REVIEW ARTICLE

The complex microbiota of raw milk

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Abstract

Here, we review what is known about the microorganisms present in raw milk, including milk from cows, sheep, goats and humans. Milk, due to its high nutritional content, can support a rich microbiota. These microorganisms enter milk from a variety of sources and, once in milk, can play a number of roles, such as facilitating dairy fermentations (e.g. Lactococcus, Lactobacillus, Streptococcus, Propionibacterium and fungal populations), causing spoilage (e.g. Pseudomonas, Clostridium, Bacillus and other spore-forming or thermotolerant microorganisms), promoting health (e.g. lactobacilli and bifidobacteria) or causing disease (e.g. Listeria, Salmonella, Escherichia coli, Campylobacter and mycotoxin-producing fungi). There is also concern that the presence of antibiotic residues in milk leads to the development of resistance, particularly among pathogenic bacteria. Here, we comprehensively review these topics, while comparing the approaches, both culture-dependent and culture-independent, which can be taken to investigate the microbial composition of milk.

Introduction

Milk is a highly nutritious food that can be obtained from a variety of animal sources such as cows, goats, sheep and buffalo, as well as humans, for human consumption. However, the high nutrient content of these milks, which includes proteins, fats, carbohydrates, vitamins, minerals and essential amino acids (Supporting information, Table S1), all at a near neutral pH and at a high water activity, provides an ideal environment for the growth of many microorganisms. Some of these nutrients are directly available to all microorganisms, while others are provided following the metabolism of major components by specific populations to release components and metabolites that are used by others (Frank, 1997). It is generally accepted that the lactic acid bacteria (LAB), a group of bacteria that ferment lactose to lactate, are a dominant population in bovine, goat, sheep and buffalo milk, prior to pasteurisation. The most common LAB genera in milk include Lactococcus, Lactobacillus, Leuconostoc, Streptococcus and Enterococcus. Psychrotrophic populations, which particularly establish themselves during cold storage, are also a major component and frequently include Pseudomonas and Acinetobacter spp. Other strains of non-LAB genera are also encountered in milk, as well as various yeasts and moulds (Quigley et al., 2011). Human milk on the other hand is typically dominated by Streptococcus, Staphylococcus, Lactobacillus and Bifidobacterium spp. (Martín et al., 2007).

The specific composition of the milk microbiota directly impacts on the subsequent development of dairy products (Fig. 1). Microorganisms can bring about the fermentation of milk through the production of lactate and have a variety of different impacts on the sensory, texture, flavour and organoleptic properties of resultant products (Wouters et al., 2002). Microorganisms can also negatively impact on milk quality and shelf life; for example, psychrotolerant bacteria can proliferate during refrigeration and, through the production of extracellular lipases and proteases, result in spoilage (Desmasures & Gueguen, 1997; Hantsis-Zacharov & Halpern, 2007). The microbial composition of milk can also have health-related implications in that the consumption of raw milk contaminated with pathogens can lead to, in some cases, severe illness (Oliver et al., 2009). In contrast, it is claimed that other raw milk microorganisms can
contribute to health by aiding digestion or by reducing the frequency of allergies, including asthma and atopic diseases, in individuals who consume raw milk during the early years of life (Debarry et al., 2007; Braun-Fahrländer & Von Mutius, 2011). This review will highlight the various microbial populations found in raw milk and the methods employed for their detection. It also addresses their sources, their subsequent significance with respect to industrial applications and the contribution of specific populations to food quality and health.

Methods employed to determine the microbial composition of milk

Many microbial communities are complex; that is, they are comprised of many different taxonomical groups of microorganisms. Raw milk is an example of an environment that contains a diverse and complex microbial population (Quigley et al., 2011; Vacheyrou et al., 2011). Most of our knowledge with respect to the identity of the microorganisms that are present in raw milk, and resultant dairy products, has been gained through the growth or ‘culturing’, and subsequent analysis, of these microorganisms. The ultimate identification of these cultured microorganisms involves phenotypic and/or genotypic methods. Phenotypic methods are those which have been traditionally employed and involve the growth of microorganisms in microbiological media (either general or selective) supplemented with morphological, biochemical or physiological characterisation (Quigley et al., 2011). These testing methods are still the standard in industrial settings and typically involve tests to determine total bacteria counts, reflecting general milk quality, or to detect specific pathogens or other microorganisms, which indicate whether contamination has occurred. Populations frequently tested for include thermoduric populations (resisting pasteurisation), sulphate-reducing clostridia, Listeria monocytogenes, Salmonella, coagulase-positive staphylococci, Escherichia coli, Enterobacteriaceae, coliforms and Bacillus cereus among others. These standard methods are legislated and accredited by National and International Accreditation Boards (e.g. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0001:0026:EN:PDF; http://www.inab.ie/). These tests generally rely heavily on the use of microbiological broths or agars that selectively support the growth of the target microbial population and often include further confirmatory biochemical analysis. These approaches are usually low-tech and inexpensive but are relatively labour intensive and time-consuming, and in some cases, insufficient discriminatory power can be a problem. More recently, considerable efforts have been made to develop more rapid, high-throughput tests

Sources

Microorganisms enter milk from contact with the animal including teat, udder, faeces; also from the housing, bedding, feed, air and water. Contact with farm equipment and milking equipment as well as insufficient farm or personnel hygiene may influence the microbial content of milk.

Role/Significance

Once in the milk these microorganisms can play an important role in dairy product manufacture; they may contribute to promoting human health or enhancing food safety. On the other hand these microorganisms can lead to spoilage of milk and dairy products or they may contribute to disease and illness in humans.
that rely on DNA-based, genotypic analysis. Such technologies, which usually rely, at least to some extent, on the application of polymerase chain reaction (PCR) technology, can be used to confirm the results generated through traditional tests, but their ability to serve as an alternative to culture-based analysis is increasingly being appreciated. One of the key benefits of replacing the culturing step relates to the fact that many microorganisms are averse to isolation using common culturing methods, thus potentially leading to a significant underestimation of the microbial communities. A number of factors must be considered when applying these culture-independent methods. The selection of a protocol that efficiently extracts nucleic acids from as many of the microorganisms present as possible is critical. One must also consider the use of strategies, such as the use of DNA-binding agents or an alternative focus on RNA, to limit the risk of false positives resulting from the amplification of DNA from dead cells (Quigley et al., 2011). Finally, a decision has to be made regarding the genes to be targeted. The oligonucleotides or probes can be selected to detect target-specific genes or to provide an overview of the microbiota within a particular niche through the non-target-specific amplification of highly conserved genes, such as the 16S or 23S rRNA genes. In the latter case, amplified products can then be analysed by techniques such as denaturing gradient gel electrophoresis (DGGE), temporal gradient gel electrophoresis (TGE) or single-stranded conformation polymorphism (SSCP; Quigley et al., 2011) to highlight similarities or differences in the populations. These approaches may be used in conjunction with Sanger (first generation) DNA sequencing, to help specifically identify the populations present. More recently, there has been a rapid evolution in next-generation DNA sequencing technologies that produce millions of sequence reads in a single run, thus allowing a much more in-depth and accurate estimation of microbial diversity. The ever-increasing number and length of the sequence reads provided by these technologies coupled with the availability of databases and bioinformatic tools have been hugely beneficial with respect to the taxonomic assignment of the microorganisms present (Loman et al., 2012). Although, to date, high-throughput sequencing approaches have not been extensively applied to assess the microbiota of dairy-based environments, there have been a number of recent publications, which suggest that this situation will change dramatically in the coming years (Masoud et al., 2011, 2012; Alegria et al., 2012; Quigley et al., 2012).

**Sources of milk microorganisms**

Milk in healthy udder cells is thought to be sterile (Tolle, 1980) but thereafter becomes colonised by microorganisms from a variety of sources, including the teat apex, milking equipment, air, water, feed, grass, soil and other environments (Fig. 1; Coorevits et al., 2008; Lejeune & Rajala-Schultz, 2009; Vacheyrou et al., 2011).

The bovine teat surface can contain a high diversity of bacteria (Braem et al., 2012; Monsallier et al., 2012; Verdier-Metz et al., 2012). In one particularly detailed study, culture-dependent methods revealed that the bacteria present could be classified at the phylum level as *Firmicutes* (76%), *Actinobacteria* (4.9%), *Proteobacteria* (17.8%) and *Bacteroides* (1.3%). When this approach was supplemented by a clone library sequencing-based approach, some additional phyla, that is, *Planctomycetes, Verrucomicrobia, Cyanobacteria, Chloroflexi* and unclassified Bacteria, were detected at low levels (Verdier-Metz et al., 2012). Notably, a large percentage of the reads from this and other studies (Fricker et al., 2011) corresponded to as yet unidentified bacteria. Of those which could be identified, many corresponded to technologically important bacteria such as *Lactobacillus, Leuconostoc* and *Enterococcus* spp. Bacteria that can be involved in flavour, aroma and colour development in cheese such as coagulase-negative staphylococci as well as *Arthrobacter, Brevibacterium* and *Corynebacterium* spp. were also detected. However, some of the microorganisms detected on the teat surface, for example, *Solobacterium, Clavibacter* and *Arcanobacterium* spp., have not been identified in milk (Verdier-Metz et al., 2012), presumably reflecting a lack of competitiveness in milk environments should transfer occur. It was also noted that the composition of the microbial community on the teat surface varied qualitatively and quantitatively from one farm to another (Verdier-Metz et al., 2012). This can be attributed to many different factors; for example, microorganisms associated with bedding material can contaminate the surface of teat and thus potentially enter milk (Vacheyrou et al., 2011). Similarly, milking machines can contain a reservoir of microorganisms, and thus, unsurprisingly, differences between machines and related practices can influence the microbial population of the milk collected (Michel et al., 2006). With respect to more general environmental factors, it has been observed that the microorganisms present in cows’ milk depend on whether animals are fed indoors or outdoors, with an increase in *Staphylococcus* spp. during outdoor feeding (Hagi et al., 2010), on the location of the animals (Bonizzi et al., 2009) and on the lactation stage (Callon et al., 2007). An intense study was carried out to relate the microorganisms detected in milk to where they can be found on the farm (Vacheyrou et al., 2011). These results highlighted 141 bacterial species, representing 54 genera, from throughout the farm. There were 25 genera detected in these milk samples, and many of these, including *Aerococcus, Streptococcus, Propionibacterium, Acinetobacter, Bacillus,*
Ochrobactrum, Pseudomonas, Psychrobacter, Staphylococcus, Sphingomonas, Enterobacter, Pantoea, Brachybacterium, Corynebacterium, Kocuria, Microbacterium and Pseudoclavibacter, were also detected in different areas throughout the farm including teat surfaces, milking parlours, hay, air and dust. Also present in milk, but not detected in the farm environment, were technologically relevant bacteria such as Lactococcus, Lactobacillus and Enterococcus as well as Leuconostoc, Demococcus and Paracoccus. Similarly, a large number of other taxa were detected in the farm environment, but not in milk (Vacheyrou et al., 2011). Finally, it is notable that the implementation of strict hygiene standards brings about a reduction in the microbial load of milk, including a reduction in populations of technological importance, which can, in turn, impact negatively on cheese manufactured using traditional or artisanal approaches (Monsallier et al., 2012). Indeed, Mallet et al. (2012) recently reported a one-magnitude reduction in the levels of technologically relevant lactococci present in raw milk relative to what had been detected 15 years before in raw milk collected from the same area (Desmasures & Gueguen, 1997). These populations seem to be particularly sensitive to the evolution of farm practices, as other populations, such as Pseudomonas, Lactobacillus and yeast populations, did not differ across the two studies. While it is important to ensure that the quality of milk is maintained at high levels, producers of traditionally manufactured raw milk should be aware that certain farming practices may negatively impact on distinctive flavours and aromas as a consequence of limiting the numbers of specific microorganisms and may need to compensate through the introduction of starters and adjunct strains.

The microbial composition of different milk types

Although the largest proportion of commercially produced milk worldwide comes from cows, there are a number of other animal sources of milk that is used for human consumption. These include quite common sources such as goats, sheep, buffalo and others utilised in more specific regions such as camel milk in African and Arab countries and yak milk in Asian countries. This section will review recent findings on the microbial content of these various milks. We will also discuss an issue that has been receiving ever more attention in recent years; that is, the microbial composition of the human milk that is consumed by infants only (Box 1).

Cows’ milk

Cows’ milk is produced on a massive scale. In 2012, the EU produced c. 139 million tonnes of cows’ milk followed by the United States with 90 million tonnes (http://www.dairyco.org.uk/market-information/supply-production/milk-production/world-milk-production/). This milk is employed in many ways, including direct consumption and the manufacture of dairy products and milk powders. Raw cows’ milk has the potential to contain a diverse bacterial population as highlighted previously (Quigley et al., 2011). Typically, cows’ milk contains a significant LAB population that includes Lactococcus (8.2 × 10¹–1.4 × 10⁵ CFU mL⁻¹), Streplococcus (1.41 × 10¹–1.5 × 10⁵ CFU mL⁻¹), Lactobacillus (1.0 × 10⁵–3.2 × 10⁶ CFU mL⁻¹), Leuconostoc (9.8 × 10²–2.5 × 10³ CFU mL⁻¹) and Enterococcus spp. (2.57 × 10¹–1.58 × 10³ CFU mL⁻¹; Fig. 2). A number of other microorganisms can be present in significant proportions. These include psychrotrophs, such as Pseudomonas, Acinetobacter and Aeromonas spp., which flourish during cold storage (Raats et al., 2011). However, while the bacterial composition of cows’ milk has been extensively studied for quite some time, new developments with respect to DNA sequencing technologies have highlighted that the diversity of these bacteria is greater than that originally appreciated (Table 1). Indeed, a recent study applied high-throughput DNA sequencing to examine the bacterial population of raw cows’ milk that was to be used for cheese production (Masoud et al., 2012); 256 bacterial species were detected, of which Streptococcus thermophilus and Lactococcus lactis dominated in the milk, representing 43.7% and 19% of reads, respectively. A number of other microorganisms that had previously been associated with raw milk, including Acinetobacter, Aeromonas, Brevibacterium, Corynebacterium, Lactobacillus, Pseudoalteromonas, Pseudomonas and Staphylococcus, which represented between 1.3% and 3.7% of the total reads, were also detected. A large subpopulation of taxa, which each corresponded to <1% of the total reads, was also highlighted (Masoud et al., 2012). We have also recently compared the bacterial population present in cows’ milk pre- and postpasteurisation using high-throughput sequencing to reveal the presence of a previously unrecognised and diverse bacterial population in unpasteurised cows’ milk. While the milk, sourced from a variety of commercial producers throughout Ireland, was dominated by Lactococcus, Pseudomonas and Leuconostoc, we also detected a number of anaerobic taxa, including Bacteroides, Faecalibacterium, Prevotella and Catenibacterium, which are more typically associated with the gut microbiota and may be entering the milk through faecal contamination. Our analyses indicate that the bacterial population of pasteurised milk is more diverse than previously appreciated, but that the nonthermoduric bacteria that are present within these populations are likely to be in a damaged, nonculturable form (Quigley et al., 2013). Thus, high-throughput sequencing approaches can provide...
a detailed insight into the bacterial composition of milk, and it is likely that these technologies will be used increasingly in future to investigate the factors that influence the composition of cows’ milk.

**Goats’ milk**

Goats’ milk production represents about 2.1% of global milk production (Tsakalidou & Odos, 2012). It is an important commodity that has gained increased interest as an alternative to cows’ milk, due to evidence that it is less likely to induce allergies (Park, 1994). Goats’ milk also differs from cows’ and sheep’s milk by virtue of having greater levels of iron bioavailability (Boyazoglu & Morand-Fehr, 2001) as well as containing smaller fat globules, having a higher content of fatty acids and forming a softer curd during subsequent fermentations, in turn leading to greater digestibility (Klinger & Rosenthal, 1997). Goats’ milk is most frequently used for cheese making, usually at farm level or in small dairies. Goats’ milk cheeses are particularly common in Mediterranean countries and south-east Europe (Pirisi et al., 2007). Goats’ milk is also typically dominated by LAB, including species of *Lactococcus* (3.7 × 10⁶ CFU mL⁻¹), *Lactobacillus* (1.34 × 10⁸ CFU mL⁻¹), *Leuconostoc* (3.27 × 10⁵ CFU mL⁻¹) and *Enterococcus* (2.95 × 10² CFU mL⁻¹), as well as *Enterobacteriaceae*, *Micrococccaceae*, moulds (filamentous fungi) and yeasts (Alonso-Calleja et al., 2002; Tamagnini et al., 2006; Nikolic et al., 2008). Callon et al. (2007) relied on the use of selective microbiological media, SSCP analysis as well as restriction fragment length polymorphism (RFLP) typing of isolates to examine the microbial diversity of 118 goats’ milk samples taken from one herd throughout one lactation year to reveal the presence of a diverse bacterial population in the milk (Table 2). In addition to microorganisms commonly encountered in milk, such as those listed above, some species were identified that are not typically associated with goats’ milk or that had previously only been associated with cheeses, including a number of corynebacteria and brachybacteria. Another unexpected finding was the presence of several halophilic species not previously associated with milk, including *Jeotgalicoccus psychrophilus*, *Salinicoccus* sp., *Dietzia maris*, *Exiguobacterium*, *Ornithinicoccus* sp. and *Halella chejuensis*. The significance of the presence of these microorganisms with respect to health, safety or product development is not known. Through this approach, it was also revealed that milks collected during winter were dominated by the presence of *Lactococcus* and *Pseudomonas*, those from summer by *Pantoea agglomerans* and *Klebsiella* and those from autumn by *Chryseobacterium indologenes*, *Acinetobacter baumannii*, *Staphylococcus*, *Corynebacteria* and yeasts. While these variations can be attributed to differences in feed, the authors suggested that other factors, such as weather conditions and the health of the animal, were also important (Callon et al., 2007). There has not been an in-depth assessment of the microbiology of goats’ milk since this study, perhaps next-generation sequencing technologies could have the potential to be very revealing.
Table 1. Bacterial populations detected in raw cows’ milk using culture-dependent, culture-independent and next-generation DNA sequencing methods

<table>
<thead>
<tr>
<th>Culture-dependent*</th>
<th>Culture-independent†</th>
<th>Next-generation sequencing‡</th>
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</thead>
<tbody>
<tr>
<td>Acinetobacter species/johnsonii/juni/haemolyticus/lwoffii</td>
<td>Acinetobacter species/johnsonii/baumannii/juni</td>
<td>Acinetobacter species</td>
</tr>
<tr>
<td>Aerococcus species/viridans</td>
<td>Aerococcus</td>
<td>Aerococcus species</td>
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<tr>
<td>Bacillus species/cereus</td>
<td>Bacillus thuringiensis</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>Brevibacterium helvolum/linens</td>
<td>Brevibacterium species/samyangensis</td>
<td>Brevibacterium linens</td>
</tr>
<tr>
<td>Chryseobacterium species</td>
<td>Chryseobacterium species/joostei/bifermentans/freundii</td>
<td>Chryseobacterium piciul</td>
</tr>
<tr>
<td>Corynebacterium ammoniagenes/freneyi glutamicum/variabili/casei</td>
<td>Corynebacterium freneyi/casei/variabili/marginleyi</td>
<td>Corynebacterium casei</td>
</tr>
<tr>
<td>Enterococcus species/faecalis/gallinarum/saccharominimus</td>
<td>Enterococcus aquamarinus</td>
<td>Enterococcus faecalis</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Escherichia coli</td>
<td>Lactobacillus casei/helveticus/plantarum/sakei/hamnosus</td>
</tr>
<tr>
<td>Lactobacillus casei/curvatus/mindensis/animals/coryneformis/gluvatum/paracasei/paraplantarum/plantarum/rhamnosus/amylolysus</td>
<td>Lactobacillus lactis/garvieae</td>
<td>Lactococcus lactis</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides</td>
<td>Leuconostoc carnosum</td>
<td>Leuconostoc</td>
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<td>Staphylococcus species/capitis/cohnii/saprophyticus/equorum/glycolae/saccharolyticus/haemolyticus/hominis/epidermis</td>
<td>Staphylococcus aureus/epidermidis/fleureti/sciuri</td>
<td>Staphylococcus saprophyticus/succinus</td>
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<td>Streptococcus species/uberti/parauberis</td>
<td>Streptococcus species/uberti/dysgalactiae/parauberis/thermophilus</td>
<td>Streptococcus thermophilus</td>
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<td>Pseudomonas species/alcalophilus/stutzeri/synxanthath/fuminium/putida</td>
<td>Pseudomonas species/fragilis/psychrophila/frenneris/iynaxanthal/putfida/pectucinogena</td>
<td>Pseudomonas species/gessardii</td>
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<td>Microbacterium liquefaciens/oxydans/lacticin</td>
<td>Microbacterium species/xinjiangensis</td>
<td>Microbacterium species/xinjiangensis</td>
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<td>Rhodococcus erythropolis</td>
<td>Rothia</td>
<td>Rothia mucilaginosa</td>
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<td>Serratia liquefaciens/odorifer</td>
<td>Stenotrophomonas species/koreensis</td>
<td>Stenotrophomonas maltophilia</td>
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<td>Enterobacter species/gergoviae</td>
<td>Empedobacter brevis</td>
<td>Enterobacter cloacae</td>
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<tr>
<td>Klebsiella ozarum/oxotoca</td>
<td>Klebsiella oxytoxa</td>
<td>Kurthia gibsoni</td>
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<td>Kocuria species/caronfli/kristinae/rhizohila</td>
<td>Kocuria species/pneumoniae</td>
<td>Leptotrichia hofstadi</td>
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<tr>
<td>Frigoribacterium species</td>
<td>Fabclamia</td>
<td>Facklami tabacinasi</td>
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<td>Paracoccus species</td>
<td>Nocardioidea dubius</td>
<td>Paracoccus carotini</td>
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<td>Micrococcus species</td>
<td>Oxithinococcus species</td>
<td>Marinomonas</td>
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<tr>
<td>Ochrobactrum anthropitritica</td>
<td>Pandoranae species/noninbergensis</td>
<td>Meiothermus species</td>
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<td>Pantoea species/appinogranum</td>
<td>Phyllobacterium myrsinacearum</td>
<td>Methylobacterium extorquens</td>
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<td>Propionibacterium freudenreichii/jensenii</td>
<td>Propionibacterium</td>
<td>Pediococcus pentosaceus</td>
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<td>Providencia stuartii</td>
<td>Proteobacteria</td>
<td>Prevotella</td>
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<td>Psychrobacter species/maritimus</td>
<td>Rastronia species/beckii</td>
<td>Psychrobacter</td>
</tr>
<tr>
<td>Pseudoclavibacter species/helvolus</td>
<td>Sphingomonas melonis</td>
<td>Pseudoalteromonas agarivorans</td>
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<td>Rahellia aquatilis</td>
<td>Thauerella species</td>
<td>Ruminococcus flavifaciens</td>
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<td>Reinibacterium salmoninarum</td>
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<td>Weissella hellenica</td>
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<td>Sphingomonas species</td>
<td>Yana halotolerans</td>
<td>Actinomyces radicidentis</td>
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<td>Achromobacter deliicatus</td>
<td>Acidobacteria</td>
<td>Alisipi finegoldii</td>
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<td>Aeromonas hydrophlia</td>
<td>Adhaeribacter aquatic</td>
<td>Aeromonas species/hydrophila/papoffi</td>
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<tr>
<td>Arthrobacter species/arilaitensis/psychrophilactophilus</td>
<td>Arthrobacter species/arilaitensis/psychrophilactophilus</td>
<td>Anaerococcus octavus</td>
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<tr>
<td>Brachybacterium species/nesterenkovii</td>
<td>Bacteroidetes</td>
<td>Bacteroides</td>
</tr>
<tr>
<td>Deinococcus species</td>
<td>Bosea thiooxidans</td>
<td>Bifidobacterium species/pseudolongum</td>
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Table 1. Continued

<table>
<thead>
<tr>
<th>Culture-dependent*</th>
<th>Culture-independent†</th>
<th>Next-generation sequencing‡</th>
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<tr>
<td>Dermacoccus species</td>
<td>Bradyrhizobium species</td>
<td>Carnobacterium species</td>
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<tr>
<td>Hafnia alvei</td>
<td>Caryophanon latum</td>
<td>Empedobacter brevis</td>
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<tr>
<td>Clavibacter michiganensis</td>
<td>Delftia</td>
<td>Catenibacterium</td>
</tr>
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<td>Comamonas testosteroni</td>
<td>Clostridium perfringens/lituseburens</td>
<td>Caulobacter crescentus</td>
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<tr>
<td>Enhydrobacter aerosaccus</td>
<td>Janibacter anophelis</td>
<td>Faecalibacterium</td>
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<td>Halomonas species</td>
<td>Janthinobacterium lividum</td>
<td>Jetiagallicoccus psychrophilus</td>
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<tr>
<td>Leucothrix species</td>
<td>Paenibacillus aparius</td>
<td>Unassigned</td>
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</table>

The bacterial names emphasised in red highlight the most prevalent bacterial populations detected.
The bacterial names emphasised in blue highlight the less prevalent, but frequently isolated bacterial populations detected.
The bacterial names in black highlight the occasional bacteria that are detected.
Where there are bacterial names in bold, these are detected by two of the three methods.
Where the bacterial name is underlined, these were detected by only one of the methods.
All other bacteria were detectable by all of the methods.

References used for data in this table are Verdier-Metz et al. (2009), Raats et al. (2011), Vacheyrou et al. (2011), Mallet et al. (2012), Masoud et al. (2012) and Quigley et al. (2013).

*Culture-dependent methods are based on isolation of bacteria using agar-based methods followed by identification using phenotypic or genotypic methods.
†Culture-independent methods are based on direct extraction of bacterial DNA from the milk followed by identification using various techniques including DGGE/SSCP/clone libraries etc.
‡Next-generation sequencing methods are based on direct extraction of bacterial DNA from the milk sample followed by identification using pyrosequencing.

Sheep milk

Sheep milk is rarely consumed but still constitutes c. 1.3% of global milk production as it is often employed throughout Europe in the development of cheese (Tsakalidou & Odos, 2012). Sheep milk is dominated by LAB, with mesophilic bacteria representing $10^2 – 10^6$ CFU mL$^{-1}$, while psychrotrophic populations correspond to $10^2 – 10^4$ CFU mL$^{-1}$ (Fotou et al., 2011). Studies assessing the impact of storing sheep milk at refrigeration temperature highlighted increases in psychrophiles, but also in mesophiles. Unsurprisingly, the thermordic population did not increase. These general trends are also affected by temperature and the length of storage (de Garnica et al., 2011). Other bacteria that have been detected on occasion can include microorganisms of concern from a milk safety perspective including E. coli, Salmonella, Staphylococcus aureus, Bacillus and Clostridium perfringens (Fotou et al., 2011). The location can affect both the nutritional composition and microbial composition of sheep milk. A correlation has been noted between milks with a higher fat content and greater counts of LAB, coliforms and moulds. In populations of streptococci and S. aureus, there was an increase and a decrease in counts, respectively, in regions where the milk was more acidic and nutrient levels were lower (Yabrir et al., 2013). Some insight into the microbiology of sheep milk was also provided by a recent study of the raw sheep milk cheese, Oscypek, which is manufactured without a starter culture (Alegria et al., 2012; Table 3). As this is a naturally fermented raw milk cheese, it is likely that these cheese-associated bacteria were also present in the corresponding raw milk. A culture-based approach established that lactococci (Lactococcus lactis ssp. lactis and ssp. cremoris) dominated (c. $10^8$ CFU g$^{-1}$), with lactobacilli (Lactobacillus casei, Lactobacillus plantarum, Lactobacillus parabuchneri and Lactobacillus brevis) also being common ($10^7 – 10^8$ CFU g$^{-1}$). Leuconostoc (Leuconostoc citreum, Leuconostoc lactis and Leuconostoc mesenteroides) were detected at levels of $10^5 – 10^8$ CFU g$^{-1}$, fungal populations were present between $10^5$ and $10^6$ CFU g$^{-1}$ and Enterobacteriaceae, including Enterobacter kobei, were at $10^5 – 10^6$ CFU g$^{-1}$, but were reduced during processing. A parallel DGGE investigation confirmed the dominance of Lactococcus lactis but also highlighted the presence of a significant population of Lactococcus garvieae, which had not been detected by culturing. This approach also revealed a number of minor populations including Tetragnococcus halophilus, Streptococcus salivarius, S. thermophilus and Streptococcus vestibularis. A high-throughput sequencing–based approach revealed the presence of 40 different genera in the cheese. This included 9 dominant genera, including 6 from the order Lactobacillales (which include the lactococci, lactobacilli and related genera), which constituted 97% of assigned sequences. The other dominant genera were the Bifidobacteriaceae, Enhydrobacter and unclassified Bacilli (Fig. 2). The benefits of employing this technology were again highlighted when previously overlooked populations...
Table 2. Bacterial populations detected in raw goats milk using culture-dependent and culture-independent methods

<table>
<thead>
<tr>
<th>Culture-dependent</th>
<th>Culture-independent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td>Acinetobacter baumannii</td>
</tr>
<tr>
<td>Arthrobacter species</td>
<td>Arthrobacter species</td>
</tr>
<tr>
<td>Bacillus thuringiensis/cereus</td>
<td>Bacillus thuringiensis/cereus</td>
</tr>
<tr>
<td>Brachybacterium paracolongeratum</td>
<td>Brachybacterium paracolongeratum</td>
</tr>
<tr>
<td>Brevibacterium stationis</td>
<td>Brevibacterium stationis</td>
</tr>
<tr>
<td>Chryseobacterium indologenes</td>
<td>Chryseobacterium indologenes</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>Citrobacter freundii</td>
</tr>
<tr>
<td>Corynebacterium variable</td>
<td>Corynebacterium variable</td>
</tr>
<tr>
<td>Delftia acidovorans</td>
<td>Delftia acidovorans</td>
</tr>
<tr>
<td>Enterococcus faecalis/ saccharominimus</td>
<td>Enterococcus faecalis/ saccharominimus</td>
</tr>
<tr>
<td>Exiguobacterium</td>
<td>Exiguobacterium</td>
</tr>
<tr>
<td>Jeotgalicoccus psychrophiles</td>
<td>Jeotgalicoccus psychrophiles</td>
</tr>
<tr>
<td>Kocuria rhizophila/Kristinae carrhipha</td>
<td>Kocuria rhizophila/Kristinae carrhipha</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>Lactobacillus casei</td>
</tr>
<tr>
<td>Lactococcus lactis/garvieae</td>
<td>Lactococcus lactis/garvieae</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides</td>
<td>Leuconostoc mesenteroides</td>
</tr>
<tr>
<td>Microbacterium oxydans</td>
<td>Microbacterium oxydans</td>
</tr>
<tr>
<td>Micrococcus species/caseolyticus</td>
<td>Micrococcus species/caseolyticus</td>
</tr>
<tr>
<td>Pantoea agglomerans</td>
<td>Pantoea agglomerans</td>
</tr>
<tr>
<td>Pseudomonas species/putida/ aenoginosa/fulgida</td>
<td>Pseudomonas species/putida/aenoginosa/fulgida</td>
</tr>
<tr>
<td>Salinococcus species</td>
<td>Salinococcus species</td>
</tr>
<tr>
<td>Staphylococcus epidermidis/ simulans/caprae/equrorum</td>
<td>Staphylococcus epidermidis/ simulans/caprae/equrorum</td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>Stenotrophomonas maltophilia</td>
</tr>
<tr>
<td>Streptococcus mitis</td>
<td>Streptococcus mitis</td>
</tr>
<tr>
<td>Dietzia maris</td>
<td>Dietzia maris</td>
</tr>
<tr>
<td>Enterobacter species/absuria</td>
<td>Enterobacter species/absuria</td>
</tr>
<tr>
<td>Methanobacterium chejuense</td>
<td>Methanobacterium chejuense</td>
</tr>
<tr>
<td>Keiobella milletoxytoca</td>
<td>Keiobella milletoxytoca</td>
</tr>
<tr>
<td>Ornithinicoccus species</td>
<td>Ornithinicoccus species</td>
</tr>
<tr>
<td>Rothia species</td>
<td>Rothia species</td>
</tr>
</tbody>
</table>

Next-generation sequencing methods are not applicable in contrast to Table 1, as at the time of writing this article, next-generation DNA sequencing technology had not been applied to monitor the microbial content of raw goat milk or any source which may represent the microbial content of raw goat milk.

The bacterial names emphasised in red highlight the most prevalent bacterial populations detected.

The bacterial names emphasised in blue highlight the less prevalent, but frequently isolated bacterial populations detected.

The bacterial names in black highlight the occasional bacteria that are detected.

Where there are bacterial names in bold, these are detected by two of the three methods.

Where the bacterial name is underlined, these were detected by only one of the methods.

All other bacteria were detectable by all of the methods.

References used for data in this table are Alonso-Calleja et al. (2002), Callon et al. (2007), Goetsch et al. (2011).

*Culture-dependent methods are based on isolation of bacteria using agar-based methods followed by identification using phenotypic or genotypic methods.

†Culture-independent methods are based on direct extraction of bacterial DNA from the milk followed by identification using various techniques including DGGE/SSCP/clone libraries etc.

of Kocuria, Sanguibacter, Flavobacteria, Chryseobacterium, Exiguobacterium, Staphylococcus and Chromohalobacter were detected. Notably, a considerable proportion, c. 20%, of sequence reads could not be assigned, and so the identity of these bacteria, and the importance of the other subpopulations, will require further attention (Alegría et al., 2012; Table 3).

**Buffalo milk**

Buffalo milk is consumed in various countries around the world, with India and Pakistan being the highest consumers. It is not as common in Europe, but it does have an important market in some Mediterranean countries where it is utilised in making traditional mozzarella cheese. The microbial content of raw buffalo milk has been assessed, through culturing, and found to contain a large population of LAB, including lactococci and lactobacilli, as well as coliforms, E. coli, S. aureus and bacterial endospores, highlighting that while technologically relevant bacteria are present, microorganisms of concern with respect to quality and safety can also be found (Ercolini et al., 2004; Han et al., 2007). Culture-independent methods, that is, DGGE, have revealed that raw buffalo milk contains a rich diversity of bacteria that changes during subsequent fermentation to manufacture traditional mozzarella (Ercolini et al., 2001). More recently, high-throughput sequencing has been applied to identify the bacterial populations present in buffalo milk and throughout the manufacture of mozzarella cheese (Table 3; Ercolini et al., 2012). The dominant microorganisms in the milk were Lactococcus spp. (30%), Acinetobacter spp. (21%), Pseudomonas spp. (20%), Streptococcus macedonicus (10%) and Lactococcus lactis (10%; Fig. 2). A number of other microorganisms were detected in low abundance including Brochothrix, Carnobacterium, Chryseobacterium, Clostridium, Corynebacterium, Enterobacteriaceae, Gammaproteobacteria and Haloanella. There was also a large percentage of unassigned reads (c. 20%) corresponding to the raw milk. This percentage was much greater than that associated with the corresponding cheese (Ercolini et al., 2012).

**Milk from other animal sources**

Other milks that are consumed by humans around the world include those produced by camels, yaks, donkeys and, to a lesser extent and in only some countries, mares. Camel milk is commonly consumed in African and Arab countries where it is a valuable food resource for pastoral people. The microbial population of camel milk, like that of other milks, can play a role in subsequent fermentations, health promotion and milk spoilage. The milk is typically dominated by mesophiles, including lactobacilli (such as
Lactobacillus helveticus, L. casei ssp. casei and L. plantarum, lactococci (such as Lactococcus lactis ssp. lactis), streptococci (such as S. salivarius) and leuconostoc (all at 10^2–10^7 CFU mL^{-1}; Khedid et al., 2009). As with other milks, camel milk can also contain human pathogens, including Salmonella, Listeria and E. coli, the prevalence of which can vary with location (Abeer et al., 2012). In naturally fermented camel milk, Streptococcus infantarius ssp. infantarius dominates, with significant populations of Lactococcus lactis ssp. lactis, S. thermophilus and L. helveticus also detected (Jans et al., 2012). Another animal that is typically associated with difficult climates is the yak. c. 92% of the world’s yaks are located in China, and yak milk can be an important commodity in some regions of China. Again

| Table 3. Bacterial populations detected in raw milk of sheep or buffalo using culture-dependent, culture-independent and next-generation DNA sequencing methods |
|----------------------------------|----------------|----------------|----------------|
| Milk source | Culture-dependent* | Culture-independent† | Next-generation sequencing‡ |
| Sheep Milk | Lactococcus lactis | Lactococcus lactis/garvieae/raffiniolactis | Lactococcus |
| | Lactobacillus casei/plantarum/parabuchneri brevis | Lactobacillus planatarum/paraplanatarum/helveticus/crispatus | Lactobacillus |
| | Leuconostoc citreum/lactis/mesenteroides/pseudomesenteroides | Leuconostoc citreum/mesenteroides/pseudomesenteroides/lactis | Leuconostoc |
| | Streptococcus thermophilus/agalactiae | Streptococcus vestibularis/salivarius/uberis-paruberis/thermophilus | Streptococcus |
| | Enterococcus faecalis/durans/italicus/kobei | Enterococcus | Enterococcus |
| | Bacillus simplex | Tetragnococcus halophilus | Tetragnococcus |
| | Pedococcus | Bifidobacterium | Actinobacteria |
| | Corynebacterium species | Chromohalobacter | Chryseobacterium |
| | Staphylococcus species | | Enhydrobacter |
| | Salmonella species | | Flavobacteria |
| | Escherichia coli | | Kocuria |
| | Enterobacter kobei | | Sanguibacter |
| | | | Staphylococcus |
| | | | Unassigned |
| Buffalo Milk | Lactobacillus species/plantarum/paracasei/fermentum/delbrueckii | Lactobacillus species/delbrueckii | Lactobacillus species/kefiranofaciens |
| | Lactobacillus species | Lactobacillus lactis | Lactobacillus species/lactis |
| | Staphylococcus species | Leuconostoc lactis | Pseudomonas species/fragi |
| | Streptococcus | Streptococcus thermophilus | Streptococcus thermophilus/macedonicus |
| | Bacillus species | Enterococcus species/faecalis | Acinetobacter species/johnsonii |
| | Escherichia coli | | Camobacteria species |
| | Unassigned | | Clostridium species/hiranonis |
| | | | Corynebacteria species |
| | | | Enterobacteria species |
| | | | Unassigned |

The bacterial names emphasised in red highlight the most prevalent bacterial populations detected. The bacterial names emphasised in blue highlight the less prevalent, but frequently isolated bacterial populations detected. The bacterial names in black highlight the occasional bacteria that are detected. Where there are bacterial names in bold, these are detected by two of the three methods. Where the bacterial name is underlined, these were detected by only one of the methods. All other bacteria were detectable by all of the methods.

Reference used for data on sheep milk is Alegria et al. (2012). For buffalo milk, the references are Ercolini et al. (2001), Devirgiliis et al. (2008), Maniruzzaman et al. (2010), and Ercolini et al. (2012).

*Culture-dependent methods are based on isolation of bacteria using agar-based methods followed by identification using phenotypic or genotypic methods.
†Culture-independent methods are based on direct extraction of bacterial DNA from the milk followed by identification using various techniques including DGGE/SSCP/clone libraries etc.
‡Next-generation sequencing methods are based on direct extraction of bacterial DNA from the milk sample followed by identification using pyrosequencing.
§Sheep milk information is from naturally ripened cheeses manufactured using raw sheep milk, indicating that the microbial population is potentially from the raw sheep milk microbial communities.

Lactobacillus helveticus, L. casei ssp. casei and L. plantarum, lactococci (such as Lactococcus lactis ssp. lactis), streptococci (such as S. salivarius) and leuconostoc (all at 10^2–10^7 CFU mL^{-1}; Khedid et al., 2009). As with other milks, camel milk can also contain human pathogens, including Salmonella, Listeria and E. coli, the prevalence of which can vary with location (Abeer et al., 2012). In naturally fermented camel milk, Streptococcus infantarius ssp. infantarius dominates, with significant populations of Lactococcus lactis ssp. lactis, S. thermophilus and L. helveticus also detected (Jans et al., 2012). Another animal that is typically associated with difficult climates is the yak. c. 92% of the world’s yaks are located in China, and yak milk can be an important commodity in some regions of China. Again
**Box 1. Human milk**

Human milk has the potential to protect a newborn against infectious disease through the provision of active components including immunoglobulins, immunocompetent cells, fatty acids, oligosaccharides and glycoproteins (Saaverda, 2002; Newburg, 2005). Furthermore, breast milk is also a source of microorganisms and of growth factors that contribute to their growth in the gut. Traditionally, it has been thought that these microorganisms are transferred from the mother’s skin (West et al., 1979). However, recent studies have indicated that bacteria present in the maternal gut can reach the mammary gland by vertical transfer (Albesharat et al., 2011). This topic requires further investigation. Regardless of the origin of these microorganisms, human milk constitutes one of the primary sources of the bacteria that colonise the gut of breastfed infants. Indeed, an infant consuming c. 800 mL day⁻¹ of human milk would be predicted to ingest between 10⁶ and 10⁷ bacteria daily (Heikkila & Saris, 2003). This may explain why some studies have shown that the bacterial composition of the gut microbiota of breastfed infants closely resembles that found in the breast milk of their mothers (Albesharat et al., 2011). As for other milks, a variety of different approaches have been taken to investigate the microbiology of human milk. Traditional culture-based methods have indicated that staphylococci, LAB and propionibacteria dominate in human milk with a significant *Bifidobacterium* population also present (Table 4; Martin et al., 2009). Similarly, several studies have demonstrated the transfer of *Staphylococcus*, *Lactobacillus*, *Bifidobacterium* as well as *Enterococcus* spp. from mother to infant through breastfeeding (Martin et al., 2004, 2009; Albesharat et al., 2011). The application of culture-independent molecular techniques, and particularly those based on analysis of 16S rRNA gene, has facilitated even more detailed analyses. These approaches have provided quite similar findings, that is, a dominance of staphylococci and streptococci and the presence of LAB, propionibacteria and bifidobacteria. However, DNA from other bacterial groups, including *Weissella*, *Clostridium* and *Serratia*, was also detected (Table 4; Martin et al., 2004, 2009). More recently, through the application of high-throughput sequencing, an even more diverse population has been uncovered (Table 4; Hunt et al., 2011; Cabrera-Rubio et al., 2012). Some taxa that were consistently found across all samples included species of *Streptococcus*, *Staphylococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Ralstonia*, *Propionibacterium*, *Sphingomonas* and *Bradyrhizobiaceae* (Fig. 2; Hunt et al., 2011; Cabrera-Rubio et al., 2012). The microbial composition of breast milk changed from prelabour to postbirth. Where birth was by nonelective caesarean section, the bacterial profile was more comparable with milk from mothers who had a natural labour than that of milk from mothers who underwent elective caesarean section. These results suggest that the physiologic changes in the mother during the labour process may influence the composition of the bacterial community. Finally, as a consequence of nursing periods, the bacterial population of breast milk eventually became dominated by microorganisms from the oral cavity and skin (Cabrera-Rubio et al., 2012). Following on from these recent investigations, it will be important to continue to unravel the roles of these individual microbial components with respect to mammary gland health, colonisation of the infant gut and, subsequently, maternal and infant health. Importantly, it is already known that human milk bacteria can play important roles in the infant gut. They can contribute to a reduction in the incidence and severity of infections by different mechanisms through competitive exclusion, the production of antimicrobial compounds or improving intestinal barrier function (Olivares et al., 2006). Many human milk lactobacilli and bifidobacteria can contribute to infant digestion by aiding in the breakdown of complex foods such as proteins and sugars; some lactobacilli increase the production of functional metabolites such as butyrate, which is utilised as an energy source and can improve intestinal function (Asakuma et al., 2011; Zivkovic et al., 2011; Gil-Campos et al., 2012), while various bifidobacteria have positive effects on health, including prevention of infection by pathogenic bacteria (e.g. protection against diarrhoea), immunostimulatory and anticarcinogenic capabilities, lowering of serum cholesterol and alleviation of lactose maldigestion (Fernández et al., 2012).

While breast milk can introduce a number of potentially health-promoting bacteria, it may also contain pathogens (Jones, 2001). Mastitis can be a common disease, with incidence rates of up to 33% in lactating mothers (Foxman et al., 2002; the issue of bovine mastitis is addressed later in the review). Mastitis results in the inflammation of the mammary lobules, usually due to the presence of staphylococci, streptococci or corynebacteria. Traditionally, *S. aureus* has been considered as the main causative agent; however, *Staphylococcus epidermidis* is emerging as a leading cause of subacute and acute mastitis in both women and veterinary medicine (Delgado et al., 2009).

The possible presence of pathogenic or spoilage microorganisms in human breast milk also needs to be considered in the context that mothers may have to store milk for a variety of different reasons. Human milk may be stored at neonatal units and used as a lifesaving therapy to high-risk infants (Silvestre et al., 2006). The influence of refrigeration on the bacterial content of human milk, over a 4-day period, has recently been assessed; refrigeration prevented the growth of total aerobic bacteria, LAB and *Enterobacteriaceae*, while reducing the levels of coagulase-positive staphylococci (Giribaldi et al., 2013).

Lactobacillus fermentum, Lactobacillus kefiranofaciens, L. plantarum ssp. plantarum, L. brevis, Lactobacillus buchneri, Leuconostoc lactis, L. mesenteroides, Lactococcus lactis ssp.
cremoris, S. thermophilus, Enterococcus faecalis, Enterococcus durans and Weissella cibaria as being among the most common species in yak milk (Watanabe et al., 2008; Yu et al., 2011). Donkey milk is less commonly consumed and, for the same reason, is less extensively studied. The microbial content of donkey milk has been found to be similar to that of other milks, consisting of LAB, coliforms and fungi, with mesophiles being detected at levels up to $10^4$ CFU mL$^{-1}$ and psychrotrophs at $10^2$ CFU mL$^{-1}$ (Zhang et al., 2008b; Sarno et al., 2012).

### Table 4. Bacterial populations detected in raw human milk using culture-dependent, culture-independent and next-generation DNA sequencing methods

<table>
<thead>
<tr>
<th>Human milk</th>
<th>Culture-dependent*</th>
<th>Culture-independent†</th>
<th>Next-generation sequencing‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corynebacterium species</td>
<td>Corynebacterium species</td>
<td>Corynebacterium species</td>
<td>Corynebacterium</td>
</tr>
<tr>
<td>Enterococcus species faecium/faecalis/durans/hiraemundii</td>
<td>Enterococcus species faecalis/feacium</td>
<td>Enterococcus</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus species/acidophilus/fermentum/plantarum/gasseri/crispatus/rhamnosus/salivarius/reuteri/casei/gastricus/vaginalis/animalis/brevis/helicustus/loris</td>
<td>Lactobacillus species/fermentum/gasseri/rhamnosus</td>
<td>Lactobacillus</td>
<td></td>
</tr>
<tr>
<td>Lactococcus species/lactis</td>
<td>Lactococcus species/lactis</td>
<td>Lactococcus</td>
<td></td>
</tr>
<tr>
<td>Leuconostoc species/mesenteroides</td>
<td>Leuconostoc species/citeae/fallax</td>
<td>Leuconostoc</td>
<td></td>
</tr>
<tr>
<td>Streptococcus species/murisorum/salivarius/ons/parasanguis/lactarius/australis/gallolyticus/vestibularis</td>
<td>Streptococcus species/murisorum/parasanguis/salivarius</td>
<td>Streptococcus</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus species/epidermidis/auris/capitis/hominis</td>
<td>Staphylococcus species/epidermidis/hominis</td>
<td>Staphylococcus</td>
<td></td>
</tr>
<tr>
<td>Rothia species/mucilaginosa</td>
<td>Rothia species/mucilaginosa</td>
<td>Rothia</td>
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<tr>
<td>Pediococcus species/pentosaceus</td>
<td>Pediococcus species/pentosaceus</td>
<td>Pediococcus</td>
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<tr>
<td>Peptostreptococcus species</td>
<td>Peptostreptococcus species</td>
<td>Peptostreptococcus</td>
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<tr>
<td>Kocurna species/rhizophila</td>
<td>Kocurna species/rhizophila</td>
<td>Kocurna</td>
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<tr>
<td>Weissella species/cibaria/confusa</td>
<td>Weissella species/cibaria/confusa</td>
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<tr>
<td>Propionibacterium species/acnes</td>
<td>Propionibacterium species/acnes</td>
<td>Propionibacterium</td>
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<tr>
<td>Acinetobacter</td>
<td>Acinetobacter</td>
<td>Acinetobacter</td>
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<tr>
<td>Pseudomonas</td>
<td>Pseudomonas</td>
<td>Pseudomonas</td>
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<tr>
<td>Sphingomonas</td>
<td>Sphingomonas</td>
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<tr>
<td>Raetonia</td>
<td>Raetonia</td>
<td>Raetonia</td>
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<tr>
<td>Serratia</td>
<td>Serratia</td>
<td>Serratia</td>
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<tr>
<td>Stenotrophomonas</td>
<td>Stenotrophomonas</td>
<td>Stenotrophomonas</td>
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<tr>
<td>Veillonella</td>
<td>Veillonella</td>
<td>Veillonella</td>
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<tr>
<td>Actinomyces</td>
<td>Actinomyces</td>
<td>Actinomyces</td>
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<tr>
<td>Bradyrhizobium</td>
<td>Bradyrhizobium</td>
<td>Bradyrhizobium</td>
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<tr>
<td>Carnobacterium</td>
<td>Carnobacterium</td>
<td>Carnobacterium</td>
<td></td>
</tr>
<tr>
<td>Citrobacter</td>
<td>Citrobacter</td>
<td>Citrobacter</td>
<td></td>
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<tr>
<td>Gemella</td>
<td>Gemella</td>
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<tr>
<td>Granulicatella</td>
<td>Granulicatella</td>
<td>Granulicatella</td>
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<tr>
<td>Lysinibacillus</td>
<td>Lysinibacillus</td>
<td>Lysinibacillus</td>
<td></td>
</tr>
<tr>
<td>Prevotella</td>
<td>Prevotella</td>
<td>Prevotella</td>
<td></td>
</tr>
<tr>
<td>Unassigned</td>
<td>Unassigned</td>
<td>Unassigned</td>
<td></td>
</tr>
</tbody>
</table>

The bacterial names emphasised in red highlight the most prevalent bacterial populations detected.
The bacterial names emphasised in blue highlight the less prevalent, but frequently isolated bacterial populations detected.
The bacterial names in black highlight the occasional bacteria that are detected.
Where there are bacterial names in bold, these are detected by two of the three methods.
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All other bacteria were detectable by all of the methods.
*Culture-dependent methods are based on isolation of bacteria using agar-based methods followed by identification using phenotypic or genotypic methods.
†Culture-independent methods are based on direct extraction of bacterial DNA from the milk followed by identification using various techniques including DGGE/SSCP/clone libraries etc.
‡Next-generation sequencing methods are based on direct extraction of bacterial DNA from the milk sample followed by identification using pyrosequencing.
Technologically relevant bacteria of raw milk

As described above, raw milk can contain a diverse bacterial population. Many such bacteria can contribute subsequently to natural fermentations. In some situations, specific strains have been so successful in this regard that they have been isolated from milk and consciously added as starters or adjuncts designed to confer desirable traits on fermented products. This can be particularly important in situations where regulations require the use of pasteurised milk, and thus, the re-introduction of dairy microorganisms can compensate for the removal of commensal populations and the associated adverse effect on the flavour of resultant products. This section will review what is known about the most technologically important genera of raw milk bacteria.

Lactococcus

Lactococcus consists of seven species, two subspecies and one biovar (www.bacterio.cict.fr; as of November 2012). Of these, Lactococcus lactis ssp. lactis and Lactococcus lactis ssp. cremoris can dominate in raw milk, cheese and other (unheated) dairy products.

In dairy foods, Lactococcus lactis, and Lactococcus lactis ssp. lactis and Lactococcus lactis ssp. cremoris in particular, are primarily known for their role as starter cultures for the cheese industry. Lactococcus lactis ssp. lactis biovar diacetylactis is also recognised for its production of flavour-developing compounds (Hugenholtz & Starrenburg, 1992). These microorganisms are distinguished from one another on the basis of arginine and citrate utilisation, growth temperature and salt tolerance (Kahala et al., 2008). While these microorganisms are naturally present in raw milk and artisanally produced cheeses (Gaya et al., 2001), they are frequently added to pasteurised milk to facilitate the commercial manufacture of cheeses (Smit et al., 2005). Their primary role during cheese production is acidification through the production of L-lactate. However, they also contribute to proteolysis, the conversion of amino acids into flavour compounds (alcohols, ketones, aldehydes), citrate utilisation and/or fat metabolism (Smit et al., 2005). A comparison of 20 Lactococcus lactis strains, 10 of the ssp. lactis phenotype and 10 of the ssp. cremoris phenotype, confirmed two major subspecies lineages that were distinguished on the basis of the presence or absence of 4571 gene orthologs (Bayjanov et al., 2009; Fernández et al., 2011). Thus, it is estimated that these phenotypically similar subspecies diverged c. 17 million years ago (Bolotin et al., 2004). Sequencing of the genome of Lactococcus lactis ssp. lactis IL1403 revealed that all known genes required for energy metabolism were present, including a number of genes involved in fermentation as well as a novel gene, poxL, encoding pyruvate oxidase, which may play a role in switching between fermentation modes. Forty-three insertion elements were identified, the distribution of which suggests that recent recombination between two closely related genomes may have occurred (Bolotin et al., 2001). Sequencing of Lactococcus lactis ssp. cremoris MG1363 revealed some similarities to strain IL1403, including proteolytic systems and genes associated with the utilisation of lactose. The absence of virulence genes from these genomes is consistent with their ‘generally regarded as safe’ (GRAS) status (Wegmann et al., 2007). Genome sequencing of Lactococcus lactis ssp. cremoris strain A76 revealed that it has 99.2% identity to the industrially important strain cremoris SK11 and identified two contiguous regions associated with the ssp. lactis lineage. The first contains genes involved in cell wall biosynthesis. The second region corresponds to a prophage. The presence of these regions suggests that they were introduced through a recombination event and highlights the potential importance of such events among strains of industrial importance (Bolotin et al., 2012). The Lactococcus lactis ssp. cremoris SK11 strain contains a number of unique plasmids, pSK11A, pSK11B, pSK11L and pSK11P, which encode important traits related to dairy adaptation and utilisation, including lactose utilisation, a complex proteolytic system and an oligopeptide permease system (Siezen et al., 2005). Lactococcus lactis ssp. lactis biovar diacetylactis strain DPC 3901 contains four unique plasmids: pVF18, pVF21, pVF22 and pVF50. While sequence analysis of these plasmids has revealed that this bacterium most likely originated from a plant origin, there are some features that highlight its adaption to milk, for example plasmid pVF59, which contains genes of relevance for growth on milk. These include genes encoding a lactose phosphotransferase operon, a protein predicted to function in D-lactate utilisation, a system for uptake of oligopeptides generated from casein degradation as well as oligopeptidases, which allow this strain to utilise casein as a nitrogen source (Fallico et al., 2011).

A number of other species of Lactococcus are naturally present in raw milk. Although Lactococcus raffinolactis is typically not used by the dairy industry because of a lack of caseinolytic activity (Holler & Steele, 1995), a recent study observed synergism between L. raffinolactis and Lactococcus lactis strains, whereby L. raffinolactis improved acid production thanks to its ability to utilise metabolic products generated by Lactococcus lactis (Kimoto-Nira et al., 2012), presumably thanks to the presence of a complete set of genes for lactate fermentation and genes responsible for an oligopeptide ABC transporter (Meslier et al., 2012b). Even though L. garvieae is a recognised
fish pathogen (Vendrell et al., 2006), it has been detected in raw milk, some natural mixed starter cultures and artisanal cheeses (Fortina et al., 2007; Foschino et al., 2008). Genomic studies have shown that L. garvieae isolates from dairy and fish sources form two distinct clusters and that only the dairy isolates possess the ability to utilise lactose (Fortina et al., 2007, 2009). It is hypothesised that this key phenotype has been gained by dairy isolates through lateral gene transfer (Ferrario et al., 2012). However, strains from both clusters lack proteolytic activity (Fortina et al., 2007). Finally, Lactococcus piscium has been detected in raw milk and raw milk cheese (Carraro et al., 2011). This is typically regarded to be a salmon-associated species (Williams et al., 1990), which is psychrotrophic and has been investigated with a view to its use as a biopreservative, albeit not in a dairy context (Fall et al., 2010). Little is known regarding its contribution to dairy products and how it has adapted to grow in dairy environments.

Lactobacillus

The genus Lactobacillus is very diverse and, according to the most recent estimations, consists of 174 different species and 27 subspecies (www.bacterio.cict.fr). Lactobacilli can be found in rich, carbohydrate-containing niches, including those associated with plants, animals, silage and raw milk (Bernardeau et al., 2008). An ever greater understanding of Lactobacillus biology has led to the use of strains of Lactobacillus for an increasing range of industrial dairy applications. In particular, their proteolytic activity and ability to produce aroma compounds and exopolysaccharides can contribute to the quality and lytic activity and ability to produce aroma compounds of strains of Lactobacillus under the most recent estimations, consists of 174 different species and 27 subspecies (www.bacterio.cict.fr). Lactobacilli can be found in rich, carbohydrate-containing niches, including those associated with plants, animals, silage and raw milk (Bernardeau et al., 2008). An ever greater understanding of Lactobacillus biology has led to the use of strains of Lactobacillus for an increasing range of industrial dairy applications. In particular, their proteolytic activity and ability to produce aroma compounds and exopolysaccharides can contribute to the quality and nutritional value of dairy products (Leroy & De Vuyst, 2004). Lactobacilli that are of particular importance in the dairy industry are L. helveticus, L. delbrueckii ssp. bulgaricus and L. delbrueckii ssp. lactis (the latter two species will be referred to as L. bulgaricus and Lactobacillus lactis hereafter).

Lactobacillus helveticus was first described by Orla-Jensen in 1919 as an isolate from an Emmental cheese (Naser et al., 2006), but it has since been evident that representatives of this species are commonly isolated from raw milk and raw milk based products (Quigley et al., 2011). Lactobacillus helveticus has a number of traits that are desirable with respect to cheese production. These include rapid autolysis of the strains, which results in the release of intracellular enzymes and a reduction in bitterness and increased flavour notes in cheese (Broadbent et al., 2011). Lactobacillus helveticus is also characterised by its ability to grow at relatively high temperatures (55 °C; Kiernan et al., 2000b; Hannon et al., 2003) and is the most proteolytic of the LAB frequently used in the manufacture of dairy products. The release of free fatty acids following lipolysis introduces important flavour compounds (Hickey et al., 2007). Genome sequencing of L. helveticus DPC4571, a Swiss cheese isolate, revealed that the presence of a high percentage of pseudogenes that are associated with loss-of-function events and presumably reflect adaptation to the dairy niche (Callanan et al., 2008). The growth of L. helveticus in milk is dependent on a complex system of proteolytic enzymes, which collectively enable strains to access essential amino acids (Christensen et al., 1999). Genome sequencing of DPC4571 revealed the presence of a number of peptidase genes (pepE, pepQ, pepT and pepD) that are likely involved in the proteolytic activity of this strain. PepQ is a proline-specific peptidase, which may be significant given that milk casein has a relatively high number of proline residues. Also, PepE, an endopeptidase, may have a role in reducing bitter defects during cheese ripening. DPC4571 lacks genes for colonisation and interaction with the mucosal surface that are present in related gastrointestinal probiotic species. These traits include surface proteins, cell wall anchoring proteins and mucus-binding proteins (Callanan et al., 2008). An isolate from naturally fermented milk, L. helveticus H10, has a larger genome than DPC4571 (Zhao et al., 2011). Most of the functional genes of this strain are conserved with DPC4571, but there are also 300 unique genes and 130 genes that are absent compared with DPC4571. Lactobacillus delbrueckii can be divided into three major subspecies, that is, ssp. delbrueckii, which is plant derived, ssp. bulgaricus and ssp. lactis (Giraffa et al., 2008). Representatives of the latter two subspecies have been regularly detected in raw milk samples, as well as being dominant populations in many traditionally manufactured cheeses and protected designation of origin (PDO) cheeses (Torriani et al., 1999; Randazzo et al., 2002; Morandi et al., 2011). Both exhibit strong proteolytic activity (Giraffa et al., 2004; Kholfi et al., 2011; Agyei & Danquah, 2012). Lactobacillus bulgaricus is, as a consequence of its worldwide use in yoghurt production, one of the most important dairy-associated lactobacilli. It acts synergistically with S. thermophilus, allowing rapid growth and acidification with desired organoleptic properties (Herbe-Jimenez et al., 2009). A number of factors are known to play a role in this ‘protocooperation’ process including degradation of milk proteins by L. bulgaricus; also, the production of formate and CO2 by S. thermophilus can stimulate the growth of L. bulgaricus. Genomics has revealed important factors, which may play a role in this beneficial interaction. While L. bulgaricus encodes a full set of genes for biosynthesis of folate, it is thought that S. thermophilus is required to produce p-aminobenzoate, which feeds this pathway. Also, in silico analysis predicted the existence of a limited
number of cell wall–bound and extracellular proteins, which could contribute to direct contact between *L. bulgaricus* and *S. thermophilus* (van de Guchte et al., 2006). As with other milk-associated microorganisms, genomic studies suggest that the genome of *L. bulgaricus* is in an active state of gene elimination and size reduction but does contain genes encoding complete transport systems for lactose as well as mannose, glucose, fructose and glycerol (van de Guchte et al., 2006). As expected, strain-specific features are evident. For example, the industrially important bacterium *L. bulgaricus* strain 2038 has a number of unique features including a gene set involved in exopolysaccharide synthesis, which may improve synaeresis (separation of liquid) as well as texture, viscosity and mouthfeel of the final product. This strain also contains a larger genome than other strains (Hao et al., 2011).

There are several other lactobacilli in raw milk that increase in number during the manufacture of dairy products and can become particularly dominant during the ripening of cheese (Henri-Dubernet et al., 2008). These populations, which are often referred to as non-starter LAB (or NSLAB), have an ability to adapt to the conditions of cheese ripening, where many nutrients are depleted, pH is reduced, and moisture content is low. Here, they are able to carry out proteolysis and lipolysis to produce many end products that contribute to flavour and texture development of cheese (Smit et al., 2005). These include *L. casei*, *Lactobacillus paracasei*, *L. plantarum/paraplantarum*, *Lactococcus rhamnosus* and *Lactobacillus curvatus*, *L. brevis*, *Lactobacillus sake*, *Lactobacillus pentosus*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus johnsonii*, *Lactobacillus crispatus*, *L. fermentum*, *L. buchneri* and *Lactobacillus gasseri*. While the levels of these species can be underestimated if culture-dependent methods are relied upon exclusively, the use of culture-independent techniques such as DGGE, TTGE and qPCR, or a combination of these with culturing, has addressed this issue in a number of instances.

**Streptococcus**

The genus *Streptococcus* consists of 97 species and 17 subspecies (www.bacterio.cict.fr). Although many genera of streptococci are pathogenic, *S. thermophilus* carries a ‘GRAS’ status (Facklam, 2002) and is frequently isolated from dairy environments, including raw milk, natural starter cultures and cheese curds (Duthoit et al., 2005; Randazzo et al., 2006; Santarelli et al., 2008). Strains of *S. thermophilus* have also been detected in the teats of cows, cowsheds and dairy facilities (Vacheyrou et al., 2011; Braem et al., 2012). *Streptococcus thermophilus* is a thermophilic LAB widely used as a starter culture in the manufacture of dairy products. It is often regarded as the second most important industrial dairy starter after *Lactococcus lactis*. Its importance in dairy products is due to its ability to rapidly convert lactose to lactate; bringing about a rapid decrease in pH; and the production of important metabolites including low levels of formate, acetoin, diacetyl, acetaldehyde and acetate (Ott et al., 2000). Many *S. thermophilus* strains produce exopolysaccharide that contributes to the desirable viscous texture and rheological properties of fermented milk products, particularly yoghurt. In cheese, *S. thermophilus* is used alone or in combination with several lactobacilli and mesophilic starters, but in yoghurt, it is always used with *L. bulgaricus*. While *S. thermophilus* is, in general, readily isolated by traditional microbiological methods, there are some instances where it has been overlooked, and rapid molecular and culture-independent methods, such as SSCP (Randazzo et al., 2002) and DGGE (Duthoit et al., 2005), were required to detect its presence.

Whole-genome sequencing of *S. thermophilus* has revealed that this bacterium exhibits 80% similarity with other streptococci, indicating that it shares a substantial part of its overall physiology and metabolism with its pathogenic relatives. The analyses suggest that their common ancestor dates back 3000–30 000 years, roughly corresponding to the origin of human dairy activity (i.e. c. 7000 years ago). The species lacks a number of functional genes typically found in other streptococci, including many genes involved in carbohydrate utilisation as well as virulence-related genes, for example, genes for some surface proteins that are required for adhesion to mucosal surfaces, and antibiotic modification genes. This lack provides strong evidence to support the GRAS status of *S. thermophilus*. Genome sequencing has also revealed the presence of more than 50 insertion sequences, which have shaped the genome and, in part, are associated with genes involved in the adaptation of the species to milk. Indeed, a number of genes appear to have been acquired from other dairy microorganisms through horizontal gene transfer (Bolotin et al., 2004). *Streptococcus thermophilus* strain ND03, isolated from naturally fermented yak milk, contains unique genes not previously found in other strains. These are contained within a large insertion island and encode a transposase, a glutamate decarboxylase, an acetyltransferase, a polysaccharide biosynthesis protein and proteins associated with exopolysaccharide biosynthesis (Sun et al., 2011). A comparative genome analysis of 47 *S. thermophilus* strains has revealed that the gene content can be split into core genes (58%; present in all strains) and noncore genes. The noncore genes can be split into conserved genes (14%), variable genes (20%) and acquired genes (8%). The latter genes – in addition to those referred to above with respect to ND03 – encode, for example, bacteriocins, efflux/uptake pumps,
proteins involved in peptide metabolism, phage proteins as well as phase resistance genes (Rasmussen et al., 2008).

Other streptococci that have been associated with milk and milk products include Streptococcus uberis, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus bovis and S. macedonicus. Streptococcus macedonicus has been isolated from artisanal raw milk cheeses (Pacini et al., 2006; De Vuyst & Tsakalidou, 2008) and has displayed some desirable characteristics from a dairy technology perspective. These include the ability to acidify and to produce peptidases and the generation of inhibitory compounds while, importantly, lacking antibiotic resistance and haemolytic activity (Lombardi et al., 2004; De Vuyst & Tsakalidou, 2008). Genome sequencing of a dairy isolate of S. macedonicus revealed that the bacterium is undergoing regular genome decay as indicated by the presence of a large number of pseudogenes. The genus also appears to lack the pil1 locus that is involved in instances of infectious endocarditis caused by pathogenic relatives (Papadimitriou et al., 2012). Nonetheless, further investigations are required to definitively establish the safety status of the species with respect to its use in dairy products. An African dairy isolate, Streptococcus infantarius ssp. infantarius strain CJ18, contains plasmids with high sequence identity to Lactococcus lactis sequences. The presence of a gal-lac operon suggests an evolutionary adaptation to the dairy niche (Jans et al., 2012). More recently, comparative genomic analysis has revealed that the gal-lac operon of CJ18 has 91% identity with that of S. thermophilus. CJ18 also contains an oligopeptide transport operon, which is important during growth in milk for the uptake of peptides and amino acids, and lacks classical streptococcal virulence factors (Jans et al., 2013).

Many of the other streptococci regularly detected in bovine milk are associated with mastitis infection. Mastitis-associated pathogens typically infect the teat canal of cows and pass into the milk during milking. The presence of these microorganisms impairs milk quality and the quality of subsequent products (Barbano et al., 2006). Streptococcus uberis is an animal pathogen and one of the major causes of bovine mastitis worldwide (Bradley et al., 2007). Genomic analysis provides evidence of the nutritional flexibility of S. uberis that allows it to occupy various ecological niches, including mammary glands (Ward et al., 2009). Streptococcus dysgalactiae, a contagious and environmental pathogen, also accounts for a notable proportion of bovine mastitis infections (Todhunter et al., 1995). A number of genes are involved in its ability to adhere to the mammary gland, where it can survive and is protected from the immune system as well as therapeutics. The severity of disease differs with genotype (Beecher et al., 2012). It has been suggested that S. dysgalactiae present in the mammary gland degrades the proteose peptones of milk prior to milking and consequently results in a reduced ability of milk to coagulate during fermentations (Merin et al., 2008). Similarly, S. agalactiae is a major causative agent associated with mastitis in cows. It has been proposed that a number of genes, including a lac operon, have been acquired through lateral gene transfer to allow this bacterium to adapt to the bovine host. An unusually high number of insertion elements have also been detected suggesting frequent genomic rearrangement (Richards et al., 2011). Finally, S. bovis is an opportunistic human pathogen, which is often associated with infections in immunocompromised patients or patients with cancer. It can also be detected in other environments including fermented foods (Barile et al., 2012).

**Propionibacterium**

The genus Propionibacterium comprises two distinct groups from different habitats, that is, strains typically found on human skin, referred to as the ‘acnes group’, and strains isolated from milk and dairy products, referred to as ‘dairy’ or ‘classical’ propionibacteria (Meile et al., 2008). Notably, it has also been claimed that dairy propionibacteria possess health-promoting characteristics (Cousin et al., 2011). The dairy group of propionibacteria comprises four species: Propionibacterium freudenreichii, Propionibacterium acidipropionici, Propionibacterium jensenii and Propionibacterium thoenii (www.bacterio.cict.fr). Propionibacterium freudenreichii serves as a starter in Swiss-style cheeses. It was first isolated over a century ago from an Emmental cheese and contributes to hole or ‘eye’ formation and flavour formation in these cheeses (Langsrud & Reinbold, 1973). The other species of dairy Propionibacterium are usually isolated from milk and different cheese types (Meile et al., 2008). The characteristic trait of P. freudenreichii is the fermentation of lactate into propionate, acetate and CO₂, while associated distinctive flavours arise from the formation of fatty acids, through lipolysis, and of branched-chain acids from the catabolism of amino acids. Due to its long documented use in cheese manufacture, P. freudenreichii has a GRAS status (Cousin et al., 2011). Whole-genome sequencing of P. freudenreichii CIRM-BIA1 revealed its ability to cope with different stresses including oxidative, bile salt and temperature stresses and an ability to resist phage attack, to accumulate nutrients and to mobilise these during periods of starvation and to synthesise most vitamins and amino acids. Sequencing also revealed the presence of a number of genes that are thought to encode surface proteins potentially involved in adhesion and immunoregulatory activity. The ability of P. freudenreichii to
utilise lactose has been found to be strain dependent. CIRM-BIA1\textsuperscript{1} possesses a lactose utilisation locus encoding a β-galactosidase, a galactose transporter and an UDP-glucose isomerise. This locus is surrounded by transposable elements, is highly similar to corresponding regions in strains of Clostridium and Mannheimia and, thus, is believed to have been acquired through gene transfer to facilitate the adaptation of the microorganism to the dairy environment. Importantly, genes involved in pathogenicity of Propionibacterium acnes are absent from CIRM-BIA1\textsuperscript{1} (Falentin \textit{et al.}, 2010).

\textbf{Leuconostoc}

The genus \textit{Leuconostoc} consists of 23 species and 4 subspecies (www.bacterio.cict.fr). \textit{Leuconostoc} spp. are frequently associated with plant material, but some, and in particular the species mesenteroides and pseudomesenteroides, are also found in milk. However, it is possible that this is due to their introduction during the collection of milk or subsequent storage and processing. Notably, in this regard, \textit{Leuconostoc} spp. have the ability to survive on surfaces, tools and pasteurisers for long periods of time and to resist heat treatments and refrigeration temperatures (Hemme & Foucaud-Scheunemann, 2004). \textit{Leuconostoc} spp. grow poorly in milk due to a lack of sufficient proteolytic activity and thus require the addition, or generation by other microorganisms, of amino acids or peptides to stimulate growth (Vedamuthu, 1994; Hemme & Foucaud-Scheunemann, 2004). \textit{Leuconostoc} spp. have the ability to produce gas (CO\textsubscript{2}), which is responsible for eye formation in some artisanal raw milk or blue-veined cheeses (Cardamone \textit{et al.}, 2011); metabolise lactose and citrate; and produce lactate, acetate, ethanol, acetaldehyde, diacetyl, acetoain and 2,3-butanediol, which contribute to the organoleptic properties of fermented dairy products (Vedamuthu, 1994; Sanchez \textit{et al.}, 2005). Due to these attributes, \textit{Leuconostoc} spp. can act as beneficial NSLAB cultures. Genome sequencing of the dairy isolate \textit{L. pseudomesenteroides} strain 4882 has further highlighted the beneficial attributes, for example, genes involved in carbohydrate fermentation, protein and amino acid metabolism and a key pathway in production of aromatic compounds from citrate (Meslier \textit{et al.}, 2012a). Notably, while phenotypic assays do not always reliably differentiate between species and subspecies of \textit{Leuconostoc}, molecular methods can facilitate the rapid characterisation of \textit{Leuconostoc} to species level (Duthoit \textit{et al.}, 2003; Sanchez \textit{et al.}, 2006; Martin-Platero \textit{et al.}, 2009).

\textbf{Enterococcus}

Enterococci are the most controversial group of food-associated LAB. Enterococci occupy a diverse range of ecological niches that include the gastrointestinal tracts of humans and animals (Giraffa, 2002) and, depending on the strain in question, can be considered to be starter cultures, probiotics, spoilage or pathogenic organisms (Bhardwaj \textit{et al.}, 2009). Due to their psychrotrophic nature, ability to survive adverse conditions, including high-temperature and high-salinity environments, and adaptability to different growth substrates and growth conditions, enterococci can survive refrigeration. In laboratory-based experiments, strains of this bacterium have been shown to potentially survive pasteurisation and thus may be part of the microbial populations in both raw and pasteurised milk as well as in subsequent products (Giraffa, 2003; Ladero \textit{et al.}, 2011; McAuley \textit{et al.}, 2012). Studies on raw milk cheeses indicate that enterococci are a common, and frequently important component of the natural cultures involved in fermentations and contribute to ripening, taste and flavour (Foulquié Moreno \textit{et al.}, 2006). The most common enterococcal species in milk and dairy products are \textit{E. faecalis} and \textit{Enterococcus faecium}, but others, including \textit{E. durans} (Francisoi \textit{et al.}, 2009), \textit{Enterococcus italicus} and \textit{Enterococcus mundtii}, are also encountered. Enterococci contribute to fermentations due to their proteolytic activity; ability to hydrolyse milk fat; and contribution to the development of flavour compounds, including acetaldehyde, acetoin and diacetyl (Franz \textit{et al.}, 1999). Recent genome sequencing projects identified large gene sets related to dairy adaption, including genes involved in lactose and galacto-oligosaccharide utilisation, in \textit{E. mundtii}. Furthermore, a large number of putative antibiotic resistance determinants have also been found (Magni \textit{et al.}, 2012). Importantly, food isolates of \textit{E. faecalis} lack a large number of genes, which are present in clinical isolates. These traits (including genes for adhesion and an entire prophage) are believed to contribute to the development of human infection (Lepage \textit{et al.}, 2006).

\textbf{Other milk microorganisms with potential technological relevance}

There are some other groups of microorganisms of technological relevance, albeit not regarded as being as important as those discussed above, which are present in low quantities in raw milk. These include a number of bacteria, including Gram-positive and Gram-negative bacteria, as well as yeast and mould populations.

\textbf{Gram-positive subpopulations}

\textit{Corynebacterium} spp. were detected in milk over 40 years ago (Jayne-Williams & Skerman, 1966) and have also been found on the teat surface and throughout the farm
environment (Vacheyrou *et al.*, 2011; Braem *et al.*, 2012). Coryneform bacteria are generally regarded as being important components of the surface of smear-ripened cheese but can also be located in cheese cores (Duthoit *et al.*, 2003). These bacteria can contribute to cheese flavour and aroma due to their ability to produce volatile sulphur compounds giving notes of garlic, onion and even cabbage to the cheese. These compounds result from the production of methanethiol, sulphides, thiols and thioesters (Bloes Breton & Berger, 1997). Sequencing of *Corynebacterium casei* UCMA 3821, *Corynebacterium vari- able* DSM 44702 and *Chryseobacterium bovis* DSM 20583 has revealed genes involved in iron acquisition and uptake, an important feature of cheese surface bacteria. Also present are genes involved in utilisation of alternative carbon and sulphur sources, amino acid metabolism and fatty acid degradation. The genetic repertoire of these strains also highlights their ability to catabolise lactate and propionate; to utilise external caseins; and to produce acetoin, butanediol and methanethiol, which are important with respect to the flavour of smear-ripened cheeses (Monnet *et al.*, 2010; Schröder *et al.*, 2011, 2012).

*Arthrobacter* spp. are commonly isolated from raw milk (Verdier-Metz *et al.*, 2009; Masoud *et al.*, 2012) and are thought to enter from the dairy facility as well as the teat surface (Vacheyrou *et al.*, 2011). While little is known with respect to the influence of these bacteria on cheese development, they are an important microorganism on the surface of smear-ripened cheese where they contribute to colour, flavour and textural development. *Arthrobacter arilaitensis* is perhaps the most important cheese-associated species, and genome sequencing of strain Re117 reveals the presence of several genes that reflect its adaptation to growth in dairy/cheese environments including salt tolerance, galactose metabolism and enzymes for catabolism of fatty acids, amino acids and lactate. As with other smear-associated bacteria, *Arthrobacter* spp. possess a gene set involved in iron transport (Monnet *et al.*, 2010). Similarly, *Brevibacterium*, which is commonly detected in raw milk (Desmasures & Gueguen, 1997; Lafarge *et al.*, 2004; Raats *et al.*, 2011; Masoud *et al.*, 2012), is known for its association with characteristic taste, aroma and colour of smear-ripened cheeses. *Brevibacterium linens* is particularly important in this regard (Irlinger & Mounier, 2009). Another genus detected in raw milk, *Carnobacterium*, consists of 11 species (www. bacterio.cict.fr; Cailliez-Grimal *et al.*, 2005). The *Carnobacterium* species most frequently isolated from dairy environments is *Carnobacterium maltaromaticum*. *Carnobacterium maltaromaticum* was first isolated from milk in 1974 and was originally named *Lactobacillus malt- aromaticus* (Mora *et al.*, 2003). Carnobacteria are slow acidifiers and therefore are not suitable for the use as starter cultures but can be considered to be beneficial NSLAB due to their aromatic and flavour contributing end products (Afzal *et al.*, 2010). These include malty aromas as well as alcohol and fruity odours in cheese, although some strains have been linked with sweat, faecal and rotten-fruit-associated flavours (Marilley & Casey, 2004). The occurrence of *Carnobacterium* in dairy products is probably underreported due to the frequent use of acetate-containing media, such as MRS medium, as acetate inhibits the growth of these microorganisms (Leisner *et al.*, 2007).

*Bifidobacterium* represents an important genus, which is generally regarded as a health-promoting genus and is most commonly associated with the gastrointestinal tract of humans and animals (Lamendella *et al.*, 2008). It is also frequently detected in raw milk and fermented dairy products (Delcenserie *et al.*, 2005), despite the fact that many *Bifidobacterium* strains have stringent nutrient requirements and are generally thought to grow poorly outside of the gut (Lamendella *et al.*, 2008). In dairy products, the presence of bifidobacteria results in increased levels of lactate and acetate but does not influence sensory or textural properties (Dinakar & Mistry, 1994). Finally, the significance of coagulase-negative staphylococci (CNS) with respect to dairy fermentations has been the subject of much debate. The species typically isolated from milk include *Staphylococcus equorum*, *Staphylococcus xylosus* and *Staphylococcus carnosus*. These bacte- ria are salt tolerant and acid tolerant. Although no CNS of dairy origin have been associated with food poisoning or human pathology, a few cases of nosocomial infection caused by *Staphylococcus caprae*, *Staphylococcus capitis* or *Staphylococcus sciluri* have been reported in patients with depressed immune systems (Irlinger, 2008). A recent study of isolates from milk and cheese revealed 17 CNS species. Ten of these contained transferable antibiotic resistance genes, and one-third exhibited haemolytic activity (Ruaro *et al.*, 2012), indicating that the safety of these bacteria must continue to be assessed.

**Gram-negative subpopulations**

Gram-negative bacteria are very common in dairy foods. They can reach high levels (10⁹–10⁸ CFU g⁻¹) in cheeses and usually consist of a diverse number of species. Although Gram-negative bacteria are regularly considered as indicators of poor hygiene and may constitute a health risk if pathogenic species are present, some may play roles in dairy fermentations by contributing positively or negatively to the sensory quality of dairy products (Delbès-Paus *et al.*, 2011, 2012). These issues are addressed in greater depth in this section.

The presence of high numbers of Gram-negative bacte- ria in milk has been noted in situations where hygiene
standards are low and generally reflect poor udder preparation, poor sanitation or deficiencies with respect to the hygiene of equipment. In one instance, milk sampled at the farm, at milk collection and at milk transportation was found to be contaminated with *E. coli* (29.6%), *Pseudomonas aeruginosa* (18.5%) and *Klebsiella pneumoniae* (16.7%) and, to a lesser extent, *Enterobacter aerogenes, Alcaligenes faecalis, Proteus mirabilis* and *Citrobacter freundii*. It was noted that no Gram-negative bacteria were isolated from pasteurised milk samples (Garedew et al., 2012). In a study investigating the presence of Gram-negative bacteria in different cheeses produced in France, 173 isolates were isolated. Nearly half of all isolates were representatives of *Enterobacteriaceae*. Overall, 26 different genera were present. The most frequent isolates included *Proteus*, *Psychrobacter*, *Halomonas*, *Serratia* and *Pseudomonas*, representing almost 54% of the total isolates. Milk and cheese core samples also contained *Chryseobacterium*, *Enterobacter* and *Stenotrophomonas*, while surface samples were dominated by *Proteus*, *Psychrobacter*, *Halomonas* and *Serratia* (Coton et al., 2012). When a model cheese system was employed to assess the consequences of the presence of some of these Gram-negative bacteria, it was established that the majority had little influence on colour, odour and volatile compounds (Delbès-Paus et al., 2011). However, *Hafnia alvei* did contribute to the production of volatile compounds and of volatile sulphur compounds in particular. Furthermore, *Psychrobacter celer* was found to flourish within the cheese surface smear during ripening, contributing to the production of volatile compounds such as aldehydes, ketones and sulphur compounds (Delbès-Paus et al., 2011; Irlinger et al., 2012). Another Gram-negative species, *Proteus vulgaris* 1 M10, has been shown to produce high concentrations of flavour compounds, particularly branched-chain alcohols during ripening (Deetae et al., 2009). These studies reveal the high biodiversity of Gram-negative bacteria among raw milk and dairy products and suggest that they may play a role in dairy fermentations. However, the fact that one of the studies referred to above revealed that c. 50% of the Gram-negative strains isolated were resistant to several antibiotics is a particular cause for concern (Delbès-Paus et al., 2011). Given this observation, and the association of particular Gram-negative bacteria with milk spoilage or disease, the presence of Gram-negative bacteria in these products will in general continue to be regarded as undesirable.

**Fungal populations**

Yeasts and moulds can also be important microbial populations within raw milk. The fungal composition of raw milk can be influenced by the physiological state of the animal, as well as the weather, feeding and season (Callon et al., 2007; Vacheyrou et al., 2011). As with bacteria, the extent of the fungal population in raw milk and dairy products is often underestimated. However, the development of culture-independent DNA-based methods, such as those targeting the internal transcribed spacer (ITS) region of fungi, has addressed this issue (Callon et al., 2006; Alessandria et al., 2010). This ITS region of fungi is particularly useful because of its high copy number, phylogenetic utility and the availability of universal primers to generate PCR amplicons. This and other culture-independent approaches, such as denaturing high-performance liquid chromatography, have led to the detection of fungi, for example, *Torrubiella* and *Malassezia*, which had not previously been detected in milk (Delavenne et al., 2011). While a relatively small number of yeast species occur in raw milk, they are persistent and can be detected at relatively high levels, that is, $10^5$–$10^6$ CFU mL$^{-1}$ (Lagneau et al., 1996). Yeasts species that have been detected in raw milk include *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Rhodotorula mucilaginosa*, *Debaryomyces hansenii*, *Geotrichum candidum*, *Geotrichum catenulatum*, *Pichia fermentans*, *Candida sake*, *Candida parapsilosis*, *Candida inconspicua*, *Trichosporon cutaneum*, *Trichosporon lactis*, *Cryptococcus curvatus*, *Cryptococcus carnescens* and *Cryptococcus victoriae* (Delavenne et al., 2011). Yeasts can play a major role in dairy fermentations due to a number of their physiological and biochemical characteristics, including the ability to utilise lactose or galactose, for example, in *D. hansenii* (Van den Tempel & Jakobsen, 2000); high proteolytic or lipolytic activity, for example, in *Yarrowia lipolytica* and *G. candidum* (Sacristán et al., 2012); and the ability to grow at low temperatures and to tolerate high salt concentrations. In cheese, yeasts secrete enzymes that play a key role in texture and produce various aromas during ripening. *Kluyveromyces marxianus* is of particular interest due to its fast growth rate, thermotolerance, the ability to assimilate a wide range of sugars, the secretion of lytic enzymes and the production of ethanol by fermentation (Lane & Morrissey, 2010). Moulds are typically present at lower levels than yeasts (Arora et al., 1991). Moulds have the ability to enhance the flavour and aroma and modify the texture and structure of milk-derived products as a consequence of bringing about extensive proteolysis and lipolysis. The mould genera that are most commonly detected in raw milk include *Penicillium*, *Geotrichum*, *Aspergillus*, *Mucor* and *Fusarium* (Lavoie et al., 2012). At the species level, *Fusarium merismoides*, *Penicillium glabrum*, *Penicillium roqueforti*, *Aspergillus fumigatus*, *Engyodontium album*, as well as species of *Cladosporium* and *Torrubiella* are common (Delavenne et al., 2011).

In recent years, genome sequencing has been particularly useful with respect to enhancing our understanding
of milkborne microorganisms by, for example, highlighting the phenomenon of genomic decay and adaptation to the milk environment, revealing potential sources of evolutionary origin and identifying genes that contribute to flavour development in dairy products. This information can allow industry to develop novel starter cultures, determine how best to enhance the efficacy of existing strains and provide re-assurances in terms of the 'GRAS' status of some of these technologically important microorganisms.

**Impact of storage conditions and downstream treatments on the microbiology of raw milk**

**Cold storage**

It is important to understand the changes that can occur in the microbiology of raw milk during its storage and as a consequence of subsequent treatments. Milk is typically stored at refrigeration temperatures that reduce the growth of most bacteria, with the exception of psychrotolerant microorganisms that can proliferate under these conditions and become a major cause of milk spoilage (Eddy, 1960; Morita, 1975; De Jonghe et al., 2011). This is primarily a consequence of the production of extracellular enzymes, with lipases and proteases being most important. These lipases degrade milk fat causing rancidity, while proteases degrade casein producing a grey colour and bitter off-flavours (De Jonghe et al., 2011). Investigations into seasonal variations of microbial growth in raw milk have, unsurprisingly, established that psychrotolerant bacteria exhibit better growth and protease production in winter milk rather than in summer milk (Marchand et al., 2008). *Pseudomonas* spp., which are commonly found in raw milk, are the most common cause of milk spoilage (Ercolini et al., 2009). The *Pseudomonas* species most commonly detected in milk and cheeses are *Pseudomonas fluorescens*, *Pseudomonas gessardii*, *Pseudomonas fragi* and *Pseudomonas lundensis* (Mallet et al, 2012). These bacteria can become the predominant microorganisms in raw milk stored at low temperatures, constituting up to 70–90% of the microbial population (Sorhau & Stepaniak, 1997). Many other psychrotolerant microorganisms are present in milk but are generally less important than *Pseudomonas* with respect to milk spoilage. In one study, these were identified as being strains of *Acinetobacter*, *Microbacterium*, *Aeromonas*, *Enterobacter*, *Flavobacterium*, *Corynebacterium*, *Clostridium*, *Bacillus*, *Staphylococcus* and some LAB (Hantsis-Zacharov & Halpern, 2007). Another study, assessing the overall impact of refrigeration (24 h) on the microbial content of raw milk, particularly noted increases in the number of *Listeria innocua*, *L. monocytogenes*, *L. fermentum*, *Staphylococcus epidermidis*, *P. fluorescens*, *E. faecium*, *Enterococcus hirae*, *E. durans*, *Leuconostoc carnosum*, *S. dysgalactiae*, *H. alvei*, *Serratia marcescens*, *K. pneumoniae*, *Kocuria rosea*, propionic acid bacteria and *Aeromonas* (Lafarge et al., 2004). Notably, some of the latter would be typically regarded as thermophilic microorganisms. A similar study, but carried out over a 48-h period, specifically highlighted increases in *Pseudomonas* and *Acinetobacter* spp. (Raats et al., 2011). Newly identified psychrotrophs, such as *Chryseobacterium* (Hantsis-Zacharov & Halpern, 2007; Hantsis-Zacharov et al., 2008a, b) and *Epilithimonas* spp. (Shakéd et al., 2009), have also been detected in raw milk. However, their involvement in milk spoilage is unclear. A number of psychrotrophic spore-forming species have also been identified and will be discussed in the next section. Yeasts and moulds have also been associated with milk spoilage (Agarwal et al., 2012).

**Pasteurisation**

Pasteurisation of raw milk is carried out to reduce the microbial load of milk and, in particular, to limit the number of spoilage microorganisms and to prevent foodborne disease. However, this process also reduces the number of microorganisms that would typically contribute to desirable sensory properties associated with raw milk cheeses. In these instances, starter cultures that are known to generate desirable flavours and aromas, as discussed above, are added to the milk postpasteurisation. The typical milk pasteurisation treatment is a 'high-temperature short-time' (HTST) approach involving heating to 72 °C for 15 s. Some countries have increased the exposure temperature and/or time (Martin et al., 2011). While this can help to further reduce bacterial counts (Fromm & Boor, 2004) and to eliminate microorganisms of concern including *Mycobacterium avium* ssp. paratuberculosis (MAP; Grant et al., 2002) and *L. monocytogenes* (Doyle et al., 1987), there have also been some suggestions that this approach can encourage the activation of spores, which may be dormant in milk (Ranieri et al., 2009). The heat treatment of milk typically reduces psychrotrophic and mesophilic populations, leaving two main groups to consider thereafter, that is, thermoduric microorganisms and bacteria introduced through postpasteurisation contamination. Following pasteurisation, some microorganisms may enter into a 'viable but nonculturable' state, meaning that they may be underestimated by traditional culture methods (Bartošze, 2009). The findings of a recent culture-independent study conducted by our group are consistent with this theory, revealing a more diverse bacterial population in pasteurised milk than expected (Quigley et al., 2013).
When one considers thermoduric bacteria, it is particularly important to keep the issue of spore-forming microorganisms in mind. These bacteria may enter the milk chain from soil, silage and bedding material and, significantly, are resistant to pasteurisation. Spore formers such as Clostridium sporogenes, Clostridium butyricum and Clostridium tyrobutyricum have the potential to survive and grow at refrigeration temperature, as well as the potential to utilise carbohydrates, proteins and lactate from milk (Driehuis, 2013). Indeed, clostridia have been identified in raw milk quite frequently (Herman et al., 1995; Lopez-Enriquez et al., 2007; Cremonesi et al., 2012) and can contribute to the spoilage of subsequent cheese products by causing a late blowing defect, which is particularly associated with C. tyrobutyricum, leading to off-flavours and textural defects in cheese (Cocolin et al., 2004; Le Bourhis et al., 2005). Culture-independent DGGE/TTGE has identified C. tyrobutyricum, but also C. sporogenes, C. butyricum and Clostridium beijerinckii as possible causes. Other spore-forming contaminants of milk include B. cereus, Bacillus sporumthermodurans and Geobacillus stearotherophilus. Bacillus cereus is a major spoilage organism of pasteurised milk and milk products stored at refrigeration temperature, causing off-flavour and curdling. This bacterium is also a concern for food safety as it can produce different types of toxins and is a potential food-poisoning agent (Driehuis, 2013). In the EU in 2010, 3.8% of all milk samples tested were positive for Bacillus toxin (European Food Safety Authority, 2012).

**Bacteriophage**

Lytic bacteriophages are other spoilage agents that are naturally present in raw milk. These viruses infect bacteria and, after intracellular replication, lyse their host cells (Marcó et al., 2012). Phages can survive at low temperature; resist a variety of different treatments; and negatively affect the quality, safety and value of dairy products (cheese in particular). Phages that target important starter or adjunct species, such as Lactococcus lactis, S. thermophilus, L. helveticus and L. delbrueckii, have long been associated with causing a delay or disruption of fermentation processes, resulting in slow acidification, undesirable organoleptic properties or complete loss of batches (Emond & Moineay, 2007). Raw milk is the most prominent source of phages within the dairy environment, with concentrations ranging from $10^3$ to $10^6$ phage mL$^{-1}$ (Madera et al., 2004). Phages can also gain access to the dairy environment by aerosols, personnel, equipment, work surfaces and dairy byproducts (Verreault et al., 2011). Thus, attaining a phage-free environment is not a realistic goal. While the phage concentration is higher in raw milk products, it has also been reported that many dairy phages are able to survive the pasteurisation of milk (Suarez & Reinheimer, 2002; Abedon, 2009). Due to the severe economic loss that phages can cause, constant monitoring of the environment is required. Traditionally, standard microbiological methods were employed. However, molecular methods based on PCR and quantitative PCR allow the rapid detection and classification of phages from different dairy matrices (Binetti et al., 2008). While many dairy bacteria can be infected by phage, there are a number of industrially important strains that have inherent resistance to phage. Genome sequencing studies have revealed that a number of strains, such as L. helveticus DPC 4571, P. freudenreichii CIRM-BIA1T and some S. thermophilus strains to name but a few, possess features to help these bacteria withstand phage attack.

**Biopreservative potential of raw milk microorganisms**

The production of an antimicrobial can be regarded as a beneficial probiotic trait. The diverse populations in raw milk produce many antimicrobials including bacteriocins, antifungals, organic acids and hydrogen peroxide. As noted above, raw milk also contains phages. These might also be regarded as biopreservative agents that could be used to extend the shelf life and safety of fermented and other foods (Stiles, 1996).

Bacteriocins are antimicrobial peptides or proteins produced by bacteria and are typically active against closely related species, but can exhibit activity across broad genera. Bacteriocin producers are naturally immune to their own bacteriocins (Cotter et al., 2005). The most recent classification groups LAB bacteriocins into two classes; class I are post-translationally modified bacteriocins, and class II are unmodified bacteriocins (Rea et al., 2011). Many LAB isolated from raw milk produce putative bacteriocin-like compounds and exhibit activity against L. monocytogenes, S. aureus, C. tyrobutyricum, C. sporogenes, E. faecalis, E. faecium and E. durans (Alegria et al., 2010; Ortolani et al., 2010; Perin et al., 2012). Some potential raw milk-derived bacteriocins have been characterised in-depth. Lactococcus lactis strains produce the best characterised bacteriocin, nisin. Nisin is well known due to its use in biopreservation throughout the world because of its wide spectrum of activity (Delves-Broughton, 1990). Lactococcus lactis strains isolated from raw milk and raw milk products are capable of producing nisin with activity against L. monocytogenes as well as other pathogens including E. coli and Staphylococcus spp. (Bravo et al., 2009; Alegria et al., 2010; Ortolani et al., 2010; Cosentino et al., 2012; Perin et al., 2012). A huge variety of other bacteriocins are produced by L. garvieae (Villani et al., 2001; Florez et al., 2012), S. thermophilus
(Gul et al., 2012), S. macedonicus (Georgalaki et al., 2002, 2013), enterococci and Leuconostoc spp. (Mathieu et al., 1993; Casaus et al., 1997; Giraffa & Carminati, 1997; Achemchem et al., 2006; Izquierdo et al., 2009; Mirhosseini et al., 2010) and are active against many spoilage and pathogenic microorganisms. In addition to contributing to the control of pathogens and spoilage microorganisms in raw milk and resultant products, these bacteriocins can also be employed, through the addition of producing LAB, fermentates or semi-purified preservatives, to enhance the safety of other foods (Cotter et al., 2005; Deegan et al., 2006).

Raw milk LAB isolates also produce organic acids, hydrogen peroxide and diacetyl (Stiles, 1996). These compounds can inhibit many potential pathogens and food spoilage microorganisms (Batdorj et al., 2007). Some strains of L. acidophilus, L. casei, L. helveticus, L. bulgaricus, S. thermophilus and Lactococcus lactis are also able to transform hippuric acid, which is naturally present in milk, into benzoic acid, thereby providing another natural preservative in milk products (Garmeine et al., 2010). Finally, common food-spoiling moulds and, to a lesser extent, yeasts can be inhibited by LAB metabolites from strains of L. casei, L. reuteri, L. plantarum and L. buchneri and strains of dairy propionibacteria (Lind et al., 2005, 2007; Voulgari et al., 2010; Delavenne et al., 2011).

Human health associations

Pathogenic bacteria associated with raw milk

Milk and dairy products are important staples of a healthy diet. However, if pathogenic microorganisms are not removed by pasteurisation, consumption of these products can represent a serious health risk. As mentioned above, these pathogens can originate from the mammary gland or associated lymph nodes of cows suffering from systemic diseases or infections (Oliver & Murinda, 2011; Hunt et al., 2012) or from equipment, raw milk tankers and personnel (Rosengren et al., 2010; Teh et al., 2011; Giacometti et al., 2012). Ingestion of these microorganisms can lead to illnesses of varying severity. Typical symptoms can include fever, nausea, vomiting, diarrhoea and abdominal pains; in extreme cases, death can occur (Langer et al., 2012). Indeed, food poisoning from consumption of raw milk and such products over a period of 13 years (1993–2006) in the United States resulted in 1571 reported incidences with 202 hospitalisations and 2 deaths. The main cause of illness was consumption of raw milk products contaminated with Salmonella spp., Listeria spp., E. coli, Campylobacter spp., Brucella spp. or Shigella spp. (Langer et al., 2012). A recent review, which examined multiple reports of milkborne pathogen detection in bulk tanks throughout different countries, found that the percentage of tanks containing the different pathogens varied greatly. The occurrence of Salmonella ranged between 0% and 11.8% of milk samples in the USA/Canada and between 1.4% and 4% of samples in Asia, while the percentage of milk samples that was positive for the presence of Listeria ranged from 0% to 7% in USA/Canada and from 0% to 1.9% in Asia. Campylobacter jejuni was detected at 2% and 9.2% of milk samples from the USA, while the percentage of bulk tank milk worldwide that was positive for shiga-toxin-producing E. coli varied from 0% to 33.5%. Mycobacterium avium ssp. paratuberculosis was present in European samples at frequencies of 1.6–19.7% and in Asia at 8.6–23%. Brucella was present in milk in Africa at 0–10%, increasing with increasing farming intensity (Oliver & Murinda, 2011).

Regardless of the frequency at which they are present, these pathogens can impact, in some instances severely, on health. Staphylococcus aureus can be transferred to milk through the teat canal, equipment, the environment or human handling (Rosengren et al., 2010) and cause illness through the production of heat-stable enterotoxins, which can withstand pasteurisation (Balaban & Rasooly, 2000). Staphylococcal toxins were detected in 18.4% of cheeses assessed across the EU in 2010 (European Food Safety Authority, 2012). Coxiella burnetii, the causative agent of Q fever, can infect many animal species, and it is thought that its association with cows, sheep and goats is the main source of human infection. The infection may be acute, presenting flu-like symptoms that are self-limiting or chronic, leading to endocarditis and hepatitis (Maurin & Raoult, 1999). C. burnetii is shed by the animal host through birth products, vaginal mucus, semen, faeces, urine and milk (Guatteo et al., 2006). While this bacterium can persist in dairy cattle populations (Astobiza et al., 2012; Tilburg et al., 2012), the consumption of raw or insufficiently pasteurised milk is rarely identified as a source of Q fever (Guatteo et al., 2006). Another zoonotic bacterium of health concern is Mycobacterium bovis. This bacterium causes the disease bovine tuberculosis in animals, with symptoms including fever, weakness, emaciation, inappetence and respiratory distress, and can lead to severe economic loss yearly (Thoen et al., 2006). This pathogen can also spread to humans through the ingestion of raw milk causing zoonotic tuberculosis, which is indistinguishable from human tuberculosis (Thoen et al., 2006). Typically, this concern is removed with pasteurisation, but remains a problem in instances where raw milk is still consumed daily (Coker et al., 2006; De la Rua-Domenech, 2006). Mycobacterium avium ssp. paratuberculosis (MAP) is the causative agent of paratuberculosis or John’s disease, which primarily infects domestic animals. MAP survives and multiplies in

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the intestinal tract mucosa, where it causes both a decrease in the absorption of nutrients and chronic diarrhoea with consequent 'wasting away' of the animals. Animals may harbour this bacterium for a long period before symptoms arise, making them dangerous vectors of infection. MAP can be shed into the external environment in animal faeces or milk. Recently, there has been increased concern with the association of MAP and Crohn’s disease in humans, an inflammatory bowel disease, whose symptoms include abdominal pain, diarrhoea, vomiting and weight loss. High prevalence of MAP has been reported in raw milk in developed countries (Argentina 8.3%, Czech Republic 2%, Ireland 0.3%, UK 6.9%, USA 0–28.6%; Slana et al., 2008). Current commercial pasteurisation standards may reduce the number of viable MAP, but do not ensure destruction (Gao et al., 2002). However, the link between Crohn’s disease and MAP still remains controversial and unclear (Chiodini et al., 2012). Another, relatively new, pathogen of concern to the dairy industry is Shiga-toxin-producing E. coli (STEC). Although cows are the main reservoir for STEC, other domestic animals including goats and sheep can also harbour this bacterium in their gastrointestinal tracts without any symptoms of disease and shed them from their faeces. If hygiene standards are not sufficiently high, milk can become contaminated during milking or processing. There are nine virulence genes associated with STEC strains. Two toxin genes, stx1 and stx2, appear to be associated with bovine dairy products, while stx1c and stx2b are more frequently associated with strains from sheep and goat milk (Martin & Beutin, 2011).

Of the milkborne pathogens, L. monocytogenes, Yersinia enterocolitica and Brucella spp. are a particular cause for concern as they are able to survive and multiply at refrigeration temperatures and may cause severe diseases. Yersinia enterocolitica is a major cause of acute gastroenteritis (Schiemann & Toma, 1978). The symptoms of illness can include diarrhoea, abdominal pain and fever and may mimic appendicitis, occasionally leading to misdiagnosis (Ackers et al., 2000). Although pasteurisation will kill Y. enterocolitica, if insufficient pasteurisation or recontamination occurs, the bacterium can multiply under refrigeration temperatures (Schiemann & Toma, 1978). Similarly, during dairy product manufacture, where hygiene standards are poor, Y. enterocolitica can become prevalent (Harakeh et al., 2012). In 2010, Yersinia incidence in raw milk and low-heat-treated milk products was low, with only two positive results reported within the EU (European Food Safety Authority, 2012). Listeria monocytogenes, a common environmental isolate, causes the human disease listeriosis, which targets highly susceptible individuals, including pregnant, immunocompromised or elderly people, and has a high fatality rate. Healthy adults are typically not at risk although they may experience flu-like or gastrointestinal symptoms (Liatsos et al., 2012). There is evidence to suggest that raw milk purchased from retailers represents a greater risk of Listerial-associated illness than milk obtained directly from milk tanks on farms, most likely as a consequence of the growth of the pathogen over the extended storage period (Latorre et al., 2011). Brucella spp. primarily cause disease in animals and from there are thought to enter into the milk supply. On consumption, these pathogens can provoke brucellosis, which leads to fever, abdominal pain, headaches and personality changes (Roop et al., 2004). Like Listeria, Brucella can survive and multiply in milk (Falenski et al., 2011), also following contamination after pasteurisation (Oliver et al., 2005). Due to the severe nature of many of the illnesses caused by milkborne pathogens, it is important to test rigorously for their presence. While traditional methods can be laborious and time-consuming, newer culture-independent methods have been investigated, with quantitative PCR being particularly rapid and sensitive (Quigley et al., 2011). However, these have yet to be implemented on a large scale by the dairy industry. While detection is important, the practices that prevent or limit the presence of pathogens are more crucial. Thus, it is important to implement a hygiene system that begins at the farm level and includes a focus on cow health and hygiene, equipment cleanliness, overall farm and personnel sanitation, correct storage and subsequent processing of milk.

Mycotoxins, that is, low molecular weight compounds produced as secondary metabolites by filamentous fungi, can lead to illness in humans with symptoms such as nausea, vomiting, diarrhoea and headache (Creppy, 2002), and some mycotoxins have carcinogenic potential (Murphy et al., 2006). The most important genera of food mycotoxigenic fungi are Aspergillus, Alternaria, Fusarium and Penicillium. Examples of mycotoxins of greatest public health and agro-economic significance include aflatoxin, trichothecenes, zearalenone, fumonisins and ochratoxin. After their intake by cows, mycotoxins follow a typical pharmacokinetic cascade of uptake from the gastrointestinal tract to the blood, internal distribution, metabolism, storage, remobilisation and excretion. The rumen has an important function in the metabolism of mycotoxins with some mycotoxins being rapidly metabolised to less toxic metabolites (e.g. ochratoxin); some are transformed into equally toxic or more toxic metabolites (e.g. zearalenone), while some are not transformed at all (e.g. fumonisins). Aflatoxin B1 is transformed into aflatoxin M1 in the liver of ruminants. While M1 is less mutagenic and genotoxic than B1, the cytotoxicity of M1 and B1 is similar. Notably, aflatoxin B1 is the only mycotoxin with significant carry-over into milk. Between 1%
and 6% is excreted in milk as aflatoxin M₁. Sixty countries now have regulations with respect to the presence of aflatoxin M₁ in milk, with limits of 0.05–0.5 μg kg⁻¹; the EU has a legal limit of 50 ng L⁻¹ (Driehuis, 2013). Testing is important given that one study has shown that a high percentage (83.2%) of raw milk samples in Portugal were positive for aflatoxins (Martins & Martins, 2000) and that the levels of aflatoxin B₁ may frequently exceed recommended limits (Nordkvist & Hoofard, 2012). Finally, yeasts, and especially Candida species, can be opportunistic pathogens, causing infections in immunocompromised patients. Debaryomyces hansenii and Y. lipolytica may also be emerging dairy pathogens. These microorganisms cause rare infections in immunocompromised patients, which are generally mild and either self-limiting or easily treated (Jacques & Casaregola, 2008).

**Antibiotic residues and antibiotic-resistant bacteria in milk**

Antibiotics have been employed to treat bacterial diseases over the past 70 years. The greatest threat to the successful application of antibiotics has been the development of resistance, particularly in pathogenic bacteria. Resistance can be intrinsic or acquired. Intrinsic resistance is a natural characteristic of a microorganism that allows it to grow in the presence of the corresponding antibiotic. Acquired resistance results either from spontaneous mutation in the bacterial genome or from the acquisition of genes encoding resistance through transduction, conjugation or transformation (Davies, 1997).

Although lactococci, enterococci and lactobacilli are intrinsically resistant to some antibiotics (Mathur & Singh, 2005), the strains of these that are found in foods are typically quite sensitive to clinically important antibiotics such as ampicillin, penicillin, gentamicin and vancomycin (Franz et al., 1999; Mannu et al., 2003; Čanžek Majhenič et al., 2005; Herrerros et al., 2005; Mathur & Singh, 2005). Furthermore, Leuconostoc strains are generally sensitive to antibiotics (Swenson et al., 1990). However, it is still important to assess the frequency with which antibiotic-resistant isolates occur in milk. A recent study determined that psychrotrophs, including P. fluorescens, P. aeruginosa, Stenotrophomonas maltophilia, Burkholderia species, as well as a number of unidentified psychrotrophs, isolated from milk harbour resistance to several β-lactam and non-β-lactam antibiotics. This trait appears to increase in occurrence through the cold chain transportation of raw milk (Munsch-Alatossava & Alatossava, 2007). Bacteria of the genus Acinetobacter isolated from raw milk have also exhibited antibiotic resistance (Gurung et al., 2013). These bacteria are widespread in nature, including soil and water, and are opportunistic pathogens in humans; multi-drug-resistant strains are a serious concern (Dijkshoorn, 2013). Other antibiotic-resistant raw milk isolates include Lactococcus lactis displaying resistance to tetracycline, clindamycin and erythromycin and L. garvieae exhibiting resistance to tetracycline, streptomycin and quinupristin–dalfopristin (Walther et al., 2008).

The use of antibiotics to treat animals that are in the food chain can obviously compound this issue by selecting for the development of antibiotic resistance among food microorganisms (in particular in cows’ milk) and by exposing consumers to antibiotic residues in milk and other dairy foods (Doyle et al., 2013). The use of antibiotics to treat mastitis during lactation is common, as between 2% and 55% of cows encounter a mastitis infection during this period (Kelton et al., 1998). Notably, bacterial strains associated with bovine mastitis, including many S. aureus isolates, have demonstrated resistance to antibiotics such as penicillins, oxacillin, streptomycin and/or gentamicin (Thaker et al., 2013). These problems can be limited through the withholding of milk from sale in situations where a cow has mastitis and is being treated with antibiotics or during a compulsory withdrawal period after antibiotic treatment. Safeguards, such as those introduced by Codex Alimentarius 2009 and European Union Council Regulation 37/2010/EC, require the monitoring of milk and provide limits with respect to the concentration of antibiotic residues that are tolerated in milk for commercial use.

**Health-promoting microorganisms**

Some raw milk isolates can have health-promoting abilities; for millennia, it has been suggested that fermented milk can cure some disorders of the digestive system, and biblical scriptures highlight the use of milk to treat body ailments (Lourens-Hattingh & Viljoen, 2001). In 1907, the Russian scientist Elie Metchnikoff pointed out the benefits of consuming a diet of fermented milk (Rasić & Kurmann, 1983). Health-promoting bacteria isolated from these beverages and other sources are commonly referred to as ‘probiotics’, that is, ‘live bacteria which when administered in adequate amounts confer a health benefit on the host’ (FAO/WHO, 2002). The selection of such bacteria for commercial probiotic application relies on criteria relating to safety, technological and digestive stress survival; intestinal cell adhesion; and human origin. The latter two criteria are controversial, and it is now recognised that adhering to these criteria should not be mandatory, although may be desirable in certain instances. Notably, many raw milk isolates have desirable probiotic traits. These include the ability to survive bile juice, to tolerate gastric acid conditions and to adhere to intestinal cells (Jamaly et al., 2011). Probiotic lactobacilli typically inhibit
pathogenic organisms, reduce lactose intolerance, increase the immune response and often are gastrointestinal isolates (Kopp-Hoolihan, 2001; Maragkoudakis et al., 2006). However, there are a number of dairy lactobacilli isolates, which also have demonstrated efficiency as probiotic strains (Maragkoudakis et al., 2006). Other milk and dairy isolates that exhibit probiotic properties include strains of Lactococcus lactis as well as a variety of Pediococcus, Leuconostoc, Enterococcus and Streptococcus isolates (Kim et al., 2006; Premalatha & Dhasarathan, 2011; Espeche et al., 2012; Floros et al., 2012; Forghani et al., 2012). Strains of P. freudenreichii, and to a lesser extent P. acidipropionici, have begun to attract attention as potential probiotics as a consequence of studies revealing an ability, either alone or in combination with other probiotics, to reduce pathogen adhesion to mucus (Collado et al., 2008), increase bifidobacteria counts in the gut, aid in restoring a healthy gut microbiota, improve bowel movement, alleviate inflammatory disorders and reduce allergy development in infants (Jan et al., 2002; Cousin et al., 2012). Finally and from a fungal perspective, the dairy yeast Pichia fermentans has demonstrated some probiotic potential, and it has been suggested that together with strains of Pichia kudriavzevii and Y. lipolytica, P. fermentans could serve as probiotics that assimilate cholesterol (Chen et al., 2010). Regardless of the specific microorganism in question, dairy products are an excellent vehicle for probiotics, regardless of their source, due to their buffering capacity and fat content, which can help protect the bacteria during gastric transit.

There has been quite a degree of focus on the use of dairy microorganisms to control hypertension. The rennin–angiotensin–aldosterone system is a key factor in the maintenance of arterial blood pressure. One of the main components of this system is angiotensin-converting enzyme (ACE). As ACE plays an important role in the regulation of arterial blood pressure, inhibition of this enzyme can generate an antihypertensive effect. ACE-inhibitory drugs are commonly used to control arterial blood pressure. Raw cows’ milk can be a source of antihypertensive activity (Meisel, 2005). LAB that release bioactive peptides with this activity include strains of E. faecalis, Lactococcus lactis ssp. cremoris, L. helveticus, L. fermentum, L. rhamnosus, L. paracasei and L. acidophilus (Muguerza et al., 2006). The antihypertensive properties of these microorganisms are being investigated and exploited by industry with a view to producing health-promoting drinks. Indeed, L. helveticus is currently used in the production of fermented drinks such as Evolus (Valio Ltd., Valio, Finland) and Calpis (Calpis Food Industry Co. Ltd., Tokyo, Japan), which have properties associated with a reduction in blood pressure through the inhibition of ACE as a consequence of the production of bioactive tripeptides (Slattery et al., 2010).

Raw milk and the raw milk microbiota have also been the focus of attention with respect to alleviating allergy. Allergy to cows’ milk affects 2.5% of children below 3 years of age due to the presence of caseins and β-lactoglobulins (Cocco et al., 2003; Gaudin et al., 2008). The bacterial fermentation of milk proteins, particularly by highly proteolytic Lactobacillus populations, results in a reduction in the allergenic properties of cows’ milk (El-Ghaish et al., 2011). Others have suggested a link between farm living, including the consumption of raw milk and raw milk microorganisms, and protection against the development of asthma and atopy later in life (Debarry et al., 2007; Ege et al., 2012). If confirmed, further investigations will be required to determine whether overall microbial load or specific components of the microbial population are responsible.

Conclusion

The microbial community within raw milk is complex. The dominant, and subdominant, microorganisms present in raw milk can have a variety of influences on the flavour, taste and texture of raw milk-derived products (Fig. 1). A number of these microorganisms also have the potential to contribute to health through the production of antimicrobials or possessing other probiotic-associated traits. Through modern genomics-based analysis, it has been established that many of these microorganisms have become adapted to milk niches from various sources, including plant and gut environments, through genomic evolution and gene gain and/or loss. Despite the beneficial impact of many milk-associated microorganisms from a flavour, technological or health-related perspective, it is clear that there can be significant risks associated with the consumption of raw milk and raw milk-derived products or, more specifically, of the pathogens that can be found therein. While many of these microorganisms gain entry to the milk from equipment and/or personnel, zoonotic pathogens can also be introduced into milk from unhealthy animals. As a consequence of this risk, pasteurisation or other treatments are employed to remove disease-causing microorganisms. In the food industry, the negative impact of removing LAB and other bacteria on subsequent food fermentations has been addressed for some time through their re-introduction in the form of starter and adjunct cultures. Similarly, once established definitively, it may be possible to restore the benefits associated with the consumption of raw milk and specific microorganisms therein, through the re-introduction of these microorganisms after processing. Thus, the microbial composition of raw milk is likely to continue to be the focus of much attention into the future.
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Raw milk microbiota


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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Representative nutritional content of four major milk types consumed throughout the world.