Microbial community dynamics of a blue-veined raw milk cheese from the UK

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Interpretive Summary

Microbial community dynamics of a blue-veined raw milk cheese from the UK. By Yunita and Dodd. Raw milk may introduce many microbial species into a cheese which could be important for product characteristics. We show that whilst some raw milk species are lost during cheese fermentation and ripening, some survive. Salt addition and handling introduce new species contributing mainly to rind microbiota. In blue-veined cheeses, piercing introduces these bacteria from the surface to the core but these often fail to establish.

Title

Microbial community dynamics of a blue-veined raw milk cheese from the UK

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Running title: Blue-veined raw milk cheese microbial community

ABSTRACT

A commercial blue-veined cheese made from unpasteurized milk was examined by conventional culturing and PCR Density Gradient Gel Electrophoresis analysis of the bacterial community 16S rRNA genes using three primer sets V3, V4V5, V6V8. Genomic DNA for amplification was extracted directly from raw milk, starter culture, cheese at different stages of production, fully ripened cheese and from the cultured cells grown on various media. The outer rind was sampled separately from the inner white core and blue veins. A diverse microbiota containing *Lactococcus lactis* subsp. *lactis*, *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Staphylococcus gallinarum*, *Staphylococcus devriesei*, *Microbacterium* sp., *Sphingobacterium* sp., *Mycetocola* sp., *Brevundimonas* sp., *Enterococcus faecalis, Proteus* sp. and *Kocuria* sp. was detected in the raw milk using culturing methods, but only *Lactococcus lactis* subsp. *lactis*, *Lactobacillus plantarum* and *Enterococcus faecalis* survived into the final cheese and were detected both in the core and the rind. Using PCR Density Gradient Gel Electrophoresis analysis of the cheese process samples, *Staphylococcus equorum* and *Enterococcus durans* were found in the rind of pre-piercing samples but not in the core and veins; after piercing, these species were found in all parts of the cheese but survived only in the rind when the cheese was fully ripened. *Brevibacterium* sp., *Halomonas* sp., *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were identified only by PCR Density Gradient Gel Electrophoresis and not cultured from the samples. *Brevibacterium* sp. was initially identified in the cheese post piercing (core and veins), *Halomonas* sp. was found in the matured cheese (rind), *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were also in the pre-piercing samples (rind) and then found through the subsequent process stages. The work suggests that in

this raw milk cheese, there is a limited community from the milk surviving into the final cheese, with salt addition and handling contributing to the final cheese consortium.

Key words: raw milk, blue-veined cheese, PCR DGGE, microbial diversity

INTRODUCTION

The most strictly prescribed unpressed blue-veined cheese in the United Kingdom (**UK**) is Stilton. It is a protected designation of origin (**PDO**) product of the counties of Derbyshire, Leicestershire and Nottinghamshire, with the requirement that milk is pasteurized at the first stage. Since March 1990, a blue-veined raw milk cheese made by the same process as Stilton has been produced commercially in Nottinghamshire. This cheese has the same texture and appearance as Blue Stilton which has a creamy white curd, open texture with blue-green well-distributed veins (created by piercing the cheese during ripening) and rough brown rind (Scott et al., 1998). Both cheeses use similar lactic cheese starter cultures which consist of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. Both cheeses also contain a starter *Penicillium roqueforti* mold which starts to grow in the core of the cheese after the piercing process, due to the aerobic conditions this creates, giving the blue-vein appearance of the cheese which develops during the ripening period (Bockelmann, 2010).

The microbial community of Stilton has been previous studied and shown to present a complex consortium of bacteria (Ercolini et al., 2003) and yeasts (Gkatzionis et al., 2014). This non-starter microbiota varies in the different parts of the cheese (white core, blue veins and outer rind) and has been shown to contribute to the flavor volatile production, which also shows variation in the different regions of the cheese (Gkatzionis et al., 2013, 2014; Price et al., 2014). However, there is no published work on raw milk blue-veined cheese originally from the UK. This may differ as the microorganisms in the raw milk will be different, coming from the cow and milking

environment, and these may contribute to the final cheese community and determine sensory properties.

There is much debate amongst cheese makers on the use of raw or pasteurized milks for cheese making. The risks and benefits for traditional cheeses, mainly raw milk cheeses, has been discussed by Montel *et al*. (2014). The concern over use of unpasteurized milk in cheese production is related to health concerns caused by pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* and *E. coli* found in cheeses (Lafarge and Lahellec, 2000). Pasteurization is usually used to assure the safety of milk and is the reason why Stilton cheese makers use pasteurized milk. However, for raw milk cheese makers, pasteurization kills desirable bacteria and destroys enzymes and proteins resulting in a less tasty cheese. The problem of raw milk safety risks can be addressed, as the fermentation should kill pathogenic bacteria if it is done properly. So, the main concern of traditional cheese producers is to preserve microbial diversity and exploit its benefits.

Therefore, in the current study, we have analyzed the bacterial diversity during raw milk blue-veined cheese production using a traditional culture approach and 16S rRNA gene polymerase chain reaction denaturing gradient gel electrophoresis (**PCR DGGE**) techniques to evaluate the bacteria which contribute to production of the characteristics of the product. The presence of *Lactococcus lactis* at the end of production has also been examined by pulse-field gel electrophoresis (**PFGE**) to determine whether its origin was from the raw milk or the added starter cultures. This should create an understanding of raw milk cheese production and the microbiota components which are needed to allow cheese makers to make a consistent product.

MATERIALS AND METHODS

Sampling

The UK blue-veined raw milk cheese is made from unpasteurized milk. The process is started by pouring the milk into a stainless steel tank, heating up to 30°C and adding starter cultures (*Lactococcus lactis* and *Penicillium roqueforti*) and 90 minutes later adding rennet. The mixture is fermented at around 30-40°C. After 6 hours, the curds form and are cut by hand into small cubes to release the whey. The soft curds then are transferred manually by ladles into a second tank and left overnight. The curds are then milled, salted and poured into plastic cylindrical hoops without pressing. Then, the cheeses are left in the hastener (21°C) and turned daily for 5 days to allow the whey to drain. On the fifth day, the cheeses are removed from the hoops and the outside smoothed by knives in a process termed 'rubbing up'. The cheeses are then moved to the ripening rooms (T = 13°C; RH = 85%) and six weeks later, fresh cheese is pierced by stainless steel needles to allow development of the blue veins. Finally, after around another 6 weeks (T = 13°C; RH = 90%) the cheeses are fully matured.

Samples from bulk raw milk, frozen starter culture and a raw milk blue-veined cheese during production were taken aseptically in the spring season in Nottinghamshire. The production sites sampled were: pre-milling, post-milling and salting, pre-piercing (6 weeks), post-piercing (9 weeks) and fully ripened cheese (12 weeks). The raw milk sample was processed immediately after collecting, but the frozen starter culture was left overnight at room temperature (at around 20°C) to thaw following the manufacturer's procedure for use in cheese production. The rest of the samples were analyzed within 6 h or kept cool at 4°C for no longer than 24 h. For all cheese samples, the outer rind was separated from the inner core of the cheese and the two regions were

tested separately. The inner core was collected using a sterilized cheese corer. All samples were obtained in triplicate.

Microbiological Analysis

The cheese samples (25 g) were weighed into a stomacher bag and diluted in quarter-strength Ringer's (225 ml; Oxoid, Hampshire, UK) and homogenized in a stomacher at 230 RPM for 2 minutes (Stomacher 400 Circulator, Seward, West Sussex, UK). These samples were considered as the 10^{-1} dilution and were further diluted to 10^{-8} by tenfold serial dilutions in the same diluent. Milk and starter culture samples were directly diluted in quarter-strength Ringer's. Samples (0.1 ml) of each dilution were spread-plated in triplicate on non-selective and selective media. Mesophilic aerobic bacteria were counted on brain heart infusion (**BHI**) agar, yeasts and molds on rose bengal chloramphenicol agar (**RBCA**), lactococci on M17 agar, lactobacilli on Rogosa agar, lactic acid bacteria (**LAB**) on MRS agar, enterococci on KF Streptococcal agar (**KFSA**) and staphylococci on Baird Parker (**BP**) agar. All agars were from Oxoid. All bacterial plates were incubated at 30°C for 2 days while yeasts and molds were grown at 25°C for 5 days. LAB and lactobacilli were incubated under anaerobic conditions which were obtained by using AnaeroGen Gas Pack (Oxoid) (Conte et al., 2011). Presumptive *Staphylococcus aureus* from BP agar were confirmed by catalase (using 40% H_2O_2) and coagulase tests (Staphytect Plus Test, Oxoid) (Collins et al., 1995).

Selection and Characterization of Isolates

To analyze the cultured populations, a total of 80 isolates (41 isolates from the mature cheese and 39 isolates from raw milk) with different colony types were randomly

picked from the seven media used. Each isolate was purified by streak-plating three times and stored in Microbank™ Bacterial and Fungal Preservation System (Pro-Lab Diagnostic, Wirral, UK) at -80°C. Isolates were examined for cell morphology by Gram staining and for their ability to grow at 10°C for 4 days and 40°C for 2 days.

DNA Extraction

Bulk cell suspensions were collected from the culture plates by adding 1 ml of sterile phosphate buffered saline (Oxoid) onto the confluent growth plates (10^{-1} dilution). A DNeasy®Mericon Food Kit (Qiagen) was used to extract DNA from cheese, milk, and bulk cell suspensions. The following procedure was used with some modification of the manufacturer's instructions. Briefly, the cheese sample (200 mg), milk sample (0.2 ml) or bulk cell suspension (0.2 ml) was placed into a sterile 1.5 ml microcentrifuge tube. Then, 1 ml of Food Lysis Buffer (cetyltrimethylammonium bromide, CTAB) and 2.5 µl of proteinase K solution were added. The mix was incubated for 30 min at 60°C in a thermomixer (Eppendorf, Germany) at 1,000 RPM followed by cooling for 1 min and centrifuging at 10,000 g for 5 min. The clear supernatant (700 µl) was placed into a new tube containing 500 µl of chloroform (Fisher Scientific, UK). Then, the mix was vortexed for 15 s and centrifuged at 14,000 g for 15 min. The upper solution (250 µl) and 1 ml of Buffer PB (5M guanidine hydrochloride and 30% isopropanol) were mixed thoroughly in a new tube by vortexing then transferred into the QIAquick spin column prior to centrifuging at 17,000 g for 1 min. Buffer AW2 (a low salt buffer, 500 µl) was added onto the column and this was centrifuged at 17,000 g for 1 min. The flow-through was discarded and the column was centrifuged again at 17,000 g for 1 min. Finally, the column was transferred into a new

tube and 100 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) was added. The mix was incubated at room temperature for 1 min before it was centrifuged at 17,000 g for 1 min to elute the DNA.

DNA was extracted from reference strains (*Lactococcus lactis* NCIMB 9918, *Lactococcus lactis* NCIMB 8763, *Lactobacillus plantarum* NCIMB 138914, *Enterococcus faecalis* NCTC 775 and *Leuconostoc mesenteroides* (University of Nottingham, Division of Food Sciences strain collection) and selected pure isolates from cheese by a boiling procedure used to lyse the cells in a heating thermocycler. The procedure followed Cocolin et al. (2001).

The extracted DNA (2 µl) concentration and purity were measured at a wavelength of 260 nm by Nanodrop (ND-1000 spectrophotometer, Thermo Scientific, Wilmington, USA). A ratio of absorbance at 260 nm and 280 nm (260/280) of 1.8 is generally accepted as pure for DNA as lower ratios indicate the presence of protein, phenol or other contaminants (Nanodrop Technologies, Inc., 2007). The extracted DNA (1.5 µl) was then used as template in PCR reactions.

PCR Amplification

The V3, V4V5, V6V8 regions of 16S rRNA genes were amplified by PCR as previously reported (Muyzer et al., 1993; Nübel et al., 1996; Schwieger and Tebbe, 1998). For DGGE analysis, a GC-clamp was added to each forward primer (Muyzer et al., 1993). PCR amplification was performed with DreamTaq™ Green PCR Master Mix (2x) (Fermentas, Yorkshire, UK) in a programmable heating thermocycler (Biorad C1000TM Thermal Cycler, Hertfordshire, UK). Nuclease-free water (23.3 µl; Fermentas), 0.1 µl of each primer (MWG-Biotech, Ebersberg, Germany) and 1.5 µl of

195 sample DNA were added into the master mix (25 µl) to give a final volume of 50 µl. The
196 PCR product (5 µl) was checked by electrophoresis on a 1% TAE agarose gel (Agarose
197 3:1, Melford Laboratories Ltd., Suffolk, UK) containing ethidium bromide (0.2 µg/ml)
198 in 1X TAE running buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) at 75 V
199 for about 45 min. The size of the amplified DNA was determined against a 100 bp
200 molecular weight marker (Promega, Southampton, UK). The gel was imaged under a
201 UV transilluminator (GelDoc XR, BioRad) and gel pictures were recorded using the
202 GelDoc system (The Quantity One 4.6.5 Basic software, USA).
203
204 ***DGGE Analysis***
205 Samples of 200 bp size PCR products (using V3 primer sets) were analyzed in
206 8% (w/v) polyacrylamide gels in 1x TAE buffer while those of 400 bp sizes (using
207 V4V5 and V6V8 primer sets) were run in 6.5% (w/v) polyacrylamide gels (Ercolini et
208 al., 2003). Parallel electrophoresis was performed at 20 V for 10 min followed by 16 h at
209 50 V by using a Bio-Rad Dcode apparatus (Universal Mutation Detection System,
210 USA). The gel contained 20-80% urea formamide for which 100% denaturant solutions
211 consisted of 40% (v/v) formamide and 7 M urea. All DGGE reagents were from Severn
212 Biotech Ltd., Worcestershire, UK. The gel was stained with GelStar (Lonza Rockland,
213 ME USA) and the images recorded.
214
215 ***Sequencing of DGGE Bands***
216 Selected bands were excised from the gel using a sterile scalpel and kept at 4^{o}C
217 for 24 h in 20 µl of TE buffer (10 mM Tris Base, 1 mM EDTA; pH 7) to elute DNA into
218 the buffer. The eluted DNA (1.5 µl) was reamplified with the same PCR program but

without the GC-clamp in the respective forward primer. The PCR products (15 µl) were purified with Zymoclean Gel DNA Recovery Kit (The Epigenetics Company; Cambridge, UK) according to the manufacturer's procedures. Following purification, 15 µl of purified DNA was sequenced (MWG Laboratory, Germany). The DNA sequences were compared with those in public data libraries by using BLAST (Basic Local Alignment Search Tool) search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Preparation of Lactococcus lactis Isolates for PFGE Analysis

Lactococcus lactis was isolated from two commercial starter cultures used alternately by the cheese producers (liquid and powdered) and fully ripened blue-veined raw milk cheese (12 weeks). The added starter cultures contained *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. The liquid starter culture was used directly for isolation, while powdered starter culture was prepared in UHT milk which had been heated at 32°C for 24 h before use following the manufacturer's procedure; 0.1 g starter culture (OV100, Orchard Valley Dairy Supplies, Worchestershire, UK) in 10 ml heated milk. For the cheese, the rind was also tested separately from the core and blue veins. All samples of each dilution were spread plated (0.1 ml in duplicate) and grown aerobically on M17 agar (Oxoid CM 0785) and anaerobically (AnaeroGen kit; Oxoid AN0035A) on MRS agar (Oxoid CM 0361) at 30°C for 2 days. Twenty five isolates were picked from each sample on each media agar and were restreaked twice to get pure isolates for further characterization. Isolates with the following characteristics: Gram positive cocci, catalase negative, oxidase negative and non-group D were presumptively identified as *Lactococcus lactis* isolates and were confirmed by sequencing of V3 region.

243 *Lactococcus lactis* subsp. *lactis* NCIMB 6681, *Lactococcus lactis* subsp. *cremoris*
244 NCIMB 8662 and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* NCIMB 10484
245 were used as controls. These strains were grown in MRS broth (Oxoid CM0359) at 30°C
246 and 100 RPM (Gallenkamp) for 24 h.
247
248 ***Preparation of Digested Genomic DNA for PFGE Analysis***
249 Cells were harvested from 3 ml of an overnight culture grown in BHI broth at
250 30°C and 100 RPM agitation (Gallenkamp), by centrifugation (Eppendorf) at 13,000 g
251 for 60 s. In a microcentrifuge tube, the cell pellet was re-suspended and washed twice
252 with 1 ml of TN buffer [1M NaCl (Fisher Scientific S/316/60) and 10 mM Tris-HCl
253 (Sigma T6066)] at pH 6.7 by centrifugation (Hettich Zentrifugen Mikro200) at 13,000 g
254 for 60 s. Re-suspended cells (300 µl) in TN buffer were warmed to 55°C (Thermomixer
255 Eppendorf) and mixed with 300 µl of 1% (w/v) pulse-field certified agarose (Bio-Rad
256 161-3109) made up in TN buffer. The suspension mixture was poured into a CHEF plug
257 mould (Bio-Rad) and allowed to solidify for 15 min at room temperature (20°C). The
258 embedded cells were lysed with 4 ml of lysis buffer [6 mM Tris-HCl, 0.1 M EDTA
259 (Fisher Scientific D/0700/53) and 1% (w/v) N-lauryl-sarcosine sodium salt (Sigma
260 L9150), pH 7.6] containing 10 mg/ml lysozyme (Sigma L6876) overnight at 37°C. The
261 plugs were washed three times with 3 ml of TE buffer [10 mM Tris-HCl and 1 mM
262 EDTA, pH 7.6] for 30 min at room temperature followed by incubation in 4 ml of
263 proteinase-K buffer [0.5 M EDTA, 1% (w/v) N-lauryl-sarcosine sodium salt, and 1
264 mg/ml proteinase-K (Fermentas EO 0491), pH 8.5] for 24 h at 55°C (100 RPM;
265 Gallenkamp). The plugs were treated twice with 4 ml of TE1 buffer [10 mM Tris-HCl
266 and 1 mM EDTA, pH 8] containing 1 mM phenylmethylsulphonyl fluoride (PMSF;

Sigma P7626) shaken at 100 RPM for 1 h at 55°C. The addition of PMSF was to obtain vivid, discrete and clear bands after running the gel (Obszańska *et al*., 2015). The plugs were finally rinsed three times with TE2 buffer [10 mM Tris-HCl and 50 mM EDTA, pH 8] for 30 min at room temperature. The plugs were stored at 4°C in TE1 buffer until use.

Before digestion, the DNA plugs were rinsed in 500 µl sterile distilled water for 15 min at room temperature followed by rinsing in 200 µl restriction buffer-D [6 mM Tris-HCl, 6 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, pH 7.9; (Promega R004A)]. The digestion was carried out for 24 h at 37°C in a 100 µl of solution consisting of *Sma*I (0.4 U/µl; Promega R6121), bovine serum albumin (BSA; 0.2 µg/µl; Promega R396D) and buffer J (1X; Promega R009A). The digested plugs were removed from the reaction reagents and stored at 4°C in 0.5X TBE buffer [44.5 mM Tris-HCl, 44.5 mM boric acid (Sigma B7901), 1.25 mM EDTA, pH 8] until use.

PFGE Analysis

The electrophoresis conditions were followed from Obodai (2006) with minor modifications. Electrophoresis was run in 2 l of 0.5X TBE buffer [44.5 mM Tris-HCl, 44.5 mM boric acid (Sigma B7901), 1.25 mM EDTA, pH 8] containing 100 µM thiourea, at 14°C for 16 h at 6 V/cm, using a counter-clamped homogenous electrophoresis cell (Bio-Rad). The pulsed time used was 4-45 s and pump pressure was 80 RPM. A 50-1000 kb DNA ladder (Sigma D-2416) was used as a molecular size marker. DNA was visualized by staining in 100 ml of sterile distilled water containing 10 µl of ethidium bromide (10 mg/ml; Fisher Scientific) for 1 h at room temperature followed by de-staining in 100 ml of sterile distilled water for 30 min at room

temperature. The gels were imaged under a UV transilluminator (GelDoc XR, Bio-Rad) and recorded with Quantity One 4.6.5 Basic Gel Doc software (Bio-Rad).

PFGE Cluster and Statistical Analysis

PFGE profiles were saved as 150 dpi tiff format and were analyzed using FPquest version 4.5 software (Bio-Rad). Calculation of the similarity of the band profiles and grouping of the PFGE patterns was based on the Dice correlation coefficient and the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis. Calculation of the significance of the PFGE profiles was done by AMOVA GenAlEx 6.5: Genetic Analysis in Excel.

RESULTS

Enumeration of Microbial Population by Plate Counts

The bacterial populations of the raw-milk cheese were enumerated through the different stages of production (Table 1); pH values were also recorded at each stage. The pH of the raw milk decreased after starter addition and then changes developed through production. Interestingly, unlike with other mold-ripened cheeses where the pH of the rind is higher than that of the core because of ammonia produced by a high yeast count (Gori et al., 2007; Mounier et al., 2006) and a high mold count (Fox et al., 2004), the higher pH of the rind relative to the core of the mature cheese was not associated with a higher total yeast and mold count (RBCA) and in fact was 2 $\log_{10}$ cfu/g lower. Presumptive *Lactococcus* counts (M17 agar) increased in the early stages of cheese fermentation with higher counts developing in the rind than in the core in later production stages. The mature cheese showed similar results. Yeasts and molds were not

isolated from the raw milk but became evident after milling and salting; these counts increased through the production stages with counts highest following piercing at 9 weeks. Counts in the rind were always higher than the core, which may reflect oxygen availability. However, the total yeast and mold counts in the rind dropped dramatically in week 12 of ripening. Presumptive *Lactobacillus* (Rogosa agar) counts showed similar trends. The LAB (MRS agar), presumptive *Staphylococcus* (BP agar) and presumptive *Enterococcus* (KFSA) counts all showed a similar trend, being low in the raw milk, increasing after salting and with a peak in counts seen in the rind at 9 weeks, with core levels much lower; this difference did not appear to be related to a pH difference between rind and core samples at this stage and again may reflect differences in oxygen availability. These bacterial groups are all recognized as moderately halotolerant and hence are likely to increase after salt addition through reduction in competition with more halo-sensitive species. The mature cheese showed a lesser or no difference between rind and core for these counts despite a greater pH difference between the two samples (Table 1).

Characterization of Raw Milk and Matured Cheese Bacterial Isolates

Eighty bacterial isolates were taken from the matured cheese and raw milk culture plates and sequenced (Table 2). The 16S rRNA gene sequencing showed the presence of *Lactobacillus curvatus*, *Staphylococcus gallinarum*, *Staphylococcus devriesei*, *Microbacterium* sp., *Sphingobacterium* sp., *Mycetocola* sp. and *Brevundimonas* sp. only in the raw milk sample. Conversely, *Enterococcus faecalis* and *Proteus* sp. were found only in the matured cheese. *Kocuria* sp. was detected in the milk and the core of the cheese samples, but not in the rind. *Lactococcus lactis* subsp. *lactis*

and *Lactobacillus plantarum* were found in all samples. All isolates, except *Proteus* sp., could grow at 10°C indicating they could grow during the early ripening step of this cheese which is carried out at around 10°C. The Gram positive coccal species (with the exception of *S. gallinarum*) showed growth at 40°C together with *Lactobacillus curvatus* showing that the isolates could grow during the initial milk heat treatment prior to starter culture addition and fermentation step which is carried out at 37-40°C.

It is notable that the M17 medium showed poor selectivity with a range of other Gram positive and Gram negative genera isolated from this medium (Table 2). This medium is widely used for isolating and enumerating *Lactococcus,* however, the counts on M17 cannot be considered to reflect the levels of *Lactococcus* accurately. In contrast, Rogosa agar, which is used for the enumeration of *Lactobacillus*, showed much greater selectivity with *Lactobacillus plantarum* the main isolate, although *Kocuria* sp. were also isolated on this medium. Notably *L. curvatus* was not isolated from Rogosa agar but only on MRS agar; hence Rogosa agar may underestimate the *Lactobacillus* population present.

Bacterial Dynamics during Cheese Production

We used three sets of primers (V3, V4V5 and V6V8) to amplify the variable regions of 16S rRNA genes of the bacterial community from samples taken through production of the cheese (Figure 1); bands identified by sequencing are shown marked. The bacterial species detected varied at each production step and with each primer used, with an increase in the diversity in the final cheese. The bacterial species in the rind were more complex than those in the core (Figure 1, V4-V5, Lanes 10 and 11). In general, *Lactococcus lactis* subsp. *lactis* and *Lactobacillus* sp. were the main

representatives in all samples with *Lactococcus* (band f) present initially in the raw milk through to the final cheese. *Lactobacillus plantarum* appeared at the post milling and salting stages (Figure 1, V3, Lane 5), as suggested by culturing, and was still detected in the fully ripened cheese (Figure 1, V3, Lane 11). *Staphylococcus equorum* and *Enterococcus durans* were found in the rind of a pre-piercing sample but not in the core and veins (Figure 1, V3, Lanes 6 and 7). Then, after cheese piercing, these species were found in all parts of the cheese, suggesting redistribution by the piercing process (Figure 1, V3, Lanes 8 and 9). However, these species only survived in the rind in the final product. Similarly, bands corresponding to *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were found in the rind pre-piercing (Figure 1, V6V8, Lane 7), but, of these, only *Acinetobacter* sp. was detected in the core (Figure 1, V6V8, Lane 6). All three were present in the core post-piercing with the first two species still detected in the rind post-piercing and in the rind of the mature cheese (Figure 1, V6V8, Lanes 9 and 11). However, only *Acinetobacter* was detected in the core of the mature cheese suggesting an influence of pH or oxygen levels. *Lactobacillus casei/paracasei*, *Enterococcus faecalis* and *Brevibacterium* sp. were only found in the rind of the matured cheese suggesting surface introduction or development of these populations occurs late in production.

A comparison between the MRS, Rogosa and KFSA cultured populations with the uncultured populations from the mature cheese is shown in Figure 2. Mostly we found the same genera by direct DNA analysis of the culture plates as we identified as purified isolates (Table 2), although the exact species identified showed some variation. In particular *Enterococcus durans* and *Staphylococcus equorum* were found in samples grown on KFSA agar (Figure 2, Lane 8) and *Lactobacillus casei/paracasei* from MRS

and Rogosa agars (Figure 2, Lanes 6 and 7), although these species were not evident from analysis of the DNA extracted directly from the respective cheese samples, suggesting these species were in very low numbers in the cheese itself. *Lactococcus lactis* was detected in the DNA sample produced by direct extraction from the cheese and was also isolated from M17 and other agars (Table 2). This demonstrated that live *Lactococus lactis* cells were still evident in the final cheese. Direct DNA analysis also showed the presence of *Brevibacterium* in the mature cheese rind which was not shown by any culture plate analysis.

PFGE Profiles of Lactococcus lactis Isolates

Twenty-six *Lactococcus lactis* isolates which were obtained at different stages of cheese production and confirmed by 16S sequencing (Table 2) and three *Lactococcus lactis* controls were subjected to *Sma*I restriction and PFGE profiling to determine strain relatedness. The *Sma*I PFGE profiles are shown in Figure 3 together with a dendrogram showing relatedness of the patterns. *Lactococcus lactis* isolates produced clear profiles and were differentiated into three non-overlapping clusters with two out rider strains at 40% similarity. All isolates from the powdered starter culture were clustered together with eight isolates from the liquid starter culture, one isolate from the cheese rind (CRB11) and *Lactococcus lactis* subsp. *cremoris* NCIMB 8662 (Cluster 1). Another isolate from the cheese rind (CRB10) clustered with isolates from the liquid starter culture (Cluster 2). Two isolates found in the liquid starter culture were similar to *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* NCIMB 10484 (Cluster 3), but not similar with any isolates from the cheese core and veins or rind. Two isolates found in the core and veins of mature cheese (COB20 and COB6) had profiles different from

those obtained from the liquid and powdered starter culture isolates. One of these isolates had a similar profile with *Lactococcus lactis* subsp. *lactis* NCIMB 6681. This suggests the *Lactococcus lactis* population originated from a number of sources.

DISCUSSION

In the present study, we have examined the bacterial species present during the production of a raw milk mold-ripened cheese by using traditional culturing methods and PCR-DGGE analysis of the variable V3, V4V5 and V6V8 regions of the 16S rRNA genes. This method has previously been used successfully to identify the microbial communities in Artisanal Sicilian (Randazzo et al., 2002), Stilton (Ercolini et al., 2003), Mozzarella (Ercolini et al., 2004), Cabrales (Flórez and Mayo, 2006), Domiati (El-Baradei et al., 2007) and Lighvan cheeses (Kafili et al., 2009). The use of a non-culture based approach was chosen to allow all bacterial components present through the cheese production to be identified although these may be in low number or may be cells not readily culturable. Initially three different primer sets were used (V3, V4-5 and V6-V8) to examine the bacterial diversity present as there have been reports of differing sensitivity of these for different bacterial groups (Lorbeg et al., 2009). However, in further analysis, only the V3 primer was used. This primer was chosen because V3 was the best target for discriminating *Lactococcus lactis* whereas, when using V4-V5, this species was detected in a triplet banding pattern which could lead to a bias in interpretation (Figure 1). It is well known that some bacteria produce more than one band because a microorganism can contain multiple rDNA copies of the target gene that are heterogeneous in sequence (Florez and Mayo, 2006; Kang *et al.*, 2010) and differences in primer target may or may not identify these regions.

Compared to Stilton cheese (Ercolini et al., 2003), the viable counts of bacterial groups of this raw milk cheese were lower on M17, MRS, Rogosa and NA agars. A diverse microbiota including *Lactobacillus, Enterococcus* and *Staphylococcus* species was culturable from the mature cheese, with *Lactococcus lactis* more readily recoverable by culture than was shown from Stilton cheese. This could reflect a differing *Lactococcus* composition between the two cheeses, as the raw milk used contained an existing population which may have differing survival properties to the added starter strains used for both cheeses. The initial mixed *Lactococcus lactis* community seen in the raw milk by DGGE analysis was quite different from the final community seen in the fully ripened cheese, suggesting a selection through processing. There were at least three bands we identified in the V4V5 DGGE profile as *Lactococcus lactis* in the starter (consistent with the three known sub-species present) and only one band of *Lactococcus lactis* we identified in the raw milk; the three *Lactococcus lactis* bands were found through the further process. However, only one band of *Lactococcus lactis* which was identified both in milk and starter culture was on the final cheese, again suggesting differing strain characteristics. From the cultured bacteria (Table 2), we identified the *Lactococcus* isolates from the raw milk and all parts of the matured cheese as *Lactococcus lactis* subsp. *lactis.* The source of the *L. lactis* subsp. *lactis* in the final mature cheese in both core+veins and rind was examined by comparison of the mature cheese isolates with isolates obtained from the starter cultures using *Sma*I PFGE. Whilst isolates from the rind showed similarity to starter cultures used in the production, particularly *Lactococcus lactis* subsp. *cremoris,* the isolates in the core and veins were quite different. Rind isolates could have been reintroduced to the surface from handling during ripening (more likely as strains matching two different starter sources were

459 evident), whereas isolates in the core and viens are more likely to have been present in
460 the milk and survived the fermentation process. This is significant as the latter may
461 have novel characteristics important for product quality and could be potential starter
462 cultures for this cheese production.

463 *Lactobacillus* species were cultured from the milk and were detected through the
464 process into the mature cheese by V3 and V4V5 amplification. However, whilst
465 *Lactobacillus plantarum* was isolated by culture and identified by DGGE analysis
466 through the entire process, *Lactobacillus curvatus* was isolated only in the raw milk and
467 was not detected through the processing stages which was an interesting finding because
468 it's presence is related to undesirable biofilm formation (Somers et al., 2001) and gas
469 production (Porcellato et al., 2015). In contrast, *Lactobacillus casei/paracasei* was
470 detected by V3 DGGE only on culture plates from the mature cheese. Thus
471 *Lactobacillus plantarum* in the matured cheese is likely to have originated from the raw
472 milk, but the source of *Lactobacillus casei/paracasei* is unclear. *Lactobacillus*
473 *plantarum* has an important role not only in flavor development (Amarita et al., 2001),
474 but has also been shown to inhibit *Listeria monocytogenes* in a smear-surface soft cheese
475 (Ennahar et al., 1998), and *Staphylococcus aureus* as well as *Salmonella* Typhimurium
476 in Montasio cheese (Stecchini et al., 1991). Its presence in both the core and the rind of
477 the mature cheese could therefore constitute an important biocontrol aspect, particularly
478 in a raw milk cheese where pathogens are not eliminated by pasteurization.

479 *Enterococcus faecalis* and *Enterococcus durans* have previously been reported in
480 cheeses (Ercolini et al., 2003; Delcenserie et al., 2014; Bulajic et al., 2015), however,
481 their presence in cheese is controversial. According to Gelsomino et al. (2001),
482 *Enterococcus faecalis* in cheese is usually assumed to be from fecal contamination and

its presence is undesirable because it can cause urinary tract infections in immune compromised patients (Coque et al., 1996). A study on enterococci in milk products (Gimenez-Pereira, 2005) revealed that enterococci strains from food are generally free from toxigenic potential in humans, which is different from enterococci strains from clinical sources, although these authors advised that the enterococcal levels in milk products should not exceed 10^7 cfu/g and good manufacturing practices were needed, specifically in the ripening period when high enterococcal levels were detected. On the other hand, this species contributes to taste and flavor development through proteolysis, lipolysis and citrate breakdown (Moreno et al., 2006). It is noticeable that we detected *Enterococcus faecalis* in the mature raw milk blue-veined cheese both by DGGE and culturing. We found no evidence of these organisms at other processing stages which suggests these populations may develop towards the end of the ripening period when the pH rises.

Staphylococcus equorum in contrast was found in the rind pre-piercing when the cheese was ripened at 21°C and introduced into the core by the piercing process, although it could only be cultured from culture plates of the rind of the mature cheese. This suggests this species is pH sensitive, as the core pre-piercing was pH 3.6 in comparison to the rind pH of 4.72 and the organism appears not to have survived exposure to this low pH, resulting in its subsequent absence from the core and vein samples of the mature cheese, even though their final pH was higher (pH 6.36). These observations support the finding that the growth of *Staphylococcus equorum* could be increased by increasing the pH from 4.0 to 6.0 and increasing the temperature from 10 to 26°C (Søndergaard and Stahnke, 2002). *Staphylococcus equorum* is a well-known starter culture in traditional sausage and ham production (Leroy et al., 2009; Landeta et

al., 2011). It could inhibit *Listeria monocytogenes* (Carnio et al., 2000) and, in Tilsit cheese, the combination of *Staphylococcus equorum* and *Debaryomyces hansenii* inhibited the growth of undesirable mold (Bockelmann, 2002). The other *Staphylococcus* found was *Staphylococcus gallinarum*. It was detected in the raw milk sample only (Table 2) indicating that this bacterium could not survive the fermentation process. *Staphylococcus gallinarum* was previously reported from chickens and a pheasant (Devriese et al., 1983) although it has been isolated from both heathy humans (Ohara-Nemoto et al., 2008) and clinical patients (Morfin-Otero et al., 2012).

In Stilton and other mold-ripened cheeses, the pH of the outer rind is usually higher than that of the inner regions indicating greater mold growth in the rind than in the core, as would be expected for these aerobic organisms. During fermentation, LAB produce acid causing the pH to drop, but as the molds grow, these break down proteins to produce ammonia and utilize the lactic acid, so the pH rises again. From our preliminary findings, the pH of the rind of three commercial Stilton cheeses was in the range 6.5 to 7.1 (unpublished data) while the pH of the raw milk cheese rind was at a higher level of pH 8.21. It was surprising therefore that the RBCA counts from the rind were lower than those of the core. This may represent a difference in the mold species growing or the extent to which they grow during the ripening period. The evaluation of yeast and fungal isolates was not part of this study but in Stilton these have been shown to be important components for final cheese characteristics (Gkatzionis et al., 2013, 2014).

We also noted that the bacterial diversity of the cheese was more complex than that reported previously for Stilton which is made with pasteurized milk (Ercolini et al., 2003); this process brings about changes in protein conformation and denaturation that

may have an impact on the species which may grow as well as removing the heat sensitive components of the microbiota. Amongst the raw milk cheese bacterial community we identified *Brevibacterium* sp., *Halomonas* sp., *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei*. These microorganisms are salt-tolerant, less acid-tolerant and have been found in the smear of surface-ripened cheeses where they contribute to the sensory characteristics produced (Rattray and Fox, 1999; Maoz et al., 2003; Mounier et al., 2005, 2007). The presence of *Halomonas* is likely to be from salt addition and these appear to have grown optimally during ripening when presumably a suitable environment had been reached. The presence of *Halomonas* sp. is considered an indicator of process hygiene (Maoz et al., 2003), however it is also found in smear ripened cheese (Mounier et al., 2005), and the influence of *Halomonas* sp. on final cheese characteristics would warrant further study. *Alkalibacterium* has previously been reported from mold-ripened cheeses with sea salt a suggested route of introduction (Ishikawa et al., 2006). The development of an alkaline pH in the mature cheese rind would explain the late isolation of this moderately halophilic alkaliphile from the mature cheese and its absence from Stilton, where the rind pH is neutral. Species of this genus produce organic acids (lactic, formic and acetic) and ethanol from glucose fermentation (Ishikawa et al., 2009) and so could be active contributors to matured cheese characteristics. However cultural isolation of this organism would require more specialist conditions than used here (Ishikawa et al., 2006), indicating the value of the non-culture based approach. *Brevibacterium* and *Corynebacterium*, although widely found in cheese production, are genera associated with the skin and may have been introduced to the cheese surface during handling of the cheese for turning. This concurs with these being found only associated with the rind. *Acinetobacter* species have been

555 isolated from raw cows' milk and raw cows' milk cheeses (Rafei et al., 2015),
556 suggesting this species was derived from the raw milk used in production.
557 All these surface-ripening bacteria were detected using different amplimers,
558 demonstrating the value of analyzing 16S regions of mixed bacterial populations with
559 more than one primer set. This could introduce a bias in the interpretation of 16S rDNA
560 sequencing results depending on the primer set used. Most metagenomics studies of
561 cheese populations using new generation sequencing focus on targeting 16S rRNA genes
562 using universal primers and so primer bias cannot be discounted, although the high
563 number of reads may be favorable for identifying species present at low levels (Bokulich
564 and Mills, 2012).
565
566 **CONCLUSIONS**
567 The bacterial composition of this raw milk blue-veined cheese was more
568 complex than that of Stilton cheese. These bacteria are likely to have originated from the
569 raw milk, the salt and handling. We also showed how the piercing process can introduce
570 surface bacteria into the core of the cheese during the ripening process, although
571 survival in the core may be dependent on the pH conditions at the time of introduction.
572 *Lactobacillus casei/paracasei*, *Staphylococcus equorum*, *Bacillus* sp., *Brevibacterium*
573 sp., *Halomonas* sp., *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei*
574 were only found by molecular methods. However, using different primers to analyze the
575 16S regions picked up different groups showing the advantage of using various primers
576 to examine mixed bacterial population. Also, culturing was still valuable as it
577 demonstrated viability od particular groups and detected a large number of raw milk

578 microbiota. It was notable that *Lactococcus lactis* subsp. *lactis* was viable in the core
579 and rind of the final cheese and some of these isolates originated from the raw milk.
580
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588
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Figures and Tables

Figure 1. Comparison of bacterial DGGE profiles of PCR amplicons of community 16S rRNA genes from samples taken at different stages of raw milk blue-veined cheese production using primer sets for three different rRNA regions: V3; V4-5; V6-8. Samples in Lane: 1 and 2, Milk; 3, Starter culture; 4, Pre-milling; 5, Post-milling and salting; 6, Pre-piercing core and veins; 7, Pre-piercing rind; 8, Post-piercing core and veins; 9, Post-piercing rind; 10, Matured cheese (core and veins); 11, Matured cheese (rind). Bands identified by excision and sequencing: a, *Lactobacillus casei/paracasei*; b, *Lactobacillus plantarum*; c, *Staphylococcus equorum*; d, *Enterococcus durans*; e, *Enterococcus faecalis*; f, *Lactococcus lactis*; g, *Brevibacterium* sp.; h, *Acinetobacter* sp.; i, *Alkalibacterium* sp.; j, *Corynebacterium casei*; k, *Bacillus* sp.; l, *Halomonas* sp. Gel running conditions are given in material and methods.

Figure 2. DGGE profiles of PCR amplicons of V3 regions of community 16S rRNA genes from samples of mature raw milk blue-veined cheese and cultured cells from culture plates. Samples in Lane: 1, Mature cheese (core and veins; direct DNA extraction); 2, Cultured cells on MRS agar from mature cheese (core and veins); 3, Cultured cells on Rogosa agar from mature cheese (core and veins); 4, Cultured cells on KFSA agar from mature cheese (core and veins); 5, Mature cheese (rind; direct DNA extraction); 6, Cultured cells on MRS agar from mature cheese (rind); 7, Cultured cells on Rogosa agar from mature cheese (rind); 8, Cultured cells on KFSA agar from mature cheese (rind); 9, *Lactococcus lactis* NCIMB 9918; 10, *Lactococcus lactis* NCIMB 8763; 11, *Lactobacillus plantarum* NCIMB 138914; 12, *Leuconostoc mesenteroides*; 13,

795 *Enterococcus faecalis* NCTC 775. Bands were identified by excision and sequencing.

796 Gel running conditions are given in material and methods.

797

798 **Figure 3.** Dendrogram and *Sma*I restriction patterns of *Lactococcus lactis*. NCIMB 6681

799 is the subspecies *lactis*, NCIMB 8662 is the subspecies *cremoris*, and NCIMB 10484 is

800 the subspecies *lactis* biovar *diacetylactis*. Clustering was performed by the UPGMA

801 method. The coefficient of similarity (40%) is indicated on the figure by the bold

802 vertical line.

803 Table 1. Microbial counts[a] at different stages of raw milk blue-veined cheese production.

Samples		pH	MRS (Lactic Acid Bacteria)	Rogosa (*Lactobacillus*)	M17 (*Lactococcus*)	BP (*Staphylococcus*)	KFSA (*Enterococcus*)	BHI (Total Aerobic Counts)	RBCA (Yeasts and Moulds)
Starter Cultures		4.47 (0.08)[b]	0.00 (0.00)	0.00 (0.00)	8.46 (0.01)	0.00 (0.00)	0.00 (0.00)	9.10 (0.05)	0.00 (0.00)
Raw Milk		6.65 (0.11)	2.76 (0.28)	0.00 (0.00)	4.00 (0.07)	2.51 (0.30)	2.18 (0.54)	4.06 (0.00)	0.00 (0.00)
Pre-Milling		4.80 (0.41)	5.26 (0.99)	0.00 (0.00)	9.00 (0.07)	4.51 (0.30)	3.84 (1.01)	9.06 (0.00)	0.00 (0.00)
Post Milling and Salting		4.80 (0.13)	7.61 (0.04)	7.58 (0.06)	8.73 (0.19)	5.88 (0.02)	7.51 (0.03)	8.72 (0.08)	4.88 (0.05)
Pre-Piercing (6 weeks)	Core	3.60 (0.77)	8.13 (0.02)	7.79 (0.23)	8.16 (0.05)	2.96 (0.46)	7.98 (0.04)	8.14 (0.01)	5.49 (0.07)
	Rind	4.72 (0.27)	8.02 (0.04)	7.97 (0.02)	11.32 (0.05)	4.83 (0.04)	7.99 (0.02)	11.43 (0.03)	5.90 (0.03)
Post Piercing (9 weeks)	Core + Veins	6.70 (0.58)	7.89 (0.06)	7.32 (0.04)	8.04 (0.04)	4.45 (0.26)	7.22 (0.05)	8.03 (0.16)	7.28 (0.01)
	Rind	6.32 (0.12)	10.70 (0.09)	7.08 (0.11)	11.00 (0.05)	10.60 (0.08)	10.68 (0.07)	11.28 (0.02)	9.99 (0.04)
Mature cheese (12 weeks)	Core + Veins	6.36 (0.24)	7.41 (0.01)	6.18 (0.04)	7.83 (0.06)	5.35 (0.06)	7.44 (0.03)	8.17 (0.21)	6.66 (0.07)
	Rind	8.21 (0.31)	6.90 (0.06)	4.85 (0.05)	8.65 (0.05)	7.03 (0.03)	7.09 (0.03)	8.92 (0.02)	4.53 (0.09)

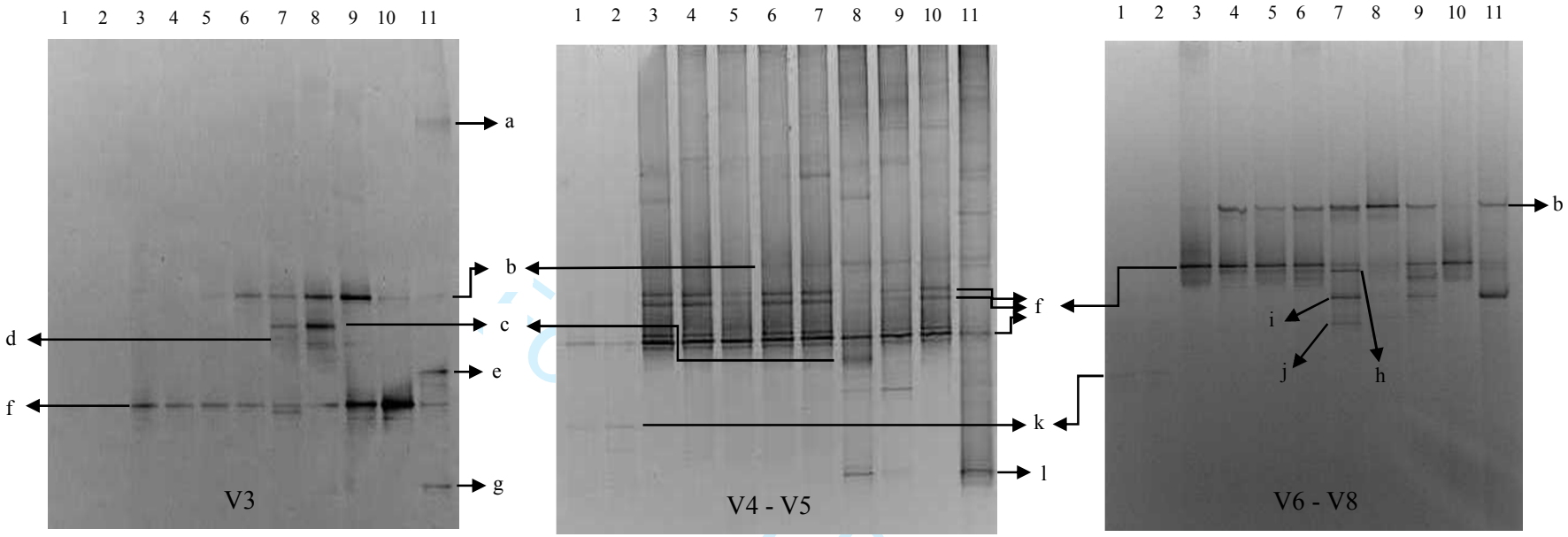
804 [a]$\log_{10}$ cfu/ml for milk and starter cultures; $\log_{10}$ cfu/g for cheese samples.

805 [b]Mean (Standard Deviation, SD).

806 Table 2. Bacteria detected in raw milk and matured raw milk blue-veined cheese using culturing methods.

Isolate identification by 16S rRNA sequencing	No. of Isolates (80)	Samples			Media (30°C)	Ability to grow at		Gram Staining	Cell Shape
		Raw Milk	Mature cheese Core and Veins	Mature cheese Rind		10°C	40°C		
Lactococcus lactis subsp. *lactis*	33	+	+	+	M17, MRS, BHI	+	+	+	Cocci, small
Lactobacillus plantarum	4	+	+	+	MRS, KFSA, Rogosa	+	-	+	Rod, small
Lactobacillus curvatus	3	+	-	-	MRS	+	+	+	Rod, big
Staphylococcus gallinarum	3	+	-	-	MRS, M17	+	-	+	Cocci, big
Staphylococcus devriesei	2	+	-	-	M17	+	+	+	Cocci, big
Microbacterium sp.	1	+	-	-	M17	+	-	+	Rod, small
Sphingobacterium sp.	3	+	-	-	M17	+	-	-	Rod, very small
Mycetocola sp.	1	+	-	-	M17	+	-	+	Cocci, very small
Brevundimonas sp.	3	+	-	-	M17	+	-	-	Rod, very small
Kocuria sp.	13	+	+	-	M17, MRS, Rogosa	+	+	+	Cocci, big
Enterococcus faecalis	13	-	+	+	KFSA, M17	+	+	+	Cocci, small
Proteus sp.	1	-	-	+	BP, M17	-	-	-	Rod, very small

807 Except for Gram staining results + : Detected , - : Not detected.


1 2 3 4 5 6 7 8 9 10 11
a
b
c
d
e
f
g
V3
1 2 3 4 5 6 7 8 9 10 11
b
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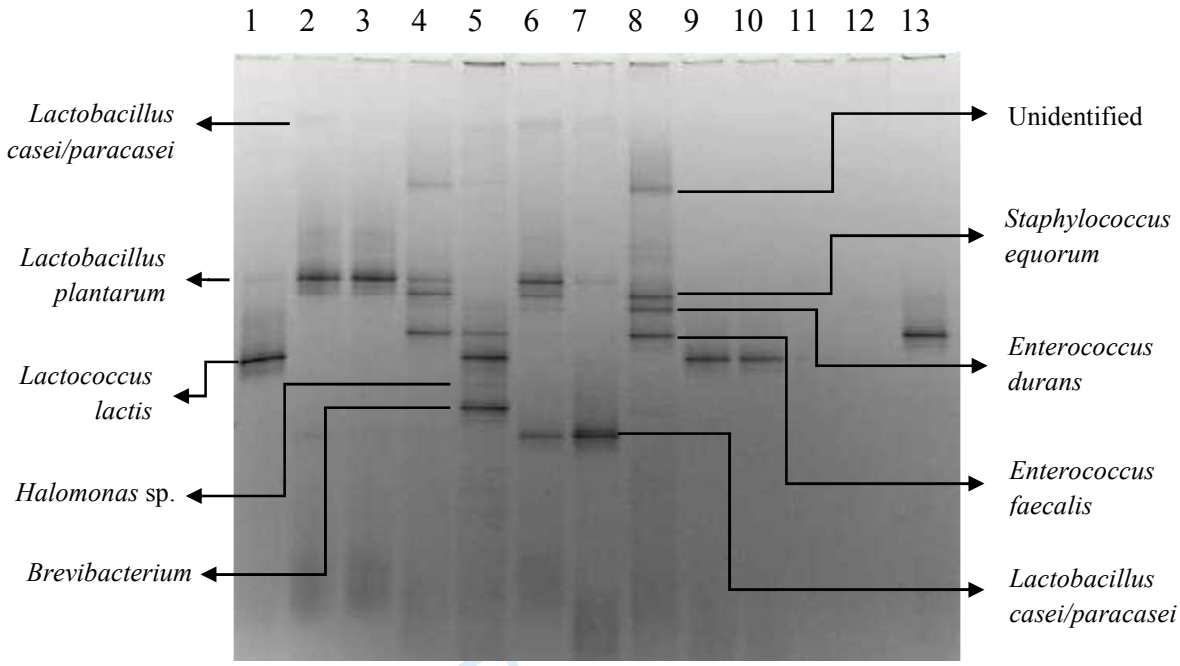
k
l
V4 - V5
1 2 3 4 5 6 7 8 9 10 11
b
f
i
j
h
V6 - V8


1 2 3 4 5 6 7 8 9 10 11 12 13
Lactobacillus casei/paracasei
Lactobacillus plantarum
Lactococcus lactis
Halomonas sp.
Brevibacterium
Unidentified
Staphylococcus equorum
Enterococcus durans
Enterococcus faecalis
Lactobacillus casei/paracasei