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Original article

MICROBIOLOGICAL AND PHYSICOCHEMICAL CHANGES DURING RIPENING IN BULGARIAN WHITE BRINED CHEESE MADE FROM RAW COW MILK

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Summary

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The main microbiological hazards of raw milk cheese are associated with *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus*. Due to its high nutritional value, cheese is an excellent medium for the growth of these pathogens. This study was aimed to observe microbial dynamics of Bulgarian white brined cheese during cheese production and ripening. Microbiological analysis included determination of *Staphylococcus aureus*, *Listeria spp.* and *Escherichia coli* counts. Some physicochemical parameters, such as total titratable acidity, sodium chloride content, water activity and pH were also examined. Results revealed statistically significant increase in bacterial counts after cheesemaking steps and decrease at the end of the ripening period. *Listeria monocytogenes* was not detected in any of the cheese samples. Raw milk cheese was of unsatisfactory quality that emphasises the need for applying and maintaining good hygiene practices along the food chain to prevent microbial contamination and growth.

Key words: *Escherichia coli*, *Listeria* spp., raw milk cheese, ripening, safety, *Staphylococcus aureus*

INTRODUCTION

White brined cheese is a traditional product of Bulgaria, which has unique flavour and nutritional value. In the past, it has been produced from raw milk but after the introduction of Bulgarian State Standard (BSS), a mandatory pasteurisation of the raw milk used is required. For all kinds of foods, the main preventive measures are based on implementation of procedures to avoid cross contamination and procedures that will secure sufficient heat treatment in order to eliminate most zoonotic bacterial pathogens such as *Salmonella spp.*, *E. coli*, *Campylobacter*, *L. monocytogenes* and *S. aureus*. Today, consumers pay more attention to healthy, high-quality and organic foods by redirecting their demands to traditionally produced raw milk

cheeses. It is believed that raw milk cheeses have stronger and richer flavour and a higher nutritional value than those made from pasteurised milk (Baran *et al.*, 2017). Therefore, the interest is focused on issues related to food safety of cheeses made from raw milk.

In recent years, there has been an increase in the practice of delivering fresh unripened cheeses to the market characterised by a higher water content, unstable consistency and poor organoleptic characteristics. Consumption of this type of cheese may pose a risk to consumers due to the possible presence of pathogenic microorganisms. The commonest pathogenic microorganisms associated with milk or dairy products are *S. aureus*, *Salmonella spp.*, *L. monocytogenes* and pathogenic *E. coli*. A source of pathogenic bacteria is the raw milk, but inappropriate handling, manufacturing, and storage are also considered significant sources of contamination. Cheeses produced in small-scale dairy processing facilities are at a higher risk for environmental contamination because of the presence of pathogenic bacteria in the farm environment that lead to milk contamination. During the cheesemaking process, microorganisms encounter different environmental stress conditions which can affect their growth, survival and adaptation. Ripening is a dynamic microbiological and enzymatic process that plays a natural selective role because of some components such as organic acids, hydrogen peroxide and bacteriocin produced by lactic acid bacteria (Arqués *et al.*, 2015). The levels of harmful microorganisms during the ripening process of cheese made from raw milk are considered to disappear or to be reduced (Fox *et al.*, 2017). The growth of a bacterial population depends on pH, water activity and food product composition. Outbreak-related illnesses caused by consumption of fresh cheese made from raw milk are reported by authors worldwide (Goulet *et al.*, 1995; Johler *et al.*, 2015). For this reason, the US Food and Drug Administration (FDA) requires a minimum of 60-day aging period of cheeses made from unpasteurised milk.

The present study aimed to examine the microbial dynamics of Bulgarian white brined cheese made from raw milk during cheesemaking and ripening, and to evaluate the potential risk of this type of cheese as a vehicle of foodborne illness. The presence and levels of *S. aureus*, *Listeria* spp. and *E. coli* in raw cow milk and cheese were investigated. The study also evaluated the impact of used raw milk on the microbiological safety of the cheese and the dynamic changes in total titratable acidity, sodium chloride content, water activity and pH throughout cheese ripening.

MATERIALS AND METHODS

Cheese production process

A small-scale dairy farm located in Haskovo province, Southern Bulgaria was chosen for raw milk cheese production. Cheese was made without the addition of starter culture. Unpasteurised (14 L) whole milk was put into a cheese vat. The milk was gradually heated to 35 °C with constant gentle agitation. Twelve milliliters rennet (activity 1:12 000, Apolon-69 EOOD, Troyan, Bulgaria) was added, agitated and milk was left 40 min for coagulation. The curd was carefully cut using curd knives (3 cm), left to settle for 30 min and then put in cheesecloth for 1 hour to separate curd from whey. The curd was pressed for 5 hours with a weight of 3 kg. The curd was cut in cheese blocks and placed in a plastic box container. Two hundred grams of salt were distributed on

the surface of the cheese and then it was immersed in 1 liter of the whey. For ripening, fresh cheese was stored at 10–12 ºC for 45 days.

Sample collection and microbial analyses

Triplicate samples (~ 50 ml or g each) were collected from raw milk before addition of the rennet, from the curd after coagulation and from the cheese on days 1, 2, 3, 4, 5, 8, 11, 14, 19, 24, 29, 33, 38 and 45 of ripening. Day 1 corresponds to the unsalted fresh cheese. For cheese samples 10 g of sample were added to 90 mL of Maximum Recovery Diluent (Merck, Germany) in a sterile Stomacher bag, and homogenised by using a stomacher (Stomacher® 400 Circulator, Seward). One milliliter of milk was diluted in 9 mL Maximum Recovery Diluent. All homogenised samples were serially diluted and 0.1 mL of the dilution was plated in duplicate on agar plates.

Total bacterial count (TBC) was determined on Plate Count Agar (Merck, Germany) at 30 °C for 72 h. Three selective culture media were used: Baird-Parker agar (Merck, Germany) with potassium tellurite and egg yolk emulsion for *S. aureus*, ENDO agar (Merck, Germany) for *E. coli* and Listeria agar (base) acc. OTTAVIANI and AGOSTI (Merck, Germany) for *Listeria spp*. The standard method according to ISO 11290-1:1996 was applied for detection of *L. monocytogenes*.

Identification of microbial pathogens

Three to five presumptive *S. aureus*, *E. coli* and *Listeria spp.* colonies were picked and repeatedly sub-cultured on Tryptic Soy agar (Merck, Germany) and incubated at 37 °C for 24 h to obtain pure cultures. Species identification of *E. coli* and *Listeria spp*. was done by colony morphology, Gram staining (HiMedia, India) and following biochemical tests: ENTEROtest 24 N (Erba Lachema s.r.o., Brno, CZ) and HiMotility^{TM} Biochemical Kit for Listeria (HiMedia, India). Staphylococcal isolates were submitted to the following tests: Gram staining, catalase reaction and coagulation of rabbit plasma (BB - NCIPD Ltd., Sofia, Bulgaria). PCR amplification of *16S rRNA* and *nuc* genes was used for identification of *S. aureus*. Specific primers were used for the detection of the genes according to Brakstad *et al.* (1992) and Monday & Bohach (1999). The genomic DNA was extracted by boiling method. PCR protocols were performed for individual genes using only one primer pair for each reaction mixture. PCR amplification was conducted in a final reaction volume of 20 µL. Each reaction mixture contained 1 µL bacterial DNA, 2 µL Reaction Buffer (Thermo Scientific, USA), 1.2 µL $MgCl_2$, 2 µL dNTPs, 0.1 µL of each of the two primers (Eurofins Genomics, Germany), 0.2 µL Taq DNA Polymerase (VWR International, Belgium) and 13.4 µL nuclease free water. DNA amplification was performed in thermocycler (QB-96, Quanta Biotech) using the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 1.30 min), ending with a final extension at 72 °C for 7 min.

The amplified PCR products were separated by gel electrophoresis in 2.5% agarose gel at 100 V for 1 hour, stained with peqGREEN (VWR International, Belgium) and finally visualised and documented under UV Transilluminator (ImageQuant 150, GE Healthcare).

Physicochemical analysis of cheese

Sodium chloride content of the cheese samples was analysed by titration with silver nitrate (Mohr's method). Total titratable acidity (TTA) was determined by titration with standard solution of sodium hydroxide, using 2–3 drops phenolphthalein as an indicator. For the pH measurement the cheese was priorly homogenised. Active acidity (pH) was measured with Sartorius Basic pH Meter with Sartorius PY-P10 ATC Combination Electrode (Goettingen, Germany). The values of water activity (a_w) in cheese were measured by a Rotronic HygroLab (Rotronic AG, Bassersdorf, Switzerland).

Statistical analyses

One-way analysis of variance (ANOVA) was used to determine statistically significant differences between the groups. All quantitative parameters were presented as mean values with standard error of mean (SEM). Pearson correlation was used to analyse the relationship between the different physicochemical parameters and the bacterial counts. All statistical analyses were performed using GraphPad InStat (Graph Pad Software, Inc).

RESULTS

Total bacterial count (TBC) in raw cow milk was 6.87±0.01 $\log_{10}$ cfu/mL that exceeded the hygienic limit. *S. aureus*, *E. coli* and *Listeria spp.* were detected in raw milk and all cheese samples (Table 1 and 2). *S. aureus* count increased significantly after cheesemaking steps ($P<0.001$) and decreased by 2.56 $\log_{10}$ cfu/g at the end of the ripening period. Differences between *S. aureus* counts were observed during the first weeks (days 2–14) and last weeks of ripening (days 30–45) ($P<0.01$). *E. coli* was the numerically predominant pathogen whose number increased after the manufacturing and decreased by 1.60 $\log_{10}$ cfu/g at the end of ripening

Table 1. *S. aureus*, *Listeria spp.* and *E. coli* counts ($\log_{10}$ cfu/mL or g) in raw cow milk, unsalted fresh and ripened cheese. Data are presented as mean ± SEM

	n	Raw cow milk	n	Unsalted fresh cheese	n	Ripened cheese
S. aureus	3	3.56 ± 0.07	3	5.10 ± 0.04***	3	2.54 ± 0.07***
Listeria spp.	3	3.46 ± 0.08	3	4.12 ± 0.02***	3	2.73 ± 0.03***
E. coli	3	6.36 ± 0.02	3	8.01 ± 0.01***	3	6.40 ± 0.01

*** $P<0.001$ vs raw milk; n= number of samples.

Table 2. Bacterial counts of *S. aureus*, *Listeria spp.* and *E. coli* in cheese samples during ripening ($\log_{10}$ cfu/g). Data are presented as mean ± SEM

	n	Days 2–14	n	Days 15–29	n	Days 30–45
S. aureus	21	3.85 ± 0.27	9	3.50 ± 0.04	9	2.57 ± 0.07**
Listeria spp.	21	4.05 ± 0.02	9	3.68 ± 0.05***	9	3.24 ± 0.14***
E. coli	21	7.59 ± 0.04	9	7.03 ± 0.05***	9	6.67 ± 0.07***

** $P<0.01$; *** $P< 0.001$ vs days 2–14; n= number of samples.

Table 3. Physicochemical analysis of Bulgarian white brined cheese made from raw cow milk during ripening. Data are presented as mean ± SEM

	n	Days 2–14	n	Days 15–29	n	Days 30–45
a_w	21	0.959 ± 0.003	9	0.935 ± 0.003***	9	0.931 ± 0.002***
pH	21	6.62 ± 0.02	9	5.57 ± 0.03	9	5.47 ± 0.02**
NaCl %	21	4.33 ± 0.16	9	5.53 ± 0.14***	9	5.15 ± 0.06**
TTA (°T)	21	106 ± 3.96	9	115 ± 1.99	9	148 ± 2.21***

a_w=water activity; TTA= total titratable acidity; ** P<0.01; *** P<0.001 vs days 2–14; n=number of samples.

(P<0.001). Decrease by 1.39 $\log_{10}$ cfu/g was observed for *Listeria spp.* count over the 45 days of ripening (P<0.001). *L. monocytogenes* was not detected in any of the cheese samples.

Results of physicochemical analysis (Table 3) showed that ripening resulted in a decrease in mean pH (P<0.01) and water activity values (P<0.001). A statistically significant difference in salt content was observed among days 2–14 and days 15–29 (P<0.001), and days 2–14 and days 30–45 of ripening (P<0.01). When the mean titratable acidity values were compared, the titratable acidity of cheese samples during the third period of ripening (days 30–45) was higher than cheese samples during the first one (days 2–14) (P<0.001).

Water activity was positively correlated with *Listeria spp.* (r=0.676, P<0.0001) and *E. coli* counts (r=0.778, P<0.0001) during cheese ripening. The survival and growth of microorganisms was affected by active acidity. pH showed positive correlation with *S. aureus* (r=0.501, P<0.01), *Listeria spp*. (r=0.589, P<0.0001) and *E. coli* counts (r=0.499, P<0.01) throughout ripening. Pearson's correlation coefficient showed negative association of NaCl content with *Listeria spp.* (r=–0.487, P<0.01) and *E. coli* counts (r=–0.723, P<0.0001). Total titritable acidity (TTA) had an inhibitory effect both on *Listeria spp.* (r=–0.700, P<0.0001) and *E. coli* (r=–0.801, P<0.0001).

DISCUSSION

In this study a combination of microbiological and physicochemical methods was used to obtain a detailed picture of potential risk of foodborne illnesses linked to raw milk cheese consumption during the ripening process.

During cheese ripening, there was a constant trend in physicochemical parameters, and at the final two weeks of ripening water activity and pH value were decreased. According to Bulgarian State Standard (BSS) 15:2010 the total titratable acidity of Bulgarian white cheese in brine after ripening is from 200 to 270 °T and salt content: 3.5 ± 0.5%. The results from the current study differed from BSS – total titratable acidity was lower but the salt content was much higher. Titratable acidity and pH values of cheese samples were not satisfactory due to the lack of starter cultures and insufficient lactose assimilation. Proper selection of starter culture is one of the most important steps in the manufacture of high-quality and

safe cheeses. The measurement of pH in cheese making is extremely important to control fermentation and hence the final quality of the product. Low pH, salt and water activity are factors which influence growth and survival of bacterial pathogens in this type of product.

Total bacterial count in raw cow milk exceeded the specific hygienic limit of 5 $\log_{10}$ cfu/mL. The high TBC is an indicator of milk quality and showed unhygienic conditions in the small dairy where the cheese was produced. Certain factors including the conditions under which milk is collected and stored prior to processing should be controlled. Microbiological results of cheese samples were compared with Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs for determination of consumer health risk associated with consumption of raw milk cheese. Although the bacterial counts of cheese were the highest during early stages of ripening only and then they declined, the presence of pathogens was still a cause for concern.

Raw milk and raw milk products are frequently implicated in foodborne illnesses caused by enterotoxigaenic *S. aureus* strains. The study of Jørgensen *et al.* (2005) on the origin of *S. aureus* in raw milk and raw milk cheese indicate shedding of the pathogen in milk from infected udder quarters of cows as the most important source of raw cow milk cheeses contamination. Staphylococcal toxins are responsible for staphylococcal food poisoning, toxic shock syndrome, several allergic and autoimmune diseases. Enterotoxigaenic *S. aureus* in fresh and short-time ripened raw milk cheeses may pose a health risk at the time of consumption if the pathogen levels exceed 5 $\log_{10}$ cfu/g (Lindqvist *et al.*, 2002). In the present work *S. aureus* counts exceeded international microbiological recommendations and EU regulations (Anonymous, 2005) in unsalted fresh cheese and in some cheese samples during the first 2 weeks of ripening, which may pose a significant risk for toxin production. Data from Bulgaria show that unripened brined cheese is often implicated in staphylococcal food poisoning, whereas there are no described cases caused by consumption of ripened cheese (Enikova, 2010). Johler *et al.* (2015) reported outbreak of staphylococcal food poisoning among children and staff members at a Swiss boarding school having consumed a soft cheese produced from raw milk. In the current work *S. aureus* grew rapidly during the first hours before immersion in brine, then the growth rate was significantly decreased ($P<0.001$). Delbes *et al.* (2006) also reported maximum *S. aureus* counts after 24 h during the manufacture of raw bovine cheese and afterward, population levels slightly decreased over the 30 days of ripening. According to Charlier *et al.* (2009), the growth of *S. aureus* is influenced by a combination of several physicochemical factors such as pH, temperature and water activity, as well as microbial competition. The positive coefficient of correlation between pH and *S. aureus* might indicate the inhibitory influence of pH on the pathogen. Water activity, NaCl content and TTA had no effect on *S. aureus* survival and growth.

The members of genus *Listeria* are widely distributed in natural environment and commonly found in dairy farms and food processing plants. *L. monocytogenes*, which is capable of growing at refrigeration temperatures, has been involved in several outbreaks attributed to soft or soft-ripened cheeses (Zottola & Smith, 1991). *L. monocytogenes* is a major cause of listeriosis in animals and severe foodborne

disease in humans, responsible for high hospitalisation and mortality rates. Problems with *L. monocytogenes* in farm cheeses exist as evidenced by outbreaks of gastrointestinal listeriosis (Danielsson-Tham *et al.*, 2004). Goulet *et al.* (1995) reported the first listeriosis outbreak linked to consumption of a raw milk cheese documented in France among pregnant women and patients with predisposing conditions. In 2017, 8 confirmed listeriosis cases were reported in the United States as a result of consumption of raw milk cheeses, and 2 deaths occurred (Anonymous, 2017). In the present survey *L. monocytogenes* was not detected unlike data reported by Jakobsen *et al.* (2011) and Kevenk & Gulel (2015) who isolated *L. monocytogenes* in 1.4% and 20% of farm cheeses tested, respectively. Şanlıbaba & Tezel (2018) reported prevalence of 24% *Listeria spp.* in homemade cheese. The significant decrease in water activity led to reduction in *Listeria spp.* count. The low pH had unfavourable influence on *Listeria spp.* growth that was confirmed by the positive coefficient of correlation. Results of correlation analysis suggested a negative correlation of *Listeria spp.* with NaCl content and TTA. That might influence survival and growth of the pathogen.

Most *E. coli* are harmless and normally live in large intestine of people and warm-blooded animals. Raw milk may become contaminated during the milking process with faecal bacteria, including *E. coli*. Contamination usually occurs through the use of contaminated water and unsanitary equipment. However, some *E. coli* have acquired specific virulence genes rendering them pathogenic, meaning they can cause intestinal and extraintestinal infections. Marier *et al.* (1973) documented occurrence of enteropathogenic *E. coli* foodborne disease in the USA due to consumption of cheese. After this outbreak the presence of these microorganisms in cheese acquired additional significance. There have been food poisoning outbreaks linked to raw milk cheeses caused by *E. coli* O157:H7 (Zottola & Smith, 1991; Currie *et al.*, 2018). In the present study the level of *E. coli* was significantly increased after cheesemaking steps and decreased at the end of ripening period to 6.40±0.01 $\log_{10}$ cfu/g, which was similar to the studies of Montet *et al.* (2009) and Peng *et al.* (2013). The cheese had substantial *E. coli* counts that suggest faecal contamination. Cattle are considered to be the main reservoir for this pathogen, and have been implicated as a source of *E. coli* infection and disease in human beings. In this work it was found that *E. coli* can pass to the milk intended for cheese and survive into cheese made from raw cow milk. This fact represents a health hazard, so the investigation of enterotoxigenic *E. coli* in cheeses should be significant for food safety. Decrease in water activity led to slight reduction in *E. coli* count. Significant positive correlation of pH and *E. coli* count during the period of ripening suggests the sensitivity of the pathogen to low pH. The change in NaCl content correlated with *E. coli* count and suggests the inhibitory effect of high salt content. The increase of TTA resulted in the decline of *E. coli* count.

Because *S. aureus*, *Listeria spp.* and *E. coli* populations increased during manufacture of raw milk cheese, it is important to control their levels in raw milk. During the first 2 weeks of ripening an active growth of target pathogens was observed. High water activity and almost neutral pH of the cheese created a favourable growth and survival environment for the pathogens. Cheese consumption dur-

ing this period hides a risk of staphylococcal food poisoning and *E. coli* enteritis. Legalisation of placing on the market unripened fresh cheeses made from raw milk for wide consumption would lead to risk of foodborne illnesses. This work shows the importance of ripening for significant reduction of pathogens in raw milk cheese. *S. aureus*, *Listeria spp.* and *E. coli* counts declined during ripening due to low pH, salt concentration and organic acids. However, the maturation period of 45 days is not sufficient to assure the safety of Bulgarian white brined cheese made from raw cow milk.

Common critical points when making cheese from raw milk are the quality of raw milk, the rate of acidification and the period of maturation. Bulgarian white brined cheese made from raw milk is matured for a long period in brine, and thus the indigenous microflora makes a significant contribution to the maturation process and regulates the quality, safety and shelf-life of the final product. In the current study, results revealed that bacterial counts significantly increased after cheesemaking steps including coagulation of milk, cutting the curd, draining the whey and final pressing. The duration and character of cheesemaking process favour the secondary contamination of milk and curd. The survival and growth of pathogens in cheese depend on many factors, such as the competing microflora, temperature, the presence of lactic acid bacteria, salts, pH, water activity, the length of maturation and the composition of the cheese. The competitive microflora in such cheese is very important in controlling the growth of possible pathogens. Bacteria probably lose their viability under the influence of metabolic products of lactic acid bacteria.

CONCLUSIONS

The results indicate that in general, the hygienic quality of raw milk cheese was of unacceptable microbiological level. *S. aureus*, *E. coli* and *Listeria spp.* were found in cheese at levels that are of concern. The microbiological quality of raw milk has also an important effect on the bacterial counts of the final product. Preventing faecal contamination of milk is an important step in reducing the prevalence of pathogens entering raw milk. However, heat treatment of raw milk is the most important process used to eliminate the risk from viable vegetative bacterial pathogens and provide a safe product. Cheese producers should be aware of raw milk cheese production by maintaining a high standard of hygiene during milking, coupled with the implementation of good hygienic practices at processing.

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