Investigating dairy microbiome: an opportunity to ensure quality, safety and typicity

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Keywords: dairy microbes, mycobiota, metagenome, meta-taxonomic, milk microbiome, microbial function, next-generation sequencing, Metagenome-assembled genomes (MAGs)

Highlights:

- Integration tools are needed to decipher what happens in a complex food ecosystem.
- An extensive culture based methodology is necessary to maintain microbial resources.
- Meta-omic approaches enable the study of the autochthonous milk/dairy microbiome
- Deciphering the microbiome may guide the right production process and ensure quality

A detailed understanding of the microbiome of cheese and dairy products is key to the optimization of flavour, appearance, overall quality and safety. Microorganisms (including bacteria, yeasts, moulds and viruses, especially bacteriophages) from the environment can enter the dairy supply chain at multiple stages with several implications. The ability to track these microorganisms and to understand their function and interaction can be greatly enhanced by the use of high-throughput sequencing. Depending on the specific production technology, dairy products can harbor several strains and antibiotic-resistance genes that can potentially interact with the gut microbiome, once the product is ingested. Milk- or cheese- associated microbial communities with their interaction, function and diversity are a key factor for the dairy industry. Multi-omics approaches have been seldom utilized in literature and they need to be further considered. Studying the role, origin, diversity and function of the microbial species involved in the complex system of dairy production can help improve processes in several fields of application. Integrating an extensive sampling procedure with an extensive culture based methodology is necessary. To this end, local producers, and in general stakeholders, should be guided to discover and maintain their microbial diversity. A better

management of microbial resources through precision fermentation processes will in turn reduce overall food losses and increase the possibility to use the microbiome in order to increase the local producers' income.

Introduction

Milk and dairy food products are complex ecosystems, susceptible to changes in the abiotic and biotic environment (including initial raw materials, process chain, ripening temperature, a_w, pH, environment and operators contamination) that alter the evolution and the mechanisms and modes of interaction within the microbiome community. The composition of the microbiome and modifications that may take place have a considerable effect on the organoleptic properties as well as on the safety of the final products. High throughput sequencing (HTS) technologies often coupled with targeted or untargeted metabolomics are used in dairy foods to evaluate:

i) Microbiota dynamics through the identification of the operational taxonomic units (OTUs) or Amplicon Sequence Variants (ASVs) of microbial communities (meta-taxonomics);
ii) Changes in microbial gene content, function and abundance *in situ* (meta-genomics and meta-transcriptomics);
iii) Metabolic changes through the profiling of enzymes, proteins and molecules (meta-proteomics and meta-metabolomics);
iv) New adaptation strategies and new genomic potential and features with fine resolution at strain-level (pangenomics);
v) The potential of autochthonous microbes that display an extensive pool of genes with adapted metabolic functions, which can be potentially used as starter culture to prevent the loss of typicity (culturomics).

With the use of one or more techniques and with the application of biostatistics and integration tools in a reasonable timeframe, we are able to decipher events and behaviors in a complex food ecosystem. In this light, the implementation of modelling analyses based on meta-omics data can help to ensure quality and safety, to prevent yield loss during production and to predict the final characteristics of products affected by a particular microbiome. Data generated by those approaches have the potential to be translated at industrial level to improve product quality by precision fermentation.

Metataxonomic approach and microbial characterization

The extensive implementation of sequencing facilities, tools and databases to compare, visualize or analyze amplicon data (QIITA, MGnify, FoodMicrobionet [1,2], GitHub, Galaxy, Anvi'o [3] or Megan) has significantly increased the number of papers and systematic reviews on the microbiota composition of milks and their derivatives [4–7].

The use of different culture independent massive sequencing technologies, of different marker genes (16S or ITS) for the whole microbiota community or of species-specific marker genes (*lacS* or *serB* genes for *Streptococcus thermophilus* or *slpH* for *Lactobacillus helveticus*) [4,8–11] has shown that microbial populations confer specific properties to milk based products. Taste, flavor, texture and nutrient composition are deeply influenced by the type of microbiota and by its network of interactions that play a central role during ripening and maturation. Of particular importance is the interaction among bacterial species, sub-species and strains due to the strong relationships between microbial dynamics and the metabolome affecting the final quality of the products. Lactobacilli, *Lactococcus*, *Leuconostoc*, *Enterococcus* and *Streptococcus* represent the core microbiota of most of the dairy products, followed by several minor populations, including both pathogenic and starter and non-starter microorganisms, depending on the animal health status (e.g. mastitis), the environment (season, farm and temperature), operators and food chain parameters (Figure 1A). These variables confer to each single matrix a particular microbiota [7]. Succession co-occurrence and interspecies interactions in such products are responsible for resilience toward colonization by undesirable microbes with negative effects on maturation and on the development of the organoleptic properties, texture and stability of the product [11]. It has been shown that in hard cheese, when the core microbiota is dominated by *Streptococcus thermophilus* and *Lacticaseibacillus rhamnosus,* spoilage phenomena mediated by *Clostridium tyrobutyricum* occur, while if *Lactobacillus delbrueckii* is dominant, *C. butyricum* appears as a spoilage species [12]. When the core microbiota of milk is simultaneously dominated by the *Lb. delbrueckii*, *Lactobacillus helveticus*, and *Lacticaseibacillus casei*, the prevalence of *Pseudomonas* and *Propionibacterium* is reduced [13]. From a spoilage perspective, the interaction that occurs between *Bacillus*, *Clostridium*, and *Pseudomonas* drives an uncontrolled fermentation with an inevitable impact on final products [14]. The metataxonomics approach has been shown to be capable to discover interactions at sub-species level that tend to dominate or codominate in the same food matrix, defining specific clusters of covariant lactobacilli [11]. The increase in the number of available datasets can help in deciphering the interactions occurring in a particular microbiota by using a machine learning approach that spans from simple correlation to probabilistic graphical models, to network-based analytical approaches. A machine

learning approach can help researchers to disentangle complex polymicrobial interactions [15]. Since the mechanisms that underlie the assembly of microbial communities remain poorly characterized, metataxonomics can bring the food industry considerably closer to the microbiome subject, pushing the possibility of using these tools to improve product quality by precision fermentation (Figure 1B) [16]. It is well known that the assemblage of microbes, apart from the initial milk microbiota, is affected by the whole chain: transport, storage, processing, cleaning and sanitation procedures, time of production, etc. [17–20]. Metataxonomics procedures can help in identifying new adaptation strategies and developing innovative process strategies to selectively modify the natural microbiome. Traditional dairy products are the result of complex and poorly defined indigenous microbial consortia activities which confer distinctive metabolic features related to the typicity and the identity of the products. It is evident that, especially for artisanal gourmet products made with natural methods, an uncontrolled fermentation can occur with all related problems (discoloration, off-flavour development, safety issue) that can cause yield and credibility losses. However, the use of commercial starter cultures, helping in standardizing the process, will inevitably carry about a loss of typicity with an impoverishment of aroma and flavour characteristics of the products. Autochthonous microbes display a vast interaction network that confer particular characteristics often preferred by the consumers. In most cases one single strain in the starter culture is not able to confer to the product the required characteristics, which instead are related to a mixture of different genetic repertoires. A correct use of the metataxonomics approach helping in describing the autochthonous microbiota can be considered as the first step in the selection of an autochthonous microbiome starter culture able to maintain the desired characteristics of traditional products and to better control the fermentation process (Figure 1B).

The mycobiota composition and its importance in dairy ecosystems

It is well known that filamentous fungi play a role in the ripening of several products (e.g., Roquefort, Stilton, Danablue, Camembert, Gorgonzola). However, mycobiota studies of dairy products lags behind that of bacterial communities, mainly due to experimental limitations. In several cases only one gene marker is not enough for taxonomic identification, taxonomy databases are incomplete, while some food related fungi may be missing in a given database [21]. These limitations have hindered the full exploitation of high throughput sequencing in the study of mycobiota.

In contrast to bacteria, it is very difficult to identify a common core mycobiota community in dairy products since their presence is highly correlated with the environment, season, atmospheric humidity and temperature. Most fungi are tolerant to high-salt and low-pH conditions and find in dairy an ecological niche, therefore the same artisanal cheese can be colonized by a different mycobiota

according to the season. The most frequent genera are *Candida*, *Trichosporon*, *Pichia*, *Saccharomyces*, *Rhodotorula*, *Yarrowia*, *Kluyveromyces, Geotrichum*, *Penicillium*, *Aspergillus* and *Debaryomyces* [22–27].

The mycobiota represents a source of several metabolites and enzymes (mainly proteolytic and lipolytic) that play an important role during ripening and maturation and confer a peculiar aromatic signature to the final products. Indigenous filamentous fungi have the ability to adapt in diverse food niches and have found the perfect environment in cheese, mainly in the rind, where they developed an adaptation strategy. This is the case of *Penicillium* were, by the use of whole genome sequencing (WGS), has been shown to have a genomic repertoire with functions involved in antagonism with other microorganisms [28]. The main attribute of fungi of relevance in cheese is their ability to produce desirable aromas (such as aldehydes, ketones, alcohols and esters) as well as the ability of some fungi to control the development of ochratoxigenic fungi, as biocontrol agents against harmful fungi and mycotoxin contamination [29]. Of particular importance is the interaction that can occur among bacteria and fungi during dairy fermentation such as the mutualistic cooperation of *Lactobacillus kefiranofaciens* producing kefiran (an exopolysaccharide), witch act as natural encapsulation material for *Kluyveromyces marxianus* and *Kazachastania khefir* [30]. The proliferation of yeasts and filamentous fungi is strictly connected with the metabolic activity of lactic acid bacteria that produce metabolites influencing the mycobiota development. The importance of studies on mycobiota of dairy foods is not only related to the potential risk of mycotoxin contamination [26,31] but also to develop strategies to reduce the prevalence of spoilage microbes (like *Corynebacterium*, *Halomonas*, *Pseudomonas*, *Pseudoalteromonas* and *Vibrio*) that are inhibited by the presence of certain autochthonous fungi [20]. Several authors report the presence of specific yeasts and filamentous fungi in PDO products and it should be recognized that some taxa to show probiotic effects, like *Galactomyces* for its capability of releasing bioactive peptides [27]. Limited information is as yet available about the mycobiota of PDO products and it is necessary to perform further studies to decipher the interactions that occur in this complex ecosystem. In addition, the analysis of the fungi can open new research horizons aiming at discovering the presence of new probiotic cultures, or the potential of bioactive compounds that might be further exploited by the dairy industry to promote specific products for their beneficial effect [27].

Zooming into functionality and strain diversity in dairy industry

Metatranscriptome and shotgun metagenome sequencing are now becoming the procedures most used to decipher the genomic potential of the whole microbiome in food systems. The decreasing cost and

171 the availability of open source platforms for data analysis are helping the researcher to study the
172 effective functions of the microbes, to discover how the process chain can be modified to drive
173 specific microbial metabolic features, to assess safety and, more recently, to directly reconstruct
174 genomes without cultivation procedures. The RNA-seq methodology is not often used in dairy studies
175 mainly because of the quite high cost and because of the RNA instability that complicates the wet lab
176 procedures. Only few studies are available but they show important results [7]. Gene expression
177 analysis by RNA-seq clearly showed that ripening temperature (in particular higher) enrich the
178 expression of genes involved in proteolysis, lipolysis, fatty acid metabolism and amino acid
179 metabolism. Enzymes leading to acetoin and diacetyl production are correlated with the temperature
180 increase with a beneficial effect on the sensorial quality of the final cheese [32]. Since also fungi
181 confer peculiar characteristic to cheeses by meta-transcriptomic approach it was possible
182 to highlight that fatty acids are late energy sources for *Geotrichum candidum* and *Penicillium*
183 *camembert* and this gene could be used as biomarker to follow this activity and to expand the
184 knowledge about fungal metabolism [33].
185 A most common approach is the DNA-seq that offers many advantages since with the same dataset
186 it is possible to profile the microbiome community (including bacteria, fungi and viruses), reconstruct
187 microbial metabolic pathways, trace genomic elements related to safety (like antimicrobial resistance
188 gene [ARGs] or virulence genes) and recover genomes at strain-level resolution. DNA-seq is also
189 useful in terms of product quality, because of the ability to identify metagenomic clusters associated
190 with the modification of color, variation of pH, and flavor development (Figure 1C) [24,34,35].
191 Finally, such approach allows to study the competition and the interaction among microorganisms,
192 as well as their strategies to develop and survive in communities (Figure 1D) [20]. Cheese microbiota
193 analysis has shown that the core microbiota is composed by few dominant taxa, but it is known that
194 strains belonging to the same species possess remarkable genomic differences [36–38]. The
195 application of computational tools to reconstruct genomes from shotgun sequencing data is helping
196 to reveal strain diversity in foods. The possibility to retrieve Metagenome-assembled genomes
197 (MAGs) has highlighted the strong correlation between abundances of specific MAGs and volatile
198 organic compounds involved in cheese aroma and emphasized the role of fungi and viruses during
199 ripening [35]. These techniques have been used profitably to confirm the transmission of potentially
200 probiotic MAGs from dairy products to gut environments [39]. In particular it was seen that dairy
201 environmental microbes that occurred in cheese, can be horizontally transmitted to human and persist
202 in the gut of those individuals with possible implications on human health [40]. However, MAGs are
203 often contaminated with sequences from other organisms, especially if samples are collected from
204 the same ecological niche. MAGs can share specific genes with plasmids, prophages or genomic

islands, which may result in false positive genomes, making the determination of the pangenome uncertain. Results can be confirmed only by an extensive culture-based approach that these studies are lacking.

The potential of DNA-seq technique is applied also to: facilitate the detection of low levels of undesirable bacteria (like spore-formers) present in these products [41]; identify foodborne pathogens by the single-nucleotide polymorphism (SNP) profiles of outbreak strain versus non outbreak strains [42]; detect mobile genetic elements as CRISPR's defense mechanism or antibiotic resistance genes [35,43]. Regarding ARGs detection, it should be pointed out that rarely LAB harbor these genes, which are often associated with environmental indigenous airborne viral populations or from members of *Enterobacteriaceae*, *Staphylococcaceae* and *Proteobacteria* [36,44,45]. Careful hygiene measures in the manufacture process should then reduce the possibility of ARG transmission through cheese.

DNA-seq technique has also the valuable ability of analyzing viruses and specifically bacteriophages or phages. Shotgun metagenomics analysis highlighted a high complexity of the viral communities both in terms of viral taxonomy and phage–host associations. Phages have a substantial impact in the dairy environment since they are used as as biocontrol agents or because in the fermentation process they can inactivate the added starter strains, leading to low-quality fermented dairy products [46,47]. Moreover phages are often involved in the mobilization of antimicrobial resistance genes among bacterial populations [44] and act as vectors for horizontal gene transfer [45].

Dairy microbiome: challenge in study design and future prospective

Several tools are now available to decipher composition, metabolites, putative functions or interaction pathways between microbes in dairy microbiome. However, defining mechanistic connections between individual microbial strains (bacteria, fungi and virus) and the final features and quality of the products remains a challenge. All the HTS techniques showed several limitations. Metataxonomics does not detect important changes at species level or is susceptible to PCR error; short sequence reads generated by WGS often provide limited resolution and are impacted by the missing details in the reference database used and by effects of genomic materials from dead cells; RNA-Seq is susceptible to handling errors, it has a sufficiently high quality but does not necessarily predict the translation into proteins: the transient nature of metabolites makes them susceptible to sampling artifacts and in addition spectra can be saturated with the highly abundant molecules from dominant species [48–50]. Based on biological questions and taking into account the issue of samples (e.g. host molecules/sequences) an appropriate study design should be based on combinations of omics tools in order to overcome those limitations.

Studies on dairy products using a multi-omics approach have been lacking to date since the vast majority of studies employed only amplicon sequencing.

In the author's opinion a better study of the microbiome of milk and dairy products requires coupling metataxonomics with an extensive culture-based approach to confirm the presence of a particular microbes/consortia. Depending on the biological question a metabolomics approach might also be added to the procedure.

If the biological question is only related to strain tracking, it is necessary to use DNA-seq plus a culture-based approach to validate and confirm the hypotheses, since putative new species obtained from assembly should be cultivated. The use of the assembly alone can only give an overview of the strain presence/association with a particular metabolite/function. To obtain a better overview of the microbial interaction in milk or dairy products DNA-seq, RNA-seq and single strain WGS should be applied. The combination of those three -omics tools will overcome the database limitation since an extensive cured database for taxonomic/function assignation is needed. If the aim is determining the genetic basis of a particular metabolite's utilization, metabolomics coupled with WGS are required.

Metagenomics and metatranscriptomics must to be coupled with proteomics followed by one or more other related approaches (lipidomics, glycomics, peptidomics and metabolomics [51]) since co-variations between molecules and microbial species is indicative of species-specific molecules. This approach can reinforce the limits of only phylogenic or genome-scale analysis to provide direct measurement of metabolic phenotypes and molecules that link the microbiome to the final products [52]. An effort in developing new strategies to cultivate microbes (especially rare species or the ones that need particular synthetic conditions) from dairy environment on the one side can guarantee biological preservation of microbe resources, and on the other side can help in deciphering genes, molecules or metabolites that are often associated as unknown by the -omics techniques. The implementation of the culture collection and single cell study is fundamental to overcome the limits in the database that often affect data interpretation of metagenomics, metaproteomics and metametabolomics studies. The rapid advance of -omics technologies requires an urgent implementation of a culture-based approach to help in data interpretation. The use of various machine learning approaches implemented with abundant data from omics tools is helping decipher the microbiome of milk and dairy foods, however strain cultivation and an extensive culture collection of autochtonous microbes must be strongly implemented.

Conclusion

The -omics approaches will open new horizons in terms of translation from research to industry. From the one side, those approaches can be directly used by industry for tracking or monitoring purposes with the use of portable devices like the MinION coupled with user-friendly software. From the other side, integrating metagenomic studies to classical microbiology and in particular the ability to replicate microbial communities *in vitro* will open new horizons in managing and using a well-defined microbiome consortium to drive the process chain. The development of new technologies and data analysis tools is helping also to choose the right production process conditions to ensure quality and safety. To this end, this flow of research and results has brought the food industry considerably closer to microbiome, pushing the use of multi omics tools to improve product quality through precision fermentation.

Conflict of interest statement

Nothing declared.

Acknowledgements

Author contributions

Luca Cocolin, Ilario Ferrocino: Conceptualization. Ilario Ferrocino: Writing- Original draft preparation. Kalliopi Rantsiou: Writing- Reviewing and Editing. Luca Cocolin: Supervision.

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*of special interest

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Figure Legend

Graphical representations of the dairy microbiome analysis workflow. A) Microorganisms from the environment (including bacteria, yeasts, moulds and viruses especially bacteriophages) can influence milk/dairy microbiome. Animal feeding and health status, pollution and environment (season and temperature) shape the initial microbiome's structure. Microbiome, is also affected by the whole chain: transport, storage, processing, cleaning and sanitation procedures, food chain parameters and time of production. B) Culturomics procedures and strains characterization can help in identifying new networks of adaptation strategies and new genomic potential and features, as well as in developing innovative process strategies in the selection of an autochthonous microbiome starter culture. C) Development of new technologies and data analysis tools can help to integrate -omics data that help in metabolic pathway reconstruction [32], MAGs reconstruction [35,39], co-occurrence of microbial communities [13], probabilistic graphical models to network-based analytical approaches [2,15]. D) Workflow illustrating integrated strategies to achieve the goal of precision fermentation. Created with BioRender.com