Study of the Fresco culture inhibitory effect against *Staphylococcus aureus* in milk and in lump cheeses

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Summary

The effect of Fresco culture on the growth of *Staphylococcus aureus* in a model medium was studied. *Staph. aureus* 2064 and Fresco culture were co-cultured in ultra high temperature treated cows' milk at 15, 18 and 21 °C. In consequence of rapid pH value decreasing down to 5.0, lactic acid bacteria (LAB) culture was able to inhibit the growth of *Staph. aureus*. The minimal Fresco culture concentration of 10^5 CFU·ml^{-1} was assumed to ensure minimal *Staph. aureus* net growth of about 1 log CFU·ml^{-1}. The potential inhibitory effect of Fresco culture was subsequently tested in the laboratory-prepared ewes' lump cheeses that were stored at 18 °C. LAB presence and their active metabolism led to a fast decrease in pH value down to 5.0 after five hours of cheese fermentation, which resulted in the inhibition of the growth of the undesirable microflora.

Keywords

lactic acid bacteria; *Staphylococcus aureus*; predictive microbiology

Original ewes' lump cheese is an artisanal full-fat, soft rennet cheese from raw ewes' milk manufactured at farm level in Slovakian mountain areas. After two weeks of ripening at temperatures from 18 °C to 21 °C, it is used for industrial production of the popular Slovakian "Bryndza" cheese. Fermentation of the lump cheese relies on native mesophilic lactic acid bacteria (LAB) such as *Lactococcus lactis, Enterococcus faecalis, Lactobacillus casei, Lb. lactis* and *Lb. plantarum*. During ripening, the essential role is played by the milk mould *Geotrichum candidum* and oxidative yeasts of the genera *Torulopsis, Candida* and *Kluyveromyces* [1, 2].

Since the presence of food-borne pathogens in raw milk cannot be completely avoided, safety of raw milk cheeses relies on the multiplication of LAB. An ubiquitous contaminant of raw milk is *Staph. aureus* that is characterized by the production of heat-stable enterotoxins. Unsatisfactory control of mastitis in herds and lack of proper hygienic measures during food preparation increases the risk of staphylococcal contamination and staphylococcal food poisoning is often associated with manually prepared food, including ewes' lump cheeses [3]. The ability to produce one of the enterotoxins was observed in more than 20% or more than 50% of *Staph. aureus* strains examined [3, 4]. Percents of 65–84% of these strains were of human origin [4]. It is neccessary to remark that once the enterotoxins are produced, they retain their activity even after killing the bacterial cells by pasteurization.

As *Staph. aureus* growth is inhibited by products of the fermentative metabolism of LAB, sufficient acidification, in particular in the early stages of cheese making, may be achieved by the use of an appropriate amount of an active starter culture [5]. The concurrent decrease in the pH value and sensorial substances production lead to the required flavour formation, shelf life prolongation and inhibition of the undesirable microflora.

Because toxinogenic *Staph. aureus* is regularly present in ewes' raw milk in low numbers and its growth during uncontrolled fermentation of milk or young cheese may be intensive, the aim of our

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study was to provide quantitative growth relation analysis between *Staph. aureus* and LAB at the temperature range related to the lump cheese fermentation or ripening conditions.

MATERIALS AND METHODS

Microorganisms

Staph. aureus 2064 originating from ewes' lump cheese was provided by Dr. Hanzélyová (State Veterinary and Food Institution, Prešov, Slovakia) and was identified by Gram staining, microscopy, catalase test, by the API Staph system (BioMérieux, Marcy l'Etoile, France) and PCR according to Pinto et al. [6]. The isolate of *Staph. aureus* was kept on the Plate Count Agar (Merck, Darmstadt, Germany) at 5 °C ± 1 °C. The mesophilic Fresco culture DVS 1010 was a commercial culture from Christian Hansen (Hørsholm, Denmark) consisting of *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *lactis* and *Streptococcus thermophilus*. The culture was stored frozen until analysis.

Inoculation and cultivation conditions

The standard suspension of microorganisms was prepared from a 24 h culture of *Staph. aureus* by standard rinsing with sterile peptone saline and aseptically inoculated into 300 ml of pre-tempered ultra high temperature treated cows' milk (1 l milk packet; pH 6.7) in order to reach as constant initial counts in each milk sample as possible. The method of inoculation that was described and validated in our recent work [7] was followed. At the same time, the Fresco culture in the initial concentration from 3–7 log CFU·ml^{-1} was inoculated into the milk. The static aerobic incubation of three parallel milk samples took place at 15, 18 and 21 °C.

Manufacture of ewes' lump cheese

The lump cheese was prepared from raw ewes' milk with or without the addition of 1% (w/v) Fresco culture according to the procedure described by Valík et al. [8]. The cheeses were kept at 18 °C for seven days.

Quantification of microorganisms in milk and in cheeses

In order to gain growth curves, the amounts of microorganisms expressed as CFU·g^{-1} or CFU·ml^{-1} were determined in pre-defined time intervals. *Staph. aureus* was enumerated on Baird-Parker agar with egg yolk and tellurite (Merck) after 24 h at 37 °C according to STN ISO 6888-1 [9]. LAB of the Fresco culture were enumerated on M17 agar (Merck) after 48 h at 30 °C and STN ISO 4833 [10].

Determination of the active acidity

In the same pre-defined time intervals as for the microbiological determination, pH values of the inoculated milk and cheese samples were measured using a pH meter (WTW 720; Inolab, Weilheim, Germany).

Fitting the growth curves and calculating the growth parameters

The growth data of the strains under study were analysed, fitted and calculated using the mechanistic modelling technique of Baranyi et al. [11], which is incorporated in the DMFit tools kindly provided by Dr. J. Baranyi (Institute of Food Research, Norwich, United Kingdom).

RESULTS AND DISCUSSION

Effect of temperature on the pH value lag phase during co-culture of *Staph. aureus* and Fresco

Due to the fermentative metabolism of LAB, lactose is largely depleted in the first hours of incubation and lactic acid is produced, which is demonstrated by a decrease in pH values. It was obvious that pH dropped after the pH lag phase and was influenced by the metabolism and the amount of present LAB. The time necessary to achieve the minimal pH value relied on LAB density and also on the temperature (Fig. 1). The most crucial part of the lump cheese manufacture is the first six hours [12, 13]. In order to reach the pH lag phase

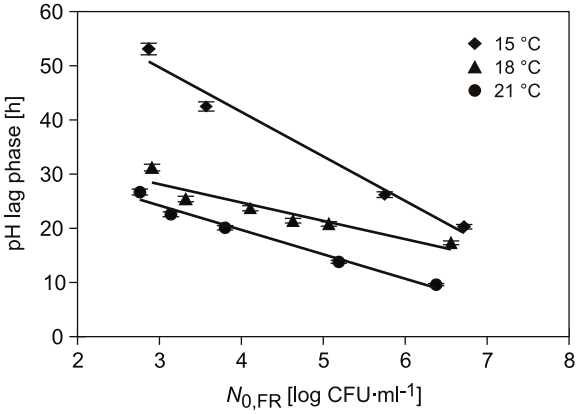

Fig. 1. Effect of initial Fresco culture density on pH lag phase in relation to the incubation temperature.

$N_{0,FR}$ – initial density of the Fresco culture.

Tab. 1. Growth parameters of *Staph. aureus* 2064 in a milk co-culture with Fresco.

Temperature	$N_{0,FR}$ [log CFU·ml^{-1}]	pH lag phase [h]	$N_{max,STA} - N_{0,STA}$ [log CFU·ml^{-1}]	Gr_{STA} [[log CFU·ml^{-1}h^{-1}]
15 °C	2.87	53.1	1.65	0.048
	3.57	42.5	1.37	0.039
	5.75	26.2	0.63	0.167
	6.72	20.3	0.25	0.014
18 °C	2.91	31.2	2.20	0.151
	3.32	25.4	1.73	0.071
	4.11	23.7	0.92	0.052
	5.07	20.8	0.85	0.035
	6.56	17.3	0.53	0.035
21 °C	2.76	26.7	2.66	0.195
	3.14	22.6	2.37	0.167
	3.80	20.1	2.01	0.154
	5.19	13.8	1.42	0.139
	3.68	9.6	0.69	0.073

$N_{0,FR}$ – initial density of Fresco culture, $N_{max,STA} - N_{0,STA}$ – increase of *Staph. aureus* counts in stationary phase, Gr_{STA} – growth rate of *Staph. aureus.*

of 6 h, there is a need of at least 7 log CFU·ml^{-1} of Fresco culture to be added at 21 °C. This condition cannot be accomplished at 18 °C at any amount of Fresco addition, even higher than 8 log CFU·ml^{-1}.

Effect of temperature and Fresco culture on *Staph. aureus* growth during co-culture at 15, 18 and 21 °C

In dependence on the type and form of the present acid, the minimal pH value for *Staph. aureus* growth is between 4.0 and 4.8 [14]. With respect to the inability of *Staph. aureus* to grow under strongly acidic conditions and the necessity of a fast pH drop to values lower than 5.0 in the early hours of fermentation, the inhibitory potential of different LAB additions against *Staph. aureus* was studied in a co-culture in milk at 15, 18 and 21 °C. These temperatures are the minimal, optimal and maximal temperatures for the proper lump cheese fermentation [15].

The effect of Fresco culture addition on *Staph. aureus* 2064 counts in milk at 15, 18 and 21 °C is presented in Tab. 1. In the absence of LAB, *Staph. aureus* grew with the growth rates of 0.057 log CFU·ml^{-1}h^{-1}; 0.118 log CFU·ml^{-1}h^{-1} and 0.210 log CFU·ml^{-1}h^{-1}, at 15, 18 and 21 °C, respectively. In stationary phase, it reached counts higher than 8 log CFU·ml^{-1} at all studied temperatures.

Duration of the pH lag phase has a direct inhibitory effect on the growth of *Staph. aureus*. It was noticed that *Staph. aureus* was able to growth only during the pH lag phase and it was effectively inhibited even at the minimal studied Fresco addition at all temperatures (about 3 log CFU·ml^{-1}, corresponding to the low LAB content in raw milk). Although the growth rate of *Staph. aureus* at 18 °C and the lowest Fresco addition was higher than in a mono-culture ($Gr_{18°C}$ = 0.118 log CFU·ml^{-1}h^{-1}), the maximal counts in stationary phase were by about 3 log CFU·ml^{-1} lower than in a mono-culture. On the other hand, at maximal Fresco addition of 7 log CFU·ml^{-1}, minimal growth rate of *Staph. aureus* (Gr_{STA} = 0.035 log CFU·ml^{-1}h^{-1}) and net growth of only 0.5 log CFU·ml^{-1} were observed. Similar results were observed also at 15 °C or 21 °C (Tab. 1). From the safety point of view, it is very important that at all studied temperatures, maximal counts of *Staph. aureus* 2064 were lower than 6 log CFU·ml^{-1} that are necessary for potential production of enterotoxins [2, 14]. The growth curves at individual incubation temperatures were presented in our previous work [5, 16].

The effect of the addition of the Fresco culture on the growth of *Staph. aureus* ($N_{max,STA} - N_{0,STA}$) at single temperature can be described by the equations 1–3 presented in Tab. 2 and depicted in Fig. 2.

The combined effect of incubation temperature and the initial density of the Fresco culture can be described by the equation 4 presented in Tab. 2. This equation enables to calculate the

Tab. 2. The effect of the addition of the Fresco culture on the growth of *Staph. aureus.*

Effect of the initial density of the Fresco culture at single temperature		Equation
15 °C	$N_{max,STA} - N_{0,STA} = -0.357 N_{0,FR} + 2.666$; ($r^2 = 0.999$)	(1)
18 °C	$N_{max,STA} - N_{0,STA} = -0.436 N_{0,FR} + 3.092$; ($r^2 = 0.765$)	(2)
21 °C	$N_{max,STA} - N_{0,STA} = -0.521 N_{0,FR} + 4.047$; ($r^2 = 0.994$)	(3)
Combined effect of incubation temperature and the initial density of the Fresco culture		
	$N_{max,STA} - N_{0,STA} = 0.274 + 0.180 T - 0.275 N_{0,FR} - 0.01181 N_{0,FR}$; ($r^2 = 0.913$)	(4)

($N_{max,STA} - N_{0,STA}$) – increase of *Staph. aureus* counts in stationary phase, $N_{0,FR}$ – initial density of the Fresco culture, T – incubation temperature.

necessary addition of Fresco culture and thermal mode during manufacture of food to ensure a minimal net growth of *Staph. aureus*.

According to the EU regulation [17], total amounts of *Staph. aureus* in raw milk cheeses should not exceed 4 log CFU·g^{-1}. Assuming properly drawn milk with 100 CFU·ml^{-1} of *Staph. aureus* [18], to keep its growth at a level lower than 2.0 log CFU·ml^{-1}, the initial Fresco density should be at least 4.0 log CFU·ml^{-1} or 2.5 log CFU·ml^{-1} at 21 °C or 18 °C, respectively.

Similarly, also culture A, which contains *Lb. acidophilus*, was able to inhibit growth of *Staph. aureus* 2064 or *Staph. aureus* D1 from maternal milk in milk co-cultures [19]. In agreement with ELKINS et al. [20] and DELBES et al. [13], the growth of *Staph. aureus* is mainly inhibited due to lactic acid production and a subsequent drop in the pH value. The ratio between the initial inoculum of *Staph. aureus* and LAB in the culture determines the efficiency of the inhibition as well.

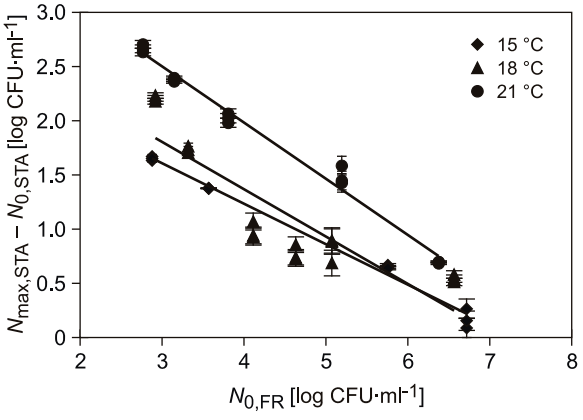

Fig. 2. Effect of initial Fresco culture density on the growth of *Staph. aureus* in relation to the incubation temperature.

($N_{max,STA} - N_{0,STA}$) – increase of *Staph. aureus* counts in stationary phase, $N_{0,FR}$ – initial density of the Fresco culture.

It was observed by CHARLIER et al. [21] that when the population of *Staph. aureus* was higher than that of *Lc. lactis*, *Staph. aureus* reached the same counts as in a pure culture. On the other hand, for ratios of 1:1 or 1:10 for *Lc. lactis, Staph. aureus* population reached lower maximal counts, of about 4 log CFU·ml^{-1} to 5 log CFU·ml^{-1}. And moreover, competition for nutrients, such as nicotinamide and biocin, may also play a significant role. Availability of these nutrients may trigger other mechanisms of inhibition, for instance, secretion of metabolites, peptides or signalling molecules [21].

Growth of *Staph. aureus* in ewes' lump cheese with or without Fresco addition at 18 °C

The competitive and inhibitory potential of Fresco culture on the growth of *Staph. aureus* was verified in ewes' lump cheeses prepared from raw milk with addition of 1% culture. This was compared with ewes' lump cheese prepared from raw milk without addition of the culture.

As presented in Fig. 3, pH of 5.0, unacceptable for growth of *Staph. aureus*, was achieved after 5 h of fermentation if there was addition of Fresco. Such short pH lag phase is crucial for the inhibition of the growth of pathogens during cheese manufacture, as it was already mentioned. Our results confirm that the higher diminution of pH during first 6 h of fermentation is achieved, the lower growth of *Staph. aureus* takes place. The growth of *Staph. aureus* in ewes' lump cheese with addition of Fresco to only about 0.96 log CFU·ml^{-1} was observed, even if the growth rate (Gr_{STA} = 0.345 log CFU·g^{-1}h^{-1}) was more than twice as high than in a milk mono-culture at 18 °C (Gr_{STA} = 0.118 log CFU ml^{-1}h^{-1}) or in cheese prepared without Fresco (Gr_{STA} = 0.117 log CFU·g^{-1}h^{-1}). In accordance with OLARTE et al. [22] and MENÉNDEZ et al. [23], due to the fast pH value decrease to 5.0, the undesirable growth of *Staph. aureus* was not observed and consequently, the EU regulation

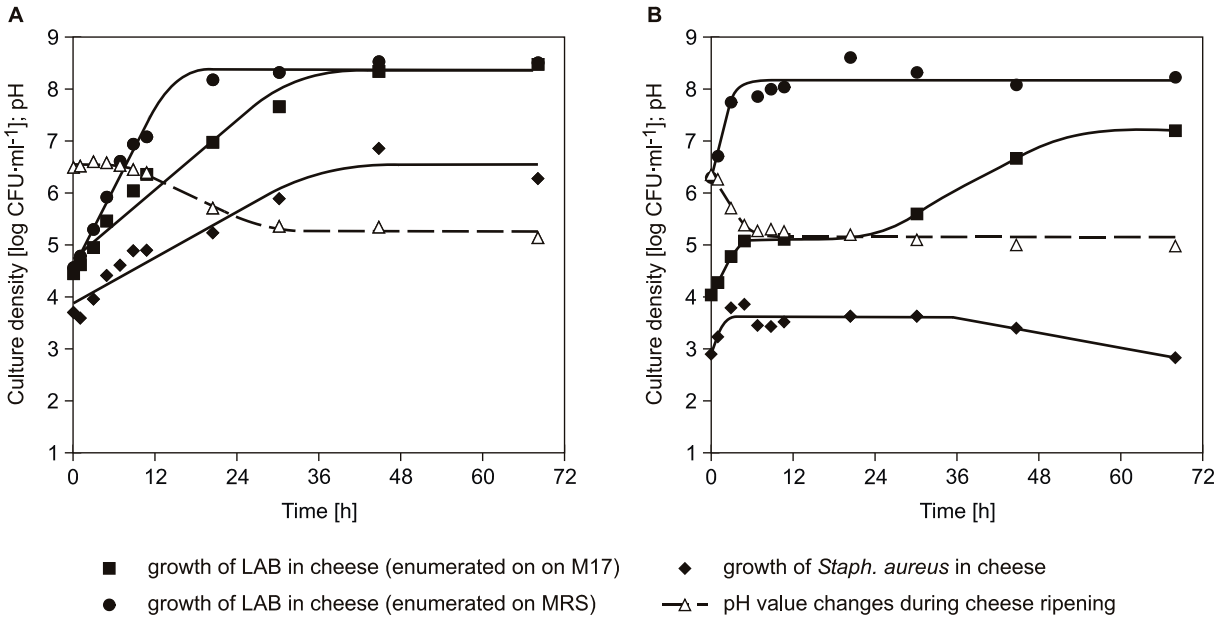

Fig. 3. Growth dynamics of microorganisms and pH value changes during fermentation of ewes' lump cheese without Fresco addition (A) and with 1% of Fresco addition (B) at 18 °C.

limit was not exceeded. Contrary to this, in cheese without Fresco the amount of *Staph. aureus* grew to about 2.9 log CFU·ml^{-1} and its maximal counts exceeded 6 log CFU·ml^{-1}.

CONCLUSION

The Fresco culture showed a strong inhibitory effect on *Staph. aureus*, at initial concentrations of at least 5 log CFU·ml^{-1} and at temperatures typical for the ewes' lump cheese production. The obtained growth data provide predictions for dairy practice to meet process hygiene criteria of the products.

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