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Original article

STUDY ON THE PREVALENCE OF LIPOLYTIC YEASTS AND MOULDS IN RAW COW MILK AND WHITE BRINED CHEESE

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Summary

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The extent of microbial contamination with moulds and yeasts of raw cow milk and white brined cheese produced in Bulgarian dairy enterprises was evaluated. The isolation and identification of microorganisms was performed using the classical microbiological methods. The samples of raw milk showed 76.6% contamination with moulds, at average amount of 3.4 $\log_{10}$ cfu/mL, maximum of 4.8 $\log_{10}$ cfu/mL. Yeasts were detected in 93.3% of the samples (average 3.5 $\log_{10}$ cfu/mL). Isolated moulds belonged to the genera *Aspergillus* (37.9%), *Geotrichum* (29.3%), *Mucor* (15.5%), *Cladosporium* (5.2%) and *Penicillium* (12.1%). Overall, 70% of studied samples were found to be contaminated with yeasts and moulds at 2.2 $\log_{10}$ cfu/g and 2.8 $\log_{10}$ cfu/g, respectively. Most commonly, isolates from white brined cheese were from the genera *Geotrichum* (32.8%), *Aspergillus* (28.1%), *Mucor* (21.8%) and *Penicillium* (9.4%). The predominating yeasts in raw cow milk and white brined cheese were *Candida spp*., *Rhodotorula spp.* and *Sacharomyces spp*. Isolates were tested for lipolytic activity on Tributyrin agar. The occurrence of lipolytic and toxigenic moulds and yeasts poses a risk for the quality and safety of milk and dairy products.

Key words: milk, moulds, white brined cheese, yeasts

INTRODUCTION

Moulds and yeasts can be found in raw milk and eliminated through pasteurisation, with their presence being an indication of secondary contamination during production (Jodral *et al.*, 1993). The contamination of dairy products with these microorganisms, more specifically of cheese, originates from the technological equipment at dairy processing plants, from water, brine and other components (Chapman & Sharpe, 1990). Moulds and yeasts grow within a wide temperature and pH range, and these factors do not limit their harmful effect on dairy products, because of which Pitt & Hocking (1997) proposed their amount as a criterion for the sanitary condition of dairy products.

The presence of moulds and yeasts in dairy products is undesirable from several perspectives, first among which are the alterations that they cause to the sensory parameters of the products. The proteolytic activity of certain species of moulds and yeasts causes imperfections in a number of flavour traits (Viljoen & Greyling, 1995). The species *Geotrichum candinum* is one of the most undesirable contaminants of cheese, similar to *Listeria monocytogenes* (Hubecova *et al.,* 2009). The presence of moulds and yeasts in ready dairy products is undesirable due to the production of metabolites, short-chain fatty acids and other chemical compounds, which harm the beneficial intestinal microflora in humans (Jacobsen & Narvhus, 1996). A significant issue in food safety is that some species of moulds produce mycotoxins, which are harmful to human health and are transferred through the food chain. Among the most dangerous ones in milk and dairy products, AFM_1 is the result of biotransformation of the AFM_1 in lactating cows, while sterigmatocystin is produced by *Aspergillus versicolor, A. nidulans*, etc. (Van Egmond *et al.*, 1997).

The moulds cause major changes in the food products during their storage, which makes them unsuitable for consumption due to reduction of their nutritional value or accumulation of mycotoxins. Mould growth on cheese is a common problem during maturation at dairy processing facilities, and also in retail products for the end user during prolonged refrigerator storage. The moulds of the *Penicillium* and the *Aspergillus* genera are the most common contaminants (Gandomi *et al.*, 2009) which could be pathogenic for humans and animals (Balajee *et al.*, 2007).

During an extended study of 297 milk samples encompassing 4 geographic regions in the Republic of Serbia, a high prevalence of moulds was established (with respective values of 91.3% in the fall and 46.64% in the winter), with the predominant species being of the *Penicillium* genus (69.3%) during the spring, the *Geotrichum* genus (27.0%) during the fall, and the *Aspergillus* genus (20.9%) during the winter (Pesic-Mikulec *et al.*, 2005). A similar study in Libya reported that 80% of the raw milk samples were contaminated with moulds, with an average quantity of 4.3×10^5 cfu/mL, whereas 50% of ready yogurt batches were contaminated at 2.1×10^4 cfu/mL, with predominant species of the *Aspergillus, Penicillium, Cladosporium, Mucor and Geotrichum* genera (El-Diasty & El-Kaseh, 2009). *Penicillium* and *Aspergillus* species were found in the traditional local Egyptian cheese, in quantities of 0.8×10^5 to 1.6×10^5 cfu/g in six different producers (El-Fadaly *et al.*, 2015).

There are no current studies in Bulgaria regarding the prevalence and species variety of moulds and yeasts in dairy products, especially in view of the technological changes that have occurred in dairy production over the last 20 years. The goal of the present study was to determine the level of contamination with moulds and yeasts of raw cow's milk and the produced batches of white brined cheese at small factories, as well as to determine the lipolytic activity of the isolated strains.

MATERIALS AND METHODS

Samples

Thirty samples of raw cow milk and thirty samples of white brined cheese were collected from the central southern region of Bulgaria during the autumn and winter periods. Raw milk samples were directly

obtained from the cooling bulk milk tank at 3 dairy farms using a central milk duct for milking of cows. Sterile 100 mL glass flasks were used for sample collection. Cheese samples were obtained from 3 small scale dairies (up to 5 000 L/day), processing milk from the region of surveyed farms. Each sample was 900 g, vacuum packed after 45 days of ripening under controlled temperature and humidity. After collection, samples were transported to the lab in a cooling bag (2–6 °C) and analysed within 24 h.

Nutrient media

Moulds and yeasts were isolated and quantitated on agar with yeast extract, glucose and chloramphenicol (YGC) (Merck Darmstadt, Germany) with final pH 6.6±0.2. Selective medium *Aspergillus flavus/parasiticus* agar (AFPA base) (Oxoid, England) with final pH 6.3±0.2 supplemented with chloramphenicol (Oxoid, England) was utilised for isolation and identification. Yeast extract sucrose (YES) agar (Samson & Hoekstra, 2000) prepared from 20 g yeast extract, 20 g/L agar-agar as well as YGC agar supplemented with 0.3% methyl-beta-cyclodextrin and 0.6% sodium deoxycholate were used for detection of aflatoxin-producing moulds (Ordaz *et al.*, 2003). The lipolytic activity of isolated moulds and yeasts was determined on tributyrin agar (Merck Darmstadt, Germany).

Sample preparation

Tenfold serial dilutions were prepared in the lab after homogenisation of the samples. After vortexing, serial dilutions of raw milk were prepared in tubes containing 9 mL sterile buffered peptone water. Ten g from the centre of the cheese samples were aseptically removed and homogenised in 90 mL sterile buffered peptone water for 5 min and thereafter, serial dilutions in tubes containing 9 mL sterile buffered peptone water were prepared. From each dilution, 1 mL were inoculated on a YGC agar plate. The presence of mould or yeast colonies was detected after 5 day-incubation of plates at 25 °C (BSS EN ISO 6611:2004). The number of colonies expressed as colony-forming units (cfu/mL or cfu/g) was calculated and presented as ($\log_{10}$ cfu/mL or $\log_{10}$ cfu/g).

Isolation and identification of mould*s*

Every morphologically distinct mould colony was reinoculated onto YGC and AFPA agar plates. YGC agar plates were incubated for 5 days at 25 °C, and those with AFPA agar – for 42 h at 30 °C. The preliminary classification of colonies on solid YGC and AFPA media was based on colony morphology traits such as pigmentation, shape, coloration on the underside, micromorphology of moulds on native preparation under oil immersion 100/1.25 (Samson & Hoekstra, 2000). The identification of *A. flavus* and *A. parasiticus* was done on AFPA agar by the presence of yellow to orange coloration of the plate underside (Pitt *et al.*, 1983).

Isolation and identification of yeasts

Colonies with yeast-specific morphology were identified using a method including successive tests for growth on Sabouraud dextrose agar, formation of ascospores, vegetative replication, fermentation and utilisation of sugars, utilisation of nitrates and urea hydrolysis (Lodder & Kriger-Van Rij, 1970).

Determination of lipolytic activity of isolated moulds and yeasts

The lipolytic activity of mould and yeast isolates was determined after individual inoculation of each isolate on tributyrin

agar plates. The plates were incubated at 30 °C for 3 days, an the lipolytic activity was evaluated by size of the transparent zone around the colony (mm). The extent of activity was interpreted as: (+) zone of 1 mm; (++) zone of 2–3 mm and (+++) zone of 3–4 mm or higher (Koburger & Jacger, 1987).

Identification of aflatoxin-producing microbial species

Aflatoxin production by moulds was detected on YES and YGC agar media (Ordaz *et al.*, 2003). Plates with tested isolates were incubated for 3 days at 28 °C and then observed under UV light (365 nm). Toxin-producing strains exhibited a white fluorescent zone around the colony.

Statistical analysis

Statistical analysis of data was done with the Statistica 6® software.

RESULTS

The examined samples of raw milk exhibited 76.6% mould contamination, indicating a mixed contamination of two or more mould species. The average extent of mould contamination was 3.4 $\log_{10}$ cfu/mL, with a maximum value of 4.8 $\log_{10}$ cfu/mL (Table 1). A total of 58 strains were isolated, belonging to the genera *Aspergillus* (37.9%), *Geotrichum* (29.3%), *Mucor* (15.5%), *Cladosporium* (5.2%) and *Penicillium* (12.1%) (Table 2). *Aspergillus* spp. was represented by the species *Aspergillus niger* (17.2%), *Aspergillus nidulans* (12.1%) and *Aspergillus flavus* (8.6%). A total of 93.3% of the milk samples were positive for yeast contamination, in the amount of 3.5 $\log_{10}$ cfu/mL. The predominant species isolated were *Candida genera* (70%), followed by *Rhodotorula spp.* (16.6%) and *Saccharomyces spp.* (13.3%) (Table 2).

From the examined samples of white brined cheese, 70% were contaminated with moulds and 63.3% with yeasts, with a mean quantity of 2.8 $\log_{10}$cfu/g and 2.2 $\log_{10}$cfu/g (Table 1). Among the moulds, the predominant species were *Geotrichum* (32.8%) followed by *Aspergillus* (28.2%) and *Mucor* (21.8%). Among the yeasts, the most prevalent were *Candida genera* (50%) and *Rhodotorula spp.* (40%) (Table 2).

The results presented in Table 2 indicate that most of the isolated moulds strains exhibited pronounced lipolytic activity, which was most obvious in the representatives of the genus *Geotrichum*, *Mucor*, as well as some *Aspergillus* species. In the *Geotrichum* genus, 17 of the isolates exhibited strong lipolytic activity, while 4 had medium values. All 14 *Mucor* isolates had strong lipolytic activity. Eight strains of the *Cladosporium spp.* exhibited a complete lack of lipolytic activity.

The *Aspergillus flavus* mould strains (n=11) isolated during the present study were tested for phenotype expression of aflatoxin-producing traits on supplemented YGC and YES agar. In 2/11 strains

Table 1. Presence of moulds and yeasts in milk and white cheese

Samples	n	Positive samples			
		Moulds		Yeasts	
		n (%)	$\log_{10}$cfu/mL	n (%)	$\log_{10}$cfu/g
Raw milk	30	23 (76.6%)	3.4±0.23	28 (93.3%)	3.5±0.42
Cheese	30	21 (70.0%)	2.8±2.23	19 (63.3%)	2.2±0.15

Table 2. Lipolytic activity of yeasts and moulds in raw milk and cheese

Species	Raw milk					White cheese				
	n (%)	+++	++	+	–	n (%)	+++	++	+	–
Moulds										
Aspergillus niger	10 (17.2%)	7	0	2	1	9 (14.1%)	5	1	2	1
Aspergillus flavus	5 (8.6%)	3	1	0	1	6 (9.4%)	4	2	0	2
Aspergilus nidulans	7 (12.1%)	4	0	1	3	3 (4.7%)	2	0	0	1
Geotrichum spp.	17 (29.3%)	10	7	0	0	21 (32.8%)	17	4	0	0
Mucor spp.	9 (15.5%)	9	0	0	0	14 (21.8%)	14	0	0	0
Penicillum spp.	7 (12.1%)	5	0	1	1	6 (9.4%)	5	0	1	1
Cladosporium spp.	3 (5.2%)	0	0	0	3	5 (7.8%)	0	0	0	5
Yeasts										
Candida genera	21 (70.0%)	16	0	3	2	25 (50.0%)	18	0	5	3
Rhodotorula spp.	5 (16.6%)	3	1	1	0	20 (40.0%)	7	5	7	1
Saccharomyces spp.	4 (13.3%)	1	3	0	0	5 (10.0%)	1	4	0	0

+++ strong; ++ average; + weak; – no activity.

Table 3. Total number of moulds and yeasts in white brined cheese samples from three different producers (log_{10}cfu/g)

Statistical parameters	Moulds	Yeasts
Producer No 1 (n=10)		
Mean	3.3	3.7
SD	2.53	2.13
Min	1.4	2.3
Max	5.3	5.7
Producer No 2 (n=10)		
Mean	3.0	3.3
SD	2.32	2.50
Min	1.2	1.6
Max	4.9	5.4
Producer No 3 (n=10)		
Mean	2.4	2.5
SD	1.97	1.94
Min	1.1	2.3
Max	5.6	5.8

(18.1%), the typical phenotype aflatoxin characteristic was found.

Samples of white brined cheese from three different milk processing facilities were examined to compare their microbiological parameters. The results for the total number of moulds showed high values in all three producers, with the highest contamination being observed in producer 1, with mean values of 3.3 log_{10}cfu/g, while for the other producers it was, respectively, 3.0 and 2.4 log_{10}cfu/g (Table 3). Total numbers of yeasts were 3.7, 3.3 and 2.5 log_{10}cfu/g for producer 1, 2 and 3 respectively.

DISCUSSION

The contamination of raw milk with moulds and yeasts is considered to be a reflection of hygienic conditions during the processes of milking and milk storage. We detected a large total amount of moulds (3.4 log_{10} cfu/mL) and yeasts (3.5 log_{10} cfu/mL) in the examined milk, exhibiting contamination with more than two taxonomic types. According to Viljoen (2001), the number of yeasts in the raw milk remains low due to the competitive utilisation of growth substrates from the fast-replicating psychrotrophic bacteria

and the secreted inhibiting bacterial metabolites. The yeast contamination, proven by our study, with a quantity of 3.5 $\log_{10}$ cfu/mL was higher than the findings in raw milk in various regions of Sardinia (2.64 $\log_{10}$cfu/mL) or Slovenia (1.7 $\log_{10}$cfu/mL) (Fadda *et al.*, 2004; Torkar & Vengušt, 2008). Separate farms from the central region of Slovenia exhibited contamination at a level close to ours (4.1 $\log_{10}$ cfu/mL), including for the values of the maximum mould contamination (3.1 $\log_{10}$ cfu/mL).

Our results regarding the isolated mould species indicated that the predominant species were *Geotrichum* (32.8%), *Aspergillus* (28.2%) and *Mucor* (21.8%). Torkar & Vengušt (2008) also found that the genus *Geotrichum* (51.5%) dominated over *Aspergilus* (33.8%) and *Mucor* (5.9%), whereas Jodral *et al.* (1993) isolated most commonly *Geotrichum* (76.5%), followed by *Fusarium* (45.3%) and *Aspergillus* (31.2). The variety of mould species established in raw milk in this study coincided with the findings of other authors regarding the mould species found in the feed and bedding. A study by O'Brien *et al.* (2005) reported that 91% of the compressed grass for feeding was contaminated with moulds belonging primarily to the *Penicillium, Geotrichum, Fusarium* and *Mucor* genera. Other authors have reported an increased amount of moulds during the autumn months, and, respectively, the highest level of mycotoxins (AFM_1) in the milk in December (Kamkar, 2005). The author points out as a primary contaminating factor directing milking at the barn, where air contamination with dust particles from the bedding and feed was high, causing them to fall in the raw milk.

Moulds in white brine cheese were found in 21 (70.0%) of the examined 30 samples, with an average quantity of 2.8 $\log_{10}$cfu/g, and high prevalence of species from *Geotrichum* (32.8%), *Aspergillus* (28.1%), *Mucor* (21.8%) and *Penicillium* (9.4%) genera. Our results were close to the contamination values obtained by Torkar & Vengušt (2008) in soft, hard and semi-hard cheese, in respective amounts of 2.8, 2.2 and 1.3 $\log_{10}$ cfu/g. Significantly higher than our values were reported by El-Diasty & Salem (2007) in Kareish cheese, indicating moulds in the amount of 3.61 $\log_{10}$cfu/g. A high contamination reaching up to 6.78 $\log_{10}$ cfu/g was reported by Khair Allah (2000). The differences in the contamination depend on the technological processes, particularly on the application of thermal processing and the employed manual labour during the process of formation and packing. Yeasts and moulds are not included in the microbiological criteria of the European Union, established through Regulation 2073 from 2005 and Regulation 1441 from 2007. Country-specific norms for microorganisms used by various countries in Europe and America suggest up to 100 cfu/g for moulds and yeasts in cheese with low water content, and up to 10 000 cfu/g yeasts and 100 cfu/g moulds in cheese with more than 50% of water content (Anonymous, 2012). In accordance with the classic technique of production in Bulgaria, white brined cheese falls within the category of ripening cheese and low water content, with the industry norms used by the Bulgarian State standards allowing up to 100 colonies of moulds and yeasts in 1 gram of product. With alternative technologies of white brined cheese production, water content can reach up to 60%. Levels of 10 to 100 cfu/g are acceptable in the dairy industry, where there are conditions with secondary contamination by moulds and yeasts, especially

when using processes with starter cultures and ripening of the cheese.

The overview made by Chapman & Sharpe (1990) reported that some types of soft cheese, cottage cheese and cream cheese exhibited quality deficiencies when subjected to high humidity, due to the spread of moulds of the *Geotrichum* genus. *Geotrichum candinum* (also known as *Oospora lactis*), often isolated from raw milk along with *Penicillium* and *Mucor*, has a negative role in the production of dairy products (Wouters *et al.*, 2002). Our study found that 32.8% of the isolated mould strains in white brined cheese belonged to the *Geotrichum* genus. Torkar & Vengušt (2008) found out a significantly higher prevalence of *Geotrichum*, reaching up to 91.9% of the isolated mould strains. Contrary to our results are the findings of Scott (1989) and Kure & Skaar (2000), who reported that the *Penicillium* genus was the most commonly encountered contaminant in cheese, followed by *Aspergillus, Cladosporium* and *Mucor*. The low percentage of isolates from the *Aspergillus* genus observed by Torkar & Vengušt (2008) was explained with the fact that milk was cooled quickly during the winter, easily reaching low temperatures, in which moulds cannot grow. According to Bullerman (1981), the *Aspergillus* species, unlike the ones of the *Penicillium* genus, cannot grow of the milk or the production environment at low temperature.

In the 122 mould strains isolated by us, the strongest lipolysis was exhibited by the representatives of the *Geotrichum*, *Mucor*, as well as few species of the *Aspergillus* genus. Strong lipolytic activity was exhibited by 17 of the *Geotrichum* isolates, while 4 had average values. Most of the isolates, or a total of 43 (74.1%) of the moulds in raw milk and 47 (73.4%) of the moulds in ready cheese were distinguished by strong lipolytic activity. Only 19 (15.5%) from a total of 122 mould strains from raw milk and cheese did not exhibit lipolytic activity. Our results for lipolysis in the isolated moulds correspond to the results of El-Diasty & Salem (2004), who found strong lipolytic activity exhibited by the species in the genus *Aspergillus, Penicillium* and *Candida spp*.

The transfer of the mould aflatoxin from the feed into the animals, along with its metabolite AFM_1 through the milk and dairy products is considered to be a major threat in the production of milk and dairy products. In our studies, a total of 11 strains were classified as belonging to the species *A. flavus* with typical growth on AFPA agar, with only 2 (18.1%) of them producing aflatoxin on supplemented YGC or YES agar. Similar results support the statement that the consumer threat of accumulating mycotoxins in the cheese has to be evaluated in accordance with the presence of the strains of *A. flavus* with a clear phenotype aflatoxin characteristic. According to Bullerman (1981) the species *A. flavus* is not found in all types of cheese, while the growth of *A. flavus* with production of aflatoxin can occur only in cheese with temperature above 10 ºC and water activity greater than 0.79. Torkar & Vengušt (2008) isolated only one *A. flavus* strain from different types of cheese, yet it had no aflatoxin-producing characteristic on YES agar.

In white brined cheese from different processing plants, we established the presence of quantitative differences in the microbial contamination with moulds and yeasts. In one of the three producers, we detected simultaneously the highest values in moulds (3.3 $\log_{10}$cfu/g) and yeasts (3.7 $\log_{10}$cfu/g) (Table 3). We believe that the observed differences in the contamination

with moulds and yeasts in the different producers were due to factors related to the quality of the raw milk used for cheese production, as well as the different sanitary conditions at the different facilities. At production plant 1, the incoming raw milk contained moulds and yeasts in the amount of 5.6 $\log_{10}$cfu/mL, whereas at plants 2 and 3 the values were 4.1 and 4.4 $\log_{10}$cfu/mL. Higher contamination with moulds and yeasts in the purchased milk leads to higher contamination of the end product. Furthermore, a similar ratio was observed between the different mould species in raw milk and the end product. For yeasts, the ratio of the different species in raw milk and the end product was different, however yeasts are easily eliminated through pasteurisation. Their repeated emergence after pasteurisation was a result of secondary contamination through the technological equipment or by the sanitary conditions of production. Viljoen *et al.* (2003) established a presence and dissemination of yeasts on the production tables (126 cfu/25 cm^2), on the doors (2105 cfu/25 cm^2), in the brine (6.6×10^4 to 2.8×10^5 126 cfu/mL) and in the air (3.0–4.0 cfu) at a facility for the production of blue cheese in Denmark. Gori *et al.* (2013) found yeasts at a highest concentration (3.7×10^6 cfu/cm^2 cheese surface) at only one plant, with the predominant species being *Yarrowia lipolytica*. At the other three production plants, the amounts of yeasts varied from 1.2×10^5 cfu/cm^2 to 7.4×10^5 cfu/cm^2, with *Geotrichum* being the predominant species only at the site with the lowest quantity of yeasts. Kure *et al.* (2001) isolated 23 mould species from 225 samples of Norwegian semi-hard cheese. *Penicillium* and *Geotrichum* made up 80.9% of the isolates. While examining cheese samples, Torkar & Vengušt (2008) found yeasts at the amounts of 2.5 $\log_{10}$cfu/g, while El-Diasty & Salem (2007) reported levels of 4.2 $\log_{10}$cfu/g of product.

CONCLUSION

Summarising the data from the studies, we conclude that the contamination with moulds and yeasts in raw milk was too high, reaching up to 76.6% in moulds and 93.3% in yeasts. Even though processing plants heat up raw milk before processing it into white brined cheese, a smilar trend was observed in the contamination of the final product, respectively in 70.0% for moulds and 63.3% for yeasts. The most commonly isolated mould species from raw milk were from the *Aspergillus* genus (37.9%), and from white brined cheese – from the *Geotrichum* genus (32.8%). Among the isolated yeasts from raw milk and white brined cheese, the *Candida* genus had the highest percentage of isolates, respectively 70.0% and 50.0%. The wide spread of moulds and yeasts with high lipolytic activity should be seen as a risk to the production process, with a need for adequate corrective actions at the technological processing level after pasteurisation. Such actions are also suggested with regard to the spread of moulds from the species *A. flavus,* identifying risk only in case of a presence of strains with a clear phenotype expression for aflatoxin production.

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