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Influence of milk used for cheese making on microbiological aspects of Camembert-type cheese

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Abstract. Camembert-type cheeses are surface mould-ripened soft cheeses obtained with *Penicillium camemberti*. Soft cheeses are more frequently associated with foodborne disease outbreaks than hard and semi-hard cheeses. During our work, three Camembert-type cheeses were prepared on a pilot/small industrial scale. The first cheese was made from bulk milk and pasteurized at 74 °C for 15 seconds. The second and third cheese were prepared from one type of milk and were heat-treated at 72 °C for 60 seconds. The microbial contamination with *Salmonella* spp. and *Staphylococcus aureus* of the three Camembert-type cheeses was evaluated. The food-related stress survival of *Salmonella* spp. and *S. aureus* isolates originated from the cheese samples was assayed. The antibiotic susceptibility of the bacterial isolates was determined by the disk diffusion method, using 12 and 16 different antibiotics respectively. Based on the results, the first cheese sample contained the highest number of *Salmonella* bacteria; *S. aureus* was detected only in the first sample. According to the results of antibiotic susceptibility of the *Salmonella*, isolates showed susceptibility to the majority of assayed antibiotics and resistance to trimethoprim, chloramphenicol, amikacin, and cefotaxime. The *S. aureus* isolates showed resistance to trimethoprim and displayed intermediate resistance to levofloxacin and ciprofloxacin.

Keywords and phrases: Camembert-type cheese, *Salmonella* spp., *Staphylococcus aureus*, food-related stress, antibiotic resistance

1. Introduction

Cheese, being a dairy product, is an integral part of human diet with its high micro- and macronutrient content and is a good source of proteins, vitamins, fat, fatty acids, and lactose. Due to the rich calcium content, it contributes to skeleton and teeth integrity (*Hamdaoui et al.*, 2021). Globally, 35% of produced raw milk

is transformed into cheese, which is equal approx. to 19×10^6 tons per year, representing a large part of the food industry (*Fox et al.*, 2017).

The Camembert-type cheese belongs to the soft surface-ripened group of cheeses, originated from the Normandy region of France from the 18th century. This speciality of cheese is manufactured all over the world. Traditionally, this type of cheese is made using raw cows' milk, obtaining a product of approximately 10 cm in diameter, having 200–250 g weight. The industrial process uses pasteurized milk (*Fox et al.*, 2017).

Soft ripened cheeses are produced by the fermentation of milk inoculated with mesophilic starter culture to lower the pH. The technology does not include the cooking and pressing steps. These specialities have two types: one with washed rind and the other one with bloomy rind. The *Penicillium camemberti* mould is responsible for the formation of the specific, roughly 3 mm thick white/grey rind. Other microorganisms as *Geotrichum candidum*, *Debaryomyces hansenii*, *Kluyveromyces* spp., and some bacterial species grow on the surface of the cheese contributing to the development of very dark orange or brown layer with a thickness of about 0.5 mm. During the ripening period (3–5 weeks), the cheese undergoes different physicochemical and biochemical changes, becoming soft, fluid, and viscous. As the result of the enzymatic activity of the surface moulds, the pH increases during ripening, which finally leads to mineral migration from the centre to the surface of the cheese. This phenomenon contributes to the swelling and hydration of the proteins (*Leclercq-Perlat*, 2011; *Spinnler*, 2017; *Batty et al.*, 2019).

The mould *P. camemberti* has a key role in the production of different aroma and flavour compounds (*Hong et al.*, 2018). Besides them, other microbes are also involved in the ripening, such as *Lactococcus lactis*, *Leuconostoc mesenteroides*, and surface bacteria *Brevibacterium linens* (*Leclercq-Perlat*, 2011).

The sensory properties of the industrial Camembert-type cheese were improved with the addition of *Lactobacillus rhamnosus* in the case of pasteurized milk (*Galli et al.*, 2019). Some of the predominant characteristics of Camembert-type cheeses include ethyl esters and the ethanol formed by fermentation, while the adjunct cultures with ester-synthesising ability contribute to the formation of the fruity flavour of cheese *(Hong et al.*, 2018).

Cheese quality is determined by multiple parameters, including the raw material, curd formation, and ripening process. Ripening is also influenced by different microbiological, physicochemical, and biochemical factors. The stabilized composition and quality of Camembert-type cheese are achieved by: the use of proper starter species; suitable fermentation conditions, including time/temperature, adequate rate of acidification; curd handling practices; cut size (*Batty et al.*, 2019).

Despite being a popular dairy product, cheese has been frequently associated with foodborne disease outbreaks. The source of pathogens could be direct contamination or cross-contamination during the manufacturing steps. Cheeses

were implicated in severe cases of salmonellosis (*Robinson et al.*, 2020). According to European Regulation No. 2073/2005, 25 g of product should not contain *Salmonella* spp.

Another foodborne pathogen with high prevalence rate in raw milk cheeses is *Staphylococcus aureus*. Their primary source is bovine mastitis, while secondarily they appear as a result of human contamination. These enterotoxin-producing bacteria favour cheese environment due to their high osmotolerance and can survive in low water activity ($a_w = 0.86$) conditions. The heat tolerant enterotoxin in milk can cause food poisoning (*Possas et al.*, 2021).

The aim of the present study was to manufacture Camembert-type cheese from pasteurized milk on a pilot/small industrial scale. To determine the effect of single source and bulk milk on cheese quality, we evaluated the presence of *Salmonella* spp. and *Staphylococcus aureus* as markers in the obtained products. Also, we determined the food-related stress tolerance and antibiotic resistance of the isolated bacteria.

2. Materials and methods

During our work, three Camembert-type cheeses were prepared on a pilot/small industrial scale. The first cheese was made using bulk milk, obtained by mixing milk from several vendors. The raw milk supposed heat treatment at 74 °C for 15 seconds. The second and third cheeses were prepared from one type of milk and were heat treated at 72 °C for 60 seconds. After one week of maturation, the third cheese was placed in a vacuum bag for further maturation.

The manufacturing procedure involved the main technological steps as follows: the refrigerated (4 °C) milk was pasteurized, the calcium chloride (200 g/1,000 L) was added to cooled milk, was inoculated with starter culture (100 DCU/1000 L), renneted (33 ml/1,000 L) and mould-inoculated. After inoculation, coagulation time was 60 minutes, whereafter the curd was cut with harps into cubes with edge lengths of 5 cm. Following the whey separation, the clot was placed into forms and rotated at regular intervals (in every 6 hours) for a day. Finally, the formed wheels were immersed in 22% brine at 14-15 °C for 45 minutes. After the drainage of the solution had taken place, the cheeses were dried on surface at 14-15 °C, relative humidity being 75-80%, and afterwards it was matured (at 16 °C, relative humidity of 80-85%) for a period of three weeks (*Molnár & Molnár*, 1999).

The microbial contamination of three Camembert-type cheeses was carried out in three steps, as follows: firstly, the stock suspension (10 g sample and 90 ml physiological solution) was obtained; secondly, the preparation of dilution series (10^{-1}–10^{-2}) was performed; thirdly, the spreading of a volume of 0.1 ml of the solutions on the used selective agar mediums was made. For detecting the presence

of *Staphylococcus aureus* and *Salmonella* spp., the following mediums were used: Mannitol Salt Agar and ChromoBio® Salmonella Base.

Bacterial isolates with characteristic colony morphology were isolated, and pure cultures were made. The isolated bacterial strains were subjected to confirmation and biochemical tests, which included: Gram-type determination with 3% KOH test, catalase test, indole test, methyl red test, lactose fermentation, gelatine hydrolysis test, and inoculation in medium with 16% NaCl content (*Dunca et al.*, 2004).

The food-related stress survival of five bacterial isolates originated from the Camembert-type cheese was assayed. Osmotic stress was determined in nutrient broth with different NaCl content as: 4%, 6%, 10%, 15%, and 20%. Each of the above mentioned broths were inoculated with 1 ml suspension of bacterial isolates (10^8 CFU/ml) taken in study and incubated at 37 °C for 24 hours. After incubation, the bacterial isolates growth was marked by + or ++ where the survival was positive (*György*, 2020).

With a view to exposing the bacterial isolates to the most selective acid stress possible, they were (10^8 CFU/ml) inoculated in nutrient broth with pH of 3 and 5.5, were adjusted with 1 M HCl with pH 3.5 and 5.5 respectively, and adjusted with lactic acid. The inoculated nutrient broth with different pH was incubated at 37 °C for 1 hour (*Horlbog et al.*, 2018). After this, an amount of 1 ml from each broth was transferred in 9 ml sterile nutrient broth and incubated at 37 °C for 24 hours. Following the incubation, the positive bacterial isolates' survival was marked by + or ++. Bacterial growth at different temperatures (8 °C, 20 °C, and 37 °C) was assayed. The bacterial isolates were inoculated on nutrient agar medium and incubated at the above-mentioned temperatures, and the growth after incubation was evaluated.

The antibiotic susceptibility of the bacterial isolates was determined by the disk diffusion method. A volume of 0.1 ml suspension of bacterial isolates (10^8 CFU/ml) was spread on Mueller–Hinton agar medium plates (Himedia), and the antibiotic disks were placed. A total of 12 different antibiotic discs containing the antibiotics imipenem 10 µg (IPM), meropenem 10 µg (MRP), tigecycline 15 µg (TGC), levofloxacin 5 µg (LE), amikacin 30 µg (AK), cefotaxime 30 µg (CTX), ofloxacin 5 µg (OF), tobramycin 10 µg (TOB), amoxyclav (amoxicillin/clavulonic acid) 30 µg (AMC), trimethoprim 5 µg (TR), gentamicin 10 µg (GEN), and chloramphenicol 30 µg (C) were used for *Salmonella* spp. isolates. For the *Staphylococcus aureus* isolates' antibiotic susceptibility study, 16 antibiotics were used: nalidixic acid 30 µg (NA), rifampicin 5 µg (RIF), tigecycline 15 µg (TGC), erythromycin 15 µg (E), levofloxacin 5 µg (LE), amikacin 30 µg (AK), linezolid 30 µg (LZ), kanamycin 30 µg (K), cefotaxime 30 µg (CTX), clindamycin 2 µg (CD), tobramycin 10 µg (TOB), ciprofloxacin 5 µg (CIP), trimethoprim 5 µg (TR), tetracycline 30 µg (TE), gentamicin 10 µg (GEN), and chloramphenicol 30 µg (C). It was incubated for 24

hrs at 37 °C. The diameter of the inhibition zone was measured, and the results of antimicrobial resistance were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines as susceptible (S), intermediate (I), and resistant (R) (*EUCAST*, 2016; *György et al.*, 2021).

3. Results and discussions

On the surface of the manufactured cheeses, the characteristic rind was properly formed by the mould, and the inside of the cheese was creamy and soft, with mushroom taste.

The pathogens and spoilage microorganisms from raw milk can survive the processing technology. In cheese, different factors contribute to the survival of pathogens, such as temperature, acidification, or the salt and moisture content (*Bachmann et al.*, 2011; *Martin et. al.*, 2021).

S*almonella* is most frequently associated with foodborne infections. Outbreaks caused by *Salmonella* represent a worldwide problem. The infectious dose depends on serotype, host defence, immune status, and, of course, the mode of transmission. The symptoms of salmonellosis include from mild to severe diarrhoeal disease (*Gharpure et al.*, 2021). All over the world, many salmonellosis outbreaks are associated with the consumption of raw milk cheese (*Lobacz & Zulewska*, 2021). The infective dose for gastroenteritis is usually > 10,000 cells, but in high-fat foods, such as cheese, it may be < 100 cells. The infective dose for enteric fever is < 1,000 cells (*Fox et al.*, 2017).

Evaluation of the microbiological quality of the three cheeses resulted that the first sample contained the highest number of *Salmonella* bacteria $3.1 \cdot 10^4$ CFU/g, while the second and third samples contained $9 \cdot 10^2$ CFU/g and $2.7 \cdot 10^2$ CFU/g. *Salmonella* species are Gram-negative, catalase-positive, non-lactose-fermenting, and not indole-producing bacteria. Gram staining and biochemical tests resulted that *Salmonella* isolates originated from cheese samples belong to the *Salmonella* group (*Table 1*).

The nutritional content of cheese favours the growth of *S. aureus.* This ubiquitous bacterium can cause food poisoning with symptoms such as abdominal cramps, vomiting, nausea, and diarrhoea. Coagulase-positive staphylococci can appear in raw milk due to cross-contamination (*Baran et al.*, 2017). Udder infections may be derived from *S. aureus.* Second transmission routes of *S. aureus* are the environment, milking equipment, human handling, and water (*Rosengren et al.*, 2010; *Bachmann et al.*, 2011). Rosengren et al. (2010) determined different types of *S. aureus* strains in cheeses. This suggests the different contamination sources such as human, animal, or environment. *S. aureus* could not be detected from the assayed Camembert-type cheese prepared from pasteurized milk. Only 33% of raw milk cheese contained it.

Table 1. Biochemical characteristics of *Salmonella spp.* isolates originated from Camembert samples

Sample	Gram staining	Catalase test	Lactose fermentation	Indole probe
Sal. 10^{-1} (1)	Gram-negative	Catalase-positive	negative	negative
Sal. 10^{-2} (1)	Gram-negative	Catalase-positive	negative	negative
Sal. A (Vák.)	Gram-negative	Catalase-positive	negative	negative

Notes: Sal. 10^{-1} (1): *Salmonella*, 10-fold dilution, first sample; Sal. 10^{-2} (1): *Salmonella*, 100-fold dilution, first sample; Sal. A (Vák.): *Salmonella*, stock solution, vacuum-packed sample; Sal. A: *Salmonella*, stock solution, second sample.

The high content (100000 CFU g^{-1}) of *S. aureus* is associated with high enterotoxin content, which remains in curd for a long time, whereas the number of *S. aureus* is decreasing during cheese ripening. Based on European Regulation (EC) No. 2073/2005, determination of coagulase-positive staphylococci is required during the manufacturing (*Bachmann et al.*, 2011).

Staphylococcus aureus was detected from the first sample – $3.1 \cdot 10^3$ CFU/g.

Characteristics of *Staphylococcus aureus* are: Gram-positive, catalase-positive, indole-negative, gelatinase-positive, and methyl red positive. Based on the results of biochemical tests, two isolates (S.a. A, S.a. 10^{-2}) were identified as *S. aureus* (notes for isolates: S.a. A: *Staphylococcus aureus*, stock, first sample; S.a. 10^{-2}: *Staphylococcus aureus*, 100-fold dilution, first sample).

The survival rate of the five bacterial isolates in different environmental stress conditions, including osmotic stress, acidic stress, and temperature, was almost the same (*Table 2*).

Table 2. Food-related stress survival of *Salmonella* spp. and *S. aureus* isolates

Bacterial isolates	NaCl					pH				Temperature		
	4%	6%	10%	15%	20%	3.5 (L.a.)	5.5 (L.a.)	3	5.5	8 °C	20 °C	37 °C
Sal. 10^{-1} (1)	++	++	-	-	-	++	++	+	++	+	++	++
Sal. 10^{-2} (1)	++	++	++	+	+	++	++	+	++	+	++	++
Sal. A (Vák.)	++	++	++	+	+	++	++	+	++	+	++	++
S.a. A	++	++	++	+	+	++	++	++	++	+	++	++
S.a. 10^{-2}	++	++	++	+	+	++	++	+	++	+	++	++

L.a.: pH adjusted with lactic acid

No remarkable differences between the isolates were observed. The tested bacteria were more tolerant to 4%, 6%, and 10% NaCl. Slower growth could be detected in the presence of 15% and 20% NaCl.

Bacteria with salt can be eliminated or its growth mitigated. The osmotic pressure effect is the disruption of the osmotic balance between the cytoplasmic and the intracellular membrane. The activation of osmoregulatory systems protects the bacteria from osmotic pressure. For example, in *S. aureus*, osmoprotective compounds are produced. Also, it has been shown that osmotic stress can contribute to the increase of antibiotic resistance (*Horn & Bhunia*, 2018). The growth and development of microorganisms in cheese are determined by different factors (*Picón*, 2018; *György & Laslo*, 2021).

None of the *Salmonella* spp. and *Staphyloccocus* isolates were inactivated by the acidic treatment. With the exception of 3 pH adjusted with 1 M HCl, the isolates grow well, whereas a slight growth was observed in the case of the three *Salmonella* isolates at the mentioned pHs. Isolates were considered as acid tolerant.

In the food matrices, microbial inactivation could be reached through acid stress. This could be achieved through fermentation, addition of organic acids as preservatives, or by acid washes. This stress may lead to cell injury, cell death, or inactivation of enzymes or may affect the transmembrane proton motive force (*Horn & Bhunia*, 2018; *Horlbog et al.*, 2018). It has been shown that *Salmonella* is an acid-adapted bacterium that can survive the acidic condition due to expression of acid shock proteins (*Horn & Bhunia*, 2018).

The bacterial isolates grow well at 20 °C and 37 °C. Also, the low temperature of 8 °C did not affect the growth. It has been shown that cold temperatures (4 °C) did not affect cell number reduction in *S. aureus*. Further, it was demonstrated that the alteration of cytoplasmic metabolites contributes to the survival of *S. aureus* in and its adaptability to different stress conditions (*Alreshidi*, 2020).

According to the results of antibiotic susceptibility, the three *Salmonella* isolates showed susceptibility to the majority of antibiotics. The isolates showed resistance to trimethoprim, chloramphenicol, amikacin, and cefotaxime. These three *Salmonella* isolates can be defined as multi-drug-resistant strains. They exhibit resistance to four classes of antibiotics, which makes them multi-drug-resistant (*Magiorakos et al.*, 2012).

The two *S. aureus* isolates showed resistance to trimethoprim and displayed intermediate resistance to levofloxacin and ciprofloxacin.

Salmonella serotypes with multiple antibiotic resistance has been detected increasingly in the food chain. It has been demonstrated that the spreading of antibiotic resistance in *Salmonella* species, the horizontal transmission of resistance genes plays a crucial role (*Nair et al.*, 2018; *Xu et al.*, 2020).

4. Conclusions

During the production of Camembert-type cheese, it is essential to follow exactly the technological process steps and to assure the proper hygienic conditions. Microbiologically, the raw material quality, the adequate heat treatment, the temperature profile during processing, and the hygienic conditions are of paramount importance.

The microbiological contamination of the cheese sample made from bulk milk was significantly higher in comparison with cheese samples made from milk from a single source. Based on the results of biochemical confirmation tests, *Salmonella* spp. was detected in all of the three cheese samples, whereas the highest cell number was detected in the first sample, in sufficient amount to infect a host. *S. aureus* was present in the first sample as well, but the infestation did not reach the threshold limit to produce enterotoxin.

The presence of *Salmonella* isolates with multiple antibiotic resistance in the cheese sample should be considered a risk indicator.

From the results of the study, it can be concluded that improper food-processing practices could contribute to the survival of foodborne pathogens. To ensure the safety of cheese, raw milk microbiological analysis and adequate pasteurization is needed.

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