Microbiological and chemical profiles of dairy farm red smear cheese made from pasteurized and un-pasteurized milk

Hansen, T.B.[1*], Larsen, L.B.[2], Hammershøj, M.[2], Nielsen, J.H.[2], Højgaard, K.S.[1] and Knøchel, S.[1]

[1]Department of Food Science, Faculty of Life Sciences, Copenhagen University, Rolighedsvej 30, DK-1958 Frederiksberg C., Denmark.

[2]Department of Food Science, Faculty of Agricultural Sciences, University of Aarhus, PO Box 50, DK-8830 Tjele, Denmark.

*Now affiliated with the National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.

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Running headline: Profiles of dairy farm red smear cheese

Abstract

A red-smear soft cheese was produced four times during a year at an organic dairy farm using pasteurized and un-pasteurized milk, respectively, from the same milking. A commercial starter culture was added. The cheeses were characterized microbiologically and chemically in order to study how heat treatment and season affected their characteristics during cheese making and ripening. Large variations between the different lots of cheeses characterized the production. However, the cheeses made from un-pasteurized milk generally had a higher lactic acid bacteria count except in cases, where the pasteurized milk was recontaminated or if acidification failure took place. Delays in acidification caused a more pronounced increase in numbers of *E.coli*, *Enterobacteriaceae* and staphylococci, as well as an increase in the plasmin and plasminogen-derived activities. A number of pre-milking and process steps were identified as important for the microbiological contamination and development in the cheeses.

1. Introduction

Although by far most cheeses in Europe are made from pasteurized milk, several traditional cheeses are made using un-pasteurized milk, so-called “raw milk cheeses”. Manufacture of these cheeses ranges from hard to fresh cheeses and from industrial scale production to the level of small farms. While pasteurization of the cheese milk has been a tremendous benefit from a food hygiene point of view, it has been claimed that it often results in less characteristic and more bland cheeses (Grappin and Beuvier 1997). Depending on the cheese type these differences may be more or less pronounced. Pasteurization will affect, not only the natural beneficial microbial flora, but also pathogenic contaminants, indigenous enzymes, and the flavour components from the raw milk, all of which contribute to the safety and quality of the cheese. While hard, long-time ripened cheeses such as Parmesan or Emmenthaler type made from un-pasteurized milk have not been associated with outbreaks of food-borne diseases, soft or unripened cheeses have been involved in a number of

cases. If the pathogens are introduced with the milk, many potentially pathogenic bacteria incl. *Salmonella* spp., *E. coli, Listeria monocytogenes*, and *Staphylococcus aureus* may survive and grow to hazardous levels during the production of soft cheeses (Johnson, Nelson and Johnson, 1990; Donnelly 2001).

In Denmark, the production of cheese based on un-pasteurized milk is subject to a very restrictive policy owing to the potential problems with microbiological safety. In order to optimize the microbiological safety of cheeses manufactured from raw milk, a number of control points in the production from raw material to the stored product must be established. The present study was undertaken to identify factors of importance for the microbiological and chemical development in red-smear cheeses made from raw or pasteurized milk. This was done by investigating parallel productions of soft surface-ripened, Münster type cheeses made from pasteurized and un-pasteurized milk, respectively, in a Danish farm dairy during several seasons.

2. Materials and methods

2.1. Cheese manufacture

Red-smear, soft, Münster-type cheese was used as test material. Cheeses made from un-pasteurized (U) and pasteurized milk (P), respectively, from the same milking were produced at an organic farm dairy in Denmark. The herd consisted of 130 piebald Holstein-Friesian dairy cattle. During a one-year-period four repetitions of the experiment were conducted; two during autumn and spring (September 2002 (test production 1), and May 2003 (test production 3)) where the dairy cows stayed outside grazing on pasture, and two in late autumn and winter (December 2002 (test production 2) and October 2003 (test production 4)), where the cows were inside a modern free stall barn with deep-bedding and were partly supplemented with silage.

For each test production 200 L of raw or pasteurized whole milk was pumped into 500-L-vats. The first lot was made with pasteurized milk, where after the raw milk was transferred through the unheated plate pasteurizer for a separate production. The pH of the milk was measured before addition of starter culture. After 19 ± 12 min at 32°C, 10 g starter culture (F-DVS *Lactococcus diacetylactis* MB-1, Chr. Hansen A/S, Hørsholm, Denmark) per 100 L was added. Approximately 73 ± 25 min after the addition of starter 30 mL rennet was added per 100 L milk and coagulation elapsed for 94 ± 8 min at 30°C. After curdling, the curd was cut (10 mm) into small pieces and stirred at 15-minute-intervals with a stainless steel stirrer for a total of 62 ± 11 min. The curd was manually distributed into 10-cm-diameter moulds with a stainless steel shovel and left to drain at approx. 25°C. After approx. 2 h the cheeses were turned, covered with a plastic shield and left for maturation and further drainage over night at room temperature. The next day the cheeses were removed from the moulds, placed on grids and manually sprinkled on the surface with grained salt. After 24 h the cheeses were smeared with a commercial red-smear culture (OFR 9, Danisco Cultor, Denmark) mixed 1:1000 with buttermilk. The smeared cheeses were placed at 16°C in a maturation room. After 7 d, each cheese was placed on parchment paper in a small wooden barrel and covered with cellophane. The cheeses were then distributed with half of the lots to Department of Food Science, Faculty of Life Sciences at University of Copenhagen for microbiological analysis, and the other half of the lots to Department of Food Science, Faculty of Agricultural Sciences at University of Aarhus for chemical and textural analyses, respectively. For the next 7 d the cheeses were ripened at 8°C and afterwards kept at 4°C for the rest of the storage period. A minimum of four duplicate samples of cheeses made from raw and pasteurized milk, respectively, was taken during the 6-week storage period of each experiment. The cheeses were analysed for the following compositional parameters in fresh and ripened (20 d) cheeses prepared from raw or pasteurised milk at Steins Laboratory (Holstebro, Denmark): Moisture (%), fat content (%), water in fat free cheese

(%), salt content (%) and average salt-in-water (%) calculated as (salt % / (salt % + moisture %)) x 100%.

2.2. Microbiological analyses

Sampling

Samples were taken of raw as well as pasteurized milk. During milking, the raw milk was continuously pumped through pipes into a cooling tank placed in adjacent dairy. All milk samples were taken during the process of filling the cheese vats and were collected in sterilized glass bottles. The temperature of the milk at the time of sampling was 30-32°C. Samples were immediately placed in a refrigerator and kept cooled down until analysed. Duplicate milk samples were manually shaken before diluted serially 10-fold in physiological saline (0.9 % NaCl; Merck, Darmstadt, Germany) with 0.1 % added Bacto-peptone (Difco, Detroit, USA) (PSP).

Cheese samples were taken from fresh cheese curd (before salting and smearing) as well as from the ripening cheese throughout the storage period. Samples were taken from the core of the cheeses in order to avoid the surface smear. A cut through the centre of the cheese was performed with a flamed cylindrical cutter. The rind in both ends was discarded and 10 g of cheese curd was aseptically transferred to filter bags, diluted 10-fold in PSP and homogenized (Stomacher 400, Seward, London, England) at low speed for 4 min., before further 10-fold serial dilution in PSP.

Environmental samples were taken from teat ends and teat cups. Sampling of these areas was done by a swab technique using sterile sampling sponges moistened with 10 mL buffered peptone water (Solar Biologicals Inc., Ogdensburg, New York). Swabs from the four teat ends on a cow were pooled. Cows showing symptoms of mastitis were omitted. Teat ends were sampled before and after a pre-milking teat treatment, which consisted of wiping the teats visually clean with a clean, wetted

cotton cloth. Teat cups were sampled by swabbing the top surface of the four ends, which had contact with the udder during milking, as a pooled sample. Teat cups were sampled before and after removal of faecal material in the milking area by the use of pressure cleaning. A total of 27 pooled teat end samples and 20 pooled teat cup samples were taken. The samples were kept refrigerated (max. 10°C) until analysis, where they were aseptically added 90 mL tryptone soya broth (Difco, Detroit, USA) (TSB) and manually shaken for 20 s before further analysis.

Surface spread-platings were performed by aseptically spreading 0.1 mL of appropriate dilutions on various indicative and/or selective agars.

Aerobic counts incl. spores

Swab samples were spread on Tryptic Soy Agar (TSA; Scharlau Chemie S.A., Barcelona, Spain), incubated aerobically at 30°C for 2 d to determine mesophilic aerobic counts. For milk and swab samples, mesophilic aerobic spore counts were determined on TSA (30°C, 2 d) after spore activation at 80°C for 10 min. Additionally, for milk and cheese the aerobic, mesophilic count incl. lactic acid bacteria , LAB, was determined on Plate Count Agar (Oxoid, Hampshire, UK) modified by adding 1 g L^{-1} skim milk powder (Oxoid, Hampshire, UK) (PCAM) and incubated aerobically at 30°C for 2 d.

Faecal counts

Total count on Xylose Lysine Desoxycholate agar (XLD; Oxoid, Hampshire, UK) incubated aerobically at 35°C for 2 d was used as indicator for the concentration of *Enterobacteriaceae*. Total count on Eosin Methylene Blue agar (EMB; Oxoid, Hampshire, UK) incubated aerobically at 35°C for 2 d was used as another indicator for the concentration of *Enterobacteriaceae,* whereas the number of greenish metallic sheen colonies with dark purple centres represented the concentration

of *Escherichia coli.* The number of purple-red colonies on Violet Red Bile agar (VRB; Oxoid, Hampshire, UK) incubated aerobically at 44°C for 24 h was used as an indicator for the concentration of faecal *E. coli*.

LAB counts

Lactococci were counted on M17 agar (M17; Oxoid, Hampshire, UK) incubated aerobically at 30°C for 24 h. Total count on De Man, Rogosa, Sharpe agar (Merck, Darmstadt, Germany), modified by lowering of pH to 5.5 with HCl (MRS), incubated at 37°C for 3 d in anaerobic jars with Anaerocult A kits (Merck, Darmstadt, Germany) was used as an indicator for the *Lactobacillus* spp. count. The concentration of lactobacilli was also determined as total count on Rogosa agar (Rogo; Oxoid, Hampshire, UK) incubated anaerobically at 30°C for 5 d.

Pathogenic bacteria counts

The number of grey-black colonies (1-3 mm diameter) on Baird Parker agar (BP; Oxoid, Hampshire, UK) incubated aerobically at 35 for 2 d was used as a measure for presumptive *Staphylococcus* spp. *Listeria* spp. were counted on Listeria Selective Agar (OX; Oxoid, Hampshire, UK) after aerobic incubation at 35°C for 2 d. Furthermore, 80 cheese samples (25 g core and rind) were analysed for the presence of *Salmonella*, *Listeria monocytogenes* and *E. coli* by the following enrichments procedures. Rappaport-Vassiliadis (RV) Enrichment Broth (Oxoid, Hampshire, UK) was used for the detection of *Salmonella*. A modification of the method described by Oxoid Limited (2006) was performed, *i.e.* TSB substituted buffered peptone water for the resuscitation and XLD substituted Brilliant Green Agar for identification of typical *Salmonella* colonies. *L. monocytogenes* was detected by the two step selective enrichment procedure using Listeria Selective Enrichment Media (UVM formulations) (Oxoid, Hampshire, UK) as described by Oxoid Limited (2006) with the exception of surface plating from the primary broth after 4 h incubation. For detection of *E. coli*

the enrichment procedure in Buffered glucose - Brilliant Green-bile broth (EE Broth; Oxoid, Hampshire, UK) for thermotrophic *Enterobacteriaceae*, described by Oxoid Limited (2006), was applied. *E. coli* was identified as greenish metallic sheen colonies with dark purple centres on EMBA or as purple-red colonies on VRB.

2.3. Chemical analyses

Preparation of cheese extracts

Cheese extracts were prepared in duplicate from 5 individual cheeses (U or P, respectively) after storage at 20, 30, 40 and 44 days. The samples were excised as cylinders from the cheeses by using a cork borer 0.5 cm diameter from the edge at two opposite sites of each cheese, and the samples (3-5 g) were weighed. The cheese extracts were prepared essentially as described (Benfeldt and Sorensen 2001) with the following modifications. Grated cheese was mixed with 2 mL 0.5 trisodium citrate, 15 mM ε-amino-n-caproic acid, per g of cheese, and after stirring at the samples were centrifuged (4°C) at 4000 x g at for 10 min to remove the fat, followed by 13.000 x g for 30 min to remove insoluble cheese and remnants of fat. The supernatant was recovered and stored at -25°C until further analysis. These supernatants were used for determination of plasmin and plasminogen, while HPLC peptide profiling were carried out on pH 4.6 soluble peptides after acidification of supernatant to pH 4.6 by addition of 25 % (v/v) acetic acid followed by centrifugation at 13.000 x g at 4°C for 10 min. The acid supernatants were stored at -25°C until further analysis.

Plasmin activity of cheese extracts

Plasmin and plasminogen-derived activities were determined in citrate cheese extracts from fresh and stored cheeses by a modification of the method described by Korycka-Dahl et al. (1983) using

the chromogenic substrate S-2403 (pyroGlu-Phe-Lys-pNA-HCl) (Haemochrom Diagnostica AB, DK-2000 Frederiksberg, Denmark). Enzyme activities were determined in thawed extracts diluted 1:10 in PBS-EDTA (50 mM NaH_2PO_4, 100 mM NaCl, 10 mM EDTA, pH 7.4). For determination of plasmin activities, wells contained 100 µL PBS-EDTA, 50 µL diluted cheese extract and 50 µL 5.6 mM substrate solution. Total plasmin plus plasminogen-derived activity was determined after preactivation of plasminogen by addition of 8 µL of a 0.01 µg/µL solution of urokinase in PBS-EDTA corresponding to 8 Plough units of urokinase (Calbiochem, San Diego, CA) to 200 µL diluted cheese extract. The reaction mixture was incubated at 37°C for 20 min. For determination of total plasmin plus plasminogen activities, wells contained 100 µL PBS-EDTA, 50 µL diluted preactivated cheese axtract and 50 µL 5.6 mM substrate solution. Substrate cleavage was determined by immediate reading of the UV-absorbance at 405 nm each minute at 37°C for 45 minutes after addition of the substrate and mixing of the reaction mixture. Rate of pNA formation was calculated from the linear portion of the absorbance versus time curve. Enzyme activity was expressed as ΔA_{405} min^{-1} mL^{-1} extract, and represents the net activity measurable in the citrate cheese extracts.

Peptide analysis by HPLC

Peptide profile of pH 4.6 soluble peptides from cheese extracts was monitored by reversed phase HPLC analysis of samples of soluble peptides prepared from fresh cheese and stored cheeses from test production 2. The extracts were analysed in duplicate. The HPLC analysis was performed on a HP 1100 Series system equipped with a variable wavelength G1314A detector (Agilent, Waldbronn, Germany). A Vydac C_{18}-column (4.6 x 250 mm, 300 Å, 5 µM, Agilent) was used for separation using the following chromatographic conditions: Solvent A was 0.1 % (v/v) tri-fluoro acetic acid (spectroscopy grade, Merck KgaA, Darmstadt, Germany) in deionised water. Solvent B

was 80 % acetonitrile (HPLC grade S, Rathburn, Scotland), 0.1 % (v/) TFA in deionised water. Samples were filtered through 0.45 μm 13 mm CE filters (Frisenette, Ebeltoft, Denmark) before application of 100 μL to the column, and eluted at a flow rate of 1 mL min^{-1} by a gradient of 0-80 % B in solvent A for 90 min. The effluent was monitored at 214 nm. For statistical analysis each chromatogram was divided into 2 elution time intervals and integrated according to the following discrimination: hydrophilic peptides 10-19 min and hydrophobic peptides 30-40 min. The intermediate part of the chromatogram (20-29 min) was not included in the further data analysis.

Extraction and detection of volatiles

The extraction and detection of volatiles from the cheeses was carried out as described by Kristensen, Hedegaard, Nielsen & Skibsted (2004), with minor modifications. Phenol and dimethyldisulphide (DMDS) accumulated in the stored cheeses were analysed by solid phase micro extraction (SPME) in combination with gas chromatography flame ionisation detection (GC-FID) from Hewlett-Packard Co (Palo Alto CA 94304, USA). Cheese (2.00 g) was filled in a 20 mL headspace vial cleaned by a gas-flame and with a glass coated stirring bar. A SPME fiber (Carboxen-PDMS, 75 µm) from Supelco (Bellefonte PA 16823-0048, USA) was inserted in the headspace of the vial, and equilibrated for 45 minutes under temperature control at 45°C**.** Analyses of the volatiles absorbed to the SPME fibre were carried out by a GC/MS-system from Varian GC 3400 in combination with a Saturn 2000 MS system using an HP-5 column (5% phenyl methyl siloxane, 30 m x 250 µm x 0.25 µm) and helium as carrier gas. Injection was performed in splitless mode with an injector temperature of 250°C, and the volatile compounds were desorbed from the SPME fibre for 5 minutes. The initial column temperature was 35°C, which was held for 1 minute and then raised with 10°C per minute to 150°C and held for 1 minute. Then the temperature was raised again to 250°C with 30°C per minute and held for 1 minute to a total run-time of 18 min. Analysis was performed by MS analysis based on library and mass spectra of external standards.

Texture analysis

The cheese texture was analysed by a uniaxial penetration test after equilibrating the cheese temperature to 20°C. A spherical stainless steel probe with Ø = 6.35 mm (1/4 inch) was used in a TA-HD*i* Texture Analyzer (Stable Micro Systems Ltd., Surrey, England) with a 100 kg load cell, 1 mN detection range, and penetration speed of 2.0 mm s^{-1}. The penetration depth was 15.0 mm vertical to the flat end of the cheese resulting in fracture of the surface and penetration into the inner cheese part. Recordings of force (N) and displacement (mm) data were obtained by 250 points per second (PPS) throughout the penetration analysis. Post penetration and surface fracture, the

modulus of the cheese texture was calculated as the maximum gradient of force per distance (N mm^{-1}), i.e. soft cheeses have high numeric values of the modulus and vice versa. In total, 5 replicates per cheese were obtained.

2.4. Data analyses

When analyses of variance were performed on microbiological data the $\log_{10}$ of bacterial counts were used as response variables. The procedure of generalized linear models (proc GLM) in SAS® 9.1 (SAS Institute Inc., Cary, NC, USA) was used for all computations. In the case of teat end samples, season and pre-milking treatment were tested as fixed effects in analysis of variance. For the samples collected from teat cups, pressure cleaning of the floor in the milking area was tested as fixed effect in a second analysis of variance. For cheese samples from test production 1, 2 and 4 the counts of lactococci (measured on M17) were response variable in a covariate analysis with storage time as the covariate, test production as blocks and pasteurization as fixed effect.

Data from chemical and microbiological analyses were evaluated by principal component analysis (PCA) on standardised variables (mean=0, SD=1) variables using the multivariate statistical software package Unscrambler ver. 9.2 (Camo Process AS, Oslo, Norway). PCA reduces the number of variables in a multidimensional data set to its most dominant features and retains a set of principal components (PCs) that capture the most related variation. The loadings represent coefficients of the PCs and reflect both how much the original variables contribute to PCs and how well PCs take into account a variable's variation over the data points. The scores are composed of the weightings for each PC and reflect the location of samples along the PCs and show sample differences or similarities. Parameters located close to each other are positively correlated, while parameters located opposite each other are negatively related. The parameters located close to (0,0) contribute less to explain the variation in the samples. The following parameters were included

representing different categories of parameters, including pH parameters: pH in milk (pH milk), pH min, drop in pH after 10 h acidification (delta pH 10), texture, pH after 24 h (pH 24h), pH cheese (pH in cheese); cheese parameters: fat % in fresh cheese (fat % start), water % in fresh cheese (water % start), calculated average salt % in water on fresh cheese (salt-in-water%); chemical parameters: plasmin activity (plasmin), plasminogen-derived activity (plasminogen), hydrophilic peptides (hydrophil. pep.), hydrophobic peptides (hydrophob. pep.), DMDS, phenol; microbiological parameters: see below.

Multivariate regression using Partial Least Square regression analysis (PLS1) was carried out using the same software, Unscrambler ver. 9.2. The response variables (Y-variables) analyzed were level of faecal *E. coli* in 20-day-old cheeses. The X-variables consisted of the 17 continuous variables belonging to one of three categories. One category described the influence of initial bacterial counts in cheese milk; a second category described the effect of different acidification parameters and a third category described the significance of various water activity measures. Initial counts (log_{10} CFU g^{-1}) of lactococci (M17 milk), lactobacilli (Rogo milk, MRS milk), staphylococci (BP milk), *Enterobacteriaceae* (XLD milk, EMBA milk) and faecal *E. coli* (VRB44 milk) were included. The following acidification parameters were used; pH in acidified cheese curd after 10 h (pH 10h), 24 h (pH 24h) and 2 d (pH 2d), drop in pH after 10 h acidification (delta pH 10h) and between 10 and 24 h acidification (delta pH 10-24h) as well as log_{10} cfu g^{-1} of starter culture in 2-day-old cheeses (M 17 2d). The water activity measures included were; initial moisture content (%) in cheeses (moisture%), initial fat content (%) in cheeses (fat%), water content (%) in fat free cheeses (water% fat free) and salt content (%) in the water phase of cheeses (salt-in-water%).

3. Results

3.1. Milking hygiene

During the four observation periods the milking area, as well as the cows, were visually most clean when the cattle was grazing. Visibly dirty hind legs were seen in 25% of the cows in grazing periods compared with 46% when the cattle were in deep straw-bedding.

Washing the teats with wetted cotton cloths followed by air-drying before milking reduced the mesophilic, aerobic count and the staphylococci on teat ends significantly ($P \leq 0.031$) with *approx.* 0.5 $\log_{10}$ CFU per cow (results not shown). The effect was even more pronounced for mesophilic, aerobic spores which were reduced from 4.1 to 3.1 $\log_{10}$ CFU per cow ($P = 0.009$). With regard to *Enterobacteriaceae* the teat washing decreased the prevalence significantly (chi-square test; $P = 0.019$) from 100% to 29% during the grazing period whereas it increased significantly ($P = 0.031$) from 67% to 100% (results not shown) in the deep bedding period. Independent of pre-milking teat treatment or type of stable, the *Enterobacteriaceae* count was *approx.* 3.5 $\log_{10}$ CFU per cow when teats were detected positive for *Enterobacteriaceae* (results not shown).

Pressure cleaning of the floor in the milking area during milking increased the faecal contamination on the outside of the teat cups considerably, whereas no effect was observed for non-faecal counts. The prevalence of faecal *E. coli* on the outside of the teat cups increased from 57% to 100% as a result of pressure cleaning, whereas the concentration of faecal *E. coli* on positive teat cups was independent on pressure cleaning ($P = 0.77$). Faecal *E. coli* counts on positive teat cups averaged 3.9 $\log_{10}$ CFU per set of cups (S.D. = 0.69). Species of *Enterobacteriaceae* were found on 17 of a total of 20 sets of cups investigated and the average concentration on positive cups increased from 3.7 to 4.4 $\log_{10}$ CFU per set of cups as a result of pressure cleaning in the milking area ($P = 0.070$).

The mesophilic, aerobic count on washed teats averaged 6.4 $\log_{10}$ CFU per cow in the winter (deep bedding stable) while this count was 1.0 $\log_{10}$ CFU lower per cow in the summer. The same tendency was found for staphylococci, whereas the mesophilic, aerobic spore count was significantly higher (P =0.034) on washed teat ends from cows that were grazing (results not shown). The prevalence of faecal *E. coli* on washed teats was lower in the grazing period (40%) compared to periods inside the deep bedding stable (73%).

The relationship between the bacterial counts on the teat cups was linearly correlated (R^2 = 0.865; P < 0.001) to the bacterial counts found on the teat ends (results not shown). The following formula described the relationship:

$$y = 1.028 + 0.868 \cdot x \qquad (1)$$

y is the average bacterial count on teat cups ($\log_{10}$ CFU per set) and x is average bacterial count on washed teat ends ($\log_{10}$ CFU per cow).

3.2. Milk

In general, there was a good correlation between bacterial counts on teat cups and bacterial counts in the raw milk (Fig 1).

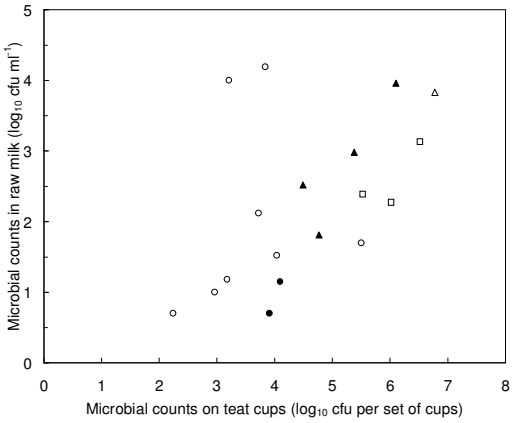

Figure 1. Relationship between various bacterial counts found on teat cups and pooled raw milk samples from the same milking. ○ faecal bacteria, ● aerobic spore counts, △ mesophilic, aerobic counts measured on TSA, ▲ lactic acid bacteria (LAB), □ staphylococci.

There were two outliers from lots 2 and 3 with much higher level of faecal bacteria in the raw milk than expected based on the faecal counts found on the teat cups. This was most likely a result of contamination from dirty dead-ends in the dairy.

The average bacterial counts in raw milk were from 0.3 to 1.3 $\log_{10}$ CFU mL^{-1} higher in raw milk originating from cows in stables with deep straw-bedding compared to grazing cows except for spore counts and counts measured on EMBA (Table 1).

Table 1. Bacterial counts ($\log_{10}$ CFU mL^{-1}) in raw milk from cows with different stable conditions and after pasteurization.

Microbial parameter	Bacterial group[a]	Raw milk[b]		Pasteurized milk[c]		
		Pasture[d]	Deep straw-bedding[e]	Average	Minimum[f]	Maximum[g]
Faecal counts	*Enterobacteriaceae* on XLD	1.42 ± 0.50	1.82 ± 0.36	1.48 ± 1.45	<1.0	3.87[h]
	Enterobacteriaceae on EMBA	4.19 ± 0.01	2.73 ± 0.78	2.40 ± 1.33	1.00	4.10[h]
	Escherichia coli on EMBA	2.15 ± 0.21	2.07 ± 0.30	2.26 ± 1.52	<1.0	4.10[h]
	Faecal *E. coli* on VRB44	1.00 ± 0.00	2.35 ± 1.91	0.80 ± 0.15	<1.0	1.00
LAB counts	Lactococci on M17	3.47 ± 0.57	3.94 ± 0.54	3.03 ± 1.24	1.30	4.34
	Lactobacilli on MRS	2.54 ± 0.40	3.39 ± 0.61	1.74 ± 0.92	<1.0	2.75
	Lactobacilli on Rogo	1.67 ± 0.25	2.26 ± 0.32	1.15 ± 0.67	<1.0	2.21
	Lactic acid bacteria on PCAM	2.97 ± 0.02	3.57 ± 0.19	2.59 ± 1.13	<1.0	3.92
Other non-LAB counts	Staphylococci on BP	2.39 ± 0.03	2.70 ± 0.50	1.41 ± 0.65	<1.0	2.38
	Total count on OX	1.34 ± 0.62	1.79 ± 0.20	<1.0	<1.0	<1.0

Spore counts	Mesophilic, aerobic spores	1.15 ± 0.21	<1.0	N.D.[h]	N.D.	N.D.

[a] See Materials & methods for detailed information on agar and incubation conditions
[b] Average and standard deviation of two to four observations
[c] Average and standard deviation of six to eight observations
[d] Results from test productions 1 and 3
[e] Results from test productions 2 and 4
[f] Results from test production 4
[g] Results from test production 2
[h] N.D.: Not determined

Although a test for alkaline phosphatase activity of the pasteurized milk showed at least a 4 $\log_{10}$ reduction of this enzyme, indicating a proper pasteurization (data not shown), counts of up to 3 $\log_{10}$ were encountered immediately after pasteurization. This indicates some degree of recontamination and it appeared to be worst in lot 2 as all maximum bacteriological counts were found in this production.

3.3. Cheese

Figure 2 shows the result of multivariate principal component analysis (PCA) of the bacteriological data collected from milk, and fresh (2 d) or ripened (20 d) cheeses.

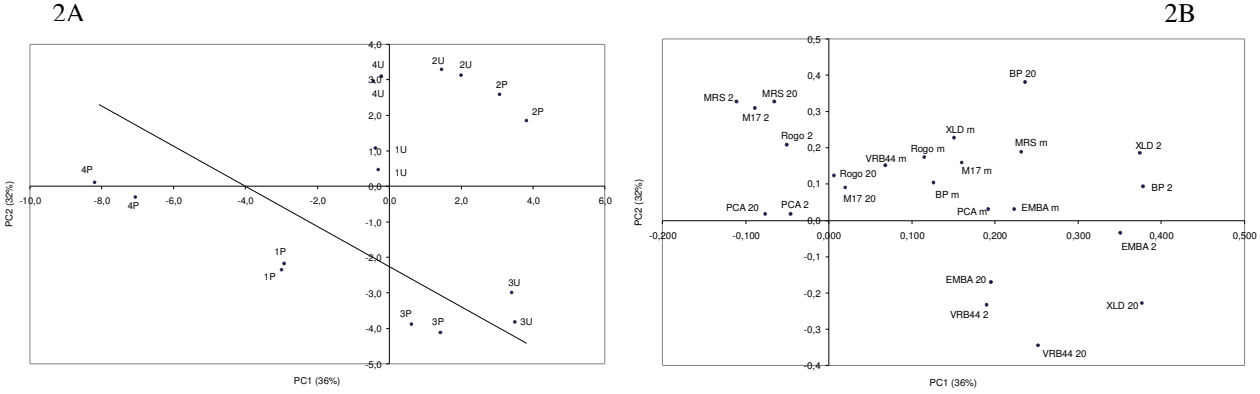

Figure 2. Principal component analysis of bacteriological counts from milk, fresh (2 d) or ripened (20 d) soft, surface-smeared cheeses manufactured from un-pasteurized or pasteurised milk (m). A) Score plot showing the groupings of samples as indicated by the straight line, and B) loading plot showing the relations of bacteriological parameters. The numbers (1, 2, 3 and 4) and the designations U or P in A indicate test productions and cheeses prepared from un-pasteurized (U) or pasteurized (P) milk, respectively. In B, m, 2 and 20 indicate measures from milk, 2 or 20 days old cheeses, respectively. For further description of variables in B), see materials and methods.

The first two PC's represented 76% of the variation in the data, with 48% explained by the first PC, and 28% by the second PC. The PCA scores plot (Fig 2A) shows some separation of the U and P samples, indicating that microbiological differences between raw or pasteurized milk and cheese could be observed. In test productions 1, 3, and 4 the pasteurized productions were in the lower part of the plot, whereas the raw productions grouped to the upper right side of the plot. The largest differences between raw and pasteurized milk or cheese were observed in test production 4. This particular production was characterized by being made after a long production stop, and the processing equipment was therefore exceptionally clean and recontamination limited. A further distinction between raw and pasteurized cheeses was that the numbers of lactic acid bacteria were higher in the raw milk cheese compared with the cheese from pasteurized milk. Very late in the storage period, from storage day 20 to 55, we found *approx.* 10^8 CFU g^{-1} of a non-starter LAB, identified as *Lactobacillus plantarum*, in the cheeses made from raw milk (results not shown). The test production 2 did not separate according to this pattern since both U and P, although separate, grouped together with other raw productions. This group (2 U and 2 P) in the upper right corner was primarily associated with high initial counts in the milk (Fig 2B). An explanation for this could be the heavy recontamination of the pasteurized milk (Table 1) observed in this particular test production, which also corresponded to a visibly less clean equipment and the dead-end close to the vat sampled. Furthermore, in this production the counts of enterobacteria were markedly higher in the pasteurized milk as compared with the raw milk indicating heavy recontamination.

Looking specifically at the loadings (Fig 2B) representing the bacteriological counts in the cheeses, it was observed that the LAB counts at day 20 (M17 20, MRS 20, Rogo 20) were placed in the upper half of the plot. Statistical analysis confirmed that raw milk cheeses had significantly ($P <$ 0.001) higher lactococcal counts throughout the ripening period compared with cheeses made from

pasteurized milk. The difference varied between 0.08 – 0.92 $\log_{10}$ CFU g^{-1} and was 0.5 $\log_{10}$ CFU g^{-1} on average.

Counts of bacteria of faecal origin in the cheeses were placed in the right side of the plot showing that lots 2 and 3 had the highest counts of faecal bacteria in the cheeses. Average count of *Enterobacteriaceae* (XLD) was 6.5 $\log_{10}$ CFU g^{-1} (S.D. = 0.88) in ripened cheeses from these lots as compared to 4.0 $\log_{10}$ CFU g^{-1} (S.D. = 1.05) for lots 1 and 4. In addition, the LAB count, 2 d after manufacture (M17 2, MRS 2, Rogo 2) (Fig 2B) which reflects the starter culture, was negatively correlated to the faecal count in the fresh cheese curd (VRB44 2, XLD 2, EMBA 2) as well as the 20-day-old cheeses (VRB44 20, XLD 20, EMBA 20). This indicates that the level of *Enterobacteriaceae* in cheeses is strongly dependent on the activity of the starter culture during the acidification step. Figure 3 presents two different acidification profiles observed. Where no measurable increase of the starter culture was observed during acidification, a $\log_{10}$-increase of up to approx. 4.5 for *Enterobacteriaceae* and 3.5 for faecal *E. coli* was seen (Fig 3). When there was an increase of more than 2 $\log_{10}$ of the starter culture during acidification, the $\log_{10}$-increase of *Enterobacteriaceae* and faecal *E. coli* was reduced to 2.2 and 2.0, respectively (Fig 3). These observations were similar for cheeses made from both pasteurized and un-pasteurized milk.

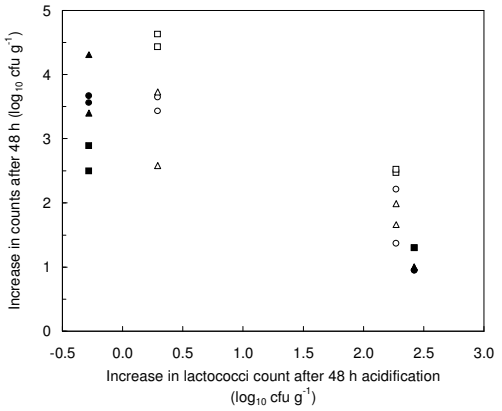

Figure 3. Correlation between the observed log_{10}-increase (log_{10}-counts per gram fresh cheese minus log_{10}-counts per g milk) in lactococci (starter) after 48 hours of acidification and the observed log_{10}-increase in faecal *E. coli* (●/○), *Enterobacteriaceae* (measured on XLD; ▲/△) and staphylococci (■/□) counts during the same period. Closed symbols are pasteurized milk, open symbols are un-pasteurized milk.

The explanation for the poor acidification turned out to be the application of an old and temperature abused starter culture (in lot 3), which also resulted in a delayed growth of lactococci (Fig 4). Where the abused starter was used, the lactococci count was observed to reach its maximum of 9.2 log_{10} CFU g^{-1} somewhere between day 2 and 10 in the case of raw milk cheese while in the case of the cheese made from pasteurized milk the maximum lactococci count (9.3 log_{10} CFU g^{-1}) was reached later, around day 14 (Fig 4). In all other lots, the lactococci counts were around 9 log_{10} CFU g^{-1} on day 2 and virtually no changes were observed during ripening for up to 40 d (results not shown).

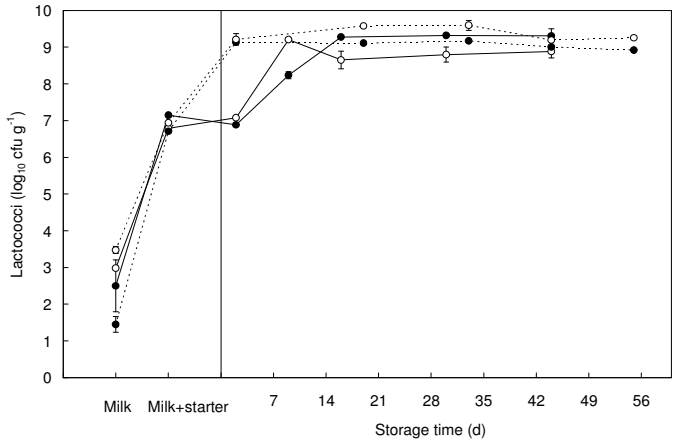

Figure 4. Counts of lactococci during manufacturing of cheeses from un-pasteurized (○) and pasteurized (●) milk. Dotted lines represent the application of a fresh starter culture and full lines the application of a temperature abused starter culture. Average and standard deviation of two samples are shown.

In the case of staphylococci in the fresh cheese curd (BP2), observations were similar to the results for *Enterobacteriaceae* (XLD2, EMBA2) (Fig 2B), *i.e.* a high activity of the starter culture reduced

the log_{10}-increase of staphylococci during acidification from 4.5 to 2.5 (Fig 3). Counts of staphylococci in 20-day-old cheeses averaged 4.8 log_{10} CFU g^{-1} (S.D. = 1.06) when made from raw milk and 3.8 log_{10} CFU g^{-1} (S.D. = 1.89) when pasteurized milk was used. None of the lots showed further growth of staphylococci during ripening.

The variations in chemical composition of the cheeses at day 20 were also large, but no systematic effect of pasteurization on the parameters was observed (Table 2). In particular moisture content as well as water content in the fat free cheese were difficult to reproduce.

Table 2. Milk pH and composition of ripened cheeses (20 d), produced from raw or pasteurized milk.

		Lot no.			
Milk	Chemical parameter	1	2	3	4
Raw	Milk pH[a]	6.74	6.71	6.68	6.66
	Cheese pH[b]	6.2 ± 0.3	5.3 ± 0.2	5.5 ± 0.1	4.9 ± 0.03
	Moisture (%)[b,c]	42.1	47.9 ± 1.1	46.9 ± 1.4	42.7 ± 2.6
	Fat content (%)[b,c]	31.5	30.6 ± 0.6	29.2 ± 0.9	33.6 ± 1.5
	Water in fat free cheese (%)[b,c]	61.5	69.0 ± 1.1	66.3 ± 1.4	64.3 ± 2.1
	Salt content (%)[b,c]	1.82	0.66 ± 0.12	0.35 ± 0.16	0.80 ± 0.23
	Calculated avg. salt-in-water (%)[d]	4.1	1.4	0.7	1.8
Pasteurized	Milk pH[a]	6.74	6.71	6.69	6.70
	Cheese pH[b]	6.1 ± 0.3	5.6 ± 0.1	4.9 ± 0.1	5.04 ± 0.1
	Moisture (%)[b,c]	47.3	48.7 ± 1.3	42.5 ± 1.3	46.2 ± 2.9
	Fat content (%)[b,c]	29.3	30.3 ± 0.8	32.3 ± 0.9	31.6 ± 1.5
	Water in fat free cheese (%)[b,c]	66.9	69.8 ± 1.1	62.8 ± 1.1	67.4 ± 2.8
	Salt content (%)[b,c]	1.76	0.25 ± 0.09	0.24 ± 0.07	0.74 ± 0.08
	Calculated avg. salt-in-water (%)[c,d]	3.6	0.5	0.6	1.6

[a] Represents one measurement on pooled milk.
[b] Average ± standard deviation of five cheeses.
[c] When no standard deviations are reported results are from pooled samples of five cheeses.
[d] Average percentage salt-in-water = (salt % / (salt % + moisture %)) x 100%.

3.4. Relations between chemical and microbiological profiles

Integrated principal component analysis (PCA) of chemical and microbiological parameters for 20-day-old cheeses was carried out to evaluate their relations in the ripened cheese. The loading plot is shown in Figure 5. The score plot in the integrated analysis of chemical and microbiological data did not give clear separation between cheeses manufactured from raw or pasteurised milk, and is

not shown. The first two components described 69 % of the variation in the data, with 45 and 24 % of the variation explained by the PC1 and 2, respectively. The parameters describing lactococci (M17) and lactobacilli (Rogo and MRS) were located close to the fat content and opposite the salt-in-water concentration of the cheese (Figure 5). The highest lactococci counts in the 20-day-old cheeses were found in the cheeses with approx. 30 % initial fat content (Figure 6) and 1 to 1.5 % salt-in-water (results not shown).

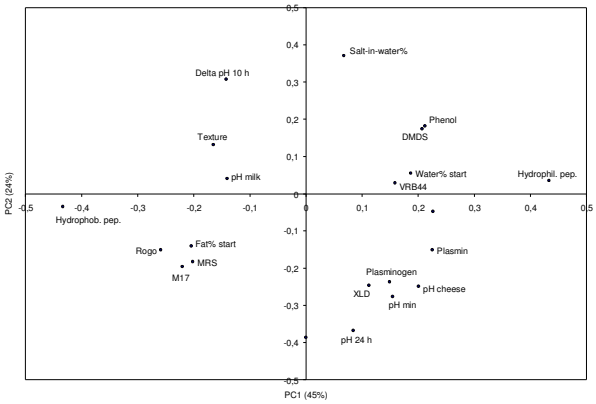

Figure 5. Principal component analysis of chemical and microbiological parameters collected from soft, surface-smeared 20-day-old cheeses. For description of variables, see materials and methods.

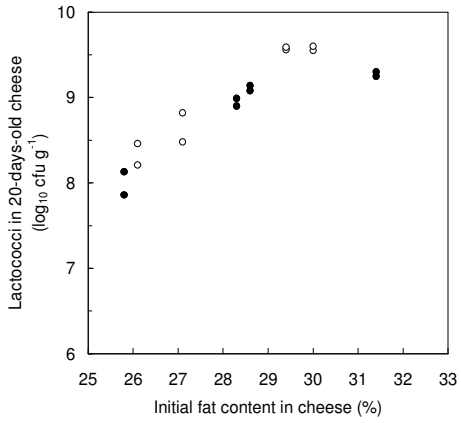

Figure 6. Relationship between counts (log_{10} CFU g^{-1}) of lactococci in 20-day-old cheeses made from un-pasteurized (○) and pasteurized (●) milk and initial fat content.

Enterobacteriaceae (XLD and EMBA) and faecal *E. coli* (VRB44) were located at the opposite plane of the PCA, opposite lactococci (M17) and lactobacilli (Rogo and MRS). *Enterobacteriaceae* (XLD and EMBA) and faecal *E. coli* (VRB44) were furthermore located together with the aroma components DMDS (dimethyl disulphide) and phenol, close to the initial moisture content (water% start) and opposite the initial salt-in-water concentration (Fig 5). The lowest counts of *Enterobacteriaceae* and faecal *E. coli* were observed at salt-in-water concentrations of 1.6% or above. Below this level, counts of *Enterobacteriaceae* (EMBA) in the 20-day-old cheeses were always above 10^6 CFU g^{-1} and up to 10^8 CFU g^{-1} were observed (results not shown). In these latter cases, it is likely that *Enterobacteriaceae* have contributed to the formation of unwanted aroma components, like DMDS, in the cheese. Furthermore, the pH parameters (pH min, pH cheese and pH 24h) are located close to *Enterobacteriaceae* (XLD), which suggests pH as another important factor in the control of *Enterobacteriaceae* in this cheese (Fig 5). The microbiological parameter describing staphylococci (BP) in the ripened cheeses was located close to pH 24 h and opposite to the pH-drop after 10 h acidification as well as the salt-in-water concentration (Fig. 5), i.e. a slow acidification profile and low amounts of salt were correlated with high level of staphylococci in the 20-day-old cheeses.

The enzymatic parameters plasmin and plasminogen as well as pH in cheese group together, but were located opposite to the variables describing texture as well as the pH-drop within the first 10 h acidification (delta pH 10h) in Figure 5. These results indicated, that a slow acidification increased the plasmin and plasminogen activities, and confirms that the activity of plasmin and plasminogen in cheeses are highly dependent on pH. Furthermore, the content of hydrophobic peptides was inversely correlated to the content of hydrophilic peptides. The observation that the lactobacilli and lactococci were located closer to the texture parameter, i.e. correlated more with a softer cheese

than plasmin, plasminogen, *Enterobacteriaceae* and faecal *E. coli*, showed that the former parameters were more crucial for soft texture development in these cheeses. The results therefore suggest proteases from lactococci and lactobacilli to be more important for soft texture development than the indigeneous protease plasmin in this type of cheese.

To identify and rank specific factors that influence the changes in the *Enterobacteriaceae* and faecal *E. coli* counts during ripening, PLS1 analyses were carried out. Results for faecal *E. coli* (VRB44) are presented in Figure 7.

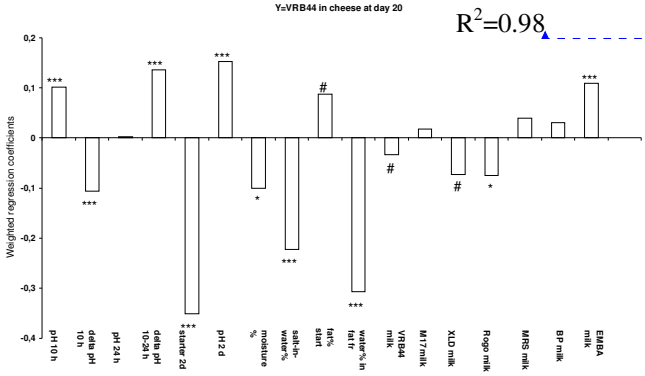

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Figure 7. Results from partial least squares regression analysis presented as the weighted regression coefficients between measured X-values and the Y-variable given as $\log_{10}$ CFU g^{-1} of faecal *E. coli* in soft, surface-smeared 20-day-old cheeses. For description of X-values, see materials and methods. #, P<0.1; *, P<0.05; **, P<0.01; ***, P<0.001. R^2 is the the correlation coefficient between measured and predicted y-variables.

The PLS1 analysis confirms acidification and water activity parameters as the prime controlling factors of the level of faecal *E. coli* in 20-day-old cheeses (Fig. 7). As indicated by an R^2 of 0.98, the model explain more than 98% of the variation in the level of faecal *E. coli*. Specifically, a high level of the starter culture 2 d after manufacture (starter 2d) and a fast pH-drop within the first 10 h acidification (delta pH 10h) correlate with a low level of faecal *E. coli* in the 20-day-old cheeses. While the initial pH drop (pH 10 h) affected the level of faecal *E. coli* significantly the pH after 24 h acidification did not appear to have any effect (Fig. 7). Of the water activity parameters,

especially a high salt-in-water content (salt-in-water%) and a high water content in fat free cheese (water% fat free) are associated with inhibition of *E. coli* growth (Fig. 7). The inhibitive effect of a high water content in fat free cheese may be an indirect effect of a high level of the starter culture, since it was strongly associated with this particular variable (results not shown). The initial level of bacteria in the milk did not appear to have similar high impact on the faecal *E. coli* count in the ripened cheeses but, as could be expected, the level of *Enterobacteriaceae* in milk (EMBA milk) was positively associated with the level of faecal *E. coli* in the 20-day-old cheeses (Fig. 7). The results of the PLS1 analysis point towards the activity of the starter culture as the most important factor.

4. Discussion

4.1 Production hygiene

In the present study, we observed a linear correlation between bacterial counts in the raw milk and bacterial counts on teat cups as well as a linear correlation between bacterial counts on teat cups and bacterial counts on teat ends. Any change in bacteriological levels observed on teat cups or teat ends may, therefore, have a direct influence on the bacteriological level of the raw milk. This observation can be useful for example in future quantitative microbial risk assessments involving raw milk or cheese. It has previously been observed that less contamination of raw milk from the cow's immediate environment takes place when the cows graze on open pasture compared with indoor housing at wet conditions (McKinnon *et al*., 1990; Slaghuis *et al*., 1991). Our results generally confirm this with a difference in bacterial counts in the order of one $\log_{10}$ CFU ml^{-1} except in the case of mesophilic, aerobic spores, *i.e. Bacillus* spores, where spores were only detected in raw milk from grazing cows. Christiansson *et al.* (1999) have shown that the prime

contamination source for *Bacillus cereus* spores is soil. Since the likelihood for contamination of teat ends is much higher in the grazing period, higher aerobic spore counts could be expected in milk from this period. *B. cereus* spores were also found in hay and dung/faeces, but in concentrations too low to be important for contamination of the milk with these spores (Christiansson *et al.*, 1999). A closer look at the bacterial counts in raw milk (Table 1), reveals that also *Enterobacteriaceae* measured on EMBA was higher in the grazing period than in the indoor housing period. However, as the same high level was also found in the pasteurized milk this specific observation appear more likely to be a result of recontamination from pipe work in the dairy.

It is not possible to obtain sterile raw milk but by adopting various hygienic measures in the milk production chain, the contamination of the raw milk can be kept at a minimum. In particular, efforts should be made to avoid contamination of the raw milk with food-borne pathogens. Apart from the obvious pre-requisite to avoid milk from cows with mastitis, the milkers took two main actions during milking in order to keep contamination of the raw milk to a minimum. They cleaned the udder and teat ends just before milking and they pressure cleaned the floor in the milking area several times during milking.

Washing of indoor housed cows with wetted, clean cotton cloths followed by air-drying before milking was observed to reduce the level of mesophilic, aerobic bacteria and staphylococci on teat ends significantly by 0.5 $\log_{10}$ CFU per cow. This was in agreement with the findings of Galton *et al.* (1986) who found a 0.7 $\log_{10}$ CFU reduction per cow for mesophilic, aerobic bacteria with a similar treatment. Even higher reductions (*approx*. 90%) can be found if disinfectants are used (Galton *et al.* 1986). During the grazing period, however, little effect was seen on the concentration of mesophilic, aerobic bacteria on the teats or in the raw milk as also reported by McKinnon *et al.* (1990).

The most pronounced effect of pre-milking teat treatment was found on *Bacillus* spores where a 1.0 $\log_{10}$ CFU reduction per cow was observed. In the study of McKinnon *et al.* (1983) it was found that the spore count in the raw milk could be reduced by 60% from washing the teats with water, an additional 10% reduction was obtained if the teats were dried with a paper towel immediately after washing, whereas there was no additional effect on spore count if sodium hypochlorite was used.

With regards to *Enterobacteriaceae* a statistically significant decrease in the prevalence on teat ends was observed as a result of teat washing in the grazing period as compared to an increase in the deep straw-bedding period. The latter could also be a result of the cleaned udder and teats being recontaminated with faecal bacteria from the teat cups. During the indoor housing period, when the cows were visibly dirtier, a much more intensive use of pressure cleaning of the floor in milking parlour took place throughout the milking hours. This frequent pressure cleaning resulted in a 100% prevalence of *Enterobacteriaceae* as well as faecal *E. coli* on the teat cups, indicating that bacteria of faecal origin were spread rather than eliminated by the splashing of water. It is, therefore, important to choose a cleaning method, such as scraping, that will minimize aerosols during milking.

From a hygienic point of view, it might be beneficial to distinguish between manufacturing of cheese from raw and pasteurized milk when choosing the pre-milking teat treatment. For production of cheese from pasteurized milk it may be fine to target the treatment specifically to reduce spores since the vegetative pathogens will be eliminated by proper pasteurization and clean equipment. For production of cheeses from raw milk it will be important to reduce the contamination of the cheese milk with pathogens of faecal origin as much as possible. Therefore, in particular for indoor-housed cows, more efficient teat cleaning should be developed. Addition of sodium hypochlorite to the

washing water followed by wiping the remaining rinse water off the teats with paper towels is more efficient but may not be acceptable for some producers.

4.2 Raw versus pasteurized

A major scientific challenge in artisanal cheese productions, such as the investigated, turned out to be the variability and the limited possibilities for reproducing whole cheese-making scenarios. Despite the fact that we had parallel productions of un-pasteurized and pasteurized cheese using milk from the exact same milking at the same farm dairy every time, we experienced high bacteriological as well as chemical variability. Especially, the variation between trials was generally higher than the variation within trials making it difficult to confirm differences between raw and pasteurized productions with classical statistical analyses, such as analysis of variance. In the present study, multivariate data analysis (Figs 2, 5 and 7) overcame some of the variability and provided some explanations for the observed variability. Overall, some discrimination of cheeses prepared from raw vs. pasteurised milk was achieved by multivariate analysis based on the microbiological parameters (Fig, 2), while integrated multivariate analysis based on both microbiological and chemical data (Fig. 5) did not result in such discrimination. This indicated that pasteurisation did not systematically affect the chemical parameters.

The most pronounced bacteriological difference between cheeses made from raw and pasteurized milk was a higher growth potential for LAB in the cheeses made from raw milk. On average ripened cheeses made from raw milk contained 9.4 $\log_{10}$ CFU g^{-1} as compared to 8.9 $\log_{10}$ CFU g^{-1} in those made from pasteurized milk, although the same amount of starter culture had been added in parallel productions. This difference is in the order of 10^9 LAB extra per gram in the cheeses made from raw milk, indicating a higher level of metabolites, and, consequently, flavour development. A higher level of lactobacilli was also observed in Swiss-type cheeses made from raw milk sompared

with cheeses prepared from pasteurised (72°C, 30 s) milk (Beauvier *et al*., 1997). The presence of high levels of *L. plantarum*, a non-starter LAB, in cheeses made from raw milk in one of the test productions confirms the potential for development of microbiologically associated sensory diversity. Also staphylococci levels tended to be higher in cheeses made from raw rather than pasteurized milk. However, levels never exceeded 10^6 CFU g^{-1}, making it unlikely for staphylococci to have contributed significantly to the sensory characteristics of the cheeses.

Pasteurization of milk has been described to enhance proteolysis with up to 30 to 40% (Noomen, 1975). We observed high variability of plasmin and plasminogen activity, but no systematic effect in relation to pasteurization. *A priori* an increase in plasmin activity by pasteurisation would have been expected (Richardson, 1983), due to a thermal instabilities of the plasmin inhibitors and plasminogen activator inhibitors. Recent reports indicate, however, that the heat stability pattern of the components of the plasmin-system in milk is more complicated, and that a delicate balance between the heat stabilities of plasmin inhibitors and plasminogen activator inhibitors determines the net measurable plasmin and plasminogen-derived activities (Burbrink and Hayes, 2006; Prado *et al*., 2006). In this study, the variability in plasmin and plasminogen-derived activities appeared to be related to differences in acidification rates (Fig 5). As the pH optimum for activity of plasmin is 7.5 (Bastian & Brown, 1996), it was observed that acidification profiles with very slowly decreasing pH resulted in higher activity, while rapid acidification resulted in lower. A higher pH leading to higher plasmin activity and resulting in texturally more hard cheeses was also found in semi-hard model cheeses (Watkinson et al., 2001), even though the texture effect in the present study was small. The water content in fat free cheese has also been observed to be higher in cheeses made from pasteurized compared to raw milk (Grappin & Beuvier, 1997), which could not be confirmed in the present study.

We have identified a number of practises in these full-scale productions, which influence the potential for growth of food-borne pathogens in cheese production. Parameters related to the acidification step were shown to be responsible for much of the experienced variation and optimization of the activity of the starter culture was essential. The most important factor was the correct storage of the starter culture. Too high a storage temperature and fluctuations in temperature and humidity due to repeated use of starter pellet from the same box reduced the activity resulting in unwanted growth of both *Enterobacteriaceae* as well as staphylococci during the acidification step (Fig 2). Our results indicated that an increase of the starter culture to at least 10^8 CFU g^{-1} in the cheese curd within 2 d after manufacture kept the increase of *Enterobacteriaceae* as well as staphylococci to a minimum. The rate of acidification, *i.e.* the pH-drop over time, was also found to be important as pH after 10 h correlated better with inhibition of *Enterobacteriaceae* and staphylococci than pH after 24 h. Therefore, it is important to optimize the growth conditions of the starter culture in order to obtain a rapid pH-drop. In our experiments slower acidification was observed in test productions with initial bacterial loads in milk above 10^4 CFU ml^{-1} (results not shown). This stresses the importance of reducing contamination of raw milk for cheese production including contamination from pipes and equipment. Another possible controlling factor is temperature. In this study, the acidification temperature was not controlled and was considerably lower than the optimum temperature for fast acid production by the added starter. The interaction between a safe pH reduction and the optimal development of flavour and texture may warrant further studies.

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