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Metagenomic Characterisation of Microbial Communities in Different Food Samples

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[1] Iorizzo M., **Bagnoli D.**, Vergalito F., Testa B., Tremonte P., Succi M., Pannella G., Letizia F., Albanese G., Lombardi S.G. and Coppola R. (2024). Diversity of Fungal Communities on Cabernet and Aglianico grapes from vineyards located in Southern Italy. *Frontiers in Microbiology;* 15:1399968.

[2] Letizia F., Albanese G., Testa B., Vergalito F., **Bagnoli D.**, Di Martino C., Carillo P., Verrillo L., Succi M., Sorrentino E., Coppola R., Tremonte P., Lombardi S.J., Di Marco R., Iorizzo M. (2022). *In vitro* assessment of bio-Functional properties from *Lactiplantibacillus plantarum* strains. *Current Issues in Molecular Biology;* 44(5), 2321-2334.

ABSTRACT

Foods are real ecosystems colonised by distinct microbial populations that, under the selective action of ecological determinants, can modify their composition by establishing interaction mechanisms that are usually only partially detectable by traditional microbiological methods of analysis. In this context, the use of culture-independent techniques, such as Next Generation Sequencing (NGS), represents an important tool for the study of microbial communities in food. Such methods are now considered complementary and supplementary to traditional culture-dependent approaches, which, while highly valuable for characterising isolated microorganisms, do not provide a complete insight into the overall composition of microbial communities. With these considerations in mind, two different food matrices were chosen during the three years of research conducted as part of the PhD course to investigate the power of culture-independent approaches for the evaluation and study of microbial consortia and their changes as a function of environmental factors.

Specifically, through high-throughput sequencing (HTS) methods, in Chapter 2 the fungi inhabiting the surfaces of grape berries of two cultivars (Cabernet and Aglianico) from different Italian vineyards located in Southern Italy were investigated, and in Chapter 3 the composition of microbial communities in sheep milk from three different dairy farms located in the Campania region were studied.

The study of fungal diversity on the surface of grape berries delineated taxa at various taxonomic levels, including *phyla* (Ascomycota, Basidiomycota and Chytridiomycota), families (Cladosporiaceae, Saccotheciaceae, Pleosporaceae, Saccharomycodaceae, Sporidiobolaceae, Didymellaceae, Filobasidiaceae, Bulleribasidiaceae and Saccharomycetaceae) and genera (*Cladosporium*, *Aureobasidium*, *Alternaria*, *Stemphylium* and *Filobasidium*). Multivariate analysis of the data revealed that some taxa are associated with specific vineyard ecosystems, with a significant impact of "vineyard location" on the composition of the fungal community, whereas the "grape variety" showed no significant effect. Furthermore, some fungi may contribute positively as fermentative yeasts, while others may have detrimental effects, potentially causing wine spoilage or vine pathogenesis. The study also clarified the main functions of the fungi core taxa, whose role in the grapevine environment is still poorly investigated. This research was recently published in the international journal *Frontiers in Microbiology*, as reported in Chapter 2.

The second study analysed some chemical features of sheep milk from the Campania region and their correlation with bacterial composition using an HTS approach. The comparative analysis of milk samples from Avellino, Caserta and Salerno provinces revealed that Avellino milk has significantly higher levels of iron, potassium and zinc. Metagenomic classification consistently identified Proteobacteria as the dominant *phylum*, followed by Firmicutes and Bacteroides. Milk from Avellino province showed a greater diversity of families and genera

than that from Caserta and Salerno provinces. The presence of metals influenced the diversity of the microbiome, with higher metal concentrations correlating with a greater diversity of species, genera and "univocal species", suggesting the influence of environmental characteristics on the microbial composition of ewe milk. Chapter 3 contains the draft of the work that will be submitted for publication in an international journal.

The final part of this PhD thesis describes the training and research activities carried out under the supervision of prof. Franca Fraternali at the Institute of Structural and Molecular Biology (ISMB), University College London (UCL), as part of a larger study concerning the emerging probiotic *Akkermansia muciniphila*.

The study of the *A. muciniphila* strain ATCC BAA-835, a gut-colonising symbiotic organism with probiotic potential, involved the simulation of gastrointestinal (GI) transit and adhesion to three different human intestinal cell lines (CaCo2, HT-29 and HT-29-MTX) at various concentrations (2 to 8 log CFU/mL). As an alternative to conventional tests, which involves separate tests, a simulation of GI transit followed by adhesion to the cell lines was also performed. The results were compared with those of *Lacticaseibacillus rhamnosus* GG ATCC 53103, a well-studied probiotic strain. *A. muciniphila* showed significantly higher survival rates after transit (about 8 log CFU/mL) than *L. rhamnosus* GG (about 3 log CFU/mL). The adhesion assays showed a trend inversely proportional to bacterial concentration. The decrease in adhesion rates for both strains after transit highlights that GI transit alters adhesion properties *in vitro*, suggesting that performing sequential assays may be a valid alternative to conventional methods of probiotic evaluation. The study is completed by an overview of protein interactions of *A. muciniphila* with human mucins, perfrmed by using different databases and softwares (GenBank and Uniprot, PSI-BLAST and CytoScape) to identify proteins with sequences homologous to *A. muciniphila* proteins involved in mucin degradation. Chapter 4 contains the draft of the work that will be submitted for publication in an international journal.

Keywords: Food, Next Generation Sequencing, Microbiome, Environmental Factors, Probiotic.

RIASSUNTO

Gli alimenti sono veri e propri ecosistemi colonizzati da popolazioni microbiche distinte che, sotto l'azione selettiva di determinanti ecologici, possono modificare la loro composizione instaurando meccanismi di interazione che di solito sono solo parzialmente rilevabili dai tradizionali metodi di analisi microbiologica. In questo contesto, l'uso di tecniche coltura-indipendenti come NGS rappresenta uno strumento importante per lo studio delle comunità microbiche negli alimenti. Tali tecniche sono oggi considerate complementari e integrative rispetto ai tradizionali metodi coltura-dipendenti che, pur essendo estremamente utili per la caratterizzazione dei microrganismi, non forniscono informazioni complete sulla composizione delle intere comunità microbiche. Alla luce di queste considerazioni, sono state scelte alcune matrici alimentari per indagare la capacità degli approcci coltura-indipendenti di valutare i consorzi microbici e i loro cambiamenti in funzione di differenti fattori ambientali.

In particolare, attraverso metodi HTS, nel Capitolo 2 sono state studiate le comunità fungine presenti sulla superficie di bacche di uva di due cultivar (Cabernet e Aglianico) provenienti da diversi vigneti del Sud Italia, mentre nel Capitolo 3 è stata studiata la composizione delle comunità microbiche di campioni di latte di pecora provenienti da tre diverse aziende casearie situate nella regione Campania.

Lo studio volto a valutare la diversità fungina delle bacche d'uva ha delineato i *taxa* a vari livelli tassonomici, tra cui *phyla* (Ascomycota, Basidiomycota e Chytridiomycota), famiglie (Cladosporiaceae, Saccotheciaceae, Pleosporaceae, Saccharomycodaceae, Sporidiobolaceae, Didymellaceae, Filobasidiaceae, Bulleribasidiaceae e Saccharomycetaceae) e generi (*Cladosporium*, *Aureobasidium*, *Alternaria*, *Stemphylium* e *Filobasidium*). L'analisi multivariata dei dati ha rivelato un impatto significativo della "posizione dei vigneti" sulla composizione delle comunità fungine, mentre il "vitigno" non ha mostrato effetti significativi. Ciò suggerisce che alcuni *taxa* sono associati a specifici ecosistemi legati alla localizzazione dei vigneti piuttosto che alla varietà delle uve. Lo studio ha evidenziato la presenza sia di lieviti fermentativi sia di funghi dannosi, questi ultimi in grado di causare il deterioramento del vino o la patogenesi della vite. La ricerca ha anche chiarito le funzioni principali dei principali *taxa* fungini rilevati, il cui ruolo è ancora poco conosciuto. Questa ricerca è stata recentemente pubblicata sulla rivista internazionale *Frontiers in Microbiology*, come riportato nel Capitolo 2.

Il secondo studio ha analizzato alcune caratteristiche chimiche del latte di pecora proveniente da allevamenti della regione Campania e le correlazioni con i consorzi microbici, investigati attraverso un approccio HTS. L'analisi comparativa dei dati sui campioni di latte provenienti dalle province di Avellino, Caserta e Salerno ha rivelato che il latte di Avellino presentava livelli significativamente più elevati di ferro, potassio e zinco. La classificazione metagenomica ha identificato i Proteobacteria come *phylum* dominante, seguiti da Firmicutes e Bacteroides. Il

latte di Avellino ha mostrato una maggiore diversità di famiglie e generi rispetto ai latte delle province di Caserta e Salerno. L'analisi dei dati ha evidenziato che la presenza di metalli ha influenzato la diversità del microbioma, con una maggiore diversità di specie, generi e "specie uniche" correlate a concentrazioni di metalli più elevate. Infatti, il latte di Avellino ha mostrato un numero maggiore di specie uniche, suggerendo l'influenza delle caratteristiche ambientali sulla sua composizione microbica. Nel Capitolo 3 è riportata la bozza del lavoro che sarà sottomesso per la pubblicazione su rivista internazionale.
La parte finale di questa tesi di dottorato descrive le attività di formazione e ricerca svolte sotto la spervisione della professoressa Franca Fraternali presso l'UCL di Londra, nell'ambito di uno studio più ampio riguardante il probiotico emergente *Akkermansia muciniphila*.
Lo studio del ceppo *A. muciniphila* ATCC BAA-835, un organismo simbiotico che colonizza l'intestino, studiato per il suo potenziale probiotico, ha previsto la simulazione del transito gastrointestinale (GI) e l'adesione a tre diverse linee cellulari intestinali umane (CaCo2, HT-29 e HT-29-MTX) a varie concentrazioni (da 2 a 8 log CFU/mL). In alternativa ai test convenzionali, che prevedono saggi separati, è stata effettuata anche una simulazione del transito GI seguita dall'adesione alle linee cellulare. I risultati sono stati confrontati con quelli di *Lacticaseibacillus rhamnosus* GG ATCC 53103, un ceppo probiotico ben studiato. *A. muciniphila* ha mostrato tassi di sopravvivenza significativamente più elevati dopo il transito (circa 8 log CFU/mL) rispetto a *L. rhamnosus* GG (circa 3 log CFU/mL). I saggi effettuati hanno inoltre evidenziato che l'adesione è inversamente proporzionale alla concentrazione batterica. La diminuzione dei tassi di adesione per entrambi i ceppi dopo il transito evidenzia che quest'ultimo altera le proprietà di adesione *in vitro*, suggerendo che l'esecuzione di saggi sequenziali può essere una valida alternativa ai metodi convenzionali di valutazione dei probiotici. Lo studio è stato completato da una panoramica delle interazioni proteiche di *A. muciniphila* con le mucine umane, realizzato utilizzando diversi *database* e *software* (GenBank e Uniprot, PSI-BLAST e CytoScape) per identificare le proteine con sequenze omologhe alle proteine di *A. muciniphila* coinvolte nella degradazione delle mucine. Il capitolo 4 contiene la bozza del lavoro che sarà sottoposto a pubblicazione su una rivista internazionale.

Parole chiave: Alimenti, *Next Generation Sequencing*, Microbioma, Fattori Ambientali, Probiotici.

Chapter 1.
SEQUENCING

1.1 DNA Sequencing

Since the discovery of the structure of DNA (Watson & Crick, 1953) and the development of methods to determine the sequence of its bases using basic approaches (Sanger and Coulson, 1975; Maxam and Gilbert, 1977), the field of DNA sequencing has developed rapidly in terms of capacity and applications. As with many technologies, advances have converged to enable routine sequencing at the genomic scale (Levy and Myers, 2016). For many years, most applications were based on the use of the Sanger method (Arora *et al*., 2019), which uses modified dideoxynucleotide trisphosphates (ddNTPs) to interrupt the DNA synthesis reaction at specific positions (Sanger *et al*., 1977). This concept allowed the development of automated sequencing systems based on this method (Smith *et al.,* 1986). It is only in recent years that NGS technologies (or, alternatively, Massive Parallel Sequencing - MPS or Second-Generation Sequencing - SGS), with their ability to economically deliver millions of DNA sequences for run, have changed the way to approach genome analysis (Mardis *et al.*, 2017). The cost and time reductions obtained through these tools have spilled over into various disciplines, such as metagenomics, cancer research, animal research and biological selection. All these fields have found NGS techniques to be powerful and valuable tools for their development.

1.2 Sanger Sequencing

Sanger sequencing, also known as first-generation sequencing, pioneered by Frederick Sanger and Coulson in 1975, delineates the nucleotide identity and sequence within DNA segments, typically spanning less than 1000 base pairs (bp). This method, acknowledged as the “chain termination method”, remains the gold standard in sequencing technology, renowned for its precision and adaptability across various research domains (Arora *et al*., 2019). Sanger’s fundamental contributions were rewarded with the Nobel Prize in Chemistry in 1980 (Adams, 2008).

The Sanger method employs ddNTPs to halt DNA synthesis selectively at predefined positions. Initially commercialized by Applied Biosystems in 1986, subsequent refinement and widespread commercial availability propelled its adoption within the scientific community (Pareek *et al*., 2011). Sanger technology was pivotal in sequencing the inaugural human genome within an industrial high-throughput (HT) setup in 2003, as part of the 13-year Human Genome Project endeavor, which incurred an approximate cost of 2.7 billion dollars (Voelkerding *et al*., 2009; Pareek *et al*., 2011).

The classical chain termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal dideoxynucleotide trisphosphates (dNTPs), and ddNTPs

(labelled with fluorescent compounds) to terminate the extension of the DNA strand (Jamuar *et al.,* 2016). Sanger sequencing starts with the denaturation of double-stranded DNA (dsDNA) (Gomes *et al.,* 2018) The DNA sample is split into four sequencing reactions containing DNA polymerase, dNTPs and only one of four ddNTPs (ddATP, ddNTP, ddCTP or ddTTP) in a stechiometrically smaller amount to allow sufficient strand elongation for analysis (Sanger *et al.,* 1977; Crossley *et al.,* 2020). The sequence will continue to elongate with dNTP until a ddNTP is attached (Gomes *et al.,* 2018). Unlike dNTPs, ddNTPs cannot bind to the next nucleotide because they lack the hydroxyl group on the ribonucleotide at the 3' position. After synthesis, the reaction products are loaded into four lanes of a single polyacrylamide gel according to the different chain-terminating nucleotides and subjected to gel electrophoresis. Depending on the size of the bands obtained, the sequence of the DNA segments is determined (Cavanaugh *et al.,* 2010).

Over the years, the Sanger method has been optimised with the use of capillary electrophoresis and the development of automated sequencers that combine the sensitivity of Polymerase Chain Reaction (PCR) with the use of ddNTPs attached to fluorescent molecules (Slatko *et al.,* 2018). The major advantage of this system is that instead of having to prepare four separate reactions, it can be performed simultaneously for all nucleotides in a single tube. Each ddNTP is assigned a different colour and a laser beam is used to determine the identity of the nucleotide as it is inserted into the PCR synthesised strand (Smith *et al.,* 1986). The result is a sequence of peaks in four different colours (electropherogram), one for each nucleotide. This method can produce a maximum read length between 800 and 1000 bp. In this way, a single fragment can be sequenced in a capillary, so the output of a run is the length of the sequenced fragment (Gupta and Verma, 2019).

Sanger sequencing is still widely used in the field of sequencing as it offers several important advantages, including good cost-effectiveness for sequencing single genes and 99.99% accuracy (Sikkema-Raddatz *et al.,* 2013). Therefore, Sanger sequencing remains very useful for applications where high throughput is not required (Slatko *et al.,* 2018).

Although Sanger's automated method can sequence large numbers of bases in a few hours, it remains an expensive sequencing technique compared to NGS. However, due to its high reliability, the Sanger method remains the technique of choice in clinical and diagnostic nucleic acid sequencing practice and it is the most useful system for validating results obtained with NGS technology (Cheng *et al*., 2023).

1.3 Next Generation Sequencing

DNA sequencing has accelerated remarkably in the last decade with the advent of NGS technologies (Bobe *et al.,* 2016). The major difference between Sanger and NGS sequencing is the sequencing volume. Whereas the Sanger method sequences one DNA fragment at a time, NGS sequences millions of smaller DNA fragments in parallel to process the sequence (multiplexing) (Meyer *et al.,* 2010; Rizzo and Buck, 2012; Attia and Saeed, 2016). This allows

a complete dataset obtained in a few hours or days at a significantly reduced cost compared to Sanger sequencing (Voelkerding *et al.,* 2009). Therefore, the main advantages of NGS over classical Sanger sequencing can be summarised as follows: low cost, speed, reduced labour, and increased accuracy. These technologies have largely replaced conventional Sanger sequencing in genomic research. However, they have not yet become a routine (Behjati and Tarpey, 2013). In fact, NGS has some limitations: it requires the initial amplification of DNA sequences, which can introduce random errors into the sequence and can lead to the loss of information that is important for understanding the function of a genomic fragment. The need for a PCR amplification step has already been overcome by the development of third-generation sequencing technologies (Cao *et al.,* 2017). In fact, the search for even more efficient technologies has stopped. Conversely, the field continues to expand into applications that integrate NGS sequencing systems to obtain high-resolution genetic information. To date, there have been at least 4 generations of DNA sequencing methods (McGinn *et al.,* 2013).

Several NGS platforms are now widely available for HT DNA sequencing using a variety of different chemistries, and further sequencing technologies are still being developed (Ansorge, 2010; Behjati and Tarpey, 2013; Fuller *et al.,* 2016; Haynes *et al.,* 2019). Although they differ in the type of biochemistry and the methods of data acquisition and processing, the various NGS platforms share a common workflow. The general steps of DNA sequencing using NGS are (i) library preparation (random genome fragmentation, ligation with appropriate adaptors), (ii) library amplification, (iii) sequencing, and (iv) bioinformatic data analysis (Ari and Arikan, 2016; Gupta and Verma, 2019). Depending on the platform used, the results generated vary in terms of read length, quality, and quantity of data (Gupta and Verma, 2019).

NGS methods include SBS (Sequencing by Synthesis) and SBH (Sequencing by Hybridization) methods (Slatko *et al.,* 2018). SBS is an evolution of Sanger sequencing without the dideoxy terminators, combined with repeated rounds of synthesis, imaging, and methods to incorporate additional nucleotides into the growing chain. These are the most widely used methods. SBH was developed in the 1980s and uses DNA oligonucleotides of a known sequence on filters that hybridise to labelled fragments of the DNA to be sequenced. By repeated hybridisation, and washing away unwanted unhybridised DNA, it is possible to determine whether the hybridised labelled fragments match the sequence of the DNA probes on the filter (Slatko *et al,* 2018). This method is relegated to technologies that depend on the use of specific probes to interrogate sequences (e.g., in diagnostic applications to identify disease-related SNPs (Single nucleotide polymorphisms) in specific genes and/or to identify severe chromosomal abnormalities (Church *et al*., 2006; Qin *et al.,* 2012).

NGS platforms parallel sequence clonally amplified or single DNA molecules immobilised in a flow cell (Voelkerding *et al*., 2009). There are several methods for immobilising DNA on a solid substrate. Based on these methods, three different technologies have been commercialised: Roche 454 Life Sciences, SOLiD (Supported Oligonucleotide Ligation and Detection) and Illumina (Cao *et al.,* 2017).

The 454 technology is derived from the convergence of emulsion PCR and pyrosequencing and

is based on the detection of chemiluminescence resulting from the release of pyrophosphate upon incorporation of dNTP by a DNA polymerase (Nyren *et al*., 1993; Diaz-Sanchez *et al.*, 2013).

The SOLiD platform is a technology for sequencing short DNA reads based on ligation reactions. Again, DNA fragments bound to adaptor oligonucleotides and immobilised on microspheres are clonally amplified by emulsion PCR (Voelkerding *et al.*, 2009). Illumina technologies are the most popular and are described in detail below.

1.4 Illumina Technologies

The most popular family of technologies is that produced by Illumina, which offers multiple sequencer options producing different amounts and lengths of reads (Table 1) (Hodkinson and Grice, 2015; Kanzi *et al.*, 2020).

Table 1. Sequencer options offered by Illumina (Haynes *et al*., 2019).

PROVIDER	SEQUENCER	MAXIMUM READ LENGTH (BP)	MAXIMUM SEQUENCE YIELD FOR RUN (GB)
Illumina	HiSeq 4000	2 × 150	1500
	HiSeq 3000	2 × 150	750
	HiSeq 2500	2 × 125	1000
	NextSeq 500	2 × 200	120
	MiSeq	2 × 300	15
	MiniSeq	2 × 150	7.5

These technologies use reversible fluorescent dideoxy terminators to sequence amplified DNA clusters on the surface of flow cells. The Illumina platforms are short-read sequencers that produce Paired-End (PE) reads of up to 2 × 300 bases (Divoll *et al.*, 2018; Haynes *et al.*, 2019). The Illumina platform uses technology first developed by Solexa (Genome Analyzer, GA) in 2006 by scientists Shankar Balasubramanian and David Klenerman. Illumina GA sequencing involves several steps, which are summarised in Figure 1.

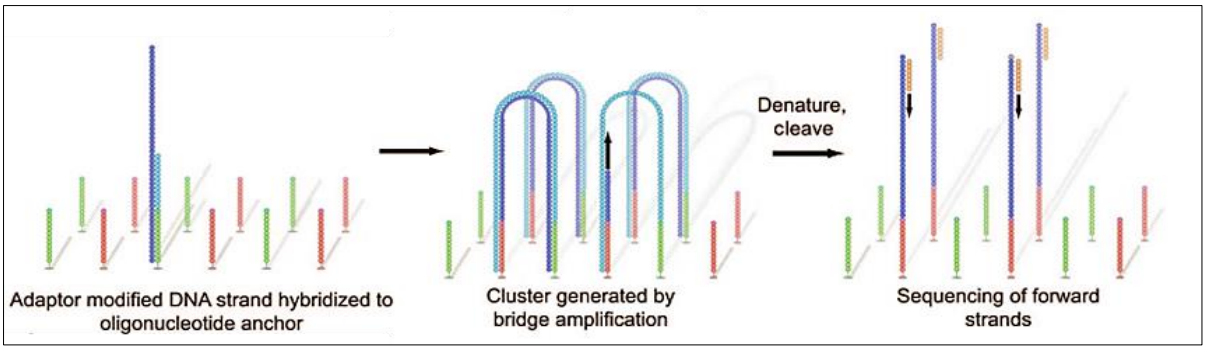


Figure 1. Flowchart Illumina GA sequencing (Voelkerding *et al*., 2009).

GA uses a flow cell consisting of an optically transparent slide with eight individual compartments to which oligonucleotide anchors are attached (Pettersson *et al.*, 2009; Morgan *et al.*, 2017).

- *Library preparation*

Genomic DNA is fragmented into segments several hundred bases long and modified to produce 5'-phosphorylated truncated ends. The polymerase activity of the Klenow fragment is used to add a single adenine base to the 3' end of the phosphorylated form DNA fragment. This prepares the DNA fragments for ligation to the adaptor oligonucleotides, which are complementary to the flow cell-anchored oligonucleotides. Single-stranded DNA bound to the adapters is added to the flow cell and immobilised by hybridisation to the anchor oligonucleotides (Ansorge *et al*., 2009; Garrido-Cardenas *et al.*, 2017).

- *Library amplification*

DNA fragments are amplified in the flow cell by "bridge amplification", which relies on capturing arcuately folded DNA strands that hybridise to an adjacent anchor oligonucleotide (Voelkerding *et al.*, 2009). Multiple cycles of amplification convert the single-stranded DNA molecule into a cluster of folded, clonally amplified fragments, each consisting of approximately 1,000 clonal amplicons (Ansorge *et al.*, 2009).

- *Sequencing by synthesis*

SBS technology uses fluorophores labelled nucleotides to sequence of clusters on the flow cell in parallel. The clusters are denatured, and a subsequent washing reaction yields only the forward strands, which are sequenced by hybridizing a primer complementary to the adapter oligonucleotide sequence. The subsequent addition of a DNA polymerase and a mixture of 4 dNTP terminators labelled allows the latter to be incorporated, based on the complementarity of the sequence of each strand, within each clonal cluster (Morozova *et al*., 2009; Liu *et al.*, 2012). After incorporation, excess reagents are removed by washing, and the fluorescence relative to each cluster is detected and recorded by the Basic Local Alignment Search Tool (CCD) camera (Voelkerding *et al.*, 2009). Successive steps remove the blocking group of reversible terminator nucleotides and remove the fluorescent label by washing, allowing the next round of sequencing to be performed (Figure 2) (Reuter *et al*., 2015; Heather and Chain, 2016).

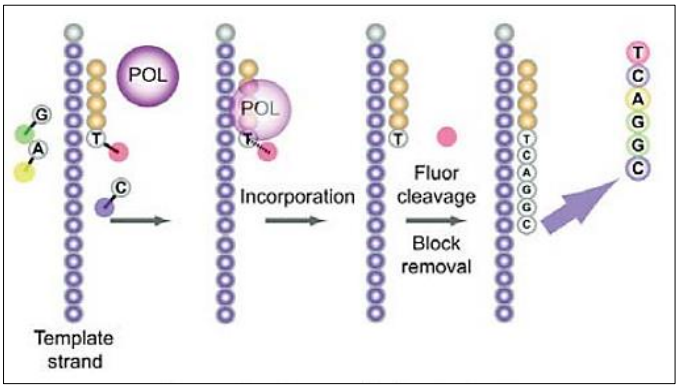


Figure 2. Base incorporation, fluorescence detection and blocking removal.

Early Illumina analysers generated 1 Gb of sequences with average reads of 35-36 bp in length and a run time of 2-3 days. Approximately 50×10^6 separate clusters can be generated in a single flow cell, producing more than a billion bases per run (Bentley *et al.*, 2008). With the introduction of HiSeq and MiSeq machines, the duration has changed to about 4 days and 24-30 hours, and the read length has increased to 250-300 bp (Bleidorn, 2015; Goodwin *et al.*, 2016).

1.5 NGS Data Analysis

The large amount of data produced by NGS technologies poses a major challenge for bioinformatics management and analysis (Kanzi *et al*., 2020). Therefore, there has been a need for the implementation of systems capable of storing increasing amounts of data and the development of advanced bioinformatics algorithms to efficiently analyse the data produced. These data require the use of specialized software for analysis, distinguished into commercial software and freely available open-source software (Haynes *et al*., 2019).

Bioinformatics analysis is divided into three steps: (i) alignment, (ii) variant calling (needed to detect mutations, SNPs, and sequencing errors), and (iii) filtering and annotation (Dolled-Filhart *et al*., 2013).

Software for analysing NGS data have different features, but in general they rely on bioinformatics pipelines that convert fluorescence images acquired by the instrument into nucleotide sequences (reads). The process of base identification (base calling) involves the assignment of a Quality Score (QS) for each read nucleotide, which measures the probability of a base being called incorrectly (Ewing *et al*., 1998a; Ewing *et al*., 1998b). The QS is assigned to each base by a phred-like algorithm, like that developed for Sanger sequencing analysis. QSs are an important tool to exclude reads and eliminate bases that show low quality and to improve alignment accuracy (Li *et al.*, 2008). In fact, the key factors in achieving adequate taxonomic targeting are the length of the reads, their quality and abundance. With the Illumina platform, the accuracy of base identification (base calling) decreases as the length of reads increases (Dohm *et al*., 2008). This is due to increased interference signals, which is a problem for the sequencing process.

The nucleotide sequence data from the reads and the QS values associated with each nucleotide are organized into output files in FASTQ format, which are used in subsequent alignment steps. Alignment is the process by which short reads are mapped to a reference genome. In this way, closely related species can be distinguished, and even phylogenetically more distant species can be deduced (Myllykangas *et al*., 2012). The alignment phase is possible thanks to the commercial availability of numerous software programs.

In targeted metagenomics, reads are grouped into OTUs (Operational Taxonomic Unit). Taxonomic assignment can then be performed, allowing classification of reads at different taxonomic levels (*phylum*, class, order, family, genus, and species) based on similarity of frequencies to those in the databases. The most current and widely used databases for taxonomic assignment of small and large rDNA sequences are Ribosomal Database Project (RDP) (Cole *et al.*, 2008), Silva (Quast *et al.*, 2012), and GreenGenes (De Santis *et al.*, 2006), while the most widely used database for taxonomic assignment of ITS rDNA sequences is UNITE (Nilsson *et al.*, 2019).

Many microorganisms have no taxonomic classification and there are no reference genomes in databases, so these organisms must be catalogued from scratch. In this case, a very common method that is used is based on genetic similarity between reads. In this way, clusters are

constructed based on sequence dissimilarity. However, this method does not provide precise information about the microorganism, limiting data interpretation.
To date, the most widely used pipelines for NGS data analysis are Mothur (Schloss *et al*., 2009) and Qiime (Caporaso *et al*., 2010). Both are open access, with manuals and example tutorials for analysis.

1.5.1 Current Applications

NGS technologies have accelerated progress in various fields of molecular biology. They are extensively applied in Whole Genome Sequencing (WGS), metagenomics, epigenetics, investigation of genome diversity, identification of non-coding RNA, exploration of protein binding sites, and profiling of gene expression through RNA sequencing (MacLean *et al*., 2009). In microbiology, aside from HT WGS, common applications of NGS techniques include the detection of novel microorganisms using metagenomic approaches and the analysis of microbial communities in environmental samples (El-Metwally *et al*., 2013).

1.5.2 NGS and Food Microbiology

NGS techniques are having a considerable influence on food microbiology (De Filippis *et al*., 2018). In this field, NGS is especially used for WGS determination of cultured isolates (e.g., bacterial colony, virus, or other organisms) and for metagenomics, by generating the sequences of all microorganisms present in the sample (Allard *et al*., 2016; Quainoo *et al*., 2017; Jagadeesan *et al*., 2019). These sequences allow the precise identification of microbial taxa, including those that cannot be cultivated and those that are present in small numbers (Mayo *et al.*, 2014). This approach is increasingly used in the characterization of food communities, such as those of cheese. Therefore, metagenomics refers to the application of HT techniques to sequence the entire DNA or RNA content found in a sample, independent of its origin.
The main advantage of metagenomic methods is their ability to characterise the bacteria in the microbiome at the species/strain level. Furthermore, they facilitate the simultaneous comparison of the relative abundance of all organisms in the microbiome, thereby facilitating the detection of potential evolutionary relationships in the sample (Cao *et al.*, 2017). The main disadvantage of the metagenomic approach is the huge amount of sequence data generated. For this reason, data analysis requires high-end bioinformatics requiring an extended term financial investment, which is feasible only in specialized laboratories. Furthermore, metagenomic approaches are unable to distinguish viable microbial populations within a microbiome (Ercolini, 2013). Alternatively, RNA can be used as a template to differentiate the viable population within a microbiome. In addition to distinguishing viable members within the population contained in a microbiome, RNA sequencing can provide a functional characterization of the different bacterial members of the microbiome. Sequencing of purified RNA from complex microbiological samples, such as food, provides a basic description of how communities interact with each other (Cao *et al*., 2017).

The application of metagenomics to characterise food samples is still in its infancy and offers interesting opportunities to predict the presence of typicality indicators that distinguish the product. As of this day, most NGS related food microbiome studies focused on fermented foods, such as cheese, sausages, and kimchi (Patra *et al*., 2016; De Filippis *et al*., 2017). With respect to such foods, studies to characterize the microbiome of unfermented foods are poor (Cao *et al*., 2017).

1.6 Third and Fourth Generation Sequencing

Beyond NGS, TGS (Third Generation Sequencing) technologies have emerged in recent years that can sequence single molecules, eliminating the need for DNA amplification, common to all previous technologies (Mitsuhashi *et al.,* 2014; Heather and Chain, 2016).

The two main TGS platforms include PacBio's Single Molecule Real-Time (SMRT) technology and Oxford Nanopore Technology, which can detect and quantify a DNA sequence based on changes in the current flowing through nanopores (Schadt *et al.,* 2010; van Dijk *et al*., 2014).

Such techniques are characterised by long read lengths (10-100 kb), albeit with a high sequencing error rate (~15%) (Surui *et al.,* 2021).

More recent are fourth-generation sequencing technologies, also known as *in situ* sequencing technologies. These techniques use second-generation NGS chemistry to read nucleic acid composition directly in fixed cells and tissues (Ke *et al.,* 2013; Lee *et al.,* 2014; Ke *et al.,* 2016).

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Chapter 2.
GRAPE AND WINE

2.1 Introduction to Grape and Wine

Wine production is one of the most important agricultural economic activities in the world (Villano *et al.,* 2017). This popular alcoholic beverage of great cultural and economic importance is closely linked to the geographic area of production (Kioroglou *et al.,* 2019; Bubeck *et al*., 2020; Costantini *et al.,* 2022). The unique sensory characteristics of wines are influenced, in a typical and unique way, by a combination of viticultural, climatic, pedological and anthropological factors, collectively known as "*terroir*" (Bokulich *et al.,* 2014; Anagnostopoulos *et al.,* 2019). Therefore, wines made from the same grape cultivar but grown in different regions are valued for their distinctive characteristics (Van Leeuwen and Seguin, 2006, Liu *et al.,* 2019). Many of the variables influencing wine composition and quality are microbiologically mediated (Aponte and Blaiotta, 2016) and the microbiological characteristics of fermenting must are mainly derived from the microbial populations present on the surface of grapes (Belda *et al.,* 2017a).

Most research therefore focuses on the grape berry, a natural reservoir of microorganisms that have various effects on must fermentation (Liu *et al*., 2017). However, grapes, like all fruits, are not a year-round habitat (Morrison-Whittle and Goddard, 2018), so each year the berries are colonised by fungi and bacteria from elsewhere in the vineyard. In fact, soil microorganisms have been shown to colonise different parts of plants, including leaves and fruit, and in the case of grapes, berries (Belda *et al*., 2017b).

The grapevine (*Vitis*) is recognised as one of the world's most important fruit crops in terms of area and economic importance. Grapes are grown throughout the world for consumption as fresh fruit and for the production of juice and wine. They serve as the primary reservoir of microorganisms critical for wine fermentation, with the microbial population on ripe grape surfaces estimated to be 10^3 to 10^6 CFU/g, consisting predominantly of yeasts (genera non-*Saccharomyces*), along with bacteria and filamentous fungi (Martins *et al*., 2012; Kioroglou *et al.,* 2019). In contrast, the predominant fermentative agent, *S. cerevisiae*, is either absent or present in very low concentrations on grape berries. Grape berries are rich in bioactive compounds, especially polyphenols, which impart numerous properties and are transferred to the wine during processing (Vorobiev *et al*., 2020).

Wine grape cultivars (*Vitis vinifera* L.) are renowned for their capacity to yield high-quality wines. These wines, esteemed for their excellence, originate from a select number of cultivars that enjoy significant consumer approval and are firmly entrenched in the market through varietal branding and the association of specific wine styles and regions with specific cultivars (Bouquet *et al*., 2006).

In European Union legislation (Regulation EU No. 1308/2013), wine is defined as a "*product*

obtained exclusively by total or partial alcoholic fermentation of fresh grapes, crushed or uncrushed, or of grape must". It is a popular alcoholic beverage cherished globally for its versatile aroma and complexity, derived from fermenting the must of *Vitis vinifera* L. grapes, encompassing numerous varieties corresponding to different wines (Iocco *et al*., 2001; Lustrato e*t al*., 2003; Gambuti *et al*., 2010). Consequently, the quality of wine hinges on the grape's composition and variety (Artero *et al.,* 2015). Although wine production and consumption span the globe, regional wine varieties hold significant lineage and geographical designations (Controlled Designation of Origin, CDO, Controlled and Guaranteed Designation of Origin, CGDO, and Protected Geographical Indication, PGI) to safeguard the authenticity of local products (Bubek *et al.,* 2020).

Thanks to the diversity of its territory, Italy is rich in grapes and wines. About 600 grape varieties are registered in the National Register of Grape Varieties (Registro Nazionale delle Varietà di Vite, 2022). This makes Italy the largest wine producer in the world, producing more than 50 million hectolitres of wine annually (Pittari *et al.,* 2023). The Italian wine industry is known for its high degree of diversification, which is an advantage in terms of biodiversity (Pittari *et al.,* 2023). Unlike other countries where grape cultivation and wine production are limited to a few areas compared to the size of the country, in Italy each region produces wine with its own typical grape varieties and local wines that aren't found in other regions.

Among various red grape variety (*Vitis vinifera* L.) produced in Southern Italy, Aglianico is widely cultivated and used to produce wines with different phenolic and aromatic characteristics (Gambacorta *et al*., 2019). It is a late-ripening variety that can adapt to different environments and growing conditions (Alba *et al*., 2011; D'Agata, 2014). It prefers a sunny climate and adapts very well to hilly, clay, calcareous and volcanic soils. The first written references to its cultivation date back to the 16th century (De Lorenzis *et al*., 2013). Traditionally, the main growing areas for Aglianico are Taburno and Taurasi in Campania and the Vulture district in Basilicata, which produce CGDO wines considered among the best red wines in Italy (De Lorenzis *et al*., 2013; Boscaino *et al*., 2019), but it is also cultivated in Puglia and Molise. Aglianico is characterised by a high content of polyphenol. The high content of flavonols and anthocyanins, which have a strong antioxidant effect, gives it high nutraceutical benefits (De Nisco *et al*., 2013), while the high tannin content gives the resulting wine an acidic and astringent character (Rinaldi *et al.,* 2014).

Cabernet Sauvignon is onother widely planted red grape in the world due to its adaptability to different soils and climatic conditions (Bezerra *et al.,* 2019). Produced from *Vitis vinifera* grapes of French origin, it is a late-ripening grape, grown with good results preferably in warm climate zones. In Italy, it is mainly cultivated i in Veneto, Sicily and Tuscany regions. Cabernet Sauvignon has a bunch with rather small berries and a thick skin, rich in colouring substances and tannins, in high relation to juice (Yao *et al*., 2021; Ren *et al.*, 2023). It has relatively high acidity and this characteristic, together with the high tannin content, allows its wines to be aged for long periods, ranging from 3 to 20 years and more. First introduced in Piedmont in 1820, Cabernet Sauvignon tends to produce full-bodied wines with a high tannin content and

considerable acidity.

2.2 Grape and Wine Microbiota

Grapes host a wide range of microbes from the vineyard environment, many also of which are recognized for their role in the must fermentation process that shapes wine quality. Identifying these microbial communities is particularly important for spontaneously fermented wines, where the native microbiota contributes to the unique flavors and aromas of wines (Ballantyne *et al*., 2019).

Grapes harbor a diverse microbial community of filamentous fungi, yeasts and bacteria, each with different physiological characteristics that affect wine production. Some species are exclusive to grapes, such as parasitic fungi and environmental bacteria. Others can thrive in wine and contribute to its microbial consortium, which includes yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Barata *et al.*, 2012).

The microbiota found in whole berries primarily consists of Basidiomycetes like *Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp., and the yeast-like fungus *Aureobasidium pullulans*. However, as grapes ripen and more nutrients become available, the population shifts towards oxidative or weakly fermentative Ascomycetes such as *Candida* spp., *Hanseniaspora* spp. and *Metschnikowia* spp. (Barata *et al.*, 2012).

NGS techniques applied to grape berries by various Authors showed a higher abundance of filamentous fungi than yeasts, especially for species associated with the berry surface. The most commonly identified filamentous fungi include *Aspergillus, Alternaria, Penicillium, Cladosporium, Botrytis, Erypse* and *Lewia,* while the most commonly identified yeasts include *Hanseniaspora, Issatchenkia, Pichia, Candida, Rhodotorula, Lachancea, Metschnikowia, Cryptococcus, Filobasidiella, Sporobolomyces* and *Torulaspora* (Morgan *et al*., 2017). The ability of the fungal genera *Alternaria, Aspergillus, Botrytis, Cladosporium, Oidium, Penicillium* and *Rhizopus* to colonise grapes has also been highlighted in works conducted by Welke (2019) and Abdo *et al.* (2020). The presence of different bacterial species is strongly influenced by both the production area and agricultural practices. Among the dominant bacterial species, members of several genera have been isolated: *Pseudomonas, Sphingomonas, Curtobacterium, Bacillus, Enterobacter, Acinetobacter, Erwinia, Citrobacter* and *Frigoribacterium* (Morgan *et al*., 2017). Ubiquitous environmental bacteria of the genus *Enterobacter* spp., *Enterococcus* spp., *Bacillus* spp., *Burkholderia* spp., *Serratia* spp., *Staphylococcus* spp. isolated from grapes do not have the ability to grow in wines (Barata *et al*., 2012). Damaged grape skins provide a favourable environment for the growth of ascomycetes such as *Pichia* spp. and *Zygoascus hellenicus* due to the high sugar content of the grape surface. This environment also favours the proliferation of problematic wine-altering yeasts such as *Zygosaccharomyces* spp. and *Torulaspora* spp. and AAB such as *Gluconobacter* spp. and *Acetobacter* spp. (Barata *et al*., 2012).

Recent amplicon sequencing studies have provided insights into the diversity, composition and

geographical distribution of fungal and bacterial microbiota in wine (Piao *et al*., 2015; Bokulich *et al*., 2016; Mezzasalma *et al*., 2018; Morgan *et al*., 2019).

The microbial communities involved in winemaking are diverse and change throughout the fermentation process. While *Saccharomyces cerevisiae* typically dominates alcholic fermentation (AF), other non-*Saccharomyces* yeasts and bacteria also play an active role in shaping the unique characteristics of wine. Non-*Saccharomyces* yeasts, from genera *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Hansenula, Klyveromyces, Pichia, Rhodotorula, Saccharomyces, Schizosaccharomyces, Sporidiobolus, Torulaspora, Zygoascus* and *Zyogosaccharomyces*, have been isolated from grapes and must during fermentation (Donahue, 2016; Vyviurska and Špánik, 2020). Instead, identified bacteria that take part in malo-lactic fermentation (MLF) and wine maturation belong to the genera *Lactobacillus, Leuconostoc, Oenococcus and Pediococcus* (James *et al.,* 2023). Differences in wine microbiota profiles have been associated with vines, including distinct cultivars, plant organs and vintages (Bokulich *et al*., 2014; Zarraonaindia *et al*., 2015; Portillo *et al*., 2016; Marasco *et al*., 2018).

The parameters that influence the composition of the epiphytic microflora of grapes are many, with climatic conditions, vineyard treatments and biotic factors being the most important (Bettenfeld *et al*., 2022). The composition of the microbial population is influenced by climatic factors such as temperature, UV exposure, rain, sunlight and wind, as well as microclimatic differences both between different vineyards and within the same vineyard. For example, the position of the plant within the vineyard determines differences in the amount of sunlight reaching the grapes, which influences the presence and prevalence of pigmented yeasts, such as those belonging to the genera *Rhodotorula* and *Sporobolomyces* (Liu *et al*., 2019). Agronomic practices and the use of chemicals such as fungicides and insecticides affect both the number and biodiversity of the grape microbiota (Escribano-Viana *et al*., 2018). Heavy rainfall can increase the microbial population of grapes due to increased nutrient availability, while other research has observed lower microbial populations in wet years. Organic crops have greater microbial biodiversity than conventional crops (Vashishth *et al*., 2022). Insects, especially fruit flies, can increase the microbial population and act as vectors for microorganisms (both apiculate yeasts such as *C. stellata*, *P. membranifaciens*, *M. pulcherrima* and LAB and AAB). The variety and ripeness of the grape, the position of the berry in the cluster and the thickness of the waxy layer on its surface influence the composition of the microbiota (Kioroglou *et al*., 2019). Inadequate cleaning of equipment during harvesting can lead to contamination of grapes and juice (Malfeito-Ferreira, 2011).

Grape must is an ideal nutrient environment for the development of yeasts, containing all the elements essential for their growth, such as easily fermentable sugars, mineral salts and vitamins. However, its chemical and physical characteristics, such as its low pH and high sugar concentration, exert a strong selective pressure on the micro-organisms, allowing only a few species to proliferate (Pretorius, 2000). The addition of sulphur dioxide contributes to this selectivity by inhibiting undesirable microorganisms (Guerrero and Cantos-Villar, 2015).

Factors influencing the composition of the must microbiota include the method of harvesting and transporting the grapes, the initial characteristics of the grapes and the pre-treatments carried out on the must, such as aeration, enzymatic treatment and sulphite addition (Malfeito-Ferreira, 2011).

The factors that influence the presence and development of yeasts during AF are many and include the initial population composition and variability of the species present in the must, the inoculation of the must with selected starter cultures, the chemical composition of the must, including the presence of fungicide or pesticide residues, and parameters related to winemaking technology, such as the addition of sulphur dioxide and winemaking temperature. Factors influencing yeasts development are divided into chemical, physical and biological factors which include nutrients, sugars, sulphur dioxide, temperature, osmotic pressure and interactions with other microorganisms (Fleet, 1990; Maicas, 2020).

Climate influences viticulture and wine quality through temperature, rainfall, and solar radiation (Van Leeuwen and Seguin, 2006; Drappier *et al.*, 2019; Arias *et al*., 2022). Cooler climates are more suited for the production of light and delicate wines, whereas warmer climates tend to form heavy and rich heavy and rich flavor profiles (Liu *et al*., 2019).

In viticulture, climatic influences are recognized at multiple geospatial scales (macroclimate, mesoclimate and microclimate). Macroclimate, also called regional climate, is determined by latitude and elevation, and can be influenced by the presence of water from seas or lakes (White, 2015). The mesoclimate, which represents the local climate of a particular vineyard, is determined by the topography including the altitude, aspect and slope of the vineyard which influence wine quality (Bramley *et al*., 2011). This seems to show weaker influences on microbial distribution, especially for bacteria. The microclimate is determined by temperature and humidity and solar variations within the vineyard. It is partly influenced by sound conditions and shading from the positions of the vine leaves. Microclimate is the environment most likely to influence the presence, growth and activity of microbes. Modification of the grapevine leaf area alters canopy microclimate, specifically the solar radiation onto grapes and leaves, and the air movement in the leaf canopy and temperature (Reynolds and Heuvel, 2009). In addition, the effects of light in the microclimate have been reported to affect a variety of aspects of berry composition and can have a strong influence on the color and flavors in the finished wine (Liu *et al*., 2015). However, little research has been done on how microclimate variability affects microbial communities.

On the other hand, numerous studies have been conducted on the effect of global warming on changes in grapevine phenology (life cycle/plant development stages), which are considered to be a clear consequence of it. Recent studies show that the temperature increase is highly correlated with the early onset of many vine growth stages and that the shortening of the duration of most phenological stages can negatively affect the composition and quality of grapes and wine (Bock *et al.*, 2011; Alikadic *et al*., 2019; Venios *et al.*, 2020).

RESEARCH AIM

Culture-dependent methods have long been used to study the diversity of the grape wine microbiota (Barata *et al*., 2012; Takahashi *et al.* 2014; Camilo et *al*., 2022). However, these methods suffer from limitations due to the need to cultivate microorganisms (Alain *et al*., 2009). In this regard, they do not allow the detection of most microbial species present on grapes, such as rare taxa, low abundance taxa and live but non-viable microorganisms (Bodor *et al.*, 2020). These issues have led to the development of HTS-based approaches (Salvetti *et al*., 2016; Morgan *et al*., 2017; Dissanayake *et al.*, 2018) which were recently applied to the assessment of grape microbial communities (Kamilari *et al*., 2021). The aim of the study presented below focused on the possibility of using HTS techniques for the investigation of fungal consortia inhabiting the grape berries surfaces of two cultivars (Cabernet and Aglianico) from three different South Italian vineyards. The research is published in the international journal *Frontiers in Microbiology*.

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Diversity of fungal communities on Cabernet and Aglianico grapes from vineyards located in Southern Italy

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Grape-associated microbial community is influenced by a combination of viticultural, climatic, pedological and anthropological factors, collectively known as *terroir*. Therefore, grapes of the same cultivar grown in different areas can be appreciated for their distinctive biogeographic characteristics. In our previous study, we showed that the phenotypic response of Aglianico and Cabernet grapevines from Molise and Sicily regions is significantly influenced by the prevailing pedoclimatic conditions, particularly soil physical properties. However, the scale at which microbial communities differ could be important in clarifying the concept of *terroir*, including whether it is linked to the grape variety present in a particular vineyard. To explore this further, in the research presented here, a comparative study on the fungal communities inhabiting the berry surfaces of Cabernet and Aglianico cultivars was conducted on different vineyards located in Southern Italy (Molise, Sicily and Campania regions, the first two of which had been involved in our previous study) by using high-throughput sequencing (HTS) and multivariate data analysis. The descriptive approach through relative abundance analysis showed the most abundant *phyla* (Ascomycota, Basidiomycota, and Chytridiomycota), families (*Cladosporiaceae*, *Saccotheciaceae*, *Pleosporaceae*, *Saccharomycodaceae*, *Sporidiobolaceae*, *Didymellaceae*, *Filobasidiaceae*, *Bulleribasidiaceae*, and *Saccharomycetaceae*) and genera (*Cladosporium*, *Aureobasidium*, *Alternaria*, *Stemphylium* and *Filobasidium*) detected on grape berries. The multivariate data analysis performed by using different packages (phyloseq, Vegan, mixOmics, microbiomeMarker and ggplot2) highlighted that the variable "vineyard location" significantly affect the fungal community, while the variable "grape variety" has no significant effect. Thus, some taxa are found to be part of specific vineyard ecosystems rather than specific grape varieties, giving additional information on the microbial contribution to wine quality, thanks to the presence of fermentative yeasts or, conversely, to the involvement in negative or detrimental roles, due to the presence of grape-deriving fungi implied in the spoilage of wine or in grapevine

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pathogenesis. In this connection, the main functions of core taxa fungi, whose role in the vineyard environment is still poorly understood, are also described.

KEYWORDS

Aglianico, Cabernet, biogeography, fungi, grape microbiota, NGS, *terroir*, metataxonomic

1 Introduction

The grapevine (*Vitis vinifera*) phyllosphere is colonized by bacteria and fungi that substantially modulate grapevine health, fruit development and ripening, as well as the quality of grapes and wine (Barata et al., 2012; Martiniuk et al., 2023). The microbiome of a grapevine plant has direct and indirect relationships with its host and specific microbial biodiversity linked to a particular vineyard location is reported to be a crucial aspect, in conjunction with edaphic, climatic and human factors, in the concept of wine *terroir* (Gilbert et al., 2014). The concept of *terroir* is essential in viticulture since it links the sensory qualities of wine to the environmental conditions in which the grapes are grown (Van Leeuwen and Seguin, 2006).

Several scientific studies have shown that non-random biogeographic distribution patterns of microbial communities of grape surface in vineyards can be modulated by a combination of numerous factors, including geographical location, farming system, soil, cultivar, vintage and climate at various levels (Bokulich et al., 2014; Martins et al., 2014; Knight et al., 2015; Singh et al., 2018). These factors also affect the biology of the vines and the composition of the grapes, which in turn affect the microbiota, and, consequently, the qualitative properties of grapes and wine (Belda et al., 2017). This cycle has given rise to the concept of microbial-wine-terroir, intended as the close correlation between microbes, wine and territories (Gilbert et al., 2014; Knight et al., 2015).

To know the biogeographical distribution of vineyard microbial communities in specific regions, it is important to understand whether the vines themselves select different microorganisms according to their physiological responses, different environmental conditions and viticultural practices (Gilbert et al., 2014; Zarraonaindia et al., 2015; Pacifico et al., 2019; Vitulo et al., 2019). Therefore, in recent years, there has been a surge in the search for regional microbial signatures and microbial biogeography of wine and we now have a greater understanding of microbial diversity among wine-producing regions to begin to understand how this biodiversity can contribute to wine quality and regional characterization (Bokulich et al., 2014, 2016). Native yeasts, residing in a niche site, represent an important component of the microbiota of a vineyard, as they can influence the chemical and sensory profile and the so-called typicality of wine in a unique, reproducible, and identifiable way (Belda et al., 2017). Several studies have shown that the microbiota involved during the early fermentation stages, which is partially determined by endophytic plant-borne yeasts and bacteria, can comply with a well-delineated biogeography reflecting the signatures of different winegrowing regions with a minor influence from the grape variety and vintage year (Barata et al., 2012; Bokulich et al., 2014; Pretorius, 2020; Kamilari et al., 2021).

The diversity of the grape microbiota has long been studied using culture-dependent techniques (Barata et al., 2012; La Hens et al., 2014; Takahashi et al., 2014; Testa et al., 2014; Camilo et al., 2022), which have the disadvantage of being limited by the cultivability of microorganisms (Cocolin et al., 2013). Indeed, although these techniques are widely used for rapid and sensitive profiling of microbial communities, they are unable to detect a significant fraction of the microbial species present on grapes as rare taxa, low-abundance taxa and living but non-viable microorganisms (Bodor et al., 2020). The limitations of the conventional methods have led to the development of high-throughput sequencing (HTS)-based approaches to study the structure of microbial communities (Bleidorn, 2015). These innovative techniques allowed the scientific community to retrieve novel information about microbial communities in all different kinds of environments. As a result, the application of HTS methods for in-depth assessment of the grape and wine microbiome has increased in recent years (Salvetti et al., 2016; Morgan et al., 2017; Dissanayake et al., 2018; Kamilari et al., 2021). In a previous study we already assessed the impact of pedoclimatic conditions on the enological performance of two red cultivars grown throughout Southern Italy (Iorizzo et al., 2023). In this study, we obtained new information by adding other wine-grape samples of two cultivars (Cabernet and Aglianico) to determine relationships between spatial proximity and fungal composition through amplicons sequencing of the internal transcribed spacer (ITS2) region using a HTS approach combined with bioinformatics.

2 Materials and methods

2.1 Berry sample collection and processing

Grape berries from cultivars Aglianico and Cabernet were collected in two different vineyards of Southern Italy (Sicily and Molise, 37° 40′; 400–700 m a.s.l. and 41° 42′; 606 m a.s.l., respectively) at the right maturation time, following the BBCH scheme described by Lorenz et al. (1995) (BBCH stage 89, berries ripe for harvest), during the 2020 growing season. Grape barriers from a third farm located in the Campania region (41° 13′; 100 m a.s.l.) and devoted to the production of Aglianico and Cabernet were also withdrawn in the same period. For each farm and for each cultivar, 30 clusters were harvested from different positions

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of the vineyard and from random positions on the plant to ensure the representation of the entire vineyard as previously described (Iorizzo et al., 2023). Briefly, at least ten berries were randomly selected from different parts of the cluster, avoiding those with visible damage and/or signs of pathogen infection, and pooled with berries from the other plants. For subsequent analyses, two different samples of 50 whole berries were taken and immediately transported to the laboratory, frozen in liquid nitrogen, and stored at −80°C for subsequent analyses.

2.2 Metataxonomic and bioinformatic analyses of grape fungal communities

Fungal community composition was analyzed by a culture-independent approach using next generation sequencing (NGS), as described in a previous study (Iorizzo et al., 2023). Total genomic DNA was extracted using the Stool DNA Isolation Kit (Norgen, Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. Two replicates were produced for each sample. DNA was extracted from 200 mg of the homogenized sample. Extracted DNA was verified by agarose gel electrophoresis. The purity and amount of DNA was measured using a NanoDrop (NanoDrop 2000/2000c, Thermo Fisher Scientific, Italy) and standardized to a concentration of 10 ng/μl. The ITS2 region of the rRNA was amplified using primers 2024F and 2409R (White et al., 1990; Zhang et al., 2018). Amplicons were sequenced using the Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) at Biodiversa s.r.l. (Rovereto, Italy). Raw paired-end reads obtained from NGS were demultiplexed using the QIIME 2 (ver. 2022.2) pipeline (Caporaso et al., 2010) and then were analyzed to evaluate the quality of sequencing with the DADA2 plugin (*qiime dada2 denoise-paired*) in QIIME2 (Callahan et al., 2016). For the quality control of reads, the Quality Score value of 25 was used as a threshold and the low-quality regions of sequences were removed. Reads were truncated at position 300 and 254 for the forward and reverse read, respectively. Moreover, to remove the base that belongs to the primer sequences, the first 5 nucleotides of reads were removed. At the end of DADA2 analysis, an amplicon sequence variant (ASV) table was obtained which recorded the frequencies of any ASV observed in each sample. The ASV table was filtered with the plugin qiime *feature-table filter-features* to discard features with frequency lower than 10 (0.001%).

ASVs were classified using the QIIME2 naïve Bayes classifier (*qiime feature-classifier fit-classifier-naive-bayes*), trained on the UNITE database (ver. 9.0, release 16-10-2022) (Abarenkov et al., 2022).

The new raw data of Campania samples were deposited in Mendeley Data with the accession number doi:10.17632/6jcyrwj2gh.

2.3 Statistical analysis

Statistical analysis and graph processing were conducted on R (Version 4.2.3) using RStudio software (Version 2022.07.0). Several packages were used in the analysis: phyloseq (Version 1.42.0) to facilitate the import, storage, handling and analysis of the microbiome data (McMurdie and Holmes, 2013), as well as to determine alpha diversity. For the latter, both Chao1 and Shannon indexes were calculated. Vegan (Version 2.6-4) package was used for the permutational multivariate analysis of variance (PERMANOVA) using the Bray Curtis metric distance. The mixOmics package was used for sparse partial least squares discriminant analysis (Rohart et al., 2017) and the microbiomeMarker (Version 1.4) package for the Linear Discriminant Analysis (LDA) effect size (SE). The ggplot2 (Version 3.4.4) package was used for graphical representation of the data.

3 Results

3.1 Bioinformatic analysis

In the year 2020 two biological replicate samples of grapes were collected from two vineyards (Aglianico and Cabernet) located in Southern Italy (Sicily, Campania and Molise regions, **Supplementary Table 1**). Surface fungi community composition of grapes was analyzed through high-throughput amplicon sequencing of the ITS2 region.

A total of 923,559 paired-end sequences were obtained from sample sequencing. The average of reads per sample was 76,963, ranging from 54,059 to 123,628. After reads quality check, denoising and chimera filtering (**Supplementary Figure 1**), 465,805 paired-end sequences were obtained (**Supplementary Table 2**) corresponding to 419 ASVs. The ASVs were filtered with the aim to discard features with frequency lower than 10. At the end of bioinformatic analysis, a feature table of 465,469 reads, corresponding to 323 ASVs, was obtained and used for the subsequent analysis. The rarefaction curves reached the plateau for all assayed samples, suggesting that the sequencing depth was appropriate (**Supplementary Figure 2**).

3.2 Grape fungi community profile

From the taxonomy classification 3 *phyla*, 17 classes, 45 orders, 96 families, 122 genera and 172 species were identified. In general, the analysis of fungal community confirmed our previous findings (Iorizzo et al., 2023). In fact, Ascomycota remained the most abundant *phylum*, representing 87.9% ± 9.4% of total reads, followed by Basidiomycota 11.8% ± 9.3% and Chytridiomycota 0.02% ± 0.08% in all analyzed samples. The remaining 0.3% ± 0.2% of the reads were represented by unclassified fungi (**Figure 1A** and **Supplementary Table 3**).

The new data processed in this study considered two variables: vineyard location (VL) and grape variety (GV). Regarding the results of relative abundance with respect to the variable VL (**Figure 1B** and **Supplementary Table 4**), we found that the grape samples from Campanian vineyard were characterized by 90% of reads belonging to the Ascomycota and about 9.7% of reads were referable to the Basidiomycota. Similarly, in the grape samples from Sicilian vineyard, an average of 94% of the reads were associated with the *phylum* Ascomycota and only 5.6% of them belonged to the Basidiomycota. A slightly different result was found in the Molisian samples as Ascomycota accounted for

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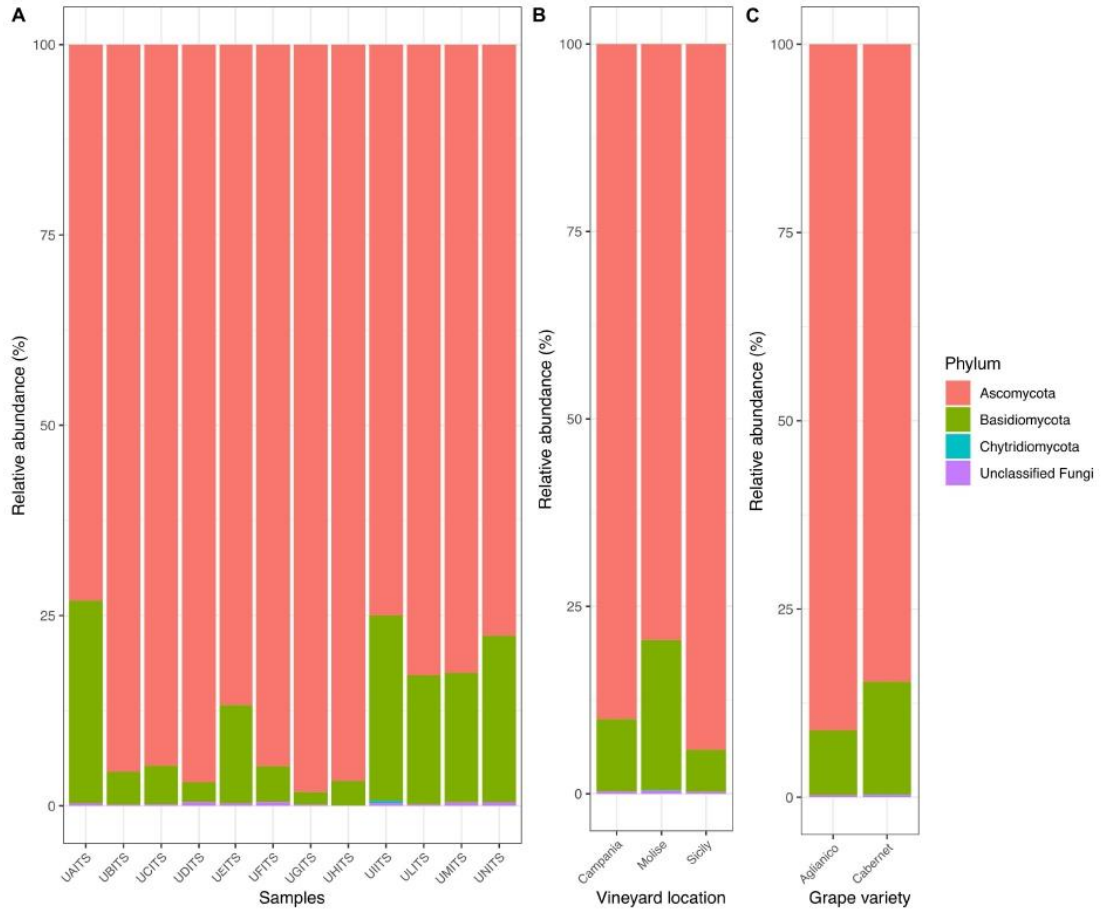


FIGURE 1
Stacked bar plot illustrates the distribution of the relative abundance of the grape fungi community at the *phylum* level. The relative abundance is reported for panel **(A)** single samples, **(B)** samples grouped by vineyard location (VL), and **(C)** samples grouped by grape variety (GV). The sample codes are specified as follow: UAITS, Cabernet_Campania_1; UBITS, Cabernet_Campania_2; UCITS, Aglianico_Campania_2; UDITS, Aglianico_Campania_3; UEITS, Cabernet_Sicily_1; UFITS, Cabernet_Sicily_3; UGITS, Aglianico_Sicily_1; UHITS, Aglianico_Sicily_2; UIITS, Cabernet_Molise_2; ULITS, Cabernet_Molise_3; UMITS, Aglianico_Molise_1; UNITS, Aglianico_Molise_2. See **Supplementary Table 1** for more details.

around 74% of the reads, while around 20% of the reads belonged to Basidiomycota. When the samples were grouped according to the GV (**Figure 1C** and **Supplementary Table 5**), we found that Ascomycota represented 91.1 and 84.6% of the *phyla* for the Aglianico and Cabernet cultivars, respectively. Basidiomycota, on the other hand, were around 8.6% in the Aglianico samples and 15% in the Cabernet samples.

Regarding the classification at the family level, out of 96 families identified, only 9 of them enclosed about 95% of total reads. In detail, *Cladosporiaceae* was the most abundant family, representing an average of 28.4% ± 13.5 of the total reads, followed by *Saccotheciaceae* 24.8% ± 14.0%, *Pleosporaceae* 15.9% ± 8.3%, *Saccharomycodaceae* 12.3% ± 18.2%, *Sporidiobolaceae* 7.40% ± 8.80%, *Didymellaceae* 2.7% ± 4.5%, *Filobasidiaceae* 1.70% ± 1.53%, *Bulleribasidiaceae* 1.2% ± 1.9%, and *Saccharomycetaceae* 0.9% ± 0.9% (**Figure 2A** and **Supplementary Table 6**). With regards to the distribution of the 9 most abundant families according to the VL (**Figure 2B** and **Supplementary Table 7**), we observed that *Cladosporiaceae* were present with a prevalence of about 20.6, 34.7, and 29.9% in vineyards of Campania, Molise and Sicily regions, respectively. *Saccotheciaceae* showed a prevalence of 33.6% in samples from Campanian vineyard, 11.7% in Molisian vineyard and 29% in Sicilian vineyard. Reads associated with *Pleosporaceae* were present at levels of 17.4, 22.1, and 8.3% in samples from vineyards located in Campania, Molise and Sicily regions, respectively. The results regarding the distribution of *Saccharomycodaceae* among the samples were particularly interesting. In fact, a consistent presence of reads attributable to *Saccharomycodaceae* were detected only in Campanian (14.4%) and Sicilian (22.1%) vineyards. On the contrary, only 0.4% of *Saccharomycodaceae* were observed in Molisian vineyard. *Sporidiobolaceae* were detected at relevant presence in Campanian (6.9% ± 10.9%) and Molisian (15.2% ± 3.4%) vineyards, while their presence on grapes from Sicilian farm was only 0.1%. Another interesting result concerns the *Didymellaceae* family. In this regard, a consistent presence

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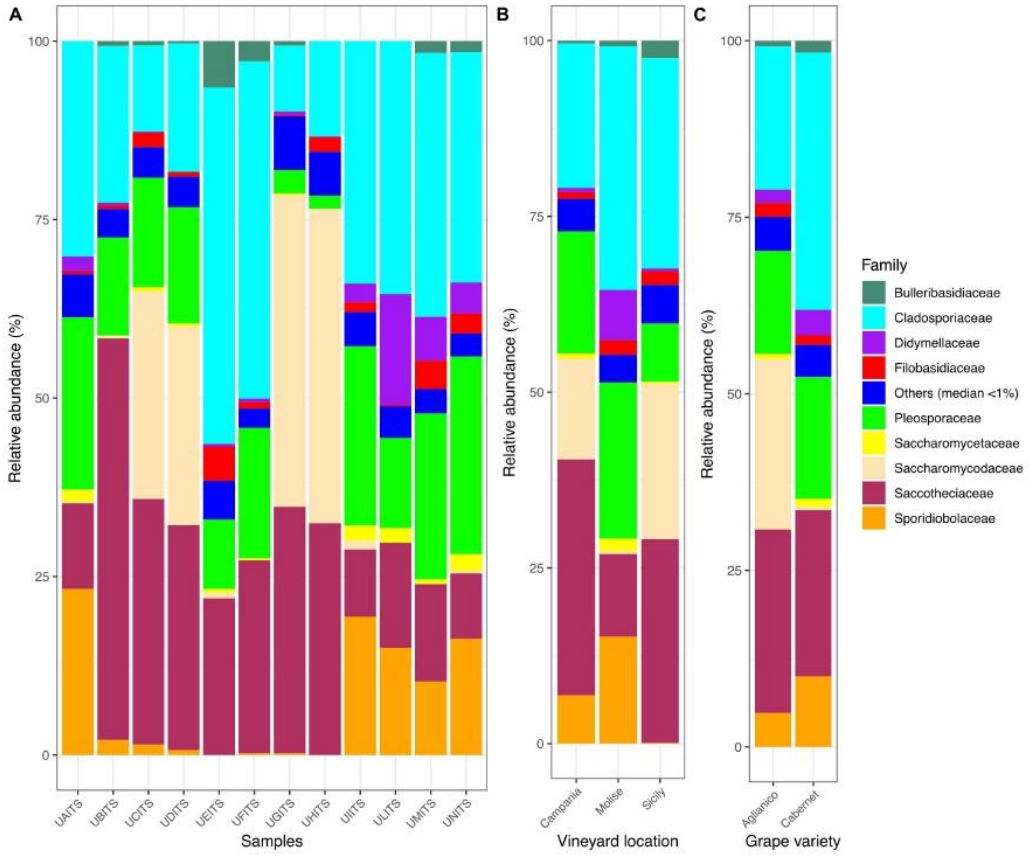


FIGURE 2
Stacked bar plot illustrates the distribution of the relative abundance of the grape fungi community at the family level. The group indicated as "Others (median < 1%)" encompasses families that show a relative abundance below median value 1. The relative abundance is reported for panel **(A)** single samples, **(B)** samples grouped by vineyard location (VL), and **(C)** samples grouped by grape variety (GV). The sample codes are specified as follow: UAITS, Cabernet_Campania_1; UBITS, Cabernet_Campania_2; UCITS, Aglianico_Campania_2; UDITS, Aglianico_Campania_3; UEITS, Cabernet_Sicily_1; UFITS, Cabernet_Sicily_3; UGITS, Aglianico_Sicily_1; UHITS, Aglianico_Sicily_2; UIITS, Cabernet_Molise_2; ULITS, Cabernet_Molise_3; UMITS, Aglianico_Molise_1; UNITS, Aglianico_Molise_2. See **Supplementary Table 1** for more details.

of reads was observed only in Molisian vineyard (7.2%), while a poor relative abundance was detected in the other samples (0.6% Campania and 0.4% Sicily).

Investigating the distribution of the nine families in relation to the variable VL (**Figure 2C** and **Supplementary Table 8**), we observed that *Saccharomycodaceae* were particularly abundant (24.1%) in Aglianico grapevine and almost undetectable in Cabernet samples (0.4%).

In relation to genera classification, out of 122 genera detected, only 10 groups were present in almost all samples (**Figure 3A** and **Supplementary Table 9**). In detail, *Cladosporium* was present with an average of 28.3%, followed by *Aureobasidium* (24.8%), *Alternaria* (13.5%), *Hanseniaspora* (12.3%), *Sporobolomyces* (7.3%), unclassified *Didymellaceae* (2.7%), *Stemphylium* (2.3%) *Filobasidium* (1.7%), *Vishniacozyma* (1.1%) and *Saccharomyces* (0.9%). Genera grouped as "Others (median < 1)" showed about 5% of average relative abundance. Grouping the genera distribution according to the VL variable (**Figure 3B** and **Supplementary Table 10**), it was observed that in Campanian vineyard the genus *Aureobasidium* was the most abundant (33.5%), followed by *Cladosporium* (20.5%), *Hanseniaspora* (14.4%), and *Alternaria* (14.3%). Samples from Molise vineyard were also characterized by the high presence of *Cladosporium* (34.4%), *Alternaria* (18.5%) and *Aureobasidium* (11.7) genera, but contrary to the Campania samples, a high amount of *Sporobolomyces* (15.2%) and a low presence of *Hanseniaspora* (0.4%) was observed. With regard to the samples from Sicilian vineyard, *Cladosporium* (29.8%), *Aureobasidium* (29.0%) and *Hanseniaspora* (22.1%) were the most abundant genera, instead *Alternaria* (7.5%) and *Sporobolomyces* (0.1%) were present in a small number. When the dataset was stratified according to the GV variable (**Figure 3C** and **Supplementary Table 11**), *Cladosporium* was the most abundant genus (34.3%) in the Cabernet grape variety, followed by *Aureobasidium* (18.3%) and *Alternaria* (14.6%), while in the Aglianico samples, a significant amount of reads attributable to the genus *Hanseniaspora* (28.4%) was recorded in addition to a high presence of *Aureobasidium* (32.0%), *Cladosporium* (15.7%) and *Alternaria* (14.6%).

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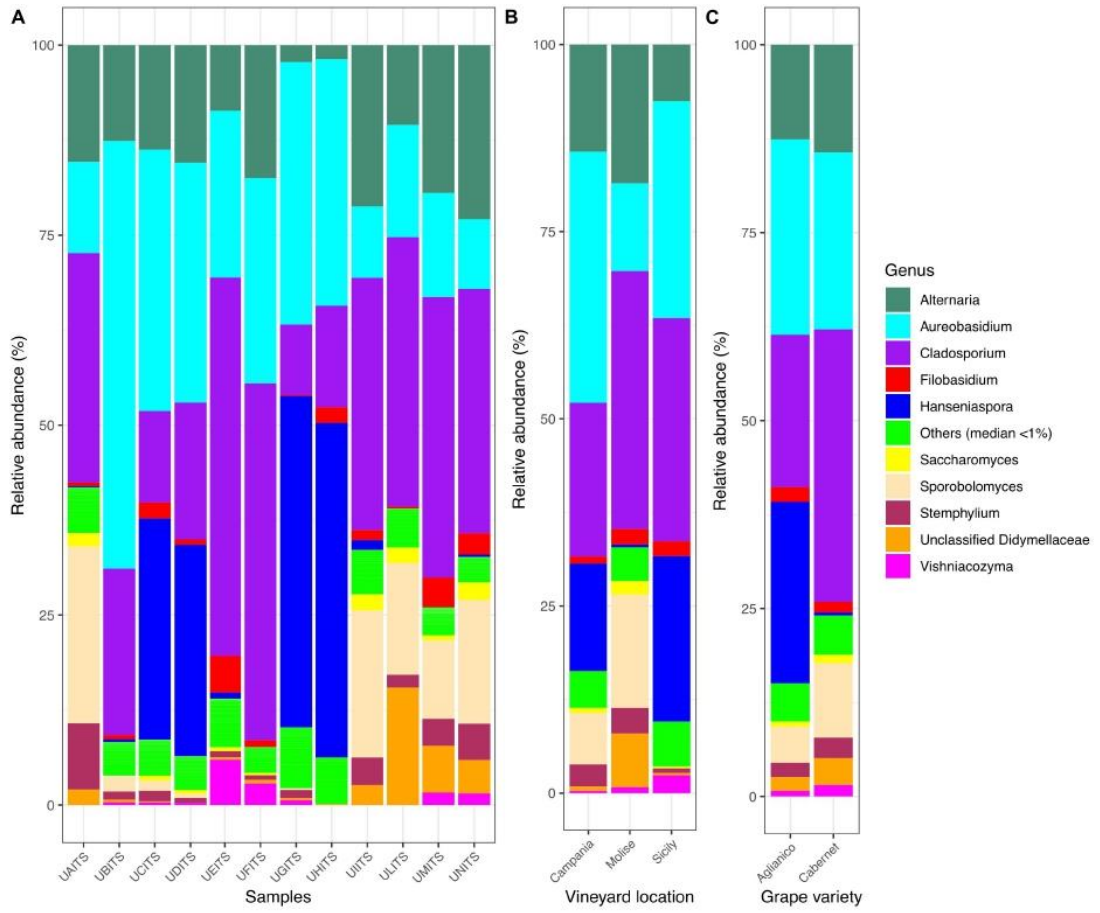


FIGURE 3
Stacked bar plot illustrates the distribution of the relative abundance of the grape fungi community at the genus level. The group indicated as "Others (median < 1%)" encompasses genera that show a relative abundance below median value 1. The relative abundance is reported for panel **(A)** single samples, **(B)** samples grouped by vineyard location (VL), and **(C)** samples grouped by grape variety (GV). The sample codes are specified as follow: UAITS, Cabernet_Campania_1; UBITS, Cabernet_Campania_2; UCITS, Aglianico_Campania_2; UDITS, Aglianico_Campania_3; UEITS, Cabernet_Sicily_1; UFITS, Cabernet_Sicily_3; UGITS, Aglianico_Sicily_1; UHITS, Aglianico_Sicily_2; UIITS, Cabernet_Molise_2; ULITS, Cabernet_Molise_3; UMITS, Aglianico_Molise_1; UNITS, Aglianico_Molise_2. See **Supplementary Table 1** for more details.

3.3 Alpha diversity of grapevine according to the vineyard location and grape variety

The estimation of intragroup diversity (alpha diversity) was carried out to verify the hypothesis that the fungi richness and biodiversity vary within the VL (Campania, Molise, and Sicily) and the GV (Aglianico and Cabernet). For this purpose, Chao1 index was used to estimate the species richness and the Shannon index was used to estimate the diversity. Results are reported in **Figure 4A** and show that there were no significant differences (Anova test, $p = 0.516$) in richness (Chao1) between GV in the three vineyards. In contrast, a significant difference (Anova test, $p = 0.021$) of diversity (Shannon) between VL was found. Specifically, the *post hoc* test showed that the Molisian vineyard was characterized by a high diversity (TukeyHSD test, $p = 0.018$) compared to the Sicilian one. No significant differences (TukeyHSD test, $p > 0.05$) were found between vineyards from Molise and Campania regions or between Campania and Sicily regions. Regarding the alpha diversity calculated from the two GV, results showed that there were no significant differences (t-test, $p > 0.05$) for species richness and diversity (**Figure 4B**).

3.4 Beta diversity

Beta diversity based on Bray Curtis distance was calculated to evaluate the degree of variation in species composition among the samples. Results showed that the fungal population segregated mainly according to the VL and slightly according to the GV (**Figure 5**). In detail, the PCoA plot shows a good separation of samples along axis 1 (explaining 47% of variation), while a less pronounced separation was observed along axis 2 (explaining 22% of variation). The effect and the significance of the variable VL (Campanian, Molisian and Sicilian vineyards) and GV

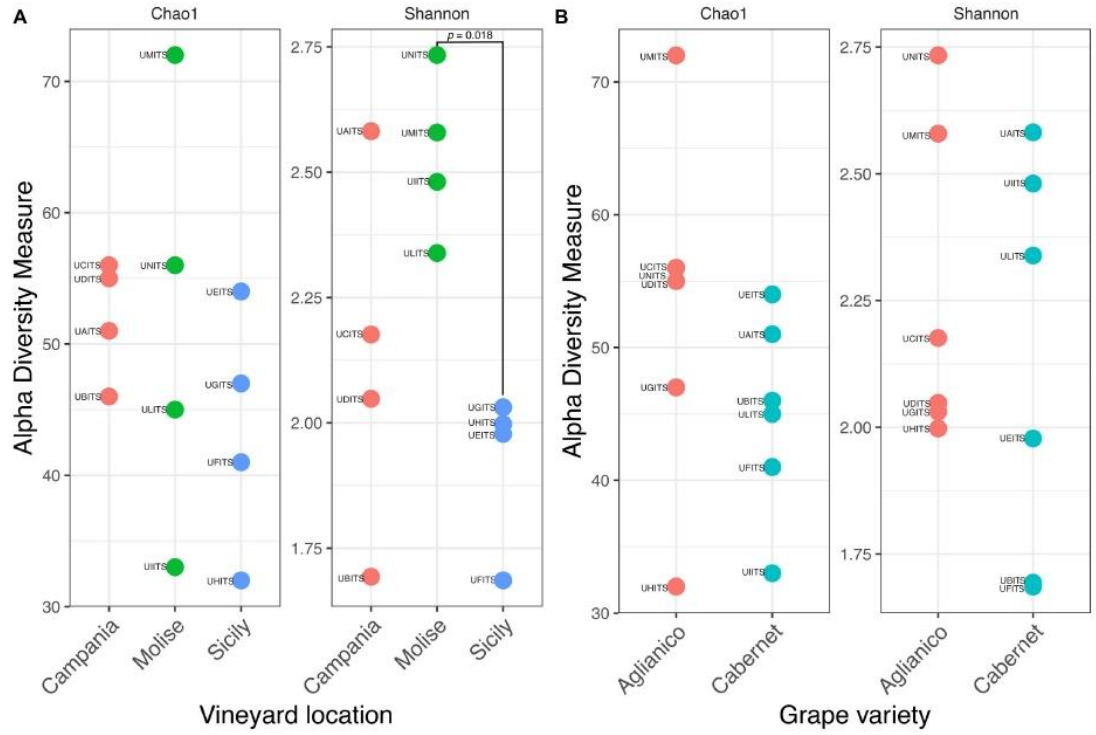


FIGURE 4
Alpha diversity of grapes calculated according to the variables **(A)** vineyard location (VL) and **(B)** grape variety (GV). Species richness and diversity were calculated with Chao1 and Shannon indexes, respectively. The sample codes are specified as follow: UAITS, Cabernet_Campania_1; UBITS, Cabernet_Campania_2; UCITS, Aglianico_Campania_2; UDITS, Aglianico_Campania_3; UEITS, Cabernet_Sicily_1; UFITS, Cabernet_Sicily_3; UGITS, Aglianico_Sicily_1; UHITS, Aglianico_Sicily_2; UIITS, Cabernet_Molise_2; ULITS, Cabernet_Molise_3; UMITS, Aglianico_Molise_1; UNITS, Aglianico_Molise_2. See **Supplementary Table 1** for more details.

(Aglianico and Cabernet) on the fungi was investigated through the Permutational Multivariate Analysis of Variance (PERMANOVA). The results revealed that the variable VL significantly ($p = 0.0001$) affected fungal communities, while the variable GV had no significant effect ($p > 0.05$). However, the interaction VL: GV showed a significant ($p = 0.001$) effect on the overall variance of the dataset (**Supplementary Table 12**). From multiple comparison tests, it was highlighted that samples from Molise vineyard were significantly different ($p = 0.039$) from those of Campanian and Sicilian vineyards. No differences ($p > 0.05$) were found between Campania and Sicily vineyards.

3.5 Discriminant analysis

The taxonomic groups driving differences between fungal communities were investigated with the sparse Partial Least Squares Discriminant Analysis (sPLS-DA). The results show that the variable VL influences the entire community (**Figure 6**). Grape samples from Molise vineyard separated from those of Sicilian vineyard along component 1 (X-variate 1), while grape samples from Campania vineyard separated from those of the other vineyards along component 2 (X-variate 2).

Taxa contributions on the first 2 components are reported in **Figures 7A, B**. Fungi that mainly increased in abundance in samples from Molise vineyard included *Sporobolomyces roseus*, *Alternaria metacromatica*, and *Cladosporium herbarum*, whilst fungi associated to samples from Sicilian vineyard belonged to the genus *Hanseniaspora*. Regarding taxa associated to Campanian vineyard, sPLS-DA analysis reported *Phaeosphaeriaceae* family and *Pichia terricola, Zygoascus meyerae, Papiliotrema flavescens* and *Hanseniaspora* spp. as the top 5 most abundant groups.

Overall, the results obtained from sPLS-DA were supported by the Linear discriminant analysis (LDA) effect size (LEfSe). LEfSe results revealed that 20 fungal taxa were mainly present in the samples of the three vineyards under study with significant differences ($p < 0.05$) at a threshold value of 1.5. In detail, one fungal taxon was associated with the Sicilian vineyard, three fungal taxa with the Campanian vineyard and 16 fungal taxa were significantly associated with the Molisian vineyard (**Figure 8**). In particular, according to results obtained by sPLS-DA analysis, the *Phaeosphaeriaceae* family was associated with the vineyard located in Campania region, while *Phaeosphaeria caricicola,* that resulted also associated with Campanian samples though LEfSe analysis, was not confirmed with sPLS-DA analysis. Regarding the samples belonging to the Molise vineyard, both LEfSe and sPLS-DA analysis confirmed *Cladosporium herbarum, Sporobolomyces roseus*, and *Alternaria metachromatica* significantly correlated to VL. Instead, *Didymellaceae* family, *Sthemphylium* genus, *Saccharomycetaceae* family and *Pyrenophora* genus resulted correlated to the Molise

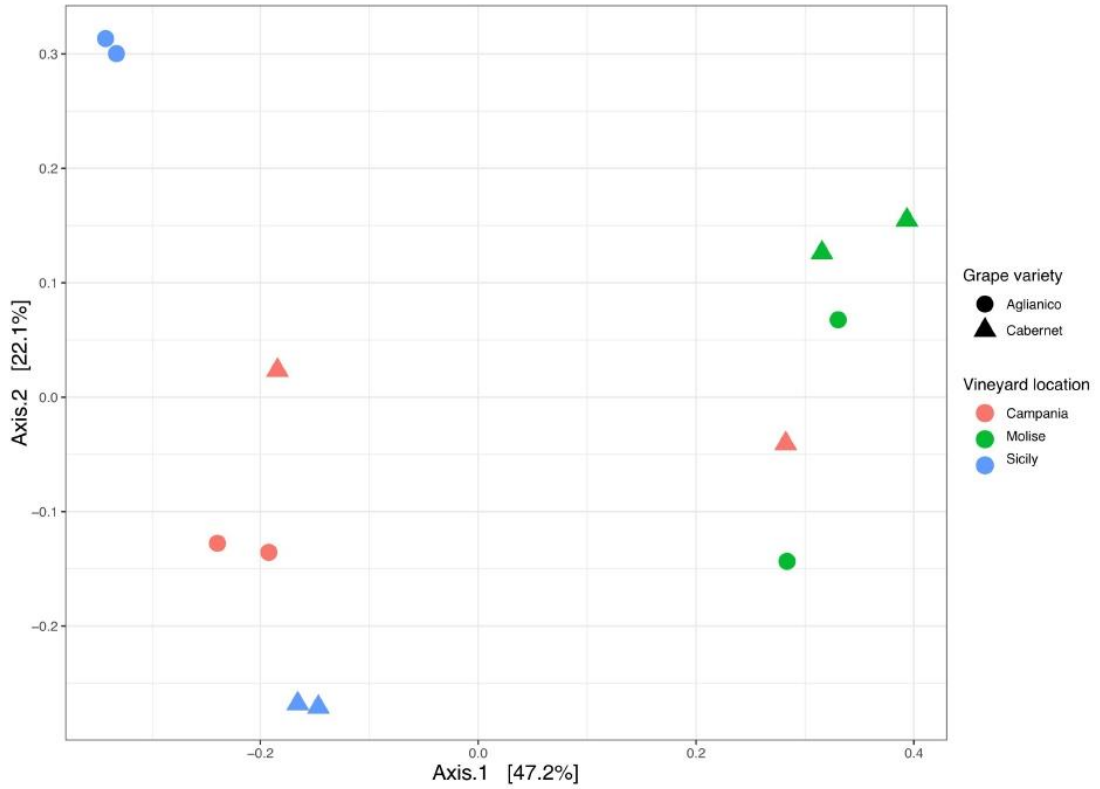


FIGURE 5
Principal Coordinate Analysis (PCoA) based on Bray Curtis distance generated with ASVs (at 99% identity) present in samples grouped according to vineyard location (VL) and grape variety (GV).

vineyard only when LEfSe analysis was applied. With regard to samples belonging to the Sicilian vineyard, LEfSe analysis reported that only Leotiomycetes was associated with the VL, contrarily to what it was obtained from the sPLS-DA analysis.

4 Discussion

Microorganisms have a key role in the winemaking process as they drive fermentation and are able to influence the aroma and quality of wine (Verginer et al., 2010; Belda et al., 2017; Sherman et al., 2020). Grape berries are one of the main sources of microbial communities that affect the winemaking process, therefore the study of the microbial diversity of wine grapes is of particular interest (Morrison-Whittle and Goddard, 2018). The structure of microbial communities could be, in turn, influenced by several factors such as geographical location, climatic conditions, grape variety, viticultural practices (e.g., fungicides, herbicides, fertilizers) and many others (Bokulich et al., 2014; Pinto et al., 2014; Setati et al., 2015; Grangeteau et al., 2017; Vitulo et al., 2019). As far as wine is concerned, individual vineyards are the smallest scale through which wine diversity can be studied (Knight et al., 2020) and understanding the scale at which microbial communities differ could be important in clarifying the concept of *terroir*.

Due to the complexity of the microbial population found on grape berries, it is evident that traditional culture-dependent methods have limitations in studying microbial diversity. On the contrary, high-throughput sequencing (HTS), coupled with multivariate data analysis, represents a valid method for studying microorganisms in a variety of contexts. In the present study, we used both HTS and multivariate data analysis to investigate the fungi community structure of grape samples collected in three different vineyards located in Campania, Molise and Sicily regions (Southern Italy) and belonging to two red grape varieties (Aglianico and Cabernet). In general, the descriptive approach through relative abundance analysis confirmed what we already found in our previous study (Iorizzo et al., 2023), but in the present research, new information is derived from the addition of new samples from the Campanian vineyard. Moreover, the statistical approach used furnished new important information on the structure of fungal communities as related to vineyard location and grape variety.

In detail, we found that the main *phyla* identified on grape berries were represented by Ascomycota, Basidiomycota and Chytridiomycota. In particular, the most abundant *phylum* in our samples was Ascomycota (~ 88%), followed by Basidiomycota (~ 12%). Similarly, Gao et al. (2019) reported that Ascomycota, Basidiomycota and Zygomycota were the main *phyla* found on the surface of grape berries from six wine regions in Xinjiang -

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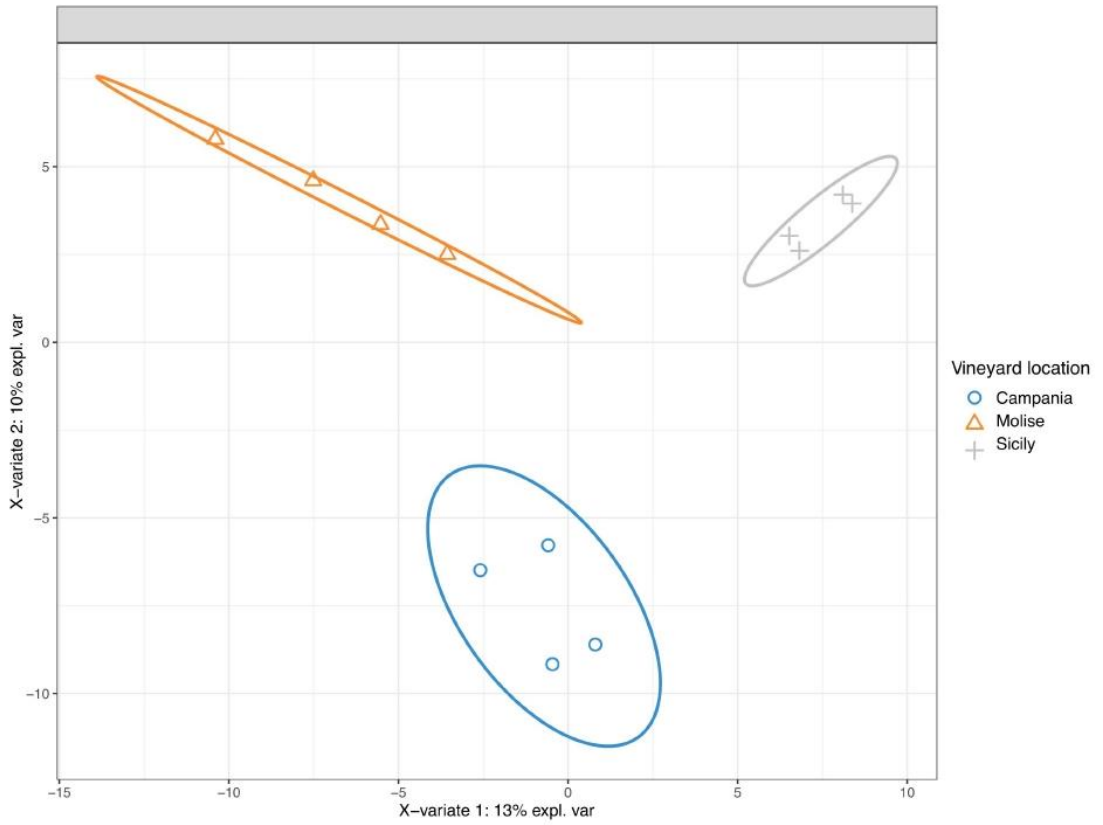


FIGURE 6
Sparse partial least-squares discriminant analysis (sPLS-DA) of read counts transformed using cumulative sum scaling normalization at the sequence variant level. The ellipse shows the 95% confidence level.

China. In addition, other authors have also found Ascomycota and Basidiomycota as the dominant fungal *phyla* on grape berries (Morrison-Whittle et al., 2017). Recently, Xu et al. (2022) conducted a study in which they reported that Ascomycota and Basidiomycota were the dominant fungi present on wine-grape surfaces.

Regarding the fungal community structure at the family level, we showed that *Cladosporiaceae*, *Saccotheciaceae*, *Pleosporaceae*, *Saccharomycodaceae* and *Didymellaceae* were the most representative families within the *phylum* Ascomycota, while *Sporidiobolaceae*, *Filobasidiaceae* and *Bulleribasidiaceae* were the most abundant ones among the *phylum* Basidiomycota. Similarly, in previous studies, other authors reported that *Pleosporaceae* and several members of *Saccotheciaceae* and *Cladosporiaceae* were the dominant fungi in Riesling grape berries (Kecskeméti et al., 2016). Interestingly, stratifying the dataset according to the variable VL, we observed that *Cladosporiaceae*, *Saccotheciaceae*, *Pleosporaceae*, and *Filobasidiaceae* were present in all samples ($n = 12$), suggesting that these 4 families represent the core taxa. Similar results were also obtained by Gao et al. (2019) in their work regarding the study of the fungal community structure of grape surfaces. In addition, Wei et al. (2022) reported that the core taxa of Cabernet Sauvignon grapes are retained at the genus level, although the fungi diversity changes during the berry development.

Studying the fungal community structure of grape berries at genus level, on a dataset stratified in accordance with the variable VL, we found that the main genera (about 71% of OTUs) were represented by *Cladosporium*, *Aureobasidium*, *Alternaria*, *Stemphylium,* and *Filobasidium*. Similarly, when the fungal community structure of grape berries was studied on a dataset stratified by the variable GV, it was observed that, apart from the genus *Stemphylium,* all other genera were conserved, indicating that *Cladosporium*, *Aureobasidium*, *Alternaria*, and *Filobasidium* constitute the core taxon. Some of these genera were also reported as the most common fungal organisms isolated from grapes in other parts of the world. For example, in a study conducted in different regions of Portugal, the authors reported that *Cladosporium, Alternaria,* and *Aureobasidium* were the main genera of Alvarinho grapes (Fernandes et al., 2023). In another recent work conducted in the Xinjiang region of China, the authors found that the dominant fungi on nine grape skins variety were represented by *Alternaria*, *Filobasidium*, *Aureobasidium*, *Erysiphe* and *Naganishia* (Xu et al., 2022). In Italy, Perpetuini et al. (2022) reported that the common genera of fungi detected in grape samples of Montepulciano d'Abruzzo cultivar (Abruzzo Region, Central Italy) were represented by *Hanseniaspora, Aureobasidium, Botrytis, Pichia, Cladosporium* and *Alternaria.* In another work conducted by Perpetuini et al. (2023), it was found that *Aureobasidium,*

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FIGURE 7
First five sequence variants contributing to the separation along with panel **(A)** component 1, and **(B)** component 2 of sPLS-DA from **Figure 6**. Bar length indicates loading coefficient ranked by importance, bottom to top; bar color indicates the group in which the sequence variant has the highest median abundance.

Metschnikowia, Hanseniaspora, Botrytis, Cladosporium, Pichia, Alternaria, Epicoccum, Vishniacozyma and *Candida* were the main genera detected on the minor grapevine cultivar cv. Nero Antico from Chieti (Abruzzo Region, Central Italy). Similarly, Rossetti et al. (2023) studied the fungal community associated with conventional and organic Trebbiano Abruzzese grapes from vineyards of Chieti (Abruzzo Region, Central Italy), and a core taxon of 8 genera was detected (*Zygosaccharomyces* spp., *Cladosporium* spp., *Botrytis* spp., *Hanseniaspora* spp., *Pichia* spp., *Alternaria* spp., *Candida* spp., *Aureobasidium* spp.) in all samples investigated.

For a better understanding of the fungal community structure of grape samples and to assess the hypothesis that the fungal population of grape berries differs between samples depending on vineyard location(VL) and/or grape variety (GV), we used several statistical tools based on both univariate and multivariate approaches.

In detail, the alpha diversity values indicated that there were no significant differences ($p > 0.05$) in terms of richness (Chao1) between the analyzed samples, highlighting that the number of species present in the samples was essentially the same, regardless the VL and the GV (**Figure 4**). On the other hand, alpha diversity as

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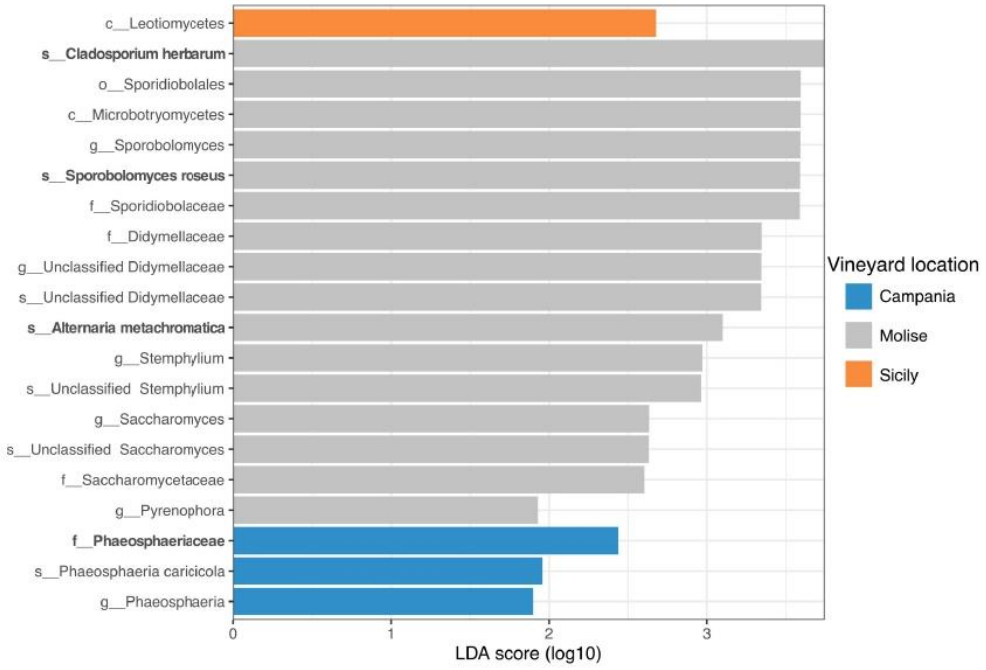


FIGURE 8
Linear discriminant analysis (LDA) effect size (LEfSe). Horizontal bars represent the effect size for each taxon. The cut off on the logarithmic LDA score for discriminative features was set to 1.5. The change of taxon relative abundance was statistically significant at $p < 0.05$. The name of the taxon level is abbreviated as p__phylum; c__class; o__order; f__family, and g__genus. Taxa name in bold were confirmed by sPLS-DA analysis reported in **Figure 7**.

measured with the Shannon index, evidenced that the VL may have a significant effect on the fungal community composition, as grapes from Molisian vineyard have a greater diversity than grapes from Sicilian one (**Figure 4**). This result indicates that there is a greater presence of rare species in grape samples from Molisian vineyard than those observed in Sicilian samples.

The effect of VL on the diversity of fungal communities among samples was also supported by the beta diversity measurement. In fact, both PCoA and sPLS-DA analyses showed that samples are mainly grouped according to the VL (**Figure 5**). These results are consistent with those reported in other studies where it was demonstrated that the diversity of the microbial community is influenced by geographical location of vineyards (Bokulich et al., 2014; Mezzasalma et al., 2017; Kioroglou et al., 2019; Papadopoulou et al., 2022; Kazou et al., 2023). Similar results were also obtained by other authors who have reported that VL has a greater impact on the fungal community than the state of ripeness (Kioroglou et al., 2019).

In the present study, a strong association between some families genera or species and their VL was confirmed by the results obtained using both sPLS-DA and LEfSe. For instance, both sPLS-DA and LEfSe analyses evidenced that *C. herbarum*, *S. roseus* and *A. methacromatica* were associated to Molisian vineyard. Similarly, the Campanian vineyard showed a strong association with the *Phaeosphaeriaceae* family, while they were weakly associated with *Pichia terricola*, *Zygoascus meyerae* and *Papiliotrema flavescens*. Regarding the Sicilian vineyard, a weak association with *Hanseniaspora* genus or with Leotiomycetes was observed since the results were not confirmed by both analyses (sPLS-DA and LEfSe).

Apart from the well-known role of *Hanseniaspora* spp. on the quality of some wines (Lombardi et al., 2018; Testa et al., 2020), the exact functions of core taxa fungi or potential biomarker fungi in wine and winemaking are unknown or poorly investigated for most of the taxa identified in the present work, while some more information, even if sometimes in contrast, is available regarding their role on grapevines. For example, some studies reported that *Filobasidium* spp. was detected on undamaged Tempranillo grapes, but it was not involved in spontaneous must fermentation (Bougreau et al., 2019). Similarly, *Aureobasidium* would appear to have no impact on the fermentation process and the wine quality (Barata et al., 2012; Setati et al., 2015; Grangeteau et al., 2017). Also, *Alternaria* spp. is generally not associated with neither fermentation or spoilage of wine (Barata et al., 2012; Setati et al., 2015; Grangeteau et al., 2017), while *Cladosporium* genus is considered a spoilage of the winery environment (Barata et al., 2012; Grangeteau et al., 2017). Both *Cladosporium* spp. and *Alternaria* spp. are endophytic fungi detected in plants showing symptoms of grapevine trunk diseases (GTDs) although their direct role in the disease was not confirmed (Patanita et al., 2022). In other studies, these genera were detected on grapevines, but no visible symptoms of plant diseases were observed (Varanda et al., 2016). Some authors reported that the presence of *Cladosporium* genus, in particular *C. herbarum*, on grape berries, is associated with the *Cladosporium* rot (Barata et al., 2012). In our study, despite the association of *C. herbarum* with

grape samples from Molise vineyard, no disease symptoms were visible on the grapes or grape plants. This could be attributed to the fact that the induction of disease may depend on many factors ascribable to the concurrent presence of this species with other microorganisms or with specific plant factors (Liu and Howell, 2021). Similar contrasting results were also reported regarding *Alternaria* spp., as some authors reported the potential role of *Alternaria* spp. as biological control agent against GTDs-associated fungi and other grapevine pathogens (Musetti et al., 2006; Bruez et al., 2014).

Sporobolomyces spp. have been detected in grape musts as well as at the beginning of wine fermentation (Li et al., 2018). These yeasts are not involved in the alcoholic fermentation, but they could contribute to the production of some metabolites that affect the aroma of wines. For instance, it is known that *Sporobolomyces roseus* can produce volatile compounds such as higher alcohols, acetate esters and thiols (Verginer et al., 2010). Moreover, there are evidence of its potential role as post-harvest biocontrol agent against *Penicillium expansum* (Sanzani et al., 2021).

5 Conclusion

Microbial *terroir* involves multiple interacting aspects, such as soil and plant-associated microbes and plant-microbe interactions. The present metataxonomic study explored the distribution of fungal communities at vineyard scale considering different vineyards located in Southern Italian regions dedicated to the production of Aglianico and Cabernet wines. Our results, although worthy of further investigation, highlighted the relevance of the vineyard location in the definition of grape-associated fungal communities. Moreover, from the results obtained in the present study, it appeared that the fungal community present on grape berries is more associated with vineyard location than with the grapevine cultivar, allowing to consider some fungal groups as territorial biomarkers regardless of the cultivar analyzed. For instance, in our study, the *Phaeosphaeriaceae* family was associated to the Campanian vineyard, while *Cladosporium herbarum, Sporobolomyces roseus*, and *Alternaria metachromatica* resulted significantly correlated to samples from Molisian vineyard and Leotiomycetes to the Sicilian one, and this for both varieties analyzed. Of course, further studies are needed, as numerous variables such as growing season, soil properties, plant status and other factors may contribute to the definition of fungal communities typical of vineyards located in near geographical areas.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://data.mendeley.com/datasets/tp2rrdz8wp/2, doi: 10.17632/tp2rrdz8wp. https://data.mendeley.com/drafts/6jcyrwj2gh, doi: 10.17632/6jcyrwj2gh.

Author contributions

MI: Conceptualization, Validation, Writing – original draft, Writing – review and editing. DB: Formal analysis, Writing – review and editing. FV: Formal analysis, Writing – review and editing. BT: Formal analysis, Writing – review and editing. PT: Conceptualization, Writing – review and editing. MS: Conceptualization, Writing – original draft, Writing – review and editing. GP: Data curation, Software, Writing – original draft, Writing – review and editing. FL: Formal analysis, Writing – review and editing. GA: Formal analysis, Writing – review and editing. SJL: Formal analysis, Writing – review and editing. RC: Conceptualization, Funding acquisition, Supervision, Validation, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1399968/full#supplementary-material

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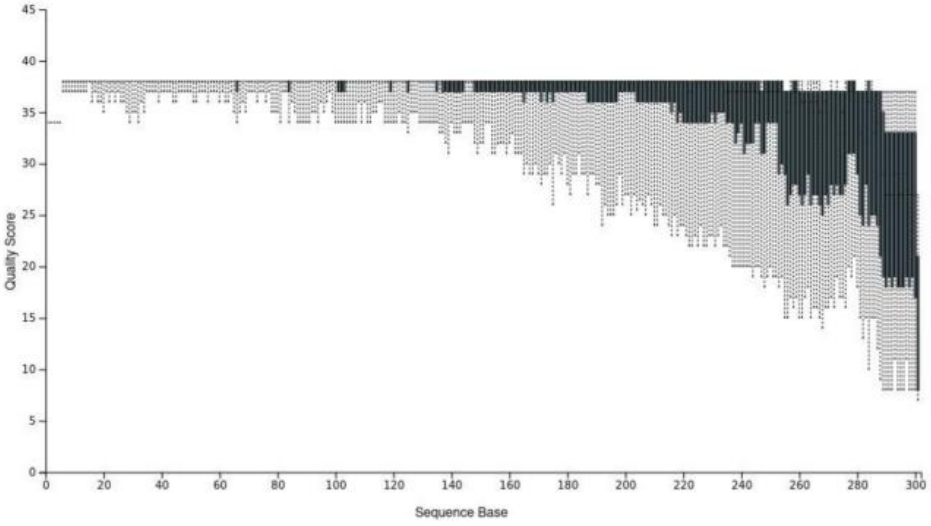
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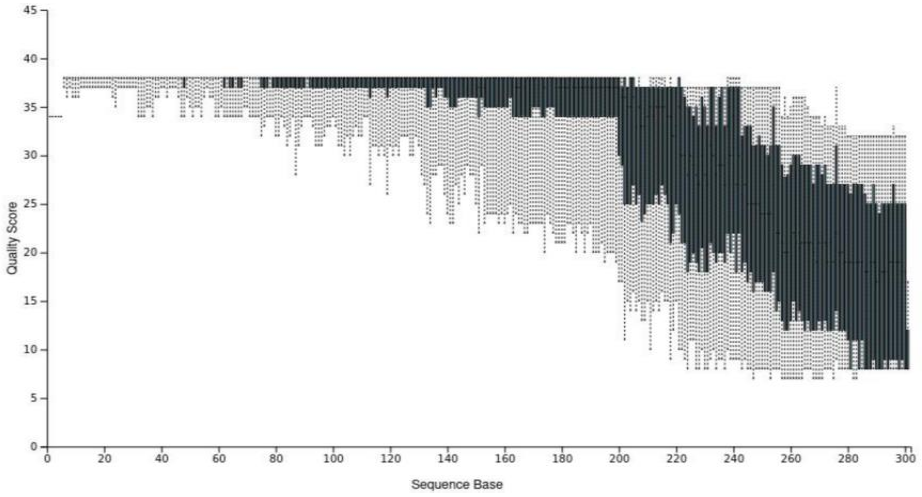
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SUPPLEMENTARY MATERIALS

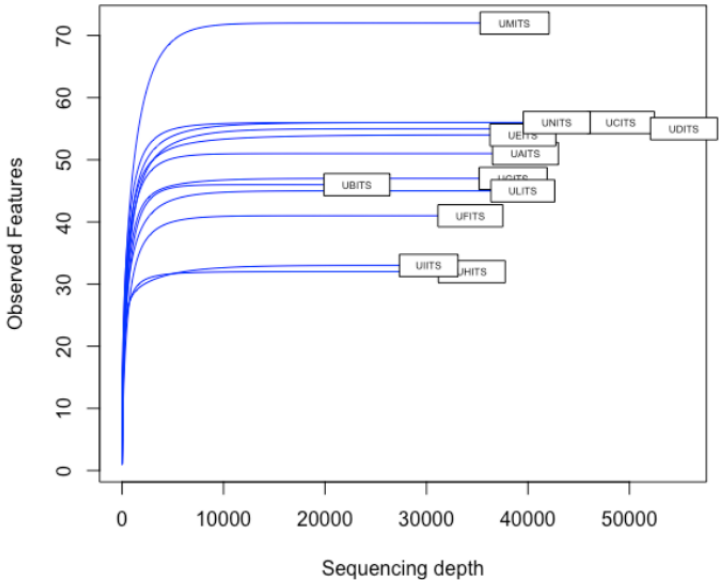
A



B



Supplementary Figure 1. Quality score plot of forward reads (A) and reverse reads (B).



Supplementary Figure 2. Rarefaction curves showing the number of observed features in relation to the number of sequenced reads (Sequencing depth).

Table S1. Sample description and metadata.

Sample ID	Samples	Sample description	Grape variety	Vineyard location
935825F894219	UAITS	Cabernet_Campania_1	Cabernet	Campania
935826F894220	UBITS	Cabernet_Campania_2	Cabernet	Campania
935827F894221	UCITS	Aglianico_Campania_2	Aglianico	Campania
935828F894222	UDITS	Aglianico_Campania_3	Aglianico	Campania
935829F894223	UEITS	Cabernet _Sicily_1	Cabernet	Sicily
935830F894224	UFITS	Cabernet _Sicily_3	Cabernet	Sicily
935831F894225	UGITS	Aglianico_Sicily_1	Aglianico	Sicily
935832F894226	UHITS	Aglianico_Sicily_2	Aglianico	Sicily
935833F894227	UIITS	Cabernet_Molise_2	Cabernet	Molise
935834F894228	ULITS	Cabernet_Molise_3	Cabernet	Molise
935835F894229	UMITS	Aglianico_Molise_1	Aglianico	Molise
935836F894230	UNITS	Aglianico_Molise_2	Aglianico	Molise

Table S2. Summary of the number of reads before and after filtering, denoising, merging and removal of chimeric sequences.

sample-id	input	filtered	percentage of input passed filter	denoised	merged	percentage of input merged	non-chimeric	percentage of input non-chimeric
935825F894219	73953	46107	62.35	45764	44989	60.83	39780	53.79
935826F894220	69566	27270	39.2	27083	24798	35.65	23151	33.28
935827F894221	104150	58687	56.35	58415	57181	54.9	49154	47.2
935828F894222	123628	64148	51.89	63929	62908	50.88	55379	44.79
935829F894223	73454	43799	59.63	42554	41843	56.96	39673	54.01
935830F894224	56392	37087	65.77	37016	36744	65.16	34337	60.89
935831F894225	78840	42800	54.29	42627	42023	53.3	38545	48.89
935832F894226	67486	36342	53.85	36200	35896	53.19	34476	51.09
935833F894227	54059	32260	59.68	32189	31861	58.94	30226	55.91
935834F894228	75193	46414	61.73	46278	45276	60.21	39513	52.55
935835F894229	68189	46528	68.23	46250	45053	66.07	38728	56.8
935836F894230	78649	49964	63.53	49704	48672	61.89	42843	54.47

Table S3. Overall descriptive statistics of the relative abundance of taxa at *Phylum* level.

Phylum	n	average	sd	min	max	median
Ascomycota	12	87.9	9.4	73.0	98.2	90.7
Basidiomycota	12	11.8	9.3	1.6	26.6	9.0
Chytridiomycota	12	0.0	0.1	0.0	0.3	0.0
Unclassified Fungi	12	0.3	0.2	0.0	0.5	0.4

Table S4. Descriptive statistics of the relative abundance of taxa at the *Phylum* level grouped according to the vineyard location (VL) variable.

Phylum	origin	n	average	sd	min	max	median
Ascomycota	Campania	4	90.0	11.4	73.0	96.9	95.1
Ascomycota	Molise	4	79.5	3.8	75.0	82.8	80.1
Ascomycota	Sicily	4	94.1	5.1	86.7	98.2	95.8
Basidiomycota	Campania	4	9.7	11.4	2.6	26.6	4.7
Basidiomycota	Molise	4	20.0	3.7	17.0	24.4	19.4
Basidiomycota	Sicily	4	5.6	5.0	1.6	12.9	4.0
Chytridiomycota	Campania	4	0.0	0.0	0.0	0.0	0.0
Chytridiomycota	Molise	4	0.1	0.1	0.0	0.3	0.0
Chytridiomycota	Sicily	4	0.0	0.0	0.0	0.0	0.0
Unclassified Fungi	Campania	4	0.3	0.2	0.2	0.5	0.3
Unclassified Fungi	Molise	4	0.4	0.1	0.2	0.5	0.4
Unclassified Fungi	Sicily	4	0.3	0.2	0.0	0.5	0.2

Table S5. Descriptive statistics of the relative abundance of taxa at the *Phylum* level grouped according to the grape variety (GV) variable.

Phylum	grape_variety	n	average	sd	min	max	median
Ascomycota	Aglianico	6	91.1	8.8	77.7	98.2	95.7
Ascomycota	Cabernet	6	84.6	9.6	73.0	95.5	84.8
Basidiomycota	Aglianico	6	8.6	8.6	1.6	21.9	4.2
Basidiomycota	Cabernet	6	15.0	9.5	4.3	26.6	14.9
Chytridiomycota	Aglianico	6	0.0	0.0	0.0	0.0	0.0
Chytridiomycota	Cabernet	6	0.0	0.1	0.0	0.3	0.0
Unclassified Fungi	Aglianico	6	0.3	0.2	0.0	0.5	0.3
Unclassified Fungi	Cabernet	6	0.3	0.1	0.2	0.5	0.4

Table S6. Overall descriptive statistics of the relative abundance of taxa at Family level.

Sample	Family	Relative abundance			
UAITS	Bulleribasidiaceae	0.0	UBITS	Filobasidiaceae	0.6
UAITS	Cladosporiaceae	30.2	UBITS	Others (median <1%)	3.9
UAITS	Didymellaceae	2.0	UBITS	Pleosporaceae	13.7
UAITS	Filobasidiaceae	0.5	UBITS	Saccharomycetaceae	0.1
UAITS	Others (median <1%)	6.0	UBITS	Saccharomycodaceae	0.4
UAITS	Pleosporaceae	24.1	UBITS	Saccotheciaceae	56.3
UAITS	Saccharomycetaceae	1.7	UBITS	Sporidiobolaceae	2.1
UAITS	Saccharomycodaceae	0.2	UCITS	Bulleribasidiaceae	0.6
UAITS	Saccotheciaceae	12.0	UCITS	Cladosporiaceae	12.1
UAITS	Sporidiobolaceae	23.3	UCITS	Didymellaceae	0.1
UBITS	Bulleribasidiaceae	0.7	UCITS	Filobasidiaceae	2.1
UBITS	Cladosporiaceae	22.0	UCITS	Others (median <1%)	4.2
UBITS	Didymellaceae	0.3	UCITS	Pleosporaceae	15.3

UCITS	Saccharomycetaceae	0.6
UCITS	Saccharomycodaceae	29.1
UCITS	Saccotheciaceae	34.4
UCITS	Sporidiobolaceae	1.5
UDITS	Bulleribasidiaceae	0.3
UDITS	Cladosporiaceae	18.0
UDITS	Didymellaceae	0.0
UDITS	Filobasidiaceae	0.7
UDITS	Others (median <1%)	4.3
UDITS	Pleosporaceae	16.2
UDITS	Saccharomycetaceae	0.5
UDITS	Saccharomycodaceae	27.8
UDITS	Saccotheciaceae	31.5
UDITS	Sporidiobolaceae	0.7
UEITS	Bulleribasidiaceae	6.5
UEITS	Cladosporiaceae	49.9
UEITS	Didymellaceae	0.4
UEITS	Filobasidiaceae	4.8
UEITS	Others (median <1%)	5.4
UEITS	Pleosporaceae	9.7
UEITS	Saccharomycetaceae	0.5
UEITS	Saccharomycodaceae	0.8
UEITS	Saccotheciaceae	21.9
UEITS	Sporidiobolaceae	0.0
UFITS	Bulleribasidiaceae	2.8
UFITS	Cladosporiaceae	47.2
UFITS	Didymellaceae	0.5
UFITS	Filobasidiaceae	0.9
UFITS	Others (median <1%)	2.7
UFITS	Pleosporaceae	18.2
UFITS	Saccharomycetaceae	0.3
UFITS	Saccharomycodaceae	0.0
UFITS	Saccotheciaceae	27.0
UFITS	Sporidiobolaceae	0.2
UGITS	Bulleribasidiaceae	0.6
UGITS	Cladosporiaceae	9.2
UGITS	Didymellaceae	0.5
UGITS	Filobasidiaceae	0.2
UGITS	Others (median <1%)	7.6
UGITS	Pleosporaceae	3.3
UGITS	Saccharomycetaceae	0.3
UGITS	Saccharomycodaceae	43.6
UGITS	Saccotheciaceae	34.6
UGITS	Sporidiobolaceae	0.2
UHITS	Bulleribasidiaceae	0.0
UHITS	Cladosporiaceae	13.3
UHITS	Didymellaceae	0.1
UHITS	Filobasidiaceae	2.1
UHITS	Others (median <1%)	6.1
UHITS	Pleosporaceae	1.8
UHITS	Saccharomycetaceae	0.0
UHITS	Saccharomycodaceae	44.0
UHITS	Saccotheciaceae	32.5
UHITS	Sporidiobolaceae	0.0
UIITS	Bulleribasidiaceae	0.0
UIITS	Cladosporiaceae	33.9
UIITS	Didymellaceae	2.6
UIITS	Filobasidiaceae	1.4
UIITS	Others (median <1%)	4.8
UIITS	Pleosporaceae	25.1
UIITS	Saccharomycetaceae	2.1
UIITS	Saccharomycodaceae	1.3
UIITS	Saccotheciaceae	9.4
UIITS	Sporidiobolaceae	19.4
ULITS	Bulleribasidiaceae	0.0
ULITS	Cladosporiaceae	35.4
ULITS	Didymellaceae	15.5
ULITS	Filobasidiaceae	0.2
ULITS	Others (median <1%)	4.4
ULITS	Pleosporaceae	12.6
ULITS	Saccharomycetaceae	2.1
ULITS	Saccharomycodaceae	0.0
ULITS	Saccotheciaceae	14.8
ULITS	Sporidiobolaceae	15.0
UMITS	Bulleribasidiaceae	1.7
UMITS	Cladosporiaceae	37.0
UMITS	Didymellaceae	6.1
UMITS	Filobasidiaceae	4.0
UMITS	Others (median <1%)	3.4
UMITS	Pleosporaceae	23.2
UMITS	Saccharomycetaceae	0.7
UMITS	Saccharomycodaceae	0.0
UMITS	Saccotheciaceae	13.6
UMITS	Sporidiobolaceae	10.3
UNITS	Bulleribasidiaceae	1.5
UNITS	Cladosporiaceae	32.3
UNITS	Didymellaceae	4.4
UNITS	Filobasidiaceae	2.8
UNITS	Others (median <1%)	3.2
UNITS	Pleosporaceae	27.7
UNITS	Saccharomycetaceae	2.4
UNITS	Saccharomycodaceae	0.3
UNITS	Saccotheciaceae	9.2
UNITS	Sporidiobolaceae	16.3

Table S7. Descriptive statistics of the relative abundance of taxa at the Family level grouped according to the vineyard location (VL) variable.

origin	Family	n	average	sd	min	max	median
Campania	Bulleribasidiaceae	4	0.40	0.31	0.00	0.67	0.46
Campania	Cladosporiaceae	4	20.55	7.59	12.06	30.16	19.99
Campania	Didymellaceae	4	0.63	0.95	0.00	2.04	0.24
Campania	Filobasidiaceae	4	0.96	0.76	0.49	2.10	0.63
Campania	Others (median <1%)	4	4.62	0.94	3.94	6.02	4.25
Campania	Pleosporaceae	4	17.35	4.62	13.74	24.10	15.78
Campania	Saccharomycetaceae	4	0.72	0.73	0.05	1.75	0.53
Campania	Saccharomycodaceae	4	14.36	16.28	0.17	29.08	14.09
Campania	Saccotheciaceae	4	33.55	18.13	11.99	56.27	32.96
Campania	Sporidiobolaceae	4	6.88	10.95	0.66	23.27	1.78
Molise	Bulleribasidiaceae	4	0.80	0.93	0.00	1.66	0.77
Molise	Cladosporiaceae	4	34.66	2.02	32.26	36.97	34.70
Molise	Didymellaceae	4	7.16	5.73	2.64	15.49	5.26
Molise	Filobasidiaceae	4	2.09	1.63	0.24	3.96	2.07
Molise	Others (median <1%)	4	3.96	0.76	3.22	4.81	3.91
Molise	Pleosporaceae	4	22.16	6.61	12.64	27.70	24.16
Molise	Saccharomycetaceae	4	1.80	0.75	0.70	2.36	2.08
Molise	Saccharomycodaceae	4	0.40	0.61	0.00	1.28	0.17
Molise	Saccotheciaceae	4	11.74	2.88	9.16	14.76	11.52
Molise	Sporidiobolaceae	4	15.22	3.77	10.29	19.37	15.62
Sicily	Bulleribasidiaceae	4	2.50	2.95	0.00	6.52	1.74
Sicily	Cladosporiaceae	4	29.93	21.63	9.23	49.90	30.29
Sicily	Didymellaceae	4	0.36	0.17	0.13	0.49	0.42
Sicily	Filobasidiaceae	4	1.99	2.05	0.17	4.83	1.48
Sicily	Others (median <1%)	4	5.45	2.04	2.71	7.57	5.77
Sicily	Pleosporaceae	4	8.27	7.48	1.85	18.24	6.49
Sicily	Saccharomycetaceae	4	0.28	0.22	0.00	0.52	0.29
Sicily	Saccharomycodaceae	4	22.11	25.07	0.00	44.02	22.22
Sicily	Saccotheciaceae	4	28.99	5.68	21.93	34.55	29.75
Sicily	Sporidiobolaceae	4	0.11	0.13	0.00	0.23	0.11

Table S8. Descriptive statistics of the relative abundance of taxa at the Family level grouped according to the grape variety (GV) variable.

grape_variety	Family	n	average	sd	min	max	median
Aglianico	Bulleribasidiaceae	6	0.79	0.67	0.00	1.66	0.62
Aglianico	Cladosporiaceae	6	20.31	11.54	9.23	36.97	15.66
Aglianico	Didymellaceae	6	1.88	2.68	0.00	6.15	0.30
Aglianico	Filobasidiaceae	6	1.96	1.38	0.17	3.96	2.08
Aglianico	Others (median <1%)	6	4.81	1.70	3.22	7.57	4.25
Aglianico	Pleosporaceae	6	14.60	10.40	1.85	27.70	15.78
Aglianico	Saccharomycetaceae	6	0.73	0.84	0.00	2.36	0.53
Aglianico	Saccharomycodaceae	6	24.14	19.81	0.00	44.02	28.45
Aglianico	Saccotheciaceae	6	25.96	11.42	9.16	34.55	32.00
Aglianico	Sporidiobolaceae	6	4.82	6.84	0.00	16.26	1.07
Cabernet	Bulleribasidiaceae	6	1.67	2.62	0.00	6.52	0.34
Cabernet	Cladosporiaceae	6	36.45	10.53	21.97	49.90	34.70
Cabernet	Didymellaceae	6	3.56	5.92	0.34	15.49	1.26
Cabernet	Filobasidiaceae	6	1.40	1.73	0.24	4.83	0.73
Cabernet	Others (median <1%)	6	4.54	1.16	2.71	6.02	4.60
Cabernet	Pleosporaceae	6	17.26	6.32	9.71	25.09	15.99
Cabernet	Saccharomycetaceae	6	1.13	0.94	0.05	2.10	1.14
Cabernet	Saccharomycodaceae	6	0.44	0.51	0.00	1.28	0.27
Cabernet	Saccotheciaceae	6	23.56	17.30	9.40	56.27	18.35
Cabernet	Sporidiobolaceae	6	9.99	10.46	0.00	23.27	8.53

Table S9. Overall descriptive statistics of the relative abundance of taxa at Genus level.

Genus	Sample	Relative abundance			
Alternaria	UAITS	15.4	Aureobasidium	UHITS	32.5
Alternaria	UBITS	12.6	Aureobasidium	UIITS	9.4
Alternaria	UCITS	13.8	Aureobasidium	ULITS	14.8
Alternaria	UDITS	15.5	Aureobasidium	UMITS	13.6
Alternaria	UEITS	8.6	Aureobasidium	UNITS	9.2
Alternaria	UFITS	17.5	Cladosporium	UAITS	30.2
Alternaria	UGITS	2.2	Cladosporium	UBITS	21.9
Alternaria	UHITS	1.8	Cladosporium	UCITS	12.1
Alternaria	UIITS	21.2	Cladosporium	UDITS	18.0
Alternaria	ULITS	10.5	Cladosporium	UEITS	49.8
Alternaria	UMITS	19.5	Cladosporium	UFITS	46.9
Alternaria	UNITS	22.9	Cladosporium	UGITS	9.2
Aureobasidium	UAITS	12.0	Cladosporium	UHITS	13.3
Aureobasidium	UBITS	56.3	Cladosporium	UIITS	33.2
Aureobasidium	UCITS	34.4	Cladosporium	ULITS	35.4
Aureobasidium	UDITS	31.5	Cladosporium	UMITS	36.9
Aureobasidium	UEITS	21.9	Cladosporium	UNITS	32.2
Aureobasidium	UFITS	27.0	Filobasidium	UAITS	0.5
Aureobasidium	UGITS	34.6	Filobasidium	UBITS	0.6

Filobasidium	UCITS	2.1	Sporobolomyces	UBITS	2.1
Filobasidium	UDITS	0.7	Sporobolomyces	UCITS	1.4
Filobasidium	UEITS	4.8	Sporobolomyces	UDITS	0.6
Filobasidium	UFITS	0.9	Sporobolomyces	UEITS	0.0
Filobasidium	UGITS	0.2	Sporobolomyces	UFITS	0.0
Filobasidium	UHITS	2.1	Sporobolomyces	UGITS	0.2
Filobasidium	UIITS	1.4	Sporobolomyces	UHITS	0.0
Filobasidium	ULITS	0.2	Sporobolomyces	UIITS	19.4
Filobasidium	UMITS	4.0	Sporobolomyces	ULITS	14.7
Filobasidium	UNITS	2.8	Sporobolomyces	UMITS	10.3
Hanseniaspora	UAITS	0.2	Sporobolomyces	UNITS	16.3
Hanseniaspora	UBITS	0.4	Stemphylium	UAITS	8.7
Hanseniaspora	UCITS	29.1	Stemphylium	UBITS	1.1
Hanseniaspora	UDITS	27.8	Stemphylium	UCITS	1.4
Hanseniaspora	UEITS	0.8	Stemphylium	UDITS	0.6
Hanseniaspora	UFITS	0.0	Stemphylium	UEITS	0.8
Hanseniaspora	UGITS	43.6	Stemphylium	UFITS	0.6
Hanseniaspora	UHITS	44.0	Stemphylium	UGITS	1.1
Hanseniaspora	UIITS	1.3	Stemphylium	UHITS	0.0
Hanseniaspora	ULITS	0.0	Stemphylium	UIITS	3.6
Hanseniaspora	UMITS	0.0	Stemphylium	ULITS	1.7
Hanseniaspora	UNITS	0.3	Stemphylium	UMITS	3.6
Others (median <1%)	UAITS	6.0	Stemphylium	UNITS	4.8
Others (median <1%)	UBITS	4.4	Unclassified Didymellaceae	UAITS	2.0
Others (median <1%)	UCITS	4.8	Unclassified Didymellaceae	UBITS	0.3
Others (median <1%)	UDITS	4.5	Unclassified Didymellaceae	UCITS	0.1
Others (median <1%)	UEITS	6.4	Unclassified Didymellaceae	UDITS	0.0
Others (median <1%)	UFITS	3.4	Unclassified Didymellaceae	UEITS	0.4
Others (median <1%)	UGITS	8.0	Unclassified Didymellaceae	UFITS	0.5
Others (median <1%)	UHITS	6.1	Unclassified Didymellaceae	UGITS	0.3
Others (median <1%)	UIITS	5.8	Unclassified Didymellaceae	UHITS	0.1
Others (median <1%)	ULITS	5.2	Unclassified Didymellaceae	UIITS	2.6
Others (median <1%)	UMITS	3.6	Unclassified Didymellaceae	ULITS	15.5
Others (median <1%)	UNITS	3.3	Unclassified Didymellaceae	UMITS	6.1
Saccharomyces	UAITS	1.7	Unclassified Didymellaceae	UNITS	4.4
Saccharomyces	UBITS	0.0	Vishniacozyma	UAITS	0.0
Saccharomyces	UCITS	0.6	Vishniacozyma	UBITS	0.4
Saccharomyces	UDITS	0.5	Vishniacozyma	UCITS	0.4
Saccharomyces	UEITS	0.5	Vishniacozyma	UDITS	0.3
Saccharomyces	UFITS	0.3	Vishniacozyma	UEITS	6.0
Saccharomyces	UGITS	0.0	Vishniacozyma	UFITS	2.8
Saccharomyces	UHITS	0.0	Vishniacozyma	UGITS	0.6
Saccharomyces	UIITS	2.1	Vishniacozyma	UHITS	0.0
Saccharomyces	ULITS	2.1	Vishniacozyma	UIITS	0.0
Saccharomyces	UMITS	0.7	Vishniacozyma	ULITS	0.0
Saccharomyces	UNITS	2.4	Vishniacozyma	UMITS	1.7
Sporobolomyces	UAITS	23.3	Vishniacozyma	UNITS	1.5

Table S10. Descriptive statistics of the relative abundance of taxa at the Genus level grouped according to the vineyard location (VL) variable.

Origin	Genus	n	average	sd	min	max	median
Campania	Alternaria	4	14.3	1.4	12.6	15.5	14.6
Campania	Aureobasidium	4	33.5	18.1	12.0	56.3	33.0
Campania	Cladosporium	4	20.5	7.6	12.1	30.2	19.9
Campania	Filobasidium	4	1.0	0.8	0.5	2.1	0.6
Campania	Hanseniaspora	4	14.4	16.3	0.2	29.1	14.1
Campania	Others (median <1%)	4	4.9	0.7	4.4	6.0	4.6
Campania	Saccharomyces	4	0.7	0.7	0.0	1.7	0.5
Campania	Sporobolomyces	4	6.8	11.0	0.6	23.3	1.7
Campania	Stemphylium	4	2.9	3.9	0.6	8.7	1.2
Campania	Unclassified Didymellaceae	4	0.6	1.0	0.0	2.0	0.2
Campania	Vishniacozyma	4	0.3	0.2	0.0	0.4	0.3
Molise	Alternaria	4	18.5	5.5	10.5	22.9	20.3
Molise	Aureobasidium	4	11.7	2.9	9.2	14.8	11.5
Molise	Cladosporium	4	34.4	2.2	32.2	36.9	34.3
Molise	Filobasidium	4	2.1	1.6	0.2	4.0	2.1
Molise	Hanseniaspora	4	0.4	0.6	0.0	1.3	0.2
Molise	Others (median <1%)	4	4.5	1.2	3.3	5.8	4.4
Molise	Saccharomyces	4	1.8	0.8	0.7	2.4	2.1
Molise	Sporobolomyces	4	15.2	3.8	10.3	19.4	15.5
Molise	Stemphylium	4	3.4	1.3	1.7	4.8	3.6
Molise	Unclassified Didymellaceae	4	7.2	5.7	2.6	15.5	5.3
Molise	Vishniacozyma	4	0.8	0.9	0.0	1.7	0.8
Sicily	Alternaria	4	7.5	7.3	1.8	17.5	5.4
Sicily	Aureobasidium	4	29.0	5.7	21.9	34.6	29.7
Sicily	Cladosporium	4	29.8	21.5	9.2	49.8	30.1
Sicily	Filobasidium	4	2.0	2.0	0.2	4.8	1.5
Sicily	Hanseniaspora	4	22.1	25.1	0.0	44.0	22.2
Sicily	Others (median <1%)	4	6.0	1.9	3.4	8.0	6.3
Sicily	Saccharomyces	4	0.2	0.3	0.0	0.5	0.2
Sicily	Sporobolomyces	4	0.1	0.1	0.0	0.2	0.0
Sicily	Stemphylium	4	0.6	0.4	0.0	1.1	0.7
Sicily	Unclassified Didymellaceae	4	0.3	0.1	0.1	0.5	0.3
Sicily	Vishniacozyma	4	2.4	2.7	0.0	6.0	1.7

Table S11. Descriptive statistics of the relative abundance of taxa at the Genus level grouped according to the grape variety variable (GV) variable.

grape_variety	Genus	n	average	sd	min	max	median
Aglianico	Alternaria	6	12.6	8.8	1.8	22.9	14.6
Aglianico	Aureobasidium	6	26.0	11.4	9.2	34.6	32.0
Aglianico	Cladosporium	6	20.3	11.5	9.2	36.9	15.7
Aglianico	Filobasidium	6	2.0	1.4	0.2	4.0	2.1
Aglianico	Hanseniaspora	6	24.1	19.8	0.0	44.0	28.4
Aglianico	Others (median <1%)	6	5.1	1.8	3.3	8.0	4.6
Aglianico	Saccharomyces	6	0.7	0.9	0.0	2.4	0.5
Aglianico	Sporobolomyces	6	4.8	6.9	0.0	16.3	1.0
Aglianico	Stemphylium	6	1.9	1.9	0.0	4.8	1.2
Aglianico	Unclassified Didymellaceae	6	1.8	2.7	0.0	6.1	0.2
Aglianico	Vishniacozyma	6	0.8	0.7	0.0	1.7	0.5
Cabernet	Alternaria	6	14.3	4.7	8.6	21.2	14.0
Cabernet	Aureobasidium	6	23.6	17.3	9.4	56.3	18.3
Cabernet	Cladosporium	6	36.2	10.5	21.9	49.8	34.3
Cabernet	Filobasidium	6	1.4	1.7	0.2	4.8	0.7
Cabernet	Hanseniaspora	6	0.4	0.5	0.0	1.3	0.3
Cabernet	Others (median <1%)	6	5.2	1.1	3.4	6.4	5.5
Cabernet	Saccharomyces	6	1.1	0.9	0.0	2.1	1.1
Cabernet	Sporobolomyces	6	9.9	10.5	0.0	23.3	8.4
Cabernet	Stemphylium	6	2.7	3.1	0.6	8.7	1.4
Cabernet	Unclassified Didymellaceae	6	3.6	5.9	0.3	15.5	1.3
Cabernet	Vishniacozyma	6	1.5	2.4	0.0	6.0	0.2

Table S12. PERMANOVA on the whole dataset. Test was conducted using the adonis2 function of the Vegan package, 10000 permutations and the Bray Curtis metric.

	Df	SumOfSqs	R2	F	Pr(>F)
vineyard_location (VL)	2	0.3827	0.2615	1.984	0.0094
grape_variety (GV)	1	0.1361	0.093	1.4112	0.1375
vineyard_location:grape_variety (VL:GV)	2	0.366	0.2501	1.8974	0.013
Residual	6	0.5786	0.3954		
Total	11	1.4634	1		

Chapter 3.
SHEEP MILK AND CHEESE

3.1 Introduction to Sheep Breeding and Ovine Milk Consumption in Italy

Sheep milk is an important source of bioactive substances that perform beneficial functions for the body health (Flis and Molik, 2021). Its valuable composition is due to high content of fatty acids (FA), immunoglobulins, proteins, hormones, vitamins and minerals. Today, the consumption of sheep milk as such is not very common, as it is almost exclusively used for dairy processing. It accounts approximately 1.3% of the global milk production and is used for cheese production throughout Europe (Quigley *et al*., 2013).

In Italy, the sheep dairy sector is characterised by strong regional disparities (Figure 1), with Southern regions and islands showing a clear tendency to become more important in this sector. In the South, there are indeed localised realities with a relevant environmental and socio-economic role of sheep farming (Pulina *et al*., 2018).

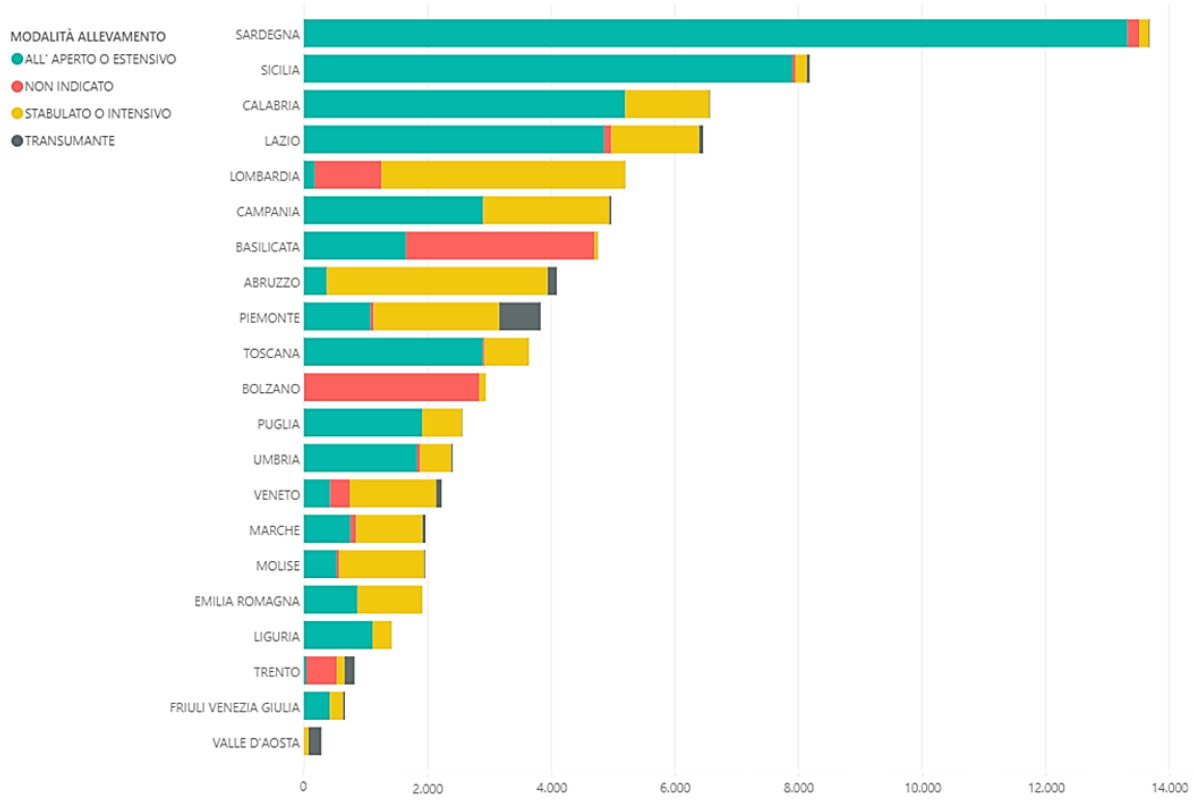


Figure 1. Sheep farms on Italian territory (processed on 30/06/2023).
Source: https://www.vetinfo.it/j6_statistiche/#/report-pbi/29.

However, many factors contribute to the decline of a sector with very high potential, but poorly organised. Added to this are the difficulties of adapting to hygiene and sanitation standards, finding affordable pastureland, the instability of raw material prices, and finding a sufficiently

specialised workforce willing to accept living conditions that are not always easy. In the light of these premises, it would be useful to improve the competitiveness of the sector by developing innovative actions aimed at promoting the quality of ovine milk and cheese, strengthening their link with the territory and biodiversity, while guaranteeing high standards of food safety and consumer health.

Italy plays a key role in the European sheep sector in terms of milk and cheese production. Indeed, it is the third largest producer of sheep milk (with 21% of European production), after Greece (31.7%) and Spain (27.3%) and the largest producer of sheep cheese (with 32% of European production) (Pulina *et al.*, 2018). Spain is the main competitor of Italian products on foreign markets, both in terms of product characteristics and price, although in 2021 it recorded a sharp fall in production of both raw materials and cheese compared with the previous year.

The sheep sector in Italy represents little more than 1% of the value of national agricultural production, but it continues to play an important role in the manufacturing sector. Sheep farming is mainly concentrated in central and Southern Italy. Half of the sheep reared today are in Sardinia region, and almost half of the value of meat and milk production is concentrated there. The rest of the sheep flocks and related production are in Sicily, Calabria and Latium regions, and to a lesser extent, in the rest of Italy (see Figure 1).

The sheep sector is characterised by a steady decline in the number of people involved in breeding, due to the gradual abandonment of this activity. However, while the percentage of farms is decreasing, their conversion to intensive or semi-intensive systems is increasing.

According to the annual census of the National Livestock Register (formally, Anagrafe Nazionale Zootecnica), in 2021 more than 80% of sheep farms were geared towards a mixed orientation, i.e. meat and milk production, the latter being used almost exclusively for cheese production (Biegalski *et al.*, 2021).

3.2 Sheep Milk Composition

The different composition of sheep milk compared to cow milk is reflected in the different physico-chemical properties. Sheep milk has a porcelain white colour, lighter than cow' milk due to the lower presence of β-carotene, and higher viscosity due to the higher dry matter content (Raynal-Ljutovac *et al.*, 2008). It also has a higher specific gravity, refractive index and titratable acidity (Wendorff and George, 2017). Sheep milk is also an important source of bioactive compounds that have beneficial effects on human health, the cardiovascular system and cancer prevention (Mendoza Guzmán *et al.*, 2011; Flis and Molik, 2021). It has been shown to contain Conjugated Linoleic Acid (CLA), Polyunsaturated Fatty Acids (PUFA, especially ω3) and fat-soluble vitamins A and E, known for their antioxidant properties (Puppel *et al.*, 2017).

The levels of these nutrients are higher in sheep milk than in milk from other ruminants and cow milk, showing a higher nutritional value (Balthazar *et al.*, 2017). Sheep milk is rich in minerals, containing more calcium, phosphorus, magnesium, zinc, manganese and copper than

cow milk (Wendorff and George, 2017). In addition, it promotes less allergic sensitisation than goat and cow milk (Masoodi and Shafi, 2010) and is free of agglutinins with higher digestibility than cow milk (Park *et al*., 2007; De Devitiis *et al*., 2023).

Considering the sheep milk composition, physico-chemical and microbiological properties, its technological behaviour differers from that of cow milk. The main differences between sheep's milk and cow's milk are in the fats and proteins. These differences are fundamental from a technological point of view, as they have a decisive influence on the processing of the milk into its derivatives, the process parameters to be used and the cheese-making yields (Silanikove *et al*., 2016). Moreover, the chemical composition of sheep milk (as well as cow milk) is subject to considerable variability caused by various factors such as diet, type and location of farming, genetic factors and others (Kasapidou *et al.,* 2021). Fat is present in sheep milk in the form of globules with an average size of 4.5 μm, surrounded by a protein membrane with a complex structure (Ramos and Juarez, 2003). Milk fat is composed of lipids such as mono-, di- and triglycerides (representing 98% of fats), cholesterol, phospholipids and sterols. The FA content of sheep milk is higher than that of cow milk. It differs significantly in the distribution of its lipid components, being much richer in medium-chain triglycerides (C6:0-C14:0) and monounsaturated, polyunsaturated and essential FA (Wendorff and George, 2017). Saturated FA C6:0, C8:0 and C10:0 are highly digestible. They therefore provide a rapid energy supply, especially for people suffering from malnutrition or fat malabsorption syndrome and ensure a low tendency to store fat. They also help to reduce total circulating cholesterol, particularly low-density lipoprotein (LDL) (Recio *et al.,* 2009). Sheep milk has a very similar FA content to cow milk with respect to butyric acid (C4:0), the low concentrations of which contribute to the *in vitro* inhibition of cancer cell lines (Rasmussen *et al.,* 2010). The lipid composition of sheep milk fat is influenced by feeding regimes and different feed compositions. In fact, according to the study by Ramos and Juarez (2003), adding concentrates and reducing the amount of forage in the sheep daily diet can reduce medium chain FA. Exposure to solar radiation and high temperatures can also reduce the profile of mono- and polyunsaturated FA (Sevi *et al.,* 2002). Adequate supply of fibre-rich forages to sheep is crucial for maintaining good milk fat levels of short-chain FA (Wendorff and George, 2017).

The average protein content of sheep milk is higher than that of cow milk. This content varies widely within the species and is influenced by breed, stage of lactation, diet, climate, season and udder health (Park *et al*., 2007). The total protein content of milk is essentially determined by its nitrogen content, multiplied by a factor of 6.38, which represents the average nitrogen content of protein (Rafiq *et al*., 2016). The true protein content is the total protein minus the non-proteins, which can make up between 5 and 7% of the total nitrogen in milk, consisting of urea (45%), amino acids (16%), creatine (2%), creatinine (2%), uric acid (2%), ammonium (1%), orotic acid, hippuric acid and others. Protein in sheep milk accounts for approximately 95% of the total nitrogen and 5% is non-protein nitrogen (Park *et al*, 2006). Milk proteins occur in two different phases, an unstable micellar phase composed of caseins and a soluble phase composed of whey proteins (Lima Nascimento *et al*., 2023). Caseins (αs1-CN, αs2-CN, β-CN

and κ-CN) make up about 76-80% of the total proteins in sheep milk from healthy udders (Marina *et al*., 2020). The main whey proteins are α-lactalbumin and β-lactoglobulin, but sheep milk also contains immunoglobulins, serum albumin, lactoferrin, peptones and some other minor proteins in small amounts (Ramos and Juarez, 2003). All these proteins make up the remaining 20-24% of total proteins (Landi *et al.*, 2021). The caseins αs1-CN and αs2-CN are lower in sheep milk than in cow milk, whereas β-CN is higher in sheep milk. For this reason, sheep milk coagulates faster and has a greater consistency than cow milk. The percentage of serum proteins is higher in sheep milk than in cow milk.

3.3 Ewe Cheeses

Sheep milk can be successfully used to make cheese with almost any recipe used for cow milk (Kalyankar *et al*., 2016). Thanks to its composition, rich in fat, protein and mineral salts, it guarantees the production of cheeses of different types, but all of them particularly tasty and full-bodied. The processing of this cheese involves the same basic operations as for other cheeses, which give rise to different products. Cheeses made from sheep milk differ in consistency, curdling temperature and maturation. The most important sheep milk cheese is Pecorino, the term used to refer to hard or semi-hard, semi-cooked Italian cheeses made from whole sheep milk and without the addition of starter cultures (Aquilanti *et al*., 2007). This term derives from "pecora" (Gaglio *et al*., 2021). The broader tradition of processing raw sheep milk is concentrated in the central and southern regions (Centi *et al.*, 2017). Pecorino cheeses, however, are produced almost throughout Italy, with names strictly referring to the geographic origin. These cheeses are considered typical products because they're processed by applying traditional production methods in specific geographical areas (Todaro *et al.*, 2011; Gaglio *et al.*, 2021). Most of them enjoy Protected Designation of Origin (PDO) status, a quality recognition granted by the European Community to protect certain agricultural products and foodstuffs produced in a specific area and according to the corresponding production protocols (Regulation EU No. 1151/2012 of the European Parliament). These designations constitute an element of protection of biodiversity, i.e. of the territory, animals, microbes, production practices and systems (Pirisi *et al.*, 2011). To date, there are thirteen Italian pecorinos with PDO status (Table 1).

Sheep cheese, considered one of the first cheeses invented by man, belongs to the centuries-old tradition of transhumance. It is characterised by a different ripening period that varies from 8 to 12 months (Di Cagno *et al.*, 2003; Schirone *et al.*, 2012). During the ripening process, the paste, which is soft and elastic in the younger cheeses, increases significantly in consistency, and the rind gradually changes from a light straw colour to its typical brown colour and becomes thicker. The taste, which is sweet and aromatic in the first months, also becomes much more piquant and characteristic. The weight of the sheep cheese varies from 1.5 to 5 kg.

Table 1. List of PDO Italian pecorino cheeses.

NAME	PRODUCTION ZONE	PDO STATUS REG.*
Canestrato Pugliese	Bari, Foggia	EC Reg. No. 1107, 12.06.1996
Pecorino Romano	Frosinone, Latina, Roma, Viterbo, Cagliari, Nuoro, Sassari, Grosseto	EC Reg. No. 1107, 12.06.1996 EC Reg. No. 1030, 29.10.2009
Pecorino Siciliano	Catania, Enna, Trapani, Caltanissetta, Palermo, Ragusa, Siracusa, Messina, Agrigento	EC Reg. No. 1107, 12.06.1996
Pecorino Toscano	Arezzo, Pisa, Massa Carrara, Livorno, Grosseto, Firenze, Prato, Lucca, Pistoia, Siena, Terni, Perugia, Viterbo	EC Reg. No. 1263, 01.07.1996
Pecorino Sardo	Cagliari, Nuoro, Oristano, Sassari	EC Reg. No. 1263, 10.07.1996
Fiore Sardo	Cagliari, Nuoro, Oristano, Sassari	EC Reg. No. 1263, 10.07.1996
Pecorino di Filiano	Potenza	EC Reg. No. 1485, 14.12.2007
Pecorino Crotonese	Crotone, Catanzaro, Cosenza	Reg. (EU) No 1262, 8.11.2014
Formaggio di Fossa di Sogliano	Forlì-Cesena, Rimini, Ravenna and part of the territory of the province of Bologna, provincial districts of Ancona, Macerata, Pesaro-Urbino and Ascoli Piceno	EC Reg. No. 1183, 30.11.09
Pecorino di Picinisco	Province of Frosinone	EC Reg. No 1161/2013
Pecorino delle Balze Volterrane	Municipalities Volterra, Pomarance, Montecatini Val di Cecina, Castelnuovo Val di Cecina, Monteverdi, all located in the province of Pisa	EC Reg. No. 271/2015
Pecorino from Monte Poro	27 municipalities in the province of Vibo Valentia	EC Reg. No. 2020/974
Piacentinu Ennese	Enna, Aidone, Assoro, Barrafranca, Calascibetta, Piazza Armerina, Pietraperzia, Valguarnera, Villarosa.	EC Reg. No 132, 14.02.2011

*Regulations supplementing the annexes of the relating to the registration of geographical indications and designations of designations of origin under the procedure laid down in Article 17 of Regulation (EEC) n. 2081/92.

The processing of the sheep cheese is carried out in full accordance with the tradition of cheese making from which it originates. Thus, even today it is produced according to traditional protocols and artisanal procedures that foresee the use of rennet paste from the rennet stomachs of lambs or goats, which contain enzymes that intervene in the coagulation phase and in the lipolytic and proteolytic processes during ripening (Santillo *et al*., 2012).

3.4 Pecorino Cheeses from Campania Region

Concerning the sheep sector in Campania, which will be discussed in more detail in the next paragraphs, dairy farming is widespread in this region. The most important sectors are buffalo and cattle, but the sheep sector is not to be underestimated. According to the National Zootechnical Register, there are about 171492 sheep in Campania. Of the more than 5030 sheep farms, only about 41 (7311 sheep) are dedicated exclusively to milk production, while about half are dedicated to meat production and the rest to both meat and milk production (report

updated 31/12/2022). In recent years, however, there has been a growing awareness that sheep farming could be a potential resource for local development and against the depopulation of mountain areas, with the consequent safeguarding of the link with the territory, support for breeders and promotion of the multifunctionality of the farm. In particular, the sheep sector, whose milk is used to make cheeses that are mainly sold on regional markets, is one of the least valued in Campania, as in all areas of central and Southern Italy. The animals are reared mainly on pasture. The farms are mostly located inland, and the milk is often processed in small cheese dairies attached to family farms, which often double as farm shops.

In Campania different types of pecorino cheeses are produced, depending on the breed or genetic type of sheep that provide the milk, the essences that characterise the different wild pastures, and the curd used. These include Pecorino Bagnolese, Pecorino di Laticauda Sannita and Pecorino Carmasciano. All of them bear the designation PAT- Prodotti Agroalimentari Tradizionali (Traditional Food Products) (Legislative Decree No. 173 of 1998) and are consumed fresh or after maturing.

Pecorino Bagnolese, also known as "casu'r pecora", is a hard cheese made from the raw milk of sheep of the native sheep breed "Bagnolese". This sheep cheese is typical of the area of the Picentini Mountains, especially of the area around the plateau of Lake Laceno at an altitude of over 1000 m and of the hills and mountains of Salerno on the slopes of Monte Marzano.

The milk used to produce Pecorino Bagnolese comes from herds raised in the wild on natural pastures. Milking is done by hand and the milk must be processed no later than 24 hours after the first milking. Locally produced lamb rennet is added to the milked milk. After the curd is formed and broken, the mass is separated from the whey, which is used to make fresh ricotta or its salted version. The ripening process takes place in cool rooms, where the cheese is frequently turned, and lasts between one month (fresh cheese) and three months (semi-matured cheese). Maturation results in a brown crust with a compact shape and a straw-yellow paste that has a uniform appearance and the right degree of greasiness on the palate. Pecorino Bagnolese is compact when cut, has a typical smell of milk and fresh grass and a rare spicy flavour.

Laticauda Sannita sheep cheese is made exclusively from the milk of Laticauda sheep, a breed native to Campania with dual aptitude (milk and meat), whose name derives from the shape of its wide, fat tail (*latis cauda*), capable of accumulating fat during the period when pastures are abundant and of utilising it during periods of drought. This sheep cheese, whose goodness is mainly due to the spontaneous grasses of the mountain pastures, such as the Ladino clover, is produced only in the breeding area of this sheep, namely in the mountain and hill communities of the provinces of Avellino, Benevento and Caserta. The different production of Pecorino di Laticauda Sannita depends on the different duration of the ripening, which varies from 2 (fresh) to 120 days (ripe). Fresh pecorino is usually flavoured with wild herbs, while mature pecorino has a hard, sometimes floury consistency, does not stick to the knife when cut, has a fine grain and a flaky curd, and has no internal cavities or imperfections. The colour varies from straw yellow to light yellow, with a pleasant and intense smell of sheep milk and a slightly spicy taste.

Carmasciano is a pecorino cheese produced in an area of Irpinia in the province of Avellino

from raw milk of Laticauda sheep on soils with volcanic activity (Coppola *et al*., 2006; Mercogliano *et al*., 2010). The characteristics of the cheese depend on the quality of the sulphurous grasses consumed in the Carmasciano area; therefore, Pecorino Carmasciano is produced in very limited quantities by small family farms and sold at a high price. Since 2009, Pecorino Carmasciano has been recognised by the Italian Ministry of Agriculture, Food and Forestry as PAT. It is a mature cheese with a dark, hard, wrinkled rind and a smooth, dry, slightly oily, dark yellow surface. The interior of the cheese has scattered holes (1-5 mm in diameter) and is pale yellow, uniform, compact, hard, crumbly, with fine to medium holes that diminish over time (Marrone *et al*., 2014). It has a typical taste with hints of milk and grass and an aftertaste that becomes spicier and saltier as it matures. These characteristics are primarily due to the inherent and heterogeneous microbial flora involved in the ripening process, which is responsible for metabolic processes that shape the properties of the final product (Capobianco *et al.*, 2005; Coppola *et al.*, 2006). The production area of Pecorino Carmasciano includes the municipalities of Guardia dei Lombardi and Rocca San Felice (Carmasciano is a hamlet located between these two municipalities), Torella dei Lombardi, Sant'Angelo dei Lombardi, Morra de Sanctis and Frigento. The production area, delimited by a circle with a radius of about 4 km, coincides with the hills that surround the Asanto Valley, described by Virgilio in the Eneide as an enchanted land. In fact, the valley is characterised by the presence of the Mefite, a lake of sulphurous origin fed by sulphurous basins that boil due to gas emissions from underground. The sulphur emanating from the water characterises the forage essences of the area such as mint, thyme, oregano, wild fennel and, above all, clover, which in turn give the milk a very particular and complex flavour (Mercogliano *et al.,* 2010). Sulphur fumes contribute to the characteristic spicy flavour and yellow colour of pecorino. The production of Pecorino Carmasciano is exclusively artisan. The cheese is made in the kitchen or in the cellars from raw whole sheep milk, milked in the evening and in the morning. About 40 L of milk are processed per day, the yield depending on the processing time. The milk (without the addition of ferments) is put into the "caccavo" (copper kettle), heated over a wood fire to 40-45°C for 15-20 min and coagulated with homemade lamb or goat rennet (Mercogliano *et al.,* 2010). About 30 min after the rennet is added, the curd is broken into grains the size of rice grains and settles to the bottom of the kettle. After removing the whey used to make ricotta, the curd is worked by hand until a compact mass is obtained, which is placed in the "fascedda" (a small wicker basket) where it rests for 48 h. Then the cheese is scalded in hot whey, rubbed with salt (dry salted after 24 h) and after 10 days it is brushed with olive oil, white wine, and vinegar. This helps to obtain a dark, firm rind and prevents the formation of mould. The ageing process lasts between 3 and 15 months, during which the cheese is stored in the dark on wooden tables under controlled humidity and temperature conditions (Mercogliano *et al.,* 2010). Pecorino Carmasciano reaches its maximum sensory expression after about 12 months of ageing.

3.5 Sheep Milk and Cheese Microbiota

The study of microbial communities in milk has gained increasing interest in recent years. The development of culture-independent techniques, in particular 16S rRNA gene sequencing, has led to new studies aimed at understanding the composition, diversity and biological role of the bacterial community in milk from different dairy species (Addis *et al*., 2016; Esteban-Blanco *et al*., 2019, 2020). Milk serves as a reservoir for a diverse community of microorganisms acquired from numerous sources. These indigenous raw milk microbes can be beneficial or harmful to both milk and cheese and subsequently, once consumed, to human health (O'Sullivan and Cotter, 2017). The microbiota enclosed in milk plays a key role throughout the cheese making process and contributes to the development of the flavor and texture typical of different types of cheeses. So, understanding the microbial community associated with milk and cheese is now considered a key factor in the dairy industry as it allows us to discover how all steps of the cheese production process affect the hosted microbiota (Tilocca *et al*., 2020).

Milk has a higher microbial diversity than cheese (Porcellato *et al*., 2018). Estaban-Blanco *et al.* (2019) used NGS of amplicons from the 16S rRNA gene to characterise the microbiome of "Churra" sheep milk, showing that the core microbiota was composed by only two taxa: *Staphylococcus* and *Escherichia/Shigella*. *E. coli*, *Salmonella*, *Staphylococcus aureus*, *Bacillus* and *Clostridium perfrigens* were also found in in the study of Fotou *et al.,* 2011. These data should not be overlooked, considering that in most cases pecorino cheese is produced with raw milk, posing health risks for the consumer in case of fresh cheese consumption. In other studies, the microbial consortia detected in ewe milk were very complex. For instance, in the study by Endres *et al.* (2021), a total of 14 *phyla* (with a relative abundance >0.1%) and 87 bacterial genera were identified in sheep milk. The most abundant *phyla* were Proteobacteria (58.03%), Firmicutes (39.93%), Actinobacteria (1.79%) and Bacteroidetes (0.17%), while the most abundant genera were *Phyllobacterium* spp. (42.59%), *Staphylococcus* spp. (29.20%), *Pseudomonas* spp. (10.96%), *Lactococcus* spp. (5.10%) and *Acinetoacter* spp. (1.38%). Esteban-Blanco *et al.* (2020) detected the presence of 43 phyla, two of which belong to the Archea. The most represented phyla were Firmicutes (64.44%), Actinobacteria (14.25%), Proteobacteria (9.08%), Acidobacteria (2.7%) and Bacteroidetes. The dominant genus in ewe milk samples was *Staphylococcus*, followed by *Lactobacillus*, *Corynebacterium*, *Streptococcus* and *Escherichia/Shigella*. In the study from Davati and Hesami (2021) a high biodiversity of bacteria in ewe milk was highlighted, with Proteobacteria as the most represented *phylum*.

The high variability in the composition of microbial communities in sheep milk depends on several factors. Upon excretion, milk is immediately colonised by a complex microbiota, including microorganisms that naturally inhabit the skin and epithelial lining of the teat canal, mainly represented by the genera *Streptococcus* spp. *Staphylococcus* spp., *Micrococcus* spp. and *Pseudomonas* spp. (Bortoluzzi and Menezes, 2014). The presence of the genus *Staphylococcus* spp. is considered to be indicative of mastitis in sheep, as it is considered to be the main aetiological agent of intramammary infections. However, as some *Staphylococcus*

species are part of the natural microbiota of many mammals, this microorganism has also been frequently observed in the raw milk of healthy sheep (Esteban-Blanco *et al*., 2019; Gonzales-Barron *et al*., 2017). Moreover, sheep milk is an important source of probiotic microorganisms. In the study by Chen *et al.* (2020), four LAB strains with potential probiotic functions (including three *Lactobacillus lactis* strains and one *Leuconostoc lactis* strain) were isolated and tested for probioticity with the aim of using them in the future for the production of various food and pharmaceutical products.

Other factor able to affect the microbial communities in ewe milk are the locations of animals in different geographical area, their health conditions and their breeding and feeding conditions. With regard to the latter factor, rumen microbial metabolism is known to influence milk biosynthesis (Xue *et al*., 2019), thus determining its composition, which in turn can influence the composition of the microbiota in milk. Moreover, microorganisms present in feed can directly pass in milk. For instance, the use of silage may increase the probability of finding *Clostridium* species in milk (Bencini *et al*., 2010).

HTS of DNA is also used to study the microbiota of different cheeses during processing, as the composition and functional properties of the microbiota give different cheeses unique characteristics (Nam *et al.,* 2021). The cheese microbiota involved in the cheese ripening process is dominated by LAB, particularly the genus *Lactococcus*, which grows slowly together with the genus *Lactobacillus* during the first weeks of cheese ripening until it dominates the microbiota at the end of the ripening process (Centi *et al.*, 2017). *Lactobacillus* spp. play a crucial role in cheese ripening by releasing bioactive peptides, vitamins, and oligosaccharides (Santiago-Lopez *et al.,* 2018). In a study by Endres *et al.* (2021), the microbiota of sheep cheese was predominantly composed of LAB, specifically *Streptococcus* spp. (73.15%), *Lactobacillus* spp. (19.91%), *Lactococcus* spp. (2.46%), and *Staphylococcus* spp. (1.74%). *Streptococcus* spp. and *Lactobacillus* spp. were also frequently identified as the predominant bacteria in cheeses in other studies (Aldrete-Tapia *et al.,* 2014; Castellanos-Rozo *et al*., 2020) and are associated with cheese flavours and textures. These genera can produce bacteriocins, which act as bioprotective agents against pathogenic and spoilage microorganisms (Kaya and Simsek, 2019; Gontijo *et al*., 2020). The presence of *Staphylococcus* spp. in ewe cheese microbiota may be of concern, as certain species may pose health risks. Their presence may be related to animal health status, handling practices and contamination during cheese production (Aldrete-Tapia *et al*., 2014).

The sensory characteristics of cheese can be influenced by various environmental and technological factors (Fox *et al.,* 2017; Serrapica *et al.,* 2020; Uzun *et al.,* 2020). The nature of the pasture contributes significantly to the definition of milk and cheese aroma (Alothman *et al*., 2019). Indeed, as already reported, nutrition plays a central role in influencing milk characteristics, which in turn determine cheese quality characteristics (Sabia *et al.,* 2020). This is possible because feeds, the main components of ruminant diets, can confer specific nutritional and organoleptic properties to milk that help define the "*terroir*" and digestibility of cheese (Giaccone *et al.,* 2016). Several studies have addressed the effects of botanical diversity and forage storage methods on the quality of different cheeses (Martin *et al.,* 2005; Uzun *et al.,*

2018; Giorgio *et al*., 2019). It has been shown that pasture and fresh grass can positively change the FA composition of milk fat in terms of PUFA (Fusaro *et al.,* 2020). These are transferred to the corresponding dairy products and influence the taste and texture of cheese (Alothman *et al.,* 2019). Therefore, the sensory characteristics of cheese reflect the characteristics of fresh pastures (Viallon *et al.,* 2000; Bugaud *et al.,* 2001) and the contribution of the natural microbiota of the animal and dairy environment.

In southern Italian regions, sheep farms are mainly dedicated to the production of milk, which is processed into different sheep cheeses associated with local traditions. Pecorino Carmasciano is an example of a traditionally aged cheese produced in Campania. In these areas, pastures are characterised by strong seasonal variations in botanical composition and productivity (Scocco *et al*., 2016). As a result, the management system changes seasonally from extensive to semi-extensive or indoor. Therefore, these cheeses often have unique flavours that result from the characteristics of native plant species in local pastures and the interaction of these plants with the soil and climate of the area (Carpino *et al*., 2004).

RESEARCH AIM

Milk and cheese derived from sheep constitute under-exploited resources of microbial genetic diversity (Endres *et al*., 2021). The microbial communities in ewe milk play a crucial role in the production of traditional cheeses made from raw milk, as they actively influence the processing through metabolic and growth progressions. The specific composition of the milk microbiota also has a direct impact on the subsequent ripening of dairy products (Quigley *et al.*, 2013). The study of microbial communities in dairy products has made significant progress in recent years. This has been driven by the development of HTS technologies, which allow the composition and function of microbial communities to be studied on a much larger scale than previously possible (Silva *et al*., 2023). Indeed, the use of classical microbiological analyses based on plate count techniques does not allow the taxonomic identification of microbes, nor can it detect species present in low numbers or in a non-culturable state (Cocolin *et al*., 2007; Neviani *et al*., 2013). In light of previous findings, the research reported below focused on the investigation and comparison of the microbial communities in samples of sheep milk from three different dairy farms in the Campania region by using a culture-independent approach. Some chemical features of ewe milk and the possible relationship between these features and bacterial composition were also investigated.

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Chemical Features and Microbial Consortia of Raw Sheep Milk from the Campania Region with a Focus on the Production of Pecorino Carmasciano Cheese

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Abstract

This study provides new insights into the determinants of the distinctive quality of Pecorino Carmasciano by examining the chemical characteristics of the sheep milk used for its production and evaluating the correlation between these characteristics and the bacterial composition, analysed using a high-throughput sequencing (HTS) approach.

Milk samples from three Campania provinces (Avellino, Caserta and Salerno) were analysed. Milk from the province of Avellino showed significantly higher concentrations of iron, potassium and zinc compared to the other sheep milk studied. The pipelines used for metagenomic classification highlighted the predominant phyla, families and genera in milk from the different provinces, with Proteobacteria as the dominant *phylum*, followed by Firmicutes and Bacteroides. Metagenomic analysis revealed a greater diversity of families and genera in the Avellino milk sample than in the Caserta and Salerno samples. The presence of metals had an impact on the microbiome. Increased metal concentrations in the milk samples induced an increase in the diversity of genera and species. Univocal bacterial species were observed in each milk sample, representing the main microbiome of each milk. Avellino milk showed a higher number of univocal species, linked to the high presence of metals, highlighting the influence of the environment on its composition.

Keywords: Ewe milk, Metals, Microbiome, PDO cheese, Mefite D'Ansanto.

1. Introduction

Over the past 20 years, several researchers have studied the characteristics of a particular pecorino cheese, named "Carmasciano" from its production area. In fact, Carmasciano is a small district located in Alta Irpinia, in the province of Avellino (Italy), and the interest in this cheese (Coppola *et al.*, 2006; Mercogliano *et al*., 2010; Marrone *et al.*, 2014; Ricciardi *et al*., 2014) is linked to the fact that sheep graze largely on Mefite D'Ansanto, considered as the largest non-volcanic environment on the Earth characterised by natural emission of low temperature CO_2 rich gases, followed by minor amounts of non-atmospheric N_2, H_2S and CH_4 (Chiodini *et al*., 2010). Carmasciano cheese, produced from ewe raw milk, is characterised by

strong flavour and taste for the use of lamb past chyimosin, and a slight piquant flavour that appears during ripening.

Due to the peculiar geomorphology of Mefite, several authors have tried to find specific markers that can correlate the characteristics of this particular site with ewe milk and cheese produced here. For example, Cozzolino *et al.* (2021) assessed the presence of more than 80 volatile organic compounds (VOCs) in samples of Pecorino Carmasciano, but there were no sulphur compounds among them, and statistical analysis revealed no significant differences in VOCs compared to other Italian Pecorino cheeses. Marrone *et al.* (2014) studied the fatty acid profile and cholesterol content of Pecorino Carmasciano during ripening. They concluded that the relatively high level of polyunsaturated fatty acids, the ratio of n-6 to n-3 fatty acids, and the low cholesterol content, together with a good presence of conjugated linoleic acid, make Pecorino Carmasciano a case for consideration by the Council of the European Union's Regulation for the awarding of a PDO.

With regard to the microbial composition of Carmasciano milk and cheese, so far mainly culture-dependent approaches have been used. Coppola *et al*. (2006) reported the presence of both non-lactic and lactic acid bacteria (LAB), with the highest counts registered in cheeses between 3 and 21 d of ripening for all searched microbial groups. Ricciardi *et al*. (2014) used a PCR-DGGE based approach for the identification of predominant LAB strains after simulated digestion of Pecorino Carmasciano, concluding that one gram of fresh Carmasciano cheese can supply the human colon with approximately the same amount of vital LAB as a probiotic drink.

With regard to the investigation of microbial populations through the use of High Throughput Sequencing (HTS), these methodologies were proposed for their potential to show how the microbiome of milk and cheese can influence the ripening process and the sensory characteristics of cheese, suggesting their application as an additional tool to identify the key microbial species that contribute to PDO designation (Kamilari *et al*., 2019). For instance, De Pasquale *et al.* (2016) correlated VOCs with the metabolically active microbiota in three Italian PDO ewes milk cheeses (Fiore Sardo, Pecorino Siciliano and Pecorino Toscano) using pyrosequencing of the 16S rRNA targeting RNA.

In the work by Gonçalves Dos Santos *et al*., 2017, Authors studied the fungal communities of Serpa is an artisanal PDO pecorino cheese from Portugal. The HTS analysis of fungal communities in both PDO and non-PDO Serpa cheeses highlighted that production practices and the environment have a significant influence on the final yeast population.

To the best of our knowledge, to date no research investigated the microbiome of milk used for the production of Pecorino Carmasciano by animal grazing on Mefite. Instead, a very recent study (De Castro et al., 2023) profiled the diversity of microorganisms residing in Mefite D'Ansanto through an HTS study of 16S rRNA and ITS2, showing that biogeochemical characteristics of this unique environment, such as pH, temperature, salts, trace elements and organic compounds, significantly influence the composition of microbial communities.

In this study, we obtained new information on factors affecting Pecorino Carmasciano tipicity by investigating some chemical features of ewe milk for its production, and the possible

relationship between these features and bacterial composition, studied through amplicons sequencing of the 16S rRNA region using an HTS approach.

2. Materials and Methods

2.1 Milk Sample Collection

Ewe milk samples were obtained from 90 animals, 30 from each of three different farms in Campania region located in Avellino (AV), Caserta (CE) and Salerno (SA) provinces. The flocks were all composed of different breed animals (Sarda, Lacaune and Laticauda) and the sheep from which the milk was collected were all free of disease and had been lambing for about two months. Milk was collected in June from mainly pasture-fed animals, and the pasture in the province of Avellino was situated predominantly in the area of Mefite D'Ansanto. The bulk milk from each farm was transported under refrigerated conditions to the dairy company Carmasciando s.r.l. (Guardia dei Lombardi, AV, Italy). Following the procedure for the production of Pecorino Carmasciano, milk was left to rest overnight at room temperature. The next day, the evening milk was mixed with the corresponding fresh milk from the same farm. Prior to cheese making, 100 mL of the bulk milk from each farm (M_Av, M_Ce, and M_Sa) were transferred into sterile containers and sent to the laboratory of Food Microbiology - University of Molise - in refrigerated conditions.

2.2 Microbiological and Physico-Chemical Analyses of Milk

Fat, lactose, protein, urea and dry matter in milk were determined by mid infrared spectrometry (Method ISO 9622:2013, IDF 141:2013). Microorganisms at 30°C (Method ISO 4833-1:2013 and ISO 7218:2007/Amd 1:2013), somatic cells (Method ISO 13366-2:2006, IDF 148-2:2006), acidity (ISTSAN Report 96/34, p. 229) and antibiotics (Method AOAC DSM 930704 1993) were also determined.

2.3. Detection of Trace Elements in Milk

Calcium, Iron, Manganese, Nickel, Potassium, Copper, Sodium, Strontium and Zinc were determined according to the method ISO 21424:2018 on milk samples. Analysis was performed by Inductively Coupled Plasma (ICP)-MS using an Agilent 7800 ICP-MS equipped with autosampler and Integrated Sample Introduction System. A multi-element stock standard solution (Multi-Element Standard) was purchased from the manufacturer. Briefly, aliquots of 0.5 g of each sample were added with 2 mL concentrated HNO_3 into cleaned digestion tube (Pyrex 50 mL culture tubes). For digestion, tubes were left overnight at room temperature, followed by a digestion at 120°C until the liquid dried out. Two additional mL of HNO_3 was added and heated to dryness, this step was performed until samples resulted clear. Additional digestion was done by adding 1 mL solution of HNO_3 and $HClO_4$ (50:50) and leaving the tubes for 2 hours at 180°C. The temperature was increased to 220°C and the solution was heated to dryness. Tubes were then cooled to room temperature, and the ash obtained was dissolved in 1

mL concentrated HCl and 10 mL of 5% HNO_3. Samples were injected into ICP-MS (Agilent 7800) to determine the mineral content. Minerals were expressed in ppm (mg/Kg). Analyses were repeated in triplicate for each sample.

2.4 Metagenomic Analysis

Total genomic DNA from the samples were extracted using the "Stool DNA Isolation Kit" (Norgen, Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. DNA, extracted from 200 µL of homogenised sample, was standardised at a concentration of 10 ng/µl for metagenomic sequencing. Bacterial communities were accessed by sequencing the V3-V4 region of 16S rRNA amplified with universal primers 341F (5'-CCTACGGGNBGCASCAG-3') and 805R (5'-GACTACNVGGGTATCTAATCC-3') (Herlemann *et al.*, 2011; Klindworth *et al.*, 2013; Takahashi *et al.*, 2014). Amplicons were sequenced using the Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) were performed through an external service (Biodiversa s.r.l., Rovereto, Italy). Raw PE reads were analysed using the QIIME2 (v. 2020.8) pipeline and DADA2 (v. 1.16). For the quality control of reads, the QS value of 20 was used as a threshold and the low-quality regions of sequences were removed. ASVs were classified using the DADA2 and QIIME2 naïve Bayes classifier (qiime feature-classifier fit-classifier-naive-bayes), trained on the GreenGenes database (v. 13_8) (McDonald *et al.*, 2012).

2.5 Statistical Analysis

Data were elaborated with SPSS (IBM SPSS Statistics, Version 2019) by using ANOVA coupled with the TukeyHSD post-hoc test. The significance level for the statistical tests was set to an α of 0.05. Pearson's correlation coefficient (r) was used to calculate linear correlation between variables.

3. Results and Discussion

3.1 Physico-Chemical and Microbiological Characteristics of Milk

Values obtained by the ewe milk composition (Table 1) highlighted the good quality of the three milk under analysis, with parameters fitting with those recorded by other Authors who studied Campanian sheep milk (Landi *et al.*, 2021), even if fat and protein content detected in this study resulted higher than those reported in other works (Serrapica *et al.*, 2020). These differences are, however, to be considered normal, since milk characteristics vary considerably depending on several factors, such as season, lactation, feed, management system and breed. Specifically, ewes selected for this study were mainly of Lacaune breed in the three flocks located in AV, CE and SA provinces of the Campania region; this choice was made to reduce the variability of milk samples. For this reason, the different milks had a similar composition, reflecting fat, protein and lactose content recorded in Lacaune sheep milk (Pulina *et al.*, 2005; Castillo *et al.*, 2019), with a few, physiological, differences among samples. The absence of

antibiotic underlined the good healthy properties of milk produced in Campania region and the good health status of the animals which furnished the milk.

Table 1. Chemical and microbiological characteristics of ewe milk from Avellino (M_Av), Caserta (M_Ce) and Salerno (M_Sa) provinces. Values are reported as average of three determinations and ± standard deviation (SD). Letters are significant differeces within each row.

	M_Av	**M_Ce**	**M_Sa**
UREA (mg/100ml)	37.70±0.36^{a}	43.07±0.66^{b}	40.73±0.15^{c}
Fat (%w/w)*	7.15±0.15^{a}	7.15±0.13^{a}	7.20±0.10^{a}
Proteins (%w/w)*	5.72±0.12^{b}	5.34±0.12^{a,b}	5.15±0.11^{a}
Lactose (%w/w)*	4.26±0.04^{a}	4.41±0.09^{a}	4.32±0.10^{a}
Dry matter (%w/w)	10.77±0.2^{a}	10.45±0.11^{a}	10.17±0.13^{a}
Acidity (°SH)	7.97±0.12^{a}	8.53±0.08^{b}	8.51±0.10^{b}
Somatic cells (cells/ml)	448400±3143^{b}	672666±4163^{c}	213900±16703^{a}
Total bacterial Count (Log CFU/ml)	7.84±0.45^{a}	8.40±0.25^{a}	7.98±0.17^{a}
Freezing point (°C)	-0.55±0.01^{a}	-0.55±0.01^{a}	-0.56±0.01^{a}
Antibiotics (presence/absence)	absence	absence	absence

The presence of metals in ewe milk samples is reported in Table 2. Only Ca, Fe, K, Na and Zn were detected in all samples, whereas Sr was found only in milk from the Salerno province (M_Sa). In particular, milk from Avellino province (M_Av) contained the highest amount of Iron, Potassium and Zinc. These values were also higher than those reported by other Authors for sheep milk (Raynal-Ljutovac *et al*., 2008; Yabrir *et al*., 2014; Siddiqui *et al*., 2023). An important factor of variability of these elements in ewe milk is the different lactation stage, the health status and the quality of feed and pasture, which is associated with the vegetation and environmental conditions (Michlová *et al*., 2016). However, as reported above, in our study we tried to minimize most of the variables by choosing animals mainly of the same breed, all free of disease, and with similar lactation stage.

Table 2. Presence of trace elements inewe milk from Avellino (M_Av), Caserta (M_Ce) and Salerno (M_Sa) provinces. Values (mg/Kg) are reported as average of three determinations and ± standard deviation (SD). Letters are significant differeces within each row.

	M_Av	**M_Ce**	**M_Sa**
Ca	1816.39±33.2^{a}	1792.92±34.58^{a}	2020.5±27.05^{b}
Fe	1.17±0.08^{b}	0.75±0.05^{a}	0.54±0.06^{a}
Mn	ND	ND	ND
Ni	ND	ND	ND
K	2112.41±25^{c}	1402.34±27.7^{a}	1523.73±23.2^{b}
Cu	ND	ND	ND
Na	467.43±12.14^{a}	768.76±13.7^{c}	603.68±14.3^{b}
Sr	ND	ND	ND
Zn	8.36±0.3^{b}	5.81±0.4^{a}	3.97±0.7^{a}

3.2 NGS Analysis

A total of 233729 PE sequences were obtained from the metagenomic sequencing of the

samples. With QIIME2, after quality control of the reads, denoising and filtering of the chimeras performed with DADA2, 103868 PE sequences were obtained (44.6% on average), which were used for the subsequent analyses (Table 3), while 81141 PE sequences (27.05%) were obtained with the DADA2 pipeline (Table 4).

Table 3. Summary of the number of reads before and after filtering, denoising, merging and removal of chimeric sequences with QIIME2 pipeline.

Sample-id	Input	Filtered	Denoised	Merged	Non-chimeric
M_Av	97228	48158	47754	46598	44884
M_Ce	52905	27503	27104	26094	24458
M_Sa	83596	52009	51725	49073	34526

Table 4. Summary of the number of reads before and after filtering, denoising, merging and removal of chimeric sequences with DADA2 pipeline.

Sample-id	Input	Filtered	Denoised-F	Denoised-R	Merged	Non-chimeric
M_Av	97228	93426	79125	88015	64068	43976
M_Ce	52905	51308	42089	47693	36900	16136
M_Sa	83596	82109	74454	81209	54604	21029

The rarefaction curves showed saturation for all sample types, indicating sufficient sequencing coverage to estimate microbial composition and diversity. To avoid problems of methodological bias and to obtain as many unique species as possible, in this study the taxonomic analysis of sheep milk samples was carried out with two different pipelines. As shown in Figure 1 and 2, DADA2 revealed the presence of 3 *phyla*, 8 classes, 14 orders, 18 families and 20 genera, while the taxonomic analysis of milk samples with QIIME2 revealed the presence of 16 *phyla*, 23 classes, 35 orders, 64 families and 83 genera.

It is evident that the analysis with QIIME2 returned more *phyla*, classes, orders, families and genera than DADA2. In the study conducted by Xue *et al.* (2018) on the influence of DNA sequencing and analysis methodologies on bacterial 16S rRNA gene community in dairy products, DADA2 showed better sequence annotation than QIIME2, leading to a more accurate representation of dairy product community standards. In our research, the use of the two different data processing pipelines produced almost overlapping results regarding the predominant *phyla*, family and genera in the analysed samples. This could be due to the fact that DADA2 is used as part of the QIIME2 pipeline for processing the amplicon sequencing data. Therefore, the denoising step will be essentially the same as using DADA2. Specifically, QIIME2 facilitated the identification of a greater number of bacterial classes, orders, and genera compared to DADA2. However, these identifications were associated with notably low, sometimes negligible, rates of identification (<1%). This phenomenon may be due to the inclusion of additional steps and analytical methods within the QIIME2 pipeline, despite the potential overlap in denoising results between the direct application of DADA2 and its integration within the QIIME framework. QIIME2 offers a wider range of functionality,

including taxonomic assignment, which can potentially identify more genera depending on the choice of reference database and taxonomic assignment method. Ultimately, it could be beneficial to try out both tools on a dataset (or a subset of it) and compare the results to see which one better meets the expected requirements.

Proteobacteria were the predominant *phylum* in all sheep milk, followed by Firmicutes and Bacteroides. Proteobacteria showed abundance percentages ranging from 94.95% to 98.85% using DADA2 and from 90.95% to 98.48% using QIIME2 (Figure 1). Similarly, Firmicutes showed abundances ranging from 1.15% to 2.19% and 1.17% to 4.38% using DADA2 and QIIME2 respectively. Bacteroides were detected only in M_Av with DADA2 whereas ranged between 0.12% and 3.54% with QIIME2.

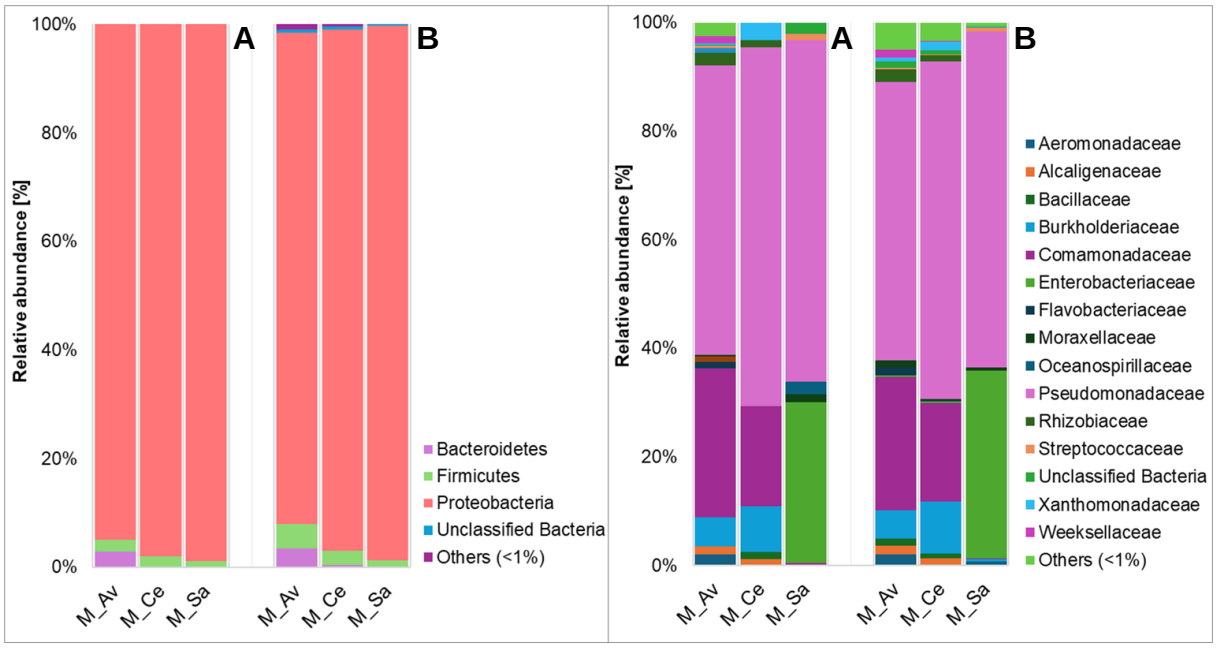


Figure 1. Distribution of relative abundance of taxa at *phylum* (left) and Family (right) level. Taxonomic analysis was performed with DADA2 (A) and QIIME2 (B). The group "Others (<1%)" includes *phyla* with a relative abundance of less than 1%.

The data obtained are consistent with those reported in the literature, according to which Proteobacteria is the dominant *phylum* in raw milk (Fuka *et al.*, 2013; Endres *et al.*, 2021; Yan *et al.*, 2022; Yuan *et al.*, 2022; Guo *et al.*, 2023). With regard to the results at the family and genus level, QIIME2 performed again better than DADA2. In particular, a total of 56, 44 and 24 families were identified in the M_Av, M_Ce and M_Sa milks, respectively, and 60, 47 and 23 genera in the M_Av, M_Ce and M_Sa milks, respectively.

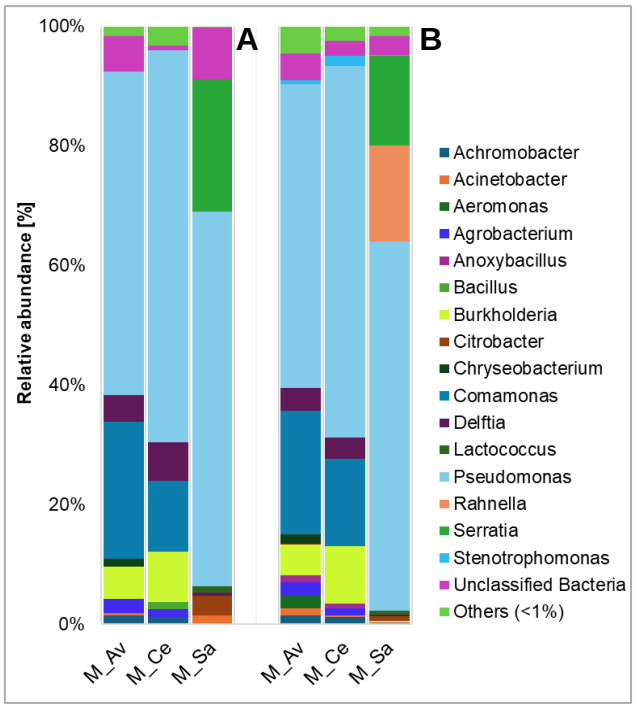


Figure 2. Distribution of relative abundance of taxa at genus level. Taxonomic analysis was performed with DADA2 (A) and QIIME2 (B). The group "Others (<1%)" includes genera with a relative abundance of less than 1%.

3.3 Data Analysis

From the data reported so far, milk from the province of Avellino (M_Av) was found to be particularly rich in Iron, Potassium and Sodium, with values, as mentioned above, higher than those reported in literature for these metals in ewe milk. In addition, metagenomic analysis conducted with NGS showed a greater number of families and genera in this milk sample than in the samples taken from the provinces of Caserta (M_Ce) and Salerno (M_Sa). In light of these observations, a correlation study was conducted to investigate the influence of the presence of metals on the microbiome. As shown in Figure 3, correlation analysis showed an increase in the number of different genera and species within the milk samples as the presence of some metals increased.

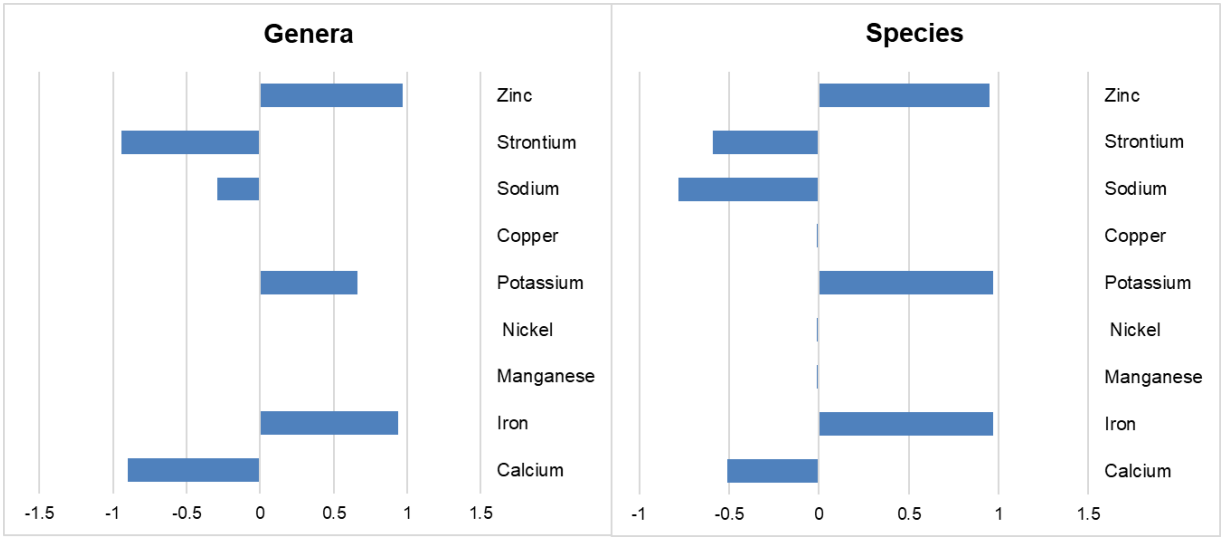


Figure 3. Correlation analysis showing bacterial genera and species detected in milk samples as function of trace elements.

The distribution (%) shown in Figure 4 confirms this observation. In fact, as the distribution of Zn, Fe and K decreased, so did the number of different genera and species. So, the milk sample with the lowest distribution of these metals (M_Sa) was characterised by the lowest distribution of different genera and species. Conversely, the milk sample with the highest distribution of Zn, K and Fe (M_Av) was also characterised by the highest distribution of different genera and species.

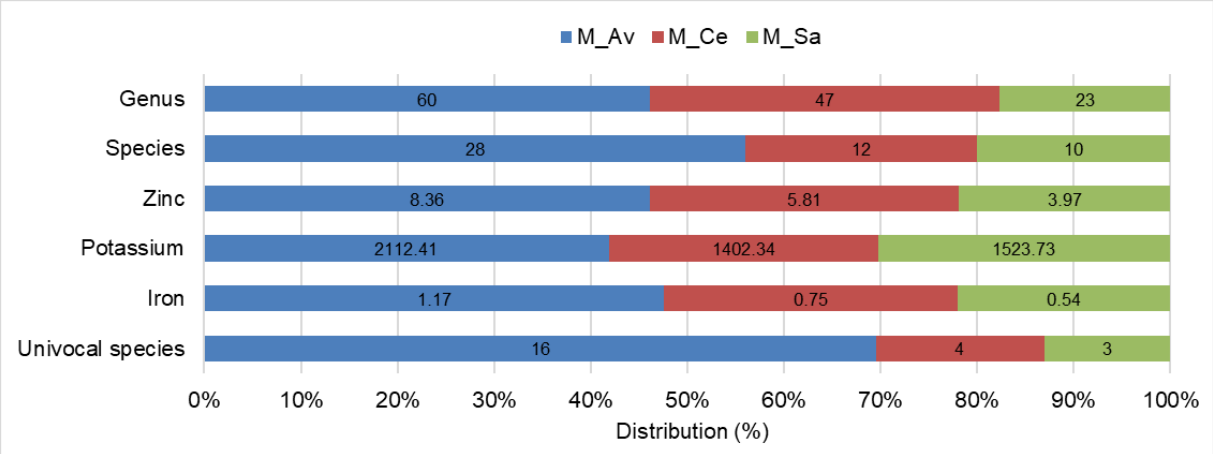


Figure 4. Distribution (%) of the main variables representing ewe milk samples.

Interestingly, the same trend was observed for "univocal species", i.e. those bacterial species that were uniquely present in each sample and can be considered as the "*core microbiome*" of each milk (Table 5). Most of these species are normally found in raw milk. In a large study comprising 142 samples of bovine raw milk from China, *Pseudomonas*, *Acinetobacter*, *Streptococcus* and *Lactobacillus* were detected as the most common genera using 16S rRNA amplicon sequencing (Yuan *et al*., 2022). Indeed, in our study *Acinetobacter*, *Streptococcus* and *Lactobacillus* were detected with very low abundance, whereas *Pseudomonas* spp. was present with abundance between 48.65% and 57.72%. With regard to univocal species, those from M_Sa were *Macrococcus caseolyticus* and *Pseudomonas* spp., also detected in cheeses produced with raw ewe milk (Fuka *et al*., 2013), and *Rahnella aquatilis*, identified as a spoilage agent in chilled chocolate milk products (Jensen *et al.*, 2001). In sample M_Ce, univocal species were *Fibrobacter succinogenes*, previously isolated from sheep rumen microbiome (Christodoulou *et al*., 2023), *Myroides odoratimimus* an opportunistic pathogen commonly found in the environment and in milk (Oleghe *et al*., 2020), *Pseudomonas viridiflava*, a bacterium associated with plants and found in milk samples from cows with mastitis (Fursova *et al*., 2021) and *Rothia nasimurium*, commonly found as commensal bacteria in the upper respiratory tract and gut of humans and other animals (Guo *et al*., 2021).

In ewe milk from the Avellino province (M_Av) most of the species annotated were commonly detected in the dairy environment. Other species have different habitats. For instance, *Alkalibacterium iburiense* is an obligately alkaliphilic bacterium that exists ubiquitously (Nakajima *et al.*, 2005); *Anoxybacillus kestanbolensis* is a moderately thermophilic bacterium

isolated from extreme environments (Valenzuela *et al.*, 2024); *Petrobacter succinatimandens* is a thermophilic heterotrophic denitrifying bacterium active in thermal environments (from 20°C to 75°C), such as hot springs, compost, freshwater lakes and hot desert (Wang *et al.*, 2023).

Table 5. Univocal species found in sheep milk samples.

Species	M_Av	M_Ce	M_Sa
Acinetobacter johnsonii	0.85		0.62
Acinetobacter lwoffii	0.22	0.25	
Acinetobacter guillouiae	0.16		
Alcaligenes faecalis	0.08	0.09	
Alkalibacterium iburiense	0.02		
Anoxybacillus kestanbolensis	0.03		
Bdellovibrio bacteriovorus	0.03		
Brevundimonas diminuta	0.1	0.16	
Brevundimonas vesicularis	0.04		
Caldinitratiruptor microaerophilus	0.03	0.06	
Corynebacterium variabile	0.14		
Fibrobacter succinogenes		0.05	
Flavobacterium succinicans	0.12		0.04
Jeotgalicoccus psychrophilus	0.02	0.04	
Kocuria koreensis	0.11		
Lactobacillus zeae	0.05		
Macrococcus caseolyticus			0.28
Myroides odoratimimus		0.04	
Paracoccus aminovorans	0.01		
Petrobacter succinatimandens	0.03		
Prevotella copri	0.03		
Propionibacterium acnes	0.02		
Pseudomonas fragi			50.07
Pseudomonas veronii	48.62	57.62	1.25
Pseudomonas viridiflava		0.1	
Pseudomonas umsongensis	0.03		2.39
Rahnella aquatilis			15.95
Rothia nasimurium		0.02	
Sphingobium yanoikuyae	0.03	0.14	
Staphylococcus equorum	0.51		
Stenotrophomonas geniculata	0.4	1.23	0.03
Stenotrophomonas acidaminiphila	0.24		
Streptococcus luteciae	0.02		
Thermoanaerobacterium saccharolyticum	0.08		
TOTAL UNIVOCAL SPECIES	**16**	**4**	**3**

4. Conclusion

This study highlighted remarkable differences in metal content of milk samples withdrawn in different farms located in three provinces of the Campania region. In particular, the milk sample

from the province of Avellino resulted particularly rich in Iron, Potassium and Zinc, and a correlation between metal content and numbers of different bacterial genera and species was detected. A "*core microbiome*" was individuated for each milk, mainly composed by typical genera and species belonging to the dairy environment. However, in the milk sample from Avellino province, some species, such as *Anoxybacillus kestanbolensis* and *Petrobacter succinatimandens*, appeared related to the specific area, that is, the Mefite D'Ansanto Valley, where sheep are taken to pasture. The combination of these distinctive traits is particularly interesting as this milk is used for the production of Pecorino Carmasciano, a typical Campanian cheese awaiting the award of PDO. In conclusion, results reported in this research highlighted that the peculiar characteristics of Mefite can influence the chemical and microbiological composition of milk. Of course, further studies are needed, as numerous variables may contribute to the definition of the chemical and microbiological features of milk from this specific area.

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Chapter 4.

AKKERMANSIA MUCINIPHILA

4.1 Introduction

Akkermansia muciniphila is a gram-negative, oval-shaped, non-motile and strictly anaerobic bacterium. First discovered in 2004 from a human faecal sample (Derrien *et al*., 2004), *A. muciniphila* is the only member of the *phylum* Verrucomicrobia found in the human gut (Derrien *et al*., 2010). *A. muciniphila* thrives in the host intestinal mucosal layer, predominantly in the caecum, and it is ubiquitous in the gastrointestinal tract (GIT) of healthy individuals and infants, constituting 1-4% of the total gut microbiota from early life (Derrien *et al.,* 2008; Geerlings *et al*., 2018). This bacterium establishes a stable colonisation in the human gut within the first year after birth, with an abundance similar to that of healthy adults, although it gradually decreases in the elderly (Collado *et al.*, 2007; Derrien *et al.*, 2008; Zhang *et al*., 2019). Metagenomic analyses consistently place *A. muciniphila* among the top 20 most aboundant species inhabiting the human GIT (Thomas *et al.*, 2014; Drell *et al.*, 2015). Its presence has also been documented in human breast milk, suggesting a potential route of transmission from mother to infant and subsequent colonisation of the GIT during the neonatal period (Collado *et al*., 2008). During this critical developmental period, *A. muciniphila* demonstrates proficient colonisation of the GIT, facilitated by an active acid resistance system and its ability to degrade human milk oligosaccharides in the neonatal stomach (Bosscher *et al*., 2001; Collado *et al*., 2012). Interest in this bacterium is also due due to its ability to secrete enzymes in the intestinal tract that contribute to the regulation of mucin, a primary component of the mucous layer covering the gastrointestinal (GI) mucosa (Corazziari, 2009; Karamzin *et al*., 2021). In fact, *A. muciniphila* uses mucin as a primary source of carbon and nitrogen (Derrien *et al*., 2004; Iwaza *et al.*, 2022). This ability to degrade mucin, which is modulated by nutrients in the mucosal layer near the intestinal epithelium (Belkaid and Hand, 2014), allows for competitive inhibition against of other mucin-using pathogenic bacteria (Belzer and de Vos, 2012), highlighting its potential as a probiotic candidate.

A. muciniphila has attracted attention as a promising candidate for advanced therapeutic probiotics (Collado *et al*., 2007; Derrien *et al.,* 2008; Belzer and de Vos, 2012; Cani and de Vos, 2017). Studies have elucidated the role of *A. muciniphila* in regulating metabolic and immune functions and providing protection against high-fat diets in murine models (Derrien *et al.*, 2011; Everard *et al*., 2013). Recently, the European Food Safety Authority (EFSA) validated the safety of pasteurised *A. muciniphila* ATCC BAA-835 as a novel food, potentially facilitating its commercialisation as a dietary supplement. Nevertheless, there are currently no globally available commercial *A. muciniphila* products, and the precise mechanisms underlying its interactions with the host are still poorly understood.

4.2 Colonization of *A. muciniphila* and its Interaction with the Host

The adhesive property of *A. muciniphila* to the mucus layer in the intestine is recognized as a beneficial probiotic characteristic (Derrien *et al*., 2010; Everard *et al.,* 2013; Chelakkot *et al.*, 2018; Hänninen *et al*., 2018). This ability contributes to the maintenance of intestinal barrier function by preventing microbial invasion, thus promoting intestinal health. The presence of *A. muciniphila* in GIT is influenced not only by nutrient intake and metabolism, but also by the integrity of the mucous layer and associated immune responses (Everard *et al.,* 2013; Reunanen *et al*., 2015). During nutritional deficiencies, such as fasting or malnutrition, the property of *A. muciniphila* to degrade mucin provides a competitive advantage, as demonstrated by the increase in its abundance after fasting in mice (Sonoyama *et al*., 2009; Cani *et al.,* 2022).

Adhesion to the intestinal mucosa may promote intestinal retention and interaction with host epithelial and immune cells (Jensen *et al.,* 2104). One possible mechanism for bacterial adhesion and host colonization involves the binding of microbial cell surface molecules to the protective layer of mucus covering the epithelial cells of the GIT, composed mainly of mucins (van Passel *et al.,* 2011). Although no specific mucus-adherent microflora has been identified, there is evidence that bacteria can bind directly to mucins by expressing specific proteins, pili, fimbriae, and flagella (Sicard *et al.,* 2017). Examples are provided by the distribution of mucus-binding proteins (MUB) in lactobacilli and the SpaC protein of *Lacticaseibacillus rhamnosus* GG (Kankainen *et al*., 2009; Jensen *et al*., 2014).

The colonisation of *A. muciniphila* in the sparse mucus layer facilitates its proximity to intestinal epithelial cells, allowing its metabolites, such as propionic and butyric acids, to exert immunomodulatory effects (Le Poul *et al*., 2003; Maslowski *et al.,* 2009). Simultaneously with the production of metabolites that promote intestinal colonisation, mucin degradation induces an increase in mucin secretion. The increased thickness and density of the mucus layer prevents pathogens from reaching and persisting on intestinal epithelial surfaces (Liu *et al.,* 2022).

Although *in vitro* investigations reveal that metabolites and short-chain fatty acids produced during mucin degradation by *A. muciniphila* play vital roles in nutrient exchange, immune system development, signal transduction and resistance to pathogen invasion (Lukovac *et al*., 2014), the intricate causal relationship between *A. muciniphila* and host genomes merits further investigation (Wang *et al.,* 2018 a,b).

In vitro adhesion studies of *A. muciniphila* to human cell lines are conducted on CaCo2 and HT-29 colon rectal adenocarcinoma cells (Reunanen *et al.,* 2015; Haddad *et al.,* 2023).

Current research on *A. muciniphila* has mainly focused on elucidating its associations with various diseases, with causality studies lacking. Direct intervention studies with *A. muciniphila* have mainly used animal models (Everard *et al*., 2013; Chelakkot *et al.,* 2018; Hänninen *et al*., 2018). Despite the lack of published open clinical trials in humans, preliminary studies suggest the safety of *A. muciniphila* for human interventions. For example, Dubourg *et al*. (2013) reported no adverse events even when *A. muciniphila* abundance reached 60% after treatment with broad-spectrum antibiotics. Furthermore, Plovier *et al.* (2017) found both live and

pasteurised *A. muciniphila* to be well tolerated and safe in overweight individuals following a 2-week oral administration.

A. muciniphila has not been conclusively linked to any disease or pathology (Derrien *et al.*, 2010). On the contrary, its presence has been associated with a healthy gut and its abundance in the human intestinal tract has been inversely correlated with various disease states (Jakobsson *et al.*, 2015). Its potential pathogenicity is primarily due to its adhesion to and degradation of the intestinal mucus layer, which can exhibit some initial pathogenic behaviour (Tuomola *et al.*, 2001; Derrien *et al.*, 2010). However, unlike pathogens, *A. muciniphila* resides primarily in the outer mucosal layer and does not penetrate the inner layer (Zhang *et al*., 2019).

Although mucin degradation has similarities to pathogenic behaviour, it contributes to the balance of intestinal self-renewal and the production of beneficial by-products. Notably, there is no evidence that *A. muciniphila* alone causes pathogenicity, although its potential synergistic effects with other bacteria remain uncertain. Despite containing lipopolysaccharide (LPS), *A. muciniphila* is not associated with endotoxemia and can even reduce endotoxin levels associated with high-fat diets in mice (Everard *et al.*, 2013). Mucin degradants regulate the host immune system through various signals, including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-10 (IL-10), and IL-4 (Derrien *et al.*, 2011; Collado *et al*., 2012; Andersson *et al.*, 2013).

Recent shifts in microbiome research focus on exploring causal relationships rather than mere associations. Therapeutic benefits have been observed upon administration of viable bacteria, highlighting the potential of *A. muciniphila* as a biomarker for assessing host health status and disease progression (Rodríguez-Daza *et al*., 2022). Surprisingly, pasteurised *A. muciniphila* has shown efficacy in preventing obesity and related complications, potentially surpassing the efficacy of live bacteria. This result highlights its therapeutic potential. Further investigation revealed that an outer membrane protein, Amuc_1100, interacts with Toll-like receptor 2 (TLR2) to improve intestinal barrier function and exert probiotic effects, even in pasteurised form (Wang *et al.*, 2021). In addition, Amuc_1100 activates TLR2 and TLR4 to modulate immune responses and intestinal barrier function, providing a theoretical basis for clinical applications (Han *et al.*, 2023).

Numerous human and animal studies have investigated the association between *A. muciniphila* levels and various disorders and diseases, particularly metabolic and inflammatory conditions (Schneeberger *et al*., 2015; Macchione *et al*., 2019; Roshanravan *et al*., 2023). Reduced levels of *A. muciniphila* have been associated with the development of obesity, type 2 diabetes, inflammatory bowel disease (IBD), autism and atopy (Zhou ans Chen, 2020, Ghaffari *et al*., 2023).

Metabolic disorders such as obesity and type 2 diabetes have been extensively studied in relation to the presence of *A. muciniphila* (Roshanravan *et al.*, 2023). It has shown promise in the prevention and treatment of these conditions by improving glucose tolerance, reducing adipose inflammation and improving insulin sensitivity (Zheng *et al.*, 2023).

In immune diseases, decreased *A. muciniphila* abundance has been associated with atopic

diseases and IBD. *A. muciniphila* has shown immunomodulatory effects by interacting with intestinal epithelial cells and regulating intestinal immunity and homeostasis (Lopez-Siles *et al.*, 2018).

In the field of cancer treatment, *A. muciniphila* has emerged as a compelling candidate for enhancing the efficacy of immune checkpoint inhibitors, such as anti-PD-1 antibodies (Zhou *et al.*, 2022). Elevated levels of *A. muciniphila* have been associated with favourable responses to immunotherapy, suggesting its potential contribution to cancer treatment (Adachi and Tamada, 2018).

Taken together, *A. muciniphila* shows diverse therapeutic potential across a range of diseases, from metabolic disorders to cancer. Continued investigation is therefore essential to elucidate its mechanisms of action and refine its clinical use.

RESEARCH AIM

Akkermansia muciniphila is emerging as a promising probiotic due to its numerous physiological benefits documented in both animal and human studies (Cheng *et al*., 2021). Its presence indicates a healthy gut, and its higher abundance in the human intestinal tract is linked to lower incidences of disease states (Jakobsson *et al*., 2015). This establishes it as a crucial biomarker for maintaining gut homeostasis (Luo *et al*., 2022). However, before *A. muciniphila* can be considered as a next-generation probiotic, further *in vitro* and *in vivo* clinical studies are needed to better understand its properties. The aim of this study was to evaluate the gastrointestinal tract simulation survivability of the *A. muciniphila* strain ATCC BAA-835 and the ability to adhere to three human intestinal cell lines (CaCo2, HT-29 and HT29-MTX) at different concentrations. In order to more closely replicate the *in vivo* conditions, the tests for the simulation of gastrointestinal passage and adhesion capacity were performed in succession.

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Simulated Gastrointestinal Transit and Adhesion to Human Cell Lines of *Akkermansia muciniphila* ATCC BAA-835

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Abstract

According to FAO/WHO guidelines, *in vitro* criteria for the selection of probiotics include the evaluation of their ability to survive in the gastrointestinal tract (GIT) and their ability to adhere to human epithelial cells. These attributes were analysed for the *Akkermansia muciniphila* strain ATCC BAA-835, a symbiotic organism known for its ability to colonise the gut, making it a potential candidate for use as a probiotic. The simulation of gastrointestinal (GI) transit followed by adhesion to human intestinal cell lines (CaCo2, HT-29 and HT-29-MTX) was studied as an alternative approach to conventional tests, performed individually. The adhesion percentage of bacteria after GI transit was compared to the percentages obtained from an adhesion assay conducted using the same bacterial concentrations not subjected to GI transit. All results were compared with those related to *Lacticaseibacillus rhamnosus* GG ATCC 53103, one of the most studied probiotic strains belonging to a species recognized as safe (GRAS). The survival rate after GI transit, as determined by both plate counts and fluorescent probes, was significantly higher for the *A. muciniphila* strain (about 8 Log CFU/mL) than for *L. rhamnosus* GG (about 3 Log CFU/mL). In contrast, adhesion rates to human cell lines, which were lower for strains with GI transit than for those without GI transit, were significantly higher for *L. rhamnosus* GG. Thus, adhesion assays were performed for both strains at different concentrations (2 to 8 Log CFU/mL). In general, adhesion rates were inversely proportional to the added bacterial concentrations. The adhesion of *A. muciniphila* at different concentrations was significantly higher than that of *L. rhamnosus* GG. The best results for *A. muciniphila* were recorded for the HT-29-MTX line, which showed percentages above 100% for concentrations from 5 to 2 Log CFU/mL. In conclusion, this work demonstrates that the strains and that the adhesion rate of strains after transit to cell lines is inversely proportional to the initial bacterial load and simulated GI transit changes the in vitro adhesion properties of strain. We believe that conducting the two tests in sequence is a viable alternative to conventional testing.

Keywords: *Akkermansia muciniphila*, *Lacticaseibacillus rhamnosus* GG, simulation gastrointestinal tract, adhesion, CaCo2, HT-29, HT29-MTX, potential probiotic.

1. Introduction

Akkermansia muciniphila is a gram-negative, anaerobic, oval-shaped bacterium belonging to the *phylum* Verrucomicrobia (Cozzolino *et al*., 2020). It is one of the normal gut symbionts throughout our life. This bacterium colonises the human gut stably within a year of birth, due to its transfer through breast milk (Collado *et al*., 2012). Its abundance in the gut reaches high levels in healthy adults, representing approximately 1 to 3% of the total microbiota, and then gradually decreases in the elderly (Collado *et al*., 2007; Derrien *et al.*, 2008; Zhang *et al.*, 2019). The strain ATCC BAA-835 was isolated in 2004 by Derrien *et al.* from a faecal sample of a healthy man (Derrien *et al*., 2004) and nowadays it is one of the most studied human intestinal colonizers due to its intriguing beneficial features. *A. muciniphila* can use mucin as carbon and nitrogen source, which gives it a competitive advantage during nutrient deficiencies in the gut (Van Passel *et al*., 2011; Derrien *et al.*, 2017; Karamzin *et al*., 2021).

Several studies showed that *A. muciniphila* is abundant in biopsies of healthy subjects and reduced in those from patients with inflammatory bowel disease, hypertension, obesity, atopic diseases, and autism (Png *et al*., 2010; Derrien *et al*., 2011; Wang *et al*., 2011; Candela *et al*., 2012; Dao *et al*., 2016). Therefore, the presence of this bacterium is considered as a significant biomarker of intestinal homeostasis and host physiology (Hansen *et al*., 2012; Le Chatelier *et al.*, 2013). Such positive actions in humans can be attributed to its ability to produce metabolites, compete with other dangerous bacteria, and maintain the balance of immunity and integrity of the intestinal barrier (Everard *et al*., 2013; Li *et al*., 2017).

The use of the strain ATCC BAA-835 as beneficial bacterium is currently limited in pasteurised form as novel food for a target population composed of general adults, excluding pregnant and lactating women, whereas its use as probiotic is still under consideration (EFSA, 2021).

The current guidelines indicate various criteria for evaluating *in vitro* and/or *in vivo* the efficacy of candidate probiotic organisms (Byakika *et al*., 2019). Among the major criteria to characterise potential probiotic bacteria there are the GIT simulation and the adherence human epithelial cells and cell lines (FAO/WHO, 2020).

Several studies have evaluated the resistance of probiotic strains to gastric acidity and biliary toxicity, as well as their ability to colonise the GI system and produce antimicrobial compounds and modify immune responses (Succi *et al.,* 2017; Galdeano *et al.,* 2019; Letizia *et al.,* 2022).

Once ingested, probiotics pass through the GIT, from the mouth through the stomach to the small intestine and colon. The probiotic must tolerate oral enzymes, in particular lysozyme, at the time of administration and resist the antimicrobial elements present in the stomach (low pH, acidic gastric fluid and pepsin) and intestine (pancreatin and bile salts), in order to temporarily persist in this environment and exert its health-promoting effects.

Probiotic adhesion relies on surface chemistry and membrane interactions, crucial for effective microbe-cell binding and intestinal colonization (Boonaert and Rouxhet, 2000; Duary *et al*., 2011).

The human intestinal cell lines CaCo2, HT-29 and HT-29-MTX have been used to identify and

subsequently evaluate microrganisms potential probiotic based on their adhesion properties (Mantzourani *et al.,* 2019; Rocha-Mendoza *et al*., 2020).

To the best of our knowledge, no information is available concerning the influence of GI transit simulation on the subsequent adhesion capacity of probiotic strains. Assays simulating GIT and the adhesion capacity of potentially probiotic bacteria are, in fact, performed individually. In this work, the tests were performed consecutively. We believe that conducting the two tests sequentially is a valid alternative to conventional tests, as performing an adhesion test with bacteria that have previously undergone GIT stress represents a condition more similar to what usually occurs *in vivo*.

2. Materials and Methods

2.1 Bacterial Strains

Bacterial strains used in this study were *Lacticaseibacillus rhamnosus* GG ATCC 53103 (ATCC, Valio Ltd, Helsinki, Finland) and *Akkermansia muciniphila* ATCC BAA-835 (ATCC, Valio Ltd, Helsinki, Finland). Strains, stored at -80°C in glycerol (Succi *et al*., 2017), were propagated at 37°C for 48-72 h in anaerobiosis (Gas Pack AnaeroTM, Oxoid™), using as growth substrates de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Milan, Italy) for *L. rhamnosus* GG ATCC 53103 and BHI (Oxoid, Milan, Italy) for *A. muciniphila* ATCC BAA-835.

2.2 Gastrointestinal Transit Simulation

In new model proposed, it is simulated the digestion and adhesion to intestinal, sequentially. Survival during simulated GI transit was evaluated following the protocol described by Vergalito *et al*., 2020.

L. rhamnosus GG ATCC 53103 and *A. muciniphila* ATCC BAA-835 were grown overnight at 37°C in 150 mL of MRS and BHI broth, respectively. The cultures were centrifuged at 8000 rpm for 10 min at 4°C (Centrifuge 5415 R; Eppendorf, Hamburg, Germany), and the pellet, washed twice, was resuspended in the same volume of sterile saline solution. One mL of each strain was taken to perform microbial counts on the respective media (Oxoid, Ltd. Hampshire, UK) at 37°C for 72 h under anaerobic conditions (AnaeroGen, Oxoid Ltd., Hampshire, UK).

Thirty-three mL of a sterile electrolyte solution (NaCl 6.2 g/L, KCl 2.2 g/L, $CaCl_2$ 0.22 g/L, and $NaHCO_3$ 1.2 g/L) simulating the chewing process and lysozyme from chicken egg white (Sigma-Aldrich) were added at a final concentration of 0.01%. After a 2 min incubation, microbial counts were performed again.

The solution containing each bacterium was then divided into four sterile bottles (portions of 45 mL each). To simulate the gastric environment, 8.25 mL of electrolyte solution containing 0.3% pepsin from porcine gastric mucosa (final concentration) (Sigma-Aldrich) was added to each bottle. Two batches (consisting of three bottles each) were obtained for each strain: the pH was lowered to 2.0 in the first (batch pH 2) and to 3.0 in the second (batch pH 3) by adding 1.0

N HCl. Aliquots of each batch were collected after 30 and 90 min and used for microbial counts. To simulate intestinal stress, oxygen was replaced by nitrogen in each bottle to achieve an anaerobic atmosphere, and the pH value was adjusted to 5.0 with a saturated sodium bicarbonate solution (8 g sodium bicarbonate in 100 mL distilled water, sterilized at 121°C for 15 min). Then 8.25 mL of a sterile electrolyte solution containing 0.45% porcine bile bovine and 0.1% pancreatin from porcine pancreas (final concentration, both produced by Sigma-Aldrich) were added to each bottle. The pH was then adjusted to 6.3 and slowly increased to 7.5 until the end of the intestinal stress test (4 h). Aliquots from each batch were collected and used for microbial counts, performed as described above. During the simulation of transit in the intestinal tract, the bottles were shaken at 150 rpm with digital orbital shaker (Cueva *et al*., 2019). Solutions were freshly prepared each day and the entire study was conducted at 37°C.

At the same time that samples were taken for plate counts, aliquots (1.5 mL) were taken to perform the cell viability assays, Live/Dead described below.

At the end of the GIT simulation, 1 mL of the bacterial suspension was collected for both *L. rhamnosus* GG and *A. muciniphila* for microbial counts and 2 mL for the intestinal cell adhesion assay.

2.3 Propagation, Maintenance of Cell Lines and Adhesion Assay

Three immortalised human cell lines were used in this study: CaCo2 (ATCC HTB-37, human colon adenocarcinoma), HT-29 (ATCC HTB-38, human colon adenocarcinoma), both purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and mucus secreting HT-29-MTX, purchased from the European Collection of Authenticated (ECACC) cell cultures (Health Security Agency, UK).

Cells were cultured in 5% CO_2 enriched atmosphere for CaCo2 and HT-29 cell lines and 8% for HT-29-MTX cell lines at 37°C in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories Inc, Logan, UT, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone) (56°C for 30 min), 2mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/mL) and streptomycin (100 mg/mL) (Biological Industries, Kibbutz Beth Haemek, Israel) in a 25 cm^2 culture flask.

The growth medium was replaced every other day until confluence was reached. Cells were harvested by adding 3 mL of 0.25% trypsin-EDTA solution at 37°C. After approximately 60% cell detachment, assessed using an inverted microscope (Olympus, model IMT2), 7 mL of complete DMEM was added to each flask. The cell suspensions were centrifuged at 1200 rpm for 5 min at room temperature and the pellets were resuspended in complete DMEM (Duary *et al.*, 2011).

Instructions given by the Providers (ATCC and ECACC) were followed obtain mature cells (15 days after seeding for CaCo2 cells and 21 days for both HT-29 and HT-29-MTX cells). Before each assay, the cells were re-observed under an inverted microscope to correctly assess confluency.

Cell cultures of each line were divided into aliquots in 6-well plates (concentration of $1x10^5$ cells/2mL DMEM) and incubated as described above. When the cells reached confluence, the medium was changed daily until the adhesion assay. The depleted DMEM was removed 24 h before the adhesion assay and DMEM without antibiotics was added (Duary *et al*., 2011).
Strains of *L. rhamnosus* GG ATCC 53103 and *A. muciniphila* ATCC BAA-835 were subjected to the adhesion assay with and without GI transit. The bacteria, after undergoing GI transit, were centrifuged (8000 rpm for 15 min at 4°C), washed twice and resuspended in DMEM without antibiotics (Figure 1).

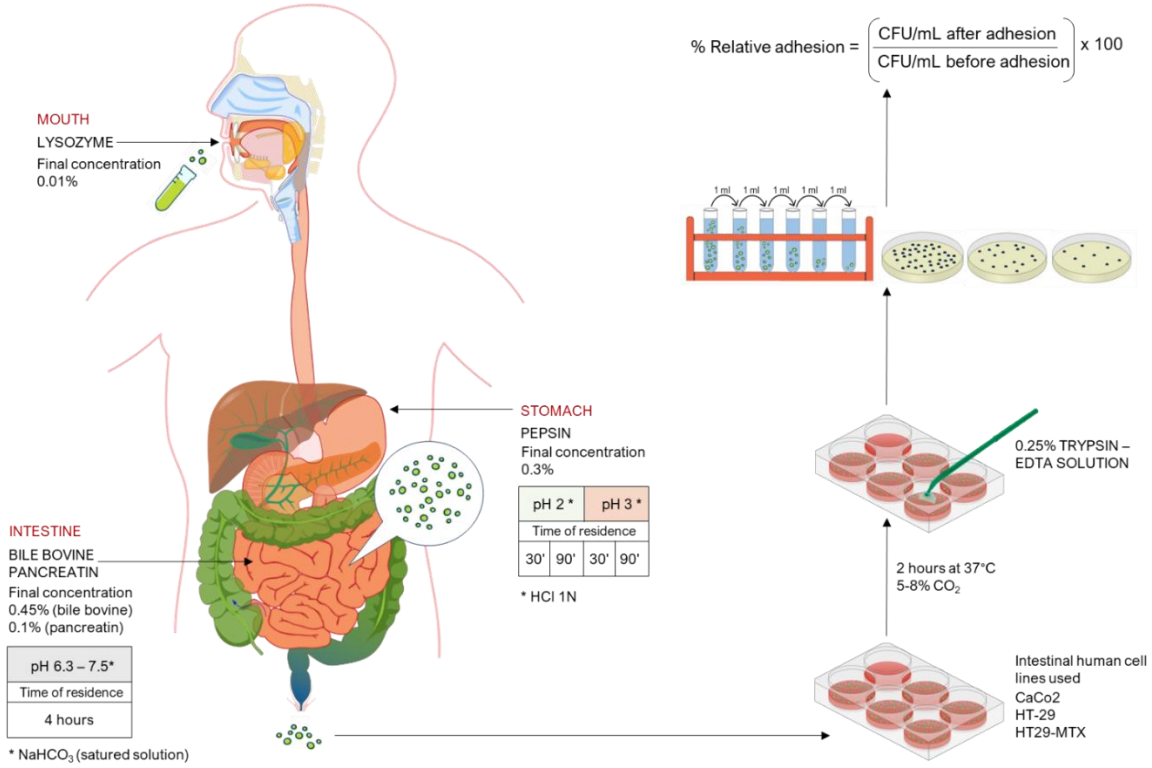


Figure 1. Schematic representation of the simulation of GI transit and subsequent adhesion to human intestinal cell lines (CaCo2, HT-29 and HT-29-MTX).

In the case of cell adhesion without transit, the bacteria were centrifuged (8000 rpm for 15 min at 4°C), washed twice and resuspended in sterile phosphate-buffered saline (PBS, pH 7) at 0.5 MacFarland Optical Density Scale (OD580, Multilabel Counter-PerkinElmer 1420, San Jose, CA, USA) to standardise the bacterial density to 10^8 CFU/mL. Subsequent dilutions in DMEM without antibiotics resulted in final bacterial loads of 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 CFU/mL. For both assays, in order to accurately assess the initial bacterial load, the strains were subjected to plate counting.
Each cell line was washed twice with 3 mL PBS (pH 7.4). Bacterial strains with and without GI transit (at different concentrations) were added to the respective wells. The 6-well plates were incubated at 37°C in 5-8% CO_2 (depending on the cell line) for 2 h. At the end of the incubation period, the DMEM was aspirated and the monolayers were washed 3 times with

sterile PBS. The cells in each well were lysed with trypsin-EDTA (0.25%) at 37°C.
To enumerate adherent bacteria, cell lysates were serially diluted with sterile saline solution (NaCl 0.9%) and plated on appropriate culture media. The percentage of adherence was calculated as follows (Valeriano *et al*., 2014):
% Relative adhesion = [(CFU/mL after adhesion)/(CFU/mL before adhesion) x 100
Assays were performed in triplicate.
The adhesion percentage achieved was contrasted with that of each strain which did not undergo the GI transit before the adhesion assay. This comparison was exclusively conducted based on the identical initial concentration.

2.4 Live/Dead Assay

In addition to plate counts, bacterial viability during GI transit was assessed with the Live/Dead assay (BacLight™ Bacterial Viability Assay, L-13152, Invitrogen™, Molecular Probes®, Eugene, Oregon, USA).
Equal volumes of SYTO 9 and propidium iodide (PI) were mixed as working solution to obtain a final concentration of each equal to 6 µM SYTO 9 and 30 µM PI. 140 µL of the mixture was added to a 500 µL aliquot of the sample. After incubation in the dark at room temperature for 15 min, all samples were washed to remove unbound dye and were filtered through Nuclepore Black Polycarbonate Membrane filters (Sigma Aldrich, Steinheim, Germany) with 0.2 µm pore size and 25 mm diameter. Each filter, containing labeled bacteria, was placed on a slide with 2 drops of BacLight mounting oil and covered with a glass coverslip. The number of live and dead bacteria was estimated from a count of 5 or more randomly chosen microscopic fields (1000X); at least 400 bacteria were counted for each sample. Sample counts were performed using the Optic Ivymen® System model 3002-F epifluorescence microscope (Comecta, Abrera, Spain) under blue excitation beams (450-490 nm) with a 100 W mercury light source (Hu *et al*., 2017).
Images were captured with a digital camera (ZLD Industrial Digital Camera) and associated software (ZLD ToupView version 3.7). Image analyses were performed using Fiji Software (version 2.9.0).
The percentage of live cells was calculated as follows:
% live cells = [live cells/(live cells + dead cells)] x 100

2.5 Study of Protein Interaction of *A. muciniphila* with Human Mucins

To study the interactions of *A. muciniphila* with human mucins, the GenBank and Uniprot databases were consulted and used to explore the genome and proteome of *A. muciniphila* and PSI-BLAST to identify proteins with sequences homologous to *A. muciniphila* proteins involved in mucin degradation. CytoScape software was used to visualise and analyse the molecular interaction networks elucidated from the data obtained in the previous steps.

2.6 Statistical Analysis

Statistical analysis and data visualization were carried out in R environment (R Core Team, 2022). Data were evaluated for normal distribution using Shapiro-Wilk test and expressed as means ± standard deviation (SD). Unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by post-hoc Turkey HSD (Honestly Significant Difference) test for multiple comparisons were used to evaluate differences in adhesion rate among groups. Graphics were generated with ggplot2 (Whickam, 2016) package. P-values ≤ 0.05 were considered statistically significant.

3. Results

3.1 Gastrointestinal Transit Simulation with Plate Count and Live/Dead Assay

The study compared the survival rates of *A. muciniphila* and *L. rhamnosus* GG strains through GI transit using both plate count and fluorescent probes. *A. muciniphila* consistently exhibited higher survival rates than *L. rhamnosus* GG, with an approximate survival count of 8 Log CFU/mL compared to 3 Log CFU/mL, respectively. Notably, fluorescent probes consistently detected higher percentages of microbial charges compared to plate counts.

Figure 2 shows the results of the GI transit performed on the *A. muciniphila* ATCC BAA-835.

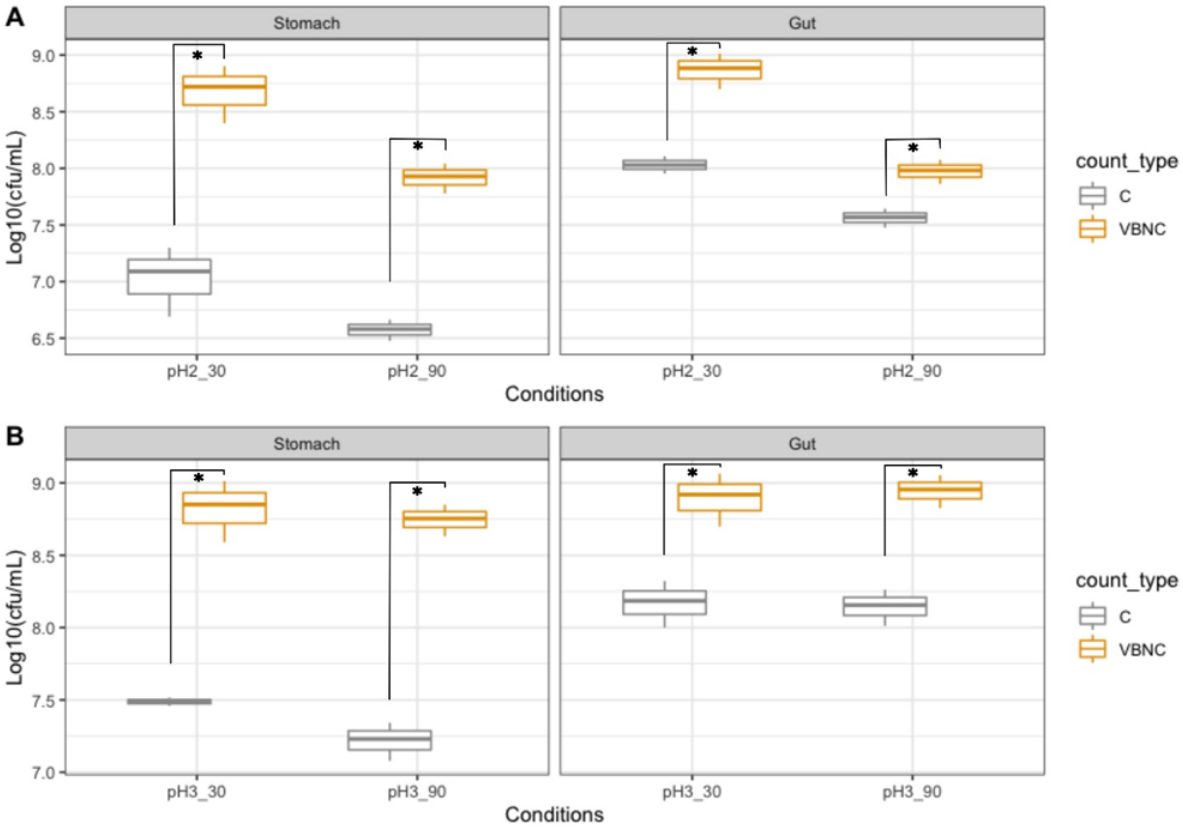


Figure 2. Results of GI transit with plate count (C) and Live/Dead test (VBNC) of *A. muciniphila* ATCC BAA-835 at pH 2 (A) and pH 3 (B) for 30 and 90 min.

Initial *A. muciniphila* load was 8.97 Log CFU/mL, decreasing to 7.09 Log CFU/mL (1.2%) after 30 min in gastric conditions (pH 2), as per plate count, while the Live/Dead indicated a higher count of 8.7 Log CFU/mL (51.1%). Transitioning to intestinal conditions saw an increase to 8.03 Log CFU/mL and 8.9 Log CFU/mL (74.6%) with the plate count and Live/Dead assay, respectively. At pH 2 after 90 min in gastric conditions, *A. muciniphila* loads were 6.6 Log CFU/mL (0.37%) via plate count and 7.9 Log CFU/mL (8.3%) with the Live/Dead assay. After intestinal transit, counts increased to 7.6 Log CFU/mL (3.60%) and approximately 8 Log CFU/mL (9.3%), respectively.

At pH 3, after 30 min, *A. muciniphila* loads were 7.5 Log CFU/mL (3%) by plate count and approximately 8.9 Log CFU/mL (69%) with the Live/Dead assay. In intestinal conditions, loads were 8.2 Log CFU/mL (14.9%) and 8.9 Log CFU/mL (80.8%) respectively. After 90 min at pH 3, *A. muciniphila* exhibited loads of 7.2 Log CFU/mL (1.65%) and 8.7 Log CFU/mL (55.3%) by plate count and Live/Dead assay respectively (Figure 3).

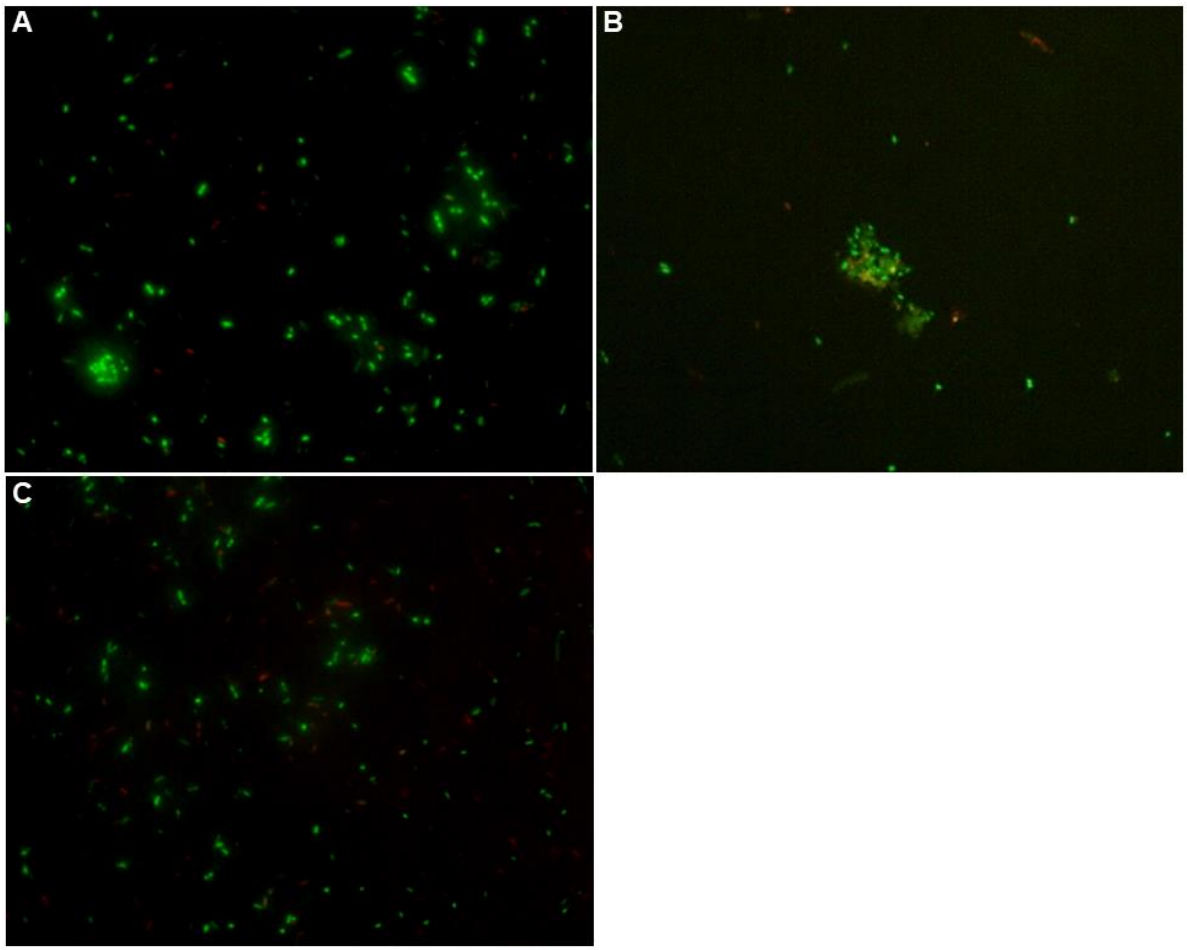


Figure 3. Fluorescence microscopy images of *A. muciniphila* obtained with the Live/Dead assay during GI transit at time 0 (A), under gastric conditions (B) and under intestinal conditions (C).

Figure 4 shows the results of the GI transit performed on the *L. rhamnosus* GG ATCC 53103 strain.

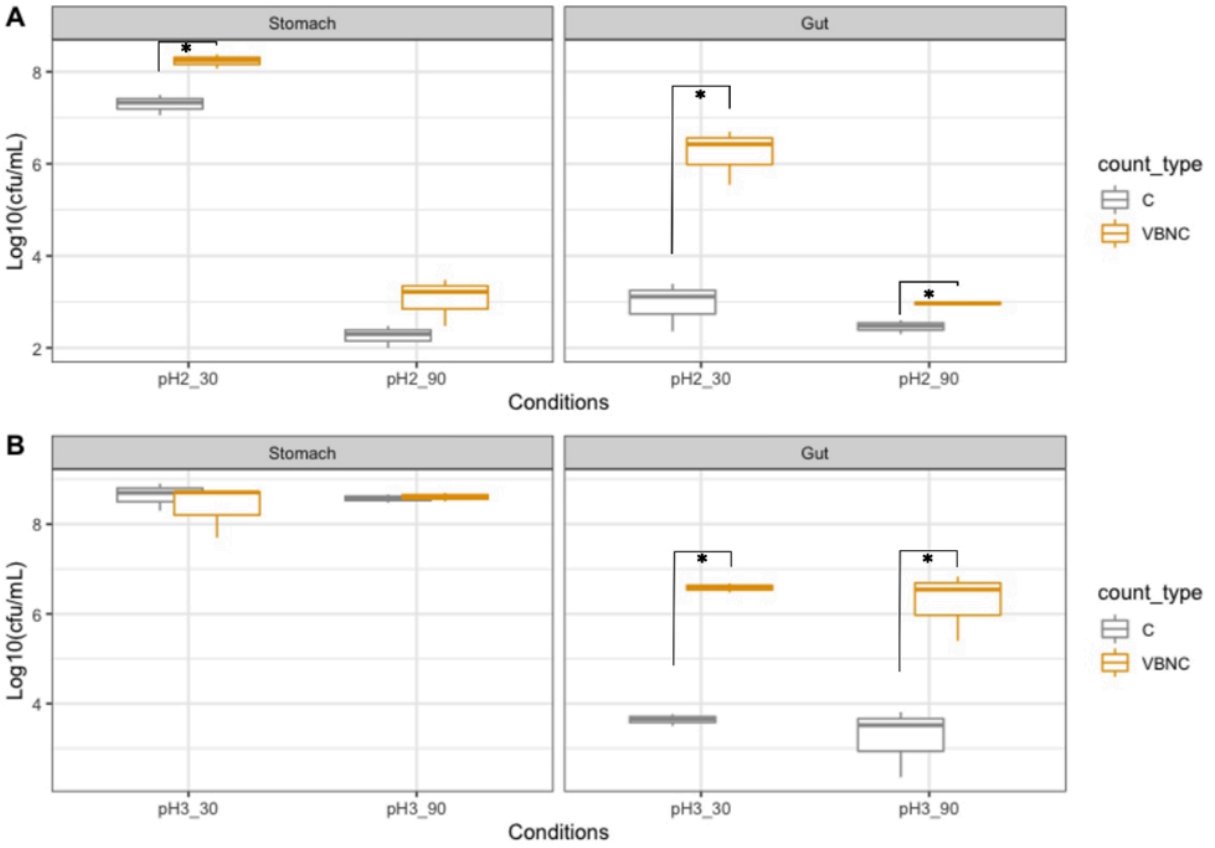


Figure 4. Results of GI transit with plate count (C) and Live/Dead test (VBNC) of *L. rhamnosus* GG ATCC 53103 at pH 2 (A) and pH 3 (B) for 30 and 90 min.

L. rhamnosus GG also showed consistently higher loads via the Live/Dead assay compared to plate count. After 30 min at pH 2, plate count recorded 7.3 Log CFU/mL (3.7%), while the Live/Dead assay indicated 8.3 Log CFU/mL (30.9%). After 90 min at pH 2, counts decreased significantly to 2.3 Log CFU/mL (0.00003%) and 3.2 Log CFU/mL (0.0003%) respectively. In intestinal conditions, plate counts further reduced to 3.1 Log CFU/mL (0.0002%), while the Live/Dead assay showed higher loads of 6.4 Log CFU/mL (0.46%). Overall, charges were higher at gastric pH 3 than pH 2, with similar levels in intestinal conditions regardless of initial gastric pH. After 30 min at pH 3, both plate count and Live/Dead assay recorded similar charges of 8.7 Log CFU/mL (86.7% and 88.6% respectively). After 90 min, plate count recorded 8.6 Log CFU/mL (65.9%), slightly lower than the Live/Dead assay's 8.7 Log CFU/mL (71.25%). Simulation at pH 3 in the intestinal tract showed comparable plate counts of 3.7 Log CFU/mL (0.001%) and 3.5 Log CFU/mL (0.001%) at 30 and 90 min, while the Live/Dead assay detected higher loads of 6.6 Log CFU/mL (0.68%) and 6.5 Log CFU/mL (0.61%) respectively (Figure 5).

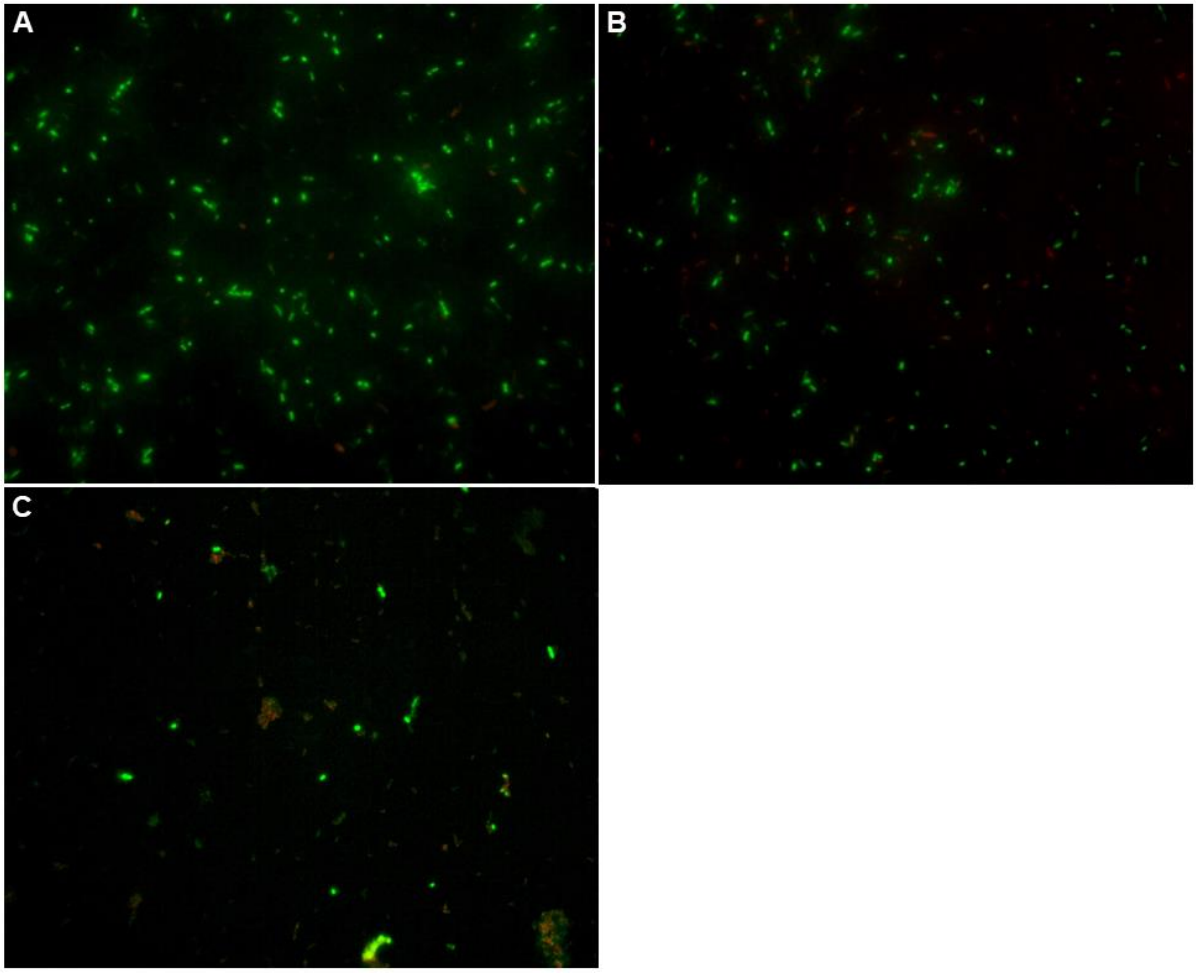


Figure 5. Fluorescence microscopy images of *L. rhamnosus* GG obtained with the Live/Dead assay during GI transit at time 0 (A), under gastric conditions (B) and under intestinal conditions (C).

3.2 Adhesion Assay at Different Concentrations

As general considerations, we noted that the adhesion rate of *A. muciniphila* ATCC BAA-835 and *L. rhamnosus* GG ATCC 53103 to CaCo2, HT-29 and HT-29-MTX cells was inversely proportional to the added bacterial concentration. Moreover, the initial bacterial concentration equal to 8 Log CFU/mL of each tested strain did not display significative differences (P-value > 0.05) in the adhesion percentage to the three cell lines (Figure 6 and Figure 7). In addition, the adhesion of *A. muciniphila* to HT-29 and HT-29-MTX cell lines was always significantly higher than that presented by *L. rhamnosus* GG (P-value ≤ 0.05), except for the combination 6 Log CFU/mL HT-29 cell line, where the adhesion rate was not different between tested strains in terms of P-value (Supplementary Table S1).

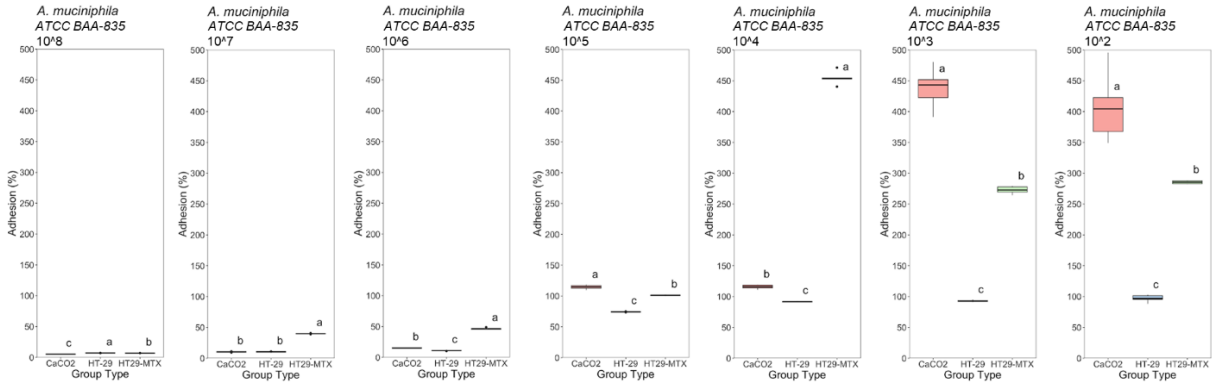


Figure 6. Adhesion percentages of *Akkermansia muciniphila* ATCC BAA-835 added at different concentrations (from left to right: 8 to 2 Log CFU/mL) to CaCo2, HT-29 and HT-29-MTX cell lines. Data with different superscript letters are significantly different (P-value ≤ 0.05) according to the post hoc ANOVA statistical analysis.

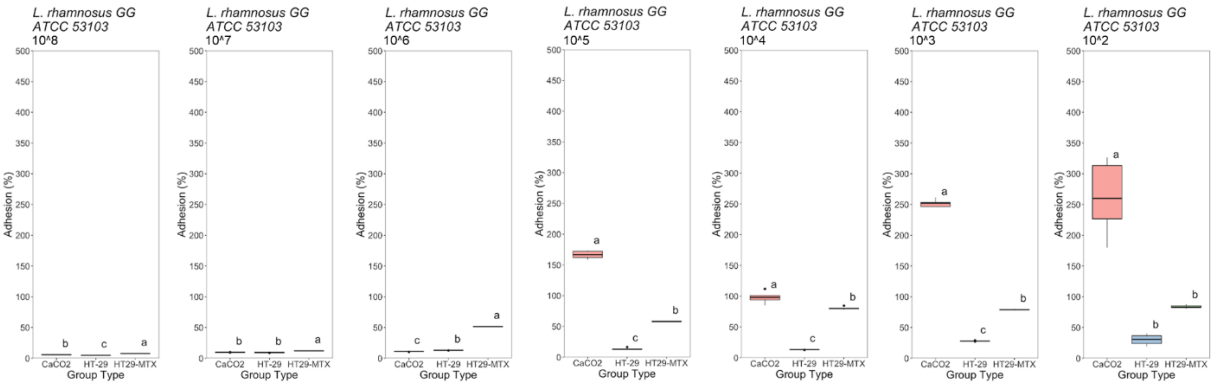


Figure 7. Adhesion percentages of *Lacticaseibacillus rhamnosus* GG ATCC 53103 added at different concentrations (from left to right: 8 to 2 Log CFU/mL) to CaCo2, HT-29 and HT-29-MTX cell lines. Data with different superscript letters are significantly different (P-value ≤ 0.05) according to the post hoc ANOVA statistical analysis.

More specifically, adhesion of *A. muciniphila* to the CaCo2 cell line showed percentages of 6.80%, 10.20% and 15.20% at initial concentrations of 8, 7 and 6 Log CFU/mL, respectively (Figure 6). Starting with concentrations of 5 Log CFU/mL, adhesion percentages higher than 100% were recorded, and the highest adhesion was detected when 2 Log CFU/mL were added (470%).

A similar trend was observed with the cell line HT-29 (Figure 6). However, the adhesion was generally lower in comparison with CaCo2 cells, especially when microbial loads below 6 Log CFU/mL were used. In fact, at initial concentrations of 8, 7 and 6 Log CFU/mL, an adhesion of 7.08%, 10.40% and 11.00%, respectively, was detected. When 5, 4, 3 and 2 Log CFU/mL where used, increasing adhesion percentages (about 74%, 92% 93% and 97%, respectively) were recorded.

The adhesion of *A. muciniphila* to the HT-29-MTX cell line showed the highest adhesion rate starting from added cells at 7 Log CFU/mL. In fact, as previously stated, the initial concentration of 8 Log CFU/mL was substantially similar, in term of adhesion percentage, to that reported with the other tested cell lines (6.82%). However, starting from a concentration of 7 Log CFU/mL up to 3 Log CFU/mL, we found significantly higher adhesion percentages in

comparison with those detected by using the other cell lines (Figure 6).
The adhesion of *L. rhamnosus* GG ATCC 53103 to CaCo2 cells (Figure 7) showed percentages of 5.84%, 9.65% and 10.90% at initial concentrations of 8, 7 and 6 Log CFU/mL, respectively. Hereinafter, adhesion percentages higher than 100% were recorded.
The HT-29 cell line in contact with *L. rhamnosus* GG showed adhesion rates of 5.86%, 9.34% and 10.91% when bacterial concentrations of 8, 7 and 6 Log CFU/mL, respectively, were used. In this case, concentrations in the range 5-2 Log CFU/mL showed adhesion percentages increasing from 13.50% to 30.10% (Supplementary Table S1).
Finally, the adhesion of *L. rhamnosus* GG to HT-29-MTX cells was lower (P-value ≤ 0.05) than that detected with CaCo2 cells, except when a bacterial concentration of 7 Log CFU/mL was used and, more markedly, with 6 Log CFU/mL (Figure 7 and Supplementary Table S1).

3.3 Comparison of Adhesion Assays on Human Intestinal Cell Lines with and without Gastrointestinal Transit

Comparative analysis of adhesion assays performed on three different human intestinal cell lines (CaCo2, HT-29 and HT-29-MTX) with and without simulation of GI transit of the *A. muciniphila* and *L. rhamnosus* GG strains revealed notable differences (Figure 8). Across all cell lines and GI transit scenarios, a consistent trend of reduced adhesion was observed for bacteria subjected to GI stress.

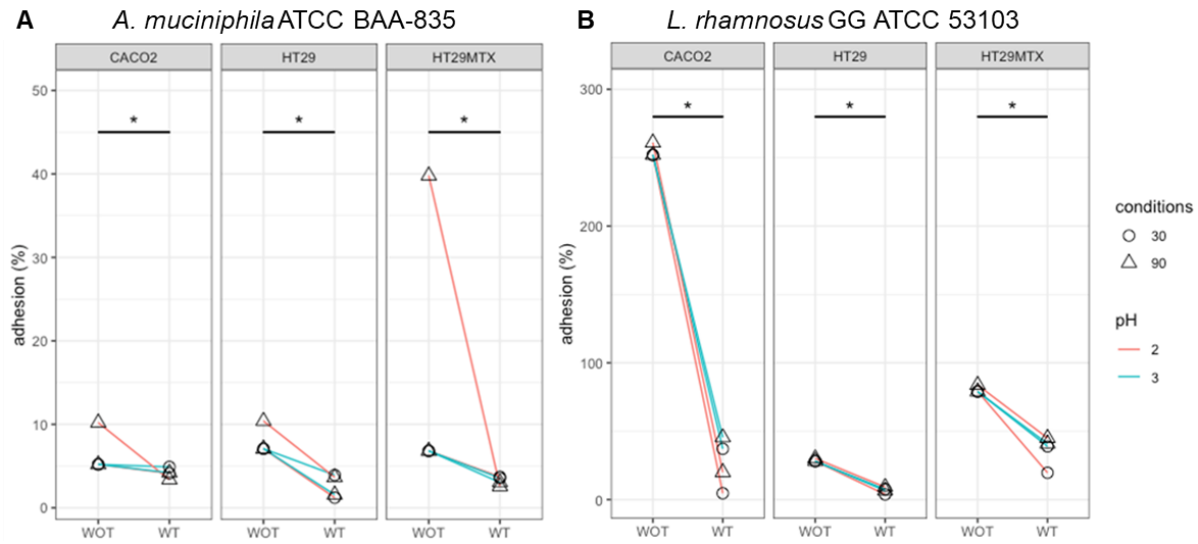


Figure 8. Comparative analysis of adhesion assays performed on human intestinal CaCo2, HT-29 and HT-29-MTX cell lines with (WT) and without (WOT) GI transit of *A. muciniphila* ATCC BAA-835 (A) and *L. rhamnosus* GG ATCC 53103 (B).

Specifically, on the CaCo2 cell line, adhesion percentages of *A. muciniphila* following GI transit at pH 2 for 30 min and pH 3 for 30 and 90 min were 4.13%, 4.94% and 4.20% respectively, whereas adhesion without transit was measured at 6.8% for the same bacterial concentration (Figure 8A). The percentage of adhesion decreased to 3.38% for bacteria expose to pH 2 transit for 90 min, compared to 10.20% for bacteria without transit.

On the HT-29 cell line, *A. muciniphila* adhesion percentages after GI transit at pH 2 for 30 min, pH 3 for 30 min and pH 3 for 90 min were 1.21%, 3.92% and 1.57% respectively, compared to 7.08% adhesion without transit at equivalent concentrations. Adhesion decreased to 3.65% after transit at pH 2 for 90 min, compared to 10.40% for non-transit bacteria.
On the HT-29-MTX cell line, *A. muciniphila* adhesion percentages after GI transit at pH 2 for 30 min and pH 3 for 30 and 90 min were 3.71%, 3.56%, and 3.04%, respectively, whereas adhesion without transit was 6.82% at the same concentrations. After transit at pH 2 for 90 min, adhesion decreased to 2.57% compared to 39.8% for non-transit bacteria.
Overall, the decrease in adhesion rates after GI transit was most pronounced for *A. muciniphila* subjected to pH 2 transit for 90 min across all cell lines.
On the CaCo2 cell line, adhesion rates of *L. rhamnosus* GG after GI transit at pH 2 for 30 min and pH 3 for 30 and 90 min were 4.62%, 37.12%, and 45.59% respectively (Figure 8B). Conversely, adhesion without transit was notably higher at 252% for the same bacterial concentration. Notably, exposure to transit at pH 2 for 90 min resulted in a adhesion percentage of 20% compared to 261% for bacteria without transit.
Similarly, on the HT-29 cell line, adhesion percentages post-GI transit at pH 2 for 30 min, and at pH 3 for 30 and 90 min were 3.77%, 9.56%, and 6.52% respectively, in contrast to 28.10% adhesion without transit at equivalent concentrations. Following transit at pH 2 for 90 min, adhesion was further reduced to 9.17% compared to 30.10% for bacteria without transit.
Moreover, on the HT-29-MTX cell line, adhesion percentages subsequent to GI transit at pH 2 for 30 min and pH 3 for 30 and 90 min were 19.62%, 38.89%, and 40.91% respectively, while adhesion without transit was measured at 79% at the same concentrations. Notably, after transit at pH 2 for 90 min, adhesion was 45.0% compared to 83.80% for bacteria without transit. These findings suggest a significant influence of GI transit conditions on the adhesion of *L. rhamnosus* GG to human intestinal cell lines.

3.4 Study of Protein Interactions of *A. muciniphila* with Human Mucins

The genome of *A. muciniphila* (strain ATCC BAA-835/ DSM 22959/JCM 33894/BCRC 81048/CCUG 64013/CIP 107961/Muc) consists of a circular chromosome of 2664102 bp with an average GC content of 55.8%. It consists of a total of 2210 genes and 12 pseudogenes. *A muciniphila* also possesses two CRISP (clustered regularly interspaced short palindromic repeats) loci that represent primitive and adaptive primitive immune systems in bacteria against invading agents such as bacteriophages or plasmids. The genome of *A. muciniphila* possesses two genes associated with resistance to β-lactam antibiotics (Amuc_0106 and Amuc_0183), a gene coding for an antibiotic resistance protein 5-nitroimidazole (Amuc_1953) and a gene coding for a putative antibiotic biosynthesis monooxygenase (Amuc_1805).The genome of this bacterium contains more than 300 genes (about 11%) for supposed proteins involved in mucin degradation, but the nature and function of most of these proteins are unknown.
The proteome of *A. muciniphila* (strain ATCC BAA-835/DSM 22959/JCM 33894/BCRC

81048/CCUG 64013/CIP 107961/Muc) is compound of 2137 proteins. Proteomic analysis revealed 80 GI mucin-degrading enzymes, such as glycosidases, sulfatases, proteases and sialidases, hexosaminidases, galactosidase, known as "mucinases" (Table 1).

Table 1. List of candidate *A. muciniphila* (ATCC strain BAA-835/DSM 22959/JCM 33894/BCRC 81048/CCUG 64013/CIP 107961/Muc) mucinases searched in the Uniprot database.

Entry Name	Protein names	Gene Names	Amino acids
B2UPI7_AKKM8	Peptidase M60 domain-containing protein	Amuc_0627	506
B2UQK5_AKKM8	Peptidase M60 domain-containing protein	Amuc_0908	747
B2ULE8_AKKM8	Peptidase M60 domain-containing protein	Amuc_1514	660
B2UR60_AKKM8	Peptidase_M43 domain-containing protein	Amuc_1119	385
B2UME7_AKKM8	Peptidase M23	Amuc_0253	289
B2UNB8_AKKM8	Peptidase M23	Amuc_0391	225
B2UNJ1_AKKM8	Peptidase M23	Amuc_0465	347
B2UR47_AKKM8	Peptidase M24	Amuc_1106	427
B2UM82_AKKM8	Peptidase M28	Amuc_0187	356
B2ULX6_AKKM8	Peptidase S1 and S6 chymotrypsin/Hap	Amuc_0176	334
B2UMQ3_AKKM8	Peptidase S1 and S6 chymotrypsin/Hap	Amuc_1791	254
B2UL29_AKKM8	Peptidase S11 D-alanyl-D-alanine carboxypeptidase 1	Amuc_1480	323
B2UN08_AKKM8	Peptidase S15	Amuc_1801	359
B2UP79_AKKM8	Oligopeptidase A (EC 3.4.24.70)	Amuc_2040	726
B2UM07_AKKM8	Carboxyl-terminal protease (EC 3.4.21.102)	Amuc_1631	748
B2ULK5_AKKM8	Peptidase M22 glycoprotease	Amuc_1571	222
B2ULJ2_AKKM8	Zinc metalloprotease (EC 3.4.24.-)	Amuc_1558	481
B2UNU1_AKKM8	Aspartyl protease	Amuc_0473	323
B2UNU3_AKKM8	Methyltransferase	Amuc_0475	191
B2UP74_AKKM8	ULP_PROTEASE domain-containing protein	Amuc_2035	251
B2UPX2_AKKM8	Trypsin-like protein serine protease typically periplasmic contain C-terminal PDZ domain-like	Amuc_0670	526
B2UQG2_AKKM8	Sulfatase	Amuc_0864	572
B2UQQ0_AKKM8	Sulfatase	Amuc_0953	1414
B2UQX4_AKKM8	Sulfatase	Amuc_1033	581
B2UR15_AKKM8	Sulfatase	Amuc_1074	552
B2UR59_AKKM8	Sulfatase	Amuc_1118	628
B2URC2_AKKM8	Sulfatase	Amuc_1182	465
B2ULS2_AKKM8	Sulfatase	Amuc_0121	526
B2UM30_AKKM8	Sulfatase	Amuc_1655	554
B2UML7_AKKM8	Sulfatase	Amuc_1755	562
B2UNH7_AKKM8	Sulfatase	Amuc_0451	719
B2UNV9_AKKM8	Sulfatase	Amuc_0491	500
B2UPC5_AKKM8	Sulfatase	Amuc_0565	542
B2UPP3_AKKM8	Formylglycine-generating enzyme, required for	Amuc_2112	969
B2ULI1_AKKM8	Sialidase domain-containing protein	Amuc_1547	595
B2UN42_AKKM8	Exo-alpha-sialidase (EC 3.2.1.18)	Amuc_1835	674
B2UPI5_AKKM8	Exo-alpha-sialidase (EC 3.2.1.18)	Amuc_0625	419
B2UPI3_AKKM8	Glycosyl hydrolase BNR repeat-containing protein	Amuc_0623	436
G1091_AKKM8	Glycosyl hydrolase family 109 protein 1 (EC 3.2.1.-)	Amuc_0017	481
G1092_AKKM8	Glycosyl hydrolase family 109 protein 2 (EC 3.2.1.-)	Amuc_0920	473
B2UKY7_AKKM8	Glycosyl hydrolase family 98 putative carbohydrate binding module	Amuc_1438	639

B2UQG1_AKKM8	Glycosyl hydrolase family 88	Amuc_0863	744
B2UPN9_AKKM8	Glycoside hydrolase family 16	Amuc_2108	319
B2UMS6_AKKM8	Glycoside hydrolase family 2 sugar binding	Amuc_0290	986
B2UQH3_AKKM8	Glycoside hydrolase family 16	Amuc_0875	313
B2UM81_AKKM8	Glycoside hydrolase family 95	Amuc_0186	788
B2UR61_AKKM8	Glycosyl hydrolase	Amuc_1120	796
B2UPU3_AKKM8	Glycoside hydrolase family 18	Amuc_2164	1233
B2UNV0_AKKM8	Hydrolase of the alpha/beta superfamily	Amuc_0482	272
B2UQU5_AKKM8	Hydrolase of the alpha/beta superfamily	Amuc_1003	377
B2ULB7_AKKM8	Alpha-N-acetylglucosaminidase (EC 3.2.1.50)	Amuc_0060	848
B2URG0_AKKM8	Alpha-N-acetylglucosaminidase (EC 3.2.1.50)	Amuc_1220	852
B2UPZ9_AKKM8	Beta-glucanase	Amuc_0697	381
B2UQ00_AKKM8	Beta-glucanase	Amuc_0698	369
GLAA_AKKM8	Alpha-1,3-galactosidase A (EC 3.2.1.n1) (Exo-alpha-	Amuc_1463	596
GLAB_AKKM8	Alpha-1,3-galactosidase B (EC 3.2.1.n1) (EC 3.2.1.n2) (Exo-alpha-galactosidase B) (EC 3.2.1.22)	Amuc_0480	794
B2UQF3_AKKM8	Alpha-galactosidase (EC 3.2.1.22)	Amuc_0855	716
B2URC7_AKKM8	Alpha-galactosidase (EC 3.2.1.22) (Melibiase)	Amuc_1187	533
B2UM40_AKKM8	Beta-galactosidase (EC 3.2.1.23) (Lactase)	Amuc_1666	1289
B2UM60_AKKM8	Beta-galactosidase (EC 3.2.1.23)	Amuc_1686	780
B2UQ71_AKKM8	Beta-galactosidase (EC 3.2.1.23)	Amuc_0771	643
B2UQ26_AKKM8	Glucan endo-1,3-beta-D-glucosidase (EC 3.2.1.39)	Amuc_0724	290
B2UQC2_AKKM8	beta-galactosidase (EC 3.2.1.23) (Lactase)	Amuc_0824	1264
B2UL68_AKKM8	Alpha-L-fucosidase (EC 3.2.1.51)	Amuc_0010	538
B2ULU6_AKKM8	Alpha-L-fucosidase (EC 3.2.1.51)	Amuc_0146	711
B2UNB9_AKKM8	Coagulation factor 5/8 type domain protein	Amuc_0392	709
B2UQE4_AKKM8	Coagulation factor 5/8 type domain protein	Amuc_0846	704
H2018_AKKM8	Beta-hexosaminidase Amuc_2018 (EC 3.2.1.52) (Beta-N-acetylhexosaminidase Am2301)	Amuc_2018	493
H2136_AKKM8	Beta-hexosaminidase Amuc_2136 (EC 3.2.1.52) (Beta-N-acetylhexosaminidase Am2136)	Amuc_2136	756
H0868_AKKM8	Beta-hexosaminidase Amuc_0868 (EC 3.2.1.52) (Beta-N-acetylhexosaminidase Am0868)	Amuc_0868	549
B2UM43_AKKM8	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Amuc_1669	547
B2UN02_AKKM8	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Amuc_0369	665
B2UN22_AKKM8	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Amuc_1815	728
B2UNC4_AKKM8	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Amuc_0397	722
B2UNM1_AKKM8	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Amuc_1924	664
B2UP58_AKKM8	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Amuc_2019	504
B2UPP0_AKKM8	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Amuc_2109	353
B2UPS8_AKKM8	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Amuc_2148	549
B2UQX3_AKKM8	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Amuc_1032	518
B2UPX9_AKKM8	Beta-hexosaminidase	Amuc_0677	159
B2UPX2_AKKM8	Trypsin-like protein serine protease typically periplasmic contain C-terminal PDZ domain-like	Amuc_0670	526

The analysis also detected the presence of a mucin-associated surface protein (MASP; UniProtID: B2UQG4, is encoded by the Amuc_0866 gene) and a pili assembly protein (PilO; UniProtID: B2UQG4, encoded by the Amuc_1100 gene). The sequence matches of the MASP and PilO proteins were searched in depth in the protein database. More than 500 similar protein

sequences were found, however, mainly belonging to the *phylum* Verrucomicrobia and the genus *Akkermansia*. Similar proteins with very low identity percentages (<20%) were also found in bacteria of the *phylum* Firmicutes and Armatimonadota.

The GroEL protein of *A. muciniphila* of has an identity percentage of 60.07% with the GroEL protein of *Lactobacillus johnsonii*. This suggests that this protein might have the same function in A. muciniphila, i.e. interacting with human mucins in the stomach, preventing *Helicobacter pylori* from binding with the mucins MUC5AC and MUC1 and promoting their aggregation and elimination (Van den Brink *et al.,* 2000; Bergonzelli *et al.,* 2006; Lillehoj *et al.,* 2012).

Following the integration of the database of human mucin interactions with bacterial proteins (found in the literature and online databases), a graphic interaction network was created (Figure 9).

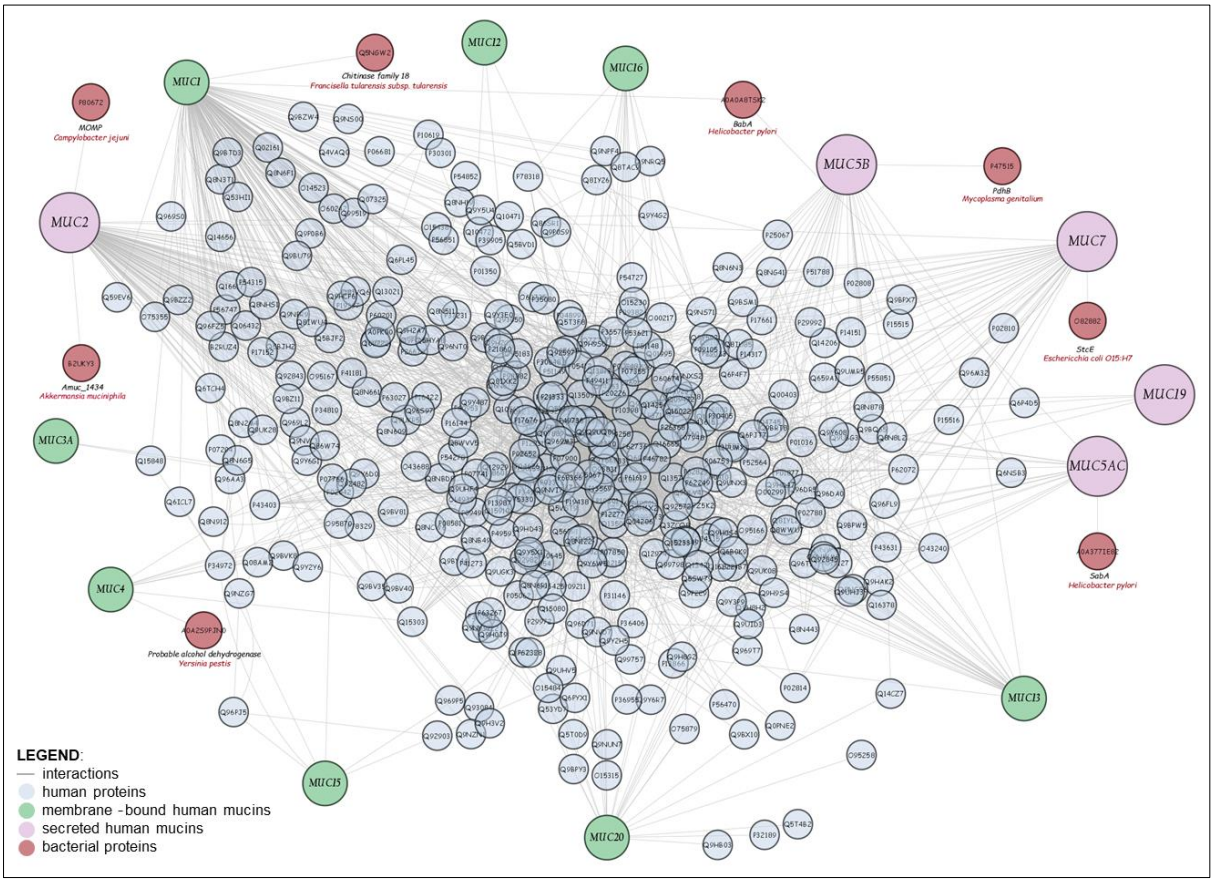


Figure 9. Interaction networks between human mucins and human and bacterial proteins.

4. Discussion

The effectiveness of probiotic microorganisms in providing health benefits depends on the amount of viable bacteria that successfully pass through the GI and reach the colon. Therefore, the ability of probiotic or potentially probiotic microorganisms to survive the GI transit is a critical criterion for ensuring sufficient colonisation. After passage through the different gastric conditions, the *A. muciniphila* strain revealed a decrease in charge of between 1.5 Log CFU/mL (at pH 3 for 30 min) and 2.4 Log CFU/mL (at pH 2 for 90 min) with the plate count, while with

the Live/Dead assay the recorded charge was similar to the starting level. For all conditions, after standing for 4 h in gut conditions, the *A. muciniphila* load increased by more than 1 Log CFU/mL. Behaviour similar to that of *A. muciniphila* during simulated transit has already been observed by other authors for LAB (Faye *et al*., 2012; Possemiers *et al*., 2010; Succi *et al*., 2017).

The *L. rhamnosus* GG strain under different gastric conditions showed a reduction in charge only at pH 2 for 30 min (reduction of 1.4 Log CFU/mL) and for 90 min (reduction of 6.4 Log CFU/mL). Under gastric conditions at pH 3, the charges were equal to the initial charge. After gastric conditions, a significant reduction in charge was recorded in all cases, with a minimum value of 1.5 Log CFU/mL for the sample under gastric conditions of pH 2 for 90 min and 3.65 Log CFU/mL for the sample under gastric conditions of pH 3 for 30 min. Even for the *L. rhamnosus* GG strain, counting with fluorescent probes always resulted in higher charge percentages. Some researchers have suggested that a portion of the bacterial population may enter a state known as viable but non-culturable (VBNC) when subjected to gastric stress (Possemiers *et al*., 2010). Within the gut environment, under less hostile conditions, some VBNC cells could revert to a culturable state, potentially accounting for the observed increase in viability following simulation of intestinal transit (Faye *et al.,* 2012; De Palencia *et al.,* 2008). Additionally, de Palencia *et al.* (2008) noted that gastric stress induced by low pH levels leads to the formation of cell aggregates, which may result in the formation of a single colony during viable plate counts, thus leading to an underestimation of viable cells. Conversely, these aggregations tend to dissipate as pH conditions become more conducive, such as those encountered during intestinal transit.

Nowadays, the most widely and versatile used models for evaluating the adhesion of microorganisms are tissue cultures (Bermudez-Brito *et al*., 2013; Martìnez-Maqueda *et al*., 2015), considering their capacity of mimicking various parts of the intestinal epithelium. Enterocytes and globet cells constitute the two major cell types of the intestine, and CaCo2 and HT-29 cell lines, both deriving from colon adenocarcinoma, display similar structural and functional features of enterocytes. Specifically, HT-29 cells are characterised by a high degree of heterogeneity due to the formation of absorptive and goblet cells mixture. In contrast to HT-29, CaCo2 cells exhibit many properties of the small intestinal epithelium when grown in culture. They form a well-differentiated polarized monolayer of columnar absorptive cells that express brush border with typical small intestinal enzymes and transporters. However, this cell line lacks the typical mucus layer of the intestinal epithelium. The cell line HT-29-MTX derives from HT-29 cells. They differentiate into mucus secreting globet cell types during cultivation, forming a mucus layer on the top of the epithelial cells resembling the colon.

In this research, we used highly characterised intestinal models, i.e. the mucus-secreting CaCo2, HT-29 and HT-29-MTX cell lines (Haddad *et al*., 2023), to test the adhesive capacity of *A. muciniphila*, as this characteristic is still considered one of the main selection criteria for probiotics (FAO/WHO, 2020). In a previous study by Reunanen *et al.* (2015), the adhesion of *A. muciniphila* and *L. rhamnosus* GG was assessed on CaCo2 and HT-29 cells. In line with our

results, they reported a greater adhesion to CaCo2 cells than that detected to the HT-29 cell line for both *A. muciniphila* and *L. rhamnosus* GG. In the same work, Authors reported the ability of *A. muciniphila* to bind both undifferentiated and mature enterocytes, strengthening their integrity. To the best of our knowledge, no information is available on the adhesion ability of *A. muciniphila* to a mucus secreting cell line, that is, HT-29- MTX.

In our research, *A. muciniphila* showed, in most cases, better adhesion to all used cell lines than *L. rhamnosus* GG, and the adhesion of *A. muciniphila* to HT-29-MTX cells was always higher than that detected by using HT-29 and CaCo2 cells. This result could be due to the ability of this bacterium to bind mucus (Karamzin *et al*., 2021) and to use mucin as the sole carbon and nitrogen source (van Passel *et al.,* 2011), even if these aspects are still debated. In fact, in our study the high adhesion rate of *A. muciniphila* to non- and low-mucus producing CaCo2 and HT-29 cell models, in particular to CaCo2 cells, still demonstrated that the attachment is not necessarily mediated by the presence of a mucus layer. In this context, the analysis of *A. muciniphila* proteome revealed the lack of canonical mucin-binding domains involved in adhesion to the intestinal mucus layer, whereas about 80 enzymes for mucin degradation were found. The functional properties of these putative mucinolytic proteins and their role in mucin degradation are still unclear (Meng *et al*., 2020). Similar results were obtained by other Authors. For instance, in past years, van Passel *et al.* (2011) sequenced and annotated *A. muciniphila* genome, showing the presence of numerous candidate mucinase-encoding genes, but not of genes encoding canonical mucus-binding domains. The study of Png *et al.* (2010) showed the good ability of *A. muciniphila* to degrade both porcine mucin and human MUC2 after 48 h of culture. Derrien *et al*. (2010) highlighted the presence of numerous mucin-degrading enzymes identified in the human digestive tract related to *A. muciniphila*, including α- and β-D-galactosidase, α-L fucosidase, α- and β-N-acetylgalactosaminidase, β-Nacetylglucosaminidase, neuraminidase and sulfatase, but no information regarding the mechanisms of its mucus binding was reported. In a recent work by Zhang *et al.* (2019), a critical analysis of the current knowledge on *A. muciniphila* was given, but no information on its adhesion ability was furnished. Only a few studies investigated the role of some *A. muciniphila* ATCC BAA-835 proteins as mucus-binding candidates in mice. In the study by Yoon *et al.* (2021), the mucinase Amuc_1631 protein was shown to bind intercellular adhesion molecule 2 (ICAM2) and induced an increase in thermogenesis and secretion of glucagon-like peptide-1 (GLP-1), a therapeutic target for type 2 diabetes. The study by Meng and co-authors (2020) identified the Amuc_1434 protein (DUF6268 domain-containing protein) as a member of the aspartic protease family. This protein promotes adhesion to the LS174T (colon cancer) cell line, which expresses high levels of MUC2, demonstrating the ability of this bacterium to bind MUC2 in mouse colon. Since human mucin-2 shares more than 76% identity with mouse mucin-2, it can be assumed that the Amuc_1434 protein of *A. muciniphila* also binds the human MUC2 protein.

By using three different cell lines, our study showed the ability of this bacterium to adhere either to the mucin covering the epithelial cell layer or directly to the enterocytes, explaining its detection in all parts of the intestinal tract (Derrien *et al*., 2010). A further information on the

adhesion to intestinal cell lines was given by evaluating different bacterial concentrations to determine the binding affinity to cells. Our results highlighted low adhesion rates when bacterial concentrations were between 8 and 7 Log CFU/mL, with scarce differences between cell lines as well as between tested bacteria. Important differences were, however, appreciated starting from concentrations of 6 Log CFU/mL. This fact confirms that in this kind of studies it is important to not saturate the cells binding sites, otherwise a progressively smaller fraction of probiotics will bind, and a lower adhesion rate will be observed (Ouwehand and Salminen, 2003). Also, considering that GI secretions can dilute probiotics up to 100 times, the consumption of 10^8 CFU/mL can become 10^5 - 10^6 CFU/mL in the intestines (Ouwehand and Salminen, 2003). The results obtained in our study confirm the above: higher bacterial concentrations of *A. muciniphila* and *L. rhamnosus* GG showed lower adhesion rates than those recorded for lower concentrations, as observed for all cell lines.

This research also highlighted that adhesion was generally greater than 100% for bacterial concentrations between 10^5 and 10^2 Log CFU/mL. This fact was particularly evident for *A. muciniphila* assessed on CaCo2 and HT-29-MTX cells and for *L. rhamnosus* GG tested on CaCo2 cells. This is probably due to an increase in bacterial charge after 2 h of incubation, that is, the time generally required to perform the adhesion test (Ouwehand and Salminen, 2003). Obviously, this behaviour is not expected in human intestinal conditions, where a harsh environment is present.

To date, the use of *A. muciniphila* as live probiotic bacterium is still under evaluation, and the EFSA Panel on Nutrition, Novel Foods and Food Allergens has currently approved its consumption in pasteurised form to be used as a food supplement at max 5 x 10^{10} cells/day by adults excluding pregnant and lactating women, under the provision that the number of viable cells in the novel food is < 10 colony forming units (CFU/g) (i.e. limit of detection) (EFSA, 2021).

It was also shown that simulating GI transit changes the *in vitro* adhesion properties of strains to human intestinal cell lines. Indeed, the adhesion after transit of the *A. muciniphila* and *L. rhamnosus* GG strains were, for each GI transit condition examined in our study, significantly lower than the adhesion without transit at the respective concentrations. Similar results were reported in the study by de los Reyes-Gavilàn *et al.* (2011) who examined the adhesion capacity of *Bifidobacterium* strains after simulation of GI transit using human gastric juices.

The reduced adhesion of the studied strains to human cell lines after the simulation of GI transit could be induced by the various environmental stresses (pH changes, exposure to bile salts and enzymatic degradation) to which they are subjected during the simulation of GI transit. Loss of viability also has a direct impact on the ability of bacteria to adhere to human cell lines (de los Reyes-Gavilán *et al.,* 2011).Exposure to GI conditions can alter the surface characteristics of bacteria. Changes in surface proteins or carbohydrate structures, for example, can have an impact on the interaction between bacteria and cell lines.

Despite the decreased rate of adhesion after the simulation of GI transit, it is important to note that this approach provides a more physiologically relevant assessment of the behaviour of

probiotics in the gut. The conditions encountered during GI transit are essential factors to consider when evaluating the efficacy of probiotics *in vivo*.

Overall, although the adhesion rate of probiotics to human cell lines may be lower after simulation of GI transit, this approach provides valuable insights into the behaviour of probiotics in the complex gut environment, contributing to the development of more effective probiotic formulations for the gut microbiota.

5. Conclusion

A. muciniphila, an intestinal symbiont that colonises the mucus layer, is considered a promising probiotic and is currently approved for consumption in pasteurised form. *A. muciniphila* reaches the gut at high loads, showing high resistance to gastric stress. The high rate of attachment to mucus-producing and non-mucus-producing cell models demonstrated that attachment is not necessarily mediated by the presence of a mucus layer, and the evaluation of this ability in relation to different bacterial concentrations provided new data useful for the characterisation of this potential probiotic bacterium. In particular, we have shown that *A. muciniphila* is able to bind to different intestinal cell lines, even when tested at concentrations below 6 Log CFU/mL, i.e. lower than those generally adopted in this type of test. However, adhesion to different cell models merits further study. Considering that for both strains adhesion after GI transit was consistently lower, we believe that performing the two tests sequentially is a valid alternative to conventional testing.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1. Comparison of adhesion percentages to CaCo2, HT-29 and HT29-MTX cell lines of *Akkermansia muciniphila* ATCC BAA-835 and *Lacticaseibacillus rhamnosus* GG ATCC 53103 tested at concentrations of 8, 7, 6, 5, 4, 3 and 2 Log CFU/mL.

		Adhesion (%) mean ± SD		T-test
Cell line	**Bacterial concentation (Log CFU/mL)**	***A. muciniphila* ATCC BAA-835**	***L. rhamnosus* GG ATCC 53103**	**P-value**
CaCo2	8	6.80 ± 0.185	5.84 ± 0.058	4.18E-06
	7	10.20 ± 0.73	9.65 ± 0.26	1.50E-01
	6	15.20 ± 0.202	10.9 ± 0.363	1.38E-06
	5	114.00 ± 3.51	167.0 ± 6.30	2.16E-07
	4	116.00 ± 3.30	170.0 ± 16.90	1.04E-04
	3	438.00 ± 33.3	252.0 ± 6.04	1.80E-06
	2	470.00 ± 21.6	261.0 ± 60.80	8.93E-05
HT-29	8	7.08 ± 0.172	5.86 ± 0.179	4.18E-06
	7	10.40 ± 0.324	9.34 ± 0.485	3.20E-03
	6	11.00 ± 0.536	10.91 ± 0.293	8.32E-01
	5	74.30 ± 0.524	13.50 ± 1.66	7.83E-13
	4	91.70 ± 0.61	13.10 ± 0.29	2.20E-16
	3	92.80 ± 1.41	28.10 ± 0.599	1.76E-13
	2	97.10 ± 5.79	30.10 ± 8.58	5.13E-07
HT29-MTX	8	6.82 ± 0.165	5.75 ± 0.082	1.17E-06
	7	39.80 ± 0.581	12.10 ± 0.582	1.08E-12
	6	66.60 ± 1.40	51.60 ± 0.538	1.70E-08
	5	131.00 ± 0.822	58.10 ± 0.247	6.57E-16
	4	455.00 ± 11.0	80.60 ± 2.37	1.19E-12
	3	465.00 ± 7.91	79.00 ± 0.822	5.78E-14
	2	482.00 ± 10.0	83.80 ± 2.60	3.74E-13