The role of copper in the manufacture of Finnish Emmental cheese

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ABSTRACT

The effects of added copper in the manufacture of Finnish Emmental cheese were studied. Consequently, cheeses were produced with or without the copper supplement and a facultative heterofermentative strain, *Lactobacillus rhamnosus* Lc705, which is currently utilized as a protective culture in large-scale manufacture in Finland. Cheeses were examined at 1, 7, 30, 60, and 90 d from the microbiological, chemical, and sensory points of view. Organic acid production was affected by the presence of copper in the cheeses. The addition of copper to cheesemilk increased the level of primary proteolysis and slowed secondary proteolysis as measured by nitrogen content in different extracts after citrate fractionation of cheeses, in pH 4.4-soluble nitrogen and 5% phosphotungstic acid-soluble nitrogen, respectively. The presence of copper appears to positively regulate the sensory characteristics of the cheese produced in our conditions; in particular, consistency was affected significantly. The role of the *Lb. rhamnosus* Lc705 protective strain has not been shown to have important effects on most of the parameters that influence the final quality of the cheeses. Although the traditional plating systems for revealing bacterial populations during cheese manufacture did not reveal any drastic differences caused by the presence of copper, the results from chemical and sensory analyses suggest that its use plays a significant role in the regulation of bacterial physiological and biochemical activities, which in turn affect the sensory quality of Emmental cheese.

Key words: copper, Emmental cheese, starter and adjunct culture, cheese quality

INTRODUCTION

Emmental cheese, also known as Swiss cheese, is a widespread cheese type that is produced in many industrialized countries; however, its manufacture may

vary depending on the place of origin. Production of Swiss Emmental uses copper vats, whereas in Finland, the manufacture of Emmental cheese is carried out in stainless steel cheese vats. Finnish Emmental includes the addition of Cu as a Cu sulfate solution at a level that will ensure concentrations of Cu in the final cheese close to that of traditional Swiss Emmental. In many other countries, stainless steel cheese vats are used but without the addition of copper.

The inhibitory effects of copper on the starters and adjunct cultures related to Emmental cheese production have been demonstrated in a species- and strain-dependent manner (Maurer et al., 1975; Mato Rodríguez and Alatossava, 2008). On the other hand, it is claimed that during the making of raw milk cheese, copper regulates an important part of the ripening process by influencing proteolytic enzymes as well as the activity of the microorganism present (Sieber et al., 2006). Copper levels that are too low result in cheese with poor flavor, in part because the ripening process is too rapid (Sieber et al., 2006). Although many cheese makers believe that Cu has a beneficial effect on the final quality of Emmental, few studies have been carried out to clarify the role of Cu in the final quality (Mueller et al., 1952; Kiermeier et al., 1961; Maurer et al., 1975). These studies suggest that under- or overdosing of copper may cause quality defects, predominantly through its effects on propionic bacterial fermentation.

The principal starters used in the production of Emmental cheese are thermophilic lactic acid bacteria (LAB) and a mixture of *Streptococcus thermophilus* spp. and *Lactobacillus* spp., including *Lactobacillus helveticus* or *Lactobacillus delbrueckii* ssp. *lactis*. Facultative heterofermentative lactobacilli (FHL) are currently used as adjunct cultures to slow propionic acid fermentation. Propionibacteria, including the species *Propionibacterium freudenreichii* ssp. *shermanii* and *P. freudenreichii* ssp. *freudenreichii*, are used as secondary starters to achieve the characteristic eyes and nutty flavor (Fröhlich-Wyder and Bachmann, 2004).

In the manufacture of Emmental cheese, strong proteolysis, in addition to intense propionic acid fermenta-

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tion, may be the primary causes of late fermentation (Baer and Ryba, 1999). *Lactobacillus helveticus* strains are responsible for the liberation of a large quantity of small peptides in Emmental cheese, and simultaneously, the presence of small peptides and free amino acids stimulates the growth of propionic acid bacteria (Baer and Ryba, 1999). Because of its intensive peptidolytic activity, *Lb. helveticus* has been replaced in traditional Swiss cheese by *Lb. delbrueckii* ssp. *lactis*. However, the strong proteolytic activity of *Lb. helveticus* is preferred by many manufacturers around the world to accelerate proteolysis and shorten the ripening period, thereby lowering the cost of production (Fröhlich-Wyder and Bachmann, 2004). The manufacture of Finnish Emmental also differs from the traditional Swiss process because it employs pasteurization (72°C/15 s) of the milk and uses *Lb. helveticus* strains. A previous study (Mato Rodríguez and Alatossava, 2008) investigated the effect of Cu on starters and adjunct cultures used in the manufacture of Emmental cheese. That study revealed that among 3 *Lb. helveticus* strains tested, 2 were significantly more sensitive to Cu than those of the *Lb. delbrueckii* species included in the study. The most sensitive species among the 3 species tested was *Lactobacillus rhamnosus*. The production of Emmental cheese in Finland also includes the addition of certain FHL strains, belonging to *Lb. rhamnosus* species, as protective strains especially against clostridia such as *Clostridium tyrobutyricum* spoilage bacteria. Cheeses produced with FHL (*Lb. casei* and *Lb. rhamnosus*) show an inhibition of propionibacteria up to 80% compared with controls (Jimeno et al., 1995). The citrate metabolism of FHL seems to cause this inhibition, because citrate-negative mutants show a much lower inhibitory effect (Jimeno, 1997). Perez Chaia et al. (1987) postulated that the ratio of citrate to copper concentration plays an important role in the inhibition of propionibacteria.

In this study, we aimed to elucidate the roles of Cu in the manufacture of Finnish Emmental. Consequently, cheeses were produced with or without the copper supplement and with or without an FHL strain of *Lb. rhamnosus* that is currently used as a protective culture in large-scale manufacture of Finnish Emmental.

MATERIALS AND METHODS

Cheese Manufacture

Small-scale experimental Emmental cheeses were produced in stainless steel vats from mildly pasteurized (72°C/15 s) milk (100 L), using the procedure of the dairy pilot plant of the Food and Environmental Sci-

ence Department at the University of Helsinki (Figure 1). The general protocol used in the production was as follows: 0.2% (wt/vol) of *Streptococcus thermophilus* T101 and 0.1% (wt/vol) of *Lb. helveticus* K16 starters were added to 100 L of pasteurized milk following the addition of 30 g of CaCl₂. *Lactobacillus rhamnosus* Lc705 was added as a protective strain at 0.01% (wt/vol) followed by addition of a secondary culture of *P. freundereichii* ssp. *freundereichii* P131 at 0.01% (wt/vol). A solution of copper sulfate (1% wt/vol CuSO₄·5H₂O) was added to obtain a final concentration of 1 to 1.5 mg/L in the cheesemilk. The milk was stirred for 22 min before the addition of 9.5 g of liquid rennet Chy-Max (600 international milk clotting units/mL average activity) from Chr. Hansen (Hørsholm, Denmark). Following curd setting (32°C/35 min), cutting (32°C/13 min), and stirring (32°C/25 min), 30% of the whey was removed and replaced with 25% (vol/vol) water. The whey-curd mixture was scalded gradually to 51 to 53°C for 50 min before molding the curd at 49°C. After the curd was pressed for 18 h and salted in brine (density 22° Baumé) for 12 h, the maturation schedule was as follows: 12°C for 10 d, 23°C for 25 d, and 5°C until the end of ripening.

Raw milk used for cheese production was obtained from the dairy company Valio Ltd. (Helsinki, Finland). The concentrated starters used were also provided by Valio Ltd. Two sets of cheeses were produced on 2 consecutive days during 2 wk. The same batch of raw milk was used for 1 wk of cheese production. During the first week, one set of cheeses was made with the addition of copper in the cheesemilk as an aqueous solution of copper sulfate stock solution (1%, wt/vol), and the other set was produced without the addition of copper. In the second week, the same procedure was applied, but the protective strain *Lb. rhamnosus* Lc705 was not included in the manufacture. The cheeses were designated as follows: **ChCu+Lc705+** = cheese produced with the addition of copper and the protective strain Lc705 into the cheesemilk; **ChCu–Lc705+** = cheese produced without the addition of copper but with the addition of the protective strain Lc705 into the cheesemilk; **ChCu+Lc705–** = cheese produced with the addition of copper but without the addition of the protective strain Lc705 into the cheesemilk; and **ChCu–Lc705–** = cheese produced without addition of copper or the protective strain Lc705 into the cheesemilk. From the 2 test cheeses (about 5 kg each) produced from each cheese vat (100 L milk), one test cheese was used for sampling for the different analyses and the other one was kept intact for sensory evaluation after 90 d of ripening. Representative samples from each test cheese were taken after pressing (d 1), during

Figure 1. Flow diagram for the manufacture of Emmental test cheese.

cold room storage for ring formation (d 7), close to the end of warm room fermentation (d 30), and during cold room ripening (d 60 and 90).

Microbiological Analysis of Milk and Cheeses

Microorganisms were enumerated in raw milk, pasteurized milk, milk before rennet addition, and cheeses at 1, 7, 30, 60, and 90 d (of ripening). To measure starter viability in cheeses, 10 g of the cheese sample was homogenized using a stomacher (Colworth stomacher 400, Seward Ltd., London, UK) for 3 min in 90 g of trisodium citrate solution (20 g/L). Subsequent dilutions were prepared using a 0.85% (wt/vol) saline solution. Corresponding serial dilutions were inoculated on different media that are currently used for revealing different species in cheeses. All microbiological sampling and measurements in milk and cheeses were performed in duplicate and the mean values results are reported.

The total bacterial counts of raw and pasteurized milk were enumerated on plate count agar (Lab M Ltd., Lancashire, UK), and the plates were incubated at 30°C for 48 h. Coliforms were enumerated in pasteurized milk and in fresh cheeses using violet red bile lactose agar (Lab M Ltd.), and the plates were incubated at 30°C for 24 h. Yeast and molds were enumerated in pasteurized milk and in cheese at different sampling points using yeast glucose chloramphenicol agar (Merck, Darmstadt, Germany), and the plates were incubated at 25°C for 5 d. *Streptococcus thermophilus* was enumerated using M17 lactose (M17-L) agar (Lab M Ltd.) after the addition of starter to the milk and cheeses at the different sampling points during ripening. The lactose concentration in the medium was adjusted to 2% (wt/vol) after autoclaving. Plates were incubated anaerobically at 42°C for 48 h. The total number of lactobacilli was enumerated using de Man, Rogosa, and Sharpe (MRS) agar (Lab M Ltd.) after the addition of starter to the

milk and cheeses at the different sampling points during ripening. The plates were incubated anaerobically at 37°C for 48 h. Nonstarter lactobacilli were enumerated using MRS agar supplemented with vancomycin (50 mg/L of agar) after the addition of starter to the milk and cheeses at the different sampling points during ripening time. Plates were incubated anaerobically at 37°C for 48 h. Vancomycin is used in MRS agar to inhibit the growth of certain species of lactobacilli such as *Lb. helveticus*. However, the growth of other *Lactobacillus* species such as *Lb. rhamnosus* is possible in the presence of vancomycin. We used MRS agar supplemented with vancomycin at 50 mg/L to count the levels of the protective strain *Lb. rhamnosus* Lc705 in test cheeses and to elucidate the effects of the presence of Cu on growth and survival of *Lb. rhamnosus* Lc705. We also used MRS-vancomycin agar by comparing the counts on MRS and MRS-vancomycin for the other set of cheeses in which the protective strain was not added to evaluate the evolution of *Lb. helveticus* during ripening. The levels of *Lb. helveticus* are masked when both starter and protective strains of *Lactobacillus* are present at high levels in cheesemilk, especially at the beginning of the process. *Lactobacillus helveticus* strains are autolytic, and they are not usually revealed after a certain period of ripening. However, *Lactobacillus* species that belong to nonstarter LAB, usually *Lb. casei*, *Lb. rhamnosus*, and *Lb. plantarum*, remained at high levels during the process (Kocaoglu-Vurma et al., 2008). Propionibacteria were enumerated using Na-lactate agar (Tuomola et al., 1999) after addition of the starter to the milk and cheeses at different sampling points during ripening. The medium consisted 5 g of tryptone, 10 g of yeast extract, 10 g of β -glycerophosphate, 16.8 mL of 50% (wt/wt) Na-lactate solution, 16 g of agar, and 1,000 mL of distilled water; the pH after autoclaving was 7.2 ± 0.2 . The plates were incubated anaerobically at 30°C for 5 d.

Chemical Analysis of Milk and Cheeses

The total composition of raw and standardized milk was measured using a MilkoScan Minor type 78100 (Foss Analytical A/S, Hillerød, Denmark). pH measurements were performed in cheeses at all sampling points using a waterproof multi parameter PCS tester 35 (Oakton Instruments, Vernon Hills, IL). The Cu content, degree of proteolysis, and concentration of organic acids were measured in 7-, 30-, 60-, and 90-d-old cheeses. The cheese samples collected at each sampling point for Cu content and proteolysis analysis were grated, divided into portions, stored in crimp-neck vial glasses (50-mL capacity) and frozen at -20°C . The copper content was determined at MTT Agrifood Research Finland (Jokio-

inen, Finland) by inductively coupled plasma emission spectrometry with Thermo Jarrell Ash Iris Advantage (Thermo Jarrell Ash, Franklin, MA) at emission spectra of 327.3 nm, following the procedure of Kumpulainen and Paakki (1987). Proteolysis was performed using the fractionation of a citrate dispersion of cheese (IDF, 1999), and the nitrogen content in the different soluble fractions obtained from the fractionation protocol was determined by the method of Kjeldahl (AOAC, 2000; method 33.2.11, 991.20). For organic acids determination, grated cheese was stored in 20-mL crimp-neck amber vials, injected with nitrogen, and frozen at -20°C until the analysis was performed. Organic acid analysis was performed by HPLC using the general protocol reported by Mullin and Emmons (1997) and Akalin et al. (2002) with some modifications: a cheese sample (2 g) was homogenized in a centrifuge tube for 1 min with 10 mL of 0.005 N H_2SO_4 using a Ystral homogenizer (Ystral, Dottingen, Germany). The homogenized sample was then extracted in a magnetic stirrer for 1 h. After extraction, the sample was centrifuged at $6,000 \times g$ for 5 min and filtered into an autosampler vial through 0.45- and 0.2- μm membrane filters. The organic acids were analyzed at MTT Agrifood Research Finland by using an Agilent 1100-series HPLC (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector. The analytical column was a Rezex RFQ Fast Acid (100 $\times$ 7.8 mm, Phenomenex, Torrance, CA), and the mobile phase was run under 0.005 N H_2SO_4 at 0.5 mL/min. Organic acids were quantified at 210 nm. Compositional analyses were carried out in 90-d-old cheeses, and they included the determination of total solids content (IDF, 1982) fat content by the Gerber van Gulik method (ISO, 1975), total nitrogen content by the Kjeldahl method (AOAC, 2000), and salt content, which was determined using a chloride analyzer (model 926S, Sherwood Scientific Ltd., Cambridge, UK) following the procedure of Johnson and Olson (1985).

Sensory Analysis of Cheeses

The sensory analysis was performed using a quality scoring by the FIL-IDF 99C method (IDF, 1997), a grading method using a 5-point quality scale for the appearance of the attributes, consistency, and flavor. The cheeses were assessed by an expert panel ($n = 5$) of the Finnish Food Safety Authority Evira in Helsinki. The panel consists of experts who have been performing quality grading of different cheeses for several years (>3 yr). The descriptions of the scores in the scale are as follows (whole numbers are used): 5 = conformity with the pre-established sensory specification, 4 = minimal deviation from the pre-established sensory specifica-

Table 1. General composition and microbiology of raw and pasteurized milk used for the manufacture of Emmental test cheeses

Item ¹	Cheesemilk ²			
	ChCu+Lc705+	ChCu–Lc705+	ChCu+Lc705–	ChCu–Lc705–
Composition (%)				
Fat	3.00	2.99	3.00	2.99
Protein	3.48	3.48	3.50	3.48
Lactose	4.57	4.56	4.58	4.59
Milk SNF	8.91	8.90	8.93	8.92
PM counts (log cfu/mL)				
Total count	2.62	2.65	2.72	2.96
Coliforms	<10	<1	<1	<1
Yeast and molds	<10	<1	<1	<1
PMS counts (log cfu/mL)				
M17-lactose agar	6.77	6.86	6.65	6.69
de Man, Rogosa, and Sharpe (MRS) agar	6.71	6.85	6.78	6.88
MRS-vancomycin agar	6.79	6.83	<1	<1
Na-lactate agar	5.69	5.77	5.73	5.69

¹PM = pasteurized milk; PMS = pasteurized milk after starter addition.

²ChCu+Lc705+ = cheese produced with the addition of copper and the protective strain *Lactobacillus rhamnosus* Lc705; ChCu–Lc705+ = cheese produced without the addition of copper but with the addition of the protective strain Lc705; ChCu+Lc705– = cheese produced with the addition of copper but without the addition of the protective strain Lc705; and ChCu–Lc705– = cheese produced without the addition of copper or the protective strain Lc705.

tion, 3 = noticeable deviation from the pre-established sensory specification, 2 = considerable deviation from the pre-established sensory specification, 1 = very considerable deviation from the pre-established sensory specification, and 0 = unfit for human consumption. If a panelist gives a score of 3 or less, a deviation from the specification (defect) must be described. The nomenclature of terms is defined in the method. The samples were evaluated at $14 \pm 2^\circ\text{C}$ and coded with a 3-digit random number.

Statistical Analysis

The results of the proteolysis analysis were analyzed by 2-way ANOVA (factors: copper and Lc705, and co-variant: age), and the results of the quality scoring were analyzed by 1-way ANOVA. Statistically significant differences ($P < 0.05$) in quality scoring were tested by Tukey's honestly significant difference test. Principal component analysis (PCA) was used to visualize the differences between cheeses in the quality scoring. Statistical tests were gained by PASW Statistics (18.0.0, 2009, SPSS Inc., Chicago, IL), Statistics for Windows (7.0, 2000, Analytical Software, Tallahassee, FL), and Unscrambler (9.2, 2005, CAMO ASA, Oslo, Norway).

RESULTS

Composition, Microbiological Quality, and Level of Starters in Cheesemilk

The raw milk used for making cheese had the following composition: fat: $4.57 \pm 0.10\%$ (wt/wt), protein:

$3.40 \pm 0.01\%$ (wt/wt), lactose: $4.45 \pm 0.09\%$ (wt/wt), and milk solids nonfat: $8.79 \pm 0.05\%$ (wt/wt). The total bacterial count in raw milk for the first set of cheeses produced was 4.23 log cfu/mL, on average, and that for the second set of cheeses was 3.90 log cfu/mL. The average values of the gross composition, microbiological quality, and starter levels in pasteurized milk used for making cheese are shown in Table 1.

Effect of Copper on the Starters and Adjunct Cultures

As shown in Figure 2a, the counts on M17-L agar did not show important differences with the presence of Cu in young cheeses, although some differences could be observed in cheeses made without the protective strain Lc705, in which counts were lower compared with the cheeses produced with this strain. Nevertheless, the counts on M17-L agar remained much higher in cheeses from 60 to 90 d of ripening when produced without the protective strain Lc705 compared with cheeses of corresponding age produced with the protective strain (Figure 2a).

As can be seen in Figure 2b, the counts on MRS were similar for all cheeses at different sampling points, with the exception of the 60- and 90-d samples, in which the counts were much lower in cheeses produced without *Lb. rhamnosus* Lc705.

Cheeses produced with or without Cu did not differ substantially, as revealed by the counts on MRS-vancomycin agar (Figure 2c). In cheeses without the protective strain, the values were negative at d 1 for cheeses made with Cu, but a few colonies were observed

Figure 2. Counting results in (a) M17-lactose (M17-L) agar, (b) de Man, Rogosa, and Sharpe (MRS) agar, (c) MRS-vancomycin agar, and (d) Na-lactate agar at different ripening periods. ChCu+Lc705+ = cheese produced with the addition of copper and the protective strain *Lactobacillus rhamnosus* Lc705 (Lc705); ChCu-Lc705+ = cheese produced without the addition of copper but with the addition of the protective strain Lc705; ChCu+Lc705- = cheese produced with the addition of copper but without the addition of the protective strain Lc705; and ChCu-Lc705- = cheese produced without the addition of copper or the protective strain Lc705.

at this sampling point in cheeses made without Cu. After 7 d, the counts gradually increased in these 2 sets of cheeses, which could be attributed either to some nonstarter LAB that can be found in this type of cheese during ripening or to the possibility that contamination with the same strain of *Lb. rhamnosus* Lc705 occurred at some point. However, no differences in the counts were seen when comparing ChCu+Lc705- and ChCu-Lc705- cheeses from 30 to 90 d of ripening (Figure 2c).

The milk used in our manufacturing process was submitted to a mild pasteurization, similar to that used in Emmental production on an industrial scale in Finland, and propionic bacteria were inoculated into the cheese vat at the level of 5 to 6 log-units. Propionic acid fermentation begins after the cheeses have been placed in the warm room, and the counts of this population increase to levels between 8 and 9 log-units and usually remain at high levels until the end of the ripening process. The counts on Na-lactate agar did not reveal any effect of the presence of Cu on the propionic bacteria

population; the counts were very similar throughout the ripening process (Figure 2d). *Lactobacillus rhamnosus* Lc705 is also used to slow propionic fermentation by controlling growth of propionibacteria, but no differences were found in counts between cheeses produced with or without this strain. A slight decrease in the propionic bacterial population in ChCu-Lc705- was observed only after 60 d of ripening, which accounts for 1 log-unit difference compared with ChCu+Lc705+ (Figure 2d).

Evolution of Chemical Composition During Ripening

During the cheesemilk coagulation period and curd treatment in the cheese vat, the pH remained constant and close to that of the initial pH in cheesemilk because the process was performed in such a way that no acidification was allowed during this period. A decrease in pH is usually observed during pressing time. In our process, the pH was determined in the cheeses after 2 h

of pressing. At this point, pH values were slightly lower in cheeses produced without Cu and higher in cheeses produced without *Lb. rhamnosus* Lc705 compared with the cheeses produced with Lc705 (pH 5.70 for ChCu+Lc705+, pH 5.65 for ChCu–Lc705+, pH 6.00 for ChCu+Lc705–, and pH 5.94 for ChCu–Lc705–). At the end of the pressing period, the pH differences were a little more pronounced between cheeses made with or without Cu, especially in those cheeses made with the *Lb. rhamnosus* Lc705 supplement (pH 5.46 and 5.29, respectively).

The average pH values, copper content, and composition at the final ripening point (90 d) for all cheeses are shown in Table 2. During cheese ripening, the pH values in cheeses containing added copper remained higher after 7 d in cold storage (the period for cheese rind formation), especially in cheeses produced with the *Lb. rhamnosus* Lc705 strain added. Later, during ripening, these pH differences disappeared. The pH values of cheeses produced without the *Lb. rhamnosus* Lc705 protective strain were higher at all timepoints compared with the set of cheeses produced with this strain. However, the differences in pH values were not very pronounced between ChCu+Lc705– and ChCu–Lc705– cheeses.

As shown in Table 2, the Cu content was slightly higher in the set of cheeses produced with the *Lb. rhamnosus* Lc705 strain. The set of cheeses made with or without Lc705 were produced using different sources of raw milk. The higher background Cu content in the cheesemilk may explain the higher content in the first set of cheeses; the corresponding values in the first and

second sets of cheesemilk were 0.11 and 0.07, respectively.

Emmental cheese is characterized by the resulting organic acids, which are produced from propionic acid bacterial fermentation. This fermentation plays a critical role in the ripening quality of this type of cheese by producing propionate, acetate, and CO₂. The acids are important flavor components, and the eyes in the cheese are formed due to CO₂ production (Piveteau, 1999).

To determine the effect of Cu on propionic bacterial activity, the content of the main organic acids that are metabolized during growth were monitored at different ripening points (Figure 3). The content of lactic acid at the 7-d sampling point was not very different when comparing the same set of cheeses produced with or without Cu (Figure 3a). At this point, the cheeses produced with the protective strain *Lb. rhamnosus* Lc705 had higher values of lactic acid compared with the cheeses without Lc705. By the end of the propionic acid fermentation period (30-d-old cheeses), the lactic acid content was considerably higher in cheeses containing added Cu, independently of the initial values measured (7 d). These values remained higher until the end of maturation (90 d) compared with the Cu-negative cheeses, with the exception of the 90-d-old ChCu–Lc705–, which showed a slight increase in lactic acid content for unknown reasons (Figure 3a). Lower consumption of lactic acid by propionic acid bacteria suggests some inhibition in their activity.

Pyruvic acid content was very similar at the 7-d ripening point in all cheeses, but an increase was ob-

Table 2. Values of pH and copper content at different ripening times (d 1 to 90) and composition ($\pm$ SD) of 90-d-old Emmental cheeses

Item	Cheese sample ¹			
	ChCu+Lc705+	ChCu–Lc705+	ChCu+Lc705–	ChCu–Lc705–
pH				
d 1	5.46	5.29	5.56	5.51
d 7	5.67	5.47	5.71	5.60
d 30	5.53	5.59	5.63	5.61
d 60	5.70	5.68	5.79	5.72
d 90	5.68	5.66	5.74	5.74
Cu content (mg/kg)				
d 7	15.0	1.56	14.1	1.10
d 30	15.2	1.69	15.0	1.14
d 60	14.8	1.63	14.4	1.14
d 90	15.4	1.60	14.2	1.13
Composition (%) of 90-d-old cheeses				
Fat	31.25 $\pm$ 0.35	29.50 $\pm$ 0.00	31.00 $\pm$ 0.00	30.25 $\pm$ 0.35
Protein	28.69 $\pm$ 0.66	30.14 $\pm$ 0.52	28.69 $\pm$ 0.30	29.30 $\pm$ 0.18
Moisture	36.71 $\pm$ 0.05	37.65 $\pm$ 0.20	36.64 $\pm$ 0.15	36.10 $\pm$ 0.06
NaCl	0.8 $\pm$ 0.24	0.70 $\pm$ 0.15	0.85 $\pm$ 0.15	0.92 $\pm$ 0.25

¹ChCu+Lc705+ = cheese produced with the addition of copper and the protective strain *Lactobacillus rhamnosus* Lc705 (Lc705); ChCu–Lc705+ = cheese produced without the addition of copper but with the addition of the protective strain Lc705; ChCu+Lc705– = cheese produced with the addition of copper but without the addition of the protective strain Lc705; and ChCu–Lc705– = cheese produced without the addition of copper or the protective strain Lc705.

Figure 3. Development of organic acids in test cheeses at different ripening points: (a) lactate, (b) pyruvate, (c) propionate, and (d) acetate. ChCu+Lc705+ = cheese produced with the addition of copper and the protective strain *Lactobacillus rhamnosus* Lc705 (Lc705); ChCu-Lc705+ = cheese produced without the addition of copper but with the addition of the protective strain Lc705; ChCu+Lc705- = cheese produced with the addition of copper but without the addition of the protective strain Lc705; and ChCu-Lc705- = cheese produced without the addition of copper or the protective strain Lc705.

served at the end of warm-room fermentation in cheeses containing supplemented Cu (Figure 3b). The accumulation of this acid in cheeses at 30 d was followed by a gradual decrease until the end of maturation. A significant relative increase of this acid was detected in ChCu-Lc705- at 90 d.

The formation of propionate in the cheese during warm-room fermentation is shown in Figure 3c. Cheeses with supplemented Cu had lower contents of propionic acid at this sampling point, and in cheeses without Cu, the amount of propionic acid remained higher until the end of ripening. The absence of the protective strain Lc705 in the second set of cheeses did not have any noticeable effect on the level of propionic acid formation. Similarly, the levels of acetic acid after 30 d of fermentation were higher in cheeses where Cu was not added to the cheesemilk, but in this case the difference was more pronounced when *Lb. rhamnosus* Lc705 was also absent (Figure 3d). However, the higher content of acetic acid observed at this point for ChCu-Lc705- disappeared later during maturation when increases in

acetic acid content were observed in the rest of the cheeses. At the end of ripening (90 d), the acetic acid contents were similar in all cheeses (Figure 3d). The ratio between propionate and acetate was similar in all cheeses at 90 d of ripening, at around 1:0.8, with the exception of ChCu+Lc705-, in which the ratio was 1:1.

The results obtained from the statistical analysis of the data are shown in Figure 4, which was generated from the proteolysis analysis of cheeses when using the citrate fractionation method at 4 ripening times. The levels of pH 4.4-soluble nitrogen (**4.4-SN**) in Figure 4a, TCA-soluble nitrogen (**TCA-SN**) in Figure 4b, and phosphotungstic acid-soluble nitrogen (**PTA-SN**) in Figure 4c are expressed as proportions of total nitrogen (**TN**) in cheeses. The mean and standard deviation of TN of all cheeses was 45.49 ± 0.94 g/kg. This is in the same range as reported for Finnish Emmental cheese (Pillonel et al., 2005). As expected, the age of the cheese mostly influenced the N levels of all fractions.

Copper had a significant effect on the level of 4.4-SN ($P < 0.05$). Supplemented Cu increased the level of 4.4-

Figure 4. Mean and standard deviations of different nitrogen fractions in cheeses with (gray bar) or without (white striped bar) added copper: (a) pH 4.4-soluble nitrogen, (b) TCA-soluble nitrogen from pH 4.4 soluble nitrogen, and (c) phosphotungstic acid-soluble nitrogen. TN = total nitrogen.

SN at every stage of ripening (Figure 4a). In addition, the level of TCA-SN increased in Cu-supplemented cheeses at d 30 to 90, although the increase was not statistically significant (Figure 4b). On the contrary, the addition of Cu significantly decreased ($P < 0.05$) the level of PTA-SN in cheeses during ripening.

The use of the Lc705 protective strain did not have an effect on proteolysis at any stage (data not shown). In addition, the interaction of Cu and Lc705 was statistically insignificant in every fraction and at every stage.

Sensory Analysis of Cheeses

The results derived from the sensory evaluation of the cheeses are shown in Figure 5. The PCA revealed that all the cheeses differed from each other (Figure 5a). Principal component 1 (PC1) explained 68% of the variance, and samples were mainly distributed along PC1 according to the supplement of Cu. Moreover, consistency was observed with Cu-positive samples, indicating that added Cu predominantly influenced the consistency positively. This was also seen in the ANOVA results, because the presence of added Cu had a statistically significant effect on the consistency of the cheeses (Figure 5b). Principal component 2 explained 28% of the variance, and samples were distributed along it according to the addition of the *Lb. rhamnosus* Lc705 protective strain. The effect of the protective strain on the appearance of the cheese was statistically significant in cheeses produced without Cu (Figure 5b); the differences in appearance were mostly attributed to eye formation in the cheeses. The addition of either Cu or the protective strain did not have statistically significant effects on the flavor of the cheeses. All the cheeses were given a low score on appearance, mainly because of problems with rind formation. However, the situation was similar for all cheeses and did not influence the evaluation of consistency and flavor.

DISCUSSION

According to the results of this study, the addition of copper during cheese-making influences the final characteristics of Emmental cheese to a certain extent. This holds true for the levels of the main organic acids (Figure 3), the degree of proteolysis (Figure 4), and the consistency of the cheeses (Figure 5). The presence of Lc705 protective strain appeared to influence primarily the appearance of the cheeses, particularly in those cheeses produced without Cu.

The results obtained from the microbiological analysis of cheeses at the different sampling points did not reveal many differences in the cheeses produced with or without Cu in any of the counting systems used. These results could be attributed in part to the lack of specificity of the plating systems and to the incubation conditions used to account for the specific populations of starters and adjunct cultures. The M17-L agar is currently used for enumerating *Lactococcus* and *Streptococcus* species in cheeses and other dairy fermented products, but it is not totally selective, similar to most of the media used for revealing certain types of bacteria in food matrices (Tharmaraj and Shah, 2003). A decline in bacterial counts after 60 d of ripening to around 5 log cfu/mL is regularly seen for *Strep. thermophilus*

Figure 5. Results of sensory evaluation: (a) principal component analysis and (b) mean and standard deviations. ChCu+Lc705+ = cheese produced with the addition of copper and the protective strain *Lactobacillus rhamnosus* Lc705 (Lc705); ChCu-Lc705+ = cheese produced without the addition of copper but with the addition of the protective strain Lc705; ChCu+Lc705- = cheese produced with the addition of copper but without the addition of the protective strain Lc705; and ChCu-Lc705- = cheese produced without addition of copper or the protective strain Lc705. Means with different letters differ significantly ($P < 0.05$). Color version available in the online PDF.

in this type of cheese because lysis in the different starter populations during cool-room ripening is more pronounced (Valence et al., 2000). Our results with the set of cheeses produced with *Lb. rhamnosus* Lc705 are in agreement with this decline. de Man, Rogosa, and Sharpe agar is the most commonly used medium for cultivation and screening of *Lactobacillus* and, although it mostly enables enumeration of this group of starters under certain incubation conditions (Tharmaraj and Shah, 2003), it is also a nonselective medium.

As noted in the Results section, the pH value after pressing in cheeses produced with Cu was higher, especially in cheeses produced with *Lb. rhamnosus* Lc705. We expected to observe more pronounced differences at the d 1 sampling point based on the results of our previous experiments, in which the pH values corresponding to Cu-positive cheeses were 2 decimal points higher than the pH of cheeses produced without Cu addition (unpublished data). The counting results in M17-L agar at this point (d 1) showed a difference of only 0.5 log units (Figure 2a); this may suggest that the activity, rather than the viability, of *Strep. thermophilus* was affected by the presence of Cu at concentrations of approximately 15 mg/kg. The influence of Cu on acid production by *Strep. thermophilus* has been reported

previously by Mueller et al. (1952), who observed that titratable acidity decreased with increasing Cu concentrations ranging from 0 to 8 mg/kg.

Although the results of plating counts in Na-lactate agar did not show differences in cheeses with different Cu contents (Figure 2d), the level of organic acids indicated that the presence of Cu markedly influenced propionic acid production and, to a lesser extent, acetic acid production (Figure 3c and d). The accumulation of lactic acid and especially pyruvic acid suggests that Cu has an inhibitory effect on the lactate metabolism of the strain of propionibacteria used in our Emmental production (Figure 3a and b). During growth of propionibacteria on lactate, the lactate is initially oxidized to pyruvate (Piveteau, 1999). The high levels of pyruvate after warm-room fermentation in cheeses produced with added Cu could be a result of excretion by the cells because of the accumulation of pyruvate to levels that may be toxic for the cell. This mechanism has been suggested as one mechanism used by *P. freundreichii* ssp. *shermanii* to regulate the intracellular pool of pyruvate in lactate metabolism (Deborde and Boyaval, 2000). Pyruvate accumulation inside the cell could occur because of the inhibition of catabolism of pyruvate to propionate, acetate, and CO₂ at one or more steps.

As described in the Results section, the presence of Cu did not appear to influence the propionate:acetate ratio. The quality of Emmental cheese can be related to the extent of propionic acid fermentation, not only in terms of the actual amounts of propionate, acetate, and CO₂, but also in terms of their relative proportions. In Swiss-type cheese, the theoretical equation (3 lactate → 2 propionate + 1 acetate + 1 CO₂ + 1 H₂O), which resulted from the formulation of Fitz (Boyaval and Core, 1995), is rarely found, and the relative product concentrations may vary significantly (Piveteau, 1999). The propionate:acetate ratio in commercial Finnish Emmental was reported to be approximately 1:0.7 (Pillonel et al., 2002). This value is in agreement with those obtained in this study (Figure 3c and d). Maurer et al. (1975) reported that added Cu in Emmental cheeses had no influence on acetic and propionic acid production. However, in the same study, the propionate:acetate ratio was 1:1 in most of the commercial cheeses but significantly higher in Iowa-style cheese, which differs from commercial ones by the fact that one-third of the whey is replaced by water during cheese-making.

The presence of added Cu increased the degree of proteolysis, as measured by N content in pH 4.4-SN (Figure 4a). This fraction contained proteins (except intact caseins), all peptides, amino acids, and smaller N compounds such as amines, urea, and ammonium (IDF, 1999). Previous studies (Maurer et al., 1975) found that, in Swiss commercial cheeses, proteolysis was considerably higher in cheeses in which Cu was added to the milk compared with controls. However, because these authors were using the method reported by Vakaleris and Price (1959), our results obtained from the pH 4.4-SN fraction should be compared with caution. The opposite occurred when proteolysis was measured by the level of soluble N after fractionation with 5% phosphotungstic acid (Figure 4c). The N compounds found in this fraction are attributed to small peptides, amino acids, and smaller N compounds such as amines, urea, and ammonium (IDF, 1999). The presence of Cu appears to regulate secondary proteolysis; one explanation for these results could be the possible inhibition of certain peptidases from LAB and non-starter LAB. The strong inhibition of aminopeptidase activities of *Strep. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* in the presence of Cu²⁺ has been observed (Tsakalidou et al., 1993). One of the desired characteristics of *Lb. helveticus* strains for the manufacture of cheeses is their high proteolytic activity; the activity of a tripeptidase from industrial *Lb. helveticus* strain 53/7 was completely inhibited by the presence of Cu (Savijoki and Palva, 2000).

The presence of Cu appeared to positively influence the sensory characteristics of the cheese produced un-

der our conditions; both consistency and flavor received higher scores in cheese with added Cu compared with the cheeses without Cu (Figure 5). In particular, consistency was affected significantly. The texture of cheeses, which denotes consistency and structure of the cheese body, are closed related to the degree of primary proteolysis. Insufficient proteolysis may cause a flat taste and a longer body consistency that is usually described as rubbery. On the other hand, excessive proteolysis results in a sharp taste and shorter body (Fröhlich-Wyder and Bachmann, 2004). The correlation between the degree of proteolysis, as measured by pH 4.4-SN and TCA-SN (Figure 4a and b), and consistency in the cheeses supplemented with Cu (Figure 5a) suggests a positive role of the presence of Cu in our manufacturing conditions. The production of Emmental cheeses in Cu vats or the addition of Cu into the cheesemilk when stainless steel cheese vats are used is reported to have positive effects on cheese proteolysis (Sieber et al., 2006).

The role of the *Lb. rhamnosus* Lc705 protective strain was not shown to have important effects on most of the parameters that influenced the final quality of the cheeses (Figure 5a). However, based on the enumeration results on MRS-vancomycin plates (Figure 2c), it is difficult to determine its role in this study. In any case, the addition of this strain had a negative effect on cheese appearance, mainly because of low eye formation when Cu was not added (Figure 5a).

CONCLUSIONS

The addition of Cu to the cheesemilk at the levels used in Finland for industrial-scale production had some positive effects on the final quality of Emmental cheese. Although the traditional plating systems for enumerating bacterial populations during cheese manufacture did not reveal any drastic differences caused by the presence of Cu, the chemical and sensory analyses suggest that utilization of Cu plays a significant role in regulation of bacterial physiological and biochemical activities, which in turn affect the sensory quality of Emmental cheese.

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