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Development of Yeast Populations during Processing and Ripening of Blue Veined Cheese

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Summary

Varieties of blue veined cheese were analyzed regularly during different stages of manufacturing and ripening to determine the origin of contaminating the yeasts present in them, their population diversity and development until the end of the storage. Yeast diversity and development in the inner and outer core of the cheeses during ripening were also compared.

Air samples revealed few if any yeasts whereas the samples in contact with the equipment and the surroundings revealed high number of yeasts, implicating it as the possible main source of post-pasteurization contamination, as very few yeasts were isolated from the milk and cheese making process itself. Samples from the inner and outer core of the maturing cheeses had typical survival curves. The number of yeasts on the outer core was about a 100-fold more than of those in the inner core.

The most abundant yeasts isolated from the environment and ripening cheeses were identified as *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Trichosporon beigelii*, *Candida versatilis* and *Cryptococcus albidus*, while the yeasts *Candida zeylanoides* and *Dekkera anomala* were additionally isolated from the environment. Yeasts were present in high number, making their occurrence in blue-veined cheeses meaningful.

Key words: blue cheese, yeasts, lactic acid bacteria, identification

Introduction

The occurrence of yeasts in dairy products like cheese is not unexpected as these products have various properties that encourage the proliferation of yeasts such as high acidity, storage at low temperature, low moisture content and elevated salt concentrations (*1,2*). Their presence is of major importance as they can be beneficial or detrimental, being a major component of the microflora that contribute to the ripening and flavour development (*1,3*) as well as causing spoilage in cheese (*4,5*). Cheeses such as blue cheese varieties have further unique physical and chemical properties, which select the growth and prevalence of specific yeast species (*6–8*) such as high fat and protein concentrations, residual unfermented lactose, high concentrations of lactate, and the presence of citric and acetic acids.

The yeasts most frequently isolated from blue cheeses include *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Kluyveromyces lactis* and *Candida* spp. (*6,7,9,10*). These yeasts form an integral part of the microflora of blue cheese where they contribute to the ripen-

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ing process by flavour formation through lipolytic and proteolytic activity (*11*), the production of aroma compounds, gas production which assists the development of *Penicillium roqueforti* and the increase of pH on the surface of the cheese resulting in the development of secondary microflora, like *Brevibacterium linens* (*12,13*).

Traditionally, raw milk was used for the manufacture of blue cheese varieties, which is still the case for some varieties (*9*). Currently, however, cheese processors prefer pasteurized milk to minimize the microbial diversity (*1*). This resulted in several advantages, like the improvement in the bacteriological quality and consequently also the quality of the cheese, with less defects incurred by the poor quality of the raw milk (*9*). Also, pasteurized milk resulted in a less diverse population of yeasts, usually considered as undesirable and a sign of poor hygiene (*1,5*). As yeasts are not able to survive pasteurization temperatures, any yeast present in the cheese will be due to post-pasteurization contaminants (*1,12*).

Sources of contamination within the dairy environment responsible for the contamination of milk and cheese during processing are primarily factory surfaces such as the floors, walls, equipment and brine (*14–16*). The contribution of these surfaces to contamination, however, varies from factory to factory, depending on the sanitizing practices (*17*). Brine contains yeast populations (*14*) ranging from 10^4 up to 10^6 cfu/mL (*7*). Because of its high salt content, a specific microflora, and consequently halotolerant yeasts like *D. hansenii* and *Y. lipolytica* are frequently encountered in the brine (*7,16*). Secondary sources include equipment, air and workers' hands and aprons (*15,18*). Spoilage in most dairy products becomes evident when the yeast population reaches 10^5–10^6 cells/g (*1*), but counts as high as 10^8 cells/g were detected in cheeses without any visible defects.

The size and composition of the yeast populations during the ripening process on the exterior and interior of the cheese differ dramatically within blue cheese (*12*). A 10-fold difference in yeast population size was encountered between the exterior and the interior, with the exterior exhibiting higher yeast populations (*12*). The interior yeast population showed a less diverse group compared to the exterior and consisted of only a few species like *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Candida versatilis* (*12*). The yeast population representative of the exterior comprised a wider diversity and in addition included strains of *Candida sake*, *Candida intermedia* and *Yarrowia lipolytica* (*12*). These populations remained viable until retail although minor differences may be expected (*19*).

Although literature (*4,6,8*) indicated the occurrence and numbers of yeasts in blue cheeses, no studies were performed to investigate the growth and development of yeast populations during manufacturing and ripening. Consequently, in this study we endeavored to determine the origin of contaminating yeasts in a blue cheese plant as well as their development during processing and maturation.

Materials and Methods

Blue-veined cheese manufacturing

Blue type cheeses (Gorgonzola-style and Danish-style) were manufactured on four occasions during the summer in a commercial cheese factory in the Western Cape region of South Africa. The basic cheese making procedure for Danish- and Gorgonzola-style was the same and carried out as described by Mistry and Kosikowski (*20*).

The main differences between the two types of cheese are the type of mould spores and starter culture used (spores of Danish-style are more proteolytic and of Gorgonzola-style more lipolytic), fat content (Gorgonzola, 65 %; Danish-style, 45 %), the formation of the mould during ripening and the time of ripening.

Sampling methods and selection of isolates

On all four occasions, several surfaces (Table 1) were sampled in duplicate by means of RODAC contact plates (*21*) using De Mann Rugosa and Sharpe agar (MRS) (Merck, C86 – pH=6.5, Darmstadt, Germany) for the analyses of lactic acid bacteria, and Dichloran Rose Bengal Chloramphenicol (DRBC) (Oxoid, Basingstoke, UK) agar for yeasts. Air was sampled by using standard settle plates (the same media in 90-mm Petri dishes) with an exposure time of 5 min.

Samples were also taken during the manufacturing of the blue cheeses at selected points as indicated in Table 2; the basic process for Danish- and Gorgonzola-style was the same. Liquid samples were taken by aseptically scooping with a sterile McCartney bottle in the cheese vat, and solid samples by aseptically cutting and transferring into sterile bags.

The mass of 10 g of solid sample portions was weighed into 90 mL of sterile peptone water and homogenized in a Colworth 400 stomacher (London, UK) for 2 min. Further decimal dilutions of the suspensions and liquid samples were performed in 9 mL of sterile peptone water. Aliquots (0.1 mL) of the dilutions were spread inoculated over the surface of plates containing the media. DRBC plates were aerobically incubated at 25 °C for 4 days, and MRS plates at 25 °C for 48 h.

Sampling during ripening

Gorgonzola-style and Danish-style blue cheeses from the same batch were kept under controlled conditions at 4 °C at the site of cheese production and sampled directly after processing at consecutive intervals on a weekly basis during ripening for over a 13-week period for Gorgonzola-style and for 26 weeks for Danish-style. Cheese samples were prepared for microbiological analysis by aseptically cutting a cheese wedge from the cheese. A 10 g of cheese sample was taken from the surface and the center of the cheese wedge respectively, and further microbiologically analyzed as described earlier.

Enumeration and isolation

All plates containing between 25 and 250 colony-forming units (cfu) (or the highest dilution if below 25) were enumerated and the mean values determined from duplicate samples. Results were recorded as the mean value of two trails, from duplicate plate samples originating from duplicate cheese samples from the same batch (2×2×2).

Yeast colonies were isolated from the highest dilution plates on DRBC plates. The yeast isolates were sub-cultured on Malt Extract agar (MEA) (Merck, C10 – pH=5.4), incubated for 48 h at 25 °C and checked for purity by colony morphology and microscopy. Pure cultures were stored on Yeast Malt Extract (YM) agar slants at 4 °C (22) during the period of investigation, until characterization.

Yeast identification

Individual yeast isolates were identified by conducting physiological, sporulation and morphological tests as described by Kurtzman and Fell (*23*). Data were interpreted using the keys of Kurtzman and Fell (*23*) and the computer program of Barnett *et al.* (*24*). Each isolate was inoculated into 6 sugar fermentation media and 32 carbon source assimilation media (*25*). Additional tests performed included growth at 37 °C, and in 50 % D-glucose medium, urea hydrolysis and assimilation of nitrogen compounds, performed by means of auxanographic method (*26*), were also included.

Ascospore formation was examined on McClary's acetate agar, potato glucose agar, Gorodkowa agar, corn meal agar and MEA (*27*). The inoculated media were incubated at 18 °C for 4 weeks and examined at 4-day intervals. Cell morphology and the mode of reproduction were examined on MEA (Biolab, Merck, Darmstadt) and on Dalmau plates (*27*). The formation of pseudo- and true mycelium was examined on corn meal agar according to the Dalmau plate technique (*22*).

Results and Discussion

Yeast development during processing

Air samples in the vicinity of processing equipment, revealed low numbers from the brine and cheese ripening rooms (Table 1), and no yeasts from the production room. In contrast to the low yeast counts observed from air samples, higher counts of bacteria were present in the air, especially in the brine room. The low number of yeasts corresponds to earlier data (*18,28*), indicating that the air contributes very little to yeast contamination in the dairy industry.

Contact samples taken within the factory environment associated with the equipment revealed limited growth of yeasts and bacteria (<10^3 cfu/25 cm^2), mainly attributed to a successful cleaning program. The stainless steel equipment, if properly cleaned, usually harbors low numbers of yeasts and bacteria, which consequently contribute little to post pasteurization contamination (*28*). However, these microbial numbers increased during processing and successive batches (*15*). Building surfaces, including the floors, walls and doors as well as the shelves in the second ripening room exhibited high numbers of yeasts whereas low numbers were detected on the surfaces in the processing room (Table 1). Higher yeast counts observed in the second ripening room are attributed to the usage of wooden shelves, which maintain higher loads of microbial contamination due to difficulty for proper cleaning. Movement between the rooms, as indicated by the high yeast counts on the doors leading to the ripening rooms (>2000 cfu/25 cm^2) also played an important role in the spreading of yeasts within the plant. Surfaces like walls and floors, therefore, make an important contribution to yeast contamination (*28*). Similar results were obtained during the making of Gouda and Cheddar and other cheeses (*4,28*).

Table 1. Enumeration of yeasts and bacteria from environmental samples in cheese plant (results are the means of duplicate samples)

SAMPLE	*N*(yeast)	*N*(bacteria)
Air (cfu/90 mm Petri dish)		
Production room	0	2.0
Brine room	3.0	22.5
1st ripening room	4.0	11.0
2nd ripening room	2.5	5.0
Equipment surfaces (cfu/25 cm^2)		
Cheese vat	0	0
Net	75.0	0
Machinery	0	25.0
Table	126.0	0
Cheese moulds	0	980.0
Humidifier outlet	0	212.0
Building surfaces (cfu/25 cm^2)		
Floor – Production room	0	0
Floor – Brine	52.0	1300.0
Floor – 1st ripening	259.0	0
Wall – Production	0	0
Wall – Brine	0	0
Wall – 1st ripening	0	0
Wall – 2nd ripening	27.5	0
Door 1	1900.0	1070.0
Door 2	2105.5	2700.0
Shelf – 1st ripening	0	880.5
Shelf – 2nd ripening	375.0	1200.0
Brine (cfu/mL)		
Foam	$2.8\cdot10^5$	$1.8\cdot10^5$
Collective outlet	$6.6\cdot10^4$	$1.9\cdot10^4$
Collective tank	$1.3\cdot10^5$	$2.3\cdot10^4$

The brine has been shown to be a major source of yeast contamination (10^4 to 10^5 cfu/mL) (Table 1) (*14*). Some of these yeasts, like *Debaryomyces hansenii*, which showed to have an enhanced resistance to such adverse conditions like high salt concentrations, are likely to increase in numbers at later stages of the production or during the ripening period (*28*). Despite the frequent occurrences of yeasts in the brine, it must be kept in mind that the numbers and diversity may vary between dairy plants and even between consecutive days in the same plant, due to variation of salt concentrations and age of the brine (*14,28*).

Very low counts of yeasts were recorded during the manufacturing process (Table 2). Counts of yeasts generally tended to slightly increase towards the end. The highest count recorded was 70 cfu/g at the end of the process, during moulding. The only yeast species isolated during the processing stage was a representative of *Torulaspora delbrueckii*.

Table 2. Enumeration of lactic acid bacteria and yeasts during the basic manufacturing process of Blue-type cheeses (results are the means of duplicate samples)

Elapsed time (h)	Procedure	*N*(lactic acid bacteria)/(cfu/g)	*N*(yeasts) /(cfu/g)
00:00	Milk in vat	2500	0
01:00	Mould and starter culture added	$1.09 \cdot 10^5$	0
01:30		$5.21 \cdot 10^8$	0
02:00		$2.62 \cdot 10^8$	0
02:40	Curd cutting	$3.60 \cdot 10^8$	0
02:45	Stir starts	$1.50 \cdot 10^7$	0
03:45		$7.30 \cdot 10^7$	10
04:00		$1.00 \cdot 10^8$	0
04:30		$1.10 \cdot 10^8$	0
05:00	Draining	$6.10 \cdot 10^7$	0
05:15	Dry salting	$9.10 \cdot 10^7$	10
06:00	Scooping/ Moulding	$1.39 \cdot 10^8$	0
06:30	Turning	$6.60 \cdot 10^7$	0
07:00		$1.12 \cdot 10^8$	0
07:30		$9.40 \cdot 10^7$	60
07:45	End of turning	$6.18 \cdot 10^8$	70

Yeasts isolated from the dairy environment are listed in Table 3. The occurrence of *Debaryomyces hansenii* in all occasions during environmental samplings was expected as the species has been isolated from the dairy environment by numerous researchers (*4,28*). *Torulaspora delbrueckii* and *Cryptococcus albidus* were found predominantly on the equipment, whereas, the former was found infrequently in the air. Both species have been isolated from the dairy environment at limited numbers (*28*). Despite the presence of *Dekkera anomala* and *Dekkera bruxellensis* on the walls and equipment, these species have not been isolated previously from the dairy environment, and were not recovered during processing or maturation. Therefore, their presence might be regarded as accidental (*29*). *Candida* spp. usually account for a small percentage of yeasts isolated from the dairy environment, which corresponds to our data.

Table 3. Yeasts associated with blue cheese manufacture and their sources

Isolates	Air	Floors/ Walls	Equip- ment	Brine
Candida versatilis				+
Candida zeylanoides				+
Cryptococcus albidus			+	
Dekkera anomala		+	+	
Debaryomyces hansenii	+	+	+	+
Dekkera bruxellensis		+		
Rhodotorula glutinis	+			
Torulaspora delbrueckii	+		+	
Trichosporon beigelii		+	+	

Yeast development during the ripening period

Survival of yeasts in the interior and exterior of Danish- and Gorgonzola-style blue cheese during ripening is summarized in Figs. 1 and 2, respectively. The yeast count includes the total number of yeasts present.

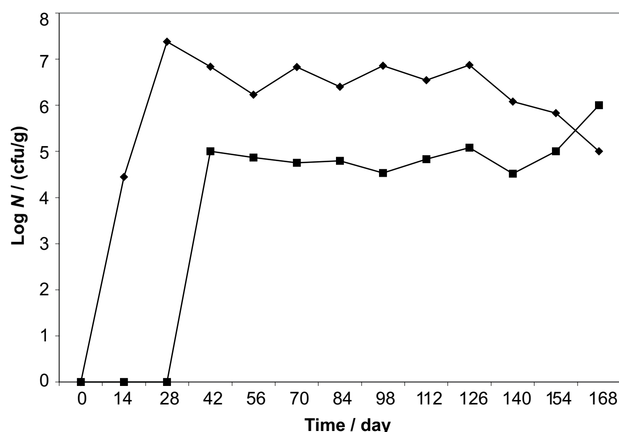

Fig. 1. Survival of total yeasts in the interior (■) and on the exterior (◆) of Danish-style blue cheese during ripening

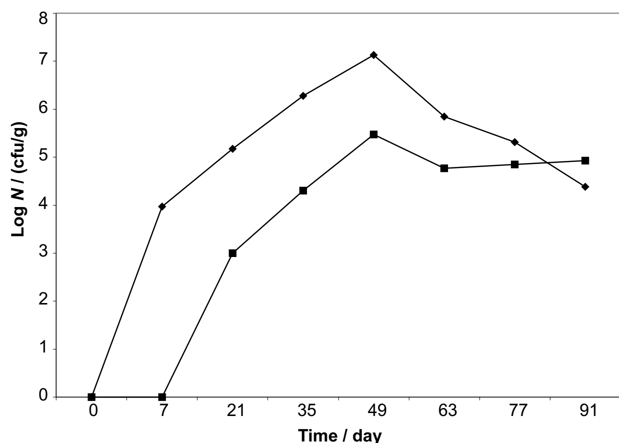

Fig. 2. Survival of total yeasts in the interior (■) and on the exterior (◆) of Gorgonzola-style blue cheese during ripening

Danish-style blue cheese

Initially, the yeast count, directly after processing, on the surface and in the interior of the cheese, revealed no yeasts, but within four weeks numbers rapidly increased on the exterior in the excess of 10^7 cfu/g ($2.39 \cdot 10^7$ cfu/g). Yeasts in the interior, however, remained absent for the initial four weeks, following a substantial increase in yeast numbers reaching counts as high as 10^5 cfu/g within the next two weeks (Fig. 1). These excessive increases in yeast numbers during the initial stages may be attributed to competitive conditions for the yeasts such as temperature (8–10 °C) during the first ripening period (*1*), which suppresses the growth of lac-

tic acid bacteria (*19*) and the high acidity of the curd, caused by fermentation of lactose by lactic acid bacteria (*1*), while yeasts originating as contaminants from the brine and the environment are halotolerant (*14*).

The development of yeasts during the whole ripening period exhibited similar patterns between the exterior and the interior, although a substantial difference of more than one log unit in the number of yeast populations on the exterior compared to the interior of the cheese (Fig. 1) was evident. After the excessive increase in yeast numbers at the initial stages of the ripening, the numbers remained more or less stable for the next 84 days. For the next period until the end of ripening, yeast numbers representative of the interior gradually increased to $>10^6$ cfu/g, whereas the numbers on the surface decreased to $>10^5$ cfu/g.

The high incidence of yeasts on the exterior, which are furthermore exposed to aerobic conditions, originates from direct contact with the environment (*12*), whereas the yeasts in the interior have limited access to oxygen (*1,29*) and consequently, the former has a competitive advantage reaching higher numbers (*12*). According to literature, the proportion between the yeast numbers on the exterior and in the interior during ripening is about 100 to 1 (*19,30*). In contrast, however, yeasts representative of the interior of the cheeses revealed an enhanced number of different species compared to the exterior (Table 4). The increased number may be attributed to a lower salt concentration in the core allowing yeasts with lower salt tolerance to grow.

Table 4. Distribution of yeast populations in interior and exterior of Danish-style and Gorgonzola-style blue cheeses

Yeast	Interior population fraction/%	Exterior population fraction/%
DANISH-STYLE		
Candida versatilis	13	12
Cryptococcus albidus	10	ND
Debaryomyces hansenii	53	50
Saccharomyces cerevisiae	10	18
Torulaspora delbrueckii	13	20
GORGONZOLA-STYLE		
Candida versatilis	19	25
Candida zeylanoides	4	ND
Cryptococcus albidus	11	ND
Debaryomyces hansenii	30	35
Rhodotorula glutinis	7	ND
Saccharomyces cerevisiae	13	ND
Torulaspora delbrueckii	14	25
Trichosporon beigelii	ND	15

ND = Not detected

Table 4 shows the composition and proportionate representation of yeasts during the ripening period on the surface and the interior of the cheese. A total of 30 strains were isolated from the interior whereas 34 strains were isolated from the surface. The most predominant species on both was *Debaryomyces hansenii*, representative of >50 % of the isolates on the surface and interior. All other species accounted for less than 20 % on all occasions. In other studies (*6,9,10*), *Debaryomyces hansenii* was the most frequently occurring species in blue cheese. The overall predominance of *Debaryomyces hansenii* can be attributed to the species' salt resistance (*1*), growth at low temperatures (*15*), proteolytic and lipolytic activity (*28*), the ability to utilise lactate and citrate (*6*) and the frequent association with environmental samples (Table 3).

Torulaspora delbrueckii was found to be the second most abundant yeast species on the exterior and in the interior of the Danish Blue-style cheese (Table 4). This species has been typically found in the raw milk of several dairies and according to van den Tempel and Jakobsen (*7*) there is a possibility that it might not be killed through pasteurization. Despite the absence of yeasts in the pasteurized milk during our survey, the species has been isolated from the equipment (Table 3) and therefore it is possible that the species became established through post pasteurization contamination. Welthagen and Viljoen (*28*) found that *Torulaspora delbrueckii* was present in 40 % of hard- to soft-cheese varieties surveyed. *Candida versatilis* was present on the exterior and in the interior of the Danish Blue-style cheese, originating from the brine (Table 3). *Candida versatilis* has been isolated from pickling brines (*31*) and to a lesser extent in cheese samples (*12*).

Cryptococcus albidus was only found in the interior of the blue cheeses. The most likely source of contamination was the processing equipment (Table 3), which corresponds with the data as indicated by Welthagen and Viljoen (*15*). The species has been recovered from different dairy products such as ice cream, butter and cheese (*4,28*) as well as blue cheese-varieties where it was predominant (*6*). Its ability to grow at low temperatures and lipolytic activity mainly contribute to its presence (*28*).

Saccharomyces cerevisiae strains were present in the interior and on the surface of the cheeses despite their absence in the environmental samples during this survey. According to Roostita and Fleet (*6*), *S. cerevisiae* is sensitive to high salt concentrations, but capable of growth in dairy products with reduced salt values utilizing cheese components as growth substrates. The occurrence of this yeast species in blue cheese is probably based on the utilization of protein and fat breakdown products from other species (*6*) but its origin is unknown.

Gorgonzola-style blue cheese

Yeasts associated with Gorgonzola-style blue cheese (Fig. 2) showed similar growth patterns as exhibited by the Danish blue-style cheese (Fig. 1). Yeasts present on the surface of Gorgonzola-style blue cheese rapidly increased directly after processing reaching a maximum of $1.34\cdot10^7$ cfu/g after 49 days of ripening (Fig. 2). In the interior, yeast numbers remained limited for the initial 7 days after processing followed by a substantial increase to reach a maximum of $2.99\cdot10^5$ cfu/g after 49 days. Low storage temperatures (8–10 °C) and high acidity of the curd selected the yeast contaminants originated from the environment. Similar to the Danish-style blue

cheese, yeasts on the surface were significantly higher compared to the interior.

After 49 days of ripening, yeast numbers on the exterior decreased gradually until the end of ripening at day 91 prior to packaging to $2.4 \cdot 10^4$ cfu/g (Fig. 2). A similar decreasing profile was followed by the yeasts present in the interior except for a slight increase at the end of the ripening period resulting in a final count of $7.1 \cdot 10^4$ cfu/g.

The most frequently occurring yeast species on the exterior of Gorgonzola-style blue cheese were representatives of *Debaryomyces hansenii*, *Candida versatilis*, *Trichosporon beigelii* and *Torulaspora delbrueckii*. In the interior, an enhanced diversity in the yeast population was obtained (Table 4). Again, *Debaryomyces hansenii* clearly predominated on the exterior and in the interior of the cheese represented by more than 30 % of the population (Table 4). *Candida versatilis* was the second most abundant yeast species on the exterior and in the interior of the cheese, whereas, *Torulaspora delbrueckii* strains were also frequently encountered. *Saccharomyces cerevisiae, Rhodotorula glutinis, Candida zeylanoides* and *Cryptococcus albidus* were only found in the interior of the Gorgonzola-style blue cheese. Species recovered from Gorgonzola- and not from Danish-style, were *Trichosporon beigelii, Rhodotorula glutinis* and *Candida zeylanoides* (Table 4). *Trichosporon* species, associated with dairy products, have been frequently recovered from cheeses (*28*,*32*), raw milk and pasteurized milk (*6*), and the brine (*14*) originated from the floors and walls, and equipment (Table 4). *Rhodotorula glutinis* and *Candida zeylanoides* isolated from cheeses (*18*) originated from the air, equipment surfaces (*28*) and brine (*14*). The ability of *Rhodotorula glutinis* to grow at low temperatures and its strong ability to hydrolyse fat contributes to its presence in cheese (*4*).

Conclusion

The results obtained clearly indicated that the development of yeasts within the blue cheeses originated as yeast contaminants present in the environment. With the exception of *Saccharomyces cerevisiae*, all the other yeast species were isolated from the immediate environment. Similar species were obtained in Australian and European blue cheese varieties (*6*), which can be contributed to the generalized usage of pasteurized milk for manufacturing of the cheeses (*7*). At the end of ripening period, yeasts were present in both cheese varieties at high numbers, making their occurrence in South African blue cheese varieties meaningful. Those species that proved to be dominant at all times were naturally selected based on environmental influences or competition between species for survival.

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Razvoj populacije kvasaca tijekom proizvodnje i zrenja plavo prošaranog sira

Sažetak

Tijekom raznih stadija proizvodnje i zrenja analizirane su vrste plavo prošaranih sireva da bi se utvrdilo podrijetlo prisutnih kontaminirajućih kvasaca te njihova raznolikost i razvoj populacije do kraja skladištenja. Također je uspoređena njihova raznolikost te praćen razvoj kvasaca u unutrašnjoj i vanjskoj kori sira tijekom zrenja. U uzorcima zraka kvasci nisu uopće pronađeni ili u samo maloj količini. U mlijeku i u samom procesu proizvodnje sira nađeno je vrlo malo kvasaca, dok su uzorci uzeti s uređaja i iz okoline sadržavali veliki broj kvasaca, što pokazuje da je to mogući glavni izvor kontaminacije nakon pasterizacije. Uzorci iz unutrašnje i vanjske kore sazrelih sireva pokazivali su tipičnu krivulju preživljavanja. Broj kvasaca u vanjskoj kori bio je oko 100 puta veći nego u unutrašnjoj. Najrašireniji kvasci izolirani iz okoline i iz sazrelih sireva utvrđeni su kao *Debaryomyces hansenii, Saccharomyces cerevisiae, Torulaspora delbrueckii, Trichosporon beigelii, Candida versatilis* i *Cryptococcus albidus*, dok su kvasci *Candida zeylanoides* i *Dekkera anomala* bili dodatno izolirani iz okoline. Kvasci prisutni u najvećem broju važni su za kakvoću plavo prošaranih sireva.