Goat Cheese: a Model for Studying the Functional Microbiota in a One-health Context

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Abstract

Background

Goat cheese is an important element of the Mediterranean diet, appreciated for its health-promoting features and the typical gustatory essences. A pivotal role in the development of these characteristics is attributed to the hosted microbiota and its continuous remodeling over the space and time. Nevertheless, a thorough study of the cheese-associated microbiota by two meta-omics approaches is still missing. Also, the study of these model systems is important in the One Health context as they enable the development of systems for monitoring environmental micro-organisms and their biological variability. In this study we employed 16S rRNA gene sequencing and metaproteomics to explore the microbiota of a typical raw goat milk cheese at diverse ripening timepoints and depths of the cheese wheel.

Results

findings from 16S rRNA gene sequencing and metaproteomics described a stable microbiota ecology across the selected ripening timepoints, providing evidence on the microbiologically driven fermentation of the goat milk products. On the other hand, important modeling of the microbiota harbored in the surface and core of the cheese mass are highlighted both in compositional and functional terms.

Conclusion

observed outcomes portrait the rind microbiota struggling for the maintenance of the cheese biosafety through competition mechanisms and/or preventing the cheese colonization by pathobionts of animal or environmental origin. Efforts in microbial competition are also accomplished in the core microbiota, although its further focusing on other biochemical routes supports the role of this microbiota in the development of both the health beneficial effects and the pleasant gustatory nuances of the goat cheeses.

Background

Goat cheese is a key element in the Mediterranean diet and is among the most consumed dairy products all over the world. The variety of cheese textures, flavours and organoleptic properties depends on several factors including -but not limited- to the cheese-making process, the animal breed and breeding management. Emerging evidence underlines the pivotal role of the microbiota, and its continuous shaping, in the conditioning of the cheese peculiarities. Here, the complex microbial diversity harboured in the milk converts the milk components, mainly carbohydrates and proteins, into secondary products and/or substrates that further trigger the growth and the metabolism of third microorganisms. This results in the continuous restructuring of the microbiota and the accumulation of the myriads of molecules, of microbial origin, constituting the cheese mass such as fatty acids, volatile organic

compounds (VOCs), amines, ketones, free amino acids, phenols, alcohols, aldehydes, lactones, sulphur compounds *etc* [1–4]. On the other hand, the presence of harmful specimens either of animal origin or acquired from the environment poses health and hygiene problems. As of today, most commercial and large-scale cheese factories accomplish milk treatment procedures (*e.g.*, thermization, pressurization) to standardize the milk quality along with drastically reducing the milk microbial load and diversity [5]. Contrarywise, the great majority of the traditional dairy products are still produced by using raw milk [6], thus, benefitting from wide microbial biodiversity operating over the stages of the cheesemaking process. Nevertheless, higher hygienic standards and precautions are required throughout the whole production process [7, 8].

Milk and cheese act as the “point of contact” between animal-, human- and environmental health; therefore, the accurate assessment of the hosted microbiota is an important tool of One-Health relevance, besides being of great importance in diverse other aspects such as biosafety, technological processing and nutritional and nutraceutical value, among the others. The rapid advances of the meta-omics disciplines enabled the fine characterization of the microbial communities harbored by virtually all ecological niches. Metagenomic and metabolomics investigations are the most employed approaches in cheese microbiota research, providing information on the composition and genetic potential of the sampled microbial community along with the overall array of metabolites produced by the consortia of microorganisms associated with cheese. Complementation of this information by metaproteomics is desirable as it would provide valuable information on the protein repertoire and the biochemical pathways being effectively performed by the microbiota components at the sampling conditions. Nevertheless, metaproteomics investigation of the milk-by products is rare owing to the technical difficulties which prevent the common adoption of the metaproteomics approach in the characterization of the cheese microbiota. To the best of our knowledge, only a couple of studies available in the literature employed metaproteomics for the investigation of the cheese microbiota [9–11] and no metaproteomics studies are available on raw goat milk cheese.

Acknowledged the potential held by studying the typical cheese microbiota, and the paucity of cheese metaproteomics studies, we complemented 16S rRNA gene sequencing and metaproteomics to draw a comprehensive picture of a typical raw goat cheese microbiota, in terms of both composition and activity. This is the first meta-omics-based study on a typical raw goat milk cheese investigating the microbial community dynamics at the rind and core of the cheese wheel, across different ripening periods. Moreover, we provide insights on the microbial interactions occurring among the naïve and environmental bacteria and their biochemical strategies to warrant biosafety of the typical raw milk-by products.

Methods

Cheese samples and experimental design

The present work explores the microbial community associated with a typical raw goat milk cheese. Caprino Nicastrese cheese is employed as the reference sample kind of a traditional raw goat milk

cheese. Following collection, the goat raw milk is coagulated for 60 minutes at 36°C using 0.4 g/L goat rennet and without the addition of any starter culture. The resulting curd is manually cut into rice-sized pieces and formed and stored at room temperature for 48 hours to drain out the residual whey. Cheese wheels are then salted for 24 hours in brine with 30% (w/v) NaCl. Finally, the cheese is ripened in wooden axes in the storage basement of the cheese farms at 10–15°C and 70–85% humidity.

Samples from the surface and inner mass (*i.e.* rind and core, respectively) of the cheese wheels are aseptically collected with a sterile knife from 30, 60 and 90 days-ripened cheese wheels. Biological replicates were sampled as defined in Fig. 1 and brought on ice to the laboratory for the subsequent isolation and analysis of the harboured microbiota.

Bacterial fraction enrichment

To avoid alterations of the microbiota composition and/or activity, all the steps of the bacterial fraction enrichment were performed at 4°C, keeping under strict control the temperature. Briefly, independent aliquots of 0.5 g of each biological replicate per sample kind were finely grated and homogenized with 15 mL buffer containing 50 mM Na_2HPO_4, 0.1% Tween 80, pH 8.0. Samples were then shaken on an orbital shaker at 1600 rpm for 10 minutes. Following, the samples were centrifuged for 20 minutes at 2500× g. The supernatant was collected in a new tube and subjected to four more rounds of shaking/centrifuge/resuspension; whereas the pellet from each step was gently resuspended and collected in a single clean “pool vial”. The “pool vial” was finally centrifuged at 12,000× g for 20 min, resulting in a bacterial pellet collected from an original amount of 0.5g cheese aliquots [1, 12, 13].

The enriched bacterial fractions represented the common starting point for the two analytical approaches employed in the present study: 16S rRNA gene sequencing and metaproteomics (Fig. 1).

16S rRNA gene sequencing and metataxonomic analysis

DNA extraction and library preparation. Cheese DNA was extracted from 9 rind and 9 core samples, respectively, 3 for each ripening time point (Fig. 1) according to EZ1 DNA Tissue protocol (Qiagen, Germany). Starting from 40 mg, 190 µl of Buffer G2 and 10 µl of proteinase K solution were added to each sample aliquot, incubating at 56°C in an Eppendorf® Thermomixer until complete sample lysis and vortexing 2–3 times per hour to disperse the sample. Two hundred µl of supernatant were transferred to a new 2 ml sample tube and the automated EZ1 extraction was finalized. Amplification of the V3–V4 variable region from the bacterial 16S rRNA gene (~460 bp) was carried out using the primers 16S_F 5'-(TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG)-3' and 16S_R 5'-(GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C)-3', according to MiSeq rRNA Amplicon Sequencing protocol (Illumina, San Diego, California, USA). The PCR reactions were set up using the 2× KAPA Hifi HotStart ready Mix kit (KAPA Biosystems Inc., Wilmington, Massachusetts, USA). DNA amplicons were cleaned-up by CleanNGS kit beads (CleanNA, Coenecoop 75,

PH Waddinxveen, Netherlands). A second amplification step was performed to obtain a unique combination of Illumina Nextera XT dual-indices for each sample. The final libraries were cleaned-up using CleanNGS kit beads, quantified by Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and normalized to 4 nM. To generate paired-end 250x2 bp-length reads, normalized libraries were pooled together and run on the Illumina MiSeq platform, according to manufacturer's specifications.

Biocomputational and Statistical Analysis for cheese microbiota Profile Analysis

QIIME2 was used to analyze the Paired-end sequencing reads [14]. Quality control, denoising, chimera removal, trimming and construction of the Amplicon Sequence Variant (ASV) table were performed by the means of DADA2 implemented as a plugin in QIIME2 [15]. The taxonomy was assigned by using a Naive Bayes model pre-trained on SILVA through the QIIME2 plugin q2-feature classifiers [16]. Alpha- and beta-diversity were computed by skbio.diversity using analysis of variance (ANOVA test) and permutational analysis of variance (PERMANOVA test), respectively; the latter was applied on phylogenetically informed weighted and unweighted Unifrac and Bray-Curtis distance matrices [17] with 9999 permutations to perform paired comparison of rind and core samples at different time points. Principal coordinate analysis (PCoA) plots were used to illustrate the beta diversity of samples. The ASV table was normalized using the Cum Sum Scaling (CSS) methodology [18], hence Kruskal-Wallis test was applied to compare taxonomic differences at phylum level (L2), family (L5), and genus (L6) level. Python 3.7 was used to perform ecological statistical analyses. Three different levels of statistical significance was identified based on different p-values ($p \leq 0.001$) and false discovery rate (FDR) thresholds ($p \leq 0.05$, $p \leq 0.001$) [19]. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [20], exploiting the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO) database was used to determine ASVs and their microbiome functions. In addition, LEfSe (Linear discriminant analysis Effect Size) were independently used to determine the features most likely explaining differences between rind and core of the cheese wheel at 30-, 60- and 90-day ripening times.

Metaproteomics analysis

Metaproteome extraction and quantification

Bacterial pellets from the above protocol of bacterial fraction enrichment were resuspended in protein extraction buffer (7M UREA, 2M Thiourea, 4% CHAPS) and subjected to 6 cycles of 1 min bead beating (Minilys, Bertin Tecnologies, FR) interspersed by 1-minute rest on ice. Bead beating steps were performed by shaking each sample at 4000 rpm with an equal amount (1:1 v/w) of 0.1 mm zirconium-silica beads. Following bead-beating, the samples were heated up to 60°C for 10 minutes and centrifuged for 20 min at

12,000× g at 4°C. The supernatant containing the extracted metaproteome was collected in a clean tube and further processed for the metaproteomic analytical workflow.

Extracted proteins were quantified with the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, USA) following the manufacturer's instructions. Approximately 50 µg of the extracted proteins were precipitated by incubation (30 min at 4°C) with precooled 20% trichloroacetic acid (TCA) and kept for further processing.

Trypsin Digestion and Mass Spectrometry Analysis

Precipitated proteins were digested *in solution*. Briefly, 50 µg of total proteins for each sample was treated for disulfide bond reduction with 10 mM DTT for 1 h at + 37° C and alkylated with 20 mM IAA at + 37° C for 1 h in the dark. Iodoacetamide excess was removed by incubation of the sample with 1.61 mM DTT at + 37° C for 20 min. Sample digestion was carried out overnight at + 37° C using trypsin in 1: 50 (w/w) ratio with respect to the protein content. Enzymatic digestion was stopped by addition of 0.1% FA (v/v).Tryptic peptides were purified and desalted by using self-assembled C18 Stage Tips [21]. Tips containing the C18 membranes with the bounded peptide mixture were eluted with 5% acetonitrile (5% ACN/ 0.1% TFA), dried at the vacuum centrifuge, and stored at -20°C until mass spectrometry measurements.

Prior MS/MS measurement, the dried peptide mixture was suspended in 0.1% FA and loaded onto a precolumn Acclaim PepMap100 C18, 5 µm, 100 Å, 300 µm i.d. x 5 mm (Thermo Scientific, San Jose, CA). Following 5 minutes of trapping, operating at 10 µL/min in eluent A, peptides were separated by a column Easy-Spray PepMap C18 (2 µm 100 Å 15 cm x 50 µm ID) with a Thermo Scientific Dionex UltiMate 3000 RSLC nano system (Sunnyvale, CA).

Analyses were performed using aqueous solution of FA (0.1%, v/v) as eluent A and ACN/FA (99.9 : 0.1, v/v) as eluent B in the following gradient elution: (i) 5% of eluent B (7 min), (ii) from 5 to 35% of eluent B (113 min), (iii) from 35 to 99% of B (15 min), (iv) 99% of B (10 min), (v) from 99 to 5% of B (2 min), (vi) 5% of B for column conditioning (13 min). The column was kept at 35°C and operated at a flow rate of 300nL/min; the injection volume was set at 5.0 µL.

Peptides were directly eluted into Orbitrap Elite nanoESI-MS/MS (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Tandem mass spectrometry measurements were performed in positive Full Scan acquisition mode in the 350-2,000 m/z range and with a resolution power of 60,000. The nanoESI tuning parameters were capillary temperature 250°C, source voltage 1.5 kV, sheath gas 0, auxiliary gas 0, and S-lens RR level 50%. MS/MS analyses were performed in data-dependent scan (DDS) mode by selecting and fragmenting the twenty most intense multiple-charged ions of the collected Full Scan spectra by using collision-induced dissociation (CID, 35% normalized collision energy) with a resolution power of 60,000. Only precursors with a charge state 2–7 and intensity above the threshold of $5x10^3$ were collected for MS/MS. The DDS set parameters were the following: exclusion mass width relative

reference mass in the low and high range 10 ppm, minimum signal threshold (counts) 500, default charge state 2, activation time 10 ms [22].

Bioinformatics data analysis and data integration

Protein identification and quantification

MS raw spectra were processed through Proteome Discoverer and MaxQuant software following a two-step database-dependent search (DDS) approach as reported previously [23]. Briefly, raw files were, at first, processed by Thermo Proteome Discoverer software (v.2.2) and searched against the UniProt KB bacteria database. Methionine oxidation was set as variable modification and carbamidomethylation of cysteine as fixed modification. The SequestHT nodes thresholds were set to “Automatic”, and a filter considering only entries with at least one peptide per protein was chosen. All other filters and settings of the software were kept as default, including protein grouping with peptide confidence set on “high” and delta Cn of 0.1. The Percolator node supporting a strict maximum parsimony principle was activated with a false discovery rate of 1%.

The first DDS enable the assessment of the microbial community composition at the family level, leading to the construction of a smaller *in-house* database accounting for the bacterial families identified in both the metaproteomics and 16S rRNA gene sequencing-based investigations. The customized database was employed in the second DDS of the MS raw data performed onto MaxQuant (v 1.6.17.0) set on LFQ modality for peptide identification and protein inference and quantification. Cysteine carbamidomethylation was set as fixed modification and methionine oxidation as variable modification. Two missed cleavage sites were allowed for *in silico* protease digestion and peptides had to be fully tryptic. All other parameters of the software were set as default, including peptide and protein FDR < 1%, at least 1 peptide per protein, precursor mass tolerance of 4.5 ppm after mass recalibration and a fragment ion mass tolerance of 20 ppm. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD032280.

Ecological and functional characterization of the microbiota by metaproteomics

Information on the taxonomic composition of the microbiota, as assessed by the identified protein repertoire, was gathered from the protein annotation of the UniProt KB database, whereas the quantitative microbiota composition was determined based on the LFQ intensities relative to each bacterial member, on a family basis. Logarithmic transformation of the cumulative intensities on a family basis was accomplished while comparing the microbiota composition in the diverse sample groups.

Identified protein repertoires were functionally categorized into biological processes and molecular functions of the Gene Ontology (GO) data repository. Abundance profiles of the identified proteins (LFQ values) were subjected to statistical investigation using Primer7 v.7 statistical software (PRIMER-E,

Plymouth, UK). Principal Component analysis (PCO) was calculated on the square root transformation of the protein LFQs. Statistical differences across the samples were calculated by performing ANOVA and PERMANOVA. Parametric T-test assessing the discriminating role of the bacterial families on microbiota composition is calculated and visualized into iMetalab using shiny apps (https://shiny.imetalab.ca/). Linear discriminant analysis Effect Size (LEfSe) was calculated in the galaxy platform (https://usegalaxy.org). Heat maps visualizing microbial community composition across the samples and functional classification of the identified proteins were drawn using heatmap.2 provided by the gplots package implemented in R v.4.2.0 software (http://www.R-project.org). Correlation analysis was performed through the corrplot package implemented in R v.4.2.0 software (http://www.R-project.org).

Results

Metataxonomic analysis of the microbiota

Sequencing of the V3-V4 regions of 16S rRNA gene identified an average of 27500 reads per sample (20000–35000 reads). The median Goods coverage of approximately 0.985 for both the rind and core sample (Additional File 1) groups is indicative of satisfactory nucleic acid extraction performance and only a minor load of the 16S-based information is neglected with the present approach. Analogous analytical performances were scored for the samples labelled on a ripening timepoint basis (Additional File 1). Sequencing reads were quality filtered and trimmed before being binned into ASVs.

Data from the DNA-based investigations were assembled in data matrices according to the Bray-Curtis similarity and the weighted and unweighted UniFrac, as depicted in the PCO plots of Fig. 2. Here, sample ordination visualizes a scattered spreading of the 16S rRNA profiles, preventing a clear distinction between the microbial community harbouring the rind and the core samples, nor the microbiota at the diverse cheese ripening stages ($p > 0.05$).

Identified ASVs, sorted on a sample basis, portray a stable microbiota composition across the three ripening periods as supported by the ANOVA analysis (30, 60 and 90 ripening days, $p > 0.05$). On the other hand, sorting the OTUs based on the cheese wheel depths (*i.e.* core and rind) reveals a tendentially different microbiota composition at the taxonomic level of family and genus (Fig. 3), with the genus *Lactobacillus*, family *Lactobacillaceae*, being overrepresented in the core associated microbiota; thus, driving the structural alteration in the comparative evaluation of the microbial communities.

Taxonomic composition of the microbial communities is further employed as input for the assessment of the functional potential of the microbiota as of the PICRUST analysis (Additional File 2). Altogether, no massive differences are underlined in the comparative evaluation of the functional potential of the microbiota colonizing the rind and the core regions as well as among microbial communities at the different ripening timepoints. These observations are statistically supported as shown by the resemblance matrices drawn according to Bray-Curtis and the Euclidean distances of the microbial communities' functional profiles (Additional File 3).

Metaproteomics investigation of the microbial communities

Microbiota investigation through metaproteomics relies on the identification of approximately 3500 proteins among the samples kept at the different ripening timepoints and cheese wheel depths. Here, a variable number of proteins was uniquely identified in each sample group; whereas most of the identified proteins are shared in the diverse combinations of sample pairs as depicted in Fig. 4, panel A. In line with the DNA-based investigations, the depiction of the metaproteomic dataset onto a PCO plot reveals a scattered distribution of the samples as the function of the ripening periods. Also, sample labelling according to the cheese wheel depth displays a clear separation of the metaproteomic profiles along the PCO1 axis (Fig. 4, panel B). Clustering of the rind and core samples is also supported by the ANOVA statistical test, scoring a $p < 0.01$.

Taxonomic composition of the microbial communities by metaproteomics portrays a higher bacterial diversity, at the family level, when compared with the companion DNA-based approach. An overview of the diversity indexes calculated for the microbial communities over the diverse cheese wheel depths and ripening timepoints is provided in Additional File 4. The cumulative abundance of the proteins relative to each bacterial family reveals a different composition (ANOVA, $p < 0.05$) of the rind and core-associated microbiotas. Whilst the overall microbiota architecture in the diverse ripening timepoints is stable as assessed by both the ANOVA and the pairwise PERMANOVA statistical tests ($p > 0.05$). Considering the above observations, we focused on the rind and core microbiota to specifically define the bacterial families driving the differences in the taxonomic structures of these microbial communities. Regardless of the ripening timepoints, abundance of the proteins belonging to the families *Bacillaceae, Rhizobiaceae, Clostridiaceae, Streptococcaceae, Caulobacteriaceae, Enterobacteriaceae, Moraxellaceae, Mycobacteriaceae, Paenibacillaceae, Pseudomonadaceae* and *Staphylococcaceae* are overrepresented in the core samples ($p < 0.05$) as reported in the volcano plot of Fig. 5. Similarly, evaluation of the protein abundance profiles by Least Common Ancestor as of the Linear Discriminant Analysis (LDA) analysis depict *Paenibacillaceae* and *Vibrionaceae* as the major discriminating families, with the first being overrepresented in the core samples; whereas Vibrionaceae are more abundant in rind-associated microbiota (Additional File 5).

The functional featuring of the cheese microbiota was accomplished by sorting the identified protein repertoire into a variety of protein ontology data repositories such as Gene Ontology (GO), PFAM and TIGRFAM. A detailed list of the functional classification of the identified protein repertoire is provided in Additional File 6. Altogether, functional classification reveals different concerns of the rind and core-associated microbiota ($p < 0.01$) in the diverse functional classification data repositories. Besides ANOVA, further statistical evaluations underline the “blind” grouping of the microbial communities harbouring the rind and core of the cheese wheel according to their different functional profiles. In line with the previous observation, no statically significant differences were observed between microbial communities at the selected ripening timepoints ($p > 0.05$) (Additional File 7). Protein sorting based on the GO biological

processes provides an overview of the major functional concern of the microbiota in the rind and core samples. Here, the rind-associated microbiota is intensively involved in the “antibiotic catabolic process”, “cellulose biosynthetic process”, “glutamine metabolic process” and “histidyl-tRNA aminoacylation” as compared with the core counterpart. On the other hand, the core microbiota is exclusively involved in biological processes such as “polyketide metabolism” and the “siderophore biosynthesis”. Also, core-microbiota is much concerned with biological processes such as the “carbohydrate derivative metabolism”, “cell division” and “phosphorelay signal transduction system”, among others (Fig. 6). Further deepening into the functional data elucidates the bacterial families principally involved in the biological process peculiar to each microbiota. Here, in the rind microbiota, the family *Rhizobiaceae* is the main player in the “antibiotic catabolic process” suggesting the bacterial specimens of environmental origin as the principal target of the antibiotic-based competition. The “cellulose biosynthetic process” is, instead, led by the *Enterobacteriaceae*; whereas, in the rind microbiota, the “histidyl-tRNA aminoacylation” biological process is accomplished by the *Bacillaceae* and *Clostridiaceae* (Fig. 6).

Functional characterization of the core microbiota highlights the families *Paenibacillaceae* and *Bacillaceae* as the major contributors to the biological processes “antibiotic biosynthetic process” and “siderophore biosynthesis” suggesting a role of these specimens in maintaining food biosafety by outcompeting pathobionts. Also, an overall involvement of the core microbiota in developing the cheese organoleptic properties is performed by *Bacillaceae* and *Rhizobiaceae* since strictly involved in the “lipid metabolic process”; whereas the biological process “arginine biosynthesis” is mostly accomplished by *Vibrionaceae* and *Enterobacteriaceae*. Also, members of the family *Pseudomonadaceae* are the major contributor to the “7,8-dihydroneopterin 3-triphosphate biosynthetic process” (Fig. 6).

Acknowledged the dynamic structure and functions of the microbial communities in the diverse cheese wheel regions, the quantitative metaproteome was used to elucidate the correlation network occurring among the bacterial families in the cheese core and rind samples.

Interestingly, the bacterial families *Enterobacteriaceae, Rhizobiaceae, Bacillaceae* and *Clostridiaceae* show a strong positive correlation with each other, supporting the previous observations on both structural and functional shaping of the microbial community harbouring the surface region of the cheese wheel (Fig. 7A).

Regarding the core microbiota, correlogram analysis displays the family *Paenibacillaceae* being negatively correlated with most of the identified bacterial specimens of likely environmental origin (e.g. *Desulfuromonadaceae, Mycobacteriaceae, Ricketsiaceae*) whilst it positively correlates with other families such as *Lactobacillaceae, Enterococcaceae* and *Bacillaceae*. The family *Bacillaceae*, in turn, support the microbiota biodiversity by positively correlating with most of the bacterial families identified in the survey. In addition, the bacterial families retained to play a role in the development of the cheese organoleptic properties (e.g. *Bacillaceae*, *Rhizobiaceae, Vibrionaceae* and *Enterobacteriaceae*) are linked by positive correlations, either strong or weak, among each other (Fig. 7B).

Discussion

Microbial consortia of the milk and its by-products are important bioindicators of the animal health status and the microbial exchange occurring through the human-animal-environment overlap. The fine orchestration of the microbial metabolic functions is the foundation of the cheesemaking technological process, including the development of the gustatory and/or olfactive nuances peculiar to each cheese, besides the maintenance of the biosafety of the dairy products. Most typical cheeses are made with raw, unprocessed milk carrying a high microbial diversity whose importance is still largely debated. On one hand, employing milk with rich microbial biodiversity enables the control of the food biosafety along with the development of unique essences by exploiting variable and versatile arrays of metabolic routes. Contrarywise this, a higher microbial diversity might host pathobionts and/or spoilage specimens; thus, the reduction of the naïve milk-associated microbial flora is thought the key to guaranteeing the quality and biosafety of the milk and its by-products [8, 24]. Although both arguments are scientifically sounding, the myriads of influencing variables, on both the structural and functional networks of the microbiota, make unpredictable the effect of the microbial consortia in the diverse aspects of the cheesemaking process. This raises the need to thoroughly investigate each microbiota and elucidate how the microbial interconnections are shaped over the experimental conditions.

In this study, we present the first meta-omics survey of the microbiota associated with a traditional cheese made with raw goat milk. The cheese of choice, as the study model, is the Caprino Nicastrese, an artisanal goat cheese produced in Calabria, south of Italy. To the best of our knowledge, two studies have been so far performed by “pre-omics” approaches to evaluate the presence of selected bacterial specimens [25, 26] but a comprehensive analysis of the typical goat cheese microbiota is lacking. The sampling strategy we adopted relies on our initial hypothesis that cheese regions with diverse physical-chemical features (*e.g.* oxygen availability) host different microbial communities, whose overall metabolism is likely pivotal to specific aspects of the cheese. The ripening timepoints selected in our study mirror the variants of the cheese currently traded (*i.e.* Caprino Nicastrese ripened at 30, 60 and 90 days).

Both DNA and protein datasets depict distinct microbiotas in the rind and core of the cheese wheel. The core-associated microbiota is featured by the emergence of new bacterial families (*e.g. Brevibacteriaceae, Micrococcaceae*) along with the increased abundance of the other bacterial families such as *Lactobacillaceae* and *Paenibacillaceae*. Altogether, this suggests how the ecological niche (*i.e.* cheese rind or core) would shape the microbiota’s architecture and mould microbial metabolism to accomplish the dairy product transformation and its preservation from spoilage. Current observations depart from the unique descriptions made on this cheese ‘microbiota identifying *Lactobacillus spp* and *Enterococcus spp*. as the most abundant specimens [25, 26]. The diverging microbiota composition is primarily imputed to the different investigation methods. Pino and colleagues [25] employed culture-dependent methods which intrinsically overestimate the most common bacterial specimens at the expense of the other ones. Also, farm-to-farm variability is to be expected due to the lack of a stiff production specification and the changing environmental variables influencing the microbiota composition [25]. Moreover, the previous

investigation undistinguished core and surface microbiota while assessing the microbial consortia composition. Nevertheless, as the current study shows, the different cheese wheel depths are associated with specific microbiota compositions and functions. Altogether, these make the studies poorly comparable; rather, an integration and complementation of the outcomes are to be considered.

A diverse general microbiota composition is drawn by the 16S rRNA gene sequencing and metaproteomics approaches. For instance, the family *Streptococcaceae* is identified as the most abundant member in core and rind microbiota by the DNA-based approach, whereas the most abundant protein profile belongs to the family *Bacillaceae* regardless of the cheese wheel depths. Also, the taxonomic assessment by metaproteomic identified a higher bacterial heterogeneity at the family level than the 16S rRNA gene sequencing. The divergent observations within the study rely on the different principles these methods are based on. Both methods target different biological macromolecules and thus, are destined to diverse technical drawbacks [27]. In addition, we retain that metaproteomics enables the identification of a higher bacterial complexity since the changes in the abundance of expressed proteins are detected earlier than changes in the number of the DNA copies targeted by 16S rRNA gene sequencing [23, 27–29].

Surprisingly, a stable microbiota composition was described by both 16S rRNA gene sequencing and metaproteomics in the samples stratified according to the ripening timepoints. Such observation was to these authors unexpected. In light of this, we believe that the major structural rearrangements have occurred in the early stage of the cheesemaking process (*i.e.* before 30 days, the time of our earliest sampling time point) and only minor shaping occur after the “microbiologically-driven” ripening, but still unable to score statistically significant differences in the whole microbial consortia composition at the diverse timepoints. An alternative/complementary interpretation of this outcome supports the slow and continuous shaping of the microbiota so that only a longer ripening window can highlight statistically significant structural changes. This view is also supported by a recent study performed on Cheddar cheese made from raw milk. Here, major shaping of the microbial consortia occurred in the very early stages of the cheese production and a rather stable microbiota composition is reported over the next 26 weeks of ageing [30]. Another recent study on 5–15 days of ripened raw goat cheese reported a stable microbiota composition, supporting the view of a slow-but-continuous shaping of the microbial consortia [31].

Functional characterization of the microbial communities supports the previous observations by grouping the samples according to the cheese wheel depth only. This clustering is supported by all the functional categorization of the protein repertoire, underlining the different functional concerns of the microbiota harbouring the rind and core of the cheese wheel.

Ontology of the rind protein repertoire depicts a microbial community mostly involved in the maintenance of the food biosafety by preventing the cheese surface colonization from foreign microbial specimens such as pathobionts or spoilage bacteria. This microbial consortium is, indeed, involved in “cellulose biosynthesis”; a biological process carried out by aerobic acetic acid bacteria that perform oxidative

fermentation of a variety of third sugar substrates, once exhausted the lactose as the main carbon source, and producing cellulose as by-products [32]. The bacterial cellulose produced in the rind surface “wrap” the dairy product, providing physical support and facilitating symbiotic interconnections among microorganisms that preserve the food from outer microorganisms’ colonization [33]. In line with the above, the overexpression of the “antibiotic catabolic process” provide support to the competition mechanisms occurring among the naïve and the environmental flora, suggesting the bacterial families encoding for this biological process as the recipient of the chemical attack. Along, the increased abundance profile of proteins belonging to the “glutamine metabolic process” might indicate an effort of the rind microbiota in the biosynthesis of nitrogen-containing compounds whose biological role is attributed to several physiological and technological functions such as antimicrobial properties and development of typical organoleptic features [34].

By comparison to the rind microbiota, the microbial community in the core of the cheese wheel is more heterogeneous, also in functional terms. The bacterial effort for the maintenance of the product biosafety is kept, although other biochemical routes are employed. In addition, core microbiota seems focused on more complex and diverse biological functions ranging from the conserved bacterial metabolism to the array of processes involved in the development of the so-called “added values” of the typical cheese in both nutraceutical and sensorial terms. Conserved metabolic processes operated by the core microbiota indicate higher participation of this microbial community in biological processes such as DNA replication, protein biosynthesis and cellular respiration processes. The latter activity is interestingly represented by the H_2O_2 catabolic processes. Besides the well-known role of hydrogen peroxide in microbial interactions [35, 36], it is also one of the major metabolic by-products of many lactic acid bacteria [37] as these often lack the respiratory chains and opt to reduce molecular oxygen to recycle NAD^+ from NADH, with increased energetic yield as compared to the classical fermentation process. Analogous cases of hybrid metabolism have been recently reported by Marco et.al [38] in *Lactobacillus plantarum*, a microorganism with pivotal roles in the technology of fermented foods production. Here, the authors discuss that combining features of respiration and fermentation would improve lactic acid bacteria functions; thus, amended products in biosafety and quality terms [38]. Resistance of the lactic bacteria to the own hydrogen peroxide is granted by the absence of oxidant-sensitive dehydratases and mononuclear Fe(II) enzymes [37, 39]. Instead, a high concern of the core microbiota in the biosynthesis of Fe(III)-chelating substances made by aerobic or facultatively anaerobic bacteria (i.e. siderophores), suggests the activation of the hybrid metabolism in the core microbial consortium, although tailored investigations to confirm this unconventional metabolic route are desirable.

The core microbiota is also focused on biological processes linked to the development of the typical nutraceutical and gustatory essences of the dairy products as it is supported by the overall microbiota concern on the biosynthesis and/or transformation of a variety of proteins, lipids and amino acids. Specifically, it has been already described how the bacterial activities in the lipids metabolism and fatty acids biosynthetic process are linked to amended organoleptic properties of the cheese and dairy products [40, 41]. In addition, microbiota involvement in the “arginine biosynthetic process via ornithine”

indicates the continuous control of the whole microbiota composition and the development of nutraceutical features acknowledged the role of ornithine in the production of bacteriocins and natural antibiotics. Also, Arginine has effects on a variety of human physiological processes such as growth/tissue repair, immune supportive effect and cellular communications [42]. Moreover, microbiota engagement in the 7,8-dihydroneopterin 3'-triphosphate biosynthetic process is suggestive of B-group vitamins and folate production whose health-promoting effects span from anticarcinogenic activity to the reduced risk of cardiovascular diseases [43, 44]. In line with biopterin production, thiamine production further supports the beneficial effects exerted by the core-associated microbial community on the cheese organoleptic properties and its health-promoting effects [45, 46].

Conclusion

This is the first meta-omics-based study on a typical raw goat milk cheese. Goat cheeses are commonly consumed, and popular, for their gustatory and beneficial properties. The complementation of 16S rRNA gene sequencing and the metaproteomic approach enabled a deep characterization of the composition and activity of the microbiota at the diverse cheese wheel depths and provides suggestive insights on the structural dynamics of the microbial community during ripening. Altogether, this explorative study provides a basic knowledge on the microbial community harboring this fascinating dairy product and deliver inputs for further objective-tailored research lines. Biological functions expressed by the investigated microbiotas is certainly of interest in the context of the biological safety of the traditional products, including the development of strategies and precautions to keep at minimum the risk of zoonoses and/or foodborne diseases. In addition, understanding the contribution of the microbiological footprint on the development of the flavor and texture of this cheese would greatly influence the cheesemaking technology by operating on the microbiota modulation, aimed at amending quality and standardization of the typical dairy products.

Declarations

Ethics approval and consent to participate:

Not applicable

Consent for publication:

Not applicable

Availability of data and materials:

The datasets supporting the conclusions of this article are available in the PRIDE repository, [PXD032280; https://www.ebi.ac.uk/pride/archive/].

Competing interests:

The authors declare that they have no competing interests

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Authors' contributions:

BT, AU, DF and PR conceived and designed the study. AS, MVR, GM, VG, CP performed the bioinformatic and statistical analyses. All authors visualized and interpreted the data. BT, CC, VG, FI and PR. wrote the manuscript. LB, LP, DB, AAS, VMM, and CP revised the manuscript. All the authors read and approved the final manuscript.

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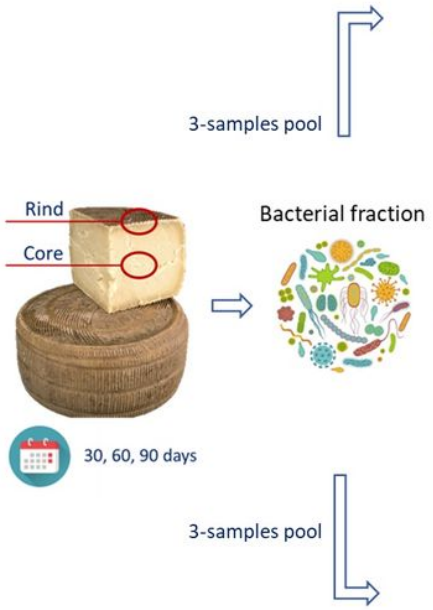
Figures



Sample pool (#)	Cheese wheel depth (pool #)	Ripening timepoints (pool #)	
18	Rind:9	30 days:3	16S rRNA gene Sequencing
		60 days:3	
		90 days:3	
	Core:9	30 days:3	
		60 days:3	
		90 days:3	
10	Rind:4	30 days:2	Metaproteomics
		60 days:1	
		90 days:1	
	Core:6	30 days:2	
		60 days:2	
		90 days:2	

Figure 1

Experimental design and sampling strategy.

Bacterial fractions were isolated from the rind and core of the cheese wheel at 30, 60 and 90 days of ripening. Pooling was performed as each pool included the bacterial extracts from three samples. Eighteen pools were subjected to 16S rRNA gene sequencing, 9 from the rind and 9 from the core. Each depth was composed of 3 sample pools taken from cheese wheels ripened for 30, 60 and 90 days. The metaproteomics survey relied on a total of 10 sample pools, 4 for the rind and 6 for the core depth. Two pools were considered from each ripening timepoint of the core depth; whereas two pools were excluded in the rind groups due to technical issues encountered during the analytical workflow. Specifically, one pool was omitted from the pool at 60 days and one from the group at 90 days of ripening.

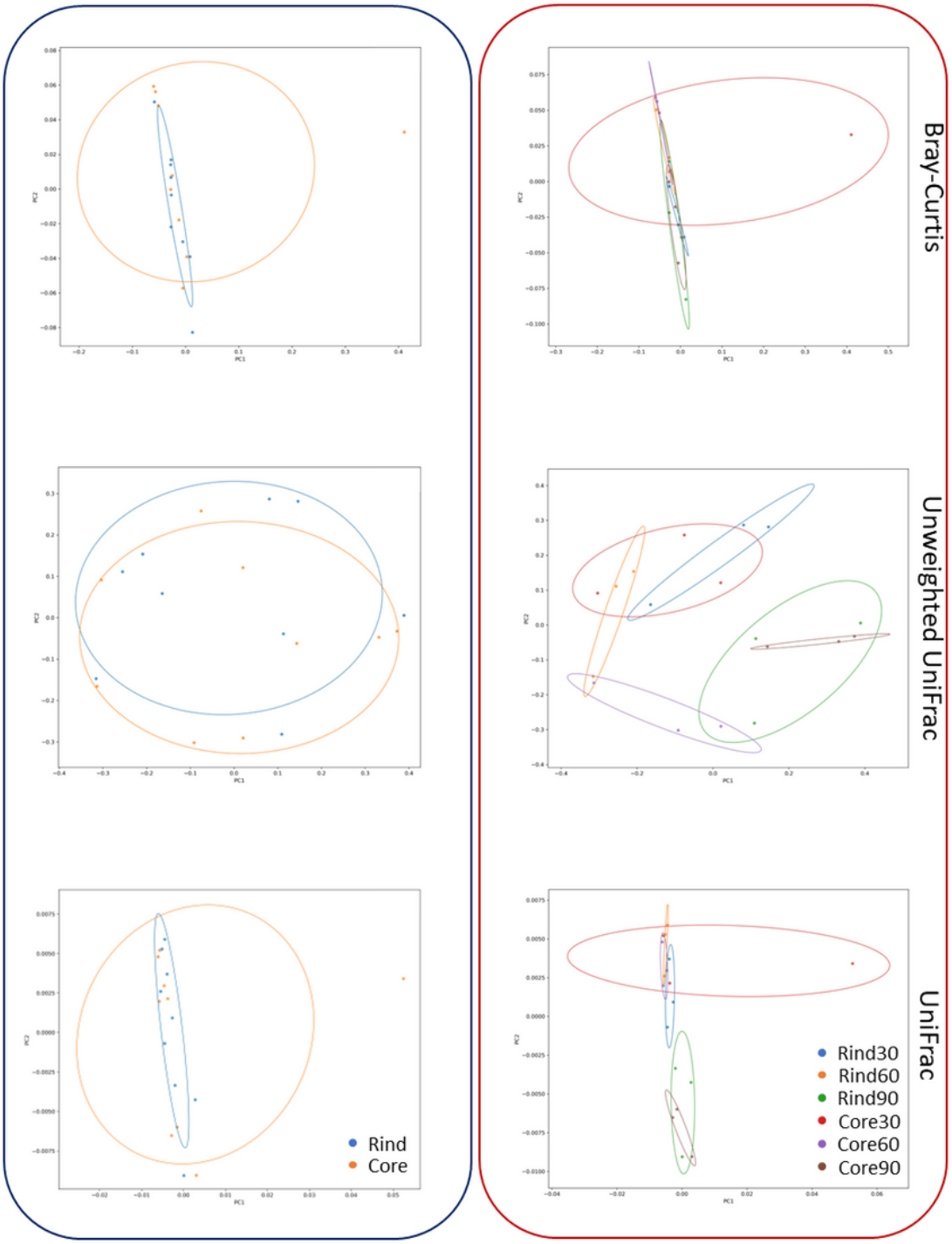


Figure 2

PCO plots of the 16S rRNA gene sequencing data. The overall DNA-based dataset is visualized into PCO plots according to Bray-Curtis, Unweighted UniFrac and UniFrac. Blue framed plots are relative to the samples sorted according to the cheese wheel depth (i.e. rind and core). Red-framed plots regard the samples stratified according to the cheese wheel depth and ripening timepoints.

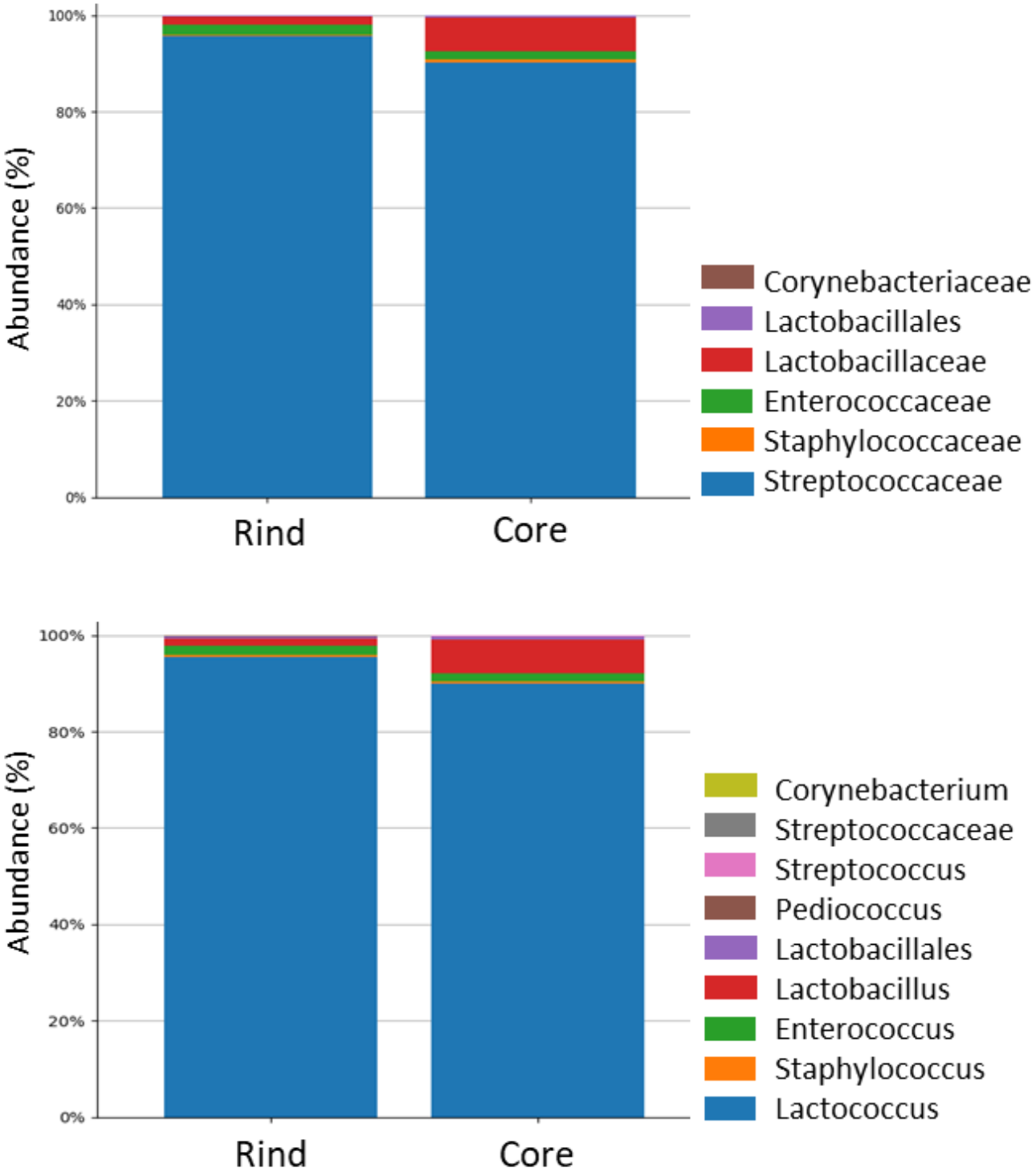


Figure 3

Microbiota composition assessment by 16S rRNA gene sequencing. The bar charts visualize the composition of the microbial community harboured in the rind and core of the cheese wheel regardless of the ripening timepoints. A panel defines the microbiota composition at the family taxon level. B panel describes the microbiota composition at the genus level. Higher taxonomic levels are displayed in both

panels when the OTU's taxonomic attribution is not possible to either family or genus levels, respectively in A and B panel.

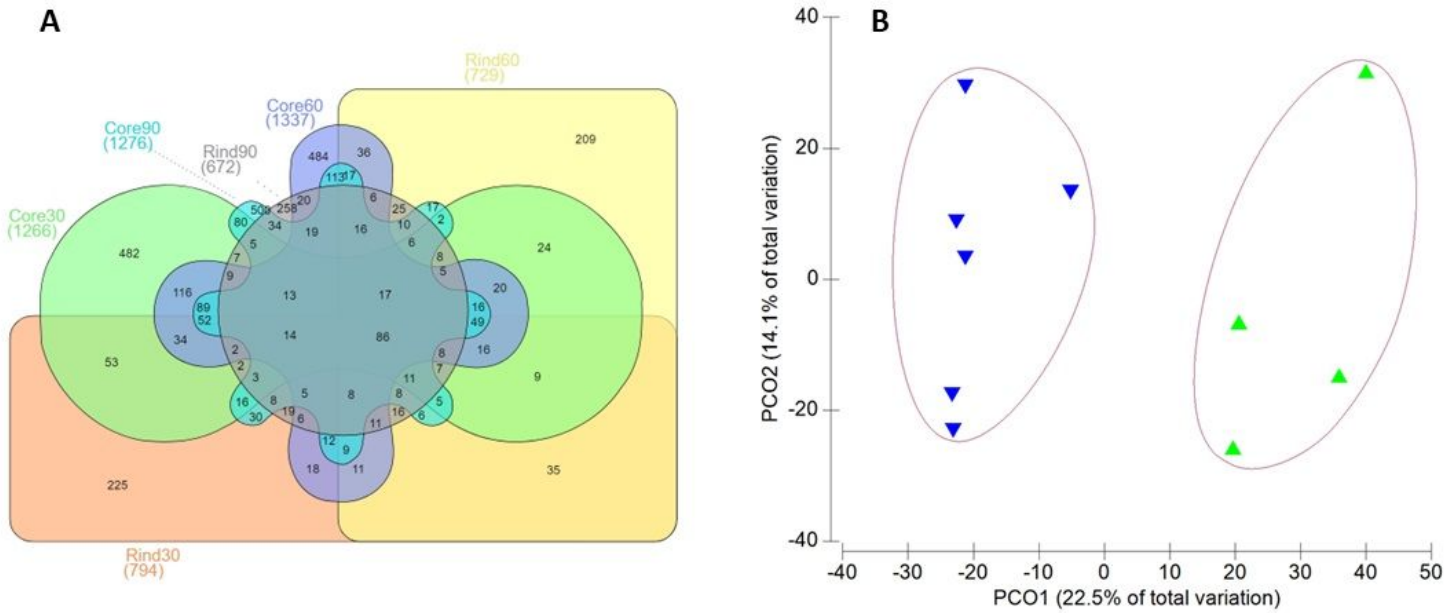


Figure 4

Metaproteomics dataset. A-panel depicts the identified protein list and their sorting into the cheese wheel depths and ripening timepoints. B-panel visualizes the dataset ordination in a PCO plot and highlights the clear separation of the rind and core samples according to the profiles of the identified protein repertoire.

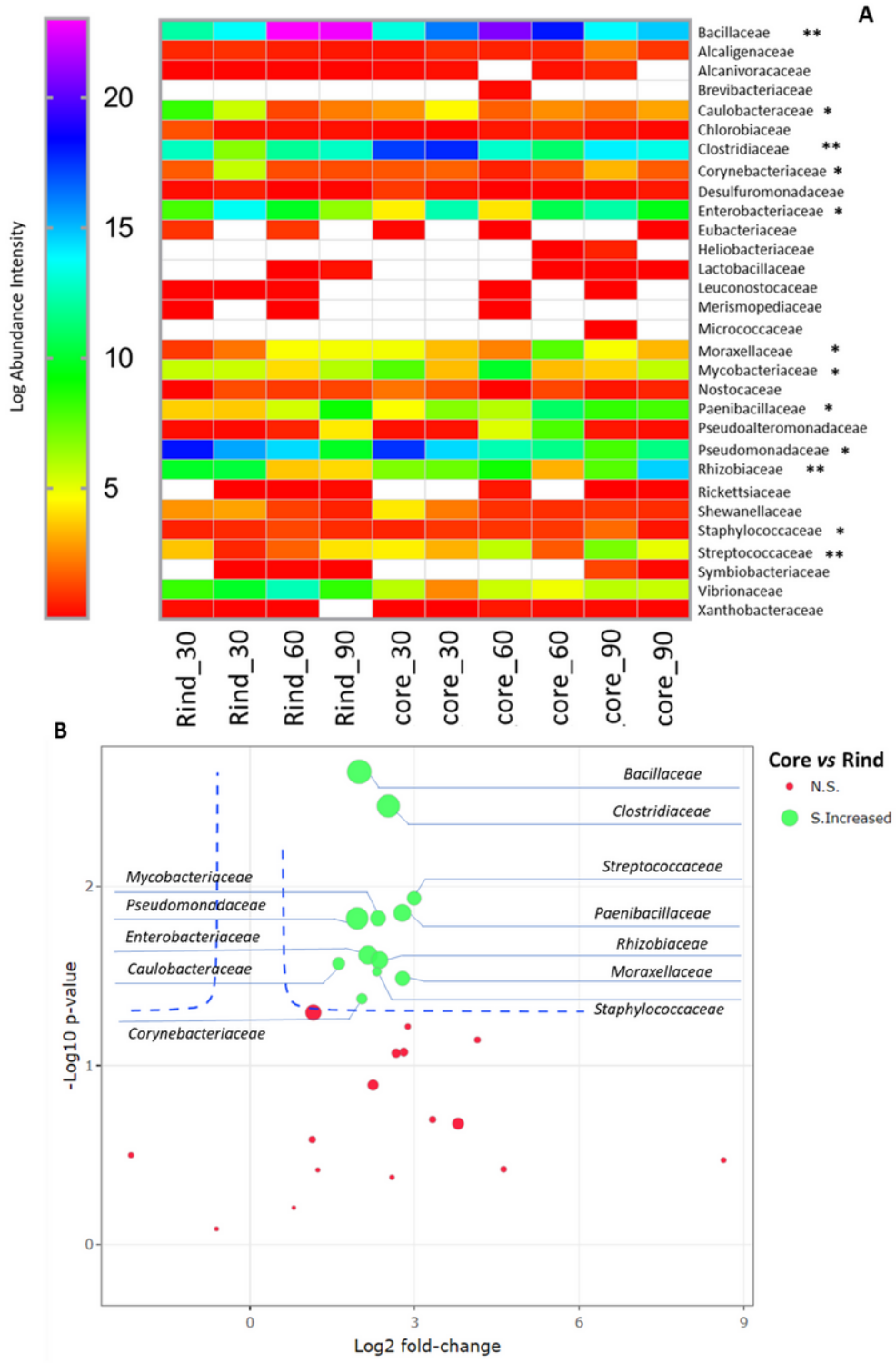


Figure 5

Microbiota composition as of the identified protein repertoire. The A-panel visualizes the quantitative composition of the microbial communities harboured in the rind and core of the cheese wheel at 30-, 60- and 90-days ripening times. T-test on a sample group basis (*i.e.* rind and core) is performed to highlight the contributors to the statistically different structure of the microbiota in the rind and the core. Single and double stars are relative to a p<0,05 and p<0,01, respectively. B-panel displays the volcano plot

summarizing the bacterial families significantly overrepresented in the Core samples (green shapes) as of the T-test analysis. Non statistically significant bacterial families are referred by the red shapes. Average intensity of each bacterial family is shown by the shapes diameter.

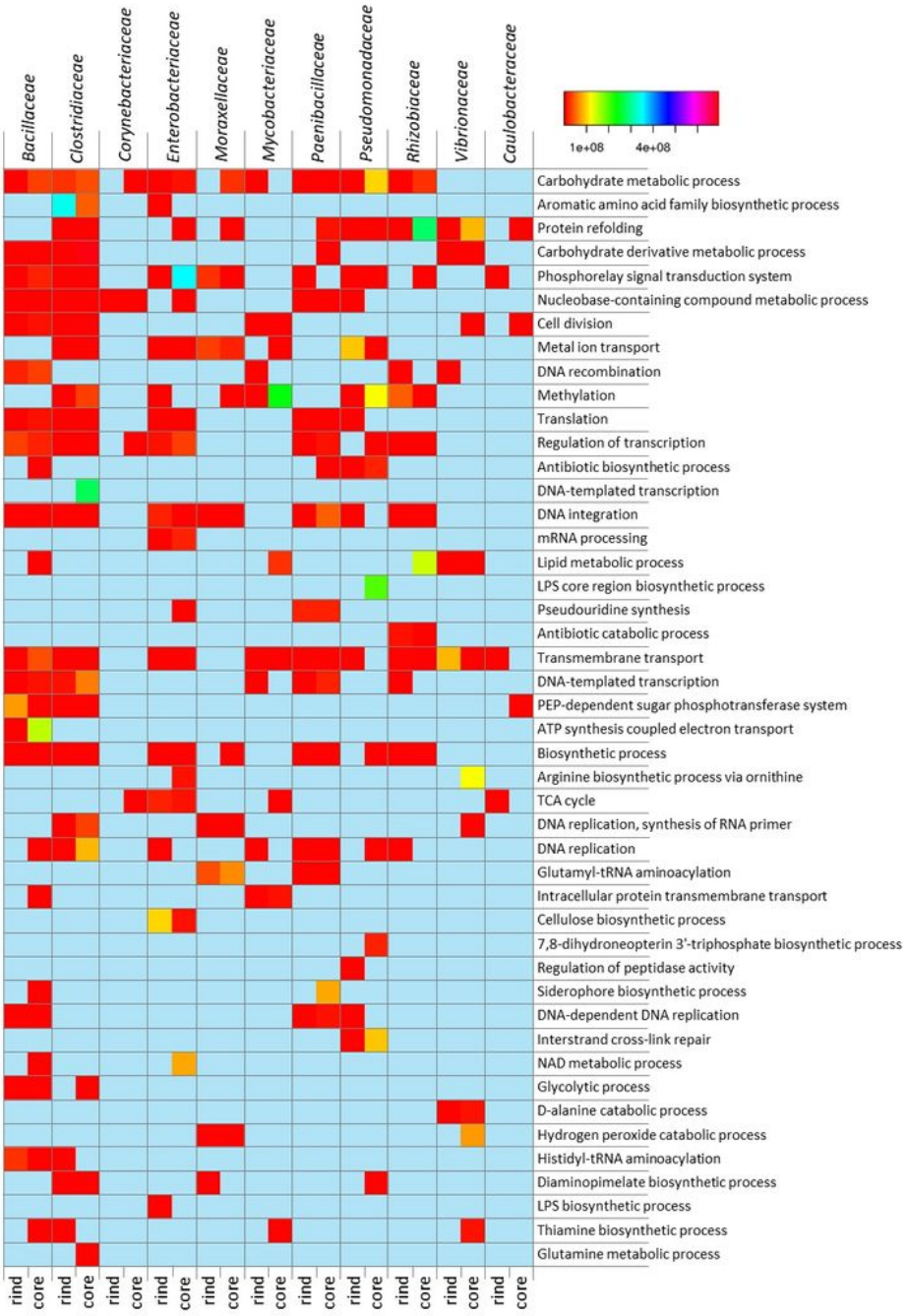


Figure 6

Functional characterization of the rind and core microbiota. The heatmap display the biological processes the microbiota of the core and rind are involved in. The figure details the functional concern of the bacterial families whose cumulative protein abundance is above the pre-fixed threshold of 1% total protein abundance intensity. The colour scale is relative to the protein abundance intensity.

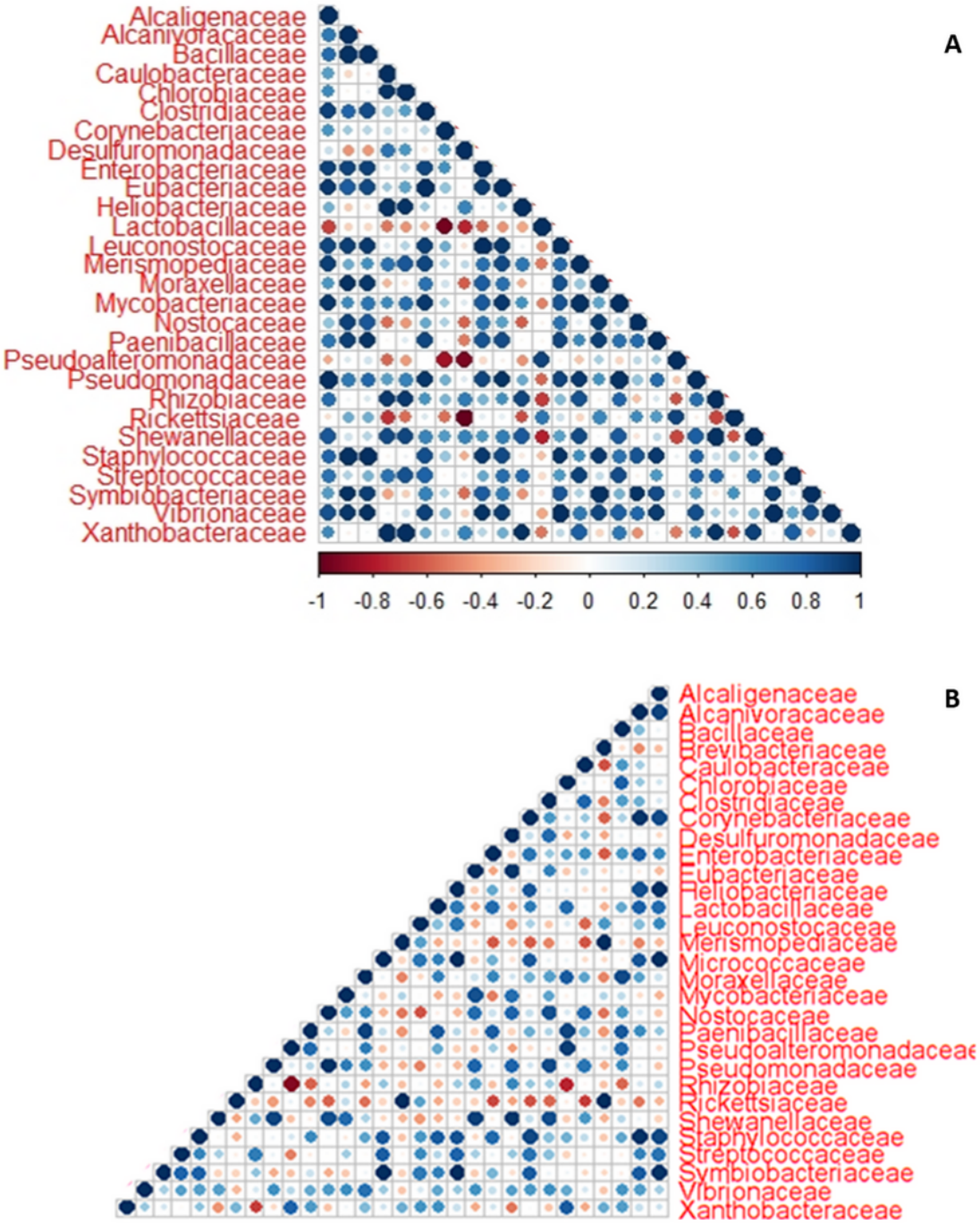


Figure 7

Correlogram analysis. The correlation computed among the members of the microbial communities harboured in the rind and core of the cheese wheel is depicted respectively in panels A and B.

Supplementary Files

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