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Microbial Quality of Regularly Consumed Imported and Local Bulgarian Cheeses of Upper Price Segment

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Abstract

The rich nutritional content of cheese favors the development both of beneficial and food-borne pathogenic microorganisms. The study aimed to evaluate the microbial quality of seven most frequently consumed cheeses, imported and local Bulgarian. The total number of bacteria, faecal enterococci, staphylococci, *Clostridium perfringens*, *E. coli*, yeasts, and molds were assessed according to ISO standards and EC № 1441 using selective and differential chromogenic media. Our study demonstrated that some cheeses complied with the national and international microbiological standards but some exceeded them. The highest bacterial content was found in sample № 4 of imported goat cheese. Faecal enterococci were detected in all samples except sample № 2 of imported cheese. The highest number of enterococci was established in sample № 7 of local Bulgarian buffalo cheese. Fungi were absent in three samples but their content was 10^2-10^4 CFU/g in four samples. Noble edible molds were detected in the imported cheeses (№ 1-4). Most of the samples were free of staphylococci but in three samples (№ 1, 5, 7) their amount varied between 10^2-10^5 CFU/g product. These findings suggest that raw milk came from cows and she-buffaloes affected by mastitis. *C. perfringens* was absent in all samples except the sample of № 4, imported goat cheese. All samples were free of *E. coli* except sample № 4 (imported goat cheese), where they were detected in a high amount. It is possible that some of the cheeses were contaminated with bacteria and fungi due to improper storage and packaging.

Keywords: cheese, food-borne pathogens, microbial food control

Резюме

Богатото хранително съдържание на сиренета благоприятства развитието както на полезни, така и на патогенни микроорганизми. Изследването ни имаше за цел да анализира микробното качество на седем най-често консумирани вносни и местни български сирена и кашкавали. Общият брой на бактериите, фекалните ентерококи, стафилококите, *Clostridium perfringens, E. coli*, дрождите и плесените беше определен съгласно стандартите ISO и ЕС № 1441, като бяха използвани селективни и диференциални хромогенни хранителни среди. Нашите резултати показаха, че някои от анализираните сирена отговарят на националните и международните микробиологични стандарти, докато други не ги покриват. Установено е, че най-високо бактериално съдържание има проба №4 от вносно козе сирене. Фекални ентерококи бяха установени във всички проби с изключение на проба № 2 от вносно сирене. Най-голям брой ентерококи е установен в проба №7 от местен български биволски кашкавал. В три проби гъбички липсват, но в четири проби съдържанието им е 10^2-10^4 КФЕ/г. Във вносните сирена (№ 1-4) са установени благородни ядливи плесени. Повечето от пробите са без стафилококи (№ 2, 3, 4, 6), но в три проби (№ 1, 5, 7) количеството им варира между 10^2-10^5 КФЕ/г продукт. Резулаттатите касаещи стафилококите предполагат, че суровото мляко идва от крави и/или биволици, страдащи от мастит. *C. perfringens* отсъства във всички проби с изключение на проба № 4, вносно козе сирене. Всички проби са чисти от *E. coli* с изключение отново на проба № 4, където те са открити във високо количество. Възможно е някои от сиренета да са били заразени с бактерии и гъбички поради неправилно съхранение и опаковане.

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Introduction

Nowadays, about 1.3 billion tons of food per year become spoiled or squandered worldwide, which represents about one-third of all food produced for human consumption (Gustavsson, 2011). Along with chemical and physical spoilage, microbiological spoilage can be especially detrimental. Bacteria and fungi are the main agents responsible for microbial spoilage and lead to numerous outbreaks (Garnier *et al*., 2017; Feuerstein *et al*., 2022). Cheese plays a significant role in human nutrition, being one of the most popular ready-to-eat foods produced worldwide. Cheese is rich in microbiota with more than 500 species of LAB (Lactic Acid Bacteria), Gram (+), and Gram (-) bacteria, yeasts, and molds identified in it so far (Almeida *et al*., 2014; Giraffa, 2002; Nunes *et al*., 2016). The rich nutritional content of cheese also favors the development of food-borne pathogenic bacteria. Recently, food-borne pathogens were determined in cheese produced from cow, ewe, and buffalo milk (Resende *et al*., 2018; Klempt *et al*., 2022; Pascu *et al*., 2022; Pasquali *et al*., 2022).

The most frequent food-borne bacteria found in cheese are *E. coli*, staphylococci, and enterococci (Jamet *et al*., 2012; Camargo *et al*., 2021; Feuerstein *et al*., 2022). These bacteria cause human skin lesions, gastrointestinal inflammation, lesions of the internal organs, and renal failure. The increased consumer demand for less preservatives in foods leads to the development of pathogenic bacteria, some of them harmful to immunocompromised consumers (transplanted, hemodialyzed persons with chronic diseases), pregnant women, and children, with an adverse effect on their health.

Besides the presence of pathogenic microorganisms, cheese harbors antimicrobial-resistant bacteria, and yeasts. Feeding animals with antibiotic-supplemented feed causes the emergence of antibiotic-resistant bacteria, which further enter the human gastrointestinal tract via dairy or meat products (Emes *et al.,* 2022). Antibiotic-resistant bacteria found in cheese were reported by Feuerstein *et al.* (2022). Antifungal-resistant yeasts were described by Bintsis *et al.* (2021) and Emes *et al.* (2022).

Our study aimed to enumerate different food-borne microorganisms in imported and local Bulgarian cheeses of the higher price segment.

Materials and Methods

Sample collection and processing

Samples were taken from seven types of high-end quality cheese purchased in big retail stores in Sofia, Bulgaria. Four kinds of cheese were imported from the EU, and three kinds of cheese were local products of Bulgaria (№ 1 - imported soft blue cow cheese, № 2 - imported soft blue cow cheese, № 3 - imported soft white goat cheese, № 4 - imported soft white goat cheese, № 5 – local yellow cow cheese, № 6 - local yellow sheep cheese, and № 7 – local yellow buffalo cheese). Bulgarian yellow hard cheese is also popular as "kashkaval". All samples were analyzed before their expiry date. Samples were prepared as follows: 10 g of each sample were aseptically cut and homogenized in 90ml sterile Ringer's solution. The solution contained NaCl 7.2g , $CaCl_2$ 0.17 g, and KCl 0.37 g dissolved into reagent-grade H_2O and brought to 1L. The solution pH was adjusted to 7.3-7.4. The obtained mixture was then heated to 44°C until complete fat dissolution. Afterward, serial dilutions (10^{-1} to 10^{-4}) were prepared, and 0.1ml of each dilution was spread on selective or differential agar.

Permissible bacterial concentrations or prohibited bacterial groups are set out in the Standard International Microbiological Criteria for Dairy Products, EC Directive 92/46/EEC: Cheeses made from raw milk, and from thermalized milk, and in the Standard US FDA „Grade A" Pasteurized Milk Ordinance 2015 Revision (EC Directive 92/446/EEC: Cheeses made from raw milk and fermented milk).

Total number of viable bacteria

The total number of viable bacteria was examined on Nutrient agar (NA) (Oxoid, England; Hi-Media, India). NA was autoclaved at 121°C /1 atm/20min. Cultivation of inoculated Petri dishes was carried out at 30°C for 48h, and enumeration was done using Koch's method and following ISO 4833-1:2013. According to the standards, viable bacteria counting higher than 10^5-10^6 CFU/g is evidence of poor manufacturing hygiene.

Faecal enterococci

KEAA (Kanamycin-Esculin-Azide agar) (Merck, Germany) was used for the detection of faecal enterococci. The medium was autoclaved under standard conditions. As the enterococci grow, they turn the white-colored agar black due to the metabolism of esculin. Enterococcal colonies grew as transparent to white colonies on it. The addition of kanamycin to the medium inhibited the growth of other Gram (+) bacteria. The medium was autoclaved at 121°C/1 atm /20 min. After plating 0.1ml of each dilution on agar, the Petri dishes were in-

cubated for 48 h at 30°C. The count of enterococci was assessed using the method of Koch, according to ISO/CD 21722.

Fungi (*Candida spp*.)

Both Candida conventional (Hi-Media, India), and differential chromogenic agar (Hi-Media, India) were used for detecting *Candida* spp. Colonies grew black-colored on white agar when using conventional agar, and white-to-rose colored when using differential chromogenic agar. While the conventional agar was autoclaved, the chromogenic agar was prepared by boiling according to the manufacturer's instructions. Incubation of the inoculated plates lasted 7 days at 28°C, and the enumeration was done according to Koch's method, and following ISO 21527-1:2008.

Staphylococci

MSA (Manitol-Salt agar, 6.5% NaCl) (Merck, Germany) was applied in the study of the genus *Staphylococcus*. The medium was autoclaved at 121°C/1atm/20 min. On the orange-pink-colored agar, staphylococcal colonies grew in golden color surrounded by a well-defined yellow halo. Inoculated Petri dishes were incubated for 48h at 30°C, and enumerated by the method of Koch, and according to ISO 6888-1:2021.

Clostridium perfringens

The pathogenic *C. perfringens* was enumerated on SPS agar (Sulphite-Polymyxin-Sulphadiazine Agar) (Hi-Media, India). The medium was autoclaved at standard conditions. 1ml inoculum of each decimal dilution was poured into the bottom of the Petri dish, and then covered with 20 ml of SPS agar for deep seeding, creating in this way anaerobic conditions necessary for clostridia. Incubation of the inoculated Petri dishes lasted 48 h at 37°C. Enumeration was carried out using the method of Koch and in concordance with ISO 7937:2004.

E. coli and coliforms

VRBA agar (Violet Red Bile Agar) (Sigma-Aldrich) was used to differentiate the number of *E. coli* and coliforms. The medium was autoclaved at standard conditions. Incubation was performed for 48h at 30°C, and the number of *E. coli* and coliforms was assessed using the method of Koch and ISO 16649-1:2018. All experiments were performed in triplicate and repeated three times with similar results.

Results and Discussion

Although European dairy products meet high technological standards, there are quality issues regarding milk production, transportation, storage, and handling, which may reduce the final product quality. The most commonly consumed cheeses in Bulgaria are soft and yellow ones produced from cows, sheep, buffalo, and goat milk. The selection of studied cheeses was made based on being frequently purchased by Bulgarians, representing cheeses of the high-price segment. It should be mentioned that the diversity of cheese in Bulgaria is not high.

Studies on the microbial quality of varieties of cheese in Austria (Schornsteiner *et al*., 2014), Brazil (Nunes *et al*., 2016; Camargo *et al.,* 2021), France (Jamet *et al*., 2012), Germany (Resende *et al*., 2018), Italy (Almeida *et al*., 2014), Mexico (Escobar-Zapeda *et al*., 2016), Portugal (Costa *et al*., 2022; Riquelme *et al*., 2015), Romania (Pascu *et al*., 2022), Spain (Nieto-Aribas *et al.*, 2011), Serbia (Bulajik *et al*., 2017) have been recently reported. Investigations are focused on the assessment of certain pathogenic microorganisms in cheese. Cheese microbiota counts are presented in Table 1.

Total number of viable bacteria

Our investigation revealed that the total number of bacteria in the examined goat, cow, sheep, and buffalo cheese samples was between $6.9x10^5$ and $1.4x10^9$ CFU/g. Considerable bacterial content exceeding the permissible limits was determined in sample № 4 – $1.4x10^9$ CFU/g (Table 1). As recommended by international standards, (EC Directive 92/446/EEC: Cheeses made from raw milk and from fermented milk; Standard EC Regulation № 2073/2005; Standard US FDA "Grade A" Pasteurized milk Ordinance 2015 Revision) the upper limit of total bacteria count in raw unpasteurized milk is $1x10^5$ -$1x10^6$ CFU/g. Cheese produced from unpasteurized milk is abundant in bacteria coming from the gastrointestinal tract of animals as well as from industrial equipment. Montel *et al.* (2014) reported 8.2 $x10^5$ CFU/g, and Moraes *et al.* (2012) described even larger than 10^6 CFU/g total mesophilic, and aerobic mesophiles in cheese sample from Brazil. The possible reasons for the high total number of bacteria in some of the samples analyzed are the inappropriate conditions during transportation and storage of the goods.

Faecal enterococci

In terms of fecal enterococci, our results exhibited a large variation in their number among the cheese samples 0 – $1.1x10^6$ CFU/g. As seen in Table 1, the highest number of enterococci was detected again in sample № 4 - >10^5 CFU/g, and in sample

Table 1. Enumeration of bacteria, yeasts, and molds in imported and local Bulgarian cheeses

№	Sample	Total number of bacteria	Faecal enterococci	*Staphylococci*	*E. coli*	*C. perfringens*	Yeasts and Molds
1	Blue cow cheese Imported	$5.3x10^6$	$3.6x10^4$	$\geq 10^4$	0	0	*P. roqueforty*
2	Blue cow cheese Imported	$2.2x10^5$	0	0	0	0	*P. roqueforty*
3	White goat cheese Imported	$7.1x10^6$	$2.4x10^2$	0	0	0	*P. camemberti*
4	White goat cheese Imported	$1.4x10^9$	$\geq 10^5$	0	$> 10^5$	$< 10^2$	*P. camemberti*
5	Yellow cow cheese (kashkaval), Bulgaria	$6.9x10^5$	$2.8x10^5$	$\geq 10^5$	0	0	$5.0x10^2$ (yeasts)
6	Yellow sheep cheese (kashkaval), Bulgaria	$2.4x10^7$	$6.1x10^3$	0	0	0	$4.9x10^3$ (yeasts)
7	Yellow buffalo cheese (kashkaval). Bulgaria	$3.0x10^7$	$1.1x10^6$	$2.0x10^2$	0	0	$1.3x10^6$ (yeasts)

№ 7– 10^6 CFU/g. Only one sample - №2 (imported blue cow cheese) was free of enterococci. Enterococci in Mozarella were reported by Giraffa (2002). Only one sample was enterococci-free: № 2 (imported soft blue cow cheese). No maximum permissive limits of enterococci in cheese are mentioned in the EC Regulation 1441. It is so because enterococci were long time considered only probiotic bacteria but the recently increased number of papers reported them as the cause of numerous enterococcal outbreaks. Enterococcal isolates obtained in our study - *E. durans* and the two strains of *E. faecalis* were recovered from goat and cow cheeses (Table 2). *E. faecalis*, *E. durans,* and *E. casseliflavus* have been reported by Gelsomino *et al.* (2002), Pascualli *et al*. (2022), and found in Cheddar cheese produced from cow milk, and Italian cow artisanal cheese. Besides *E. faecalis* and *E. caseliflavus* Resende *et al.* (2018) found *E. faecium* in Brazilian Minas soft cow cheese, and *E. durans* in artisanal cheese from the Western Balkans (Jahansepas *et al*., 2022). Antibiotic resistance of enterococci was reported in a series of papers published by Giraffa (2002), Jamet *et al.* (2012), Perin *et al.* (2014), Gaglio *et al.* (2014), Pasquali *et al.* (2022). Apart from their wide antibiotic resistance, enterococci were proven to infect immunocompromised patients (Chajecka-Wierzchowska *et al*., 2017). Malti *et al.* (2015) even described that enterococci are capable of crossing the brain barrier, and can be responsible for brain abscesses.

Staphylococci

Regarding the staphylococci, the results showed the absence of staphylococci in four out of seven samples. Three samples contained staphylococci (samples № 1, 5, and 7). It is worth highlighting that staphylococci were found in an unusually increased concentration of $\geq 10^5$ CFU/g in only one sample - № 5. One more sample possessed a relatively high staphylococcal amount - sample № 1 $\geq 10^4$ CFU/g. Four cheese samples - № 2, 3, 4, and 6 were staphylococci free. According to EC Standard 2073/2005, the limit for staphylococci in cheeses produced from raw milk is $1x10^4$-$1x10^5$ CFU/g. Serrano *et al.* (2018) reported $8.8x10^4$ CFU/g of staphylococci in seven Swiss cheeses. *Staphylococcus aureus* has been manifested as a controversial food-borne pathogen due to its resistance to methicillin (MRSA). MRSA are associated with severe infections in humans, and animals, such as bacteremia, wound infections, pyogenic lesions, and mastitis (Podkowik *et al*., 2013; Sergelidis *et al*., 2017). MRSA staphylococci were recently identified in cheese, as reported by Steinka *et al*. (2018). Additionally, staphylococci isolated from traditional raw milk cheeses in Serbia were reported by Bulajic *et al.* (2017). The research group of Casaes Nunes (2016) documented enterotoxin production and the presence of antimicrobial-resistant staphylococci in Frescal cheese from Brazil, one of the most popular cheeses in this country.

During our experiments, one isolate of *Staphylococcus simulans* was obtained from sample № 5 (local cow yellow cheese, "kashkaval"). Recently, this bacterium has been considered as an emerging cutaneous pathogen. Generally, *S. simulans* is a well-established animal pathogen affecting cows, sheep, goats, and horses and can be easily transmitted to food of such origin. Besides, it is known as the causative agent of bovine mastitis. Bridget

et al. (2016) described severe osteoarthritis, and 5-month swelling of the right toe of a farmer due to *S. simulans*. Another report of *S. simulans* as a skin infectious agent and an authentic pathogenic agent of osteoarticular infections was described by Mallet *et al.* (2011). *S. simulans* grew in the synovial fluid and blood cultures in patients in France. It was also recently described as strongly associated with endocarditis both in broilers and in humans. A putative reason for the high amount of staphylococci found in cheese sample № 5 (local cow yellow cheese) is the collection of raw milk from cows affected by mastitis.

C. perfringens

The analysis of *C. perfringens* was unambiguous and showed that all samples except sample №4 were free of *C. perfringens*. In sample № 4 (imported goat cheese) *C. perfringens* was enumerated as 10^2 CFU/g. It is known that 1 million people worldwide are annually affected by *C. perfringens*. The presence of *C. perfringens* in cheese samples is not routinely investigated during food analyses although it is one of the most common causative agents of food poisoning. This bacterium is a Gram (+), motile, spore-forming, and aerobic pathogen. *C. perfringens* causes tissue necrosis, bacteremia, emphysematous cholecystitis, and gas gangrene. The mechanism of intoxication with this pathogen involves the ingestion of 10^6-10^7 living cells per gram of food. The harmful effect of *C. perfringens* is mainly due to its enterotoxin, produced in the large human intestine during sporulation of the microorganism, and released upon lysis of its sporangia. Many countries do not have microbial criteria for *C. perfringens* in foods. Very strict criteria are applied in South Korea (n=5, o=0, m=0 for 25 g).

E. coli and coliforms

Our results demonstrated that *E. coli* and other coliforms were absent in six out of seven cheese samples. Again one sample - № 4 (imported goat cheese) contained a high number of *E. coli* exceeding the standard. (>10^5 CFU/g). According to the EC standard, the limit for *E. coli* is 1x10^4-1x10^5 CFU/g. All studied cheese samples except one were *E. coli*-free. A high level of *E. coli* at a concentration of 1x10^5 CFU/g was found in sample № 4 (white goat cheese, imported). Total coliforms to the amount of 1x10^3 CFU/g were reported for Brazilian cheeses by Moraes *et al.* (2012). *E. coli* itself was between 1x10^2 and 3.5x 10^6 CFU/g.

Yeasts and molds

The counts of yeasts and molds varied among the studied cheese samples. The blue and white cheeses (№ 1, 2, 3, and 4, imported) showed growth solely of noble molds, and no yeasts. Two noble molds that improve the cheese flavor and taste grew as a diaper in all four imported cheeses were detected - *Penicillium roqueforti* (№ 1 and № 2) and *P. camemberti* (№3 and №4). In samples № 5, 6, and 7 only yeasts and no molds were found. Yeasts in the latest three samples were enumerated as follows: 5.0.x10^2, 4.9.x10^3, and 1.3x10^6 CFU/g, respectively. Our results revealed that in three samples (№ 1, 2, and 5) fungi (*Candida* spp.) were absent, while in the other four samples (№ 3, 4, 6, and 7) they were found in amounts of 10^4, 10^4, 10^2 and 10^3 CFU/g, respectively. The highest amount of yeasts was found in sample 7 - local Bulgarian buffalo yellow cheese. The lack of yeasts in the blue and white soft cheeses is explainable as these cheeses undergo additional processing with inoculation of molds. Molds were not detected in samples of yellow cow, sheep, and buffalo cheeses "kashkaval", as this kind of cheese ripens without the addition of noble molds. Pathogenic molds were not detected in them.

Yeasts are part of our normal microflora, and invasive infections only occur as a result of impaired immune function. Entering the bloodstream, they cause fungemia. By contrast, molds are ubiquitous, and as their conidia are inhaled daily, infections generally occur in the airways. As described by Garnier *et al*. (2017) yeasts and molds can grow in a wide variety of foods, including both raw foods such as fruits, cereals, vegetables, meat, and milk, and processed foods. *Candida spp*. are frequent spoilers of fresh dairy products like fresh cheese, cream, yogurt, and olives (Satchanska *et al.,* 2019). As reported by Bintsis (2021), total raw milk yeast counts are generally in the range of 10–10^3 CFU/ml, and yeast genera commonly identified in raw milk include *Candida*, *Cryptococcus*, *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Trichosporon*, *Pichia,* and *Rhodotorula*. The same author reported at least six *Candida* species found in cheese: *Candida catenulate*, *C. etchellsii, C. glaebosa, Candida lambica, C. parapsilosis,* and *C. zeylanoides*.

Usually, heat treatment eliminates yeast contamination of milk. Garnier *et al.* (2017) reported a disturbing finding that certain yeast species are highly resistant to pasteurization. The high number of yeasts frequently observed in cheese could be related to their ability to develop at low temperatures, ferment lactose, and assimilate organic acids, and most importantly, they are resistant to high salt concentrations.

Conclusions

Our study demonstrated that some analyzed cheeses complied with the national and international microbiological standards but some exceeded them. The highest bacterial content was found in sample № 4 of imported goat cheese. Faecal enterococci were detected in all samples except sample № 2 of imported cheese. The highest number of enterococci was established in sample № 7 of local Bulgarian buffalo cheese. Fungi were absent in three samples but their content was 10^2-10^4 CFU/g in four samples. Noble edible molds were detected in the imported cheeses (№ 1-4). Most of the samples were free of staphylococci (№ 2, 3, 4, 6) but in three samples (№ 1, 5, 7) their amount varied between 10^2-10^5 CFU/g product. These findings concerning staphyloccci suggest that raw milk came from cows and she-buffaloes affected by mastitis. *C. perfringens* was absent in all samples except the sample of № 4, imported goat cheese. All samples were free of *E. coli* except sample № 4 (imported goat cheese), where they were detected in a high amount - 10^5 CFU/g. It is possible that some of the cheeses were contaminated with bacteria, and fungi due to improper storage and packaging.

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