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1 Cheese rind microbial communities: diversity, composition and origin

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12 Abstract

Cheese rinds host a specific microbiota composed of both prokaryotes (such as Actinobacteria, Firmicutes and Proteobacteria) and eukaryotes (primarily yeasts and moulds). By combining modern molecular biology tools with conventional, culture-based techniques, it has now become possible to create a catalogue of the biodiversity that inhabits this special environment. Here, we review the microbial genera detected on the cheese surface and highlight the previously unsuspected importance of non-inoculated microflora - raising the question of the latter's environmental sources and their role in shaping microbial communities. There is now a clear need to revise the current view of the cheese rind ecosystem (*i.e.* that of a well-defined, perfectly controlled ecosystem). Inclusion of these new findings should enable us to better understand the cheese-making process.

24 Introduction

25 Cheese is one of the oldest fermented foods created by man. It has been produced and 26 consumed for thousands of years and has been adapted to match the technical, social and 27 economic conditions in various parts of the world. Consequently, the cheese fermentation

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process is strongly linked to culture and tradition - especially in rural households and village communities. Worldwide, there are about 1,000 distinct types of (most artisanal) cheese, with a remarkable variety of textures, visual aspects, aromas and flavours. The organoleptic richness and diversity of these cheeses (all of which are prepared from the same raw material, *i.e.* milk) can be explained by the action of a large number of microorganisms that flourish in cheese and that specifically degrade the components of the curd during the cheese-making process (Montel et al., 2014).

The cheese rind's characteristics help to define the type of cheese and largely determine its flavour. Furthermore, cheese rind constitutes a man-made ecosystem that has resulted from the selection and establishment of functional microbial communities. Cheese rind communities are less complex than natural environments, such as soil or the lumen of the digestive tract. Moreover, the cheese rind ecosystem is in contact with the external environment and consequently differs dramatically from the cheese core in terms of microbial composition and biochemical characteristics (Almena-Aliste & Mietton, 2014). The surface microflora has a major organoleptic impact on cheese thanks to its various enzymatic activities and its role as a barrier against pathogens and spoilage microorganisms (Irlinger & Mounier, 2009). Consequently, studying and understanding this particular ecosystem is of great interest from both public health and economic perspectives.

Our knowledge of microbial diversity in cheese rinds is still fragmented but has expanded significantly over the last decade, due to the use of molecular biology techniques to profile microbial communities. Here, we review and summarize recent literature on the microbial diversity of cheese rinds. This analysis enabled us to establish an up-to-date catalogue of the microbial taxa associated with this special environment. Furthermore, we draw a distinction between technological and adventitious microflora, and focus on the latter's possible origins. This evaluation should be of immense help in exploring unstudied cheese environments and retrieving isolates belonging to taxa not previously observed on the cheese surface.

55 The distribution of bacterial and fungal genera within rind communities

The composition of the cheese surface microbiota has been studied for several decades via the application of conventional, culture-based analyses (Valdès-Stauber *et al.*, 1997; Maoz *et al.* 2003; Viljoen *et al.*, 2003; Feurer *et al.*, 2004a; Mounier *et al.*, 2005; Callon *et al.*, 2006;

Larpin et al., 2006; Lopandic et al., 2006; Florez & Mayo, 2006; Rea et al., 2007; Goerges et al., 2008; Mounier et al., 2009; Bleicher et al., 2010; Panelli et al., 2012; Roth et al., 2010; Larpin-Laborde et al., 2011; Amato et al., 2012; Lavoie et al., 2012; Gori et al. 2013; Cogan et al., 2014; Gkazionis et al., 2014) and, since the end of the 20th century, by using cultureindependent molecular biology methods based on the direct extraction of DNA and/or RNA from the matrix. The first culture-independent methods to be applied to cheese microbial communities were based either on molecular fingerprinting (such as PCR- denaturing gradient gel electrophoresis, PCR- temperature gradient gel electrophoresis and PCR-single strand conformation polymorphism analysis) or cloning and sequencing of target rRNA sequences after PCR amplification (Ercolini et al., 2003; Feurer et al., 2004a; Ogier et al., 2004; Callon et al., 2006; Parayre et al., 2007; Dolci et al., 2009; Mounier et al., 2009; Fontana et al. 2010; Roth et al., 2010; Feligni et al., 2012; Dolci et al., 2013; Gori et al. 2013, Gkazionis et al., 2014; Hermet et al., 2014; Schornsteiner et al., 2014). The recent development of high-throughput sequencing (HTS) technologies has dramatically changed our view of microbial communities inhabiting fermented food products. In particular, metabarcoding analysis (e.g. deep sequencing of phylogenetic biomarker amplicons, such as variable regions of rRNA genes or intergenic transcribed spacers amplified using universal PCR primers) now enable microbial communities to be profiled with unprecedented depth (Ercolini 2013; Bokulich and Mills, 2012). To the best of our knowledge, only four publications have described the application of HTS techniques to cheese rind samples (Ouigley et al., 2012; Bokulich et al., 2013; Wolfe et al., 2014; Delcenserie et al., 2014). However, it is likely that greater use of HTS techniques in the future will result in large-scale, highly detailed descriptions of the microbial communities from cheese rinds.

The literature on the microbial diversity of cheese rinds (studied with both culture-dependent and -independent approaches) is summarized in Tables 1 and 2. Although this list is not exhaustive, it encompasses most of the results generated over the last fifteen years. Four main cheese types have been considered: i) unpressed, uncooked soft cheeses with bloomy or washed rinds, (ii) pressed, uncooked, semi-hard cheeses with natural or washed rinds, (iii) pressed, cooked hard cheeses with washed rinds and (iv) blue-veined cheeses. The production processes for these cheeses differ in terms of three critical steps (namely coagulation, draining and ripening), which means that cheese varieties have very different formats and physicochemical characteristics (Almena-Aliste & Mietton, 2014). Accordingly, a great variety of species assemblages has been observed. The picture that has emerged from the

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published data is that microbial populations may differ extensively from one cheese variety to another but also within a given variety, as a function of the place of production or the season (Rea et al., 2007; Viljoen et al., 2003). Nonetheless, the specific signature of a cheese surface community is due to the presence of a set of different microbial species, rather than a particular species. Many phylotypes are common to most cheese varieties but have varying populations and levels of complexity. The microbial communities of cheese rinds range from simple to complex assemblages harbouring Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, yeasts and moulds. These microorganisms vary in terms of their abundance and diversity during the cheese ripening process, depending on the type of rind (bloomy, washed or natural) and the technology used (soft, hard or semi-hard). At the beginning of the cheese-making process, lactic acid bacteria (LAB) from starter cultures are dominant in terms of cell counts. Over the first few days of ripening, yeasts and/or moulds colonize the cheese surface. The viable cell count for yeast reaches around 6-8 \log_{10} cfu/cm² and then remains fairly constant until the end of ripening. The progressive deacidification of the cheese surface by the yeasts and/or moulds favours the establishment of an acid-sensitive, salt-tolerant bacterial community. The final bacterial cell count is 1 to $2 \log_{10}$ units higher than the yeast cell count (Cogan et al., 2014). After reviewing 33 cheese rind studies, we identified 104 bacterial genera (1 Acidobacteria, 28 Actinobacteria, 5 Bacteroidetes, 24 Firmicutes and 46 Proteobacteria) and 39 fungal genera (21 moulds and 18 yeasts). On average, 10.2 bacterial genera (ranging from 3 to 30, depending on the cheese variety) and 4.5 fungal genera (ranging from 1 to 11) were detected in cheese rinds (Tables 1, 2 and 3). We did not take account of the overall diversity studies performed by Wolfe et al. (2014) and Quigley et al. (2012) using HTS technologies because the types and varieties of cheese were not specified. Among the yeasts, Debaryomyces, Yarrowia, Candida and Geotrichum were the most frequently detected genera (present in 86%, 57%, 54% and 49% of the cheeses, respectively), followed by Kluvveromyces (32%) and Pichia (22%). Of the filamentous fungi, Penicillium was most frequently detected (in 19% of the cheeses), followed by Scopulariopsis and Fusarium (both 8%). Staphylococcus was the most frequently found Firmicutes genus (present in 78% of the cheeses). Other abundant Firmicutes were the LAB Lactococcus, Enterococcus, Lactobacillus, Streptococcus and Vagococcus, present in 50%, 41%, 25%, 22% and 16% of the cheeses respectively, and halophilic lactic bacteria such as Marinilactibacillus and Facklamia (present in 22% and 16% of the cheeses, respectively). Considering the Actinobacteria, Brevibacterium, Corynebacterium and Arthrobacter were the most frequent genera (present in 75%, 75% and 66% of the cheeses, respectively), followed by

Brachybacterium, Microbacterium, Agrococcus and Micrococcus (present in 38%, 38%, 19%
and 19% of the cheeses, respectively). The genera Psychrobacter, Halomonas,
Pseudoalteromonas and Vibrio (which are all halotolerant Proteobacteria) are also major
cheese rind microorganisms, since they were detected in 33%, 31%, 22% and 19% of the
cheeses, respectively.

Similar profiles have been obtained in recent, large-scale HTS studies. For example, Wolfe et al. (2014) characterized the fungal and bacterial diversity of 137 different cheese rinds collected in the United States and 9 European countries, and Quigley et al. (2012) studied bacterial communities from 11 Irish cheese rinds. In Wolfe et al.'s study (2014), 14 bacterial and 10 fungal dominant genera (representing >1% of the overall dataset) were distributed across three different rind biofilms (bloomy, washed and natural) with varying abundance. Of these, only two fungal genera (Aspergillus and Sporandonema) and one bacterial genus (*Nocardiopsis*) were not detected in the other studies shown in Tables 1 and 2. This observation shows that conventional culture-based and culture-independent techniques detect the same dominant populations as HTS, leading to the conclusion that all dominant microorganisms are cultivable and the selective media used for enumerating and isolation of cheese strains are appropriate and effectively allow the highlighting of the whole rind biodiversity. It is noteworthy that representative isolates from 24 dominant genera detected by sequencing were easily retrieved from dilutions of rind samples (Wolfe et al., 2014). On average, there were 6.5 dominant bacterial genera and 3.2 dominant fungal genera per rind sample (Table 3). These values are slightly lower than those estimated in our review on the basis of either culture-dependent or culture-independent techniques - almost certainly because (i) only dominant genera were considered in the calculation and (ii) Wolfe et al. (2014) excluded LAB genera (Lactococcus, Streptococcus, Leuconostoc and Lactobacillus) from their analysis. Indeed, 54 subdominant genera (44 bacteria and 10 fungi) were detected in the study by Wolfe et al. (2014) (Table 4), including genera commonly found on the cheese surface such as the eukaryotes Mucor, Kluyveromyces, Yarrowia and Pichia and the bacteria Leucobacter and Microbacterium. Moreover, Wolfe et al. (2014) also detected 4 fungal genera and 27 bacterial genera that had never been mentioned in other reports. This finding reflects the powerful ability of HTS technologies to access the diversity of low-abundant taxa for the first time. In terms of LAB (often considered to be cheese curd contaminants), the current literature suggests that these bacteria are indeed found in the cheese rind itself (Table 1). Furthermore, Quigley et al. (2012) estimated that LAB taxa account for between 2% and

4.8% of the total bacterial community in washed rinds and up to 98% in some natural rinds. The reported presence of LAB in cheese rinds may also be due to inter-study differences in the sampling technique (i.e. the thickness of the rind samples, or whether the rinds are scraped or cut off), since this may result in inadvertent sampling of the cheese core and thus contamination of the rind by LAB. By combining the literature results, we found that culturemethods and culture-independent molecular biology techniques dependent are complementary, rather than contradictory or exclusive). On one hand, culture-based techniques are easily able to characterize the individual properties of dominant members of the cheese rind microflora (e.g. genomic repertoires, metabolic capacities, and growth kinetics). On the other hand, molecular biology methods (especially those based on HTS technologies) are valuable for highlighting the existence of previously unexpected subdominant populations. In turn, this information will be very helpful in designing specific culture media for the isolation of novel members of the cheese microbiota.

173 Ripening adjunct cultures versus house microbiota

If we take into account Bourdichon et al.'s 2012 inventory of microbial food cultures (an update of the 2002 International Dairy Federation/European Food and Feed Cultures Association list of cultures with "technological beneficial use"), some of the above-mentioned genera may be considered as starters or ripening cultures that are deliberately inoculated to produce cheeses under well-controlled ripening conditions. Thus, the filamentous fungal species that are highly adapted to the cheese surface include Penicillium camemberti (a species only found in cheese, derived from *Penicillium commune*), the filamentous yeast Geotrichum candidum and Fusarium domesticum. These species are commonly used and found in cheeses requiring a velvety appearance, such as Camembert, Brie, Taleggio, Reblochon, Saint-Nectaire, Tilsit, Limburger, Brick and Raclette (Arteau et al., 2010). The species F. domesticum (also referred to as "Anticollanti" and formerly assigned taxonomically to the *Trichotecium domesticum* species or the *Cylindrocarpon* genus) is able to favour drying of the cheese surface and thus a reduction in the latter's stickiness (Bachmann et al., 2005; Ropars et al., 2012). The presence of G. candidum (an anamorph of Galactomyces candidus) does not necessarily result from deliberate inoculation. Indeed, G. candidum is known to be ubiquitous and appears rapidly on the cheese surface, regardless of geographical distribution or the type of technology (Wolfe et al., 2014). Nevertheless, G. candidum's growth is

inhibited in cheeses with a high salt content (Boutrou & Gueguen, 2005). Penicillium roqueforti is a major ripening species in blue-veined cheeses such as Roquefort, Gorgonzola, Stilton, Danish Blue and Cabrales. In Stilton, it has been shown that P. roqueforti grows within the veins and not on the surface (Gkatzionis et al., 2014). Scopulariopsis flava is abundant in the washed rinds of Danish Harvati cheese, Swiss Tilsit, Austrian Vorarlberger Bergkäse hard cheeses, and French tommes from the Pyrenees and Ossau-Iraty (Amato et al., 2012; Gori et al., 2013; Schornsteiner et al., 2014; Ropars et al., 2012). It is also used as a ripening culture (Bourdichon et al., 2012; Hermet et al., 2012; Ropars et al., 2012). Wolfe et al. (2014) also observed that members of the Scopulariopsis genus are frequently detected in dry natural rinds and that the genus' presence is negatively correlated with surface moisture levels. Several Mucor species (M. mucedo, M. plumbeus and M. racemosus) can be used as ripening cultures in some cheeses with washed or natural rinds, including Saint-Nectaire, Tomme de Savoie and Taleggio (Barrios et al., 1998; Hermet et al., 2012). The most common commercial ripening yeasts are Kluyveromyces lactis, Kluyveromyces marxianus and Debaryomyces hansenii (Lavoie et al., 2012). Given that D. hansenii is ubiquitous and highly salt tolerant, it may be present at the surface of most cheese varieties - even in cases in which it has not been added deliberately. This species is very common in blue-veined cheeses but is also found in washed and bloomy rinds (Gkatzionis et al., 2014; Schornsteiner et al., 2014; Cocolin et al., 2009; Valdès-Stauber et al., 1997). Kluyveromyces lactis and K. marxianus are involved in the very early steps of ripening (thanks to their ability to ferment lactose) but generally disappear after a few days of ripening (Arteau et al., 2010; Larpin et al., 2006; 2011).

When considering the *Firmicutes*, the LAB *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. cremoris are the major components of the mesophilic lactic starter cultures widely used in cheese-making. They are frequently combined with other mesophilic (Leuconostoc mesenteroides, L. pseudomesenteroides) or thermophilic (Streptococcus thermophilus) LAB. The non-starter LAB (NSLAB) mainly correspond to facultative heterofermentative lactobacilli (Lactobacillus paracasei, L. casei, L. rhamnosus, L. plantarum, L. curvatus, and Pediococcus acidilactici). In some technologies, NSLAB are added as adjunct cultures to accelerate the ripening process; they help to produce the free amino acids that improve cheese flavour and reduce harshness and bitterness. Beside LAB, other components of commercial cheese-making cultures include Propionibacterium freudenreichii (an anaerobic actinobacterium involved in flavour and eye formation in Swiss-type cheeses), coagulase-

negative staphylococci (mostly S. xylosus and S. equorum) and other aerobic Actinobacteria (Brevibacterium aurantiacum, Brevibacterium linens, Arthrobacter arilaitensis, Arthrobacter sp., Corynebacterium casei, Corynebacterium variabile, Brachybacterium alimentarium and Microbacterium gubbeenense) that contribute to the flavour and colour of surface-ripened cheeses (Bockelmann, 2010). The only Gram-negative bacterium used in commercial cultures is the enterobacterium Hafnia alvei. However, this species can also be adventitious. When H. alvei is inoculated into smear-ripened or mould-ripened cheeses manufactured with pasteurized milk, it increases the level of volatile sulphur compounds and therefore improves the cheese flavour (Irlinger et al., 2012).

It is noteworthy that the strains from commercial ripening cultures used for the manufacturing of cheeses made from pasteurized milk are not necessarily found to be the dominant surface microorganisms (Feurer et al., 2004b; Goerges et al., 2008; Larpin et al., 2011; Rea et al., 2007; Gori et al., 2013). For example, the species Brevibacterium aurantiacum (formerly assigned to *B. linens* and frequently used for the manufacturing of smear-ripened cheeses) is only sporadically found on the surface of these cheeses (Larpin-Laborde et al., 2011; Goerges et al., 2008; Mounier et al., 2005; Rea et al., 2007; Brennan et al., 2002). According to most of the studies considered in Tables 1 and 2, the cheese surface is dominated by adventitious microflora and by microorganisms from starter and secondary cultures. In a study of the microbial diversity of 137 cheese rind communities (Wolfe et al. 2014), it was estimated that 60% of the bacterial genera and 25% of the fungal genera detected did not originate from starter or secondary cultures. Many of the "house" flora cited in Tables 1 and 2 correspond to environmental genera, such as marine halotolerant Proteobacteria (Psychrobacter, Pseudoalteromonas, Halomonas, Vibrio and Advenella), and marine halophilic and alkaliphilic LAB (Vagococcus, Facklamia and Marinilactibacillus) (Wolfe et al., 2014; Quigley et al., 2012; Schornsteiner et al, 2014; Ishikawa et al., 2007; Amato et al., 2012; Mounier et al, 2009; Roth et al., 2011; Bokulich et al., 2013; Bleicher et al., 2010; Gori et al., 2013).

Among the yeasts, *Yarrowia lipolytica* is ubiquitous and grows spontaneously on cheese
surfaces. It has been shown that other dairy yeast species (including *D. hansenii* and *G. candidum*) can be outgrown by *Y. lipolytica* (Mounier *et al.*, 2008; Viljoen *et al.*, 2003).
Several species of *Candida* and *Pichia* are also frequently found on the rind of European smear-ripened cheeses (Larpin *et al.*, 2006, 2011; Mounier *et al.*, 2009; Cogan *et al.*, 2014).
Some species have been detected only in specific rinds. For example, members of the *Yaniella*

genus have been detected in Swiss and Austrian washed rinds (Schornsteiner *et al.*, 2014;
Wolfe et al, 2014). The fungal airborne genus *Cladosporium* is commonly detected in the rind
of blue-veined cheeses (*e.g.* Taleggio) and the rind of mould-ripened cheeses (*e.g.*Camembert) (Panelli *et al.*, 2012, Arteau *et al.*, 2010).

Furthermore, several studies have shown that the bacterial flora dominating cheese surfaces belong to well-known inoculated species assigned to genera Staphylococcus, Corvnebacterium, Brachybacterium, Arthrobacter, Microbacterium, Brevibacterium, Debaryomyces and Geotrichum, but that the dominating strains tend to originate from the "house" microflora rather than the added ripening culture (Gori et al., 2013; Mounier et al., 2006; Goerges et al., 2008; Rea et al., 2007; Feurer et al., 2004b). These findings emphasize that (i) the microbial composition of cheese rind is strongly influenced by the environmental communities present in the area of cheese production and (ii) microorganisms selected as ripening cultures for their expression of defined technological functions often behave differently in complex microbial communities, probably due to their poor adaptation to cheese-making processes and their lack of competitive advantage over indigenous microbiota.

273 Reservoirs of microbial diversity

The presence of adventitious microorganisms in rind raises several questions: (i) how do they colonize the cheese-making environment, (ii) how do they transfer to the cheese surface and (iii) which technological parameters favour or hamper their development? Adventitious microorganisms may be present through the entire production chain, from the farm to the final product. This is especially true of dairy environments such as raw milk, dairy utensils (e.g. wooden shelves, vat or brines) and the atmosphere of farms or the ripening cellar - all of which act as potential vectors (Figure 1). Although many studies have focused on microbial diversity in raw milk (reviewed in Quigley et al., 2013), few studies have looked at the impact of cheese-processing environments on microbial community assemblages in specific production units. However, all the available studies show that each manufacturing unit has a specific house microflora, which is dependent on the environmental conditions prevailing during the cheese-making and is characterized by a typical, stable and recurrent microbiota that drives the ripening and potentially has a role in the development of the cheese's organoleptic properties (Feligini et al., 2012; Mounier et al., 2006; Viljoen et al., 2003; Panelli et al., 2012; Bokulich et al., 2013; Schirmer et al., 2013). The microbial diversity in

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raw milk is substantial, since a single milk sample can contain as many as 36 dominant microbial species (for a review, see Montel *et al.* (2014)). The microbial diversity in milk varies from farm to farm and is strongly influenced by the overall farm management system. The teat surface is the main source of useful cheese-making bacteria present in milk (Vachevrou *et al.*, 2011; Verdier Metz *et al.*, 2012). The fungal species most frequently present in raw cow's milk sampled at different locations and different farms have been assigned to Penicillium commune, Yarrowia lipolytica, Debaryomyces hansenii, Kluvveromyces marxianus and several species of Candida (Panelli et al., 2013; Lavoie et al., 2012; Vacheyrou et al., 2011; Fleet, 2007; Viljoen et al., 2003). While no significant geographical influence was established, there is a difference in microbiota composition between farm milk and the dairy tank milk. The former is dominated by Gram-positive Corvnebacterium, bacteria (Staphylococcus, Macrococcus, Kocuria. Lactococcus. Lactobacillus and Enterococcus), and the latter is dominated by Gram-negative bacteria (Pseudomonas, Acinetobacter, Chryseobacterium, Achromobacter, Halomonas and Psychrobacter) (Fricker et al., 2011; Vacheyrou et al., 2011; Delbes et al., 2007; Raats et al., 2011; Rasolofo et al., 2010). This disparity may be attributed to differences in storage temperature and storage time. During cheese-making, the main shifts in rind microbial composition occurred during curd production and ripening. This observation shows that the two key driving forces for microbial growth are pH (which decreases during curd production and increases at mid-ripening) and the salt content (Figure 1). These physical-chemical attributes exert selective pressure on the microbiota and consequently favour microbial species that are specifically adapted to these environmental constraints (Irlinger & Mounier, 2009). For example, the deacidification of the cheese surface (due to the consumption of lactate and the production of ammonia by moulds and yeasts) stimulates the growth of acid-sensitive aerobic bacteria, such as Actinobacteria and Proteobacteria. Although there are too few data on the presence of strains related to alkaliphilic LAB (e.g. Marinilactibacillus, Vagococcus, Facklamia) to form an opinion on the latter's practical significance and possible origin, the environmental study by Bokulich *et al.*, (2013) has shown that γ -*Proteobacteria* (e.g. Psychrobacter, Pseudoalteromonas, Halomonas, and Vibrio) present in cheese rinds also dominated the washed-rind maturation room surfaces (*i.e.* the wash bucket, draining table, brine tank, aging rack and drains) in one of the two dairy facilities studied. These genera are known to be halophilic (or halotolerant) and psychrotolerant, which could explain their ubiquitous distribution in food products (cheese, meat and fish) and in natural milieus such as sea water and soil (Nychas et al., 2008, Olofson et al., 2007, Margesin et al., 2011). In this

respect, the salting step (which consists in rubbing salt on the cheese surface, or soaking cheeses in a brine solution) generates a high salt concentration that favours the growth of these microorganisms or other halotolerant microorganisms such as Staphylococcus, Brevibacterium, Arthrobacter, Corynebacterium and Leucobacter (Ishikawa et al., 2006; Mounier et al., 2005; Mounier et al., 2006; Bokulich et al., 2013; Cogan et al., 2014; Feligini et al., 2012). Wooden processing surfaces (including aging shelves and vats) are also rich sources of microorganisms, which generally produce biofilms that are stable over several seasons. These biofilms are dominated by yeasts, moulds and Actinobacteria, and correlations have been observed with the microbial groups in cheese rinds (Mounier et al., 2006; Mariani et al., 2007; Soaeres et al., 2011; Didienne et al., 2012).

334 Conclusions and future perspectives

Thanks to efforts over the past decade, we are starting to obtain a reliable, in-depth picture of the microbial diversity of cheese rinds. In this review, we emphasized that the cheese rind environment hosts a variety of microorganisms from several sources (including commercial ripening adjunct cultures and adventitious flora). Indeed, cheese production and aging environments may be a rich source of microbes throughout the course of fermentation. However, little attention has been paid to the characterization of facility-specific "house" microbiota and how the latter are selected, survive, and colonise cheese rinds. This is a topic that needs to be explored further.

We have summarized the literature data at the genus level, and have thus shed light on a common set of a dozen genera distributed across many cheese varieties. These findings suggest the existence of a core microbiota that has adapted to the cheese surface. However, large-scale studies of phylotypes at the species or strain level are now required. Recent advances in HTS technologies have made this type of study feasible - providing that similar progress in the tools' taxonomic resolution can also achieved. Given that HTS and cloning-dependent approaches are only semi-quantitative, it might also be interesting to use quantitative PCR to more precisely quantify the abundance of certain phylotypes. Lastly, there is a need to focus on the species' ecological adaptation, colonization capabilities, ability to resist and/or adapt to disturbances (e.g. environmental changes, or contamination by a pathogen) and mutual interactions. In particular, the food industry is looking for powerful

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tools that enable the in-depth, reliable characterization of food microbial communities and thus provides better control of production processes.
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361 362	Table 1. The presence of bacterial genera in cheese rinds, as determined in selected biodiversity studies ¹
363 364	A total of 77 bacterial genera are listed (1 <i>Acidobacteria</i> , 18 <i>Actinobacteria</i> , 3 <i>Bacteroidetes</i> , 19 <i>Firmicutes</i> and 36 <i>Proteobacteria</i>).
365 366 367 368 369 370 371	¹ The bibliographic references for the data in this table are as follows: Cogan <i>et al.</i> (2014); Goerges <i>et al.</i> (2008); Valdès-Stauber <i>et al.</i> (1997); Larpin <i>et al.</i> (2006); Larpin-Laborde <i>et al.</i> (2011); Mounier <i>et al.</i> (2009); Mounier <i>et al.</i> (2005); Ogier <i>et al.</i> (2004); Feurer <i>et al.</i> (2004); Bleicher <i>et al.</i> (2010); Maoz <i>et al.</i> (2003); Parayre <i>et al.</i> (2007); Rea <i>et al.</i> (2007); Gori <i>et al.</i> (2013); Fontana <i>et al.</i> (2010); Feligni <i>et al.</i> (2012); Bockulich <i>et al.</i> (2013); Amato <i>et al.</i> (2012); Roth <i>et al.</i> (2010); Dolci <i>et al.</i> (2009); Dolci <i>et al.</i> (2013); Schornsteiner <i>et al.</i> (2014); Ercolini <i>et al.</i> (2003); Quigley <i>et al.</i> (2012); Wolfe <i>et al.</i> (2014) and Delcenserie <i>et al.</i> (2014).
372	² Various methods were used to detect dominant genera in cheese rind samples:
373 374	• Culture-dependent (CD) methods (traditional plating and isolation of bacteria, followed by genotyping).
375 376 377	• Culture-independent (CI) methods (direct extraction of microbial DNA from cheese rinds, followed by identification via molecular biology methods such as PCR-DGGE/TGGE, PCR-CE-SSCP, and 16S rDNA cloning/sequencing.
378 379	• High-throughput sequencing (HTS, direct extraction of microbial DNA from cheese rinds, followed by identification via V4/V1-V3 16S rDNA amplicon sequencing (454 pyrosequencing or Illumina)).
380 381	³ The frequency calculation takes account of 31 different cheese varieties from all separate studies except Quigley <i>et al.</i> , 2012 and Wolfe <i>et al.</i> , 2014.
382 383	⁴ The frequency calculation takes account of 137 different cheese rinds from a single study (Wolfe <i>et al.</i> , 2014).
384	⁵ Acidobacteria
385	⁶ Bacteroidetes
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387 388	Table 2. The presence of fungal genera in cheese rinds, as determined in selected biodiversity studies ¹
389	A total of 35 fungal genera are listed (17 molds and 18 yeasts)
390 391 392 393 394 395	¹ The bibliographic references for the data in this table are as follows: Cogan <i>et al.</i> (2014), Goerges <i>et al.</i> (2008), Valdès-Stauber <i>et al.</i> (1997), Larpin <i>et al.</i> (2006), Mounier <i>et al.</i> (2009), Mounier <i>et al.</i> (2005), Hermet <i>et al.</i> (2014), Gori <i>et al.</i> (2013); Lopandic <i>et al.</i> (2006), Panelli <i>et al.</i> (2010), Viljoen <i>et al.</i> (2003), Lavoie <i>et al.</i> (2012), Bockulich <i>et al.</i> (2013), Callon <i>et al.</i> (2006), Amato <i>et al.</i> (2012), Dolci <i>et al.</i> (2009), Schornsteiner <i>et al.</i> (2014), Gkazionis <i>et al.</i> (2014), Florez & Mayo (2007) and Wolfe <i>et al.</i> (2014).
396	² Various methods were used to detect dominant genera in cheese rind samples:

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3	397	• Culture-dependent (CD) methods (traditional plating and isolation of fungi, followed by genotyping).
4 5 6	398 200	• Culture-independent (CI) methods (direct extraction of microbial DNA from cheese rinds, followed by identification via molecular biology methods such as PCP_DCCE/TCCE_PCP_CE_SSCP_ and 18S
7 8	400	rDNA cloning/sequencing;
9	401	• High-throughput sequencing (HTS, direct extraction of microbial DNA from cheese rinds, followed
10 11 12	402 403	by identification via internal transcribed spacers amplicon sequencing (454 pyrosequencing or Illumina)).
13 14	404	³ The frequency calculation takes account of 33 different cheese varieties from 19 separate studies.
15	405	⁴ The frequency calculation takes account of 137 different cheese rinds from a single study (Wolfe <i>et</i>
16 17	406	<i>al.</i> , 2014).
18 19	407	
20	100	Table 3 Comparison of games righness in different types of chaoses, using culture dependent
21 22 23	408 409	and culture-independent methods.
24 25	410	nd: not determined.
26 27	411	+X: number of genera listed as being subdominant (<i>i.e.</i> abundance <1%)
28 29	412	DGGE: denaturing gradient gel electrophoresis
30 31	413	TGGE: temperature gradient gel electrophoresis
32 33	414	SSCP: single strand conformation polymorphism
34 35 26	415	ITS: intergenic transcribed spacers
30 37 38	416	HTS: high-throughput sequencing
39 40	417	
40 41	418	Table 4. The subdominant bacterial and fungal genera in cheese rinds, as determined by an HTS study
42	419	of 137 European and American cheeses (Wolfe <i>et al.</i> , 2014).
43 44	420	¹ Bacteroidetes
45	421	² The frequency calculation takes account of 137 different cheese rinds from a single study (Wolfe et
40 47 49	422	<i>al.</i> , 2014)
40 49	423	A total of 44 subdominant bacterial genera (15 Actinobacteria, 2 Bacteroidetes, 6 Firmicutes and 21
50	424	Proteobacteria) and 10 subdominant fungal genera (6 moulds and 4 yeasts) were detected in the 137
51 52	425	cheese rinds
53	426	* A total of 23 genera (5 Actinobacteria, 1 Firmicute, 11 Proteobacteria, 2 moulds and 4 yeasts) were
54 55	427	found to be dominant in other biodiversity studies.
56 57 58 59	428	
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4 5 6 7	430 431	Figure 1. Schematic overview of potential reservoirs of the different microbial groups colonising cheese rinds.
8	432	LAB, lactic acid bacteria;
10 11	433	HALAB, halophilic and alkalophilic LAB;
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15 16 17 18 19 20 21 22 32 42 526 27 28 29 30 132 33 435 36 37 38 940 41 42 43 44 50 51 52 53 45 56 57 58 59	436	
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452	• High-throughput sequ	uencing (HTS,	direct extraction	of microbial DNA	from cheese rinds	, followed
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- 453 by identification via internal transcribed spacers amplicon sequencing (454 pyrosequencing or
- 454 Illumina)).
- ³ The frequency calculation takes account of 33 different cheese varieties from 19 separate studies.
- ⁴ The frequency calculation takes account of 137 different cheese rinds from a single study (Wolfe *et al.*, 2014).
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Table 3. Comparison of genus richness in different types of cheeses, using culture-dependent and culture-independent methods. 460 Number of fungal genera found by: Type and number (n) of cheeses Molds Yeasts Total Average value of Richness index Culture-dependent method (CD) Soft cheeses n = 187 15 22 4.5 Semi-hard cheeses n = 42 12 14 6.25 Blue cheeses n = 3 2 6 8 3.3 Total CD 17 26 9 4.6 Culture-independent method (CI) Soft cheeses n = 41 3 4 2.8 PCR-DGGE. TGGE. SSCP / Cloning Semi-hard cheeses n = 3 3 8 11 6,0 Hard cheeses 5 3 8 4,0 n = 2Blue cheeses 5 6 4.5 n = 2Total CI 7 10 17 4.2 Total (CD + CI) 18 29 4.5 11 Culture-independent method (HTS) 5 2 7 5.5 Soft cheeses n = 2HTS - ITS amplicon sequencing 10(+10)cheese rinds n = 137 7 (+6) 3 (+4) 3.2 (nd) **Total HTS** 7 (+8) 4 (+4) 11 (+12) Total (CD + CI + HTS) 35 (+4) 17 (+4) 18 Number of bacterial genera found by: Average value of Richness index Type and number (n) of cheeses Acidobacteria Actinobacteria Firmicutes Proteobacteria Bacteroïdete. Total Culture-dependent method (CD) Soft cheeses n = 1312 10 18 40 8.6 Semi-hard cheeses n = 3 9 8 6 23 12.7 15 12 47 Total CD 20 9.4 Culture-independent method (CI) Soft cheeses 7 9 25 7.7 n = 7 9 PCR-DGGE. TGGE. SSCP / Cloning Semi-hard cheeses 6 8 2 16 8.0 n = 4Hard cheeses 10 4 6 2 22 22.0 n = 1 7 Blue cheeses n = 25 4.0 1 Total CI 2 12 13 14 41 8.3 Total (CD +CI) 17 15 26 2 60 8.8 Culture-independent method (HTS) 5 2 37 Soft cheeses n = 41 13 16 24.5 HTS - 16S rDNA amplicon sequencing Irish cheese rinds n = 11 4 (nd) 7 (nd) 8 (nd) 19 (nd) nd 6 (+21) 14 (+44) cheese rinds n = 137 6(+15)1 (+6) 1(+2)6.5 (nd) Total HTS 1 6(+10)8 (+7) 8 (+19) 2 (+2) 63 Total (CD + CI + HTS) 1 18 (+10) 19 (+5) 36 (+10) 3 (+2) 77 (+27) 461

462 nd: not determined.

463 +X: number of genera listed as being subdominant (*i.e.* abundance <1%)

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5	464	DGGE: denaturing gradient gel electrophoresis
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- **Table 4.** The subdominant bacterial and fungal genera in cheese rinds, as determined by an HTS study
- 472 of 137 European and American cheeses (Wolfe *et al.*, 2014).

		Frequency (%) ⁴			Frequency (%) ⁴
	Actinomyces	0.3		Eremascus	0.6
	Agrococcus *	6.4	s	Ilyonectria	3.3
	Agromyces	14.1	old	Mucor*	5.2
	Amycolatopsis	0.8	Μ	Nectria*	2.8
	Citricoccus*	0.6		Oidiodendron	0.8
ria	Dermacoccus	0.8		Trichotecium	1.7
ctei	Janibacter	1.7	ß	Cryptococcus*	<0.1
bba	Kocuria*	5.2	eas	Kluyveromyces*	5.5 1.1
tinc	Leucobacter* Mierobacter*	1.1	y	Tricnosporon* Vannowia*	1.1
Acti Agru Agru Agru Agru Agru Amy Citr Der Jan Koc Leuu Mic Nes Prot Ren Stree Ren Stree Ren Bac Stree Pep Pep Pep Pep Alco Ave. Bar Stree Bar Myr Bac Chr Myr Bac Stree Chr Bac Stree Pep Pep Pep Pep Alco Ave. Bar Nes Stree Ren Stree Pep Pep Pep Pep Pep Pep Pep Pep Pep Alco Ave. Stree Stree Chr Bar Stree Pep Pep Pep Pep Pep Pep Pep Pep Pep P	Nastarankonia	17		Number of genera	1.9
	Duomianomonoanona	0.2		Number of genera	10
	Promicromonospora	0.3			
	Pseudoclavibacter	1.1			
	Renibacterium	1.7			
	Streptomyces	2.2			
~~	Chryseobacterium	0.6			
Acta Agr Agr Agr Agr Agr Amy Citt Der Jan Koo Leu Mic Ness Pro Pse Ren Stre Chr Myn Bac Stre Pro Pse Ren Myn Chr Myn Chr Bac Oer Pep Pep Alco Ave Bor But Chr Bac Stre Chr Hao Cor Pro Pse Ren Stre Chr Myn Cor Pro Pse Ren Stre Chr Myn Cor Pep Pep Pep Pep Pep Pep Pep Chr Bac Stre Chr Myn Cor Pro Pep Pep Pep Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Pep Pep Pep Pep Chr Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Pep Pep Pep Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Bac Stre Chr Stre Chr Bac Stre Chr Bac Stre Stre Chr Stre Stre Stre Stre Stre Stre Chr Stre Stre Stre Stre Stre Stre Stre Str	Myroides	0.8			
	Bacillus	3,0			
sə	Jeotgalicoccus	2.5			
icu	Macrococcus*	0.3			
irm	Oerskovia	0.8			
E	Peptoniphilus	0.8			
	Peptostreptococcus	0.6			
	Alcaligenes*	1.7			
	Averyella	8.6			
	Bordetella	0.3			
	Buttiauxella	0.3			
	Citrobacter*	0.8			
	Cobetia*	4.7			
	Chromohalobacter	0.3			
	Enterobacter*	0.6			
ria	Haerehalobacter	0.3			
ctei	Idiomarina*	0.3			
pqa	Ignatzschineria	0.8			
ətec	Lysobacter	1./			
Prc	Marinobacter* Marinomonac*	0.9			
	Marmomonas Morganella*	3.3			
	Decenisphaera	0.6			
	Ducui den ci n*	0.0			
		1.4			
	Kaoultella*	4.1			
	Stenotrophomonas*	0.6			
	Tatumella	0.3			
	Xenorhabdus	0.3			
	Number of genera	44			

 474 ¹Bacteroidetes

- ² The frequency calculation takes account of 137 different cheese rinds from a single study (Wolfe et al., 2014)
- A total of 44 subdominant bacterial genera (15 Actinobacteria, 2 Bacteroidetes, 6 Firmicutes and 21 Proteobacteria) and 10 subdominant fungal genera (6 moulds and 4 yeasts) were detected in the 137 cheese rinds
- * A total of 23 genera (5 Actinobacteria, 1 Firmicute, 11 Proteobacteria, 2 moulds and 4 yeasts) were found to be dominant in other biodiversity studies.

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77 bacterial genera (1 Acidobacteria, 18 Actinobacteria, 3 Bacteroidetes, 19 Firmicutes and 36 Proteoba

 A total of 77 bacterial genera are listed (1 Acidobacteria, 18 Actinobacteria, 3 Bacteroidetes, 19 Firmicutes an ¹ The bibliographic references for the data in this table are as follows: Cogan *et al.* (2014); Goerges *et al.* (20

² Various methods were used to detect dominant genera in cheese rind samples:

• Culture-dependent (CD) methods (traditional plating and isolation of bacteria, followed by genotyping).

• Culture-independent (CI) methods (direct extraction of microbial DNA from cheese rinds, followed by ident

• High-throughput sequencing (HTS, direct extraction of microbial DNA from cheese rinds, followed by ident

³ The frequency calculation takes account of 31 different cheese varieties from all separate studies except Qui

⁴ The frequency calculation takes account of 137 different cheese rinds from a single study (Wolfe *et al.*, 201-

⁵ Acidobacteria

⁶ Bacteroidetes

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1d 36 Proteobacteria).
08); Valdès-Stauber *et al.* (1997); Larpin *et al.* (2006); Larpin-Laborde *et al.* (2011); Mounier *et al.*

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