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FOOD MOLECULAR IDENTIFICATION AND CHARACTERIZATION: TOWARDS GEOGRAPHICAL TRACEABILITY

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1. SUMMARY

Food safety and quality depend on raw material characteristics and on the chemical, physical and biotechnological approaches adopted during manufacturing and transformation processes. Since a huge number of microorganisms are involved in food production, most products should be considered as complex matrices where any microbial component has a precise role and evolves in response to changes in physical and chemical features of the whole system. So, understanding the dynamics of microbial community involved in a food supply chain is useful to reduce food spoilage outbreaks, enhance industrial processes and extend product's shelf-life. The analysis of food microbiota is also pivotal to improve biotransformation processes, like winemaking. From time immemorial, the wine industry has been selectively growing grapevine cultivars showing different traits (e.g., grape size, color and flavor), and small variations in soil composition, irrigation and climate, have long been associated with shifts in these traits. The microbial flora coexisting with the plant may be one of the key factors influencing these traits. Despite long-time difficulties in analyzing single bacterial strains, the High Throughput Sequencing technologies (HTS) are nowadays an emerging and widely adopted tool for microbial characterization, even in food matrices. To clarify the contribution of the microbiome of grape during wine fermentation steps, I used an HTS-based approach to identify bacteria and fungi communities associated to berries and musts of Cannonau cultivar from four vineyards belonging to different regions in Sardinia. Cannonau is the most important grapevine cultivar of Sardinia (Italy), where most vineyards are cultivated without phytochemical treatments. Bioinformatics analyses suggested that microbiome colonizing berries collected at the four different localities shared a core

composition characterized by Enterobacteriales, Pseudomonadales, Bacillales, and Rhodospirillales. However, any area seems to enrich berries microbiome with peculiar microbial traits. For example, berries belonging to the biodynamic vineyards of Mamoiada were rich in Bacillales bacteria typical of manure. During vinification processes, performed at the same wine cellar under controlled conditions and without using any yeast starter, more than 50% of bacteria groups of berries reached musts, and each locality had its own private bacteria signature. This work suggests that natural berries microbiome could be influenced by pedoclimatic and anthropological conditions (e.g., farming management), and that fruits' microorganisms persist during the fermentation process. One of the main open questions about grape microbiome, regards the active role of grapevine cultivars in modelling microbial community. To investigate the relationship between plant genotype, its microbiome and the contribution of field environmental and pedoclimatic conditions, I planned sampling activities to collect 3 different grapevine cultivars Sauvignon Blanc, Syrha, Cabernet Sauvignon and soil samples from 3 different geographical area Pavia (Northern Italy), San Michele all'Adige (Northern Italy, close to the Alps) and Logroño (Spain). The HTS analysis of collected samples allowed to characterize bacterial profiles and the correlation between plant, fruit microbiome and the environment. This is a first step towards the understanding of the role of *terroir* and plant genotype in shaping the microbiome and quality of grapevine fruit and related products (i.e., must and wine). On the whole, such work provides clear evidence that the biogeographic characteristics of field's microorganisms may lead to regional properties associated to valuable crops. Human microbiome is

changing the face of medicine. Similarly, future research efforts should be more and more focused on the analysis of crop and environmental microbial communities to change the face of conventional agriculture.

2. INTRODUCTION

2.1 Genomics, metagenomics and food quality

2.1.1 The application of DNA analysis in the food sector

The industrial sector finds a solid support in genetic and biomolecular techniques to develop new analytical tools for improving food quality and safety. The continuous advances in molecular technologies, as well as the increase of information, dissemination and communication about DNA tools efficacy caused a progressive interest on food genetic analysis also by non-specialists stakeholders. The application of molecular techniques was used firstly for raw material identification and subsequently for genetic traceability, especially in the agro-food sector. In this context, DNA markers represent a powerful tool to verify the authenticity of products and to protect both producers and consumers, ensuring freedom of choice, accuracy of labelling and avoiding the fraudulent food alterations, such as the partial or complete replacement of a certain food item with cheaper components.

Today, DNA extraction can be successfully and reliably achieved in several kinds of foodstuffs and therefore molecular authentication of raw material and processed food can be routinely used at different steps of the food supply chain. In this way, DNA-based characterization and traceability of food components can improve the transparency and the protection of local and industrial food production systems. Moreover, this trend meets the consumer's demand. Consumers are more and more aware and informed about food quality and safety; they read products' label carefully and prefer those items having a well-declared origin and composition. In addition, increased human intolerances and allergies and the healthy lifestyles have been contributing to increase the public

attention towards a critic selection of food products. Taking advantage of the modern traceability systems, it is nowadays easier to trace each single step of an articulated food supply chain, thus allowing consumers to have accurate information and control on the product, but also facilitating the withdrawal of foodstuffs in case of problems. All food products must comply with the description provided by the manufacturers or processors, with reference to the origin of the ingredients, details of the transformation process, the geographic origin and the identity of the species, breeds or varieties used.

The validation of food authenticity relies mostly on the analysis of metabolites, proteins and/or DNA sequences. The protein-based methods are conducted by using on immunological [1] or electrophoretic and chromatographic assays [2] while the metabolite analysis is based mainly on HPLC [3,4], NMR [5,6] and MS [7,8]. While being effective in testing fresh products, chemical and protein-based approaches can be biased by the strong food manufacturing processes, the limited number of detectable isozymes, or the high tissue and developmental stage specificity of the markers. DNA markers are more informative than protein or chemical based methods, because DNA better resists physical and chemical industrial processes [9]. DNA is also detectable in presence of small traces of organic material therefore permitting the detection of low-concentration biological adulterants. As a consequence, DNA markers and in particular PCR-based methods have rapidly become the most used tools in the field of food control. Among these, discontinuous molecular markers such as RAPDs, AFLPs, and their variants (e.g., ISSR, SSAP) as well as sequencing-based systems such as SNPs and SSRs have been successfully adopted for the characterization of food raw

materials. However, being highly species specific, these approaches require access to the correct DNA sequence of the organisms and their application is often limited to a single species.

Therefore, DNA markers offer a powerful tool to address the validation of food authenticity and traceability of primary products entering the food chains both in fresh and processed food.

2.1.2 DNA barcoding

In the last decade, DNA barcoding, a standardized method providing species identification through the analysis of the variability in a short genomic region – the "barcode", was proposed as a universal DNA-based tool for species identification [10]. It has been extensively used in animal and plant biodiversity analysis, as well as the genetic traceability of livestock, crop species, and their related food products [11, 12], but also those minor crops and local products still lacking of a reference genetic fingerprinting [13]. As an example, DNA barcoding was extensively applied in the last decade to verify the origin of seafood [14] and to exclude commercial frauds occurring in its production and distribution [15]. Several scientific works were published concerning the use of this approach on botanical product, investigating plant species involved in human poisoning cases, due to contamination or substitution with plants potentially poisonous [16].

The analytical procedure consists in a robust, efficient and standardized system with the aim of species identification (Fig. 1), leading towards a certification of both origin and quality of food raw materials and detection of food adulteration or species substitution in the industrial food chain.

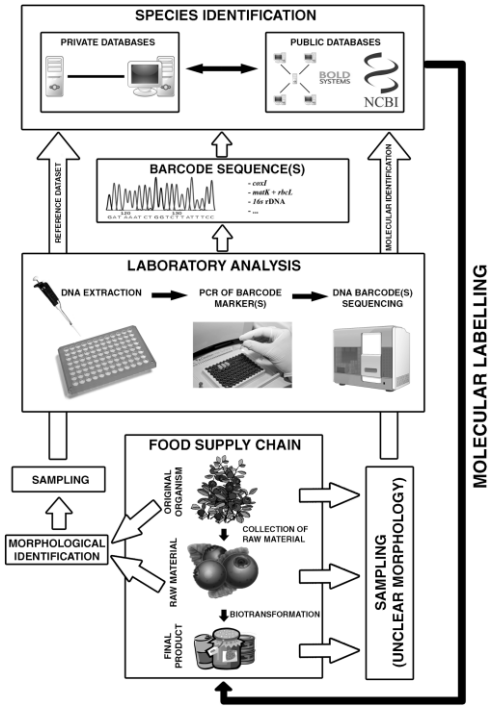

Figure 1. Workflow of DNA barcoding approach, to provide a proper labelling of any foodstuff.

The principal keystone of this approach is the amplification and sequencing of standard and universal DNA regions (usually referred to as barcodes) shared by all the organisms as a marker to identify species. For example, in 2009 the CBoL (Consortium for the Barcode of Life) Plant Working Group [17] suggested the combination of two plastidial loci (*rbcL* and *matK*) as core-barcode regions due to the straightforward recovery rate of *rbcL* and the high resolution of *matK*. Among other potential barcodes, the *trnH-psbA* intergenic spacer is easily amplified and has a high genetic variability among closely related taxa [18, 19]. The nuclear ITS region, and specifically the ITS2 portion, was also indicated as a supplementary DNA barcode region due to its higher

evolution rate [20, 21]. Along with universality, resolution, and standardization of the chosen marker regions, the strength of the DNA barcoding relies on the availability of reference DNA barcoding archives that successfully address taxonomic assignments of plants. For example, the International Barcode of Life Project (iBOL) coordinates the repository BOLD (barcode of life database) that supports the collection of DNA barcodes for creating a reference library for all living species [22].

In addition, DNA barcoding is a sensitive, fast, cheap and reliable approach, able to identify and tracking a wide panel of raw materials and deriving food commodities. The cost and time-effectiveness of DNA barcoding and the recent development of innovative sequencing technologies allow a certain degree of automation in species identification, which is particularly useful in simultaneous monitoring activities of multiple foodstuffs and batches.

DNA barcoding approach is not free of pitfalls and it should be taken into account that failures are mainly in the essence of biological species rather than in the method. As an example, the method cannot yet be easily applied to the differentiation of GM (genetically modified) food raw materials, based on the standard molecular markers. In fact, the genetic modification usually does not involve the plastidial or nuclear regions analysed in a classical DNA barcoding approach. However, a panel of additional markers (i.e., promoters, reporter genes) could be applied in combination with classical DNA barcodes, in order to design a fast and reliable traceability system for these kinds of products. Another concerning issue in using DNA barcoding is that a reliable identification requires a well-populated reference database. Many animal and plant taxa

are almost completely characterized under this point of view but many others still require an in-depth analysis to provide useful reference, especially when species from underinvestigated regions of the world are involved.

In conclusion, in the modern context of food traceability, DNA barcoding allows the characterization not only of food raw materials but also of the associated microbial communities, essential for their fermentation or preservation. A correct evaluation of the origin and safety of food components is also essential for new foodstuffs (e.g., the modern functional foods), where the microbial component plays a key role in enhancing their nutritional value.

2.1.3 Other DNA markers

DNA markers usually permit the identification of variations of the nucleotide sequence that can highlight inter and intra-species diversity. In certain conditions, such differences provide high information due to their moderate or high frequency of occurrence and their stability through generations. The analysis of polymorphic DNA markers is applicable in a wide range of applications, even including the evaluation and characterization of genetic variation. According to their throughput features, the most common DNA markers can be classified into three major groups: low-, medium- and high-throughput [23]. The former is generally referred to hybridization-based markers such as Restriction Fragment Length Polymorphisms (RLFP), the medium-throughput include Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR), also known as microsatellite, and DNA barcoding, while the latter

category includes sequence-based markers like Single Nucleotide Polymorphism (SNP). RFLP was the first approach used to analyze inter and intra-species genetic variability at genomic level. DNA variations can be highlighted by comparing the digested DNA patterns of different samples (i.e., species or varieties). RFLP markers have been widely employed for several purposes, ranging from the construction of linkage maps in several botanical species [24] to the authentication of seafood products [25]. However, the detection of RFLPs is a labour- and time-consuming process, not amenable to automation, and it is, presently, considered obsolete. PCR-based methods involve the amplification of DNA fragments using specific or arbitrary primers. Amplicons can be separated by electrophoresis and visualized by different technologies. RAPDs are able to detect, concurrently, loci in several regions of a genome. RAPD analysis has been widely used for taxonomic and phylogenetic studies [26] for species differentiation [27] and to study phylogeographic patterns of genetic variations [28]. DNA polymorphisms can also be revealed by AFLPs; unlike RAPDs, this technique is highly reproducible as it combines restriction digestion and PCR.

SSR markers are composed by tandem repeated motifs of 2–6 bp, representing the core of the microsatellite, that can be amplified using the unique flanking region for primers annealing. SSRs are highly reproducible, highly polymorphic, and appropriate to automation [29]. They were successfully employed in varietal identification and proved to be very effective for the authentication of food components, both of animal and plant origin [30–36].

Recently, SNP markers, caused by the replacement of a single nucleotide, have become the most used markers in genetic characterization studies as well as in translational genomic [37]. SNPs are, in fact, the most abundant forms of genetic variation among individuals of a species.

Beside nuclear genome, organellar genomes, have been proposed as a valuable tool for species discrimination. DNA markers developed from mitochondrial genome were proposed as DNA barcode that is a standard region of the genome, which is usually characterized by a high inter-specific, and low intra-specific variability [38].

2.1.4 DNA analysis of processed food: the case of fermented products

Fermented foods have been produced and consumed since the beginning of civilization and they constitute a significant proportion of human diet. Actually, thousands of different types of fermented foods or beverages are consumed globally, making up 5-40% of the human diet. Fermentation represents mankind's oldest means of food preservation, but also fermented foods benefit consumers through enhanced nutritional content, digestibility, microbial stability, and in some cases detoxification [39-43]. In addition, these foods often serve as vehicles for beneficial microorganisms that play a beneficial role in human health, as well as prebiotic substances, which promote the growth and health modulating activities of beneficial microbes in the human body [44].

The quality, authentication and traceability of fermented food product should consider both the raw original matrices (e.g., grape and must) and the microbial community involved in fermentation processes.

Many efforts are directed toward the description of microbial communities responsible for different food fermentations to ensure

process efficiency, product quality and safety. Molecular methods, relying on detection of nucleic acid sequences, have progressively replaced traditional, culture-based analytical methods for microbial community profiling due to their greater speed and accuracy. Most culture media are indeed inadequate for the growth of specific organisms, leading to an unreal structure of the microbial communities, even in simple biological systems, such as food fermentations. Additionally, the stressful conditions of some food systems, as in the case of alcoholic fermentations, can induce a viable-but-not-culturable state in microorganisms, preventing culture-based detection.

As a result, several novel approaches have been developed to perform these microbial analyses. Such modern advances have been referred to as 'next-generation sequencing' (NGS) and, more recently, 'high-throughput sequencing' (HTS). HTS techniques are able to provide sequence data around a hundred times faster and cheaper than the conventional Sanger approach. Sequencers from 454 Life Sciences/Roche (producing about a million sequences of length 800–1000 base pairs), Solexa/Illumina and Applied Biosystems SOLiD technology (producing over a billion sequences of length 50–500 base pairs) were produced as second-generation technologies and other competitive instruments appeared on the market such as the Ion Torrent and PacBio. Prior to reaching a taxonomic assignment of the whole biological content of a food ecosystem (i.e. including raw materials and food-borne microorganisms and viruses), sequences generated with HTS have to be filtered, denoised and analysed using bioinformatic tools. Another advantage of using HTS technologies refers to the possibility of preparing several DNA samples, from different extracts and marked with

different DNA tags, at the same time. Thanks to these practical advantages, it is possible to analyse in parallel a very high number of samples and hence lower the analysis cost. The reduction in cost and time for generating DNA sequence data has resulted in a range of new successful applications.

For example, high-throughput sequencing (HTS) technologies have been widely applied to the characterization of the complete spectrum of ingredients in complex food matrices, as well as alterations or peculiarities in their microbial composition and the monitoring of microbial dynamics in food fermentative processes. Two different HTS approaches can be used. The most common one involves that DNA markers are amplified from genomic DNA (or RNA, after a reverse-transcription step) through PCR and sequenced. Taxonomic relevant genes are usually sequenced through this approach, leading the taxonomic composition of the microbial community and the relative abundance of its members. In metagenomics and metatranscriptomics studies, no PCR is performed and total DNA or cDNA is sequenced. Besides the taxonomical composition of the community, this approach allows obtaining the abundance of all microbial genes. The study of microbial ecology is relevant in biotechnology as it is the basis for not only the development of fermentations but also for the comprehension of the microbial interactions that drive a premium quality process. The availability of such a powerful tool box offers tantalizing opportunities to study food microbes and understanding how their potential functions can be changed or modulated with the ultimate scope of improving food quality.

Amplicon-based HTS targeting at genes used for taxonomic studies has become the most widely exploited approach in food microbial ecology. In the past decade, it was widely used to monitor microbial communities during fermentation of different types of foodstuffs and beverages. Several questions can be addressed by the description of microbial communities during fermentations. For example, an in-depth characterization of the normal or abnormal microbial consortia at different stages of fermentations is important in order to evaluate lot-to-lot consistency, identify biomarkers for product quality or spoilage, and learning how to manipulate fermentation conditions to improve the process control.

Dairy is the most explored environment and a broad variety of cheeses and other food items are studied through amplicon-based HTS, allowing monitoring of curd fermentation [45, 46] or cheese ripening [47-51] and exploring the spatial distribution of microbes in different parts of the same cheese [49, 51, 52].

In many cases, the study of food microbiota highlighted possible relationships between microbial community structure/dynamics and physicochemical parameters, such as pH, water activity (aw), salt concentration and temperature [51, 53-57]. In other studies, the microbiota was related to raw material origin [58, 59] or quality [49, 50, 60], as well as to development of flavour-impact compounds [48, 51, 52]. Moreover, food-related environments were found to harbour a resident microbiota, beneficially involved in dairy [61-63], alcoholic [64, 65] and sourdough [57] fermentations, although the presence of potential spoilers was also emphasized in some cases [62, 66].

Although fungi can be very important in some kinds of food fermentations, there is a huge difference in the number of published studies describing fungal and bacterial communities through HTS. While 16S ribosomal RNA (rRNA) is the common choice for bacteria, more variability at the target gene was highlighted for fungi. The most frequently used region is the nuclear internal transcribed spacer (ITS) due to the availability of a well-curated database (https://unite.ut.ee). Therefore, the use of different targets would be also advisable, such as the 26S [62, 67, 68] or the 18S rRNA [57, 69] genes.

After a bioinformatic pipeline, the huge amount of sequencing data need to be filtered using quality criteria and then clustered to group sequences into operational taxonomic units (OTUs). Clustering is conducted according to similarity based on an established threshold (usually 97%). The final step consists in the taxonomical assignment.: for each OTU, a representative sequence is selected randomly and classified by the comparison with updated databases. However, the obtained data need to be interpreted being aware of culture-independent PCR biases that have been reviewed elsewhere [71], such as the possibility of preferential amplification, due to the different efficiency of primer pairs targeting at the selected species, that may result in the under-representation of some clades [72].

Notwithstanding the thick body of literature accumulated on food microbial communities assessed by amplicon sequencing, most of the studies are basically descriptive. In addition, well-known microbial players have been identified and thus limited new information was provided on food fermentative processes. A picture of the entire microbial community can be obtained, tracking and comparing the

abundance of bacteria and other organisms at the same time, although the methods have to be chosen carefully, in order to avoid preferential nucleic acids extractions from bacterial cells.

Food fermentations are often complex phenomena, involving several microbial species and strains. The most widely used application in food microbiology is the use of amplicon-based sequencing, leading to an in-depth description of the ecosystem studied. This can be undoubtedly useful in order to understand microbial dynamics and evolution during food production, as well as to identify the presence of possible spoilers. Nevertheless, the real advance led by HTS is the application of shotgun metagenomics and metatranscriptomics. These approaches are still underexploited in food microbial ecology. Their application to food fermentations may be extremely useful in order to explore microbial functions directly in the food matrix and understand microbial behaviour in response to different process conditions. Moreover, recovering microbial genomes from the metagenomes allows to monitor the evolution of different strains during the process and to compare their genomic potential. These tools promise to be an invaluable help to better understand and possibly tune microbial activities in order to ensure process efficiency, product quality and safety. Moreover, recent scientific works underline the role of microbial component as a direct link with the territory, as well as cultivation features and characteristics of the production area. One of the most appropriate example is wine and winemaking process that are really influenced by the microbial component (soil, environment, grape, closer plants, management) at the level of product quality and organoleptic properties, but also at the level of relation with the territory.

2.2 *Vitis vinifera* L. and wine production

2.2.1 *Vitis vinifera* L.: origin and domestication

The history of the grapevine, *Vitis vinifera* L., is enormously long. In particular, it is much old, and date back to around 100 million years ago. The first Vitaceae fossils date back to the Cretaceous roughly 65 million years ago [72]. While, the earliest fossil findings of the genus *Vitis*, dating back to Cenozoic (Tertiary), have been found in continental Europe, England, Iceland and North America. Particularly in the Miocene (between 24 and 5 million years ago) there are numerous ancestral *Vitis* spp. that, thanks to a mild climate, spread widely even in areas where they are today absent. The ancestral life most directly related to the present European vines is *Vitis praevinifera* L., which represents a stage of approaching the grapevine of our continent, *Vitis vinifera* L [73, 74].

The last two glaciations (Riss from 200,000 to 130,000 and Wurm from 110,000 to 12 000 years ago) occurred on the Earth, leading to the disappearance of a large number of species, but thanks to the presence of "climate refugees" located in countries facing the Mediterranean basin, in the eastern United States, Asia in the East and in the Caucasus, some could save themselves. The effect of these geological events caused the separation of the two Vitaceae into two different variability centres [75]. The first includes the great peninsulas and islands of the Mediterranean, Lower Asia and North Africa, while the latter stretches from the Black Sea Mountains to India. The consequence of glaciation was also a homogeneous distribution of species belonging to the genus *Vitis*; in Europe the only *Vitis vinifera* L. spread, while other species settled in

North America and Asia. Currently, the wildlife diffusion area includes the Mediterranean area up to the Black Sea and reaches the southern part of the Caspian Sea shown in Figure 2.

Figure 2. Map showing the distribution ranges of wild grapevine in the Mediterranean basin [76].

Another effect probably attributable to glaciations is the great intra-specific variability that we find today in cultivated vines, expressed as inter and intra-varietal variability. The presence of ice in Europe led to an extremely heterogeneous distribution of surviving individuals who colonized a substantial part of the continent and moved to the Middle and Middle East where they developed colonies of relatively isolated *Vitis vinifera* L. colonies that soon became populations with its own genetic characteristics.

From the analysis of the oldest remains, it is understood that *Vitis vinifera* L. ssp. *silvestris*, the wild grapevine, is the subspecies that appears earlier in the Mesolithic and only between the end of the Bronze Age and the beginning of the Iron Age it was possible to find the subspecies *Vitis*

vinifera L. ssp. *vinifera*, the cultivated vine. The first vineyards, however, date back to the end of the Neolithic period, brought to light in the excavations of Crete and other areas of Greece, while in Europe they appear only at the end of the Bronze Age (1700-1500 BC).

The phylogenetic studies of the last decades show that the origin of cultivated grapevines was done by domestication of *Vitis vinifera* L. ssp. *silvestris* [77] with a selection continued for millennia.

2.2.2 The process of domestication

Domestication is a man-made selection process with the aim of adapting plants and animals to their own needs. It is interesting to note that this process was conducted for about 10'000 years, after the last ice age, in several regions independently [78]. At least seven areas of domestication from Vavilov [79] have been identified, including Mesoamerica (Mexico), Andean Highlands, Southeast Asia, Far East, fertile Crescent area (Mesopotamia, Syria, Egypt), Mediterranean, the Ethiopian plateau and the Arabian Peninsula [78, 79]; currently this number is extremely grown. Agriculture is therefore one of the few "inventions" that can be traced back to more places and outbreaks, has progressively spread to other regions, including, for example, Europe and North America [80, 81].

Despite the diverse geographic distribution of domestication centres, it is possible to identify a number of similar characteristics that have been selected in very different crops. All these characteristics form "domestication syndrome" [82] and result from the selection of spontaneous mutants in wild populations that have been selected at various stages of wildlife growth or after harvest [83].

Among these modifications, it is easy to remember, for example, the case of the tomato in which the wild progeny appears tiny compared to the cultivated fruit or the maize in which the wild progenitor (teosinte) is characterized by long and numerous branches and branches while the cultivated one has a single stem. Another example is wild rice which has an ear that is easily broken up, while the cultured individuals have a rigid ear that facilitates collection [81]. Many of the traits selected by man during the transition from cultivated wild plants are currently in the wild. It follows that fully-grown crops cannot survive in the natural environment without human intervention.

All this entails a close mutualism between man and the plants he has modified to ensure mutual survival [80, 81].

2.2.3 The domestication of the *Vitis vinifera* L.

The domestication of *V. vinifera* L. from the need to maximize the production of berries in order to guarantee greater yield to growers. Wildlife, as mentioned above, is almost entirely characterized by dioecious plants. The different populations are composed of non-fruiting male individuals, female individuals and few hermaphrodate individuals (about 1%) able to self-fertilize and are more fruit-bearing than females [84, 85]. It is therefore considered that the prehistoric farmer has only selected female plants and rare hermaphrodite plants to propagate; the result of this slow artificial selection process was a progeny consisting predominantly of fruiting hermaphrodites. It is extremely difficult to accurately date the phenomenon of passing from the wild to the domestic grapevine. Some documents [86] come from Sitagroi finds in eastern Macedonia where, over a period of two thousand years, from 4700 BC to

1900 BC, there was a slow domestication that can be detected by the size of the grapes that pass from a typical length/width ratio of wildlife to that of the domestic grapevine.

It would appear that wildlife protection/selection activities began close to the villages at the beginning of the 4th millennium BC in regions where they grow spontaneously such as the Gulf of Alexandre, South-eastern Anatolia and the high Tigris-Euphrates (Circum-northwest Mesopotamia).

The intense and dynamic cultural relations of the North Circum-Mesopotamian regions with those of the South East Circum-Mediterranean must also have spread to the latter, the cultivation of the grapevine, which confirms that in the North Circum-Mesopotamian incubation of the grapevine culture has started at least a few centuries before.

If we define "wine civilization" as the domestic life has clearly been established, it is clear that it is in the Greek world that the expansion of wine culture in the western Mediterranean is to be expanded.

It is thanks to the economic expansion of society in pre-classical Greece [87], which began massive transport of grapevines and wine in that area. The viticulture introduced became, in a few centuries, sporadic and relatively marginal cultivation, the basis of the agriculture of grape and olive culture, featuring Magna Grecia, the Etruscan area and the big islands. In Italy, the beginning of the "wine civilization" can be dated with the advent of the Oenotrians (Enotri), an ancient population set up in Lucania, before the middle of the 5th century AC and with the colonization of the west western Po Valley, which began in the first half of the last millennium BC but spread rapidly also in the nearby areas

[88]. It is at the beginning of the civilization of iron which, as Pallottini [89] pointed out, began to form the various regional ethnic groups, each characterized by its own specific culture and hence its own type of agriculture, with particular varieties of cultivated plants. Consequently, at that time, the primates of the ancestors of the grapevines and therefore the corresponding local wines develop [90]. Based on historical-archaeological information describing the various stages as finds of plant remedies and tools, a grapevine pattern of Europe has been developed [84]. According to this scheme, it is assumed that *Vitis vinifera* L. ssp. *vinifera* (or *sativa*) appears in the Near East [91-94] and in transcaucasian regions [95] from the second half of the IV millennium BC then spread to the eastern Mediterranean, Palestine, southern Lebanon and Jordan [92, 96, 97]. In the first half of the III millennium BC is present in lower Asia, in southern Greece, Crete, Cyprus and Egypt. Around the first half of the II millennium a.C. the cultivated grapevine is found in the south of the Balkans [98, 99] and appears in southern Italy in the second half of the millennium. The last stage of this quest lies in northern Italy, southern France, Spain and Portugal, regions gained by grapevines in the second part of the I millennium [77, 85, 100]. A crucial, controversial and currently unresolved issue is the need to clarify whether the cultivated grapevine has been generated in a single domestication center (primary) or whether this phenomenon has occurred in different places (secondary domestication centers); that is to say, if the modern European grapevines were born, they were only spontaneous and indigenous plants of the Transcaucasia and fertile crescent or whether wild grapes have been contributed to this process in all European wine-growing regions. Historical data and archaeological finds cannot clarity on the affair, on

the one hand McGovern [93] and collaborators support a monocentric origin of cultivated grapevine, Rivera-Nunez and Walker [101] and Bakels [102] they support the hypothesis of secondary domestication processes occurring in different areas of the Mediterranean. Even from a morphological and taxonomic point of view, opinions are conflicting; Zeven and Zhukosky [103] proposed at least three large domestication centers for grapevine (near East, Central Asia, and Mediterranean), while according to Zohary and Spiegel-Roy [92] and Olmo [95], the domestication center is only one: near East and Transcaucasia. It is therefore evident that the use of historical, cultural, morphological and ampelographic information is useful but insufficient to provide clear evidence of this phenomenon [104-106]. The results obtained from recent molecular analyses carried out on wild and cultivated grapevines of different geographic areas [106-109] agree with the historical and archaeological information confirming a correlation between genetic similarity and territorial proximity. On the basis of this data it could be argued that the cultivation of the cultivated grapevine was carried out by means of a "progressive wave" mechanism and that the different cultivars were carried, for later stages, from the eastern parts to the west (Spain, France and Italy).

On the other hand, molecular data also show genetic similarities between localized varieties in geographically distant geographies and genetic variability in the areas west of the distribution site larger than the one found in the east. This fact could be explained by the fact that they have secondary domestication phenomena in western regions [108, 110] and genetic erosion phenomena in Eastern ones [111, 112] but there is no evidence to clarify which of the two hypotheses is the correct one. This

scenario complicates, therefore, the understanding of the evolutionary history of cultivated grapevine. In particular, the continuous exchange of plant material (mostly cuttings) in different historical periods and the possible accidental breeding of wild vines and cultivated vines does not allow a complete clarification of the diffusion and distribution of the species [108].

2.2.4 Grapevine biodiversity in the Sardinia region

Historically Sardinia has occupied a highly strategic position for commercial routes in the Mediterranean Sea. *Vitis vinifera* L. is one of the most important species that grows on the island both as a grapevine plant (V. *vinifera* ssp. *silvestris*) and cultivated (*V. vinifera* ssp. *vinifera*). The large number of grapevine varieties [112, 113] is the result of a long history of colonization by many different human populations. In 238 BC, the island became part of the Roman Empire and subsequently conquered by various Mediterranean populations including Spanish from 1479 to 1714. During this period, cultural and commercial exchanges with other Mediterranean civilizations led to a substantial modification of the agricultural products of the island.

As far as grapevine cultivation is concerned, many similarities are known between Spanish and Sardinian varieties, often called by similar names, for example "Bobal" and "Bovale", "Cariñena" and "Carignano", "Garnacha" and "Granaccia" [112]. It is assumed that invaders influenced Sardinian viticulture by introducing new varieties, improving the genetic composition of the local germplasm. At the same time, certain Sardinian cultivars, some derived from the domestication and cultivation of wild local grapevines, could have been introduced into Spain. For example,

the cultivar ‘Cannonau’ is one of the most important grape varieties cultivated in Sardinia. Ampelographic and historical information show ‘Cannonau’ as a synonym of several Spanish red cultivars such as ‘Garnacha Tinta’, ‘Garnacho’, ‘Tinto Aragón’, ‘Alicante’, and ‘Garnatxa Negra’ [113, 114]. In Spain, there are several accessions of ‘Garnacha’, including a white cultivar ‘Garnacha Blanca’. Besides the supposed synonymy with ‘Cannonau’,Martinez and coworkers [115] suggest a relationship between ‘Garnacha’ and ‘Mencia’. In addition, in France, USA, and Australia, ‘Garnacha Tinta’ is called ‘Grenache’ [116]. Based on these considerations, Zecca and coworker [117] defined synonyms and false homonyms of ‘Cannonau’ and ‘Garnacha’ using DNA molecular markers and analysed the origins of accessions of ‘Cannonau’ and ‘Garnacha’ by studying their genetic relationships with wild grapevines in Sardinia.

A SSR analysis of the viticultural history of Sardinia and Spain. confirmed the synonymy between ‘Cannonau’ and several ‘Garnacha Tinta’ accessions.

Since Cannonau is one of the core products of Sardinian wine industry and Garnacha Tinta, in Spain, is a blending wine, the genetic origin of cultivars has to be only one of the many elements that characterize a wine. Wine quality relies on a precise equilibrium among different factors and it is largely influenced by the environmental conditions of the production area.

2.3 Discovering the *terroir’s* secrets

2.3.1 The *terroir* concept

Defining the *terroir* is not trivial. If we use the conventional definition, we should define it as '*an area where the natural, physical and chemical conditions, the geographic area and the climate allow the production of a wine with a specific identity and unique characteristics linked to its territory*'. Although this definition provides many elements of territoriality, there are different limits when applied to the wine context. First of all, it excludes the role of man and agricultural practices that can substantially modify the cultivation of a grapevine and the production of a wine. In addition to the climatic and pedological conditions, other elements of tradition and culture of the area should be considered, such as local food preferences, integration with local cuisine and products, art and much more. An extreme example of the human impact on the production of a wine with peculiar traits closely linked to the territory, is that of Bruschette, a typical wine of the area of Bitti (Nuoro, Sardinia). This wine is made with grapes that have not completed their ripening yet and are vinified hastily without waiting for the completion of the fermentation process. The result is an acidic, low-alcoholic wine with a pale pink color since the maceration has not been completed, so many metabolites that give color to the wine remain in the skins. According to the standards of a wine, Bruschette is definitely a really complex product to be consumed. This choice, however, blends with local culture and tradition. The grape harvest is in fact carried out about two weeks before a local festival "Festa del Miracolo". This example denotes how human habits can have an extreme influence on the final product. Without going to an early harvest or partial vinification, the agricultural choices, ranging from land management strategies to pruning the vineyard, as well as their

technology of the winery, make a major mark on the wine and make it part of his territory.

Grapevine and wine have a long tradition closely linked to the people who have discovered, consumed and appreciated it. Nowadays, wine production is often influenced by consumer trends and therefore by market demand. From the fashion of barricaded wines to fresh and scented wines, from international grapevines to valorisation of local ones, from wine as an aperitif to wine for meditation. It is also important pointing out that there is a close relationship between grapevine, field and grower and only an intimate understanding of these relationships allows to obtain a product of high value, that is not only pleasing to the palate but is unable to leave an indelible imprint of its *terroir*, that makes it so special.

2.3.2 The study of "*terroir*"

Discovering the secrets of a territory in the widest sense of the term and thus including traditions and culture is very difficult but is certainly the most important element to characterize the typicality. For example, Cabernet Sauvignon and Chardonnay are among the most cultivated and consumed grapevines in the world, able to adapt to different soils and highly varied production technologies. However, for the same cultivars there may be very different viticultural products. This does not mean that the cultivar does not play an essential role in viticulture production. Cabernet Sauvignon is an exceptional grapevine with a fruit rich in secondary metabolites able to give color, smell and texture to the wine. However, we know that these metabolic traits do not only express themselves through the genome expression of Cabernet Sauvignon, but

they are the result of a complex interaction between the environment and the plant. Climate, solar exposure and water availability are just some of the elements capable of acting on the gene expression and biochemistry of the fruit of the grapevine and thus modifying the final metabolic profile [118]. The winemaker has a comprehensive knowledge of his/her plants and knows how to care for them to maximize their production quality. He/She knows the climate, the orientation of vineyard row and their sunlight exposure, and which are the best moments to harvest. This is exactly what is usually intended as intimate relationship between man, environment and plant. How can we understand the terms of this relationship and codify the *terroir* is a major challenge of viticulture and wine study.

The strategy to adopt is to study the individual components and then develop an integrated analytical process that is able to give a good measure of each element. In the past, many studies have been conducted to evaluate environmental components in order to maximize the expression of a grapevine. Through the practice of zoning, a territory was divided into areas based on the pedological, climatic and agronomic characteristics in order to identify and describe the environmental and crop factors that contribute to determining the production capacity of a grapevine, the composition of the grapes and the characteristics of the wine. Although this practice has produced excellent results in various areas and has contributed to the quality of many wines, the approach adopted only considered the abiotic component of a territory. Only recently, numerous emerging studies [58, 119, 120] emphasized the role of the microbial ecosystem, associated with soil and grapevine plant [121], as an essential element not only in contributing to the growth and

development of the plant, but also in the giving an imprinting of uniqueness to the wine obtained from these grapes.

2.3.3 The microbiome: an element of relationship between environment and plant.

Knowledge of the microbial ecosystem of a given environmental matrix is really interesting for understanding the relationship between the environment and higher living organisms. In this sense, it is interesting to know which bacteria, yeasts, protozoa or algae characterize the soil of a vineyard, and what effects may they have on the grapevine plant. Agrarian microbiologists have often been used to isolate soil microorganisms and to know their metabolism in order to evaluate the advantages offered to the grapevine plant [122]. Certainly, there have been so many researches aimed at studying pathogenic microorganisms or those capable of infecting grapevine plants with negative effects on production. Developing fast diagnostic systems for identifying possible microbial pathogens is essential to timely intervene on the vineyard and eradicate the disease.

However, the study of agronomic microbiology has undergone a great revolution thanks to new technological approaches that allowed the analysis of an entire microbial ecosystem through DNA. This is the so-called metagenomic approach. Thanks to the modern sequencing technologies known as High Throughput Sequencing (Fig. 3) it is possible to sequence the genome of the microorganisms of a certain area (metagenome) and through their DNA imprint (DNA fingerprint) it is possible to recognize and study their properties. By means of the metagenomic analysis, we obtain the knowledge of the microbiome of a

given geographic region or gene sequences that can be traced back to each bacterium that characterizes a given microbial environment.

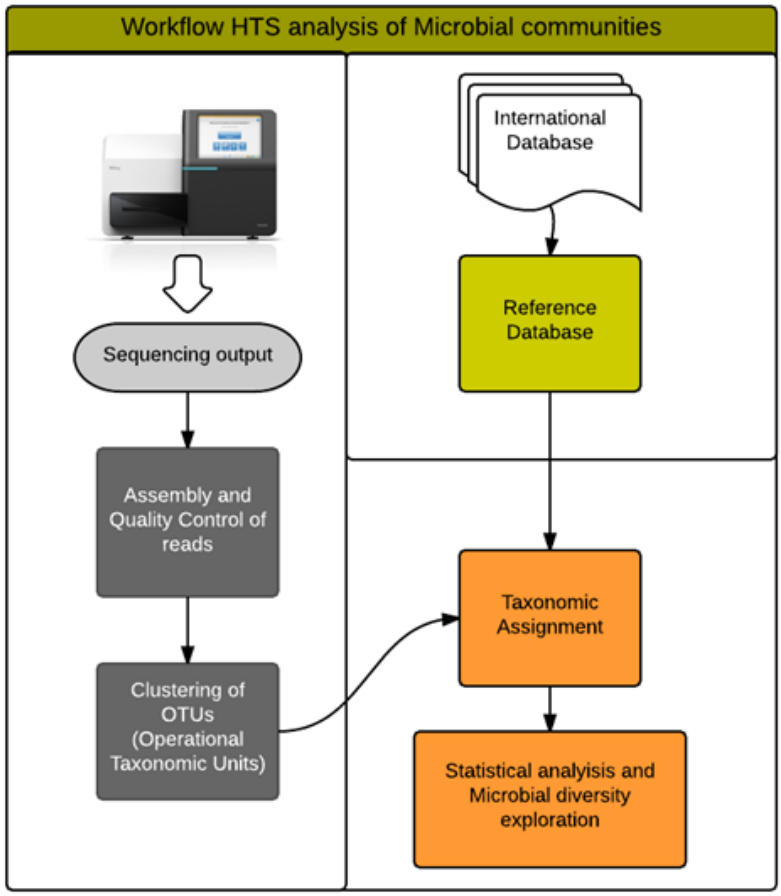

Figure 3. HTS analytical workflow for studies of microbial ecology.

This technological revolution has allowed, besides analyzing a large number of organisms at the same time, to study many environmental microbes, which are notoriously difficult to cultivate in the laboratory due to their particular environmental and metabolic needs.

Thanks to this approach, very exhaustive studies have taken place on the vineyard microbiome, as well as on the musts and wines, and it has emerged that the bacterial population is extremely rich and varied and that there is close relationship with the environment. If concentrated on the plant, the various analytical techniques have allowed quantification of the microbial component of the fruit consisting of a large amount of yeast (10^2 to 10^4 cells/g) and a more modest number of bacteria (e.g. 10^2 cells/g of *Lactobacillus* spp.). Fruit conditions can greatly modify these relationships and the taxonomic composition of the microorganisms involved: for example, berry spoilage and sugar juice leakage promotes the development of bacteria responsible for acetic fermentation that can also reach values of 10^6 cells per gram [123].

Zarraonaindia and collaborators [124] highlighted the intimate relationship between soil and plant microbiology. Research findings show that the phylum level most represented on the different organs of the plant is Proteobacteria (fruits 80.7%, leaves 90%, flowers 98%). The distribution of the different species on the plant is not random. In some cases, there are groups shared between leaves and berries where there are Firmicutes, Acidobacteria, and Bacteroidetes, but there is also some uniqueness such as in the case of flowers where bacteria of the genus *Pseudomonas* and *Erwinia* are dominant. Concerning fungi, there are several mild species populating the different organs of the plant, although the dominant phylum is represented by Ascomycota with genera *Aureobasidium*, *Sporormiella*, *Alternaria* and *Guignardia* [119].

The origin of microorganisms that populate the grapevine plant is undoubtedly the soil [124]. Soil characteristics affect its microbial composition [125], however, only a small portion of the soil bacteria

reaches the air part of the plant as leaves, flowers and fruits. But what are the effects these microorganisms have on the plant? In some cases, the grapevine can only be a growth surface without any relationship between plant and bacteria or yeast. In other cases, bacteria may have different effects on the grapevine; for example, it is known that species belonging to the genera *Pseudomonas* and *Sphingomonas* can affect the health and productivity of the plant, while the presence of *Methylobacterium* spp. on the leaves helps to stimulate the growth of the plant through the production of phytohormones. The presence of radically rooted *Steroidobacter* spp. appears to be essential to stimulate rootstock elongation, vascular tissue differentiation, and later stimulate fruit maturation. The microbiome of the plant changes with the growth and development of the plant itself and this is closely related to the resources the grapevine can offer to the microworld. Berries still unrefined are poor in sugars and have an uncharted exocarp. Under these conditions the microbiome is rich in Basidiomycetes invading the whole plant together as well as lactic bacteria such as *Lactobacillus* spp., and *Oenococcus oeni*. When the berry matures and then begins to supply more resources, appear ascomycetes such as *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia* that accompany the ripening of the fruit. When the fruit is mature and is rich in sugary nutrients, it is invaded by mild fermentations of the genus *Pichia*, *Zygosaccharomyces*, *Zygoascus*. and *Torulaspora* spp and bacteria capable of fermentation of acetic and lactic acid.

With increasing knowledge, more and more details on the plant, environment and microbial relationship can be added and this will provide viticulture an important tool for improving yields, fortifying

plants, reducing chemical fertilization and enhancing the peculiarities of a wine.

There are two more points to point out. The first is related to the imprint that plant microbiome can provide to the must and then to the wine produced with those grapes. According to Bokulich [58], that studied the Californian wines, there would be a close relationship between the biogeography of the vineyard and the microbiome, and this 'microbial impression' would find itself an element of uniqueness even in wines with possible effects on the organoleptic properties of the wine itself. According to Portillo [120], however, the biogeographic imprint would be influenced by many factors closely related to the single vineyard and could change consistently within the vineyard itself. Exposure, availability of resources as well as management practices can certainly select some bacteria rather than others.

The second aspect to be considered is that of the cellar's role. To date, many studies have been focused on yeasts and it is clear that *S. cerevisiae* is certainly the yeast that plays a prominent role in the fermentation processes since the early stages. However, other organisms are pivotal to shape the aroma of a wine [126] belonging to very different genera such as *Candida*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Pichia*, *Lachancea*, *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Zygosaccharomyces* and *Saccharomycodes*. According to metabolic studies, these yeasts are able to produce aromatic compounds capable of modifying the organoleptic properties of wine [127, 128]. As for bacteria, an important role is played by lactic bacteria such as *Oenococcus oeni* rather than bacteria responsible for acetic fermentation such as *Gluconobacter* spp and *Acetobacter* spp. However,

microorganisms belonging to *Pedobacter*, *Sphingomonas*, *Janthinobacterium* and *Pseudomonas* were also found, which can play an important role in fermentative processes.

What remains to be clarified is how much the microbial component of wine is linked to the field and to the plant and how much it is affected by the wine cellar.

2.4 Project aims

The general aim of this Ph.D. project is to develop reliable and universal molecular-based systems for the characterization and traceability of agricultural food products. A key aspect of this research is the use of High Throughput technologies such as DNA sequencing (HTS) systems able to analyse not only raw materials but also complex matrices and processed food products.

From the technical point of view, my research activities have been focused on the application of HTS processes to analyse grape and wine microbiome. In this framework, the first objective was to understand whether or not, universal DNA barcoding markers or other loci traditionally used to achieve identification purposes, were able to characterize bacteria and yeasts colonizing fruit and must. In a second step, the reliability of such identification system has been tested by accessing publicly available molecular databases.

Based on obtained results, an experimental plan has been developed to use a DNA metabarcoding approach to characterize the microbiome of fruit and musts belonging to different geographic areas. Specifically, I tried to answer two main questions: i) do microorganisms influence grape

and wine? ii) Can these microorganisms be used as fingerprint to trace wine origin? To achieve these issues, the work has been planned to understand which variables, in addition to the pedoclimatic ones, could have an influence on grape and wine microorganisms' community in the field and in wine cellar.

Such an analysis has also been compared with those obtained by other HTS technologies to understand whether and how an integrative approach (e.g. DNA-based and chemical) could lead to a better product's traceability and quality valorisation.

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3. CHAPTER 1

Emerging DNA-based technologies to characterize food ecosystems

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3.1 Abstract

Food safety and quality depend on raw materials characteristics and on the chemical, physical and biotechnological processes adopted during food transformation. Since a huge number of microorganisms are involved in food production, foodstuffs should be considered as complex matrices where any microbial component have a precise role and evolves in response to physical and chemical composition of food. Moreover, knowing the dynamics of microbial community involved in a food supply chain it is useful to reduce food spoilage, enhance industrial processes and extend products shelf-life. In a more comprehensive vision, a precise understanding of the metabolic activity of microorganisms can be used to drive biotransformation steps towards the improvement of quality and nutritional value of food. High Throughput Sequencing technologies (HTS) are nowadays an emerging and widely adopted tool for microbial characterization of food matrices. Differently from traditional culture-dependent approaches, HTS allows the analysis of genomic regions of the whole biotic panel inhabiting and constituting food ecosystems. Our intent is to provide an up-to-date review of the principal fields of application of HTS in food studies. In particular, we devoted major attention to the analysis of food microbiota and to the applied implications deriving from its characterization in the principal food categories to improve biotransformation processes.

3.2 DNA barcoding to characterize food raw material and derived products

Along the food supply chain, characteristics of raw materials strongly influence the quality of final food products. This is a postulate of traditional and modern food-related disciplines. In this perspective, the selection of high-quality vegetables, meat or fish and the availability of suitable tools for their traceability represented so far the main goals of food producers (Aung & Chang, 2014; Imazio et al., 2002; Opara & Mazaud, 2001). The demand for reliable traceability systems is indeed essential to authenticate the geographical provenance of foodstuffs (also in the case of protected designation of origin products, PDO), and to prevent commercial frauds and adulteration cases. Such emerging topics addressed the scientific research, hence producing different analytical approaches to the problem (El Sheikha et al., 2009; Mafra, Ferreira, & Oliveira, 2008; Myers, 2011).

The validation of food authenticity relies mostly on the analysis of chemical compounds, proteins and/or DNA sequences. While being effective in testing fresh products, chemical and protein-based approaches can be biased by the strong food manufacturing processes, the limited number of detectable isozymes, or the high tissue and developmental stage specificity of the markers. DNA markers are more informative than protein or chemical based methods because DNA better resists physical and chemical industrial processes (Madesis, Ganopoulos, Sakaridis, Argiriou, & Tsaftaris, 2014). DNA is also detectable in presence of small traces of organic material therefore permitting the detection of low-concentration biological adulterants.

Table 1
List of references concerning the DNA barcoding characterization of raw materials or processed food products.

Foodstuff category	Raw Material / Food Product	References
Fruit	Mango	(Hidayat, Kusumawaty, & Pancoro, 2013)
	Citrus species	(Yu, Yan, Lu, & Zhou, 2011)
	Goji	(Xin et al., 2013)
	Berries	(Jaakola, et al., 2010)
	Pineapple	(Hidayat, Abdullah, Kuppusamy, Samad & Wagiran, 2012)
	Olives and Olive oil	(Agrimonti, Vietina, Pafundo, & Marmiroli, 2011; Ganopoulos, et al., 2013)
	Cocoa	(Kane et al., 2012)
	Dates	(Enan & Ahmed, 2014)
Vegetables	*Capsicum* cultivars	(Jarret, 2008)
	Legume seeds	(Ganopoulos, Madesis, Darzentas, Argiriou, & Tsaftaris, 2012; Madesis, Ganopoulos, Anagnostis, & Tsaftaris, 2012)
	Soybean and other crops	(Kim Y.H. et al., 2014)
Aromatic plants	Fresh and processed spices	(De Mattia et al., 2011; Federici et al., 2013; Gismondi, Fanali, Labarga, Caiola, & Canini, 2013; Kojoma et al., 2002; Parvathy et al., 2014; Theodoridis et al., 2012; Wang et al., 2013)
Herbal Infusions	Tea	(Stoeckle et al., 2011)
	Plant-based beverages	(Li et al. 2012)
Mushrooms	Wild and cultivated mushrooms	(Dentinger, Didukh, & Moncalvo, 2011; Khaund & Joshi, 2014; Raja, Baker, Little, & Oberlies, 2014)
Honey	Honey	(Bruni et al., 2015; Valentini, Miquel, & Taberlet, 2010)
Jams	Fruit Jams	Arleo et al., 2012
Medicinal plants	Medicinal plants	(Pansa et al., 2011; Zuo et al., 2011)
Seafood	Various fishes	(Ardura, Linde, Moreira, & Garcia-Vazquez, 2010; Ardura, Planes, & Garcia-Vazquez, 2013; Carvalho et al., 2015; Galal-Khallaf, Ardura, Mohammed-Geba, Borrell, & Garcia-Vazquez, 2014; Lamendin, Miller, & Ward, 2015).
	Tuna and other scombrid	(Abdullah & Rehbein, 2014; Botti

	species	& Giuffra, 2010)
	Smoked fish products	(Smith, McVeagh, & Steinke, 2008)
	Crab meat products	(Haye, Segovia, Vera, Gallardo, & Gallardo-Escàrate, 2012)
	Philippines fish products	(Maralit et al., 2013)
Meat	Bovidae species	(Cai et al., 2011)
	Bovine, Ovine, Caprine meat	(Saderi, Saderi, & Rahimi, 2013)
	Game Meat	(D'amato, Alechine, Cloete, Davison, & Corach, 2013).
Dairy products	Milk source	(Gonçalves, Pereira, Amorim, & van Asch, 2012; Guerreiro, Fernandes, & Bardsley, 2012)
	Plant traces in milk	(Ponzoni et al., 2009)

As a consequence, DNA markers and in particular PCR-based methods have rapidly become the most used tools in the field of food control. Among these, discontinuous molecular markers such as RAPDs, AFLPs, and their variants (e.g., ISSR, SSAP) as well as sequencing-based systems such as SNPs and SSRs have been successfully adopted for the characterization of food raw materials. However, being highly species specific, these approaches require access to the correct DNA sequence of the organisms and their application is often limited to a single species. In the last decade, DNA barcoding, a standardized method providing species identification through the analysis of the variability in a short DNA gene region – the "barcode", was proposed as a universal DNA-based tool for species identification (Hebert, Ratnasingham, & deWaard, 2003).

Recently, Galimberti et al. (2013) reviewed the usefulness of DNA barcoding to certify food identity by tracking origin and provenance of raw materials at different levels of their transformation. DNA barcoding permits to discriminate biological entities analyzing the variability in a single or in a few standard molecular marker(s) (Casiraghi, Labra, Ferri, Galimberti, & De Mattia, 2010). In this context, DNA sequence(s)

identify different food products in the same way a supermarket scanner uses the black stripes of the UPC barcode to identify any purchase. The application of this tool opened new opportunities to track not only common crops and breeds, but also those minor crops and local products still lacking of a reference genetic fingerprinting (Galimberti et al., 2014). As an example, DNA barcoding was extensively applied in the last decade to verify the origin of seafood (Becker, Hanner, & Steinke, 2011) and to exclude commercial frauds occurring in its production and distribution (Barbuto et al., 2010; Carvalho, Palhares, Drummond, & Frigo, 2015; Cutarelli et al., 2014). The success of seafood molecular identification allowed the US Food and Drug Administration to propose DNA barcoding as a routine approach for the authentication of fish-based commercial products (Yancy et al., 2008). Both consumers and foodstuff producers may take advantage of a DNA barcoding screening, especially concerning items distributed as shredded or powered material, which otherwise result as unidentifiable by a simple morphological analysis (Cornara et al., 2013). Among these, promising results were obtained in studies on commercial spices (De Mattia et al., 2011), herbal teas (Li et al., 2012) and fruit juices (Faria, Magalhães, Nunes, & Oliveira, 2013). Table 1 provides an updated list of case studies on identification and traceability of raw materials / processed foodstuffs by using DNA barcoding. Analysis of the case studies provided in Table 1 suggests that DNA barcoding is a sensitive, fast and cheap approach, able to identify and tracking a wide panel of raw materials and deriving food commodities. The cost and time-effectiveness of DNA barcoding and the recent development of innovative sequencing technologies allow a certain degree of automation in species identification, which is

particularly useful in simultaneous monitoring activities of multiple foodstuffs and batches.

Moreover, works listed in Table 1 highlight the principal advantages of using DNA barcoding for both producers and consumers. The firsts can value their products by certifying composition and provenance of raw materials and can have access to a sort of universal certification system (a pivotal requisite as we are in the era of globalization). On the other hand, consumers can defend they themselves against frauds and species substitution cases, as well as knowing the full composition of foodstuffs. This growing awareness is useful in mitigating the health impact of allergenic reactions, intolerances and other outbreaks, as also outlined by international regulations (e.g. the recently adopted Reg. (EU) No 1169/2011; European Commission, 2011).

International agencies or institutions, which are responsible for quality control of raw materials or food commodities, can cooperate by exchanging their data, hence creating reference databases, the lack of which is the main limit of the method. In fact, whereas some groups of organisms (e.g. fish) are well represented, a lot of work is required to provide reference DNA barcoding data for poorly investigated taxonomic groups (e.g., minor crops).

As a diagnostic tool, DNA barcoding approach can be more or less fallacious, and it should be taken into account that failures are mainly in the essence of biological species rather than in the method (Casiraghi et al., 2010). DNA barcoding performance is strongly influenced by the molecular variability of the organisms. As an example, the method cannot to date being easily applied to the differentiation of GM (Genetically Modified) food raw material, breeds and cultivars, basing on

the standard molecular markers. The modified genomic tracts usually do not involve the plastidial or nuclear regions analysed in a classical DNA barcoding approach. However, given the increasing demand of a fast and reliable traceability system for these kinds of products, a panel of additional markers (i.e. promoters, reporter genes) could be applied in combination with classical DNA barcodes. As an example, very recent studies showed the potential of High-Resolution-Melting (HRM) analyses when coupled to the investigation of DNA barcoding markers (bar-HRM) to differentiate cultivars and closely related species and to authenticate Protected Designation of Origin (PDO) of some food products (Druml & Cichna-Markl, 2014; Ganopoulos, Bazakos, Madesis, Kalaitzis, & Tsaftaris, 2013; Ganopoulos, Madesis, Darzentas, Argiriou, & Tsaftaris, 2012; Jaakola, Suokas, & Häggman, 2010).

3.3 The complex ecosystem of food biotransformation processes

Food quality does not rely on raw material characteristics only, but also on manufacturing and biotransformation processes involved during their conversion into final food products. Since time immemorial, biotechnological procedures are involved in food production. These, take advantage of environmental microorganisms such as bacteria and yeasts and of their metabolisms, transforming raw materials into enriched foodstuffs. Well-known examples refer to the production of wine, beer and other alcoholics, where biotransformation increases their organoleptic properties and extends their shelf-life; yogurt and dairy products, where microorganisms transform milk into products exhibiting

peculiar sensory and functional (e.g., probiotics) characteristics; bread and other bakery products obtained by the fermentation activity of selected yeasts. Pools of microorganisms can modify chemical and physical features of raw materials to get new metabolites and materials and therefore influencing sensory, safety and nutritional properties of the final transformed food products (Bull, Plummer, Marchesi, & Mahenthiralingam, 2013; Caplice & Fitzgerald, 1999).

Generally, in food industries physical and chemical modifications of raw materials are well calibrated at any step of the production chain to preserve organoleptic properties of the final product (De Filippis, La Storia, Stellato, Gatti, & Ercolini, 2014; Doyle & Buchanan, 2013). However, the calibration of biotransformation procedures is even more difficult.

Before discussing the complexity of microbial ecosystems, it is necessary to describe the three main categories of food biotransformation processes: fermentation, biopreservation, and functionalisation.

The fermentation process consists in the oxidation of carbohydrates to obtain major end products such as alcohol and carbon dioxide, as well as vitamins and secondary metabolites, thanks to the metabolic pathways of microorganisms (Ray & Daeschel, 1992). In the last 20 years, due to the continuous discoveries in biotechnology and genetic engineering, fermentation has definitively moved to industrialized and life-science driven technology (Waites, Morgan, Rockey, & Hington, 2009). Nowadays, there is an astonishing variety of fermented foods covering a broad range of food substrates (e.g., plants, milk, and many others). Considering that fermented foods constitute 1/3 of the human diet (Cambell-Platt, 1994) and due to the importance of this process in many

industrial compartments, the next chapters of this review will focus on case studies and novel techniques to explore microbial ecosystems involved in this biotransformation process.

Concerning biopreservation, most of food and beverages, require treatments that elongate their shelf-life, in order to maintain an acceptable level of quality and safety from manufacturing to consumption. Modern food preservation approaches are based on the use of microorganism producing antimicrobial compounds (i.e., organic acids, ethanol, hydrogen peroxide and bacteriocins) able to inhibit or contrast food spoilage (Ross, Morgan, & Hill, 2002). For example, a considerable number of starter strains used mainly in fermented foods derives from the activity of lactic acid bacteria (LAB). LAB are able to produce antimicrobial metabolites such as lactic acid, acetic acid and other organic acids therefore determining a low pH environment that prevents the growth of several pathogenic and spoilage microorganisms (Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013; Crowley, Mahony, & Sinderen, 2013). Nowadays, more than 170 bacteriocins have been described and are used for food preservation purposes (Hammami, Zouhir, Le Lay, Hamida, & Fliss, 2010). The last frontier of biopreservation is the use of microbial antagonistic molecules to functionalize food packages (Appendini & Hotchkiss, 2002). Active packaging systems include natural antimicrobials as additives, among which nisin, one of the most studied and commercialized bacteriocins. As an example, bacteriocins applied to food packaging materials were found to inhibit Listeria monocytogenes on meat products (Gàlvez, Abriouel, López, & Omar, 2007). The exploitation of such naturally biopreservation strategies holds great potentials, especially in the last

years, as the awareness of the consumer towards the so-called "green technologies" (i.e., minimally processed foods, free from chemical and harmful preservatives) is growing and growing.

Functionalisation is the production of new metabolites or functions mediated by microorganisms which can be delivered to the consumer through diet. These kinds of food, known as functional foods or nutraceuticals (Shah, 2007), share three basic characteristics: they derive from naturally occurring ingredients; they have to be consumed as a part of daily diet and they have significant benefits for human health. The most common functional foods can be grouped into three categories: probiotics, prebiotics and synbiotics (Pfeiler & Klaenhammer, 2013). A probiotic is a live microorganism that confers a health benefit on the host when administered in adequate amount. Prebiotics are non-digestible food ingredients that stimulate growth and/or activity of other bacteria, with positive effects on host's health. When both prebiotics and probiotics are present in the same food product, those functional foods are referred to as synbiotics. As a direct consequence of this new nutritional trend, a wide panel of functional foods became suitable for large-scale industrial production (Stanton, Ross, Fitzgerald, & Sinderen, 2005). A great number of genera of bacteria are used as probiotics, but the main species showing probiotic characteristics are Lactobacillus acidophilus, Bifidobacterium spp., and L. casei (Bull et al., 2013). Also yeasts play an important role as probiotics, with Saccharomyces boulardii as the most known probiotic fungus which has been successfully used for curing intestinal diseases (Czerucka, Piche & Rampal 2007). Several applications of probiotics and /or prebiotics have been studied: from the

enhancement of immune response to positive effects in contrasting allergies and even AIDS or other pathologies.

Fermentation, biopreservation, and functionalisation processes involve microorganism communities, sensitive to different environmental parameters (Bokulich, Thorngate, Richardson, & Mills, 2014; Minervini, De Angelis, Di Cagno, & Gobbetti, 2014). Moreover, community structure and relationships among different bacteria, yeasts and other microorganisms undergo substantial changes during biotransformation. Thus, only an exhaustive evaluation of microbial community structure and of its dynamics during food production could help optimizing industrial transformation steps in order to get high-quality products.

Except for traditionally biotransformed foods and beverages, an astounding number of edible products, including the emerging 'functional foods', involves the activity of microorganisms during at least one step of their industrial production. Thus, several microorganisms gained an important role in human food production and this trend rapidly increased with the advances and industrialization of modern food manufacturing procedures (Betoret, Betoret, Vidal, & Fito, 2011; Roberfroid, 2000). For this reason, at the industrial level, biotransformation steps could be partially controlled by using selected microorganisms as reaction starters. For example, in the case of wine-making, selected S. cerevisiae strains are used for activating the alcoholic fermentation of must. However, others microorganisms naturally inhabiting raw materials or the surrounding environment, could also be involved during food transformation. Again, in the case of wine, the wine cellar yeasts and bacteria could actively contribute to the chemical modification of grape juice to obtain wine with specific organoleptic properties (Bokulich,

Ohta, Richardson, & Mills, 2013; David et al., 2014). Environmental microorganisms represent an important source of biodiversity to differentiate a certain food product from the others, even at a reduced spatial production scale (Quigley et al., 2012; Riquelme et al., 2015). For these reasons, modern food companies should not underestimate the importance of knowing the composition of microbial community accompanying food from farms to consumer's fork or glass.

Moreover, during food production, undesirable microorganisms could also enter into the food supply chain (Bondi, Messi, Halami, Papadopoulou, & Niederhausern, 2014; Newell et al., 2010). External microbial components can reduce the quality of food products (spoilage microorganisms) or even negatively affect their safety (foodborne pathogens) (Doyle & Buchanan, 2013). In these cases, an in-depth analysis of food microbial community is essential to assess safety of raw materials and related final products (Fusco & Quero, 2014; Solieri, Dakal, & Giudici, 2013).

Given the complex dynamics occurring in food ecosystems, one of the emerging topics of food science is the development of revolutionary analytical systems able to characterize the microbial community as well as the DNA barcoding approach is able to characterize raw materials.

Nowadays, the occurrence and abundance of microbes in a given food ecosystem can be evaluated by studying its microbiota (Ercolini, 2013), which refers to the sum of microscopic living beings and their genomes (i.e., the microbiome) in the environment under investigation. In this review, we discuss the potential of modern technological advances in the molecular characterization of food-related microorganisms. Only the combination of high quality raw materials with fine regulated

biotransformation processes will lead to the improvement of food nutritional quality.

3.4 Novel molecular approaches to investigate food ecosystems

Since the advent of disciplines devoted to the study of food, the investigation of microbial ecology has dramatically changed and this process is in constant evolution (Solieri et al., 2013). For a long period, food-associated microorganisms and their dynamics have been studied through culture based-methods (Doyle & Buchanan, 2013). However, these revealed to be often weak to accomplish a complete microbial characterization of many ecosystems, among which foodstuffs (Ceuppens et al., 2014). Problems and shortcomings of culturing methods basically involve the underestimation of microbial diversity, and even the failure of a precise detection of some species or genera.

Following the advent of molecular biology, a plethora of laboratory techniques have been developed and most of these are now extensively adopted in food control activities (see for example, Ercolini, 2013 and Solieri et al., 2013). Molecular approaches permit to identify food-related microorgranisms and estimate their relative abundance, providing a fast, accurate and economic detection tool. Most techniques rely on the analysis of genetic DNA markers and become increasingly important in food microbiology. They identify microorganisms rapidly and accurately, complementing or substituting classical methods (Ceuppens et al., 2014; Chakraborty, Doss, Patra, & Bandyopadhyay, 2014).

Denaturing gradient gel electrophoresis (DGGE) is one of the most used fingerprinting techniques in food microbiology. It is based on the separation of polymerase chain reaction (PCR) amplicons of the same size but different sequences. Fragments are separated in a denaturing gradient gel based on their differential denaturation (melting) profile (Ercolini, 2004). In recent years, PCR-DGGE has been largely used to characterize bacteria and yeasts in fermented products (Muyzer, De Waal, & Uitterlinden, 1993; Peres, Barlet, Loiseau, & Montet, 2007) and to define the origin of raw material starting from the characteristics of its yeast or bacterial communities as in the case of fruit (El Sheikha, Bouvet, & Montet, 2011; El Sheikha, Durand, Sarter, Okullo, & Montet, 2012; El Sheikha, Métayer, & Montet, 2011) and fish (El Sheikha & Montet, 2014; Le Nguyen, Ngoc, Dijoux, Loiseau, & Montet, 2008). However, it is not always possible to resolve DGGE fragments when the difference in sequence is not wide enough or when different DNA fragments have identical melting behavior (Ercolini, 2004).

Since advances in technology have always driven discoveries and changes in microorganism taxonomy, taxonomic identification is an issue of primary importance when approaching the study of food microbiota. In this scenario, genomics now underlies a renaissance in food microbiology therefore accelerating food safety monitoring and food production processes (Ceuppens et al., 2014). Considering bacteria, the present taxonomy is still a complex topic for biologists as well as an area of growing interest, because the definition of microbial species as a taxonomic unit lacks a commonly accepted theoretical basis (Felis & Dellaglio, 2007). Microbial taxonomy directly influences a number of basic scientific and applied fields where microorganisms are involved

(Tautz, Arctander, Minelli, Thomas, & Vogler, 2003) including food production, conservation and probiotic activity. Depending on the level of investigation required, the taxonomic resolution of microorganisms can vary. For example, the genus rank could be sufficient when monitoring changes in microbial community during a biotransformation or treatment process of food raw material (e.g., fermentation, pasteurization) (Quigley et al., 2012). In contrast, species or strains have to be precisely identified in case of pathogen detection analyses, or to assess the efficacy of a certain probiotic.

Aiming to differentiate microorganisms at the species level, methods based on DNA sequencing are currently the most adopted. In many cases, when a fast and accurate response is needed, a 'DNA barcoding-like' approach is the most reliable (Chakraborty et al., 2014). Many scientists used 16S rRNA gene as a universal marker for species-level typing of microorganisms (Bokulich, 2012; Claesson et al., 2010; Janda & Abbot, 2007). This genomic region is considered a 'bacterial barcode' due to its peculiar properties (Patel, 2001): it is present in all the bacterial species, it contains sufficient information (1500 bp long) to differentiate species and, in some cases, strains (Muñoz-Quezada et al., 2013) and finally, the 16S rRNA relies upon an impressive archive of reference sequences such as Greengenes (De Santis et al., 2006) and SILVA (Pruesse et al., 2007). Amplicons belonging to whole genomic extraction conducted on the matrices under investigation (e.g., food products) are sequenced and reads are compared to reference databases to identify the Operational Taxonomic Units - OTUs (Sandionigi et al., in press).

Several studies test analytical approaches for the DNA-based detection of emergent food microbial contaminants in a wide panel of food products

(see for example Fusco & Quero, 2014; Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010 and related references). Such techniques allow to detect specific bacteria and strains in different steps of the food supply chain as reported for example in the cases of seafood and meat manufacturing (Amagliani, Brandi, & Schiavano, 2012; Norhana, Poole, Deeth, & Dykes, 2010; Zbrun et al., 2013). In international trade, major food categories like those cited above, and are commonly shipped very long distances and are therefore exposed to various contaminants such as Salmonella, Listeria and Campylobacter. PCR and Real-Time PCR based methods are nowadays routinely used for the detection of these pathogens. Primer combinations also permit the simultaneous identification of a panel of foodborne pathogens in a single reaction (see for example Jofré et al., 2005).

Progresses in sequencing technologies and bioinformatics analysis of data, led nowadays to a more complex scenario of food control activities. Detection approaches targeting one or few microorganisms are not sufficient to have a reliable characterization of quality and safety of foodstuffs. Recent technological advances offer a panel of analytical tools able to screen the whole microbial community of food matrices. The use of universal markers produces several DNA barcode fragments, corresponding to the each bacterial species present in a food sample. With the ultimate goal of characterizing the complete spectrum of microorganisms, the traditional Sanger sequencing approach results inadequate to uncover this huge diversity. To date, several novel approaches, referred to as ‘Next Generation Sequencing’ (NGS) and, more recently, ‘High Throughput Sequencing’ (HTS), have been developed (Ercolini, 2013; Mayo et al., 2014; Solieri et al., 2013).

HTS techniques are able to provide sequence data around a hundred times faster and cheaper than the conventional Sanger approach. Sequencers from 454 Life Sciences/Roche (producing about a million sequences of 800 to 1000 base length), Solexa/Illumina and Applied Biosystems SOLiD technology (producing over a billion sequences of 50 to 500 base length) were produced as second generation technologies and other competitive instruments appeared on the market such as the Ion Torrent and PacBio. HTS technologies also permit to prepare several DNA samples from different extracts and to mark them with different DNA tags, mixed and processed at the same time. Thanks to these practical advantages, it is possible to analyze in parallel a very high number of samples, and hence lower the analysis cost. The reduction in cost and time for generating DNA sequence data has resulted in a range of new successful applications, including food traceability and especially food microbiology (Madesis et al., 2014).

Table 2 encompasses recent and emblematic case studies concerning the adoption of HTS approaches to study the microbial ecosystem (in terms of diversity and dynamics) of different food categories. In most cases, the obtained results could be of great impact on the food supply chain to improve industrial biotransformation processes, enhance quality of final products, extend the shelf-life and valuating local productions.

In the following sections, we selected two of the most representative food categories to highlight the role of novel molecular approaches in characterizing food microbial ecosystems. The first category refers to foodstuffs having plant organisms as starting raw material and where HTS analyses was used to characterize the microbiota of some food products from field to table. Similarly, the second section describes

emblematic case studies involving dairy products, which are characterized by complex and sometimes unconventional biotransformation processes.

3.5 Microbiota composition and dynamics in plant fermentation processes

Fermentation is considered one of the oldest biotechnological methods to convert sugars, starches, or other carbohydrates, into alcohol, and organic acids, by microorganisms. Archaeologists have found molecular evidence for the production of fermented beverages dated back to 7000 and 5400 BC. In the Neolithic, fermentation ensured vegetable preservation (McGovern, Glusker, Exner, & Voigt, 1996; Ross et al., 2002) and was based on spontaneous microorganisms inhabiting fruits and seeds. Nowadays, many selected strains of microorganisms are used to transform raw materials in foodstuff having additional nutritional properties. HTS analyses also clarified the key role of spontaneous microorganisms in biotransformation processes (Table 2).

Table 2

Case studies concerning the use of emerging DNA-based technologies to characterize food microbiota. Potential implications for the food supply chain are reported for each food category.

Raw material / Food category	Aims	Implications for the food supply chain	References
Grapevine	Study of bacterial consortia inhabiting	Valuing cultivars and wine production at the regional scale	(Bokulich et al., 2014)

	grapevine surfaces		
Must and Wine	Study of microbial community of must and its dynamics during alcoholic fermentation	Improvement of wine fermentation processes	(Bokulich et al., 2012; Bokulich et al., 2013; David et al., 2014)
Beer	Study of microbial community involved during beer production	Improvement of brewery at both artisanal and industrial scale	(Bokulich, Bamforth, & Mills, 2012; Jung, Nam, Roh, & Bae, 2012)
Soybean, rice and vegetables	Study of microbial community of fermented products	Quality improvement of final foodstuffs. Valorization of production by enhancing sensorial characteristics of local and commercial products	(Jung et al., 2011; Kim et al.,2011; Nam, Lee, & Lim, 2012; Sakamoto, Tanaka, Sonomoto, & Nakayama, 2011; Park et al., 2012)
Olives	Study of olive fermentation dynamics and bacterial biodiversity	Improvement of the sensory quality of table olives	(Cocolin et al., 2013)
Raw milk	Assessing the effects of cattle's diet on milk quality	Enhance and preserve organoleptic quality and shelf-life of raw milk and dairy products by calibrating cattle diet	(Kuehn et al.2013, Masoud et al., 2012; Zhang et al., in press)
Processed Milk	Influence of milk origin and treatments on	Selection of new strains or strains with novel properties for their use as dairy starters	(Delgado et al., 2013; Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011; Leite et al., 2012)

	microbiota		
PDO cheeses	Characterization of the microbiota involved in cheese production	Improvement of fermenation processes to obtain high quality cheese	(Aldrete-Tapia et al., 2014; Alegría, Szczesny, Mayo, Bardowski,& Kowalczyk, 2012; De Filippis et al., 2014; De Pasquale et al., 2014; Lusk et al., 2012; Quigley et al., 2012; Riquelme et al., 2015)
Seafood	Study of microbial community of fermented seafood	Improvement of fermentation and conservation processes	(Roh et al., 2009; Koyanagi et al., 2011)
Seafood	Investigating relationships between seafood microbiota and products' shelf-life	Shelf-life extension of seafood products	(Broekaert, Heyndrickx, Herman, Devlieghere, & Vlaemynck, 2013; Chaillou et al., 2014; Kim H.J. et al., 2014; Koyanagi et al., 2011)
Meat	Characterization of microbial communities and dynamics associated to meat products	Improvement of organoleptic characteristics and quality of typical products.	(Chaillou et al., 2014; Nieminem et al., 2012; Polka, Rebecchi, Pisacane, Morelli, & Puglisi, 2015)

The equilibrium among spontaneous and commercial microorganisms during fermentation depends on many factors, including the microbial biodiversity present in the food and the environmental conditions occurring during biotransformation. An HTS approach allows to study the evolution of food microbiota in time and in response to different parameters such as temperature, pH, substrate chemical compositions and others. For example, David et al. (2014) mapped microbial population dynamics in wine musts (organic and conventional) and showed substantial changes during each biotransformation phase in response to musts characteristics. These data could be used by winemakers to drive fermentation processes and to set up the most suitable environmental conditions to enhance wine characteristics (Bokulich, Joseph, Allen, Benson, & Mills, 2012). Similar analyses were conducted for brewing. Data suggested that beer is characterized by consistent modification in microbial activity at every stage, from raw material production and malting to stability in the package. Again, HTS approach allowed to evaluate this diversity and to exclude the presence of undesirable bacteria (Vriesekoop, Krahl, Hucker, & Menz, 2012).

In table olive fermentation, HTS techinques were used to evaluate the impact of NaOH treatment (Cocolin et al., 2013). No treated olives were characterized by the presence of halophilic bacteria, which were substituted by Lactobacillus at the later stages of the fermentation, whereas Enterobacteria were dominant when the olives were treated with sodium hydroxide. Higher biodiversity was found for Lactobacillus plantarum isolated during untreated fermentation: different biotypes were found on the olive surface and in the brines. When the debittering process was carried out, a decrease in the number of L. plantarum biotypes was

observed and those originating from the surface of the olive did not differ from those occurring in the brines. These changes in microbiata structure could lead to a modification of sensory quality of olives.

In plant products, the microbial community of cultivation area could also influence the quality and nutritional value of final food products. Using HTS analyses, Bokulich et al. (2013), identified the “wine microbial terroir" and elucidated the relationship between production region, climate, and microbial patterns. This information may help to enhance biological control of vineyard, improving the wine supply and to enhance economic value of important agricultural commodities, as also suggested by Baldan et al. (in press).

Microbiome analysis could also be used to evaluate and enhance the nutritional value of food products. For example, analysis performed on different commercial brands and local productions of doenjang, a traditional fermented soybean product, revealed consistent differences in microbial community structure (see Table 2 for references). Such differences largely influence the flavor and nutritional properties of doenjang (Nam, Park, & Lim, 2012). Commercial brands contain simple microbial communities dominated by Tetragenococcus and Staphylococcus that homogenize the taste and composition of the product. In contrast, local products showed conspicuous variability in microbial populations, providing products of completely different fermentations.

The analysis of spontaneous microbiota associated with original raw materials and the evaluation of antimicrobial components is another important element to drive biotransformation processes. For example, the consistent demands of new flours from cereals and other crops lead to the

test of different mixtures. Chestnut flour was considered one of the most interesting raw materials due to its content of proteins with essential amino acids (4–7%), mineral salts and vitamins; however, the occurrence of phenolic compounds with antimicrobial activity prevents the use of this raw material for the fermented products (De Vasconcelos, Bennet, Rosa, & Ferreira-Cardoso, 2010). The combination of chestnut flour with wheat (Dall'Asta et al., 2013), rice (Demirkesen, Mert, Sumnu, & Sahin, 2010) and rye flours could reduce the chestnut antibacterial components. A mix of raw materials resulted in a mix of microbiota that can contribute to improve the efficacy of biotransformation (Aponte et al., 2014).

Finally, the modern molecular approaches to study microbial ecosystems of plant-derived foods could also reduce food spoilage occurrence due to undesirable microorganisms. In general, food alteration derives from contamination mediated by specific microorganisms, but sometimes several pathogens can simultaneously contaminate a food matrix (Fusco & Quero, 2014). For example, brewing could be negatively affected by different classes of bacteria such as lactic acid bacteria, acetic acid bacteria, Enterobacteriaceae, Zymomonas (Vriesekoop et al., 2012) that can coexist. In these cases, HTS analysis is reliable to identify any undesirable microorganism and could be used to enhance food sanitation and preservation measures.

3.6 The evolution of microbial community in artisanal and industrial dairy production

Dairy products are the result of a long history and local traditions (Cordain et al., 2005) that led nowadays to the recognition of hundreds of

Protected Designation of Origin (PDO) products. Such brand refers to peculiarities in their flavor, consistency and methods of production that are characteristics of a certain geographical site and increase their market value. Due to the economic relevance, health and social issues related to this category of foodstuff, many DNA-based techniques are currently available to assess authenticity and adulteration of milk-derived food (Mafra, et al., 2008). Among the applications of these molecular tools, there is the possibility of detecting the adulteration of higher value milk by nondeclared cow's milk (Galimberti et al., 2013) and even to detect traces of feed-derived plant DNA fragments in raw milk and in its fractions (Ponzoni, Mastromauro, Giani, & Breviario, 2009). In contrast, the characterization of their microbial component is much more difficult. Microbial dynamics occurring within major ingredients involved in the manufacturing of typical cheeses (i.e., milk, rennet, salt) shape the production of the different varieties and can contribute to aroma and taste defects. As a result, the microbiota of different cheeses varies considerably depending on the type of fermentation adopted (Quigley et al., 2012). Due to the complexity of biotransformation processes, diversity, not only at the species level but also at the strain one is pivotal for industrial purposes. This aspect requires the availability of reliable methods for strain discrimination and monitoring (De Filippis et al., 2014). Indeed, a deep knowledge of raw materials indigenous microbiota could permit a proper selection and dosage of a starter culture to enhance the transformation steps and increase sensorial properties of the final product (See Table 2 for examples).

Microbial populations in cheese can be split into two distinct groups i.e., starter and non-starter microorganisms. Homofermentative lactic acid

bacteria (LAB) are the dominant and most important component of the microbiota of fermented milk products as they act as starter cultures, causing rapid acidification via the production of lactic acid. In some fermented dairy products, additional yeasts, molds, as well as bacteria such as non-starter lactic acid bacteria (NSLABs), are involved for the production of flavor compounds or carbon dioxide (De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti, 2014; Fox, Guinee, Cogan, & McSweeney, 2000; Quigley et al., 2012). However, they can also be associated with the occurrence of defects. The relative importance of the starter culture and other added microorganisms varies from product to product (Johnson & Steele, 2013), as well as the microbial composition in different parts of a ripened product (e.g., internal part, rind). A precise control of microbial strains and their proportions is fundamental to minimize cheese defects and enhance its quality (O'Sullivan, Giblin, McSweeney, Sheehan, & Cotter, 2013).

The basic goal of characterizing microbial diversity and community dynamics in relation to dairy microbiology is to understand the relationships between microorganisms and their impact on food sensorial properties and safety (Solieri et al., 2013). The modern molecular approach to study microbiota composition can contribute to clarify the role of raw milk quality and added ingredients in dairy transformation processes. Many studies showed how cheese microbiota structure can vary according to the animal origin of the milk (Coppola, Blaiotta, Ercolini & Moschetti, 2001; Quigley et al., 2012), its preliminary treatments (e.g., pasteurization, Delgado et al., 2013) and additional ingredients used during production (Ercolini, De Filippis, La Storia, & Iacono, 2012). In a survey based on HTS analyses conducted on the

microbiota of 62 artisanal Irish cheeses, Quigley et al. (2012), provided evidence for a different microbial richness (in terms of genera of bacteria) in milk of different sources, with a maximum (i.e., 21 genera) for cow milk cheeses and a minimum (i.e., 2 genera) for sheep milk cheeses. They also highlighted, in some cheeses, a negative effect of salt content on the presence of certain genera (e.g., Leuconostoc and Pseudomonas) as well as a different microbial community structure when herbs and species were involved during cheese manufacturing.

In 2012, Ercolini et al., demonstrated the importance of the microbiota of natural whey colture (NWC) added to raw milk to drive fermentation processes and shaping the final bacterial community of water buffalo mozzarella, a highly appreciated Italian nonripened cheese. Although completely different production technologies are employed, some products such as Grana Padano, Parmigiano Reggiano and other PDO cheeses share the use of the NWC as starter for the curd acidification. Studies on their microbial communities and dynamics revealed by HTS approach (e.g. De Filippis et al., 2014), showed how, starting from similar NWCs, temperature and pH drive selection of a characteristic core microbiota, responsible in the achieving the typical sensory characteristics of each cheese type.

Animal diet was thought to be of primary importance for determining milk composition, microbial structure and quality. Using a 454 pyrosequencing approach, Zhang, Huo, Zhu, & Mao (2014), found that high-concentrate feeding had significant effects on shaping the milk microbial community of dairy cows. This kind of diet resulted in a greater proportion of psychrotrophic bacteria in milk, such as Pseudomonas, Brevundimonas, Sphingobacterium, Alcaligenes,

Enterobacter and Lactobacillus. A possible conclusion was that inappropriate cattle feeding may lower the organoleptic quality of raw milk and dairy products, also limiting the shelf-life of processed fluid milk.

HTS analysis of microbiota composition can also give information about the dairy production methods. Generally, traditional manufacturing processes (i.e., artisanal production) are characterized by a complex microbial community. In contrast, industrially obtained foods are characterized by more-simple microbial consortia (De Filippis et al., 2014; Ercolini, 2013).

Several researches also revealed that different cheese-making units within the same broad geographic area share a common core microbiota (see for example De Filippis et al., 2014; Quigley et al., 2012). A precise knowledge of such bacterial consortia may help in transferring certain productions from the artisanal to the industrial level with consequent economical benefits.

However, in dairy production, one of the possible risks occurring in the passage from artisanal to industrial manufacturing could be the loss of flavors and aromas which are characteristic of the product. This goal requires the standardization of cheese production process, using for example pasteurized milk instead of the raw one. The standardization of fermented dairies manufacturing is not trivial because different products, similar in appearance can exhibit unique bacterial profiles and unique sensorial properties (Lusk et al., 2012). In a recent study, Aldrete-Tapia, Escobar- Ramírez, Tamplin, & Hernández-Iturriaga (2014), used HTS techniques to establish the denomination of origin for the Mexican artisanal Poro cheese: they provided an insight of the composition and

dynamics of bacterial communities present during its production and ripening. Since molecular data determined the relative composition and bacterial species in artisanal production process of Poro cheese, it could be possible to identify not only the microbial communities but also those bacteria that could be potentially used in starter cultures.

Another emblematic case is that of Pico Cheese, an artisanal dairy cattle product manufactured by few Azorean (Portugal) producers without the addition of starter cultures (Riquelme et al., 2015). Given the ongoing loss of local producers and the necessity of preserve its peculiarity and enhance its marketability even at a semi-industrial scale production, Riquelme et al. (2015) examined in depth the microbiota diversity and dynamics during ripening of Pico Cheese. Researchers characterized the core bacterial components (Lactococcus, Streptococcus and some unclassified Enterobacteriaceae) of artisanal Pico cheese microbiota, a first step to recreate certain conditions for a potential industrial production.

The microbiota of the processing environment also influences the microbial community and its succession of fermented dairy products. During manufacturing, raw milk and its fermented intermediates, encounter many different surfaces, all acting as potential vectors for microbes. HTS analyses conducted by Bokulich & Mills (2013) on two artisanal cheesemaking plants revealed that similar communities of microbes occupied the same surface types, reflecting the selection for distinct communities on the basis of the production stage. Such a situation may play an important role in populating cheese microbial communities, beneficially directing the course of sequential fermentation and the quality of final products (see for example the cases of water

buffalo mozzarella and other artisanal cheeses: Aldrete.-Tapia et al., 2014; Mauriello, Moio, Genovese & Ercolini, 2003; Randazzo, Pitino, Ribbera, & Caggia, 2010). Interestingly, De Filippis et al. (2014), in a study on three highly-appreciated PDO Italian cheeses, found many sub-dominant OTUs of environmental provenance, probably arising from soil and agricultural environment and established into the final product.

The spatial distribution of microbes in foods is also a very interesting issue. It was demonstrated that structurally complex foodstuffs can host a different microbiota within their parts, such as the crust, veins, and core in a blue cheese (Ercolini, 2013). The use of HTS technologies is successful in assessing the location of different microbes across food matrices (Gkatzionis, Yunita, Linforth, Dickinson, & Dodd, 2014) and this information can have important consequences in understanding and enhancing ripening and flavoring processes of high-value products.

3.7 Conclusions

High throughput sequencing technologies are nowadays an emerging and widely adopted tool for microbial characterization of a huge number of matrices and ecosystems, among which foodstuffs. In the field of food quality and safety assessment, the vast majority of published studies focus on fermented beverages and dairy products, in spite of their relevance and economic value in the global market. Other food categories such as meat and seafood are widely distributed worldwide but many aspects of their microbial ecology are largely unknown. In recent years, thanks to the growing accessibility of modern analytical technologies (i.e.

HTS), the first studies on these apparently less complex food matrices are emerging.

In contrast to environmental microbiology, few studies have been conducted to identify the metabolic pathways and active compounds involved during the main food transformation processes. A more detailed knowledge on the role of different microorganisms in food would help in enhancing production processes, reducing wastes and extending products shelf-life. In this context, recent advances in 'omic' can have great relevance in food science. In the very next-future an effective integration among different sources of biological information is auspicable in order to better understand and manipulate flavor formation, taste and the nutritional quality of foodstuff.

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4. CHAPTER 2

Towards a Universal Approach Based on Omics Technologies for the Quality Control of Food

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4.1 Abstract

In the last decades, food science has greatly developed, turning from the consideration of food as mere source of energy to a growing awareness on its importance for health and particularly in reducing the risk of diseases. Such vision led to an increasing attention towards the origin and quality of raw materials as well as their derived food products. The continuous advance in molecular biology allowed setting up efficient and universal omics tools to unequivocally identify the origin of food items and their traceability. In this review, we considered the application of a genomics approach known as DNA barcoding in characterizing the composition of foodstuffs and its traceability along the food supply chain. Moreover, metabolomics analytical strategies based on Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS) were discussed as they also work well in evaluating food quality. The combination of both approaches allows us to define a sort of molecular labelling of food that is easily understandable by the operators involved in the food sector: producers, distributors, and consumers. Current technologies based on digital information systems such as web platforms and smartphone apps can facilitate the adoption of such molecular labelling.

4.2 The Demand for Universal Analytical Tools to Characterize Foodstuffs

The globalization of the food market has led to a corresponding increase in issues concerning the authenticity and safety of imported foods. Consumers are susceptible to any form of food alteration that may occur

during artisanal or industrial manufacturing processes and pay attention to food ingredients as these can influence nutritional and health conditions [1–3]. The consumer's awareness in terms of food quality and safety is growing and growing and implies the search for products with exhaustive labelling reporting details about the original raw materials and with assurances about the absence of harmful chemical and microbial contaminants [4–6]. These topics drove the development of new analytical tools in the context of food science [7]. A relevant section of approaches was the one devoted to the screening of undesired microorganisms, often occurring in foodstuffs, to ensure human safety and preventing food spoilage and/or the spread of foodborne disease outbreaks [8, 9]. Foodborne pathogens, as well as spoilage microorganisms, can already be present in the indigenous microbiota of raw materials or colonize the final food product by contamination during manufacturing [10]; therefore, laboratory analyses must be conducted both on raw materials and transformed food items. There is a great number of microorganism taxa traditionally associated with human diseases and for which every food product should be tested in order to ensure their absence. *Salmonella* spp. is one of the major pathogens responsible for foodborne disease outbreaks throughout the world and *S. enterica* is the most frequently isolated species [11]. Other important and frequently reported foodborne pathogens belong to the genera *Campylobacter*, *Yersinia*, *Shigella*, *Vibrio*, *Clostridium*, *Bacillus*, *Listeria*, and *Staphylococcus* [12, 13]. Most of these microorganisms are not easily detectable with culture dependent approaches, but DNA-based tests that improve their detection have been developed. Most of these are

based on the simultaneous detection of a wide panel of entities by using universal DNA marker regions such as the 16s rDNA or the ITS [14, 15]. DNA-based approaches have acquired a growing importance also to respond to another consumer's request that is the authentication of both raw materials and processed food products [1]. Such a demand arose due to different factors: (i) the globalization of the food market that caused a longer and more articulated food supply chain, where raw materials are globally exported and processed in countries different from the origin; (ii) the industrialization of manufacturing processes (e.g., fermentation, biopreservation, and functionalization [16]) that are becoming more and more complex and largely unknown to the consumers; (iii) the strong modifications to which foodstuffs are subject before being sold (e.g., slicing and powdering) that impede a correct identification of the original raw materials by the consumer; (iv) the growing occurrence of allergies and intolerances related to certain foods or components of processed foodstuffs, typical of western countries. A plethora of molecular-based tools has been developed to characterize food composition and validate food authenticity [1], most of which relying on the analysis of proteins [17] and/or DNA sequences [18]. Protein based approaches are useful in characterizing the composition of fresh products; however, these methods can be biased by several factors such as the strong food manufacturing processes, the limited number of detectable isozymes, or the high tissue and developmental stage specificity of the markers [19]. DNA markers were definitely proven to be more informative than protein-based methods because DNA better resists industrial processes such as shredding, boiling, pressure cooking, or transformations mediated by chemical agents [20, 21]. This property allows a successful identification

of animal, plant, or fungi raw materials, even when they are present at small traces. Moreover, the availability of advanced technologies and efficient commercial kits for DNA extraction permits obtaining an acceptable yield of genetic material from processed or degraded biological material [8, 22, 23].

DNA analyses in food science are based on specific genome regions used as "identity markers" easily detectable by Polymerase Chain Reaction (PCR) [18]. Discontinuous molecular markers such as Amplified Fragment Length Polymorphisms (AFLPs), as well as their variants (i.e., ISSR, SSAP, and SAMPL), have been successfully used in the characterization of several food raw materials [18, 24]. Moreover, species specific makers have been developed for the most important and traded categories of animal and plant raw materials. This is the case of Single Nucleotide Polymorphisms (SNPs) and Simple Sequence Repeats (SSRs) that are largely used because of their high level of polymorphism and high reproducibility [25]. These approaches are used both in the identification of plant cultivars [26, 27] and animal breeds [28, 29] and to prevent fraudulent commercial activities [30, 31]. However, being highly species-specific, these approaches require a deep knowledge of the genotypes of the organisms and their application is often limited to a single taxon, or to a few closely related taxa. Nowadays, producers, manufacturers, distributors, and consumers advocate the development and adoption of universal tools to assess not only the origin and traceability of raw materials and derived food products but also the inadvertent occurrence of other species (i.e., contamination) or cases of species substitution (i.e., frauds). The development of innovative food-

related universal tools based on DNA analysis will be the first issue treated in this paper.

However, the DNA certification of identity and origin of foodstuffs are not necessarily synonyms of food quality. As an example, the genetic identity of a vineyard influences some aspects of wine quality [32] but other environmental factors could affect the plant phenotype and therefore the wine organoleptic properties [33–35]. For these reasons, the DNA-based analysis should be combined with a precise evaluation of chemical food characteristics. The second section of this paper will be devoted to the analysis of modern metabolomics techniques in the field of food science.

Both DNA-based and metabolomics approaches can be simultaneously performed through the so-called omics platforms [36], the use of which is expected to progressively become a routine in the context of food control. Given the recent bioinformatics advances, omics platforms are able to process huge amounts of data and combine information belonging to different analytical approaches. Hence, the technological innovations concerning food quality lie in both the development of universal and more accurate analytical systems and their reciprocal integration.

4.3 DNA Barcoding: A Universal Approach for Food Characterization

As discussed in the previous chapter, an aspect of primary importance in food science is the need to identify the origin of food raw materials, as well as tracing food products along the entire food supply chain by using universal, rapid, and inexpensive tools. In the last decade, “DNA

barcoding" was proposed as a universal method to identify living organism including edible plants and animals [37]. The rationale of this approach consists in the analysis of the variability at one or a few standard region/s of the genome (i.e., DNA barcodes) occurring in the whole panel of organisms constituting the raw materials and their derived food products [38].

The 5'-end portion of mitochondrial *coxI* gene was suggested as standard DNA barcode region for metazoans. In plants, mitochondrial DNA has slower substitution rates and shows intramolecular recombination [39], therefore impeding a reliable species identification. The research for an ideal DNA barcode in terrestrial plants has focused on two plastid DNA regions (i.e., *rbcL* and *matK*) considered as the "core-barcode" [40]. These can be supported by other regions, such as the *trnH-psbA* intergenic spacer, due to their higher variability among congenerics [41, 42]. Internal transcribed spacer regions of nuclear ribosomal DNA (ITS) were also recommended as additional markers in angiosperms [39].

Although there is still much debate on the identification performances of these markers, DNA barcoding showed its effectiveness when used to characterize unknown specimens based on the comparison with reference sequences [42, 43], especially for edible organisms used in food production [44–47]. The efficacy of DNA barcoding is supported by the availability of a comprehensive and continuously growing public library of DNA barcodes, the Barcode of Life Data System (BOLD), which provides a global identification system that is freely accessible [48, 49]. This platform consists of several components, including the Identification Engine tool (BOLD-IDS), which works with DNA barcode sequences

and returns a taxonomic assignment at the species level whenever possible.

A case in which DNA barcoding works well is the analysis of seafood [50], where *coxI* showed higher discrimination ability and in several cases allowed the identification of the origin of certain fish stocks. Moreover, in the modern market, many seafood species are sold as fillets or slices, therefore hindering the application of classical identification approaches. In such cases, the molecular analysis is the only reliable strategy to identify species [51]. Given its efficacy, DNA barcoding was adopted by the US Food and Drug Administration for the authentication of fish-based commercial products [52].

A limited success of the method was achieved concerning meat identification, especially concerning farmed species. The main reason of this pitfall lies in the scarce variability of the conventional barcode region among animal breeds and in the frequent occurrence of hybridization events [53]. In contrast, regarding dairy products, DNA barcoding has been proven efficient in characterizing composition and origin of milk. Indeed, the plastidial *rbcL* barcode marker was found to be able to detect traces of food-derived plant DNA fragments in raw cow milk [54, 55], thus opening new perspectives for the traceability of milk and dairy products in general.

Among plant-based foodstuffs, the DNA barcoding approach has been used for many applications [56] and to investigate the genetic relationships between wild and cultivated plants, as well as their origin. As an example, DNA barcoding was used to characterize the bean germplasm (*Phaseolus vulgaris* L.) and was found able to distinguish among different haplotypes of bean accessions from the Mesoamerican

and Andean areas [57]. Similarly, the DNA barcoding approach was adopted to assess the origin and quality of spices [44, 58], herbal products [59, 60], and naturally processed plant products such as multiflower honey [61]. Other studies investigated the ability of DNA barcoding in discerning toxic plants from edible species: cultivated species of the genera *Solanum* and *Prunus* were successfully distinguished from their toxic congenerics [62] and from some frequent plant species misidentifications that cause poisoning in human [63].

On the whole, the most important innovation introduced by DNA barcoding is the merging in a single approach of three characteristics typical of molecular analytic tools: (i) the *molecularization* of identification processes (i.e., the investigation of DNA variability to discriminate among taxa); (ii) the *standardization* of molecular marker/s and of analytical procedures; (iii) the *data computerization* of identification results (i.e., the not redundant transposition of the data using informatics) [64]. This last element is fundamental to make the analytic DNA-based tool accessible to the different actors involved in the food supply chain. Table 1 provides an updated list of DNA barcoding case studies dealing with raw materials and foodstuffs with a clear indication of the beneficiary subjects of the analysis: producer, distributor, and consumer. Although DNA barcoding largely demonstrated its high sensitivity and reliability in the authentication of food products, it should be specified that most food products are composed of a mix of organisms. In these cases, the use of universal primers and standard sequencing approaches, based on the traditional Sanger technology, are inefficient to discriminate among the single components. As a result, the requirement for high-throughput sequencing

techniques grew by an unpredicted extent [106]. Several novel approaches evolved to replace the traditional Sanger sequencing method; these modern advances have been referred to as "high-throughput DNA sequencing" (HTS). HTS techniques are able to provide billion sequence data several times faster and cheaper than the conventional Sanger approach.

Table 1: Updated list of DNA barcoding case studies in the field of food science and principal stakeholders. Producers are interested in valuing their crops or breeds by molecular certification; distributors are mainly interested in the traceability and authentication of traded products; the interest of consumers is to avoid commercial frauds/species substitutions and have an assurance on food provenance.

Food category	Target analysis	Interested stakeholders			References
		Producer	Distributor	Consumer	
Plants	Identification of species and provenance of *Mangifera* species	X	X	X	[65]
	Traceability of *Lycium barbarum* (Goji)		X	X	[66]
	Authenticity analyses of berry species	X	X	X	[67]
	Molecular identification of pineapple cultivars	X			[68]
	Identification of cocoa (*Theobroma* spp.; *Malvaceae*) cultivars	X			[69]
	Identification of date cultivars	X	X		[70]
	Identification of *Capsicum* species		X	X	[71]
	Authentication of PDO Fava Santorini (*Lathyrus clymenum*)	X	X	X	[72]
	Identification of Mediterranean bean species			X	[73]
	Identification and authentication of some Lamiaceae species		X	X	[44]
	Identification of *Thymus* species			X	[74]
	Authentication of saffron		X	X	[75]
	Authentication of black pepper powder		X	X	[76]
	Identification of *Salvia* species	X	X	X	[77]
	Authentication of herbal teas	X	X	X	[78]
	Authentication of turmeric powder (Zingiberaceae)		X	X	[79]
	Identification of herbs in beverages			X	[80]
	Authentication of fruits in jams		X	X	[81]
Mushrooms	Mushrooms identification		X	X	[82, 83]
Honey	Characterization of monofloral or multiflower honey	X		X	[42, 61]
Fishes and seafood	Identification of commercial fish species		X	X	[84–86]
	Identification of processed fish products			X	[87–92]
	Labelling authentication of fish products		X	X	[47, 51, 93–96]
	Identification of poisonous seafood species		X	X	[97]
	Identification of crab meat products			X	[98, 99]
	Origin and Authentication of Hairtail Fish and Shrimp	X	X	X	[100]
	Identification of *Octopus* species			X	[101]
Meat	Labelling authentication of game meat species			X	[45, 102, 103]
	Identification of ground meat products		X	X	[104]
	Identification of bovid species	X	X	X	[105]

The reduction in cost and time for generating DNA sequence data has resulted in a range of new successful applications, including food traceability and especially food microbiology [16, 107]. As an example, HTS techniques have been used to identify fruit species in yogurts [108] and pollen composition in multiflower honeys [109].

Nowadays, the use of DNA barcoding in the food sector moved from the academic research to a real application. The "molecular labelling" provided by DNA barcoding has benefits for both consumers (who are ensured on the origin, quality, and safety of food items) and producers (who can give an additional value to their products or have an assurance on the quality of starting raw materials). Concerning the analytical feasibility of the method, the DNA barcoding tool is easily accessible due to the availability of public molecular reference databases and a lot of equipped public or private laboratories able to perform the analysis. Newmaster and colleagues, in a publication dated 2009, estimated the cost of a single analysis in a few Euro and very short times of response [110]. Federici and colleagues demonstrated that portions of the standard DNA barcodes could be chosen as SCAR markers to discriminate in less than three hours between edible plant species from poisonous ones [63]. These characteristics make DNA barcoding a diagnostic method suitable for food control analyses by national and international agencies. As previously underlined, to assess the origin of food items, DNA-based analyses should be combined with the characterization of food metabolites to obtain an exhaustive molecular label.

4.4 Innovative Applications of Metabolomics Tools for an Exhaustive Food Labelling

The analysis of food metabolome represents a new frontier in the evaluation of food quality [111]. The metabolome consists of low molecular weight entities (i.e., <1,000 Da) [112] belonging to a wide range of chemical classes, occurring at different concentrations. In general, these metabolites are the final downstream products of the genome and of its interactions with the environment. For this reason, the analysis of genotype only (e.g., DNA barcoding) is certainly important but not exhaustive to evaluate the overall quality of food items.

In food chemistry, some molecules such as sugars are common and abundant, whereas minor compounds like vitamins occur at smaller amounts or even at trace concentrations (e.g., femtomolar). In addition, the physicochemical properties of some groups of molecules, or the patterns of reciprocal interaction, could pose problems to their fine characterization and quantification. Thus, efficient and sensitive analytical tools are required for a reliable characterization of food metabolome. Whilst in DNA fingerprinting approaches the identification is based on the reading of short nucleotide DNA sequences, a metabolomics fingerprinting analysis aims at establishing the patterns of metabolites belonging to different chemical classes and that are correlated to certain characteristics. Thus, one of the main challenges in food metabolomics is facing the complex networks of molecules (e.g., sugars, amino acids, peptides, organic acids, phenols, terpenes, or steroids) occurring in a particular food item. For these reasons, two approaches (*profiling* and *fingerprinting*) can be used to characterize the

food metabolome. Profiling is a targeted strategy focused on the analysis of a group of related metabolites, often belonging to the same chemical class. An example of this approach is the discrimination between Arabica and Robusta coffee origins, based on the identification and quantification of a specific class of molecules, including 16-O-Methylcafestol, by NMR spectroscopy [113]. In addition, very recently, Monti and coworkers discriminated among different peach qualities and level of ripening, which depend on the abundance of several metabolites, including amino acids, sugars, and organic acids [114]. The second approach (fingerprinting), is an untargeted strategy based on comparing patterns of metabolites among different samples using chemometric tools. The main aim of fingerprinting is not to identify all the involved compounds but to establish patterns among them; this approach enables the simultaneous detection of a wide class of metabolites. Examples of metabolic fingerprinting on different foodstuffs include grape and wine [115, 116], orange [117], saffron [118], olive oil [119], and wheat and bread [120]. Profiling and fingerprinting can offer complementary information and thus can be used alone or in combination [121, 122].

Independently from the adopted strategy, a reliable tool to analyse the metabolome of a certain food should ideally meet some features: (i) the possibility of recognizing a variety of chemical structures, (ii) the possibility of dealing with large range of concentrations at which metabolites are present in a matrix, (iii) the capability of the analytical platforms, and (iv) the availability of reference databases with extensive details and descriptors [123].

Today, there are two analytical platforms meeting these criteria: Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS)

[121]. The application of NMR and MS techniques greatly increased in the last years (Figure 1(a)) and this research field covers several subject areas and disciplines (Figure 1(b)).

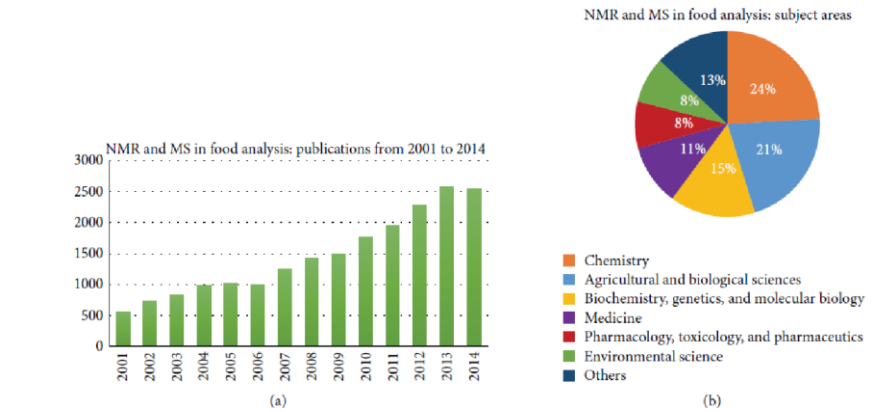

Figure 1: (a) Studies published in the area of food research, based on NMR and/or MS analyses, from2001 to 2014. (b)NMR- and/or MS-based studies published from 2001 to 2014, divided for subject area. Source: Scopus (entries: NMR, food or Mass Spectrometry, food).

A good advantage of both techniques is the "highthroughput" capability of spectroscopic and structural information that permits characterizing a wide range of metabolites simultaneously, with high analytical precision. Compared to NMR, MS is more sensitive and can be used alone or combined with gas chromatography, liquid chromatography, or capillary electrophoresis to provide a higher sensitivity for metabolites present at low or even at trace concentrations [124–127]. However, even though MS-based analytical methods can detect hundreds of metabolites, many others could remain unidentified. On the other side, the main advantages of NMR are the ease sample preparation and the determination of very different chemical species in a single experiment. In addition, the

identification of molecules is easier and more straightforward than in the case of MS. Other important advantages of NMR are its inherently quantitative signals and its nontargeted and non destructive nature with regard to the specimen of the technique. Thus, in case of an initial metabolomics study where the composition of the metabolite pool is not known, a NMR approach is useful and can inform future studies by targeted GC-MS metabolomics or other approaches to look for specific low-concentration metabolites (targeted strategy). NMR sensitivity is considered one of the main limitations in its application to metabolomics analysis, especially when compared to MS. However, continuous developments in hardware (e.g., magnet strength, probe head design, and console electronics) have allowed and will allow a growing sensitivity of NMR. Also, a rapid growth in new, potent algorithms for multivariate data analysis facilitates the use of NMR spectroscopy as a competitive, complementary analytical platform for investigating the food metabolome (Table 2).

Table 2: Examples of NMR and MS application in the field of food science.

Scope	Food category	Aim of the analysis	Analytical tool	References
Food traceability, authenticity, and safety	Saffron (*Crocus sativus* L.)	Quality and geographical origin	NMR	[118]
	Orange	Geographical origin	UPLC-qTOF-MS	[117]
	Raw milk	Safety: drug residues and other contaminants	UPLC–ESI–MS/MS	[128]
	Apple, hazelnuts, maize, green pepper	Safety: fungal and bacterial metabolites	LC–MS/MS	[129]
	Buffalo's mozzarella	Quality and traceability	NMR	[130]
	Olive oil	Geographical origin	NMR	[119]
	Wheat and bread	Geographical and varietal origin	NMR and IRMS	[120]
Food composition and physical characteristics	Grape	Effects of agronomical practices on composition	NMR	[116]
	Pork meat	Fatty acid chain composition	NMR	[131]
	Onion	Metabolic profiling	NMR and HPLC-MS	[132]
Food processing and storage	Wine	Effects of fermentation and aging	NMR	[115]
	Tea	Processing (variety)	LC–DAD-MS	[133]
	Beer	Profiling of raw materials for beer production	HS-SPME-GC-MS	[134]
	Coffee	Roasting process	NMR	[135]
Food and health	*Salvia sclareoides*	Compounds against neurodegenerative disease	STD-NMR	[136]
	Green tea	Compounds against neurodegenerative disease	STD-NMR	[137]
	Litchi (*Litchi chinensis Sonn.*)	Identification of bioactive compounds	NMR and MS	[138]

The most important innovation provided by metabolomics tools is their standardization and the universality of the procedures. The amount of data generated by these analyses is enormous. For this reason, several chemometric tools [139, 140] are employed. In fact, to analyze food metabolomics data, some intermediate steps are necessary, including peak detection, spectra normalization, integration, and data alignment before multivariate statistical analysis.

Based on these aspects, it is currently possible to create a molecular label, which combines the genetic profile of a certain food item and its metabolic content. The advantages of such integration are relevant and would certainly constitute a real innovation in food science. One example is the case of wine, which can be putatively characterized with both DNA analysis of the original grape cultivar (e.g., [141, 142]) and the metabolic profile to identify wine characteristics, such as fermentation behaviours and antioxidant properties. Indeed, the analysis of metabolome was shown successful in identifying specific chemical compounds strictly related to the geographic production areas [115, 143]. The origin of wine could also be supported by the DNA-based analysis of must/wine microbiome [144–146]. Merging these three sources of data would result in a molecular label that is truly exhaustive and follows the Protected Designation of Origin (PDO) of wine.

Another application of metabolomics was on olive oil. Longobardi et al. [119] used a ^{1}H NMR fingerprinting combined with multivariate statistical analysis to authenticate extra virgin olive oils from seven different Mediterranean regions, demonstrating the possibility to predict the origin of olive oil samples with a very high confidence (>78%). At

the DNA level, DNA barcoding cannot distinguish among different olive cultivars, whereas other genomics markers such as SSR and SNP were successful in achieving this goal [147]. DNA barcoding, combined with HRM (High Resolution Melting) analysis, was used instead to detect adulteration of olive oil with other oils [148]. Also in this case, genomics and metabolomics analyses could be complementary, to offer to the producer/consumer a comprehensive certification of origin and quality of oil.

An important aspect of food metabolome is that of flavour and aroma determination, which is often linked to the composition in volatile molecules. Dynamic headspace solid-phase microextraction (HS-SPME) followed by GC separation and high-resolution MS analyser can be exploited to characterize the volatile components of some foodstuffs. With this approach, the volatile metabolomics pattern of beer raw materials has been defined in a recent paper [134]. Similar results were obtained with aromatic spices [149, 150] that have been also characterized using DNA barcoding approaches [44, 76]. In a strict sense, these results indicate that in the case of spices it is possible not only to identify the species but also the peculiar aromatic components responsible for their flavour and scent. Such combined analytical system can be seen as a way to also evaluate the efficacy of the processing of spices-based products along the entire supply chain (e.g., harvesting, exsiccation, grinding, and packaging).

Taking advantage of all these features and tools, NMR and MS are today able to answer most issues related to food analysis: (i) food traceability, authenticity, and safety, (ii) food composition and physical characteristics, (iii) food processing and storage, and (iv) food and health.

Thus, the study of the whole metabolic profile of food products can help defining quality features that make certain foods unique and can bring information on food safety and authenticity. For example, genetic modification, microorganism's colonization, and other food characteristics of major concern for human health are likely to influence large portions of the raw material or processed food molecular profile.

Another advantage of including the characteristics of the metabolome in the molecular label of a certain food is the potential of metabolomics in evaluating critical steps of the supply chain such as production, storage, and distribution. In 2014, Gallo and colleagues [116] described an interesting NMR application to study the influence of agronomical practices on the chemical composition of commercial table grapes. Specifically, the variability of the grape metabolome composition was evaluated considering primary metabolites, the compounds directly involved in the growth, and development of fruits. The authors found glucose, fructose, arginine, and ethanol as compounds quantitatively influenced by farming practices. Moreover, the comparison between organic and conventional productions showed a higher sugar content for the latter, resulting in a higher sugar-to-acid ratio [116].

In such a context, a metabolomics approach is complementary to a DNA barcoding analysis in evaluating the production processes as well as in monitoring the occurrence of alterations and species substitutions cases. For example, in 2015, Cagliani et al. [118] published an interesting application of metabolomics to characterize saffron, a very expensive and PDO spice. By using a multivariate statistical analysis of NMR data, they identified reliable biomarkers, specifically picrocrocin and crocins that

permit distinguishing Italian products from other commercial varieties, where these peculiar compounds are less abundant (or even absent) [118]. The availability of an analytical platform based on the combination of genomics and metabolomics tools will have great potential in terms of food safety. As underlined in the first chapter, since its introduction in the 90's, the DNA-based diagnostics has developed different strategies to detect food pathogenic organisms. A DNA barcoding approach, combined with the use of HTS technologies, could certainly provide great advantages in this field because it would permit obtaining a comprehensive vision of all the putative food-related pathogens. However, this integrative panel of data would not be completely exhaustive because some microorganisms could be dead or inactive or become pathogenic only when they release specific toxins or metabolites [151, 152]. In this context, a metabolomics analysis based on MS/NMR approaches could provide important information regarding the occurrence of these metabolites or other compounds of major concern (e.g., antibiotics and pesticides) in foodstuffs. A rapid and simple analytical method, able to identify 255 veterinary drug residues in raw milk, was developed by Zhan and coworkers [128]. Their method was based on a two-step precipitation and ultra performance liquid chromatography coupled with electrospray ionization and tandem Mass Spectrometry (UPLC–ESI–MS/MS). Malachov´a et al. [129] optimized and validated in 2014 a LC–MS/MS method for the detection of 295 fungal and bacterial metabolites in four different types of food matrices: apple puree for infants (high water content), hazelnuts (high fat content), maize (high starch and low fat content), and green pepper (difficult or unique matrix).

Finally, recent studies have shown the possibility to link the metabolic profiling and characterization of foodstuffs to the screening of food matrices, aiming at the identification of small molecules able to bind and modulate the activity of a target protein (often involved in the etiology of specific pathologies). Techniques such as Saturation Transfer Difference-(STD-) NMR [153–155] and trNOESY NMR experiments [156, 157] allowed the identification of natural ligands present in Salvia sclareoides [136] and green tea [137], able to recognize, bind, and modulate the activity of A peptides (whose aggregation processes are considered among the main biochemical events leading to Alzheimer's disease).

In conclusion, the future of food analysis will necessarily be based on the exploitation of integrative approaches, including both genomics and metabolomics. If in the past this was not feasible because of the lack of expertise and technical limitations, the current technological advances offer high performances in terms of standardization and universality to investigate a wide panel of food items. The spread of omics platforms, able to simultaneously process different matrices with a multiapproach strategy [111], unified under the control of bioinformatics tools, is boosting this revolution.

4.5 From Omics to Foodomics

The use of omics platforms to assess important aspects of food items (i.e., contaminants and bioactive molecules) is essential to obtain an exhaustive characterization of food quality and safety or to assess the effect of food on human cells, tissues, and organs as well. The availability of such platforms responds to a general trend in food science

about the linking between food and health [7]. Nowadays, food is more and more considered not only as a source of energy but also as an affordable way to prevent future diseases. In this scenario, human health should be considered as a dynamic position in a multidimensional space [158] that spans from growth to development to reproduction. Early nutritional events (i.e., since the embryonic state) and food imprinting can define the trajectories of development and contribute to the wellness or the insurgence of noncommunicable diseases such as allergy, diabetes, and obesity [159]. In the development and maintaining ages, a proper nutrition could offer the better cost effective way to prevent such noncommunicable diseases [160]. Furthermore, undernutrition and overweight are global problems. The "global nutrition report" of 2013 highlights how the world is off-track to meet the 2025 World Health Assembly targets for nutrition [161]. Apart from social and economical issues, from the scientific point of view, nutrition research can furnish the keys for defining the characteristics of a proper nutrition. Therefore, a new discipline known as "foodomics" has been defined to study the food and nutrition domains through the application of advanced omics technologies to improve consumer's well-being, health, and confidence [162, 163]. Thanks to foodomics, many issues related to food could be addressed such as the evaluation of the effects of certain bioactive food components on biochemical, molecular, and cellular mechanisms, or the identification of gene-based differences among individuals in response to a specific dietary pattern [164–166]. Foodomics tools could permit identifying molecular biomarkers strictly related to the genes involved in the early stages of a certain disease and to elucidate the effect of bioactive food constituents on crucial molecular pathways for preventing

future diseases with an adequate diet [166–168]. For example, a foodomics analysis was used to evaluate the effect of dietary polyphenols against colon cancer [169]. Ibáñez and coworkers [169] tested the chemopreventive effect of polyphenols from rosemary on the total gene, protein, and metabolite expression in human HT29 colon cancer cells. The results obtained from each component of the omics platform (i.e., transcriptomics, proteomics, and metabolomics) were integrated to estimate which cellular pathways were activated in response to polyphenols. Data suggests that polyphenols bring about an induction of cell-cycle arrest, an increase of apoptosis, and an improvement of cellular antioxidant activity. The genes, proteins, and metabolites involved in these three processes were identified thanks to the multiparameter omics analysis. It is important to underline the fact that the induction of apoptosis is especially relevant in colon cancer, since the renewal of the colon epithelium via apoptosis is the way used by the organism to eliminate deteriorated cells that can mutate to carcinogenic. Therefore, a diet rich in polyphenols plays an important role in the prevention of colon cancer.

Foodomics is a powerful discipline to identify the adding value properties of food items, as well as to detect food related toxins and allergens or to assess the effects of food on human metabolism by evaluating cell-response [170, 171]. The efficacy of omics in the food sector also meets the emerging needs related to personalized nutrition [172]. A number of recent studies underlined the enormous variability of individual response to the same diet or food components: it is well known that food ingredients have effects that are unique to each individual, as unique as is its own transcriptome, proteome, and metabolome [158]. The role of

foodomics does not finish once a personalized diet has been identified. Indeed, an exhaustive evaluation of the factors altering the metabolic properties of food components should also be taken into account. These factors include production process, methods, and duration of conservation, interaction with other components, cooking procedures, digestion, and interaction with microbiome [173]. The advantages of foodomics are relevant not only for producers but also for consumers to encourage a healthy diet and to reduce educational, behavioural, and economic barriers to accessing wellness. In this context, recent smartphone "apps" are becoming a powerful tool to promote the consumption of high-quality foodstuffs and in particular the consumption of those food items able to prevent diseases [174–177]. Such informative tools (including online portals and dissemination web sites) can be useful for different stakeholders to translate a molecular label based on omics approaches in a more understandable language for the whole category of consumers. The molecular labelling that combines DNA barcoding and metabolomics data with the information of foodomics represents a precious source of data to meet consumer requirements. In this sense, smartphone apps represent a simple tool able to share and translate molecular data to the various stakeholders of the food supply chain.

4.6 References

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5. CHAPTER 3

Grape microbiome as a reliable and persistent signature of field origin and environmental conditions in Cannonau wine production

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5.1 Abstract

Grape berries harbor a wide range of microbes originating from the vineyard environment, many of which are recognized for their role in the must fermentation process shaping wine quality. To better clarify the contribution of the microbiome of grape fruits during wine fermentation, we used high-throughput sequencing to identify bacterial and fungi communities associated with berries and musts of Cannonau. This is the most important cultivar-wine of Sardinia (Italy) where most vineyards are cultivated without phytochemical treatments. Results suggested that microbiomes of berries collected at four different localities share a core composition characterized by Enterobacteriales, Pseudomonadales, Bacillales, and Rhodospirillales. However, any area seems to enrich berries microbiome with peculiar microbial traits. For example, berries belonging to the biodynamic vineyards of Mamoiada were rich in Bacillales typical of manure (i.e. *Lysinibacillus*, *Bacillus*, and *Sporosarcina*), whereas in the Santadi locality, berries showed soil bacteria such as Pasteurellales and Bacteroidales as well as Rhodospirillales and Lactobacillales which are commonly involved in wine fermentation. In the case of fungi, the most abundant taxa were Dothioraceae, Pleosporaceae, and Saccharomycodaceae, and although the proportion of these families varied among localities, they occurred ubiquitously in all vineyards. During vinification processes performed at the same wine cellar under controlled conditions and without using any yeast starter, more than 50% of bacteria groups of berries reached musts, and each locality had its own private bacteria signature, even if *Saccharomyces cerevisiae* represented the most abundant fungal species.

This work suggests that natural berries microbiome could be influenced by pedoclimatic and anthropologic conditions (e.g., farming management), and the fruits' microorganisms persist during the fermentation process. For these reasons, a reliable wine genotyping should include the entire holobiont (plant and all its symbionts), and bioprospecting activities on grape microbiota could lead to improved viticulture yields and wine quality.

5.2 Introduction

To date, at least 5000-8000 grape cultivars showing particular traits (grape size, shape, color, and flavor) have been selected by viticulturists [1, 2]. Despite this huge diversity, variations in environmental conditions (i.e., soil composition, water management, and climate) and fermentation processes shape the contribution of these traits and modify the quality of the resulting wines. The identification of key environmental elements involved in the regional variation of grape and wine quality characteristics is a critical feature for improving wine production in terms of consumer preference and economic appreciation [3].

Several studies showed the effects of abiotic conditions on grapevine growth and fruit development, such as UV solar radiation [4], water availability [5], and nitrogen sources [6]. At the same time, biotic factors are also involved, since *Vitis vinifera* L. naturally hosts a reservoir of microorganisms [7-9] that interact with the plant and affect both the qualitative and quantitative scale of wine production.

The occurrence and effects of regional-specific microbiota in defining wine characteristics is a more controversial issue. Experimental analyses

suggest that microbes colonizing berries could significantly affect grapevine and fruit health and development [10]. However, grapevine bacteria and yeasts also contribute to shaping phenotypic characteristics, such as flavour, colour, and sugar content [11], thus influencing the winemaking process as well [12, 13].

Recently, High-Throughput DNA Sequencing techniques (HTS) have being used to characterize bacterial communities of different grapevine plant portions, such as leaves and berries [14] and to assess the provenance in terms of plant portion and farming region of some microbial groups [15, 16]. Metagenomic analyses suggested that soil serves as a primary source of microorganisms with edaphic factors influencing the native grapevine microbiome, since the microbial communities of soils from the same viticultural region are quite heterogeneous [9]. Bokulich [15] showed that *Vitis* microbial biogeography is non-randomly associated with regional, varietal, and climatic factors across multiscale viticultural zones. Moreover, in 2016, the same research team [17] suggested a strong association involving grapevine microbiota, fermentation characteristics, and wine chemical composition.

Viticulturists are aware that the ground where plants grow imparts a peculiar metabolic trait on grapes and wine; this concept is usually referred to as *terroir*. In vineyards several variables could affect grape characteristics, such as soil composition and structure topography, climate conditions, and agricultural practices. Moreover, these conditions could also influence the plant microbiome. During vinification, other variables could act on the biotransformation of grape juice, such as the environmental conditions of the wine cellar [11, 17], as well as chemical

and microbial processes involved in the fermentation processes. On the whole, wine characteristics (color, flavor, fragrance, sugar content) result from the complex interaction between abiotic and biotic elements occurring in two distinct environments: vineyards and wine cellars. To better clarify the diversity and dynamics of the microbiome belonging to these two environments and its role during wine production, we analyzed grapes and musts of cv. *Cannonau*, one of the most important black grape varieties cultivated in Sardinia (Italy).

Sardinia is the second largest Italian island located in the western Mediterranean to the south of Corsica between the Italian peninsula, Spain, and Tunisia. This island is ideally suited for viticulture [18], and it is characterized by a huge number of grapevine cultivars with different morphological and chemical characteristics [19]. Due to peculiar pedoclimatic conditions occurring in different parts the island, this model offers a great opportunity to study the relationships and changes occurring at both the environmental and grape microbiome.

In the present study, we investigated the bacterial and fungal microbiome of Cannonau berries cultivated from different localities and musts produced, under controlled conditions, at the same wine cellar. This presents the opportunity to track the microbial community from grapes to wine cellar. Specifically, the goals of our work were to: i) evaluate the microbial diversity at the vineyard level in response to different environmental conditions and farming management (e.g., biodynamic) of Sardinian localities ii) study the dynamics of microbial diversity from the vineyard to the wine cellar to estimate the impact of field bacteria on wine must.

5.3 Materials and methods

5.3.1 Samples collection and wine production

Given its pedoclimatic conditions and geographical isolation, Sardinian viticulture does not demand phytochemical treatments. For this reason, the island represents a suitable area to investigate the effect of natural field characteristics on the microbiome of grapes and therefore on the resulting must. To perform our experiments, we selected four Sardinian localities: Alghero (ALG), Mamoiada (MAM), Mores (MOR), and Santadi (SAN) (Fig 1).

Climatic and pedological conditions and the managed condition of vineyards for each locality are provided in Table 1.

Fig 1. Map of Sardinia showing sampling vineyard localities. ALG (Alghero lat. 40.650 N, lon. 8.244 E), MOR (Mores lat. 40.517 N, lon. 8.806 E), SAN (Santadi lat. 39.090 N, lon. 8.793 E), MAM (Mamoiada lat. 40.222 N, lon. 9.309 E).

Table 1. Vineyard characteristics: for each investigated locality, geographical, pedoclimatic, and farming systems information are provided.

Name	Geographical coordinates	Climatic condition	Pedological condition	Elevation (s.l.m.)	Orientation	Farming system
Alghero (ALG)	40.650°N, 8.244°E	Coastal Mediterranean plain	calcareous	70	70	conventional
Mamoiada (MAM)	40.222°N, 9.309°E	Mediterranean Mountain area	granites	760	760	biodynamic
Mores (MOR)	40.517°N, 8.806°E	Mediterranean interior plain	calcareous	280	280	conventional
Santadi (SAN)	39.090°N, 8.793°E	Mediterranean interior plain	schists-granites	230	230	conventional

During the harvest of 2015, mature grapes (20 degrees Brix) of the most diffused cultivar '*Cannonau*' were collected as bunches bulks (100 berries) at each locality. One degree Brix is 1 gram of sucrose in 100 grams of solution and the scale is used as a proxy for grape maturation and fermentation progress. Sampling was performed in collaboration with specialized technicians of Agricultural Research Agency of Sardinia (AGRIS) at vineyards of the four localities. Although no specific authorization was required for sampling activities, vineyards owners gave permission to conduct the study on these sites. Field studies did not involve endangered or protected species. The collected Grape samples (G) were immediately frozen, shipped on ice, and stored at −80 °C. These

samples were used to characterize the berries microbiome (i.e. bacteria and yeast communities) of each locality.

To evaluate the effect of the environmental microbial community on must, we performed a wine-making process in controlled conditions starting from mature grapes collected from each locality. This was carried out at the experimental winery of AGRIS in a scale of 100 L per locality, without wine yeast starter and sulfur dioxide treatment. These conditions provide a natural fermentation process without any forced microbial selection [20]. This strategy is an emerging trend of winemaking to enhance natural interactions between microorganisms occurring during vinification [21]. To assess the characteristics and changes of must microbiome, we identified two phases based on the analysis of fermentation curves (data not shown), namely initial must (iM) and end must (eM). The former has been collected about six hours after pressing, when the must shows the highest level of glucose (at least 20 degrees Brix) and the lowest level of ethanol (100 ml of iM for each locality). End must has been collected at 7 days after the winemaking process has started and when glucose level is lower than 2 degrees Brix and ethanol reach 12% v/v (100 ml of iM for each locality). In the case of iM samples, we were interested in evaluating the effect of the wine cellar on the original grape microbiome, whereas in the case of eM samples, we tested the dynamics of microbiome composition during the fermentation process. Each sample was stored at – 80 °C.

5.3.2 DNA extraction

Microbial biomass recovery from G samples was obtained starting from twenty berries randomly selected from each vineyard. These were thawed

and placed in 500 mL sterile Erlenmeyer flasks. Berries were washed with 100 mL of isotonic solution (0.9% w/v NaCl) for 3 h with agitation at 150 rpm. The obtained cell suspensions were separated from the berries by centrifugation at 6,000 × g for 15 min. Pellets were stored at -20° C until DNA isolation.

In the case of must, microbial biomass was obtained from 10 mL of iM and eM samples. These were thawed and centrifuged at 6,000 × g for 15 min, washed three times in ice-cold isotonic solution. Pellets were stored at -20° C until DNA isolation. G, iM and eM samples were processed in duplicate.

Total genomic DNA were obtained from pellets using PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions with modifications specific for wet soil samples.

Before libraries preparation, the obtained genomic DNA extracts were purified using Zymo Research DNA Clean and Concentrator-10 (Zymo Research, Irvine, CA, USA) to remove PCR inhibitors.

5.3.3 Library preparation and sequencing

For each DNA sample, two independent DNA libraries, for bacteria and fungi, were prepared following Illumina guidelines (16S Metagenomic Sequencing Library Preparation, Part #15044223 Rev. B) with modifications. Bacterial V3 and V4 16S rRNA genes were amplified using primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 [22] with the addition of the Illumina overhang adapter sequences.

Fungal internal transcribed spacer (ITS) 1 loci were amplified with primers BITS and B58S3 [23], with the supplement of the Illumina

overhang adapter sequences. Before amplification, DNA extracts were normalized by means of Quantitative real-time PCR (qPCR) Ct values with the same amplification primer pairs and the same protocols described by Bruno and colleagues [24, 25]. Finally, the obtained libraries were submitted to Polo d'Innovazione Genomica, Genetica e Biologia Società Consortile R.L. (POLO-GGB, Perugia, Italy) for Illumina paired-end library preparation, cluster generation, and 2 x 300-bp paired-end sequencing (MiSeq Reagent Kit v3) on an Illumina MiSeq instrument.

5.3.4 Microbial composition and community structure analysis

Analysis of bacterial and fungal communities were performed using scripts of the QIIME pipeline [26]. Raw Illumina reads were paired and pre-processed using USEARCH merge pairs algorithm [27]. During the Quality filter step reads were filtered out if: 1) ambiguous bases were detected, 2) lengths were outside the bounds of 250 bp and/or 3) average quality scores over a sliding window of 40 bp dropped below 25.

Bacterial reads were then processed by VSEARCH 1.1.8 software version [28], which removed noise and chimeras prior to performing de novo clustering into OTUs at 97% sequence identity and discarding those OTUs represented by less than 75 sequences. The cluster centroid for each OTU was chosen as the OTU representative sequence. The taxonomic assignment of the representative sequences was carried out using the RDP Bayesian Classifier [29] against the SILVA SSU non-redundant database (version 119 release) adopting a consensus confidence threshold of 0.8. The RDP classifier was then used for the taxonomic assignment of OTUs.

Fungal reads were cleaned concerning the noise and the chimera using the same workflow adopted for bacteria reads. Before OTUs clustering, ITSx extractor [30] was used in order to filter non-fungi contaminant reads. De novo OTUs were calculated, as in the case of bacteria, using the VSEARCH cluster algorithm at 97% sequence identity with the cluster centroid for each OTU as the OTU representative sequence. The taxonomic assignment of the representative sequences was carried out using the RDP Bayesian Classifier against UNITE fungal database [31].

For both communities, a rarefaction table was calculated for each sample to determine the suitable sequencing depth that covers the extant microbial diversity.

The intra group diversity estimation (alpha diversity) was calculated using the number of observed OTUs and the Shannon index. Community analyses (beta-diversity) were performed with qualitative (Jaccard and unweighted UniFrac for fungi and bacteria respectively) and quantitative (Bray-Curtis and weighted UniFrac for fungi and bacteria respectively) distance metrics [32] using QIIME and *phyloseq* R package for statistical computing [33, 34]. Statistical significance among groups was determined by the ADONIS (permutation based ANOVA (PerMANOVA)) functions of the *vegan* R Package [35]. PerMANOVA Pairwise contrast was performed with R script [36].

The phylogenetic tree necessary to calculate UNIFRAC distances and based on the alignment of OTUs representative sequences was built using RAxML version 7.4.2 [37] with the GTRGAMMA model bootstrapping (1’000 replicates) best maximum likelihood tree inference. Multibar plots were generated with QIIME.

A Venn diagram was created with the online tool [38] by calculating the number of shared and unique OTUs in the different datasets.

5.4. Results and discussion

5.4.1 Sequence analysis

To characterize the microbial consortia associated with grapes and musts of Cannonau vineyards by HTS approach, a total of 1'600'000 and 5'000'000 quality-filtered sequences were obtained for the 16S rRNA and ITS1 marker, respectively. After the removal of low quality reads sequences failing the alignment or annotated as host or mitochondrial or chloroplast sequences, and singleton sequences, a total amount of 235'371 16S rRNA V3-V4 amplicon sequences belonging to the three fermentation steps (G, iM, eM) for 24 samples were considered for further bioinformatics analyses. These sequences had an average of 430 bp (ranging from 400 to 438 bp) with primer removal and clustered into 264 OTUs. Moreover, for the same set of samples and adopting the same procedure, a total of 216 ITS1 OTUs were supported by sequences with an average of 400 bp (ranging from 390 to 405 bp) (see for more details S1 Table).

5.4.2 Bacteria and fungi OTU diversity

OTUs diversity of bacteria and fungi was analyzed separately and described in Table 2. Considering that the two replicates did not statistically differ for each sample (R2 P v>0, data not shown), we decided to combine replicates to calculate alpha diversity. Concerning

bacteria, the observed OTUs ranged from 50-113 for G samples, 46-103 for iM, and 67-70 for eM.

Table 2. Number of observed bacteria and fungi OTUs and related Shannon index for each sample typology at each sampling site.

Samples	Bacteria		Fungi	
	Observed OTU	**Shannon Index**	**Observed OTU**	**Shannon Index**
ALG G	**55**	2.175450	**110**	2.653449
ALG iM	**59**	2.229309	**108**	2.547885
ALG eM	**70**	2.217277	**36**	2.261021
MOR G	**84**	3.299958	**117**	2.269252
MOR iM	**94**	1.763944	**69**	1.290504
MOR eM	**67**	2.858682	**26**	2.464016
SAN G	**113**	2.366787	**133**	1.882989
SAN iM	**103**	2.101003	**63**	1.271993
SAN eM	**69**	2.764250	**27**	1.971678
MAM G	**50**	3.483344	**94**	1.892080
MAM iM	**46**	2.362264	**52**	2.254171
MAM eM	**68**	3.150848	**21**	2.391844

Data suggests that all grape samples significantly differ from each other (see data in S1 Text) with the exception of ALG-MAM (p-value = 1). This was also confirmed by Shannon Indexes (Table 2) (see data in S1 Text). The diversity among localities decreases in must samples; in the case of iM samples, only those from SAN significantly differ from ALG and MAM, but they do not differ from those of MOR (p-value = 1). Significant differences were also detected between MOR iM and MAM iM (ANOVA F=-0.5552, p= 0.00006). Finally, the OTUs diversity among localities was reduced in the case of eM samples, and no significant differences were observed among musts belonging to all localities.

In the case of fungi, all the localities share the same trend: a high number of OTUs were detected in G samples and were lower in iM and eM

samples. This could suggest that several fungal OTUs occurring at fruit levels do not reach the wine cellar or do not resist the wine fermentation process as previously shown by Gilbert and colleagues [39]. Moreover, fungi species occurring in wine cellars do not largely enrich the wine microbiome in terms of OTUs diversity [23].

To better estimate the microbial dynamics of grapes from fields to the wine cellar in the four study areas, we calculated the beta diversity. Fig 2A shows the Unweighted and Weighted UniFrac PCoA plot obtained by 16S rRNA data. As previously suggested, the two sample replicates showed good overlapping (PerMANOVA pairwise test results are shown in data in S2 Text).

This confirms that the sampling strategy was adequate to depict the microbiome heterogeneity of the considered vineyard. Fig 2A showed clear differences between G and musts samples. Moreover, this analysis showed that the bacterial OTUs on fruit surfaces were very different among the four localities, and this information is well represented by the unweighted non-metric multidimensional scaling NMDS (ADONIS $R2=0.34$ $p\text{-value}<0.001$). The iM and eM samples clustered closer. These data support the hypothesis that most of the field bacteria taxa growing on fruit surfaces are not able to persist into the wine cellar environment. This condition could be related to the fact that most of these bacteria cannot resist the change from grape aerobic to must anaerobic conditions, as well as the antimicrobial effect of secondary metabolites and ethanol occurring in must.

However, during fermentation a different bacterial community arises in must probably originating from the wine cellar. These considerations agree with previous studies [40, 41] suggesting that different

microorganisms diffused in the wine cellar play peculiar roles in the specific steps of the winemaking process [11] and that their mutual interaction could affect wine characteristics.

Fig 2B shows the beta diversity of fungi OTUs. Considering the relative abundance (Bray-Curtis analysis), a pattern similar to bacteria was observed, but in this case, differences between iM and eM are more consistent. This could further suggest that wine cellar environment influences the must microbiome during the fermentation process [42]. This hypothesis is particularly supported by looking at *S. cerevisiae*; although this yeast was not added as a commercial starter in the 4 analyzed musts, it also occurred in iM and eM samples. The wine cellar could represent a primary source of this yeast and probably the other non-*Saccharomyces* yeasts, however their occurrence and development in must is related to the complex relationships among microorganisms during different fermentation phases [11, 20, 43].

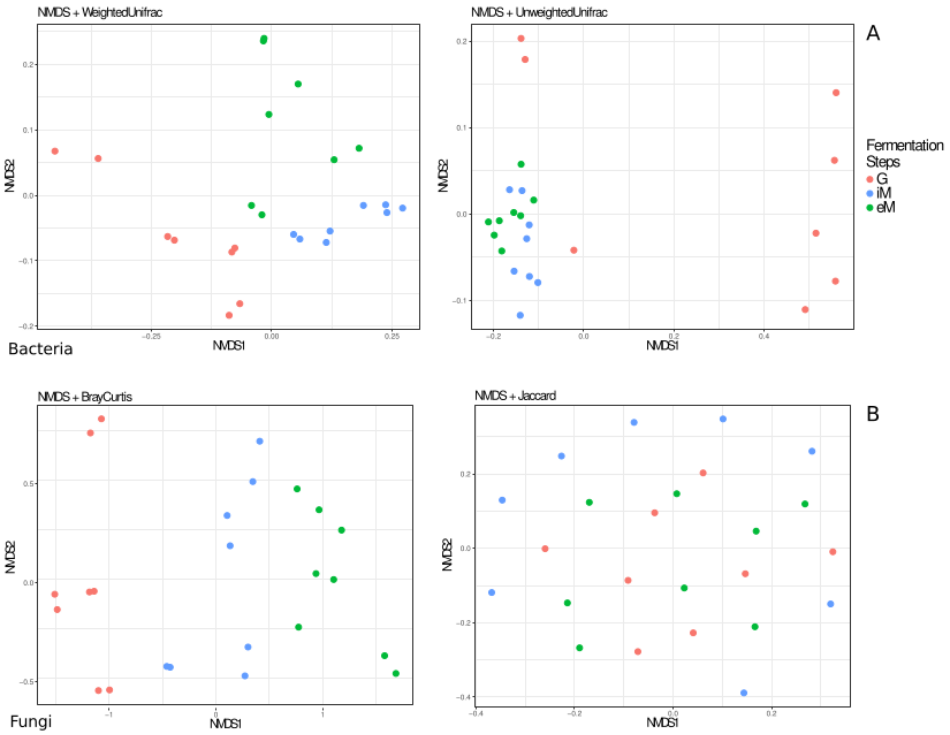


Fig 2. Microbial beta diversity at the four sampling localities. (A) Non-metric multidimensional scaling (NMDS) using unweighted and weighted UNIFRAC distance matrices as measures of beta diversity in bacterial communities. (B) NMDS using the Jaccard and Bray Curtis distance matrix coefficients to estimate the beta diversity of fungi communities. Red dots indicate grape samples (G), blue dots the initial must (iM) samples, and the green dots the end of must samples (eM).

To better illustrate the variation of fungi OTUs from field to wine cellar, we produced a heatmap analysis (Fig 3). This clearly showed a drop in the number of OTUs in the winery, from the initial phase of must (iM) and which became more appreciable in eM samples, as was also supported by the alpha diversity data for each locality (Table 2).

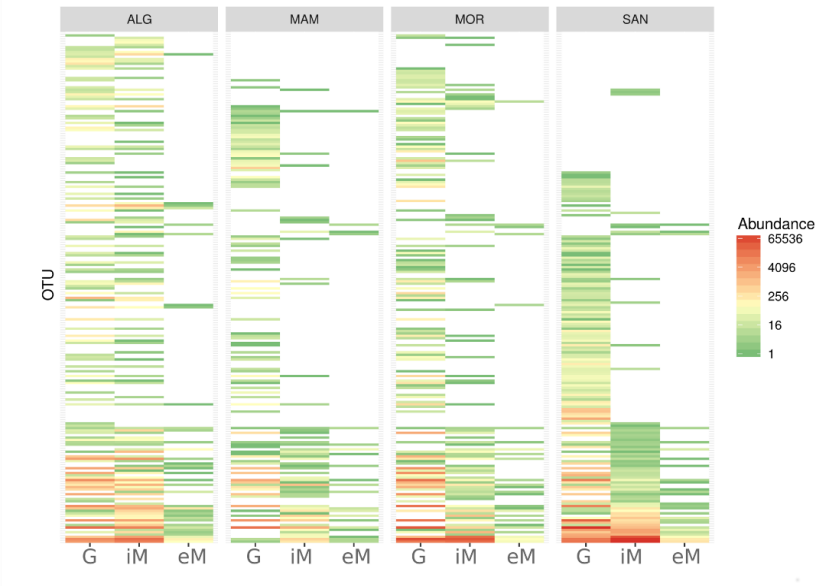


Fig 3. Heatmap of fungal OTUs. The figure shows the distribution of fungi OTUs for each sample typology (G, iM, and eM) at each sampling locality (ALG, MOR, SAN, and MAM).

5.4.3 Microbial taxonomy diversity

To understand the occurrence and the role of different microorganisms during the wine process and their origin, we analyzed the microbial taxonomy of the grape and must of *Cannonau* at different Sardinian localities.

The taxonomic assignment of sequences was performed using the RDP Classifier for both bacteria and fungi. PE sequences matching those in Silva and UNITE databases exceeding the 0.8 confidence threshold were classified and assigned to a taxonomic rank. Concerning bacteria, the 264 different OTUs were assigned to 13 predominant phyla, 44 orders, and 73 families. About of 80% of OTU were identified at the genus level. In the

case of fungi, a total of 216 OTUs were detected and corresponded to 4 phyla, 35 orders, and 48 families.

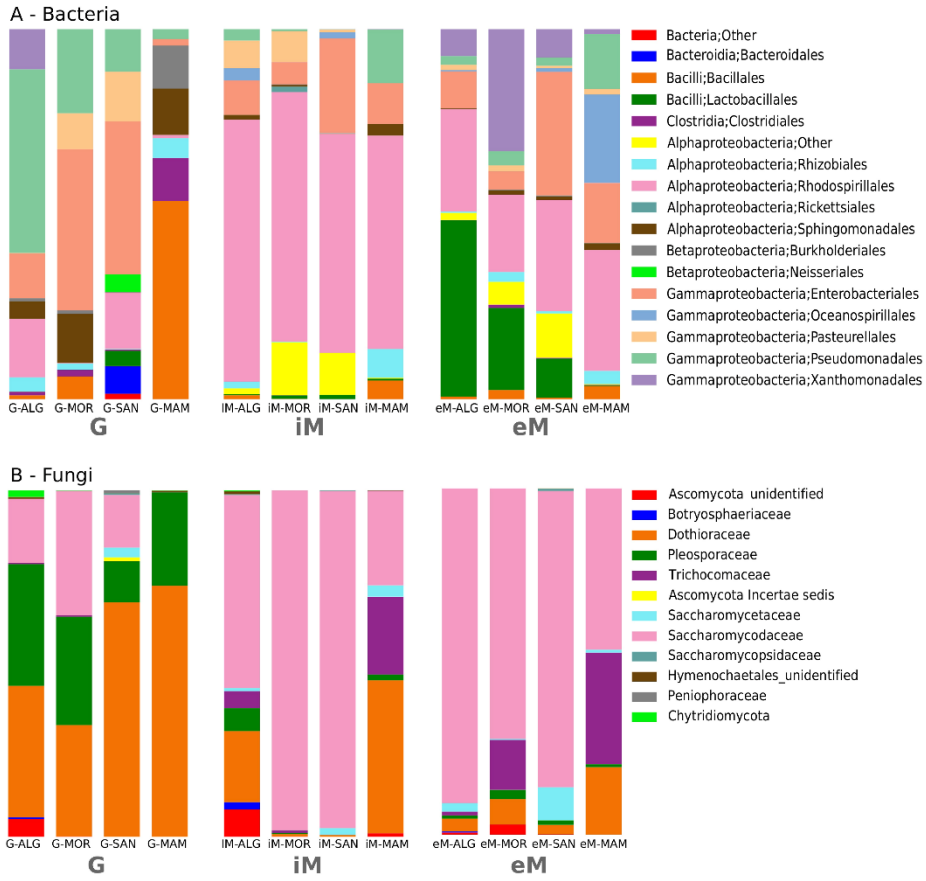


Fig 4. Distribution of bacterial and fungal assigned taxa. Relative abundance of bacterial orders (A) and fungi families (B) recovered in G, iM, and eM samples at each sampling locality. Where the assignment to the Order (bacteria) or Family (fungi) rank failed, the nearest taxonomic level with supported assignment has been reported.

Fig 4A describes the distribution of bacterial orders having a relative abundance > 0.01% determined by summing the counts derived from the two biological replicates for each sample distinguished in G, iM, and eM.

Results confirm that G sample bacterial communities varied greatly across the four localities and were constituted predominantly by Enterobacteriales (19.5%), Pseudomonadales (17.5%), Bacillales (11.8%), and Rhodospirillales (8.8%). This finding agrees with data obtained by microbiome analysis performed on Grenache, one of the synonyms of Cannonau [44]. However, these four shared predominant bacteria orders should not be considered a private fingerprint of this genotype, because the same taxa were already detected on several other grapevine cultivars, such as Chardonnay, Cabernet, and Zinfandel [15]. These bacteria can be then considered a 'common microbiome' of vineyard soil, and they seem to not respond to pedological and environmental conditions [9, 16, 39]. The consistent presence of bacteria belonging to these orders on grape fruits could be explained by microbial migration through rain splash, winds, and insects as supported by Martins and co-workers [8], as well as by taking into account their adaptations to fruit characteristics [16]. Although the effect of these microorganisms on grape fruits and wine are unclear so far, we can conclude that a stable core microorganism of vineyards could be considered a basal biotic element able to influence different grape organs and any plant growth stages [45].

A second biotic element consists of the 'peculiar microbial' community that is characteristic of each vineyard and is influenced by environmental conditions and anthropogenic factors. As shown in Fig 4A, all 4 analyzed vineyards have different microbiomes at the berries level. However, samples of SAN and MAM localities showed the most peculiar microbial diversity in comparison to other localities. In SAN G samples, a consistent occurrence of Rhodospirillales (14.5%), Pasteurellales

(13.1%), Bacteroidales (7.6%) was detected. Members of these taxa have been previously described in the vineyard soil microbiome. Specifically, Zarraonaindia [9] detected them in five vineyards in Long Island (NY, US) characterized by granitic soil. The acidic conditions of the soil in SAN, due to the occurrence of schists-granites soil (Table 1), could support the development of similar traits to the microbiome of Long Island. Therefore, 'peculiar' berries microbiome could be directly influenced by soil characteristics, such as pH and soil nutritional resources [46].

Another characteristic trait of the SAN G microbiome is the modest presence of Acetobacteraceae (Rhodospirillales 14.5%) and Lactobacillaceae (1.8%), which are typically involved in the glucose fermentation of must. The occurrence of these bacteria could suggest an over-ripening of grape fruits in this vineyard, which it is generally accompanied by fruit softening and partial degradation of exocarp resulting in leakage of the grape's sugary juice. However, all sampling was performed at 20 degrees Brix, thus excluding over-ripening. Another possibility could be related to the damage of fruits. SAN samples were characterized by a relevant percentage (i.e. > 30%) of berries damaged by insects, probably belonging to Hymenoptera (AGRIS Personal communication). This event causes the fruit juice, rich in sugars content, to come out and promotes bacterial growth. In this case, external biologic factors might have influenced the berries microbiome with possible consequences on the final wine, because these microorganisms are able to actively and prematurely begin the fermentation process.

Concerning MAM samples, the collected fruits were dominated by Bacillales (32%) including members of *Lysinibacillus*, *Bacillus*

(Bacillaceae), and *Sporosarcina* (Planococcaceae). These bacteria were found in the manure of cows, pigs, and poultry [47, 48, 49], and their occurrence in MAM could be explained by considering the biodynamic farming system applied in this vineyard. Specifically, in MAM a cow horn filled with manure was buried for maturation and subsequently was activated with water during the spring (dynamization), and the resulting product was sprayed into the field. This strategy is declared to improve soil quality, as well as enhancing plant growth and resistance to pathogens. Recent works suggested that the effect of biodynamic management could also be explained by the modification of the plant microbiome [7, 50, 51], because bacteria can act as biological disease suppression agents and could also stimulate plant growth and have an effect on plant health.

The biodynamic practice could also explain the conspicuous presence of Clostridia bacteria (4.5%) with members of Peptostreptococcaceae and Clostridiaceae. Both of these bacteria have also been detected in manure [52] and could support the beneficial effect on plant growth as well.

We conclude that in MAM the characteristic traits of the fruits microbiome are largely influenced by agricultural management and that bacteria originally related to animal manure is also able to grow on grape fruits. However on MAM G samples, we also observed members of Burkholderiales (i.e. *Massilia* sp.) and Rhizobiales (*Rhizobium* sp.) which are typically diffused in soil rich in organic matter fractions. Also, these bacteria are important players for viticulture soils, since they are able to promote plant growth [53]. On the whole, we can hypothesize that the berries microbiome characteristics reliably reflects the soil fertility of the

vineyard. This is also evident in the plant of MAM vineyards, which were highly vigorous in vegetative organs, such as leaves and branches.

Concerning ALG and MOR vineyards, G samples shared the same microbial orders with a large abundance of Pseudomonadales and Enterobacteriales. This condition probably resulted from similarities in pedoclimatic characteristics: e.g. the same localities showed calcareous soil. However, some differences between these localities were detected: in MOR G samples, members of Pasteurellales were detected, whereas in ALG G samples, Xanthomonadales occurred.

Concerning the yeast diversity of G samples, the most abundant taxa were Dothioraceae (*Aureobasidium*, 49.86%), Pleosporaceae (*Alternaria*, 18.43%; *Pleospora*, 6.63%), and Saccharomycodaceae (*Hanseniaspora*, 17.63%). Although the proportion of these families changed among localities, their presence was ubiquitous in all vineyards. Berries of MAM were characterized by the absence of Saccharomycodaceae, whereas the group Saccharomycetaceae was detected in SAN, but not in the other localities.

The taxonomic diversity of musts was more moderate than that of G samples with a consistent relative abundance of Rhodospirillales (from 67.2% in ALG to 50.3%% in MAM), represented by the genera *Gluconobacter* and *Gluconacetobacter*, involved in the initial steps of the fermentation process. Members of Pseudomonadales, Bacillales, and Enterobacillales occurring in G samples, dropped in musts, because they did not have the ability to grow during the wine fermentation processes [10]. Our analysis suggests moderate differences of the microbiome among samples coming from the four localities. We underline that all the collected berries were processed at the same experimental wine cellar;

therefore, all musts could be influenced by the same wine cellar's microbiome. Moreover, the winemaking process was performed under the same chemical-physical parameters. For these reasons, the only appreciable differences lay in the persistence of microorganisms deriving from the vineyard, like the Rhizobiales and Pseudomonadales (i.e., *Acinetobacter*) in MAM samples.

In eM samples, the microbiome was more variable among the samples rather than in iM samples. We detected *Gluconobacter* and *Gluconacetobacter* (Rhodospirillales) involved in must fermentation as well as *Lactobacillus* (Bacillales) involved in malolactic fermentation. In general, malolactic fermentation is most often performed shortly after the end of the alcoholic fermentation, and for this reason, it is active during eM and not in iM. In the MAM eM samples, we did not detect Lactobacillales, but there were some species described in the must, such as bacteria belonging to *Carnomonas* (Oceanospirillales) [54].

In the case of fungi, the most abundant species in must was represented by S. cerevisiae. Although this organism did not occur on the grape surface, starting from the first fermentation steps, it became dominant in must due to its higher fermentative ability, growth rate, and tolerance to ethanol [55]. The primary source of this yeast is crush equipment and barrel room surfaces [23], and this explains its presence in our must samples treated without any use of commercial starter. Starting from the first phases of fermentation, *S. cerevisiae* supplants the various non-*Saccharomyces* yeasts and modifies the must's characteristics with consequences on the whole microbiome [23]. To better assess the peculiar yeasts occurring in the iM and eM samples of the four localities, the bar chart of Fig 4B was computed without *S. cerevisiae* OTUs. Data

suggest that the second abundant yeast family is represented by Saccharomycodaceae with Hanseniaspora uvarum and other fermentative yeasts, such as members of Trichocomaceae (i.e. *Aspergillus* spp. in MOR and MAM) and Saccharomycetaceae (i.e. *Candida* spp. in SAN). Although these yeasts were less represented than S. cerevisiae, they are important microbial actors involved in wine fermentation, and they are able to modify wine aroma and other organoleptic characteristics through the production of a greater range of sensory-active compounds. For example, *Hanseniaspora uvarum* products 2-phenylethyl acetate, which contributes to the rose, honey, fruity and flower aromas of wines [56 57, 58]. The origin of these yeasts is partially unclear. Some of these are detected on grapes [10], while others could be resident microorganisms of wine cellar [42]. Considering that in our study all the wine juices were fermented in the same wine cellar, we could hypothesize that differences in yeasts occurring in musts could derive from the field. Although these yeasts were not detected on the berries surfaces (e.g., *Hanseniaspora uvarum*, *Aspergillus* spp. and *Candida*), we cannot exclude their presence as spores not detectable by our NGS sequencing analysis, but they may become appreciable in must where they are able to germinate and proliferate. Nowadays, it is critical not only to define which microbes contribute to create a high-quality wine, but also how their metabolisms can influence wine organoleptic characteristics. Integrated databases, based on HTS and biochemical data, will permit in the very next future to analyze in depth the effects of a certain microbiome on metabolome. For example the WineSeq® platform (Biome Makers, Inc.) [59] revealed that the occurrence in G samples of some yeasts could have important effects on wine quality and human health. This is the case of the detected

Hanseniaspora sp. yeast is related to a potential sensory profile enhancement on wine flavour.

5.4.4 From field to wine cellar

Many studies suggest that the grapevine's microbiome influences the plant's physiology, and it can then also determine some aspects of the secondary metabolites' profile shown by fruits [11, 17]. A challenging issue to address is the impact of the berries' indigenous microbiota on wine fermentation and the consequent effect on the sensory complexity of wines [20]. Yeasts play important roles during the alcoholic fermentation step and also have a significant impact on wine quality. However, in our experimental conditions, bacteria represented the most relevant elements of differentiation among localities (108 private OTUs out of 176 detected on G samples); therefore, we studied the persistence of the characteristic field microbiome of different localities in must samples. We underline that although bacteria are not the main driving force shaping wine characteristics and quality, they do have a significant effect on the final product. For example, lactic acid bacteria are known to convert L-malic acid to lactic acid through MLF and to impart flavor complexity, while acetic acid bacteria produce acetic acid, which is a key factor in wine spoilage. Similarly, we can expect that the bacteria occurring on Cannonau berries at different localities may play a key role in must fermentation and wine quality. However, only the investigation of bacteria communities' dynamics during Cannonau fermentation could assess the influence of each microbial group on wine characteristics.

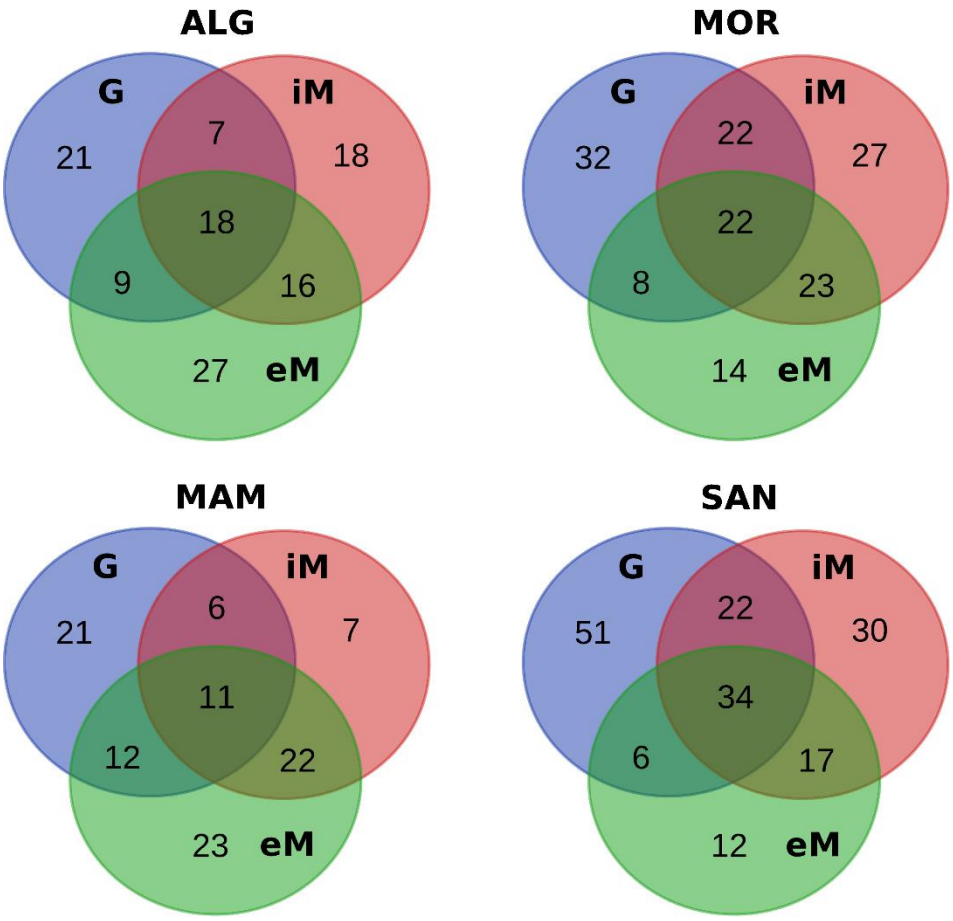


Fig 5. Venn diagram of shared OTUs. The figure shows the number of shared OTUs among sample typologies belonging to the four sampling localities.

A Venn graph (Fig 5) shows that more than 50% of G OTUs reach the wine cellar in all localities. As suggested by Bokulich [17], vineyard-specific microbial signatures diminished during fermentation (Fig 4A, Fig 4B and Fig 5) as the growth of fermentative organisms reshaped the community structure, richness, and diversity of the wines. However, our data showed that more than 50% of shared OTUs between G and iM persisted in the eM phase. These bacteria could modify wine traits [11, 17, 60] not only at the field level, but also by active metabolism in must

[11, 46, 53]. Fig 6 shows that those OTUs shared by field and wine cellar environments (Venn center diagram) correspond to several bacterial orders and most of these are shared by two localities at least (Fig 6).

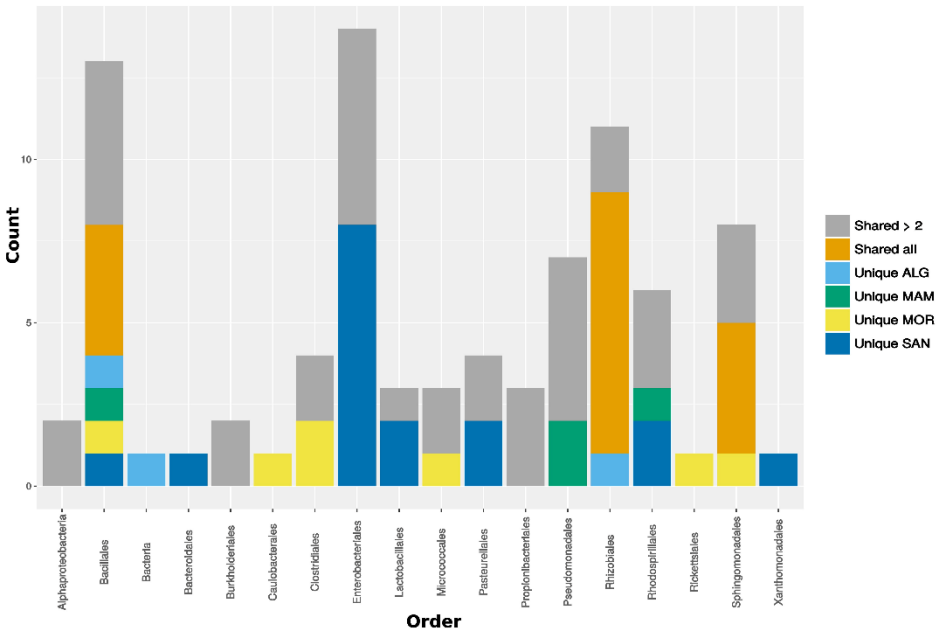


Fig 6. Barplot showing the distribution of unique bacterial OTUs (y axis) in G samples. On the x axis, bacterial orders are reported. Gray bars indicate the number of OTUs belonging to a specific Order shared by more than two localities. Orange bars indicate how many OTUs are shared among all four sampling localities. The other colors indicate the number of unique OTUs belonging to different orders.

In any case, each locality has its own private OTUs. Among the investigated localities, SAN showed the highest number of unique microbial traits (see S2 Table) and representatives of Enterobacteriales, Pasteurellales, Rhodospirillales, and Lactobacillales reach the wine cellar. As previously discussed, some of these bacteria are most active during fermentation, such as *Gluconobacter* (Rhodospirillales) and

Lactobacillus (Lactobacillales). A few OTUs were detected as unique fingerprints of ALG and MAM, but some of these were very active in must fermentation, such as the *Acetobacter* (Rhodospirillales) detected in MAM. Finally, a microbial fingerprint of MOR grape and must was characterized by several orders including the member of Caulobacteriales and Clostridiales with genera involved in plant growth stimulation [61]. Their role in wine fermentation is still unclear, but we cannot exclude that these bacteria could be able to modify some wine's metabolites. In conclusion, we can affirm that microbiomes found at the four investigated Sardinian localities can have an impact on fruit and, must. In addition, metabolism of different microorganisms could positively enhance some wine flavor traits, but also emphasize negative organoleptic characteristics.

5.5 Conclusions

In this study, we demonstrated a distinct microbial composition of *Cannonau* fruits from different Sardinian localities with consequent effects also on the musts' microbiome. To date, the role of grapevine microbes in the field has been largely ignored, with the only exception of microbial pathogens, mainly because the available technologies did not exist, and this prevented examining the community structure of the multitudes of bacterial and fungal species associated with each plant at any real depth or breadth [39]. Thanks to the HTS approach, we can now evaluate the microbial community of the grape and wine also in response to different environmental conditions and farming practices [46]. Probably, in the very next future, this technology will be also used to

deeply investigate viruses and phytoplasma that largely influence vineyard sector [62].

Emerging work clearly show that pedoclimatic conditions could affect wine characteristics not only due to the abiotic characteristics (i.e. soil, sun exposition), but also at the grape microbiome that is able to influence plant growth and development [10]. Bokulich [15] demonstrated the existence of regional microbiome fingerprints in California vineyards; Portillo [44] showed that several environmental variables, such as vineyard altitude and the geographical orientation of the plant could also affect the grape microbiome. Our study confirms that pedoclimatic characteristics could modify the fruit microbiome and underlines that agricultural practices, such as biodynamics, as well as the occurrence of opportunistic insects, such as hymenopterans, can have a consistent effect on the bacterial communities of berries and corresponding must. These results suggest that the role of the field environmental microbiome is not limited to promoting grape fruit maturation and enhancing the occurrence of some secondary metabolites strictly related to wine color and flavor, but it is also an important source of microorganisms that are able to influence wine fermentation and metabolic composition.

Characteristics of the cultivars' genotype play an important role in viticulture, so starting from the 1990's, DNA fingerprinting approaches were used to identify synonymous cultivars and to unmask incorrect attributions. Our team [63], demonstrated a complete genetic identity between Cannonau and Spanish Grenache by using SSR markers. However, the microbiomes of these cultivars are very different [44], and appreciable differences were also observed among Grenache localities. This finding suggests that the value of the cultivar genotype is somewhat

relative. A reliable genotyping should include the entire holobiont (i.e. the plant and all its symbionts [64]) of a specific Cannonau or Garnacha plant. The study of the grapevine microbiome does not represent a simple element of the product's traceability and identity. We should consider that bioprospecting activities on grape microbiota could led to the discovery of several species with positive enological properties, as recently documented by the WineSeq® project [59]. Occurrence and abundance of these species could be easily monitored by using conventional cultivation strategies and target PCR approaches (Real-Time and Digital PCR) to be used for improving wine quality, to enhance immune capability, and reduce the use of agrochemicals. Nowadays, only an exhaustive knowledge about the vineyard, the winery and their inhabitants could permit real advancements in management activities aimed towards a better sustainable system without any loss in terms of yields and product quality.

5.6 Supporting information

S1 Text. ANOVA result on alpha diversity values for 16S and ITS1.
The ANOVA and Pairwise Post-hoc test results performed for each locality considering the three fermentation steps.

Significant alpha diversity interactions considering Observed OTUs

Among G different Localities

16S

	diff	**lwr**	**upr**	**p adj**
G:Mores-G:Alghero	2.750000e+01	0.1976667	54.802333	0.0477842

G:Santadi-G:Alghero	5.400000e+01	26.676667	81.302333	0.0001744
G:Mores-G:Mamoiada	2.850000e+01	1.1976667	55.802333	0.0379752
G:Santadi-G:Mores	2.650000e+01	-0.8023333	53.802333	0.0600727
G:Santadi-G:Mamoiada	5.500000e+01	27.6976667	82.302333	0.0001453

All G sample are significantly different from each other, the only exception is ALG and MAM (p-value = 1).

ITS1

	diff	**lwr**	**upr**	**p adj**
G:Mores-G:Mamoiada	22.0	1.659009	42.340991	0.0299666
G:Santadi-G:Mamoiada	33.5	13.159009	53.840991	0.0010065

Among iM different Localities

16S

	diff	**lwr**	**upr**	**p adj**
iM:Santadi-iM:Alghero	4.450000e+01	17.1976667	71.802333	0.0011070
iM:Mores-iM:Mamoiada	3.650000e+01	9.1976667	63.802333	0.0061444
iM:Santadi-iM:Mamoiada	5.550000e+01	28.1976667	82.802333	0.0001327

ITS1

	diff	**lwr**	**upr**	**p adj**
iM:Mamoiada-iM:Alghero	-52.0	-72.340991	-31.659009	0.0000123
iM:Mores-iM:Alghero	-39.0	-59.340991	-18.659009	0.0002371
iM:Santadi-iM:Alghero	-42.0	-62.340991	-21.659009	0.0001134

Among eM different Localities

There are no differences among eM that come from different cultivation sites for both markers.

Considering same locality among G-iM-eM

16S

ALG

There are no significant differences

MOR

There are no significant differences

SAN

	diff	**lwr**	**upr**	**p adj**
eM:Santadi-G:Santadi	-4.050000e+01	-67.8023333	-13.197667	0.0025636
iM:Santadi-eM:Santadi	3.450000e+01	7.1976667	61.802333	0.0096204

MAM

There are no significant differences

ITS1

ALG

	diff	**lwr**	**upr**	**p adj**
eM:Alghero-G:Alghero	-66.0	-86.340991	-45.659009	0.0000009
iM:Alghero-G:Alghero	-1.5	-21.840991	18.840991	1.0000000
iM:Alghero-eM:Alghero	64.5	44.159.009	84840991	0.0000012

MOR

	diff	**lwr**	**upr**	**p adj**
eM:Mores-G:Mores	-82.0	-102.340991	-61.659009	0.0000001

iM:Mores-G:Mores	-48.5	-68.840991	-28159009	0.0000258
iM:Mores-eM:Mores	33.5	13.159009	53.840991	0.0010065

SAN

	diff	lwr	upr	p adj
eM:Santadi-G:Santadi	-93.0	-113.340991	-72.659009	0.0000000
iM:Santadi-G:Santadi	-63.0	-83.340991	-42.659009	0.0000015
iM:Santadi-eM:Santadi	30.0	96.59009	50.340991	0.0026950

MAM

	diff	**lwr**	**upr**	**p adj**
eM:Mamoiada-G:Mamoiada	-64.0	-84.340991	-43.659009	0.0000013
iM:Mamoiada-G:Mamoiada	-39.5	-59.840991	-19.159009	0.0002092
iM:Mamoiada-eM:Mamoiada	24.5	4.159009	44.840991	0.0138796

Significant alpha diversity interactions considering Shannon Index

Among G different Localities

16S

	diff	**lwr**	**upr**	**p adj**
G:Mamoiada-G:Alghero	1.163710802	0.90875985	1.418661755	0.0000000
G:Mores-G:Alghero	1.110571214	0.85562026	1.365522167	0.0000000
G:Santadi-G:Mamoiada	-0.925280294	-1.18023125	-0.670329341	0.0000003
G:Santadi-G:Mores	-0.872140706	-1.12709166	-0.617189753	0.0000005

All G sample are significantly different from each other, the only exception is MOR=MAM and SAN=ALG (p-value = 1)

ITS1

	diff	**lwr**	**upr**	**p adj**
G:Mamoiada-G:Alghero	-0.757906867	-0.94694729	-0.568866441	0.0000001
G:Mores-G:Alghero	-0.395509932	-0.58455036	-0.206469506	0.0000993
G:Santadi-G:Alghero	-0.762850078	-0.95189050	-0.573809652	0.0000001
G:Mores-G:Mamoiada	0.362396935	0.17335651	0.551437361	0.0002374
G:Santadi-G:Mores	-0.367340146	-0.55638057	-0.178299720	0.0002078

Among iM different Localities

16S

	diff	**lwr**	**upr**	**p adj**
iM:Mores-iM:Alghero	-0.437990490	-0.69294144	-0.183039537	0.0006802
iM:Mores-iM:Mamoiada	-0.555282653	-0.81023361	-0.300331700	0.0000659
iM:Santadi-iM:Mores	0.342933945	0.08798299	0.597884898	0.0058456

Mores is the locality that maintain an isolation level.

ITS1

	diff	**lwr**	**upr**	**p adj**
iM:Mamoiada-iM:Alghero	-0.304900154	-0.49394058	-0.115859728	0.0012181
iM:Mores-iM:Alghero	-1.262674217	-1.45171464	-1.073633791	0.0000000
iM:Santadi-iM:Alghero	-1.277719280	-1.46675971	-1.088678854	0.0000000
iM:Mores-iM:Mamoiada	-0.957774063	-1.14681449	-0.768733637	0.0000000

iM:Santadi-iM:Mamoiada	-0.972819126	-1.16185955	-0.783778699	0.0000000

Among eM different Localities

16S

	diff	**lwr**	**upr**	**p adj**
eM:Mamoiada-eM:Alghero	0.834182690	0.57923174	1.089133643	0.0000008
eM:Mores-eM:Alghero	0.581140835	0.32618988	0.836091788	0.0000412

ITS1

	diff	**lwr**	**upr**	**p adj**
eM:Santadi-eM:Alghero	-0.279319142	-0.46835957	-0.090278716	0.0026526
eM:Santadi-eM:Mamoiada	-0.355149535	-0.54418996	-0.166109109	0.0002892
eM:Santadi-eM:Mores	-0.420928151	-0.60996858	-0.231887724	0.0000525

Considering same locality among G-iM-eM

16S

ALG

There are no significant differences

MOR

	diff	**lwr**	**upr**	**p adj**
eM:Mores-G:Mores	-0.466189949	-0.72114090	-0.211238996	0.0003759
iM:Mores-G:Mores	-1.490951796	-1.74590275	-1.236000843	0.0000000
iM:Mores-eM:Mores	-1.024761847	-1.27971280	-0.769810894	0.0000001

SAN

	diff	**lwr**	**upr**	**p adj**
eM:Santadi-G:Santadi	0.355425427	0.10047447	0.610376380	0.0043508
iM:Santadi-G:Santadi	-0.275877146	-0.53082810	-0.020926193	0.0298689
iM:Santadi-eM:Santadi	-0.631302573	-0.88625353	-0.376351620	0.0000173

MAM

	diff	**lwr**	**upr**	**p adj**
iM:Mamoiada-G:Mamoiada	-0.988808731	-1.24375968	-0.733857778	0.0000001
iM:Mamoiada-eM:Mamoiada	-0.722521049	-0.97747200	-0.467570096	0.0000041

ITS1

ALG

	diff	**lwr**	**upr**	**p adj**
eM:Alghero-G:Alghero	-0.421837257	-0.61087768	-0.232796831	0.0000514
iM:Alghero-eM:Alghero	0.324306862	0.13526644	0.513347289	0.0006891

MOR

	diff	**lwr**	**upr**	**p adj**
iM:Mores-G:Mores	-0.964694680	-1.15373511	-0.775654254	0.0000000
iM:Mores-eM:Mores	-1.079976364	-1.26901679	-0.890935937	0.0000000

SAN

	diff	lwr	upr	p adj
iM:Santadi-G:Santadi	-0.612399597	-0.80144002	-0.423359170	0.0000009
iM:Santadi-eM:Santadi	-0.674093276	-0.86313370	-0.485052849	0.0000003

MAM

	diff	lwr	upr	p adj
eM:Mamoiada-G:Mamoiada	0.411900003	0.22285958	0.600940429	0.0000657
iM:Mamoiada-G:Mamoiada	0.355476318	0.16643589	0.544516744	0.0002867

S2 Text. ADONIS test results. Results of ADONIS test performed to explore beta diversity patterns.

Type= fermentation steps
Cultivation_Site= ALG, MOR, SAN, MAM

16S
##unweighted unifrac distance matrix
Call:
*adonis(formula = unweight.dist ~ Type * Cultivation_Site, data = df.wine)*

Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

	Df	*SumsOfSqs*	*MeanSqs*	*F.Model*	*R2*	*Pr(>F)*
Type	*2*	*1.1219*	*0.56096*	*11.7381*	*0.34629*	*0.001 ****
Cultivation_Site	*3*	*0.6617*	*0.22058*	*4.6156*	*0.20425*	*0.001 ****
Type:Cultivation_Site	*6*	*0.8827*	*0.14711*	*3.0783*	*0.27245*	*0.001 ****
Residuals	*12*	*0.5735*	*0.04779*		*0.17701*	
Total	*23*	*3.2398*			*1.00000*	

---
*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

##weighted unifrac distance matrix

Call:
*adonis(formula = wine.dist ~ Type * Cultivation_Site, data = df.wine)*

Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

	Df	*SumsOfSqs*	*MeanSqs*	*F.Model*	*R2*	*Pr(>F)*
Type	*2*	*0.95041*	*0.47520*	*115.181*	*0.48852*	*0.001 *****
Cultivation_Site	*3*	*0.42870*	*0.14290*	*34.637*	*0.22036*	*0.001 *****
Type:Cultivation_Site	*6*	*0.51686*	*0.08614*	*20.880*	*0.26567*	*0.001 *****
Residuals	*12*	*0.04951*	*0.00413*		*0.02545*	
Total	*23*	*1.94548*			*1.00000*	

---
*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

ITS1

##jaccard distance matrix

Call:
*adonis(formula = jaccard.dist ~ Type * Cultivation_Site, data = df.wine)*

Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

	Df	*SumsOfSqs*	*MeanSqs*	*F.Model*	*R2*	*Pr(>F)*
Type	*2*	*-1.08959*	*-0.54479*	*-2.48194*	*-0.82683*	*0.999*
Cultivation_Site	*3*	*-0.20219*	*-0.06740*	*-0.30705*	*-0.15343*	*0.954*
Type:Cultivation_Site	*6*	*-0.02447*	*-0.00408*	*-0.01858*	*-0.01857*	*0.987*
Residuals	*12*	*2.63404*	*0.21950*		*1.99883*	
Total	*23*	*1.31779*			*1.00000*	

>

##Bray-Curtis distance matrix

Call:
*adonis(formula = bray.dist ~ Type * Cultivation_Site, data = df.wine)*

Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

	Df	*SumsOfSqs*	*MeanSqs*	*F.Model*	*R2*	*Pr(>F)*
Type	*2*	*2.3259*	*1.16293*	*112.118*	*0.47443*	*0.001 *****
Cultivation_Site	*3*	*1.5923*	*0.53077*	*51.171*	*0.32480*	*0.001 *****
Type:Cultivation_Site	*6*	*0.8598*	*0.14331*	*13.816*	*0.17539*	*0.001 *****
Residuals	*12*	*0.1245*	*0.01037*		*0.02539*	
Total	*23*	*4.9025*			*1.00000*	

---

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

S1 Table. Run performance detail Table.

16S	quality-filtered sequences + singleton	no Eucariota, chloroplast, mitocondria and Unclassified < 0.1%	no Eucariota, chloroplast, mitocondria and Unclassified < 0.1% + Only Villasor wine making
Num samples	32	32	24
OTU	702	264	264
total count	1583562	304829	235371
Min reads x sample	4816	176	180
Max reads x sample	117534	79666	79666

ITS1	quality-filtered sequences + singleton	No *Saccharomyces cerevisiae*	No *Saccharomyces cerevisiae* + Only Villasor wine making
Num samples	32	32	24
OTU	222	216	216
total count	5162064	1303420	1041606

Min reads x sample	14638	104	104
Max reads x sample	328759	166307	166307

S2 Table. Table of unique OTUs distribution. Dataframe of unique OTUs distribution for localities based on Venn diagrams analysis.

OTU	Area	Unique	Phylum	Class	Order	Family	Genus
B.OTU_241	alghero_core	shared	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	
B.OTU_307	alghero_core	shared	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Gluconobacter
B.OTU_311	alghero_core	shared	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadales	
B.OTU_325	alghero_core	shared	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
B.OTU_48	alghero_core	shared	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Tatumella
B.OTU_538	alghero_core	shared	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria	Alphaproteobacteria	
B.OTU_65	alghero_core	shared	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
B.OTU_67	alghero_core	shared	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Kluyvera
B.OTU_70	alghero_core	shared	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Orbus
B.OTU_82	alghero_core	shared	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas

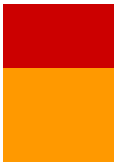
B.OTU_97	alghero_core	shared	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea
B.OTU_263	mamoiada_core	shared	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Propionibacterium
B.OTU_66	mamoiada_core	shared	Firmicutes	Bacilli	Bacillales	Bacillales	
B.OTU_84	mamoiada_core	shared	Firmicutes	Bacilli	Bacillales	Bacillales	
B.OTU_241	mores_core	shared	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	
B.OTU_263	mores_core	shared	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Propionibacterium
B.OTU_307	mores_core	shared	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Gluconobacter
B.OTU_311	mores_core	shared	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadales	
B.OTU_371	mores_core	shared	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	g
B.OTU_63	mores_core	shared	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia
B.OTU_65	mores_core	shared	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
B.OTU_66	mores_core	shared	Firmicutes	Bacilli	Bacillales	Bacillales	
B.OTU_70	mores_core	shared	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Orbus
B.OTU_82	mores_core	shared	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
B.OTU_84	mores_core	shared	Firmicutes	Bacilli	Bacillales	Bacillales	
B.OTU_263	santadi_core	shared	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Propionibacterium
B.OTU_307	santadi_core	shared	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Gluconobacter

B.OTU_311	santadi_core	shared	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaldes	
B.OTU_325	santadi_core	shared	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
B.OTU_371	santadi_core	shared	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	g
B.OTU_48	santadi_core	shared	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Tatumella
B.OTU_538	santadi_core	shared	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria	Alphaproteobacteria	
B.OTU_63	santadi_core	shared	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia
B.OTU_65	santadi_core	shared	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
B.OTU_66	santadi_core	shared	Firmicutes	Bacilli	Bacillales	Bacillales	
B.OTU_67	santadi_core	shared	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Kluyvera
B.OTU_81	santadi_core	shared	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
B.OTU_97	santadi_core	shared	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea
B.OTU_305	all	shared_all	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas
B.OTU_313	all	shared_all	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium
B.OTU_358	all	shared_all	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
B.OTU_86	all	shared_all	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus
B.OTU_282	alghero_core	u_alg	Bacteria				
B.OTU_310	alghero_core	u_alg	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium

B.OTU_61	alghero_core	u_alg	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus
B.OTU_329	mamoiada_core	u_mam	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter
B.OTU_53	mamoiada_core	u_mam	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
B.OTU_54	mamoiada_core	u_mam	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
B.OTU_74	mamoiada_core	u_mam	Firmicutes	Bacilli	Bacillales	Bacillaceae	
B.OTU_228	mores_core	u_mor	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	
B.OTU_231	mores_core	u_mor	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	g1
B.OTU_321	mores_core	u_mor	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
B.OTU_334	mores_core	u_mor	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas
B.OTU_336	mores_core	u_mor	Proteobacteria	Alphaproteobacteria	Sphingomonadales		
B.OTU_370	mores_core	u_mor	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	g
B.OTU_89	mores_core	u_mor	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina
B.OTU_104	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
B.OTU_112	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
B.OTU_113	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Orbus
B.OTU_122	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia
B.OTU_124	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	

B.OTU_125	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia-Shigella	
B.OTU_127	santadi_core	u_sant	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Leuconostoc	
B.OTU_133	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
B.OTU_138	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
B.OTU_139	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
B.OTU_142	santadi_core	u_sant	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	
B.OTU_187	santadi_core	u_sant	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
B.OTU_308	santadi_core	u_sant	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Gluconacetobacter	
B.OTU_328	santadi_core	u_sant	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Asaia	
B.OTU_341	santadi_core	u_sant	Firmicutes	Bacilli	Bacillales	Planococcaceae	Incertae_Sedis1	
B.OTU_56	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
B.OTU_98	santadi_core	u_sant	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Orbus	

		unique per area	core	%	unique per G	%xG	observed otu	%xtot
	santadi	17	34	50	71	23,94366197	113	15,0442
	mores	7	22	31,818182	23	30,43478261	84	8,3333
	mamoiada	4	11	36,363636	6	66,66666667	50	8



alghero	3	18	16,666667	8	37,5	55	5,45455	
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6. CHAPTER 4

Soil and vineyard biodiversity shape grape microbiome regardless of cultivar

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6.1 Abstract

Recent studies have highlighted the role of the grapevine microbiome in addressing a wide panel of features, ranging from plant growth and development to wine quality. Conversely, the influence of cultivar and vineyard environmental conditions in shaping the grape microbiome have been poorly investigated. In this study, we selected three international diffused grapevine cultivars (Cabernet Sauvignon, Sauvignon Blanc, and Syrah) growing under three different geographical and climatic conditions (Northern Italy NI, Italian Alps AI, and Northern Spain NS). The soil and grape microbiome was characterized by 16s rRNA High Throughput Sequencing (HTS), and the obtained results showed that all grape samples shared some abundant bacterial classes, regardless of sampling locality (e.g., Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Clostridia, Bacilli, Actinobacteria). However, rare Operational Taxonomic Units (OTUs) act as geographical signatures, which was also confirmed by PCoA analysis. Interestingly, we did not find a significant relationship between each cultivar and its microbiome profile. Concerning the origin of the grape microbiome, our study confirms that vineyard soil represents a primary reservoir for grape associated bacteria with almost 60% of genera shared between the soil and grape. At each locality, grapevine cultivars shared a core of bacterial genera belonging to the vineyard soil, as well as from other local biodiversity elements such as arthropods inhabiting or foraging in the vineyard. Finally, a machine learning analysis showed that it was possible to predict the geographical origin and cultivar of grape starting from its microbiome composition with a high accuracy (9 cases out of 12

tested samples). Overall, these findings open new perspectives for the development of more comprehensive and integrated research activities to test which environmental variables have an effective role in shaping the microbiome composition and dynamics of cultivated species over time and space.

6.2 Introduction

In the last 10 years, due to the advances in metagenomics, it has become clearer and clearer that plants host a wide panel of bacteria and yeasts (Melcher et al., 2014), most of which are not cultivable and therefore are almost unknown at the taxonomic and metabolic levels. Such microorganisms interact with the plant organs and are able to influence plant nutrition, development, productivity, and stress responses (White et al., 2014; Bacon and White, 2016).

Soil acts as a microbial reservoir for plants, especially concerning underground plant microbiota (Barata et al., 2012; Bacon and White, 2016). Usually, microbial diversity is higher at the roots than at aboveground organs due to the mostly selective nutrient-poor conditions and high exposure to variable abiotic factors (i.e. temperature, humidity, and UV radiation intensity) of the leaves, flowers, and fruit (Ottesen et al., 2013). The origin of the microbial community in aboveground plant organs is less studied than that of the root microbiome, and many issues are unknown (Berg et al., 2014).

It is even clearer that environmental microorganisms are essential for ensuring ecosystem equilibrium and are able to influence the relationships between plants and abiotic (e.g. soil, water, and solar light)

and biotic (e.g., other microbial organisms) elements. Understanding how microbial assemblages colonizing the whole plant can play a key role in ecosystem and agroecosystem management is a challenging issue of emerging concern (Van der Heijden and Wagg, 2013). In the last few years, one of the main research goals was discovering the origin of microbial community that colonizes crops and its direct influence on plant productivity, stress tolerance, and resistance (Morgan et al., 2017). This information may provide biological targets for future biotechnological applications, as well as basal information to control field microbial diversity for enhancing production yield (Finkel et al., 2017).

In the case of the grapevine (*Vitis vinifera* L.), the role of plant microbiota is much more relevant, because it is involved in the concept of terroir, and field microorganisms have a documented effect during wine production (Mezzasalma et al., 2017; Grangeteau et al., 2017). The rationale is that the grapevine naturally hosts a rich community of microorganisms that interact with plant organs, including fruit, and they can be transferred to the winery where, ultimately, they may affect wine production and influence its quality (Bokulich et al., 2016; Belda et al., 2017; Morgan et al., 2017). Recently, DNA HTS studies supported that the grape microbiome is related to vineyard location, climatic conditions, and other vineyard-related factors (Bokulich et al., 2016). Mezzasalma and co-workers (2017) also showed that agronomical practices, such as biodynamic management are able to modify the microbiome of grape fruits and must. Similarly, other authors suggested that the occurrence of specific bacteria in must and wine has an effect on wine characteristics and typicity (Belda et al., 2017; Liu et al., 2017).

One of the main questions regards how the influence of grapevine cultivar and plant organs model the grape microbiome. Martins and colleagues (2013) showed that some epiphytic bacteria were shared by aerial plant portions and the soil. This finding led them to propose that the physical proximity between soil and the plant might facilitate microbial migration through rain splash, winds, pollinators and other foragers, and parasites. Compant (2011) showed that grapevine's aboveground organs might also be colonized by bacteria from other plants species. However, the molecular and physical mechanisms involved in plant-microbial interactions are not completely clear. Moreover, any grapevine cultivar will show peculiar secondary metabolites, and most of these are concentrated in the fruit. Some of these metabolites have antimicrobial properties (Chong et al., 2009; Katalinić, et al., 2010) and could influence the composition of grape microbiome both quantitatively and qualitatively. Based on these assumptions, we hypothesize that each cultivar could have an active and specific role in the interaction with and selection of its microbial community.

In this work, we investigated the microbiome composition of three international grapevine cultivars (i.e., Cabernet Sauvignon, Sauvignon Blanc, and Syrah) growing under three different geographical and environmental conditions. We characterized the composition of the grape microbiome of each cultivar and evaluated the influence of vineyard soils and grape characteristics in shaping plant epiphytic bacteria.

6.3 Materials and methods

6.3.1 Plant and soil sampling

Cabernet Sauvignon (CS), Syrah (SY), and Sauvignon Blanc (SB) were selected as candidate cultivars to evaluate the role of plants in selecting surface bacteria due to the differences in their bunch and berry characteristics (Table 1).

Table 1: Morphological characteristics of bunch and grape of the three studied cultivars. Data obtained from the Organisation Internationale de la Vigne et du Vin (OIV, http://www.oiv.int) and the Italian Vitis Database (VitisDB, http://www.vitisdb.it). Ampelometric characteristics were also verified by field visual inspection.

	Bunch			**Berry**				
Cultivar	**Compactness**	**Size (length/ width)**	**Shape**	**Size (length/ width)**	**Thickness of skin**	**Bloom**	**Consistency**	**Colour**
Cabernet Sauvignon	medium/ dense	short/ medium	conical	short/ medium	medium	high	soft	blue - dark
Syrah	medium/ dense	short/ medium	funnel shaped	medium/ medium	medium	medium /high	medium-hard	blue - dark
Sauvignon Blanc	Dense	medium/ medium	conical	medium/ medium	medium	medium	soft	green-yellow

To better estimate the role of environmental conditions on berry microbiome, a total of 45 grape samples (5 bunches*3 cultivars*3

geographical localities) were collected at three germplasm collections having similar pedological features (i.e., gravelly sandy soil, with good drainage and permeability to water and air). The first sampling site was the germplasm collection of E. Mach Foundation (Lat 46°18’37"N; Lon 11°13'4"E) at the foot of the Italian Alps (hereafter AI). The second site was the Lombardy Regional Collection in Northern Italy (Lat 44°58’35’’N, Lon 9°5’61’’E) characterized by mild continental climatic conditions (hereafter NI), and the last site was the experimental collection of Government of La Rioja (Lat 42°28′N, Lon 2°27′W) located in Northern Spain (hereafter NS) and characterized by a continental climate.

During the harvest of 2016, mature grapes (20 degrees Brix) for each cultivar were selected for microbial analysis. One degree Brix is 1 gram of sucrose in 100 g of solution and the scale is used as a proxy for grape maturation and fermentation progress. Sampling was performed in collaboration with specialized technicians from research institutes. Five samples from each cultivar were collected as bunches (100 berries for each sample). Grapes were immediately frozen and stored at −80 °C until DNA isolation.

Concerning soil, a total of 15 samples (i.e., 5 soil samples*3 geographical localities) were also collected. Each sample consisted of three cores (top-layer 0-10 cm) pooled to make a single comprehensive sample. Soil sampling was performed at germplasm collections along with grape sampling. Samples were stored at -80° C until DNA isolation.

6.3.2 DNA extraction

Microbial biomass recovery from grape samples was obtained starting from twenty berries randomly selected from each cultivar of each sampling site. Berries were thawed and placed in 500 mL sterile Erlenmeyer flasks and washed with 100 mL of isotonic solution (0.9% w/v NaCl) for 3 h with agitation at 150 rpm. The obtained cell suspension was separated from the berries by centrifugation at 6,000 × g for 15 min. Pellets were stored at -20° C until DNA isolation. Total genomic DNA were obtained from pellets using PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions with modifications specific for wet soil samples.

The same commercial kit was adopted to extract soil DNA starting at 0.25 g of soil for each collected sample. Before library preparation, the obtained genomic DNA extracts were purified using Zymo Research DNA Clean and Concentrator-10 (Zymo Research, Irvine, CA, USA) to remove PCR inhibitors.

6.3.3 Library preparation and sequencing

DNA libraries for each sample were prepared following Illumina guidelines (16S Metagenomic Sequencing Library Preparation, Part #15044223 Rev. B) with modifications. Bacterial V3 and V4 regions of the 16S rRNA gene were amplified using primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth et al., 2013) with the addition of the Illumina overhang adapter sequences.

Before amplification, DNA extracts were normalized by means of Quantitative real-time PCR (qPCR) Ct values with the same

amplification primer pairs and the same protocols described by Bruno and colleagues (2016; 2017). Library sequencing was performed through Illumina MiSeq instrument using MiSeq Reagent Kit v3 (2 x 300-bp paired-end sequencing). The library preparation and the sequencing process were conducted by the Center for Translational Genomics and Bioinformatics of Hospital San Raffaele (Milan, Italy).

6.3.4 Microbial composition and community structure analysis

Analysis of bacterial communities was performed using the plugins of the QIIME2 suite (Caporaso et al., 2010). Raw Illumina reads were paired and pre-processed using VSEARCH v2.5.0 *--merge pairs* algorithm (Rognes et al., 2016). Reads were filtered out if ambiguous bases were detected and lengths were outside the bounds of 250 bp. Moreover, an expected error=1 was used as an indicator of read accuracy.
Bacterial features were obtained using *--cluster_fast* algorithm with a 97% sequence identity with at least a depth of 75x for each feature. The cluster centroid for each feature was chosen as the representative sequence of the cluster. The taxonomic assignment of the representative sequences, to obtain the OTUs, was carried out using the VSEARCH Classifier against the SILVA SSU non-redundant database (128 release) adopting a consensus confidence threshold of 0.8. A rarefaction table was calculated for each sample to determine the most suitable sequencing depth that covers the extant microbial diversity.

The intra group diversity (alpha diversity) was calculated using the number of observed OTUs and the Faith's Phylogenetic Diversity (Faith,

1992). The Kruskal-Wallis (pairwise) test was used to test for associations between discrete metadata categories and alpha diversity data.

Community analyses (beta-diversity) were performed with qualitative (Jaccard and unweighted UniFrac) and quantitative (Bray-Curtis and weighted UniFrac) distance metrics using QIIME2 plugins. Statistical significance among groups was determined by the ADONIS (permutation-based ANOVA (PerMANOVA)) test (Anderson, 2005). PerMANOVA Pairwise contrast was performed by the beta-group-significance command of "diversity" plugin. The phylogenetic tree necessary to calculate UniFrac distances and based on the alignment of OTUs representative sequences was built using RAxML version 7.4.2 (Stamatakis, 2006) with the GTRGAMMA model bootstrapping (1,000 replicates) the best maximum likelihood tree inference. Multibar plots were generated with the QIIME2 dedicated plugin.

The Venn diagrams were created with the online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) by calculating the number of shared and unique genera in the different datasets.

The Random Forest classifier implemented in the *qiime sample-classifier classify-samples* QIIME2 plugin was used to predict a categorical sample metadata category (i.e. Geographical origin, Cultivar, and the combination of the two variables). The number of trees to grow for estimation was set to 1000. Overall accuracy (i.e., the fraction of times that the tested samples are assigned the correct class) was calculated for

each factor. K-fold cross-validation was performed during automatic feature selection and parameter optimization steps. A five-fold cross-validation was also performed.

6.4 Results

6.4.1 Sequence analysis

Samples of grape and soil were sequenced in replicate. After filtering and primer removal, the remaining sequences were of high quality and had an average length of 430 bp (range: 400 - 438 bp) and clustered into 1154 OTUs (Supplementary Data S1). To characterize the microbial consortia associated with grapes of the three cultivars (45 samples) and soil samples (15 in total), 2,056,066 and 1,450,304 quality-filtered 16S rRNA sequences were obtained respectively. After the removal of sequences corresponding to the grapevine genome (mitochondrial and chloroplast genomes included), and singleton sequences, a total of 818,076 and 1,001,230 sequences were used to describe the microbial profile of grape and soil samples.

6.4.2 Grape microbiome diversity and distribution

The microbial taxonomic composition of grapes, summarized at phylum level, is shown in Figure 1. Overall, a total of 18 phyla (Bacteria domains), 55 classes, 98 orders, 197 families, and 374 genera were found within all grape samples. Regardless of provenance and cultivar, bacterial communities were dominated by Proteobacteria (71.4%), Firmicutes (12.7%), Actinobacteria (9.6%), Bacteroidetes (3.4%).

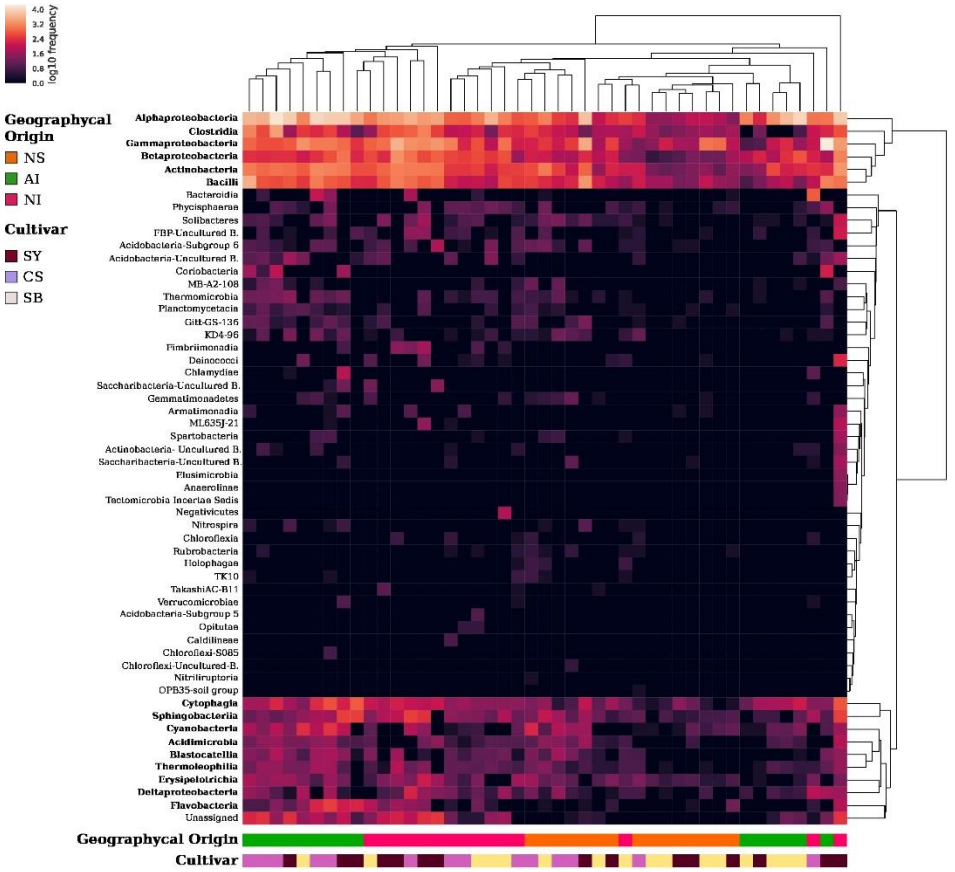

Figure 1: Heat map showing the relative abundance of the most abundant bacterial classes identified in grape samples. Colour shading in the heat map indicates the abundance (expressed as log10 frequency) for each class in the sample. The upper cladogram, shows clusters of grape samples based on classes distribution, whereas the right cladogram shows groups of bacteria classes based on their distribution among samples. Bottom coloured bars show samples groups based on locality or cultivar.

Complete taxonomic assignments for each detected OTU are shown in Supplementary Data S1.

Concerning microbial diversity distribution among the investigated samples, the heat map (Figure 1) shows that the six most abundant

classes of bacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Clostridia, Bacilli, and Actinobacteria, were shared by all cultivars and sampling localities. Conversely, other nine less abundant classes, Cytophagia, Sphingobacteria, Cyanobacteria, Acidimicrobia, Blastocatellia, Thermoleophilia, Erysipelotrichia, Deltaproteobacteria, and Flavobacteria, were shared mainly by grapes from Italian Localities NI and AI (see also Supplementary Data S1).

At the geographical level, the heat map (Figure 1) distinguished the analysed grape accessions in two main clusters where microbial groups of NS grapes grouped entirely in the second one, while AI and NI accessions were shared between both clusters. Cultivar microbial traits were distributed in both clusters. The alpha diversity analysis suggested that, in general, Spain grape samples (NS) show the lowest microbial diversity (PD metric. AI: (mean +/- sd) =18.99 +/- 9.14; NI: (mean +/- sd) =13.71 +/- 3.39; NS: (mean +/- sd) =6.71+/- 4.55; H=2.76; p=0.009).

To better explore the microbial differences among localities and grape cultivars, we computed beta diversity metrics and generated Principal Coordinates Analysis (PCoA). In order to normalize the variance during the analysis, we set the even sampling depth to 1000. The script that calculates beta diversity metrics uses this parameter to subsample the counts in each sample without replacement, so each sample in the resulting table has a total count of 1000. If the total count for any sample is smaller than 1000, the samples are dropped for the diversity analysis. Using this value, we lost two Sauvignon Blanc and three Syrah samples from NS. The PCoA clearly shows a significant geographical structuration of the studied accessions for all the cultivars at the three

localities (NI vs AI, pseudo-F= 9.73, p <0.001; NI vs NS, pseudo-F= 4.46, p <0.001; AI vs NS, pseudo-F= 7.91, p<0.001) (Figure 2A).

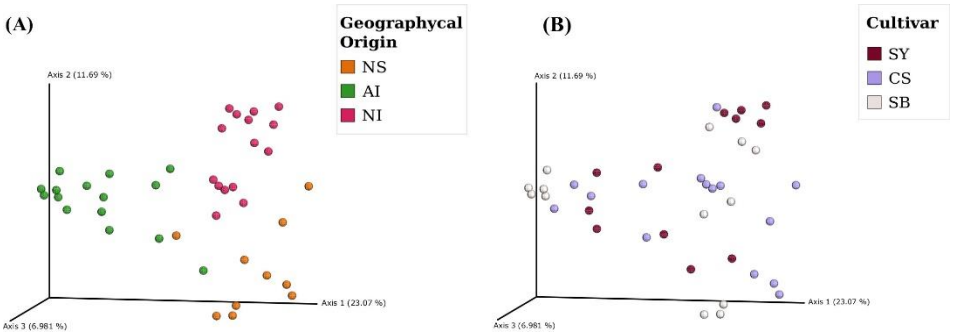

Figure 2. PCoA Emperor plots based on Bray-Curtis diversity metric. **(A)** Comparison of grape microbiota communities among sampling localities. **(B)** Comparison of grape microbiota community among investigated cultivars.

Conversely, there is no significant correlation between cultivars and their microbiome profile as shown in Figure 2B (p>0.05), (see also Supplementary Data S2 for complete PERMANOVA pairwise test results).

6.4.3 The origin of the grape microbiome

Figure 3A shows the distribution of bacterial classes per grape having a relative abundance > 0.005%. Cultivar, geographical provenance variables, and microbiome data from soil samples are included as well. Compared to grape, soil was richer in terms of microbial diversity (Figure 3B; PD metric (mean +/- sd): grape = 9.56 +/- 4.13; soil = 15.3 +/- 1.05; H=20.4; p < 0.0001). Overall, 22 phyla (Archaea and Bacteria domains), 64 classes, 111 orders, 203 families, and 365 genera were found within the 15 analysed soil samples. Several of the most abundant

bacterial classes are shared between soil and grape samples (i.e. Alphaproteobacteria, Gammaprotecobacteria, Actinobateria, and Betaproteobacteria) (Figure 3A). However, microorganisms belonging to Bacilli and Clostridia (Firmicutes) occur more frequently on grape surface then in soil samples.

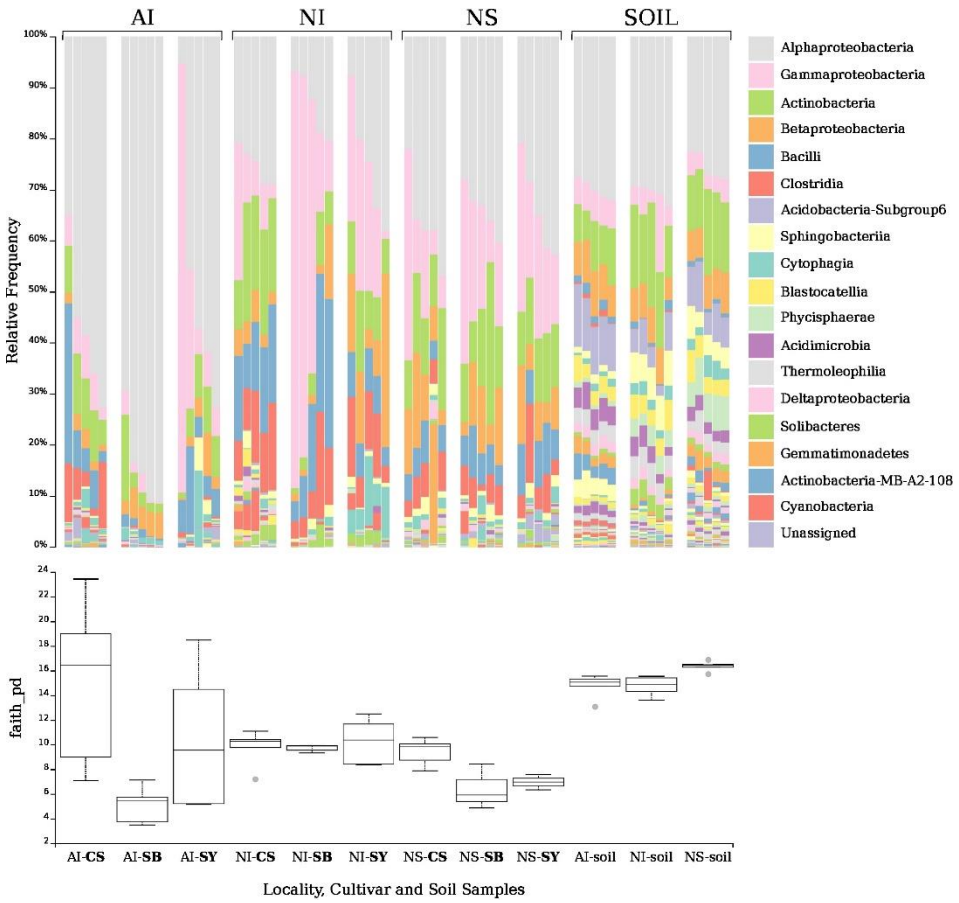

Figure 3: Barchart analysis depicting the relative abundance and distribution of the OTUs assigned to class taxonomic rank **(A)**. The legend lists the 18 most abundant Classes. Boxplots **(B)** show the Faith's Phylogenetic Diversity (a qualitative measure of community richness incorporating phylogenetic relationships) for each cultivar's grape and soil sample at each sampling site.

Results confirmed that some samples of NS (Sauvignon Blanc) had a microbial diversity lower than others (Figure 3B). Moreover, the abundance of some bacterial classes such as Clostridia (Firmicutes) was lower in NS samples of Sauvignon Blanc and Syrah (Figure 3, Pairwise Krustal-Wallis test results are reported in Supplementary Data S3).

In general, the microbiome differences among cultivars and localities are attributed to the less abundant OTUs. To assess which bacterial genera were exclusive of a certain cultivar and/or locality and to evaluate the influence of soil bacteria in modelling grape microbiome, we estimated the portion of shared genera between soil samples and related grape cultivars at each sampling site.

Venn diagrams confirmed that each cultivar shared almost 60% of genera with soil (Figure 4A). Specifically, in the case of AI the number of soil genera shared with cultivars ranged from 186 in CS to 123 in SB. At NI, they ranged from 189 in CS to 139 in SY and at NS, and 188 and 93 genera were shared between soil and CS and SB respectively. Interestingly, some unique microbial traits were found. Most bacterial genera were shared by all cultivars but were exclusive to a certain sampling locality; however, other genera were unique to single cultivars. For example, in the case of AI, 27 genera were shared among the three cultivars but not with the soil microbiome, while 7, 18, and 6 were unique to SB, CS, and SY, respectively. A similar situation occurred for NI vineyards where 20 bacterial genera were shared among the three cultivars while 8, 6, and 14 were unique to SB, CS, and SY respectively.

Concerning NS, 17 genera were shared among the three cultivars while 2, 7, and 11 were unique to SB, CS, and SY, respectively (Figure 4A).

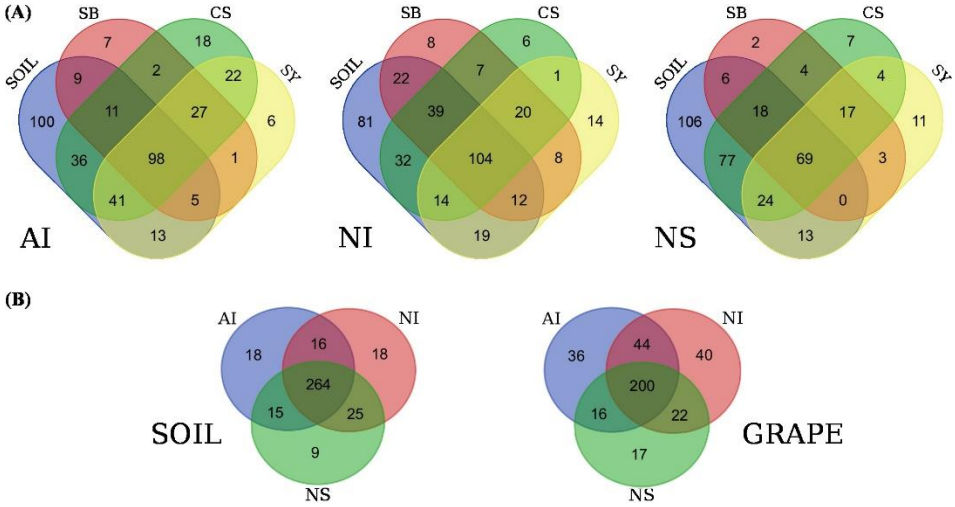

Figure 4. Venn diagrams showing the number of shared bacterial genera among grape cultivars and soil at each locality **(A)**. In **(B)**, the diagrams show the number of shared bacterial genera among soil samples and grape samples (regardless of cultivar) from the three localities.

Figure 4B shows the number of shared bacteria genera among all soil samples and among grape samples (regardless of cultivar). Overall, data show that soils of the three localities share a greater proportion of bacterial genera than those shared among cultivars at the same site. At the taxonomic level, grape samples of the three localities show 36, 40, and 17 genera exclusive to AI, NI, and NS respectively. The complete list of these particular bacteria is reported in Supplementary Data S4.

6.4.4 Machine learning analysis

A random forest was used as supervised learning classifier to predict cultivar identity and provenance of a certain grape sample based on its microbiome composition. Taxonomic diversity at the genus level was used as a trainer for the classifier. At the geographical level, the comparison between 'true label' vs 'predicted label' showed the highest probability to correctly predict the geographical origin of NS and NI grape samples, while the overall accuracy reached a value of 0.75 in the case of AI accessions (Figure 5A).

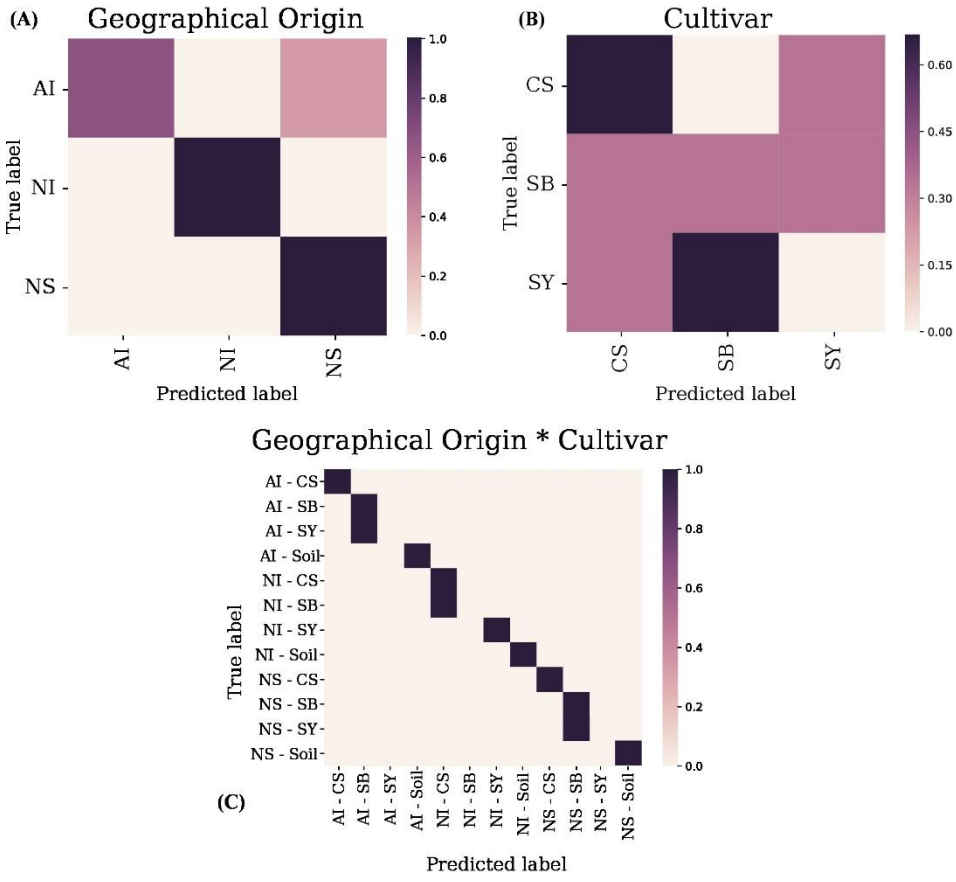

Figure 5: Machine learning analysis performed at geographical level **(A)**, Cultivar identity **(B),** and the combination of two factors **(C)**. Features table was collapsed to genus level. Overall accuracy levels are indicated as a scatter plot showing predicted vs. true values for each tested sample, along with a linear regression line fitted to the data with 95% intervals (grey shading).

Conversely, the prediction level for cultivar identity showed higher uncertainty with the only exception of CS which reached about 0.6 of overall accuracy (Figure 5B). When combining the two factors (i.e., geographical origin and cultivar identity), the machine learning tool correctly predicted 9 cases out of 12 with high accuracy (Figure 5C). Accuracy values for each of the three tested models are reported in Supplementary Data S5.

6.5 Discussion

6.5.1 Vineyards as a complex and dynamic ecosystem

This study supports the hypothesis that vineyard soil represents a primary reservoir for grape associated bacteria, most of which are involved in processes ranging from plant nutrition and development to the modification of grape and wine quality (Bokulich et al. 2016; Belda et al., 2017). For example, in our samples, several members of Alphaproteobacteria (e.g., Rhizobiales, Rhodobacterales, Sphingomonadales) were shared between the soil and grape at each investigated locality. Rhizobiales (e.g., *Bradyrhizobium*) contribute to plant nutrition, since they are involved in nitrogen fixation. Although in many cases these bacteria form root nodules, some species may be found in other plant portions and could provide nutrients to the plant even

though it lacks nodules (Bacon and White, 2016). Among Rhodobacterales and Betaproteobacteria, we detected members of *Craurococcus* (Acetobacteraceae) and *Massilia* that are involved in the metabolism of phosphate and in plant growth promotion respectively (Ofek et al., 2012; Kecskeméti et al., 2016). Other important microbial protagonists found on the grape surface and belonging to soil were Bacillales and Clostridiales (Zarraonaindia et al., 2015). In some cases, the occurrence of these bacteria is related to the fertilization strategy, including the use of manure (Ding et al, 2014). Their role on fruit it is not clear yet; however, it is known that these microorganisms also persist during vinification, thus it is expected that they can influence fermentation processes and wine quality (Piao et al., 2015). Furthermore, the soil bacteria *Methylobacterium* and *Gluconobacter* (Alphaproteobacteria) were also found in our grape samples at all the investigated localities. These are expected to play an important role during wine fermentation depending on the developmental phase of grape at the moment of harvest and could affect wine quality as well.

Given the pivotal and renowned importance of the soil microbiome in the era of precision agriculture, any tool able to enhance the occurrence of key microorganisms on grape surfaces could really have an impact on wine quality. For example, Martins (2013) suggested that soil bacteria could easily reach the grape surface during rain or when transported by wind (Martins et al. 2013). Therefore, the currently adopted precision irrigation systems could enhance or reduce soil microorganism colonization rate (Campos et al., 2000) and favour the movement of bacteria from soil to the fruit. Other practices, such as the use of cover

crops could also influence soil microbial ecology and indirectly grape microbiome (Ingels et a., 2005).

The findings discussed here provide new information concerning the microbial diversity of vineyard soils. Microbial diversity was high in our samples, but there were a few differences among the three geographically distant sampling areas. Particularly, differences were due to bacteria involved in processes such as degradation organic matter (e.g., *Azoarcus*) and fertilization (e.g. *Larkinella*). Moreover, parasitic bacteria (e.g. *Burkholderia* and *Serratia*) also occurred. This condition agrees with the idea that vineyard soils could share a core of bacteria but differ in those microbial groups more influenced by the biotic/abiotic factors of the vineyard, including farming management (Pinto and Gomes, 2016). In general, these 'extra-core' bacteria do not largely influence the grape microbiome. For example, among the 36 microbial genera unique to AI grape samples, only 3 (*Luteibacter*, *Spirosoma* and *Taibaiella*) were shared with the soil. In the cases of NI and NS samples, we did not find any shared microbial genus between soil and grape within those bacterial genera exclusive to each site. Conversely, the microbial core of soil could have a greater influence on the bacterial genera on grape at each locality, as we found from 41% to 88% genera of AI and NI grape respectively, to belong to soil-core OTUs. The remaining genera could have an extra-soil origin. Some of these bacteria (e.g., *Wolbachia*, *Cardinium*, *Rickettsia*, and *Hamiltonella*) could belong to arthropods in vineyards (Delort and Amato, 2017), thus supporting the hypothesis of a functional role played by local biodiversity in transferring microbial organisms to the grape (Gilbert et al., 2014). Vineyard structure and management could

indirectly act on the process of microbial transfer by influencing the communities of potential vectors inhabiting this agroecosystem, such as insects (Sanguankeo and León, 2011; Caprio et al. 2015) and birds (Assandri et al., 2017a; 2017b), at a multilevel scale. These animals use the vineyard as part of their home range since trophic and reproductive niches favouring the introduction of microbes also belong in surrounding habitats.

6.5.2 The passive role of the grapevine in the selection of the fruit microbiome

One of the aims of this study was to evaluate the role of the grapevine in selecting the epiphytic microbial community of grape berries. We hypothesized that when microorganisms reach the berry, they establish and start to interact with fruit skin. These dynamics occur between the external waxy layer (bloom) of the berry, which is useful for preventing water loss through evaporation, and the hypodermis layer (Knoche and Lang, 2017). It is known that the number of skin layers of grape berries and their thickness are cultivar-specific. Although in our case, the thickness of the three selected cultivars was similar, the natural waxy coat of CS is more abundant in comparison to SB, while SY shows an intermediate value (OIV, 2015). These physical features could influence the contact and permeability of the grape berry cuticle to different microorganisms as observed for some pathogens, such as *Botrytis cinerea* (Herzog et al., 2015). Moreover, also the occurrence of anthocyanins could have a role in shaping the grape microbiome due to the antimicrobial properties of this group of molecules (Cisowska et al., 2011; Apolinar-Valiente et al., 2017). In this study, anthocyanins occur

only in the two dark berry cultivars in CS and SY (Table 1). Concerning bunch features, the three cultivars showed different densities, sizes, and shapes (Table 1); therefore, we expected that these traits could also have an influence on the access and permeability of microorganisms to the bunch. However, our results do not support this hypothesis. The PCoA and the machine learning analysis support the evidence that geographical origin plays a major role in selecting the microorganisms on grape surfaces rather than the plant ampelometric characteristics. Therefore, we suggest that the plant should be considered passive in selecting its fruit ectophytic bacteria. Probably, local environmental conditions combined with agronomic management characteristics are more able to modify the berries microbiome, at least much more than the genetic characteristics of plants. This could explain why all grape cultivars at each locality shared a different fraction of soil core bacteria.

6.6 Conclusions

In the past, grapevine management and wine production exploited the experience and knowledge of wine growers and enologists who worked to optimize production based on agronomic and chemical parameters. Although the general principles of fermentation were known, wine organoleptic properties were usually attributed to the geographical origin of grape. In the last few years, the development and higher affordability of HTS technologies allowed a better understanding about the microbial dynamics involving the grapevine, from the field to the barrel. By taking advantage of HTS technologies in this study, we here suggest a key-role of soil and vineyard biodiversity and a passive, marginal role of the grapevine in influencing the grape microbiome. Although this kind of

research could provide valuable information on wine origin, the interpretation of HTS microbiome data deserves caution, because there are still unknown interactions between plants and environmental microorganisms. Further difficulties reside in the possibility of recovering a large amount of data that is representative of seasonal and geographical changes. It should also be highlighted that the analytical potential of molecular tools and the standardization of bioinformatics pipelines combined with the emerging machine learning approaches offer new opportunities to develop wider and integrated research activities to test which variables have an effective role in shaping microbiome composition and dynamics over time and space. These perspectives will also permit an efficient integration with metabolome features of grapevine accessions to uncover the intimate sensorial characteristics of grapes and wine.

6.7 Supporting Information

Supplementary_Data_S1.csv

OTUs Assignment. For each unique OTU, (Feature ID), assigned Taxonomy and the confidence value of the assignment are reported.

Supplementary_Data_S2.csv

PERMANOVA pairwise results considering as response variable Bray-Curtis dissimilarity matrix.

Supplementary_Data_S3.csv

Krustal-Wallis pairwise test with measure of Faith PD metric as response variable. Samples are clustered for Cultivar and Geographic Origin.

Supplementary_Data_S4.csv

Multi-sheet file including all the Venn diagram results. The results of each diagram depicted in Figure 4 are reported in a separate sheet.

Supplementary_Data_S5.csv

Machine learning overall accuracy. Classification accuracy results for the three tested models showed in Figure 5.

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7. DISCUSSION AND CONCLUSIONS

7.1 *Vitis vinifera* L. microbiome: from grape to wine.

In the last decades, food science has greatly developed and the concept of food itself has shifted, from considering it as a mere source of energy to a growing awareness on its importance for health prevention. Such vision led to an increasing attention towards the origin and quality of the raw materials and their derived food products. The continuous advances in molecular biology allowed setting up efficient and universal 'omics' tools to unequivocally identify the origin of food and its traceability. In this PhD project, I adopted bio-molecular techniques such as DNA barcoding and metabarcoding to characterize the composition and traceability of foodstuffs.

I took advantage of the study of variability at conserved genomic regions, also using high-throughput technologies, to study microbial communities of grape and wine. It is important to underline that similar strategies can also be used to characterize several other matrices and food ecosystems (e.g., dairy, fish, and meat products).

In contrast to environmental microbiology analysis, a few studies have been conducted to identify the metabolic pathways and active compounds of food, also considering chemical modification during food transformation processes [1-3]. A more detailed knowledge on the role of different microorganisms in food and their metabolic pathway would help in enhancing food production processes and quality and extending the product shelf-life.

My study was mainly focused on grapevine. In the last twenty years, many studied applied DNA markers to characterize grapevine features and to define the relationships among different cultivars [4, 5]. However, in most

cases, cultivar identification does not represent the key element to characterize wine and to connect this food commodity with its geographical origin. In this context, I studied the microbial grape ecosystem. Grape harbours a wide range of microbes originating from the vineyard environment, many of which play a critical role in grape health and quality, which decisively influence the winemaking process.

The first aim of my research project was to better clarify the contribution of grape's microbiome during wine fermentation. To achieve this ambitious goal, I used HTS technologies to identify bacterial and fungi communities associated with berries and musts of Cannonau, the most important cultivar-wine of Sardinia (Italy) where most vineyards are cultivated without phytochemical treatments. Previously, our research group evaluated the genetic structure of grapevine germplasm of Sardinia, even in relation to Spanish cultivars [6]. Such comparison showed many genetic identities, suggesting the occurrence of several cases of synonymy, and one of these is between Cannonau and 'Garnacha Tinta' that share the same SSR profile. However, while Cannonau is one of the core products of Sardinian wine industry, Garnacha Tinta, in Spain, is a blending wine. Such a condition lead to the following question: does the genetic identity of grape correspond to wine identity? Clearly, the genetic origin of cultivars is only one of the many elements useful for characterizing wine. Wine quality relies on a precise equilibrium among several factors and it is largely influenced by the environmental conditions of the production area.

The findings of my first research case suggested that microbiomes of berries collected at four different localities share a core composition characterized by Enterobacteriales, Pseudomonadales, Bacillales, and

Rhodospirillales. However, any area seems to enrich berries microbiome with peculiar microbial traits. For example, berries belonging to the biodynamic vineyards of Mamoiada (Sardinia) were rich in Bacillales typical of manure (i.e. *Lysinibacillus*, *Bacillus*, and *Sporosarcina*), whereas in another Sardinian locality, Santadi, berries showed soil bacteria such as Pasteurellales and Bacteroidales as well as Rhodospirillales and Lactobacillales which are commonly involved in wine fermentation. In the case of fungi, the most abundant taxa were Dothioraceae, Pleosporaceae, and Saccharomycodaceae, and although the proportion of these families varied among localities, they occurred ubiquitously in all vineyards. During vinification processes, performed at the same wine cellar under controlled conditions and without using any yeast starter, more than 50% of bacteria groups of berries reached musts, and each locality had its own private bacteria signature, even if *Saccharomyces cerevisiae* represented the most abundant fungal species.

This analysis suggests that natural berries microbiome could be influenced by pedoclimatic and anthropologic conditions (e.g., farming management), and fruit microorganisms persist during the overall fermentation process.

I also demonstrated a distinct microbial composition of *Cannonau* fruit from different Sardinian localities with consequent effects also on the musts' microbiome. To date, the role of grapevine microbes in the field has been largely ignored, with the only exception of microbial pathogens, mainly because the available technologies did not exist, and this prevented examining the community structure of the huge number of bacteria and fungi species associated to each plant at any real depth or breadth [7]. Using HTS approach, I succeeded in evaluating the microbial community of grape and wine samples, also in response to different environmental

conditions and farming practices [8]. Probably, in the very next future, this technology will be also used to deeply investigate viruses and phytoplasma that largely affect the vineyard sector [9].

Emerging researches clearly showed that pedoclimatic conditions can affect wine characteristics not only due to the abiotic characteristics (e.g., soil and sun exposition), but also at the grape microbiome that is able to influence plant health, growth and development [10]. Bokulich [11] demonstrated the existence of regional microbiome fingerprints in California vineyards; Portillo [12] showed that several environmental variables, such as vineyard altitude and the geographical orientation of the plant could also affect the grape microbiome. My study confirms that pedoclimatic characteristics have a real influence on fruit microbiome and underlines that agricultural practices, such as biodynamics, as well as the occurrence of opportunistic insects, such as hymenopterans, can have a consistent effect on the bacterial communities of berries and corresponding must. Like previous researches [11, 12], this study showed non-random distribution of grape bacteria across different vineyards, which allows proposing that these peculiar microbial traits could be used to obtain specific wine organoleptic features and naturally enforce distinctive *terroir* characteristics in local winery production. Moreover, these results suggest that the role of the field environmental microbiome is not limited to promoting grape fruit ripening and enhancing the occurrence of some secondary metabolites strictly related to wine color and flavor, but it is also an important source of microorganisms that are able to influence wine fermentation and metabolic composition. The diversity and abundance of microorganisms that are able to establish in a niche in the soil and on the vine will determine both the grape's quality and the variability of

microorganisms that will enter the winemaking steps, thus affecting the final products [10]. Taking into account microbiome information, it could be possible to prevent fermentation problems, volatile acidity increases, *Brettanomyces* contamination and biogenic amines production, other than reducing chemical treatments and perform them only when necessary, especially for particular items such as organic wine.

7.2 Effects of vineyard environmental features and cultivar on grape microbiome.

In the case of the grapevine, the role of plant microbiota is very relevant to wine production as it seems to be correlated to the concept of 'terroir'. The significant regional differences in vineyard biodiversity were hypothesized to be responsible for regional differences in wine style and character, commonly referred to as the microbial aspect of the 'terroir' concept [11]. However, despite a number of studies explored this issue [7, 13], an important aspect about grape microbiome still remains unknown or, at least, poorly investigated. This regards the potential active role played by grape cultivars and plant organs in modelling their microbial community. To shed light on this topic, I investigated, during my PhD project, the relationship between the plant genotype and its microbiome considering field environmental characteristics and pedoclimatic conditions. I performed 16S metagenomic analysis of three different grapevine cultivars Sauvignon Blanc, Syrha, Cabernet Sauvignon, and related soil samples, from three different geographical area of the Mediterranean basin, Pavia and San Michele all'Adige (Italy) and Logroño (Spain). The HTS output showed peculiar

bacterial profiles and led to clarify the correlation between plant, fruit microbiome and the environment.

The bioinformatics analysis supported a significant correlation between microbiome of grape samples of the same area, underlining a strong effect of vineyard and a lower contribution of cultivar genetic identity. This was further emphasized by the relevance of about 60% of bacterial genera shared by soil and grape samples belonging to the same vineyard, supporting the hypothesis that soil represents a primary reservoir for grape associated bacteria, most of which are involved in processes ranging from plant nutrition and development to the modification of grape and wine quality [14, 15]. In fact, some of bacteria shared between grape and soil are involved in nitrogen fixation and metabolism of phosphate. Some other bacteria are related to the fertilization strategy, including the use of manure [16]. Although their role on fruit it is not clear, however, it is known that these microorganisms also persist during vinification, thus it is expected that they can play an important role during wine fermentation and could affect wine quality as well [17].

Bringing microbial ecology into agriculture represents an innovative way to provide mechanistic understanding for observations that farmers and viticulturists have been making for millennia [7]. Since the soil microbiome has a great importance, the current precision agriculture can use any tool able to enhance the occurrence on grape of those microorganisms with a positive impact on wine quality. For example, we ascertained that soil bacteria could reach the grape surface during rain or when transported by wind [18]. Therefore, the currently adopted precision irrigation systems and practices like the use of cover crops could also influence soil microbial ecology and indirectly grape microbiome [19, 20].

The findings discussed here provide new information concerning the microbial diversity of vineyard soils. Microbial diversity was high in our samples, but there were a few differences among the three geographically distant sampling areas. This condition agrees with the idea that vineyard soils could share a core of bacteria but differ in those microbial groups more influenced by the biotic/abiotic factors of the vineyard, including farming management [21]. At each locality, the microbial core of soil could have a greater influence on the bacterial genera on grape, as we found about 60% genera belonging to soil. The remaining genera could have an extra-soil origin: some of these bacteria (e.g., *Wolbachia*, *Cardinium*, *Rickettsia*, and *Hamiltonella*) could belong to arthropods in vineyards [22], thus supporting the hypothesis of a functional role played by local biodiversity in transferring microbial organisms to the grape [7]. Vineyard structure and management could indirectly act on the process of microbial transfer by influencing the communities of potential vectors inhabiting this agroecosystem, such as insects [23, 24] and birds [25, 26], that use the vineyard as part of their home range favouring the introduction of microbes.

In this study I wanted also to evaluate the role of the grapevine in selecting the epiphytic microbial community of grape berries. I hypothesized that when microorganisms reach the berry, they establish and start to interact with fruit skin. These dynamics occur between the external waxy layer (bloom) of the berry, which is useful for preventing water loss through evaporation, and the hypodermis layer [27]. It is known that the number of skin layers of grape berries and their thickness are cultivar-specific. Although in this case, the thickness of the three selected cultivars was similar, the natural waxy coat of Cabernet Sauvignon is more abundant in

comparison to Sauvignon Blanc, while Syrah shows an intermediate value [28]. These physical features could influence the contact and permeability of the grape berry cuticle to different microorganisms as observed for some pathogens, such as *Botrytis cinerea* [29]. Moreover, also the occurrence of anthocyanins could have a role in shaping the grape microbiome due to the antimicrobial properties of this group of molecules [12, 30]. In this study, anthocyanins occur only in the two dark berry cultivars (Cabernet Sauvignon and Syrah). Concerning bunch features, the three cultivars showed different densities, sizes, and shapes; therefore, I expected that these traits could also have an influence on the access and permeability of microorganisms to the bunch. However, the results do not support this hypothesis, but showed the evidence that geographical origin plays a major role in selecting the microorganisms on grape surfaces rather than the plant ampelometric characteristics. Therefore, I suggest that the plant should be considered passive in selecting its fruit ectophytic bacteria. Probably, local environmental conditions combined with agronomic management characteristics are more able to modify the berries microbiome, at least much more than the genetic characteristics of plants.

7.3 Grapevine as holobiont.

Characteristics of the cultivars' genotype play an important role in viticulture, thus starting from the 1990's, DNA fingerprinting approaches were used to identify synonymous cultivars and to unmask incorrect attributions [31, 32]. Our team [6] demonstrated a complete genetic identity between Cannonau and Spanish Grenache by using SSR markers. However, the microbiomes of these cultivars are very different, and

appreciable differences were also observed among Grenache localities. This finding suggests that the value of the cultivar genotype is somewhat relative. Overall, a reliable genotyping should include the entire holobiont (i.e. the plant and all its symbionts [33]) of a specific Cannonau or Garnacha plant. The study of grape microbiome does not represent a simple element to achieve the product's traceability and identity. We should consider that bioprospecting activities on grape microbiota could lead to improved viticulture yields and wine quality, through the discovery of several microbial species with positive enological properties, as recently documented by the WineSeq® project [34]. Occurrence and abundance of these species could be easily monitored by using conventional cultivation strategies and target PCR approaches (Real-Time and Digital PCR) [35, 36] to improve wine quality, to enhance immune capability, and reduce the use of agrochemicals. Nowadays, only an exhaustive knowledge about the vineyard, the winery and their inhabitants could permit real advancements in management activities aimed at creating more sustainable systems without any loss in terms of yields and product quality.

In the past, grapevine management and wine production exploited the experience and knowledge of wine growers and enologists who worked to optimize production based on agronomic and chemical parameters. Although the general principles of fermentation were known, wine organoleptic properties were usually attributed to the geographical origin of grape. In the last few years, the development and higher affordability of HTS technologies allowed a better understanding about the microbial dynamics involving the grapevine, from the field to the barrel [37-39]. By taking advantage of HTS technologies in this study, I here suggest a key-

role of soil and vineyard biodiversity and a passive, marginal role of the grapevine in influencing the grape microbiome.

Also the machine learning analysis supports the evidence that geographical origin has a major influence on microbial composition of grape surfaces. A random forest was used to predict cultivar identity and provenance of a certain grape sample based on its microbiome composition. At the geographical level, the comparison showed the highest probability to correctly predict the geographical origin of grape samples. Conversely, the prediction level for cultivar identity showed higher uncertainty. When combining the two factors (i.e., geographical origin and cultivar identity), the machine learning tool correctly predicted 9 cases out of 12 with high accuracy.

Although our research could provide valuable information on wine origin, the interpretation of HTS microbiome data deserves caution, because there are still unknown interactions between plants and environmental microorganisms. Further difficulties reside in the possibility of recovering a large amount of data that is representative of seasonal and geographical changes. It should also be highlighted that the analytical potential of molecular tools and the standardization of bioinformatics pipelines combined with the emerging machine learning approaches offer new opportunities to develop wider and integrated research activities to test which variables have an effective role in shaping microbiome composition and dynamics over time and space. For example, to test if edaphic factors, climate, and vineyard structure and management influence microbiome composition, or if other sources of bacteria like water, surrounding plants, animals that live or pass through the vineyard could play a significant role on the final microbiome of grape. These additional information will help

defining a comprehensive vision on the complex network of interactions involving the crop, the territory and the deriving food items. For viticulture and other agricultural fields where the crop is also associated to particular flavor properties that may also be manipulated, understanding how the bacteria, fungi and viruses influence the development and hence chemical makeup of the crop is essential.

In conclusion, the approach used and proposed in this PhD study can represent a reliable starting point for improving viticulture management and wine production, but also for agriculture and agro-food industry. This study represents a step forward in the context of agro-food geographical traceability. Additionally, in accordance with an industry 4.0 vision, plant and soil microbiome analyses can represent a valuable tool for defining appropriate farming practices and for monitoring agriculture productivity. Citing Gilbert and co-authors, "*There is a long way to go, but such work provides us tantalizing evidence that the biogeographic characteristics of terrestrial microorganisms may indeed lead to regionalized properties associated with valuable crops. Future work will build on this ecological observation to change the face of agriculture, much as the human microbiome is changing the face of medicine*" [7].

7.1 References

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8. OTHER SCIENTIFIC CONTRIBUTIONS

8.1 Toward a better understanding of *Apis mellifera* and *Varroa destructor* microbiomes: introducing "*PhyloH*" as a novel phylogenetic diversity analysis tool

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8.2 Evaluation of the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains and their in vitro effect

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8.3 Chemical, molecular, and proteomic analyses of moss bag biomonitoring in a petrochemical area of Sardinia (Italy)

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8.4 Evaluating the efficacy of restoration plantings through DNA barcoding of frugivorous bird diets

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8.5 A Randomized, Double-Blind, Placebo-Controlled Trial: The Efficacy of Multispecies Probiotic Supplementation in Alleviating Symptoms of Irritable Bowel Syndrome Associated with Constipation

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8.6 Poisonous or non-poisonous plants? DNA-based tools and applications for accurate identification

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8.7 Orally administered multispecies probiotic formulations to prevent uro-genital infections: a randomized placebo-controlled pilot study

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8.8 A DNA barcoding approach for identifying species in Amazonian traditional medicine: The case of Piri-Piri

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8.9 DNA Barcoding Meets Nanotechnology: Development of a Universal Colorimetric Test for Food Authentication

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