materials and starter culture preparation

The ewe's whole milk used in this study was obtained from "Valle del Belìce" sheep reared in several farms within Agrigento province (Sicily, Italy). The collected milk was transported in a temperature-controlled environment to "Cooperativa Agricola Tumarrano" located in Cammarata (Agrigento, Italy) where it was transformed. Raw milk before pasteurization was characterized as follows: pH 6.63, lactose 4.50%, fat 6.22%, protein 5.72%, casein 4.35%, and urea 32.55 mg/dL. Pasteurization treatment occurred at 72 °C for 15 s in a P75 50/2 pasteurizer (Tecnolat S.p.a., Nocera Inferiore, Italy). Lactic acid fermentation took place using freeze-dried commercial starters consisting of *Streptococcus*

thermophilus (LYOBAC-D GDA). To obtain the typical eyes expected in a Swiss-type cheese, propionic acid bacteria (PAB) were inoculated, and propionic fermentation occurred by means of freeze-dried commercial starters consisting of *Propionibacterium freudenreichii* (LYOBAC-D PROPRIONIBACTERIUM). Both freeze-dried cultures were purchased from the enterprise Alce International s.r.l. (Quistello, Italy) and were inoculated directly into the tank milk, after reactivation was performed following manufacturer's instruction. Briefly, lyophilized cells (5 units) were revitalized in 2 L of ewe's milk and kept under gentle agitation for 10 min before addition to the final milk volume.

Ewiss cheese production and sample collection

The experimental plan involved two distinct productions (Figure 1). The first production was carried out using raw milk (RM), while the second production employed pasteurized milk (PM). The novel ewe's cheese manufactured using Swiss-type cheese technology was named "Ewiss cheese" (EC).

The production of this novel cheese followed the protocol outlined in Figure 2. Specifically, a volume of 500 L of ewe's milk was heated to 40 °C using a Pol-P-Polivalente (Inox Art Production S.r.l., Cammarata, Italy). After heat treatment, starter inoculation was performed through direct inoculum into the tank. Curdling of the milk took place by adding 245 g of extra lamb rennet paste (Caglificio Clerici, Cadorago, Italy), title 1: 10,000. The curd was mechanically broken down to the size of grains and cooked at 55 °C for 5 min under slow agitation as reported by Gaglio et al. [9]. The clot was then transferred into circular moulds that were pressed to facilitate the draining of the whey and

stewed at 45 °C, turning upside down in intervals of 10 min until pH values reached 5.15–5.20. Brining was carried out by immersing the cheeses in saturated brine at 8–12 °C for 7 d. After this, cheese ripening took place at low temperatures (4–7 °C) for 2 wk for acidification, then at higher temperatures (approx. 20–22 °C) for 8 wk for the development of PAB, and again at lower temperatures until 9 mo of ripening for the settlement of the eyes.

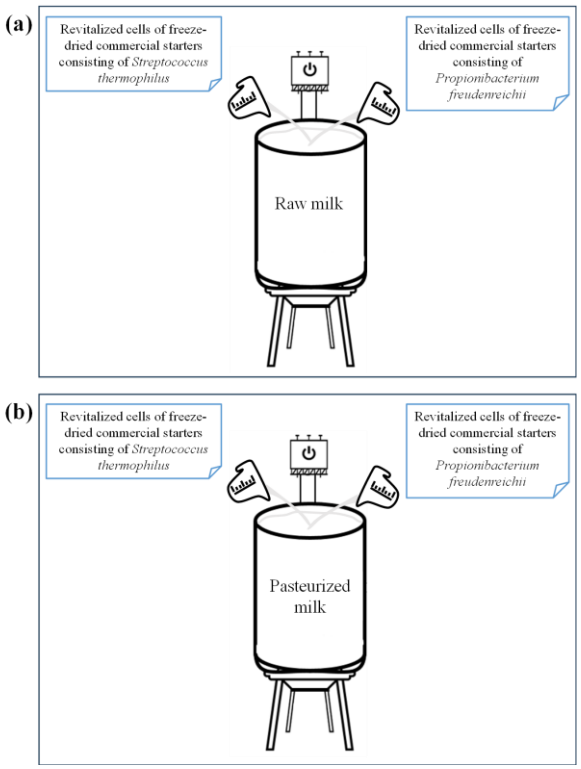

Figure 1. Experimental design of cheese productions. (a) production performed with raw milk and commercial starter cultures; (b) production performed with pasteurized milk and commercial starter cultures.

Cheeses productions were performed in triplicate over three consecutive months (three independent experimental replicates). Samples of commercial starter cultures (CSC), RM, PM, inoculated milk (IM), curd (C) and cheese (Ch) from RM-EC and PM-EC productions after 3, 6 and 9 mo were collected for analysis. All analyses performed on cheese at 9 mo of ripening were compared to those of a commercial cow's Swiss cheese at 9 mo of ripening (C-SC) (Lactalis Suisse, Küssnacht, Swiss).

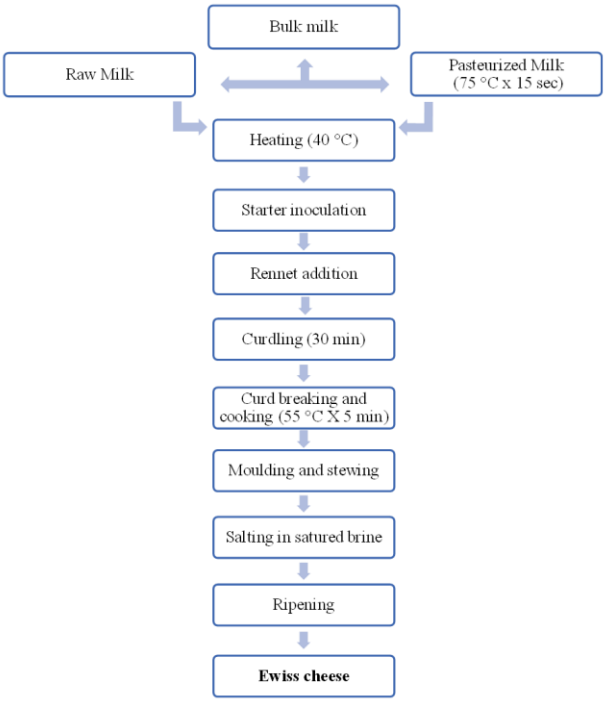

Figure 2. Flowsheet of Ewiss cheese production.

Monitoring of physical parameters

All samples collected throughout the cheese-making process were kept under refrigerated conditions during transport to the laboratory of Agricultural Microbiology (University of Palermo) where they were analyzed. The changes in pH were monitored using a portable Hanna HI98165 pH meter (Hanna Instruments, Woonsocket, RI, USA). The temperature of the cheeses was monitored during the entire process, from the molding until the 9th mo on ripening, using Thermo Button 22 T 8 K data loggers (VWR International S.r.l., Milan, Italy) which were put inside the cheese core at the molding step.

Microbiological analyses

The number of viable cells of all samples collected along the production chain of RM-EC and PM-EC productions were estimated using the serial decimal dilution method. To

perform this analysis, 1 mL of milk samples were directly serially diluted in Ringer's solution (Oxoid, Basingostoke, UK) while 10 g of freeze-dried commercial starters, curd and cheese samples were weighed aseptically and added with 90 mL of 2% sodium citrate solution. The samples were then homogenized in a stomacher BagMixer® 400 (Interscience, Saint Nom, France) at maximum speed for 2 min. Further serial dilutions were carried out in Ringer's solution [10].

The following groups of microorganisms were enumerated: total mesophilic and psychrophilic microorganisms on Plate Count Agar (PCA) with 1 g/L skimmed milk (SkM) incubated at 30 °C for 72 h and 7 d, respectively; mesophilic and thermophilic cocci on M17 agar with cycloheximide and anaerobically incubated at 30 and 44 °C, respectively, for 48 h; mesophilic lactobacilli on de Man-Rogosa-Sharp medium (MRS) agar acidified at pH 5.4 with lactic acid (5 mol/L) anaerobically incubated at 30 °C for 48 h; thermophilic lactobacilli on Whey-Based Agar Medium (WBAM) prepared as described by Settanni et al. [11] anaerobically incubated at 44 °C for 48 h; PAB on Pal Propiobac Plus media (PPP) incubated at 30 °C for 7 d, in anaerobic jars; enterococci on Kanamycin Aesculin Azide (KAA) agar incubated at 37 °C for 24 h; total coliforms on Violet Red Bile Agar (VRBA) incubated at 37 °C for 24 h; members of Enterobacteriaceae family on Violet Red Bile Glucose Agar (VRBGA) incubated at 37 °C for 24 h; *Escherichia coli* and *Salmonella* spp. was plated on Hektoen Enteric agar (HEA) and incubated at 37 °C for 24 h; coagulase-positive staphylococci (CPS) on Baird Parker (BP) agar with rabbit plasma fibrinogen, incubated at 37 °C for 48 h; *Listeria monocytogenes* was investigated by plating on *Listeria* selective agar base (LSAB) with SR0140E supplement; pseudomonads on *Pseudomonas* Agar Base (PSAB) with selective supplement incubated at 25 °C for 48

h; yeasts on Yeast Peptone Dextrose (YPD) agar with chloramphenicol (0.1 mg/mL) and incubated aerobically at 25 °C for 48 h; moulds on Potato Dextrose Agar (PDA) media incubated aerobically at 25 °C for 7 d; clostridia were estimated using the most probable number (MPN) technique inoculated into reinforced clostridial medium (RCM) supplemented with 1.4% (v/v) Na-lactate and incubated at 37 °C for 7 d.

All media and supplements used in this study were purchased from Oxoid, except PPP provided by Laboratoires Standa (Caen, France). All plate counts were performed in duplicate.

Monitoring of commercial starter culture and identification of autochthonous milk LAB

All presumptive LAB and PAB developed on MRS, M17 and PPP, respectively, inoculated with the cell suspensions of RM, IM and cheese samples at 9 mo of ripening were collected, purified, and tested for Gram reaction and catalase activity [12]. Differentiation of the isolates collected and dominance of *S. thermophilus* and *P. freudenreichii* (added as fermenting agents) over indigenous raw milk LAB were carried out by random amplification of polymorphic DNA (RAPD)-PCR analysis as described by Garofalo et al. [13]. Briefly, RAPD profiles obtained from LAB isolated from RM before commercial starter culture addition were compared with pure cultures of *S. thermophilus* and *P. freudenreichii* strains originating from freeze-dried commercial starters. Genotypic identification of the LAB resistant to the pasteurization process was performed at the AGRIVET Centre (Palermo, Italy) following the approach reported by Gaglio et al. [14].

Physicochemical composition of cheeses

The physicochemical properties of the cheeses at the different stages of ripening were investigated. The pH was measured by immersing a portable Hanna HI98161 pH meter (Hanna Instruments, Woonsocket, RI, USA) into homogenized cheese sample. Measurements were taken in three different parts, and the results were averaged. Dry matter, fat, ash content, and total acidity were detected according to the AOAC International method [15–18]. The salt content was determined following the method described by Hooi et al. [10]. Determination of nitrogen fractions was performed according to the IDF standard [19]. All compositional analyses were performed in duplicate.

The color of cheeses was detected by using a tristimulus chromometer Minolta CR-400 (Minolta, Osaka, Japan). L* (lightness), a* (redness/greenness), and b* (yellowness/blueness) were measured. The Chroma value was calculated as: $Chroma=\sqrt{(a^2+b^2)}$ according to the standard CIE [20].

Texture analysis, free fatty acid profile and organic acids composition

Texture analysis was performed on all cheese samples, while the fatty and organic acid profile was investigated in cheese samples after 9 mo of ripening. Cheese texture was measured by means of the TA.XTplusC Texture Analyser (Stable Micro Systems, Godalming, UK). The analysis included the measurement of primary parameters such as hardness, adhesiveness, springiness, cohesiveness and resilience, as well as secondary parameters such as chewiness and gumminess. Cheese samples (3 cm× 3 cm× 3 cm in size) were taken from the center of the cheese using a sharp knife. Samples were tested after they reached room temperature. The employed compression ratio was 50% from the initial

height of the samples using two compression cycles at a constant crosshead speed of 2 mm/s. The texture analyses were performed twice for two independent samples from each batch of cheese. A total of four readings were taken for each treatment. Measured parameters were obtained by the Exponent software (Stable Micro Systems) version (6.0.6.0) from TPA curves.

The fatty acid profile of cheeses was determined by Gas Chromatography (GC-MS/MGas Chromatography- Mass Spectrometry (GC-MS/MS) on 10 g of grated cheese samples with the esterification of fatty acids to their methyl esters based on the methodology of De Jong and Badings [21] with modification. Briefly, the sample injected (1 µL) with a split ratio of 1:40, through a GC-MS/MS (Agilent, 7890B GC -7010B MS Agilent Technologies Inc., Santa Clara, CA, USA) with a flame ionization detector with an autosampler (Gerstel, Germany). The separation of the fatty acids was achieved in a capillary Agilent J& W DB-WAX column (60 m x 0.25 µm x 0.25 µm) using helium as the carrier gas (1 mL/min). The oven temperature was held at 50 °C for 1 min, raised to 200 °C at a rate of 25 °C/min held for 10 min and then to 230 °C at a rate of 3 °C/min held this temperature for 26 min. The inlet temperature and detector were set to 250 °C and 300 °C, respectively. Fatty acid identification was verified by comparing the sample peak retention times with the reference standards (Supelco 37 Component FAME Mix, Sigma-Aldrich, St. Louis, MO, USA).

Organic acids and lactose were determined on a homogeneous puree of cheese samples through an HPLC Shimadzu Nexera 2 (Kyoto, Japan), as described by Manolaki et al. [22] with some modifications. In brief, the chromatograph included a ICSep ICE-Coregel 87H column (30 cm x 7.8 mm x 9µm, Transgenomic, USA) and a Refractive Index Detector

(RID). The mobile phase was 4 mM H2SO4 (0.4 mL/min). The data were recorded and analysed by LabSolutions software, and the concentrations were tentatively calculated using standard curves.

Detection of volatile compounds

The volatile composition of Ewiss cheese was determined by the headspace solid-phase microextraction method (HS-SPME) and gas chromatography/mass spectrometry (Agilent 7890B GC, 7010B MS triple quadrupole, MS Agilent Technologies Inc.) after 9 mo of ripening. The samples were kept at 60 °C for 15 min and the volatile compounds were adsorbed with a coated fiber (Carboxen TM/PDMS StableFlexTM) for 45 min. The samples were then injected into a capillary column (60 m x 0.25 mm i.dx 0.25µm, J&W Scientific, Folsom, CA, USA) with a desorption time of 5 min. The column temperature was raised to 90 °C with an increase of 3 °C per min after waiting 4 min at 40 °C and then maintained at 130 °C for 4 min with an increase of 4 °C per min. Finally, the temperature was raised to 240 °C increasing by 5 °C and held at this temperature for 8 min. The carrier gas was He (1 mLvb/min). The energy of the electrons was 70 eV and the mass range was 30–600 m/z. The partition ratio was 1:10. The volatile compounds were identified using the NIST library and expressed as percentages of the peak area of the total ion chromatogram in a heat map.

Sensory evaluation

A sensory evaluation was conducted in a sensory analysis room allocated in the Department of Agricultural, Food and Forest Science (SAAF) of the University of Palermo

under artificial light. Panel members (n = 14, 8 females, 6 males, ages ranging from 25 to 54) were recruited from students and staff of the same department. To recognize specific attributes of the Swiss cheese typology, assessors were instructed to use procedures consistent with international standards for the training of descriptive panels [23]. All samples were cut 24 h before evaluation, placed into the evaluation containers, and refrigerated overnight. Samples were removed from refrigeration 1 h before evaluation to allow equilibration to room temperature. Cheeses (15 g) were served in plastic dishes coded with three random digits. Panelists used unsalted crackers and water to clean their palates between samples and were unaware of the tested sample. Samples were analyzed for their olfactory traits (odor intensity, butter odor, milk odor, unpleasant odor), appearance attributes (rind, color, eye size, eye distribution, elasticity, and firmness), and gustatory features (saltiness, sweetness, bitterness, typical Swiss cheese flavor, persistence of taste, unpleasant flavors, and overall satisfaction). All attributes were rated on a 9 cm line scale, in which direction was from left to right with increasing intensities from “low” at 0 to “high” at 9. The results obtained by the panelists were averaged.

Statistical Analyses

Microbiological counts, and physicochemical parameters were analyzed by analysis of variance (ANOVA); the model included the effects of milk treatment (raw and pasteurized). Tukey’s post hoc test was used to estimate significant differences. Statistical significance was set at $p < 0.05$. The concentrations of Volatile Organic Compounds (VOCs) emitted from ovine cheeses were visually depicted using a heat map generated through ascending hierarchical clustering. The color gradient represented the VOC

concentrations, transitioning from yellow (indicating lower concentrations) to red (indicating higher concentrations). All statistical processing was performed with XLStat software version 2020.3.1 for Excel (Addinsoft, New York, NY, USA).

Results

Acidification process, ripening temperature, and microbiological characteristics

During cheese making, the acidification process was followed in both RM-EC and PM-EC trials, and the pH kinetics were quite similar. Raw milk had an initial pH of 6.7 and reached a pH of 6.4 at molding. Temperature was monitored both during production and during ripening. Data loggers present in the cheese paste allowed to monitor core temperature throughout the ripening period. The results showed an average temperature of 44.7 °C immediately after molding. After 24 h, the temperature reached 16 °C and decreased slowly until the third mo of ripening with an average temperature of 14 °C. Data loggers recorded a slow rise in temperature from the 3rd mo of ripening and the temperature registered at the 9th mo was 18 °C.

Table 1 shows the levels of microbiological counts. Raw milk hosted levels of total mesophilic microorganisms (TMM), mesophilic coccus and rod LAB at 10^6 CFU/mL and thermophilic coccus and rod LAB at 10^4 CFU/mL. Following pasteurization, the levels of these bacteria decreased by approximately 3 Log cycles. Regarding undesired bacterial groups, especially total coliforms, members of the Enterobacteriaceae family, pseudomonads, and *E. coli* they were found at 10^3 CFU/mL in RM and completely disappeared in PM. The dried starter cultures LYOBAC-D GDA and LYOBAC-D PROPRIONIBACTERIUM showed a dominance of thermophilic LAB cocci and PAB,

respectively, with levels above 10 Log CFU/g. After addition, the resulting levels of thermophilic LAB cocci and PAB were approximately 10^7 CFU/mL in both RM and PM. The curds of the two cheese trials showed significant differences in TMM. The curds obtained from RM-EC production achieved higher values and showed a rising trend up to the 6th mo of ripening in the cheese, after which it decreased slightly. The same behavior was found for PM-EC production, but in the two productions, the levels differed by about 1 Log cycle. Total psychrophilic microorganisms showed a decreasing trend in 9-mo ripened cheeses. Among the heterogeneous group of LAB, mesophilic cocci dominated samples of both trials, whereas rod-shaped thermophiles presented the lowest values in cheeses at the end of maturation. Additionally, 6- and 9-mo ripened cheeses obtained by PM exhibited slightly higher values than the respective cheeses processed by RM.

In PM-EC production, PAB reached the highest levels in the Ch-3 samples and remained high (around 10^7 CFU/g) even in the Ch-9 samples. Enterococci differed significantly among cheese productions and were below the detection limits (<2 Log CFU/g) in curd from PM-EC production.

Among the undesirable bacteria investigated, curd and cheese samples from PM-EC production did not show detectable levels. However, total coliforms and enterobacteria were detected in curd (5.15 and 5.39 Log CFU/g, respectively) and Ch-3 (2.87 and 3.87 Log CFU/g, respectively) from RM-EC production, but were no longer counted in Ch-6 and Ch-9 samples. No pathogenic microorganisms of dairy interest such as *L. monocytogenes*, *Salmonella* spp. and CPS were ever found in any sample.

Table 1. Microbial loads[a] of samples.

Samples	Microorganisms[b]													
	TMM	TPM	Mesophilic cocci	Mesophilic rod	Thermophilic cocci	Thermophilic rod	Propionic acid bacteria	Enterococci	Total coliforms	Enterobacteriaceaee	*E. coli*	Pseudomodads	Yeasts	Moulds
RM	5.95[a]	4.83[a]	5.86[a]	5.91[a]	4.36[a]	4.84[a]	<2[a]	2.41[a]	3.03[a]	3.25[a]	2.91[a]	3.39[a]	2.79[a]	2.15[a]
PM	3.17[b]	2.11[b]	3.07[b]	3.21[b]	1.94[b]	1.55[b]	<2[a]	<1[b]	<1[b]	<1[b]	<1[b]	<1[b]	<1[b]	<1[b]
SEM	0.382	0.375	0.382	0.370	0.334	0.355	-	0.331	0.415	0.445	0.426	0.506	0.383	0.296
P - value	<0.0001	0.001	<0.0001	<0.0001	0.001	<0.0001	-	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001
C RM-EC	7.54[a]	4.59[a]	7.45	5.14	6.84[a]	4.86[a]	7.64[a]	5.24[a]	5.15[a]	5.39[a]	4.04[a]	4.51[a]	3.57[a]	1.65[a]
C PM-EC	7.37[b]	4.24[b]	7.33	5.05	6.42[b]	4.15[b]	5.94[b]	<2[b]	<1[b]	<1[b]	<2[b]	<2[b]	<2[b]	<2[b]
SEM	0.028	0.056	0.021	0.030	0.063	0.103	0.234	0.718	0.706	0.738	0.553	0.618	0.489	0.226
P - value	0.04	0.03	0.061	0.423	0.012	0.005	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Ch-3 RM-EC	7.75	4.32[a]	7.40[b]	7.78[a]	7.63	6.15[b]	8.23[b]	6.85[a]	2.87[a]	3.87[a]	<2	<2	<2	<2
Ch-3 PM-EC	7.42	3.35[b]	8.12[a]	6.92[b]	7.59	6.87[a]	8.71[a]	3.72[b]	<1[b]	<1[b]	<2	<2	<2	<2
SEM	0.058	0.134	0.104	0.118	0..009	0..105	0.069	0.430	0.394	0.530	-	-	-	-
P- value	0..071	<0.0001	0.004	<0.0001	0.196	0.005	0.003	<0.0001	<0.0001	<0.0001	-	-	-	-
Ch-6 RM-EC	8.01[a]	4.34[a]	7.53[b]	7.76[b]	6.21[b]	6.89[b]	7.21[b]	4.44[a]	<1	<1	<2	<2	<2	<2
Ch-6 PM-EC	7.46[b]	3.54[b]	8.25[a]	8.24[a]	7.47[a]	7.57[a]	8.20[a]	3.67[b]	<1	<1	<2	<2	<2	<2
SEM	0.079	0.112	0.100	0.068	0.173	0.094	0.136	0.107	-	-	-	-	-	-
P - value	0.003	0.001	<0.0001	0.002	<0.0001	<0.0001	<0.0001	<0.0001	-	-	-	-	-	-
Ch-9 RM-EC	7.07	4.35[a]	6.44[b]	7.17	6.15[b]	6.10[b]	7.08[a]	4.04[a]	<1	<1	<2	<2	<2	<2
Ch-9 PM-EC	6.93	3.09[b]	7.09[a]	7.12	6.54[a]	6.87[a]	6.86[b]	3.21[b]	<1	<1	<2	<2	<2	<2
SEM	0.032	0.174	0.091	0.040	0.065	0.108	0.033	0.118	-	-	-	-	-	-
P - value	0.215	<0.0001	0.001	0.744	0.044	0.001	0.014	0.002	-	-	-	-	-	-

Results indicate mean values of four plate counts (carried out in duplicates for two independent productions). [a] $\log_{10}$ cfu/g for curd and ripened cheeses. [b] Abbreviations: TMM, total mesophilic microorganisms; TPM total psychrotrophic microorganisms; *E.*, *Escherichia*. RM, raw milk; PM, pasteurized milk; C, curd; EC, Ewiss cheese; Ch-3, cheese ripened 3 mo; Ch-6, cheese ripened 6 mo; Ch-9, cheese ripened 9 mo; n.e., not evaluated. SEM, standard error of the mean. On the column: a, b = $p < 0.05$.

Persistence of commercial starter cultures and identification of autochthonous milk LAB

After enumeration, 52 presumptive LAB (Gram positive and catalase negative) and 61 presumptive PAB (Gram positive and catalase positive) were isolated from commercial starter cultures, RM, RM-EC and PM-EC at 9 mo of ripening. All isolates were subjected to RAPD-PCR to follow the added commercial starter strains throughout the production of cheeses by polymorphic profile comparison. The dendrogram reported in Figure 3 shows only 12 of the 113 isolates analyzed. These 12 isolates represent bacteria collected from various samples at least once. The remaining 101 strains were excluded from Figure 3 because they exhibited identical RAPD profiles as other cultures from the same sample. Two major RAPD clusters which included the strains *S. thermophilus* and *P. freudenreichii*, both isolated from freeze-dried commercial starters, were identified. In particular, only *P. freudenreichii* were detected both in PM and RM cheeses at 9 mo of ripening.

Four different strains (ESC1, ESC2, ESC17 and ESC22), isolated from RM, were identified by 16S rRNA gene sequencing as *Enterococcus faecalis* (Ac. No. PP430126 and PP430128) and *Lactococcus lactis* (Ac. No. PP430125 and PP430127). As reported in Figure 3, these strains were not detected in any of the samples analyzed after milk inoculation with commercial starter cultures. These results confirmed the dominance of the

added *P. freudenreichii* strain, originating from commercial starter culture, over autochthonous milk LAB.

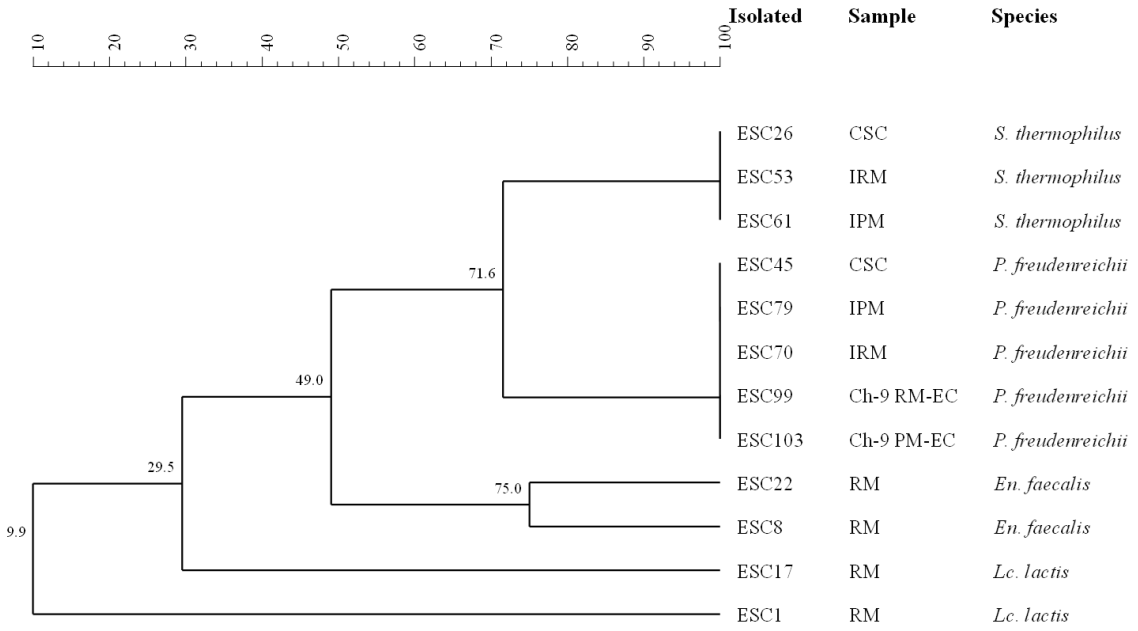

Figure 3. Dendrogram obtained with combined RAPD-PCR patterns generated with three primers for LAB and PAB strains isolated during cheese productions. Abbreviations: CSC, commercial starter culture; RM, raw milk; IRM, inoculated raw milk; IPM, inoculated pasteurized milk; Ch-9 RM-EC, Ewiss cheese at 9 mo of ripening produced with raw milk; Ch-9 PM-EC, Ewiss cheese at 9 mo of ripening produced with pasteurized milk; *En.*, *Enterococcus*; *Lc.*, *Lactococcus*.

Physicochemical characteristics

The results of the physicochemical analysis are shown in Table 2. During ripening, pH increased in both productions by about half a point. Moreover, no statistically significant differences with commercial cow's cheese were observed in the pH value of the ewe's cheese. A similar trend was observed for dry matter content. RM-EC production, however, had higher values compared to PM-EC production. Notwithstanding, both deviated significantly from the C-SC, which resulted in a much lower value. Fat content, instead, firstly increased in both productions by 2 percentage points then decreased in the 9-mo ripened cheese within a value superimposable to that of commercial cheese. Protein values

were almost similar for all cheeses. Throughout the ripening process, the behavior of the two cheeses diverged in terms of salt content. For RM-EC, the salt content increased until the 6th mo and remained stable thereafter. However, in the case of PM-EC, salt content showed a slight decline at the 6th mo, followed by a subsequent rise of nearly one point by the 9th mo of ripening. In terms of total acidity, the maximum percentage (3.65%) was observed in the 9th mo ripened PM-EC sample.

Colorimetric parameters (Table 2) indicated that, considering cheeses at the end of ripening, lightness was comparable with the commercial cow's cheese; instead, the redness attribute was higher in the PM-EC cheese (78.62) whilst C-SC registered the highest value for yellowness parameters (22.39). Lastly, Chroma and Hue angle were significantly higher in cow's commercial cheese (22.54 and -0.12, respectively) than in ewes' cheeses. During the aging period these two parameters both tended to increase in novel cheeses.

Table 2. Physicochemical parameters of Ewiss cheeses.

Samples	Parameters											
	pH	Dry matter (%)	Fat (% in DM)	Protein (% in DM)	Salt (% in DM)	Ash (% in DM)	Acidity (%)	Lightness (*L**)	Redness (*a**)	Yellowness (*b**)	Chroma	Hue angle
Ch-3 RM-EC	4.75^{a}	67.86^{a}	53.42^{a}	29.87^{b}	3.46^{a}	7.30^{a}	1.89^{b}	76.61	-3.60^{b}	11.90^{b}	12.43^{b}	-0.30
Ch-3 PM-EC	4.67^{b}	64.19^{b}	48.55^{b}	43.50^{a}	2.52^{b}	6.30^{b}	2.03^{a}	75.75	-4.75^{a}	14.81^{a}	15.56^{a}	-0.32
SEM	0.50	6.62	5.19	4.05	0.31	0.69	0.23	7.73	0.44	1.37	1.44	0.196
P - value	0.01	<0.0001	0.023	<0.0001	<0.0001	<0.0001	0.40	0.284	0.009	0.01	0.01	0.03
Ch-6 RM-EC	5.11	69.97^{b}	55.02^{a}	31.77^{b}	3.54^{a}	6.86^{a}	1.72^{b}	81.80	-3.13^{b}	13.00^{b}	13.37^{b}	-0.24
Ch-6 PM-EC	5.00	66.42^{b}	51.19^{b}	39.42^{a}	2.28^{b}	6.28^{b}	2.62^{a}	82.61	-3.88^{a}	14.68^{a}	15.19^{a}	-0.26
SEM	0.46	6.25	4.88	3.54	0.31	0.60	0.23	7.54	0.36	1.29	1.34	0.03
P - value	0.218	<0.0001	0.031	<0.0001	<0.0001	<0.0001	0.004	0.616	0.005	0.001	0.001	0.196
C-SC	5.56^{b}	63.93^{c}	48.88	28.64^{b}	2.21^{c}	5.52^{c}	2.08^{c}	79.16	-2.67^{c}	22.39^{a}	22.54^{a}	-0.12^{c}
Ch-9 RM-EC	5.52^{b}	76.00^{a}	51.31	29.30^{b}	3.54^{a}	6.72^{a}	2.98^{b}	77.09	-3.39^{b}	14.95^{b}	15.33^{b}	-0.23^{b}
Ch-9 PM-EC	5.65^{a}	67.99^{b}	50.38	34.36^{a}	3.10^{b}	6.40^{b}	3.65^{a}	78.62	-4.15^{a}	16.26^{b}	16.79^{b}	-0.26^{a}
SEM	0.02	1.55	0.46	0.81	0.17	0.16	0.20	0.50	0.19	1.01	0.97	0.02
P - value	0.001	<0.0001	0.171	<0.0001	<0.0001	<0.0001	<0.0001	0.351	0.001	<0.0001	<0.0001	<0.0001

Results indicate mean values of three determinations carried out in duplicate for each of the two independent cheese-making.
Abbreviations: RM, raw milk; PM, pasteurized milk; EC, Ewiss cheese; Ch-3, cheese ripened 3 mo; Ch-6, cheese ripened 6 mo; Ch-9, cheese ripened 9 mo; DM, dry matter; SEM, standard error of the mean. On the column: a, b = $p < 0.05$.

Textures, fatty acids profile and organic acids composition

Texture profile analysis of the Swiss-type cheeses is presented in Table 3. The texture of Ewiss cheeses changed during ripening, specifically hardness, gumminess and chewiness increased from the 3rd mo of ripening until the end of maturation. Moreover, all three of these characteristics exhibited significant differences compared to the C-SC. No significant differences were found between the 9-mo ripened cheeses in terms of adhesiveness, springiness and cohesiveness. Lastly, resilience was lowered during ripening in the experimental ewe's cheeses, leaving the C-SC a significantly higher value with respect to others.

Table 3. Texture attributes in Ewiss cheeses.

Samples	Texture (N/mm)						
	Hardness (N)	Adhesiveness (N.sec)	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
Ch-3 RM-EC	150.77^{a}	-5.80^{a}	0.79	0.70	105.95^{a}	84.10^{a}	0.37
Ch-3 PM-EC	93.85^{b}	-5.22^{b}	0.83	0.75	70.86^{b}	58.80^{b}	0.40
SEM	13.35	0.62	0.07	0.06	8.87	7.14	0.03
P - value	<0.0001	<0.0001	0.196	0.074	0.001	0.008	0.140
Ch-6 RM-EC	158.11	-2.66	0.83	0.59^{b}	92.98	77.34	0.29
Ch-6 PM-EC	157.4	-8.55	0.84	0.67^{a}	106.10	89.03	0.32
SEM	13.89	0.98	0.07	0.05	8.76	7.37	0.03
P - value	0.972	0.070	0.482	0.028	0.363	0.318	0.060
C-SC	127.39^{b}	-53.77	0.86^{a}	0.75^{a}	96.10^{b}	83.04	0.41^{a}
Ch-9 RM-EC	284.29^{a}	nd	0.77^{c}	0.48^{b}	136.44^{ab}	105.61	0.21^{c}
Ch-9 PM-EC	222.15^{a}	-11.91	0.80^{b}	0.65^{a}	144.61^{a}	115.79	0.30^{b}
SEM	18.24	9.29	0.01	0.03	6.93	4.85	0.02
P - value	0.002	0.182	<0.0001	0.001	0.04	0.086	<0.0001

Results indicate mean values of four determinations carried out in duplicate for each of the two independent cheese-making.
Abbreviations: RM, raw milk; PM, pasteurized milk; EC, Ewiss cheese; Ch-3, cheese ripened 3 mo; Ch-6, cheese ripened 6 mo; Ch-9, cheese ripened 9 mo; SEM, standard error of the mean. On the column: a, b = $p < 0.05$.

The fatty acid profile is shown in Table 4. Only cheese at the end of aging was assayed. Palmitic acid (C 16:0) was the most abundant fatty acid in all the cheeses found in the C-SC at 35.91%, in RM-EC at 34.81% and in PM-EC at 26.88%. It was followed by oleic acid cis (C 18:1) in C-SC and PM-EC cheeses. Meanwhile, RM-EC featured a higher myristic acid (C 14:00) content (14.83) than oleic acid (12.33). Other fatty acids detected were similar among cheeses, except for capric acid (C 10:0), occurring in low amounts in C-SC (3.31) and in considerably higher amounts in ewe's milk cheeses (11.17 and 9.68 in RM-EC and PM-EC, respectively).

Table 4. Free fatty acid profile in Ewiss cheeses.

Fatty acids	**Samples**				
	C-SC	RM-EC	PM-EC	SEM	*P* - value
C6	2.09^{c}	3.30^{a}	2.86^{b}	0.09	<0.0001
C8	1.38^{c}	3.39^{a}	3.11^{b}	0.16	<0.0001
C10:0	3.31^{c}	11.17^{a}	9.68^{b}	0.60	<0.0001
C12:0	4.01^{c}	6.46^{a}	5.53^{b}	0.18	<0.0001
C14:0	13.32^{b}	14.83^{a}	12.44^{c}	0.18	<0.00010
C15:0	1.23	1.21	1.18	0.01	0.105
C16:0	35.91^{a}	34.81^{a}	26.88^{b}	0.72	<0.0001
C16:1	1.76^{a}	1.17^{b}	1.01^{b}	0.06	<0.0001
C18:0	9.87^{a}	5.82^{b}	9.57^{a}	0.33	<0.0001
C18:1 *cis*	19.65^{a}	12.33^{c}	17.00^{b}	0.54	<0.0001
C18:1 *trans*	1.66^{b}	0.90^{b}	2.85^{a}	0.15	0.002
C18:2	1.95	1.60	3.12	0.17	0.145
C18:3 n3	0.45^{c}	0.60^{b}	1.11^{a}	0.05	<0.0001

Results indicate mean values of six determinations (carried out in triplicate for two independent productions). Data within a line followed by the same letter are not significantly different according to Tukey test. Abbreviations: C-SC, commercial Swiss cheese; RM-EC, raw milk Ewiss cheese; PM-EC, pasteurized milk Ewiss cheese; SEM, standard error of the mean. On the row: a, b, c = $p < 0.05$.

As stated previously, the composition of organic acids is given only for cheese at the end of maturation (Table 5). When analysed, the three cheeses showed statistically significant differences in all organic acids. Lactic acid concentration was more abundant in

RM-EC production (3467.2 ppm). The butyric acid concentration in ewe's cheeses (326.1 and 312.3 ppm in RM-EC and PM-EC, respectively) was nearly double that found in C-SC (151.4 ppm). Cheese made from PM showed a higher concentration of propionic acid (1978.9 ppm) as well as fumaric acid (502.4 ppm); this latter acid, however, was not found in C-SC.

Table 5. Organic acids profile in ripened Ewiss cheeses.

Organic acids	**Samples**				
	C-SC	RM-EC	PM-EC	SEM	*P* - value
Lactic acid	576.5^{c}	3467.2^{a}	741.4^{b}	234.32	<0.0001
Butyric acid	151.4^{c}	326.1^{a}	312.3^{b}	14.03	<0.0001
Fumaric acid	nd	367.3^{b}	502.4^{a}	37.53	<0.00010
Propionic acid	1525.7^{b}	715.7^{c}	1978.9^{a}	92.37	0.105
Glucose	157.4^{a}	20.5^{c}	42.6^{b}	10.61	<0.0001

Results are expressed in parts per million (ppm). Data within a line followed by the same letter are not significantly different according to Duncan test. Abbreviation: C-SC, commercial Swiss cheese; RM-EC, raw milk Ewiss cheese; PM-EC, pasteurized milk Ewiss cheese; SEM, standard error of the mean; nd, not detected. On the row: a, b, c = $P < 0.05$.

Composition of volatile organic compounds

The volatile organic compounds (VOCs) of the three kinds of cheese were analyzed using SPME-GC/MS, and the results are shown in Figure 4. The study evidenced differences in quality between the C-SC, RM-EC, and PM-EC cheeses. A total of 46 compounds were identified among the chemical classes of carboxylic acids, aldehydes, ketones, esters, alcohols, and other compounds. Carboxylic acids were the main class of the VOC in all samples analysed, followed by ketones. Esters were not found in the C-SC, but were found in the RM-EC (8.25) and PM-EC (3.04) samples. The PM sample had 33.10% propionic acid, which was the most abundant compound among the acids found in

percentage terms. Acetoin was the most abundant ketone found in the C-SC, while the experimental cheeses had a higher percentage content of 2-heptanone. Benzaldehyde was the most notable constituent within the group of aldehydes found in C-SC. However, a negligible amount was found in sheep's milk cheeses. The study found that the presence of ethyl propionate, ethyl caproate, and ethyl caprylate was significantly higher in RM cheese than in PM sheep cheeses. Conversely, ethyl butyrate and propyl propionate were only detected in PM sheep cheeses. Alcohols were the most abundant group in the RM sample, with ethyl alcohol, (S)-(+)-2-heptanol, and 2-butanol as the most abundant components.

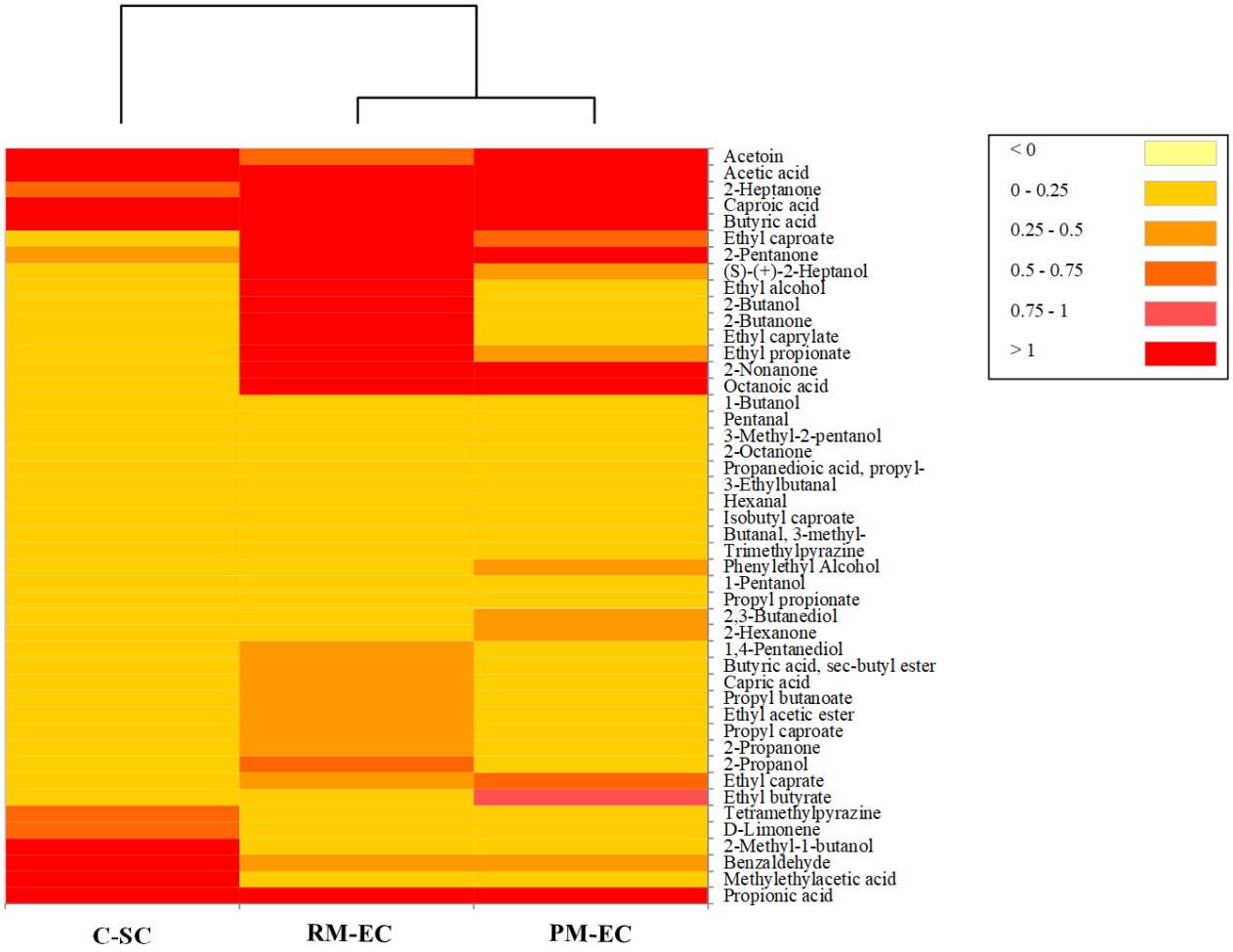

Figure 4. Volatile compounds of Ewiss cheeses using HS-SPME-GC-MS analysis. The heat map plot depicts the relative concentration of each VOCs. Abbreviations: C-SC, commercial Swiss cheese; RM-EC, raw milk Ewiss cheese, PM-EC, pasteurized milk Ewiss cheese.

Sensory assessment

The spider plot in Figure 5 shows the sensory assessment of the new ewe's milk cheese and that of the commercial cheese. The sensory characteristics of the cheeses were evaluated at the end of ripening. Ewe's cheeses were compared to C-SC, and the results did not reveal statistically significant differences ($p > 0.05$) for most of the attributes considered. Overall, none of the cheeses exhibited unpleasant odors, and the sensory characteristics varied slightly among the cheeses evaluated. Cheeses made from RM showed a higher odor intensity, firmness, and saltiness. Sweetness and milk odor were predominant in C-SC, while cheese made from PM received the lowest scores for color and eye distribution. Statistically significant differences ($p < 0.01$) were detected in the size of the eyes, elasticity of the cheeses, and typical Swiss cheese flavor; as expected, C-SC received the highest scores, while RM-EC the lowest. Even though there were no statistical differences, the PM cheeses were the most appreciated by the judges.

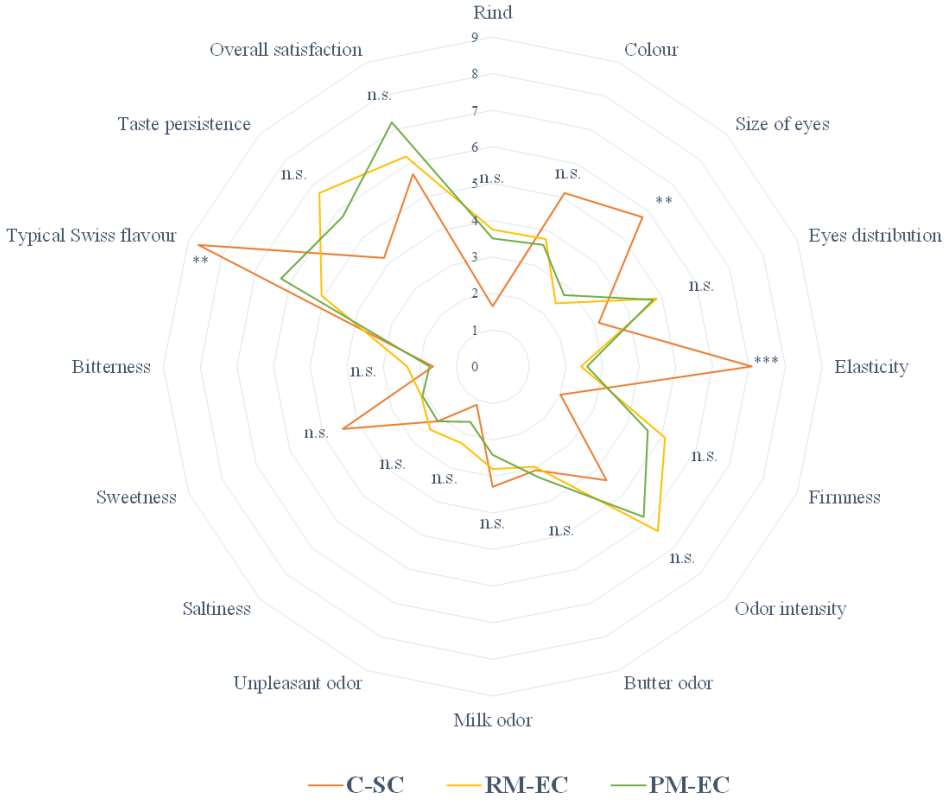

Figure 5. Sensorial assessment of Ewiss cheeses. Abbreviations: C-SC, commercial Swiss cheese; RM-EC, raw milk Ewiss cheese; PM-EC, pasteurized milk Ewiss cheese. *p* value: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s., not significant.

Discussion

Ewe's cheese products are an integral part of the cultural heritage of many Italian regions. Hard and semi-hard cheeses, with a medium to long ripening time, are the most representative. The use of raw milk ties these products to their specific production areas and ensures a high microbiological biodiversity. The production and marketing of these dairy products contribute significantly to the country's economy [24]. However, managing the sanitary quality of ewe's milk poses many difficulties, such as low per animal, milking systems, and herd rearing conditions and to control the microbiological quality of the milk, pasteurization treatment is often applied to ewe's milk.

The aim of this work was twofold: i) to develop a new ewe's milk product using the technology of a well-known and appreciated cheese such as Swiss-type cheese; and ii) to compare the new cheeses by processing both RM and PM. Considering that cheeses are unique ecosystems that contain different types of microorganisms [25], the microbiological parameters were assessed first by plate counts.

The plate count analysis performed on milk samples revealed that RM hosted consistent levels of TMM and LAB, while PM was characterized by cell densities of these bacteria at three orders of magnitude lower. Similar results were previously reported by Garofalo et al. [13] and Barbaccia et al. [26] in RM and PM used to produce ovine pressed and stretched cheeses. Both PM and RM cheeses resulted safe in terms of health and hygiene. Although RM-EC initially showed the presence of undesirable bacteria in RM and curd samples, these were undetectable by the end of the ripening. Reassessing the safety of hard raw milk cheeses such as Cheddar, Swiss and Italian hard cheeses, when matured for a given period revealed that this process does not favor the survival of pathogens, as observed in previous studies [27–29]. Regarding LAB, the two experimental cheeses exhibited differences in the microorganisms detected. In RM cheese, there was a higher occurrence of PAB (7.08 Log CFU/g), which are typical in Swiss-type cheeses [30]. In contrast, the thermophilic cocci and rod-shaped lactic acid bacteria had a higher load in the PM cheese sample (6.54 and 6.87 Log CFU/g, respectively). Moreover, RM-EC reported a higher cell density of enterococci, which are non-starter LAB. These enterococci play a significant role in the development of sensory characteristics during cheese ripening [31], consistent with the findings of Terzić-Vidojević et al. [32] who recognized enterococci as an essential part of the natural microbial population of raw milk.

A total of four LAB strains isolated from RM were identified and allotted into *En. faecalis* and *Lc. lactis* species. These LAB species are typical of ovine milk microbiota [33]. However, none of these strains was found in RM-EC and PM-EC after 9 mo of ripening, confirming the ability of the added *P. freudenreichii* strain to persist and dominate during the ripening period [34].

The physicochemical composition of the cheeses was comparable in terms of fat content – 51.31% in RM-EC and 50.38% in PM-EC (both measured in dry matter). Ewe's milk is known for its richness in fat, protein, minerals, and vitamins, setting it apart from milk of other species [35]. However, protein content and acidity were higher in PM-EC. A similar finding was observed by Rezaei et al. [36] when investigating the physicochemical properties of Motal cheese to assess the impact of pasteurization. Interestingly, cheese color did not exhibit significant differences between the two types; both displayed similar lightness, yellowness, and chroma index.

Understanding the rheological properties of cheese is crucial for assessing its consistency, structure, and overall quality. These properties are influenced by factors such as composition, processing techniques, and storage conditions. Notably, the composition of cheese, including its fat, protein, and moisture content, significantly impacts its texture and mouthfeel [37]. In the context of this study, a texture analysis was conducted on two types of cheeses: RM-EC and PM-EC. Interestingly, they exhibited similar hardness and chewiness parameters. However, when considering other measured parameters—such as springiness, cohesiveness, and gumminess—differences emerged. PM-EC displayed slightly higher values in these aspects. This variation may be attributed to proteolysis, a

process affecting the texture of the experimental cheese, as also observed by Awad et al. [38].

Cheese not only provides essential compounds like calcium, proteins and vitamins, but it also contains numerous bioactive molecules. Among these, fatty acids play a crucial role [39]. The fatty acid profiles of raw and pasteurised milk cheeses exhibit significant differences across nearly all examined fatty acids. In both cheese types, capric acid (C10:0), myristic acid (C14:0), palmitic acid (C16:0), and oleic acid (C18:1 cis) dominate. However, the RM-EC sample has a higher proportion of these compounds. This variation is attributed to metabolic activities by the natural microbiota in raw milk [40]. Unfortunately, pasteurization leads to the inactivation of these bacteria and enzymes, which are characteristic of raw milk [41].

Organic acids play a pivotal role in determining cheese quality and serve as valuable indicators of starter activity during the ripening process. In ewe's milk cheese, water-soluble and short-chain organic acids arise through microbial metabolism. These acids include lactic, acetic, pyruvic, propionic, formic, and butyric acids, all of which contribute to the distinctive flavor and aroma of the final cheeses [42]. An intriguing finding from our research is the elevated propionic acid content observed in cheese made from PM. This particular acid is responsible for the characteristic eyes and nutty flavor commonly associated with Swiss artisanal cheese [30,43]. Despite pasteurization inactivating approximately 95% of enzymes, their activity remains significant in long-ripened cheeses.

The production of cheese involves several factors that impact its aroma. Among these factors, the origin of the milk and its treatment—whether raw or pasteurized—are crucial parameters [44]. According to existing literature, cheeses made from PM exhibit less

development of volatile compounds during the ripening process compared to their counterparts made from raw milk. Interestingly, when comparing raw ewe's milk cheese to cheeses made from raw milk of other species, the former displays the highest volatile content [45]. Our research aligns with these findings. In our study, the RM-EC cheese demonstrated remarkable versatility in terms of volatile compounds analyzed. It exhibited elevated levels of caproic and capric acids, which significantly contribute to the taste and aroma of cheese [46]. Additionally, higher amounts of 2-butanone and 2-heptanone, important ketones, impart a buttery smell and herbaceous notes in cheese [47]. However, it remains evident that the unique aroma of Swiss cheese is challenging to replicate [48].

Swiss cheese is characterized by several key volatile compounds, including acetic acid, butyric acid, ethyl butyrate, ethyl caproate, propanoic acid, and tetramethylpyrazine [49]. Interestingly, our study found that these compounds were present in both types of Ewiss cheeses (from RM and PM). However, contrary to our expectations, the PM-EC sample exhibited higher levels of some of these acids. In addition, it is worth noting that PM-EC also displayed elevated concentrations of ethyl butyrate and ethyl caproate, both of which significantly contribute to the flavor profiles of various cheeses, including Cheddar, Emmental, Grana Padano, and Pecorino. Even at low concentrations, these esters impart fruity notes to the cheese [47,50].

Flavor plays a pivotal role in the dairy industry, significantly impacting consumer acceptance and preference. The milk source (mainly cow, goat, or sheep) directly influences the taste and aroma of dairy products. Cheese aroma, in particular, results from a delicate balance of various aromatic compounds that, individually, cannot fully capture the complete sensory experience [51]. Considering the exceptional quality of ewe's milk—

known for its low allergenic activity and rich concentration of nutraceutical compounds—ewe's milk cheeses, whether raw or pasteurized, resonate with health-conscious consumers [52]. This underscores the importance of sensory analysis as an essential evaluation criterion. A product's global competitiveness hinges on its acceptance by discerning consumers. Our research reveals minimal differences between RM-EC and PM-EC cheeses. However, in terms of overall acceptance, the PM-EC sample received higher ratings from judges.

Variations in eye size and the distinctive Swiss flavor, likely influenced by milk type, suggest ample room for improvement in new cheese products. Swiss cheese production typically involves clarifying the milk before pouring it into the vat, a step that enhances eye development and distribution [53]. This clarification process may also account for the observed differences. Despite these nuances, parameters such as color, absence of unpleasant odor, and taste persistence present a positive outlook for these novel cheeses. These attributes align with modern consumer expectations [54]. Importantly, our findings encourage the production of ewe's milk cheeses, a category that remains relatively unknown and underappreciated. These cheeses, although associated with a robust animal aroma, hold promise even among young consumers unfamiliar with their unique qualities.

Conclusions

This research conducted an extensive analysis of the microbiological, physicochemical, VOC profile, and sensory characteristics of the novel Ewiss cheese. The cheese was manufactured from ewe's milk processed at an industrial level using freeze-dried

commercial starters, following the Swiss cheese-making method. Notably, the microbiological analysis revealed no statistically significant differences between RM-EC and PM-EC productions in terms of LAB composition. LAB cocci predominantly drove the acidification process. Chemical and textural analyses highlighted distinctions between the experimental Ewiss cheese and the commercial Swiss cheese, primarily attributed to the higher protein content in ewe's milk. Impressively, sensory evaluation indicated that the new cheese received high praise from judges. In the realm of developing ewe's milk products for modern consumers, this study stands as the pioneering successful attempt to create an ewe's cheese by adapting the well-established and beloved Swiss cheese-making process.

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Ending notes

Cheese, a globally consumed food, is produced through farming activities that yield high-quality traditional products. Beyond its culinary value, cheese contributes to ecological and non-market services (biodiversity conservation, flood prevention, water purification, etc.). Moreover, it fosters social cohesion in rural areas and preserves local cultural heritage. The rearing of sheep not only provides income and employment opportunities in disadvantaged, hill, and marginal areas, but also plays a crucial role in areas where the rural economy lacks diversification. However, the sheep sector has experienced significant changes over the past two decades. Cheese producers now face ongoing challenges that demand adaptation and innovation to maintain competitiveness. Strategic focus lies in enhancing farm technology and promoting innovation across practices, processes, and products within the small ruminant sector. To ensure the sheep sector's continued integration in the market, embracing more innovation and technology becomes essential, even as acceptance of these innovations remains a key sustainability challenge.

The primary objective of this doctoral program was to enhance the competitiveness of Sicilian dairy farms, enabling them to withstand the competitive pressures of emerging economies. Over the course of three years, different experimental cheese-making initiatives were carried out leveraging innovative technological processes. These trials resulted in the production of novel products, utilizing sheep's milk sourced from native Sicilian breeds. The product innovation was facilitated through the application of LAB selected from the Sicilian sheep dairy context. Additionally, the use of by-products from the supply chain such as grape pomace, and incorporated EOs was explored. All newly

developed products underwent comprehensive evaluation, considering microbiological, physicochemical, and sensory aspects.

Our findings led to the development of new products, totally connected to the territory of origin, starting from the raw material obtained by processing the milk of indigenous breeds, to the use of lactic acid bacteria isolated within the context of the Sicilian sheep chain. Each product was found to be safe from a microbiological point of view, rich in volatile organic compounds, and most importantly appreciated by the consumer, who is now open to new market trends and sensitive to the issues of sustainability and product authenticity.

As we conclude this research endeavor, it is evident that sheep's milk processing holds the potential to yield innovative products that contribute to the local economy and can emerge as strong contenders in the national market.

Papers published in the three-year PhD course

Publications in international journals:

1. Gaglio, R.*, Botta, L., **Garofalo, G.**, Guida, G., Settanni, L., Lopresti, F. (2020). *In vitro* antifungal activity of biopolymeric foam activated with carvacrol. Journal of Food Quality and Hazards Control, 7, 136–141. https://doi.org/10.18502/jfqhc.7.3.4145.
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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.

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1. **Garofalo, G.**, Gaglio, R., Sardina, M.T., Portolano, B., Settanni, L. Development of new dairy products using raw ewe's milk to produce a "Swiss" type cheese. 6th International Conference on Microbial Diversity 2021. Convegno online, 14-15 Dicembre 2021. 78.
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1. Liguori, G., Greco, G., Cannatella, M., **Garofalo, G.**, Settanni, L., Inglese, P. (2021). Valutazione dell'applicazione di edible coating sulla qualità dei frutti di ficodindia in IV gamma. XIII Giornate Scientifiche SOI. Catania, 22-23 giugno 2021. 54.
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1. **Garofalo, G**. L'innovazione di settore e di processo nel settore lattiero-caseario ovino. Convegno "Triprol@c - Nuove tecnologie per le produzioni lattiero-casearie ovine". Palermo (PA), 19 Giugno 2023.
2. **Garofalo, G**. Application of innovative production protocols and selected lactic acid bacteria for the enhancement of dairy products. In Virtual Workshop on the Development in the Italian PhD Research on Food Science, Technology and Biotechnology. Convegno online, 14-15 Settembre, 2022.