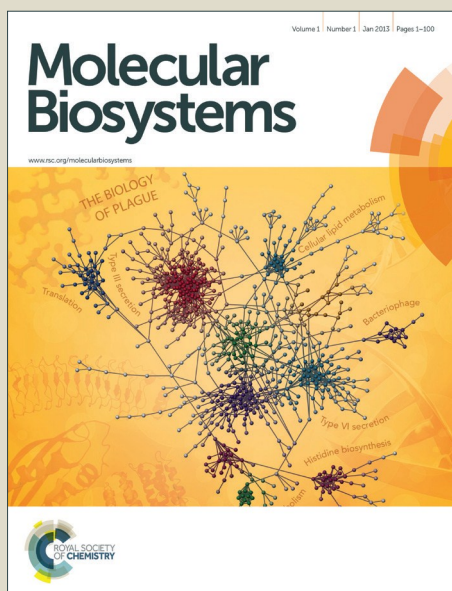


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1 The bovine milk microbiota: insights and perspectives from -omics studies

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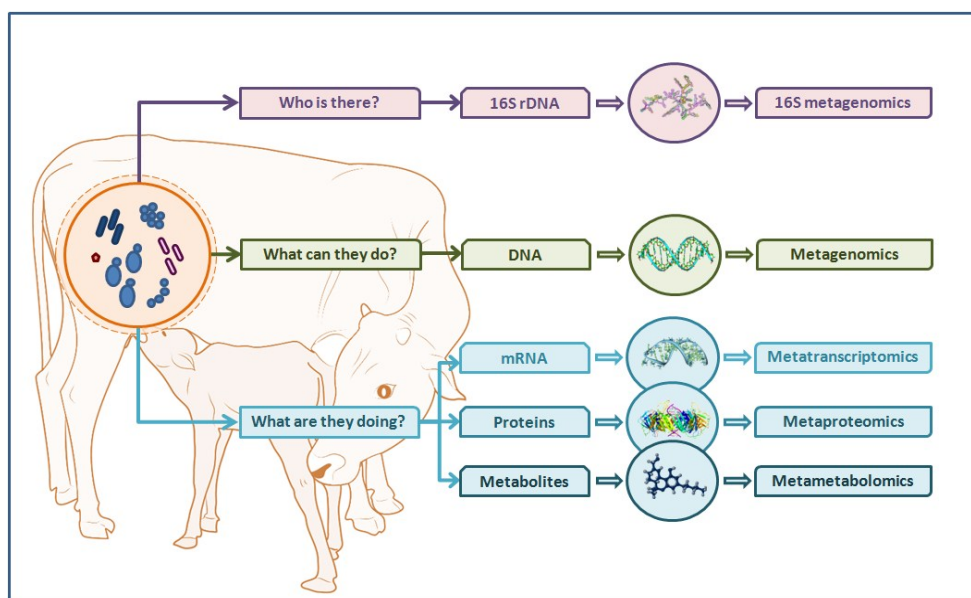
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11 **Recent findings and future perspectives of -omics studies on the bovine milk microbiota,**
12 **focusing on its impact on animal health**

13 Summary

14 The recent and significant progresses in culture-independent techniques, together with the parallel development
15 of -omics technologies and data analysis capabilities, have led to a new perception of the milk microbiota as a
16 complex microbial community with great diversity and multifaceted biological roles, living in an environment that
17 was until recently believed to be sterile. In this review, we summarize and discuss the latest findings on the milk
18 microbiota in dairy cows, with a focus on the role it plays in bovine physiology and health.

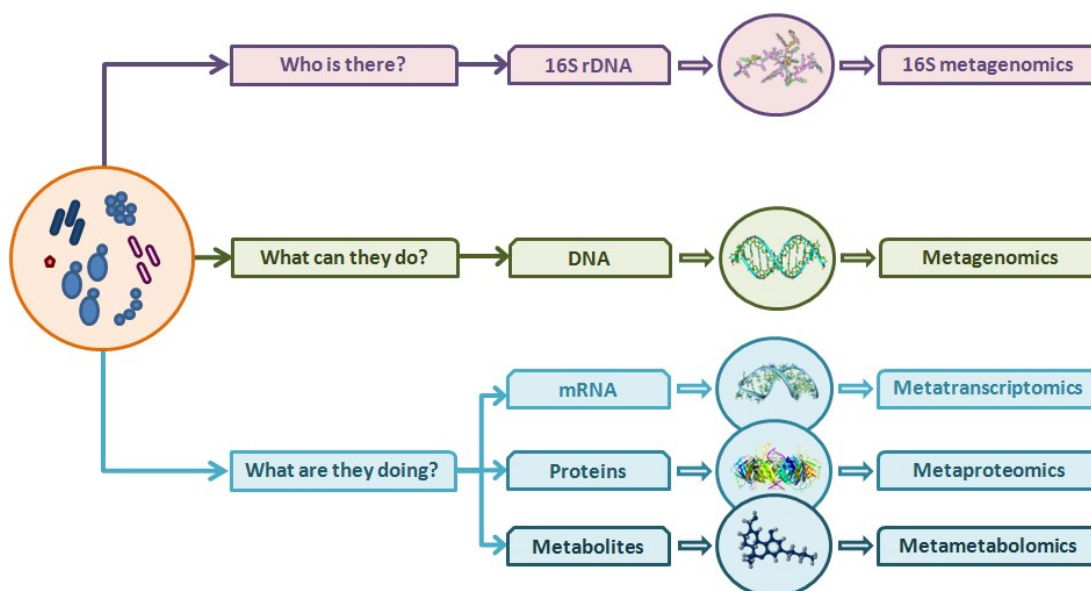
19 Following an introduction on microbial communities and the importance of their study, we present an overview of
20 the -omics methods currently available for their characterization, and outline the potential offered by a systems
21 biology approach encompassing metatranscriptomics, metaproteomics, and metametabolomics. Then, we review
22 the recent discoveries on the dairy cow milk microbiome enabled by the application of -omics approaches.
23 Learning from studies in humans and in the mouse model, and after a description of the endogenous route
24 hypothesis, we discuss the role of the milk microbiota on both the mother and the offspring physiology and health,
25 and report how it can be changed by farming practices and during infection. In conclusion, we shortly outline the
26 impact of the milk microbiota on quality of milk and of dairy products.
27

28

View Article Online
DOI: 10.1039/C6MB00217J29 **Microbial communities and the milk microbiota**

30 The complex living entities defined as microbial communities, or microbial consortia, have gained increasing
 31 interest in the recent years, and the evolution of advanced molecular methods has spurred a significant wave of
 32 studies dedicated to their detailed understanding. Learning from these studies, we have now become aware that
 33 animals host a wide diversity of microbial communities that have evolved with them as a result of complex and
 34 mutualistic interactions, and that play crucial roles in their biology and health status.^{1,2} The paradigm of a highly
 35 evolved, complex, and tightly host-interconnected microbial community is the gastrointestinal microbiota,³⁻⁶ but
 36 in the recent years the microbial communities of diverse anatomical sites have been characterized, ranging from
 37 more obvious districts such as the skin and the genitourinary tract, to less obvious ones such as the airways, and
 38 including areas that were previously considered as absolutely devoid of microorganisms, such as the placenta and
 39 the fetus.^{7,8} Until recently, the mammary gland and the milk contained in it were also believed to be sterile,⁹ and
 40 microorganisms found in milk were thought to be the result of an external contamination. However, this belief has
 41 recently been challenged, as a result of the integration of culture-based methods with more sensitive molecular
 42 methods.¹⁰

43 Due to its importance for animal health and its correlations with quality and safety of dairy productions, the
 44 interest in understanding the origin and composition of the milk microbiota has significantly grown in the last
 45 decade.¹¹ As a result of the rapid evolution of meta-omics sciences, a wide range of approaches is now available
 46 for its detailed characterization, enabling to gather information ranging from its taxonomic composition, to its
 47 functional potential, to the molecules it produces as a result of its functioning (Figure 1).
 48



49

50 **Figure 1.** Outline of the approaches available for studying the milk microbiota.

51

52 **Approaches to understanding the milk microbiota: 16S metagenomics and shotgun metagenomics**

53 The characterization of the whole set of microbial genomes, the metagenome, might be based on target
 54 sequencing of the 16S rDNA or supported by shotgun, genome wide, sequencing. The former approach relies on a

55 combination of PCR amplification and sequencing of a 16S rRNA gene fragment (16S metagenomics).^{12,13}
56 Therefore, it allows the characterization of the bacterial component in the microbial community. The rRNA genes
57 are the most conserved genes in all bacteria, yet they carry hypervariable regions, where sequences have diverged
58 over evolutionary time. In 16S rDNA sequencing studies, a pair of so called “universal” primers is designed to bind
59 to conserved regions and amplify variable regions that capture the taxonomic information. Sequencing of the
60 amplified pool of 16S rDNA fragments enables the most accurate assignment of each read to its specific taxon.
61 Then, the relative abundance of each taxon can be estimated.¹⁴
62 However, amplicon-based metagenomics suffers several limitations, including the loss of diversity due to PCR
63 biases,^{15–18} and variability in diversity estimates.¹⁹ For instance, different 16S rDNA variable loci have differential
64 capacity in resolution of taxa, and the number of 16S rRNA gene copies in bacterial genomes varies quite
65 considerably. In addition, amplicon sequencing gives information on the taxonomy of the community, but not on
66 its biological functions.^{19–21} Although phylogenetic reconstruction may provide hints into this latter aspect,²² its
67 accuracy is linked to the correct representation of the microbial diversity in the genome sequence databases and is
68 hampered by the functional gene heterogeneity between strains of the same species due to horizontal gene
69 transfer.²³
70 To extend the information captured by 16S metagenomics, shotgun metagenomics provides a further approach to
71 study the non-culturable microbiota, offering a wider perspective on microbial diversity.¹⁷ In this case, instead of
72 amplifying a specific target locus, the whole metagenomic DNA is extracted, reduced into fragments, and
73 sequenced. This produces a great number of genomic sequences, that align to genomic locations in all the DNA
74 genomes of the whole community, including DNA viruses and yeasts. As a result, it becomes possible to
75 interrogate these data either by sampling taxonomically informative loci, such as the 16S rDNA, or by analyzing
76 those sequences that provide information on the functional potential of the metagenome, that is, understand who
77 is in the community, but also what the community is capable of doing. Interestingly, the metagenome of a complex
78 microbial community (e.g. human feces) has been reported to be linearly correlated with the metatranscriptome,
79 indicating that the measured potential and actual activity of the microbiota share many similarities.²⁴
80 Of course, this huge potential brings several challenges.^{17,25–29} The first and most obvious one is represented by the
81 extreme complexity and dimension of the data generated. In addition, being the metagenome a collection of
82 genomes highly diverse in abundance, less represented genomes may be only partially sequenced, and difficulties
83 often arise in obtaining extended sequences assembly and alignment.³⁰ The vast amount of data generated, then,
84 needs to be interrogated in order to obtain meaningful results. This presents problems both in terms of
85 computational power and of dedicated informatics software for analysis and interpretation of results. In addition,
86 unwanted host DNA may be present, often in significant amounts, requiring the application of molecular and
87 bioinformatic methods for its removal.^{31–33} A wide and constantly evolving range of bioinformatic tools for
88 taxonomy and functional analysis is available in free software platforms, such as mothur, QIIME, and UniFrac for
89 16S, MGRAST, Kraken, and MEGAN for metagenomics, and LEfSe for differential analysis. Statistical analysis can
90 then be carried out in packages such as R, Metastats, or Primer-E.^{17,29,34}
91 As a final consideration, generating metagenomic data is relatively more expensive, although the rapid progresses
92 in DNA sequencing technologies are improving this aspect. Several different platforms are available.³⁵
93 Pyrosequencing with the Roche/454 GS-FLX is a reliable system that provides long reads (500 bp), but newer NGS

platforms, such as Illumina's HiSeq and MiSeq and Life Technologies' Ion Torrent, have elevated sequencing potentials. In bacterial microbiota studies, the HiSeq can provide the highest data output with the lowest costs, but MiSeq is preferable when short turn-around times are desired.^{36,37} The Ion Torrent (Ion PGM™ Sequencer and Ion Proton™ Sequencer) is also a valid low-cost, scalable and high-throughput alternative, providing up to 400 bp sequence reads.³⁸ To date, high-throughput sequencing has not been extensively applied to assess the ruminant milk microbiota, but that will likely change significantly in the years to come.^{11,39–41}

Beyond metagenomics: metatranscriptomic, metaproteomic, and metametabolomic methods

As stated above, the genomic content of a microbial community gives insights about its functional potential, but no information can be inferred about the functional activities that the microbiota is actually accomplishing in a particular condition or time point. To reach this goal, additional -omics data should be collected from the microbial community by means of metatranscriptomics, metaproteomics and metametabolomics (Figure 1 and Table 1).⁴²

Table 1. Features of the -omics approaches available for studying microbial communities.

Approach	Target molecule(s)	Information provided	Drawbacks
16S metagenomics	16S rRNA gene (or its hypervariable regions)	Taxonomic distribution	Only bacteria are characterized
Metagenomics	Community DNA	Taxonomic distribution and gene potential	Issues with sequence annotation and costs
Metatranscriptomics	Community RNA (or mRNA)	Taxonomic distribution and gene expression	Issues with RNA stability and data analysis
Metaproteomics	Community proteins	Taxonomic distribution and protein expression	Issues with protein dynamic range and data analysis
Metametabolomics	Community metabolites/ organic compounds	Metabolic fluxes	No direct link between metabolite and microbial taxonomy

Metatranscriptomics analyzes the RNA transcript pool expressed by a microbial community at a specific point in time,⁴³ thus allowing a simultaneous investigation of the gene expression (mRNA) and abundance (rRNA) of microorganisms.⁴⁴ When 16S rDNA data are already available or not necessary, several strategies can be applied to enrich for prokaryotic mRNA molecules and reduce the rRNA fraction of metatranscriptomes,⁴⁵ such as selective nuclease degradation of rRNA,⁴⁶ rRNA depletion by capture with commercial kits,⁴⁷ and polyadenylation and enrichment of mRNA.⁴⁸ After extraction, RNA is subjected to reverse transcription to cDNA, and cDNAs are analyzed by high-throughput sequencing technologies (RNA-seq).^{49,50} Quality assessment and decontamination from host/rRNA sequences can be performed using standard metagenomics tools. Sample preparation issues due to the low stability of RNA and bioinformatic issues related to sequence reconstruction, annotation and statistical analysis can be considered as the main challenging aspects in a metatranscriptomic investigation.⁵¹

Metaproteomics encompasses the large-scale study of the whole protein complement of a microbiota, providing a direct measure of the functional activity of a microbial community.^{13,43} (Meta)proteomic approaches also enable the analysis of splicing variants and co- and post-translational modifications, as well as the detection of protein-protein interactions and protein complexes.⁵² The analytical requirements for metaproteome characterization

124 include high sensitivity and broad dynamic range in peptide identification.⁵³ In view of this, coupling effective liquid chromatography (LC) separation systems with high-resolution mass spectrometers (MS) represents the state-of-the-art technique for metaproteomics.⁵⁴ In a typical metaproteomic experiment, the extracted proteins are therefore digested with proteolytic enzyme(s) to generate a complex peptide mixture, which is eventually analyzed by LC-MS. The presence of contaminating proteins (e.g. from the host), the huge dynamic range in protein abundance, and - even more importantly - the bioinformatic analysis issues (especially related to construction and annotation of sequence databases for peptide identification) are the most difficult tasks in metaproteomic studies.^{55,56} Notably, the availability of (meta)genomic sequences from the community being studied is vital for efficient protein identification and annotation.⁵⁷⁻⁵⁹

133 Metametabolomics refers to the systematic analysis of the metabolite complement produced by microbial communities. Metabolites are typically in a state of flux, which implies that their abundance varies as a function of time within the ecosystem.⁶⁰ The most common analytical techniques used to characterize a microbial metabolome are MS and proton nuclear magnetic resonance (NMR), each one with its respective advantages and disadvantages: NMR is a non-destructive, non-selective and cost-effective approach, while MS offers better sensitivity and, if coupled to separation techniques (as LC or gas chromatography), is capable to detect a broader range of molecules.^{61,62} Specific issues concerning metametabolomic analysis are due to the non-uniformity of the molecules to be profiled (spanning a broad range in hydrophobicity and molecular weight), as well as to the impossibility to directly link the particular metabolite detected to a specific microbial taxonomy.^{51,63}

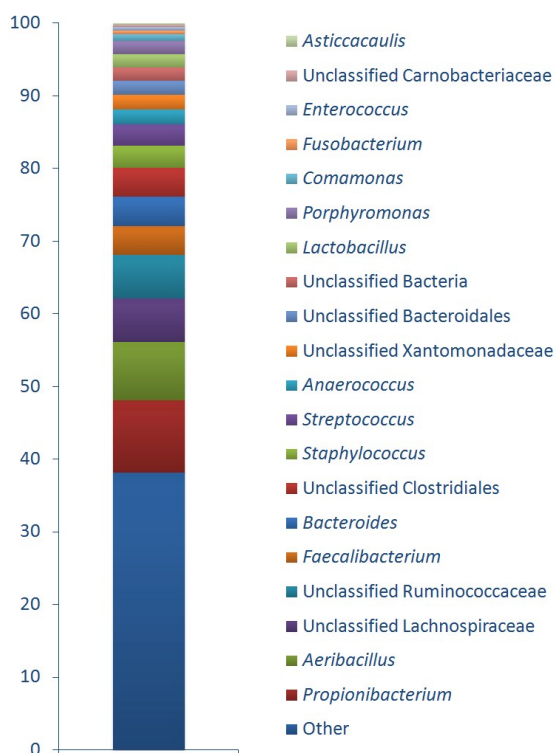
142 The application of a systems biology approach - comprising metatranscriptomics, metaproteomics and metametabolomics - to the study of the milk microbiota in the years to come is expected to provide a much wider and sharper picture of the functional activity of milk microbial communities, compared to the information that one would infer from DNA sequence alone. Each -omics technology provides a unique perspective, and, by integrating these large-scale datasets, scientists can investigate microbial community dynamics and interactions at an unprecedented level (Table 1).⁶⁴

149 **The healthy milk microbiota**

150 Milk is a complex, species-specific biological fluid aimed to satisfy the nutritional requirements of the mammalian offspring, but it does also exert numerous functional roles along offspring development.^{2,65-67} The biological actions of milk are due to presence of immune cells and of an assortment of active molecules, including sugars, nucleotides, lipids, immunoglobulins, antimicrobial proteins, cytokines, and other immuno-modulatory factors.^{66,68-71} In addition, milk contains a complex and varied community of bacteria, with an abundance estimated in approximately 10^3 - 10^4 colony-forming units per milliliter in human milk.⁷²

156 The human milk microbiota has been the subject of different studies in the recent years, aimed to understand its role in physiology and health of both the nursing mother and her infant.^{65,66} On the other hand, most studies on the dairy ruminant microbiota have been carried out with a focus on how the microbial flora of milk changes when it becomes a food product, either for direct consumption or for transformation into dairy products. That is, by considering microbial ecology of raw milk, rather than how the milk microbiota behaves in the context of animal health and physiology.¹¹ To date, only few studies have been carried out in cows with this purpose. Kuehn et al.

162 used pyrosequencing of bacterial 16S rRNA genes to investigate bacterial DNA diversity in 10 mastitic, culture
 163 negative, milk samples.⁷³ In this work, the microbiota of milk samples obtained from healthy quarters from the
 164 same cows was also described for comparison purposes. The authors were able to show significant differences
 165 among the microbial profiles of healthy milk samples. The most abundant genera were: *Ralstonia*, *Pseudomonas*,
 166 *Sphingomonas*, *Stenotrophomonas*, *Psychrobacter*, *Bradyrhizobium*, *Corynebacterium*, *Pelomonas*, and
 167 *Staphylococcus*. Abundances of *Pseudomonas*, *Psychrobacter*, and *Ralstonia* were significantly higher in healthy
 168 samples comparing to the mastitic ones. In a more recently published study, Oikonomou et al. described in detail
 169 the microbial diversity of 144 bovine milk samples derived from clinically unaffected quarters across a range of
 170 somatic cell count values.⁷⁴ Four bacterial genera were present in all the samples obtained from healthy quarters
 171 (*Faecalibacterium*, unclassified *Lachnospiraceae*, *Propionibacterium* and *Aeribacillus*) and could be considered part
 172 of a healthy milk core microbiota. Other genera found to be prevalent in most of the milk samples with very low
 173 somatic cell counts were: *Bacteroides*, *Staphylococcus*, *Streptococcus*, *Anaerococcus*, *Lactobacillus*,
 174 *Porphyromonas*, *Comamonas*, *Fusobacterium* and *Enterococcus* (Figure 2). Certain bacterial genera (e.g.
 175 *Lactobacillus*, *Paenibacillus*) were associated with healthier udder quarters.



177
 178 **Figure 2.** Distribution of the twenty most prevalent bacterial genera found in 50 healthy quarter milk samples with
 179 less than 20,000 cells/mL.⁷⁴

181 Zhang et al. described the effects of different dairy cattle diets (high concentrate versus low concentrate diet) on
 182 milk microbial communities using pyrosequencing of the 16s rRNA genes.⁷⁵ Despite the small number of animals
 183 enrolled in their study (n=4) the authors were able to suggest diet associated differences in milk microbial
 184 communities. In the work of Falentin et al.,⁷⁶ milk from healthy quarters was associated to a high proportion of the

185 Clostridia class, the Bacteroidetes phylum and the Bifidobacteriales order. Table 2 summarizes the current findings
 186 on composition of the healthy cow milk microbiota.

187

188 **Table 2.** Composition of the healthy cow milk microbiota.

Study	Most prevalent genera
Kuehn et al. ⁷³	<i>Ralstonia</i> , <i>Pseudomonas</i> , <i>Sphingomonas</i> , <i>Stenotrophomonas</i> , <i>Psychrobacter</i> , <i>Bradyrhizobium</i> , <i>Corynebacterium</i> , <i>Pelomonas</i> , <i>Staphylococcus</i>
Oikonomou et al. ⁷⁷	<i>Propionibacterium</i> , <i>Aeribacillus</i> , unclassified <i>Lachnospiraceae</i> , <i>Faecalibacterium</i> , <i>Bacteroides</i> , unclassified <i>Clostridiales</i> , <i>Staphylococcus</i> , <i>Streptococcus</i> , <i>Anaerococcus</i> , <i>Unclassified</i> <i>Xanthomonadaceae</i> , unclassified <i>Bacteroidales</i> , <i>Unclassified Bacteria</i> , <i>Lactobacillus</i> , <i>Porphyromonas</i> , <i>Comamonas</i> , <i>Fusobacterium</i> , <i>Enterococcus</i> , unclassified <i>Carnobacteriaceae</i> , <i>Asticcacaulis</i>
Zhang et al. ⁷⁵	<i>Chryseobacterium</i> , <i>Streptococcus</i> , <i>Enterococcus</i> , <i>Stenotrophomonas</i> , <i>Brevundimonas</i> , <i>Lactococcus</i> , <i>Sphingomonas</i> , <i>Prevotella</i> , <i>Sphingobacterium</i> , <i>Helcococcus</i> , <i>Leucobacter</i> , <i>Butyrivibrio</i> , <i>Atopostipes</i> , <i>Bosea</i> , <i>Alcaligenes</i> , <i>Ruminococcus</i> , <i>Facklamia</i> , <i>Actinomyces</i> , <i>Sphingobium</i> , <i>Trueperella</i> , <i>Pseudomonas</i> , <i>Enterobacter</i> , <i>Comamonas</i> , <i>Megasphaera</i> , <i>Salinicoccus</i> , <i>Ochrobactrum</i> , <i>Lactobacillus</i> , <i>Mogibacterium</i> , <i>Peptococcus</i> , <i>Succiniclasticum</i> , <i>Myroides</i>

189

190 In dairy ruminant species other than cows, studies have been carried out almost exclusively for purposes of dairy
 191 production, and not for investigating mammary health or offspring health. Therefore, experimental design and
 192 sampling procedures may not be adequate for extracting information on the *sensu stricto* milk microbiota.¹¹

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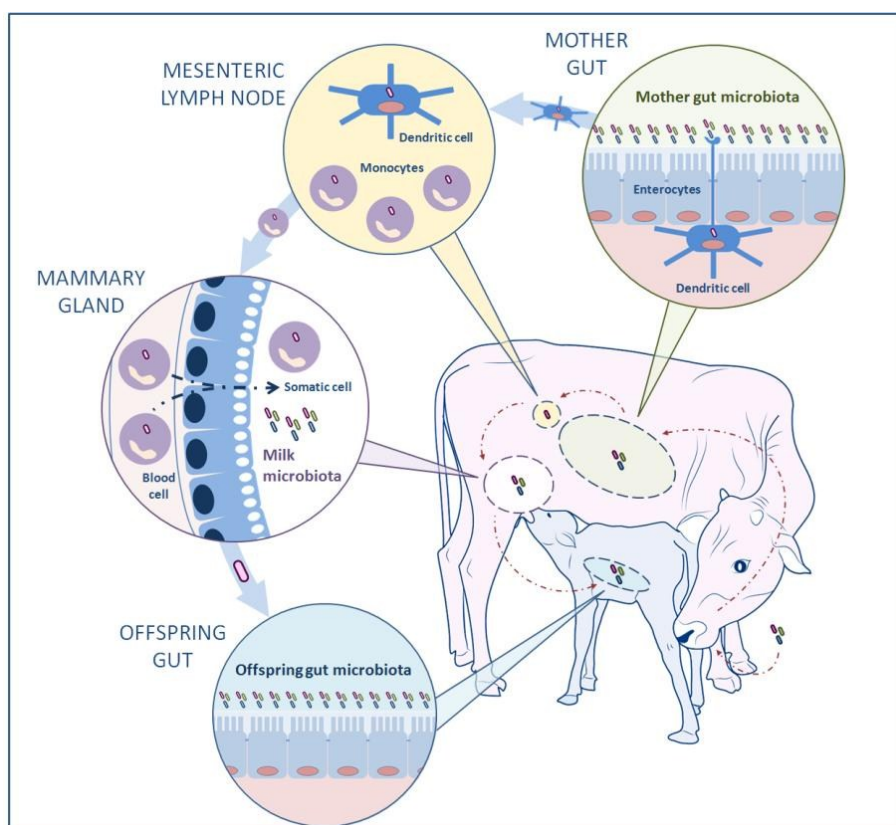
194 **Origin of the milk microbiota: the endogenous route hypothesis**

195 Traditionally, it is believed that bacteria found in milk result from contamination by the external environment, the
 196 mammary gland skin, or the oral cavity of the offspring. However, several studies support the hypothesis that
 197 presence of bacteria in milk is not the mere result of an external colonization. It has been demonstrated that,
 198 adding to their different composition in terms of bacterial taxa, bacterial isolates present in the mammary gland
 199 are genotypically different from those found in skin, within the same host and the same bacterial species.⁷⁸

200 Therefore, the udder skin and teat canal cannot be considered as the sole contributors to shaping the milk
 201 microbiota.^{65,79} Adding to this, bacteria such as bifidobacteria are strictly anaerobic, making skin an unlikely
 202 source.⁸⁰ These and other observations have led to consider the possibility of an endogenous route. In fact,
 203 ecological niches in the host microbiota do not constitute separate environments, but are rather a network of
 204 inter-related communities undergoing constant exchanges.⁸¹ Therefore, microorganisms from other anatomical
 205 locations may in some way make it to enter the mammary gland. More specifically, several authors described the
 206 existence of an entero-mammary pathway, based on the ability of some microbes to leave the intestinal lumen,
 207 travel through the mesenteric lymph nodes, and reach the mammary gland.^{65,71,78,82–85}

208 The suggestion of an endogenous origin of the milk microbiota has been corroborated by different studies carried
 209 out in mice.^{71,86–89} Although the mechanisms by which microbes get to cross the intestinal barrier and reach other
 210 body sites has not been completely clarified, it is likely that this may involve immune cells, especially Dendritic

211 Cells (DC).^{71,82} In fact, DCs are able to sample intestinal contents by opening the tight junctions among enterocytes,
 212 and reach the lumen with their dendrites without damaging the epithelial barrier integrity.^{85,90} As a result of this
 213 sampling ability, these cells can harbor live commensal bacteria, and carry them to the mesenteric lymph
 214 nodes.^{91,92} Once there, bacteria remain viable for up to several days, and have the chance to spread to other
 215 distant mucosal surfaces, including the lactating mammary gland, by means of the mucosal associated lymphoid
 216 system. In fact, during lactation, cells from gut-associated lymphoid tissue travel to the breast via the lymphatic
 217 and peripheral blood circulations. Donnet-Hughes et al. showed that, during lactation, human peripheral blood
 218 mononuclear cells and breast milk cells contain bacteria and their genetic material.⁸⁵ In addition, the presence of
 219 viable lactic acid bacteria in the bloodstream of human subjects has been reported,^{93–95} further showing that some
 220 members of the intestinal microbiota may have a rather underrated ability to travel to distant extra-intestinal
 221 locations of their host in a viable form.⁶⁵ The authors also showed an increase in bacterial translocation from the
 222 mouse intestine during pregnancy and lactation and the presence of bacterially loaded DCs in lactating breast
 223 tissue.



224
 225 **Figure 3.** The entero-mammary pathway hypothesis in ruminants and the mother-offspring microbial flow.

226
 227 The hormonal and physiological changes occurring during late pregnancy and lactation influence and condition
 228 permissivity of this bacterial transport.⁶⁶ It is believed that, adding to the transport of viable members of the
 229 intestinal microbiota, this mechanism has the role of educating the offspring's immune system to recognize
 230 molecular patterns associated to commensal microorganisms, in order to develop an appropriate response to
 231 them.⁸⁵ This migration may occur either selectively, that is, certain strains may be recognized by immune cells and
 232 transported into milk, while others may not, or immune cells may take up all microorganisms, but only those able
 233 to escape killing would be transported to the mammary gland.⁹⁶

234 A recent article by Young et al. reported the transfer of intestinal bacteria to the mammary gland in cows,
235 supporting the existence of an endogenous entero-mammary pathway also in ruminants.⁹⁷ The authors have
236 investigated the microbial composition and diversity of feces, milk leukocytes and blood leukocytes in healthy
237 lactating cows by pyrosequencing barcode-tagged 16S rDNA amplicons, demonstrating the shared presence of a
238 small number of bacterial OTUs belonging to the *Ruminococcus* and *Bifidobacterium* genera and to the
239 Peptostreptococcaceae family in all three samples from the same animals. In order to avoid external
240 contamination and to prevent stretching or damaging of the teat canal, the authors used a catheter for collecting
241 milk by gravity into a sterile container. The presence of these bacteria in the three environments supports the
242 occurrence of a mechanism responsible for migration of some components of the intestinal microbiota to the
243 mammary gland via circulating white blood cells. However, the cell types responsible for the trafficking of
244 microbiota from the mesenteric lymph nodes to milk remain to be established.
245 Further research will be needed to dissect the mechanisms by which intestinal bacteria are transported to the
246 circulation and to the mammary gland of ruminants, as well as to understand the implications that this can have
247 for the health of the lactating animal, her offspring, and the human consumer. The current knowledge on the
248 entero-mammary pathway hypothesis in ruminants is outlined in Figure 3.

250 **Functions of the milk microbiota: lessons learned from human milk and the mouse model,** 251 **and hints about its impact on the offspring ruminant health**

252 As stated above, most of the studies on the physiological milk microbiota of dairy ruminants have been carried out
253 with a focus on how the microbial flora of milk evolves when it ceases to become a *sensu stricto* biological fluid to
254 become a processed food or a dairy product.¹¹ Therefore, most of the insights on the physiology of the mother's
255 milk microbiota and on its influences on the offspring development and health have been gathered from studies
256 on humans and on the mouse model.

257 The milk microbiota exerts many short and long term influences on both the mother and the offspring
258 physiology.^{71,72,98-101} One of these is the transmission of microbes to the developing offspring gastrointestinal tract
259 (Figure 3).^{65,78,80,102-104} The role of the milk microbiota as a "seed" for the developing intestinal microbiota is also
260 evident in their close similarity; it is only after weaning that a significant diversification of the two communities
261 takes place.¹⁰⁵ As an example of the complex interaction among milk molecules, milk microbiota and offspring
262 intestinal microbiota, it has been demonstrated that the abundant oligosaccharides present in human milk (HMOs,
263 human milk oligosaccharides) are not digestible for the lactating infant. Instead, these are fermented by specific
264 phylotypes of bifidobacteria and lactobacilli.¹⁰⁶⁻¹⁰⁹ In this way, HMOs provide a selective advantage to the milk and
265 intestinal microbes that are able to metabolize them, and to thrive in the acidic environment generated by their
266 digestion. In turn, this developing, selected microflora acts as a competitive "guard" to the blooming of adverse
267 microbes. Although in lower concentration than human milk, bovine milk does also contain complex milk
268 oligosaccharides analogous to HMOs, the bovine milk oligosaccharides (BMOs).¹¹⁰⁻¹¹³ However, the role that these
269 BMOs play on the milk microbiota of the cow mammary gland and of the intestinal microbiota has not been
270 investigated yet.

271 Milk influences other health promoting bacteria, including *Lactobacillus*, *Bacteroides*, and *Clostridium* species that
272 can influence mucin production, mucosal permeability, T-cell balance, and dampening of mucosal
273 inflammation.¹¹⁴⁻¹¹⁹ Studies carried out in germ-free mice have revealed that the development of a fully functional
274 immune system requires early life colonization.¹²⁰ All this considered, milk bacteria can be crucial for programming
275 the appropriate functionality of the immune system against food antigens, pathogens, and commensal bacteria.
276 Therefore, the intestinal microbiota of the offspring, and the evolution of its immunity, are shaped by the
277 “seeding” operated by the milk microbiota, deriving from the mother’s entero-mammary pathway, by the infant’s
278 environment, and by the continuous crosstalk between the mother’s mammary gland and the suckling infant oral
279 microbiota, with their synchronized development and evolution throughout lactation. In support of this latter
280 observation, Cabrera-Rubio et al. have demonstrated that the milk microbiota of healthy women evolves along
281 lactation, and undergoes a series of changes as lactation proceeds.⁷⁹

282 In ruminants, the role of the milk microbiota in shaping the intestinal microbiota of the newborn takes a further
283 implication. In fact, these animals harbor an additional, very complex microbial community, that has the crucial
284 role of carrying out plant digestion and converting otherwise non-digestible material into useful chemical
285 compounds: the rumen microbiota.¹²¹ Microbial colonization of the rumen occurs almost immediately; bacteria
286 with cellulolytic capabilities are already present in calves of 3-5 days of age, and are abundant in 2-3 week old
287 calves.^{122,123} Recently, a study on ruminal bacterial communities has demonstrated that pre-ruminant calves
288 harbor bacteria and functions that are present in mature animals.¹²⁴ By using a pyrosequencing approach, Jami et
289 al. have demonstrated that cellulolytic bacterial species are already present in the rumen of newborn calves as
290 early as 1 day after birth, and at increasing abundance on the third day.¹²¹ This is reinforced by Fonty et al. and
291 Minato et al., who isolated cellulolytic bacteria from the rumen in the first week after birth.^{122,123} Jami et al.
292 demonstrated that establishment in the rumen of crucial bacterial species begins on the first day of life, when the
293 animals are still being fed exclusively colostrum, that is, before the intake of plant material.¹²¹ This notion has also
294 been advanced for microbial communities in the developing human infant’s intestinal microbiota.¹²⁵ Although the
295 authors do postulate that this primary bacterial community might be transmitted from the mother, they propose
296 that this may occur via skin, the birth canal, or saliva.¹²⁶ However, the role of the mother entero-mammary
297 pathway in seeding the microbiota of the young ruminant might deserve further investigation.

298

299 **The milk microbiota and mammary gland infection**

300 Mastitis due to intramammary infection is a highly prevalent disease in dairy cows and it is arguably the most
301 important one for the dairy industry worldwide, causing economic losses due to reduced milk production,
302 discarded milk, lower probability of conception, premature culling, and treatment cost.¹²⁷ The decrease in milk
303 production per cow resulting from mastitis has been well-studied, and is estimated to impact on approximately
304 15% of the milk production potential of the affected cow.¹²⁸ Mastitis is also a serious animal welfare issue as it is
305 associated with pain, reduced well-being and behavioural changes of the affected animals.¹²⁹ Defined as
306 inflammation of the mammary tissue, it can be characterized by the movement of leukocytes and serum proteins
307 from the blood to the site of infection. As a consequence, mastitis is typically monitored by using as an indicator
308 the number of cells present in a milliliter of milk, defined as the somatic cell count, although novel, potentially

309 highly sensitive, protein markers are emerging to aid its detection.^{130–137} Intramammary infection can be
310 categorized into subclinical and clinical disease; the former is thought to be 3–40 times more prevalent than the
311 latter and is defined as the presence of infection without clinical signs of local inflammation, whilst clinical mastitis
312 involves an inflammatory response causing visibly abnormal milk, sometimes accompanied by swelling and/or
313 redness of the mammary glands, and by an increase in the somatic cell count.

314 Identification of the bacteria responsible for intramammary infection is an important component of eventual
315 clinical resolution of the disease. Currently, bacterial culture is the gold standard method for identification of
316 mastitis-causing microorganisms. However, limitations of classical bacterial culture, such as 48 hours to obtain
317 results, or the fact that in approximately 25% of milk samples from clinical mastitis cases bacteria are not detected
318 in conventional culture have spurred investigations of culture independent, molecular techniques for mastitis
319 diagnosis.¹³⁸ Methods such as real-time PCR¹³⁹, multiplex PCR (mPCR)¹⁴⁰, denaturing gradient gel electrophoresis
320 (DGGE) PCR¹⁴¹, and PCR single-strand conformation polymorphism (SSCP)¹⁴² are now being used to identify
321 bacterial DNA in milk samples. Molecular epidemiological studies have greatly contributed in advancing our
322 knowledge of bovine mastitis, and have been extensively used for over two decades now.¹⁴³

323 Bhatt et al. performed metagenomic analysis of milk samples collected from Kankrej, Gir (*Bos indicus*) and
324 crossbred cattle affected with subclinical mastitis using shotgun sequencing and 454 GS-FLX technology.¹⁴⁴ Their
325 metagenomic approach came to confirm culturing results, but was also able to produce a significant amount of
326 additional information. A total of 56 different species with varying abundance were detected in the subclinically
327 infected milk, together with several bacteriophages. The authors concluded that subclinical mastitis is a
328 polymicrobial disease, a conclusion that was not well supported by their data mainly because samples from
329 unaffected quarters were not obtained for comparison purposes.

330 Oikonomou et al. used metagenomic pyrosequencing of bacterial 16S rDNA genes to investigate bacterial DNA
331 diversity in milk samples of mastitic and healthy dairy cows and compared the results with those obtained by
332 classical bacterial culture.¹⁴⁵ One hundred and thirty-six milk samples were collected from cows showing signs of
333 mastitis and used for microbiological culture. The mastitis pathogens identified by culture were generally among
334 the most frequent organisms detected by pyrosequencing, and in some cases (*Escherichia coli*, *Klebsiella* spp. and
335 *Streptococcus uberis* mastitis) the single most prevalent microorganism. *Trueperella pyogenes* sequences were the
336 second most prevalent sequences in mastitis cases diagnosed as *Trueperella pyogenes* by culture, *Streptococcus*
337 *dysgalactiae* sequences were the second most prevalent sequences in mastitis cases diagnosed as *Streptococcus*
338 *dysgalactiae* by culture, and *Staphylococcus aureus* sequences were the third most prevalent in mastitis cases
339 diagnosed as *Staphylococcus aureus* by culture. In samples that were aerobic culture negative, pyrosequencing
340 identified DNA of bacteria that are known to cause mastitis, DNA of bacteria that are known pathogens but have
341 so far not been associated with mastitis, and DNA of bacteria that are currently not known to be pathogens.
342 Additionally, a high number of anaerobic bacterial sequences (with sequences belonging to *Fusobacterium*
343 *necrophorum* being highly prevalent) were identified in all mastitis cases, regardless of the culture-based diagnosis.
344 On the other hand, *Fusobacterium necrophorum* sequences were practically absent in the 20 samples that were
345 derived from healthy, low somatic cell count quarters, while *Porphyromonas* spp. sequences were detected but in
346 low prevalence comparing to their prevalence in the mastitic samples. Therefore, a possible role of certain
347 anaerobic bacteria as opportunistic pathogens was speculated. This study showed that the use of metagenomic

348 pyrosequencing of the 16S rDNA should be considered an important tool to advance our knowledge regarding the
349 pathogenesis of bovine mastitis and could be developed as a diagnostic tool. However, being a cross-sectional
350 prevalence study, it lacked the ability to show a proper time order to infer a cause and effect relationship. By using
351 pyrosequencing of bacterial 16S rDNA genes, Kuehn et al. described the bacterial communities in culture negative
352 mastitic milk samples, showing significant differences with healthy milk samples. Principal coordinates analysis
353 suggested that non-clinical and clinical samples generally fell within separate clusters.⁷³ In the study by Oikonomou
354 et al., adding to bacterial genera present in all the samples obtained from healthy quarters (*Faecalibacterium*,
355 unclassified Lachnospiraceae, *Propionibacterium* and *Aeribacillus*), *Streptococcus uberis* sequences were found in
356 all groups of samples, with a lower prevalence in low somatic cell counts groups. This was considered unexpected
357 by the authors as this bacterial species is generally recognized as a major mastitis pathogen. It was hypothesized
358 that *Streptococcus uberis* may, although in small quantities, be part of the normal milk microbiota, and therefore
359 clinical mastitis may in such cases be a dysbiosis, rather than a simple primary infection.⁷⁴ In the Falentin et al.
360 study, quarters with a mastitis history showed a higher proportion of the Bacilli class (*Staphylococcus*) and
361 Chlamydiia class.⁷⁶ Concerning dairy ruminant species other than cows, there are basically no -omics studies on
362 how the milk microbiota changes in mastitis.

363 From the studies carried out in women on the role of the milk microbiota in intramammary infections and mastitis,
364 we may gather useful hints on the possible role of the intestinal microbiota as a reservoir for mastitis-causing
365 bacteria. On the other hand, mechanisms such as nutrient competition, bacteriocins and antimicrobial molecules
366 released by specific members of the community in milk may play a role in repressing the blooming of potential
367 pathogens, and contrast intramammary infections.¹⁰⁰ Hunt and coworkers have reported the host-dependence of
368 the milk microbiota in women, and have suggested that its composition may play a role in determining whether
369 they will suffer or not from mastitis.⁷² As reviewed above, HMOs have the ability to modulate the intestinal
370 microbiota of the breastfed infant, and structurally analogous oligosaccharides, BMOs, are present in cow milk.^{110–}
371 ¹¹³ As such, it can be speculated that BMOs may also impact bacterial communities of the cow mammary gland.⁶⁶
372 Interestingly, HMOs fall within milk group categories that mirror blood group characteristics, and are under genetic
373 control.¹⁴⁶ It has been demonstrated that some strains of *Staphylococcus*, the leading cause of mastitis in women,
374 bind only to selected HMO types.¹⁴⁷ This would suggest that susceptibility to mastitis might be conditioned not
375 only by the bacterial composition of milk or by exposure to specific pathogens, but also by the genetic makeup of
376 the animal and the corresponding type of BMOs present in milk.⁶⁶

377 The existence of an entero-mammary pathway in ruminants⁹⁷ (Figure 3) opens several interesting speculations
378 concerning possible alternative ways to antibiotics for contrasting mastitis. In women, an effective mastitis
379 treatment has been provided by the oral administration of probiotics, including *Lactobacillus salivarius* CECT5713
380 and *L. fermentum* CECT5716.^{88,89} These impacted the milk microbiota by lowering the total bacterial count by 2 log
381 and replacing mastitis-causing *Staphylococcus* species with *Lactobacillus* species. This was also shown to facilitate
382 breastfeeding, leading to health benefits for both mother and infant. The possibility of influencing the milk
383 microbiota through the oral administration of pre- or probiotics may open interesting perspectives in reducing the
384 risk of mastitis in dairy cows.¹⁴⁸ These examples emphasize the possible magnitude of the milk microbiota
385 influence on dairy ruminant health, demanding future investigations.

386

387 **The impact of farming practices on the mother/offspring microbiota crosstalk, and the waste** 388 **milk issue**

389 Current farming practices pose several hindrances to the finely evolved crosstalk between the mother and the
390 offspring microbiota. In fact, although calf management procedures can slightly vary among commercial dairy
391 farms,¹⁴⁹ calves are removed from their dams after birth, and administered colostrum, pooled colostrum, or
392 colostrum substitutes. Then, they are typically fed whole bulk tank milk, milk replacer, or a combination of them,
393 together with a starter feed. Therefore, the mother/offspring microbiota axis, with its reciprocal crosstalk, is
394 disrupted. In ruminants that are left with their mothers, the mother/offspring crosstalk may play a relevant role in
395 evolution of both the mother's milk and the intestinal microbiota of the offspring along lactation.

396 In dairy calf management, attention should be paid to the quality of colostrum and milk that are administered in
397 the farm, when considering that a healthy, well-balanced, microbiota-competent mother's milk is crucial for a
398 correct development of the offspring's immune system. In fact, an imbalance in the intestinal microbiota is seen
399 when calves are under stress conditions, such as in intensive rearing systems, with a reduction of *Lactobacillus* and
400 *Bifidobacterium* species and an increase in pathobiont microorganisms. It is also interesting to notice that feeding
401 whole milk to calves improved the lactic acid bacteria to coliforms ratio, further demonstrating the complex action
402 exerted by milk on the intestinal microbiota.¹⁵⁰

403 Much care is given to providing clean and high quality colostrum to newborn calves within 6 hours from birth.
404 However, numerous farms use unsaleable, waste milk, for post-colostrum calf feeding. Waste milk is represented
405 by milk which cannot be sold for human consumption, and it is typically derived from cows with high somatic cell
406 counts and from cows treated with antibiotics.¹⁵¹ Feeding waste milk to preweaned calves is a widespread
407 phenomenon, if one considers that, in 2002, it was practiced in 87.2% of all US dairy farms.¹⁵² Although the use of
408 waste milk is economically advantageous for the farmer, and it is generally believed to be a safe and better
409 alternative to milk replacers, especially after pasteurization, it can raise some concerns. In fact, waste milk can be
410 heavily unbalanced in terms of milk microbiota, be contaminated with potentially harmful pathogens,¹⁵³ or contain
411 antibiotic residues, with possible consequences on the future animal well-being.¹⁵⁴

412 These issues have been examined by different research groups. Edrington et al. evaluated the effect of feeding
413 waste milk on the bacterial diversity of the dairy calf fecal microbiota.¹⁴⁹ The authors applied 16S rDNA bacterial
414 tag-encoded FLX amplicon pyrosequencing to fecal samples from one week to six month old dairy calves fed
415 pasteurized or nonpasteurized waste milk. As a result, bacterial diversity in terms of total number of different
416 species was higher in calves fed pasteurized waste milk, and increased with age in both groups. Concerning specific
417 microorganisms, *Salmonella* was detected in calves fed unpasteurized waste milk, and *Treponema*, an important
418 beneficial bacterium in rumen, was higher in the pasteurized waste milk group, becoming higher with age in the
419 same group. The consistent detection of *Salmonella* only in young calves fed unpasteurized waste milk was an
420 important finding related to this practice. In conclusion, therefore, pasteurization of waste milk was advised. The
421 impact of feeding bulk milk or waste milk on calf performance and health was evaluated also by Aust and
422 coworkers. According to these authors as well, pasteurized waste milk can be considered an acceptable feed.¹⁵⁵

423 A more significant problem concerning the use of waste milk, however, may be represented by presence of
424 antimicrobial residues, and the potential enrichment in the antibiotic resistance gene (ARG) pool available for

425 transfer to pathogens, the “resistome”.¹⁵⁶ In addition, continuous antibiotic pressure may increase opportunities
426 for horizontal ARG transfer.^{157–159} It should also be considered that the intestinal microbiota resistome is largely
427 studied with culture-based or PCR-based experiments, with a consequent underestimation of novel resistance
428 genes.^{160–163}

429 An important aspect that needs to be taken into account when examining literature data is the administration
430 route. In this respect, mouse models can provide useful indications on the impact of antibiotics fed to young calves
431 through waste milk consumption, since in the case of infant mice antibiotics are administered through the
432 mother’s milk.^{154,164} In support of this observation, significant differences were observed upon oral versus
433 intravenous administration of ampicillin and tetracycline. Oral administration resulted in a 4-log increase in
434 ampicillin and 2-fold increase in tetracycline resistance gene copy number over intravenous administration. This is
435 also probably due to the fact that intravenously administered ampicillin is cleared through urine and does not
436 interact with the gut microbiota.¹⁶⁵

437 Adding to enrichment and selection of ARGs, antibiotics can affect specific phylogenetic subgroups of the intestinal
438 microbiota. Preterm human infants treated with different antibiotics have an increased load of potentially
439 pathogenic (pathobionts) Enterobacteriaceae, and a lower number of Bifidobacteriaceae, Bacilli, and
440 Lactobacillales, that are connected to a healthy microbiota.^{166–168} In mice exposed to subtherapeutic doses of
441 antibiotics in drinking water, there was a significant decrease in the ratio of Bacteroides to Firmicutes, although
442 this may depend on the specific spectra of antibiotics used.¹⁶⁴ In another study, administration of cefoperazone
443 was associated to a loss in microbial diversity without recovery at six weeks.¹⁶⁹ Therefore, in mice, even low
444 antibiotic dosages have long-term consequences on microorganisms associated with healthy microbiota, including
445 *Lactobacillus* spp., Bifidobacteriaceae (lowered) and Enterobacteriaceae (increased).^{164,167}

446 Limited information is currently available on the impact of drug residues on the microbiota using *in vivo* natural
447 models. Van Vleck Pereira et al. evaluated the effect on the calf fecal microbiota of feeding raw milk spiked with
448 antibiotic concentrations below the safe levels limit established by the Federal Department of Agriculture (FDA).¹⁷⁰
449 Sequencing of the microbial 16S rRNA genes was conducted using the Illumina MiSeq on calf feces collected along
450 six weeks of age. The study demonstrated that the presence of drug residues in the milk affects the composition of
451 the microbial population in the feces. In fact, the weekly fecal microbial profile of the two calf groups was easily
452 discriminated at the genus level, although no significant differences were seen for higher taxonomic levels. The
453 authors postulated that even minimal antibiotic concentrations may have a selective impact on the competition
454 among microbes, by influencing the final balance between sensitive and resistant microbial populations. That is,
455 residues can exert a selective pressure on immature microbiota that have none or very low resistance to
456 colonization by foreign microbes, resulting in an abrupt transition to a microbial profile that is most commonly
457 found in older preweaned calves. In fact, when microbes are exposed to sub-minimal inhibitory concentrations of
458 antibiotics, these will not kill all susceptible bacteria, but will impair their growth, providing a selective advantage
459 to microbes that carry ARG with a low fitness costs, contributing to their persistence even when the antibiotic is
460 removed.¹⁷¹

461 The occurrence of changes in the fecal microbiota of young calves upon parenteral antibiotic administrations was
462 also seen by Oultram et al. in a preliminary study.¹⁷² One week post treatment the groups showed the greatest
463 difference in the fecal microbiota composition, while two weeks post-treatment they became more similar,

464 showing a recovery of microbial diversity in the treated group. *Lactobacillus* species were the most affected by
 465 antibiotic. Further studies will be needed, and are advised, to clarify the impact of antibiotic residues in milk on the
 466 correct maturation and health of the dairy ruminant microbiota.

467 Another farming practice potentially interfering with the milk microbiota balance is represented by the
 468 intramammary antibiotic therapy administered to cows at dry-off or during lactation. In fact, many dairy herds are
 469 routinely treated in every quarter with antibiotic at drying off. This is defined as “blanket” approach, and is
 470 considered more effective than selective treatment in preventing new infections early in the dry period, without
 471 requiring laboratory screening procedures to decide which cows and quarters to treat. Lactation intramammary
 472 antibiotic tubes are the most common treatment for mild and moderate cases of mastitis, and are usually given
 473 without knowing the type of bacteria that is causing the infection.^{173,174} However, when subclinical mastitis in a
 474 herd is very low level (every cow has SCC below 100,000 cells/ml), intramammary antibiotic administration only to
 475 selected higher risk cows is considered appropriate by some dairy farmers and veterinarians. Because of concerns
 476 about selection for antimicrobial resistance, the blanket approach has not been implemented in the Nordic
 477 European countries for decades and it is increasingly abandoned in The Netherlands. The impact of this practice on
 478 the physiological milk microbiota and on the potential selection for ARG may deserve further investigation.

479

480 **Raw milk microbial ecology and its impact on dairy products**

481 Being a rich and nutritious fluid, milk supports the growth of many microorganisms. Therefore, adding to its
 482 endogenous microbiota, once milked it is rapidly colonized by a variety of other microbes coming from the teat
 483 canal, udder skin, milking machine, containers and tanks used for its storage, reflecting also the farm and the
 484 pasture environment. Adding to the contribution that these can exert on milk fermentation by transforming
 485 lactose in lactate, they can bring about a variety of attributes that impact on the sensory and textural
 486 characteristics of the dairy products derived from it.¹⁷⁵ Furthermore, contamination with, and subsequent growth
 487 in milk of potentially pathogenic bacteria (or with toxins produced by them) can have implications for human
 488 health and is therefore a relevant issue to consider. And, it is also important to assess how the composition of the
 489 microbiota evolves in raw milk during milking, transport, storage, and dairy processing, and how it impacts on the
 490 composition and quality of dairy products (Table 3).

491 Table 3. Sources and impact of exogenous microorganisms found in raw milk.¹¹

Sources	Impact			
	Food Technology	Health Promotion	Spoilage	Human illness
Udder and teat	<i>Lactococcus</i>	<i>Lactococcus</i>	<i>Pseudomonas</i>	<i>Listeria</i>
Hides	<i>Lactobacillus</i>	<i>Lactobacillus</i>	<i>Acinetobacter</i>	<i>Staphylococcus</i>
Feces	<i>Streptococcus</i>	<i>Streptococcus</i>	<i>Chryseobacterium</i>	<i>Escherichia</i>
Housing	<i>Leuconostoc</i>	<i>Leuconostoc</i>	<i>Clostridium</i>	<i>Campylobacter</i>
Bedding	<i>Enterococcus</i>	<i>Enterococcus</i>	Phages	<i>Mycobacterium</i>
Feed/Pasture	<i>Propionibacterium</i>	Yeast species		Fungi - aflatoxins
Air				
Water				

492

493 These studies have been recently covered in a complete and extensive review by Quigley and coworkers, and we
 494 refer the readers to their work for a detailed description of the recent literature on this subject.¹¹ In their review,

495 the authors describe the current knowledge on the microorganisms that can be found in raw milk of the main dairy
496 ruminant species.

497

498 **Conclusion**

499 The tremendous evolution of molecular and -omics technologies has enabled numerous breakthroughs in the
500 study of microbial communities, making us aware of the varied and complex assortments of microbes that inhabit
501 living animals, and of the reciprocal interactions that these entertain among themselves and with their hosts.
502 Following the unexpected acknowledgement that even the healthy mammary gland, and the milk contained within
503 it, are colonized by a variety of microbes, -omics approaches have already been used to enable their
504 characterization in humans, as well as to understand the role they play in both the mother and the offspring
505 health. Following the studies on raw milk microbial ecology, -omics approaches are now beginning to be applied
506 also to the *sensu stricto* milk microbiota of dairy ruminants. As a result, its relevant interactions with the
507 physiology and health of the lactating dam and the suckling offspring are becoming more and more evident. When
508 considering the significant economical implications that this can have for dairy ruminant farming, the application
509 of -omics sciences to the milk microbiota is expected to improve our understanding of open questions and
510 challenges such as the etiology and dynamics of sub-clinical and culture-negative mastitis, the impact of farming
511 management decisions on the mammary gland health and offspring health, the role of the intestine as a mastitis
512 pathogen reservoir, the development of novel strategies for preventing and contrasting mastitis management, and
513 the control of antibiotic resistance.

514

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