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

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

14

15 Highlights:

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

20

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

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

38

39 **Introduction**

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

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

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

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69 **Metataxonomic approach and microbial characterization**

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

75 The use of different culture independent massive sequencing technologies, of different marker genes
76 (16S or ITS) for the whole microbiota community or of species-specific marker genes (*lacS* or *serB*
77 genes for *Streptococcus thermophilus* or *slpH* for *Lactobacillus helveticus*) [4,8–11] has shown that
78 microbial populations confer specific properties to milk based products. Taste, flavor, texture and
79 nutrient composition are deeply influenced by the type of microbiota and by its network of
80 interactions that play a central role during ripening and maturation. Of particular importance is the
81 interaction among bacterial species, sub-species and strains due to the strong relationships between
82 microbial dynamics and the metabolome affecting the final quality of the products. Lactobacilli,
83 *Lactococcus*, *Leuconostoc*, *Enterococcus* and *Streptococcus* represent the core microbiota of most of
84 the dairy products, followed by several minor populations, including both pathogenic and starter and
85 non-starter microorganisms, depending on the animal health status (e.g. mastitis), the environment
86 (season, farm and temperature), operators and food chain parameters (Figure 1A). These variables
87 confer to each single matrix a particular microbiota [7]. Succession co-occurrence and interspecies
88 interactions in such products are responsible for resilience toward colonization by undesirable
89 microbes with negative effects on maturation and on the development of the organoleptic properties,
90 texture and stability of the product [11]. It has been shown that in hard cheese, when the core
91 microbiota is dominated by *Streptococcus thermophilus* and *Lacticaseibacillus rhamnosus*, spoilage
92 phenomena mediated by *Clostridium tyrobutyricum* occur, while if *Lactobacillus delbrueckii* is
93 dominant, *C. butyricum* appears as a spoilage species [12]. When the core microbiota of milk is
94 simultaneously dominated by the *Lb. delbrueckii*, *Lactobacillus helveticus*, and *Lacticaseibacillus*
95 *casei*, the prevalence of *Pseudomonas* and *Propionibacterium* is reduced [13]. From a spoilage
96 perspective, the interaction that occurs between *Bacillus*, *Clostridium*, and *Pseudomonas* drives an
97 uncontrolled fermentation with an inevitable impact on final products [14]. The metataxonomics
98 approach has been shown to be capable to discover interactions at sub-species level that tend to
99 dominate or codominate in the same food matrix, defining specific clusters of covariant lactobacilli
100 [11]. The increase in the number of available datasets can help in deciphering the interactions
101 occurring in a particular microbiota by using a machine learning approach that spans from simple
102 correlation to probabilistic graphical models, to network-based analytical approaches. A machine

103 learning approach can help researchers to disentangle complex polymicrobial interactions [15]. Since
104 the mechanisms that underlie the assembly of microbial communities remain poorly characterized,
105 metataxonomics can bring the food industry considerably closer to the microbiome subject, pushing
106 the possibility of using these tools to improve product quality by precision fermentation (Figure 1B)
107 [16]. It is well known that the assemblage of microbes, apart from the initial milk microbiota, is
108 affected by the whole chain: transport, storage, processing, cleaning and sanitation procedures, time
109 of production, etc. [17–20]. Metataxonomics procedures can help in identifying new adaptation
110 strategies and developing innovative process strategies to selectively modify the natural microbiome.
111 Traditional dairy products are the result of complex and poorly defined indigenous microbial
112 consortia activities which confer distinctive metabolic features related to the typicality and the identity
113 of the products. It is evident that, especially for artisanal gourmet products made with natural
114 methods, an uncontrolled fermentation can occur with all related problems (discoloration, off-flavour
115 development, safety issue) that can cause yield and credibility losses. However, the use of commercial
116 starter cultures, helping in standardizing the process, will inevitably carry about a loss of typicality with
117 an impoverishment of aroma and flavour characteristics of the products. Autochthonous microbes
118 display a vast interaction network that confer particular characteristics often preferred by the
119 consumers. In most cases one single strain in the starter culture is not able to confer to the product
120 the required characteristics, which instead are related to a mixture of different genetic repertoires. A
121 correct use of the metataxonomics approach helping in describing the autochthonous microbiota can
122 be considered as the first step in the selection of an autochthonous microbiome starter culture able to
123 maintain the desired characteristics of traditional products and to better control the fermentation
124 process (Figure 1B).

126 **The mycobiota composition and its importance in dairy ecosystems**

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

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

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

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

166

167 **Zooming into functionality and strain diversity in dairy industry**

168

169 Metatranscriptome and shotgun metagenome sequencing are now becoming the procedures most used
170 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

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

208 The potential of DNA-seq technique is applied also to: facilitate the detection of low levels of
209 undesirable bacteria (like spore-formers) present in these products [41]; identify foodborne pathogens
210 by the single-nucleotide polymorphism (SNP) profiles of outbreak strain versus non outbreak strains
211 [42]; detect mobile genetic elements as CRISPR's defense mechanism or antibiotic resistance genes
212 [35,43]. Regarding ARGs detection, it should be pointed out that rarely LAB harbor these genes,
213 which are often associated with environmental indigenous airborne viral populations or from
214 members of *Enterobacteriaceae*, *Staphylococcaceae* and *Proteobacteria* [36,44,45]. Careful hygiene
215 measures in the manufacture process should then reduce the possibility of ARG transmission through
216 cheese.

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

224 225 **Dairy microbiome: challenge in study design and future prospective**

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

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

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

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

253 Metagenomics and metatranscriptomics must to be coupled with proteomics followed by one or more
254 other related approaches (lipidomics, glycomics, peptidomics and metabolomics [51]) since co-
255 variations between molecules and microbial species is indicative of species-specific molecules. This
256 approach can reinforce the limits of only phylogenic or genome-scale analysis to provide direct
257 measurement of metabolic phenotypes and molecules that link the microbiome to the final products
258 [52]. An effort in developing new strategies to cultivate microbes (especially rare species or the ones
259 that need particular synthetic conditions) from dairy environment on the one side can guarantee
260 biological preservation of microbe resources, and on the other side can help in deciphering genes,
261 molecules or metabolites that are often associated as unknown by the -omics techniques. The
262 implementation of the culture collection and single cell study is fundamental to overcome the limits
263 in the database that often affect data interpretation of metagenomics, metaproteomics and
264 metametabolomics studies. The rapid advance of -omics technologies requires an urgent
265 implementation of a culture-based approach to help in data interpretation. The use of various machine
266 learning approaches implemented with abundant data from omics tools is helping decipher the
267 microbiome of milk and dairy foods, however strain cultivation and an extensive culture collection
268 of autochthonous microbes must be strongly implemented.

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273 **Conclusion**

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

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286 **Conflict of interest statement**

287 **Nothing declared.**

288

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290

291 **Author contributions**

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

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307 **References and recommended reading**

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309 *of special interest

310 **of outstanding interest

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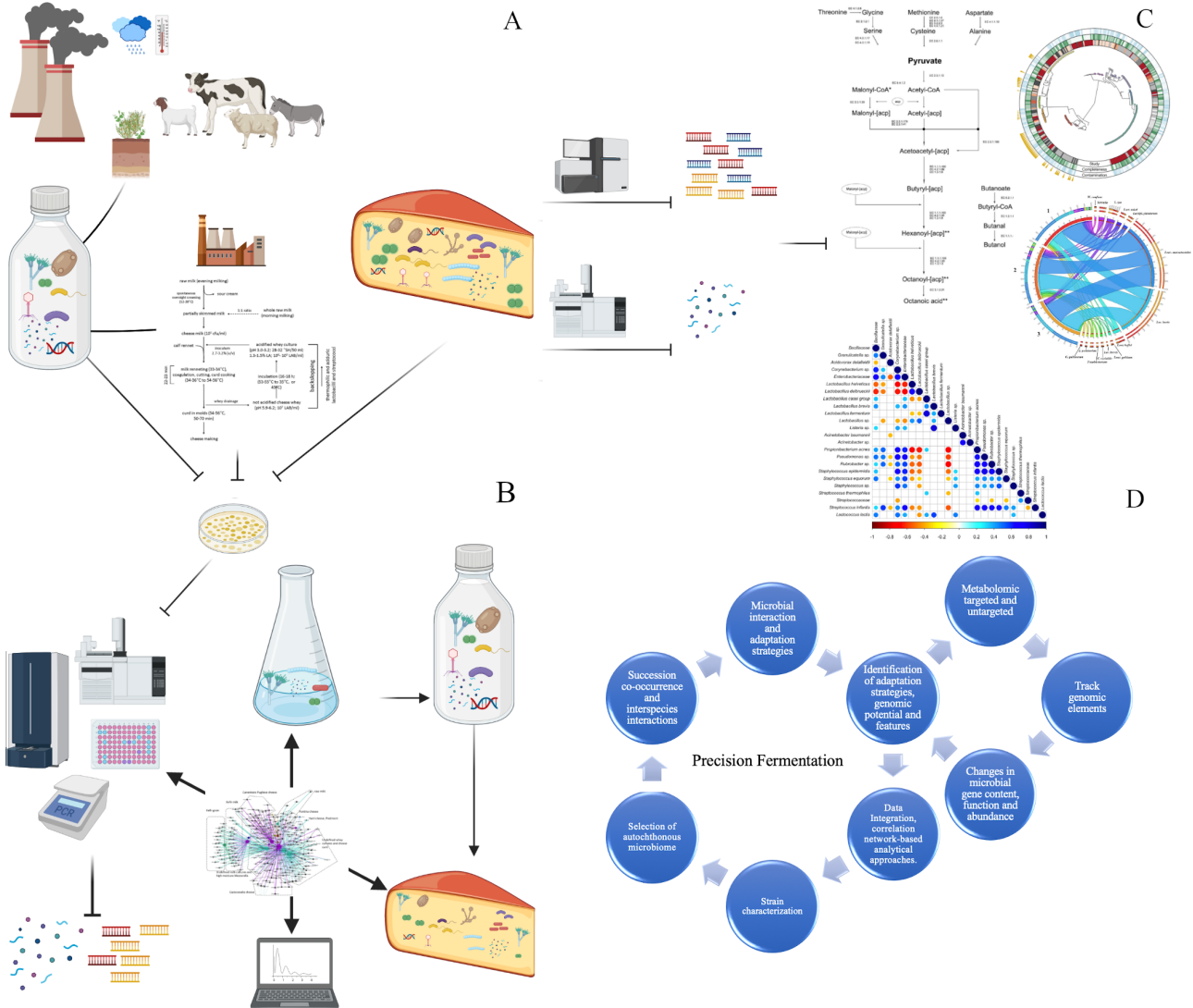
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477 **Figure Legend**

478 Graphical representations of the dairy microbiome analysis workflow. A) Microorganisms from the
 479 environment (including bacteria, yeasts, moulds and viruses especially bacteriophages) can influence
 480 milk/dairy microbiome. Animal feeding and health status, pollution and environment (season and
 481 temperature) shape the initial microbiome's structure. Microbiome, is also affected by the whole
 482 chain: transport, storage, processing, cleaning and sanitation procedures, food chain parameters and
 483 time of production. B) Culturomics procedures and strains characterization can help in identifying
 484 new networks of adaptation strategies and new genomic potential and features, as well as in
 485 developing innovative process strategies in the selection of an autochthonous microbiome starter
 486 culture. C) Development of new technologies and data analysis tools can help to integrate -omics data
 487 that help in metabolic pathway reconstruction [32], MAGs reconstruction [35,39], co-occurrence of
 488 microbial communities [13], probabilistic graphical models to network-based analytical approaches
 489 [2,15]. D) Workflow illustrating integrated strategies to achieve the goal of precision fermentation.
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