

High-Throughput Sequencing for Detection of Subpopulations of Bacteria Not Previously Associated with Artisanal Cheeses

Lisa Quigley,^{a,b} Orla O'Sullivan,^a Tom P. Beresford,^b R. Paul Ross,^{a,c} Gerald F. Fitzgerald,^{b,c} and Paul D. Cotter^{a,c}

Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland^a; Microbiology Department, University College Cork, Cork, Ireland^b; and Alimentary Pharmabiotic Centre, Cork, Ireland^c

Here, high-throughput sequencing was employed to reveal the highly diverse bacterial populations present in 62 Irish artisanal cheeses and, in some cases, associated cheese rinds. Using this approach, we revealed the presence of several genera not previously associated with cheese, including *Faecalibacterium*, *Prevotella*, and *Helcococcus* and, for the first time, detected the presence of *Arthrobacter* and *Brachybacterium* in goats' milk cheese. Our analysis confirmed many previously observed patterns, such as the dominance of typical cheese bacteria, the fact that the microbiota of raw and pasteurized milk cheeses differ, and that the level of cheese maturation has a significant influence on *Lactobacillus* populations. It was also noted that cheeses containing adjunct ingredients had lower proportions of *Lactococcus* species. It is thus apparent that high-throughput sequencing-based investigations can provide valuable insights into the microbial populations of artisanal foods.

High-throughput sequencing has revolutionized the field of microbial ecology, allowing for a more accurate identification of microbial taxa, including those which are difficult to culture and/or are present in low abundance (43). These technologies have provided detailed insights into the microbial compositions of a wide variety of different ecosystems, including sea (43), soil (38), and gut environments (2, 9), as well as that of a relatively small selection of food-associated niches (17, 33, 39). One group of complex microbial environments not assessed, to date, in this way are artisanal cheeses. The complex, fermentation-based nature of cheese means that the microbiota of different cheeses vary considerably. Many of these microbes are also hugely influential with respect to the textural and organoleptic properties of a cheese (31). Thus, unsurprisingly, there have been considerable efforts made to characterize the microbial populations of cheeses. Traditional culture-independent molecular methods, most frequently the analysis of 16S rRNA genes through denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) (21, 35), single-stranded conformation polymorphisms (SSCP) (7), and/or Sanger sequencing (20), have improved our understanding of cheese microbial populations (36). However, we anticipated that the application of high-throughput sequencing could provide an even more detailed understanding of the microbial composition of cheese. Thus, we have applied this technology to investigate the microbiota of 62 soft, semihard, and hard artisanal cheeses, manufactured from unpasteurized or pasteurized cow, goat, and sheep milk, and of 11 associated naturally developed or smear-ripened rinds.

MATERIALS AND METHODS

Cheese collection and nucleic acid extraction. A total of 62 handmade cheeses, including 18 soft cheeses, 31 semihard cheeses, and 13 hard cheeses, manufactured from unpasteurized or pasteurized cow, goat, or sheep milk were obtained from artisanal cheese producers and farmer's markets throughout Ireland (see Table S1 in the supplemental material). To facilitate the culture-independent analysis of the bacterial compositions of these cheeses, their associated rinds, naturally developed or smear-ripened cheese rinds, were also analyzed. One gram of cheese or 1g of cheese rind (6, 13, 16, 20, 22) was combined with 9 ml 2% trisodium citrate and

homogenized before DNA was extracted using the PowerFood microbial DNA isolation kit (MoBio Laboratories Inc.).

PCR amplification of the microbial community 16S rRNA genes. The DNA extracts were used as a template for PCR amplification according to the methods described by Quigley et al. (36). Here, universal 16S primers targeting the V4 region (239 nucleotides long) predicted to bind to 94.6% of all 16S genes were incorporated, i.e., the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers, R1 (5'-TACCRGGGTHCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC), and R4 (5'-TACNVGGGTA TCTAATC) (RDP pyrosequencing pipeline; <http://pyro.cme.msu.edu/pyro/help.jsp>). The primers incorporated a proprietary 19-mer sequence (GCCTGCCAGCCCGCTCAG) at the 5' end to allow emulsion-based clonal amplification for the 454 pyrosequencing system. Unique molecular identifier (MID) tags were incorporated between the adaptamer and the target-specific primer sequence, to allow identification of individual sequences from pooled amplicons. The PCR mixture contained 25 μ l GoTaq Green master mix (Promega), 1 μ l of each primer (200 nmol liter⁻¹), 5 μ l DNA template, and nuclease-free H₂O to give a final reaction volume of 50 μ l. PCR amplification was performed using a G-Storm thermal cycler (Gene Technologies, United Kingdom). The amplification program consisted of an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min. A final elongation step at 72°C for 2 min was also included. Amplicons were cleaned using the AMPure XP purification system (Beckman Coulter, Takeley, United Kingdom). The quantity of DNA extracted was assessed using the Quant-It Picogreen dsDNA reagent (Invitrogen) in accordance with the manufacturer's instructions and a Nanodrop 3300 fluorospectrometer (Thermo Fisher Scientific Inc.).

High-throughput sequencing and bioinformatics analysis. The 16S rRNA V4 amplicons were sequenced on a 454 genome sequencer FLX platform (Roche Diagnostics Ltd., Burgess Hill, West Sussex, United

Received 21 March 2012 Accepted 28 May 2012

Published ahead of print 8 June 2012

Address correspondence to Paul D. Cotter, paul.cotter@teagasc.ie.

Supplemental material for this article may be found at <http://aem.asm.org/>.

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doi:10.1128/AEM.00918-12

TABLE 1 Sequencing richness, diversity, and coverage of artisanal cheeses^a

Data set	Result for cheese type		
	Soft	Semihard	Hard
Similarity (%)	97	97	97
Chao1 richness estimation	295	315	254
Shannon index for diversity	3.8	4.3	3.7
Good's coverage (%)	92	90	91

^a Average results of the statistical analysis of artisanal cheese, at two different similarity levels, for sequencing richness, diversity, and coverage as analyzed with MOTHUR software. The analyses were separated on the basis of cheese type.

Kingdom) according to Roche 454 protocols. Read processing was performed using techniques implemented in the RDP pyrosequencing pipeline (11). Sequences not passing the FLX quality controls were discarded, the 454-specific portions of the primers were trimmed, the raw sequences were sorted according to tag sequences, and reads with low quality scores (quality scores below 40) and short lengths (less than 150 bp for the 16S rRNA V4 region) were removed, as were reads that did not have exact matches with respect to primer sequence. Statistical analysis to measure the sequencing diversity, included Chao1 richness, Shannon diversity, and Good's coverage results, as well as monitoring results for sequencing abundance using rarefaction, were performed using the MOTHUR package (42). Principal coordinate analysis (PCoA), measuring dissimilarities at phylogenetic distances based on weighted UniFrac analysis, was performed using the QIIME suite of programs (8). Trimmed Fasta sequences were assessed by BLAST analysis (1) against information in a previously published 16S rRNA-specific database (45) using default parameters. The resulting BLAST output was parsed using MEGAN (27). MEGAN assigns reads to NCBI taxonomies by employing the lowest common ancestor algorithm, which assigns each RNA tag to the lowest common ancestor in the taxonomy from a subset of the best-scoring matches in the BLAST result. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarization (absolute cutoff, BLAST bit

score of 86; relative cutoff, 10% of the top hit) (45). Statistical significance was determined by using the nonparametric Kruskal-Wallis test (29) in the Minitab statistical package.

RESULTS

Sequencing and bioinformatic analysis. DNA was extracted from a 1-g samples from 62 cheeses and from the rinds of 11 of the cheeses (see Table S1 in the supplemental material). Following total genomic DNA extraction, amplicons of the V4 16S rRNA gene were generated, and a total of 116,238 pyrosequencing reads were obtained through 454 sequencing, corresponding to 32,322, 48,388, and 18,340 reads from soft, semihard, and hard cheeses, respectively, and 17,188 reads corresponding to cheese rinds. Diversity, richness, and coverage estimations were calculated for each data set (Table 1; individual sample diversity results are presented in Table S2 of the supplemental material). The Chao1 estimator of species richness indicated good sample richness throughout. The Shannon diversity index, a measurement of overall diversity, indicated a diverse microbiota, while the Good's coverage result, an estimator of completeness of sampling, highlighted good overall sampling, with levels of 89 to 95%. Rarefaction curve analysis, which assesses species richness from the results of sampling, showed all samples approached being parallel with the x axis, revealing that the overall bacterial diversity was well represented (Fig. 1). Principal coordinate analysis, which clusters the communities according to different parameters, in this case cheese type, animal source of milk, and whether the milk was pasteurized, was conducted according to weighted UniFrac distances (Fig. 2). Regardless of the community parameters, there was no definitive split in the microbiota of the cheeses. However, the most extreme outliers generally tended to be cheese rinds from cow's milk cheeses. No statistical differences were found in operational taxonomic units at the phylum level; however, a number of

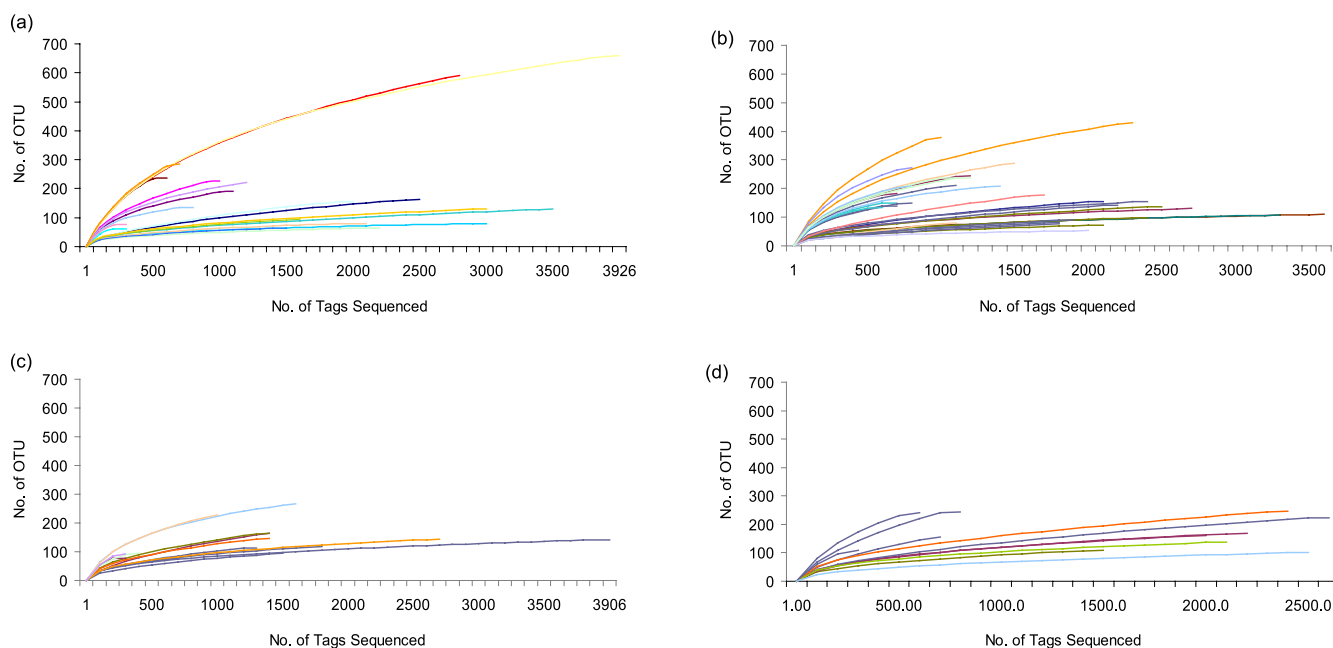


FIG 1 Rarefaction curves of microbial populations from artisanal cheeses and cheese rinds. Each line represents a cheese sampled and sequenced. (a) Soft cheeses; (b) semihard cheeses; (c) hard cheeses; (d) cheese rinds. The curvature of a line toward the right (or x) axis shows that a reasonable number of sequences were obtained, and thus sampling was sufficient.

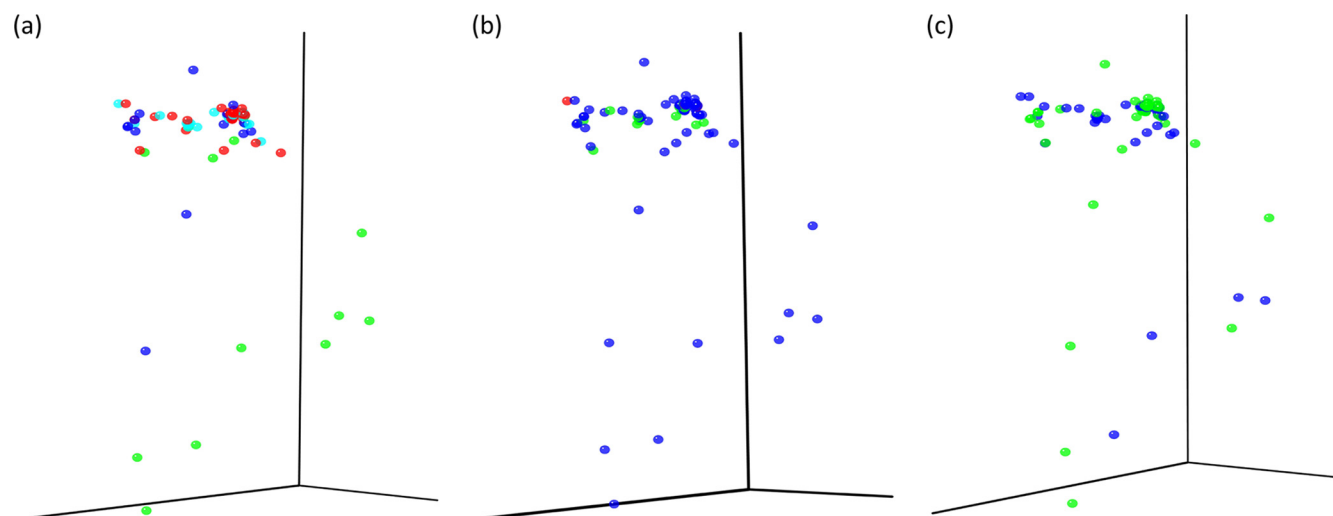


FIG 2 Principal coordinate analysis graphs for weighted UniFrac results. Samples were assessed for different community parameters. (a) Cheese type. Soft cheeses, light blue; semihard cheeses, red; hard cheeses, dark blue; rinds, green. (b) Animal sources. Cow, dark blue; goat, green; sheep, red. (c) Milk. Unpasteurized, green; pasteurized, blue.

statistical differences were determined at the genus level. The gene sequence information has been summarized in a MiXS-MIMARKS metatable (46), which is shown in Table S3 of the supplemental material.

Microbial compositions of artisanal cheeses as revealed by pyrosequencing. *In silico* analysis of high-throughput sequence data revealed microorganisms corresponding to five phyla in the soft, semihard and hard cheeses (Table 2). These phyla were representatives of four bacterial phyla, i.e., *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. Surprisingly, a fifth phylum detected was the fungal phylum *Ascomycota*. The latter was detected occasionally throughout the cheese samples at a subdominant level, i.e., 0.37 to 0.50%, and at the genus level its detection corresponded almost exclusively with *Penicillium*. Further examination of the *Penicillium* sequence established that it corresponds to that of the mitochondrial 16S rRNA gene of *Penicillium*. Of the four bacterial phyla, *Firmicutes* dominated in the three cheese types, corresponding to 96%, 95%, and 91% of the reads from the soft, semihard, and hard cheeses, respectively. *Proteobacteria* (0.91, 3.31, and 1.79%), *Bacteroidetes* (0.27, 0.25, and 0.21%), and *Actinobacteria* (1.22, 0.12, and 4.45%) were detected at various levels throughout (Table 2).

The bacteria detected corresponded to 21 different genera (Fig. 3; Table 2), with *Lactococcus* dominating. At the depth of analysis carried out, a total of eight genera were found to be common in all three cheese types (soft, semihard, and hard). In addition to *Lactococcus* (89.93, 84.45, and 49.56%), *Lactobacillus* (0.65, 7.30, and 17.8%), *Leuconostoc* (1.79, 0.51, and 1.8%), *Pseudomonas* (0.11, 0.03, and 0.49%), *Psychrobacter* (0.58, 0.02, and 0.53%), *Staphylococcus* (0.06, 0.17, and 0.73%), *Arthrobacter* (0.28, 0.08, and 1.1%) and *Faecalibacterium* (0.02, 0.08, and 0.05%) were also identified. Statistically significant differences were observed for the levels of *Lactococcus* ($P = 0.031$) and *Lactobacillus* ($P = 0.010$), with the level of lactococci increasing and the level of lactobacilli decreasing between soft, semihard, and hard cheese types (Fig. 3). *Vibrio* were found in soft cheeses only (0.02%), and *Helcococcus* (0.07%), *Halomonas* (0.25%), and *Streptococcus* (0.04%) were

found in the semihard cheeses only, while *Enterococcus* (0.1%), *Tetragenococcus* (0.05%), and *Clostridium* (0.06%) were found in hard cheeses only. Three genera were shared between soft and semihard cheeses. These were *Pseudoalteromonas* (0.06 and 0.03%), *Pediococcus* (0.03% and 0.27%), and *Bifidobacterium* (0.02% and 0.03%). *Brevibacterium* (0.81% and 2.10%) was the only genus shared between soft and hard cheeses, and *Prevotella* (0.15% and 0.34%) was the only genus common to semihard and hard cheeses.

Some interesting observations were made regarding the influence of the animal source of milk and pasteurization on the microbial populations present in the resultant cheeses. We noted that cow's milk cheese contained 21 different bacterial genera (Table 2), whereas goat milk contained only 8 different bacterial genera, and only two bacterial genera, *Lactococcus* and *Lactobacillus*, were detected in sheep's milk cheese. Also, by comparing the bacterial genera present in artisanal cheeses manufactured from unpasteurized milk relative to those made from pasteurized milk, it was apparent that *Halomonas*, *Helcococcus*, *Streptococcus*, *Enterococcus*, and *Tetragenococcus* were detected in raw milk cheeses only and that *Clostridium* and *Vibrio* were detected from pasteurized milk cheeses only. A significant difference was noted in the levels of *Lactococcus* ($P = 0.025$) and *Lactobacillus* ($P = 0.002$) between unpasteurized and pasteurized milk cheeses. Further comparisons provided some interesting findings. Cheeses S2 and S3 were produced with milk from the same herd using similar protocols, but they differed in that S3 was a feta-style cheese and thus had a higher salt content, which may explain the absence of *Leuconostoc* and *Pseudomonas* from this cheese. Similarly, S7 and H9 were produced in the same farmhouse but differed with respect to the level of maturation, with associated differences in the proportions of *Lactobacillus* (0.65% in soft cheese, 17.8% in hard cheese). This is reflective of the aforementioned overall greater number of lactobacilli in hard relative to semihard and, in turn, soft cheeses. This pattern was also apparent when cheeses SH1 and H3 to H6, all from the same producers, were compared. In addition, the results of a specific comparison of H4 and H5 were also interesting, as these cheeses differed solely on the basis of H5 containing

TABLE 2 Summary of reads calculated from total phylum reads for each variable assessed

Phylum or genus	% of reads in the phylum or genus per:								
	Cheese type				Animal source			Milk type	
	Soft	Semihard	Hard	Rind	Cow	Goat	Sheep	Unpasteurized	Pasteurized
Phyla									
<i>Proteobacteria</i>	0.91	3.31	1.79	26.99	95.5	89.6	98.6	96.1	93.1
<i>Firmicutes</i>	96.03	95.49	91.41	32.14	0.69	1.4	0	0.74	0.83
<i>Acintobacteria</i>	1.22	0.12	4.45	26.02	1.4	6.8	0	1.9	2.5
<i>Bacteroidetes</i>	0.27	0.25	0.21	5.47	0.19	1.1	0	0.22	0.48
<i>Ascomycota</i>	0.49	0.37	0.50	7.13	0.50	0.89	1.38	0.61	0.60
Genera									
<i>Lactococcus</i>	89.83	84.45	49.56	25.80	77.2	76.0	98.5	72.8	84.4
<i>Leuconostoc</i>	1.79	0.51	1.80	2.57	1.0	2.2	0	1.2	1.1
<i>Lactobacillus</i>	0.65	7.30	17.80	0.20	8.1	1.0	0.08	11.3	0.82
<i>Pseudomonas</i>	0.11	0.03	0.49	0.14	0.07	0.64	0	0.09	0.25
<i>Psychrobacter</i>	0.58	0.02	0.53	9.92	0.28	0	0	0.21	0.23
<i>Staphylococcus</i>	0.06	0.17	0.73	1.98	0.31	0	0	0.30	0.17
<i>Arthrobacter</i>	0.28	0.08	1.10	4.14	0.39	0.85	0	0.49	0.39
<i>Pseudoalteromonas</i>	0.06	0.03	0	3.80	0.03	0	0	0.03	0.02
<i>Vibrio</i>	0.02	0	0	2.84	0.004	0	0	0	0.008
<i>Faecalibacterium</i>	0.02	0.08	0.05	0	0.07	0	0	0.07	0.04
<i>Bifidobacterium</i>	0.02	0.03	0	0	0.02	0	0	0.02	0.009
<i>Brevibacterium</i>	0.02	0	2.10	9.22	0.57	5.4	0	0.82	1.9
<i>Brachybacterium</i>	0.81	0	1.45	3.56	0.39	0.50	0	0.57	0.17
<i>Pediococcus</i>	0.03	0.27	0	0	0.12	0	0	0.17	0.01
<i>Prevotella</i>	0	0.15	0.34	0.03	0.07	0.048	0	0.10	0.16
<i>Halomonas</i>	0	0.25	0	2.46	0.12	0	0	0.1	0
<i>Enterococcus</i>	0	0	0.10	0	0.02	0	0	0.03	0
<i>Helcococcus</i>	0	0.07	0	0	0.03	0	0	0.05	0
<i>Tetragenococcus</i>	0	0	0.05	0.18	0.01	0	0	0.02	0
<i>Corynebacterium</i>	0	0	0	1.20	0	0	0	0	0
<i>Streptococcus</i>	0	0.04	0	0.24	0.01	0	0	0.02	0
<i>Clostridium</i>	0	0	0.06	0	0.01	0	0	0	0.02
<i>Facklamia</i>	0	0	0	0.60	0	0	0	0	0
<i>Flavobacterium</i>	0	0	0	0.19	0	0	0	0	0
<i>Cronobacter</i>	0	0	0	0.05	0	0	0	0	0
<i>Penicillium</i>	0.49	0.37	0.51	12.96	0.50	0.89	1.38	0.61	0.60
Other genera	5.16	6.14	23.34	17.91	10.67	12.47	0.04	11.1	9.7

an adjunct ingredient, fenugreek seeds, the presence of which coincided with a reduction in the proportion of lactococci (from 61 to 2%) and increase in lactobacilli (from 34 to 95%). The inclusion of herbs, spices, or seaweed was also found to coincide with

reduced proportions of lactococci in sample SH8 relative to SH10 and SH11 and in SH28 relative to SH26.

Revealing the microbial compositions of the rinds of artisanal cheeses. We again used high-throughput sequencing to an-

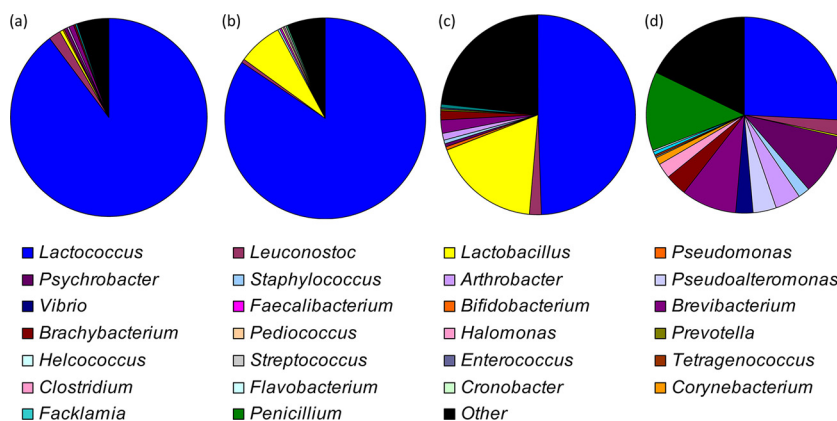


FIG 3 Assignment of cheese microbiota at the genus level, according to MEGAN. (a) soft cheese; (b) semihard cheese; (c) hard cheese; (d) cheese rinds.

alyze the microbiota of 11 rinds of the artisanal cheeses (samples R1 to 11) (Fig. 3; see Table S1). These included smear/wash-ripened rinds, i.e., R1, R7, R8, and R9; naturally developed rinds, i.e., R2 to R6 and R11; and one mold-ripened rind, R10. *In silico* analysis of sequence data revealed the presence of 19 different genera (Fig. 3; Table 2). While some of these genera, including *Lactococcus*, *Leuconostoc*, and *Lactobacillus*, corresponded to those also detected in the cheese core, a selection was identified in the cheese rinds only. These included *Corynebacterium* (1.2%), *Facklamia* (0.60%), *Flavobacterium* (0.19%), and *Cronobacter* (0.05%). While *Lactococcus* remained the most common genus in cheese rinds, the relative proportions of this genus were significantly lower in the rind than in the core. Generally, smear/wash-ripened rinds had particularly low levels of lactococci (1.9 to 4.8%), while naturally developed rinds had levels of lactococci of up to 98%. It was also apparent that *Psychrobacter* and *Brevibacterium* represented a considerable proportion, i.e., 0.29 to 57% and 0.67 to 54.6%, respectively, or ~10% on average, of the total population. The other genera detected, i.e., *Leuconostoc*, *Lactobacillus*, *Pseudomonas*, *Psychrobacter*, *Pseudoalteromonas*, *Brachybacterium*, *Prevotella*, *Arthrobacter*, *Streptococcus*, *Tetragenococcus*, and *Facklamia*, corresponded to between 0.03 and 4.14% of reads (Table 2). *Brevibacterium* and *Brachybacterium* were present at significantly different levels in the rinds of soft, semihard, and hard cheeses compared to cheese core ($P = 0.040$ and $P = 0.014$, respectively). *Penicillium* was also detected in the cheese rind and in higher proportions than were detected in the cheese core. Finally, we also detected the presence of *Prevotella*, a genus that has previously not been detected in cheese or cheese rinds, and we noted that *Vibrio* was only identified in rinds of the smear/wash-developed variety ($P = 0.009$).

DISCUSSION

In this study, pyrosequencing-based 16S rRNA profiling provided detailed insights into the complex microbiota of artisanal cheeses. Its use effectively revealed the presence of a number of taxa not previously associated with specific cheese types or, indeed, of any cheeses. Among those identified for the first time were the genera *Prevotella* and *Faecalibacterium*. *Prevotella* spp. are Gram-negative bacteria from the phylum *Bacteroidetes* that thrive in anaerobic environments. They are commensals of the rumen and hind gut in cattle and sheep, but they can also be the cause of periodontal disease as well as other human infections. Members of the genus *Faecalibacterium* are strict anaerobes and have been shown to produce butyrate, D-lactate, and formate, as well as utilize acetate (18). While butyrate can contribute positively to cheese development, at high levels this product can induce the late-blowing defect in cheese (10). D-Lactate and acetate are also produced during the development of cheese (25). Further investigations will be required to determine if, at the levels present in cheese, these microbes contribute flavor in a significant way. A third genus which is typically associated with anaerobic gastrointestinal environments, *Helcococcus*, was also detected, but only in one cheese, a semihard cheese made from unpasteurized cow milk. *Helcococcus* spp. have been associated with clinical problems in humans (12), cows (30), sheep (47), and horses (41) and thus, in this instance, may reflect the sourcing of contaminated milk from an infected animal. Given that in this study these insights were gained through the analysis of merely a 1-g sample per cheese, it may be that further investigations of even larger sample sizes and at a greater

depth of sequencing will uncover additional genera not previously associated with cheese. Nevertheless, the detection of these anaerobes reveals that the microbiota of cheeses are more diverse than previously appreciated, thus further highlighting the benefits of high-throughput sequencing investigations.

We also detected a number of genera not previously associated with specific cheese types. *Arthrobacter* and *Brachybacterium* were detected for the first time in goat cheese; these are commonly detected on the cheese surface but not, to our knowledge, in the core of goat cheese. The presence of *Pseudoalteromonas* in soft and semihard cow milk cheeses, as well as cheese rinds, was also unexpected. *Pseudoalteromonas* species are usually regarded as marine bacteria (5) and have been detected on the surface of a smear-ripened cheese on only one previous occasion (23). This is the first instance in which this genus has been detected in a cheese core.

In addition to identifying taxa not previously associated with cheeses or specific cheese types, there were a number of other interesting observations. We noted that the milk source impacted the number of genera detected, i.e., 21 genera from cow milk cheeses, 8 from goat milk cheeses, and 2 from sheep milk cheeses, although this observation may have been influenced by differences in the number of samples within each group. Notably, a number of studies have previously established that milk source can influence the type and number of microbes present in a cheese (13, 37). Previous studies have also highlighted the dramatic impact of milk pasteurization on the microbiota of resultant cheeses (4, 13, 19). Here, through the use of high-throughput sequencing, we also observed differences in cheeses produced from raw and pasteurized milks (Table 2) in that, for example, significant differences in levels of *Lactococcus* and *Lactobacillus* organisms were apparent when these cheese types were compared. For further studies to investigate these differences, it would be interesting to focus on RNA (and thus cDNA), or to employ stains that inactivate DNA from cells that have been killed by the temperature treatment, to determine if such approaches provide different results.

Several previous studies have employed other technologies to investigate the impact of salt (26), ripening (32), and additional ingredients (3, 14, 44) on the microbiota of cheese. Here we noted that neither *Leuconostoc* nor *Pseudomonas* was detected in cheeses with a high salt content, that significantly increased *Lactobacillus* populations were detected in cheeses from the same farmhouse but which had been ripened to various degrees, and that the inclusion of adjunct ingredients, such as herbs, spices, or seaweed, did impact microbial composition.

As a consequence of its exposure to the external environment and, in some cases, steps taken during the manufacturing process, the microbiota of the rind of a cheese will frequently differ dramatically from that of the rest of the cheese (24). This presumably reflects the exposure of the cheese rind to the environment. Many of the bacterial genera detected are commonly identified in cheese rinds and, indeed, *Corynebacterium*, *Arthrobacter*, *Brevibacterium*, and *Halomonas* have previously been identified on the surface of Irish artisanal cheeses (34). The high proportions of *Psychrobacter* and *Brevibacterium* in the cheese rinds studied here were particularly notable. *Brevibacterium* is known to be involved in the development of cheese rind flavors and smear rind color (28). Although *Psychrobacter* is frequently detected on cheese surfaces, its specific role is unclear. It may contribute to flavor, given the ability of

strains from this genus to produce branched-chain aldehydes, alcohols, and esters (15). The impact of the presence of such high proportions of these bacteria on the cheese surface will require further investigation. The previously unreported or rare phenomenon of *Prevotella*, *Facklamia* (40), or *Vibrio* being detected on cheese rinds further highlights the benefits of employing high-throughput sequencing to investigate these populations.

Thus, in conclusion, we have employed high-throughput sequencing to investigate the microbiota of 62 Irish artisanal cheeses in a greater depth than ever before. We have highlighted for the first time the presence of a number of genera previously undetected in cheeses, as well as detecting genera not typically associated with specific cheese types. These analyses also provide insights into the influence of different factors on the composition of the artisanal cheese microbiota, which can now be investigated in greater depth through the study of cheeses prepared in the laboratory.

ACKNOWLEDGMENT

Lisa Quigley is funded by the Teagasc Walsh Fellowship Scheme.

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