AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF FOOD SCIENCE & HUMAN NUTRITION
LABORATORY OF MICROBIOLOGY & BIOTECHNOLOGY OF FOODS

POSTGRADUATE STUDIES PROGRAM
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Postgraduate Diploma Thesis

Identification of microbiota of Sicilian artisanal raw milk cheese

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Χαρακτηρισμός της μικροχλωρίδας παραδοσιακού τυριού της Σικελίας από νωπό γάλα

Panagiota N. Xenou

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MSc Food Safety & Quality Management Systems
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ABSTRACT

Several studies performed in the sector of ruminant husbandry have shown the crucial role of feeding strategies in influencing the overall quality of animal-based foods, especially dairy foods, with the development of volatile organic compounds that influence aroma and taste. To evaluate the effects of dietary olive cake, a source of bioactive phenolic compounds, as feed supplementation of lactating dairy cows, on the microbiological profile and structure of the LAB bacterial community, as well as on its distribution at the end of maturation of Caciocavallo or in the Sicilian dialect Cosacavaddu Ibleo, control (CTR) and experimental (EXP) cheesesamples, produced in two cheesemaking trials, were subjected to plate counting and PCR-DGGE (denaturing gradient gel electrophoresis) analyses. In addition, the LAB population from analyzed cheeses was characterized through preliminary in vitro safety tests. Microbiological data showed the absence of *Salmonella spp., L. monocytogenes, E. coli, and E. coli O157* in all cheese samples. The lactic acid bacteria (LAB) trend was quite similar in both control and experimental cheeses, reaching a value above 7.00 $\log_{10}$ CFU/g. Both mesophilic and thermophilic lactococci showed a similar trend, except for the CTR sample in the second cheesemaking where mesophilic lactococci reached the value of 8.78 $\log_{10}$ CFU/g the highest value ever. PCR-DGGE fingerprinting revealed slight microbial dynamics differences among the two cheesemaking trials, but a more biodiverse profile for experimental cheese samples, probably related to cows' feed supplementation that during the ripening process selected certain microbial species. Another important issue, all tested LAB isolates, later more in-depth tested, fulfilled the safety assessment, and could represent a promising source of probiotic strains.

Moreover, 42 LAB isolates were analyzed with rep-PCR fingerprinting method using the BOXAIR primer to characterize them at species level. Results showed that the prevailing LAB species were *Pediococcus acidilactici* (31%)*, Limosilactobacillus fermentum* (31%)*, Lacticaseibacillus paracasei* (7%)*, Lacticaseibacillus rhamnosus* (10%)*, Lactobacillus delbrueckii* (19%) και *Streptococcus mitis / Streptococcus oralis* (2%)*.*

This study highlighted the persistence of the resilient core microbiota of Cosacavaddu Ibleo cheeses at the end of ripening and a cow-feeding influence on the biodiversity of the LAB community of EXP cheese samples, confirming the sustainable use of food by-products in farming practices to obtain a more sustainable and biodiverse dairy product with a lower environmental impact, able also to preserve its typical traits. However, further studies are necessary to identify the dominant microbial species highlighted in the DGGE profiles.

Scientific area: Food Microbiology

Keywords: Traditional Sicilian cheeses, Caciocavallo or cosacavaddu cheese, microbiome, lactic acid bacteria, olive paste, cow's food supplement, PCR-DGGE analysis, rep-PCR fingerprint method.

Χαρακτηρισμός της μικροχλωρίδας παραδοσιακού τυριού της Σικελίας από νωπό γάλα

ΠΜΣ Συστήματα Διαχείρισης Ποιότητας & Ασφάλειας Τροφίμων
Τμήμα Επιστήμης Τροφίμων & Διατροφής του Ανθρώπου
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ΠΕΡΙΛΗΨΗ

Αρκετές μελέτες που πραγματοποιήθηκαν στον τομέα της εκτροφής μηρυκαστικών, έχουν δείξει τον κρίσιμο ρόλο της διατροφής στον καθορισμό της συνολικής ποιότητας των ζωικών τροφίμων, ιδιαίτερα των γαλακτοκομικών, με την ανάπτυξη πτητικών οργανικών ενώσεων που επηρεάζουν το άρωμα και τη γεύση. Σκοπός της παρούσας μελέτης ήταν η αξιολόγηση της επίδρασης της πάστας ελιάς, ως συμπλήρωμα διατροφής σε αγελάδες γαλακτοπαραγωγής, στο μικροβιολογικό προφίλ και στη δομή της βακτηριακής κοινότητας των οξυγαλακτικών βακτηρίων στο τέλος της ωρίμανσης, στο παραδοσιακό τυρί Caciocavallo ή Cosacavaddu Ibleo από την περιοχή της Σικελίας. Συγκεκριμένα, τυριά από δύο διαφορετικές τυροκομήσεις υποβλήθηκαν σε μικροβιολογική ανάλυση.

Επιπλέον, ο πληθυσμός των οξυγαλακτικών βακτηρίων από τα υπό εξέταση τυριά χαρακτηρίστηκε μέσω προκαταρκτικών δοκιμών ασφάλειας *invitro* προκειμένου να διαπιστωθεί η δυνατότητα χρήσης τους ως καλλιέργειες εκκίνησης. Τα αποτελέσματα της μικροβιολογικής ανάλυσης έδειξαν την απουσία των παθογόνων βακτηρίων *Salmonella* spp., *L. monocytogenes*, *E. coli* και *E. Coli* O157 σε όλα τα δείγματα τυριού. Ο πληθυσμός των οξυγαλακτικών βακτηρίων ήταν παρόμοιος, τόσο στα δείγματα ελέγχου (χωρίς τη χορήγηση πάστας ελιάς στο σιτηρέσιο) όσο και στα πειραματικά δείγματα τυριού (με χορήγηση πάστας ελιάς), ανήλθε σε 7,00 $\log_{10}$ CFU/g περίπου. Τόσο οι μεσόφιλοι όσο και οι θερμόφιλοι γαλακτόκοκκοι εμφάνισαν παρόμοιο πληθυσμό (περίπου 7.00 $\log_{10}$cfu/g), εκτός από το δείγμα ελέγχου στη δεύτερη τυροκόμηση όπου οι μεσόφιλοι γαλακτόκοκκοιέφτασαν την τιμή των 8,78 $\log_{10}$ CFU/g που ήταν η υψηλότερη τιμή. Τα αποτελέσματα της ανάλυσηςPCR-DGGEεμφάνισαν μικρές διαφορές μεταξύ των δύο

δοκιμών τυροκόμησης, αλλά μεγαλύτερη βιοποικιλότητα για τα πειραματικά δείγματα τυριού (EXP), που σχετίζεται πιθανότατα με την ενίσχυση του σιτηρεσίου των αγελάδων με διατροφικό συμπλήρωμα πάστας ελιάς. Επιπλέον, τα απομονωμένα στελέχη οξυγαλακτικών βακτηρίων έδωσαν αρνητικά αποτελέσματα στη δοκιμή της αιμόλυσης και θα μπορούσαν να αποτελέσουν μια υποσχόμενη πηγή προβιοτικών καλλιεργειών.

Επιπλέον, 42 απομονώσεις οξυγαλακτικών βακτηρίωναναλύθηκαν με τη μέθοδο rep-PCR χρησιμοποιώντας τον εκκινητή (BOXAIR) προκειμένου να πραγματοποιηθεί ταυτοποίηση σε επίπεδο είδους. Μετά τη διαδικασία ηλεκτροφόρησης που πραγματοποιήθηκε στο ενισχυμένο DNA, προέκυψε δενδρόγραμμα ομαδοποίησης των στελεχών σε 15 ομάδες με χρήση του προγράμματος Biomumerics. Από κάθε ομάδα επιλέχθηκαν αντιπροσωπευτικά στελέχη για ενίσχυση του γονιδίου 16SrRNA. Με την ενίσχυση του γονιδίου αυτού πραγματοποιήθηκε η ταυτοποίηση σε επίπεδο είδους μέσω του αλγόριθμου BLAST. Τα αποτελέσματα έδειξαν ότι στα δείγματα τυριού βρέθηκαν τα είδη *Pediococcus acidilactici* (31%)*, Limosilactobacillus fermentum* (31%)*, Lacticaseibacillus paracasei* (7%)*, Lacticaseibacillus rhamnosus* (10%)*, Lactobacillus delbrueckii* (19%) και *Streptococcus mitis / Streptococcus oralis* (2%)*.*

Αυτή η μελέτη έδωσε πληροφορίες για τη βιοπικοιλότητα των οξυγαλακτικών βακτηρίων στο τέλος του σταδίου ωρίμανσης του τυριού CosacavadduI bleo με την προσθήκη πάστας ελιάς στο σιτηρεσίο που χορηγήθηκε σε αγελάδες γαλακτοπαραγωγής, επιβεβαιώνοντας τη βιώσιμη χρήση των υποπροϊόντων του αγροδιατροφικού τομέα στις γεωργικές πρακτικές. Έτσι, αποκτάται ένα πιο βιώσιμο και με μεγαλύτερη βιοποικιλότητα γαλακτοκομικό προϊόν, με χαμηλότερο περιβαλλοντικό αποτύπωμα, ικανό επίσης να διατηρήσει τα τυπικά του χαρακτηριστικά.

Επιστημονική περιοχή: Μικροβιολογία Τροφίμων

Λέξεις κλειδιά: Παραδοσιακά τυριά Σικελίας, Caciocavallo or Cosacavaddu cheese, μικροβίωμα, οξυγαλακτικά βακτήρια, πάστα ελιάς, συμπλήρωμα διατροφής αγελάδων, ανάλυση PCR-DGGE, rep-PCR.

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Preface

Within ruminant-based food production, the need to both solve the problem of the disposal of waste material and reduce production costs for livestock feeding, without compromising animal wellness and quality food products, made the use of agro-industrial by-products as feed supplements in animal nutrition an interesting and successful adopted strategy. Indeed, such a system could contribute to providing opportunities to spare arable land, freshwater, or fertilizers, exploit side streams more efficiently (residues of food, biofuel, or wood production), mitigate methane emissions, and influence the chemical and nutritional profiles of animal-based foods.

Indeed, it was widely demonstrated that the use of by-products of the olive oil industry as feed supplements, including olive cake, essentially composed of olive pulp and peel, wastewater and free of woody parts, can improve the nutritional quality of animal products, inducing, especially in dairy foods, the development of volatile organic compounds that influence aroma and taste. In the Italian context, where cheeses are considered the largest and most diversified group of dairy products, an in-depth study of the relationship between nutrition management strategies and the nutritional and nutraceutical properties of dairy products is also desirable to respond to the current trend in consumer choices towards high-quality foods. In Sicily, a sector of great importance is certainly linked to traditional cheeses, recognized by the majority of consumers as foods of excellent sensorial quality, able to offer distinctive aromas determined by traditions born and handed down in a specific territory and inextricably linked. Although the production of these cheeses, carried out using raw milk and without the addition of starter culture, with the use of local raw materials in specific rural areas, manages to contribute to the maintenance of a sustainable environment and production, in line with the current trend of developing a circular economy, the effects of animal supplementation with olive oil industry residues on their typical microbiota need to be explored further.

1. Introduction

1.1 The suitability of agro-industrial by-products

Approximately, 67% of the global production of olive oil is primarily produced, consumed, and exported by European nations. About 4 million square kilometres are used for the cultivation of olive trees in the countries of the Mediterranean region of the European Union, combining traditional, extensive, and super-intensive groves. Greece has the highest per capita consumption of olive oil in the EU, consuming about 12 kg per person annually, while Italy and Spain are the biggest users, consuming about 500,000 tons annually each (Foti et al., 2022). Consequently, the processing of olive oil leads to the inevitable production of by-products that pose serious environmental problems for their phenolic components, such as oleuropein, hydroxytyrosol, and tyrosol, and economic problems linked to their disposal.

However, olive oil by-products can be treated and properly exploited in different fields for their health-promoting properties, and they represent great potential for the food and beverage, cosmetic, and pharmaceutical industries (Foti et al., 2022). Several chemical, physical, and biological processes have been evaluated to improve their nutritional value, and numerous efforts have been made to increase the value-adding of these by-products, through their stabilization to create chemicals added-value extract that can either be directly added as functional ingredients or completely processed.

The suitability of agro-industrial by-products as feed supplements in animal nutrition was extensively evaluated as a promising strategy to reduce animal feeding costs satisfying the nutritional needs of livestock (Biondi et al., 2019; Dorbane et al., 2019; Liotta et al., 2019), as well as overcome both environmental and economic problems (Awawdeh, 2011).

1.2 Olive cake in feed

Utilizing agroindustrial by-products as food for animals has become a popular tactic in the last few decades to decrease feeding expenses and deal with the issue of recycling waste, which can be expensive to dispose of. In Mediterranean nations, the olive (*Olea europaea*) and olive oil industries are significant to their economy and

society. According to whether olive oil is extracted or table olives are processed, the extraction process produces solid residue and contaminated waste water, both of which have significant negative effects on the environment.

Ground olive pulp and stones make up the 90% to 92% dry material (DM) known as the exhausted olive cake. This by-product has high levels of cellulose (9% to 13% DM), hemicellulose (13% to 17% DM), and lignin (28% to 32% DM). There are several ways to incorporate olive by-products into an animal's diet; these include giving it fresh, ensiled, dried, or as a concentrate component. (Tufarelli et al., 2013).

The partial replacement of any of the most popular vegetable oils in aquaculture feed—such as soybean, linseed, sunflower, rapeseed palm oil, and olive oil—can only be suggested if the diet has enough fatty acids to fulfil humans' essential fat requirements. It is shown that adding olive products at 8% improves the product's cardioprotective qualities.

Many studies were performed to evaluate any change in the flavor profile of milk or dairy products obtained from cows given olive products. The outcomes demonstrated that olive products did not affect the product's sensorycharacteristics. Numerous studies have demonstrated that the milk produced by buffalo and sheep improved the nutritional value of the final product. (Foti et al., 2022)

The majority of research on olive cakes has been on how they affect feed value quality assessment and enhancement, as well as animal product quality and fattening efficiency. Given that enough research has been done on the nutritional attributes of olive cake, more attention should be paid to comprehending how olive cake affects blood metabolites, meat quality, and the composition of fatty acids in the meat. (Ozdogan et al., 2017).

Several studies have shown that adding olive by-products to the diet improves the health of consumers by raising the amounts of monounsaturated fatty acids (MUFAs) and lowering those of saturated fatty acids (SFAs) in ruminant milk and meat. Simultaneously, there are fewer negative effects on ruminal fermentation, nutrient utilization, growth performance, carcass characteristics, milk output, and composition, and feeding expenses and environmental impact are decreased. (Tzamaloukas et al., 2021). The fatty acid composition of cow's milk and cheese stated that enriched feed enhanced the amount of monounsaturated and polyunsaturated fatty acids (Foti et al., 2022).

Furthermore, it has been proved that adding olive products to feeds, is improving the meat's oxidative stability. Additionally, there were found to be phenols in the meat, which improved the product's sensory qualities and caused a 5% drop in saturated fat. Several studies have demonstrated that adding olive products to animal feed benefits the animals as well as increasing the quantity and nutritional value of the by-products, such as meat, milk, cheese, and eggs (Foti et al., 2022)

Additionally, the volatilome composition and microbiological quality of the experimental cheeses improved, which was associated with a reduction in several microbiological groups connected to spoilage and did not influence the primary species. The intentional administration of using the olive cake by-product in farming techniques helps to produce a dairy food product that is safer, more sustainable, and has less of an impact on the environment. These results imply that olive products can enhance milk's and cheese's nutritional qualities without degrading their taste (Calabrese et al., 2023).

2. Typicality of dairy products

According to the definition by Guerrero et al. (2009), a traditional food product is "*a product frequently consumed or associated with specific celebrations and/or seasons, normally transmitted from one generation to another, made accurately in a specific way according to the gastronomic heritage, with little or no processing/manipulation, distinguished and known because of its sensorial properties and associated with a certain local area, region or country*". Raw milk cheeses and, in general, all cheeses made on farms or at small dairies fit this definition perfectly as each one is produced in a specifically defined geographical area using specific know-how and skills and with little or no prior processing of the milk, or using also thermized or pasteurized milk inoculated with various starter combinations and allowing the growth and expression of ripening microbiota (Montel et al., 2014). Traditional cheeses are recognized for their diverse and distinctive sensory properties, derived from adapted manufacturing practices to the characteristics of the vat milk day by day.

Southern Mediterranean countries, especially the neighbouring countries, share the same dietary habits and food culture. Milk has a symbolic value of life and fertility in the lives of people residing there, but it is a highly perishable product. Fermentation is one of the oldest food preservation techniques.

2.1 Fermented dairy products

The first records of fermented dairy products trace back to our ancestors' domestication of the first milk animals approximately 8000 years ago. The craft of producing cheese has spread quickly over the ages due to a variety of factors, such as climate, geography, cheese-associated microbiota, and cultural customs. These factors have produced a wide range of flavors, textures, and tastes that are uniquely tied to the region of production and regional traditions (Zinno et al., 2022)

Due to their extended shelf lives and the high perishability of milk, fermented dairy products are valuable providers of nutrition and energy during times of crisis. The distinctive organoleptic characteristics, nutritional worth, and medicinal qualities of traditional fermented milk products are well-known. There are many different types of artisanal dairy products, and each region has its own designations and production

methods. These products can be divided into drinkable yogurt, spoonable yogurt, butter, and cheese. They also vary in taste and consistency. They are created by utilizing rennet addition, back-slopping fermentation, spontaneous fermentation, or a particular starter to ferment raw milk at room temperature.

Traditional fermented products are heritage foods with a long history of use. Passing this know-how through generations contributed to the preservation of the organoleptic quality and authenticity of fermented products. Thus, the protection of the gastronomic heritage is deemed necessary. Product registration and certification could contribute to setting strict criteria for the quality and the geographical origin of the product, its production, and its formulation. This would protect and give more value to the traditional fermented dairy products in the global market (Mefleh et al., 2022)

Fermentation is a straightforward procedure that enhances organoleptic characteristics and increases shelf life without requiring a lot of work. Common fermented goods are made with complex microbial communities that exist naturally and offer several health benefits. Probiotic bacteria (*Lactobacillus, Bifidobacterium, Streptococcus, Leuconostoc, Propionibacterium, Bacillus*, and *Enterococcus*) and their by-products, which include fatty acids, amino acids, minerals, and vitamins, are linked to the health benefits of fermentation. Since fermented dairy products have a pH of less than 4.5, they are usually regarded as microbiologically safe. Near that threshold, the majority of microorganisms experience growth inhibition. Due to their desired sensory qualities and health-promoting properties, a number of traditional goods are becoming more and more popular, leading to their industrialization and global commercialization. Nevertheless, the use of modern processing and commercial starter cultures to recreate the authentic taste and flavor is challenging, mainly because traditional fermented products are heritage foods with a long history of use.

Microorganisms can be naturally occurring on the raw material, or they can be introduced as a starting culture. They can also be found in the environment, on or in the ingredients. Due to the scientific and technological revolution in Western countries, fermentation became a controlled process appropriate for large-scale manufacturing systems. Traditionally, co-fermentation with different lactic acid bacteria (LAB) and yeast has been used to produce spontaneously fermented dairy products, which give a variety of distinct flavors and substances (Yu et al., 2021)

One of the most popular ways of preservation is fermentation. In addition to ensuring the food is stored longer, it also has other advantages like as preventing the growth of harmful microbes, enhancing the organoleptic qualities and digestibility of the product, and serving as a useful source of beneficial microorganisms. Functional strains are becoming increasingly popular because, in addition to being conventional probiotic strains, they can help treat a wide range of illnesses, including digestive system issues, mental illnesses, and immune system stimulation. A lot of food products, including cheese, kefirs, and yogurts, are made from milk—typically cow's milk—through the fermentation process. The diversity of the microbiome allows for the execution of several fermentation pathways, resulting in the creation of numerous final products (like cheeses) with unique consistency, flavor, and smell (Skowron et al., 2022).

It is critical to comprehend how the distinct physio-chemical and sensory qualities of fermented dairy products are influenced by their microbial communities. Characterizing their microbial structure and the variables influencing the microbiota has become essential (Yu et al., 2021). Defenders of traditional cheeses recommend maintaining high taxonomic diversity in indigenous cheese microbial communities and diverse cheese-making practices. Their arguments rely on the fact that a high diversity of microbial activities, combined with particular cheese manufacturing methods, is the key to allowing traditional cheeses to develop their particular characteristics, including low pathogen risk and diversification of gustatory characteristics. The raw milk microbiota is an important part of the microbiota of many traditional cheeses (Montel et al., 2014).

2.2 Cosacavaddu cheese

A huge variety of cheeses is produced in Italy according to local traditions due to different climates, cultures, and history. One of the most characteristic traditional cheeses is Caciocavallo or Cosacavaddu cheese in the Sicilian dialect. It is considered a ripened Pasta Filata cheese type. Pasta-filata cheeses include Provolone, Mozzarella and Caciocavallo. It's about a group of cheeses manufactured from the milk of cows, buffalo, goats, or sheep.

Caciocavallo is manufactured by almost the same technological processes as

Provolone cheese. This semi-hard cheese is produced with wooden tools (Busetta et al., 2023). Traditionally, it was extensively manufactured in southern Italy and that's why it is well known as the cheese of the Kingdom of the Two Sicilies. Nowadays, several countries, especially the Mediterranean, produce this type of cheese. It is more often produced in small-scale artisanal dairies and is mainly sold on local markets. This is the exact reason that its current production cannot be exactly quantified. Caciocavallo from Molise, especially the home-style varieties, is considered to be a great typical production, worth protection, and worldwide known, both for its top quality and also for its economic value as it is widely consumed and appreciated around the world.

Even though there is a long tradition of this cheese and a big consumer interest, only two varieties are labelled as Protected Designation of Origin (PDO). The first was established in 1993 by the Italian legislation named "Caciocavallo Silano" (IT/ PDO/0117/000) and the second one is named Ragusano (IT/PDO/0017/ 1505). Subsequently, there is no protection of the generic designation 'Caciocavallo'.

Unlike the DOP, the Ibleo caciocavallo was extensively manufactured in southern Italy and nowadays, several countries, especially the Mediterranean, produce this type of cheese. It is more often produced in small-scale artisanal dairies and is mainly sold on local markets. This is the exact reason that its current production cannot be exactly quantified.

The Ibleo caciocavallo is one of the oldest cheeses in Sicily, where it is produced all year round, using raw cow's milk, rennet lamb, and/or kid paste. The production area falls within the territory of the County of Modica and, in particular, of the entire territory of the province of Ragusa and the southeastern side of the Iblei Mountains relating to the province of Syracuse. The cheese-making and maturing processes vary depending on the weight of the cheese, and it is traditional for the use of wooden tools ("tina", "staccio", etc.). Salting is carried out in brine, for a variable time based on the size of the wheels, or dry, during production (Corfilac.it). Usually, the ripening time takes place on wooden shelves for small cheeses, or astride a beam for heavier cheeses intended for prolonged maturation, in natural rooms, not subjected to control, and are subject to seasonal temperature variations (Corfilac.it).The common parallelepiped shape of Caciocavallo varies according to weight (2 - 10 kg), and is typically rind, generally not capped, and never smoked.

Usually, the ripening time is shorter than six months but, in many cases, the aging can last until three years (Busetta et al., 2023). The moisture level is medium (35%–55%) and it has a medium–hard texture. It has a homogeneous body with few small eyes or none at all and its color is more or less intense yellow, according to animal feed and aging. It has a sweet taste that tends to be more piquant while ripening.

Caciocavallo is produced by coagulation of raw cow milk, at a natural pH. At this pH, a natural whey starter and liquid calf rennet, it is cooked at about 85 °C and manually stretched and shaped.

Stretched curd cheeses are characterized by the stretching process which takes place using hot water at 70-80 °C. Stretched curds include hard cheeses with less than 40% humidity, semi-hard cheeses with humidity between 40 and 45%, and soft cheeses with humidity greater than 50%.

More specifically, Caciocavallo's cheesemaking consists of two main phases. The first phase is named curd formation and the second phase is named stretching of acidified curd. In this phase, the forming, salting, and ripening take place in the production cycle. First of all, the milk is heated to 36–38 °C and inoculated with a starter culture. After 30-40 minutes the consistency of the curd is considered to be appropriate and the coagulum can be cut into fine particles (6–8 mm). Then, the whey is partially removed, the fresh curd is been acidified under heated whey or ona table and the ripened curd is manually stretched and shaped. After that, Caciocavallo is left in cool water. In the last step, it is salted in brine and left to ripen (Figure 1) (Uzun et al., 2019).

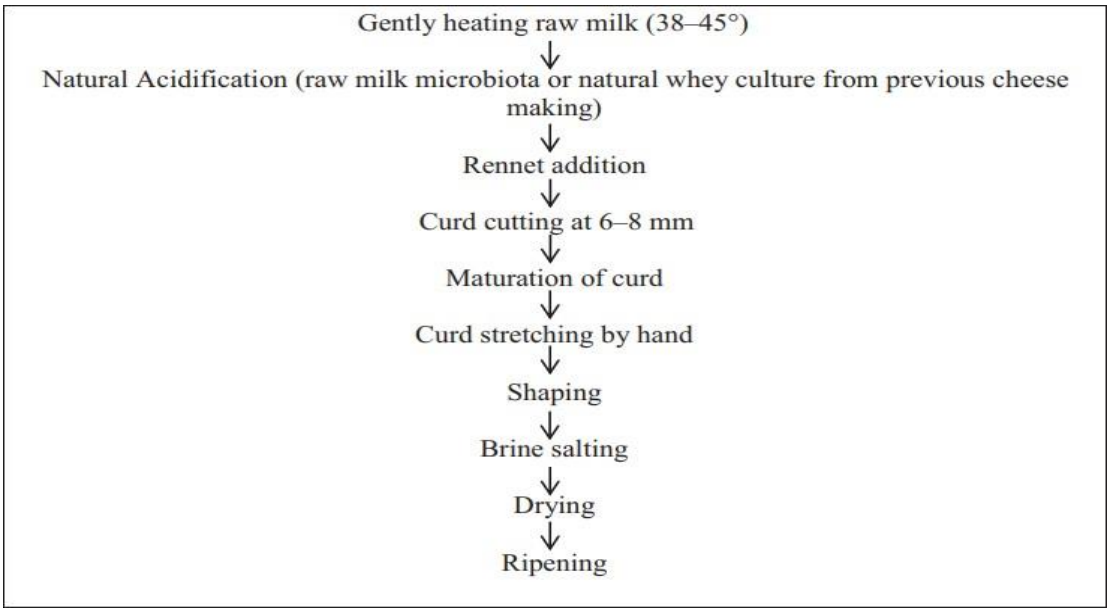


Figure 1: Caciocavallo production flow sheet

2.3 Microbiota in dairy products

Dairy products offer a favorable environment for the growth and survival of numerous microorganisms, including fungi, because of their physical and chemical properties (pH, aw, and redox potential). The fungal microbiome can be determined to enhance consumer safety, dairy product quality, and manufacturing methods. The quantity and diversity of fungus found in dairy products, as well as the microbiological quality of the milk utilized, the heat treatment, the production process, and the ripening environment, all differ greatly. Particular cheeses are madewith the help of particular yeasts and molds, which also help the cheeses' flavor and aroma grow. The most well-known varieties are blue cheeses such as Gorgonzola, Stilton, Camembert, and Roquefort. These are manufactured from two different species of *Penicillium: P. camemberti*, which is used to make Camembert, and *P. roqueforti*, which is used to make other blue cheeses. On the other hand, dairy products can also deteriorate due to yeasts and molds (Cenci-Goga et al., 2021).

A variety of characteristics, such as animal health (including metabolic disorders and mastitis) and the ensuing effects on the quality of milk and dairy products can be predicted based on the general composition of microbiota in dairy products. Furthermore, the gastrointestinal flora, nutritional characteristics, and consumer well-being are impacted by these elements. They are also essential to the development of

niche goods and dairy products unique to a given area (Tzora, 2023)

It is widely acknowledged that, before pasteurization, the lactic acid bacteria (LAB), a class of bacteria that ferments lactose to lactate, constitutes the majority of the population in milk from cows, goats, sheep, and buffalo. *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus* are the most prevalent LAB genera in milk. The subsequent development of dairy products is directly impacted by the uniqueness of the milk microbiota. Lactate generation by microorganisms can cause milk to ferment, and the resulting products' flavor, texture, and organoleptic qualities can be affected in many ways. Microorganisms can also have a detrimental effect on the shelf life and quality of milk. For instance, extracellular lipases and proteases produced by psychrotolerant bacteria can cause spoiling when they multiply under refrigeration (Quigleyet al., 2013).

Traditional cheeses are valued by customers for their unique organoleptic characteristics and authenticity. They are a food category with economic significance for specific geographic locations. To ensure safety, the majority of these products in Italy are made from raw milk, either with or without natural whey cultures added, by EU regulations. Naturally occurring lactic acid bacteria (LAB) play a major role in inhibiting the growth of pathogenic and undesirable microbes through their antagonistic effects. Nonetheless, fluctuations in the native microbiota's makeup may lead to instability in LAB's safety-promoting operations. Safety could be increased by adding cultures chosen from autochthonous LAB without changing the standard manufacturing procedure or sensory attributes (Aprea et al., 2021)

The Greek word "probiotic" means "for life." The term "probiotic" has taken on many different meanings over time. Probiotics are live microbiota cultures that, when given to humans or animals, improve the natural microbiota's characteristics. These bacteria can be used in the food industry and are widely distributed in nature. Some foods—including dairy products like yogurt—are thought to be excellent sources of probiotics. Microorganisms that replenish and provide beneficial maintenance to the digestive tract are frequently associated with milk and milk products. Gram-positive lactic acid bacteria are defined by their lack of a cytochromesystem and inability to generate pores. They are also catalase-negative. Probiotics can be found in a variety of dairy-based foods, such as cheese, yogurt, and milk. The *Lactobacillus* and its many strains are present in the majority of processed milk products.

Probably the most obvious advantages of LAB fermentation are improved food taste and longer shelf life. It is generally accepted that using bacteria is safe, because LABs can produce bacteriocins and offer health benefits, like reducing blood ammonia levels, controlling intestinal infections, improving lactose utilization, and effectively resisting bile and gastric acid, affecting the immune system and lowering blood cholesterol levels. Probiotic usage of various LAB strains has increased especially Lactobacilli and Bifidobacterium, which are adult human gut bacteria with beneficial medicinal properties.

The term "probiotic" was originally used in 1908 by Metchnikoff, who proposed that using fermented dairy products would prolong their shelf life. Lilly and Stillwell concluded in 1956 that a bacterium released certain growth stimulators for another germ. It is believed that Lactobacillus was the first probiotic ever described. Gram-positive bacteria belonging to the LAB category comprise this genus. These rod-shaped bacteria, of which there are over 183 known species, are widely employed in a variety of industrial food processing procedures. One bacterium that benefits the host is Lactobacillus acidophilus. Recent studies have looked into the discovery of certain species of normal vaginal bacteria that produce hydrogen peroxide.

Lactobacilli are isolated from milk products, particularly from cheese. According to Piewengam's research, probiotic formulations are thought to improve human health through immunestimulant effects or interbacterial competition between beneficial and dangerous bacteria. Probiotic usage was considered a promising strategy for managing and preventing several infectious diseases (Kishwer et al., 2022).

The resident bacteria influence every stage of cheese processing, from raw milk pasteurization to aging, and are significantly responsible for the gastronomic value, quality, and safety of cheese (Reuben et al., 2023). Several factors such as geography, manufacturing processes, climatic conditions, season, and the use of raw or pasteurized milk influence the microbial community of the cheeses, which in turn contribute to cheese’s characteristics, quality, and safety (Nam et al., 2021; Yeluri Jonnala et al., 2018).

Starter cultures, whose selection depends on the type of cheese and cheese-making practice, usually include one or more of *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii*. During the

cheese aging step, non-starter bacteria cultures are introduced into the cheese matrix, because it is able to promote a complex series of biochemical reactions with starter cultures, and contributing to the development of the flavor and texture of cheese. The secondary microbial population is composed of a combination of bacteria, yeasts, and molds, generally associated with the type of cheese (Beresford et al., 2001), which are introduced into cheese either from the ingredients or from the environment. The union of both microbial communities allows the improvement of the cheese quality, through the production of specific microbial metabolites such as lactic acid, free amino acids, and other volatile compounds(Nam et al., 2021),that influence the cheese microbiota and its flavor. Contrarily, the artisanal cheese varieties, made from raw milk and following specific protocols according to traditional heritage, have a complex and autochthonous non-starter microbiota, characterized by the succession of different microorganisms throughout cheesemaking. These microorganisms are an essential component of all ripened cheeses and play an important role in cheese ripening by influencing the sensorial and physicochemical characteristics of the final product.

Cheese produced from raw milk is often characterized by a richer and more distinctive flavor than its counterpart produced from pasteurized milk, mainly related to the greater diversity of microorganisms in cheese produced from raw milk.Usually, the microbiota of raw milk includes *Lactococcus* spp., *Leuconostoc* spp., *Enterococcus* spp., *Streptococcus* spp., *Micrococcus* spp., *Staphylococcus* spp., *Arthrobacter* spp., *Corynebacterium* spp., *Brevibacterium* spp., *Enterobacter* spp.,*Citrobacter* spp., and *Acinetobacter* spp., whose succession during cheese ripening is related to their ability to adapt to specific environmental conditions that affect cheese characteristics. Indeed, microbial populations differ among raw milk cheeses depending on the manufacturing process, but the bacteria in the cheese core are dominated by LAB belonging to the genera *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, and *Leuconostoc* (Quigley et al., 2013). LAB bacteria can modulate cheese appearance, texture, aroma, nutrient composition, quality, and shelf-life, though highly variable between varieties, reaching a concentration in ripened cheesegreater than 10^9 colony-forming units (CFU)/g, while those of yeasts and filamentous fungi range widely between 10^2 and 10^7CFU/g (Mayo et al., 2021). Depending on the microbial taxon, maximum numbers are reached by the end of the fermentation (e.g., *Lc. lactis*),

between day 7 to 17 (e.g., *Lactobacillus* spp.) after one to two months of ripening (e.g., *filamentous fungi*). Once the highest level is reached, numbers decline slightly but consistently afterward. However, variations in the composition and/or dynamics of the microbial communities making up the typical microbiota of a given cheese, as well as the upper hand of undesirable microorganisms can lead to serious technological and sensorial defects and even pose food safety risks. Indeed, the growth dynamics of strains of raw milk during production and maturation are difficult to predict, and consequently also the reproducibility of consistent flavor and quality.

Although the naturally occurring LAB plays a major role in inhibiting the growth of pathogenic and undesirable microorganisms through their antagonistic effects, the fluctuations in the native microbiota can lead to instability in LAB's safety- promoting operations, as well as to off-flavors and/or texture deficiencies. Moreover, dairy products offer a favorable environment for the growth and survival of numerous microorganisms, including fungi, because of their physical and chemical properties (pH, aw, and redox potential) (Beniamino et al., 2021). Therefore, sometimes the unique sensorial characteristics and authenticity of traditional cheeses, highly appreciated by consumers, are to the detriment of safety. With the growing concern over food safety, the European Union EC 853/2004 regulation set limits on the total plate count (TPC) and somatic cell count (SCC) of bacteria in raw milk from cows at 10^5 CFU/mL and < $4 \cdot 10^5$ for cows, respectively, at 30 °C. For other mammal species, such as goats and sheep, the TPC limit is increased to $1.5 \cdot 10^6$ CFU/mL. Additionally, several methods are employed to ensure milk security and reduce the microbial load, and among them, the most often used, are high-pressure and heat treatments (Tilocca et al., 2020).

3. Lactic acid bacteria (LAB)

For years, researchers have been interested in lactic acid bacteria (LAB), a class of Gram-positive microorganisms that are naturally present in fermented food products and are utilized as probiotics. LABs are a powerful source of bioactive chemicals with a variety of roles and activities, even if their exact nature is still unknown. Promising anticancer potential has been shown by metabolites of LAB, including bacteriocins, exopolysaccharides, and short-chain fatty acids. Studies examining the relationship between immunological systems and the bioactive metabolites of LAB have shown that these compounds have a substantial immunomodulatory effect, which explains their extensive therapeutic potential (Garbacz, 2022).

Lactic acid bacteria (LAB) constitute a homogenous group of microorganisms, accompanied by the ability to synthesize lactic acid, as the primary product of carbohydrate fermentation. LAB are catalase-negative rods or cocci, mainly from the Lactobacillales order. Generally, LABs do not produce spores and are non-mobile, anaerobic, or microaerophilic. They are acidophilic, which allows them to survive and replicate in environments with relatively low pH, between 4.5 and 7.0, and have a low number of G+C pairs in their genome (Garbacz, 2022; Khalid, 2011).

LAB belong to the Firmicutes phylum, Bacilli class, and Latobacillales order, and are classified based on their cell morphology, type of glucose fermentation, the range of growth temperatures, and fermented sugars (Quinto et al., 2014). Based on their fermentation products, LAB can be divided into homofermentative, when the primary product of fermentation by the former is lactic acid, and heterofermentative, when both lactic acid and other products, such as acetic acid, alcohol, and carbon dioxide were synthesized. The best-known genera of LAB are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*, with the *Lactobacillus* being the largest among the genera listed above, with more than 100 described species (Quinto et al., 2014). Other LAB genera include *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*.

As it was mentioned in fermented foods, lactic acid bacteria (LAB) are important probiotic organisms that are usually regarded as harmless. LAB improves plant health, detoxifies dangerous pollutants, and controls soil organic matter and the biochemical cycle. Decomposing plants, traditional fermented milk products, and thenatural flora

in the human gastrointestinal tract are all sources of them. InvestigatingLAB found in unidentified areas could result in the isolation of rare species. They are acclimated to acidic conditions and high sugar concentrations, although their classification is rather complicated. LAB strains improve soil fertility and health, making them attractive options for sustainable agriculture (Raman et al., 2022).

To produce energy, lactic acid bacteria ferment carbohydrates, utilizing endogenous carbon sources as the final electron acceptor rather than oxygen. They are aerotolerant and they have peroxidases to shield them from oxygen by-products like hydrogen peroxide. Most LABs are non-motile, and except for pediococci, cell division takes place in a single plane. The most popular approach for identifying LAB has been phenotypic methods; however, more recently, molecular techniques like 16S rDNA sequencing have been developed, allowing for more accurate and consistent identification of particular strains.

Decomposing plants and fruits, dairy products, fermented meat and fish, cereals, beets, pickled vegetables, potatoes, sourdough, silages, fermented drinks, juices, sewage, and the cavities of people and animals are all sources of lactobacilli. They are the predominant organisms in the vagina and are found mostly in the ileum, colon, and oral cavity of humans. As of right now, the LAB group is categorized under the order Latobacillales, class Bacilli, and phylum Firmicutes. As mentioned before, some LABs are employed as probiotics because of their health benefits. Probiotics are living organisms, such as yeast or bacteria that enhance the health of people or animals. They can be found in nutritional supplements that include bacteria to support the development of the gut microbiota, as well as in fermented foods like yogurt and cheese. Probiotics are defined as organisms that are fundamentally non-pathogenic, generally recognized as safe (GRAS), tolerant of low pH, able to withstand high quantities of conjugated and de-conjugated bile salts, immune system tolerant, and should not produce antibodies. Furthermore, these organisms must not use horizontal gene transfer to pass on antibiotic resistance genes to prospective pathogens.

Antibacterial compounds are produced by LAB isolated from homemade fermented vegetables, which are effective against common foodborne bacterial infections that are both Gram-positive and, crucially, Gram-negative. Due to their wide range of inhibition, these LAB strains may be useful as organic biopreservatives in a variety of food products and may even aid in the fight against human diseases.

(Mokoena et al., 2017).

In individuals suffering from diarrhoea, lactic acid bacteria are more important probiotic organisms to preserve and replenish the gut microbiota. LAB are very promising biotechnological organisms because they can synthesize a wide range of metabolites under fermentation conditions, including vitamins, lactic acid, reuterin, bacteriocins, reutericyclin, and antioxidants. Food products that have been fermented by LABs can have some anticancer effects, while biosynthesizedmetabolites like lactic, propionic, and acetic acid have antifungal qualities (Sylvester et al., 2023)

3.1 Technological role of LAB

LAB, normally present in milk, utensils, and surfaces of dairy farms, play an important role in cheesemaking, both in the early stages of milk fermentation and during cheese ripening (Nicosia et al., 2023). In traditional cheeses, the complex microbiota present milk and equipment, characterized by the indigenous LAB population, usually consists of streptococci, enterococci, and lactobacilli, which is considered a source of enzymatic activity involved in flavor formation (Coelho et al., 2022). Indeed, compared with cheeses manufactured from pasteurized milk, traditional cheeses have more intense and unique flavors all related to the technological properties determined by the complex dynamics and interaction among cheese indigenous LAB (Nicosia et al., 2023). During the initial step of cheese-making, some of these microorganisms, contribute to the rapid acidification of milk, a desirable property of LAB in the production of dairy products, fermenting the lactose, and producing high concentrations of lactic acid, without the need to add starter cultures. In the later stages, the complex LAB community present depends on their ability to survive heat and acidity, to grow during ripening with energy sources other than carbohydrates present in milk, and to grow and tolerate low water activity (Gatti et al., 2014), is involved during the ripening process for their ability to contribute to the desired flavor, and in general to the final structure of the cheese. Three primary LAB metabolic pathways, such as lactate, free fatty acid release metabolisms, and proteolysis, are mainly involved in the definition of the sensory profile of the final product (Nam et al., 2021). Proteolysis is of great importance because it contributes to degradation products, such as peptides and even aminoacids play an active role as

substrates for several secondary reactions, which give rise to many important flavor compounds (Murtaza et al., 2014) as well as the fatty acids released by lipolysis. Indeed, LAB possesses a complex enzymatic system that includes proteinases and peptidases, able to hydrolyze small peptides starting from long peptide chains (Nicosia et al., 2023).

In addition, through the fermentation of citrate, glucose, lactose, and other carbon sources, the production of diacetyl and acetoin confer desirable sensory features to ripened cheeses (Pino et al., 2019). Furthermore, another activity performed by LAB is the production of exopolysaccharides (EPS) to improve texture through the increase of the firmness of the casein network by binding water and interacting with other milk components, such as whey proteins and casein micelles, along with promoting antimicrobial and antioxidant activities in dairy products (Nicosia et al., 2023; Coelho et al., 2022). Indeed, LAB can also be used as protective cultures to control the contamination of either pathogenic bacteria or produce bacteriocins, which reduce the risk of pathogen growth and survival or explain their possible probiotic properties, including resistance to gastric acidity, digestive enzymes, and bile salts (Coelho et al., 2022; Kanmani et al., 2013). Finally, LAB may also have health-promoting potential, either by degrading nutrient-damaging compounds (e.g. biogenic amines and cholesterol), or by increasing the number of beneficial compounds (e.g., antihypertensive peptides, γ-aminobutyric acid (GABA), short-chain fatty acids (SCFA), and conjugated linoleic acid (CLA) (Coelho et al., 2022; Ogawa et al., 2005).

3.2 Lactococcus and Lactobacillus genera

Thanks to the recent application of high-through put DNA sequencing (HTS), which allowed an in-depth analysis of the composition and functional potential of traditional cheeses, it was widely demonstrated that bacterial communities differ among raw milk cheeses depending on the manufacturing process, but that exists a cheese core bacteria, dominated by LAB belonging to the genera *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, and *Leuconostoc* (Coelho et al., 2022; Nero et al., 2021; Biolcati et al., 2020).

The genus *Lactococcus* is characterized by Gram-positive cocci that occur singly,

in pairs, or chains, non-spore-forming, non-motile, facultatively anaerobic, non-β-hemolytic, and catalase-negative, with an optimal growth temperature comprised between 10 °C and at 40 °C, and able to tolerate the NaCl (Coelho et al., 2022). The genus consists of 17 species that have a fermentative metabolism, with *Lc. lacti s*pecies is the most commonly found in raw milk and dairy products. Lactococci boast an established use as starter cultures for dairy products (Bintsis, 2018), mainly for their metabolic stability resistance to bacteriophages, and ability to produce unique compounds, many of which are derived from amino acid degradation (Vinderola et al, 2019).

Lactobacilli are Gram-positive, non-spore-forming, catalase-negative bacilli or coccobacilli (Hammeset al., 1995), strictly fermentative, aerotolerant or anaerobic, and with glucose as a carbon source, can be homofermentative and produce more than 85% lactic acid, or heterofermentative and produce lactic acid, CO_2, ethanol, and/or acetic acid (Coelho et al., 2022).

As per the latest recent estimates, the genus Lactobacillus comprises 261 different species, making it an extremely diverse genus. Rich carbohydrate- containing niches, such as those connected to plants, animals, silage, and raw milk, are the common habitat of the lactobacilli group. The utilization of *Lactobacillus* strains for a growing number of industrial dairy applications is a result of our growing understanding of Lactobacillus biology. Specifically, their capacity to generate exopolysaccharides and fragrance compounds, as well as their proteolytic activity, can improve the nutritional content and quality of dairy products.

By-products with bioactivity and a wide range of health-promoting benefits are frequently produced by LAB-driven fermentations. These benefits include immunity modulation, anti-allergenic, anti-obesity, anti-oxidant, and anti-anxiety effects, as well as protection against infectious agents (Mathur et al., 2020).

4. Culture-dependent methods for microbiota characterization

Culture-dependent procedures refer to conventional culture-based techniques combined with the genotypic (species-specific PCR or RAPD) and phenotypic (physiological and biochemical) analyses required for accurate identification and typing. The majority of the times, cultivation techniques are limited to defining groups or genera; hardly ever do they accurately characterize the species. To improve the sensitivity of the analyses, colonies must be isolated from the plate and identified by biochemical and/or molecular typing.

The phenotypic description of pure cultures of microorganisms, isolated using selective culture media having specified concentrations of hydrogen and carbon or other energy sources, is the only basis for identification when utilizing these procedures (Bergey's Manual). Phenotypic characteristics encompass the examination of cellular morphology, physiology, and metabolic fermentation processes.

A microorganism's morphology comprises its cell characteristics (shape, gram staining, endospore and/or flagella presence) as well as the colony characteristics (color, size, and shape); an examination of the physiological and biochemical characteristics reveals the microorganism's capacity to grow at various temperatures, pH levels, salt concentrations, in the presence of different substrates, antimicrobial agents, and specific environmental conditions. These techniques cannot be regarded as capable of making species- or strain-level distinctions, and most importantly, they are not able to reveal information about the phylogenetic relationships between different bacterial groups, even though they are typically accurate enough to distinguish some groups of bacteria.

Furthermore, because the cultivable strains represent a very small fraction of the possible microbial population present in the various matrices, there are many issues with microbe identification when standard cultivation methods are applied (Giraffa and Neviani, 2001). In actuality, even if they are viable, a large number of the microorganisms that comprise an ecosystem's population might not becultivable. Negative environmental factors, such as low temperatures, insufficient nutrients, or other environmental stressors, can cause cells that are cultivable in and of themselves to enter a state known as "vitality but non-cultivable" (VNC), where they exhibit active metabolism but do not form colonies in standard culture media.

Thus, using fingerprinting techniques is crucial in this project to be able to estimate the "real" microflora linked with a particular habitat. The advancement of molecular approaches that fall under the category of "independent cultivation," meaning that they do not necessitate the prior cultivation of microorganisms, has marked a significant turning point in the investigation of microbial ecosystems to accurately comprehend their evolution and function.

5. Culture-independent methods

The techniques for analyzing microbial communities have seen significant developments in the last 20 years in the field of microbial ecology. The necessity of a culture-independent molecular approach has increased due to the disparity between the variety observed in vivo and that arising using standard culture methods.

This shift was initially made possible by examining fatty acid profiles, but more recently, DNA has emerged as the molecule under investigation of choice. Molecular methods began to open up new microbial ecology research avenues around the end of the 1990s. These techniques, which are referred to as "culture-independent," are typified by the fact that the microorganisms are immediately identified in the sample using DNA and RNA analysis, rather than being cultured and isolated.

RNA analysis can be used to determine whether species are metabolically active, while DNA analysis can be used to determine the number and kind of microbial species present in a given sample (Coppola et al., 2001). Microbial ecology studies rely on culture-independent techniques such as automated ribosomal intergenic spacer analysis (ARISA), cloning and sequencing of 16S rRNA libraries, temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), terminal restriction fragment length polymorphism (TRFLP), and single strand conformation polymorphism (SSCP). Double-stranded DNA fragments of the same length but distinct sequences can be separated using gel electrophoresis under denaturing conditions (DGGE) or TGGE method (Randazzo et al., 2009).

When chemical (urea and formamide) or physical (temperature) agents are used to create a linear denaturation gradient, the molecules exhibit distinct denaturation behaviours that lead to fingerprinting. Using universal primers, which have the potential to amplify all of the DNA or RNA of specific microbial groups, nucleic acids are exposed to gene amplification in PCR-DGGE. The more complex the DNA molecule mixes produced after amplification, the higher the microbial biodiversity in the system under study. The DNA molecules slow down their electrophoretic migration until they stop in a particular location on the gel when they come into contact with the denaturing gradient, which specifies an opening of the double helix.It is possible to distinguish DNA from complicated microbial communities by observing the varied

migration behaviours that different conformations induce in the gel. Following extraction from the acrylamide gel, specific bands of interest can beidentified and sequenced, just like with DGGE and TGGE. Next, a vector must becreated by cloning and reamplifying the single strand. The high rate of single-strandre-annealing during migration is a drawback of this method. The 16S rRNA generestriction fragment length polymorphism (RFLP) approach, also known as amplifiedribosomal DNA restriction analysis (ARDRA), is based on the electrophoreticseparation of the fragments in agarose after the PCR products are digested with restriction enzymes.

6. Thesis purpose

Considering the biological relevance of the by-products of the olive industry in animal nutrition, as an interesting and promising strategy both to minimize their ecological footprint and to improve the quality of products of animal origin, the objective of the present study was to study the effects of lactating dairy cows feed supplementation with olive cake, an important source of bioactive phenolic compounds, on the diversity and microbial dynamism of the traditional mature Cosacavaddu Ibleo cheese.

For this purpose, cheese samples, deriving from milk produced by control cows (fed with conventional feed) and that produced by cows fed with experimental feed (supplemented with 8% olive cake), and subjected to two different cheese-making trials, for up to 180 days of maturation, were analysed microbiologically. In detail, a polyphasic approach was employed, involving the use of culture-dependent and independent microbiological methods, to evaluate both the biodiversity of the cultivable microbiota and the structure of the bacterial community, as well as its distribution during maturation.

7. Materials and methods

7.1 Analysed samples

Samples of Cosacavaddu Ibleo, obtained from milk taken from Control Group (CTR) cows (fed conventionally) and Experimental (EXP) Group cows (feed with 8% *olive cake* supplementation) and from two different cheese-making processes, were collected at the end of considered maturation period (180 days) and transported, under refrigerated conditions, to the Food Microbiology laboratory of the Department of Agriculture, Food and Environment (Di3A) for microbiological analyses.

7.2 Microbiological analyses of cheese samples

Microbiological analysis of 180-day-old CTR and EXP cheese samples, relating to both cheesemaking trials, was performed. In detail, 25 g of both core and surface sections of cheese were weighed in a stomacher bag and added of 225 ml ofpeptone water. Serial dilutions were made and 100 µl were put into the following media: Kanamycin Aesculin Azide (KAA) Agar, aerobically incubated at 37 °C for 24– 48 h, for enterococci count; Mannitol Salt Agar (MSA), incubated at 32°C for 48 h, for staphylococci count; M17, incubated at 30 °C and 42 °C for 24–48 h, for lactococci and thermophilic lactococci, respectively; de Man Rogosa and Sharpe agar (MRS) and Rogosa, incubated at 32°C for 72 h under microaerophilic condition, for LAB and lactobacilli, respectively; and Sabouraud Dextrose Agar (SDA), incubatedat 25 °C for 3–5 days, for yeasts and moulds count. Finally, Violet Red Bile Glucose Agar (VRBGA) and Plate Count Agar (PCA) were used for Enterobacteriaceae, and total mesophilic aerobic bacteria count, respectively, and incubated at 32 °C for 48
h. All media were purchased from Liofilchem srl (Italy). Moreover, for both Listeria monocytogenes and Salmonella spp. detection, according to the International Organization for Standardization (ISO), a two-stage enrichment method was used.

7.3 LABs isolation and phenotypic characterization

To characterize the lactic population, a representative number of colonies was randomly picked from each MRS, Rogosa, and M17 (at both 32 °C and 42 °C) agar

plates and each colony was purified by streaking three times. All isolates were subsequently checked for catalase reaction and Gram straining before being stored at -20 °C, with 20% glycerol, until further analysis.

7.4 Safety tests

The selected strains were subjected to phenotypical assays to characterize their safety. In detail, for hemolytic activity, the cultures were streaked onto blood agar medium, containing sheep blood. After incubation at 37 °C for 24-48 h, the hemolytic activity of each isolate was measured and classified as total or β- hemolysis (clear halos around the colonies), partial or α-hemolysis (greenish halos around the colonies), or γ- hemolysis (absence of hemolysis). *Proteus mirabilis* was used as the positive control. For gelatinase production, 10-µL aliquots of the examined cultures were spotted on gelatinase agar media. After incubation at 37 °C for 48 h, the plates were maintained at 4 °C for 4 h, and gelatin hydrolysis was recorded by the formation of opaque halos around the colonies. DNAse activity was identified by spotting 10-µL aliquots of the cultures on the surface of DNAse methyl green agar (BD). After incubation at 37 °C for 48 h, the formation of clear halos around the colonies was identified as a positive result. All assays were conducted in two independent repetitions.

7.5 Genotypic characterization of isolated strains

To genotypically confirm that the isolated strains belong to the genus Lactobacillus, a genus-specific PCR colony was performed, using Lac1 and Lac2 primers.

7.6 Total DNA extraction from cheese samples

Total DNA extraction from dairy samples was performed according to the protocol previously described by Randazzo et al. (2002), with some modifications. In detail, dairy samples (5 g) were incubated at 45 °C for 30 min with 20 mL of sodium citrate solution (2%, w/v, trisodium citrate dihydrate) and glass pearls (diameter, 3 mm). The suspensions were mixed with a vortex mixer for approximately 5 min, the large

material was left to settle, and the supernatants were transferred to clean tubes. After centrifugation for 10 min at 8.000 rpm, the fat layer at the top was removed with a cotton tip. The cell pellets were resuspended in 1 mL of TE buffer and centrifuged at 8.000 rpm for 10 min. The supernatant fluid (approximately 900µl) was removed, and the remaining 100 µL and pellet were mixed and transferred to a 2-mL screw-cap tube containing 0.3 g of zirconium beads and 150
µL of phenol-TE (phenol equilibrated with TE; Life Technologies, Gaithersburg, Md). The samples were treated at 5.000 rpm for 5min in a bead beater. After the addition of 150 µL of CI solution, consisting of chloroform and isoamyl alcohol at a 24:1 (v/v) ratio, the tubes were vortexed briefly and centrifuged at 13,000 rpm for 5 min. From the aqueous phase DNA isolation was performed using phenol-chloroform extractions with 150 µL of phenol-TE and 150 µL of CI solution until a clear interface was obtained. After a final CI extraction, the DNA was precipitated by the addition of 2 volumes of ethanol (-20 °C) to the aqueous phase. After incubation at -20 °C for 30 min, the DNA was collected at 13,000 rpm for 20 min, washed briefly with 70% ethanol, and resuspended in 500 µL of TE. Five units of DNase-free RNase (Promega) were added, and the sample was incubated at 37 °C for 15 min. Two CI extractions were performed until a clear interface was obtained. The DNA was precipitated with 3 M sodium acetate (pH 5.2) and 96% ethanol (-20 °C) and stored at -20 °C for 30 min. DNA was collected by centrifugation and resuspended in 50 µL of TE buffer. The amount and integrity of the nucleic acids were determined visually after electrophoresis on a 1.2% agarose gel containing Gel Red Nucleic Acid Stain (Biotium, Italy) and comparison to standard concentrations of DNA markers.

7.7 PCR-DGGE analysis of 16S rDNA

PCR amplification was performed with the DreamTaq 2X PCR Master mix (Thermo Fisher Scientific), added with 5 pmol of each primer, and 1 µL of appropriately diluted template DNA in a final volume of 50 µL. To investigate the dominant bacterial communities by DGGE analysis PCR products were generated using PCR primers U968-GC and L1401-r to amplify the V6 to V8 region of eubacterial 16S rDNA (Nubel et al., 1996). The 40-nucleotide GC-rich sequence at the 5' end of primer U968-GC improves the detection of sequence variations of amplified DNA fragments by

subsequent TGGE/DGGE (Muyzer et al., 1993). The samples were amplified in a Perkin–Elmer Applied Biosystem GenAmp PCR System 9700 (Foster City, CA, USA) programmed as follows: initial denaturation of DNA for5 min at 94 °C; 35 cycles each consisting of 30 s at 94 °C, 30 s at 56 °C and 40 s at 68 °C; and extension of incomplete products for 7 min at 68 °C. DGGE analysis of PCR amplicons was performed on the Dcode System apparatus (BioRad, Hercules, CA), as previously described (Muyzer et al., 1993). Electrophoresis was performed ina 0.8 mm-thick polyacrylamide gel 8% [w/v], acrylamide: bisacrylamide [37.5:1] containing a urea plus formamide gradient from 30% to 60%, increasing in the direction of the electrophoresis run. Optimal separation was achieved with a 40– 60% urea-formamide denaturant gradient, increasing in the direction of electrophoresis. A 100% denaturant corresponds to 7 M urea and 40% (v/v) formamide. The gels were subjected to a constant voltage of 85 V and at the temperature of 60 °C for 15 h in 0.5×TAE buffer. The DNA bands were visualized by silver staining and developed as previously described (Sanguinetti et al., 1994).

7.8 Bacteria DNA extraction and Rep-PCR fingerprinting

Genomic DNA of bacteria isolates was extracted from a 2 mL overnight culture using NucleoSpin® Microbial DNA kit (Macherey-Nagel, Dürer, Germany). DNA concentration and quality of bacteria isolates was measured using a Quawell Q5000 Read First photometer (Quawell Technology Inc., San Jose, CA, United States). Fingerprinting of the isolates was performed by repetitive element palindromic PCR (rep-PCR). Amplification was performed in 25 µL final PCR reaction volume containing 50 ng genomic DNA, 7.5 pmolBOXA1R primer (5'- CTACGGCAAGGCGACGCTGACG-3') and 12.5µLOneTaq-Quick Load 2× Master Mix with Standard Buffer (New England Biolabs, UK). PCR was performed using a Mastercycler Gradient thermal cycler (Eppendorf, Germany) as follows: initial denaturation at 92 °C for 2 min, 35 cycles with denaturation at 92 °C for 30 s, primerannealing at 40 °C for 2 min, and primer extension at 72 °C for 3 min, followed by final extension at 72 °C for 15 min. PCR products were separated electrophoretically at 60 V on a 1.0% (w/v) agarose (Sigma-Aldrich, Steinheim, Germany) gelin TAE (Tris-acetate-EDTA) (40 mM Tris-acetate, 1 mM EDTA, pH 8,2) buffer for 2.5 hours. Molecular sizes of amplified DNA fragments were estimated by comparison with a 1 kb DNA ladder plus (Nippon Genetics Europe

GmbH, Germany). Rep-PCR fingerprints were clustered using BioNumerics v. 6.0 (Applied Maths, Ghent, Belgium).

7.9 Identification of bacteria isolates

Selected bacterial isolates based on the rep-PCR fingerprints were identified at the species level by sequencing the 16S rDNA gene using the primer pair 16SF (5'-GGA GAG TTA GAT CTT GGC TCA G-3')/16SR (5'-AGA AAG GAG GTG ATC CAG CC-3'). Amplification was performed in 50 µL final PCR reaction volume containing 200 ng genomic DNA, 10 pm of each primer and 25 µL OneTaq-Quick Load 2×Master Mix with Standard Buffer (New England Biolabs). PCR was performed using a Mastercycler Gradient thermal cycler (Eppendorf) as follows: initial denaturation at 94 °C for 2 min, 30 cycles with denaturation at 94 °C for 30 s, primer annealing at 56
°C for 30 s, and primer extension at 72 °C for 80 s, followed by final extension at 72 °C for 5 min. PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Duren, Germany). Sequences were identified by comparing them with the sequences that are deposited in the GenBank database using the Basic Local Alignment Tool for nucleotide sequences (BLASTn) search engine tool of the National Center for Biotechnology Information (NCBI).

8. Results

8.1. Cultivable microbiota

Results of microbiological analysis carried out on both 180-day-old CTR and EXP cheese samples, expressed as $\log_{10}$ CFU/g (± standard deviation), are shown in Table 1. Overall, *Salmonella* spp., *L. monocytogenes*, *E. coli*, and *E. coli* O157 were never detected in all analyzed samples. Relating to the first cheesemaking, LAB achieved an average value of 7.62 $\log_{10}$ CFU/g in CTR samples and 7.42 $\log_{10}$ CFU/g in EXP cheeses, while the lactococci group showed the same average value (about 7.00 $\log_{10}$ CFU/g) in the same samples. Thermophilic lactococci and staphylococci groups were more numerous in the olive paste treated sample, reaching a value of 7.19 and 7.11 $\log_{10}$ CFU/g, respectively (Table 1). A similar microbial abundance was observed for Enterobacteriaceae and total mesophilic bacteria, while enterococci and eumycetic population were less numerous in the EXP samples.

Regarding the second cheesemaking trial, both the LAB and thermophilic lactococci densities were around 7.00 $\log_{10}$ CFU/g in both cheese samples. Contrarily, the *Lactococcus* group reached a value of 8.78 $\log_{10}$ CFU/g, almost two log units higher than the first cheesemaking, in the CTR sample, while slightly decreased in EXP cheese. The Enterobacteriaceae group showed the same density, as well as the eumycetic population, while enterococci, coagulase-positive staphylococci, and total mesophilic bacteria are always more abundant in EXP samples.

Table 1: Microbial counts expressed as $\log_{10}$ CFU/g (± standard deviation) in control samples (CTR) and experimental samples (EXP), during the first (I) and the second (II) cheesemaking trials.

Samples	*Enterococcus* spp.		Enterobacteriaceae		Coag.+ staphylococci		Total mesophilic bacteria	
	I	II	I	II	I	II	I	II
CTR	4.85±1.14	2.81±0.05	3.06±0.03	3.00±0.00	6.54±0.59	5.39±1.16	5.65±0.49	5.71±0.79
EXP	2.63±0.89	4.40±0.28	3.09±0.02	3.00±0.00	7.11±0.05	6.23±0.39	5.72±0.17	6.41±0.41

Samples	Yeasts&moulds		*Lactococcus* spp.		Thermophilic lactococci		LAB	
	I	II	I	II	I	II	I	II
CTR	7.54±0.33	6.89±0.02	7.07±0.10	8.78±0.06	6.60±0.17	7.23±0.33	7.62±0.46	7.18±0.14
EXP	6.60±0.69	6.74±0.41	7.13±0.18	6.91±0.38	7.19±0.41	7.17±0.12	7.42±0.39	7.03±0.33

8.2. LAB strains characterization

Overall, 71 strains presumably attributable to the LAB group were isolated. In detail, 49 strains were catalase-negative and Gram-positive rods, and 22 were catalase-negative and Gram-positive cocci. All the examined strains confirmed as LAB strains, through PCR analyses (Figure 2).

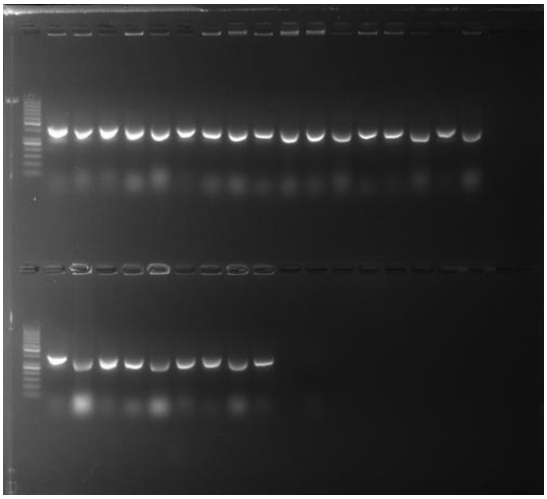
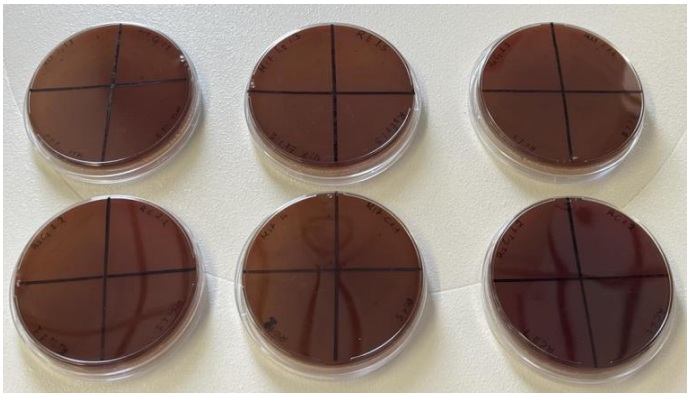


Figure 2: Electrophoretic run of genus-specific PCR

As it was mentioned before, dairy products, such as cheese, contain a high amount of probiotics and that's why they are considered as a good source. To be taken into consideration as an acceptable probiotic, every bacterial strain needs to possess certain specific characteristics. The FAO/WHO recommendations state that each possible probiotic strain needs to be accurately identified before undergoing a number of in vitro tests, to determine its functional characteristics. This is due to the fact that probiotic qualities vary depending on the strain, environment, and dosage. (Shokryazdan et al., 2017) So, in this study many tests were conducted in order to examine the safety of the strains and if they can be considered suitable for use as probiotics or as starters cultures.

Hemolytic activity was evaluated on blood agar plates containing sheep blood. Based on the appearance of a clear zone, green halo or no zones around colonies, the hemolytic activity was visually detected and distinguished as β-hemolysis, α-hemolysis, or γ-hemolysis. All the 83 strains were negative in the hemolytic test and showed γ-hemolysis. (Figure 3)

Positive control (proteus)



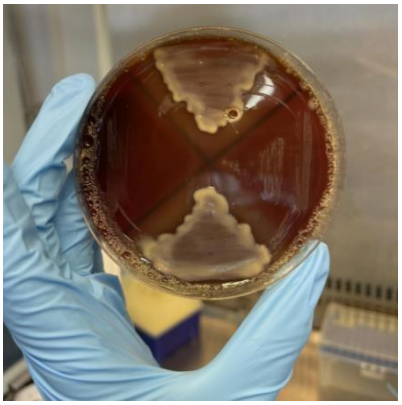
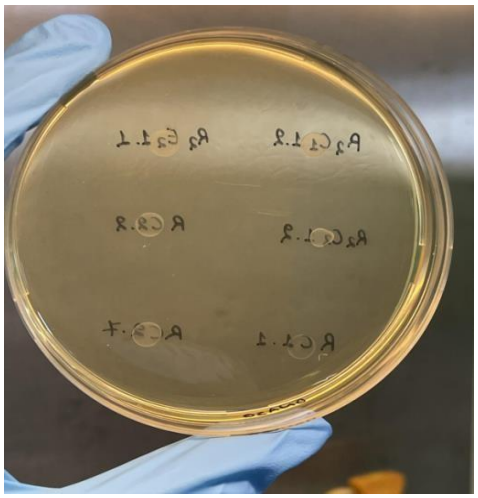


Figure 3: Hemolytic test (blood agar)

From the overnight cultures in the tubes (5 mL of MRS broth) 5 µL were inoculated in DNA agar media, for the DNase test, and 5 µL were inoculated in gelatinase agar media, for the gelatinase test. After 48 hours of incubation, the results were again negative of all 83 strains, in both tests (Figure 4, 5).



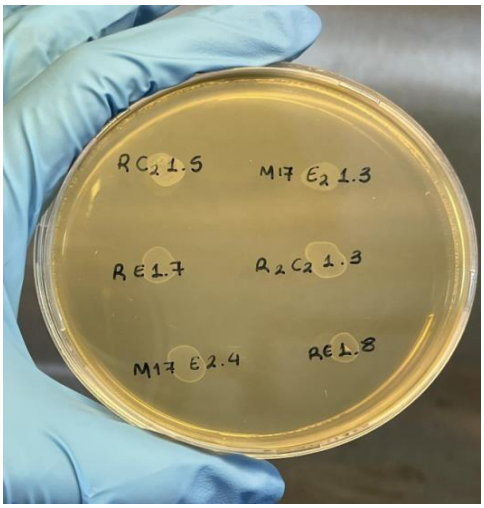


Figure 4: DNase test

Figure 5: Gelatinase test

As a result, the strains were also considered safe because they showed neither hemolysis halos after growth on blood agar plates (Figure 3) nor zones of gelatin hydrolysis on gelatin agar plates overlaid with saturated ammonium sulfate solution (Figure 5). In addition, none of the strains showed DNAse activity (Figure 4).

8.3. PCR-DGGE of cheese samples

PCR-DGGE fingerprinting revealed differences in microbial dynamics among CTR and EXP cheese samples after 180 days of ripening for both cheesemaking trials. The appearance and disappearance of amplicons in the DGGE pattern indicate important shifts in the microbial community structure, while the intensity of an individual band is a semi-quantitative measure for the relative abundance of this sequence in the population (Muyzer et al., 1993).

Analysis of samples showed some differences in the profiles of CTR and EXP cheeses, which remained quite similar for the two cheesemaking trials. Although with a different intensity, many amplicons at the same position in all four cheese samples (bands A, B, and C, lines 1, 2, 3 and 4) dominated all analyzed samples (Figure 4), strongly suggesting similarity in the microbial composition, while other bands appeared only in specific kinds of cheese. Indeed, concerning the CTR samples, a profile less biodiverse was observed, with higher bands (bands D, lanes 1 and 2) appearing at the bottom of the gel. The profiles of EXP cheeses showed a much more densely populated profile, containing new distinct dominant amplicons (bands E, F, and G, lanes 3 and 4), with greater intensity, and other bands differentlydistributed in the gel (band H, lanes 3 and 4).

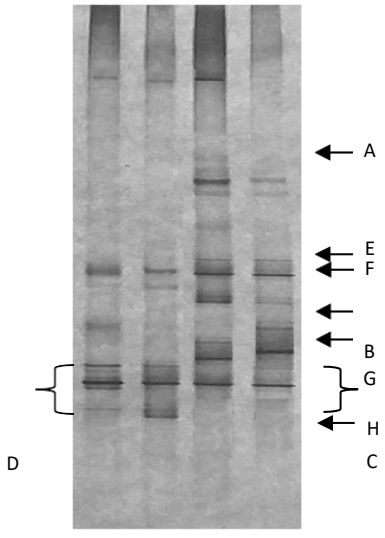
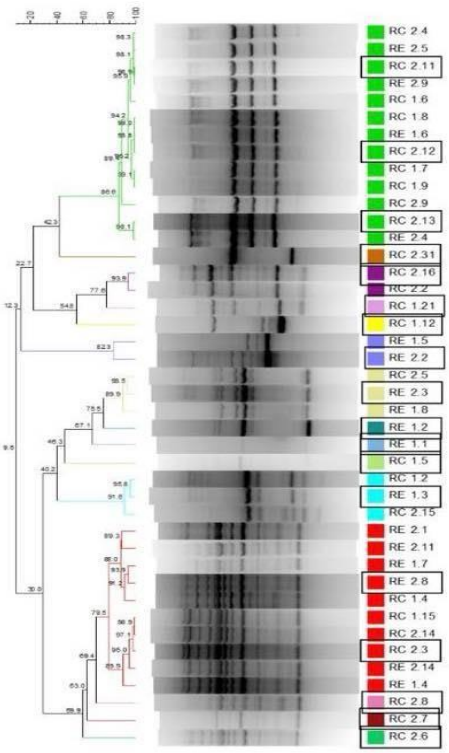


Figure 6: PCR – DGGE analysis

8.4. Results from the identification of bacteria isolates

Dendrogram Rep – PCR of 42 isolates



Selected sample	Bacteria Species
RC 2.13	*Pediococcus acidilactici*
RE 2.2	*Limosilactobacillus fermentum*
RC 2.16	*Lacticaseibacillus paracasei*
RC 2.31	*Lacticaseibacillus rhamnosus*
RC 2.3	*Limosilactobacillus fermentum*
RC 2.7	*Limosilactobacillus fermentum*
RE 2.8	*Limosilactobacillus fermentum*
RE 2.3	*Lactobacillus delbrueckii*
RC 2.11	*Pediococcus acidilactici*
RC 2.12	*Pediococcus acidilactici*
RC 2.6	*Limosilactobacillus fermentum*
RE 1.3	*Lacticaseibacillus rhamnosus*
RC1.5	*Lactobacillus delbrueckii*
RE 1.1	*Lactobacillus delbrueckii*
RC1.21	*Lacticaseibacillus paracasei*
RE 1.2	*Lactobacillus delbrueckii*
RC 2.8	*Limosilactobacillus fermentum*
RC 1.12	*Streptococcus mitis / Streptococcus oralis*

RC : Control cheese

RE : Experimental cheese

Figure 7: rep-PCR fingerprinting of bacteria isolates using the BOXAIR primer. Image analysis was performed using BioNumerics v. 6.0. Based on rep-PCR clustering, specific isolated were selected for 16S rRna gene sequencing.

As mentioned before, 49 colonies were catalase-negative and Gram-positive rods. However, 7 strains could not be revitalized, therefore 42 bacteria isolates were analyzed with rep-PCR fingerprinting method using the BOXAIR primer. The 42 isolates were clustered in 15 groups and 18 representative isolates of all groups were selected and subjected to 16S rRNA gene sequencing. According to the sequencing results, among the 42 bacterial isolates, 13 isolates were identified as *Pediococcus acidilactici,* 13 as *Limosilactobacillus fermentum,* 3 as *Lacticaseibacillus paracasei,* 4 as *Lacticaseibacillus rhamnosus, 8* as *Lactobacillus delbrueckii* and 1 as *Streptococcus mitis / Streptococcus oralis.* Although the isolates were characterized as Gram-positive rods, according to the sequencing results 13 isolates were identified as *pediococci* and 1 as *streptococci*, indicating either potential contamination or incorrect assessment of their cell morphology.

Table 8: Isolates grouped in Rep – PCR Clusters and Species.

ISOLATE	Rep – PCR Cluster	Species identification by 16S rRNA gene sequencing
RC 2.11	**1**	***Pediococcus acidilactici***
RC 2.4	1	*Pediococcus acidilactici*
RE 2.5	1	*Pediococcus acidilactici*
RE 2.9	1	*Pediococcus acidilactici*
RC 1.6	1	*Pediococcus acidilactici*
RC 1.8	1	*Pediococcus acidilactici*
RE 1.6	1	*Pediococcus acidilactici*
RC 2.12	**1**	***Pediococcus acidilactici***
RC 1.7	1	*Pediococcus acidilactici*
RC 1.9	1	*Pediococcus acidilactici*
RC 2.9	1	*Pediococcus acidilactici*
RC 2.13	**1**	***Pediococcus acidilactici***
RE 2.4	1	*Pediococcus acidilactici*
RC 2.16	**2**	***Lacticaseibacillus paracasei***
RC 2.2	2	*Lacticaseibacillus paracasei*
RC 1.21	**3**	***Lacticaseibacillus paracasei***
RE 2.3	**4**	***Lactobacillus delbrueckii***
RC 2.5	4	*Lactobacillus delbrueckii*
RE 1.6	4	*Lactobacillus delbrueckii*
RC 1.5	**5**	***Lactobacillus delbrueckii***
RE 1.1	**6**	***Lactobacillus delbrueckii***
RE 1.2	**7**	***Lactobacillus delbrueckii***
RC 2.31	**8**	***Lacticaseibacillus rhamnosus***
RE 1.3	**9**	***Lacticaseibacillus rhamnosus***
RC 1.2	9	*Lacticaseibacillus rhamnosus*
RC 2.15	9	*Lacticaseibacillus rhamnosus*
RE 2.2	**10**	***Limosilactobacillus fermentum***
RE 1.5	10	*Limosilactobacillus fermentum*
RE 2.8	**11**	***Limosilactobacillus fermentum***
RE 2.1	11	*Limosilactobacillus fermentum*
RE 2.11	11	*Limosilactobacillus fermentum*
RE 1.7	11	*Limosilactobacillus fermentum*
RC 1.4	11	*Limosilactobacillus fermentum*
RC 1.15	11	*Limosilactobacillus fermentum*
RC 2.14	11	*Limosilactobacillus fermentum*
RC 2.3	**11**	***Limosilactobacillus fermentum***
RE 2.14	11	*Limosilactobacillus fermentum*
RE 1.4	11	*Limosilactobacillus fermentum*
RC 2.8	**12**	***Limosilactobacillus fermentum***
RC 2.7	**13**	***Limosilactobacillus fermentum***
RC 2.6	**14**	***Limosilactobacillus fermentum***
RC 1.12	**15**	***Streptococcus mitis / Streptococcus oralis***

*** 18 representative isolates were subjected to 16S rRNA gene sequencing**

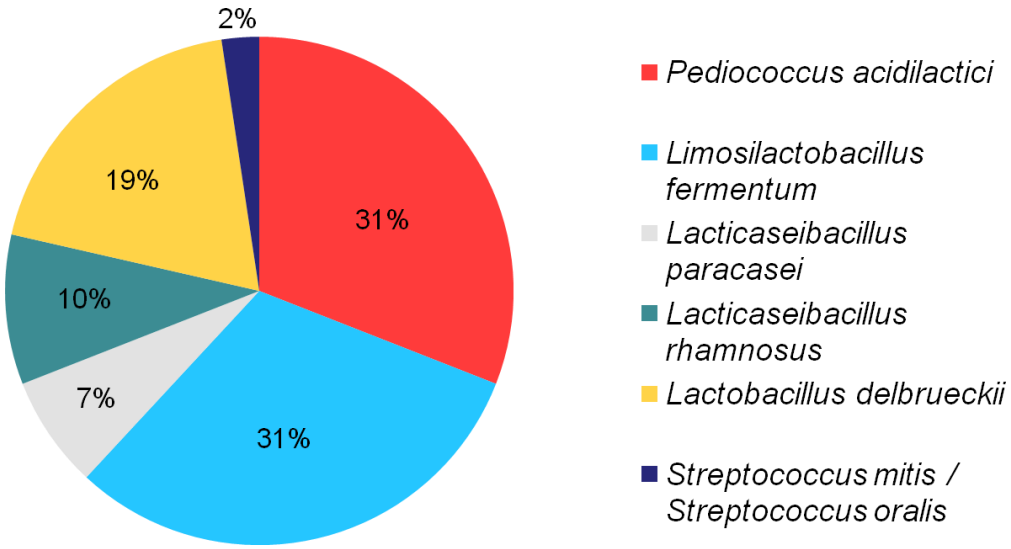


Figure 9: The percentage of species identified in Caciocavallo cheese from both cheesemaking process and experimental trials.

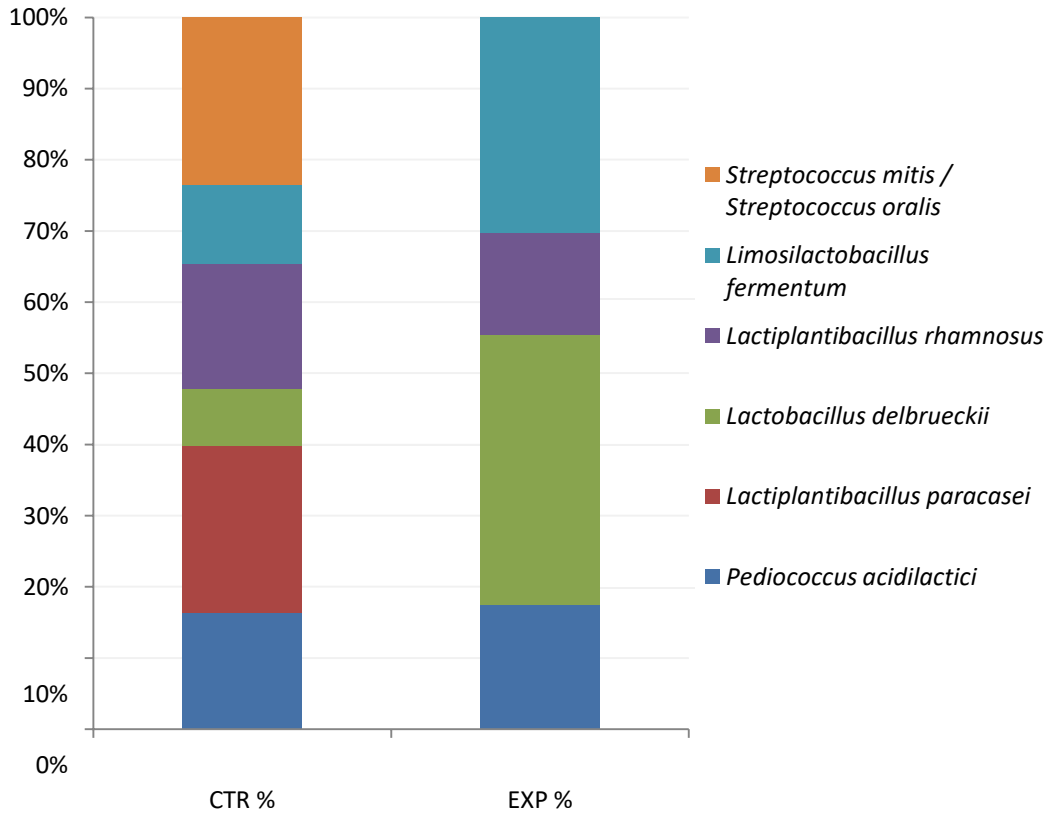


Figure 10. The percentage of LAB species identified by Rep-PCR in Caciocavallo cheese from control (CTR) and experimental (EXP) samples.

The characterization of the LAB isolates within the cheese samples is presented in Figure 10. From the overall number of 42 LAB isolates that were previously characterized phenotypically as lactobacilli, 25 were attributed to samples derived from cows' milk that received no dietary intervention (control samples), whereas 17 derived from samples with prior dietary intervention with olive cake in the cows' feed. Based on Figure 10, it can be concluded that 2 species were detected only in the control samples, namely *Lacticaseibacillus paracasei and Streptococcus mitis / Streptococcus oralis,* and 4 species were detected in both control and experimental samples (*Pediococcus acidilactici, Lactobacillus delbrueckii, Lactiplantibacillus rhamnosus, Limosilactobacillus fermentum).*

Pediococcus spp. are lactic acid bacteria (LAB) that are classified as facultative anaerobes, Gram-positive, non-motile, spore-forming, coccus-shaped (organized in tetrads), and catalase-negative. They are also commonly referred to as probiotics. The potent antilisterial bacteriocin pediocin is produced by a variety of *Pediococcus* strains. *Pediococcus* isolated from various biological habitats has been used in a number of experiments to perform fermentation operations for the production of Pediocin or *Pediococcus* cells (Porto et al., 2017). Significant probiotic characteristics have been demonstrated by *Pediococcus acidilactici*. While this strain was responsive to antibiotics, low pH, high bile salts, and NaCl could not have a major effect on it. It also possessed strong antimicrobial properties. (Khaleghi et al., 2023).

Limosilactobacillus fermentum is a lactic acid bacterium (LAB), Gram-positive, non-spore-forming, rod-shaped that produces organic acid from the fermentation of carbohydrates. *L. fermentum* can also inhibit the growth of food-borne pathogens in food products, and it possesses the "generally recognized as safe" (GRAS). Foods obtained from fermentation by *L. fermentum* usually possess palatability, high sensory quality, texture, stability, and nutritional properties (Pakroo et al., 2022).

The isolates of *Lacticaseibacillus paracasei* that dominated the cheeses over the course of 63 weeks of maturation displayed a variety of phenotypic traits, such as optimal growth temperature, salt tolerance, growth on N-acetylglucosamine and N-acetylgalactosamine, as well as delayed growth on D-ribose, carbon sources that are probably present in cheese because of bacterial autolysis (Shah et al., 2021).

Lactobacillus delbrueckii ssp. is a lactic acid producing bacterium that is commonly employed by the dairy industry, particularly in the manufacturing of yogurt and cheese. In the dairy business, *Lactobacillus delbrueckii and Streptococcus thermophilus* are employed to ferment milk into yogurt. Studies on *Lactobacillus delbrueckii* spp. have concentrated on their probiotic and biological characteristics. After incubation in a simulated gastric solution (pH 2.0), viable cell counts decreased moderately, although the addition of milk had a protective effect on the strains. The majority of the strains were able to grow in the presence of fermented prebiotics and bile salts. Certain strains exhibited elevated levels of β-galactosidase activity and hydrophobicity. The potent antibacterial activity directed against microorganisms resulted from the synthesis of lactic acid. (Guglielmotti et al., 2007).

9. Discussion

In the present study, the evaluation of the effects of dietary olive cake as feed supplementation of lactating dairy cows on microbiological profiles of Caciocavallo cheese was performed. We applied culture-dependent and culture-independent methods to evaluate the microbiological biodiversity and safety of CTR and EXP CosacavadduIbleo cheeses, after 180 ripening days. In addition, the LAB population from analyzed cheeses was characterized through preliminary *in vitro* tests to assess the safety traits.

Overall, microbiological data revealed no significant effects of the experimental diet on cultivable microbiota, during the two cheesemaking trials. We hypothesize that in addition to the shared milking, and cheesemaking, the utilization of traditional tools contributed to concealing the effect of diet strategy. According to previous reports describing the LAB abundance of different raw milk cheeses produced under traditional practices, in Cosacavaddu cheese lactobacilli dominated the non-starter lactic acid bacteria (NSLAB) population (Pino et al., 2019; Caggia et al., 2015; Solieri et al., 2014), together to both mesophilic and thermophilic lactococci, reaching the average level of 7.40-7.22 (lactobacilli, in CTR and EXP, respectively), 7.92-7.02 (mesophilic lactococci in CTR and EXP, respectively), and 6.91-7.18 $\log_{10}$ CFU/g (termophilic lactococci in CTR and EXP, respectively), which was by several orders of magnitude higher compared to *Enterococcus* that did not exceed 3.83 and 3.51 $\log_{10}$ CFU/g in CTR and EXP samples, respectively. Moreover, yeasts and molds were also detected as part of the dominant cheese microbiota, playing an important role during the ripening stages, for producing enzymes with lipolytic and proteolytic activity that contribute to determining the aroma and texture of the cheese and increase the shelf-life of the product (Rohm et al., 2002). However, in our study, all analyzed samples showed *S. aureus* levels exceeding the limit of 10^5 CFU/g which is considered to introduce a significant risk of production of enterotoxins, especially in the case of a fairly advanced maturation such as the one under consideration. Since the microbial diversity and quality of cheese result from the quality of the raw material and hygienic conditions during milk processing, and the metabolic activity of microorganisms originating from both the raw material and the processing environment impacts the sensory quality and durability of cheese, itis necessary to

apply stringent good handling practices during the production of this type of cheese. However, the presence of the total mesophilic bacterial load, which is attested to the values observed and could provide insight into the hygienic accuracy of the operations adopted, did not appear to have promoted the increase in the risk of swelling or hindered the normal development of the dairy microbial population (O' Sullivan et al., 2013).

In our analysis, through the culture-independent methods able to detect the DNA of the microbiota of interest, not discriminating between live and dead cells (Aquilanti et al., 2016), we used a primer pair universal for LAB bacteria, which allowed us to characterize the DNA of lactic population present in tested samples. Although no differences in cultivable microbiota counts were detected, PCR-DGGE analysis revealed differences in microbial dynamics in the LAB structure of the CTR and EXP cheese samples, independently of the cheesemaking trials. Indeed, a more biodiverse profile was observed for experimental cheese samples, probably related to cows' feed supplementation that, during the ripening process, selected certain microbial species. Similar discrepancies in the LAB diversity obtained by culture- dependent and culture-independent identification were observed by others (Aquilanti et al., 2016; Ercolini et al., 2001). Taking into account that the species composition revealed by using the PCR-DGGE method shows both species that are active in the tested cheese samples as well as species that were present in the raw material and in the products at any stage of the manufacturing process, but not necessarily active in the final product, we can support the statement that the use of both approaches gives the most complete picture of the microbial composition that was and/or is currently active in the product. However, further studies are necessary to identify the dominant microbial species highlighted in the DGGE profiles.

In the present study, 71 LAB strains isolated from traditional Cosacavaddu Ibleo cheese were preliminary screened for safety aspects. None of the strains exhibited either hemolytic, gelatinase, or DNAse activity, reaffirming what as previously reported (Pino et al., 2019; Caggia et al., 2015), about the traditional cheeses as alternative and readily available source of lactobacilli with promising functional properties, which could impact positively human health. As is well known, although lactobacilli are food-grade organisms witnessing a long history of safe use, receiving most of them the Qualified Presumption of Safety (QPS) status (EFSA, 2012), the

safety assessment represents one of the key criteria that should be taken into account for the selection of probiotic strains since it is considered an important factor to ensure consumers' safety (Castro-López et al., 2023).

Analyzed with rep-PCR fingerprinting method using the BOXAIR primer 42 bacteria isolates took place for 42 isolates. As a result, those isolates were clustered in 15 groups and 18 representative isolates of all groups were selected and subjected to 16S rRNA gene sequencing. According to the bibliography all the identified species (*Pediococcus acidilactici, Limosilactobacillus fermentum, Lacticaseibacillus paracasei, Lacticaseibacillus rhamnosus and Lactobacillus delbrueckii*) are often isolated in dairy products.

More specifically, raw milk has been found to contain about 400 different types of bacteria, including lactic acid bacteria, yeasts, molds, and Gram- and catalase-positive and negative bacteria. This biodiversity increases on the cheese surfaces, where many different species of bacteria are present, but declines in the cheese cores, where a few number of lactic acid bacteria species are numerically dominating.

Both propionic acid bacteria in cheeses (Switzerland-style cheeses) and LAB predominate at all phases of the cheese process in the cores of traditional raw milk cooked hard cheeses and pasta filata cheeses (also prepared from raw milk). Despite partial lysis and partial unculturability from the earliest days of processing, thermophilic starter LAB constitute the predominant viable microflora from the commencement of cheese-making to at least six months of ripening. According to the bibliography, the primary thermophilic LAB species, which can differ even within a single cheese type, are *Lactobacillus delbrueckii, Streptococcus thermophilus, Lactobacillus helveticus*, and *Lactobacillus fermentum*. As ripening advances, *L. helveticus* levels decrease more quickly than those of *L. delbrueckii.*

Thermophilic LAB are derived from raw milk, wooden vats, or whey cultures. *L. delbrueckii* from milk only becomes dominant when during cheese maturation. Only after 10 to 30 months of ripening can mesophilic viable/cultivable non-starter lactobacilli (LAB), mostly *Lactobacillus paracasei* but also *Lactobacillus rhamnosus*, become prominent. Depending on the kind and starting concentrations of the strains, microbial development and the balance between cultivable LAB fluctuate from just below the surface to the center of the core. Once the curd temperature reaches 47–48 °C, viable *Macrococcus caseolyticus*, *Rothia* spp., *Leuconostoc lactis*, *Lactococcus*

lactis, and psychrotrophic bacteria (*Chryseobacterium* spp., *Pseudomonas* spp.) vanish.

Differences across vat raw milk batches and/or whey cultures, as well as variations in ripening circumstances are the primary causes of variation in active cell counts, species balance, and strain composition among cheeses of the same type, depending on the dairy and time period. *S. thermophilus, L. paracasei*, and/or *L. delbruekii* strains can coexist in Caciocavallo, Gruyère, and Comté PDO cheeses in various amounts. It is possible that specific circumstances develop throughout the cheese process because the predominant strains of *Lactobacillus casei* in cheese cores differ often from those in raw milks. Non-starter LAB predominate in the centers of most types of cheese, mostly mesophilic *lactobacilli* but also *leuconostocs*, *enterococci*, and *pediococci* for some cheese variations. (Montel et al.,2014).

Despite the need for further studies, the traditional fermented dairy products manufactured using ancestral knowledge and artisanal techniques transmitted across generations by various ethnicities remain the potential natural source of these beneficial probiotic candidate strains, since the isolation and characterization of new ones are still desirable (Castro-López et al., 2023; Kaur et al., 2022).

To conclude bibliography confirms the results of this research because *Pediococcus acidilactici, Limosilactobacillus fermentum, Lacticaseibacillus paracasei, Lacticaseibacillus rhamnosus* and *Lactobacillus delbrueckii* are predominant strains in similar cheeses.

Conclusion

The obtained results showed the complementary of the applied methods for the evaluation of bacterial diversity of cheeses. By using classical methods, we attested the absence of pathogenic microorganisms and the predominance of lactic acid bacteria and eumycetic population in all cheese samples, highlighting a low variability between the various samples analyzed. With DNA-based methods, we analyzed the structure of the LAB population that confirmed the previous obtained data, showing a low-variable lactic population between the same cheese samples in relation to the cheesemaking trials. However, a more biodiverse profile was observed for experimental cheese samples, probably related to cows' feed supplementation that during the ripening process selected certain microbial species.Additionally, according to the sequencing results, the predominant species were identified as *Pediococcus acidilactici, Limosilactobacillus fermentum, Lacticaseibacillus paracasei, Lacticaseibacillus rhamnosus and Lactobacillus delbrueckii.* Another important issue, all tested LAB isolates, later more in-depth tested, fulfilled the safety assessment, therefore the population of lactic acid bacteria found in regional cheeses could represent a promising source of probiotic strains.

In this study, although the persistence of a *core microbiota* of Cosacavaddu during ripening has been attested, cow-feeding influence has been noted as acting on the biodiversity of the LAB community. Therefore, the use of *olive cake* in agricultural practices represents a valid strategy to obtain a more sustainable and biodiverse dairy product with a lower environmental impact, able also to preserveits typical traits.

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