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Cow Teat Skin, a Potential Source of Diverse Microbial Populations for Cheese Production

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The diversity of the microbial community on cow teat skin was evaluated using a culture-dependent method based on the use of different dairy-specific media, followed by the identification of isolates by 16S rRNA gene sequencing. This was combined with a direct molecular approach by cloning and 16S rRNA gene sequencing. This study highlighted the large diversity of the bacterial community that may be found on teat skin, where 79.8% of clones corresponded to various unidentified species as well as 66 identified species, mainly belonging to those commonly found in raw milk (*Enterococcus*, *Pediococcus*, *Enterobacter*, *Pantoea*, *Aerococcus*, and *Staphylococcus*). Several of them, such as nonstarter lactic acid bacteria (NSLAB), *Staphylococcus*, and *Actinobacteria*, may contribute to the development of the sensory characteristics of cheese during ripening. Therefore, teat skin could be an interesting source or vector of biodiversity for milk. Variations of microbial counts and diversity between the farms studied have been observed. Moreover, *Staphylococcus auricularis*, *Staphylococcus devriesei*, *Staphylococcus arlettae*, *Streptococcus bovis*, *Streptococcus equinus*, *Clavibacter michiganensis*, *Coprococcus catus*, or *Arthrobacter gandavensis* commensal bacteria of teat skin and teat canal, as well as human skin, are not common in milk, suggesting that there is a breakdown of microbial flow from animal to milk. It would then be interesting to thoroughly study this microbial flow from teat to milk.

Thirty years of milking practices driven by a hygienic strategy have led to the presence of very low levels of all microbial populations in raw milk, including those of interest for cheesemaking. Indeed, more than 90% of milk in France presents a level of total microbiota below 10^5 CFU ml^{-1} or lower (5). This is of concern to cheese makers as it causes a loss of diversity and richness of the sensory properties of raw-milk cheeses. However, more than 150 species were identified in milk and raw-milk cheeses combining culture-independent and culture-dependent methods. To identify the possibilities of action needed to maintain microbial milk diversity while eliminating pathogens, a better knowledge of the farm environment as a reservoir of biodiversity is required.

In the udder cells of healthy cows, milk is sterile; however, it is enriched with microorganisms during its passage through the teat canal (26) by contact with the surface of teat skin, the surrounding air, and other environmental factors on the farm (52). Housing conditions (bedding material, litter management) and milk house water supply can also influence the quality of the milk (27, 29, 43). Moreover, according to the maintenance and the microbial diversity of milk, biofilms formed in the milking machine can inoculate the milk (9).

The teat cistern, teat canal, and teat apex can be colonized by a variety of microorganisms including pathogenic bacteria (26, 47). The microbial population of the teat canal has been described by cloning and sequencing of 16S rRNA genes (26). On the other hand, teat end (47) and teat skin microbial populations (58) have been described only by applying culture-based methods. Teat skin has been described as the first reservoir of microbial diversity that can be found in milk during milking (40, 58), but these studies report only cultivable microorganisms and not the entire diversity.

Therefore, the aim of this study was to create an inventory of microbial populations commonly found on the teat skin of dairy cows. For this purpose, a direct molecular approach using 16S rRNA gene cloning and sequencing methods was combined with a culture-dependent method on different types of media, followed by molecular identification of isolates by 16S rRNA gene sequencing.

MATERIALS AND METHODS

Teat skin samples and microbial analyses. Seventeen farms spread over the entire department of Cantal (Massif Central, France) were studied between February and April when the cows were kept indoors at all times. An 18th farm was studied during the summer when cows were outside. These farms were representative of Cantal dairy herds with regard to cow numbers (between 30 and 60 Holstein or Montbeliarde cows) and cattle housing (mats or free stalls with or without straw), feeding (hay or silage), and milking (pipeline).

The anterior right and posterior left teats were swabbed with a single sterile swab (Ecolab Dermasoft) moistened with 5 ml of sterile NaCl (9 g $liter^{-1}$)-Tween 80 (1 g $liter^{-1}$). Each time, the teat surface in contact with the liner, as well as the teat end, was carefully sampled using sterile gloves. Two samples from six healthy (free of mastitis) dairy cows, randomly selected from each of these 18 farms, were taken at an interval of one per week. The sampling procedure was systematically performed before any wash of the teats by the farmer at the beginning of milking. After sampling, each swab was placed in an individual stomacher bag filled with 10 ml of NaCl-Tween 80 solution, and 0.5% sterile G milk was added (Standa Industrie, Caen, France). The samples were then stored at 4°C until they were blended for 4 min with a stomacher (bag filter and bag system; Interscience, St. Nom la Bretèche, France). Individual swab suspensions

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were extracted, frozen in 10% glycerol, and kept at −20°C until analyzed, despite the risk of underestimation of biodiversity. On the day of analysis, after samples were thawed at 25°C, the microbial populations of each sample from each cow were counted on several media. Considering the specificity of the media used for milk and cheese, the bacterial populations enumerated were presumed to be only as follows: total bacterial count on plate count agar (PCA) medium (32), facultative heterofermentative lactobacilli on FH agar medium (36), enterococci on Slanetz and Bartley (SB) agar (54), yeasts and molds on oxytetracyclin glucose agar (OGA) medium (31), dextrane-negative bacteria on Mayeux-Sandine-Elliker (MSE) agar (38), *Pseudomonas* on cetrimin-fucidin-cephalosporin (CFC) medium (34), and coagulase-negative staphylococci on rabbit plasma fibrinogen (RPF) agar (2). The presumed ripening bacteria were counted on cheese-ripening bacterial medium (CRBM) (16), the presumed *Enterobacteriaceae* were counted on violet red bile lactose (VRBL) medium (33), and the presumed Gram-negative bacteria (Gram$^-$) were counted on PCA medium with vancomycin and purple crystal added (PCAi). All culture media were purchased from Biokar Diagnostics (Beauvais, France). All microbiological analyses were performed in duplicate.

Statistical analyses. Ascending hierarchical classification (AHC) using Euclidean distances and Ward's method was performed with Stastistica software (StatSoft, Inc., Tulsa, OK). The average of the individual microbial counts of the OGA, CRBM, FH, SB, and CFC media were independent variables. The dendrogram obtained made it possible to significantly display four groups of farms at a threshold aggregation distance of less than 5. This analysis was followed by *k*-means clustering to establish the microbial characteristics of the four groups. The average of individual microbial enumerations from teat suspensions obtained using all bacterial media were analyzed by monofactorial (farm group defined by AHC) variance analysis (StatSoft, Inc., Tulsa, OK).

Culture-dependent bacterial inventory. Out of 18 farms, 1 farm was randomly selected from each statistically similar group of farms, and the six teat skin samples from the six dairy cows of each farm were pooled and plated on five culture media. From each medium, a number of colonies, equal to the square root of the count of colonies, were randomly selected for amplification. PCR amplifications of 16S rRNA genes (1,450 bp) were carried out directly from 1 μl of water suspension from each colony using the universal primers W02 (5′-GNTACCTTGTTACGACTT-3′) and W18 (5′-AGAGTTTGATCMTGGCTCAG-3′) as previously described by Callon et al. (7). All amplifications were performed with a GeneAmp PCR System 9700 (Applied Biosystems, Courtaboeuf, France). The 309 amplified products (64 colonies from CRBM, 82 from MSE medium, 80 from PCA medium, 40 from PCA medium with vancomycin, and 43 from SB medium) were purified and sequenced using the W18 primer by LGC Genomics, GmbH (Berlin, Germany). The 700 bp of the 5′ ends obtained for the 16S rRNA genes of the isolates were compared to sequences available in the GenBank database, using the BLASTn, version 2.2.24, program (1). Sequences with a percentage similarity of 98% or higher were considered to be representative of the same species.

DNA extraction. Two milliliters of six-cow pools of teat samples from three selected farms was centrifuged at 6,000 × *g* for 10 min to concentrate the sample to 0.5 ml. Twenty microliters of lysozyme (100 mg ml^{-1}), 20 μl of lysostaphin (1 mg ml^{-1}), and 2 μl of pronase (10 mg ml^{-1}) were added, and each sample was incubated for 2 h at 37°C. Forty microliters of 10% SDS was then added to each sample, which was then incubated for 1 h at 55°C. Total bacterial DNA was then extracted using mechanical zirconium bead crushing and the phenol-based purification method as previously described by Delbès et al. (15). The DNA pellet was resuspended in 50 μl of water, and the DNA solution was stored at −20°C.

PCR amplification, cloning, and sequencing of 16S rRNA genes. The DNA samples extracted from the teat skin samples were used to amplify 16S rRNA genes with the universal bacterial primers W18 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1406R (5′-ACGGGCGGTGWGTRCA A-3′). Each PCR consisted of 25 μl (total volume) and contained 2.5 μl of 10× buffer, 1.5 of $MgSO_4$ (25 mM), 2.5 μl of deoxynucleoside triphosphates (dNTPs; 10 μM), 2.5 μl of each primer (10 mM), and 0.2 μl (1 U) of *Tfl* or *Taq* polymerase (Promega Corp., Madison, WI). Reactions were performed in a Thermocycleur Mastercycler gradient (Eppendorf, France). PCR conditions were 5 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, with a final extension of 7 min at 72°C. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), cloned using a PCR TOPO-Blunt cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), and then transferred into *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA). Bacterial colonies were isolated after a 24-h incubation on agar plates (100 μM kanamycin) at 37°C. Then, they were selected, and the recombinant vectors were tested using M13 PCR. Sequencing reactions were performed using M13 forward primer (LGC Genomics, Berlin, Germany).

16S rRNA genes clone libraries. After clone sequencing, the 16S rRNA gene library was established with the 1,172 obtained sequences (850 bp on average). The sequences were cleaned automatically; i.e., TOPO-Blunt vectors sequences and low-quality sequences and sequences of less than 500 bp in length were excluded from the analysis using self-developed software. The clone library was screened for chimeric sequences with the Bellerophon program (30 [http://foo.maths.uq.edu.au/~huber/bellerophon.pl]. Thirty-four sequences identified as chimeras were excluded. The remaining 1,091 bacterial sequences were analyzed.

Phylogenetic analysis. Multiple alignments were performed with the program MUSCLE (20). Phylogenetic relationships between sequences were determined by calculating the distance matrix using the dnadist program within the PHYLIP software package (version 3.5c) and using the Jukes-Cantor correction for multiple substitutions (J. Felsenstein, 1993, Department of Genetics, University of Washington, Seattle, WA). Library diversity was then studied by calculating diversity statistics and rarefaction curves using a distance-based OTU and richness program (DOTUR), which assigns sequences to operational taxonomic units (OTUs) based on genetic distances (50). Sequences with identities of ≥97% were assumed to belong to the same OTU. Coverage values were calculated by the following equation: $C = 1 - (n/N) \times 100$, where n is the number of the OTUs, N is the number of clones examined, and C is the percent coverage (39). Sequences were queried in GenBank using BLASTn, version 2.2.24 (1), in September 2010. Sequences with less than 97% similarity to any previously identified sequence were classified as unidentified and analyzed by the ribosomal Database Project Classifier program, version 10 (10, 59), to determine the phylogenetic placement of OTUs with ≥80% confidence.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences obtained in this study have been deposited in the GenBank database under accession numbers JN834092 to JN835182 (clones) and JN852991 to JN853299 (isolates).

RESULTS

Culture-dependent bacterial inventory. As a first step, the 18 farms were assigned into four groups by an ascending hierarchical classification based on the average of individual microbial counts of teat skin on five culture media (OGA, CRBM, SB, CFC, and FH) and followed by *k*-means clustering. These four groups of farms differed by the average levels of teat skin microbial populations enumerated on several microbial media (Table 1). Levels of Gram-negative bacteria and molds were similar in the four groups of farms. Among the four groups, the farm groups A ($n = 2$ farms) and D ($n = 3$ farms) had the highest microbial counts on PCA, MSE, SB, CFC, and RPF media, with group A having a higher level of yeasts than group D (+1.24 $\log_{10}$ CFU ml^{-1} of teat suspension). The ripening bacteria enumerated on CRBM and the coagulase-negative staphylococci enumerated on RPF medium were also dominant in these two farm groups. In the group C farms ($n = 9$ farms), cow teat skin showed the lowest bacterial counts on all

TABLE 1 Microbial counts on different media

Microbial flora	Medium	No. of bacteria by farm group[a]				P^b
		A ($n = 2$)	B ($n = 4$)	C ($n = 9$)	D ($n = 3$)	
Total mesophilic bacteria	PCA	6.61 C	5.47 DE	5.02 E	6.03 CD	***
Presumed Gram-negative bacteria	PCAi	2.94	2.09	1.54	2.46	NS
Mold	OGA	2.46	2.67	2.08	3.11	NS
Yeast	OGA	2.68 C	1.87 CD	1.07 E	1.44 E	*
Presumed ripening flora	CRBM	6.50 C	5.44 CD	4.75 E	6.05 CD	**
Dextrane-negative lactic acid bacteria	MSE	4.65 C	3.34 D	2.41 D	4.57 C	***
Enterococcus	SB	4.72 C	3.38 D	1.39 E	4.27 C	***
Pseudomonas	CFC	2.96 C	1.83 D	1.68 D	2.93 C	**
Coagulase-negative *Staphylococcus*	RPF	6.20 C	4.45 D	3.97 D	5.92 C	**
Heterofermentative facultative *Lactobacillus*	FH	2.70 C	2.80 C	1.35 D	2.10 C	*
Enterobacteriaceae	VRBL	1.38	1.32	0.7	1.37	NS

[a] Results are expressed mean $\log_{10}$ CFU ml^{-1} of teat suspension. *n*, number of farms in each group. Results with different letters (C, D, and E) are significantly different by the statistical Newman-Keuls test.

[b] NS, nonsignificant; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

culture media. Finally, in group B ($n = 4$ farms) cow teat counts of yeasts and counts on PCA, CRBM, MSE, SB, CFC, and FH were higher than those of group C but lower than those of groups A and D except for FH counts.

Four farms, FA, FB, FC, and FD, were randomly selected from farm groups A, B, C, and D in order to study the microbial community of cow teats. A six-cow pool of teat skin samples from each representative farm was inoculated on five culture media (CRBM, SB, MSE, PCA, and PCAi). Among a total of 309 colonies sequenced from these five media, 169 colonies came from FD, 52 were from FC, 51 were from FA, and 37 were from FB. All of the 16S rRNA gene sequences showed a similarity above 98% with a GenBank entry. Affiliations of the 16S rRNA gene sequences of the 309 colonies (Tables 2 and 3) revealed the presence of members of four phyla corresponding to *Firmicutes* (235 colonies, or, 76%), *Proteobacteria* (55 colonies, or 17.8%), *Actinobacteria* (15 colonies, or 4.9%), and *Bacteroidetes* (4 colonies, or 1.3%). The major phylum, *Firmicutes*, was composed of at least 21 different species mainly affiliated with the genus *Staphylococcus* (140 colonies, or 59.6%), followed by the genera *Aerococcus* (58 colonies, or 24.7%), *Enterococcus* (26 colonies, or 11%), and *Bacillus* (6 colonies, or 2.55%). The remaining 2.15% consisted of isolates affiliated with the genus *Paenibacillus* and family *Lactobacillaceae*. Among *Proteobacteria*, 38.2% were assigned to the genus *Pantoea*, 10.9% to the genus *Sphingomonas*, 10.9% to the species *Escherichia coli*, and 9% to the genus *Enterobacter*. Within the *Actinobacteria* phylum, 53.3% of colonies belonged to the family *Microbacteriaceae*: they were assigned to *Clavibacter michiganensis*, *Curtobacterium herbarum*, *Microbacterium* sp., and *Plantibacter agrosticola*. In total, at least 46 different genera or species were identified among the 309 isolates obtained from teat skin.

The microbial populations varied among the four farms studied. In farms FC and FD, 22 and 19 different species were identified, respectively, and 13 different species were found in farms FA as well as FB. The majority of the colonies from FA teat skin (82.3%) belonged to the *Firmicutes* phylum, of which 73.8% were assigned to the genus *Aerococcus* sp. or to *Aerococcus viridans*, and 14.3% were assigned to *Staphylococcus xylosus* or *Staphylococcus arlettae*. This was in accordance with the high levels of the coagulase-negative staphylococci and of the ripening bacteria enumerated on CRBM. The isolates from FB were mainly assigned to two phyla: *Firmicutes* (48.6%), with the dominance of the genus *Enterococcus* (66.6%), and *Proteobacteria* (45.9%), with five genera (*Afipia* genosp. 1, *Bradyrhizobium* sp., *Enterobacter* sp., *Sphingomonas* sp., and uncultured *Burkholderia* sp.). The FC teat skin sample was mostly composed of *Proteobacteria* (34.6%), of which 66% were represented equally by *Escherichia coli*, *Ochrobactrum*, and *Pantoea* sp.; the rest were assigned to the genus *Staphylococcus* (32.7%) and five species of *Actinobacteria* (17.3%). Despite the low count of coagulase-negative staphylococci in farm FC, the genus *Staphylococcus* was divided into three species (11.7% *Staphylococcus haemolyticus*, 5.9% *Staphylococcus saprophyticus*, and 5.9% *Staphylococcus xylosus*) and a high percentage of unidentified staphylococci (76.5%). The FD teat skin bacteria were composed of 88.7% *Firmicutes* mainly affiliated to the genus *Staphylococcus* (76.7%) and to the genus *Aerococcus* (14.7%). In addition to 48.7% of unidentified staphylococci, the FD teat skin sample presented a great diversity of *Staphylococcus* species, with 40% *Staphylococcus xylosus*, 4.3% *Staphylococcus vitulinus*, 3.5% *Staphylococcus haemolyticus*, 1.7% *Staphylococcus pasteuri*, 0.9% *Staphylococcus fleurettii*, and 0.9% *Staphylococcus succinus*.

Culture-independent bacterial analyses. Culture-independent analyses were done on three of the four farms: FA, FC, and FD. FB was excluded from analysis because its microbial diversity was low after culture-dependent analyses. The three different libraries, FA, FC, and FD, were composed of 332, 394, and 365 clones, respectively.

The samples from FA, FC, and FD were pooled into a single library in order to better estimate the bacterial diversity of cow teat skin. The rarefaction curve, used in order to evaluate the richness of the clones generated in this study using 97% as the taxonomic unit cutoff, did not plateau, suggesting that inclusion of a larger number of clones would have provided a better knowledge of bacterial species diversity (see Fig. S1 in the supplemental material for data). However, the level of coverage was 65.6% and 88.5% at the 0.1 and 0.2 distance levels, respectively, thus suggesting that the library (1,091 clones) was sufficient to give a good picture at family and order levels of the very substantial bacterial diversity present on cow teat skin. Of the three farms, the FC clone library had the lowest level of coverage at species level (FC, 297 OTUs, or 24.6%; FA, 224 OTUs, or 32.5%; and FD, 203 OTUs, or 44.4%). For these three farms, rarefaction curves did not plateau at the

TABLE 2 Number of bacterial sequences identified by the culture-dependent method

Phylum	Genus and/or species	No. of sequences by farm				Total no. of sequences
		FA	FB	FC	FD	
Actinobacteria	*Arthrobacter gandavensis*			3		3
	Brevibacterium sp.				1	1
	Clavibacter michiganensis			1	2	3
	Curtobacterium herbarum	1				1
	Isoptericola sp.				1	1
	Microbacterium sp.			2		2
	Plantibacter agrosticola			2		2
	Rothia sp.	1				1
	Sanguibacter soli			1		1
	Total for group	2		9	4	15
Bacteroidetes	Uncultured *Bacteroidetes*		2		2	4
Firmicutes	*Aerococcus* sp.	21	1	3	16	41
	Aerococcus viridans	10	1		6	17
	Bacillus pumilus			2	2	4
	Bacillus safensis			2		2
	Enterococcus faecium		7			7
	Enterococcus hirae	2				2
	Enterococcus lactis	1				1
	Enterococcus sp.		5		11	16
	Leuconostoc mesenteroides	1				1
	Paenibacillus sp.			1		1
	Pediococcus pentosaceus		3			3
	Staphylococcus arlettae	1				1
	Staphylococcus fleurettii				1	1
	Staphylococcus haemolyticus			2	4	6
	Staphylococcus pasteuri				2	2
	Staphylococcus saprophyticus			1		1
	Staphylococcus sp.			13	56	69
	Staphylococcus succinus		1		1	2
	Staphylococcus vitulinus				5	5
	Staphylococcus xylosus	5		1	46	52
	Streptococcus equinus	1				1
	Total for group	42	18	25	150	235
Proteobacteria	*Afipia* genosp.1		2			2
	Bradyrhizobium sp.		2			2
	Burkholderia sp.		2			2
	Enterobacter amnigenus			1		1
	Enterobacter kobei			1		1
	Enterobacter sp.		1	2		3
	Escherichia coli	1		4	1	6
	Ochrobactrum			4		4
	Pantoea agglomerans	2		2	5	9
	Pantoea sp.	4		2	6	12
	Pseudomonas sp.				1	1
	Rahnella aquatilis			1		1
	Serratia sp.			1		1
	Sphingomonas sp.		6			6
	Uncultured *Burkholderia* sp.		4			4
	Total for group	7	17	18	13	55
	Total for all	51	37	52	169	309

0.03 distance level (see Fig. S2 for data). Farm FC also had the lowest level of coverage at the 0.1 and 0.2 distance levels, with 56% and 80%, respectively, versus 63.3% and 88% for farm FA and 72.3% and 87.5%, respectively, for farm FD.

Affiliations of the 1,091 16S rRNA gene sequences revealed the presence of members of nine phyla (Table 3). Within these nine phyla, the clones were grouped in 754 OTUs based on a cutoff value of 97%. The distribution of OTUs and clones into microbial classes and orders is shown in Table S1 in the supplemental material. A total of 618 out of the 754 OTUs (i.e., 82%) were singletons, demonstrating the high diversity of this bacterial community. Two major phyla were found corresponding to *Firmicutes*

TABLE 3 Phylum compositions as revealed by culture-dependent and -independent approaches

Phylum	Composition (% of sequences) as determined by:	
	Culture-independent method	Culture-dependent method
Firmicutes	74.2	76.05
Actinobacteria	14.1	4.85
Bacteroidetes	3.7	1.3
TM7	3.4	0
Proteobacteria	1.6	17.8
Planctomycetes	0.8	0
Verrucomicrobia	0.7	0
Cyanobacteria	0.1	0
Chloroflexi	0.1	0
Unclassified bacteria	1.3	0

(522 OTUs with 810 clones, or 74.2%) and *Actinobacteria* (123 OTUs with 155 clones, or 14.1%), followed by *Bacteroidetes* (39 OTUs with 40 clones, or 3.7%), TM7 (26 OTUs with 37 clones, or 3.4%), and *Proteobacteria* (17 OTUs with 18 clones, or 1.6%). The other phyla represented less than 1% of the total isolates: *Planctomycetes* (0.8%), *Verrucomicrobia* (0.7%), *Cyanobacteria* (0.1%), and *Chloroflexi* (0.1%). The remaining 1.1% of the total clones appeared as unclassified bacteria.

Species diversity was also investigated. Among the 1,091 sequences, 285 sequences had a similarity of >98% with a GenBank entry (Table 4). A total of 140 of the 285 identified sequences corresponded to well-known cultivable species, whereas 145 were affiliated to genera but did not correspond to any identified species.

Among the identified clones, the majority of *Firmicutes* belonged to the genus *Facklamia* (49 clones, of which 39 were assigned to *Facklamia tabacinasalis*), followed by the genera *Clostridium* (46 clones, comprising at least 4 species), *Turicibacter* (19 clones, of which 15 were assigned to *Turicibacter sanguinis*), the species *Aerosphaera taetra* (13 clones), and the genus *Staphylococcus* (7 clones, of which 5 were assigned to *Staphylocaccus devriesei*, 1 was assigned to *Staphylococcus aureus*, and 1 was assigned to *Staphylococcus auricularis*). The majority of clones identified as *Actinobacteria* belonged to the *Corynebacterium* genus (36 clones) and included at least four species, with a majority belonging to *Corynebacterium xerosis* (15 clones).

The distribution of microbial populations varied among the three farms studied. The 59 identified sequences from farm FA were distributed into 30 OTUs. The 111 identified sequences from farm FC were distributed into 53 OTUs. In farm FD, among a number of identified sequences similar to sequences in FC (115 sequences), only 15 OTUs were found. Farm FA was characterized by a high diversity of sequences identified as *Actinobacteria* (32 clones, 16 OTUs), with a majority belonging to *Corynebacterium* (15 clones, belonging at least to four species), followed by the *Firmicutes* (23 clones, 11 OTUs), with 7 clones assigned to *Facklamia* and 5 clones to *Aerosphaera taetra*. Farm FC showed a high diversity of identified sequences in all phyla. Among *Firmicutes* (52 clones, 25 OTUs), 17 clones were assigned to at least three *Clostridium* species; *Staphylococcus* (7 clones, 3 species) bacteria were found only in that farm, while no sequence was affiliated to *Facklamia*. Among the three farms, most sequences identified as *Bacteroidetes* and *Proteobacteria* were found in FC (18 clones and 12 OTUs in total), while the TM7 phylum was found only in FC. Finally, farm FD showed the lowest diversity in all phyla. It was characterized by a high number of sequences assigned to *Firmicutes* (94 clones, 10 OTUs), dominated by *Facklamia tabacinasalis* (33 clones), followed by *Clostridium* sp. (25 clones), *Turicibacter sanguinis* (12 clones), and *Aerosphaera taetra* (8 clones). Sequences identified as *Actinobacteria* (21 clones, 5 OTUs) belonged mainly to the genera *Corynebacterium* (17 clones) and *Dietzia* (3 clones).

DISCUSSION

The combination of culture-dependent and-independent methods gives a new vision of the microbial diversity existing on the surface of the teat skin, compared to the study of Vacheyrou et al. (58), where a higher number of farms was analyzed using only a culture-dependent method. In our study, among 29 species identified by the culture-dependent method, 10 of them were also found by the culture-independent method. In addition, 27 other bacterial species were found only by the culture-independent method. However, the majority of clones (79.8%) corresponded to unidentified and/or uncultured species.

Our results confirm that teat skin may be considered a possible microbial source for milk (40, 58) that must be controlled to avoid the presence of animal- or human-pathogenic bacteria. Teat skin (microbial diversity) may also be used as a source of bacteria useful for cheesemaking. The sensory qualities of raw milk cheeses result from a balance between different microbial populations (lactic acid bacteria, Gram-negative bacteria, *Staphylococcus*, high-GC Gram-positive bacteria, etc.), with each having a potential role, not yet fully understood, in aromatic compound production in cheese (14, 19). Lactic acid bacteria such as leuconostocs (28), enterococci (22), or nonstarter lactic acid bacteria (NSLAB) (4), which ensure acidification and/or produce antipathogenic and aromatic compounds in cheeses, were detected on teat skin in this study. The mesophilic *Lactobacillus* count was between 1.35 and 2.70 $\log_{10}$ CFU ml^{-1} of teat suspension, and the count of *Enterococcus* bacteria on SB medium was between 1.39 and 4.72 $\log_{10}$ CFU ml^{-1} of teat suspension. *Enterococcus faecium*, *Enterococcus hirae*, *Enterococcus lactis*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Streptococcus* sp., and *Streptococcus bovis* identified on teat skin have also been identified in milk (7, 8), suggesting that these species might be inoculated in milk by teat skin and not only by the teat canal (58). However, *Lactococcus lactis* (21, 23, 45), *Streptococcus thermophilus*, *Streptococcus parauberis*, and *Streptococcus dysgalactiae* (15, 25, 45), which have been identified in cow milk, have not been found on teat skin yet (58) or in the teat canal (26).

As noted by Vacheyrou et al. (58), *Aerococcus* sp. (particularly *Aerococcus viridans*) and *Staphylococcus* sp. were found dominant from culture-based methods, especially in farm FD. Similarly, *Staphylococcus arlettae*, *Staphylococcus fleurettii*, *Staphylococcus haemolyticus*, *Staphylococcus pasteuri*, *Staphylococcus saprophyticus*, *Staphylococcus succinus*, *Staphylococcus vitulinus*, *Staphylococcus xylosus*, *Staphylococcus aureus*, *Staphylococcus auricularis*, *Staphylococcus devriesei* and *Dietzia maris*, similar to *Staphylococcus*, identified in our study, are commonly found in milk or raw-milk cheeses (8, 15, 56). *Staphylococcus equorum* found in milk, cheese, and on cow teat (12, 35, 58) was not retrieved from teat skin in our study, but this species may have been misidentified

TABLE 4 Number of clones at the species level based on 16S rRNA gene sequence analysis

Phylum	Genus and/or species	No. of clones by farm			Total no. of clones
		FA	FC	FD	
Actinobacteria	*Aeromicrobium* sp.		1		1
	Arcanobacterium pyogenes	6			6
	Arthrobacter sp.		2		2
	Brachybacterium sp.	1			1
	Corynebacterium freneyi	2			2
	Corynebacterium pilosum	3			3
	Corynebacterium sp.	8	3	3	14
	Corynebacterium vitaeruminis	1	1		2
	Corynebacterium xerosis	1		14	15
	Curtobacterium sp.		1		1
	Dietzia cinnamea	1			1
	Dietzia maris			1	1
	Dietzia sp.			2	2
	Eggerthella hongkongensis strain		2		2
	Enterorhabdus caecimuris		1		1
	Janibacter melonis		1		1
	Knoellia subterranea		1		1
	Leucobacter aridicollis	1			1
	Leucobacter komagatae	1			1
	Marmoricola sp.		1		1
	Microbacterium phyllosphaerae	1			1
	Microbacterium sp.	1	4		5
	Uncultured *Microbacterium* sp.	1			1
	Nocardioides sp.		3	1	4
	Olsenella sp.	2	6		8
	Uncultured *Olsenella* sp.	1			1
	Propioniferax sp.	1			1
	Terrabacter terrae		1		1
	Tetrasphaera elongata		1		1
	Total for group	32	29	21	82
Bacteroidetes	Uncultured *Alistipes* sp.		2		2
	Uncultured *Bacteroides* sp.	1	4		5
	Parabacteroides merdae		1		1
	Uncultured *Parabacteroides* sp.		2		2
	Porphyromonas levii		1		1
	Uncultured *Chryseobacterium* sp.		1		1
	Total for group	1	11		12
Firmicutes	*Aerococcus* sp.		3		3
	Aerosphaera taetra	5		8	13
	Bacillus oleronius		1		1
	Bacillus sp.		1		1
	Uncultured *Bacillus* sp.		1		1
	Enterococcus sp.			1	1
	Facklamia tabacinasalis	6		33	39
	Facklamia sp.	1			1
	Uncultured *Facklamia* sp.			9	9
	Uncultured *Lactobacillus* sp.		1		1
	Sharpea azabuensis	1			1
	Staphylococcus aureus		1		1
	Staphylococcus auricularis		1		1
	Staphylococcus devriesei		5		5
	Streptococcus bovis	1		1	2
	Uncultured *Streptococcus* sp.		1		1
	Uncultured *Anaerovorax* sp.		1		1
	Butyrivibrio sp.		1		1
	Clostridium alkalicellulosi		1		1
	Clostridium jejuense		1		1
	Clostridium orbiscindens		1		1
	Clostridium sordellii	2			2
	Clostridium sp.	2	5	3	10
	Uncultured *Clostridium* sp.		9	22	31
	Coprococcus catus	1	2		3
	Eubacterium tenue	2		1	3
	Eubacterium sp.		4		4
	Uncultured *Ruminococcus* sp.		1		1
	Syntrophococcus sucromutans		1		1
	Uncultured *Erysipelothrix* sp.		1		1
	Holdemania filiformis		2		2
	Solobacterium moorei	1	3		4
	Turicibacter sanguinis	1	2	12	15
	Uncultured *Turicibacter* sp.			4	4
	Selenomonas ruminantium		2		2
	Total for group	23	52	94	169
Proteobacteria	*Bosea* sp.		1		1
	Uncultured *Devosia* sp.		1		1
	Methylobacterium sp.	2	1		3
	Paracoccus sp.		2		2
	Rhizobium sp.	1			1
	Uncultured *Acidovorax* sp.		1		1
	Uncultured *Stenotrophomonas* sp.		1		1
	Total for group	3	7		10
TM7	TM7 phylum sp.		12		12
Total identified		59	111	115	285
Total unidentified		273	283	250	806
Total of all		332	394	365	1091

since its 16S rRNA gene sequence is very similar to the sequences of *Staphylococcus xylosus* and *Staphylococcus saprophyticus* (35, 57). Coagulase-negative staphylococci (*Staphylococcus xylosus* and *Staphylococcus equorum*) can be involved in flavor and aroma formation of cheeses (35). *Aerococcus viridans* has some antilisterial properties (6).

Actinobacteria affiliated to the *Arthrobacter*, *Brevibacterium*, and *Corynebacterium* genera contribute to cheese texture, flavor, and color formation during ripening (17). *Corynebacterium variabile* is frequently found on cheese (21, 23, 45); however, it was not found in this study or in the one from Vacheyrou et al. (58). Sequences affiliated to *Corynebacterium xerosis*, as well as *Facklamia tabacinasalis*, *Aerosphaera taetra*, and *Turicibacter sanguinis*, are frequent among clones in the 16S rRNA library and were also detected in milk or cheese (15, 23, 46). All of these species were dominant among clones identified in farm FD, where the microbial community was not very diversified. FD differed from the other farms by its hygiene practices, which were not as stringent as in the other farms and could promote these species. The following species, less frequently detected on the surface of cow teats, were

also present in milk or cheese: *Bacillus oleronius* and *Bacillus pumilus* (11), *Leucobacter komagatae*, and *Eubacterium tenue* (15, 51), and Gram-negative bacteria such as *Enterobacter*, *Pseudomonas*, *Selenomonas*, *Pantoea agglomerans*, *Serratia*, *Escherichia coli*, and *Rahnella aquatilis* (13). Gram-negative bacteria may have a positive or negative effect on the flavor of cheeses, depending on their ratio in the microbial community (41, 42).

The species of *Clostridium* predominantly detected using the direct molecular method were not the most common species found in milk (i.e., *Clostridium lituseburense* and *Clostridium perfringens*) (15, 45).

In addition, other species which are not commonly found in dairy products were detected in our study on cow teat skin and were present on the surface of human or cow skin and in the cow teat canal. The coagulase-negative staphylococci including *Staphylococcus auricularis* (60) were considered the most common bacteria from skin of various animals (18, 53). *Streptococcus bovis* and *Streptococcus equinus* are other examples of species found on cow teat skin (49). *Staphylococcus devriesei* was identified on teat apices (56), *Staphylococcus arlettae* was found in the teat canal (26) or on human skin (24), and *Clostridium sordellii* was found in human intestinal microbiota and enteric lesions of cows (44).

Similarly, among species present on teat skin and uncommon in milk, many were detected in different types of environments: *Aerosphaera taetra* was previously identified from soil, *Clavibacter michiganensis* was from plants (3), *Arcanobacterium pyogene*s and *Arthrobacter gandavensis* were from cow uteri (48, 55), and *Coprococcus catus* (61) and *Solobacterium moorei* were found in feces (37). It is therefore not surprising to find these species and the numerous unidentified sequences or sequences identified as uncultured bacteria, such as those affiliated to the TM7 phylum in our study. They may originate from the components of the environment in contact with teat skin, such as soil (since the sampling was made before cleaning of the teat skin).

In consequence, teat skin may be a particularly interesting source of biodiversity for milk. It may be noted that the composition of the microbial community varied qualitatively and quantitatively from one farm to another. The variation of teat microbiota in link with milking production practices was underlined by Monsallier et al. (F. Monsallier, I. Verdier-Metz, C. Agabriel, B. Martin, and M. C. Montel, submitted for publication). The milking hygiene practices of farm FC were close to those of farm FA; nevertheless, their degrees of microbial diversity were different. Indeed, the microbiota from FC cow teat skin was the most diverse, with species (*Plantibacter agrosticola* and *Curtobacterium herbarum*) from an environment like the phyllosphere of grasses. Moreover the microbial diversity of cow teat skin from that farm may be underestimated, as indicated by rarefaction curves. FC was the only farm where the animals grazed a very diverse pasture every day. Then, it would be interesting to investigate more thoroughly the relationship between animal grazing and the diversity of the teat microbiota. As suggested in our study and in the one of Vacheyrou et al. (58), teat is not the only microbial source for milk; others have already been described, such as dust, air, and hay. On the other hand, some species found on teats had not yet been detected in milk. Further investigations at the species and strain levels will be necessary to better compare the microbial populations of cow teat skin and milk and to evaluate the microbial flow from environments to animals and from animals to milk.

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