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Coexistence of Lactic Acid Bacteria and Potential Spoilage Microbiota in a Dairy Processing Environment

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Microbial contamination in food processing plants can play a fundamental role in food quality and safety. In this study, the microbiota in a dairy plant was studied by both 16S rRNA- and 26S rRNA-based culture-independent high-throughput amplicon sequencing. Environmental samples from surfaces and tools were studied along with the different types of cheese produced in the same plant. The microbiota of environmental swabs was very complex, including more than 200 operational taxonomic units with extremely variable relative abundances (0.01 to 99%) depending on the species and sample. A core microbiota shared by 70% of the samples indicated a coexistence of lactic acid bacteria with a remarkable level of *Streptococcus thermophilus* and possible spoilage-associated bacteria, including *Pseudomonas*, *Acinetobacter*, and *Psychrobacter*, with a relative abundance above 50%. The most abundant yeasts were *Kluyveromyces marxianus*, *Yamadazyma triangularis*, *Trichosporon faecale*, and *Debaryomyces hansenii*. Beta-diversity analyses showed a clear separation of environmental and cheese samples based on both yeast and bacterial community structure. In addition, predicted metagenomes also indicated differential distribution of metabolic pathways between the two categories of samples. Cooccurrence and coexclusion pattern analyses indicated that the occurrence of potential spoilers was excluded by lactic acid bacteria. In addition, their persistence in the environment can be helpful to counter the development of potential spoilers that may contaminate the cheeses, with possible negative effects on their microbiological quality.

Cheese manufacture and ripening are affected by the metabolic activity of different types of microorganisms. When milk of optimal hygienic quality is used, the dairy microbial consortia can be simple when starter cultures are employed, or a higher degree of complexity can occur in the case of natural fermentations. The environmental microbiota from the processing plant has been often addressed as a source of microbes that may play a role in the cheese making (1–4). When lactic acid bacteria (LAB) are included in the environmental microbiota, they may actively contribute to fermentation and ripening of cheese. Conversely, when potential spoilage organisms contaminate the environment, these organisms can play a crucial role because they can be transferred from the environment to intermediates of production and may negatively affect the cheese production process and the quality of the final products. It has been demonstrated that the microbial populations involved in fermentation and ripening are often found on the processing surfaces (1, 3, 5, 6), highlighting the importance of the plant environment in potentially contributing to the microbiota of cheese. Depending on the nature of the microorganisms, the environmental microbiota can exert functional activities important for the fermentative and/or the ripening process but sometimes may also be a hazard for cheese quality and safety (7).

The study of the microbial ecology of foods has undergone a major revolution, and the advent in microbial ecology of sensitive culture-independent tools allows a rapid and effective evaluation of microbial contamination in many sorts of environments (8). Optimal conditions for microbial growth can occur in food processing facilities. The growth of microorganisms in a food processing environment and the establishment of certain microbial communities can lead to the development of a well-defined environmental microbiota. Various microbial contamination sources can be identified in a dairy processing plant, including the tank, cheese vat, bench, cloths, knives, and other tools (9). In the food industry, the resident microbial communities may create a persistent source of product contamination (5), causing food spoilage (10) and leading to serious hygienic problems (11, 12) and also economic losses (10, 13). Organic residues from food processing can create microenvironments for growth and accumulation of microorganisms and can represent a relevant source of cross-contamination (10, 13, 14). Improperly cleaned or sanitized equipment is usually considered the major source of milk and dairy product contamination (15). Exploring the relationships between fermentation and facility environment can be very useful to clarify whether the processing environment can actually influence the standard development of the cheese production. Microbial loads in the milk, as well as strain and species richness (16), increase after the milk is poured into a vat (4, 6, 16). Bokulich and Mills (1) demonstrated that fermentation-associated microbes dominate most surfaces in dairy environments and can be transferred to the product, influencing the course of the fermentation. The species composition of vat surface biofilms was found to be stable over several seasons but varied widely between vats (4, 17). Sometimes, facility-resident biotypes can outcompete the commercial strains.

Received 20 July 2015 **Accepted** 1 September 2015

Accepted manuscript posted online 4 September 2015

Citation Stellato G, De Filippis F, La Storia A, Ercolini D. 2015. Coexistence of lactic acid bacteria and potential spoilage microbiota in a dairy processing environment. Appl Environ Microbiol 81:7893–7904. doi:10.1128/AEM.02294-15.

Editor: C. A. Elkins

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02294-15.

TABLE 1 Description of dairy and environmental samples analyzed in this study[a]

Sample	Sampling method[b]	Description
Brine Caciocavallo	i	Liquid brine used for salting of Caciocavallo cheese
Caciocavallo after molding	ii	Caciocavallo cheese obtained after stretching and molding steps
Caciocavallo t_0	ii	Caciocavallo cheese obtained after stretching, molding, and brining; ready to start the maturation process
Caciocavallo t_{30}	ii	Caciocavallo cheese after 30 days of ripening
Chopper	iii	Steel machine used for curd shredding
Chopper 2	iii	See above
Curd bench	iii	Steel table where the curd is left to drain
Grancacio	ii	Pasta-filata cheese with production technology similar to Caciocavallo but characterized by a larger size
Hand	v	Hand of the stretching operator
Hook t_0	vi	Tool used to hang the Caciocavallo by a rope during ripening, sampled at the start of the maturation step
Hook t_{30}	vi	Hook sampled after 30 days of cheese ripening
Knife curd	vii	Knife used to cut the curd for the draining
Mold Grancacio	iv	Mold used for molding of Grancacio
Mold mozzarella	iv	Mold used for molding of mozzarella
Mold ricotta	iv	Mold used for molding of ricotta
Molder	iii	Steel machine used to mold the stretched curd for the production of mozzarella and Caciocavallo
Molder 2	iii	See above
Mozzarella	ii	Mozzarella cheese
Ricotta	ii	Fresh, soft cheese that does not undergo fermentation or ripening
Rope	ii	Used to tie Caciocavallo cheese molds at the neck in order to hang it for ripening
Scamorza	ii	Pasta-filata cheese with a semisoft texture and a typical pear shape
Stretcher	iii	Steel machine used to stretch the shredded curd
Stretcher 2	iii	See above
Tank curd	iii	Vat where milk is heated and rennet is added to obtain the curd
Tank ricotta	iii	Vat where whey is heated to be curdled to obtain ricotta cheese
Tank Scamorza	iii	Tank containing the brine for Scamorza cheese salting
Vat	iii	Vat containing the milk to be curdled

[a] The environmental samples were collected at least 1 h after ordinary cleaning procedures. Ordinary cleaning was performed within 1 h after cheese making. This process consisted of cleaning the floor surfaces and equipment with detergent and water (40°C), repeated rinsing with hot (40°C) and cold (20°C) water, and the use of disinfectants and rinsing with cold water. An additional weekly cleaning was applied to refrigeration and ripening rooms, staff rooms, and hallways.

[b] Sampling was performed using one of the following methods: (i) 50-ml portions of liquid brine were sampled by sterile vessels; (ii) representative longitudinal sections were sampled; (iii) the steel surface was sampled by rubbing it with a sterile cotton-tipped swab vertically, horizontally, and diagonally across the sampling site (100 cm^2) delineated by a template; (iv) mold was sampled by rubbing it with a sterile cotton-tipped swab vertically, horizontally, and diagonally across the sampling site (25 cm^2); (v) the operator's palm was sampled by rubbing it with a sterile cotton-tipped swab vertically, horizontally, and diagonally; (vi) the hook was sampled by rubbing it with a sterile cotton-tipped swab across all surfaces; (vi) both blades were sampled by rubbing them with a sterile cotton-tipped swab across all surfaces; or (vii) a rope 25 cm in length (the standard length used to tie the Caciocavallo) was taken.

In fact, the bacteria found in ripened washed-rind cheeses were different from those inoculated (7, 18).

It can be supposed that an equilibrium exists between dairy products and plant environment where microbial transfer occurs from both parts. Consequently, the dairy-environment relationship has the potential to affect the dairy process dynamics and the quality of the final products.

In this study, environmental swabs were collected in a dairy plant, and they were analyzed by using a culture-independent amplicon sequencing approach in order to describe the microbiota populating the dairy environment. Moreover, the microbiota of the cheeses produced was also assessed with the additional aim of investigating the existing overlap between environmental and cheese microbiota and how such a relationship may influence the quality of the manufactures.

MATERIALS AND METHODS

Sampling and DNA extraction. The dairy plant considered in the present study is located in the Campania region (Southern Italy) in the province of Salerno, and it is involved in the production of different dairy products: ricotta (R), mozzarella (M), Caciocavallo (C), Grancacio (G), and Scamorza (S) cheeses. All of these cheeses except R are produced by using natural whey cultures (NWCs) as a natural starter for the fermentation according to a back-slopping procedure (19). Sample collection was replicated twice at 3-week intervals. More details regarding the samples and sampling procedures are provided in Table 1. Across all areas, surface swab samples from work surfaces and from all tools usually used during the production process were taken. In addition, samples from the R, M, C, G, and S cheese manufactures were collected. The sampling took place on the surfaces after routine cleaning (Table 1) and before the start of production. Surfaces were sampled with sterile cotton-tipped swabs that were moistened with sterile phosphate-buffered saline (PBS) and rubbed vertically, horizontally, and diagonally across the sampling site (100 cm^2) delineated by a template, rotating the swab to ensure full contact of all parts of the swab tip and surface (1).

After sample collection, the swabs were placed into sterile, 10-ml polyethylene tubes containing 1 ml of sterile PBS, cooled at 4°C for the necessary time of transport to the laboratory. The cheeses were sampled after production, transferred into sterile plastic bags, and transported to the laboratory under refrigeration. Environmental swabs and cheese samples were analyzed within 6 h.

Prior to DNA extraction, the tubes were vigorously stirred in a vortex to transfer the cells from the swab to solution. Total DNA extraction from the swab and cheese samples was carried out using a Biostic Bacteremia DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The extraction protocol was applied to the pellet (12,000 × *g*) obtained from 2 ml of suspension for the swab samples or from 2 ml of a homogenized 2-fold

TABLE 2 Observed diversity and estimated sample coverage for 16S and 26S rRNA amplicons analyzed in this study

Sample	OTUs		Chao1		Shannon index (%)		ESC[a] (%)	
	16S	26S	16S	26S	16S	26S	16S	26S
Brine Caciocavallo	93	8	155.50	8.50	3.51	0.19	98	99
Caciocavallo after molding	67	18	86.25	18.00	1.94	3.18	99	99
Caciocavallo t_0	42	20	50.63	21.00	3.03	2.43	99	99
Caciocavallo t_{30}	58	22	71.34	22.00	3.60	2.98	99	99
Chopper	79	13	119.89	16.00	3.18	1.21	99	99
Chopper 2	81	20	110.86	26.00	3.44	2.83	99	98
Curd bench	98	18	124.69	20.50	3.75	1.47	98	99
Grancacio	65	16	79.62	19.00	2.13	1.18	99	99
Hand	120	18	142.47	19.50	4.73	1.83	99	99
Hook t_0	83	3	115.21	3.00	2.85	0.03	99	99
Hook t_{30}	71	4	101.86	4.00	2.99	1.51	98	100
Knife curd	127	10	154.77	11.00	4.22	0.82	98	99
Mold Grancacio	85	11	108.54	12.00	3.42	0.88	99	99
Mold mozzarella	60	27	96.50	34.50	2.56	2.65	98	99
Mold ricotta	98	12	130.75	12.00	4.15	2.70	99	99
Molder	92	22	115.79	22.60	3.22	3.04	99	99
Molder 2	128	18	171.02	18.00	3.62	3.13	99	99
Mozzarella	62	20	77.55	20.50	1.70	2.39	99	99
Ricotta	114	11	160.43	14.00	4.50	1.91	99	99
Rope	102	17	139.53	17.50	3.93	2.19	99	99
Scamorza	79	21	100.08	22.50	2.22	2.65	99	99
Stretch dipper	61	23	81.60	26.00	2.93	3.24	99	99
Stretcher	68	22	112.52	22.00	2.86	3.82	99	99
Stretcher 2	82	23	102.56	29.00	2.96	3.61	99	99
Tank curd	111	7	131.03	8.00	4.60	0.82	99	99
Tank ricotta	79	12	115.73	12.00	2.48	2.53	99	99
Tank Scamorza	90	21	139.83	22.50	3.44	2.77	99	99
Vat	96	24	122.09	25.00	3.86	3.15	99	99

[a] ESC, estimated sample coverage (Good's coverage).

dilution of the cheese samples in one-quarter-strength Ringer's solution (Oxoid, Milan, Italy). All of the samples were collected and used under the surveillance of the dairy manager. No animals were involved in the present study; only animal products were employed.

16S and 26S rRNA gene amplicon library preparation and sequencing. The bacterial diversity was studied by pyrosequencing of the amplified V1-to-V3 region of the 16S rRNA gene amplifying a fragment of 520 bp (20). 454 adapters (454 Life Sciences, Roche, Italy) were included in the forward primer, followed by a 10-bp sample-specific multiplex identifier (MID). Each PCR mixture (final volume, 50 μl) contained 60 ng of template DNA, 0.1 μM concentrations of each primer, 0.50 mmol of each deoxynucleoside triphosphate liter^{-1}, 2.5 mmol of $MgCl_2$ liter^{-1}, 5 μl of 10× PCR buffer, and 2.5 U of native *Taq* polymerase (Invitrogen, Milan, Italy). The following PCR conditions were used: 94°C for 2 min, 35 cycles of 95°C for 20 s, 56°C for 45 s, and 72°C for 5 min, with a final extension at 72°C for 7 min. The fungal community was studied by sequencing of the D1-D2 domain of the 26S rRNA gene amplifying a fragment of 540 bp using the primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) (21) as recently reported (54). The 454 adapters were included in the forward primer, followed by a 10-bp sample-specific MID. PCR mixtures were prepared as described above. The following PCR conditions were used: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 52°C for 45 s, and 72°C for 1 min, and then a final extension at 72°C for 7 min and holding at 4°C (54).

After agarose gel electrophoresis, PCR products were purified twice using an Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and quantified using the QuantiFluor system (Promega, Milan, Italy), and then an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions, using Titanium chemistry (20).

Bioinformatics and data analysis. In order to remove sequences of poor quality, raw reads were first filtered according to the 454 processing pipeline. Sequences were then analyzed and further filtered using QIIME 1.8.0 software (22) and a previously described pipeline (23). The reads were excluded from the analysis if they were shorter than 300 or 450 bp for the 16S or 26S rRNA gene, respectively. Sequences that passed the quality filter were chosen and singletons were excluded. Operational taxonomic units (OTUs) defined by 99% similarity were picked using the UCLUST method (24), and the representative sequences were submitted to the RDPII classifier (25) to obtain the taxonomy assignment and the relative abundance of each OTU. The Greengenes 16S rRNA gene database (26) was used for the taxonomic assignment of bacteria, and the deepest level of assignment was interpreted as a putative species identification. For the 26S rRNA gene, the centroids of each sequence cluster (i.e., the longest sequence) were compared to the sequences reported in GenBank by using the BLAST (Basic Local Alignment Search Tool) algorithm, in order to obtain the taxonomic assignment. Alpha and beta diversities were studied through QIIME as previously described (23). Statistical analysis and plotting was carried out in the R environment (http://www.r-project.org). Venn diagrams were obtained by using Bioinformatics and Evolutionary Genomics software (27) in order to describe the microbial community shared by different sets of samples.

Metagenome predictions. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States [http://picrust.github.io/picrust/]) (28) was used to predict the functional profiles in the microbial communities of environmental swabs and cheese samples. For the analysis with PICRUSt, the OTU levels at 97% identity were picked by the closed reference method against the Greengenes database (version 05/2013) using QIIME 1.8. The data were normalized for 16S rRNA gene copy numbers, and the metagenomes were predicted. From the inferred metagenomes, KEGG orthologs were identified, and the table obtained

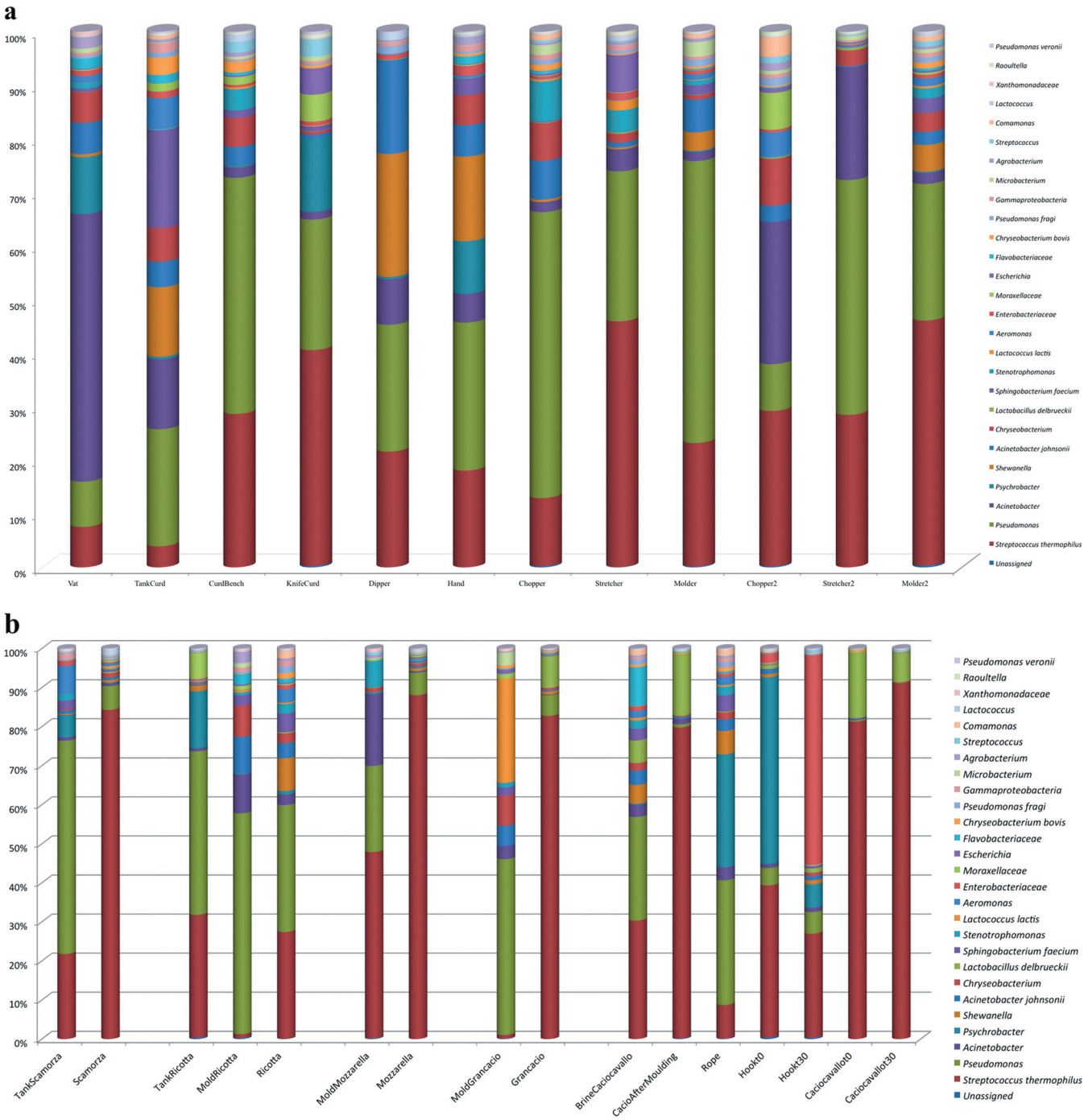

FIG 1 Abundances of bacterial genera and species in environmental swabs from the dairy plant (a) and in cheeses with their relative specific tools and equipment (b). Only OTUs occurring in 70% of the samples are reported.

was rarefied at the lowest number of sequences per sample. KEGG orthologs were then collapsed at level 3 of hierarchy, and the resulting table was imported in R (www.r-project.org). Nearest sequenced taxon index (NSTI) values were calculated in order to evaluate the accuracy of metagenome predictions, which depends on how closely related the microbes in a given sample are to microbes with sequenced genome representatives; NSTIs with lower values indicate a closer mean relationship (28).

Nucleotide sequence accession number. The 16S and 26S rRNA gene sequences are available at the Sequence Read Archive of the NCBI (accession number SRP058584).

RESULTS

Sequencing data analysis and alpha and beta diversity. A total of 117,490 reads passed the filters applied through the QIIME split_library.py script, with an average length of 454 bp. The results of the alpha diversity analysis are reported in Table 2. For both 16S and 26S rRNA genes, Good's estimated sample coverage (ESC) indicated that a satisfactory coverage was reached for all of the samples (an ESC of 99% in most cases). The diversity indices varied greatly depending on the samples, and there was a signifi-

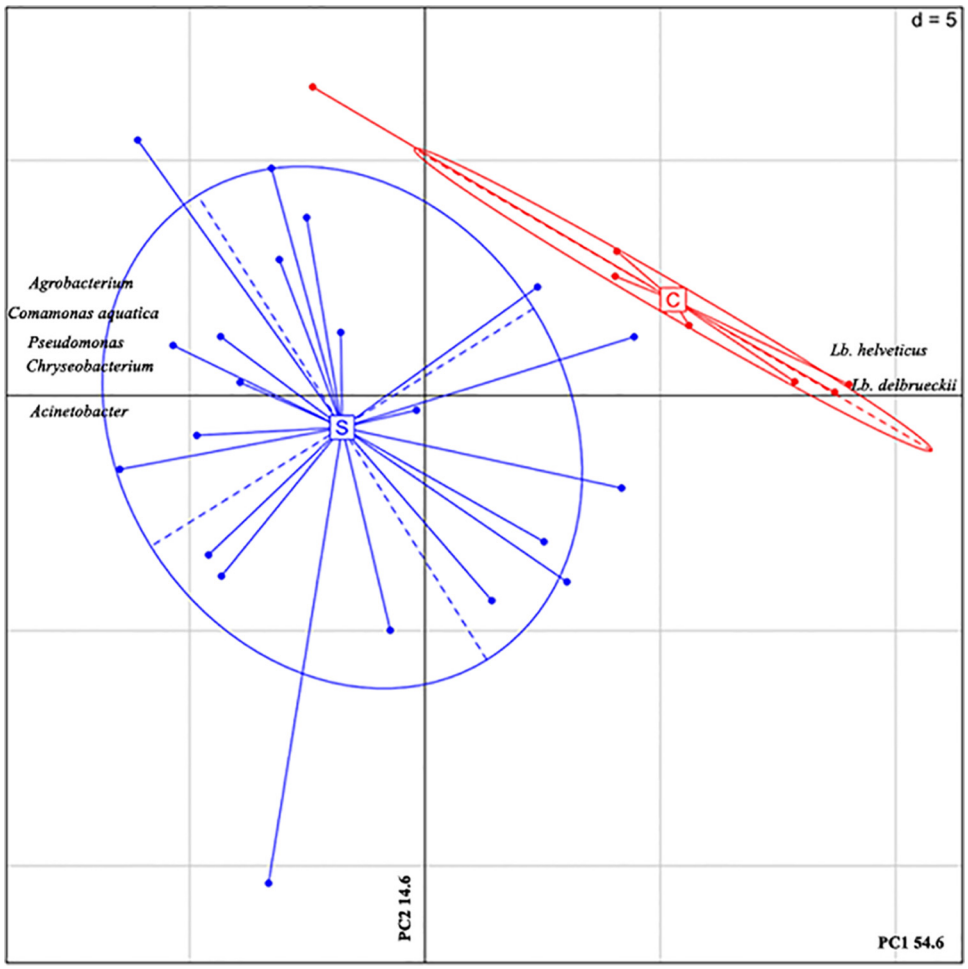

FIG 2 Principal component analysis based on the species-level microbiota. The two principal components were plotted using the Made4 package in R. The center of gravity for each cluster is marked by a rectangle indicating the sample type (S, swabs; C, cheeses). Only OTUs that showed a loading score of >0.7 are shown in the figure.

cant association between the sample type (cheese or environmental samples) and diversity indices, as confirmed by the Adonis and Anosim statistical tests run by QIIME ($P < 0.001$).

The microbial diversity in the different samples was quite heterogeneous for the 16S rRNA, but overall a higher level of diversity was shown in the swabs than in the cheese samples, with average values of 90 and 70 OTUs, respectively. The principal coordinate analysis (PCoA) based on weighted Unifrac distance showed a clear separation between cheese and environmental microbiota, except in the case of the ricotta cheese, which clustered with the swab samples (see Fig. S1a in the supplemental material). A lower level of microbial diversity was found in 26S data, as shown by the lower number of fungal OTUs detected (Table 2); however, an analysis of the fungal community confirmed the microbiota-driven differences between cheeses and environmental swabs (see Fig. S1b in the supplemental material). All of the bacterial and fungal taxa identified in the present study are reported in Tables S1 and S2 in the supplemental material.

Bacterial diversity in cheeses and dairy plant. Two different samplings in the same dairy were carried out. Since the Kendall's correlation between the matrices obtained in the two samplings was high (Kendall's tau = 0.71; $P = 2 \times 10^{-16}$), the average OTU abundance was used for the subsequent analyses. A common microbiota, including OTUs occurring in 70% of the samples, was shared among the samples, though taxa with variable relative abundances were included (Fig. 1). The most abundant members of the core microbiota were *Streptococcus thermophilus*, *Pseudomonas*, *Acinetobacter* spp., *Acinetobacter johnsonii*, and *Psychrobacter* spp., which occurred in 99% of the samples. A predominance of *Streptococcus thermophilus* was found in the cheese samples (average, 70%), except for the ricotta, where the abundance was 24%. In the environmental samples, the abundance of *S. thermophilus* was extremely variable, ranging from 3% (tank curd) to 43% (stretcher). *Pseudomonas* was found in all of the samples; however, in the cheese samples, it was a minor contaminant (never above 5%), except for the ricotta, where its abundance reached 30%. Moreover, *Pseudomonas* showed a remarkable presence in the environmental samples, achieving the highest levels in the molder and chopper (50%), followed by curd bench, chopper 2, tank, and mold dedicated to ricotta cheese making (average, 30%).

Acinetobacter occurred in all of the samples, displaying higher levels in vat specimens (33%) and a lower average abundance of 20% in other environmental samples (Fig. 1). Moreover, some

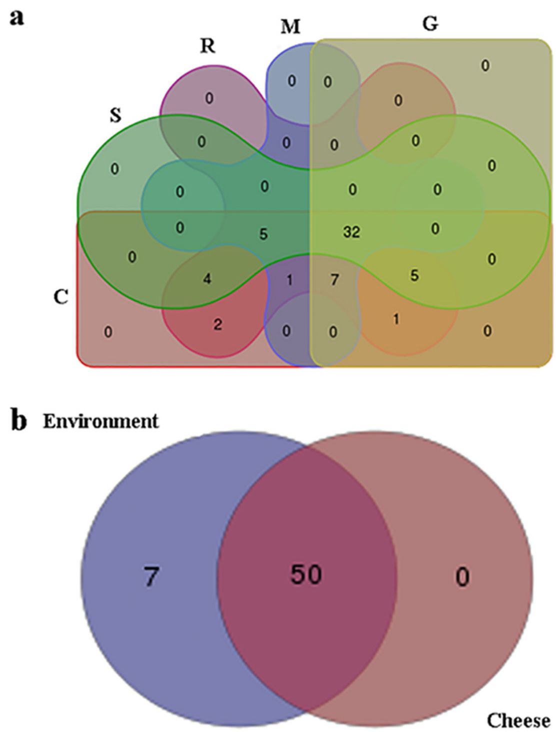

FIG 3 Venn diagram showing the number of shared OTUs between samples obtained by 16S rRNA gene pyrosequencing analysis. Samples were grouped by cheese type, including relative environmental samples from the dedicated equipment (a) and their combination, separating environmental swabs from cheese samples (b). C, Caciocavallo; S, Scamorza; R, ricotta; M, mozzarella; G, Grancacio.

OTUs prevailed only in specific samples. For example, *Lactobacillus delbrueckii* occurred in 70% of the samples, but it was particularly abundant in Caciocavallo samples at the beginning of the manufacture (15%), as well as *Lactococcus lactis*, which had a remarkable abundance in mold Grancacio (20%) but in the rest of the samples was never above 1%. A principal component analysis of the species-level microbiota clearly grouped cheeses and environmental swabs separately (Fig. 2). Many OTUs of the *Firmicutes* phylum, such as *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactobacillus casei* group, *Pediococcus* sp., *Leuconostoc* sp., *Weissella* sp., *Lactococcus garviae*, and *S. thermophilus*, had significantly higher abundances in cheeses ($P < 0.01$), whereas *Corynebacterium variabile*, *Kocuria* sp., *Microbacterium* sp., *Pseudoclavibacter helvolus* (*Actinobacteria*) and *Pseudomonas* spp., *Psychrobacter* sp., *Acinetobacter* sp., and *Paracoccus marcusii* (*Proteobacteria*) were more abundant in environmental swabs ($P < 0.01$).

With regard to genus-level OTUs found in at least 30% of the samples, the numbers of genera shared between the different groups of samples are represented in Fig. 3. The samples were grouped by cheese type, and for each cheese type the environmental samples from the specific equipment were included. Thirty-two OTUs were common to all of the groups, and, interestingly, no microbial genus was specifically associated with a single-cheese group of samples (Fig. 3a). Remarkably, seven genera were specific for the environmental samples, while none was exclusively associated with cheeses (Fig. 3b).

Fungal diversity in cheese and dairy plant. Thirty-six fungal OTUs were identified, and they were distributed among the samples with different relative abundances (Fig. 4). Overall, the most abundant species in environmental swabs were present in the corresponding cheese samples, although they were never predominant. The most abundant were *Kluyveromyces marxianus*, *Yamadazyma triangularis*, *Trichosporon faecale*, and *Debaryomyces hansenii*, occurring in 90% of the samples. A predominance of *Y. triangularis* was found in the cheese samples (average, 56%), except for the Caciocavallo cheese, where *K. marxianus* was the most abundant; this difference probably caused the differentiation in the PCoA plot showing the separation of samples based on 26S rRNA data (see Fig. S1b in the supplemental material). On the other hand, in the swab samples, the abundance of *Y. triangularis* was extremely variable, ranging from 0.7% (knife curd) to >90% (brine and hook t_0). *T. faecale* was found in 95% of the samples, although it was never >2% in cheese samples and it was much more abundant in the curd-related environmental swabs (Fig. 4). *D. hansenii* had a 90% occurrence rate, with higher levels of relative abundance in R, M, and related environmental samples.

Also, in the case of fungal communities, most of the OTUs were shared by the different cheese groups. Twelve genera were common to all cheese groups and related equipment. In this case, some group-specific genera could be identified (Fig. 5a), although they never displayed >1% relative abundance values. Eighteen genera were shared by the environmental swabs and cheese samples (Fig. 5b). Only *Candida sake* was found exclusively in cheese samples, although its average abundance was low (0.01%).

OTU cooccurrence and/or coexclusion. With regard to the 16S rRNA gene data, OTU cooccurrence was investigated by considering the genus-level taxonomic assignment and including OTUs with at least 0.1% relative abundance in at least five samples and significant correlations with a false discovery rate (FDR) of <0.05 (Fig. 6a). *Lactobacillus* showed the highest number of negative correlations, including the core OTUs of *Pseudomonas*, *Acinetobacter*, and *Agrobacterium*, whereas it cooccurred with *Streptococcus*. The analysis of the relationships within the fungal microbiota showed a coexclusion between *Saccharomyces* and *Debaryomyces* and between *Yamadazyma* and *Trichosporon* (Fig. 6b).

Diversity of predicted metagenomes. The PICRUSt tool was used to predict the metagenomes from the 16S rRNA gene sequence database (28). The weighted NSTI for the samples of the present study was 0.015 ± 0.013. More precisely, the cheese samples had the lowest NSTI values (0.003 ± 0.07), whereas environmental swabs had an average NSTI of 0.018 ± 0.013. A clear separation between cheese and environmental samples was achieved also by considering the predicted metagenomes (Fig. 7). In particular, cheeses were characterized by a lower abundance of KEGG pathways belonging to xenobiotic biodegradation and metabolism and biosynthesis of other secondary metabolites. In contrast, galactose metabolism, glycolysis, pentose phosphate pathways, and activities related to several amino acid metabolisms were more abundant in cheeses than in environmental samples.

DISCUSSION

In this study, the microbiota in a dairy processing plant was studied by rRNA gene-based culture-independent high-throughput sequencing to describe the bacterial patterns characterizing the environment and the different cheese manufactures. The microbiota of the environment was very complex, including more than 500 taxa at the genus/species level. Other studies describing the microbial community across surfaces in cheese-making plants re-

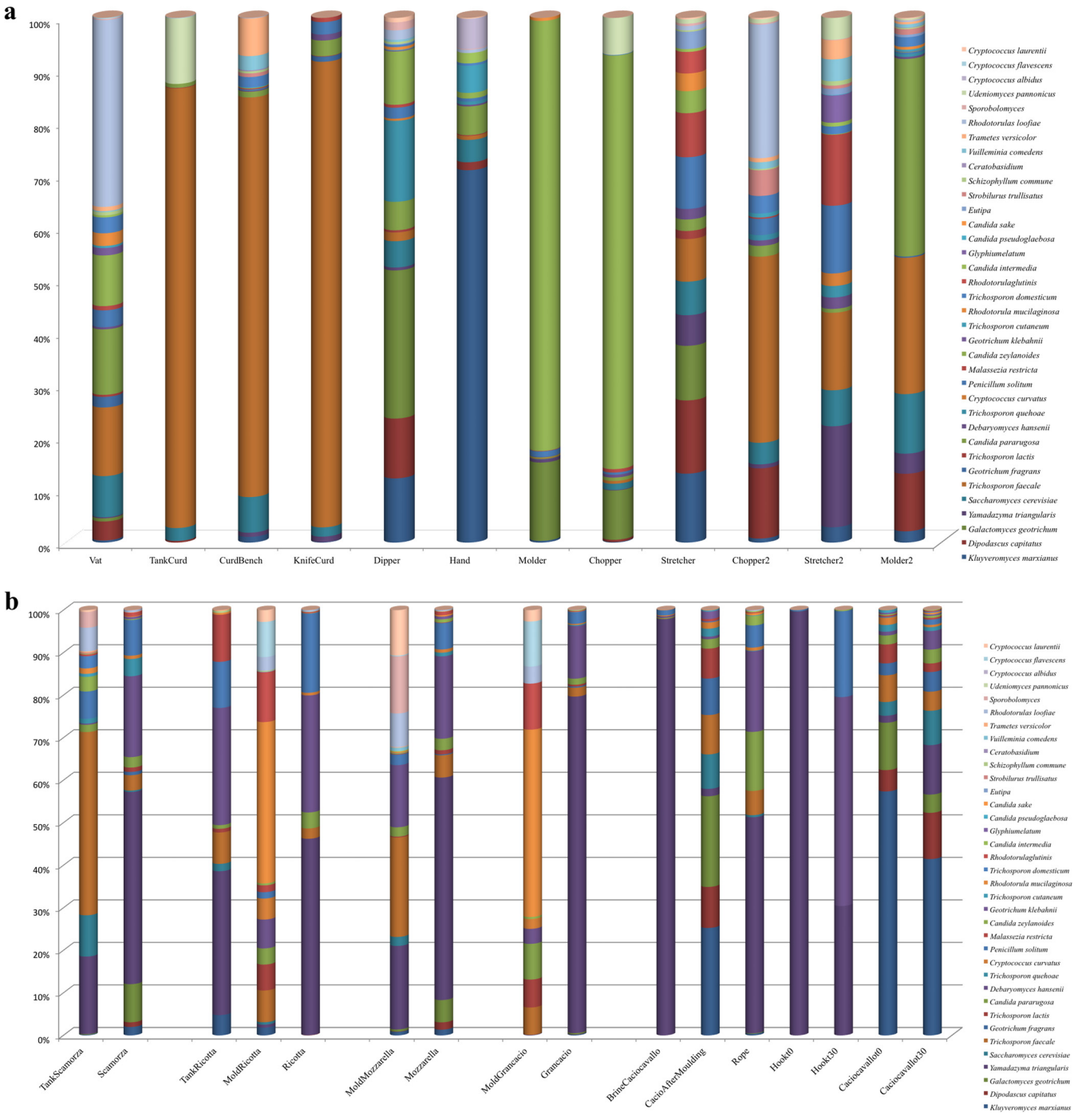

FIG 4 Abundance of fungal genera and species in environmental swabs from the dairy plant (a) and in cheeses with their relative specific tools and equipment (b). Only OTUs occurring in 70% of the samples are reported.

ported that the microbiota from surfaces had a high variability in composition, and most of the OTUs identified in the cheese manufacture originated from the processing environment (1, 29, 30). Our microbial community structure had several microbial species in common with the previous studies, although the relative abundance of the species can depend remarkably on the specific manufacture studied.

The settlement of the resident microbiota can depend on the characteristics of the surfaces, ecological factors, nutrient availability and composition, and the ability of microbes to develop biofilms, as well as on operators and cleaning procedures (2, 30, 31). The cleaning procedures used in the dairy plant constantly ensure, according to the producers, a standard quality of the cheeses; no spoilage case has been reported in the last 5 years. The most abundant bacteria in the dairy environment were *Streptococcus thermophilus*, *Pseudomonas* spp., and *Psychrobacter* spp., while

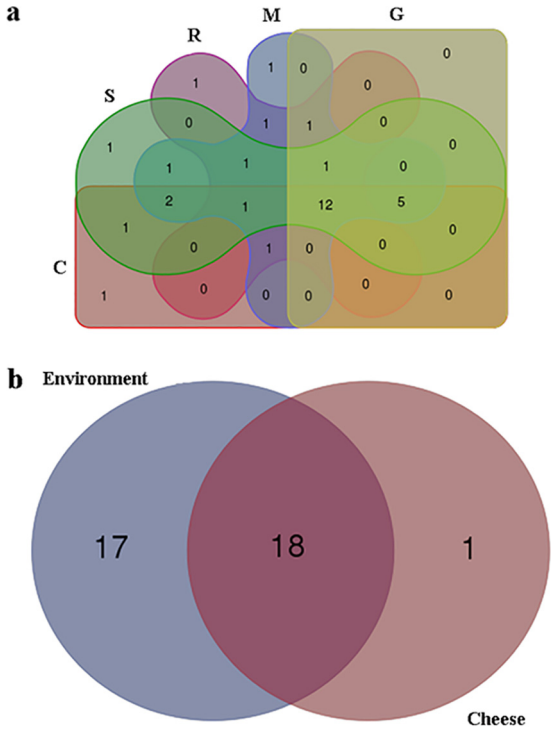

FIG 5 Venn diagram showing the number of shared OTUs between samples obtained by 26S rRNA gene pyrosequencing analysis. Samples were grouped by cheese type, including relative environmental samples from the dedicated equipment (a) and their combination separating environmental swab from cheese samples (b). C, Caciocavallo; S, Scamorza; R, ricotta; M, mozzarella; G, Grancacio.

Debaryomyces, *Yamadazyma*, and *Galactomyces* spp. prevailed among the fungi. These bacterial and fungal genera (excluding *Yamadazyma*) were already reported in other studies describing the microbiota of cheese-making plant surfaces (1, 3, 31, 32). They coexisted in the same environment and are spread both across surfaces and in the dairy intermediates and products. Differences in relative abundance were mainly due to fermentation and processing, with *S. thermophilus* more abundant in the cheeses and the potential spoilers *Pseudomonas* and *Psychrobacter* more abundant on the surfaces. Similarly, *Y. triangularis* and *D. hansenii* were more abundant in cheese, whereas *T. faecale* was more abundant on surfaces. This is likely due to selective conditions imposed by curd fermentation and heating steps (such as curd stretching for the pasta-filata cheese) that are unfavorable to the persistence of the spoilers. Such distribution suggests that specific care should be taken in the ordinary cleaning of tools and surfaces that are involved in the postfermentation steps of production. Interestingly, some of the above-mentioned species coexcluded in the dairy environment. *Streptococcus* and *Lactobacillus* indeed had the highest numbers of coexclusions with contaminating and potential spoilage bacteria, and the same behavior was noticed for *Yamadazyma*. All of the cheeses produced in the two visits to the dairy had standard quality and did not present any defects. It is interesting to speculate that the establishment of LAB in the environment could have a valuable effect toward protection against spoilage bacteria, as supported by our coexclusion patterns, and protection against spoilage could be obtained when LAB colonize the environment. Such coexclusion may be due to differential colonization capability, which depends on several factors (2, 30, 31). Our evidence is based on only one dairy, although it comes from repeated samplings; therefore, other surveys involving other dairies using natural starters will be important to further support our theory. Cocultivation assays involving LAB and cheese spoilage bacteria would be valuable for studying competition mechanisms in depth, although these trials are not always fully descriptive of the actual relationships that are established in real cheeses (55). The LAB species identified here come from the NWCs used for the fermentation, although strain-level identification was not performed in the present study. Indeed, *Streptococcus thermophilus* and species of *Lactobacillus* are frequently found in the NWCs for cheese production (20, 23, 33, 34). However, these species are persistently found in the environment even after cleaning, as shown by our results, and this is consistent with the current literature reporting *S. thermophilus* (16) and other LAB to be persistent in the environment (6, 17, 35). LAB use in manufacture and their occurrence in the environment can be regarded as beneficial for both cheese quality and environmental protection. This is further confirmed by the fact that ricotta, the only cheese analyzed that is not derived by a fermentation procedure, although it comes from the heat treatment of the whey resulting from other manufactures, showed a high microbial diversity, which made it indistinguishable from the environmental samples. In addition, the lower incidence of LAB in ricotta was associated with a higher relative abundance of contaminating bacteria from the surfaces and the related tools of production. The gaskets used for molding and as containers for ricotta are made of plastic material, which is more porous, less adequate for a thorough cleaning, and sensitive to hot water, which can affect the mechanical properties of the plastic, in turn permitting corrosion, leading to an increased possibility of adherence by bacteria (33, 39, 40). Consistently, all of the cheese molders made of stainless steel showed a lower microbial diversity (in terms of number of OTUs) than the plastic ones, suggesting that these should replace the plastic gaskets for improved quality and safety of the unfermented dairy productions. The variability of the alpha diversity parameters indicated that surface contamination is strictly dependent on the surface material, which represents a fundamental issue for the maintenance of standard hygiene in food processing plants (31).

Pseudomonas in the environmental samples likely originates from raw milk, which can be contaminated by water and soil or by inappropriate sanitizing of milking surfaces, or contamination can occur during storage and while transporting equipment (30). In addition, persistence on surfaces can be facilitated by the well-known ability of *Pseudomonas* to adhere to food processing surfaces (30, 38–40), with the organism growing very rapidly on dairy plant equipment (36, 41). The psychrotrophic nature of *Pseudomonas* can help it to withstand the competing microbial populations in milk and in fresh cheeses (42–45), possibly determining changes in food structure or discoloration, such as the case of "blue mozzarella cheese" (46, 47). *Acinetobacter*, *Pseudomonas*, and *Psychrobacter* spp. can be involved in food spoilage, and they are recognized as undesirable bacteria in food processing environments (48). In the present study, these bacteria were found to be abundant in several samples, confirming some of our previous evidence suggesting that they could be members of the resident microbiota of food processing plants (49, 50).

The predominance of some specific yeasts can reduce the risk of spoilage by other microorganisms. This was demonstrated in

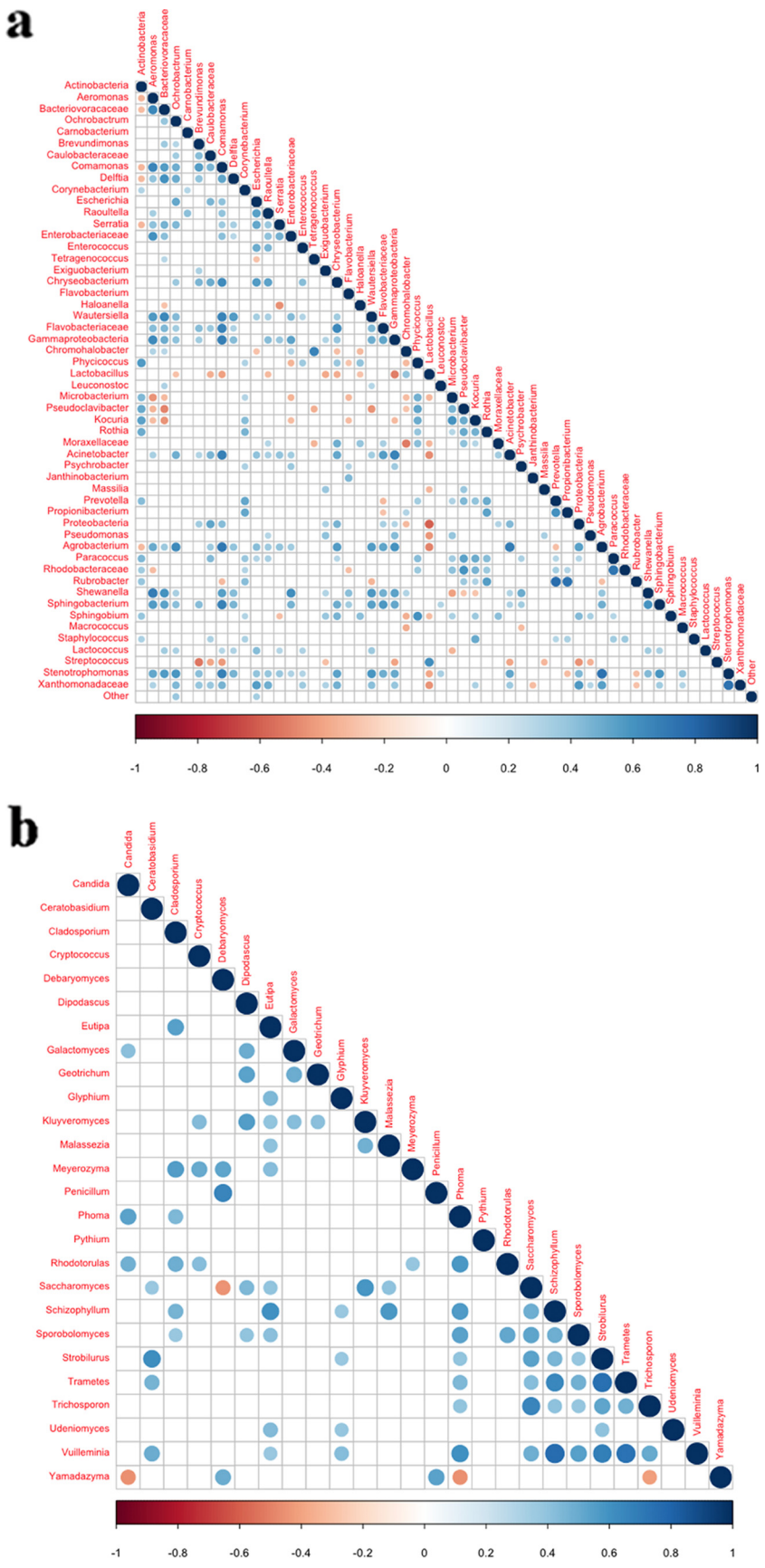

FIG 6 Spearman rank correlation matrix of bacterial (a) and fungal (b) OTUs with a >0.1% abundance in at least five samples. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colors of the scale bar denote the nature of the correlation, with "1" indicating a perfectly positive correlation (dark blue) and "−1" indicating a perfectly negative correlation (dark red) between two microbial genera. Only significant correlations (FDR < 0.05) are shown.

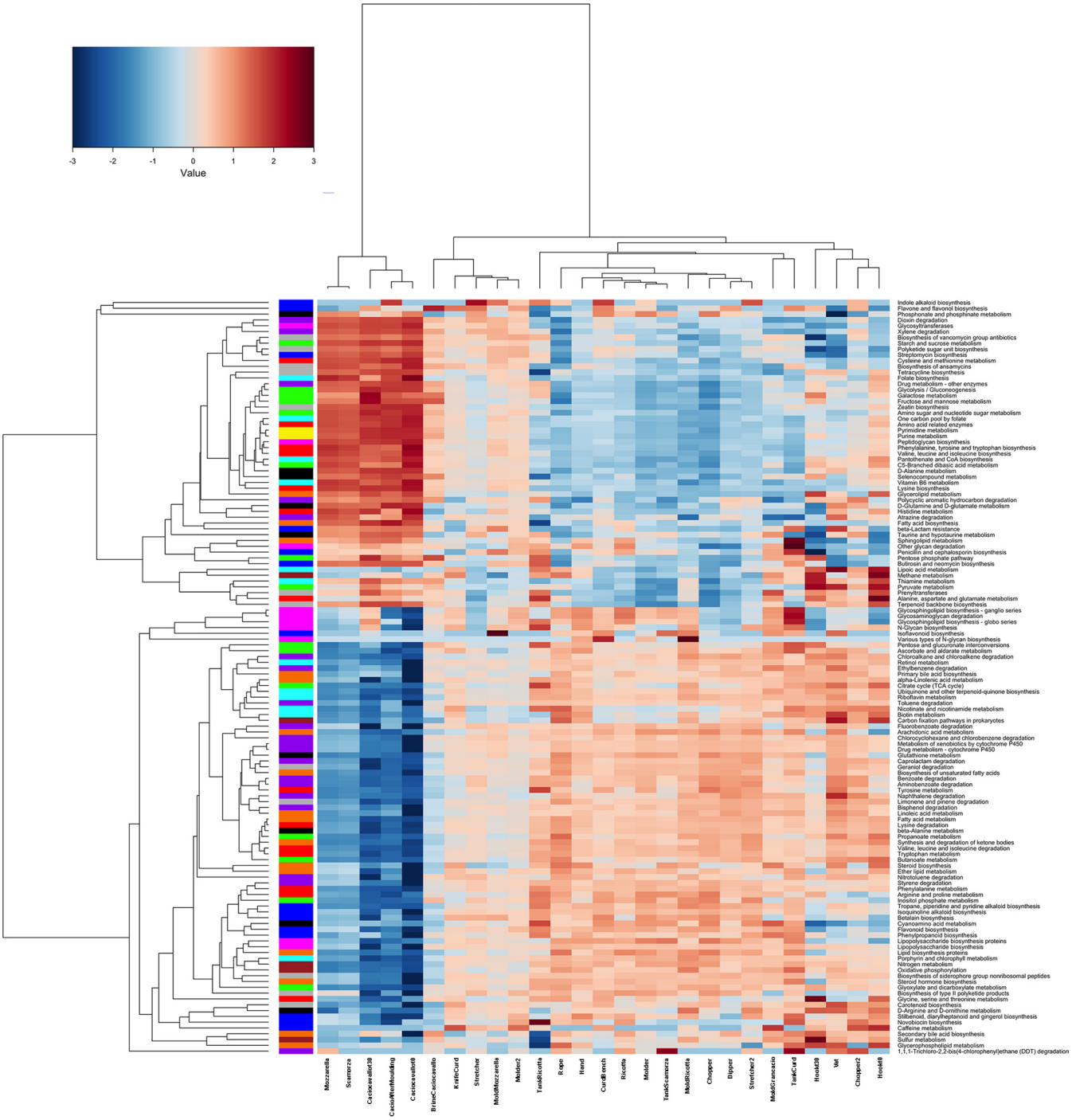

FIG 7 Average linkage clustering based on the Spearman correlation coefficients of the proportion of KEGG orthologs collapsed at level 3 of the hierarchy, filtered for a subject prevalence of at least 10%. Row bar colors denote the higher level of hierarchy (L2): carbohydrate (green), amino acid (red), energy (brown), cofactor and vitamin (cyan), terpenoid and polyketide (gray), lipid (orange), xenobiotic (purple), nucleotide (violet), and other amino acid (black) metabolisms; biosynthesis of other secondary metabolites (blue); glycan biosynthesis and metabolism (magenta). Only KEGG orthologs belonging to the metabolism category were considered. The color scale represents the scaled abundance of each gene, denoted as the Z-score, with red indicating high abundance and blue indicating low abundance.

the case of *D. hansenii* (a member of our core microbiota), which was able to control contamination by clostridial species through the production of antibacterial metabolites (51). Previous studies indicated that *D. hansenii* originates from the dairy house microbiota (1, 29, 52), and this further highlights the possible impact of the environmental microbiota on food processing.

Cleaning procedures can have an impact on the spatial distribution of the microbial communities in food processing plants

(51, 53, 56). Our samplings were performed after the cleaning routine. The results of replicate samplings showed consistent microbial profiles and suggest that these communities are established on the surfaces and on the equipment in spite of frequent cleaning and sanitation. Indeed, our study focused on one dairy plant only, and different results may be obtained in other processing environments where different starter cultures, raw materials, and cheese-making protocols are used. However, the persistence of dairy bacteria in the environment and the occurrence of a cheese environment core microbiota are consistent with previous reports (1), suggesting that this may be the general case.

Environmental microbiota in food processing plants can be very important for the achievement and maintenance of food quality. The persistence of LAB in the environment can be helpful to contrast the development of potential spoilers, and the use of natural starters may represent a valuable source of robust LAB that can spread in the environment.

Facility ecosystem surveillance by mapping the microbiota may become a valuable approach to monitor environmental contamination in order to support the overall quality management in the dairy plants. Moreover, understanding the interactions between cheese and specific environmental microbiota can represent a crucial step to ensure cheese manufacture of a standard quality level.

ACKNOWLEDGMENTS

This study was supported by a grant from the Ministero dell'Istruzione, dell'Università e della Ricerca within the program PON01_02863 Research and Competitiveness 2007–2013, under the project "Encapsulation of Active Ingredients for Improving the Quality and Food Safety." F.D.F. was supported by a grant from Campania Region within the program "POR CAMPANIA FSE 2007/2013" (project CARINA: Safety, Sustainability, and Competitiveness of the Agro-Food Production in Campania).

We thank the dairy Campolongo S.r.l., Montesano sulla Marcellana, Italy, for providing samples for this study.

REFERENCES

1. **Bokulich NA, Mills DA.** 2013. Facility specific "house" microbiome drives landscapes of artisan cheesemaking plants. Appl Environ Microbiol **17:**5214–5223.
2. **Ksontini H, Kachouri F, Hamdi M.** 2013. Dairy biofilm: impact of microbial community on raw milk quality. J Food Qual **36:**282–290. http://dx.doi.org/10.1111/jfq.12036.
3. **Irlinger F, Mounier J.** 2009. Microbial interactions in cheese: implication for cheese quality and safety. Curr Opin Biotechnol **20:**142–148. http://dx.doi.org/10.1016/j.copbio.2009.02.016.
4. **Didienne R, Defargues C, Callon C, Meylheuc T, Hulin S, Montel MC.** 2012. Characteristics of microbial biofilm on wooden vats ('gerles') in PDO Salers cheese. Int J Food Microbiol **156:**91–101. http://dx.doi.org/10.1016/j.ijfoodmicro.2012.03.007.
5. **Lindsay D, Holy A.** 2006. What food safety professionals should know about safety professionals should know about bacterial biofilms. Br Food J **108:**27–37. http://dx.doi.org/10.1108/00070700610637616.
6. **Lortal S, Di Blasi A, Madec MN, Pediliggieri C, Tuminello L, Tanguy G, Fauquant J, Lecuona Y, Campo P, Carpino S, Licitra G.** 2009. Tina wooden vat biofilm: a safe and highly efficient lactic acid bacteria delivering system in PDO Ragusano cheese making. Int J Food Microbiol **132:**1–8. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.02.026.
7. **Mounier J, Georges S, Gelsomino R, Vancanneyt M, Vandemuelebroecke K, Hoste B, Brennan NM, Scherer S, Swings J, Fitzgerald GF, Cogan TM.** 2006. Sources of adventitious microflora of a smear-ripened cheese. J Appl Microbiol **101:**668–681. http://dx.doi.org/10.1111/j.1365-2672.2006.02922.x.
8. **Ercolini D.** 2013. High-throughput sequencing and metagenomics: moving forward in the culture independent analysis of food microbial ecology. Appl Environ Microbiol **79:**3148–3155. http://dx.doi.org/10.1128/AEM.00256-13.
9. **Kousta M, Mataragas M, Skandamis P, Drosinos EH.** 2009. Prevalence and sources of cheese contamination with pathogens at farm and processing levels. Food Control **21:**805–815.
10. **Brooks JD, Flint SH.** 2008. Biofilms in the food industry: problems and potential solutions. Int J Food Sci Technol **43:**2163–2176. http://dx.doi.org/10.1111/j.1365-2621.2008.01839.x.
11. **Carpentier B, Cerf O.** 1993. Biofilms and their consequences with particular references to hygiene in the food industry. J Appl Bacteriol **75:**499–511. http://dx.doi.org/10.1111/j.1365-2672.1993.tb01587.x.
12. **Kumar CG, Anand SK.** 1998. Significance of microbial biofilms in food industry: a review. Int J Food Microbiol **42:**9–27. http://dx.doi.org/10.1016/S0168-1605(98)00060-9.
13. **Zottola EA, Sasahara KC.** 1994. Microbial biofilms in the food processing industry: should they be a concern? Int J Food Microbiol **23:**125–148.
14. **McLandsborough L, Rodriguez A, Pérez-Conesa D, Weiss J.** 2006. Biofilms: at the interface between biophysics and microbiology. Food Biophys **1:**94–114. http://dx.doi.org/10.1007/s11483-005-9004-x.
15. **Srey S, Jahid IK.** 2012. Biofilm formation in food industries: a food safety concern. Food Control **31:**572–585.
16. **Settanni L, Di Grigoli A, Tornambé G, Bellina V, Francesca N, Moschetti G, Bonanno A.** 2012. Persistence of wild *Streptococcus thermophilus* strains on wooden vat and during the manufacture of a traditional Caciocavallo type cheese. Int J Food Microbiol **155:**73–81. http://dx.doi.org/10.1016/j.ijfoodmicro.2012.01.022.
17. **Licitra G, Ogier JC, Parayre S, Pediliggieri C, Carnemolla TM, Falentin H, Madec MN, Carpino S, Lortal S.** 2007. Variability of bacterial biofilms of the "Tina" wood vats used in the Ragusano cheese-making process. Appl Environ Microbiol **73:**6980–6987. http://dx.doi.org/10.1128/AEM.00835-07.
18. **Goerges S, Mounier J, Rea MC, Gelsomino R, Heise V.** 2008. Commercial ripening starter microorganisms inoculated into cheese milk do not successfully establish themselves in the resident microbial ripening consortia of a South German red smear cheese. Appl Environ Microbiol **74:**2210–2217. http://dx.doi.org/10.1128/AEM.01663-07.
19. **Parente E, Cogan TM.** 2004. Starter cultures: general aspects, p 123–148. *In* Fox PF, McSweeney PLH, Cogan TM, Guinee TP (ed), Cheese: chemistry, physics. Microbiology, vol 1. Elsevier/Academic Press, Inc, San Diego, CA.
20. **Ercolini D, De Filippis F, La Storia A, Iacono M.** 2012. "Remake" by high-throughput sequencing of the microbiota involved in the production of water buffalo mozzarella cheese. Appl Environ Microbiol **78:**8142–8145. http://dx.doi.org/10.1128/AEM.02218-12.
21. **Kurtzman CP, Robnett CJ.** 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek **73:**331–371. http://dx.doi.org/10.1023/A:1001761008817.
22. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knight D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods **7:**335–336. http://dx.doi.org/10.1038/nmeth.f.303.
23. **De Filippis F, La Storia A, Stellato G, Gatti M, Ercolini D.** 2014. A selected core microbiome drives the early stages of three popular Italian cheese manufacture PLoS One **9:**e89680. http://dx.doi.org/10.1371/journal.pone.0089680.
24. **Edgar RC, Baterman A.** 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics **26:**2460–2461. http://dx.doi.org/10.1093/bioinformatics/btq461.
25. **Wang Q, Garrity GM, Tiedje JM, Cole JR.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol **78:**5261–5267.
26. **Mc Donald D, Price MN, Goodrich J, Nawrocki EP, De Santis TZ, Probs A, Andersen GL, Knight R, Hugenholtz P.** 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J **6:**610–618. http://dx.doi.org/10.1038/ismej.2011.139.
27. **Shade A, Handselman J.** 2012. Beyond the Venn diagram: the hunt for a core microbiome. Environ Microbiol **14:**4–12. http://dx.doi.org/10.1111/j.1462-2920.2011.02585.x.

28. **Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C.** 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol **31:**814–821. http://dx.doi.org/10.1038/nbt.2676.
29. **Gori K, Ryssel M, Arneborg N, Jespersen L.** 2013. Isolation and identification of the microbiota of Danish farmhouse and industrially produced surface-ripened cheeses. Microb Ecol **65:**602–615. http://dx.doi.org/10.1007/s00248-012-0138-3.
30. **Andreani NA, Martino ME, Fasolato L, Carraro L, Montemurro F, Mioni R.** 2015. Reprint of "Tracking the Blue: an MLST Approach to Characterise the *Pseudomonas fluorescens* Group." Food Microbiol **45:**148–158. http://dx.doi.org/10.1016/j.fm.2014.11.011.
31. **Marchand S, De Block J, De Jonghe V, Coorevits A, Heyndrickx M, Herman L.** 2012. Biofilm formation in milk production and processing environments; influence on milk quality and safe. Comp Rev Food Sci Food Safety **11:**133–147. http://dx.doi.org/10.1111/j.1541-4337.2011.00183.x.
32. **Quigley L, Sullivan O, Beresford T, Ross R, Fitzgerald G, Cotter P.** 2012. High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. Appl Environ Microbiol **78:**5717–5723. http://dx.doi.org/10.1128/AEM.00918-12.
33. **Ercolini D, Frisso G, Mauriello G, Salvatore F, Coppola S.** 2008. Microbial diversity in natural whey cultures used for the production of Caciocavallo Silano PDO cheese. Int J Food Microbiol **124:**164–170. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.03.007.
34. **Di Grigoli A, Francesca N, Gaglio R, Guarrasi V, Moschetti M, Scatassa ML.** 2015. The influence of wooden equipment employed on the characteristics of a traditional stretched cheese during ripening. Food Microbiol **46:**81–91. http://dx.doi.org/10.1016/j.fm.2014.07.008.
35. **Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM.** 2001. Recent advances in cheese microbiology. Int J Dairy Microbiol **11:**259–274. http://dx.doi.org/10.1016/S0958-6946(01)00056-5.
36. **Bremer PJ, Seale B, Flint S, Palmer J.** 2009. Biofilms in dairy processing, p 396–431. *In* Fratamico PM, Annous BA, Gunther NW, IV (ed), Biofilms in the food and beverage industries. Wood Head Publishing, Ltd, Oxford, United Kingdom.
37. **Faille C, Carpentier B.** 2009. Food contact surfaces, surface soiling and biofilm formation, p 304–308. *In* Fratamico PM, Annous BA, Gunther NW, IV (ed), Biofilms in the food and beverage industries. Wood Head Publishing, Ltd, Oxford, United Kingdom.
38. **Van Houdt R, Michiels CW.** 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. Int J Food Microbiol **4:**1117–1131. http://dx.doi.org/10.1111/j.1365-2672.2010.04756.x.
39. **Myszka K, Czaczyk K.** 2011. Bacterial biofilms on food contact surfaces: a review. Pol J Food Nutr Sci **61:**173–180.
40. **Cleto S, Matos S, Kluskens L, Vieira M.** 2012. Characterization of contaminants from a sanitized milk processing plant. PLoS One **7:**e40189. http://dx.doi.org/10.1371/journal.pone.0040189.
41. **Scott SA, Brooks JD, Rakonjac J, Walker KMR, Flint SH.** 2007. The formation of thermophilic spores during the manufacture of whole milk powder. Int J Dairy Technol **60:**109–117. http://dx.doi.org/10.1111/j.1471-0307.2007.00309.x.
42. **De Jonghe V, Coorevits A, Van Hoorde K, Messens W, Van Landschoot A, De Vos P, Heyndrickx M.** 2011. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Appl Environ Microbiol **77:**460–470. http://dx.doi.org/10.1128/AEM.00521-10.
43. **Franciosi E, Settanni L, Cologna N, Cavazza A, Poznanski E.** 2011. Microbial analysis of raw cow's milk used for cheese-making: influence of storage treatments on microbial composition and other technological traits. World J Microbiol Biotechnol **27:**171–180. http://dx.doi.org/10.1007/s11274-010-0443-2.
44. **Martin NH, Murphy SC, Ralyea RD, Wiedmann M, Boor KJ.** 2011. When cheese gets the blues: *Pseudomonas fluorescens* as the causative agent of cheese spoilage. J Dairy Sci **94:**3176–3183. http://dx.doi.org/10.3168/jds.2011-4312.
45. **Morales P, Garcia EF, Nunez M.** 2005. Volatile compounds produced in cheese by *Pseudomonas* strains of dairy origin belonging to six different species. J Agric Food Chem **53:**6835–6843.
46. **Caputo L, Quintieri L, Bianchi DM, Decastelli L, Monaci L, Visconti A, Baruzzi F.** 2014. Pepsin-digested bovine lactoferrin prevents Mozzarella cheese blue discoloration caused by *Pseudomonas fluorescens*. Food Microbiol **46:**15–24. http://dx.doi.org/10.1016/j.fm.2014.06.021.
47. **Chiesa F, Lomonaco S, Nucera D, Garoglio D, Dalmasso A, Civera T.** 2014. Distribution of *Pseudomonas* species in a dairy plant affected by occasional blue discoloration. Int J Food Safety **3:**245–248.
48. **Doulgeraki AI, Ercolini D, Villani F, Nychas GJE.** 2012. Spoilage microbiota associated to the storage of raw meat in different conditions. Int J Food Microbiol **157:**130–141. http://dx.doi.org/10.1016/j.ijfoodmicro.2012.05.020.
49. **De Filippis F, La Storia A, Villani F, Ercolini D.** 2013. Exploring the sources of bacterial spoilers in beefsteaks by culture-independent high-throughput sequencing PLoS One **8:**e70222. http://dx.doi.org/10.1371/journal.pone.0070222.
50. **Stellato G, La Storia A, Cirillo T, Ercolini D.** 2015. Bacterial biogeographical patterns in a cooking centre for hospital foodservice. Int J Food Microbiol **193:**99–108. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.10.018.
51. **Prillinger H, Molnàr O, Lechner FE, Lopandic K.** 1999. Phenotypic and genotypic identification of yeasts from cheese. Van Leeuwenhoek J Microb **75:**267–283. http://dx.doi.org/10.1023/A:1001889917533.
52. **Petersen KM, Westall S, Jespersen L.** 2002. Microbial succession of *Debaryomyces hansenii* strains during the production of Danish surface-ripened cheeses. J Dairy Sci **85:**478–486. http://dx.doi.org/10.3168/jds.S0022-0302(02)74098-8.
53. **Simões M, Simões LC, Vieira MJ.** 2010. A review of current and emergent biofilm control strategies. J Food Sci Technol **43:**573–583.
54. **Garofalo C, Osimani A, Milanović V, Aquilanti L, De Filippis F, Stellato G, Di Mauro S, Turchetti B, Buzzini P, Ercolini D, Clementi F.** 2015. Bacteria and yeast microbiota in milk kefir grains from different Italian regions. Food Microbiol **49:**123–133. http://dx.doi.org/10.1016/j.fm.2015.01.017.
55. **Wolfe BE, Button JE, Santarelli M, Dutton RJ.** 2014. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. Cell **158:**422–433. http://dx.doi.org/10.1016/j.cell.2014.05.041.
56. **Parkar SG, Flintand SH, Brooks JD.** 2004. Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. Int J App Microbiol **96:**110–116. http://dx.doi.org/10.1046/j.1365-2672.2003.02136.x.