Stability of the Biodiversity of the Surface Consortia of Gubbeen, a Red-Smear Cheese

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ABSTRACT

A total of 1,052 bacteria and 828 yeasts were isolated from the surface flora of 6 batches of Gubbeen cheese made in 1996–1997 and 2002–2003. Stability of the microflora was evaluated over time and also during ripening at 4, 10, and 16 d (batches 4, 5, and 6) or at 4, 16, 23, and 37 d (batches 1, 2, and 3). Bacteria were identified using pulsed-field gel electrophoresis, repetitive extragenic palindromic-PCR, and 16S rRNA gene sequencing, and yeasts were identified by Fourier transform infrared spectroscopy. The bacteria included at least 17 species, of which the most common were *Staphylococcus saprophyticus* (316 isolates), *Corynebacterium casei* (248 isolates), *Brevibacterium aurantiacum* (187 isolates), *Corynebacterium variabile* (146 isolates), *Microbacterium gubbeenense* (55 isolates), *Staphylococcus equorum/cohnii* (31 isolates), and *Psychrobacter* spp. (26 isolates). The most common yeasts were *Debaryomyces hansenii* (624 isolates), *Candida catenulata* (135 isolates), and *Candida lusitanae* (62 isolates). In all batches of cheese except batch 2, a progression of bacteria was observed, with staphylococci dominating the early stages of ripening and coryneforms the later stages. No progression of yeast was found. Pulsed-field gel electrophoresis showed that several different strains of the 5 important species of bacteria were present, but generally only one predominated. The commercial strains used for smearing the cheese were recovered, but only in very small numbers early in ripening. Four species, *B. aurantiacum*, *C. casei*, *C. variabile*, and *Staph. saprophyticus*, were found on all batches of cheese, but their relative importance varied considerably. The results imply that significant variation occurs in the surface microflora of cheese.

Key words: smear cheese, yeast and bacteria, coryneform, staphylococci

INTRODUCTION

Red-smear cheeses are an important part of European cheese production and are characterized by the development of microbial consortia composed of yeast and bacteria on the cheese surface early in ripening, which give the cheese its characteristic red, glistening color (Brennan et al., 2004). These consortia are mainly responsible for the development of flavor in these cheeses. Smear cheeses include hard (e.g., Gruyère), semihard (e.g., Tilsit), and soft (e.g., Reblochon) varieties, which are ripened at relatively high temperatures of 10 to 14°C and at high relative humidities of >95%. Such conditions promote the growth of microorganisms, which may be present on the cheese surface. The cheese may be deliberately inoculated with strains of *Brevibacterium linens* and *Debaryomyces hansenii*, and in some countries, “old-young” smearing is also carried out in which old (ripened) cheese is washed with saline and the young cheeses are then smeared with the mixture. Soft and semisoft smear cheeses are particularly prone to contamination with listeria; therefore, this practice has been questioned because it can result in inoculation of the surface of the green cheese with pathogens and other undesirable bacteria. These cheeses are also washed with dilute saline several times in the early stages of ripening to spread any microcolonies of yeast or bacteria that have developed on the cheese surface.

Little is known about the microbiology of the surface of these cheeses. Historically, *B. linens* was considered to be the important bacterium on these cheeses, but more recent information suggests that it comprises <15% of the microflora (Eliskases-Lechner and Ginzinger, 1995a). Several coryneforms including *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, and *Microbacterium* spp. are important components of the cheese

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surface flora (Eliskases-Lechner and Ginzinger 1995a; Valdes-Stauber et al., 1997; Bockelmann and Hoppe Seyler, 2001). In these studies, the bacteria were identified phenotypically. Identification based solely on phenotype is especially difficult with corynebacteria, suggesting that many of the isolates may have been misidentified; for example, strains CA12 and CA15, which were isolated from smear cheese, were first described as *Arthrobacter nicotianae*, then *Microbacterium barkeri*, and finally, after molecular characterization, *Microbacterium gubbeenense* (Bockelmann et al., 2005). Molecular probes for *Brevibacterium*, *Microbacterium*, and *Arthrobacter* have been developed (Kollöffel et al., 1997; Gelsomino et al., 2004). Brennan et al. (2002) and Mounier et al. (2005) used a combination of classical and molecular techniques to identify the bacteria and yeasts. The latter workers identified 14 species in 4 varieties of smear cheeses, but only 2 species, *Corynebacterium casei* and *Arthrobacter arilaitensis*, both of which have only recently been described (Brennan et al., 2001a; Irlinger et al., 2005), were common to all cheeses. A combination of culture-dependent and culture-independent and molecular methods was used by Feurer et al. (2004) to identify the bacteria on the surfaces of a raw and a pasteurized milk cheese. The raw milk cheese had a more diverse flora, including (in order of importance) *Corynebacterium casei*, *B. linens*, *Marinus psychrotolerans*, *Lactobacillus curvatus* ssp. *curvatus*, *Streptococcus thermophilus*, *M. gubbeenense*, *Brachybacterium tyrofermentans*, and *Arthr. arilaitensis*. In contrast, the pasteurized milk cheese contained only *Arthr. arilaitensis*, *Corynebacterium maltaromaticum*, *Lactococcus lactis* ssp. *lactis*, *Strep. thermophilus*, and *B. linens*. Coagulase-negative staphylococci, including *Staphylococcus equorum*, *Staphylococcus vitulinus*, *Staphylococcus xylosus*, *Staphylococcus saprophyticus*, and *Staphylococcus lentus*, have been isolated from numerous French cheeses (Irlinger et al., 1997). Recently, several new species have also been isolated from the surface of cheeses, including *Arthrobacter bergerei* and *Arthr. arilaitensis*, from Reblochon and Camembert cheeses, respectively (Irlinger et al., 2005), *C. casei*, *Corynebacterium mooreparkense*, and *M. gubbeenense* from Gubbeen cheese (Brennan et al., 2001a,b), and *Staphylococcus succinius* ssp. *casei* and *Staph. equorum* ssp. *linens* from Swiss cheese (Place et al., 2002, 2003). *Corynebacterium mooreparkense* was later shown to be a heterotypic synonym of *Corynebacterium variabile* (Gelsomino et al., 2005).

Yeasts are also an important part of the flora of these cheeses, and Eliskases-Lechner and Ginzinger (1995b), Valdes-Stauber et al. (1997), and Prillinger et al. (1999) have shown that *D. hansenii*, *Candida catenulata*, *Yar-*

rowia lipolytica, and *Geotrichum candidum* are important on German and Austrian smear cheeses. The contribution all of these microorganisms make to the surface growth of cheeses is not clear because no systematic study of isolates from cheeses during ripening has been made, except for the study by Brennan et al. (2002). Those authors showed that *C. casei* and *C. variabile* dominated the bacterial flora of one batch of Gubbeen cheese after 4, 16, 23, and 37 d of ripening; they did not identify the yeast flora.

Nothing is known of the stability of the microflora within a smear cheese factory, except for the report by Maoz et al. (2003), who showed that the dominant organisms in 2 red-smear cheeses were similar over a 6-mo period. In the present study, a systematic analysis was undertaken of the dominant bacteria and yeasts on the surface of 6 batches of Gubbeen cheese during ripening, over 2 time periods, 1996–1997 and 2002–2003, using both phenotypic and genotypic techniques.

MATERIALS AND METHODS

Cheese

Six batches of Gubbeen cheese were studied, all made and ripened in the same plant. These cheeses were made in October 1996 (batch 1), October 1997 (batch 2), November 1997 (batch 3), November 2002 (batch 4), and January 2003 (batches 5 and 6). Batches 5 and 6 were manufactured 9 d apart. A description of the cheese and its manufacturing and ripening process can be found in Brennan et al. (2002). All cheeses were smeared with a surface ripening culture 1 to 2 d after manufacture. These were *B. linens* BL2 (Chr. Hansen Laboratory, Hørsholm, Denmark) in the case of batches 1 to 3 and OFR9 (Danisco Cultures, Copenhagen), which is a mixed culture of *brevibacteria*, yeast, and *G. candidum*, in the case of batches 4 to 6.

Isolation of Microorganisms

Bacteria and yeasts in the cheese were quantified as described by Brennan et al. (2002). For batches 1, 2, and 3, isolates were made after 4, 16, 23, and 37 d of ripening and after 4 (early), 10 (middle), and 16 d (late) of ripening for batches 4, 5, and 6. Eighty bacteria and 50 yeasts were isolated at each time point from batch 1, 50 bacteria and 30 yeasts from batches 2 and 3, and 50 of each organism from batches 4 to 6. Isolates were purified and stored in a 1:1 mixture of trypticase soy broth and glycerol at -80°C (bacteria) or 4°C (yeasts).

Phenotypic Characterization of the Bacteria

The bacteria were examined microscopically (phase-contrast), tested for catalase and oxidase, and stained

for their gram reaction by the usual methods. The ability to undergo a rod-coccus transformation was examined on mineral base E, yeast extract, glucose agar after incubation at 30°C for 12 h, and 1, 3, and 7 d, as described by Cure and Keddie (1973). Strains that were rod-shaped in young cultures, and cocci in older cultures were considered to undergo this transformation. The rod-shaped organisms were not tested further, but the cocci were tested for their resistance or sensitivity to furazolidone (100 µg/disc) and lysostaphin (0.2 mg/mL), ability to oxidize glycerol in the presence of erythromycin (0.4 µg/mL), and ability to grow in Brewer's thioglycollate broth. Identification of the bacteria, but not the yeast from batch 2 cheese, was published previously (Brennan et al., 2002) and is included in this paper where comparisons among batches are necessary.

Pulsed-Field Gel Electrophoresis

Each bacterial isolate was subjected to pulsed-field gel electrophoresis (PFGE) analysis by the method described by Brennan et al. (2002) using *SpeI* and *AscI* as the restriction enzymes for the coryneforms and *SmaI* for the coccal-shaped organisms. Strains that had identical band patterns or that differed by only 1 or 2 bands were considered to be identical (Tenover et al., 1995). Representatives of each pattern were identified by repetitive extragenic palindromic (rep)-PCR.

DNA Extraction and rep-PCR

Representatives of each PFGE profile were given an R-number (Research-Collection, Laboratorium of Microbiology, University of Ghent, Belgium) and stored at -80°C. Isolates from stock were streaked on trypticase soy broth (BBL, Sparks, MD) containing 1.5% agar plates (Oxoid, Basingstoke, UK) for 1 to 2 d at 30°C. The DNA of rod-shaped isolates was extracted according to Gevers et al. (2001). The only difference in the protocol was the use of lysostaphin (5,000 U/mL, Sigma, St. Louis, MO) instead of mutanolysin during extraction of DNA from coccus-shaped isolates. The set of reference strains included for identification by rep-PCR was that of Mounier et al. (2005) and was chosen according to the most common species present on red-smear cheeses. Polymerase chain reaction was performed according to Versalovic et al. (1994) with the primers BOXA1R and (GTG)₅ for rods and cocci, respectively. If a strain gave an unsatisfactory band pattern with one primer set, the other primer set was tested. Band patterns were analyzed using the Pearson coefficient, the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis, and BioNumerics software (Applied Maths, St-Martens-Latem, Belgium).

16SrRNA Gene Sequence Analysis

Genomic DNA obtained for the rep-PCR was also used for 16S rRNA gene sequence analysis. The 16S rRNA genes were amplified by PCR using the primers described by Coenye et al. (1999). Polymerase chain reaction-amplified 16S rDNA were purified using the NucleoFast 96 PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and purified using the Montage SEQ₉₆ Sequencing Reaction Cleanup kit (Millipore, Bedford, MA). Sequencing was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The primers described by Coenye et al. (1999) were used to obtain a partial sequence (~700 bp), ensuring highly reliable assembled data. Sequence assembly was performed by using the program AutoAssembler (Applied Biosystems). Phylogenetic analysis was performed using the software package BioNumerics after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL. This alignment was calculated pairwise using an open gap penalty of 100% and a unit gap penalty of 0%. A similarity matrix was created by homology calculation with a gap penalty of 0% and after discarding unknown bases. The resulting tree was constructed using the neighbor-joining method.

Fourier Transform Infrared Analysis

Each yeast isolate was subjected to Fourier transform infrared (FTIR) analysis according to the methods described by Kümmerle et al. (1998), which resulted in both dereplication and identification of the yeasts. Yeasts were streaked as a lawn of cells on yeast extract glucose chloramphenicol agar (YGCA) containing 5.0 g of yeast extract, 20.0 g of glucose, 0.1 g of chloramphenicol, and 15.0 g of agar/L (Merck, Darmstadt, Germany) and were incubated at 27°C for 24 h. Spectra were recorded and evaluated according to Kümmerle et al. (1998) using an IFS-28B FTIR spectrometer (Bruker, Karlsruhe, Germany). For identification, a special reference database, maintained at the Technical University of Munich and comprising >2,500 strains of yeast, was used.

Chemical Analysis of Cheese

Moisture and salt were determined by standard methods (Lynch et al., 1997). The pH was determined by placing the electrode directly into the grated cheese.

Figure 1. pH and salt and moisture contents of batches 2, 3, 4, 5, and 6 of Gubbeen cheese during ripening. Note differences in time axes.

RESULTS

Cheese Composition

The composition of batch 1 cheese was not measured. The variations in salt, pH, and moisture in the other batches during ripening are shown in Figure 1. In batches 2 and 3, a significant decrease in the moisture content on the surface occurred during ripening, and in batch 2, but not batch 3 cheese, a small increase in the salt content of the surface occurred. These effects were not observed in batches 4, 5, and 6, probably because the latter cheeses were examined over a shorter ripening time of 16 d, compared with 37 d in the case of batch 2 and 3 cheeses. The pH increased from ~5 to

5.5–6 in all batches, although some variation occurred in the case of batch 3. The increase was more rapid in batch 4 cheese than in cheeses from batches 5 and 6.

Development of Bacteria and Yeast

The growth of yeasts and salt-tolerant bacteria on the surface of each batch of cheese during ripening is compared in Figure 2. Growth was followed for 44 d in the case of batches 1 to 3 but for only 16 d in the case of batches 4 to 6. In all cheeses, the bacteria reached higher numbers ($\sim 10^8$ cfu/cm²) than the yeasts ($\sim 10^6$ cfu/cm²), and both groups of microorganisms were lower in batch 1 cheese than in the other batches. In batches

Figure 2. Counts of bacteria and yeast on each batch of Gubbeen cheese during ripening. Note differences in x axes for batches 1, 2, and 3 and batches 4, 5, and 6.

4, 5, and 6, the number of yeasts was similar at each time point, indicating that yeast growth had finished before d 4. Exponential growth of the bacteria ceased around d 10, which is the reason that growth was followed for only 16 d in batches 4, 5, and 6.

Identification of the Yeasts

The total number of yeasts isolated was 828, and the number of different species found at each ripening point is shown in Table 1. The most numerous species was *D. hansenii* (624 isolates), followed by *C. catenulata* (135

isolates), *Candida lusitanae* (62 isolates), *Trichosporon ovoides* (3 isolates), *Candida parapsylosis* (2 isolates), *Candida intermedia* (1 isolate), and *Pichia guilliermondii* (1 isolate). *Debaryomyces hansenii* was the dominant yeast in all cheeses except in batch 2, which was dominated by *C. catenulata*. *Debaryomyces hansenii* was also found in batch 2 and *C. catenulata* in batch 3, whereas *C. lusitanae* was found in batches 1 and 2. The other species in Table 1 were found sporadically at different stages of ripening. Generally, the levels of the 3 most abundant species did not vary to any great extent during ripening, except for *D. hansenii* in

Table 1. Identification of the yeast isolates from 6 batches of Gubbeen cheese during ripening

Species	Ripening time, d																				Total	
	Batch 1				Batch 2				Batch 3				Batch 4			Batch 5			Batch 6			
	4	16	23	37	4	16	23	37	4	16	23	37	4	10	16	4	10	16	4	10		16
<i>Debaryomyces hansenii</i>	50	37	7	8	5		5		18	17	22	21	47	48	45	49	50	45	50	50	50	624
<i>Candida catenulata</i>		3		1	11	27	21	25	11	13	8	9	1	2	3							135
<i>Candida intermedia</i>													1									1
<i>Candida parapsylosis</i>		2																				2
<i>Clavispora lusitaniae</i>		8	18	11	13	3	3	5	1													62
<i>Trichosporon ovoides</i>				2			1															3
<i>Pichia guilliermondii</i>					1																	1
Total	50	50	25	22	30	30	30	30	30	30	30	30	49	50	48	49	50	45	50	50	50	828

Table 2. Identification of bacterial isolates from 6 batches of Gubbeen cheese during ripening

Organism	Ripening time, d																								Total
	Batch 1				Batch 2				Batch 3				Batch 4			Batch 5			Batch 6						
	4	16	23	37	4	16	23	37	4	16	23	37	4	10	16	4	10	16	4	10	16				
Staphylococci																									
<i>Staphylococcus saprophyticus</i>	27	1	8	3	1	2		2	50	10	12	1	49	2		37	34	6	32	32	7	316			
<i>Staphylococcus equorum/cohnii</i>	3	10	13									1					1	1	1			30			
<i>Staphylococcus hominis</i>	1																					1			
<i>Staphylococcus epidermidis</i>	5	5	4	1								2										17			
<i>Staphylococcus capitis</i>	4																					4			
<i>Staphylococcus aureus</i>				1																		1			
Coryneforms																									
<i>Agrococcus casei</i>			2	4											2							8			
<i>Arthrobacter arilaitensis</i>																1	6	3	5		2	17			
<i>Brevibacterium linens</i>	1																					1			
<i>Brevibacterium aurantiacum</i>	9	48	38	58	1					6	3	4			1			2		1	16	187			
<i>Brevibacterium casei</i> group ¹		1								1								1				3			
<i>Corynebacterium casei</i>	1				26	24	18	28		16	18	31	1	12	19		8	18	3	15	10	248			
<i>Corynebacterium variabile</i>	4	10	2		3	13	23	6		8	5	2		34	22			10		4		146			
<i>Corynebacterium flavescentis</i>				1		5																6			
<i>Curtobacterium flaccumfaciens</i>				2						6		2										10			
<i>Microbacterium gubbeenense</i>	1		7	2	7	6	7	12		2	3	5		1	2							55			
<i>Pseudoclavier helvolus</i>																9			6			15			
Others																									
<i>Psychrobacter</i> sp.	20	1	2																		3	26			
<i>Halomonas venusta</i>																	1	6			1	8			
Lactic acid bacteria																2			2	1	1	6			
<i>Micrococcus luteus</i>											3											3			
Unidentified	4	3	3	6						1	2	2		1	4	1		3	1	1	3	35			
Not recovered after freezing		1	1	2							3	1									3	11			
Overall total	80	80	80	80	38	50	48	48	50	50	50	50	50	50	50	50	50	50	50	50	50	1,154			

¹Probably *Brevibacterium antiquum*.

batch 1 cheese, which showed a continuous decrease during ripening to d 23, after which it remained constant. The yeasts present at low levels were found more frequently in cheeses from batches 1 and 2 than in any of the others. Compared with batches 1, 2, and 3, batches 4, 5, and 6 were much more homogeneous and were dominated by *D. hansenii* (434 isolates), with very low levels of *C. catenulata* (6 isolates) and *C. intermedia* (1 isolate), all of which were found in batch 4 cheese.

Identification of the Bacteria

The total number of bacteria isolated from the 6 batches of cheese was 1,154, and 1,052 (96%) of them were identified (Table 2). The bacteria were dominated by 2 gram-positive groups, *Staphylococcus* (369 isolates) and coryneforms (*Agrococcus*, *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Microbacterium*, and *Pseudoclavibacter* spp.; 696 isolates); small numbers of gram-negative bacteria, including *Psychrobacter* spp. and *Halomonas venusta* were also found. The data for the isolates in batch 2 were published previously (Brennan et al., 2002) and are included for completeness. *Staphylococcus saprophyticus* was the dominant *Staphylococcus* (316 isolates) in all

batches (even though only 5 isolates were found in batch 2), followed by *Staph. equorum/cohnii* (30 isolates), *Staphylococcus epidermidis* (17 isolates), *Staphylococcus capitis* (4 isolates), *Staphylococcus hominis* (1 isolate), and *Staphylococcus aureus* (1 isolate). The latter 4 species were found only in batch 1 cheese. We have no explanation for this result. In this batch also, *Staph. saprophyticus* was present on d 4, 23, and 37 but not on d 16. *Staphylococcus equorum* and *Staph. cohnii* could not unequivocally be differentiated from each other by the different methods used in this study.

Corynebacterium casei (248 isolates) was the dominant coryneform, followed by *Brevibacterium aurantiacum* (187 isolates), *C. variabile* (146 isolates), and *M. gubbeenense* (55 isolates). Other bacteria were found in low numbers, such as *Agrococcus casei* (Bora et al., 2007) in batches 1 and 4, *Arthr. arilaitensis* and *Pseudoclavibacter helvolus* in batches 5 and 6 and *Psychrobacter* spp. in batches 1 and 6. Thirty-five isolates (3%) were not identified.

Interbatch Variation in Bacteria

Batch 1 contained 16 species of bacteria, batches 3 and 6 contained 10 species each, batch 5 contained 9

species, and batches 2 and 4 had 6 species each (Table 2). Four species, *B. aurantiacum*, *C. casei*, *C. variabile*, and *Staph. saprophyticus*, dominated the surface microflora. However, their relative importance varied considerably. *Brevibacterium aurantiacum* was the dominant coryneform (153 isolates) in batch 1 cheese, but only 1 isolate was found in batches 2 and 4, 2 isolates in batch 5, 3 in batch 3, and 17 in batch 6. Similarly, 96 isolates of *C. casei* were found in batch 2, compared with only 1 isolate in batch 1, 26 in batch 5, 28 in batch 6, 32 in batch 4, and 65 in batch 3. The number of isolates of *C. variabile* varied from 4 to 56 in batches 6 and 4, respectively, and those of *Staph. saprophyticus* varied from 5 to 77 in batches 2 and 5, respectively. Overall, *C. casei*, *C. variabile*, and *Staph. saprophyticus* were more evenly distributed on the cheese surface than was *B. aurantiacum*. *Microbacterium gubbeenense* was found in small but significant numbers in batches 1 to 4, but not in batches 5 and 6. The other species listed in Table 2 were found in much smaller numbers and, in most cases, only sporadically.

Progression of Bacteria

A progression of bacteria occurred during ripening. Staphylococci dominated the early stage (d 4) of ripening of all batches except batch 2, whereas coryneforms (*Agrococcus*, *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Microbacterium*, and *Pseudoclavibacter* spp.) dominated the later stages (d 16, 23, and 36) of all batches (Figure 3). Only 5 *Staphylococcus* isolates out of a total of 184 were found in batch 2 cheese. Isolates from d 10 were made only in batches 4, 5 and 6 and, in these batches, staphylococci dominated batches 5 and 6 and coryneforms batch 4 (Figure 3). No progression of yeast was observed.

Recovery of the Surface Ripening Cultures

Two commercial cultures, *B. linens* BL2 and OFR9, were used to deliberately inoculate the cheeses surfaces of batches 1 to 3 and batches 4 to 6, respectively, 1 to 2 d after manufacture. *Brevibacterium linens* BL2 was identified by rep-PCR as a strain of *B. aurantiacum*. The OFR9 culture is a mixed-species culture that contains *P. helvolus*, a *Brevibacterium* spp., and a yeast that could not be identified from the FTIR database of >2,500 strains (Mounier et al., 2006). Two bacterial isolates from d 4 of batch 1 cheese had the same PFGE patterns as *B. aurantiacum* BL2, and 15 bacterial isolates from d 4 of batches 5 and 6 had the same PFGE patterns as *P. helvolus*. Isolates with profiles similar to these 2 strains were not subsequently recovered at any other time point. Isolates with PFGE patterns simi-

Figure 3. Number of strains of staphylococci and corynebacteria (*Agrococcus*, *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, and *Microbacterium* spp.) isolated from each batch of cheese at each time point during ripening.

lar to that of the *Brevibacterium* spp. from the OFR9 culture were not recovered at any stage.

Strain Identifications

Pulsed-field gel electrophoresis patterns showed that several strains of *B. aurantiacum*, *C. casei*, *C. variabile*, *M. gubbeenense*, and *Staph. saprophyticus* were present on the cheese. This is shown in Figure 4 for *C. casei*, *C. variabile*, and *Staph. saprophyticus*. In all species, one strain was dominant, strain 3.23.14 in the case of *C. casei*, strain 3.23.28 of *C. variabile*, and strain 6M34 of *Staph. saprophyticus*. Each was found in 5 of the 6 batches of cheese. Two other fairly dominant strains of *C. variabile* (strains 4M2 and 4L1) were also found in batches 4, 5, and 6 and of *Staph. saprophyticus* (strains 1.4.39 and 1.4.47) in batches 1, 2, and 3 (Figure 4). The dominant strain of *M. gubbeenense* was found only in batches 1, 2, and 3 and that of *B. aurantiacum* overwhelmingly (82% of isolates) in batch 1 and in small

Figure 4. Pulsed-field gel electrophoresis patterns of the different strains of *Corynebacterium casei*, cut with *AscI*; *Corynebacterium variable*, cut with *SpeI*; and *Staphylococcus saprophyticus*, cut with *SmaI*. The number of strains with that particular profile isolated from each batch of cheese is also indicated.

numbers in batches 3 and 6 (data not shown). The other strains of each species were found sporadically.

Only one PFGE pattern was found among the 9 isolates of *A. casei* from batches 1 and 4, from the 17 isolates of *Arthr. arilaitensis* from batches 5 and 6, from the 15 isolates of *P. helvolus* from batches 5 and 6, from the 4 isolates of *S. capitatis* from batch 1, and from the 8 isolates of *H. venusta* from batches 5 and 6, indicating that each of these species was present as a single clone. The 23 isolates of *Psychrobacter* spp. from batch 1 all had the same PFGE pattern, which was different from the 3 isolates from batch 6, which comprised 2 profiles (data not shown). Two different PFGE patterns were found for the 28 isolates of *Staph. equorum/cohnii* from batch 1, but one type predominated (26 isolates) and was found in batches 1, 3, 5, and 6; the other profile (2 isolates) was only found in cheese from batch 1. In contrast, 4 different PFGE patterns were found among the 16 isolates of *Staph. epidermidis*, all except 2 isolates of which were from batch 1; 3 different PFGE patterns were found among the 10 isolates of *Curtobacterium flaccumfaciens* from batches 1 and 3.

DISCUSSION

In total, 828 yeasts and 1,152 bacteria were isolated at either 3 or 4 different time points during ripening from the 6 batches of cheese. The methodology used allowed us to isolate the dominant microflora, because all isolates were made from plates containing countable numbers of bacteria. The results showed little intra-batch variation but considerable interbatch variation. The probable reason for this variation is the lack of adequate control of temperature and humidity during ripening of Gubbeen cheese, both of which are important in controlling the growth of the surface microflora (Leclercq-Perlat et al., 2000; Bonaiti et al., 2004).

Debaryomyces hansenii was the dominant yeast in all batches of cheese except batch 2, in which *C. catenulata* dominated (Table 1). The latter species and *C. lusitaniae* were also important members of the yeast microflora in batch 3; *C. lusitaniae* was also found in batch 1 cheese.

The dominant bacteria were *B. aurantiacum*, *C. casei*, *C. variable*, and *Staph. saprophyticus*, and there was much greater diversity in the bacteria isolated (17 species) than in the yeasts (6 species). In addition, the ratio of the dominant bacteria to each other was much greater than that of the yeast (Table 2). For example, *B. aurantiacum* was present in large numbers in batch 1 but was only a minor component of the other 5 batches of cheese. In addition, *C. casei* was the dominant coryneform in batches 2, 3, and 6 but *C. variable* dominated

batch 4. *Microbacterium gubbeenense* was a small but important member of the microflora of batches 1 to 4, and *Arthr. arilaitensis* was found only in batches 5 and 6. *Staphylococcus saprophyticus* was present in all batches in high numbers, except in batch 2; the remaining species listed in Table 2 were found only sporadically. Whether these differences in microflora also resulted in differences in the flavor of the cheeses was not determined. *Arthrobacter arilaitensis* and *C. casei* have also been found on other Irish smear-ripened cheeses (Mounier et al., 2005).

Very few reports are available in the literature on the systematic changes that occur in the microbial flora on the surface of smear cheeses during ripening. The results generally show that a progression of species occurs during ripening, with staphylococci dominating the earlier stages, but not exclusively so, and coryneforms, particularly *C. variable* and *C. casei*, dominating the later stages of ripening. When d 4 was excluded, *C. casei*, *C. variable*, and *M. gubbeenense* were generally found in about the same numbers at each ripening point within a batch of cheese; however, in batches 5 and 6 *C. variable* was an exception. Similar results were also found by Rademaker et al. (2005). Why staphylococci are generally replaced by coryneforms during ripening is not clear. One older report suggests that the yeasts produce vitamins, pantothenic acid, niacin, and riboflavin, which stimulate the growth of *B. linens* on cheese (Purko et al., 1951). It is possible that the *Staph. saprophyticus* produces some growth factors for the coryneforms, but what they are is not clear. They are unlikely to be vitamins, because milk is a source of many vitamins and many lactic cultures used in cheese manufacture also produce vitamins. Staphylococci and cheese coryneforms are quite salt tolerant (Brennan et al., 2002), so salt is not involved. It is also possible that some component is produced by the coryneforms that inhibits the staphylococci.

The results of the overall changes in yeast and bacterial numbers during ripening are 5- to 10-fold lower than those of Tilsit cheeses (Eliskases-Lechner and Ginzinger, 1995a,b). In addition, the increases in the surface pH of Gubbeen cheese are not as large as those in Tilsit, and some variation was evident in different batches. The smaller change in pH may be due to the loss of moisture from the surface of Gubbeen cheese, with a consequent decrease in water activity and inhibition of microbial growth.

Only 2 of the 5 commercial cultures deliberately inoculated onto the cheese surface were subsequently found on the cheese surface, 2 isolates of *B. linens* BL2 from batch 1, and 15 isolates of *P. helvolus* from batches 5 and 6. The latter all had the same PFGE pattern, and all were isolated within 2 d of inoculation but were not

subsequently isolated during cheese ripening. These results imply that the commercial cultures do not readily implant on the surface. Historically, *B. linens* was considered to be the dominant organism on the surface of smear cheeses. The present study shows that *B. linens* is not an important organism on the cheese surface, confirming the results of Eliskases-Lechner and Ginzinger (1995a) and Mounier et al. (2005). The deliberately inoculated strains may be present as subpopulations, but they are not part of the dominant microflora. This result contrasts with that of Rademaker et al. (2005), who, using a terminal RFLP fingerprint analysis, found that the deliberately inoculated strains were present on the cheese surface throughout an 8-wk ripening period.

These results raise the question of the source of the organisms on the cheese surface. The fact that many isolates of the same species had the same PFGE patterns, and that dominant strains of those species were present and showed several PFGE patterns would suggest that a house microflora is associated with this particular cheese. This has recently been confirmed by Mounier et al. (2006), who, in a detailed analysis of the sources of contamination, identified the brine as a significant source of *Staph. saprophyticus* and the hands of the personnel as important vectors for the coryneforms. Some bacteria present in low numbers were monoclonal, indicating the presence of a single focus of contamination.

Staphylococcus saprophyticus is a class 2 organism that causes urinary tract infections in women (Hedman et al., 1990). This raises the question of the safety status of the cheese. *Staphylococcus saprophyticus* has also been isolated from other cheeses (Vernozy Rozand et al., 1996; Irlinger et al., 1997; Delbès and Montel, 2005) and from the surface of fermented meat products, where the curing process and the salt are considered to be the main sources of contamination (Vilar et al., 2000; Gardini et al., 2003). Thus, the organism would appear to be common in fermented foods.

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