UNIVERSITÀ DEGLI STUDI DI PADOVA
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in Italian Food and Wine

Cheese Genomics from the Region: A Comprehensive HTS Study on Grana Padano Authentication

Supervisor
Prof. Luca Fasolato
Co-supervisor
Dr. Sarah Currò

Submitted by
Mahsa Akbarzadehjavan
Student no.
2040540

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I want to express my gratitude to everyone who played a role in helping me get to where I am today. Some joined me along the way, while others are no longer beside me, but each person has been instrumental in my journey.

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Summary

The intricate interplay between cheese microbiota and the quest for authenticity not only illuminates the fascinating microbial ecology shaping cheese flavor and quality but also underscores the pivotal role of microbiota analysis in preserving traditional cheese-making techniques, ensuring product integrity, and fostering a deeper understanding of culinary heritage.

The concept of food authenticity revolves around legally acknowledging distinctive traits upheld by a meticulous and bureaucratic certification process involving various entities. Geographical origin authentication is presented as a critical pillar not only for ensuring food quality and safety but also for economic growth. The alignment with regional natural capital goals emphasizes the connectivity of environmental, economic, and regulatory considerations in building sustainable rural landscapes. Preserving the natural capital of different regions is crucial to prevent potential damage to the environment, society, and economy. To achieve this objective, various analysis methods are employed, and ongoing research endeavors aim to refine methodologies for greater precision. One notable approach that is gaining attention is DNA sequencing. This analysis method is rooted in the genomic examination of microorganisms in agricultural products.

This review highlights the analytical techniques of microbiota examination as fortifying defences against food fraud. Emphasizing geographical identification and authenticity serves as a linchpin, fostering consumer confidence, streamlining regulatory compliance, and contributing to the integrity of the global food supply chain. The subsequent literature review focuses on PDO labelling, with a specific emphasis on Grana Padano PDO cheese and the application of High-Throughput Sequencing (HTS) in analysing cheese microbiota to determine its geographic origin and prevent fraudulent labelling in accordance with PDO regulations.

Introduction

The adulteration of food poses a potential threat to human health, making food safety and quality control crucial aspects in the realm of food chemistry and related fields. Consequently, key stakeholders in the food supply chain, including regulatory bodies, food processors, retailers, and consumers, express significant interest in certifying the authenticity of food products. Various analytical techniques have become increasingly attractive for this purpose. However, food chemists, scientists, and technologists often deal with monitoring and developing innovative products, as well as identifying and preventing food fraud. (Galanakis, 2021) This ongoing process is essential for validating the authenticity of specific products and plays a pivotal role in supporting law enforcement actions.

The surge in food frauds and the production of counterfeit products for economic gain has emerged as a significant concern over the past decade. In 2022, the Alert and Cooperation network received a significant number of notifications, totalling at 4361. Among them, 600 were related to agri-food frauds, emphasizing the critical role of food supply chains and the necessity for more severe monitoring and enforcement systems (Figure 1). These figures serve as a reminder of the importance of taking proactive measures to prevent fraud and safeguard the safety and quality of food products for consumers. (European Commision 2022)

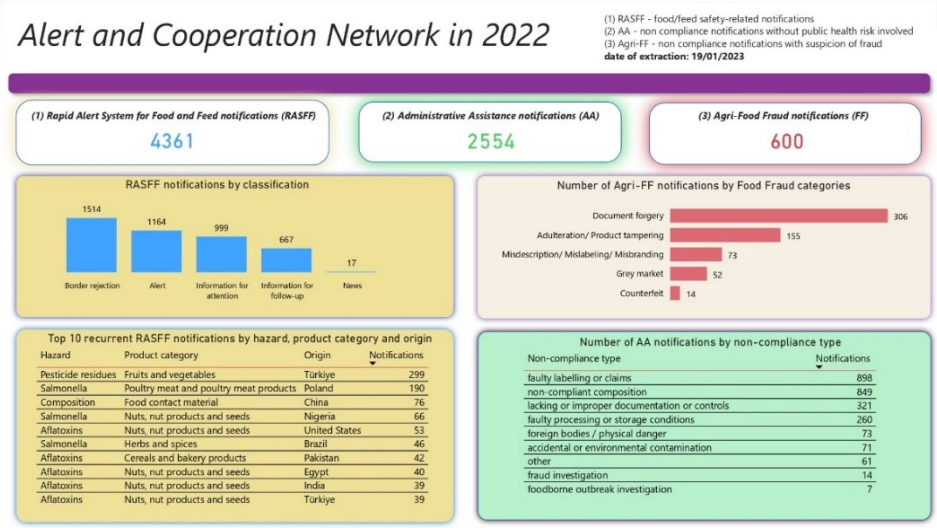

Figure 1: Annual Food fraud notifications received in 2022. There were 600 Agri-Food notifications received. Document forgery had the highest number of notifications, followed by adulteration and tampering. (European Commision 2022)

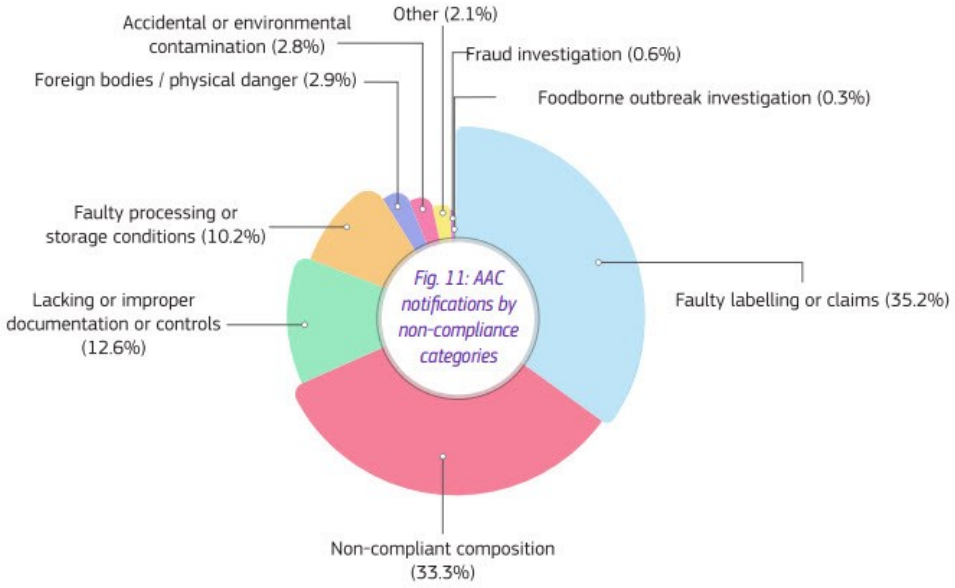

Figure 2:Annual food Fraud reports of 2022. The top reported type of violation was faulty labelling or claims (35.2%).(European Commission 2022 annual report)

Annual food Fraud reports of 2022. The top reported type of violation was faulty labelling or claims (35.2%).(European Commission 2022 annual report)

In the food safety industry, cheese notifications have been consistently reported as the most common for both safety alerts associated with pathogenic microorganisms and fraud incidences related to fraudulent documentation. This information has been sourced from the Rapid Alert System for Food and Feed (RASFF) and HorizonScan, two prominent databases that track food safety incidents globally. (Montgomery et al., 2020) As an example, Italian authorities seized three tons of cheese in May 2019, which had expired several months prior and was stored in adverse conditions. The cheese labels indicated that it was "caciocavallo podolico irpino di cave," a type of caciotta. However, it was discovered that the cheese was produced with common cow's milk, pasteurized and bovine rennet instead of using whole raw milk and the more valuable lamb or kid rennet. According to the findings reported in the press release, some of the

mature cheeses were produced in locations other than those shown on the label (European Commission Newsletter, 2019) Also, during 2022, the ICQRF office in Sardinia investigated to protect Fiore Sardo PDO cheese. This investigation activity discovered that some important Italian cheese producer have obtained Fiore Sardo PDO cheese not respecting the product specifications. In fact, the chemical analisys performed by ICQRF Lab of Perugia had as results that the Fiore Sardo PDO cheese was produced using thermic treated milk as row material, instead “crude milk” as required by the product specifications. (ICQRF ,2023)

Evaluating the safety and authenticity of food serves as a potent strategy to address this issue and safeguard public health. However, the increasing complexity of fraudulent activities necessitates an ongoing enhancement and refinement of analytical methodologies to effectively combat these practices.

The concept of food authenticity revolves around legally acknowledging the distinctive traits of a legitimate food product, encompassing its uniqueness, quality, safety, and traceability, all upheld by a regulatory certification framework. Numerous organizations have been formed for establishing standards and preventing fraud including European Commission (EC), Denominazione di Origine Controllata, and European Food Safety Authority (EFSA). Typically, the certification process is meticulous and bureaucratic, requiring the involvement of various entities, such as the scientific community, governmental organizations, as well as food producers and consumers.

Geographical origin authentication, the process of verifying that food matches its label description, emerges as a critical pillar, not only ensuring the quality and safety of food products but also playing a pivotal role in boosting economies. Beyond safeguarding consumer interests, this authentication mechanism serves as a robust tool for enforcing both national and international legislation. The regional capability to valorise natural capital aligns with the broader goals of geographical origin authentication, emphasizing the interconnectedness of environmental, economic, and regulatory considerations in shaping sustainable and resilient rural landscapes.

The restoration and improvement of natural capital (NC) in rural areas constitute a key objective of the EU's rural development policy (RDP). This initiative aims to yield environmental and biodiversity benefits while positioning NC as a vital territorial asset. NC also serves as a foundation for generating socio-economic second-order effects that enhance economic competitiveness and rural viability. However, the ability of regions to leverage natural capital for economic gain relies on specific contextual factors, needs, potentials, and targeted policy support. (Zasada et al., 2018)

The researchers developed various scientific techniques to protect consumers and screen for adulteration in the food chain. Using a non-targeted approach to analyse food fraud involves utilizing an analytical technique that produces a clear and distinct signal of the authentic sample being analysed. While the signal may differ between techniques, it can still be used to compare the sample's signal profile with a library of previously gathered samples that are relevant to the analysis at hand. These samples serve as a reference for the analysis and help to identify any discrepancies. (Jiménez-Carvelo et al., 2021)

Compared to targeted methods, non-targeted analysis is gaining importance in the food industry due to the complexities of authentication issues such as origin and production methods. To ensure proper procedures, standardized analytical methods and consistent data evaluation, interpretation, and reporting are crucial in legal disputes for non-targeted approaches. (Ballin & Laursen, 2019)

By delving into analytical techniques for food authentication, we not only fortify our defences against food fraud but also elevate the registration processes and marketing decision-making associated with typical products. The emphasis on geographical identification and authenticity thus emerges as a linchpin, fostering confidence among consumers, streamlining regulatory compliance, and ultimately contributing to the integrity of the global food supply chain.

In this literature review, we discuss general definitions and methodologies related to PDO labelling, with a specific focus on cheese production, notably Grana Padano PDO. Furthermore, we explore the application of High-Throughput Sequencing (HTS) in analyzing the microbiota content of cheese. This study aims

to demonstrate how HTS sequencing can be used to determine cheese's geographic origin and avoid fraudulent labels in accordance with PDO regulations.

The primary objective of this investigation is to assess the feasibility of utilizing microbial communities present in cheese products as indicators of their geographical origin by employing microbiota DNA sequencing techniques and conducting comparative analyses of the microbial compositions in various cheeses.

Methodology

The aim of this methodology is to provide a comprehensive and in-depth examination of the existing literature on Cheese genomics, focusing particularly on Grana Padano. This will involve synthesizing significant findings and perspectives on the topic, thereby enhancing our understanding of this area of research.

The methodology employed for this bibliographic thesis is grounded in a systematic and in-depth exploration of scientific literature pertaining to cheese genomics and microbiota, with a specific focus on studies utilizing high-throughput sequencing (HTS) technologies. The following detailed description of key phases reflects the approach taken:

An extensive search was conducted on academic databases, scientific journals, and official publications. Search terms included relevant keywords such as "cheese genomics," "Grana Padano," and "DNA sequencing."

Strict criteria were defined to select literature based on its relevance to the specific theme of Grana Padano genomics and the use of HTS. Only peer-reviewed scientific articles published within a specified timeframe were included. Each included article underwent critical analysis, evaluating the methodology employed, results obtained, and conclusions drawn. Efforts were made to identify emerging trends, gaps in existing research, and potential controversies.:

The literature review was organized around key themes, following a logical structure to enhance reader comprehension. Information was synthesized clearly, with particular attention to connections between studies and implications for Grana Padano's genomics.

Papers cited in this review mainly discuss cheese, milk and authentication analysis. All sources that were used in this thesis have been properly cited according to the accepted citation standards used in scientific publications. A comprehensive bibliography is included at the end of the thesis. All sources used in the thesis were cited correctly, following the accepted citation standards in scientific publications.

1. Authentication and Cheese Production

According to the Cambridge dictionary, authenticity refers to "the quality of being real or true."

Food authenticity refers to the process of verifying that food matches its label description. This involves checking the quality of the food, including its sensory and physiochemical attributes, as well as its origin, such as species and geographic identifications. It also involves verifying the production technologies used, such as traditional or organic methods, and the processing techniques employed, such as freezing, irradiation, or microwave heating techniques.

Ensuring that high-value food products meet specific quality attributes is especially important, as these types of products are often targeted by fraudulent labelling (Danezis, Tsagkaris, Camin, et al., 2016; El Sheikha, 2021)

The definition of authentication is to return food to its reference. Hence, the criteria that define food authenticity are numerous and vary from product to product. Figure 3 shows some of these criteria.

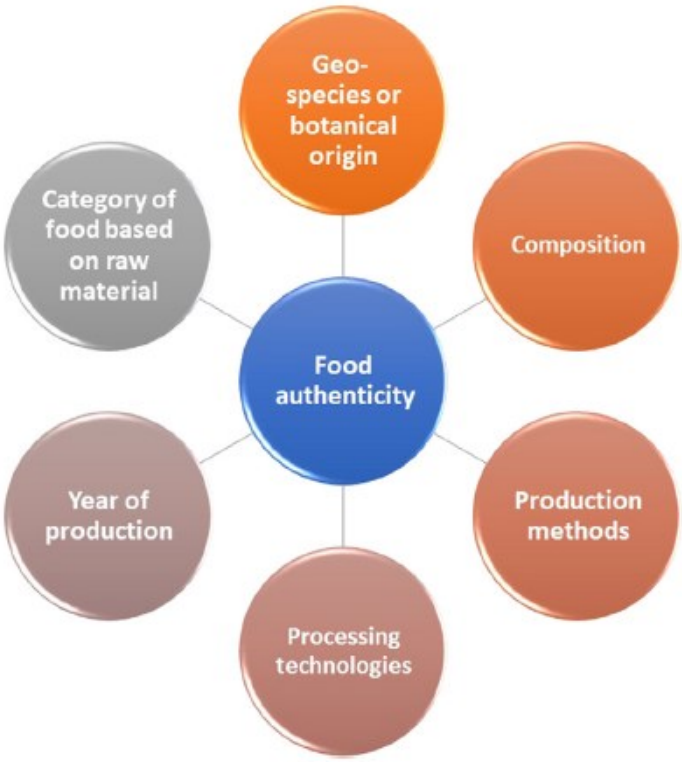

Figure 3:Food authenticity is determined by a set of criteria that play a vital role in authentication analysis. Each of these criteria is of paramount importance, as they help establish the genuineness and quality of food products. (El Sheikha, 2021)

1.1 PDO Authentication

The fraudulent manipulation of food products poses a significant risk to global food safety and has the potential to inflict severe harm on human health. Food fraud is a serious issue that has been prevalent in the food industry. Some examples of food fraud include the addition of melamine to Chinese milk products to increase the apparent nitrogen content and implying a higher protein content,(Gossner et al., 2009) the dilution of extra virgin olive oils with cheaper, lower quality oils to reduce manufacturing costs(Jabeur et al., 2014), and the 'Horse meat scandal' that was discovered in several British and Irish markets in 2013. These cases have led to increased consumer awareness and regulatory improvements.(Brooks et al., 2021)

However, there is still more work to be done to combat food fraud and ensure the safety and authenticity of the food we consume. Various food authorities worldwide express heightened concerns regarding such fraudulent practices. Regulatory bodies, including the FDA, WHO, and the European Commission, have instituted distinct legislations and delineated definitions aimed at addressing and preventing food fraud. The EU Agri-Food Fraud Network is responsible for addressing food fraud in Europe in collaboration with various bodies such as the EC Knowledge Centre for Food Fraud and Quality, OLAF, and Europol. The cooperation between officials with agri-food chain knowledge, police, customs officers, judges, and prosecutors' administrations is crucial to combat fraud at both national and EU levels.(European Commission, n.d.)

Nowadays, fraudulent activities extend beyond compromising health considerations. The current emphasis on utilizing organic, bio, traditional, and artisanal products, coupled with a heightened appreciation for preserving traditional methods in the food sector, underscores a newfound significance in the pursuit of authenticity and the prevention of adulteration compared to previous periods.

In 1992, the first European legislation for agricultural products and foodstuffs was adopted, covering PGI and PDO labels. The EU quality policy is designed to safeguard the identities of specific products, endorsing their distinctive features tied to both their geographical origin and traditional expertise. The designation of

a 'geographical indication' (GI) is granted to product names with a specific connection to their production location. This GI recognition not only instils consumer trust and facilitates the differentiation of high-quality products but also aids producers in enhancing their marketability. The list of products under consideration or already bestowed with GI recognition is documented in geographical indications registers, which also detail the geographical and production specifications for each item. Geographical indications, acknowledged as intellectual property, play an increasingly pivotal role in trade negotiations between the EU and other nations. Furthermore, additional EU quality schemes underscore the significance of traditional production processes or products originating from challenging natural landscapes such as mountains or islands (European Commission, 2023.).

In the European Union (EU), food certification legislation is defined by the European Commission (EC) Directives and Regulations produced by the European Food Safety Authority (EFSA) (EFSA, 2023). Established through EU Regulation, EFSA operates under a framework of laws and guidelines known as General Food Laws (Regulation EU 178/2), primarily focused on ensuring the quality and safety of food. The EU regulation requires all food products to be safe for consumption. This is mandatory and applies to all food sold in the EU market. Article 16 outlines clear regulations for the labelling, advertising, and presentation of food and feed products. The regulations aim to prevent the adulteration of labelling and ingredient composition. Food and feed labels, advertisements, and packaging should not confuse or deceive consumers. This includes the shape, appearance, and materials used in the packaging, as well as how they are displayed and the information provided about them. All information should be clear and truthful and not mislead consumers through any medium.

The PDO, PGI and TSG schemes were introduced, not only as a way to support consumers' decisions, but also as a mean of food control (Dias & Mendes, 2018) In a study, Italy secured the second position in awareness regarding quality schemes among EU countries, trailing behind Greece, with a recorded awareness level of 16% .There is also evidence of regional variation, with higher

levels of awareness in regions where protected products have their origin. (Grunert & Aachmann, 2016)

According to eAmberosia, the EU Commission's Geographical Indications register reports a total of 1,656 registered products. Among these, those originating from Italy account for approximately 20.2%, with a specific count of 335. Notably, this food product category includes 16.7% dedicated to the diverse offerings of 56 varieties of cheeses. (European Commission, December 2023)

Protected Denomination of Origin cheeses (PDO) are products of high commercial value limited to legislative and proper labelling rules. Their popularity is growing and therefore it is crucial to protect the interests of the producers and to protect the consumers.

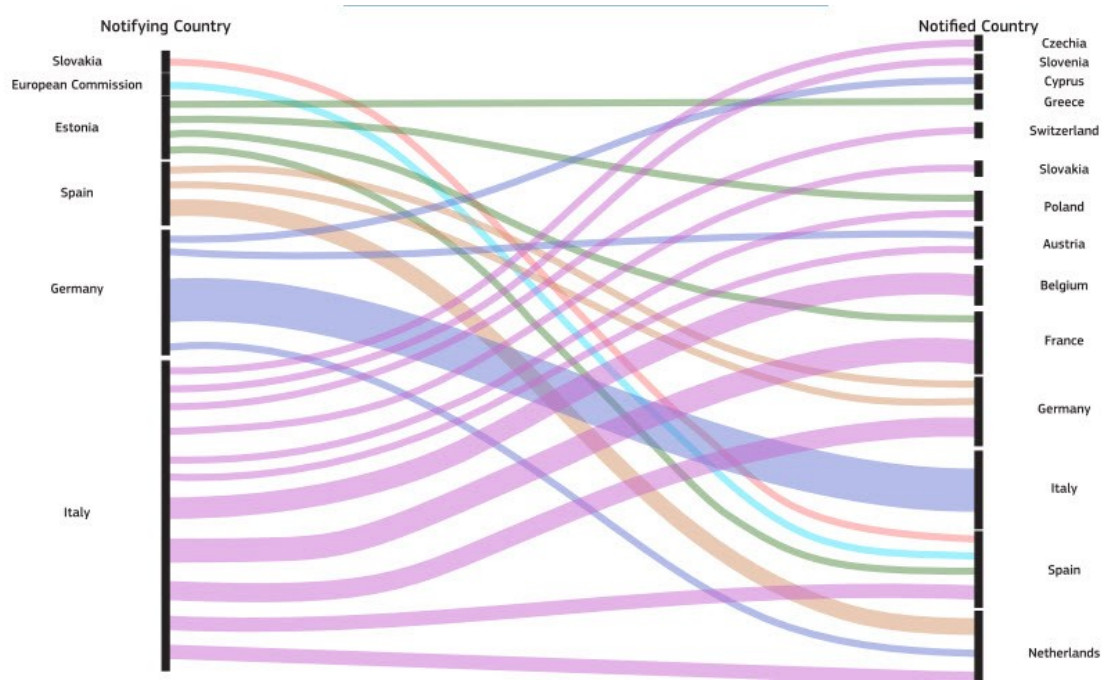

Figure 4:In the course of 2022, 37 cases (30 AAC and 7 FF notifications) were received on PDO and PGI with fraud suspicion elements. Italy received noticible requests, created by Germany.(EU commission annual report,2022)

European producers are aware of these schemes and consumers are showing renewed interest in traditional food. On one hand, agricultural producers in the EU are increasingly interested in using geographical indications to differentiate their products in international markets, and thereby improve their competitiveness and profitability. On the other hand, consumers' growing interest in quality and traditional products creates a demand for agricultural products and foodstuffs with

specific, identifiable characteristics, particularly those that are linked to their geographical origin and their production method (Dias & Mendes, 2018)

Overall, these Schemes are spreading worldwide, especially in EU and they have an important influence on different aspects of the food industry and economy. With increasing public awareness and impact on governments, there is a need to focus on detecting frauds related to regional productions.

Establishing proof of origin is essential for safeguarding consumers, ensuring food quality and safety, and adhering to both national and international laws, standards, and guidelines. With the globalization of food markets and the increased diversity of food products worldwide, there's a risk of fraudulent labelling due to the assumed quality of these products. Numerous organizations have been formed for establishing standards and overseeing the origin of components and production processes. Examples include the Institut National des Appellations d'Origine (INAO) in France, Denominazione di Origine Controllata in Italy, and Denominación de Origen in Spain. Committing to these standards ensures the production of high-quality final products, deserving fair pricing at the point of sale. However, their quality has made them targets for counterfeiters and illegal food traders. Consequently, authenticating food is crucial both for its provenance and quality.(Danezis, Tsagkaris, Camin, et al., 2016; El Sheikha, 2021)

In Europe, food authenticity is a significant concern, particularly regarding origin. Legislation within the European Union has reserved specific names for foods and beverages of particular quality or reputation, following the Council Regulation EEC No 2081/92. These laws established a regulatory framework for quality schemes for food products like Protected Designation of Origin (PDO), linking them to specific geographical areas of production. (Danezis, Tsagkaris, Camin, et al., 2016)

Several methods have been developed to evaluate products and ensure their geographical origin, as discussed in the next chapters.

1.2 Cheese Products

Cheese, a popular dairy product enjoyed globally in various types has become a significant market. In 2022, the global cheese market reached a value of around

83.4 billion U.S. dollars, with projections indicating it will surpass 120 billion U.S. dollars by 2028 (Statista, August 31, 2023).

Despite the widespread popularity of cheese, the dominant player in global dairy production is cow milk. In the past year, the world produced over 544 million metric tons of cow milk, greatly exceeding the approximately 22 million metric tons of cheese. Cheese production has shown a gradual increase each year since 2015, and in 2022, the European Union accounted for about half of the global cheese production (Statista, 2023).

While cows contribute the majority of commercially produced milk globally (Figure 5), there are other animal sources of milk used for human consumption. These include goats, sheep, buffalo, and other mammals specific to certain regions. In 2022, the European Union alone produced 160.0 billion tonnes of raw milk, with cow's milk accounting for 154.3 billion tonnes, while the rest is shared among mammals other than cows (Eurostat,2023).

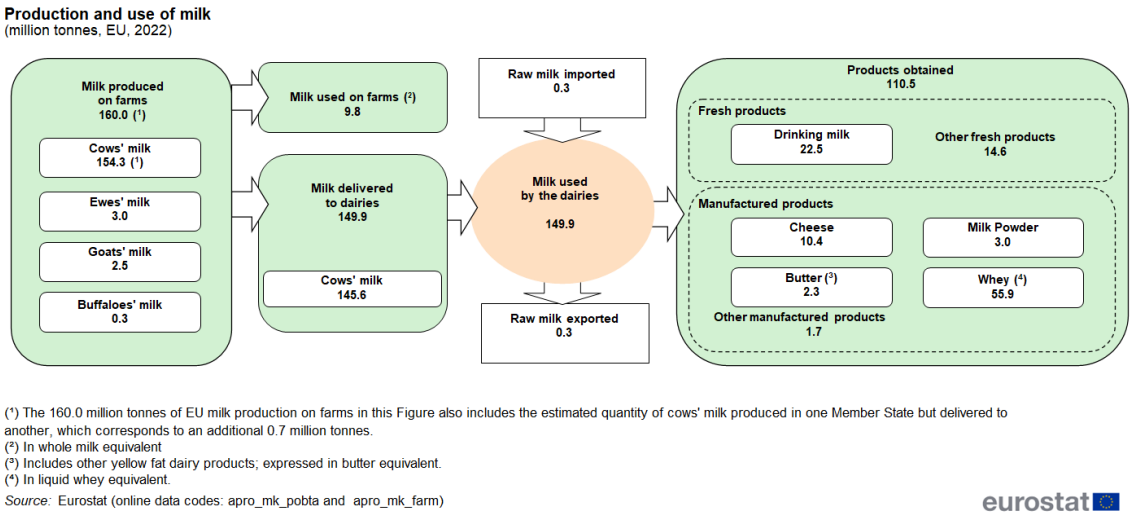

Figure 5 : Production and use of milk in EU by 2022 (Eurostat, December 2023)

Italy has a notable place in European dairy industry and it is the largest producer of PDO Cheeses. In Italy, more than 80% of the produced milk is processed into cheese, among which about 44% are Protected Designation of Origin (PDO) cheeses and the remaining part is mostly used to produce local and typical cheeses (Figure7). (Molle et al., 2023)(clal, Retrieved January 2024)

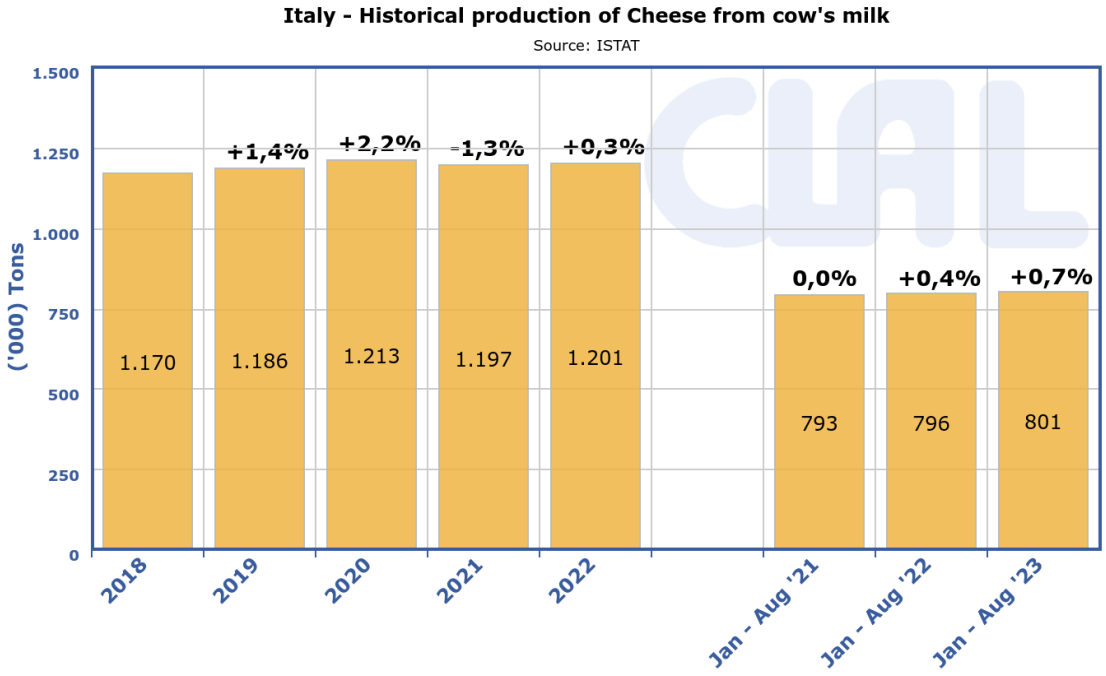

Figure 6: Production of Cheese from Cow's milk in Italy (clal, January 2024)

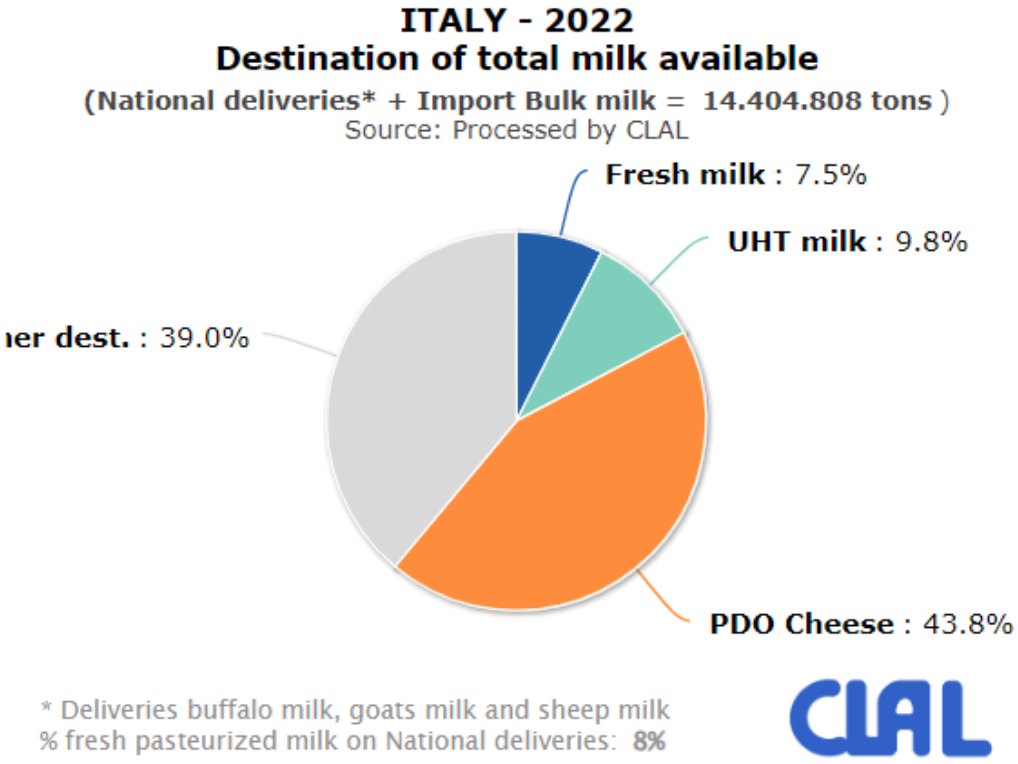

Figure 7:Destination of total milk available in Italy. 43.8% of the milk is used for Production of PDO cheese. (CLAL,2024)

Cheese appeal to consumers hinges significantly on specific sensory characteristics, particularly flavour and aroma. These attributes are tied to the diversity of compounds and molecules present in cheese, such as fatty acids, volatile organic compounds (VOCs), amines, ketones, free amino acids, phenols, alcohols, aldehydes, lactones, sulphur compounds, and more. The interplay of these molecules is influenced by various factors, including the type of cheese, the technological aspects of the cheese-making process, and the conditions during ripening (Pino et al., 2018)

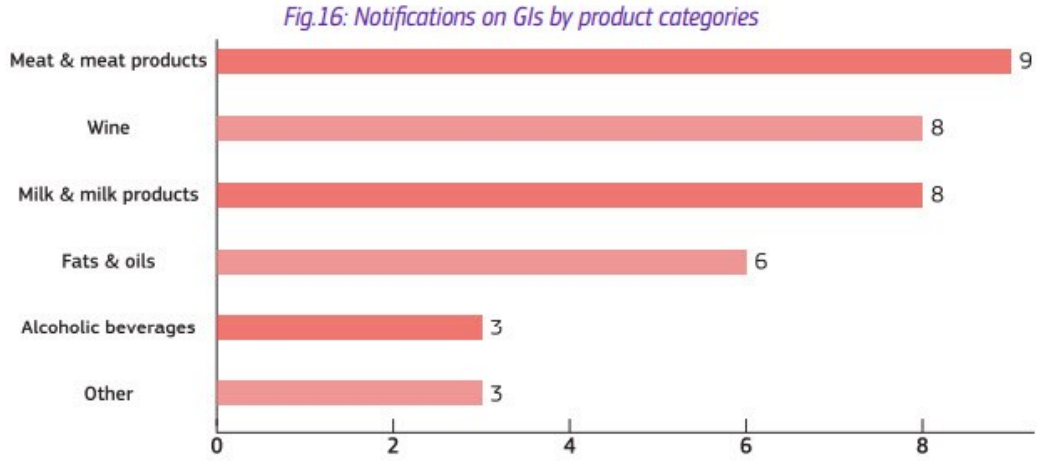

Figure 8:In the most reported product category of food frauds, milk and milk products (21.6%, e.g. Parmigiano Reggiano PDO-IT-0016) are in the third place. (European Commission 2022 annual report)

The wide range of flavors and textures found in various types of cheese can be attributed to the distinct behaviors exhibited by the same species of bacteria in different cheese varieties. The diverse stages of production have varying impacts on the microbiota community, leading to the final product's attributes. Raw milk cheeses, characterized by a more pronounced and complex flavour, contrast with processed counterparts. This distinction can be attributed to the thriving native microbiota present in raw milk cheeses, a feature which is less apparent in cheeses produced from pasteurized or micro filtered milk. (Neviani et al., 2013)The microbiota in raw milk plays a significant role in shaping the microbial community found in many traditional cheeses.

The physical properties and sensory characteristics of cheeses result from the fermentation carried out by a diverse natural microbial community. In cheese-

making, microorganisms actively shape both the composition of the cheese as well as the structure of the associated microbiota. Additionally, the composition of milk, and its derivatives during processing, provides a nutrient-rich source for diverse microorganisms' optimal growth. (Tilocca et al., 2020) The milk-associated microbiota plays a crucial role in shaping the distinctive characteristics of each cheese. The intricate composition of milk, and its subsequent derivatives during processing, serves as a nutrient-rich environment, fostering the optimal growth of a diverse range of microorganisms from various sources. Specifically, the microbial metabolism of certain colonizing bacteria, such as Lactic Acid-producing Bacteria (LAB), transforms milk components, primarily carbohydrates and proteins, into secondary products. These by-products, in turn, serve as substrates for the growth and metabolism of other microorganisms, significantly influencing the composition of the final product (Alessandria et al., 2016; Tilocca et al., 2020)

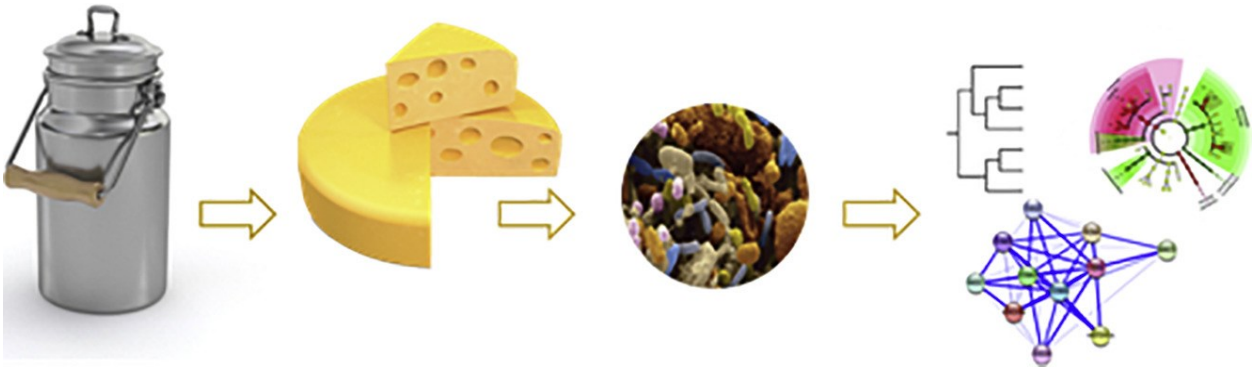

Figure 9: A general overview of the microbiota's path in to make the final attributes. Milk is the primary source of microbiota. During cheese ripening, microbiota contribute to the final chemical composition, aroma, and flavor. (Tilocca et al., 2020)

Throughout the cheese-making process, if the cheese microbiota experience influences from physicochemical stress or pressure arising from various technological phases, it can change the whole production and final product attributes. Thus, microbiota preservation is important also in this manner.

Recognizing the importance of microbiota in cheese-making emphasizes the need to carefully manage and protect it for successful production. This is crucial to avoid mistakes, loss of production, and potential spoilage. Furthermore, we

can utilize the microbiota for additional analytical purposes, which we will delve into in later chapters.

Various analytical methods are available for studying the microbial communities of cheese. Species identification in cheese microbiology can be done through culture-dependent or culture-independent methods. Culture-dependent methods involve isolating and culturing microorganisms before identifying them based on their characteristics. These methods have revealed insights into microbial populations during cheese production but are time-consuming and may not accurately represent the community's diversity.

Culture-independent methods, on the other hand, directly analyze DNA or RNA without culturing. Total DNA or RNA is extracted from the substrate and subjected to global analysis, allowing for the study of total diversity in a single step. These methods, often using PCR amplification, are faster and more exhaustive, making them suitable for monitoring microbial communities over time. However, they may have biases that need to be addressed.

Overall, culture-independent methods offer a promising avenue for studying cheese microbiota dynamics in detail, overcoming some limitations of culture-dependent approaches.(Jany & Barbier, 2008) Among them, The application of high-throughput sequencing has proven invaluable in exploring microbial communities within ecological environments, including food products. This method, known for its ability to swiftly and accurately identify microorganisms, is particularly useful for those challenging to culture and those existing in low abundance (Jin et al., 2018). Researchers have employed this approach to investigate the bacterial diversity of different types of cheeses in order to find possible fraud or contamination.

1.2.1 Grana Padano Cheese

Grana Padano is crafted within a designated geographical region situated in northern Italy, notably spanning provinces such as Alessandria, Asti, Biella, Cuneo, Novara, Torino, Verbania, Vercelli, Bergamo, Brescia, Como, Cremona, Lecco, Lodi, Mantova, Milano, Monza, Pavia, Sondrio, Varese, Trento, Padova,

Rovigo, Treviso, Venezia, Verona, Vicenza, Bologna, Ferrara, Forlì Cesena, Piacenza, Ravenna, and Rimini. Additionally, specific districts in the Bolzano province, including Ante-rive, Lauregno, Proves, Senale-S. Felice, and Trodena, contribute to its production. (Figure 10)

The production area for ‘Grana Padano’ PDO is largely the same as the region of the Po Valley, that is to say the geographical area of the Po riverbed, characterised by alluvial, fluvioglacial floodplain soils, well-supplied with water: this is one of the most fertile areas of the world and among the best suited for growing forage. (eAmbrossia, 2023)

Figure 10:Reigions of Grana Padano Production (consortium of Grana padano; January 2024)

The several steps involved in producing this cheese have been refined over the centuries and, since 1996, are regulated by the European Union. (Molle et al., 2023) Grana Padano holds the P.D.O. (Protected Designation of Origin) status, subject to regulation by European directives. These regulations outline essential procedures in cheese processing, define the product characteristics, and mandate the issuance of a "quality seal" by the "Consorzio per la tutela del Grana Padano," an authority responsible for upholding quality standards. (Clal. January 2024; eAmbrossia. January 2024)

The manufacturing of Grana Padano constitutes approximately 22% of Italy's total milk output. In 2023, Italy saw a notable production of 5,456,349 wheels of Grana Padano, marking a significant increase of 4.7% compared to the previous

year. Moreover, March 2023 witnessed a record-setting production, reaching its highest point in the last five years. (Clal. January 2024)

Grana Padano production involves the use of unpasteurized cow's milk sourced from local farms adhering to well-defined and strictly regulated processes. The cows, which are milked only twice a day, have access to an automatic milking system. The raw milk is collected from herds in various provinces of the Po Valley. It undergoes storage at a minimum temperature of 8°C and is then skimmed through natural creaming. The homogenous mixture is achieved in 1000-L traditional bell-shaped copper cooking cauldrons (or vats, each cauldron produces two wheels of cheese, called “twin wheels”) in which an aliquot of whey from the previous day is added as a starter to lower the pH. This whey is rich in Lactic acid bacteria and triggers the milk to transform into cheese. The temperature is raised to a range of 31 to 33°C, at which point calf rennet is introduced. Following milk coagulation, the curd is cut into small granules to enhance whey expulsion with a giant whisk (spino) into pieces which are as small as grains of rice. Subsequently, the curd undergoes heating until reaching 56°C to facilitate further whey expulsion.

The curd is allowed to rest in the vat, immersed in whey, for a maximum of 70 minutes post-cooking to form a compact mass. This mass is then divided into two portions, extracted from the vat by using a sort of a wooden shovel (pala) and a linen cloth (schiavino), the curd mass is raised from the bottom, and placed into cylindrical molds. During this phase, the cheese undergoes pressing to eliminate excess whey and compact the mass. After spending two days in the molds, the cheese undergoes immersion in brine, a process lasting 14 to 30 days, depending on the saline solution and cheese wheel size. Following brining, the wheels are taken into a “hot room” (camera calda) where they will dry for a few hours. Finally, the cheese wheels are moved to ripening rooms. Grana Padano must undergo a minimum ripening period of 9 months, while certain varieties can age for up to 20 months or more. (Molle et al., 2023)(Grana Padano Consortium. January 2024)

1.3 Microbial Community of Cheese and Microbiota

In 1988, Whipps and colleagues, who were studying the ecology of microorganisms in the rhizosphere, were the first to point the term "microbiome." They defined it as a "distinctive community of microorganisms" that inhabit a "specifically defined habitat with unique physio-chemical properties," which serves as their "theater of activity."(Berg et al., 2020)

Over the last 20 years, numerous definitions have been proposed for the term microbiome. One of the most cited, albeit not the earliest, was that introduced by Joshua Lederberg(Lederberg & Mccray, 2001), He referred to the microbiome as "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space." (Del Frari & Ferreira, 2021)

The microbiome is a term used to describe all living organisms that constitute the microbiota. Figure 11 provides a detailed etymology and differentiation between these two terms. The microbiome includes various members such as bacteria, archaea, fungi, algae, and small protists, which most microbiome researchers agree with. However, the inclusion of phages, viruses, plasmids, and mobile genetic elements remains a controversial topic in defining the microbiome. (Marchesi & Ravel, 2015)The food microbiome is affected by many intrinsic factors like the microbiota present in the raw materials used, the microbiota existing in different production lines and equipment, and the air present in the processing areas. (Michailidou et al., 2021)

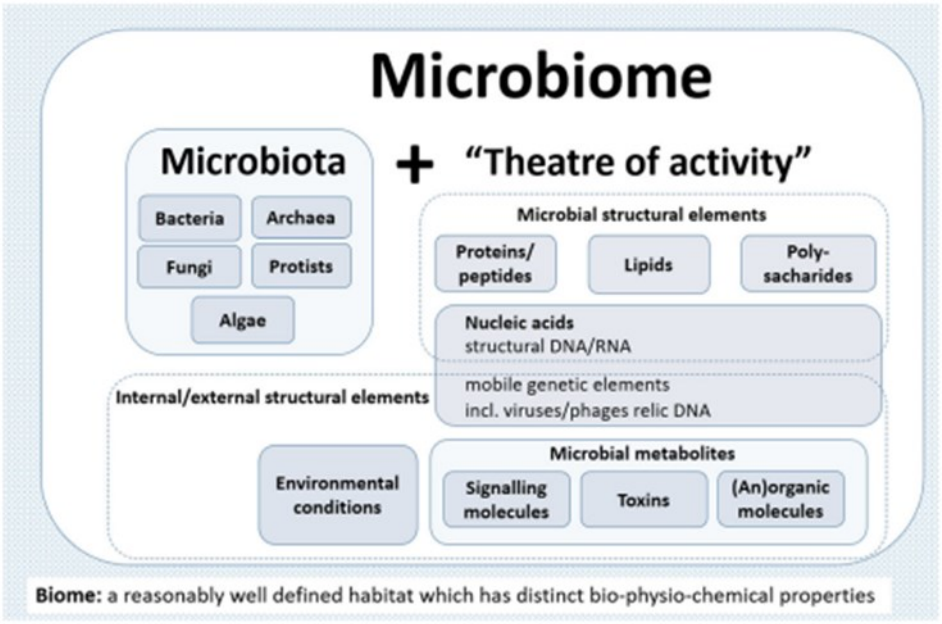

Figure 11: A schematic Figure highlighting the composition of the term microbiome containing both the microbiota (community of microorganisms) and their "theatre of activity" (structural elements, metabolites/signal molecules, and the surrounding environmental conditions. (Michailidou et al., 2021)

Each type of cheese possesses a distinct collection of microbes, The microbiome of each variety of fermented cheese is distinct, shaped by a combination of regional factors and variations in the specific production process and comprising a unique pool. This pool includes the dominant population, primarily composed of lactic acid bacteria, which thrives in the cheese environment and colonizes it successfully. The subdominant microbes exist in lower abundance, limited due to the strong presence of LAB. Additionally, there is a group of low-abundance species, mainly present in the cheese due to environmental contamination. (Kamilari et al., 2019)

There is a direct correlation between the microbiota in milk and the subsequent development of dairy products. Through the fermentation of milk by microorganisms, the production of lactate occurs, impacting the sensory qualities, texture, flavor, and overall organoleptic characteristics of the resulting products. Environmental factors unquestionably contribute significantly to the variability in the composition of the milk microbiota. Fresh milk harbours a diverse range of microorganisms of animal origin. The mammary glands surface serves as the

primary source of bacterial contamination, encompassing phyla such as Actinobacteria, Firmicutes, and Proteobacteria.

Various factors, including the type of forage provided to animals (such as grass, silage, and hay), the hygienic conditions of the animals, and milk storage, play a crucial role in shaping the composition of the raw milk microbiota. Previous studies examining the microbial profile of diverse pastures have reported a high presence of Enterobacteriaceae, coryneform bacteria, staphylococci, and yeasts, coupled with a reduced count of lactic acid bacteria (LAB) in grasslands (Tilocca et al., 2020)The initial prevalence of Starter Lactic Acid Bacteria (SLAB) is attributed to their metabolic capacity to efficiently ferment lactose. However, in the case of long-ripened cheeses, other Non-Starter Lactic Acid Bacteria (NSLAB) with enhanced proteolytic and lipolytic abilities, capable of metabolizing carbon sources beyond lactose, can dominate the cheese microenvironment. This dominance has a significant impact on the flavor and texture of the cheeses. (Kamilari et al., 2019)

Traditionally, cheese production involved the direct curding of whole raw milk without any preventive treatment. Even today, the majority of artisanal cheeses continue to be crafted using raw milk. In contrast, many commercially and large-scale produced cheeses undergo milk treatments, facilitating the manipulation of milk characteristics, the standardization of milk quality, and the reduction of the milk microbial load.

Parmeggiano Reggiano is a perfect example of this dichotomy, as the specified method emphasizes that "the milk must not undergo any heat treatment or physical or mechanical treatments such as centrifugation, bactofugation, or microfiltration, and additives may not be used" (eAmbrosia).

Similar rules are considered for Grana Padano PDO. The milk must be partially skimmed by natural surface skimming at a temperature of between 8 and 20 °C. From the barn to the production line, it cannot undergo any physical, mechanical or thermal treatments that might modify its status of natural raw milk. There are more specific conditions in the criteria of milk used for Grana Padano, which the Regulation governs the terms and conditions for granting to companies, licensed to package Grana Padano PDO cheese, the use of the logo "Il Nostro Latte” (Our Milk), created and deposited by the Consortium for the Protection of Grana

Padano Cheese (or Grana Padano cheese Protection Consortium) and reproduction parameters should be considered in all respects an integral and substantial part of the regulation itself.

Within traditional raw milk cheeses, lactic acid bacteria (LAB) strains play a crucial role in shaping the characteristics and overall quality of the cheese. One study shows that there is a strong, negative relationship between richness and LAB abundance (Reuben et al., 2023). Additionally, Lactobacillus species such as *L. parabuchneri (now Lentilactobacillus parabuchneri), L. parafarraginis (now Lentilactobacillus parafarraginis), L. hilgardii (now Lentilactobacillus hilgardii), L. diolivorans (Lentilactobacillus diolivorans), and L. nasuensis (now Lacticaseibacillus nasuensis)* are found in GP cheese, potentially due to the use of corn silage in cow diets. (Giraffa, 2021) (LactoTax, n.d.)

The microbial composition of cheeses is subject to variations influenced by factors such as the type of cheese, environmental conditions, ecological considerations, and processing factors, including the milk source, starter type, heat treatments (e.g., refrigeration, pasteurization, cooking), and ripening conditions (Yeluri Jonnala et al., 2018). Various criteria, including the quantity of *Enterobacteriaceae*, the count of coagulase-positive *Staphylococcus aureus*, and the overall bacterial count, can be utilized to assess the microbiological quality of raw milk.

The microbial composition and diversity differ not only between raw and pasteurized milk but also among curd, whey, and cheese. In raw milk, the microbiota is influenced by microorganisms present in the teat canal, on the teat skin's surface, hygiene practices, animal handlers, and the indigenous microbiota of equipment and storage containers. The diversity levels are impacted by the milk source, with cow's milk exhibiting greater diversity than that of goats and sheep (Yeluri Jonnala et al., 2018). Remarkably, in one study, 27% of bacteria detected in raw milk cheese were also identified on the teat surface. These bacteria, including species like *Brevibacterium linens, Staphylococcus equorum*, and Lactic Acid Bacteria (LAB) such as *Lactococcus lactis*, play a role in flavor, aroma, and color development in the cheese production process.

1.4 Factors influencing Microbial community of Cheese

As Mentioned, Variations in microbial composition and diversity are evident across different stages, such as raw and pasteurized milk, as well as between curd, whey, and the final cheese product.

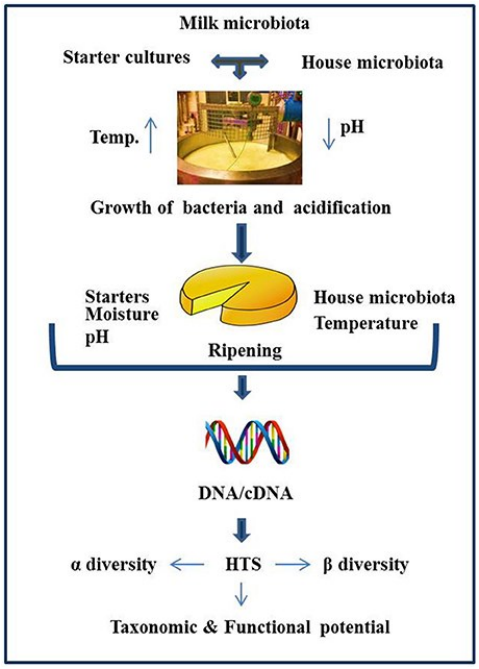

Figure 12:Schematic representation of factors influencing the cheese microbiota, as revealed by HTS. (Yeluri Jonnala et al., 2018)

Some of important factors contribute to the diversity of the cheese microbial community are indicated below:

Milk: The microbiota is the first source of cheese microbiota composition. The microbiota of raw milk has a important role in the development of cheese flavour, as well as implications for shelf life and safety and it is shaped by microorganisms present in the teat canal, on the surface of teat skin, hygiene protocols, the handlers of the animals, and the native microbiota found in equipment and storage containers. (Frétin et al., 2018)

Additionally, the source of the milk appears to play a role in determining its diversity levels, with cow's milk exhibiting a higher diversity compared to that of goats and sheep (Yeluri Jonnala et al., 2018)

Heat treatments: High-temperature treatment (when pasteurized milk is used) and low pH also contribute to the selection of specific bacteria in some artisanal cheeses that are made in the absence of starter bacteria.

***Producers and Production Practices*:** The microbial composition of cheeses can vary among producers, influenced by production methods and the presence or absence of starters (Yeluri Jonnala et al., 2018). High-throughput sequencing (HTS) analyses conducted on samples from nine different producers revealed the consistent presence of the genera *Lactococcus, Lactobacillus, and Streptococcus* which most of their mutants are resistant to heat, in all samples. However, these genera were found in different proportions, contributing to variations in the specific characteristics of Plaisentif cheese.(Dalmasso et al., 2016) It's noteworthy that even in large-scale industrial production , which tries to reduce the scale of variation by using specific starters, differences in the associated microbiota can still be observed.

***Production Environment*:** The indigenous microbiota presents in the production environment, which colonizes equipment, brine tanks, vats, wooden surfaces, knives, and various other surfaces within production facilities, significantly influences the microbial communities of cheese. The colonization dynamics hinge upon surface characteristics, nutrient availability and composition, microbial capacity to form biofilms, ecological factors, as well as the practices of operators and the efficacy of cleaning processes.

***Cheese Rinds*:** The microbiota present in cheese rinds exhibits a high level of complexity in comparison to core samples. This microbial composition varies across different types of rinds, degrees of ripening, and environmental conditions. High-throughput sequencing (HTS) analyses conducted on 11 artisanal Irish cheeses, including soft, hard, and semi-hard varieties, identified 19 genera. Notably, *Lactococcus, Leuconostoc, and Lactobacillus* were found in both rind and core samples, while Corynebacterium, Facklamia, Flavobacterium, and Cronobacter were exclusively detected in rind samples. The washing of the rind in the cheese production process contributes to this distinct microbial presence.

Furthermore, a study observed that rind samples from cheeses produced in different geographical regions exhibited similar patterns of microbial clusters, suggesting that, in this particular case, geographical distance did not impact the microbial community.

***Starter Culture*:** Primary contributors to the microbial populations in fermented cheeses are starter cultures, although many artisanal cheeses are crafted without their addition. Italian PDO cheeses, such as Asiago Pressato, Gorgonzola, Asiago d'Allevo, Quartirolo Lombardo, Taleggio, Raschera Montasio, and Mozzarella, utilize undefined natural milk starter cultures (NMC). These NMCs are produced through the heat treatment of raw milk (60–63°C, 20–30 min) followed by incubation at elevated temperatures (39–42°C). Additionally, the inclusion of natural whey cultures (NWC), containing thermophilic lactic acid bacteria (LAB), has been a traditional practice in curd fermentation for various PDO cheeses, including Parmigiano Reggiano, Grana Padano, Provolone Valpadana, Mozzarella di Bufala Campana, and Caciocavallo Silano. The addition of NWC is considered essential for the flavor development of these cheeses. NWC formation involves heating raw milk at 60–63°C for 20–30 min and subsequent incubation at temperatures of 39–42°C. 16S rRNA sequencing analysis has indicated that NWC is predominantly dominated by Streptococcus thermophilus and *Lactobacillus delbrueckii* (Kamilari et al., 2019).

The isolation, identification, and characterization of microbial strains predominant in Natural Whey Cultures (NWC) and Natural Milk Cultures (NMC), crucial for cheese ripening, have facilitated their use as starter cultures. Traditional artisan cheese-making industries often apply selected bacterial strains from these cultures as starting cultures in cheese ripening, resulting in their dominance in the microbiome of the cheeses.

Various Natural Milk Cultures and Natural Whey Cultures exert distinct influences on the bacterial community of Italian PDO Cheeses. In the case of Grana Padano, a whey starter culture is employed. The whey generated on a given day is rich in lactic acid bacteria, making it an ideal catalyst for initiating the transformation of milk into cheese. (Yeluri Jonnala et al., 2018)(Grana Padano Consortium)

Brine: The microbial diversity and composition of brine are contingent upon the type of cheese, the specific cheese plant, and salinity concentrations. Adventitious bacteria found in brine, such as *Staphylococcus equorum*, exhibit robust antibacterial activity against *L. monocytogenes* on cheese surfaces, influencing the flavor and color properties of smear-ripened cheeses.(Yeluri Jonnala et al., 2018)

However, contaminated brine poses a risk of contaminating the cheese core and surface, with Mozzarella being susceptible to such spoilage. *Listeria monocytogenes* is a foodborne pathogen for humans, given its capacity to grow in refrigerated conditions. Cheese can become contaminated with L. monocytogenes through cross-contamination during the cheese-making process such as packaging materials, racks for product transport, hand tools, gloves, among others. Due to its capability to survive in high salt concentrations and form biofilms, the cells that contaminate the brine and fluids can endure and be carried to the food product, particularly Mozzarella di Bufala Campana PDO cheese.(Ricci et al., 2022; Silva et al., 2023)

Effective cleaning procedures are crucial for eliminating spoilage bacteria present on surfaces in dairy plants, with the material of the surface playing a significant role in bacterial adherence.(Yeluri Jonnala et al., 2018)

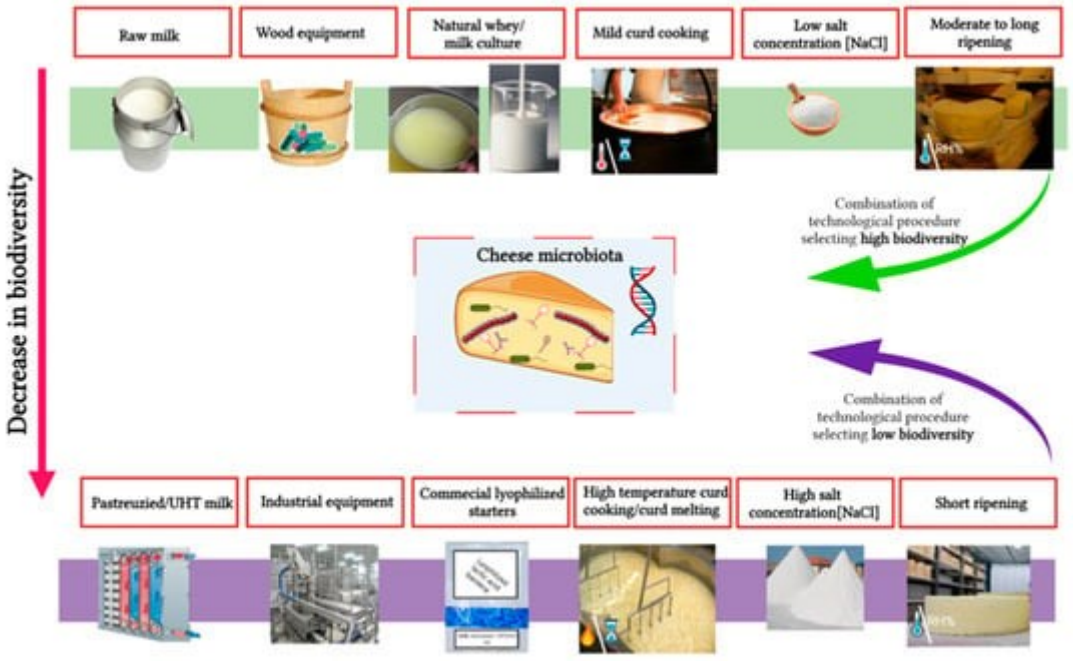

Figure 13:Technological factors affecting cheese microbiota biodiversity.(Cardin et al., 2022)

2. Methodes of Cheese Authentication

Analytical technologies (physicochemical techniques, biological approaches) are the most effective means of detecting adulteration or mislabelling in food products.(Danezis, Tsagkaris, Brusic, et al., 2016; Danezis, Tsagkaris, Camin, et al., 2016; El Sheikha, 2021) Various methods rely on the presence of organic compounds to determine the primary components of the sample or to identify organic compounds that are specific to a PDO. These methods analyse multiple signals to obtain specific insights into the unique characteristics of a cheese that are linked to its origin. Modern techniques favour polyphasic chemical and physical analyses rather than relying on single-parameter descriptions such as dry matter, total protein, or salt content. (Armenta & de la Guardia, 2016; Cardin et al., 2022)

Analytical methods primarily rely on assessing chemical compositions, which can be similar even across different areas. Efforts have been made to identify specific components typical of certain regions or production methods. Molecular methods are also utilized, particularly when different strains or breeds of organisms are involved in production. Chromatographic and molecular methods are prominent in food authentication solutions. Additionally, isotopic, vibrational, UV & and fluorescence spectroscopy, elemental techniques, and NMR are widely used. While non-chromatographic MS, microbial fingerprinting, and sensory analysis have not been fully utilized, they are expected to gain more attention. Recent trends show an increase in the popularity of chromatographic, molecular, vibrational, and fluorescence spectroscopy techniques in food authentication.(Danezis, Tsagkaris, Camin, et al., 2016; El Sheikha, 2021)

Geographical authentication methods used for cheese authentication can be categorized as chemical-based, physical-based, and DNA-based (Cardin et al., 2022)

Chemical analyses such as stable isotope ratios, trace elements, and fatty acid profiles are traditionally used for origin authentication. However, emerging techniques like DNA-based methodologies and Near Infrared Spectroscopy are also being explored in this field.

In general, the methods encompass gas chromatography (GC), high-performance liquid chromatography (HPLC), infrared (IR) spectroscopy, solid-phase micro extraction (SPME) and purge and trap (P&T), as well as nuclear magnetic resonance (NMR) spectroscopy. (Kamilari et al., 2019)

In this discussion, we will have a view of some important methods and their applications in authenticity control. By using these authentication methods, consumers can be confident that they are receiving authentic products that meet their expectations.

2.1 Chemical based analysis

2.1.1 Isotope Ratio Mass Spectrometry (IRMS)

IRMS technology can detect the natural isotopic abundance of light and heavy stable isotopes, mainly determined by climatic or geographical factors.In food science, IRMS analysis is used for the isotopic profiling of products, the investigation of food adulteration, the tracing of geographic origin, and the authentication of organic status.(Giannioti et al., 2024)The stable isotope ratios of hydrogen, carbon, nitrogen, oxygen, and sulfur in feed and water are closely related to the local environment(Hong et al., 2017).The mineral content and trace elements found in cheese clearly reflect the soil type of the cultivation area and the environmental growing conditions used in food production. Therefore, they have been widely used to distinguish cheeses from different geographical regions. Additionally, the elemental composition of cheese, along with animal breed, plant feed, and mineral supplementation, can indicate geological and pedological traits of the environment(Armenta & de la Guardia, 2016; Cardin et al., 2024)

IRMS methods have been developed for the detection of food adulteration and geographic characterisation, such as in the case of Italian grape musts or Chinese honey and food. In particular this technique has been widely used for the verification of authenticity of honey based on the carbon stable isotope ratio analysis of the different sugars(Giannioti et al., 2024).

In case of cheeses, Isotope fingerprinting has proven to be a reliable method for authenticating the geographical origin of typical cheeses. It is worth mentioning that stable isotope ratio analyses are already being used as a traceability tool for certain PDO cheeses, such as Grana Padano and Parmigiano Reggiano.

Scientists often use isotopic analysis to determine the origins of food. Since regulations for food labelling are subject to change, and laws are continually evolving, it is crucial to improve and update testing and surveillance measures regularly. By doing so, we can detect and prevent fraud more effectively.

1.1.2 Elemental fingerprinting

The process of elemental fingerprinting is a comprehensive approach used to identify the chemical species, content, and distribution of elements within biological systems. Its importance lies in the recognition and exposure of inorganic elements (Mittal et al., 2017).

Currently, there are various analytical methods available for determining the concentration of elements, including atomic absorption spectrometry, graphite furnace atomic absorption spectrometry, inductively coupled plasma-atomic emission spectrometry, and inductively coupled plasma-mass spectrometry (ICP-MS). ICP-MS has emerged as the preferred method due to its high sensitivity and ability to determine concentrations of multiple elements simultaneously.

The elemental composition of animal products is influenced by a variety of factors, including animal species (e.g., cow, sheep or goat), mineral supplementation, drinking water, and production practices. Additionally, the elemental profile of soil and mineral pollution is associated with specific geographical areas and serves as a unique identifier of origin. Recent studies have also included Rare Earth Elements (REE) and precious metals in these analyses, which are reliable and authentic markers for various products. As a result, elemental fingerprinting is a highly efficient and effective tool that has demonstrated exceptional performance in scientific research(Cardin et al., 2024; Danezis, Tsagkaris, Camin, et al., 2016; Zhu et al., 2023)

1.1.3 Volatilome

The analysis of volatile organic compounds (VOCs) has become a prevalent method for verifying the origin of cheese, as these compounds play a crucial role in defining the unique qualities of various cheese types. The volatilome is the complete set of VOCs produced by an organism. In the case of food, the volatilome is often comprised of various chemical classes that result from the organism's primary metabolisms and reactions. In the context of cheese, these compounds are generated by the metabolic activities of the microbiota during glycolysis, proteolysis, and lipolysis, leading to a diverse range of VOCs. The metabolic activities of cheese microbiota are instrumental in shaping the distinct characteristics of each cheese type. (Cardin et al., 2024)

As volatile compounds play a significant role in defining the typicity of cheese, VOC analysis has become a prominent method for authenticating its origin. Notably, cheese origin can result in substantial differences in the relative abundances of the majority of VOCs investigated. Certain compounds found in cheese may originate from animal feed or proteolysis, which can occur during the cutting of grass or the drying process, or due to the use of coagulants. On the other hand, methyl ketones are believed to be produced during cheese ripening as a result of the metabolic activity of the dominant microbiota. (Ruiz et al., 2023)

Chromatographic resolution is a fundamental analysis method for determining the volatilomes in cheese. GC analysis is the most efficient method for determining VOCs in cheese products. (Rodríguez-Hernández et al., 2022; Squara et al., 2022)

1.1.3.1 Gas Chromatography (GC)

Gas Chromatography (GC) is widely used in various applications, especially in the analysis of fatty acids and triglycerides. For this purpose, GC with a flame ionization detector (FID) is employed, which requires prior derivatization into fatty acids methyl esters (FAME) and triglyceride trimethyl ethers (TG) . The method is particularly effective in separating, identifying, and quantifying fatty acid methyl esters (FAMEs) in dairy fat (Ahad & Nissar, 2017; Haddad et al., 2023)

In gas chromatography, the sample is first vaporized in a heated chamber. Then, the mixture components are separated as they travel through the column transported by the flow of a high-pressure inert gas such as helium, nitrogen, or hydrogen. The separation is based on the selective interaction of the components with the column material.

GC coupled with MS is the preferred method for analysing volatile compounds due to its high reproducibility. However, GC-MS analysis requires careful sample cleaning, which makes it expensive and time-consuming (Ahad & Nissar, 2017).

GC-FID can be considered an efficient and affordable tool in the authentication of cheese and dairy products. It is particularly useful for detecting subtle frauds related to the geographical origin, animal breed, and farming system (Amores & Virto, 2019). The identification and quantitation of many FAs, including minor ones and isomers that differ by the position and geometry of a double bond or the location of a lateral chain, are required for the detection of such frauds. Long GC capillary columns, with more complex and specific phases, new chromatographic arrangements in multiple dimensions, SPE, TLC, and HPLC methods for improved fractionations, and mass spectrometers to identify minor FA, make it possible to detect and quantify the vast majority of the FA present in milk (Ahad & Nissar, 2017).

In one study, GC-FID analysis of the differences between the cheese fatty acid and triacylglycerols content in different producers showed that the amount of fatty acid is connected to the animals' diet via the rumen microbiota. This suggests that GC analysis can be used for species-specific prediction models and is an effective methodology for cheese authentication (Amores & Virto, 2019). GC-FID is also the official method for foreign fat determination in milk and has been proven effective in authenticating milk and dairy products (Haddad et al., 2023).

1.2.2 Nuclear Magnetic Resonance

NMR spectroscopy is a well-established method for food metabolomic analysis. This technique relies on the absorption of radiofrequency radiation by atomic nuclei. While NMR analysis is more commonly used for examining plant-derived food, it has also shown great success in analyzing dairy and meat products (Tarapoulouzi & Theocharis, 2022; Zhang et al., 2024). Specifically, NMR technology can identify species in raw milk and analyze cheese samples from different breeds. This technology is commonly used to investigate food authenticity, with a focus on cheese and milk studies. It has proven highly effective in ensuring the authenticity and quality of dairy products and tracking product evolution throughout processing and storage.(Tarapoulouzi & Theocharis, 2022; Zhang et al., 2024)

H-NMR is a highly stable and sensitive approach for lipid analysis, capable of suppressing interferences through modern pulse sequences. Its reliability is confirmed by both NMR and statistical analysis, making it a valuable tool in food analysis. In particular, the differentiation of ripening stages in Parmigiano Reggiano cheese is of great economic importance, and this method has shown reasonably good reliability in achieving accurate results. (Lolli et al., 2018; Zhang et al., 2024)

A further distinguishing feature of H-NMR is its inherent ability to quantify based on the signal area, which directly relates to the number of protons in the chemical structure, making it particularly suitable for detecting cyclopropane fatty acids. H-NMR was employed to determine the content of CPFAs in hay milk and milk obtained from cows that were fed with silage. (Figure 14) The use of H-NMR has been effective in authenticating dairy products, such as Parmigiano Reggiano cheese, by monitoring the absence of cyclopropane fatty acids (CPFAs). (Iannone et al., 2024a)

CPFA is not present in PDO cheeses that prohibit the use of silages. However, in Grana Padano cheese samples, CPFA is always present, indicating that silages are not prohibited in their production. Therefore, CPFAs are used as a

marker to distinguish the presence of silage in the feed and for the authentication of Grana Padano and Parmigiano Reggiano cheeses.(Caligiani et al., 2016; Iannone et al., 2024b)

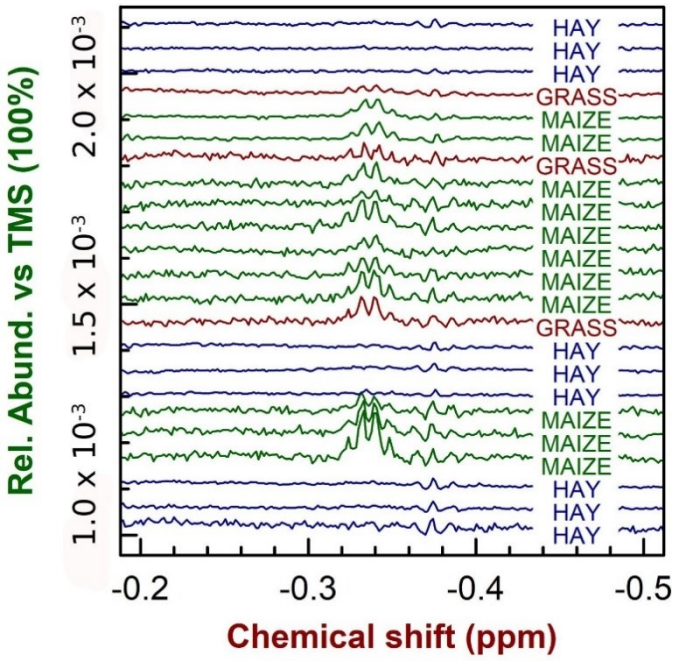

Figure 14: Representative 1H NMR spectra of silage and non silage cow fed milks.(Iannone et al., 2024b)

1.2.3 Infrared Spectroscopy

Near-infrared spectroscopy (NIR) is a rapid analysis method that requires minimal sample preparation and has proven to be effective for food industry authentication. NIR signals are linked to molecular vibrations, particularly the overtones and combinations of fundamental vibrations. NIRS technology generates a vast range of spectral data, spanning from near-infrared to far-infrared, which provides valuable chemical and physical information. This enables the use of NIRS to authenticate various aspects of cheese production, such as its geographic origin, milk type, manufacturing process, quality parameters, and composition data. Furthermore, NIRS can also detect any potential fraud, such as adulteration, ensuring the authenticity of the product.

(Cardin et al., 2024; Lei & Sun, 2019) NIR is mainly applied to oils, fruits, and cereals in plant-derived products

NIR spectra contain extensive information from overtones and combination bands of fundamental bonds, such as C-H, O-H, C-HO, and N-H, generating a complex spectrum. Near Infrared Spectroscopy (NIRS) is an emerging technique in the origin authenticating field, therefore Chemometric techniques are needed to extract desired spectral information, such as the presence of adulterants.(Abbas et al., 2018; Lobato et al., 2018)

1.3 DNA based Analysis

The genetic material of a species is contained within its cells, known as DNA. DNA is a nucleic acid that carries the genetic information for the regulation and the biological development of all cellular life forms. DNA is composed of two strands that coil around each other in the shape of a double helix. DNA is a polymer which consists of nucleotides. Each nucleotide contains a phosphate group, a sugar group (deoxyribose), and a nitrogen base. The four DNA bases are adenine (A), guanine (G). cytosine (C), and thymine (T). DNA is ubiquitously present and can be identified in any appropriate sample regardless of the tissue of origin.

DNA barcoding is a biological identification method that uses relatively short genomic DNA fragments as markers. DNA analysis has proven to be a reliable method for species identification, particularly through PCR-based assays; An exciting development in this field is DNA barcoding, which serves as a valuable supplement to traditional identification methods. DNA barcoding is utilized in various product authentication processes, including honey, spices, cheese, and dairy products, as well as for the authentication of Halal and Kosher food. (Rohman et al., 2021; Utzeri et al., 2018; Zhang et al., 2024)

DNA is a highly stable molecule and in recent years, DNA-based techniques such as PCR have become powerful tools for food dairy products authentication, namely through specific markers selection which makes this method the most reliable for food product authentication.(Figure 15) (Baptista et al., 2021)

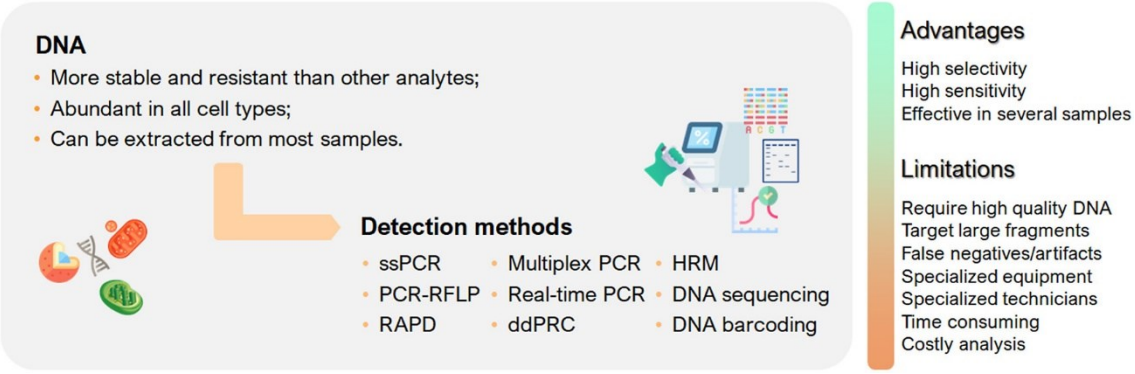

Figure 15: Advantages and limitations of DNA based methods in food authenticity (Barrias et al., 2024)

Protein-based techniques for dairy products authentication have been replaced by higher sensitive and reproducible DNA-based methods since DNA is more chemical and thermal stable than proteins and thus, withstands food processing.(Baptista et al., 2021) DNA-based methods present clear advances in relation to protein-based methods in terms of time, price, sample amount and sample processing. Moreover, the detection limit between DNA and protein-based methods is similar. PCR is the favoured molecular technique for authenticating dairy products due to its ability to analyse DNA molecules. However, next-generation sequencing methods and advanced bioinformatics tools have also offered more comprehensive insights into the composition and possible functionality of cheese microbiota.(Afshari et al., 2020; Baptista et al., 2021)

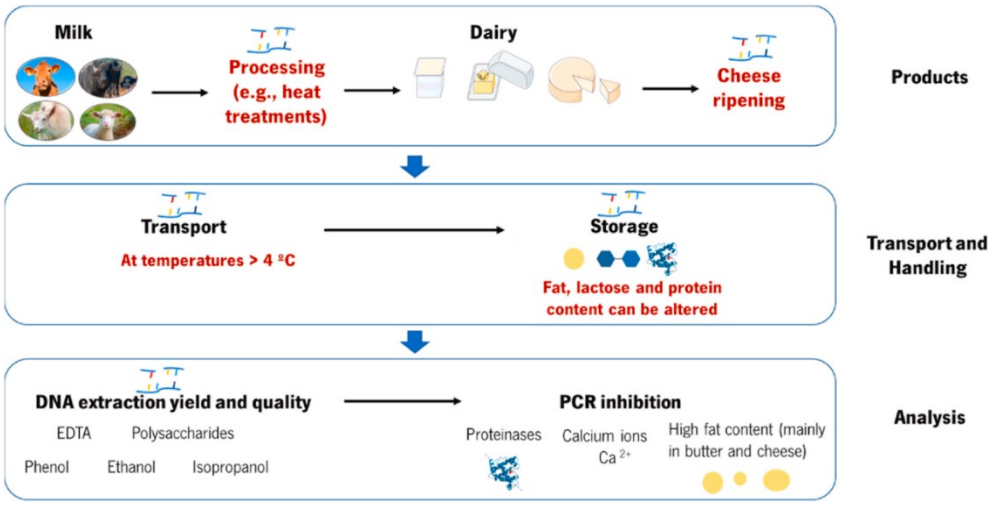

Figure 16:DNA-based authentication of milk and dairy products(Baptista et al., 2021)

3. DNA Analysis in authentication of cheese

With the introduction of HTS techniques, several studies addressed the microbiota characterization of the major PDO cheeses, aiming to improve their quality, safety, and commercial values. (Turri et al., 2021) The use of HTS for studying the microbiome community of cheese has been done with different purposes. Among them, finding the possible fraud, safety evaluation, and monitoring the ripening are the most common uses of this method. It also provides several snapshots of the intermediate steps during cheese production, enabling tracking and following the bacterial communities' progress from raw milk to the final ripened cheese. (Kamilari et al., 2019)

DNA sequencing has been used in various authentication processes, implemented at different scales. A study conducted on cheese samples from Kazakhstan, Belgium, Kalmykia, and Italy revealed the potential of DNA analysis in determining the origin of the cheese.(Li et al., 2017) The study found significant differences in bacterial composition among the 40 samples, with 43 genera showing varying abundances across groups. The study explored the relationship between sample origins and key microbial species contributing to the microbial community's structural differences. Statistically confirmed, the bacterial composition differences among the samples identified 16 key genera. It suggests that cheeses from different regions have distinct microbial profiles, highlighting the influence of geographical origin on their bacterial composition. (Li et al., 2017)This research demonstrates the potential of examining the geographical origin of cheese in different countries.

Through bacterial amplicon sequencing analysis, the dominant genera and unique bacterial community profiles between Plaisentif cheese producers were identified, allowing for the detection of fraudulent starter additions. In this study, through the data analysis, the researchers used the HTS to find the possibility of using commercial starters were added in order to standardize the production, even though it is not contemplated in the Plaisentif technical production policy. (Dalmasso et al., 2016; Kamilari et al., 2019)

The study also revealed the abundant genus present in each production stage for the different makers, highlighting the impact of bacterial community differences on the final product. The diverse microbial communities observed across all analyzed matrices were likely a result of traditional dairy practices, in line with Plaisentif's production policy. It is reasonable to believe that the rich texture and flavor of cheese from specific producers are a result of the microbial population in the milk and environmental factors.(Dalmasso et al., 2016) It also was observed that Plaisentif cheese has some uncommon genera. These genera are *Flavobacterium, Brevibacterium, Salinicoccus, Vagococcus, Anaerobacillus, and Sphingobacterium*. They can be employed as markers for identifying this particular type of cheese in future studies.

Storico Ribelle cheese has a high concentration of S. thermophilus bacteria. This is likely attributed to the customary use of wooden equipment throughout the cheesemaking process, from milk collection to ripening. Wooden poles, bands, and shelves may act as a natural reservoir for S. thermophilus strains of wooden vat origin, thus contributing to the microbial diversity in traditional dairy products. (Turri et al., 2021)

For a more precise authentication of the origin, fewer but more exclusive studies are available.

3.1 Grana Padano PDO

While our research on authentication via DNA barcoding of microbiota in Grana Padano was limited, we did come across a particularly intriguing study that sheds light on various aspects of this cheese's microbiota community. This research has been cited in literature reviews as a valuable source for verifying the authenticity of cheese. (e.g. (Cardin et al., 2022))

Zago et al., 2021 conducted a comprehensive and informative study on authentication through DNA barcoding, providing valuable insights. As previously noted, milk serves as a primary source of microbiota in cheese products, including Grana Padano, and it's worth mentioning that the use of high quality silage and fermented feeds is permitted for cow feeding of GP. (Neviani et al., 2013). Lactobacillus is a type of microbe that is commonly found in various forage

crops and silages. This particular genus plays a crucial role in the production of lactic acid and the reduction of pH during the fermentation of silage(Cai et al., 2012) Detecting the presence of this particular species during the analysis can serve as a reliable authentication method for Grana Padano cheese. This unique species is one of the key distinguishing factors in the milk used specifically for this type of cheese.

The research found that the bacterial community in GP cheese was entirely sequenced due to sufficient sequencing depth. The samples were divided into five GP production regions, namely Emilia-Romagna, Lombardy, Piedmont, Veneto, and Trentino-Alto Adige.This study showed Grana Padano produced in Trentino-Alto Adige had higher bacterial richness compared to other regions.

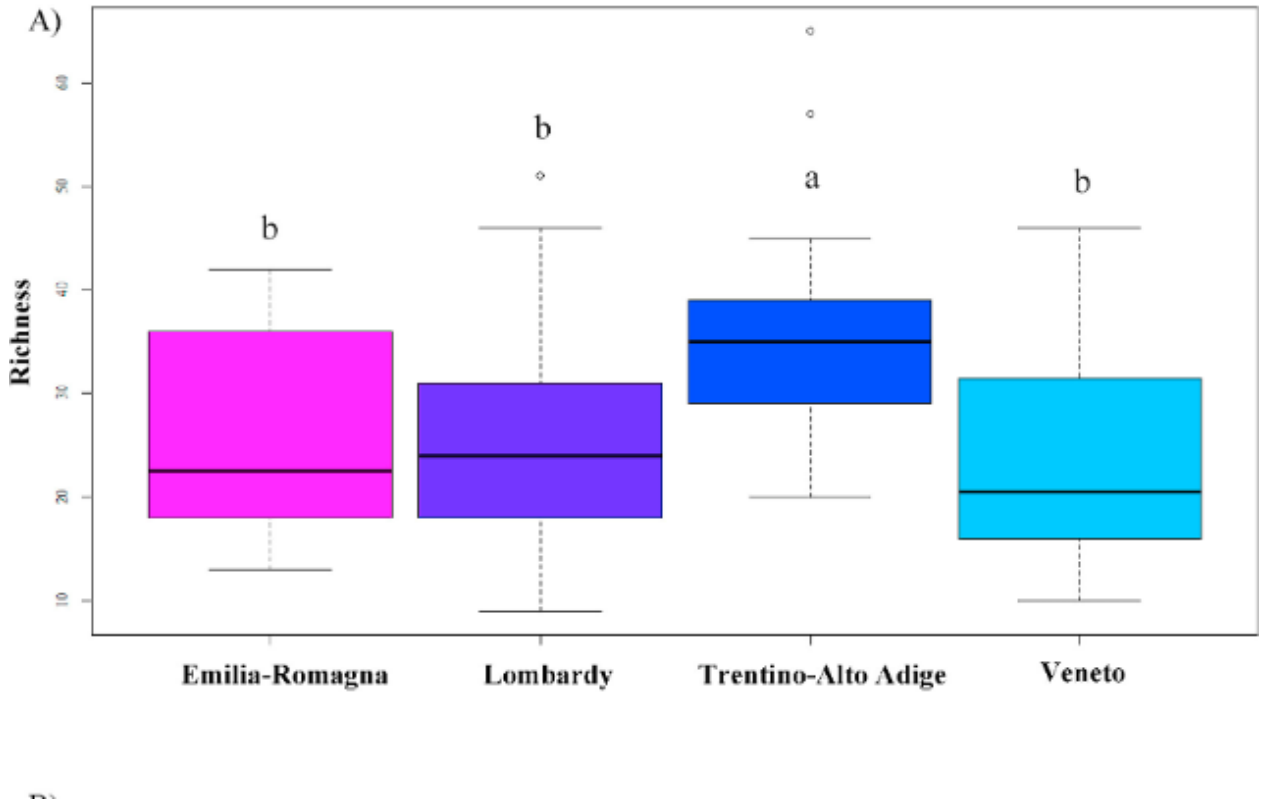

Figure 17:Microbial indices showing the species richness of Grana Padano cheeses based on the sampling region. The samples from Trentino-Alto Adige have higher richness than other regions.(Zago et al., 2021)

L. delbrueckii, Lact. rhamnosus, and Lact. casei were more prevalent in samples from Emilia-Romagna, Lombardy, and Piedmont, while L. helveticus and Lim. fermentum were more abundant in samples from Veneto and Trentino-Alto

Adige.The distribution of dominant species varied among different regions, providing insights into the microbial composition associated with specific geographical origins.

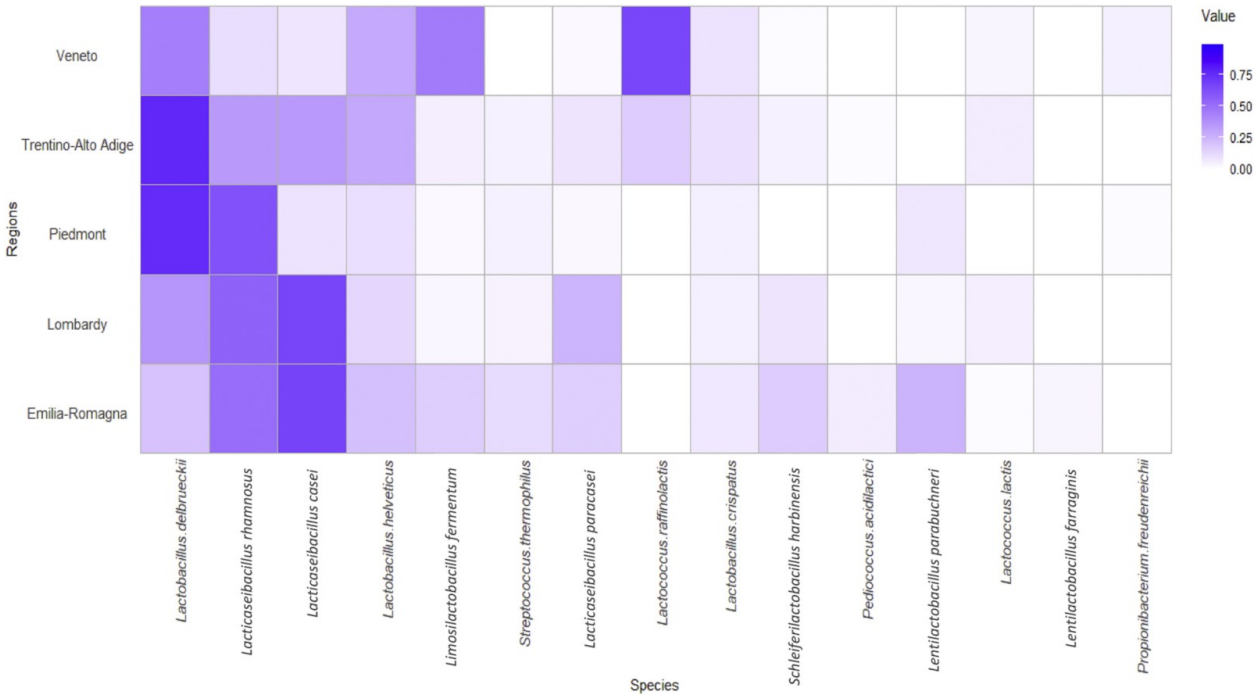

Figure 18:Heatmap showing the microbial abundance of the first 15 dominant microbial species found in Grana Padano cheese samples as a function of the geographical origin (region).(Zago et al., 2021)

The color scale indicates the species' relative abundance and more intense is the color, more abundant are the species. The sample origin is indicated. The high abundance of *Lactococcos rafinolactis* can differ the cheese from Veneto Region from others.(Zago et al., 2021)

Subdominant species in Grana Padano (GP) cheese samples also varied by region.*Lactobacillus jensenii* was present in GP samples from all five regions, while *Lact. nasuensis* was found in samples from four regions, excluding Lombardy. This shows a difference between same cheese with different geographical production site.

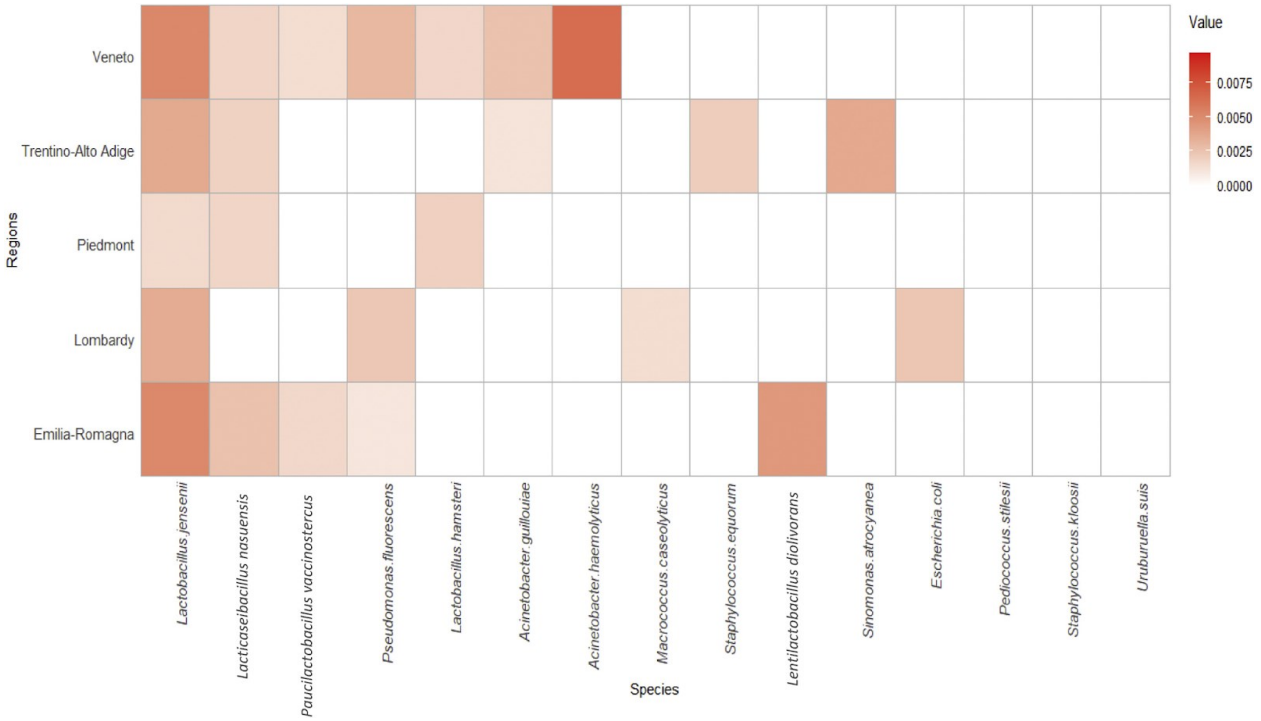

Figure 19:Heatmap showing the microbial abundance of the first 15 subdominant microbial species found in Grana Padano cheese as a function of the geographical origin (region).(Zago et al., 2021)

Figure 17 shows the microbial abundance of the first 15 subdominant microbial species found in Grana Padano cheese as a function of the geographical origin The color scale indicates the species' relative abundance and more intense is the color, more abundant are the species. The sample origin is indicated. Some dubdominant species are present in specific regions. Among those, the absence of *L. nasuensis in Lombardy is significant.* (Zago et al., 2021)

While the study has demonstrated promising data collection, it is important to note that there may be limitations that were not fully accounted for in the analysis. Therefore, it is recommended that further research be conducted in order to more comprehensively understand the efficacy and potential applications of this new approach for future analyses.

DNA analysis is highly sensitive and selective, and effective for a wide range of applications, but it comes with its drawbacks. High-quality DNA is required, and the analysis can produce false artifacts, making it a time-consuming and costly process.(Barrias et al., 2024; Domenico et al., 2017) While spectroscopic techniques, such as NIR, provides fast and low costs and a little or no need for

sample preparation before analysis.(Abbas et al., 2018; Lobato et al., 2018) Mass spectrometry and DNA analysis are highly sensitive and versatile techniques that can be applied across a wide range of fields. These techniques have become increasingly popular in recent years, particularly in the field of food authenticity studies, where their more mature applications have been reported. Scholars have developed rapid analysis techniques for food analysis based on mass spectrometry and DNA technology that improve efficiency without affecting the analysis effect. However, these techniques often result in large volumes of complex data.(Zhang et al., 2024)

Overall, DNA is one of the most reliable indicators available. Unlike other indicators, such as mineral elements and stable isotopes, DNA is consistent across different parts of organisms and maintains its consistency and thermal stability throughout the entire life cycle of plants and animals, from the farmland to the dining table. This makes it an ideal choice for food analysis and traceability purposes.(Scarano & Rao, 2014)

DNA barcoding has proven to be a valuable tool for verifying the origin and quality of raw materials as well as for detecting fraud in the industrial food chain.(Scarano & Rao, 2014) The use of NGS and DNA barcoding techniques for food authentication has been steadily increasing. However, there is still a need for more sensitive, multi-targeted, high-throughput DNA technologies to improve food authentication. Additionally, it is important to continuously develop and enhance DNA marker databases and create more appropriate primers to advance rapid DNA technology in the field of food authenticity.(Zhang et al., 2024)

Conclusion

During the fermentation process of cheeses, specific microbes play a crucial role in shaping the unique characteristics of each type of cheese. Understanding the diversity, abundance, and functional processes of microbes is crucial for determining the quality of fermented cheeses. High-throughput sequencing (HTS) technologies are valuable tools for identifying the microbial signature, supporting designations like PDO (Protected Designation of Origin), and enhancing the authentication process. While there are some limitations, advancements in HTS technologies can complement traditional methods, offering faster, more accurate, and cost-effective ways to analyze microbial communities in cheese.

Based on the research findings, it is evident that the origin of Grana Padano samples has a significant impact on the richness, evenness, and relative abundance of bacterial species(Zago et al., 2021) However, there are limitations with the metabarcoding approach, as it may not capture key biomarkers originating from the dairy environment, crucial for authenticating traditional cheese.

Integrating systems biology, combining metabolomics and metagenomics, could enhance our understanding, particularly when studying typical and industrial products. Artificial intelligence approaches, such as deep learning and machine learning, may improve classification rates for distinguishing authentic and fraudulent products. (Cardin et al., 2024; Kamilari et al., 2019)

With the use of DNA barcoding methods, advancements in research and new studies, a larger database will be available to discuss cheese authenticity fraud more accurately.

Further research comparing DNA-based analyses with established reference methods (e.g., isotope fingerprinting and trace element analysis) for cheese origin authentication is essential. Combining isotope fingerprinting or trace element analysis with metagenomics could offer a powerful approach for authenticating the geographical origin of cheese.

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