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

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ABSTRACT

A commercial blue-veined cheese made from unpasteurized milk was examined by conventional culturing and PCR denaturing gradient gel electrophoresis analysis of the bacterial community 16S rRNA genes using 3 primer sets, V3, V4V5, and 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* ssp. *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* ssp. *lactis*, *Lactobacillus plantarum*, and *Enterococcus faecalis* survived to the final cheese and were detected both in the core and the rind. Using PCR denaturing gradient gel electrophoresis analysis of the cheese process samples, *Staphylococcus equorum* and *Enterococcus durans* were found in the rind of prepiercing 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 denaturing gradient gel electrophoresis and not cultured from the samples. *Brevibacterium* sp. was initially identified in the cheese postpiercing (core and veins), *Halomonas* sp. was found in the matured cheese (rind), and *Acinetobacter* sp., *Alkalibacterium* sp., and *Corynebacterium casei* were also found in the prepiercing samples (rind) and then found through the subsequent process stages. The work suggests that in this raw milk cheese, a limited community from the milk survive to 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 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, consisting of *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis*. Both cheeses also contain a starter *Penicillium roqueforti* mold that 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 that 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 nonstarter 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, no work has been published on raw milk blue-veined cheese originally from the UK. The nonstarter microbiota may differ

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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 among cheese makers on the use of raw or pasteurized milks for cheesemaking. The risks and benefits for traditional cheeses, mainly raw milk cheeses, have 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 *Salmonella*, *Campylobacter*, *Brucella*, Shiga-toxin producing *Escherichia coli*, *Listeria monocytogenes*, and *Shigella* found in cheeses (Gould et al., 2014). Pasteurization is usually used to ensure the safety of milk and is the reason why Stilton cheesemakers use pasteurized milk. However, for raw milk cheesemakers, pasteurization kills desirable bacteria and destroys enzymes and proteins, resulting in a less tasty cheese. The safety risks of raw milk can be addressed, however, as fermentation should kill pathogenic bacteria if it is done properly. Thus, the main concern of traditional cheese producers is to preserve microbial diversity and exploit its benefits.

Therefore, in the current study, we analyzed the bacterial diversity during raw milk blue-veined cheese production using a traditional culture approach and 16S rRNA gene PCR denaturing gradient gel electrophoresis (**DGGE**) techniques to evaluate the bacteria that contribute to production of the characteristics of the product. The presence of *Lactococcus lactis* at the end of production has also been examined by pulsed-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 needed to allow cheesemakers to create 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 to 30°C, adding starter cultures (*Lactococcus lactis* and *Penicillium roqueforti*), and 90 min later adding rennet. The mixture is fermented at around 30 to 40°C. After 6 h, the curds form and are cut by hand into small cubes to release the whey. The soft curds are transferred manually by ladles into a second tank and left overnight. The curds are milled, salted, and poured into plastic cylindrical hoops without pressing. Cheeses are then left in the hastener (21°C) and turned daily for 5 d 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 (13°C, relative humidity = 85%), and 6 wk later fresh cheese is pierced by stainless steel needles to allow development of the blue veins. Finally, after around another 6 wk (13°C, relative humidity = 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 premilling, postmilling and salting, prepiercing (6 wk), postpiercing (9 wk), and fully ripened cheese (12 wk). 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 2 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, diluted in quarter-strength Ringer's (225 mL; Oxoid, Hampshire, UK), and homogenized in a stomacher at 230 rpm for 2 min (Stomacher 400 Circulator, Seward, West Sussex, UK). These samples were considered as the 10^{-1} dilution and were further diluted to 10^{-8} by 10-fold 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 nonselective 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 d, whereas yeasts and molds were grown at 25°C for 5 d. The 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., 2004).

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