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The microbiota of dairy milk: a review

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Abstract:	Since the first paper published in 2011, the number of studies which have used High Throughput Sequencing methods for the characterization of the structure and function of the microbial communities of milk and dairy products has been increasing almost exponentially. Due to the lack of consensus on laboratory procedures, sequencing approaches and bioinformatic pipelines, the comparison of raw data from different studies is still difficult, but the availability of well-structured databases with raw and processed sequences has boosted our ability to get quantitative insights in the microbiota of these important food commodities. Methods for the characterization of microbial communities of dairy foods have evolved steadily. Metataxonomic approaches targeting the 16S RNA gene for bacteria and Internal Transcribed Regions or the 18S or 28S RNA genes for fungi are used more frequently than metagenomic and metatranscriptomics approaches. Unfortunately, Standard Operating Procedures developed for human and animal microbiome or environmental microbiome studies have not been adopted by microbiologists studying dairy products, and reaching a consensus on both wet- and dry laboratory procedures and on the use of internal standards, controls and mock communities would certainly be beneficial. Further methodological issues are the need for methods with high taxonomic resolution (like single molecule real time sequencing, or sequencing of targets other than RNA genes) and the selective evaluation of the active fraction of the microbiota. The microbiota of milk is highly complex and variable and is affected by a large number of different sources of contamination and by the selective effect of storage at low temperature and heat treatments. Although the issue of existence of a microbiota of the healthy udder has not been fully clarified, it is well known that mastitis greatly affects the composition of teat and milk microbiota, with changes which often produce lasting effects. Mastitis and dysbiosis of the teat and milk microbiota are strongly connected, and mastitis usually causes a dramatic reduction of microbial diversity. The microbiota of the teat surface in healthy lactating animals is highly diverse and variable and is affected by several factors, including breed, farming system, feed, health status, etc. Members of the genus Streptococcus and Staphylococcus are both highly prevalent and abundant, while the prevalence and abundance of other Bacilli, Clostridia, Bacteroidia, Erysipelotrichia and Gammaproteobacteria is lower and more variable. Streptococcus and Staphylococcus , together with Actinobacteria and lactic acid bacteria, will persist throughout production and storage of milk and will be eventually found in cheese. The composition of the microbiota of raw milk from individual animals or of composite samples from bulk tanks at the dairy farm is affected by a large number of interacting factors, including species, breed, farming practices, bedding, feeding, washing and disinfection of the teat surface, season of the year, lactation stage and geographic location within countries. Even so, a large number of bacterial genera are both prevalent and abundant in several studies on raw cow milk, and have also been frequently found in the milk of ewes and water buffaloes, thus supporting the idea of a common core microbiome in milk. Intra-mammary infections such as mastitis have a large, and sometimes lasting, impact on milk microbiota, generally causing a reduction of diversity and a shift in the composition of the microbiota, with clear evidences of dysbiosis, even in subclinical, asymptomatic infections. Contamination from equipment at the farm and at the processing plant, temperature

	and duration of storage, and heat treatments at the processing plant will result in further dramatic changes of the microbiota, with an increase in the proportion of psychrotrophic genera, including Pseudomonas, Acinetobacter, Lactococcus and Psychrobacter , while heat treatments result in an increase in relative proportion of thermoduric and spore-formers genera, like Thermus , Bacillus , Paenibacillus , Anoxybacillus and Turicibacter , some of which can cause spoilage of liquid milk and cheese. Overall, High Throughput Sequencing methods have confirmed what was previously known from low sensitivity cultivation based and cultivation independent techniques, but have also offered a deeper insight in the source of microorganisms in milk and on the factors which shape the microbial communities.

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Prof. Effie Tsakalidou
Editor
International Dairy Journal

Prof. Thom Huppertz
Editor in chief
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Potenza, 13/3/2020

Subject: submission of a critical review on milk microbiota

Dear Effie and Thom,
please find attached the revised version of manuscript:

The microbiota of dairy milk: a review

Eugenio Parente, Annamaria Ricciardi, Teresa Zotta

We did our best to answer to answer to reviewers' comments. The review is still very long but we feel that this is justified by the complexity of the subject.
As you can see from the text and from the answers to reviewers comments several reviews have been published on milk microbiota. Most focus on veterinary aspects and we do feel that our review, which focuses more on aspects related to contamination of milk from milking to transformation might be of interest of dairy scientists. In addition, the supplementary material contains by far the most extensive annotated list of published papers on the microbiota of milk and dairy products and the version of FoodMicrobionet supplied as supplementary data is an asset for whoever wants to perform quantitative meta-analyses on the microbiota of dairy products.
We are still struggling with the idea of doing a similar work on fermented milks and cheese but, as you can imagine, this is really a daunting task. Being virtually prisoners at home helps, but not much…

Regards

Eugenio

PS you may have noticed than my affiliation is changed. I am back at the School of Agriculture

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Responses and rebuttals.

General

The manuscript was shortened: the text is 3 pages shorter. This has been achieved by removing material which was repetitious or sections which had been covered in recent reviews. The section on methods was shortened and several other sections were rearranged to improve their flow. If need be the section on methods can be completely removed (this would reduce the manuscript by approximately 6-7 pages, including references which would be eliminated), although we think that it provides useful insights.
A further literature search was performed and around 40 citations were added to Supplementary Table 1. Only a few of this were related to the main subject of this review (milk). It is worth noticing that the literature list in this manuscript (and in supplementary material) is far more extensive than in other recent reviews and we feel it provides an excellent reference for perspective readers.
The affiliation of two of the authors changed and acknowledgements for partial funding was added.
More detail was added to the abstract.

During the reviewing and revision process yet another review on milk microbiome has been published (Oikonomou, G., Addis, M., Chassard, C., Nader-Macias, M., Grant, I., Delbès, C., Bogni, C., Loir, Y., Even, S. (2020). Milk Microbiota: What Are We Exactly Talking About? Frontiers in Microbiology 11(), 60. https://dx.doi.org/10.3389/fmicb.2020.00060). This, like other reviews on milk microbiome, has a veterinary/health science perspective. We still feel that our review does provide some added value because it has a better focus on what happens after milking and, most of all, because it provides a quantitative meta-study and a tool (DairyFMBN) that other scientists can use to formulate/substantiate their own hypotheses. In addition, we feel that the availability of a large amount of metataxonomic data in the database provided as supplementary data, which is unique to this manuscript, may be a significant asset to scientists interested in carrying out quantitative meta-studies on the microbiota of milk and cheese.

Responses (and rebuttals) to reviewer's comments

Reviewer #1:

Comment: Although the work aims to describe the complexity contained in a large amount of recently published works (the last 9 years of literature in the dairy sector), the reference list, despite long, appears incomplete and lacks relevant papers from existing literature, and this compromises the overall quality of the review.

Response: Thank you for pointing up this. We are embarrassed we missed so many papers. We have performed a more extensive literature search, using not only keyword searches but looking at cross references (several papers are not indexed with the appropriate keywords in bibliographic databases and/or had so few, if any, citations that were very difficult to locate) and have indeed found a significant number of other papers which had escaped prior searches. Apart from the three papers you pointed out (see below) we have added a number of others to the Supplementary Table (almost all on fermented milks or cheese). We hope they include if not all, almost all relevant papers from existing literature.

Comment: Regarding the scientific aspects of the review, analysis of data deriving from high-throughput sequencing surveys is complicated by some factors that are clearly highlighted by the authors, among which the intrinsic variability of the raw milk microbiota, the effect of confounding factors (type of milk, type of heat treatments, breed of animals, seasonality, different types of analysis), the different methods for data collection/analysis. For this reason, numerous paragraphs of the work are limited to the presentation of the results of other works, and only in some parts of the text there is an attempt to formulate hypotheses or a synthesis of knowledge, as you should expect in a review with such a generic title.
A number of works are compared and discussed more widely, also thanks to the help of graphics (Falardeau et al., 2019; Frétin et al., 2018; Cremonesi et al., 2018; Castro et al., 2019; Catozzi et al., 2017; Doyle et al., 2017a; Li et al., 2018; Skeie et al., 2019), and are described in more detail. On the other hand, the length of certain paragraphs makes it difficult to reach overall observations, and limits the purpose of this review.

Response: see general comments. Several sections were shortened and restructured and we have tried to provide a better synthesis of knowledge and to point out more clearly at the need for further research in selected areas.

Comment: I think that the review should include a paragraph where a short description of mastitis is given, since this condition greatly affects the microbiota of raw milk, and is discussed from many of the described works, yet never illustrated in the text.

Response: we are under the impression that most, if not all, readers of the International Dairy Journal would be well aware of what mastitis is and of its consequences on the dairy industry. Nonetheless, we have added a short description and a citation, as requested

Comment: Lines 113-115: the authors state: "regions of the genome of selected bacteria have been used as a target to study the microdiversity of selected species (…)" but omit to report at least 3 recent papers using such approach that can be easily found within literature (Xie et al., 2019; Levante et al. 2017, Milesi et al., 2018).

Response: https://dx.doi.org/10.3390/genes10070530, https://dx.doi.org/10.1016/j.ijfoodmicro.2017.07.002 and **Milani et al., 2018** https://dx.doi.org/10.1128/aem.00706-18, and Milani et al., 2019 https://dx.doi.org/10.3390/microorganisms7120599 have been added to the reference list and very briefly discussed (although as for many other of the methods using targets other than 16S there has been very little further use of these targets; time will tell).
A further recent paper (https://doi.org/10.1016/j.fm.2019.03.015 using 4 proteolysis related genes was also cited

Comment: Figure captions are too concise and lack to provide a clear description of the graphs presented.

Response: Most of the graphs reflect standard statistical and graphical analysis techniques in microbiome studies and should be easy to understand. However, we did our best to improve the captions

Minor
Various abbreviations in the text are not explicated: **please note that line numbers have changed and some text may have been removed/moved**
Line 517: Define NMDS **done**
Line 673: Define ASV **done (defined before)**
Line 831: Define CIP **done**
Line 920: Define HTST **done**
Supplementary Figure 1 - Abbreviations are missing a description in figure caption **done**
Line 407: Decreases should be decreased. Correct **the sentence was changed**
Line 705 - 706 Ps. psychrohila please correct **done**

Reviewer #2:

Comment: The review analyzes the methods used to characterize the microbiota of dairy milk using high throughput sequencing approaches.
The manuscript is well-written, but some paragraphs are too long and detailed.
In my opinion, the text should be shortened.

Response: The text has significantly been shortened (3 pages), as a consequence, some of the corrections you requested below may have ended up in paragraphs which were removed or significantly shortened.

Minor points (please note that due to substantial changes in the text line numbers have changed and some of the text you required to change has been deleted)
Line 26: "are more frequently used" instead of "are most frequently used" **done**
Line 28: "therefore" instead of "thus" **done**
Line 45: "season of the year AND geographic" **done**
Lines 86-87: "the characterization of the microbiota of milk (and dairy products) and on the microbiota of milk from cows and other dairy species" what is the difference? please rephrase **corrected**
Line 105: Add the abbreviation HTS the first time **do you mean add the full definition? It was already there**
Line 116: please remove the highlighter from ; **done**
Line 134: "is the most frequently" instead of "are the most frequently" **done**
Line 135: Full name for OTU, ASV and RDP the first time mentioned **done**
Lines 155-156: However, due to the decreasing costs in sequencing and the progress" instead of "However, because of the decreasing costs in sequencing and of the progress" **done**
Line 157: "as well as the staggering amount" instead of "and the staggering amount" **done**
Line 167: "metagenomics is the inference" instead of "metagenomics if the inference"? **done**
Line 182: Full name for PMA **done**
Lines 391-406: This paragraph should be shortened, e.g. data and references as supplementary table **done**
Line 407: "decreased diversity" instead of "decreases diversity" **done**
Line 410: please rephrase "if the relative abundance of one OTU increases the abundance of the others decreases" **done**
Line 468: "significant differences." instead of "significant differences" **done**
Line 484: "and Firmicutes as well as an increase in Proteobacteria and Bacteroidetes" instead

of "and Firmicutes and and increase in Proteobacteria and Bacteroidetes" **done**
Line 497: "and" not italics **done**
Line 513: "for teat" instead of "fort teat" **done**
Line 540: <2x105 instead of <200,000 and >1x106 instead of >1,000,000 **done**
Line 590: "combined contamination" instead of "combination contamination"? **rephrased**
Line 591: "several anaerobic Firmicutes and Bacteroidetes genera were the most abundant in the" instead of "several anaerobic Firmicutes and Bacteroidetes were the most abundant genera in the" **done**
Lines 617-618: "while for individual milk samples of cows fed on pasture" instead of "while for individual milk samples of milk fed on pasture"? **done**
Line 618: "found (Doyle et al., 2017b)" instead of "found; Doyle et al., 2017b)" **done**
Line 621: "with a culturomics approach" instead of "using a culturomics approach". You had the word "using" 2 times **done**
Line 625: "Lactococcus, one Serratia" instead of "Lactococcus and one Serratia" **done**
Line 641: "SCC" instead of "somatic cell counts" **we would like to leave it as it is because the full sentence is bacterial and somatic cell counts**
Line 644: "somatic cell counts" is no needed **OK**
Line 656: "and Streptococcus" no italics for "and" **done**
Lines 669-670: "and several the abundance of several bacterial genera" please rephrase **done**
Line 673: "were also significantly different" instead of "was also significantly different" **done**
Line 674: "had the highest" instead of "has the highest" **done**
Line 697: do you have the complete name of "AT" metagenomics in the text? **AT defined at the beginning and used to replace amplicon targeted elsewhere (except in the abstract)**
Line 731: "was lower" instead of "was lowest" **done**
Line 764: "Proteobacteria" instead of "Proteo- bacteria" **done**
Line 764: "Streptococcus, Staphylococcus and Clostridiales" instead of "Streptococcus and Staphylococcus and Clostridiales" **done**
Line 792: "and Clostridium" no italics for "and" **done**
Line 792: "On the other" instead of "In the other" **In one ... in the other this refers to the two silos. We think that "In" is better here**
Line 796: "due to the variability" instead of "because of the variability" **done**
Line 797: "non-starter genera" instead of "non-starter species" as Streptococcus, Staphylococcus, Macrococcus and Corynebacterium are genera. Or non-starter species of the genera Streptococcus, Staphylococcus, Macrococcus and Corynebacterium **done**
Line 808: "when the time a given piece of" please rephrase **done**
Line 831: Complete name for "CIP" **done**
Lines 834-835: "On the latter spore formers, including Bacillus and Anoxybacillus, were abundant." please rephrase **done**
Line 912: Add the abbreviation "GI" the first time mentioned gastrointestinal **done and replaced elsewhere**
Line 920: Add the abbreviation HTST the first time mentioned the complete name **done**
Line 933: "and ewe's milk cheeses" instead of "ewe's milk cheeses" **done**
Line 949: "sequencing platforms, methods and bioinformatic" instead of "sequencing platforms and methods and bioinformatic" **done**
Line 962: "for the study" instead of "to the study" **done**
Line 970: "might contribute microorganisms relevant for" please rephrase **done**

The microbiota of dairy milk: a review

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Abstract.

Since the first paper published in 2011, the number of studies which have used High Throughput Sequencing methods for the characterization of the structure and function of the microbial communities of milk and dairy products has been increasing almost exponentially. Due to the lack of consensus on laboratory procedures, sequencing approaches and bioinformatic pipelines, the comparison of raw data from different studies is still difficult, but the availability of well-structured databases with raw and processed sequences has boosted our ability to get quantitative insights in the microbiota of these important food commodities.

Methods for the characterization of microbial communities of dairy foods have evolved steadily. Metataxonomic approaches targeting the 16S RNA gene for bacteria and Internal Transcribed Regions or the 18S or 28S RNA genes for fungi are used more frequently than metagenomic and metatranscriptomics approaches. Unfortunately, Standard Operating Procedures developed for human and animal microbiome or environmental microbiome studies have not been adopted by microbiologists studying dairy products, and reaching a consensus on both wet- and dry laboratory procedures and on the use of internal standards, controls and mock communities would certainly be beneficial. Further methodological issues are the need for methods with high taxonomic resolution (like single molecule real time sequencing, or sequencing of targets other than RNA genes) and the selective evaluation of the active fraction of the microbiota.

The microbiota of milk is highly complex and variable and is affected by a large number of different sources of contamination and by the selective effect of storage at low temperature and heat treatments. Although the issue of existence of a microbiota of the healthy udder has not been fully clarified, it is well known that mastitis greatly affects the composition of

45 teat and milk microbiota, with changes which often produce lasting effects. Mastitis and
46 dysbiosis of the teat and milk microbiota are strongly connected, and mastitis usually causes
47 a dramatic reduction of microbial diversity. The microbiota of the teat surface in healthy
48 lactating animals is highly diverse and variable and is affected by several factors, including
49 breed, farming system, feed, health status, etc. Members of the genus *Streptococcus* and
50 *Staphylococcus* are both highly prevalent and abundant, while the prevalence and
51 abundance of other *Bacilli, Clostridia, Bacteroidia, Erysipelotrichia* and
52 *Gammaproteobacteria* is lower and more variable. *Streptococcus* and *Staphylococcus*,
53 together with *Actinobacteria* and lactic acid bacteria, will persist throughout production and
54 storage of milk and will be eventually found in cheese. The composition of the microbiota of
55 raw milk from individual animals or of composite samples from bulk tanks at the dairy farm
56 is affected by a large number of interacting factors, including species, breed, farming
57 practices, bedding, feeding, washing and disinfection of the teat surface, season of the year,
58 lactation stage and geographic location within countries. Even so, a large number of
59 bacterial genera are both prevalent and abundant in several studies on raw cow milk, and
60 have also been frequently found in the milk of ewes and water buffaloes, thus supporting
61 the idea of a common core microbiome in milk. Intra-mammary infections such as mastitis
62 have a large, and sometimes lasting, impact on milk microbiota, generally causing a
63 reduction of diversity and a shift in the composition of the microbiota, with clear evidences
64 of dysbiosis, even in subclinical, asymptomatic infections.
65 Contamination from equipment at the farm and at the processing plant, temperature and
66 duration of storage, and heat treatments at the processing plant will result in further
67 dramatic changes of the microbiota, with an increase in the proportion of psychrotrophic
68 genera, including *Pseudomonas, Acinetobacter, Lactococcus* and *Psychrobacter*, while heat

69 treatments result in an increase in relative proportion of thermoduric and spore-formers
70 genera, like *Thermus*, *Bacillus*, *Paenibacillus*, *Anoxybacillus* and *Turicibacter*, some of which
71 can cause spoilage of liquid milk and cheese.
72 Overall, High Throughput Sequencing methods have confirmed what was previously known
73 from low sensitivity cultivation based and cultivation independent techniques, but have also
74 offered a deeper insight in the source of microorganisms in milk and on the factors which
75 shape the microbial communities.
76
77 **1. Introduction**.
78 In the last ten years more than 165 papers in which the microbiota of dairy products has
79 been characterized using High Throughput Sequencing (HTS) approaches have been
80 published (Supplementary Table 1). Milk and dairy products are therefore the foods for
81 which most data on the structure and functions of microbial communities involved in animal
82 health, safety, fermentation and spoilage are available (De Filippis, Parente, & Ercolini,
83 2017). Literature on the microbiome of the bovine udder and its role in the health and well-
84 being of dairy cows (Derakhshani et al., 2018a; Rainard, 2017), on the microbiome of milk
85 (Addis et al., 2016; Oikomonou et al., 2020; Quigley et al., 2013; Tilocca et al., 2020), and
86 cheese (Afshari, Pillidge, Dias, Osborn, & Gill, 2018; Yeluri Jonnala, McSweeney, Sheehan, &
87 Cotter, 2018) has been recently reviewed. However, the most recent reviews on milk
88 microbiota focus on aspects related to the role of udder and milk microbiota on the health
89 of dairy animals (Addis et al., 2016; Oikomonou et al., 2020) and on methods (Tilocca et al.,
90 2020), rather than on aspects relevant to the safety and quality of milk for the production of
91 dairy products. While the teat surface and interior are certainly a source of bacteria which
92 may play a role in safety and quality of dairy products, other factors, including further

93 contamination from milking, farm and processing plant equipment environments, growth
94 during storage and destruction of microorganisms by pasteurization will give a significant
95 contribution in shaping the microbiota of dairy and cheese milk.
96 In addition, the availability of raw or processed sequencing data in large databases (QIITA,
97 https://qiita.ucsd.edu/; MGnify, https://www.ebi.ac.uk/metagenomics/; FoodMicrobionet,
98 Parente et al., 2016a; Parente, De Filippis, Ercolini, Ricciardi, & Zotta, 2019) offers
99 unprecedented opportunities for exploring the food microbiome in meta-studies. Recently,
100 we have released version 3.1 of FoodMicrobionet (Parente et al., 2019), a database of
101 studies on the bacterial microbiota of foods, which is easily accessible through an
102 interactive app. The database is steadily growing and its latest version includes, at the time
103 of writing this article, 42 studies on the bacterial microbiome of dairy products (see
104 supplementary material and supplementary data).
105 The objective of this work is to critically review the recent knowledge on methods used in
106 the characterization of the microbiota of milk from cows and other dairy species, on its
107 potential sources and on its dynamics during storage, transport and processing in liquid
108 pasteurized milk, and make use of the data available in FoodMicrobionet to carry out meta-
109 analyses on studies on the dairy milk microbiota.
110
111 **2. Milk and dairy products illuminated: the evolution of methods**.
112 All three HTS approaches (amplicon sequencing, shotgun metagenomics and
113 metatranscriptomics; Yeluri Jonnala et al., 2018) have been used, alone or in combination,
114 to characterize the microbiome of milk. Methods for nucleic acid extraction, wet laboratory
115 stages, sequencing and bioinformatic analyses vary greatly among studies (Supplementary
116 Table 1), and current approaches have been outlined in a recent review (Tilocca et al.,

2020). We will briefly discuss some aspects which shape our ability to understand dairy microbiota by nucleic acid targeted omics approaches.

2.1 Amplicon sequencing.

Amplicon sequencing is by far the most used approach for metataxonomic studies. For bacteria, the 16S RNA gene has been most frequently targeted, but a few studies have used 16S RNA as target (Supplementary Table 1), in order to focus on the "active" fraction of the microbiota. The variable regions targeted also vary, with V1-V3 being most frequently used before the demise of the Roche 454 platforms, and V3-V4 and V4 being most frequently used with the Illumina platforms. As to fungi, Internal Transcribed Spacers ITS1 and ITS2 have been most frequently used as a target, while the use of regions of 18S and 28S is far less common. Due to the relatively short length of the regions targeted, the taxonomic resolution is often limited to the genus or, less frequently, to the species level, depending on the length and quality of sequences. However, most recently the use of single molecule sequencing (Jin et al., 2018; Li et al., 2017; Mo et al., 2019; Yang et al., 2019; Yu et al., 2018), the ability to detect oligotypes (Kamimura, De Filippis, Sant'Ana, & Ercolini, 2019) and Amplicon Sequence Variants (ASV, Callahan et al., 2016), or the availability of optimized databases (Meola et al., 2019) has been claimed to increase taxonomic resolution. Resolution at the species level or below is critical in the analysis of the microbiota of dairy products, because species belonging to the same genus may have a very different significance for the quality and safety of dairy products. For example, several genera, like *Streptococcus, Staphylococcus and Corynebacterium*, include both pathogenic and starter and non-starter microorganisms, while others, like *Lactobacillus* include species which are

140 either starter or non-starter bacteria. The use of the methods with the highest taxonomic
141 resolution should therefore be encouraged.
142 A few other coding or non-coding regions of the genome of selected bacteria have been
143 used as a target to study the micro-diversity of selected species, with a variable degree of
144 success in terms of accuracy and sensitivity: *Streptococcus thermophilus* (*lacSZ*, De Filippis,
145 La Storia, Stellato, Gatti, & Ercolini, 2014; *serB*, Parente et al., 2016b; Ricciardi et al., 2016),
146 amine producing lactic acid bacteria (*tdc* and *hdc*, O'Sullivan et al., 2015), *Lactococcus lactis*
147 (*purR, epsD*, Frantzen, Kleppen, & Holo, 2018), genus *Lactobacillus* (*groEL*, Jiang et al., 2019;
148 ITS, Milani et al., 2018), *L. casei* group (*spxB*, Levante et al., 2017), genus *Bifidobacterium*
149 (Milani et al., 2019), and members of the *Bacillus cereus* group (*panC, glpT*, Porcellato,
150 Aspholm, Skeie, & Mellegård, 2019). Proteolysis related genes of LAB (*prtP, pepN, pepX,*
151 *bcaT*) have also been used as target for metatranscriptomic studies (Pangallo et al., 2019).
152 The usefulness of protein coding genes might be limited to selected species, but it is a
153 cheaper alternative to shotgun whole genome sequencing, which might detect only the
154 dominating strains in microbial communities.
155 To date, no Standard Operating Procedures (SOPs) for AT studies in foods exist. A discussion
156 on the best approach for AT studies for milk and dairy foods is beyond the scope of this
157 review, and the factors affecting the results have been reviewed recently (Pollock,
158 Glendinning, Wisedchanwet, & Watson, 2018). However, it is quite clear that the
159 development of SOPs, and the use of negative controls and mock communities or internal
160 standards would be highly desirable and should be requested by editors and reviewers of
161 scientific journals.
162
163 **2.2 Shotgun approaches**.

To date, shotgun metagenomic studies of dairy products are comparatively rare (only 11% of studies listed in Supplementary Table 1) and meta-transcriptomic studies are even less frequent (only 4%). Due to the decreasing costs in sequencing and the progress in the development of bioinformatic pipelines for taxonomic annotation and genome reconstruction, as well as the staggering amount of information they can provide (Tilocca et al., 2020; Yeluri Jonnala et al., 2018) they are likely to become more frequent. Pipelines for metagenomic annotation and data visualization have been reviewed recently (Breitwieser, Lu, & Salzberg, 2017; Quince, Walker, Simpson, Loman, & Segata, 2017; Sudarikov, Tyakht, & Alexeev, 2017), and the choice of the pipeline has been shown to affect the results, especially in low diversity samples which are typical of cheese and fermented milks (Walsh et al., 2018). An alternative to the use of shotgun metagenomics is the inference of metagenomes using bioinformatic tools such as PICRUSt (and its most recent iteration, PICRUSt2, Douglas et al., 2019). Although this tool has performed relatively well in benchmarking (Douglas et al., 2019) it has been used relatively rarely for microbial communities of milk and dairy products (Cremonesi et al., 2018; Li et al., 2018; Ramezani, Hosseini, Ferrocino, Amoozegar, & Cocolin, 2017; Stellato, De Filippis, La Storia, & Ercolini, 2015; Yang et al., 2019).

2.3 A question of life and death.

A further issue related to the experimental approach is the inability of methods targeting DNA to distinguish active/viable members of microbial communities from those which are dead/inactive and contribute little or nothing to fermentation or spoilage. Studies using both DNA and RNA as a target are relatively rare (see Supplementary Table 1; De Filippis, Genovese, Ferranti, Gilbert, & Ercolini, 2016; Kastman et al., 2016; Sattin et al., 2016b) and

188 the use of dyes which prevent the PCR amplification of DNA from dead cells (or more
189 properly, cells with a damaged membrane), such as PMA (propidium monoazide, Emerson
190 et al., 2017), is only marginally more frequent (Erkus et al., 2016; Kable, Srisengfa, Xue,
191 Coates, & Marco, 2019; Mo et al., 2019; Porcellato & Skeie, 2016). Extensive benchmarking
192 it still needed to rule out biases due to differential ability of PMA to penetrate cell
193 membranes (Emerson et al., 2017). At any rate, whenever both the “active” and “inactive”
194 fraction of the microbiota have been targeted, significant differences have been found
195 between the two, usually with lower diversity in the “active” microbiota.
196
197 **2.4 Of experimental design (or lack thereof).**
198 Regrettably, the overwhelming majority of the studies listed in Supplementary Table 1 are
199 descriptive in nature, and even when inferential methods are used, their effectiveness in
200 detecting significant differences (because of high natural variability, and potentially high
201 type I and/or type II errors) is dubious. In fact, only in a very few cases experimental designs
202 have been used (De Filippis et al., 2016; Doyle, Gleeson, O'Toole, & Cotter, 2017b; Ganda et
203 al., 2016; Ganda et al., 2017; Guzzon et al., 2017; Porcellato & Skeie, 2016), and for most
204 studies the approach is quasi-experimental in nature, with insufficient randomization,
205 blocking and control of confounding factors. The issue of sampling effort is also critical: the
206 range for the number of samples analysed in studies shown in Supplementary Table 1 is 1-
207 1674, but 50% of the studies have used 24 samples or less. Because of the very high
208 variability of the microbiota of raw milk, due to seasonal, geographical and technological
209 factors (Kable et al., 2016; Skeie, Håland, Thorsen, Narvhus, & Porcellato, 2019) one really
210 wonders if, especially for raw milk fermented milks or cheeses produced in artisanal plants,
211 low (<50) sample numbers and low numbers of sampling locations (farms, cheesemaking

plants) are adequate to cover the expected diversity, and, even in larger studies, utmost care should be dedicated to the design of the experiments and to the analysis of the data using appropriate inferential methods.

The issue of microbial interactions in dairy ecosystems is of great interest for both scientific and practical reasons, but it has been addressed only infrequently (Frétin et al., 2018; Murugesan et al., 2018; Parente et al., 2016a; Parente, Zotta, Faust, De Filippis, & Ercolini, 2018; Wolfe, Button, Santarelli, & Dutton, 2014). Detecting true interactions among species presents several challenges (Layeghifard, Hwang, & Guttman, 2017). Unfortunately, most studies are cross-sectional in nature, and, even when they are longitudinal, the number and distribution of sampling times is insufficient for model-based methods for detection of microbial interactions (Faust & Raes, 2012). More research is definitely needed in this area and combinations of culture independent and dependent approaches (Wolfe et al., 2014) are needed to validate the nature of the microbial interactions and evaluate their significance for the quality of dairy foods.

3. The microbiota of milk: from the teat to the carton.

A large amount of data on the microbiota of raw or pasteurized milk composition or milk contact surfaces (teat and udder surface, tanks and silos at the dairy farm or at the processing plant, etc.) are available for milk from practically all dairy animals (mostly cow, but also ewes, goats, water buffaloes, yaks, camels), either as a part of studies specifically focusing on milk quality or as a part of studies on cheese microbiota (see Supplementary Table 1). Milk microbiota is undoubtedly complex and highly variable and in most studies the sampling effort is limited or the approach is merely descriptive, thus obscuring causal relationships. However, a few large, designed or quasi-experimental studies addressing one

of more aspects (effect of cow's health, feeding, farming, breed, season, geographical source of milk, effect of contamination during the production and distribution chain, effect of storage temperature) are available, and combination of data from different studies in meta-analyses may help in identifying a core microbiota or detecting wider geographical or temporal trends. In the following sections, we will review the composition of the microbiota of milk as it travels from the udder to the storage tank in processing plants and, finally to the carton of pasteurized milk.

3.1 Raw milk

3.1.1 Inside and outside the udder.

The first sources of microorganisms in raw milk are, quite obviously, the udder and the teat surface (Derakhshani, Plaizier, De Buck, Barkema, & Khafipour, 2018b). The composition of the mammary microbiota in ruminants has been recently reviewed (Derakhshani et al., 2018b; Rainard, 2017), and the mechanisms which determine its composition and dynamics are outside the scope of this review. While it is still somewhat controversial if a microbiota of the healthy mammary gland exists or if it is the result of contamination during sampling (Derakhshani et al., 2018a; Rainard, 2017), it is clear that the teat canal and apex may be colonized by bacteria and that these bacteria may contribute to the homeostasis of this niche or cause infection of the mammary gland. In fact, most of the studies using milk from individual quarters or individual animals have focused on the effect of disease (mastitis, either clinical or subclinical, subclinical acidosis) on the microbiota of milk from cows (see below and Supplementary Table 4 for a list of studies), while only a few studies are available on ewe (Castro et al., 2019; Esteban-Blanco et al., 2019), goat (McInnis, Kalanetra, Mills, & Maga, 2015) or on water buffalo milk (Catozzi et al., 2017; Patel et al., 2016; Patel, Kunjadia,

Koringa, Joshi, & Kunjadiya, 2019). This is justified by the economical and practical importance of mastitis, which is the most important disease in dairy animals in terms of both impact on milk production and quality and in terms of animal well being (Ruegg, 2017). A few studies have analysed the milk from individual healthy cows and the teat surface to investigate the sources of microorganisms, beneficial or not, and their potential effect on cow's health (Cremonesi et al., 2018; Falentin et al., 2016; Frétin et al., 2018). It is important to remember that sampling and disinfection may dramatically affect the composition of the microbiota of individual milk samples (Metzger et al., 2018a), that milk obtained aseptically or by abiding to hygienic practices has usually low counts (often less than $1x10^4$ cfu/ml), and that contamination might significantly affect the results of AT studies for low count samples (Dahlberg et al., 2019). In addition, a high number of amplification cycles may be necessary for teat milk obtained aseptically (Metzger et al., 2018a) and success rate of amplification may be relatively low for milk obtained aseptically from healthy quarters. The results of some early studies on low counts milk which did not include negative control or proper treatment for removing contamination might be therefore slightly biased.

The teat interior and surface are among the most significant sources of microorganisms for individual milk samples, and microorganisms from these sources may persist during transport and transformation of milk. On the other hand, mastitis is likely to have a larger impact compared to external contamination from the teat interior and surface. In a carefully controlled experiment (Andrews, Neher, Weicht, & Barlow, 2019), intramammary infection was found to dramatically affect the composition of the microbiota of teat cistern milk, which, compared to the milk of healthy animals, had a lower bacterial diversity, was more variable among different cows and was often enriched in pathogenic bacteria belonging to the same genus of those isolated by culturing from the affected quarters, supporting the

hypothesis that mastitis is correlated with dysbiosis of the mammary gland. In addition, the microbiota of cistern milk and teat apex of infected quarters was more similar compared to healthy quarters, while the microbiota of cistern milk and teat apex in healthy animals was also more variable in time, suggesting that it was more affected by external contamination. The effect of mastitis on the microbiota of the teat apex may be detectable even long after the demise of symptoms. Falentin et al. (2016) examined the microbiota of the teat apex (foremilk + teat apex swabs) for healthy Holstein cows from a single experimental farm. The cows had different previous histories of mastitis. The cow's teat canal microbiota was highly variable (even within the same cow) and teat microbiota from cows without and with a previous history of mastitis could be clearly differentiated, while microbiota of cows with an uncertain status tended to cluster with those of cows with a previous history of mastitis (cluster 1). Discrimination between the two clusters was due to a higher abundance of members of class *Bacilli* (with *Staphylococcus aureus* and *Staph. equorum* as the most prevalent and abundant species) in cluster 1 and higher relative abundance of a diverse array of genera belonging to the phylum *Actinobacteria* (including *Bifidobacterium*), class *Clostridia*, phylum *Bacteroidetes*, including several genera associated with the gastrointestinal (GI) tract, in cluster 2. The origin of these microorganisms may be therefore the teat canal itself, in the case of mastitis agents like *Staph. aureus,* while the potential origin of bacteria associated with the GI tract is uncertain. The procedure used in this study included thorough washing and sanitation before sampling of the teat canal, and may have reduced contamination from loosely attached bacterial cells originating from faeces or from the environment, but it might not have prevented it completely.

Data on the microbiota of the cow teat skin surface in animals showing no signs of clinical mastitis are available for three more studies (Doyle et al., 2017b; Falardeau, Keeney, Trmčić,

Kitts, & Wang, 2019; Frétin et al., 2018). Teat skin microbiota was highly diverse and variable: a prevalence and abundance plot with data extracted from FoodMicrobionet is shown in Supplementary Figure 1, and a table showing the top 50 taxa in terms of prevalence and relative abundance is provided as Supplementary Table 2. Frétin et al. (2018) sampled the teat skin of cows belonging to two breeds (Holstein and Montbeliarde) under two different farming regimes (extensive EXT, with cows feeding exclusively on pasture, and semi-extensive, SEMI, with cows feeding on pasture and concentrate) prior to evening milking (i.e. prior to washing). As a consequence, the results were probably affected by both autochthonous species and by contaminants from faeces and the farm/pasture environment. More than 300 Operational Taxonomic Units (OTUs) and 98 genera were identified, including both *Actinobacteria* and *Clostridia* as abundant members. Some OTUs (twelve, including members of the genera *Brevibacterium, Lactococcus, Lactobacillus, Streptococcus, Staphylococcus, Macrococcus, Escherichia*) persisted throughout the process, from teat skin to ripened cheese, while 201 were specific to teat skin. The microbiota of teat skin was most affected by the grazing system and by the season of sampling (July vs. September). Falardeau et al. (2019), in a large source tracking study, confirmed that teat skin (sampled prior to washing and milking) had a high microbial diversity, which however was comparable to that of teat milk and tank milk. *Clostridiales* were the most abundant members of the microbiota (17-41%), but *Actinobacteria* (*Corynebacterium* and *Brevibacterium*), *Bacteroidetes* (*Bacteroides* and *Alistipes*), and *Proteobacteria* (including *Pseudomonas* and *Acinetobacter*) were all found in the subdominant microbiota. The main difference between these two studies is the higher relative proportion of *Clostridia* and *Bacteroidia* in Falardeau et al. (2019) and the higher proportion of *Bacilli* and *Erysipelotrichia* in Frétin et al. (2018). The results of Falardeau et al. (2019) are similar to

those of Doyle et al. (2017b) who analysed, in a systematic study, the effect of farming (indoor vs outdoor), and cleaning procedure on the microbiota of teat surface, individual and bulk milk samples, and confirmed that teat swab microbiota is highly diverse and significantly affected by both farming practices and cleaning.

3.1.2 The microbiota of teat milk.

The microbiota of samples obtained by milking individual quarters or individual animals has been analysed in several studies, focusing on the effect of disease and/or disease treatment (mastitis: Angelopoulou et al., 2019; Bhatt et al., 2012; Ganda et al., 2016, 2017; Hoque et al., 2019; Kuehn et al., 2013; Metzger et al., 2018b; Oikonomou, Machado, Santisteban, Schukken, & Bicalho, 2012; Oikonomou et al., 2014; Oultram, Ganda, Boulding, Bicalho, & Oikonomou, 2017; Pang et al., 2018; Taponen et al., 2019; Vasquez et al., 2019; subclinical acidosis: Zhang, Huo, Zhu, & Mao, 2015), on sampling (Metzger et al., 2018a) or on the tracking of sources of contaminations (Cremonesi et al., 2018; Dahlberg et al., 2019; Doyle et al., 2017b; Falardeau et al., 2019; Metzger et al., 2018a). Comparing these studies is difficult, because of differences in practically all the factors which are known to affect the composition of microbiota (breed, health status, farming, bedding, feeding, lactation stage, etc.), in sampling, in methods used for the analysis of the microbiota. In particular, the composition of the microbiota of individual milk samples has been proven to be strongly dependent on the sampling procedure used (Metzger et al., 2018a) and sampling procedures must be carefully documented to allow the interpretation of results from different studies.

However, several findings have been confirmed by multiple studies: a. the microbiota of milk of healthy animals is highly diverse and variable; b. mastitis and other clinical and

subclinical conditions strongly affect the composition and diversity of the microbiota; c. a large number of other factors, including breed, parity, farming systems, feeding, bedding, season of the year, and days in milking significantly affect the composition of milk microbiota.

To illustrate the variability of the composition of teat milk samples we have compared the results for milk from healthy cows from two studies (one illustrating he effect of breed and days in milking: Cremonesi et al., 2018; the other on contamination sources from farm to fork: Falardeau et al., 2019) for which sequences are publicly available and which are included in FoodMicrobionet. The assembly of taxa for the two studies include 1184 genera (most of which with very low prevalence and abundance) belonging to 115 classes of 45 phyla. A bar plot of the relative abundance of the 20 most abundant and prevalent taxa is shown in Figure 2, while a prevalence and abundance plot and the data on prevalence on the top 50 most abundant taxa are shown in Supplementary Figure 2 and Supplementary Table 3 respectively. In both studies the most prevalent and abundant taxa belong to phyla *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, but large differences are evident both within and between studies. Cremonesi et al. (2018) compared Holstein Friesians with an Italian breed (Rendena, which shows lower prevalence of mastitis) from the same farm, from drying off, to colostrum stage and to late lactation. The number of samples in this study was relatively low, but variation over time was observed for both breeds and beginning of lactation had a significant impact on the microbiota. The composition of the microbiota of Rendena cows was more stable, and significantly different from that of Holstein cows. *Streptococcus* was the most prevalent and abundant genus in both breeds and together with *Lactobacillus* was the only genus shared by all samples. On the other hand, Falardeau et al. (2019) found that *Actinobacteria* (with genera *Kocuria, Dermatococcus* and *Dietzia*)

were by far the dominating phylum in teat milk, while *Firmicutes* (with *Lactococcus* and *Clostridium* XI as most abundant genera) and *Proteobacteria* (with *Enhydrobacter* and *Psychrobacter* as most abundant genera) were less abundant. Notably, the microbiota of teat milk for this study was quite different from that of teat skin (see above).

A seasonal effect on the composition of teat milk was also found by Metzger et al. (2018b) who monitored for over 150 days the composition of microbiota of teat milk obtained from healthy cow quarters, newly infected quarters and quarters with chronic inflammation or clinical mastitis. They found a strong effect of season of the year and of time of lactation, which resulted in increased richness from Winter to Summer in all cohorts, and in significant changes in the relative abundance of 20 OTUs (including *Fibrobacter*, *Corynebacterium*, *Arthrobacter*, *Bacteroidetes*), which they attributed to contamination from sand bedding and/or to physiological changes during lactation (*Bacteroidetes*). Interestingly, milk from quarter with chronic inflammation showed the greatest seasonal changes.

Much emphasis has been given to the comparison of the microbiota of individual milk samples from healthy cows and cows with subclinical or clinical mastitis, and in the latter, for culture positive and negative samples. Differences between healthy and diseased quarters are almost always significant (even for culture negative quarters, Kuehn et al., 2013; Oikonomou et al., 2012) and analysis of the microbiota may contribute to the diagnosis in quarters with subclinical mastitis. Dominating bacteria in mastitic milk change quite substantially in different studies, depending on the number of samples tested and on the causative agents in individual cows (Supplementary Table 4). Mastitis agents are usually abundant components of the microbiota in mastitic milk, but not necessarily the most abundant (Oikonomou et al., 2012) and their abundance may change over time (Ganda et al., 2016, 2017). In several cases, association of two or more agents of mastitis are found

(Angelopoulou et al., 2019; Bhatt et al., 2012; Oikonomou et al., 2012). Potential bacterial pathogens as *Str. uberis* and *Staph. aureus* might also be found with high prevalence in milk from healthy quarters (Oikonomou et al., 2014) while, on the other hand, some mastitis agents like *Escherichia coli* or *Klebsiella* were never found in samples from healthy quarters: this has led to speculate that bacterial mastitis can be considered as a dysbiosis rather than a primary clinical infection. This hypothesis may also be supported by the fact that species which most contribute to the discrimination between mastitic and non mastitic milk are not necessarily mastitis pathogens, although they might have been occasionally associated with mastitis (Kuehn et al., 2013). A recent study (Angelopoulou et al., 2019) has confirmed the complex, polymicrobial nature of mastitis and showed that culture based approaches and AT metagenomics complement each other. In an attempt to evaluate if significant associations could be detected in this data set between known mastitis pathogens and other bacteria, we carried out inferences of microbial association networks as described in Parente et al. (2018) (Supplementary Figure 3). Only two modules were detected, one including *Escherichia/Shigella* and the other *Staphylococcus*, two genera which include species identified by culturing as potential agents of mastitis. The two modules did not overlap but, due to the low number of samples, it is not clear if this reflect true differences in the microbiota of milk connected to infection from either *Escherichia* or *Staphylococcus*. Another frequently observed consequence of inflammation due to mastitis is a decreased diversity in the microbiota (Andrews et al., 2019; Bonsaglia et al., 2017; Ganda et al., 2016, 2017; Kuehn et al., 2013; Metzger et al., 2018b; Taponen et al., 2019; Vasquez et al., 2019), although the lower number of OTUs in mastitic milk may be simply due to the compositional nature of AT data (when the relative abundance of one OTU increases the relative abundance of the others decreases and may fall below detection limits). Decrease in alpha

428 diversity is a clear indication of a dysbiosis and in at least one study the largest reduction
429 was associated with the largest decrease in milk production (Vasquez et al., 2019). However,
430 distinguishing samples with subclinical mastitis from samples from healthy cows might not
431 be straightforward using descriptive techniques when quasi-experimental designs are used
432 (Pang et al., 2018), and in some non-severe cases it might be difficult to associate the
433 mastitis condition with significant changes in abundance of bacterial families (Ganda et al.,
434 2016; Vasquez et al., 2019). The potential impact of treatments used to control mastitis at
435 dry off is of both scientific and practical importance. Ganda et al. (2016) compared two
436 groups of Holstein cows which had been randomly allocated to a control group or to a group
437 treated with 5 day intramammary treatment with Ceftiofur (a third generation
438 cephalosporin). Antibiotic treatment did not affect the clinical or bacteriological cure rates,
439 nor bacterial clearance or bacterial load, but did reduce over time the relative abundance of
440 *Enterobacteriaceae* in quarters with *E. coli* mastitis. However, no clear effect was observed
441 on quarters without a culture diagnosis. In a subsequent study, the same group (Bonsaglia
442 et al., 2017) showed that treatment with the same antibiotic at dry-off did not change the
443 incidence of mastitis in the first 60 days post-partum for healthy cows, nor did it significantly
444 affect the composition of the milk microbiota after 7 days. The authors concluded that this
445 type of treatment does not cause dysbiosis but it does not have any therapeutic value for
446 healthy cows. However, large individual variability may have increased type II error and
447 prevented the detection of significant differences. Using the same antibiotic in a challenge
448 study with *E. coli,* Ganda et al. (2017) observed that, independently of antibiotic use, normal
449 microbiota re-established itself over a 216 h sampling time. This study is an excellent
450 example of the dynamic changes of the composition of milk microbiota prior and during
451 infection with a mastitis pathogen and shows how, after a disturbance, the microbiota may

452 shortly revert to its initial status (or to a status which is not statistically different from the
453 initial one).
454 Studies on the effect of mastitis on milk microbiota for other species are relatively rare and
455 somewhat limited in scope. Catozzi et al. (2017) investigated the teat milk microbiota in 137
456 samples of water buffalo milk obtained from healthy quarters and from quarters with
457 evidences of clinical and subclinical mastitis from 88 farms of limited area in Southern Italy.
458 They identified a core microbiota of fifteen genera, including genera commonly found in
459 cow's milk (see Figure 3). Both subclinical and clinical mastitis significantly changed the
460 composition of the microbiota, usually with a relative decrease in psychrotrophic
461 microorganism (*Pseudomonas, Psychrobacter*), a decrease in *Actinobacteria* and *Firmicutes*
462 and increase in *Proteobacteria* and *Bacteroidetes*, and clinical mastitis resulted in a decrease
463 in alpha diversity. In general, the strongest differences were found between samples with
464 low ($<1x10^5$/ml) and high ($0.5x10^6$ to $>1x10^6$) somatic cell counts (SCC). Culture results for
465 quarters with clinical mastitis confirmed the occurrence of common mastitis pathogens,
466 such as *Staph. aureus, T. pyogenes, Str. agalactiae* (alone or in combination with *S. aureus*),
467 *Ps. aeruginosa* and some coagulase negative staphylococci. Similar results (reduced
468 diversity, ability to discriminate samples from healthy animals from those with subclinical
469 and clinical mastitis) have been found for water-buffalo milk in India (Patel et al., 2016,
470 2019).
471 Results on the effect of mastitis on ewe's milk microbiota are somewhat contradictory.
472 Esteban-Blanco et al. (2019) investigated the microbiota of teat milk obtained from a
473 relatively low number (50) of healthy Assaf ewes from a single flock in Spain. Only 5 genera
474 (*Staphylococcus, Lactobacillus, Corynebacterium, Streptococcus* and *Escherichia/Shigella*)
475 were shared among all samples, and a high diversity was observed. Evidences of sub-clinical

476 mastitis were associated to a reduced diversity. Using inference of microbial association
477 networks the authors identified two modules of ASVs, and observed that the relative
478 abundance of the species in the two modules in samples without or with subclinical mastitis
479 was different: this further confirms the hypothesis that subclinical mastitis causes global
480 changes in the microbiota, which affect not only the potential causative agent but a number
481 of other taxa. Castro et al. (2019) analysed teat milk from 36 healthy Manchega ewes with
482 or without a previous history of mastitis from two farms in Spain. They found significant
483 differences between the microbiota in the two farms (with significant differences between
484 the relative abundances *Staphylococcus, Paenibacillus* and *Geobacillus*) but, contrary to
485 what had been reported for teat microbiota of cows by Falentin et al. (2016) did not find
486 any significant difference due to history of mastitis: again, it is unclear if this reflects true
487 lack of differences or is a consequence of high variability.
488 Other clinical or subclinical conditions may affect milk microbiota and susceptibility to
489 mastitis. Zhang et al. (2015) using a crossover experiment analysed pooled teat milk samples
490 from Holstein cows with or without an induced subclinical acidosis condition. Although a
491 high concentrate (HC) diet, which resulted in subclinical acidosis, did not affect microbial
492 diversity, significant differences were found between cows with or without clinical acidosis
493 with the former having a significantly higher abundance of *Proteobacteria,* and lower
494 abundance of *Armatimonadetes, Spirochaetes, Planctomycetes, Fibrobacteres, Chloroflexi,*
495 *Tenericutes, Lentisphaerae, Synergistetes, Elusimicrobia, Cyanobacteria, Verrucomicrobia*
496 and *Firmicutes*. The authors claimed that potential mastitis agents (including
497 *Stenotrophomonas maltophila*, *Brevundimonas diminuta*, *Str. parauberis* and *Enterococcus*
498 *faecalis*) were significantly more abundant in the milk of cows fed the HC diet, which also
499 resulted in an increase in the abundance of psychrotrophic organisms and this may support

500 the idea that mastitis is related to dysbiosis. On the other hand, other mastitis agents, like
501 *Strep. agalactiae* were significantly more abundant in the milk of cows fed a low
502 concentrate diet.
503 Finally, there is some limited data (Zhong, Xue, & Liu, 2018) that may support the idea that
504 udder health status may be related to the microbiota of other body sites. In fact, diversity
505 of the microbiota of rumen in cows with low ($<2x10^5$) or high ($>1x10^6$) SCC has been found
506 to be significantly different, and although no evidence of separation of the composition of
507 the microbiota of rumen in four groups of cows with different SCC was found by beta
508 diversity analysis, significant differences were found in the relative abundance of a few taxa
509 (phyla SR1, *Actinobacteria*, unclassified family *Clostridiales*, genus *Butyrivibrio*,
510 *Proteobacteria* and family *Succinivibrionaceae*). However, the authors did not present an
511 evidence of a cause effect relationships nor analysed the composition of the microbiota of
512 milk.
513 Overall, these data support the idea that clinical and sub-clinical conditions significantly
514 affect the composition and diversity of teat milk microbiota, prior to any further
515 contamination from environmental sources, and that these changes may result in dysbiosis,
516 compared to the "normal" situation characterized by a highly diverse microbiota. The
517 dysbiosis status may be more (Falentin et al., 2016) or less (Ganda et al., 2016) persistent,
518 and more complex and controlled longitudinal studies are clearly needed to clarify how the
519 homeostasis of the milk microbiota is maintained or recovered in different conditions.
520 Due to limited availability of data, to high variability, and to differences in methodologies, a
521 direct comparison of the composition of microbiota of teat milk from different dairy species
522 is difficult. The distribution of genera in teat milk obtained from cows, ewes or water
523 buffaloes is shown in Figure 3, while a NMDS (Non-metric MultiDimensional Scaling) plot is

shown in Supplementary Figure 4. Due to the low number of studies and samples shown here it is difficult to generalize, but it is clear that several of the most abundant genera, although varying in abundance, appear in the milk of the 3 species. A shared core microbiome may exist for these three species, at least when results are aggregated at the genus level. This is confirmed by the partial overlap of the confidence ellipses of the samples from different species in Supplementary Figure 4.

3.1.3 Further down the line: the microbiota of bulk tank milk.

The vast majority of studies on milk microbiota focus on composite samples obtained from bulk tanks at the dairy farm, from tanker trucks or from silos at the dairy processing plant. Apart from disease, a large number of interrelated factors has been shown to affect the composition of the microbiota for bulk milk: season of the year (Doyle, Gleeson, O'Toole, & Cotter, 2017a; Doyle et al., 2017b; Kable et al., 2016, 2019; Li et al., 2018; Porcellato et al., 2018; Zhang, Palmer, Teh, Biggs, & Flint, 2019), lactation stage (Doyle et al., 2017a), type of farming (indoor/outdoor: Doyle et al., 2017a, 2017b), geographic location within a country/region (Kable et al., 2016, 2019; Porcellato et al., 2018; Skeie et al., 2019; Zhang et al., 2019), processing environment (Kable et al., 2016), teat preparation (Doyle et al., 2017b), storage conditions (Doyle et al., 2017a).

The issue of sources of contamination of bulk tank milk is of great practical importance, since preventing and controlling contamination by selected pathogenic or spoilage organisms may contribute to improve the safety and quality of raw milk and raw milk products. Since not all studies fully document all potential sources of variation, it is difficult to track unambiguously sources of contamination and to separate the effect of contamination from that of storage (combination of time temperature), except for a few

large and structured studies (Doyle et al., 2017a, 2017b; Falardeau et al., 2019; Kable et al., 2016; Porcellato et al., 2018).

Using a source tracking approach, Doyle et al. (2017b) clearly identified teat surface and faeces as two of the major sources of microorganisms in bulk tank milk and were able to identify the contribution of other major sources of contamination (including grass for cows grazed on pasture, bedding and silage for cows housed indoor). However, the effect of housing was confounded with that of season and lactation stage since experiments were carried out on the same herd. For sources of contamination which were common to both housing regimes (faeces, teat) the composition of microbiota was affected more by the housing than by the nature of the sample (i.e. samples from the outdoor regime tended to cluster together in beta diversity analysis). In addition, the relative importance of a given source of contamination changed for the two housing regimes (with faeces giving a higher relative contribution for bulk tank milk from cows housed indoors). The effect of teat treatment (which compared no treatment with a treatment including washing with water, disinfectant and thorough washing) clearly showed an interaction with housing (indoor vs outdoor) perhaps due to the different ability of main teat contaminants to adhere to teat surface or to survive to the treatment.

These findings were confirmed by a large recent study (Falardeau et al., 2019) in which the microbiota in both environmental (soil, faeces, pasture, hay, bedding, cow environment) and food samples (individual and pooled milk samples at both the dairy farm and at the cheesemaking plant) in an artisanal cheese making facility was analysed. The microbiota of teat milk was significantly different from the microbiota of bulk tank milk (pooled pre- and post-transport milk), possibly because of both contamination from equipment and growth at refrigeration temperature. In fact, several anaerobic *Firmicutes* and *Bacteroidetes* genera

were the most abundant in the bulk tank milk, but not in the teat milk, and the authors hypothesized that their source was the milking machine environment, although this was not formally proven. At any rate, 78 out of 93 core OTUs present in milk environments were also present in the dairy farm environment, supporting the idea that the dairy farm is an important source of microorganisms in bulk tank milk. An even larger number of taxa (at the genus level or above) were shared by pasture and feed, farm environments, teat skin, teat milk and bulk tank milk (Supplementary Figure 5). However, the relative abundance of the top 25 most abundant genera varied greatly among and within different sample sources (Figure 4).

In general, source tracking studies should be taken with caution, even when longer sequences are used. Falardeau et al. (2019) used relatively short fragments (V3 region) and Doyle et al. (2017b) targeted the V3-V4 region but used OTUs inferred using a 97% similarity level, while in an amplicon targeted study with higher taxonomic resolution, Skeie et al. (2019) showed that even within the same species different ASVs may have a different distribution (see below). Metagenomic studies may reveal the composition of populations at the strain level and allow the tracking of the sources of the most prevalent and abundant strains, but the cost of studies with sufficient sample sizes would probably be unjustified.

The effect of season on the composition of bulk tank milk has been studied by several authors (Doyle et al., 2017a; Li et al., 2018; Zhang et al., 2019): all have found a significant effect, which, however, may be confounded with many other factors (days in lactation, farming system, feeding, etc.). Doyle et al. (2017a) compared samples collected in Spring and October, but, due to farming practices in Ireland, this was completely confounded with feed, lactation stage and housing. Mid-lactation samples (collected in Spring with cows feeding on pasture outdoors) were significantly different from late lactation samples

596 (collected in Autumn when at least for part of the sampling period the cows were housed
597 inside and fed a diet containing concentrate and silage): they had a higher diversity, while
598 for individual milk samples of cows fed on pasture a slightly lower diversity was found
599 (Doyle et al., 2017b), and 85 taxa showed significant differences in abundance between mid-
600 and late-lactation samples.
601 Two further studies (Li et al., 2018; Zhang et al., 2019) have confirmed that a significant
602 seasonal variation exists in the composition of milk microbiota. In both studies members of
603 the genera *Acinetobacter*, *Lactococcus* and *Pseudomonas* were found to be both abundant
604 and highly prevalent, and several genera showed changes of abundance in different seasons
605 (*Pseudomonas*, *Propionibacterium, Flavobacterium*: Li et al., 2018; *Pseudomonas*, two
606 *Lactococcus*, one *Serratia* and one *Acinetobacter:* Zhang et al., 2019). Unfortunately, both
607 studies used a descriptive approach and little or no details were provided on critical factors
608 which are likely to affect microbiota and which may be confounded with the effect of
609 season (farming, breeds, feeding, lactation stage, etc.) and the causes of the observed
610 patterns remain unclear.
611 Using a high-resolution method based on ASV inference, Skeie et al., (2019) confirmed the
612 high variability, even over short time scales, of the composition of bulk tank milk microbiota.
613 The authors analysed 135 milk samples in three samplings from 45 farms in Norway over
614 three months in Winter. The farms were located in two geographically distant areas sharing
615 similar climatic conditions, but had three different milking systems. Although milk was
616 collected on average every 3 days, bacterial counts were reasonably low, with a median
617 value of 4.25 log(cfu/ml) and only two samples exceeding 10^5 cfu/ml, with higher values
618 associated systematically with parlour farms with automatic milking systems. Beta-diversity
619 (weighted UniFrac) was significantly affected by all the variables used in this quasi-

620 experimental study (areas, sampling, farms, housing/milking systems) and the abundance of
621 several bacterial genera (including *Pseudomonas, Bacillus, Staphylococcus, Paenibacillus,*
622 *Psychrobacter, Chryseobacterium, Aerococcus,* and *Rhizobium*) was significantly different
623 among geographic areas, farms or sampling dates. The microdiversity for selected taxa, as
624 measured by the number and types of ASVs, was also affected b several factors.
625 Interestingly, *Corynebacterium*, which had the highest number of ASVs but a low average
626 abundance, had the lowest number of ASVs per farm. The composition of populations of
627 *Pseudomonas* and *Lactococcus* were significantly affected by collection day, area and
628 housing/milking system possibly because of the potential variety of environmental sources
629 and growth during refrigerated storage. *Bacillus* and *Streptococcus* populations changed
630 between collection days from the same farm and between farms and geographical areas.
631 Levels and composition of populations of *Bacillus* and *Paenibacillus*, two aerobic spore
632 formers genera which are of particular concern due to their ability to survive pasteurization
633 and grow in long shelf life pasteurized milk (Porcellato et al., 2018; Porcellato et al., 2019),
634 were different between the 2 geographical areas. Fluctuations and diversity in the
635 composition of *Streptococcus* populations were attributed to variability in the
636 contamination of the udder of individual cows, with higher variability in larger farms.
637 The temperature and duration of storage are clearly two factors which may have a dramatic
638 effect on the composition of the microbiota, with higher refrigeration temperatures
639 favouring the growth of psychrotrophic microorganisms. Doyle et al. (2017a) investigated
640 the effect of temperature (2, 4 or 6°C) on the composition of microbiota of mid- and late-
641 lactation milk stored for 5 days. There was very little increase in bacterial numbers during
642 storage, as judged by qPCR, except for late-lactation samples stored at 6°C. No significant
643 differences in composition of the microbiota was noted at the end of storage at 2°C.

644 However, at both 4 and 6°C a significant increase was found in the proportion of
645 *Streptococcus* and *Pseudomonas* for samples stored at 4°C and of *Acinetobacter* and
646 *Pseudomonas* for samples stored at 6°C. The authors did not attempt to use the qPCR data
647 in conjunction with the relative abundance data obtained by AT metagenomics and it is not
648 clear if the strong decrease in abundance for some taxa (*Staphylococcus*, *Rhodanococcus*
649 and uncultured *Ruminococcaceae*) is due to the compositional nature of abundance tables
650 or if any other taxa increased in number during refrigerated storage.
651 In an another study (Zhang et al., 2019) prolonged storage at 7°C also resulted in high
652 relative abundance of several psychrotrophic bacteria belonging to the genera
653 *Pseudomonas, Acinetobacter, Carnobacterium, Chryseobacterium, Erwinia, Hafnia,*
654 *Flavobacterium, Kluyvera* and *Lactococcus*, with some species (*Ps. fluorescens* and *Ps.*
655 *psychrophila*) reaching almost 80% of the sequences. A similar trend for increase in the
656 relative abundance of psychrotrophic bacteria was also found in the source tracking study of
657 Falardeau et al., 2019, where bulk and transport tanks contamination may have significantly
658 contributed, together with storage, to the increase of psychrotrophs, with *Pseudomonas*,
659 *Psychrobacter* and *Acinetobacter* among the most abundant genera.
660 Variations in simple milk quality parameters may be associated to changes in the microbiota
661 composition. Rodrigues, Lima, Canniatti-Brazaca, & Bicalho (2017) analysed 472 low counts
662 ($<10^4$ cfu/ml) bulk tank milk samples from dairy farms in New York state (USA) over 2
663 months and evaluate the correlation between SCC, and standard plate counts (SPC) with
664 composition of the microbiota. As usual, a high diversity and variability was found but a core
665 microbiome was identified across the 19 farms. Significant association was found between
666 the relative abundance of several genera and low or high SCC and SPC counts. In fact, high
667 SCC milk was significantly associated with increased abundance of *Corynebacterium*,

668 *Streptococcus*, *Lactobacillus*, *Coxiella*, *Arthrobacter*, and *Lactococcus* (noticeably only some
669 of this may be potentially associated with mastitis), while high SPC milk (>3.6 log cfu/ml)
670 had increased abundances of *Acinetobacter, Enterobacteriaceae, Corynebacterium* and
671 *Streptococcus* and usually lower diversity: this suggests that shifts in community
672 composition were due to bacterial growth.
673 Even with this very large variability, a core microbiota of bulk tank milk does apparently
674 exist. Using data in FoodMicrobionet, we were able to combine the results, at the genus
675 level or above, for five studies including 199 samples of bulk tank milk from different
676 geographic regions (Ireland: Doyle et al., 2017a; France: Frétin et al., 2018; New Zealand: Li
677 et al., 2018; Norway: Skeie et al., 2019; Canada: Falardeau et al., 2019). Data on prevalence
678 and abundance of the top most prevalent and abundant taxa are shown in Supplementary
679 Figure 6 and Supplementary Table 5, while the distribution of abundance for the 25 most
680 prevalent and abundant genera is shown in Figure 5. The combined diversity in these five
681 studies is impressive, with almost 2,000 taxa identified at the genus level or above.
682 However, four phyla (*Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*) include
683 the majority of the most abundant and prevalent taxa. The top 25 genera include
684 psychrotrophs (*Acinetobacter, Chryseobacterium, Pseudomonas, Psychrobacter*), bacteria
685 associated with gut (*Atopostipes, Bacteroides, Christensenellaceae, Clostridium,*
686 *Rikenellaceae, Romboutsia, Ruminococcaceae*), or with teat skin (*Staphylococcus,*
687 *Aerococcus, Turicibacter, Streptococcus, Facklamia, Corynebacterium, Bacillus*), including
688 genera with potentially beneficial microorganisms (*Lactobacillus, Streptococcus,*
689 *Lactococcus, Staphylococcus, Corynebacterium*). The intra-study variability was sometimes
690 substantial but, surprisingly enough, the composition of the microbiota for some studies,
691 even from different countries, was similar, as assessed by non-metric multidimensional

692 scaling (Supplementary Figure 7). Not surprisingly, the diversity was lower for samples
693 obtained from a single farm (ST37: Doyle et al., 2017a; ST39: Frétin et al., 2018; ST74:
694 Falardeau et al., 2019), even when samples were obtained in different seasons (Doyle et al.,
695 2017a; Frétin et al., 2018) or from cows belonging to different breeds (Frétin et al., 2018) or
696 with different feeding regimes (Doyle et al., 2017a; Frétin et al., 2018).
697 There are very limited data on the composition of bulk tank milk at the dairy farm for
698 species other than cows and the results are difficult to generalize. In two small studies on
699 jennies (de los Dolores Soto del Rio, Dalmasso, Civera, & Bottero, 2017) and on goats
700 (McInnis et al., 2015) the usual high diversity and variability was found, but any inference on
701 the potential sources of variation is impossible due to the limited scope of these studies.
702 However, in both studies a potential effect of lysozyme, which was naturally abundant in
703 jennies' milk or whose secretion was engineered in goats might have affected the
704 composition of the microbiota.
705
706 *3.1.4 From the farm to the processing plant*
707 Transfer of bulk tank milk from the dairy farm to the processing plant and further storage
708 and processing steps may alter significantly the composition of milk microbiota as a result of
709 contamination and growth. A number of recent well-structured studies have offered
710 significant insight in this area for both milk processed to become liquid milk products or
711 cheese (Falardeau et al., 2019; Kable et al., 2016, 2019; Porcellato et al., 2018, 2019).
712 All evidences confirm that contamination form transport trucks and from tanks and
713 equipment at the dairy plant, cleaning routines, heat treatments, and duration and
714 conditions of storage have a significant impact on the structure of microbial communities of
715 milk, and thus potentially affect the quality of cheese and milk due to variations in the

716 abundance of potential starter and non-starter species of the genera (*Streptococcus,*
717 *Staphylococcus, Macrococcus, Corynebacterium*, etc.) and of spoilage bacteria
718 (*Acinetobacter*, *Pseudomonas,* psychrotrophic spore-formers).
719 Kable et al. (2016) analysed the microbiota of milk from 899 tankers delivering raw cow milk
720 to two processing farms in California (USA) over three seasons. They confirmed the
721 occurrence of a high variability in the composition of the microbiota, the occurrence of
722 seasonal variations, and found a core microbiota dominated by members of the genera
723 *Streptococcus*, *Staphylococcus* and *Clostridiales*. Notably, only some of the taxa
724 (*Staphylococcus, Bacillus, Enterococcus, Streptococcus, Clostridium, Ruminococcus,*
725 *Corynebacterium, Acinetobacter*) they identified as members of the core cow milk
726 microbiota match those found as being highly prevalent and abundant in studies on bulk
727 tank milk (see Figure 5 and Supplementary Table 5), but this might have been due to
728 differences in criteria used to define the core microbiota. Significant differences were found
729 between samples collected in the three seasons. Relative abundance of *Firmicutes* was
730 significantly smaller in Spring samples, while those of *Actinobacteria* was higher, while the
731 relative abundance of *Bacteroidetes* was higher in Fall. The authors hypothesized that
732 differences in composition might be due to differential exposure to sources of
733 contamination in different seasons (with possibly more contact with soil, a potential source
734 of *Actinobacteria*, in Spring), but clear-cut evidence for this is lacking. While tanker milk
735 samples had low counts (median ~$5x10^3$ cfu/ml, as measured by qPCR), growth between
736 refilling cycles of the tankers cannot be excluded as a contributing factor to the observed
737 differences (Kuhn, Meunier-Goddik, & Waite-Cusic, 2018). This is confirmed by a later work
738 (Kable et al., 2019; see below) and by the observation that the silo used for milk storage
739 may significantly affect the composition of the microbiota of milk transferred from tanker

740 trucks, with *Pseudomonadales* and *Lactobacillales* being more abundant in the silos. Growth
741 in residual milk in the silo may be a contributing factor (higher cell counts in silos compared
742 to tankers) but stochastic patterns of contamination may contribute. Two groups of silos
743 were identified. In one the microbiota was similar to those of the tankers used to fill them,
744 and had significantly higher proportions of *Streptococcus*, *Corynebacterium, Macrococcus*
745 and *Clostridium*. In the other the microbiota of the silos was distant from those of the
746 tankers (weighted UniFrac distance), and *Acinetobacter* was significantly more abundant. In
747 a follow-up study (Kable et al., 2019) the authors carried out an in depth investigation of the
748 quantitative and qualitative variations of the microbiota in the processing plant. In addition,
749 PMA treatment was used to enrich viable cells after lethal treatments (pasteurization). They
750 confirmed that OTUs belonging to the genus *Streptococcus* (and tentatively identified as *S.*
751 *thermophilus/salivarius*) were most abundant and prevalent in milk at all stages, and
752 confirmed the occurrence of a seasonal effect, with some genera (including *Acinetobacter*
753 and *Lactococcus*) more abundant in late Summer compared to Spring. Several taxa, whose
754 overall abundance was relatively low, showed interesting time-dependent or spatial
755 patterns and were occasionally more abundant. An effect of growth on the composition of
756 bacterial communities was clearly related to the length of time a piece of equipment was
757 operated after cleaning and to heat treatments. Psychrotrophic species clearly increased as
758 storage duration increased: the relative abundance of *Acinetobacter* and *Lactococcus*
759 significantly increased over time in the raw milk silo while a single *Pseudomonas* OTU was
760 enriched in summer after a post-pasteurization concentration step. On the other hand, heat
761 treatments caused a decrease in non spore-formers and an increase of thermoduric species
762 and spore-formers, especially when the active fraction of the microbiota was targeted using
763 PMA treatments. *Anoxybacillus*, whose overall abundance was relatively low, seemed to be

764 enriched after long operation time and both *Anoxybacillus* and *Thermus* were enriched after
765 pasteurization. Composition of viable and total microbiota after pasteurization was
766 dramatically different for some steps, as shown by weighted UniFrac distance. *Turicibacter*,
767 was significantly enriched in the viable fraction, while the abundance of *Staphylococcus* was
768 significantly lower. Other spore-formers or thermotolerant genera (including *Bacillus*,
769 *Clostridium*, *Anoxybacillus* and *Thermus*) were also more abundant in the viable fraction
770 after pasteurization (the difference was not statistically significant) while several other non
771 spore-formers (*Bacilli*, *Clostridia*, and *Actinobacteria*) showed a lower abundance in the
772 viable fraction. This confirms that obtaining a realistic picture of the active fraction of the
773 microbial community by eliminating the contribution from dead or membrane damaged
774 cells is of utmost importance (Erkus et al., 2016; Porcellato & Skeie, 2016).
775 Kable et al. (2019) also evaluated the impact of time of operation of individual pieces of
776 equipment after cleaning. Within 19 h from cleaning-in-place (CIP) the numbers of viable
777 cells were low (<3,200 cells/ml) and the microbiota composition was diverse, although
778 different species prevailed in different pieces of equipment, mostly depending on whether
779 they contained raw or pasteurized milk: spore-formers, including *Bacillus* and *Anoxybacillus*,
780 were abundant in equipment containing pasteurized milk. After 19 h of operation since the
781 last cleaning, the equipment was divided in two groups. In some samples there was little
782 increase in bacterial numbers and the dominating taxa were close to those appearing in raw
783 milk. In the others, which mostly included milk feeds which had not been pasteurized, were
784 dominated by *Acinetobacter* and *Lactococcus*, while in the concentration step silos the
785 dominating genus was *Anoxybacillus*. Unfortunately, in this study relatively short sequences
786 were used (V4 region of the 16S RNA gene) and proper source tracking at or below the
787 species level is almost impossible.

788 Porcellato et al. (2018, 2019), using a similar approach, evaluated the dynamics of the
789 microbiota in a Norwegian plant producing liquid pasteurized milk as a function of season of
790 production, milk source, and time and temperature of storage of the pasteurized milk. As in
791 many other papers, a very high diversity and significant differences in the composition of
792 the microbiota between different seasons was found in silo milk, although no significant
793 difference was found for dairies located in two different areas of the country. Pasteurization
794 reduced counts by up to 2 log cycles, and only after prolonged (13-14 d, end of shelf life)
795 incubation of the pasteurized milk cartons at abuse temperature (8°C) counts exceeding 10^7
796 cfu/ml (with high presumptive *B. cereus* counts) were obtained, with significantly higher
797 abundances of *Bacillus, Paenibacillus, Solibacillus, Anoxybacillus, Geobacillus* and
798 *Jeotgalicococcus*. Although no treatment was used to separate viable from total bacteria,
799 the composition of the microbiota immediately after pasteurization was significantly
800 different from that of raw milk: 21 order level OTUs (15 of which *Clostridiales*) were more
801 abundant in raw milk and 27 (including *Lactobacillales, Clostridiales* and *Pseudomonadales*)
802 more abundant in pasteurized milk. After incubation at 4°C a seasonal variation of the
803 abundance of *Bacillus* was found in pasteurized milk, and this correlated well with the
804 seasonal variation of this genus in raw milk.
805 In a follow up work, the same authors (Porcellato et al., 2019) used a more sensitive AT
806 strategy, by using a in depth analysis of OTUs identified on the basis of 16S RNA gene
807 sequence and assigned to the genus *Bacillus* and by amplifying conserved regions of three
808 genes (pantothenate synthase, *panC*; glycerol-3 phosphate transporter *glpT*; pyruvate
809 carboxylase, *pyrC*). A single OTU belonging to the *B. cereus* group made up 99.6% of the
810 sequences of the genus *Bacillus*, while another occasionally dominated the microbiota of
811 spoiled cartons at 8°C. Use of *panC* as gene target resulted in the highest diversity. A

812 seasonal variation of the composition of the population of the *B. cereus* group was found,
813 and storage at 8°C resulted in a higher diversity compared to raw milk and milk stored at
814 4°C, perhaps simply because more strains had the opportunity to grow over the detection
815 limit. Two *panC* sequence types (ST) were found at relatively high abundance in all samples.
816 Several other papers have demonstrated that the pasteurization treatment results in an
817 enrichment of spore-formers (*Bacillus, Anoxybacillus, Turicibacter*) or thermotolerant
818 bacteria (*Thermus*) in both the viable fraction and in the total microbiota. These genera
819 have been frequently found in raw milk (see Figures 4 and 5 and Supplementary Tables 3
820 and 5) and some have been associated to spoilage (*Anoxybacillus*, Kable et al., 2019;
821 *Thermus,* Quigley et al., 2016; *Bacillus*, Sattin et al., 2016a; Porcellato et al., 2018).
822 *Turicibacter* is universally present in milk, from teat milk to pasteurized milk, but its
823 significance and its potential to grow and spoil milk are not known. *Anoxybacillus* may show
824 some potential to survive and grow in milk (Kable et al., 2019) and has been found in high
825 numbers in Ricotta cheese (Sattin et al., 2016a) where it might contribute to spoilage.
826 Another recent study (Falardeau et al., 2019) has confirmed that contamination with
827 microorganisms from storage tanks and storage at low temperatures are the main drivers of
828 changes in the composition of microbiota of bulk tank milk. In this study, the composition of
829 the microbiota of milk at the farm bulk tank was dramatically different from that of the
830 storage tank at the cheesemaking plant (with a duration of transportation of about 20 min),
831 and further incubation in a chilled room resulted in the microbiota being dominated by
832 *Pseudomonas*. On the other hand, the composition of the microbiota of the bulk tank and
833 that of the transport tank were very similar and significantly different from that of the
834 pooled tank milk pre- and post-transport.

When data from these three studies (Falardeau et al., 2019; Kable et al., 2019; Porcellato et al., 2018) are compared, a more general picture emerges. The 50 most prevalent and abundant taxa are listed in Supplementary Table 6. Several of these taxa match those found in bulk tank milk, and, although many taxa whose likely origin is the GI tract are still present at low abundance, the most abundant and prevalent taxa include *Streptococcus, Lactococcus, Pseudomonas, Acinetobacter, Lactobacillus, Staphylococcus, Psychrobacter* and *Escherichia/Shigella*. However, the prevalence and maximum relative abundance of many genera including psychrotrophs is increased compared to bulk tank milk at the farm, confirming that storage at low temperature is the main driver of the change in composition of the microbiota. Several thermoduric taxa, including spore-formers (*Turicibacter, Anoxybacillus, Bacillus, Clostridium*) are also prevalent and sometimes abundant. The distribution of abundance of the top 25 genera in raw and HTST (high temperature short time) treated milk at the processing plant is compared in Figure 6. While there is no guarantee that the nucleic acid target was from viable cells (only data for samples not treated with PMA from Kable et al., 2019 are shown), some major differences are evident between studies (some taxa which were relatively abundant in ST74 and ST87 are almost absent in ST38) and, within study, between HTST and raw milk. In the latter, the relative proportion of psychrotrophs tends to be higher, while that of thermoduric species, including both non spore-formers and spore-formers is higher.

Several papers describing the evolution of the composition of cheese microbiota report the composition of raw milk prior to starter addition. These include studies on cow milk cheeses (Alessandria et al., 2016; Bokulich & Mills, 2013; Calasso et al., 2016; Carafa et al., 2019; De Filippis et al., 2016; De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti, 2014b; Dolci, De Filippis, La Storia, Ercolini, & Cocolin, 2014; Falardeau et al., 2019; Frétin et al., 2018; Giello

et al., 2017; Masoud et al., 2011), water-buffalo cheeses (Ercolini, De Filippis, La Storia, & Iacono, 2012), ewe's milk cheeses (De Pasquale et al., 2014a), fermented yak milk products (Joang et al., 2019) and camel milk (Amrouche, Mounier, Pawtowski, Thomas, & Picot, 2020; Zhao et al., 2019). Although these studies invariably show a high diversity in milk microbiota, the description of the storage conditions and duration, and of the source of the milk is generally insufficient to allow any comparison with studies focusing on milk.

4. Conclusions

The microbiota of milk is probably one of the most complex food microbial communities, because of the multiplicity of sources of contamination (Addis et al., 2016; Derakshani et al., 2018a). The availability of HTS methods, with the ability to study in detail both the taxonomic structure and the functionality of microbial communities has revolutionized out ability to study the structure and function of food microbiomes and has undoubtedly greatly contributed to our understanding of the factors affecting contamination patterns and successions in milk and dairy foods. The availability of raw and processed sequence data (see Supplementary material and data) greatly enhances our ability of combining and analysing results from different studies, thus, facilitating metastudies. At almost 9 years from the publication of the first paper on the microbiota of dairy products, sequencing platforms, methods and bioinformatic approaches have evolved greatly and, although most recent papers use similar pipelines, there is still a need for a consensus of dairy microbiologists on SOPs, which would improve confidence in the results and comparability of studies. Careful documentation of all potential factors affecting the composition of milk microbiota is of uttermost importance for the interpretation of the results.

882 Even with these caveats, our understanding of the relationships between udder health and
883 milk quality has significantly improved. Mastitis and dysbiosis of microbial communities of
884 the udder are strongly related, although the cause-effect relationship is not completely
885 clear. In addition, HTS approaches allow to shed light on the potential causative agents in
886 culture negative and sub-clinical cases and to clarify the polymicrobial nature of some
887 mastitis cases.
888 Practically all conceivable factors related to farming and storage of raw milk have been
889 shown to affect the composition of milk microbiota, and several sources (faeces, pasture,
890 feed, milking equipment, storage tanks, etc.) might contribute microorganisms relevant for
891 the quality of dairy milk and fermented dairy products. However, the low taxonomic
892 resolution of some studies (which track genera, or at best, species, not strains) still obscures
893 the potential contribution of each source and more effort should be probably devoted to
894 disentangle the relative contribution of contamination, growth at low temperature and
895 ability to survive pasteurization treatments in determining the potential for microorganisms
896 from different sources to affect the quality of dairy products. Recent findings on the origin
897 and survival of spore-formers and other thermoduric microorganisms in the dairy plant
898 (Kable et al., 2019; Porcellato et al., 2018, 2019) have indeed provided insights which might
899 contribute to improving practices in cleaning, sanitation and heat treatment of milk.
900 However, still much remains to be done on the potential contribution of milk in terms of
901 starter and non-starter microorganisms relevant to cheese production: although HTS
902 methods are indeed much more sensitive than other cultivation dependent and
903 independent approaches, the low levels of contamination of hygienically produced milk
904 complicate the tracking of species and strains. The decreasing costs of shotgun approaches

and the availability of powerful bioinformatic pipelines might in the near future open new avenues to the study of sources of milk contamination.

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Legends to figures.

Figure 1. Stacked bar plot showing the relative abundance of bacterial classes on cow's teats in two studies (Falardeau et al., 2019; Frétin et al., 2018). Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019).

Figure 2. Stacked bar plot showing the relative abundance of the 20 most abundant taxa (at the genus level or above are shown) in individual samples from teat milk from Cremonesi et al. (2018; colostrum samples were removed) and Falardeau et al. (2019). Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019). For the Cremonesi et al. (2018) samples, HF and REN in sample names indicate samples from Holstein Fresians and Rendena cows, respectively.

Figure 3. Stacked bar plot showing the relative abundance of the 24 most abundant taxa (at the genus level or above are shown) for teat milk from cows (ST47, Cremonesi et al., 2018, colostrum samples were removed; ST74, Falardeau et al., 2019), ewes (Castro et al., 2019) and water-buffaloes (Catozzi et al., 2017; only samples from healthy quarters are shown). Samples from each study have been pooled. Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019).

Figure 4. Boxplots for the distribution of relative abundance for the 25 most abundant and prevalent genera in pasture and feed, farm environments, teat skin, teat and bulk tank milk in Falardeau et al. (2019). Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019).

Figure 5. Boxplots for the distribution of relative abundance for the 25 most abundant and prevalent genera in cow bulk tank milk from five studies (ST37 Doyle et al., 2017a; ST39 Frétin et al., 2018; ST81 Li et al., 2018; ST46 Skeie et al., 2019; ST74 Falardeau et al., 2019).

1336 Sequences were downloaded from NCBI/SRA and processed as described in Parente et al.

1337 (2019).

1338 **Figure 6.** Boxplots for the distribution of relative abundance for the 25 most abundant and

1339 prevalent genera in raw and HTST treated milk at the processing plant for three studies

1340 (ST38 Porcellato et al., 2018; ST74 Falardeau et al., 2019; ST87 Kable et al., 2019).

1341 Sequences were downloaded from NCBI/SRA and processed as described in Parente et al.

1342 (2019).

1343

Figure 1

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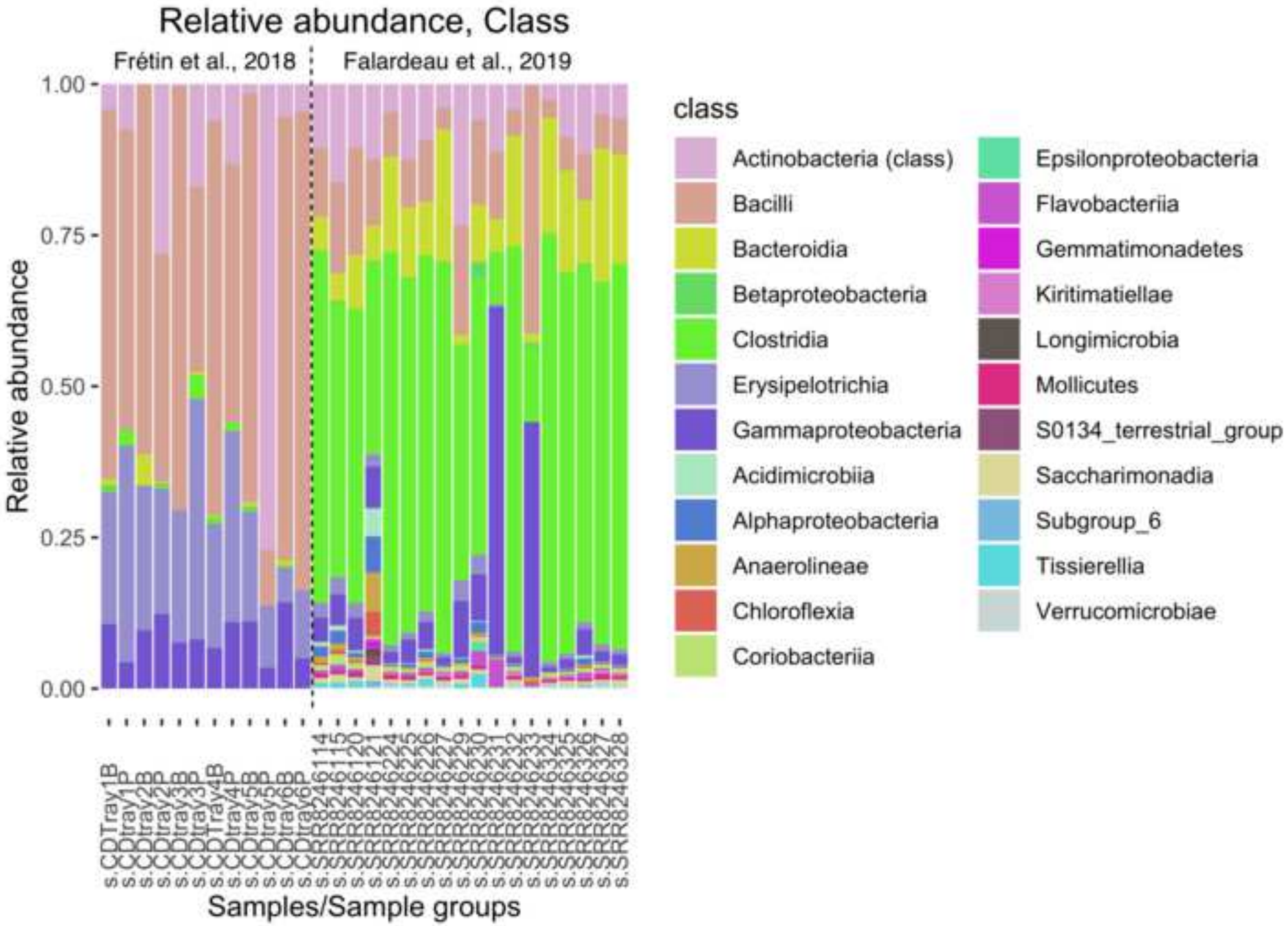

Figure 2

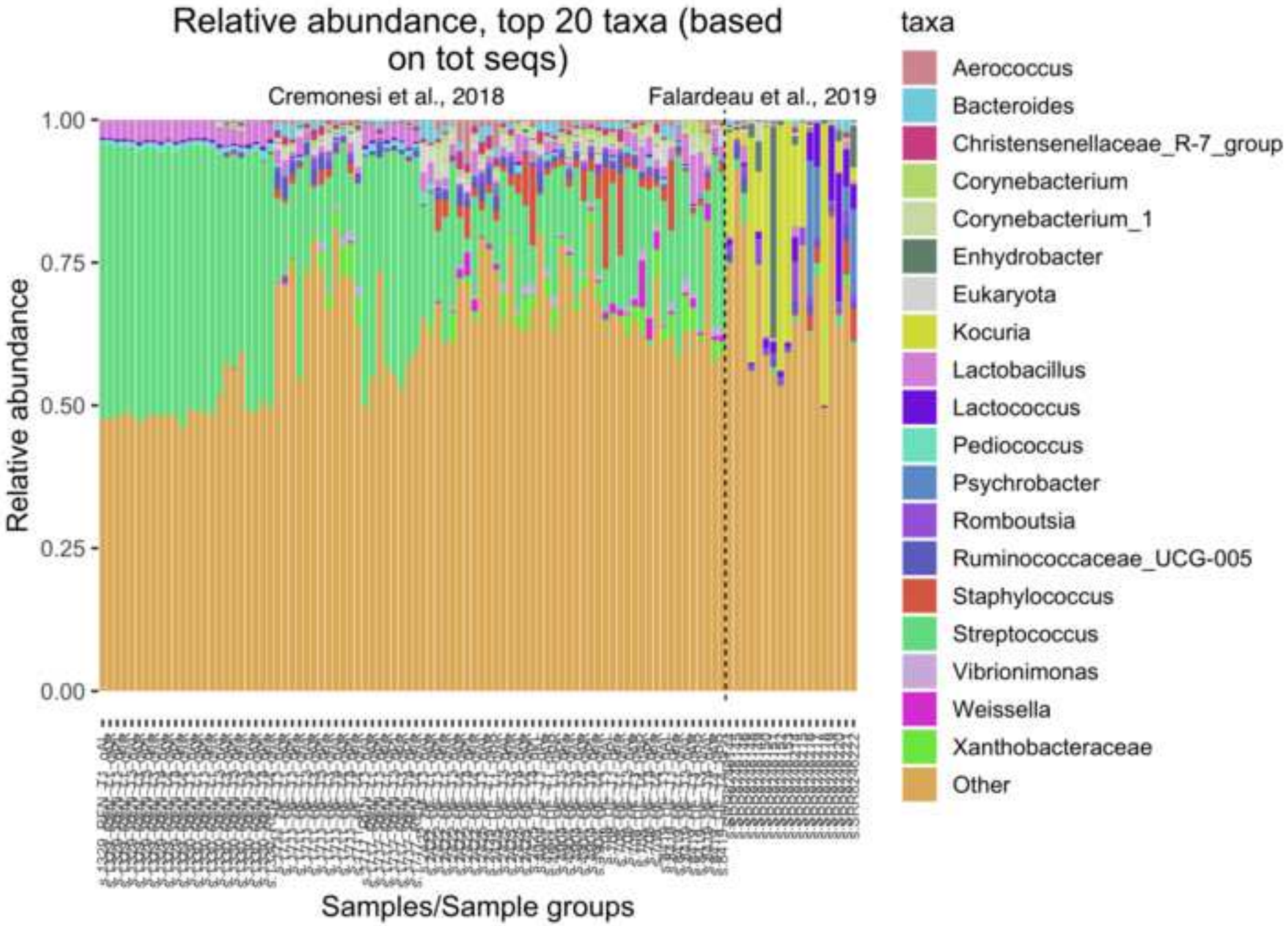

Figure 3

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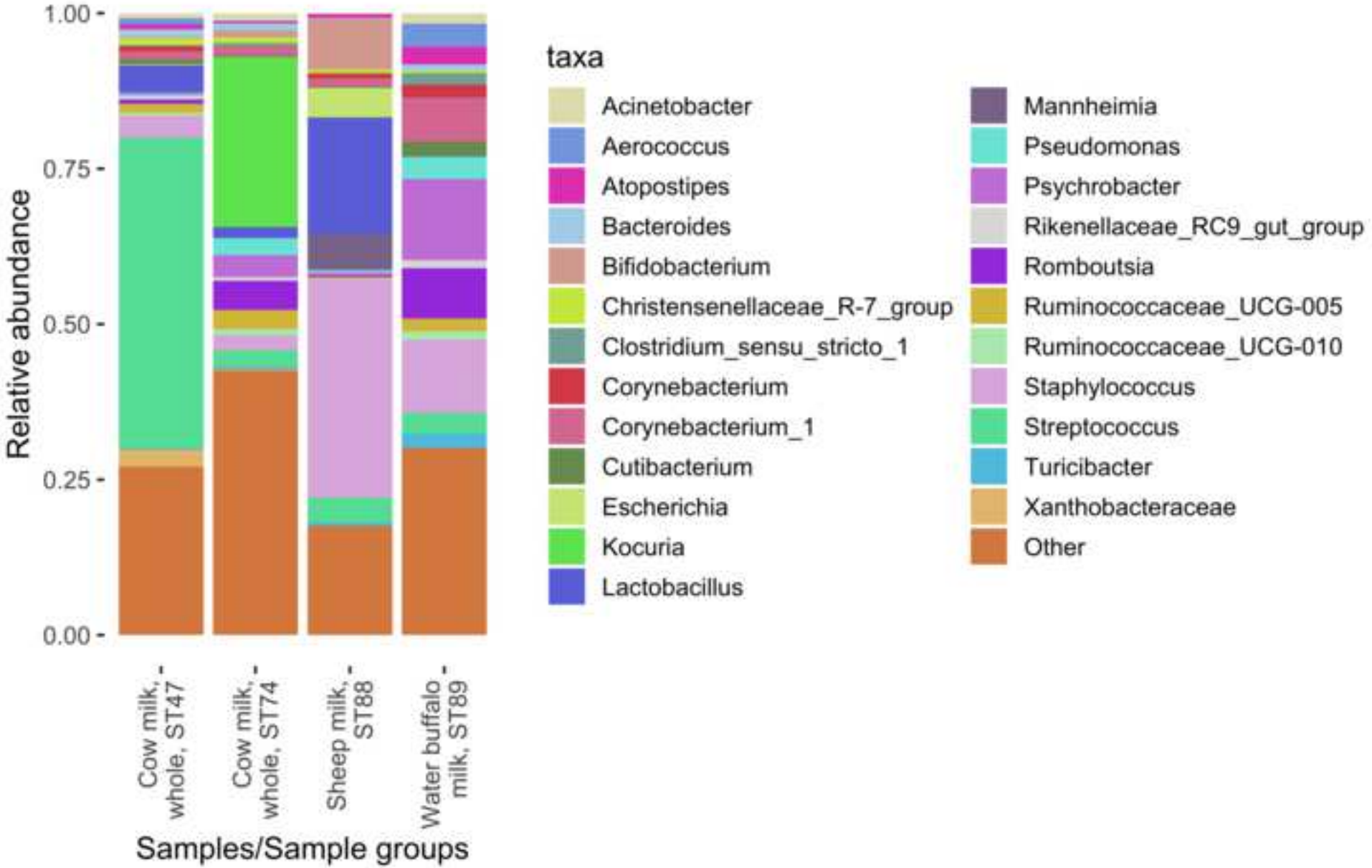

Figure 4

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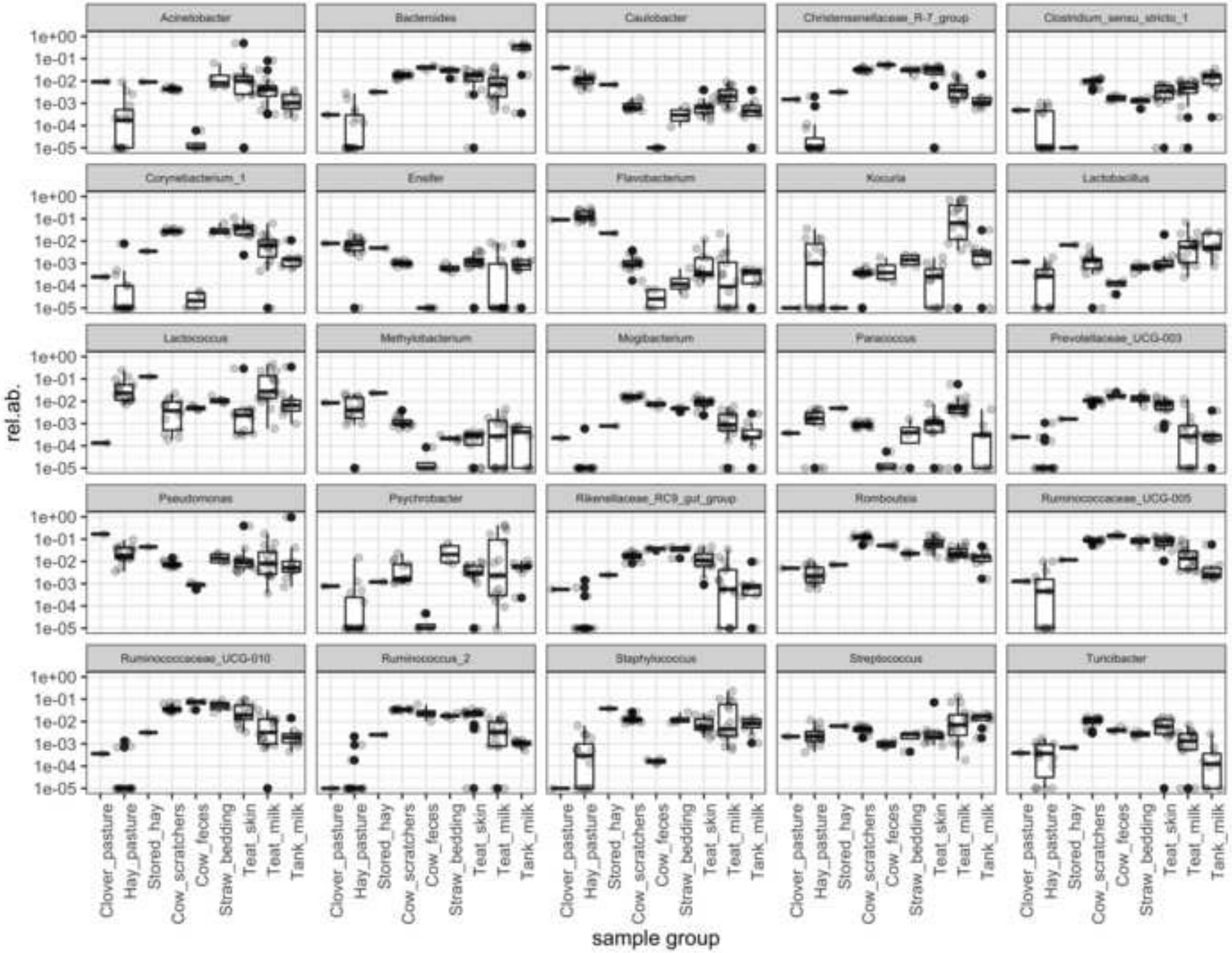

Figure 5

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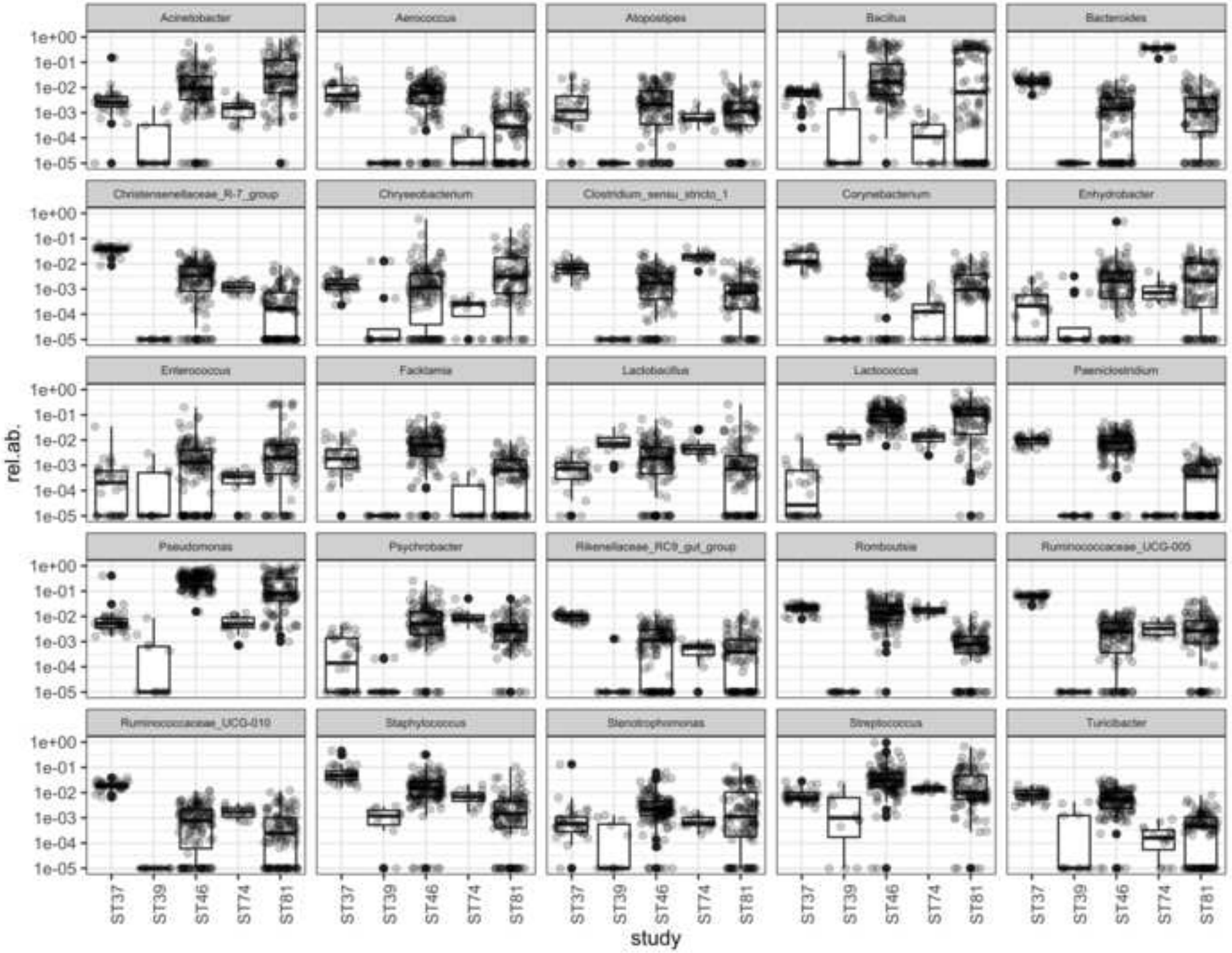

Figure 6

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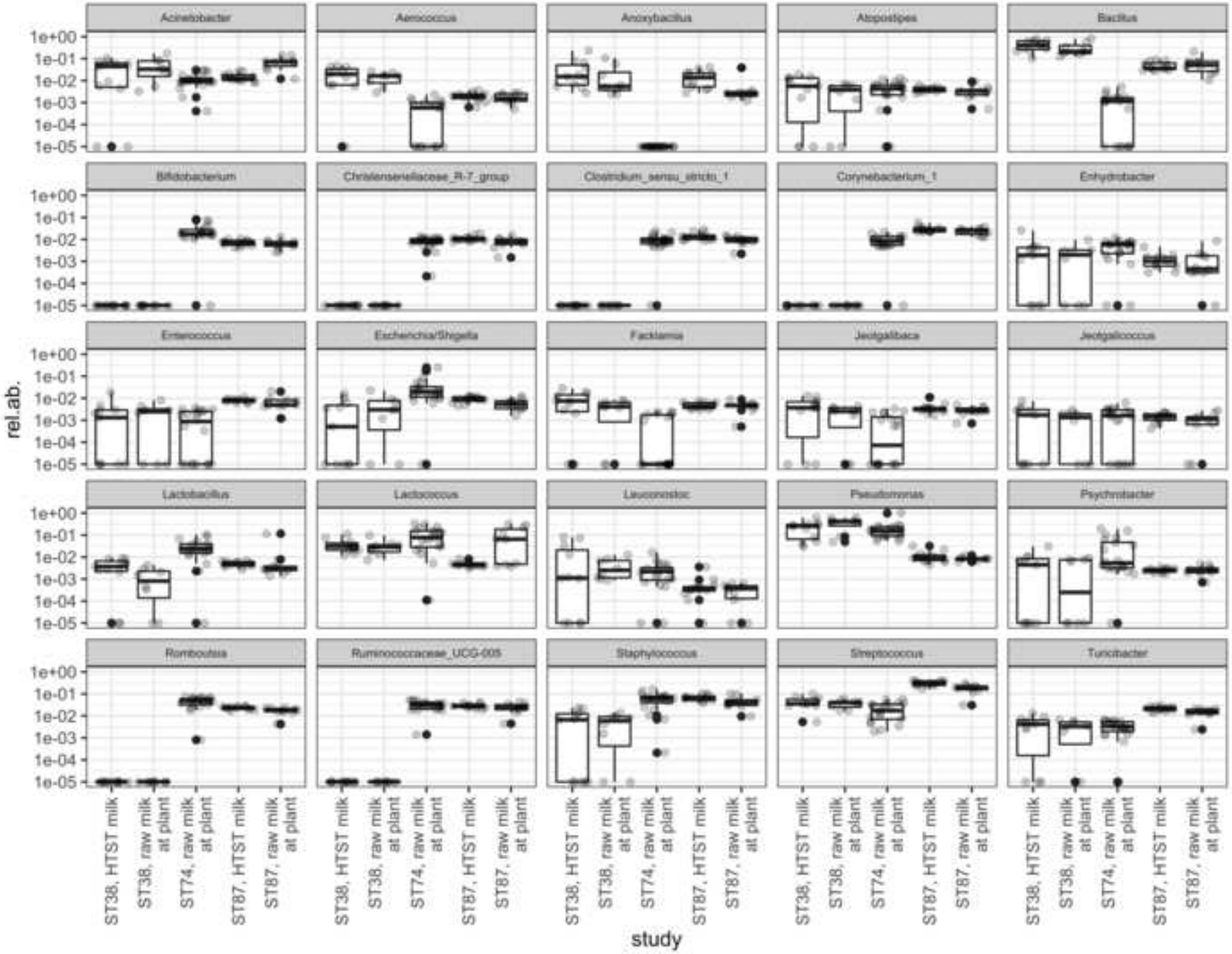

The microbiota of dairy milk: a review

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Supplementary material

Supplementary Table 1. List of studies on the characterization of microbiota of milk and dairy products with high throughput sequencing approaches (last updated February 2020).

Target											sample type					
Bacteria	Fungi	Viruses	Meta	Other	platform	Study type	Stat. an.[1]	Seq. accn.	n	food group	food	env.	short descr.	year	reference	FMBN
16S RNA gene, V4	--	--	--	--	454 GS FLX	cs	descr.	--	3	Fermented milk	x		A single Kefir grain (interior + esterior) and milk from Ireland	2011	Dobson, O'Sullivan, Cotter, Ross, Hill, 2011	--
16S RNA gene, V3-V4	--	--	--	--	454 GS FLX	ts (4)	descr.	--	11	Cheese	x		Cow milk and Danish raw milk semi-hard cheeses, made with different starter cultures	2011	Masoud et al., 2011	--
16S RNA gene, V5-V6	--	--	--	--	454 GS FLX	ts (3)	descr.	--	3	Cheese	x		Polish PDO raw ewe milk cheese produced without starter (Oscypek)	2012	Alegría, Szczesny, Mayo, Bardowski, & Kowalczyk, 2012	--
--	--	--	DNA	--	454 GS FLX	cs	descr.	--	3	Milk	x		Raw milk from cows with subclinical mastitis	2012	Bhatt et al., 2012	--
16S RNA gene, V1-V3	--	--	--	--	454 GS Junior	ts (5)	descr.	SRP014821	10	Cheese	x		Raw milk, whey culture, curd before and after fermentation and water-buffalo Mozzarella cheese from two different manufactures	2012	Ercolini, De Filippis, La Storia, & Iacono, 2012	0_6_2
16S RNA gene, V1-V2	--	--	--	--	454 GS 20	cs	descr.	SRR340041, SRR340042, SRR340043	3	Fermented milk	x		Kefir grains (3) from Brasil	2012	Leite et al., 2012	--
16S RNA gene, V1-V3	--	--	--	--	454 GS Titanium	cs	descr. + inf.	--	12	Cheese	x		Latin style soft cheese (queso fresco, quesito), after 24 h enrichment in serum dextrose broth	2012	Lusk et al., 2012	--
16S RNA gene, V1-V2	--	--	--	--	454 GS FLX	cs	descr.	--	156	Milk	x		Raw milk from mastitic (136) and healthy (20) cows	2012	Oikonomou, Machado, Santisteban, Schukken, & Bicalho, 2012	--
16S RNA gene, V4	--	--	--	--	454 GS FLX	cs	descr. + inf.	--	62	Cheese	x		Artisanal Irish soft, semi-hard and hard cheeses from raw or pasteurized cow, goat, or sheep milk	2012	Quigley et al., 2012	2
16S RNA gene, V4-V5	ITS1 gene	--	--	--	Illumina MiSeq	cs	descr. + inf.	Study 1884 (16S) and 1919 (ITS)	86	Cheese	x	x	Milk, curd, cheese and cheesemaking environment from two artisanal	2013	Bokulich & Mills, 2013	--

[1] Abbreviations: Stat. an. statistical analysis; descr. descriptive; inf. inferential; Study type: cs cross sectional, ts, time series (number of time points in parentheses) Seq. accn. sequence accession; env. environment; short descr. short description.

								(QIIME)					cheesemaking plants producing fresh, bloomy-rind, and washed-rind (smear-ripened) cheeses			
16S RNA gene, V1-V2	--	--	DNA	--	454 GS FLX	cs	descr.	SRP014212	3	Starter cultures	x		One raw milk and two selectively incubated natural milk cultures (63°C, 30 min + 42°C, 24 h)	2013	Delgado et al., 2013	3_2
--	--	--	DNA	--	454 GS FLX	ts	descr.	--	1	Starter cultures	x		Gouda cheese starter culture	2013	Erkus et al., 2013	--
16S RNA gene, V6-V9	--	--	--	--	454 GS FLX	ts (3)	descr.	--	9	Cheese	x		Croatian raw ewes' milk cheeses (3)	2013	Fuka et al., 2013	--
16S RNA gene, V6	--	--	--	--	Illumina HiSeq 2000	cs	descr.	--	6	Fermented milk	x		Kefir grains from Tibet	2013	Gao et al., 2013	--
16S RNA gene, V1-V2	--	--	--	--	454 GS FLX	cs	descr. + inf.	--	22	Milk	x		Raw cow milk from healthy and mastitic quarters	2013	Kuehn et al., 2013	--
16S RNA gene, V4-V5	ITS1 gene	--	--	--	454 GS FLX	cs	descr. + inf.	ERP002650	46	Fermented milk	x		Kefir grain and kefir milk from different sources	2013	Marsh, O'sullivan, Hill, Ross, & Cotter, 2013	0_6_2
16S RNA gene, V1-V3	--	--	--	--	454 GS FLX	ts (5)	descr.	--	30	Cheese	x		Poro (a fresh, raw milk Mexican cheese) cheese, from raw milk to mature (60 d cheese), three dairies, two seasons	2014	Aldrete-Tapia, Escobar-Ramírez, Tamplin, & Hernández-Iturriaga, 2014	--
16S RNA gene, V1-V3	--	--	--	--	454 GS Junior	cs	descr.	SRP026104	12	Cheese	x		Artisanal and industrial rennet pastes (mostly from lamb)	2014	Cruciata et al., 2014	3_2
16S RNA gene, V1-V3	--	--	--	*lacSZ* gene	454 GS Junior	cs	descr. + inf.	SRP033419	50	Cheese	x		Undefined strain starters (whey cultures) and cheese curds for water-buffalo Mozzarella, Grana Padano and Parmigiano Reggiano cheese	2014	De Filippis, La Storia, Stellato, Gatti, & Ercolini, 2014	0_6_2
16S RNA, V1-V3	--	--	--	--	454 GS FLX	ts (11)	descr. + inf.	--	11	Cheese	x		Milk, curd and Caciocavallo cheese during ripening.	2014	De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti, 2014b	0_6_2
16S RNA, V1-V3	--	--	--	--	454 GS Junior	ts (11)	descr. + inf.	SRP038100	11	Cheese	x		Milk, curd and ewe's milk Canestrato cheese during ripening.	2014	De Pasquale et al., 2014a	0_6_2
16S RNA gene, V1-V3	--	--	--	--	454 GS Junior	cs	descr. + inf.	SRP037967	22	Cheese	x		Herve cheese from raw or pasteurized milk; rind and heart were analysed for 11 different cheeses	2014	Delcenserie et al., 2014	--
16S RNA, V1-V3	--	--	--	--	454 GS Junior	cs + ts (7)	descr. + inf.	SRP040575	27	Cheese	x		Milk (from different lactation stages), curd and Fontina cheese from three different dairies	2014	Dolci, De Filippis, La Storia, Ercolini, & Cocolin, 2014	0_6_2
--	--	--	RNA	--	454 GS FLX	ts (7)	descr.	--	7	Cheese	x		Metatranscriptome of *Geotrichum candidum* and *P.*	2014	Lessard, Viel, Boyle, St-Gelais, & Labrie,	--

													camemberti in a Canadian Camembert during ripening		2014	
16S RNA, V1-V2	--	--	DNA	--	454 GS FLX	cs	descr.	--	2	Fermented milk	x		Turkish kefir grains	2014	Nalbantoglu et al., 2014	--
16S RNA gene, V1-V2	--	--	--	--	454 GS FLX	cs	descr. + inf.	SRP030032	177	Milk	x		Raw cow milk from healthy quarters and quarters with clinical and subclinical mastitis	2014	Oikonomou et al., 2014	--
16S RNA gene,V1-V2	--	--	--	--	454 GS Junior	cs	descr.	--	53	Fermented milk	x		Microbiota of Airag, Khoormog, Tarag from Mongolia	2014	Oki, Dugersuren, Demberel, & Watanabe, 2014	--
16S RNA gene, V3	ITS1 gene	--	--	--	454 GS FLX	cs	descr. + inf.	MG-RAST 2912, 4509270Ð4 509296	17	Fermented milk	x		Tarag, a fermented (acid-alcoholic) cow's milk product from Northern China and Mongolia	2014	Sun et al., 2014	--
16S RNA, V4	ITS1 gene	--	DNA	--	Illumina HiSeq	cs + ts	descr. + inf.	MG-RAST mgp8988	137	Cheese	x		Cheese rind for bloomy, washed and natural rind cheeses from several countries	2014	Wolfe, Button, Santarelli, & Dutton, 2014	--
16S RNA gene, V1-V3	18S RNA gene, V4	--	--	--	454 GS FLX	cs	descr. + inf.	MG-RAST 4570725.3–4570765.3, 4576499.3	22	Fermented milk	x		Fungal and bacterial communities in home made fermented milks in China	2014	Xu et al., 2014	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	SRP055798	40	Cheese	x		Grana Padano cheese samples with or without blowing defect, from different factories, at different ripening times, produced with and without lysozyme	2015	Bassi, Puglisi, & Cocconcelli, 2015	1_1
16S RNA gene, V4	ITS1 gene	--	--	--	Illumina MiSeq	cs	descr. + inf.	--	194	Fermented milk	x		Matsoni made in two different regions (Georgia or Armenia) with different types of milk	2015	Bokulich et al., 2015	--
--	--	--	DNA, RNA	--	SOLiD	ts (5)	descr.	PRJEB6315	15	Cheese	x		Metagenomic and metatranscriptomic analysis of a surface ripened cheese	2015	Dugat-Bony et al., 2015	--
16S RNA gene, V1-V3	26S RNA gene, D1-D2	--	--	--	454 GS Junior	cs	descr.	--	6	Fermented milk	x		Kefir grains (6) from different 5 Italian regions	2015	Garofalo et al., 2015	--
16S RNA gene, V1-V3	26S RNA gene, D1/D 2	--	--	--	454 Junior	cs	descr.	--	15	Fermented milk	x		Bacterial and fungal microbiota of kefir grains and milks from Belgium	2015	Korsak et al., 2015	--
16S RNA gene V1-V3	18S RNA gene,	--	--	--	GS FLX	cs	descr.	--	16	Fermented milk	x		Fermented yak milk from Tibet from two villages	2015	Liu et al., 2015a	--

	V4															
16S RNA gene, V1-V3	18S RNA gene, V4	--	--	--	454 GS FLX	cs	descr.	--	19	Fermented milk	x		Naturally fermented cow milks produced by Mongolian families in two regions of Russia	2015	Liu et al., 2015b	--
16S RNA gene, V4	--	--	--	--	Illumina GAIIx	cs	descr. + inf.	--	24	Milk	x		Wild type and engineered (to contain human lysozyme) raw goat milk at early, mid and late lactation	2015	McInnis, Kalanetra, Mills, & Maga, 2015	--
16S RNA gene, V4-V5	--	--	--	--	454 GS FLX	ts (4)	descr.	ERP009223	31	Cheese	x		Continental (Swiss type cheese) produced early and late in the day, core and rind samples included, different ripening times	2015	O'Sullivan, Cotter, et al., 2015a	1_1
--	--	--	--	*tdc, hdc* gene	Ion Torrent PGM	cs	descr.	--	10	Cheese	x		amplicon targeted amplification of tyrosine and histidine decarboxylase genes in semi hard and hard raw milk cheeses	2015	O'Sullivan, Fallico, et al., 2015b	--
16S RNA gene, V3-V4	--	--	--	--	454 GS FLX	ts (3)	descr. + inf.	--	28	Cheese	x		Pico (a fresh raw milk PDO cheese produced without starter in the Azorean Islands) from three manufactures, throughout ripening	2015	Riquelme et al., 2015	--
16S RNA gene, V3-V4	26S RNA gene	--	--	--		ts (9)	descr.	--	10	Cheese	x		Dynamics of the microbiota in Danbo cheese (core and surface; this is a semi-hard Danish smear cheese)	2015	Ryssel et al., 2015	--
16S RNA gene, V1-V3	26S RNA gene, D1-D2	--	--	--	454 GS Junior	cs	descr. + inf.	SRP058584	45	Cheese	x	x	Environmental swabs from an Italian dairy plant and different kind of cheeses (Mozzarella; Ricotta; Scamorza; Caciocavallo; Grancacio) produced in the same plant	2015	Stellato, De Filippis, La Storia, & Ercolini, 2015	--
16S RNA gene, V3-V5	--	--	--	--	454 GS FLX	cs	descr.	SRP056906	35	Milk		x	Microbial composition and diversity of milk and blood leukocytes and faeces of healthy lactating cows	2015	Young, Hine, Wallace, Callaghan, & Bibiloni, 2015	--
16S RNA gene, V1-V3	--	--	--	--	454 GS FLX	cs	descr. + inf.	--	24	Milk	x		Raw milk from Holstein cows with or without induced subclinical acidosis	2015	Zhang, Huo, Zhu, & Mao, 2015	--
16S RNA, V1-V3	--	--	--	--	454 GS Junior	ts (13)	descr.	SRP044294	39	Cheese	x		Piedmont hard cheese made from raw milk: milk, curd and cheese throughout ripening	2016	Alessandria et al., 2016	0_6_2
16S RNA, V1-V3	--	--	--	--	454 GS Junior	cs + ts (9)	descr. + inf.	--	48	Cheese	x	x	Environmental swabs from an Italian dairy plant and different kind of cheeses	2016	Calasso et al., 2016	1_1

													(Caciotta A02SV; Caciocavallo Pugliese A02XE) and cow milk produced in the same plant			
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs + ts (4)	descr.	--	36	Cheese	x		Dynamics of the microbiota in Plaisentif, an artisanal cheese produced in Italian Alps	2016	Dalmasso et al., 2016	--
16S RNA gene and 16S RNA, V1-V3	--	--	RNA	--	454 GS Junior	ts (8)	descr. + inf.	SRP061555	68	Cheese	x		Caciocavallo Silano cheese manufacture: starter culture, milk, curd and cheese throughout ripening.	2016	De Filippis, Genovese, Ferranti, Gilbert, & Ercolini, 2016	1_0_3
16S RNA, V1-V3	--	--	--	--	454 GS FLX	cs	descr. + inf.	PRJNA286758	30	Cheese	x		Active microbiota of Italian PDO ewe's milk cheeses	2016	De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti, 2016	--
16S RNA gene, V3-V4	ITS2 gene	--	--	--	Illumina MiSeq	cs	descr. + inf.	SRP071345	95	Cheese	x		Bacterial and fungal diversity of core and rind of 60 cheeses belonging to 12 popular surface ripened French cheese varieties (both washed rind and mould ripened)	2016	Dugat-Bony et al., 2016	3_1
16S RNA gene, V3-V4	--	--	--	--		ts (3)	descr. + inf.	--	18	Cheese	x		Evolution of the microbiota of two blue veined cheeses (PDO Bleu d'Auvergne and PDO Fourme d'Ambert) during storage under CO_2 atmosphere	2016	Duval et al., 2016	--
16S RNA gene, V1-V2	--	--	--	--	454 GS Junior	cs	descr. + inf.	--	8	Cheese		x	Floor drain water and floor drain biofilms in a cheese plant producing different types of cheese	2016	Dzieciol et al., 2016	--
--	--	--	DNA	--	Illumina HiSeq	cs	descr.	--	1	Cheese	x		A single sample (3 replicates) of 6 week-old Gouda cheese, with the main objective of seeing about the differential abundance of genes of the different lineages present in the starter	2016	Erkus et al., 2016	--
--	--	--	DNA	--	Illumina HiSeq2000/2500	--	descr.	SAMN03771674	25	Cheese	x		Mexican Cotija cheese (samples were pooled)	2016	Escobar-Zepeda, Sanchez-Flores, & Quirasco Baruch, 2016	--
16S RNA gene, V3-V4	--	--	--	--	454 GS FLX	cs	descr. + inf.	--	31	Milk		x	Cow's udder quarters with a different history of mastitis	2016	Falentin et al., 2016	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	ts (8)	descr. + inf.	--	640	Milk	x		Longitudinal description of the changes in the microbiome of cow milk associated with mastitis and antimicrobial therapy	2016	Ganda et al., 2016	--

16S RNA gene, V1-V3	--	--	--	--	454 GS Junior	cs	descr. + inf.	SRP052240	29	Cheese	x		Commercial high-moisture Mozzarella cheese produced with different acidification methods	2016	Guidone et al., 2016	0_6_2
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	ERP015209	1507	Milk	x		Tanker milk	2016	Kable et al., 2016	--
--	--	--	DNA, RNA	--		cs + ts (5)	descr.	--	25	Cheese	x		Metagenome and metatranscriptome of washed rind cheese produced from raw milk, and model systems; for one cheese analysis over ripening	2016	Kastman et al., 2016	--
16S RNA gene, V1-V3	--	--	--	--	454 GS Junior	ts (3)	descr.	--	9	Cheese	x		Microbiota of Cheddar cheese made from LTLT or thermised milk, during ripening	2016	Lee, Joung, Choi, Kim, & Oh, 2016	--
16S RNA gene, V1-V3	--	--	--	spxB	454 Junior	ts (3)	descr. + inf.	SRP063447	6	Cheese	x		Bacterial community and L. casei populations in 2 samples of Grana Padano during ripening	2017	Levante et al., 2017	--
16S RNA gene, V4	ITS	--	--	--	Illumina MiSeq	cs	descr.	--	2	Cheese	x		Bacterial and fungal microbiota of Rubing (a milk cakemade from goat milk)	2017	Liu, Kuda, Takahashi, & Kimura, 2017	--
16S RNA gene, V3-V4	--	--	RNA	--	Illumina MiSeq and HiSeq 1000	ts (4)	descr. + inf.	ERP012739	12	Cheese	x		Rind microbiota and metatranscriptome of a Reblochon type cheese during ripening (35 d)	2016	Monnet et al., 2016	--
16S RNA gene, V1-V3	--	--	--	*serB* gene	454 GS Junior	cs + ts (2-10)	descr. + inf.	SRP057506	24	Starter cultures	x		Undefined strain starters (milk cultures) for high-moisture Mozzarella cheese	2016	Parente et al., 2016	0_6_2
16S RNA gene,V1-V3, V4-V5, V6-V8					454 GS FLX	cs	descr.	--	4	Milk	x		Microbiota of milk from water-buffaloes with clinical or subclinical mastitis	2016	Patel et al., 2016	--
16S RNA gene, V3-V4	--	--	DNA	--	Illumina MiSeq	cs + ts	descr. + inf.	--	40	Cheese	x		Dutch cheese produced with different technologies (washing, scalding temperature) throughout ripening	2016	Porcellato & Skeie, 2016	--
16S RNA gene, V4-V5	--	--	DNA	--	454 GS FLX + Illumina HiSeq 2000	cs	descr. + inf.	ERP006630	59	Cheese	x		Investigating the role of the microbiota in Pink Cheese. Cheddar, Emmental and cheese coloured with Annatto, either unspoiled or spoiled.	2016	Quigley et al., 2016	2
--	--	--	--	*serB* gene	454 GS Junior	cs + ts	descr.	--		Cheese	x		Distribution of *serB* alleles in different cheeses by traditional cultivation based methods and amplicon	2016	Ricciardi et al., 2016	--

													targeted HTS			
16S RNA, V3-V4	--	--	--	--	Illumina MiSeq	ts (8)	descr. + inf.	SRP060430	46	Cheese	x		Bovine ricotta cheese (two lots, winter and spring) without or with pink discoloration, throughout storage at 8°C.	2016	Sattin, Andreani, Carraro, Fasolato, et al., 2016a	3_1
16S RNA gene and 16S RNA, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	SRP070771	30	Cheese	x		Pasteurized whey used for bovine ricotta manufacture (compliant and non-compliant samples)	2016	Sattin, Andreani, Carraro, Lucchini, et al., 2016b	3_1
16S RNA gene, V1-V2	--	--	--	--	?	cs	descr.	PRJEB11385	6	Cheese		x	Bacterial and funga (clone library)l microbiota of floor drain water and biofilms in an Austrian dairy plant	2016	Schön et al., 2016	--
16S RNA gene, V3-V4	ITS1 gene	--	DNA	--	Illumina MiSeq	ts (3)	descr. + inf.	ERP017163	17	Fermented milk	x		Microbiota of three kefirs over the course of fermentation (24 h)	2016	Walsh et al., 2016	3_1
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr.	--	?	Fermented milk	x		Bacterial composition of Malaysian kefir grains	2016	Zamberi et al., 2016	--
16S RNA gene, V3 and V1-V3	--	--	--	--	--	cs	descr. + inf.	--	85	Fermented milk, cheese	x		A reanalysis of data from several studies of the same group, including koumiss, yougurt, cheese etc-	2016	Zhong et al., 2016a	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	ts (2)	descr. + inf.	--	84	Milk	x		Impact of dry cow therapy with without antibiotics at dry off on milk microbiome	2017	Bonsaglia et al., 2017	--
16S RNA gene, V1-V2	--	--	--	--	IonTorrent PGM	cs	descr. + inf.	SRP106438	137	Milk	x		Microbiota of water-buffalo milk obtained from healthy animals and from animals with clinical and subclinical mastitis	2017	Catozzi et al., 2017	3_2
16S RNA gene, V1-V3	--	--	--	--	Illumina MiSeq	ts (5)	descr.	PRJNA300927 (no public data)	25	Cheese	x		Rind and surface of Tomme d'Orchie, a pressed uncooked raw cow milk cheese from France, throughout ripening (0-21 d)	2017	Ceugniez et al., 2017	
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	--	11	Milk	x		Donkey milk from Italian farms	2017	de los Dolores Soto del Rio, Dalmasso, Civera, & Bottero, 2017	--
16S RNA, V1-V3	ITS1 gene	--	--	--	Illumina MiSeq	cs	descr.	SRP102840	4	Fermented milk	x		Microbiota of 4 kefir grains from different regions of Turkey and volatile profile	2017	Dertli & Çon, 2017	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	ts (5)	descr. + inf.	ERP022373	72	Milk	x		Raw milk from bulk tanks, mid and late lactation, stored at different temperatures	2017	Doyle, Gleeson, O'Toole, & Cotter, 2017a	3_1
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	ERP018630	149	Milk	x	x	A study on sources of contamination of milk and	2017	Doyle, Gleeson, O'Toole, & Cotter,	--

													effect of cleaning		2017b	
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	ts (36)	descr. + inf.	--	1674	Milk	x		Longitudinal study on the changes in microbiome of cows experimentally infected with *E. coli* and treated or not with ceftiofur	2017	Ganda et al., 2017	--
16S RNA gene, V1-V3	--	--	--	--	454 GS Junior	ts (6)	descr. + inf.	SRP070077	38	Cheese	x		Caciocavallo cheese, throughout ripening, with milk obtained under different cow's feeding regimes	2017	Giello et al., 2017	3_1
--	ITS3 spacer	--	--	--	Illumina MiSeq	cs	descr.	--	8	Cheese	x		Yeast microbiota of a traditional portuguese raw ewe's milk cheese (Serpa) made in both PDO-registered and unregistered cheesemaking plants	2017	Gonçalves Dos Santos, Benito, Córdoba, Alvarenga, & Ruiz-Moyano Seco de Herrera, 2017	--
16S RNA gene, V1-V3	ITS1 gene	--	--	--	454 GS FLX+	cs	descr. + inf.	SRP051167	26	Cheese	x	x	Cheese with red-brown defect and cheese environment	2017	Guzzon et al., 2017	3_1
16S RNA gene	--	--	--	--	PacBio RS II	cs	descr. + inf.	--	6	Cheese	x		Bacterial microbiota of a Kazakhstan cheese, evaluated single molecule real time sequencing with PacBio, and compared with that of artisanal cheeses (including Belgium and Italy)	2017	Li et al., 2017	--
16S RNA, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	SRP109273	19	Cheese		x	Brine samples from cheesemaking plants in Italy, cheese type not provided	2017	Marino et al., 2017	3_1
16S RNA, V1-V3	--	--	--	--	Illumina MiSeq	cs + ts (3)	descr. + inf.	SRP110830	50	Cheese	x		Burrata cheese with or without protective lactobacilli and dietary fibers	2017	Minervini, Conte, Del Nobile, Gobbetti, & De Angelis, 2017	3_1
16S RNA gene, V3	--	--	--	--	Illumina MiSeq	cs	descr.	SRP090797	6	Fermented milk	x		Microbiota of Suero Costeño, a fermented cream from Colombia	2017	Motato et al., 2017	--
16S RNA gene, V1-V3	--	--	--	--	454 GS FLX	ts (4)	descr.	--	21	Cheese	x		Microbiota and mRNA for a Camembert type cheese throughout ripening	2017	Oh et al., 2017	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr.	--	130	Milk	x		Cow milk from paired healthy and mastitic quarters	2017	Oultram, Ganda, Boulding, Bicalho, & Oikonomou, 2017	--
16S RNA, V3-V4	--	--	--	--	Illumina MiSeq	ts (3)	descr. + inf.	SRP074051	15	Cheese	x		Liqvan, a traditional brined raw ewe's milk cheese produced in Iran, throughout ripening	2017	Ramezani, Hosseini, Ferrocino, Amoozegar, & Cocolin, 2017	3_2
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	--	472	Milk	x		Cow's bulk tank milk from 19 farms NY state over a	2017	Rodrigues, Lima, Canniatti-Brazaca, &	--

													period of 2 months, and correlation between microbiota and milk quality parameters, including somatic cell count		Bicalho, 2017	
16S RNA gene, V1-V2			DNA		Illumina MiSeq	cs	desscriptive + inf.	PRJEB20873	10	Fermented milk	x		Metagenomic analysis of nunu a fermented raw cow milk product from Ghana, made by trained and untrained operators	2017	Walsh et al., 2017	--
--	--	--	DNA	--	Illumina MiSeq	ts (4)	descr. + inf.	ERP017154	12	Cheese	x		Whole genome sequencing of Cheddar cheese inoculated with two different smear cultures	2018	Bertuzzi et al., 2018	--
--	--	DNA	--	--	Illumina MiSeq	cs	descr.	ERP024450	3	Cheese		x	Targeting airborne virome in cheese plants by shotgun metagenomics	2018	Colombo et al., 2018	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	ts (4)	descr. + inf.	SRP120497	117	Milk	x		Raw milk from healthy quarters of dairy cows from two breeds (Holsteins and Rendena, during lactation	2018	Cremonesi et al., 2018	3_2
--	--	--	DNA, RNA	--	Illumina MiSeq, SOLiD 5500XL	ts (2)	descr.	PRJEB23938	6	Cheese	x		Metagenomic and metatrascriptomic analysis of Maasdam cheese at two dimes during ripening	2018	Duru et al., 2018	
16S RNA gene, V1-V3	--	--	--	purR, epsD	Illumina MiSeq	cs	descr.	ERP105082	18	Cheese	x		Use of *purR* and *epsD* as a target for studying diversity of *Lactococcus lactis* in three undefined strain starters	2018	Frantzen, Kleppen, & Holo, 2018	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs + ts (2)	descr. + inf.	SRP126475	48	Cheese, Milk	x	x	Teat skin and Cantal cheese microbiota, as a function of grazing system	2018	Frétin et al., 2018	3_1
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr.	--	8	Cheese	x		cheese at 15 d refrigerated storage of Vastedda del Belice, a raw ewe's milk cheese made in vats obtained with seven different wood types	2018	Gaglio et al., 2014	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr.	--	8	Cheese	x		Bacterial microbiota of a traditional portuguese raw ewe's milk cheese (Serpa) made in both PDO-registered and unregistered cheesemaking plants	2018	Gonçalves et al., 2018	--
16S RNA gene, V3	ITS2 gene	--	--	--	Illumina NextSeq	cs	descr.	--	7	Cheese		x	Brines used for Danbo, a surface ripened semi-hard cheese	2018	Haastrup et al., 2018	--
16S RNA gene	--	--	--	--	PacBio RS II	cs	descr.	--	7	Cheese	x		Cottage cheese from Buryata (Russia)	2018	Jin et al., 2018	--
16S RNA	--	--	--	--	Illumina	cs	descr. +	SRP111889	112	Milk	x		Raw milk samples collected	2018	Li et al., 2018	3_2

gene, V3-V4					MiSeq		inf.						from 10 farms around Shangai over 12 months			
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	--	descr.	--	280	Milk	x		An optimization of DNA extraction methods for milk samples from cows with or without mastitis	2018	Lima, Bicalho, & Bicalho, 2018	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	SRP134034	50	Milk	x		Effect of sampling technique and bedding on the microbiota of milk of primiparous cows	2018	Metzger, Hernandez, Skarlupka, Suen, et al., 2018a	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	ts	descr. + inf.	SRP151652	408	Milk	x		Microbiota of teat milk from healthy mammary glands or mammary glands with different status of mastitis (chronical, clinical, subclinical), over 150 days	2018	Metzger, Hernandez, Skarlupka, Walker, et al., 2018b	--
16S RNA gene, V3	--	--	--	ITS (Lactobacillus)	Illumina MiSeq	cs	descr.	PRJNA434072	20	Cheese, starter cultures	x		Stool, vaginal swab, fresh Parmesan cheese (1 day of ripening, 5 samples), whey (5 samples), and cecal (from free-range chickens) samples	2018	Milani et al., 2018	--
16S RNA gene, V3-V4	18S RNA gene, V4-V7	--	--	--	Ion Torrent PGM	cs	descr. + inf.	SRP072467	17	Cheese	x		Fungal and bacterial communities in 17 Mexican soft and semi-hard cheese varieties from raw or pasteurized milk	2018	Murugesan et al., 2018	--
16S RNA gene, V4	ITS	--	--	--	Illumina MiSeq	cs	descr.	--	8	Fermented milk	x		Bacterial and fungal microbiota of traditional fermented milk products from Bangladesh (Dahi, chanar-misti, paneer, and borhani)	2018	Nahidul-Islam, Kuda, Takahashi, & Kimura, 2018	--
16S RNA gene, V1-V4	--	--	--	--	454 GS FLX	cd	descr.	--	10	Cheese	x		Microbiota of a Gouda type cheese (surface and core)after 120 d ripening	2018	Oh & Kim, 2018	--
16S RNA gene, V1-V2	--	--	--	--	Illumina HiSeq 2000	cs	descr. + inf.	--	64	Milk	x		Evaluates the teat cow milk microbiota in two farms with different incidence rate of subclinical mastitis	2018	Pang et al., 2018	--
16S RNA gene, V4					Illumina MiSeq	cs	descr. + inf.	PRJNA482953	226	Fermented milk	x	x	Lait caillé (an artisanal fermented milk) and biofil samples from containers for lait caillé from different urban and rural locations in Northern Senegal	2018	Parker et al., 2018	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs + ts (6)	descr. + inf.	ERP105036	864	Milk	x		Raw and pasteurized cow milk from two Danish dairies. Only a random subset of samples from the	2018	Porcellato et al., 2018	3_1

													study is included in FoodMicrobionet.			
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs + ts (2)	descr.	SRP103624	92	Cheese	x		Gouda cheese (15 brands), from pasteurized or raw milk. Spatial (core, surface, middle) and variability was assessed. Cheese age (2-18 mo) confounded with brand.	2018	Salazar et al., 2018	3_2
16S RNA gene, V4-V5	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	MG-RAST (4732361 - 4732414)	54	Fermented milk	x		Fermented milk products (chhurpi, churkam, dahi) from two regions of India	2018	Shangpliang, Rai, Keisam, Jeyaram, & Tamang, 2018	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	--	8	Fermented milk	x		Dadhi, a fermented milk produced from water-buffalo milk in West Sumatra	2018	Venema & Surono, 2018	--
16S RNA gene, V4	ITS1 gene	--	DNA	--	Illumina MiSeq Ion PGM	cs	descr.	--	2	Cheese		x	High-throughput amplicon and shotgun metagenomic sequencing of stable 30 y old brines from an artisanal and a large scale plant in Flanders.	2018	Vermote, Verce, De Vuyst, & Weckx, 2018	--
--	--	--	DNA	--	Illumina MiSeq, NextSeq, Ion Proton	cs	descr. + inf.	ERP104300	6	Fermented milk	x		Six kefir samples and a 13 strains mock community, used to evaluate the effect of sequencing platform and species classifiers in shotgun analyses	2018	Walsh et al., 2018	--
16S RNA gene, V3-V4	--	--	--	--	Illumina HiSeq 2500	cs	descr. + inf.	--	15	Cheese	x		Microbiota of Rushan cheese from three areas in the Yunnan province, China	2018	Xue, Yang, Wang, Guo, & Shao, 2018	--
16S RNA gene	--	--	--	--	PacBio RS II	cs	descr.	--	8	Fermented milk	x		Traditional sour cream and butter from Buryatia, Russia	2018	Yu et al., 2018	--
16S RNA gene, V4-V6	--	--	--	--	454 FLX+	cs	descr.	--	72	Fermented milk	x		Yoghurt, kefir, acidophilus milk manufactured from pasteurized or unpasteurized milk and some raw milk samples	2018	Zalewska, Kaevska, & Slana, 2018	--
16S RNA gene, V4	ITS2, ITS5	--	--	--	Illumina MiSeq	ts (5)	descr. + inf.	--	5	Cheese	x		Bacterial and fungal diversity in cheese produced in the Uighur area of China	2018	Zheng et al., 2018	--
16S RNA gene, V3-V4	--	--	--	--	Illumina HiSeq	cs	descr. + inf.	--	175	Milk environment		x	Microbiota in the rumen of cows with different levels of somatic cell counts in milk (from $<2x10^5$ to $>1x10^6$)	2018	Zhong, Xue, & Liu, 2018	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	--	114	Milk	x	x	Microbiota of the teat end skin and milk of cows with and without mastitis	2019	Andrews, Neher, Weicht, & Barlow, 2019	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs		SRP173041	50	Milk	x		Mastitic cow milk (with high CMT counts) from Ireland	2019	Angelopoulou et al., 2019	3_2

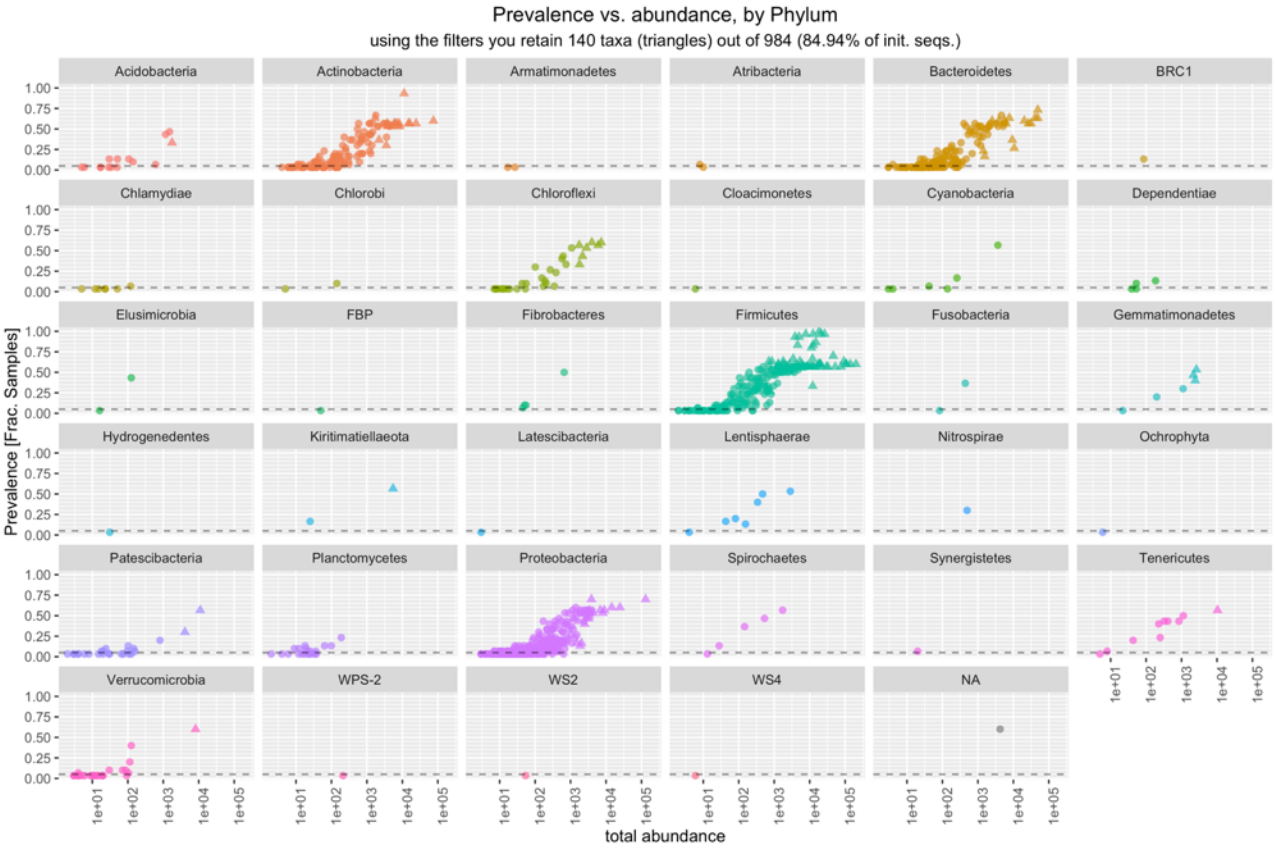
16S RNA gene, V3					Ion Torrent PGM	cs	descr.	MK789774-MK78984	6	Fermented milk	x		Microbiota of Dhanaan, a fermented milk from Ethiopia	2019	Behre et al., 2019	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	ts (3)	descr.	--	91	Starter cultures	x		Bacterial communities in Natural whey cultures for Parmigiano Reggiano, in one dairy, two lines, over several days	2019	Bertani et al., 2019	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	ts (3)	descr. + inf.	SRP166154	39	Cheese	x		Milk curd and cheese for a raw milk mini cheese enriched in GABA	2019	Carafa et al., 2019	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	SRP182770	36	Milk	x		Microbiota of teat milk from healthy Manchega ewes (with or without an history of mastitis) from 2 farms	2019	Castro et al., 2019b	3_2
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs + ts (8)	descr. + inf.	SRP156693	288	Milk	x		Low biomass cow's milk from healthy udder quarters	2019	Dahlberg et al., 2019	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr.	SRP192216	4	Fermented milk	x		Miscellaneous African fermented foods, including ghee and fermented milks	2019	Diaz et al., 2019	3_2
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	--	50	Milk	x		Microbiota of individual ewe's milk (teat milk) and its relationship with somatic cell counts for subclinical mastitis	2019	Esteban-Blanco et al., 2019	--
16S RNA gene, V3	--	--	--	--	Illumina HiSeq 2500	cs	descr. + inf.	SRP170819	375	Cheese, milk	x	x	Cow milk, cheese (4 types: Brie, Cheddar, Gruyere, Jarlsberg) and environmental samples, from farm to fork for an artisanal cheese production facility	2019	Falardeau, Keeney, Trmčić, Kitts, & Wang, 2019	3_2
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr.	--	27	Cheese	x	x	Samples from 2 Brazilian dairies, inlcuding cheese (Minas Frescal, Coahlo, a semi-hard cheese), milk, whey and food environment	2019	Frazilio, Almeida, Niño-Arias, & Martinis, 2019	--
16S RNA gene, V1-V3	ITS1	--	--	--	Illumina MiSeq	cs	descr.	--	10	Fermented milk	x		Bacterial and fungal communities from 5 kefir milks and 5 kefir grains from Tibet	2019	Gao & Zhang, 2019	--
16S RNA gene, V4					Illumina MiSeq	cs + ts(6)	descr. + inf.	--	48	Fermented milk	x	x	Laboratory made lait caillé using lahals imported from Senegal	2019	Groenenboom et al., 2019	
--	--	--	DNA	--	Illumina MiSeq	cs	descr.	PRJNA529353 (no public data)	21	Milk	x		Milk for healthy and mastitic crossbred cows collected from several farms	2019	Hoque et al., 2019	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs + ts	descr. + inf.	ERP015209	142	Milk	x		Cow milk as it passes through the collection and processing chain	2019	Kable, Srisengfa, Xue, Coates, & Marco, 2019	3_2

16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	SRP165151	200	Cheese	x		Artisanal raw milk cheeses from Brasil (11 different types from 5 geographical areas)	2019	Kamimura, De Filippis, Sant'Ana, & Ercolini, 2019	3_2
16S RNA gene, V3-V4	?	--	--	--	Illumina MiSeq	cs	descr.	--	3	Fermented milk	x		Tibetan kefir grains	2019	Liu, Zhang, Xie, Wang, & of, 2019	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	SRP156292	39	Cheese	x		High moisture Mozzarella cheese (cow or buffalo milk), produced with different types of starters	2019	Marino et al., 2019	3_2
16S RNA gene, V3	--	--	--	ITS (Bifidobacterium)	Illumina MiSeq	cs	descr. + inf.	--	21	Cheese	x		Italian raw milk cheeses from cow, goat, ewes' or waterbuffalo milk)	2019	Milani et al., 2019	--
16S RNA gene	--	--	--	--	PacBio RS II	cs	descr. + inf.	mgp87678	19	Fermented milk	x		Fermented milk products from Mongolia (including koumiss)	2019	Mo et al., 2019	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr.	SRP163054	40	Milk environment		x	Artificial biofilms on stainless steel surfaces in contact with raw milk	2019	Oliveira et al., 2019	--
16S RNA, V1-V3	--	--	--	prtP, pepN, pepX, bcaT cDNA (LAB)	Illumina NextSeq	ts (3)	descr.	--	3	Cheese	x		Metataxonomic analysis and HTS for expression of 4 genes related to N metabolism in a ewe's milk cheese (Bryndza) during ripening	2019	Pangallo et al., 2019	--
16S RNA gene,V1-V3, V4-V5, V6-V8	--	--	--	--	454 GS FLX	cs	descr.	--	18	Milk	x		Microbiota of milk from healthy water-buffaloes or from animals with clinical or subclinical mastitis	2019	Patel, Kunjadia, Koringa, Joshi, & Kunjadiya, 2019	--
16S RNA gene, V1-V3	ITS1-ITS2	--	--	--	Illumina MiSeq	ts (3)	descr.	ERP113761	3	Cheese	x		Microbiota of Halitzia, a white brined goat cheese from Cyprus	2019	Papademas et al., 2019	--
--	--	--	RNA	--	Illumina NextSeq500	ts (2)	descr.	PRJEB30420	48	Cheese	x		Metatranscriptomic analysis of a model surface ripened cheese made using a sterilized curd and synthetic microbial communities	2019	Pham, Landaud, Lieben, Bonnarme, & Monnet, 2019	--
16S RNA gene, V3-V4	--	--	--	*panC, glpT, pycA*	Illumina MiSeq	cs + ts	descr. + inf.	ERP105775	184	Milk	x		Bacillus diversity in pasteurized cow milk during refrigerated storage	2019	Porcellato, Aspholm, Skeie, & Mellegård, 2019	--
16S RNA gene, V3-V5	--	--	--	--	Illumina MiSeq	cs + ts (7)	descr. + inf.	--	91	Cheese	x		Bacterial communities in Minas cheese (Brasil) during ripening	2019	Sant'Anna et al., 2019	--
--	ITS1 gene	--	--	--	Illumina MiSeq	cs	descr. + inf.	SRP170707	60	Cheese, Fermented milk	x		Yeasts in Wagashi cheese and traditional fermented milk from Benin.	2019	Sessou et al., 2019	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs + ts (3)	descr. + inf.	ERP106513	135	Milk	x		Bulk tank bovine raw milk from 45 dairy farms in	2019	Skeie, Håland, Thorsen, Narvhus, &	3_2

													Norway, 2 geographical areas, different seasons		Porcellato, 2019	
16S RNA gene, V4	--	--	DNA	--	PacBio, Illumina MiSeq, Oxford MinION	cs	descr.	SAMN09703751, SAMN09580370	2	Starter culture	x		Metagenomic analysis (using three sequencing platforms) of two Natural whey cultures used for Gruyere cheese	2019	Somerville et al., 2019	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	--	49	Milk	x		Milk form healthy and mastitic quarters (same cow) analysed by 16S metagenomics and qPCR	2019	Taponen et al., 2019	--
16S RNA gene, V1-V2	--	--	--	--	Illumina HiSeq 2500	cs	descr. + inf.	SRP192494	12	Milk	x		Microbiome and metabolome in the teat milk of healthy cows and of cows with subclinical S. agalactiae mastitis	2019	Tong et al., 2019	--
16S RNA gene, V4	--	--	--	--	Illumina MiSeq	cs	descr. + inf.	--	85	Milk	x		Teat milk from non-severe *E. coli* mastitis in cows and follow up sples	2019	Vasquez et al., 2019	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	ts (9)	descr. + inf.	--	27	Fermented milk	x		Bacterial successions in the microbiota of Koumiss produced in Mongolia	2019	Wurihan et al., 2019	--
16S RNA gene	--	--	--	--	PacBio RS II	cs	descr.	mgp89891	12	Cheese	x		Amplicon targeted metagenomics and functional analysis using PiCRUST for 12 cheeses from different regions	2019	Yanget al., 2019	--
16S RNA gene, V3-V4	--	--	--	groEL	Illumina MiSeq	cs	descr.	--	6	Fermented milk	x	x	Feces and indigenous fermented milk (kurut) samples (6 samples for kurut 24 overall)	2019	Xie et al., 2019	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	cs	descr.	--	280	Milk	x		Refrigerated raw cow milk from different geographical areas of New Zealand, in 4 different seasons, before and after storage at 7°C	2019	Zhang, Palmer, Teh, Biggs, & Flint, 2019	--
16S RNA gene, V3-V4	ITS	--	--	--	Illumina MiSeq	cs	descr.	SRP128665	2	Milk	x		Fungal and bacterial microbiota of camel dromedary milk from Algenria	2020	Amrouche, Mounier, Pawtowski, Thomas, & Picot, 2020.	--
16S RNA gene, V3-V4	--	--	--	--	Illumina MiSeq	ts (4)	descr.	PRJEB36556	24	Cheese	x		Evolution of the microbiota of Paipa cheese (Colombia) during ripening	2020	Castellanos-Rozo, Pulido, Grande, Lucas, & Gálvez, 2020	--
16S RNA gene, V3-V4	--	--	--	groEL	Illumina MiSeq	cs	descr.	--	36	Milk, fermented milk	x		Microbiota of raw yak milk, qula, and fermented yak milk samples were collected from the Aba Tibetan autonomous region of China	2020	Jiang et al., 2020	--
16S RNA gene, V3-V4					Illumina MiSeq	cs	descr. + inf.	PRJNA526725	26	Cheese	x		Milk, ripened cheese an environmental samples for	2020	Kamimura et al., 2020	--

													Serra de Canastra, an artisanal cheese produced in Brazil			
16S RNA gene, V3-V9, V2-V8	--	--	--	--	Ion Torrent PGM	ts (3)	descr.	--	5	Cheese		x	Microbiota of Feta cheese during ripening in plastic or stainless steel containers	2020	Spyrelli, Stamatiou, Tassou, Nychas, & Doulgeraki, 2020	--
16S RNA gene	--	--	--	--	PacBio RS II	cs	descr.	--	15	Milk	x		Camel milk from Mongolia	2020	Zhao et al., 2020	--

Supplementary Figure 1. A prevalence and abundance plot for samples from teat skin from Frétin et al. (2018) and Falardeau et al. (2019). Sequences were downloaded from NCBI Short Read Archive (SRA) and processed as described in Parente, De Filippis, Ercolini, Ricciardi, & Zotta (2019). Prevalence threshold was 0.05 and abundance threshold 0.005. Triangles are taxa which pass the filter.

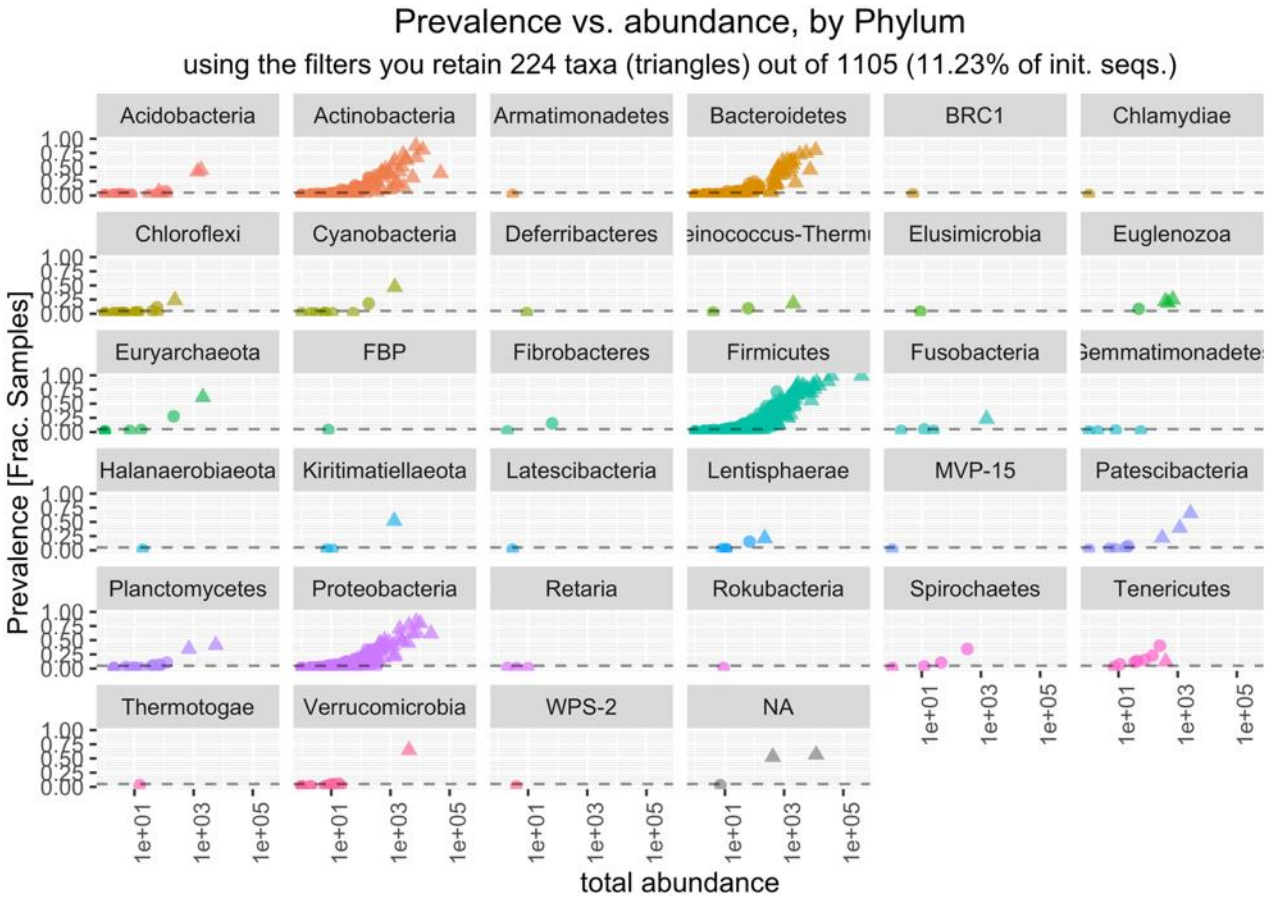


Supplementary Table 2. The top 50 taxa in terms of prevalence and abundance from cow's teat skin in the studies of Frétin et al. (2018) and Falardeau et al. (2019). Sequences were downloaded from NCBI SRA and processed as described in Parente et al. (2019).

phylum	class	genus	rel. ab.	min. rel. ab.	max. rel. ab.	rel. prev.
Firmicutes	*Bacilli*	*Staphylococcus*	0.0077	0.0013	0.4235	1.0000
Firmicutes	*Bacilli*	*Aerococcus*	0.0108	0.0000	0.2167	0.9667
Firmicutes	*Erysipelotrichia*	*Turicibacter*	0.0086	0.0000	0.3959	0.9667
Firmicutes	*Bacilli*	*Jeotgalicoccus*	0.0051	0.0000	0.2572	0.9667
Firmicutes	*Bacilli*	*Lactococcus*	0.0032	0.0000	0.2927	0.9667
Actinobacteria	*Actinobacteria*	*Bifidobacterium*	0.0046	0.0000	0.7660	0.9333
Firmicutes	*Bacilli*	*Streptococcus*	0.0021	0.0000	0.0724	0.9333
Firmicutes	*Bacilli*	*Sporosarcina*	0.0016	0.0000	0.0872	0.9333
Firmicutes	*Bacilli*		0.0062	0.0000	0.0466	0.8667
Firmicutes	*Bacilli*	*Facklamia*	0.0051	0.0000	0.0940	0.8333
Firmicutes	*Bacilli*	*Bacillus*	0.0019	0.0000	0.1727	0.8333
Firmicutes	*Bacilli*	*Jeotgalibaca*	0.0046	0.0000	0.0236	0.8000
Bacteroidetes	*Bacteroidia*	*Bacteroides*	0.0202	0.0000	0.0399	0.7333
Proteobacteria	*Gammaproteobacteria*	*Acinetobacter*	0.0536	0.0000	0.4923	0.7000
Firmicutes	*Bacilli*	*Atopostipes*	0.0190	0.0000	0.0540	0.7000
Proteobacteria	*Gammaproteobacteria*	*Luteimonas*	0.0016	0.0000	0.0135	0.7000
Firmicutes	*Erysipelotrichia*		0.0049	0.0000	0.0089	0.6667
Bacteroidetes	*Bacteroidia*	*Prevotellaceae_UCG-004*	0.0019	0.0000	0.0041	0.6667
Firmicutes	*Bacilli*	*Alloiococcus*	0.0014	0.0000	0.0773	0.6667
Actinobacteria	*Actinobacteria (class)*	*Nocardioides*	0.0007	0.0000	0.0041	0.6667
Firmicutes	*Clostridia*		0.0378	0.0000	0.0658	0.6333
Bacteroidetes	*Bacteroidia*	*Rikenellaceae_RC9_gut_group*	0.0201	0.0000	0.0451	0.6333
Bacteroidetes	*Bacteroidia*	*Alistipes*	0.0168	0.0000	0.0362	0.6333
Bacteroidetes	*Bacteroidia*		0.0032	0.0000	0.0069	0.6333
Firmicutes	*Bacilli*	*Solibacillus*	0.0022	0.0000	0.0122	0.6333
Firmicutes	*Bacilli*	*Lactobacillus*	0.0007	0.0000	0.0197	0.6333
Actinobacteria	*Actinobacteria*	*Flaviflexus*	0.0007	0.0000	0.0096	0.6333
Firmicutes	*Erysipelotrichia*	*Solobacterium*	0.0004	0.0000	0.0136	0.6333
Firmicutes	*Bacilli*	*Ignavigranum*	0.0003	0.0000	0.0094	0.6333
Firmicutes	*Clostridia*	*Ruminococcaceae_UCG-005*	0.0832	0.0000	0.1480	0.6000
Firmicutes	*Clostridia*	*Romboutsia*	0.0577	0.0000	0.1472	0.6000
Firmicutes	*Clostridia*		0.0448	0.0000	0.0707	0.6000
Firmicutes	*Clostridia*	*Ruminococcaceae_UCG-010*	0.0431	0.0000	0.1089	0.6000
Actinobacteria	*Actinobacteria*	*Corynebacterium*	0.0309	0.0000	0.1101	0.6000
Proteobacteria	*Gammaproteobacteria*	*Pseudomonas*	0.0102	0.0000	0.3950	0.6000
Firmicutes	*Clostridia*	*Mogibacterium*	0.0084	0.0000	0.0173	0.6000
Bacteroidetes	*Bacteroidia*	*Prevotellaceae_UCG-003*	0.0084	0.0000	0.0158	0.6000
Firmicutes	*Clostridia*	*Ruminococcaceae_UCG-014*	0.0079	0.0000	0.0120	0.6000
Firmicutes	*Clostridia*	*Flavonifractor*	0.0079	0.0000	0.0165	0.6000

Firmicutes	*Clostridia*		0.0072	0.0000	0.0111	0.6000
Proteobacteria	*Gammaproteobacteria*	*Marinospirillum*	0.0060	0.0000	0.0273	0.6000
Verrucomicrobia	*Verrucomicrobiae*	*Akkermansia*	0.0034	0.0000	0.0072	0.6000
Chloroflexi	*Chloroflexia*		0.0030	0.0000	0.0213	0.6000
Firmicutes	*Bacilli*		0.0025	0.0000	0.0123	0.6000
Bacteroidetes	*Bacteroidia*		0.0023	0.0000	0.0054	0.6000
Firmicutes	*Clostridia*		0.0017	0.0000	0.0031	0.6000
Chloroflexi	*Anaerolineae*		0.0016	0.0000	0.0090	0.6000
Bacteroidetes	*Flavobacteriia*	*Flavobacterium*	0.0016	0.0000	0.0108	0.6000
Firmicutes	*Bacilli*	*Salinicoccus*	0.0011	0.0000	0.0060	0.6000

Supplementary Figure 2. A prevalence and abundance plot for samples from teat milk from Cremonesi et al. (2018) (colostrum samples were removed) and Falardeau et al. (2019). Sequences were downloaded from NCBI SRA and processed as described in Parente et al. (2019). Prevalence threshold was 0.05 and abundance threshold 0.005. Triangles are taxa which pass the filter.



Supplementary Table 3. The top 50 taxa in terms of prevalence and abundance from teat milk from Cremonesi et al. (2018) (colostrum samples were removed) and Falardeau et al. (2019). Sequences were downloaded from NCBI SRA and processed as described in Parente et al. (2019).

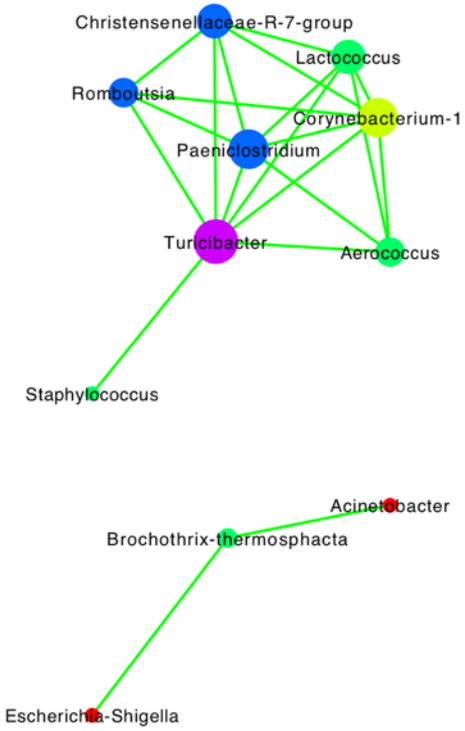
phylum	class	family	genus	rel. ab.	min. rel. ab.	max. rel. ab.	rel. prev.
Firmicutes	*Bacilli*	*Streptococcaceae*	*Streptococcus*	0.4170	0	0.8939	0.9904
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Lactobacillus*	0.0394	0	0.1626	0.9904
Firmicutes	*Bacilli*	*Staphylococcaceae*	*Staphylococcus*	0.0306	0	0.3398	0.8942
Firmicutes	*Bacilli*	*Streptococcaceae*	*Lactococcus*	0.0125	0	0.4684	0.8750
Actinobacteria	*Actinobacteria (class)*	*Propionibacteriaceae*	*Cutibacterium*	0.0072	0	0.0430	0.8750
Firmicutes	*Clostridia*	*Clostridiaceae_1*	*Clostridium_sensu_stricto_1*	0.0030	0	0.0157	0.8462
Firmicutes	*Clostridia*	*Christensenellaceae*	*Christensenellaceae_R-7_group*	0.0096	0	0.0589	0.8269
Proteobacteria	*Gammaproteobacteria*	*Moraxellaceae*	*Acinetobacter*	0.0074	0	0.1031	0.8269
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-005*	0.0153	0	0.0941	0.8173
Firmicutes	*Clostridia*	*Peptostreptococcaceae*	*Romboutsia*	0.0100	0	0.1215	0.8173
Firmicutes	*Bacilli*			0.0042	0	0.0106	0.8173
Actinobacteria	*Actinobacteria (class)*	*Corynebacteriaceae*	*Corynebacterium_1*	0.0123	0	0.0977	0.8077
Firmicutes	*Bacilli*	*Enterococcaceae*	*Enterococcus*	0.0026	0	0.0171	0.8077
Bacteroidetes	*Bacteroidia*	*Bacteroidaceae*	*Bacteroides*	0.0116	0	0.0733	0.7981
Proteobacteria	*Gammaproteobacteria*	*Moraxellaceae*	*Enhydrobacter*	0.0102	0	0.5485	0.7981
Firmicutes	*Clostridia*	*Ruminococcaceae*		0.0049	0	0.0529	0.7885
Firmicutes	*Bacilli*	*Leuconostocaceae*	*Leuconostoc*	0.0030	0	0.0287	0.7692
Firmicutes	*Bacilli*	*Streptococcaceae*		0.0026	0	0.0135	0.7692
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Pediococcus*	0.0103	0	0.0395	0.7596
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadaceae*	*Pseudomonas*	0.0042	0	0.1722	0.7596
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcus_2*	0.0037	0	0.0219	0.7596
Firmicutes	*Clostridia*	*Lachnospiraceae*		0.0064	0	0.0592	0.7500
Firmicutes	*Bacilli*	*Carnobacteriaceae*	*Atopostipes*	0.0061	0	0.0854	0.7500
Bacteroidetes	*Bacteroidia*	*Rikenellaceae*	*Rikenellaceae_RC9_gut_group*	0.0056	0	0.0441	0.7500
Bacteroidetes	*Bacteroidia*	*Rikenellaceae*	*Alistipes*	0.0034	0	0.0279	0.7212
Actinobacteria	*Actinobacteria (class)*	*Brevibacteriaceae*	*Brevibacterium*	0.0027	0	0.0250	0.7212
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-010*	0.0048	0	0.0565	0.7115
Firmicutes	*Bacilli*	*Streptococcaceae*	*Anthococcus*	0.0006	0	0.0028	0.7115
Firmicutes	*Erysipelotrichia*	*Erysipelotrichaceae*	*Turicibacter*	0.0026	0	0.0148	0.7019
Proteobacteria	*Alphaproteobacteria*	*Rhodobacteraceae*	*Paracoccus*	0.0021	0	0.0565	0.7019
Firmicutes	*Bacilli*	*Aerococcaceae*	*Aerococcus*	0.0083	0	0.1402	0.6923
Firmicutes	*Negativicutes*	*Acidaminococcaceae*	*Phascolarctobacterium*	0.0063	0	0.0430	0.6923
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-014*	0.0020	0	0.0138	0.6923
Actinobacteria	*Actinobacteria (class)*	*Corynebacteriaceae*	*Corynebacterium*	0.0075	0	0.0494	0.6731
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-013*	0.0028	0	0.0156	0.6731
Firmicutes	*Bacilli*	*Aerococcaceae*	*Facklamia*	0.0028	0	0.0324	0.6635
Actinobacteria	*Actinobacteria (class)*	*Bifidobacteriaceae*	*Bifidobacterium*	0.0033	0	0.0388	0.6538

Firmicutes	*Clostridia*	*Lachnospiraceae*	*Lachnospiraceae_NK3A20_group*	0.0028	0	0.0235	0.6538
Patescibacteria	*Saccharimonadia*	*Saccharimonadaceae*	*Candidatus_Saccharimonas*	0.0027	0	0.0154	0.6538
Firmicutes	*Clostridia*	*Family_XIII*	*Family_XIII_AD3011_group*	0.0018	0	0.0089	0.6538
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiaceae*	*Akkermansia*	0.0042	0	0.0322	0.6442
Proteobacteria	*Gammaproteobacteria*	*Moraxellaceae*	*Psychrobacter*	0.0079	0	0.4249	0.6346
Actinobacteria	*Actinobacteria (class)*	*Dermabacteraceae*	*Brachybacterium*	0.0034	0	0.1307	0.6250
Bacteroidetes	*Bacteroidia*	*Prevotellaceae*	*Prevotellaceae_UCG-003*	0.0021	0	0.0167	0.6250
Bacteroidetes	*Bacteroidia*			0.0013	0	0.0126	0.6250
Proteobacteria	*Alphaproteobacteria*	*Xanthobacteraceae*		0.0235	0	0.1191	0.6154
Proteobacteria	*Betaproteobacteria*	*Alcaligenaceae*	*Oligella*	0.0056	0	0.0898	0.6154
Euryarchaeota	*Methanobacteria*	*Methanobacteriaceae*	*Methanobrevibacter*	0.0021	0	0.0121	0.6154
Bacteroidetes	*Bacteroidia*	*Bacteroidales_RF16_group*		0.0017	0	0.0264	0.6154
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_NK4A214_group*	0.0015	0	0.0141	0.6154

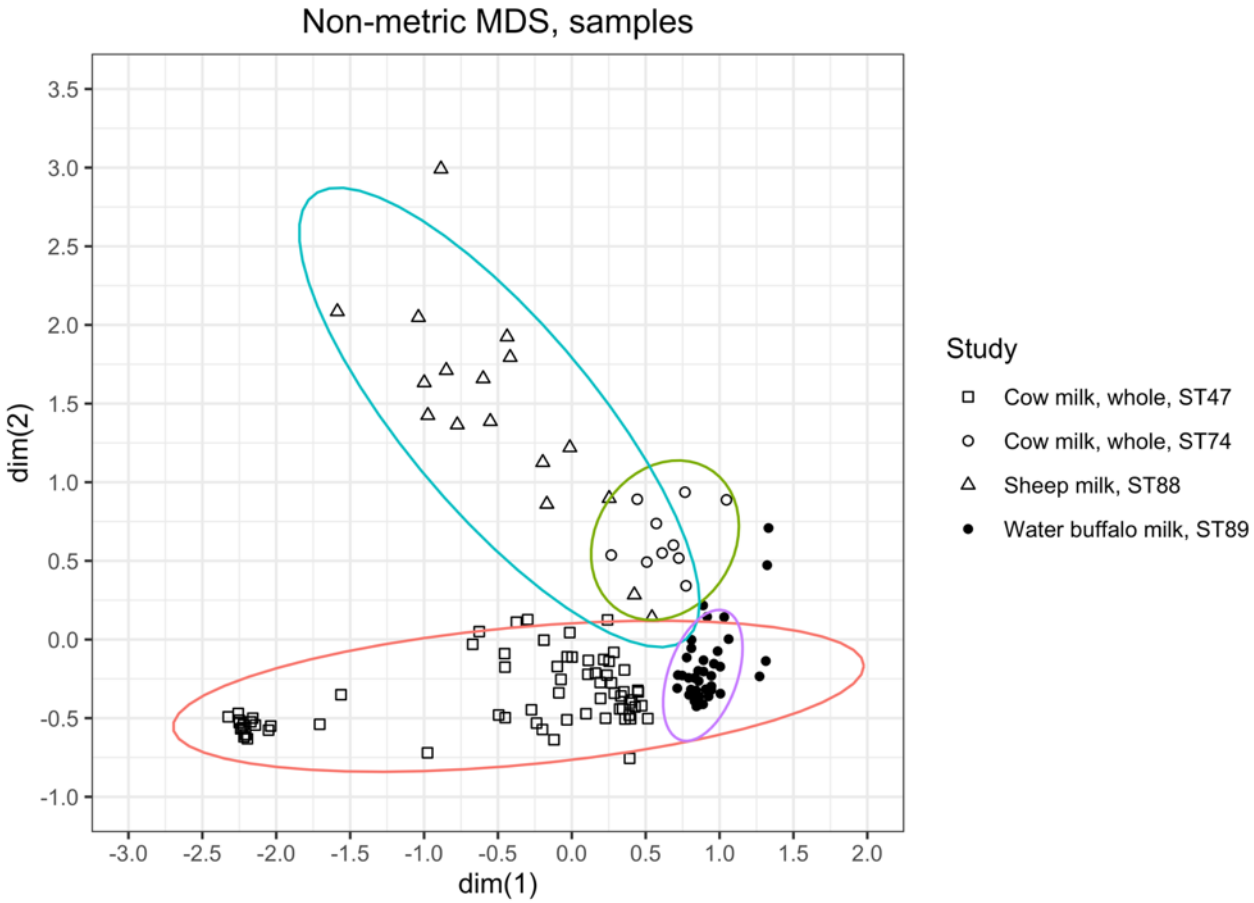
Supplementary Table 4. A compilation of dominating bacteria (and potential causative agents of mastitis) detected in the microbiota of milk from cows with clinical or subclinical mastitis.

Reference	*Bacillus*	*Campylobacter spp.*	*Corynebacteriium spp.*	*Corynebacterium bovis*	*Coxiella spp.*	*Enterococcus spp.*	*Escherichia coli*	*Fusobacterium necrophorum*	*Klebsiella pneumoniae*	*Klebsiella spp.*	*Listeria spp.*	*Mycoplasma*	*Pasteurella multocida*	*Porphyromonas levii*	*Pseudomonas spp.*	*Pseudomonas aeruginosa*	*Rhodococcus*	*Serratia spp.*	*Sneathia sanguineagens*	*Staphylococcus spp.*	*Staphylococcus aureus*	*Staphylococcus carnosus*	*Stahylococcus chromogenes*	*Staphylococci (coagulase negative)*	*Streptococcus spp.*	*Streptococcus agalactiae*	*Streptococcus dysgalactiae*	*Streptococcus uberis*	*Trueperella pyogenes*
Andrews et al., 2019															x					x					x				
Angelopoulou et al., 2019															x		x			x					x				
Bhatt et al., 2012							x		x							x					x								
Ganda et al., 2016							x			x					x														
Ganda et al., 2017							x																						
Hoque et al., 2019		x					x			x										x									
Kuehn et al., 2013	x			x						x		x	x					x			x				x	x			x
Metzger et al., 2018b					x															x									
Oikonomou et al., 2012							x	x		x				x							x						x		x
Oikonomou et al., 2014							x	x		x				x							x						x		x
Oultram et al., 2017						?	?				?						x		x			x	x				x	x	
Pang et al., 2018			x				x			x															x				
Taponen et al., 2019																					x							x	
Tong et al., 2019																										x			
Vasquez et al., 2019							x																						

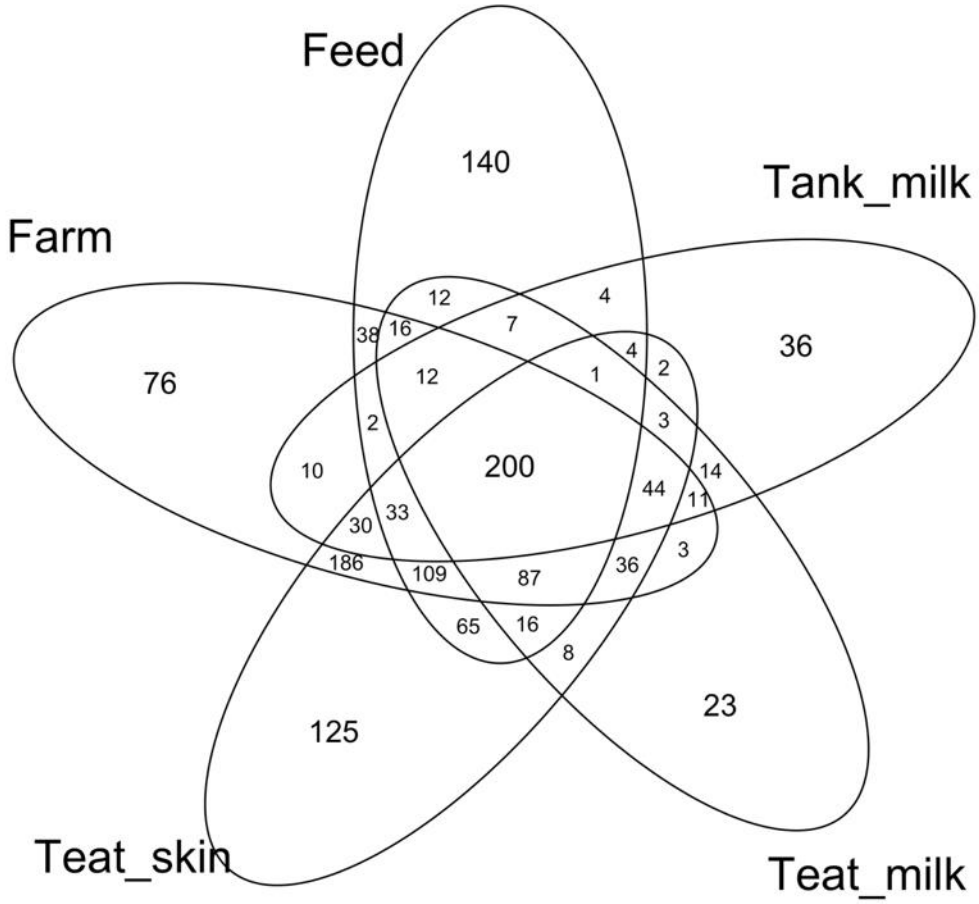
Supplementary Figure 3. Microbial association network for the data on mastitic milk from Ireland of Angelopoulou et al. (2019). Sequences were downloaded from NCBI SRA and processed as described in Parente et al. (2019) and the microbial association network was inferred using the CoNet app as described by Parente, Zotta, Faust, De Filippis, & Ercolini (2018). Colour of the nodes was assigned on the basis of the class, the size of the nodes was made proportional to the betweenness centrality, the thickness of the edges to the q values of the inferred interaction. Only co-presence edges (in green) were significant.



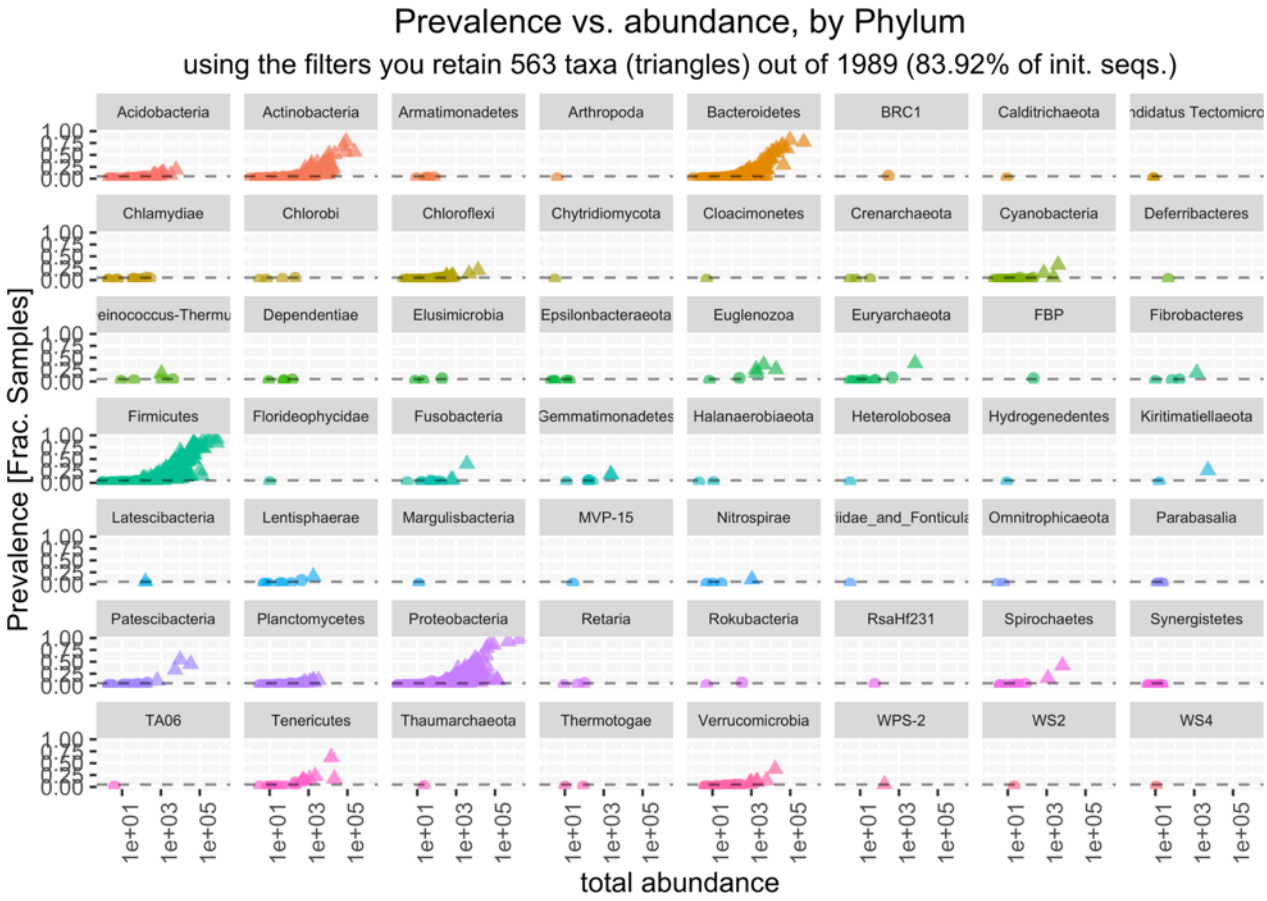
Supplementary Figure 4. Non-metric multidimensional scaling (NMDS) plots for samples obtained by calculating the Bray-Curtis distance after Wisconsin transformation for absolute abundance data of teat milk from cows (ST47, Cremonesi et al., 2018, colostrum samples were removed; ST74 Falardeau et al., 2019), ewes (Castro, Alba, Aparicio, Arroyo, Jiménez, Fernández, Arias, & Rodriguez, 2019a) and water-buffaloes (Catozzi et al., 2017; only samples from healthy quarters are shown). Sequences were downloaded from NCBI SRA and processed as described in Parente et al. (2019) and NMDS was carried out using functions of the vegan package.



Supplementary Figure 5. Venn diagram showing the taxa (at the genus level or above) which are shared between feed (clover and hay pasture and stored hay), farm environments (cow faeces, straw bedding, cow scratchers), teat skin, teat milk and bulk tank milk (cooled, pre-transport milk) in the study of Falardeau et al. (2019). Sequences were downloaded from NCBI SRA and processed as described in Parente et al. (2019).



Supplementary Figure 6. A prevalence and abundance plot for samples of cow's bulk tank milk from 5 studies (Doyle, Gleeson, O'Toole, & Cotter, 2017a; Frétin et al., 2018; Li et al., 2018; Skeie et al., 2019; Falardeau et al., 2019). Sequences were downloaded from NCBI SRA and processed as described in Parente et al. (2019). Prevalence threshold was 0.05 and abundance threshold 0.001. Triangles are taxa which pass the filter.

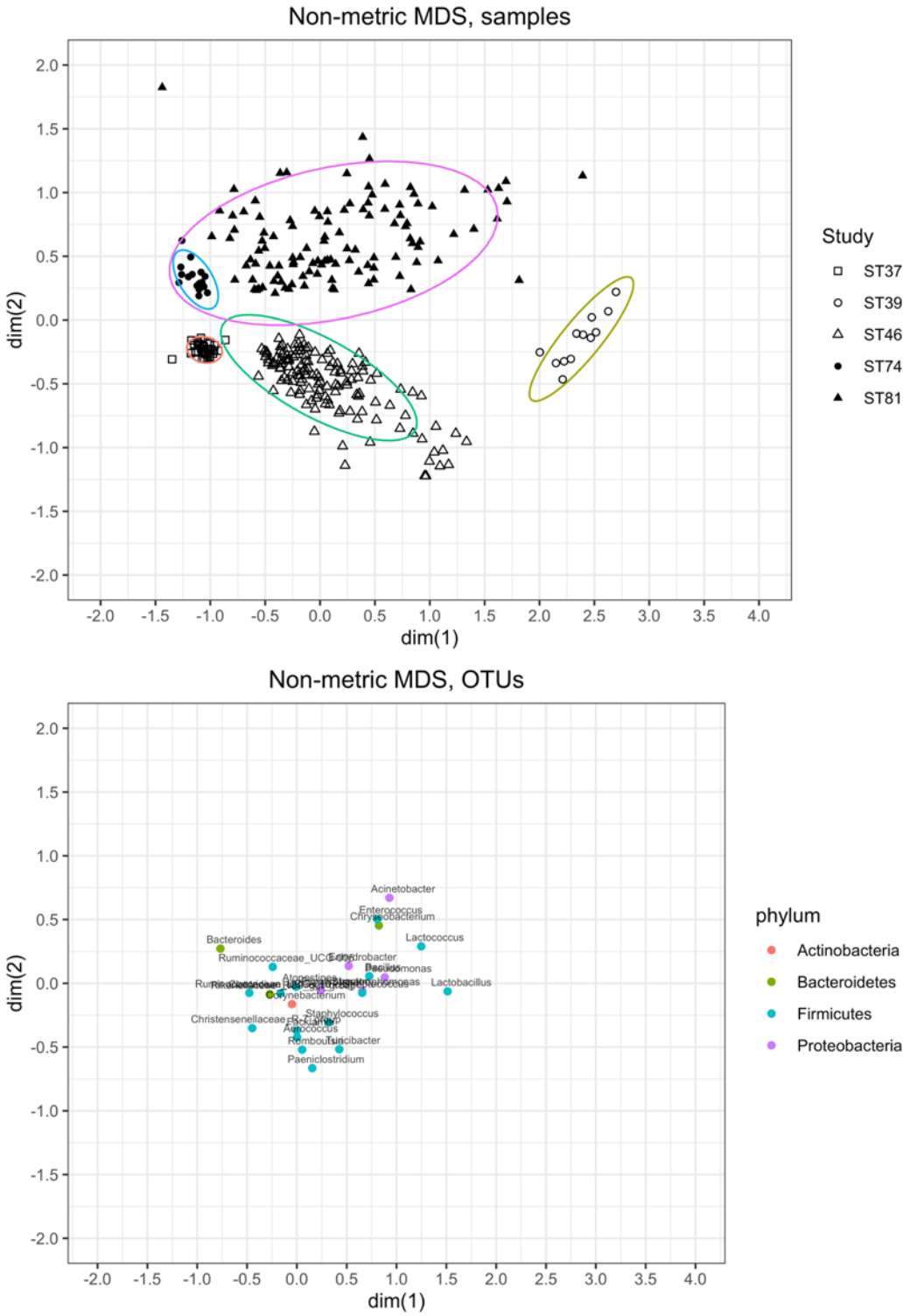


Supplementary Table 5. The top 50 taxa in terms of prevalence and abundance from cow's bulk tank milk from 5 studies (Doyle et al., 2017a; Frétin et al., 2018; Li et al., 2018; Skeie et al., 2019; Falardeau et al., 2019). Sequences were downloaded from NCBI SRA and processed as described in Parente et al. (2019).

phylum	class	family	genus	rel. ab.	min. rel. ab.	max. rel. ab.	rel. prev.
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadaceae*	*Pseudomonas*	0.1730	0	0.9604	0.9775
Firmicutes	*Bacilli*	*Streptococcaceae*	*Streptococcus*	0.0393	0	0.9546	0.9678
Firmicutes	*Bacilli*	*Streptococcaceae*	*Lactococcus*	0.0704	0	0.8214	0.9293
Proteobacteria	*Gammaproteobacteria*	*Moraxellaceae*	*Acinetobacter*	0.0472	0	0.8662	0.9293
Firmicutes	*Bacilli*	*Staphylococcaceae*	*Staphylococcus*	0.0259	0	0.4470	0.9003
Firmicutes	*Clostridia*	*Peptostreptococcaceae*	*Romboutsia*	0.0134	0	0.0870	0.8810
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadaceae*	*Stenotrophomonas*	0.0052	0	0.1300	0.8682
Proteobacteria	*Gammaproteobacteria*	*Moraxellaceae*	*Psychrobacter*	0.0080	0	0.2526	0.8585
Firmicutes	*Bacilli*	*Bacillaceae*	*Bacillus*	0.0702	0	0.7412	0.8424
Firmicutes	*Clostridia*	*Clostridiaceae_1*	*Clostridium_sensu_stricto_1*	0.0051	0	0.0447	0.8296
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-005*	0.0185	0	0.0916	0.8264
Firmicutes	*Bacilli*	*Carnobacteriaceae*	*Atopostipes*	0.0042	0	0.0348	0.8232
Firmicutes	*Erysipelotrichia*	*Erysipelotrichaceae*	*Turicibacter*	0.0047	0	0.0348	0.8199
Bacteroidetes	*Flavobacteriia*	*Flavobacteriaceae*	*Chryseobacterium*	0.0093	0	0.5929	0.8135
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Lactobacillus*	0.0044	0	0.2667	0.8135
Proteobacteria	*Gammaproteobacteria*	*Moraxellaceae*	*Enhydrobacter*	0.0036	0	0.4685	0.7910
Firmicutes	*Bacilli*	*Aerococcaceae*	*Facklamia*	0.0059	0	0.0948	0.7878
Actinobacteria	*Actinobacteria (class)*	*Corynebacteriaceae*	*Corynebacterium*	0.0083	0	0.0481	0.7846
Firmicutes	*Bacilli*	*Aerococcaceae*	*Aerococcus*	0.0068	0	0.0637	0.7749
Bacteroidetes	*Bacteroidia*	*Bacteroidaceae*	*Bacteroides*	0.0479	0	0.4979	0.7717
Firmicutes	*Clostridia*	*Ruminococcaceae*	*NA*	0.0087	0	0.0397	0.7685
Firmicutes	*Clostridia*	*Christensenellaceae*	*Christensenellaceae_R-7_group*	0.0123	0	0.0593	0.7556
Firmicutes	*Bacilli*	*Enterococcaceae*	*Enterococcus*	0.0102	0	0.2884	0.7492
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-010*	0.0063	0	0.0382	0.7428
Firmicutes	*Bacilli*	*Carnobacteriaceae*		0.0044	0	0.2956	0.7395
Firmicutes	*Clostridia*	*Peptostreptococcaceae*	*Paeniclostridium*	0.0055	0	0.0516	0.7331
Firmicutes	*Clostridia*	*Lachnospiraceae*		0.0156	0	0.0758	0.7235
Bacteroidetes	*Bacteroidia*	*Rikenellaceae*	*Rikenellaceae_RC9_gut_group*	0.0038	0	0.0176	0.7042
Actinobacteria	*Actinobacteria (class)*	*Corynebacteriaceae*	*Corynebacterium_1*	0.0060	0	0.0807	0.6881
Firmicutes	*Bacilli*	*Staphylococcaceae*	*Jeotgalicoccus*	0.0037	0	0.0404	0.6817
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-014*	0.0049	0	0.0405	0.6785
Firmicutes	*Bacilli*	*Aerococcaceae*	*Ignavigranum*	0.0009	0	0.0098	0.6720
Bacteroidetes	*Bacteroidia*	*Rikenellaceae*	*Alistipes*	0.0033	0	0.0185	0.6592
Bacteroidetes	*Flavobacteriia*	*Flavobacteriaceae*	*Flavobacterium*	0.0062	0	0.5933	0.6367
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadaceae*	*Sphingomonas*	0.0034	0	0.0247	0.6367
Bacteroidetes	*Bacteroidia*	*Bacteroidales_RF16_group*		0.0019	0	0.0102	0.6302
Tenericutes	*Mollicutes*			0.0014	0	0.0143	0.6270
Firmicutes	*Negativicutes*	*Acidaminococcaceae*	*Phascolarctobacterium*	0.0011	0	0.0076	0.6077
Firmicutes	*Clostridia*	*Family_XIII*	*Family_XIII_AD3011*	0.0049	0	0.0203	0.6013

			_group				
Firmicutes	*Clostridia*	*Family_XIII*		0.0040	0	0.0216	0.5981
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcus_2*	0.0024	0	0.0146	0.5981
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-013*	0.0049	0	0.0257	0.5949
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriaceae*		0.0019	0	0.0910	0.5949
Firmicutes	*Clostridia*	*ClostridialesFamilyXIII.IncertaeSedis*	*Mogibacterium*	0.0012	0	0.0102	0.5723
Proteobacteria	*Betaproteobacteria*	*Burkholderiaceae*	*Ralstonia*	0.0009	0	0.0169	0.5723
Actinobacteria	*Actinobacteria (class)*	*Propionibacteriaceae*	*Cutibacterium*	0.0224	0	0.9603	0.5659
Bacteroidetes	*Bacteroidia*	*Muribaculaceae*		0.0011	0	0.0093	0.5627
Actinobacteria	*Actinobacteria (class)*	*Bifidobacteriaceae*	*Bifidobacterium*	0.0076	0	0.1064	0.5595
Bacteroidetes	*Bacteroidia*	*Prevotellaceae*	*Prevotellaceae_UCG-003*	0.0019	0	0.0113	0.5595
Bacteroidetes	*Bacteroidia*			0.0013	0	0.0081	0.5498

Supplementary Figure 7. Non-metric multidimensional scaling plots for samples and "species" obtained by calculating the Bray-Curtis distance after Wisconsin transformation for absolute abundance data of cow's bulk tank milk from 5 studies (ST37: Doyle et al., 2017a; ST39: Frétin et al., 2018; ST81: Li et al., 2018; ST46: Skeie et al., 2019; ST74: Falardeau et al., 2019) Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019) and NMDS was carried out using functions of the vegan package.



Supplementary Table 6. The top 50 taxa in terms of prevalence and abundance for cow milk in silos at the processing plant. Results from three studies (Porcellato et al., 2018; Kable et al., 2019; Falardeau et al., 2019). Sequences were downloaded from NCBI SRA and processed as described in Parente et al., 2019.

phylum	class	family	genus	rel. ab.	min. rel. ab.	max. rel. ab.	rel. prev.
Firmicutes	*Bacilli*	*Streptococcaceae*	*Streptococcus*	0.0581	0.0009	0.4015	1.000
Firmicutes	*Bacilli*	*Streptococcaceae*	*Lactococcus*	0.0282	0.0002	0.5750	1.000
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadaceae*	*Pseudomonas*	0.0895	0.0000	0.9874	0.974
Proteobacteria	*Gammaproteobacteria*	*Moraxellaceae*	*Acinetobacter*	0.0321	0.0000	0.2964	0.957
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Lactobacillus*	0.0110	0.0000	0.1598	0.948
Firmicutes	*Bacilli*	*Staphylococcaceae*	*Staphylococcus*	0.0236	0.0000	0.2897	0.931
Proteobacteria	*Gammaproteobacteria*	*Moraxellaceae*	*Psychrobacter*	0.0107	0.0000	0.5481	0.888
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriaceae*	*Escherichia/Shigella*	0.0138	0.0000	0.2359	0.871
Firmicutes	*Bacilli*	*Carnobacteriaceae*	*Atopostipes*	0.0023	0.0000	0.0268	0.853
Bacteroidetes	*Bacteroidia*	*Bacteroidaceae*	*Bacteroides*	0.1239	0.0000	0.4979	0.836
Firmicutes	*Bacilli*	*Bacillaceae*	*Bacillus*	0.0286	0.0000	0.8106	0.836
Firmicutes	*Clostridia*	*Peptostreptococcaceae*	*Romboutsia*	0.0161	0.0000	0.0738	0.836
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-005*	0.0132	0.0000	0.0806	0.836
Firmicutes	*Erysipelotrichia*	*Erysipelotrichaceae*	*Turicibacter*	0.0045	0.0000	0.0318	0.836
Proteobacteria	*Gammaproteobacteria*	*Moraxellaceae*	*Enhydrobacter*	0.0020	0.0000	0.0257	0.836
Firmicutes	*Bacilli*	*Leuconostocaceae*	*Leuconostoc*	0.0016	0.0000	0.0814	0.810
Actinobacteria	*Actinobacteria (class)*	*Corynebacteriaceae*	*Corynebacterium_1*	0.0078	0.0000	0.0553	0.802
Actinobacteria	*Actinobacteria (class)*	*Bifidobacteriaceae*	*Bifidobacterium*	0.0230	0.0000	0.1064	0.793
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-010*	0.0041	0.0000	0.0385	0.793
Firmicutes	*Clostridia*	*Christensenellaceae*	*Christensenellaceae_R-7_group*	0.0046	0.0000	0.0248	0.785
Actinobacteria	*Actinobacteria (class)*	*Micrococcaceae*	*Kocuria*	0.0086	0.0000	0.2027	0.767
Firmicutes	*Clostridia*	*Ruminococcaceae*		0.0044	0.0000	0.0266	0.767
Firmicutes	*Clostridia*	*Lachnospiraceae*		0.0237	0.0000	0.0758	0.75
Actinobacteria	*Actinobacteria (class)*	*Brevibacteriaceae*	*Brevibacterium*	0.0054	0.0000	0.0918	0.750
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcus_2*	0.0027	0.0000	0.0223	0.741
Bacteroidetes	*Bacteroidia*	*Rikenellaceae*	*Rikenellaceae_RC9_gut_group*	0.0022	0.0000	0.0137	0.741
Bacteroidetes	*Bacteroidia*	*Rikenellaceae*	*Alistipes*	0.0017	0.0000	0.0133	0.724
Bacteroidetes	*Bacteroidia*	*Bacteroidales_RF16_group*		0.0011	0.0000	0.0119	0.716
Firmicutes	*Clostridia*	*Clostridiaceae_1*	*Clostridium_sensu_stricto_1*	0.0097	0.0000	0.0447	0.698
Firmicutes	*Clostridia*	*ClostridialesFamilyXIII.IncertaeSedis*	*Mogibacterium*	0.0009	0.0000	0.0074	0.681
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadaceae*	*Stenotrophomonas*	0.0090	0.0000	0.1014	0.672
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillaceae*	*Marinospirillum*	0.0018	0.0000	0.0448	0.672
Proteobacteria	*Alphaproteobacteria*	*Rhodobacteraceae*	*Paracoccus*	0.0017	0.0000	0.0114	0.672
Firmicutes	*Bacilli*	*Carnobacteriaceae*		0.0014	0.0000	0.0179	0.672
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-013*	0.0053	0.0000	0.0257	0.638
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Flavonifractor*	0.0034	0.0000	0.0099	0.638
Firmicutes	*Bacilli*	*Carnobacteriaceae*	*Jeotgalibaca*	0.0012	0.0000	0.0127	0.628
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcaceae_UCG-014*	0.0010	0.0000	0.0065	0.628

Firmicutes	*Clostridia*			0.0008	0.0000	0.0110	0.621
Bacteroidetes	*Bacteroidia*	*Prevotellaceae*	*Prevotellaceae_UCG-003*	0.0008	0.0000	0.0059	0.621
Firmicutes	*Bacilli*	*Enterococcaceae*	*Enterococcus*	0.0022	0.0000	0.0186	0.612
Firmicutes	*Clostridia*	*Family_XIII*	*NA*	0.0012	0.0000	0.0100	0.612
Firmicutes	*Clostridia*	*Family_XIII*	*Family_XIII_AD3011_group*	0.0009	0.0000	0.0059	0.612
Bacteroidetes	*Bacteroidia*			0.0007	0.0000	0.0090	0.612
Firmicutes	*Bacilli*	*Staphylococcaceae*	*Jeotgalicoccus*	0.0006	0.0000	0.0075	0.612
Proteobacteria	*Alphaproteobacteria*	*Caulobacteraceae*	*Caulobacter*	0.0106	0.0000	0.1279	0.603
Firmicutes	*Erysipelotrichia*	*Erysipelotrichaceae*		0.0009	0.0000	0.0053	0.603
Firmicutes	*Bacilli*	*Aerococcaceae*	*Aerococcus*	0.0015	0.0000	0.0480	0.595
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Roseburia*	0.0292	0.0000	0.1205	0.586
Tenericutes	*Mollicutes*			0.0006	0.0000	0.0037	0.586

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The microbiota of dairy milk: a review

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Abstract.

Since the first paper published in 2011, the number of studies which have used High Throughput Sequencing methods for the~~The literature on the s~~characterization of the structure and function of the microbial communities of milk and dairy products ~~is vast~~has been increasing almost exponentially~~, with more than 120 150 papers published in the last 9 ten years. . Although amplicon targeted approaches are most frequently used, the number of metagenomic and metatranscriptomic studies is increasing. Even if no~~Due to the lack of consensus ~~is available~~ on laboratory procedures, sequencing approaches and bioinformatic pipelines, ~~thus complicating~~ the comparison of raw data from different studies is still difficult, but the availability of well-structured databases with raw and processed sequences has boosted ~~out~~ our ability to get quantitative insights in the microbiota of these important ~~classes of~~ food ~~products~~commodities.

Methods for the characterization of microbial communities of dairy foods have evolved steadily. Metataxonomic approaches targeting the 16S RNA gene for bacteria and Internal Transcribed Regions or the 18S or 28S RNA genes for fungi are used more frequently than metagenomic and metatranscriptomics approaches. Unfortunately, Standard Operating Procedures developed for human and animal microbiome or environmental microbiome studies have not been adopted by microbiologists studying dairy products, and reaching a consensus on both wet- and dry laboratory procedures and on the use of internal standards, controls and mock communities would certainly be beneficial. Further methodological issues are the need for methods with high taxonomic resolution (like single molecule real time sequencing, or sequencing of targets other than RNA genes) and the selective evaluation of the active fraction of the microbiota.

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The microbiota of milk is highly complex and variable and is affected by a large number of different sources of contamination and by the selective effect of storage at low temperature and heat treatments. Although the issue of existence of a microbiota of the healthy udder has not been fully clarified, it is well known that mastitis greatly affects the composition of teat and milk microbiota, with changes which often produce lasting effects. Mastitis and dysbiosis of the teat and milk microbiota are strongly connected, and mastitis usually causes a dramatic reduction of microbial diversity. The microbiota of the teat surface in healthy lactating animals is highly diverse and variable and is affected by several factors, including breed, farming system, feed, health status, etc. ~~and m~~Members of the genus *Streptococcus* and *Staphylococcus* are both highly prevalent and abundant, while the prevalence and abundance of other *Bacilli, Clostridia, Bacteroidia, Erysipelotrichia* and *Gammaproteobacteria* is lower and more variable. ~~*These genera*~~*Streptococcus* and *Staphylococcus*, together with *Actinobacteria* and lactic acid bacteria, will persist throughout production and storage of milk and will be eventually found in cheese. The composition of the microbiota of raw milk from individual animals or of composite samples from bulk tanks at the dairy farm is affected by a large number of interacting factors, including species, breed, farming practices, bedding, feeding, washing and disinfection of the teat surface, season of the year, lactation stage~~,~~ and geographic location within countries. Even so, a large number of bacterial genera are both prevalent and abundant in several studies on raw cow milk, and have also been frequently found in the milk of ewes and water buffaloes, thus supporting the idea of a common core microbiome in milk. Intra-mammary infections such as mastitis have a large, and sometimes lasting, impact on milk microbiota, generally causing a reduction of diversity and a shift in the composition of the microbiota, with clear evidences of dysbiosis, even in subclinical, asymptomatic infections.

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Contamination from equipment at the farm and at the processing plant, temperature and duration of storage, and heat treatments at the processing plant will result in further dramatic changes of the microbiota, with an increase in the proportion of psychrotrophic genera, including *Pseudomonas, Acinetobacter, Lactococcus* and *Psychrobacter*, ~~followed by~~while heat treatments result in an increase in relative proportion of thermoduric and spore-formers~~ing~~ genera, like *Thermus*, *Bacillus*, *Paenibacillus*, *Anoxybacillus* and *Turicibacter*, some of which can cause spoilage of liquid milk and cheese.

Overall, High Throughput Sequencing methods have confirmed what was previously known from low sensitivity cultivation based and cultivation independent techniques, but ~~has~~ have also offered a deeper insight in the source of microorganisms in milk ~~and dairy products~~ and on the factors which shape ~~their~~ the microbial communities.

1. Introduction.

In the last ~~eight~~ ten years more than ~~120~~ 1655 papers in which the microbiota of dairy products has been characterized using ~~high~~ High ~~throughput~~ Throughput ~~sequencing~~ Sequencing (HTS) approaches have been published (Supplementary Table 1). Milk and dairy products are therefore the foods for which most data on the structure and functions of microbial communities involved in animal health, safety, fermentation and spoilage are available (De Filippis, Parente, & Ercolini, 2017). Literature on the microbiome of the bovine udder and its role in the health and well-being of dairy cows (Derakhshani et al., 2018a; Rainard, 2017), ~~of~~ on the microbiome of milk (Addis et al., 2016; Oikomonou et al., 2020; Quigley et al., 2013; Tilocca et al., 2020), and cheese (Afshari, Pillidge, Dias, Osborn, & Gill, 2018; Yeluri Jonnala, McSweeney, Sheehan, & Cotter, 2018~~)~~ has been recently reviewed. However, the most recent reviews on milk microbiota focus on aspects related to the role of

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udder and milk microbiota on the health of dairy animals (Addis et al., 2016; Oikomonou et al., 2020) and on methods (Tilocca et al., 2020), rather than on aspects relevant to the safety and quality of milk for the production of dairy products. While the teat surface and interior are certainly a source of bacteria which may play a role in safety and quality of dairy products, other factors, including further contamination from milking, farm and processing plant equipment ~~of~~ environments, growth during storage and destruction of microorganisms by pasteurization will give a significant contribution in shaping the microbiota of dairy and cheese milk.

In addition, the availability of raw or processed sequencing data in large databases (QIITA, https://qiita.ucsd.edu/; MGnify, https://www.ebi.ac.uk/metagenomics/; FoodMicrobionet, Parente et al., 2016a; Parente, De Filippis, Ercolini, Ricciardi, & Zotta, 2019) offers unprecedented opportunities for exploring the food microbiome in meta-studies. Recently, we have released version 3.1 of FoodMicrobionet (Parente et al., 2019), a database of studies on the bacterial microbiota of foods, which is easily accessible through an interactive app. The database is steadily growing and its latest version includes, at the time of writing this article, 42 studies on the bacterial microbiome of dairy products (see supplementary material and supplementary data on Mendeley https://data.mendeley.com/datasets/3cwf729p34/1).

The objective of this work is to critically review the recent knowledge on methods used in the characterization of the microbiota of milk ~~(and dairy products) and on the microbiota of milk~~ from cows and other dairy species, on its potential sources and on its dynamics during storage, transport and processing in liquid pasteurized milk, and make use of the data available in FoodMicrobionet to carry out meta-analyses on studies on the dairy milk microbiota.

2. Milk and dairy products illuminated: the evolution of methods.

All three ~~high throughput sequencing~~HTS approaches (amplicon sequencing, shotgun metagenomics and metatranscriptomics; Yeluri Jonnala et al., 2018) have been used, alone or in combination, to characterize the microbiome of milk~~, . and a variety of~~M ~~m~~ethods ~~in terms of~~for nucleic acid extraction, wet laboratory stages, sequencing and bioinformatic analyses ~~have been used~~vary greatly among studies (Supplementary Table 1), and current approaches have been outlined in a recent review (Tilocca et al., 2020). We will briefly discuss some aspects which shape our ability to understand dairy microbiota by nucleic acid targeted omics approaches.

2.1 Amplicon sequencing.

Amplicon sequencing is by far the most used approach for metataxonomic studies. For bacteria, the 16S RNA gene has been most frequently targeted ~~(96 out of 108 studies)~~, ~~while 16S RNA has been less frequently targeted (14 studies)~~but a few studies have used 16S RNA as target (Supplementary Table 1), in order to focus on the "active" fraction of the microbiota. The variable regions targeted also vary, with V1-V3 being most frequently used before the demise of the Roche 454 platforms, and V3-V4 and V4 being most frequently used ~~as the~~with the Illumina platforms ~~have become the workhorse of today's high throughput sequencing (HTS)~~. As to fungi, Internal Transcribed Spacers ITS1 and ITS2 have been most frequently used as a target, while the use of regions of 18S and 28S is far less common. Due to the relatively short length of the regions targeted ~~(<500 bp, but often <300 bp)~~, the taxonomic resolution ~~of most studies~~ is often limited to the genus or, less frequently, to the species level, depending on the length and quality of sequences.

However, most recently the use of single molecule sequencing (Jin et al., 2018; Li et al., 2017; Mo et al., 2019; Yang et al., 2019; Yu et al., 2018), the ability to detect oligotypes (Kamimura, De Filippis, Sant'Ana, & Ercolini, 2019) and Amplicon Sequence Variants (ASV, Callahan et al., 2016), or the availability of optimized databases (Meola et al., 2019) has been claimed to increase taxonomic resolution. ~~Taxonomic r~~Resolution at the species level or below is critical in the analysis of the microbiota of dairy products, because species belonging to the same genus may have a very different significance for the quality and safety of dairy products. ~~(f~~For example, several genera, like *Streptococcus, Staphylococcus and Corynebacterium*, include both pathogenic and starter and non-starter microorganisms, while others, like *Lactobacillus* include species which are either starter or non-starter bacteria~~)~~. ~~and t~~The use of ~~longer 16S RNA gene targets (V3-V4 as opposed to V3 or V4) and of bioinformatic procedures based on the estimation of ASVs should be encouraged~~the methods with the highest taxonomic resolution should therefore be encouraged. ~~In addition, a~~A few other coding or non-coding regions of the genome of selected bacteria have been used as a target to study the micro-diversity of selected species, with a variable degree of success in terms of accuracy and sensitivity: *Streptococcus thermophilus* (*lacSZ*, De Filippis, La Storia, Stellato, Gatti, & Ercolini, 2014; *serB*, Parente et al., 2016b; Ricciardi et al., 2016), amine producing lactic acid bacteria (*tdc* and *hdc*, O'Sullivan et al., 2015), *Lactococcus lactis* (*purR, epsD*, Frantzen, Kleppen, & Holo, 2018), genus *Lactobacillus* (*groEL*, Jiang et al., 2019; ITS, Milani et al., 2018), *L. casei* group (*spxB*, Levante et al., 2017)~~De Filippis, La Storia, Gatti, & Neviani, 2017)~~, genus *Bifidobacterium* (Milani et al., 2019), and members of the *Bacillus cereus* group (*panC, glpT*, Porcellato, Aspholm, Skeie, & Mellegård, 2019). Proteolysis related genes of LAB (*prtP, pepN, pepX, bcaT*) have also been used as target for metatranscriptomic studies (Pangallo et al., 2019). ~~Taxonomic resolution at the species level~~

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~~or below is critical in the analysis of the microbiota of dairy products, because species belonging to the same genus may have a very different significance for the quality and safety of dairy products (for example, several genera, like *Streptococcus, Staphylococcus and Corynebacterium*, include both pathogenic and starter and non-starter microorganisms) and the use of longer 16S RNA gene targets (V3-V4 as opposed to V3 or V4) and of bioinformatic procedures based on the estimation of ASVs should be encouraged.~~ ~~The study of the microdiversity of microbial populations is also important, both in source tracking and in studying the dynamics of microbial communities during the manufacture of fermented dairy products, but t~~The usefulness of protein coding genes might be limited to selected species, ~~and can often detect sequence variants and not strains. On the other hand,~~but it is a cheaper alternative to shotgun whole genome sequencing, ~~which~~ which might ~~at least~~ detect ~~the~~ only the dominating strains in microbial communities~~, is less common, probably because of cost issues~~.

~~The bioinformatic pipelines used for sequence analysis and taxonomic annotation in amplicon targeted (AT) studies vary greatly (data not shown). QIIME (and more recently QIIME2) are is the most frequently used, followed by Mothur. A variety of methods has also been used for taxonomic annotation of OTUs (Operational Taxonomic Units; or ASVs, Amplicon Sequence Variants): variations of BLASTn and of the RDP (Ribosomal Database Project) classifier are the most frequent, while Greengenes and Silva are most frequently used for bacteria and UNITE for fungi. Recently, an optimized database for dairy products has been proposed (Meola et al., 2019).~~ To date, no Standard Operating Procedures (SOPs) for ~~amplicon targeted~~AT studies in foods exist~~, although most authors do use procedures which are close to those developed for the earth and human microbiome (Bálint et al., 2016)~~. A discussion on the best approach for ~~amplicon targeted~~AT studies for milk and dairy

foods is ~~well~~ beyond the scope of this review, and the factors affecting the results have been reviewed recently (Pollock, Glendinning, Wisedchanwet, & Watson, 2018). However, it is quite clear that the development of SOPs, and the use of negative controls and mock communities or internal standards would be highly desirable and should be requested by editors and reviewers of scientific journals. ~~In fact, negative controls would help in detecting contamination, which is a significant issue in low DNA samples (Dahlberg et al., 2019; raw milk samples produced hygienically often fall in this category), and can be most easily addressed using statistical tools (Davis, Proctor, Holmes, Relman, & Callahan, 2018) if negative controls and mock communities are included.~~

2.2 Shotgun approaches.

To date, shotgun metagenomic studies of dairy products are comparatively rare (only ~~18~~ ~~18~~11% of studies ~~out of 122~~ ~~158~~ listed in Supplementary Table 1) and meta-transcriptomic studies are even less frequent (only ~~4~~7~~4~~%). ~~However, d~~Due to the decreasing costs in sequencing and the progress ~~However, because of the decreasing costs in sequencing and of the progress~~ in the development of bioinformatic pipelines for taxonomic annotation and genome reconstruction, ~~and~~ as well as the staggering amount of information they can provide (Tilocca et al., 2020; ~~high taxonomic resolution, reconstruction of metabolic pathways and, when using RNA as a target, of the pathways which are more expressed at a given time during production and manufacture~~Yeluri Jonnala et al., 2018) they are likely to become more frequent. ~~Again, comparing the pipelines for metagenomic and metatranscriptomic studies is beyond the scope of this review.~~ Pipelines for metagenomic annotation and data visualization have been reviewed recently (Breitwieser, Lu, & Salzberg, 2017; Quince, Walker, Simpson, Loman, & Segata, 2017; Sudarikov, Tyakht, & Alexeev,

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2017), and, as for amplicon targetedAT approaches, the choice of the pipeline has been shown to affect the results, especially in low diversity samples which are typical of cheese and fermented milks (Walsh et al., 2018). An alternative to the use of shotgun metagenomics if is the inference of metagenomes using bioinformatic tools such as PICRUSt (and its most recent iteration, PICRUSt2, Douglas et al., 2019). Although this tool has performed relatively well in benchmarking (Douglas et al., 2019) it has been used relatively rarely for microbial communities of milk and dairy products (Cremonesi et al., 2018; Li et al., 2018; Ramezani, Hosseini, Ferrocino, Amoozegar, & Cocolin, 2017; Stellato, De Filippis, La Storia, & Ercolini, 2015; Yang et al., 2019).

2.3 A question of life and death.

A further issue related to the experimental approach is the inability of methods targeting DNA to distinguish active/viable members of microbial communities from those which are dead/inactive. and Approaches based exclusively on DNA may keep detecting microorganisms which, being dead or inactive, contribute little or nothing to fermentation or spoilage. In fact, the risk of detecting the ghost of the microbiota past is quite high, especially when working with products obtained from pasteurized milk. Studies using both DNA and RNA as a target are relatively rare (see Supplementary Table 1; De Filippis, Genovese, Ferranti, Gilbert, & Ercolini, 2016; Kastman et al., 2016; Sattin et al., 2016b) while and the use of dyes which prevent the PCR amplification of DNA from dead cells (or more properly, cells with a damaged membrane), such as PMA (propidium monoazide, Emerson et al., 2017), is only marginally more frequent (Erkus et al., 2016; Kable, Srisengfa, Xue, Coates, & Marco, 2019; Mo et al., 2019; Porcellato & Skeie, 2016). The evidence that, at least in some situations, PMA can be used to detect with relatively little biases the active

~~fraction of the microbial community is quite convincing (Erkus et al., 2016). Nevertheless,~~ ~~without e~~Extensive benchmarking it still ~~not possible~~needed to rule out biases due to differential ability of PMA to penetrate cell membranes (Emerson et al., 2017). At any rate, whenever both the "active" and "inactive" fraction of the microbiota have been targeted, significant differences have been found between the two, usually with lower diversity in the "active" microbiota. ~~However, existing data definitely confirm that approaches based exclusively on DNA may keep detecting throughout the production and storage process of milk and dairy foods microorganisms which, being dead or inactive, contribute little or nothing to fermentation or spoilage.~~

2.4 Of experimental design (or lack thereof).

Regrettably, the overwhelming majority of the studies listed in Supplementary Table 1 are descriptive in nature, and even when inferential methods are used, their effectiveness in detecting significant differences (because of high natural variability, and potentially high type I and/or type II errors) is dubious. In fact, only in a very few cases experimental designs have been used (De Filippis et al., 2016; Doyle, Gleeson, O'Toole, & Cotter, 2017b; Ganda et al., 2016; Ganda et al., 2017; Guzzon et al., 2017; Porcellato & Skeie, 2016), and for most studies the approach is quasi-experimental in nature, with insufficient randomization, blocking and control of confounding factors. The issue of sampling effort is also critical: the range for the number of samples analysed in ~~the~~ studies shown in Supplementary Table 1 is 1-1674, but 50% of the studies have used 24 samples or less. Because of the very high variability of the microbiota of raw milk, due to seasonal, geographical and technological factors (Kable et al., 2016; Skeie, Håland, Thorsen, Narvhus, & Porcellato, 2019) one really wonders if, especially for raw milk fermented milks or cheeses produced in artisanal plants,

low (<50) sample numbers and low numbers of sampling locations (farms, cheesemaking plants) are adequate to cover the expected diversity, and, even in larger studies, utmost care should be dedicated to the design of the experiments and to the analysis of the data using appropriate inferential methods.

The issue of microbial interactions in dairy ecosystems is of great interest for both scientific and practical reasons, but it has been addressed only infrequently (Frétin et al., 2018; Murugesan et al., 2018; Parente et al., 2016a; Parente, Zotta, Faust, De Filippis, & Ercolini, 2018; Wolfe, Button, Santarelli, & Dutton, 2014). Detecting true interactions among species presents several challenges (Layeghifard, Hwang, & Guttman, 2017). Unfortunately, most studies are cross-sectional in nature, and, even when they are longitudinal, the number and distribution of sampling times is insufficient for model-based methods for detection of microbial interactions (Faust & Raes, 2012). More research is definitely needed in this area and combinations of culture independent and dependent approaches (Wolfe et al., 2014) are needed to validate the nature of the microbial interactions and evaluate their significance for the quality of dairy foods.

3. The microbiota of milk: from the teat to the carton.

A large amount of data on the microbiota of raw or pasteurized milk composition or milk contact surfaces (teat and udder surface, tanks and silos at the dairy farm or at the processing plant, etc.) are available for ~~cow, ewe, goat, and water buffalo and yak~~ milk from practically all dairy animals (mostly cow, but also ewes, goats, water buffaloes, yaks, camels), either as a part of studies specifically focusing on milk quality or as a part of studies on cheese microbiota (see Supplementary Table 1). Milk microbiota is undoubtedly complex and highly variable and in most studies the sampling effort is limited or the approach is merely descriptive, thus obscuring causal relationships. However, a few large~~r~~, designed or quasi-experimental studies addressing one of more aspects (effect of cow's health, feeding, farming, breed, season, geographical source of milk, effect of contamination during the production and distribution chain, effect of storage temperature) are available, and combination of data from different studies in meta-analyses may help in identifying a core microbiota or detecting wider geographical or temporal trends. In the following sections, we will review the composition of the microbiota of milk as it travels from the udder to the storage tank in processing plants and, finally to the carton of pasteurized milk.

3.1 Raw milk

3.1.1 ~~Straight form~~Inside and outside the udder.

The first sources of microorganisms in raw milk are, quite obviously, the udder and the teat surface (Derakhshani, Plaizier, De Buck, Barkema, & Khafipour, 2018b)~~, and in several papers individual (i.e. from individual cows or quarters) samples have been collected and analysed in order to study the composition of the microbiota of milk before any further contamination from operators, milking machines and milking environment, piping or tanks.~~ .

The composition of the mammary microbiota in ruminants has been recently reviewed

(Derakhshani~~, Plaizier, De Buck, Barkema, & Khafipour~~ et al., 2018b; Rainard, 2017), and the mechanisms which determine its ~~stability~~ composition and dynamics are outside the scope of this review. While it is still somewhat controversial if a microbiota of the healthy mammary gland exists or if it is the result of contamination during sampling (Derakhshani et al., 2018a; Rainard, 2017), it is clear that the teat canal and apex may be colonized by bacteria and that these bacteria may contribute to the homeostasis of this niche or cause infection of the mammary gland ~~after milking begins and that mastitis is associated to dysbiosis, although the cause-effect relationship is not completely clear (i.e. if mastitis is causing the dysbiosis of dysbiosis is favouring mastitis (Derakhshani et al., 2018b). Mastitis is the most important disease in dairy animals in terms of both impact on milk production and quality and in terms of animal well being and can be either clinical (with swelling and inflammation of the udder and visible changes in milk, besides the occurrence of high Somatic Cells Counts and possibly isolation of the causative agent) or subclinical (with no visible symptoms but high SCC) (Ruegg, 2017).~~

In fact, most of the studies ~~dealing with the microbiota of~~ using milk from individual quarters or individual animals have focused on the effect of disease (mastitis, either clinical or subclinical, subclinical acidosis) on the microbiota of milk from cows ~~milk~~ (see below and ~~below~~ Supplementary Table ~~A~~ 4 for a list of studies), while only ~~two~~ a few studies ~~on~~ are available on ewes~~'~~ ~~milk~~ (Castro et al., 2019; Esteban-Blanco et al., 2019), goat (McInnis, Kalanetra, Mills, & Maga, 2015) ~~and one~~or on water buffalo milk (Catozzi et al., 2017; Patel et al., 2016; Patel, Kunjadia, Koringa, Joshi, & Kunjadiya, 2019)~~ are available~~. This is justified by the economical and practical importance of mastitis, which is the most important disease in dairy animals in terms of both impact on milk production and quality and in terms of animal well being (Ruegg, 2017).

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~~A~~ A few studies have analysed the milk from individual healthy cows and~~or~~ the teat surface to investigate the sources of microorganisms, beneficial or not, and their potential effect on cow's health (Cremonesi et al., 2018; Falentin et al., 2016; Frétin et al., 2018). ~~However, it~~It is ~~necessary~~ important to remember that sampling and disinfection may dramatically affect the composition of the microbiota of individual milk samples (Metzger et al., 2018a), that milk obtained aseptically or by abiding to hygienic practices has usually low counts (often less than $1x10^4$ cfu/ml), and that contamination might significantly affect the results of ~~amplicon targeted~~AT studies for low count samples (Dahlberg et al., 2019)~~, with *Methylobacterium* being frequently identified as a contaminant~~. ~~Indeed~~In addition, a high number of amplification cycles may be necessary for teat milk obtained aseptically (Metzger et al., 2018a) and success rate of amplification may be relatively low for milk obtained aseptically from healthy quarters. The results of some early studies on low counts milk which did not include negative control or proper treatment for removing contamination might be therefore slightly biased.

The teat interior and surface are among the most significant sources of microorganisms for individual milk samples, and microorganisms from these sources may persist during transport and transformation of milk. On the other hand, mastitis is likely to have a larger impact compared to external contamination from the teat interior and surface. In a carefully controlled experiment (Andrews, Neher, Weicht, & Barlow, 2019), intramammary infection was found to dramatically affect the composition of the microbiota of teat cistern milk, which, compared to the milk of healthy animals, had a lower bacterial diversity, was more variable among different cows and was often enriched in pathogenic bacteria belonging to the same genus of those isolated by culturing from the affected quarters, supporting the hypothesis that mastitis is correlated with dysbiosis of the mammary gland. In addition, the

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microbiota of cistern milk and teat apex of infected quarters was more similar compared to healthy quarters, while the microbiota of cistern milk and teat apex in healthy animals was also more variable in time, suggesting that it was more affected by external contamination. The effect of mastitis on the microbiota of the teat apex may be detectable even long after the demise of symptoms. Falentin et al. (2016) examined the microbiota of the teat apex (foremilk + teat apex swabs) for healthy Holstein cows from a single experimental farm. The cows had different previous histories of mastitis. The cow's teat canal microbiota was highly variable (even within the same cow) and teat microbiota from cows without and with a previous history of mastitis could be clearly differentiated, while microbiota of cows with an uncertain status tended to cluster with those of cows with a previous history of mastitis (cluster 1). Discrimination between the two clusters was due to a higher abundance of members of class *Bacilli* (with *Staphylococcus aureus* and *Staph. equorum* as the most prevalent and abundant species) in cluster 1 and higher relative abundance of a diverse array of genera belonging to the phylum *Actinobacteria* (including *Bifidobacterium*), class *Clostridia*, phylum *Bacteroidetes*, including several genera associated with the gastrointestinal (GI) tract, in cluster 2. The origin of these microorganisms may be therefore the teat canal itself, in the case of mastitis agents like *Staph. aureus,* while the potential origin of bacteria associated with the GI tract is uncertain. The procedure used in this study included thorough washing and sanitation before sampling of the teat canal, and may have reduced contamination from loosely attached bacterial cells originating from faeces or from the environment, but it might not have prevented it completely. ~~Doyle et al. (2017b) analysed, in a systematic study, the effect of farming (indoor vs outdoor), and cleaning procedure on the microbiota of teat surface, individual and bulk milk samples. Teat swab~~

microbiota was found to be highly diverse and significantly affected by both farming practices and cleaning.

Falentin et al. (2016) examined the microbiota of the teat canal for healthy Holstein cows from a single experimental farm. The cows had different previous histories of mastitis. The authors found that the cow's teat canal microbiota is highly variable (even within the same cow) and that teat microbiota from cows without and with a previous history of mastitis can be clearly differentiated, while those with an uncertain status tended to cluster with those of cows with a previous history of mastitis. Discrimination between the two groups observed using PCoA and cluster analysis was due to a higher abundance of members of class *Bacilli* (with *Staphylococcus aureus* and *Staph. equorum* as the most prevalent and abundant species) in cluster 1 and higher relative abundance of a diverse array of genera belonging to the phylum *Actinobacteria* (including *Bifidobacterium*), class *Clostridia* (*Ruminococcus, Clostridium, Coprococcus, Oscillospira, Roseburia, Anaerovibrio, Phascolarctobacterium, Selenomonas*, etc.), phylum *Bacteroidetes* (*Prevotella, Bacteroides, Paludibacter*, etc.), including several genera associated with the gastrointestinal (GI) tract. The authors concluded that mastitis causes lasting changes in the microbiota of the teat canal, a finding that has been substantiated by other authors. The origin of these microorganisms may be therefore the teat canal itself, in the case of mastitis agents like *Staph. aureus*, or the gut, although enteromammary gland pathway does not operate efficiently in ruminants (Rainard, 2017). The procedure used in this study included thorough washing and sanitation before sampling of the teat canal, and may have reduced contamination from loosely attached bacterial cells originating from faeces or from the environment, although it might not have prevented it completely and therefore the source

~~of bacteria associated with the gastrointestinal~~ GI tract ~~in the teat canal of healthy quarters is uncertain.~~

Data on the microbiota of the cow teat skin surface in animals showing no signs of clinical mastitis are available for ~~two~~ three more studies (Doyle et al., 2017b; Falardeau, Keeney, Trmčić, Kitts, & Wang, 2019; Frétin et al., 2018). ~~For neither of these studies data on previous history of mastitis are available, although one may assume that no clinical signs of mastitis were evident at time of sampling. In both studies the microbiota of t~~Teat skin microbiota was highly diverse and variable: a prevalence and abundance plot with data extracted from FoodMicrobionet is shown in Supplementary Figure 1, and a table showing the top 50 taxa in terms of prevalence and relative abundance is provided as Supplementary Table 2. Frétin et al. (2018) sampled the teat skin of cows belonging to two breeds (Holstein and Montbeliarde) under two different farming regimes (extensive EXT, with cows feeding exclusively on pasture, and semi-extensive, SEMI, with cows feeding on pasture and concentrate) prior to evening milking (i.e. prior to washing). As a ~~result~~consequence, the ~~microbiota~~ results ~~may be~~were probably affected by both autochthonous species and by contaminants from faeces and the farm/pasture environment. ~~. The microbiota of teat skin was highly diverse (with m~~More than 300 Operational Taxonomic Units (OTUs) and 98 genera~~)~~ were identified, ~~and included~~ including both *Actinobacteria* and *Clostridia* as abundant members. ~~,~~ Some~~with 12~~ OTUs (twelve, including members of the genera *Brevibacterium, Lactococcus, Lactobacillus, Streptococcus, Staphylococcus, Macrococcus, Escherichia*) ~~persisting~~ persisted throughout the process ~~(including members of the genera *Brevibacterium, Lactococcus, Lactobacillus, Streptococcus, Staphylococcus, Macrococcus, Escherichia*)~~, from teat skin to ripened cheese, while 201 were specific to teat skin. The microbiota of teat skin was most affected by the grazing system and by the season of

sampling (July vs. September). ~~In both seasons in the EXT system a higher relative abundance of genus *Corynobacterium*, family *Lachnospiraceae*, class *Coriobacteriia* and genus *Aerococcus*, while in SEMI the relative abundance of order *Bifidobacteriales* and genus *Clostridium* was higher. The number of differentially abundant taxa was higher in July and taxa with differential abundance were not necessarily the same in the two sampling periods.~~ Falardeau et al. (2019), in a large source tracking study, confirmed that teat skin (sampled prior to washing~~,~~ and milking) had a high microbial diversity ~~(which is confirmed by the number of different taxa and their low average abundance in Supplementary Figure 1)~~, which however was comparable to that of teat milk and tank milk. *Clostridiales* were the most ~~important~~ abundant members of the microbiota (17-41%), but *Actinobacteria* (*Corynebacterium* and *Brevibacterium*), *Bacteroidetes* (*Bacteroides* and *Alistipes*), and *Proteobacteria* (including *Pseudomonas* and *Acinetobacter*) were ~~also members of~~all found in the subdominant microbiota. ~~The distribution of taxa in classes (prior to filtering for abundance and prevalence is shown in Figure 1.~~ The main difference between these two studies is the higher relative proportion of *Clostridia* and *Bacteroidia* in Falardeau et al. (2019~~; whose results are similar to those of Doyle et al., 2017b~~) and the higher proportion of *Bacilli* and *Erysipelotrichia* in Frétin et al. (2018). The results of Falardeau et al. (2019) are similar to those of Doyle et al. (2017b) who analysed, in a systematic study, the effect of farming (indoor vs outdoor), and cleaning procedure on the microbiota of teat surface, individual and bulk milk samples~~. T~~, and confirmed that teat swab microbiota ~~was~~is ~~found to be~~ highly diverse and significantly affected by both farming practices and cleaning.~~The three studies differ in several ways (including the target region for DNA, V3 and V3-V4) respectively, but feeding and farming system may have played a major role in the observed~~

~~differences in the composition of the microbiota, as shown in many studies on teat and tank milk (see below).~~

3.1.2 The microbiota of teat milk.

The microbiota of ~~milk~~ samples obtained by milking ~~from~~ individual quarters or individual animals has been analysed in several studies, focusing on the effect of disease and/or disease treatment (mastitis: Angelopoulou et al., 2019; Bhatt et al., 2012; Ganda et al., 2016, 2017; ~~Ganda et al., 2017;~~ Hoque et al., 2019; Kuehn et al., 2013; Metzger et al., 2018b; Oikonomou, Machado, Santisteban, Schukken, & Bicalho, 2012; Oikonomou et al., 2014; Oultram, Ganda, Boulding, Bicalho, & Oikonomou, 2017; Pang et al., 2018; Taponen et al., 2019; Vasquez et al., 2019; subclinical acidosis: Zhang, Huo, Zhu, & Mao, 2015), on sampling (Metzger et al., 2018a) or on the tracking of sources of contaminations (Cremonesi et al., 2018; Dahlberg et al., 2019; Doyle et al., 2017b; Falardeau et al., 2019; Metzger et al., 2018a). Comparing these studies is difficult, because of differences in practically all the factors which are known to affect the composition of microbiota (breed, health status, farming, bedding, feeding, lactation stage, etc.), in sampling, in ~~gene targets and sequencing platforms and in bioinformatic approaches~~methods used for the analysis of the mic~~i~~robiota. In ~~fact~~particular, the composition of the microbiota of individual milk samples has been proven to be strongly dependent on the sampling procedure used (Metzger et al., 2018a) and sampling procedures must be carefully documented to allow the interpretation of results from different studies~~, since they strongly affect the composition of individual milk samples (Metzger et al., 2018a).~~.

However, several findings have been confirmed by multiple studies: a. the microbiota of milk of healthy animals is highly diverse and variable; b. mastitis and other clinical and

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subclinical conditions strongly affect the composition and diversity of the microbiota; c. a large number of other factors, including breed, parity, farming systems, feeding, bedding, season of the year, and days in milking significantly affect the composition of milk microbiotab. mastitis and other clinical and subclinical conditions strongly affect the composition and diversity of the microbiota; c. a large number of other factors, including breed, parity, farming systems, feeding, bedding, season of the year, and days in milking significantly affect the composition of milk microbiota. The effect of sampling may be quite significant: Metzger et al. (2018a) compared analysis of pooled samples, with conventional aseptic sampling from individual quarters and sampling from the gland cistern using a needle and a vacuum tube. Although the results of this (and other studies analysing low biomass samples) need to be taken with care, unless potential contamination is dealt with (Dahlberg et al., 2019), significant differences were found in terms of diversity and species composition depending on sampling technique, with the highest diversity for composite samples: this is in good agreement with results of Falentin et al. (2016) who found differences in composition of the microbiota among teats belonging to the same quarter. Even with aseptic sampling techniques, a high diversity was found, with more than 4,000 OTUs detected, only 14 of which had an abundance >1%. Bedding had a significant effect on the composition of the microbiota of cisternal samples and it is unlikely that this is due to external contamination, but due to low sample number these results need to be confirmed. To illustrate the and variability of the composition of teat milk samples Figure 2 shows thewe have compared the combined results for teat milk from healthy cows from two studies (one illustrating he effect of breed and days in milking: Cremonesi et al., 2018; the other on contamination sources from farm to fork: Falardeau et al., 2019) for which sequences are publicly available and which are included in FoodMicrobionet. The assembly

of taxa for the two studies include 1184 genera (most of which with very low prevalence and abundance) belonging to 115 classes of 45 phyla. ~~a~~A bar plot of the relative abundance of the 20 most abundant and prevalent~~e~~ taxa is shown in Figure 2~~,~~ while a prevalence and abundance plot and the data on prevalence on the top 50 most abundant taxa are shown in Supplementary Figure 2 and Supplementary Table ~~2~~ 3 respectively. ~~Teat milk microbiota is highly diverse and its composition is clearly affected by several factors (including breed, lactation stage, the latter being frequently confounded with season and feeding) and may be highly variable among individual cows. Many of the genera in table 2 match those found by other authors (Bonsaglia et al., 2017) and the~~In both studies the most prevalent and abundant taxa belong to phyla *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, but large differences are evident both within and between ~~the two~~ studies.

Cremonesi et al. (2018) compared Holstein Friesians with a ~~local~~n Italian breed (Rendena, which shows lower prevalence of mastitis) from the same farm, from drying off, to colostrum stage and to late lactation. ~~Although t~~The number of samples in this study was relatively low, but variation over time was observed for both breeds~~, but~~ and b~~B~~eginning of lactation had a significant impact on the microbiota. ~~the~~ The composition of the microbiota of Rendena cows was more stable, and significantly different from that of Holstein cows. ~~Beginning of lactation had a significant impact on the microbiota.~~ *Streptococcus* was the most prevalent and abundant genus in both breeds and together with *Lactobacillus* was the only genus shared by all samples. ~~Due to low number of samples, the evidences for causal relationships are scant for this study.~~ On the other hand, Falardeau et al. (2019) found that *Actinobacteria* (with genera *Kocuria, Dermatococcus* and *Dietzia*) were by far the dominating phylum in teat milk, while *Firmicutes* (with *Lactococcus* and *Clostridium* XI as most abundant genera) and *Proteobacteria* (with *Enhydrobacter* and *Psychrobacter* as most

abundant genera) were less abundant. Notably, the microbiota of teat milk for this study was quite different from that of teat skin (see above).

A seasonal effect on the composition of teat milk was also found by Metzger et al. (2018b) ~~In a large longitudinal study,~~who ~~Metzger et al. (2018b)~~ monitored for over 150 days the composition of microbiota of teat milk obtained from healthy cow quarters, newly infected quarters and quarters with chronic inflammation or clinical mastitis. They found a strong effect of season of the year and of time of lactation, which resulted in increased richness from Winter to Summer in all cohorts, and in significant changes in the relative abundance of 20 OTUs (including *Fibrobacter*, *Corynebacterium*, *Arthrobacter*, *Bacteroidetes*), which they attributed to contamination from sand bedding and/or to physiological changes during lactation (*Bacteroidetes*). ~~The health status also greatly impacted the composition of the microbiota and caused a reduction in diversity in milk from quarters with chronic or acute inflammation.~~ Interestingly, milk from quarter with chronic inflammation showed the greatest seasonal changes.

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Much emphasis has been given to the comparison of the microbiota of individual milk samples from healthy cows and cows with subclinical or ~~subclinical or~~ clinical mastitis, and in the latter ~~case~~, for culture positive and negative samples. Differences between healthy and diseased quarters are almost always significant (even for culture negative quarters, Kuehn et al., 2013; Oikonomou et al., 2012) and analysis of the microbiota may contribute to the diagnosis in quarters with subclinical mastitis~~As it would be expected, differences between microbiota of healthy and diseased quarters are always significant (even for culture negative quarters, Kuehn et al., 2013; Oikonomou et al., 2012).; however, no differences are observed in some studies (Ganda et al., 2016), D~~and dominating bacteria in

mastitic milk change quite substantially in different studies, depending on the number of samples tested and on the causative agents ~~of mastitis~~ in individual cows (Supplementary T~~t~~able ~~AA4~~)~~*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and/or *Staph. aureus:* Bhatt et al., 2012; *E. coli, Klebsiella spp., Trueperella pyogenes, Streptococcus dysgalactiae, Staph. aureus* and anaerobic pathogenic bacteria which are known to act in synergism with aerobic mastitis agents, such as *Fusobacterium necrophorum, Porphyromonas levii:* Oikonomou et al., 2012, Oikonomou et al., 2014; coagulase negative staphylococci, *Staphylococcus aureus, Streptococcus, E. coli, Klebsiella, T. pyogenes, Corynebacterium bovis, Serratia, Bacillus, Pasteurella multocida, Streptococcus, Mycoplasma:* Kuehn et al., 2013; *Str. uberis, Str. dysgalactiae, Staph. carnosus, Staph. chromogenes, Sneathia sanguinegens, Rhodococcus,* and possibly *Enterococcus, Listeria, E. coli:* Oultram et al., 2017; *Klebsiella, Escherichia–Shigella, Streptococcus* and *Corynebacterium*: Pang et al., 2018; *Campylobacter, Klebsiella, Escherichia, Staphylococcus:* Hoque et al., 2019; *E. coli:* Vasquez et al., 2019; *Rhodococcus, Pseudomonas, Streptococcus, Staphylococcus:* Angelopoulou et al., 2019; *Coxiella, Staphylococcus:* Metzger et al., 2018b; Gram-negative pathogens, including *E. coli, Klebsiella* and *Pseudomonas:* Ganda et al., 2016; *E. coli:* Ganda et al., 2017; *Staph. aureus* and *Str. uberis:* Taponen et al., 2019; Strep.). Mastitis agents are usually abundant components of the microbiota in mastitic milk, but not necessarily the most abundant (Oikonomou et al., 2012) and their abundance may change over time (Ganda et al., 2016, 2017~~; Ganda et al., 2017~~). In several cases, association of two or more agents of mastitis are found (Angelopoulou et al., 2019; Bhatt et al., 2012; Oikonomou et al., 2012). Potential bacterial pathogens as *Str. uberis* and *Staph. aureus* might also be found with high prevalence in milk from healthy quarters (Oikonomou et al., 2014) while, on the other hand, some mastitis agents like *Escherichia coli* or *Klebsiella* were

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never found in samples from healthy quarters: this has led to speculate that bacterial mastitis can be considered as a dysbiosis rather than a primary clinical infection. This hypothesis may also be supported by the fact that species ~~that~~which most contribute to the discrimination between mastitic and non mastitic milk are not necessarily mastitis pathogens, although they might have been occasionally associated with mastitis (Kuehn et al., 2013). A recent study (Angelopoulou et al., 2019) has confirmed the complex, polymicrobial nature of mastitis and showed that culture based approaches and AT metagenomics complement each other. In an attempt to evaluate if significant associations could be detected in this data set between known mastitis pathogens and other bacteria, we carried out inferences of microbial association networks as described in Parente et al. (2018) (Supplementary Figure 3). Only two modules were detected, one including *Escherichia/Shigella* and the other *Staphylococcus*, two genera which include species identified by culturing as potential agents of mastitis. The two modules did not overlap but, due to the low number of samples, it is not clear if this reflect true differences in the microbiota of milk connected to infection from either *Escherichia* or *Staphylococcus*.

Another frequently observed consequence of inflammation due to mastitis is a ~~decreases~~ decreased diversity in the microbiota (Andrews et al., 2019; Bonsaglia et al., 2017~~)~~; Ganda et al., 2016, 2017~~; Ganda et al., 2017~~; Kuehn et al., 2013; Metzger et al., 2018b; Taponen et al., 2019; Vasquez et al., 2019), although the lower number of OTUs in mastitic milk may be simply due to the compositional nature of AT data (when the relative abundance of one OTU increases the relative abundance of the others decreases and may fall below detection limits). Decrease in alpha diversity is a clear indication of a dysbiosis and ~~In~~ in at least one study the largest reduction ~~in alpha diversity~~ was associated with ~~a~~ the largest decrease in milk production (Vasquez et al., 2019).

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~~On the other hand~~However, distinguishing samples with subclinical mastitis from samples from healthy cows might not be straightforward using ~~purely~~ descriptive techniques when quasi-experimental designs are used (Pang et al., 2018), and in some non-severe cases it might be difficult to associate the mastitis condition with significant changes in abundance of bacterial families (Ganda et al., 2016; Vasquez et al., 2019).

~~In a large longitudinal study, Metzger et al. (2018b) monitored for over 150 days the composition of microbiota of teat milk obtained from healthy cow quarters, newly infected quarters and quarters with chronic inflammation or clinical mastitis. They found a strong effect of season of the year and of time of lactation, which resulted in increased richness from Winter to Summer in all cohorts, and in significant changes in the relative abundance of 20 OTUs (including *Fibrobacter, Corynebacterium, Arthrobacter, Bacteroidetes*), which they attributed to contamination from sand bedding and/or to physiological changes during lactation (*Bacteroidetes*). The health status also greatly impacted the composition of the microbiota and caused a reduction in diversity in milk from quarters with chronic or acute inflammation. Interestingly, milk from quarter with chronic inflammation showed the greatest seasonal changes.~~

~~The agents causing mastitis are usually abundant components of the microbiota in mastitis milk, but not necessarily the most abundant (Oikonomou et al., 2012) and their relative abundance may change over time (Ganda et al., 2016; Ganda et al., 2017). In several cases association of two or more bacteria which are recognized as agents of mastitis are found (Angelopoulou et al., 2019; Bhatt et al., 2012; Oikonomou et al., 2012) and high throughput sequencing was able to detect bacteria associated with mastitis (*Str. uberis, T. pyogenes, E. coli*) in mastitic milk samples which were negative in culture based analyses. Potential bacterial pathogens as *Str. uberis* and *Staph. aureus* might also be found with high~~

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~~prevalence in milk from healthy quarters (Oikonomou et al., 2014) while, on the other hand, some mastitis agents like *Escherichia coli* or *Klebsiella* were never found in samples from healthy quarters: this has led to speculate that bacterial mastitis can be considered as a dysbiosis rather than a primary clinical infection. This hypothesis may also be supported by the fact that species that most contribute to the discrimination between mastitic and non mastitic milk are not necessarily mastitis pathogens, although they might have been occasionally associated with mastitis (Kuehn et al., 2013). A recent study (Angelopoulou et al., 2019) has confirmed the complex, polymicrobial nature of mastitis and showed that culture based approaches and amplicon targeted~~AT ~~metagenomics complement each other. In an attempt to evaluate if significant associations could be detected in this data set between known mastitis pathogens and other bacteria we carried out inferences of microbial association networks as described in Parente et al. (2018) (Supplementary Figure 3). Only two modules were detected, one including *Escherichia/Shigella* and the other *Staphylococcus*, two genera which include species identified by culturing as potential agents of mastitis. The two modules did not overlap but, due to the low number of samples, it is not clear if this reflect true differences in the microbiota of milk connected to infection from either *Escherichia* or *Staphylococcus*.~~

~~A~~The potential impact of ~~ntibiotic and other~~ treatments used to control mastitis at dry off is of both scientific and practical importance~~do not necessarily have a significant effect on milk microbiota nor on the incidence of mastitis during the same or following milking season~~.

Ganda et al. (2016) compared two groups of Holstein cows which had been randomly allocated to a control group or to a group treated with 5 day intramammary treatment with Ceftiofur (a third generation cephalosporin). Antibiotic treatment did not affect the clinical or bacteriological cure rates, nor bacterial clearance or bacterial load, but did reduce over

time the relative abundance of *Enterobacteriaceae* in quarters with *E. coli* mastitis. However, no clear effect was observed on quarters without a culture diagnosis. In a subsequent study, the same group (Bonsaglia et al., 2017) showed that treatment with the same antibiotic at dry-off did not change the incidence of mastitis in the first 60 days post-partum for healthy cows, nor did it significantly affect the composition of the milk microbiota after 7 days. The authors concluded that this type of treatment does not cause dysbiosis but it does not have any therapeutic value for healthy cows. However, large individual variability may have increased type II error and prevented the detection of significant differences. Using the same antibiotic in a challenge study with *E. coli,* Ganda et al. (2017) observed that, independently of antibiotic use, normal microbiota re-established itself over a 216 h sampling time. This study is an excellent example of the dynamic changes of the composition of milk microbiota prior and during infection with a mastitis pathogen and shows how, after a disturbance, the microbiota may shortly revert to its initial status (or to a status which is not statistically different from the initial one).

Studies on the effect of mastitis on milk microbiota for other species ~~(ewe; water buffalo)~~ are relatively rare and somewhat limited in scope. Catozzi et al. (2017) investigated the teat milk microbiota in 137 samples of water buffalo milk obtained from healthy quarters and from quarters with evidences of clinical and subclinical mastitis from 88 farms of ~~a relatively~~ limited area in Southern Italy. They identified a core microbiota of fifteen genera, including genera commonly found in cow's milk (see Figure 3) ~~as *Micrococcus, Propionibacterium, Solibacillus, Staphylococcus, Aerococcus, Facklamia, Trichococcus, Turicibacter, Clostridium, Acinetobacter, Psychrobacter and Pseudomonas*~~. Both subclinical and clinical mastitis significantly changed the composition of the microbiota, usually with a relative decrease in psychrotrophic microorganism (*Pseudomonas, Psychrobacter*), a decrease in *Actinobacteria*

and *Firmicutes* and ~~and~~ increase in *Proteobacteria* and *Bacteroidetes*~~.~~, ~~As usual,~~and clinical mastitis resulted in a decrease in alpha diversity. In general, the strongest differences were found between samples with low (<1x10^5/ml) and high (0.5x10^6 to >1x10^6) somatic cell counts (SCC). Culture results for quarters with clinical mastitis confirmed the occurrence of common mastitis pathogens, such as *Staph. aureus, T. pyogenes, Str. agalactiae* (alone or in combination with *S. aureus*), *Ps. aeruginosa* and some coagulase negative staphylococci. Similar results (reduced diversity, ability to discriminate ~~d~~samples from healthy animals from those with subclinical and clinical mastitis) have been found for water-buffalo milk in India (Patel et al., 2016, 2019).~~Since the results for mastitic milk were pooled (either in term of diagnosis, clinical vs subclinical; or in term of somatic cell counts (SCC), it is not clear if the most abundant genera in the microbial community of clinical mastitis samples matched those obtained by culturing. In general, the strongest differences were found between samples with low (<1x10^5/ml) and high (0.5x10^6 to >1x10^6) somatic cell counts~~SCC ~~(SCC).~~ ~~The authors concluded that the core microbiota of teat milk from water buffalo is different from that of cow, but this is contradicted by comparisons with multiple data sets (see below).~~

~~Available r~~Results on the effect of mastitis on ewe's milk microbiota are somewhat contradictory. Esteban-Blanco et al. (2019) investigated the microbiota of teat milk obtained from a relatively low number (50) of healthy Assaf ewes from a single flock in Spain. ~~Apparently, o~~Only 5 genera (*Staphylococcus, Lactobacillus, Corynebacterium, Streptococcus* and *Escherichia/Shigella*) were shared among all samples, and a high diversity was observed. Evidences of sub-clinical mastitis ~~(SCC>4x10^6, without signs of alteration of milk or inflammation of the udder)~~ were associated to a reduced diversity. Using inference of microbial association networks the authors identified two modules of ASVs, and observed

692 that the relative abundance of the species in the two modules in samples without or with
693 subclinical mastitis was different: this further confirms the hypothesis that subclinical
694 mastitis causes global changes in the microbiota, which affect not only the potential
695 causative agent but a number of other taxa. ~~A ASV identified as *Staphylococcus* was~~
696 ~~significantly associated with increased SCC, while six other taxa (*Corynebacterium,*~~
697 ~~*Alloiococcus, Staphylococcus, Joetgalicoccus, Salinicoccus, Carnobacteriaceae*) were~~
698 ~~associated with reduced SCC.~~
699 Castro et al. (2019) ~~analysed~~analyszed teat milk from 36 healthy Manchega ewes with or
700 without a previous history of mastitis from two farms in Spain. They found significant
701 differences between the microbiota in the two farms (with significant differences between
702 the relative abundances *Staphylococcus, Paenibacillus* and *Geobacillus*) but, contrary to
703 what had been reported for~~t~~ teat microbiota of cows by Falentin et al. (2016) did not find
704 any significant difference due to history of mastitis: again, it is unclear if this reflects true
705 lack of differences or is a consequence of high variability.
706 Other clinical or subclinical conditions may affect milk microbiota and susceptibility to
707 mastitis. Zhang et al. (2015) using a crossover experiment analysed pooled teat milk samples
708 from Holstein cows with or without an induced subclinical acidosis condition. Although a
709 high concentrate (HC) diet, which resulted in subclinical acidosis, did not affect microbial
710 diversity, significant differences were found between cows with or without clinical acidosis
711 with the former having a significantly higher abundance of *Proteobacteria*, and lower
712 abundance of *Armatimonadetes, Spirochaetes, Planctomycetes, Fibrobacteres, Chloroflexi,*
713 *Tenericutes, Lentisphaerae, Synergistetes, Elusimicrobia, Cyanobacteria, Verrucomicrobia*
714 and *Firmicutes*. The authors claimed that potential mastitis agents (including
715 *Stenotrophomonas maltophila*, *Brevundimonas diminuta*, *Str. parauberis* and *Enterococcus*

716 *faecalis*) were significantly more abundant in the milk of cows fed the HC diet, which also
717 resulted in an increase in the abundance of psychrotrophic organisms and this may support
718 the idea that mastitis is related to dysbiosis. On the other hand, other mastitis agents, like
719 *Strep. agalactiae* were significantly more abundant in the milk of cows fed a low
720 concentrate diet.
721 Finally, there is some limited data (Zhong, Xue, & Liu, 2018) that may support the idea that
722 udder health status may be related to the microbiota of other body sites. In fact, diversity
723 of the microbiota of rumen in cows with low ($<2x10^5$) or high ($>1x10^6$) SCC has been found
724 to be significantly different, and although no evidence of separation of the composition of
725 the microbiota of rumen in four groups of cows with different SCC was found by beta
726 diversity analysis, significant differences were found in the relative abundance of a few taxa
727 (phyla SR1, *Actinobacteria*, unclassified family *Clostridiales*, genus *Butyrivibrio*,
728 *Proteobacteria* and family *Succinivibrionaceae*). However, the authors did not present an
729 evidence of a cause effect relationships nor analysed the composition of the microbiota of
730 milk.
731 Overall, these data support the idea that clinical and sub-clinical conditions significantly
732 affect the composition and diversity of teat milk microbiota, prior to any further
733 contamination from environmental sources, and that ~~this~~these changes may result~~s~~ in
734 dysbiosis, compared to the "normal" situation characterized by a highly diverse microbiota.
735 The dysbiosis status may be more (Falentin et al., 2016) or less (Ganda et al., 2016)
736 persistent, and more complex and controlled longitudinal studies are clearly needed to
737 clarify how the homeostasis of the milk microbiota is maintained or recovered in different
738 conditions.

~~A~~ Due to limited availability of data, to high variability, and to differences in methodologies, a direct comparison of the ~~the~~ composition of microbiota of teat milk from different dairy species is difficult. ~~comparison of t~~The distribution of genera in teat milk obtained from cows, ewes or water buffaloes is shown in Figure 3, while a NMDS (Non-metric MultiDimensional ~~D~~Scaling) plot is shown in Supplementary Figure 4.

Due to the low number of studies and samples shown here it is difficult to generalize, but it is clear that several of the most abundant genera, although varying in abundance, appear in the milk of the 3 species. A shared core microbiome may exist for these three species, at least when results are aggregated at the genus level. This is confirmed by the partial overlap of the confidence ellipses of the samples from different species in Supplementary Figure 4.

~~A shared core microbiome may exist for these three species, at least when results are aggregated at the genus level.~~

~~Other clinical or subclinical conditions may affect milk microbiota and susceptibility to mastitis. Zhang et al. (2015) using a crossover experiment analysed pooled teat milk samples from Holstein cows with or without an induced subclinical acidosis condition. Although a high concentrate (HC) diet, which resulted in subclinical acidosis, did not affect microbial diversity, significant differences were found between cows with or without clinical acidosis with the former having a significantly higher abundance of *Proteobacteria*, and lower abundance of *Armatimonadetes, Spirochaetes, Planctomycetes, Fibrobacteres, Chloroflexi, Tenericutes, Lentisphaerae, Synergistetes, Elusimicrobia, Cyanobacteria, Verrucomicrobia* and *Firmicutes*. The authors claimed that potential mastitis agents (including *Stenotrophomonas maltophila*, *Brevundimonas diminuta*, *Str. parauberis* and *Enterococcus faecalis*) were significantly more abundant in the milk of cows fed the HC diet, which also resulted in an increase in the abundance of psychrotrophic organisms and this may support~~

~~the idea that mastitis is related to dysbiosis. On the other hand, other mastitis agents, like *Strep. agalactiae* were significantly more abundant in the milk of cows fed a low concentrate diet.~~

~~Furthermore, there is some limited data (Zhong, Xue, & Liu, 2018) that may support the idea that udder health status may be related to the microbiota of other body sites. In fact, diversity of the microbiota of rumen in cows with low (<2x10^{5}00,000) or high (>1x10^6,000,000) SCC has been found to be significantly different, and although no evidence of separation of the composition of the microbiota of rumen in four groups of cows with different SCC was found by beta diversity analysis, significant differences were found in the relative abundance of a few taxa (phyla SR1, *Actinobacteria*, unclassified family *Clostridiales*, genus *Butyrivibrio*, *Proteobacteria* and family *Succinivibrionaceae*). However, the authors did not present an evidence of a cause effect relationships nor analysed the composition of the microbiota of milk.~~

3.1.3 Further down the line: the microbiota of bulk tank milk.

The vast majority of studies on milk microbiota ~~only report on the composition of~~focus on composite samples obtained from bulk tanks at the dairy farm, from tanker trucks or from silos at the dairy processing plant. Apart from disease, a large number of interrelated factors has been shown to affect the composition of the microbiota for bulk milk: season of the year (Doyle, Gleeson, O'Toole, & Cotter, 2017a; Doyle et al., 2017b; Kable et al., 2016, 2019; ~~Kable et al., 2019;~~ Li et al., 2018; Porcellato et al., 2018; Zhang, Palmer, Teh, Biggs, & Flint, 2019), lactation stage (Doyle et al., 2017a), type of farming (indoor/outdoor: Doyle et al., 2017a, 2017b), geographic location within a country/region (Kable et al., 2016, 2019; ~~Kable et al., 2019;~~ Porcellato et al., 2018; Skeie et al., 2019; Zhang et al., 2019), processing

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environment (Kable et al., 2016), teat preparation (Doyle et al., 2017b), storage conditions (Doyle et al., 2017a). ~~and although all studies confirm a high diversity and a high variability, a core microbiota can be identified, with several genera being present in a large proportion of samples (see Supplementary Figure 5 and Supplementary Table 3).~~

The issue of sources of contamination of bulk tank milk is of great practical importance, since preventing and controlling contamination by selected pathogenic or spoilage organisms may contribute to improve~~ing~~ the safety and quality of raw milk and raw milk products. Since not all studies fully document all ~~the~~ potential sources of variation, it is difficult to track unambiguously sources of contamination and to separate the effect of contamination from that of storage (combination of time temperature), except for a few large and structured studies (Doyle et al., 2017a, 2017b; Falardeau et al., 2019; Kable et al., 2016; Porcellato et al., 2018).

Using a source tracking approach, Doyle et al. (2017b) clearly identified teat surface and faeces as two of the major sources of microorganisms in bulk tank milk and were able to identify the contribution of other ~~potential~~ major sources of contamination (including grass for cows grazed on pasture, bedding and silage for cows housed indoor). ~~, although~~ However, the effect of housing was confounded with that of season and lactation stage since experiments were carried out on the same herd. For sources of contamination which were common to both housing regimes (faeces, teat) the composition of microbiota was affected more by the housing than by the nature of the sample (i.e. samples from the outdoor regime tended to cluster together in beta diversity analysis). In addition, the relative importance of a given source of contamination changed for the two housing regimes (with faeces giving a higher relative contribution for bulk tank milk from cows housed indoors). The effect of teat treatment (which compared no treatment with a

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811 treatment including washing with water, disinfectant and thorough washing) clearly showed
812 an interaction with housing (indoor vs outdoor) perhaps due to the different ability of main
813 teat contaminants to adhere to teat surface ~~(the effect of prep on total number of bacteria~~
814 ~~in individual milk samples was higher for cows housed outdoor)~~ or to survive to the
815 treatment.
816 These findings were confirmed by a large recent study (Falardeau et al., ~~(~~2019) ~~in a large~~
817 ~~recent study analysed the~~in which the microbiota in both environmental (soil, faeces,
818 pasture, hay, bedding, cow environment) and food samples (individual and ~~-~~pooled milk
819 samples at both the dairy farm and at the cheesemaking plant) in an artisanal cheese
820 making facility was analys~~z~~ed. ~~Samples included potential sources of contamination (soil,~~
821 ~~faeces, pasture, hay, bedding, cow environment) and individual and pooled milk samples (at~~
822 ~~both the dairy farm and at the cheesemaking plant).~~ The microbiota of teat milk was
823 significantly different from the microbiota of bulk tank milk (pooled pre- and post-transport
824 milk), ~~although the latter result~~possibly because ~~might have been due to~~of ~~a combination~~
825 both contamination from equipment and growth at refrigeration temperature. In fact,
826 several anaerobic *Firmicutes* and *Bacteroidetes* genera were the most abundant ~~genera~~ in
827 the bulk tank milk, but not in the teat milk, and the authors hypothesized that their source
828 was the milking machine environment, although this was not formally proven. At any rate,
829 78 out of 93 core OTUs present in milk environments were also present in the dairy farm
830 environment, ~~confirming~~ supporting the idea that the dairy farm is an important source of
831 microorganisms in bulk tank milk~~, and~~. A~~a~~n even larger number of taxa (at the genus level or
832 above) were shared by pasture and feed, farm environments, teat skin, teat milk and bulk
833 tank milk (Supplementary Figure 5). However, the relative abundance of the top 25 most
834 abundant genera~~,~~ varied greatly among and within different sample sources (Figure 4).

~~Indeed, given the low taxonomic resolution used in this study (only the V3 region of 16S RNA gene was targeted) it is hard to infer the potential paths for contamination and the relative contribution of growth in the bulk tank milk.~~

In general, source tracking studies should be taken with caution, even when longer sequences are used. Falardeau et al. (2019) used relatively short fragments (V3 region) and Doyle et al. (2017b) targeted the V3-V4 region but used OTUs inferred using a 97% similarity level, while in an amplicon targeted study with higher taxonomic resolution, Skeie et al. (2019) showed that even within the same species different ~~sequence variant~~ASVs may have a different distribution (see below). ~~On the other hand, the cost of metagenomic~~ Metagenomic studies ~~(which~~ may reveal the composition of populations at the strain level~~)~~ and allow the tracking of the sources of the most prevalent and abundant strains, but the cost of studies with sufficient sample sizes ~~comparable sample sizes~~ would probably be unjustified.

The effect of season on the composition of bulk tank milk has been studied by several authors (Doyle et al., 2017a; Li et al., 2018; Zhang et al., 2019): all have found a significant effect ~~of the season of the year~~, ~~although this effect~~which, however, may be confounded with many other factors (days in lactation, farming system, feeding, etc.). Doyle et al. (2017a) ~~investigated the effect of season, with~~compared samples collected in Spring and October, but, due to farming practices in Ireland, this was completely confounded with feed, lactation stage and housing. ~~The combination of factors lactation stage/season/housing feed had indeed a large effect on the composition of the microbiota.~~ Mid-lactation samples (collected in Spring with cows feeding on pasture outdoors) were significantly different from late lactation samples (collected in Autumn when at least for part of the sampling period the cows were housed inside and fed a diet containing

concentrate and silage): they had a higher diversity, while for individual milk samples of ~~milk~~ cows fed on pasture a slightly lower diversity was found~~;~~ (Doyle et al., 2017b), and 85 taxa showed significant differences in abundance between mid- and late-lactation samples. Two further studies (Li et al., 2018; Zhang et al.~~,~~ ~~(~~2019) have confirmed that a significant seasonal variation exists in the composition of milk microbiota. In both studies members of the genera *Acinetobacter*, *Lactococcus* and *Pseudomonas* were found to be both abundant and highly prevalent, and several genera showed changes of abundance in different seasons (*Pseudomonas*, *Propionibacterium, Flo~~a~~vobacterium*: Li et al., 2018; *Pseudomonas*, two *Lactococcus*, one *Serratia* and one *Acinetobacter:* Zhang et al., 2019). Unfortunately, both studies used a descriptive approach and little or no details were provided on critical factors which are likely to affect microbiota and which may be confounded with the effect of season (farming, breeds, feeding, lactation stage, etc.) and the causes of the observed patterns remain unclear.~~ evaluated the seasonal variation in the composition of the microbiota (with special emphasis on psychrotrophic bacteria, which were also studied using a culturomics approach) using 240 milk samples from bulk tanks of farms from different areas of New Zealand. They found no significant differences due to geographic location but significant differences due to season, with changes in the abundance of two species of *Pseudomonas*, two *Lactococcus* and one *Serratia* and one *Acinetobacter*. Unfortunately, the approach was purely descriptive and it is unclear if the results were due to differences in temperature or to other factors. Interestingly, results for species identification differed significantly for 16S metagenomics and for the culturomics approach based on isolation and identification by MALDI-ToF, even for abundant *Pseudomonas* species. The reasons for this are not clear.~~

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~~High variability in composition and significant changes associated with seasons of the year~~Similar results ~~were also found~~ obtained ~~by~~ Li et al., ~~(~~2018~~)~~ ~~who analysed 112 samples from bulk tanks of 10 dairy farms from the province of Shangai (PRC) over a period of 12 months. Only a few genera (*Pseudomonas*, *Acinetobacter*, *Lactococcus*) were found to be abundant and present in all (or almost all) samples while many others had low abundance and prevalence. The composition of the microbiota was similar between adjacent months of the year and diversity (which was very variable among samples from the same month) was highest in June and lowest in December. In this quasi-experimental study the authors did find significant differences in genus composition between different months and attributed them to changes in humidity and temperature (although this might only be an evidence of correlation, not causation). An attempt to correlate genus abundances to quality parameters (including bacterial and somatic cell counts) was also carried out, but since p values, rather than q-values deriving from corrections for multiple testing were used the results are dubious. A sounder approach for the evaluation of the correlations between milk quality parameters (somatic cell counts, SCC, and standard plate counts, SPC, both measured by flow cytometry) and microbiota composition was used by Rodrigues, Lima, Canniatti-Brazaca, & Bicalho (2017), who analysed 472 low counts (<10^4 cfu/ml) bulk tank milk samples from dairy farms in New York state (USA) over 2 months. As usual, a high diversity and variability was found but a core microbiome comprising *Ruminococcaceae, Acinetobacter, Clostridiales, Bacteroidales*, 5–7N15, *Pseudomonas, Staphylococcus, Lachnospiraceae, Corynebacterium, Planococcaceae, Bacillus*, and *Thermoanaerobacterium* was identified across the 19 farms. Significant association was found between the relative abundance of several genera and low or high SCC and SPC counts. In fact, high SCC milk was significantly associated with increased abundance of *Corynebacterium*, *Streptococcus*,

~~*Lactobacillus, Coxiella, Arthrobacter*, and *Lactococcus* (noticeably only some of this may be potentially associated with mastitis), while high SPC milk (>3.6 log cfu/ml) had increased abundances of *Acinetobacter, Enterobacteriaceae, Corynebacterium* and *Streptococcus* and usually lower diversity: this might therefore only be a sign of shifts in community composition due to growth.~~

Using a ~~high resolution~~high-resolution method based on ASV inference, Skeie et al., (2019) confirmed the high variability, even over short time scales, of the composition of bulk tank milk microbiota. The authors analysed 135 milk samples in three samplings from 45 farms ~~2 farms~~ in Norway over ~~a period of~~ three months in Winter. The ~~45~~ farms were located in two geographically distant areas~~, shared~~ sharing similar climatic conditions, but had three different milking systems. Although milk was collected on average every 3 days, bacterial counts were reasonably low, with a median value of 4.25 log(cfu/ml) and only two samples exceeding 10^5 cfu/ml, with higher values associated systematically with parlour farms with automatic milking systems ~~(may be due to difficulties in applying strict hygienic measures of milking machine sanitation)~~. Beta-diversity (weighted UniFrac) was significantly affected by all the variables used in this quasi-experimental study (areas, sampling, farms, housing/milking systems) and ~~several~~ the abundance of several bacterial genera (including *Pseudomonas, Bacillus, Staphylococcus, Paenibacillus, Psychrobacter, Chryseobacterium, Aerococcus,* and *Rhizobium*) was significantly different among geographic areas, farms or sampling dates. The microdiversity for selected taxa, as measured by the number and types of ASVs, ~~was~~ was also affected b several factors~~also significantly different for some species~~. Interestingly, *Corynebacterium*, which ~~has~~ had the highest number of ASVs but a low average abundance, had the lowest number of ASVs per farm. The composition of populations of *Pseudomonas* and *Lactococcus* were significantly affected by collection day,

area and housing/milking system possibly because of the potential variety of environmental sources and growth during refrigerated storage. *Bacillus* and *Streptococcus* populations changed between collection days from the same farm and between farms and geographical areas. Levels and composition of populations of *Bacillus* and *Paenibacillus*, two aerobic spore formers genera which are of particular concern due to their ability to survive pasteurization and grow in long shelf life pasteurized milk (Porcellato et al., 2018; Porcellato et al., 2019), ~~,~~were different between the 2 geographical areas. ~~Clear cut attribution of these variations to specific causes was very difficult: high prevalence and abundance of *Pseudomonas* and *Lactococcus* was attributed to the potential variety of environmental sources and to growth during refrigerated storage (both genera are also found as abundant members of bulk and silo tank milk in other studies), while~~F~~-~~fluctuations and diversity in the composition of *Streptococcus* populations were attributed to variability in the contamination of the udder of individual cows, with higher variability in larger farms. ~~Several factors might have influenced the variation in composition of *Bacillus* and *Paenibacillus* populations, two aerobic sporeforming genera which are of particular concern due to their ability to survive pasteurization and grow in long shelf life pasteurized milk (Porcellato et al., 2018; Porcellato et al., 2019).~~

The temperature and duration of storage are clearly two factors which may have a dramatic effect on the composition of the microbiota, with higher refrigeration temperatures ~~in the range 2-7~~ favouring the growth of psychrotrophic microorganisms. Doyle et al. (2017a) investigated the effect of temperature (2, 4 or 6°C) on the composition of microbiota of mid- and late-lactation milk stored for 5 days. There was very little increase in bacterial numbers during storage, as judged by qPCR, except for late-lactation samples stored at 6°C. No significant differences in composition of the microbiota was noted at the end of storage

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at 2°C. However, at both 4 and 6°C a significant increase was found in the proportion of *Streptococcus* and *Pseudomonas* for samples stored at 4°C and of *Acinetobacter* and *Pseudomonas* for samples stored at 6°C. The authors did not attempt to use the qPCR data in conjunction with the relative abundance data obtained by AT metagenomics and it is not clear if the strong decrease in abundance for some taxa (*Staphylococcus*, *Rhodanococcus* and uncultured *Ruminococcaceae*) is due to the compositional nature of abundance tables or if any other taxa increased in number during refrigerated storage.

In an another study (Zhang et al., 2019) prolonged storage at 7°C also resulted in high relative abundance of several psychrotrophic bacteria belonging to the genera *Pseudomonas, Acinetobacter, Carnobacterium, Chryseobacterium, Erwinia, Hafnia, Flavobacterium, Kluyvera* and *Lactococcus*, with some species (*Ps. fluorescens* and *Ps. psychrophila*) reaching almost 80% of the sequences. A similar trend for increase in the relative abundance of psychrotrophic bacteria was also found in the source tracking study of Falardeau et al., 2019, where bulk and transport tanks contamination may have significantly contributed, together with storage, to the increase of psychrotrophs, with *Pseudomonas*, *Psychrobacter* and *Acinetobacter* among the most abundant genera.

Variations in simple milk quality parameters may be ~~reflected in~~associated to changes in the microbiota composition. Rodrigues, Lima, Canniatti-Brazaca, & Bicalho (2017) analysed 472 low counts (<10^4 cfu/ml) bulk tank milk samples from dairy farms in New York state (USA) over 2 months and evaluate the correlation between SCC, and standard plate counts (SPC) with composition of the microbiota. As usual, a high diversity and variability was found but a core microbiome ~~comprising *Ruminococcaceae, Acinetobacter, Clostridiales, Bacteroidales, 5–7N15, Pseudomonas, Staphylococcus, Lachnospiraceae, Corynebacterium, Planococcaceae, Bacillus,* and *Thermoanaerobacterium*~~ was identified across the 19 farms.

Significant association was found between the relative abundance of several genera and low or high SCC and SPC counts. In fact, high SCC milk was significantly associated with increased abundance of *Corynebacterium*, *Streptococcus*, *Lactobacillus*, *Coxiella*, *Arthrobacter*, and *Lactococcus* (noticeably only some of this may be potentially associated with mastitis), while high SPC milk (>3.6 log cfu/ml) had increased abundances of *Acinetobacter, Enterobacteriaceae, Corynebacterium* and *Streptococcus* and usually lower diversity: this ~~might therefore only be a sign of~~suggests that shifts in community composition were due to bacterial growth.

Even with this very large variability, a core microbiota of bulk tank milk does apparently exist. Using data in FoodMicrobionet, we were able to combine the results, at the genus level or above, for five studies including 199 samples of bulk tank milk from different geographic regions (Ireland: Doyle et al., 2017a; France: Frétin et al., 2018; New Zealand: Li et al., 2018; Norway: Skeie et al., 2019; Canada: Falardeau et al., 2019). Data on prevalence and abundance of the top most prevalent and abundant taxa are shown in Supplementary F~~f~~igure 6 and S~~s~~upplementary T~~t~~able ~~3~~5, while the distribution of abundance for the 25 most prevalent and abundant genera is shown in Figure 5. The combined diversity in these five studies is impressive, with almost 2,000 taxa identified at the genus level or above. However, four phyla (*Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*) include the majority of the most abundant and prevalent taxa. The top 25 genera include ~~a collection of~~ psychrotroph~~ics~~ ~~microorganisms~~ (*Acinetobacter, Chryseobacterium, Pseudomonas, Psychrobacter*), ~~genera of fecal origin~~bacteria associated with gut (*Atopostipes, Bacteroides, Christensenellaceae, Clostridium, Rikenellaceae, Romboutsia, Ruminococcaceae*), or ~~associated~~ with teat skin (*Staphylococcus, Aerococcus, Turicibacter, Streptococcus, Facklamia, Corynebacterium, Bacillus*), including genera with potentially

beneficial microorganisms (*Lactobacillus, Streptococcus, Lactococcus, Staphylococcus, Corynebacterium*). The intra-study variability was sometimes substantial but, surprisingly enough, the composition of the microbiota for some studies, even from different countries, was similar, as assessed by non-metric multidimensional scaling (Supplementary Ffigure 7). Not surprisingly, the diversity was ~~lowest~~ lower for samples obtained from a single farm (ST37: Doyle et al., 2017a; ST39: Frétin et al., 2018; ST74: Falardeau et al., 2019), even when samples were obtained in different seasons (Doyle et al., 2017a; Frétin et al., 2018) or from cows belonging to different breeds (Frétin et al., 2018) or with different feeding regimes (Doyle et al., 2017a; Frétin et al., 2018).

There are very limited data on the composition of bulk tank milk at the dairy farm for species other than cows and the results are difficult to generalize. ~~In a small study on donkey milk, the microbiota of the raw milk from healthy jennies from five farms producing very low amounts of milk was studied~~In two small studies on jennies (de los Dolores Soto del Rio, Dalmasso, Civera, & Bottero, 2017) and on goats (McInnis et al., 2015) the usual high diversity and variability was found, but any inference on the potential sources of variation is impossible due to the limited scope of these studies. However, in both studies a potential effect of lysozyme, which was naturally abundant in jennies' milk or whose secretion was engineered in goats might have affected the composition of the microbiota. ~~As for many other raw milk samples, a high diversity and variability was found, and the "core" microbiota included 24 genera (*Acinetobacter, Carnobacterium, Chryseobacterium, Citrobacter, Cupriavidus, Flavobacterium, Janthinobacterium, Lactobacillus, Lactococcus, Leuconostoc, Pseudomonas, Ralstonia, Sphingobacterium, Stenotrophomonas, Streptococcus, Veillonella, Yersinia*) many of which are commonly found in raw cow milk. The high prevalence and abundance of *Proteobacteria* (especially psychrotrophs), compared~~

~~to *Firmicutes* and *Actinobacteria* suggests that growth at refrigerated temperature may have contributed, although the high concentration of lysozyme in donkey milk may also be a factor. In fact, the composition of the microbiota of composite samples for goats which had been engineered to produce a higher quantity of lysozyme was significantly different from that of wild types (McInnis et al., 2015) (and affected by the stage of lactation) but did not necessarily show a higher abundance of *Proteobacteria* in milk with high concentration of lysozyme.~~

3.1.4 From the farm to the processing plant

Transfer of bulk tank milk from the dairy farm to the processing plant and further storage and processing steps may alter significantly the composition of milk microbiota as a result of contamination and growth. A number of ~~large and~~recent well~~-~~structured studies have offered significant insight in this area for both milk processed to become liquid milk products or cheese (Falardeau et al., 2019; Kable et al., 2016, 2019; Porcellato et al., 2018, 2019).

All evidences confirm that contamination form transport trucks and from tanks and equipment at the dairy plant, cleaning routines, heat treatments, and duration and conditions of storage have a significant impact on the structure of microbial communities of milk, and thus potentially affect the quality of cheese and milk due to variations in the abundance of potential starter and non-starter species of the genera (*Streptococcus, Staphylococcus, Macrococcus, Corynebacterium*, etc.) and of spoilage bacteria (*Acinetobacter*, *Pseudomonas*, psychrotrophic spore-formers).

Kable et al. (2016) analysed the microbiota of milk from 899 tankers delivering raw cow milk to two processing farms in California (USA) over three seasons. They confirmed the

occurrence of a high variability in the composition of the microbiota, the occurrence of seasonal variations, and found a core microbiota ~~(including members of the phyla *Firmicutes, Actinobacteria, Bacteroidetes, Proteo- bacteria,* and *Tenericutes)*~~ dominated by members of the genera *Streptococcus*, ~~and~~ *Staphylococcus* and *Clostridiales*. Notably, only some of the taxa (*Staphylococcus, Bacillus, Enterococcus, Streptococcus, Clostridium, Ruminococcus, Corynebacterium, Acinetobacter*) they identified as members of the core cow milk microbiota match those found as being highly prevalent and abundant in ~~a combination of~~ studies on bulk tank milk (see Figure 5 and Supplementary Table ~~3~~5), but this might have been due to differences in criteria used to define the core microbiota. ~~However, Kable et al., 2016 used a very strict criterion for inclusion of taxa in the core microbiota (presence in 100% of samples), which led to the exclusion of important genera, including *Pseudomonas*, which had been found in all but two of the tankers. Due to the high variability of cow milk microbiota and to methodological issues (the number of sequences in low count milk may be low or may be affected by PCR bias) it would be probably more prudent to use a more relaxed criterion for inclusion of taxa in the core microbiota.~~ Significant differences were found between samples collected in the three seasons. ~~In fact, cell numbers estimated by qPCR were low (in the order of 10^3 cells/ml) and in this study the highest and lowest diversity found in Spring and Autumn, corresponded well to small but significant differences in cell numbers found between samples obtained in these two seasons.~~ Relative abundance of *Firmicutes* was significantly smaller in Spring samples, while those of *Actinobacteria* was higher, while the relative abundance of *Bacteroidetes* was higher in Fall. The authors hypothesized that differences in composition might be due to differential exposure to sources of contamination in different seasons (with possibly more contact with soil, a potential source of *Actinobacteria*, in Spring), but ~~clearcut~~clear-cut evidence for this is

lacking. While tanker milk samples had low counts (median ~5x10^3 cfu/ml, as measured by qPCR), ~~-~~growth between refilling cycles of the tankers cannot be excluded as a contributing factor to the observed differences (Kuhn, Meunier-Goddik, & Waite-Cusic, 2018). ~~One important finding of this study is the identification of a significant contribution of large silos used for storage to the microbiota of raw milk at the processing plant~~This is confirmed by a later work (Kable et al., 2019; see below) and by the observation that the silo used for milk storage may significantly affect the composition of the microbiota of milk transferred from tanker trucks, with *Pseudomonadales* and *Lactobacillales* being more abundant in the silos. Growth in residual milk in the silo may be a contributing factor (higher cell counts in silos compared to tankers) but stochastic patterns of contamination may contribute. ~~In fact, the authors monitored the microbiota of 5 silos and of the tankers used to fill them. The microbiota in the silos was significantly different from those of the tankers, with *Pseudomonadales* and *Lactobacillales* being more abundant in the silos. This was due partly to growth (higher cell counts in silos compared to tankers).~~ Two groups of silos were identified. In one the microbiota was similar to those of the tankers used to fill them, and had significantly higher proportions of *Streptococcus*, *Corynebacterium, Macrococcus* and *Clostridium*. In the other the microbiota of the silos was distant from those of the tankers (weighted UniFrac distance), and *Acinetobacter* was significantly more abundant. ~~The authors concluded that differences in composition of the microbiota due to seasonal variation and to contamination/growth might significantly impact the quality of dairy products because of the variability in the presence or abundance of potential non-starter species (*Streptococcus, Staphylococcus, Macrococcus, Corynebacterium*, etc.) and of spoilage bacteria (*Acinetobacter*, but also *Pseudomonas*).~~ In a follow-up ~~to this work~~study (Kable et al., 2019) the authors carried out an in depth investigation of the quantitative and

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qualitative variations of the microbiota in the processing plant. In addition, PMA treatment was used to enrich viable cells after lethal treatments (pasteurization). They confirmed that OTUs belonging to the genus *Streptococcus* (and tentatively identified as *S. thermophilus/salivarius*) were most abundant and prevalent in milk at all stages, and confirmed the occurrence of a seasonal effect, with some genera (including *Acinetobacter* and *Lactococcus*) more abundant in late Summer compared to Spring. Several taxa, whose overall abundance was relatively low, showed interesting time- dependent or spatial ~~dependent~~ patterns and were occasionally more abundant. An effect of growth on the composition of bacterial communities was ~~evident when the time a given piece of equipment was operated from last cleaning and before sampling was kept into account~~clearly related to the length of time a piece of equipment was operated after cleaning and to heat treatments~~:~~. ~~for example, in~~Psychrotrophic species clearly increased as storage duration increased: ~~silos and in the separator feed~~ the relative abundance of *Acinetobacter* and *Lactococcus* significantly increased over time in the raw milk silo while a single *Pseudomonas* OTU was enriched in summer after a post-pasteurization concentration step. On the other hand, heat treatments caused a decrease in non spore-formers and an increase of thermoduric species and spore-formers, especially when the active fraction of the microbiota was targeted using PMA treatments.~~.~~ *Anoxybacillus*, whose overall abundance was relatively low, ~~-~~seemed to be enriched after long operation time and both *Anoxybacillus* and *Thermus* were enriched after pasteurization~~. *Thermus* was also enriched after pasteurization~~. ~~A single *Pseudomonas* OTU was also enriched in summer after a post-pasteurization concentration step.~~ Composition of viable and total microbiota ~~(which were separated using a PMA treatment)~~ after pasteurization was dramatically different for some steps, as shown by weighted UniFrac distance. ~~For a few genera significant differences were~~

~~found between the abundance in the viable and total fraction: *Turicibacter*~~, was significantly enriched in the viable fraction, while the abundance of *Staphylococcus* was significantly lower. Other spore-formers or thermotolerant genera (including *Bacillus*, *Clostridium*, *Anoxybacillus* and *Thermus*) were also more abundant in the viable fraction after pasteurization (the difference was not statistically significant) while several other non-spore-formers (*Bacilli*, *Clostridia*, and *Actinobacteria*) showed a lower abundance in the viable fraction.

~~Although use of intercalating dyes to discriminate viable fractions of the microbial community requires careful optimization (Emerson et al., 2017),~~ This confirms ~~other authors (Erkus et al., 2016; Porcellato & Skeie, 2016) have confirmed~~ that ~~this technique is needed to~~ obtaining a realistic picture of the active fraction of the microbial community by eliminating the contribution from dead or membrane damaged cells is of utmost importance (Erkus et al., 2016; Porcellato & Skeie, 2016).

~~In the same study, the dynamics of the composition of the microbiota in milk which had been in contact with different pieces of equipment for less or more than 19 h after cleaning in place was examined.~~ Kable et al. (2019) also evaluated the impact of time of operation of individual pieces of equipment after cleaning. Within 19 h from ~~CIP~~ cleaning-in-place (CIP) the numbers of viable cells were low (<3,200 cells/ml) and the microbiota composition was diverse, although different species prevailed in different pieces of equipment, mostly depending on whether they contained raw or pasteurized milk: ~~. On the latters s~~pore-formers, including *Bacillus* and *Anoxybacillus*, were abundant in equipment containing pasteurized milk. After 19 h of operation since the last cleaning, the equipment was divided in two groups. In some samples there was little increase in bacterial numbers and the dominating taxa were close to those appearing in raw milk ~~(*Streptococcus, Staphylococcus,*~~

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~~*Acinetobacter, Lactococcus*~~). In the others, which mostly included milk feeds which had not been pasteurized, were dominated by *Acinetobacter* and *Lactococcus*, while in the concentration step silos the dominating genus was *Anoxybacillus*. Unfortunately, in this study relatively short sequences were used (V4 region of the 16S RNA gene) and proper source tracking at or below the species level is almost impossible.

~~Several other papers have demonstrated that the pasteurization treatment results in an enrichment of sporeformers (*Bacillus, Anoxybacillus, Turicibacter*) or thermotolerant bacteria (*Thermus*) in both the viable fraction and in the total microbiota. These genera have been frequently found in raw milk (see Figures 4 and 5 and Supplementary Tables 2 and 3) and some have been associated to spoilage (like *Anoxybacillus*, Kable et al., 2019; *Thermus*, Quigley et al., 2016; *Bacillus*, Sattin, Andreani, Carraro, Fasolato, et al., 2016a; Porcellato et al., 2018). *Turicibacter* is universally present in milk, from teat milk to pasteurized milk, but its significance and its potential to grow and spoil milk are not known. *Anoxybacillus* may show some potential to survive and grow in milk (Kable et al., 2019) and has been found in high numbers in Ricotta cheese (Sattin et al., 2016a) where it might contribute to spoilage.~~

Porcellato et al. (2018, 2019), using a similar approach, evaluated the dynamics of the microbiota in a Norwegian plant producing liquid pasteurized milk as a function of season of production, milk source, and time and temperature of storage of the pasteurized milk. As in many other papers, a very high diversity and significant differences in the composition of the microbiota between different seasons was found in silo milk, although no significant difference was found ~~in~~ for dairies located in two different areas of the country. ~~The reasons for this are not clear, but may be due to the variability of microbiota composition (Skeie et al., 2019) and perhaps to the long storage time pre-transport (up to three days), which may~~

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~~have contributed to the relatively high microbial counts in silo milk (median $1.8x10^4$ and sometimes $>10^5$ cfu/ml) compared to other studies (Kable et al., 2019).~~ Pasteurization reduced counts by up to 2 log cycles, and only after prolonged (13-14 d, end of shelf life) incubation of the pasteurized milk cartons at abuse temperature (8°C) counts exceeding 10^7 cfu/ml (with high presumptive *B. cereus* counts) were obtained, with significantly higher abundances of *Bacillus, Paenibacillus, Solibacillus, Anoxybacillus, Geobacillus* and *Jeotgalicococcus*. Although no treatment was used to separate viable from total bacteria, the composition of the microbiota immediately after pasteurization was significantly different from that of raw milk~~. Even with large seasonal variability in composition, a core microbiota was found in silo milk, with the most abundant and prevalent genera being *Pseudomonas, Bacillus, Acinetobacter, Streptococcus, Lactococcus, Chryseobacterium*. Significant differences were found at the order level between raw and pasteurized milk, with~~: 21 order level OTUs (15 of which *Clostridiales*) were more abundant in raw milk and 27 (including *Lactobacillales, Clostridiales* and *Pseudomonadales*) more abundant in pasteurized milk. ~~Incubation at 4°C did not significantly change the composition of the microbiota, while significant changes were found after 13-14 d at 8°C when *Bacillus, Paenibacillus, Solibacillus, Anoxybacillus, Geobacillus* and *Jeotgalicococcus* were significantly more abundant than in milk stored at 4°C and in raw milk. At~~After incubation at 4°C a seasonal variation of the abundance of *Bacillus* was found in pasteurized milk, and this correlated well with the seasonal variation of this genus in raw milk.

~~In this work the sequencing strategy, which was based on amplification of the V4 region of the 16S RNA gene, did not allow taxonomic resolution below the genus level.~~ In a follow up work, the same authors (Porcellato et al., 2019) used a more sensitive ~~amplicon targeted~~AT strategy, by using a in depth analysis of OTUs identified on the basis of 16S RNA ~~genus~~ gene

sequence and assigned to the genus *Bacillus* and by amplifying conserved regions of three genes (pantothenate synthase, *panC*; glycerol-3 phosphate transporter *glpT*; pyruvate carboxylase, *pyrC*) ~~using degenerate primers optimized for members of the *Bacillus cereus* group~~. A single OTU belonging to the *B. cereus* group made up 99.6% of the sequences of the genus *Bacillus*, while another occasionally dominated the microbiota of spoiled cartons at 8°C. Use of ~~Of the three other gene targets, *panC*~~ as gene target ~~was the one~~ result~~inged~~ in the highest diversity~~, although, due to low numbers of members of the *B. cereus* group in most samples, it mostly gave reliable amplification in samples stored at 8°C, while only some raw milk samples and pasteurized milk samples stored at 4°C produced amplicons~~. A seasonal variation of the composition of the population of the *B. cereus* group was found, and storage at 8°C resulted in a higher diversity compared to raw milk and milk stored at 4°C, perhaps simply because more strains had the opportunity to grow over the detection limit. Two *panC* sequence types (ST) were found at relatively high abundance in all samples. ~~A high variability in ST abundance was found among replicate milk cartons of the same batch but it is not clear if this is due to stochastic differences in the inoculum or to other factors.~~

Several other papers have demonstrated that the pasteurization treatment results in an enrichment of spore-formers (*Bacillus, Anoxybacillus, Turicibacter*) or thermotolerant bacteria (*Thermus*) in both the viable fraction and in the total microbiota. These genera have been frequently found in raw milk (see Figures 4 and 5 and Supplementary Tables 3 and 5) and some have been associated to spoilage (*Anoxybacillus*, Kable et al., 2019; *Thermus,* Quigley et al., 2016; *Bacillus*, Sattin ~~, Andreani, Carraro, Fasolato,~~ et al., 2016a; Porcellato et al., 2018). *Turicibacter* is universally present in milk, from teat milk to pasteurized milk, but its significance and its potential to grow and spoil milk are not known.

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Anoxybacillus may show some potential to survive and grow in milk (Kable et al., 2019) and has been found in high numbers in Ricotta cheese (Sattin et al., 2016a) where it might contribute to spoilage.

Another recent study (Falardeau et al., 2019) has confirmed that contamination with microorganisms from storage tanks and storage at low temperatures are the main drivers of changes in the composition of microbiota of bulk tank milk. In this study, the composition of the microbiota of milk at the farm bulk tank was dramatically different from that of the storage tank at the cheesemaking plant (with a duration of transportation of about 20 min), and further incubation in a chilled room resulted in the microbiota being dominated by *Pseudomonas*. On the other hand, the composition of the microbiota of the bulk tank and that of the transport tank were very similar and significantly different from that of the pooled tank milk pre- and post-transport.

When data from these three studies (Falardeau et al., 2019; Kable et al., 2019; Porcellato et al., 2018) are compared, a more general picture emerges. The 50 most prevalent and abundant taxa are listed in Supplementary Table 46. Several of these taxa match those found in bulk tank milk, and, although many taxa whose likely origin is the GI tract are still present at low abundance, the most abundant and prevalent taxa include *Streptococcus, Lactococcus, Pseudomonas, Acinetobacter, Lactobacillus, Staphylococcus, Psychrobacter* and *Escherichia/Shigella*. However, the prevalence and maximum relative abundance of many genera including psychrotrophs is increased compared to bulk tank milk at the farm, confirming that storage at low temperature is the main driver of the change in composition of the microbiota. Several thermoduric taxa, including spore-formers (*Turicibacter, Anoxybacillus, Bacillus, Clostridium*) are also prevalent and sometimes abundant. The distribution of abundance of the top 25 genera in raw and HTST (high temperature short

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time) treated milk at the processing plant is compared in Figure 6. While there is no guarantee that the nucleic acid target was from viable cells (only data ~~without~~ for samples not treated with PMA from Kable et al., 2019 are shown), some major differences are evident between studies (some taxa which were relatively abundant in ST74 and ST87 are almost absent in ST38) and, within study, between HTST and raw milk. In the latter, the relative proportion of psychrotrophs tends to be higher, while that of thermoduric species, including both non-spore-formers and spore-formers is higher.

Several papers describing the evolution of the composition of cheese microbiota report the composition of raw milk prior to starter addition. These include studies on cow milk cheeses (Alessandria et al., 2016; Bokulich & Mills, 2013; Calasso et al., 2016; Carafa et al., 2019; De Filippis et al., 2016; De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti, 2014b; Dolci, De Filippis, La Storia, Ercolini, & Cocolin, 2014; Falardeau et al., 2019; Frétin et al., 2018; Giello et al., 2017; Masoud et al., 2011), water-buffalo cheeses (Ercolini, De Filippis, La Storia, & Iacono, 2012), ewe's milk cheeses (De Pasquale et al., 2014a), fermented yak milk products (Joang et al., 2019~~)~~) and camel milk (Amrouche, Mounier, Pawtowski, Thomas, & Picot, 2020~~Amrouche et al., 2019~~; Zhao et al., 2019). Although ~~they~~ these studies invariably show a high diversity in milk microbiota, the description of the storage conditions and duration, and of the source of the milk is generally insufficient to allow any comparison with studies focusing on milk.

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4. Conclusions

The microbiota of milk is probably one of the most complex food microbial communities, because of the multiplicity of sources ~~which contribute to~~of contamination (Addis et al., 2016; Derakshani et al., 2018a). The availability of ~~high throughput sequencing~~HTS methods,

with the ability to study in detail both the taxonomic structure and the functionality of microbial communities has revolutionized out ability to study the structure and function of food microbiomes and has undoubtedly greatly contributed to our understanding of the factors affecting contamination patterns and successions in milk and dairy foods. The availability of raw and processed sequence data (see Supplementary material and data) greatly enhances our ability of combining and analysing results from different studies, thus, facilitating metastudies. At almost 9 years from the publication of the first paper on the microbiota of dairy products, ~~(Masoud et al., 2011)~~ sequencing platforms, ~~and~~ methods and bioinformatic approaches have evolved greatly and, although most recent papers use similar pipelines, there is still a need for a consensus of dairy microbiologists on ~~Standard Operating Procedures~~SOPs, which would improve confidence in the results and comparability of studies. ~~Three points therefore require special attention: aseptic sampling of milk from the udder or the cistern, measures to deal with contamination in low DNA samples, and design of experiments and analysis of data. In fact, a further challenge is the variability in composition of the microbiota which is observed within the same animal, between animals belonging to the same farm/experimental group, and between farms in the same geographical region or during the same season, which might obscure true differences due to technologically relevant factors (farming practices, feed, cleaning methods, etc.).~~ Careful documentation of all potential factors affecting the composition of milk microbiota is of uttermost importance for the interpretation of the results.

Even with ~~this~~ these caveats, ~~high throughput sequencing (HTS) approaches to the study of milk microbiota have greatly contributed to the~~our understanding of the relationships between udder health and milk quality has significantly improved. Mastitis and dysbiosis of microbial communities of the udder are strongly related, although the cause-effect

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relationship is not completely clear. In addition, HTS approaches allow to shed light on the potential causative agents in culture negative and sub-clinical cases and to clarify the polymicrobial nature of some mastitis cases.

Practically all conceivable factors related to farming and storage of raw milk have been shown to affect the composition of milk microbiota, and several sources (faeces, pasture, feed, milking equipment, storage tanks, etc.)~~,~~ might contribute microorganisms relevant for the quality of dairy milk and fermented dairy products. However, the low taxonomic resolution of some studies (which track genera, or at best, species, not strains) still obscures the potential contribution of each source and more effort should be probably devoted to disentangle the relative contribution of contamination, growth at low temperature and ability to survive pasteurization treatments in determining the potential for microorganisms from different sources to affect the quality of dairy products. ~~However, the r~~Recent findings on the origin and survival of spore-formers and other thermoduric microorganisms in the dairy plant (Kable et al., 2019; Porcellato et al., 2018, 2019) have indeed ~~provides~~ provided insights which might contribute to improving practices in cleaning, sanitation and heat treatment of milk. However, still much remains to be done on the potential contribution of milk in terms of starter and non-starter microorganisms relevant to cheese production: although HTS methods are indeed much more sensitive than other cultivation dependent and independent approaches, the low levels of contamination of hygienically produced milk complicate the tracking of species and strains. The decreasing costs of shotgun approaches and the availability of powerful bioinformatic pipelines might in the near future open new avenues to the study of sources of milk contamination.

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Legends to figures.

Figure 1. ~~Relative~~ Stacked bar plot showing the relative abundance of bacterial classes on cow's teats in two studies (Falardeau et al., 2019; Frétin et al., 2018~~;~~). Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019).

Figure 2. Stacked bar plot showing the relative abundance of the 20 most abundant taxa (at the genus level or above are shown) ~~Abundance plot for~~in individual samples from teat milk from Cremonesi et al. (2018; colostrum samples were removed) and Falardeau et al. (2019). ~~Only the 20 most abundant taxa (at the genus level or above are shown), while the other taxa have been pooled.~~ Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019). For the Cremonesi et al. (2018) samples, HF and REN in sample names indicate samples from Holstein Fresians and Rendena cows, respectively.

Figure 3. Stacked bar plot showing the relative abundance of the 24 most abundant taxa (at the genus level or above are shown) ~~Abundance plot~~ for ~~samples from~~ teat milk from cows (ST47, Cremonesi et al., 2018, colostrum samples were removed; ST74, Falardeau et al., 2019), ewes (Castro et al., 2019) and water-buffaloes (Catozzi et al., 2017; only samples from healthy quarters are shown). ~~Only the 24 most abundant taxa (at the genus level or above are shown), while the other taxa have been pooled; a~~All ~~s~~amples from each study have been pooled. Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019).

Figure 4. ~~The~~ Boxplots for the distribution of relative abundance for the 25 most abundant and prevalent genera in pasture and feed, farm environments, teat skin, teat and bulk tank milk in Falardeau et al. (2019). Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019).

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Figure 5. Boxplots for the ~~The~~ distribution of relative abundance for the 25 most abundant and prevalent genera in cow bulk tank milk from five studies (ST37 Doyle et al., 2017a; ST39 Frétin et al., 2018; ST81 Li et al., 2018; ST46 Skeie et al., 2019; ST74 Falardeau et al., 2019). Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019).

Figure 6. Boxplots for the ~~The~~ distribution of relative abundance for the 25 most abundant and prevalent genera in raw and HTST treated milk at the processing plant for three studies (ST38 Porcellato et al., 2018; ST74 Falardeau et al., 2019; ST87 Kable et al., 2019). Sequences were downloaded from NCBI/SRA and processed as described in Parente et al. (2019).

Highlights

- the microbiota of milk from dairy species is highly complex and variable
- teat and faecal microbiota contribute significantly to this diversity
- mastitis dramatically alters the milk microbiota, with lasting consequences
- multiple sources at the dairy farm affect the composition
- contamination from equipment and growth further shape the microbiota

Credit Author Statement

Eugenio Parente: Data curation; Software; Supervision; Writing – original draft
Annamaria Ricciardi: Data curation; Writing – review & editing
Teresa Zotta: Data curation; Writing – review & editing