Application of Quantitative Approach Focused on the Competition of Lactic Acid Bacteria Culture with Coagulase-positive Staphylococci under the Conditions Related to Artisanal Cheese Fermentation

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Abstract

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A quantitative analysis of *Staphylococcus aureus* growth was carried out in milk fermented with the mesophilic mixed-strain culture of lactic acid bacteria (LAB), in relation to the conditions prevailing during artisanal ewes' lump cheese production. Both the temperature and initial volume of the culture had a dramatic effect on the behaviour of the *S. aureus* strain under study. Depending on the conditions, the growth, reaching maximal population density (N_{max}) and a strong inhibition of *S. aureus* were observed. Regression analysis of the results enabled us to explain the relationships between the parameters such as the growth or inhibition rate, lag phase and N_{max}–N_0 and the temperature and initial culture density on the other hand. The surface methodology enabled to show that the rate of *S. aureus* inhibition (r_{inh}) was affected by the temperature and volume of LAB inoculum following the equation: $r_{inh} = -0.1302 + 0.02325 \times T - 0.000975 \times T^2 - 0.00001 \times T^2 \times V_0^2$ ($R = 0.965$). The fact that *S. aureus* increased in number mostly only by about 1 log and did not reach 10^6 CFU/ml during our model experiments was the most useful for good manufacturing practice. The knowledge obtained in this study concerned with the quantitative behaviour of *S. aureus* including the information on the inhibition at the selected environmental factors (temperature, inoculum size, and pH) is essential for the improvement of the fermentation process, quality and safety of artisanal ewes' cheese production used as a raw material for industrial Bryndza cheese production.

Keywords: *Staphylococcus aureus*; lactic acid bacteria; growth competition; surface methodology

The safety and quality of fermented raw foods are generally determined by the presence of pathogenic and spoilage microorganisms, their interaction with lactic acid bacteria, intrinsic and extrinsic environmental and technological factors (Görner & Valík 2004). This fact concerns also the short ripened ewes' lump cheese traditionally produced in Slovak Republic at the upland cottages immediately after milking. The cheese is curdled with rennet, fermented by native lactic acid bacteria, and ripened shortly for 7 days to 10 days (Figure 1). Then it is usually sent to the cheese factory for the production of the soft Bryndza cheese. Despite the raw milk origin, the ewes' lump cheese is also consumed as fresh cheese (Palo & Kaláb 1984).

This work deals with the behaviour of coagulase-positive staphylococci whose population belongs to the ubiquitous microflora of ewes' milk.

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Moreover, *S. aureus* is able to multiply rapidly, especially during the initial phase of preparation when natural lactic acid bacteria are in the lag phase and a sufficient amount of lactic acid has not yet been produced. The initial period to reach pH 5.3 lasted on average up to 30 h in upland artisanal ewes' cheese production (Valík *et al*. 2004a). However, *S. aureus* is very competitive in milk and dairy environment; it is quite sensitive to higher lactic acid concentrations.

The growth of *Staphylococcus aureus* and potential production of heat-stable enterotoxins with respect to the food matrices and conditions of food preparation symbolises the potential or even real threat to public health through food poisoning outbreaks. As reported by EFSA, 18 EU Member States informed on 293 food-borne outbreaks caused by *Staphylococcus* subsp. However, only 30% outbreaks were verified, two cases from a verified one and one base from a possible outbreak having been fatal (EFSA 2011). According to the official control, the staphylococcal enterotoxins were detected/found in 9 ewes' cheeses and 13 other food samples, even heat-treated, from total 170 food samples analysed (MPRV SR 2010).

Response surface methodology (RSM) consists of a set of statistical and mathematical techniques that can be used to characterise the relationships between the response and the independent variables, alone or in combination, on the processes (Baş & Boyaci 2007). The objective of using RSM is to improve and optimise the process (depended variables). RSM has become very popular in the recent period for description and optimisation of various processes. In the predictive microbiology, for example, it is often used to describe the relationships between the combination of factors and the growth curve parameters in order to predict the growth parameters of *Leuconostoc mesenteroides* (Zurera-Cosano *et al.* 2006) or heat-resistance of *Alicyclobacillus acidoterrestis* (Bahçeci & Acar 2007).

In a previous work (Valík *et al*. 2004a), we indentified insufficient acidification rate during the ewes' cheese fermentation. Based on the above information, the aim of this work was to characterise the *S. aureus* growth during milk fermentation. Subsequently, we tried to find maximal inhibitory effect on *S. aureus* growth using various initial volumes of the starter culture and incubation temperatures. In order to prevent this toxigenic bacterium from reaching the density of 10^6 CFU/g the work also focused on using proper incubation temperature and inoculum size of mixed mesophilic LAB culture in combination. This could be essential for the improvement of the fermentation process, quality and safety of ewes' lump cheese that is used as a raw material for the industrial production of original Bryndza cheese in Slovak Republic.

MATERIAL AND METHODS

Experimental design. Triplicated milk samples were simultaneously inoculated with an 18h *S. aureus* culture to the initial density $N_0 = 3.6 \pm 0.2$ log CFU/ml together with 1–6% v/v of standard mesophilic mixed-strain LAB culture A. Static fermentation of the samples was carried out at 8, 12, 15, 18, 21, and 25°C.

Microorganisms. The strain of *Staphylococcus aureus* D1 was isolated from human milk by Sirotná, MSc. from the Public Health Authority of the Slovak Republic (Bratislava, Slovak Rwpublic). The growth of this strain as a mono-culture was described by Medveďová *et al.* (2009). The identity of *S. aureus* was confirmed by the API system (BioMérieux, Marcy l'Etoile, France). Additionally, Gram staining and catalase tests were performed. The LAB culture A consisted of mesophilic lactococci and *L. acidophilus.* In fact, the culture A that was used within the experiments was the synonym for the fresh acidophilus milk Acidko (Meggle/Rajo, Bratislava, Slovak Republic).

Inoculation and cultivation conditions. The strain of *S. aureus* D1 was maintained on the slopes of Plate Count Agar (PCA, Imuna, Šarišské Michaľany, Slovak Republic) at 5 ± 1°C. The standard suspension of the isolate was prepared from an 18h culture grown on the PCA agar at 37°C. This staphylococcal suspension was inoculated aseptically into 300 ml of pre-tempered milk in order to reach as constant initial *S. aureus* counts in each sample as possible (approximately 10^3 CFU/ml). We respected the method of inoculation that was described and validated in our recent work (Valík *et al*. 2008).

Number of S. aureus in milk. The actual counts of *S. aureus* in the samples inoculated with a constant concentration of the individual D1 strain were determined at predefined time intervals by ten-fold dilution and cultivation on the Baird-Parker Agar according to the STN ISO 6888-1 (1999) standard procedure in order to gain the growth curves. LAB were enumerated on M17

Agar (Merck, Darmstadt, Germany) after incubation at 30°C for 48 hours.

Fitting of growth curves and calculating growth parameters. The growth curves of *S. aureus* at each combination of the temperature and LAB inoculum size were primary modelled by the DMFit-model of Baranyi and Roberts (1994) which had been kindly provided by Dr. J. Baranyi (IFR, Norwich, UK). Secondary, the standard response surface methodology of the Statistica 7.1 software package (StatSoft Inc., Tulsa, USA) and linear regression tools by Steppan *et al.* (2006) were used. The response surface and response profile were generated for easier comprehension of the condition for the growth control of *S. aureus* by the growth competitiveness and lactic acid produced by culture A in co-culture.

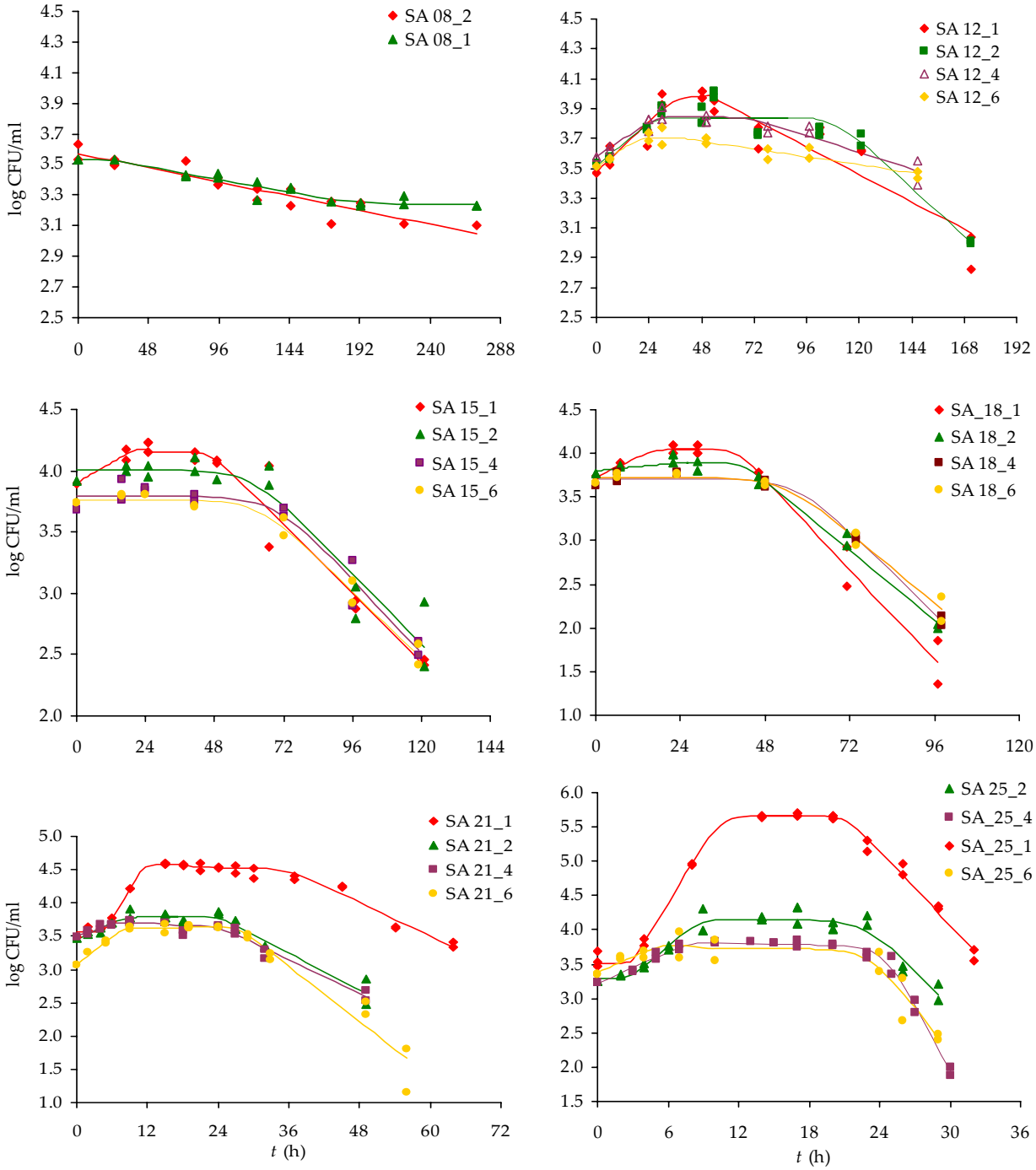


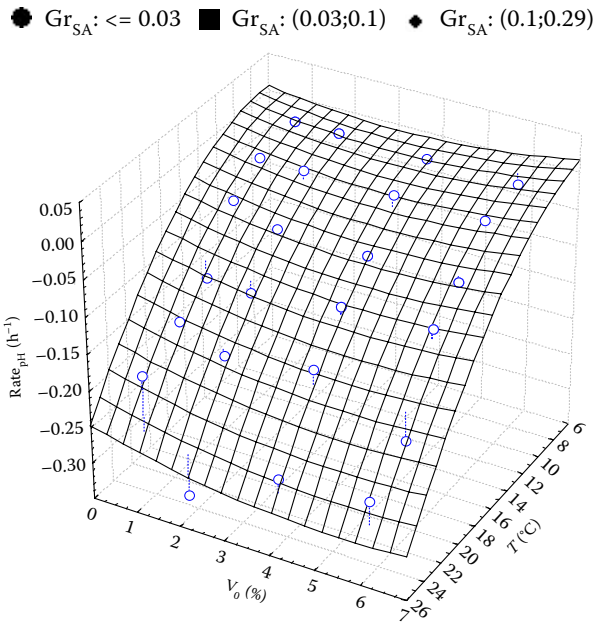
Figure 1. Plots of the growth dynamics of *S. aureus* D1 in milk as dependent on temperature and volume of the 24 h LAB culture inoculum

RESULTS AND DISCUSSION

In the previous study, we showed that acidifying of the ewes' milk curd started after 10–20 h period and went on intensively for 20 h in eight products manufactured under upland farm conditions (Valík *et al.* 2004a). Thus, the level of acidity equivalent to pH of 5.2 to 4.9 was usually reached in young cheese after 35 ± 5 h from the beginning. In those field trials, the initial numbers of *S. aureus* in ewes' milk were about 1.7×10^2 CFU/ml, but in cheese after 3 days of ripening they reached 1.5×10^6 CFU/g. Then the number of *S. aureus* declined to 5.0×10^2 CFU/ml after 6 days at optimal acidity of the cheese of pH 4.9–4.8. The fact that *S. aureus* exceeded the critical number became the main reason for next research. Regarding to these findings, the number of coagulase-positive staphylococci as the process hygiene criterion should not exceed 10^4 CFU/g (Commission Regulation (EC) No 2073/2005).

Effect of temperature and LAB inoculum size on the growth dynamic of *S. aureus* D1

The growth of *Staphylococcus aureus* D1 in relation to the incubation temperature from 8°C to 25°C and amount of LAB inoculum was studied in milk co-culture. As shown in Figure 1, most of the growth curves, except those at 8°C, comprised all the growth phases. Thus, the growth rates (Gr_{SA}), maximal density reached in the stationary phase (N_{max}) and the rates of inhibition (r_{inh}) were estimated. One of the aims of this study was to find the conditions enabling to control *S. aureus* growth during the artisanal cheese production. In the first view, this is demonstrated in Figure 1. Based on the plots above, it was supposed that all the parameters had been influenced by the incubation temperature and growth of competitive lactic acid bacteria determined as the amount of their inoculum. The greatest difference between the initial and maximal numbers of *S. aureus* (N_{max}–N_0) > 2 log CFU/ml was found only at 25°C and 1% (v/v) inoculum size of the culture. In other trials, except the case of 21°C and 1% of inoculum, the *S. aureus* growth exerted less than 1 log. Based on these results, it is strongly recommended to use the starter culture in artisanal cheese production. Even in the mountain areas, this can be performed by inoculation of LAB in the form of fresh fermented acidophilus milk Acidko. The potential of this culture for the milk acidification is demonstrated in Figure 2 showing the rate of pH decrease as statistically highly relevant to the temperature and inoculum size. This was also the reason why this culture was used in further experiments.



$$rate_{pH} = -0.0226 + 0.0088 \times T - 0.007 \times V_0 - 0.0007 \times T^2 - 0.0004 \times T \times V_0 + 0.0015 \times V_0^2$$

Figure 2. The rates of pH decrease (1/h) as dependent on temperature and inoculum size of mesophilic LAB culture during milk fermentation (R^2 = 0.963)

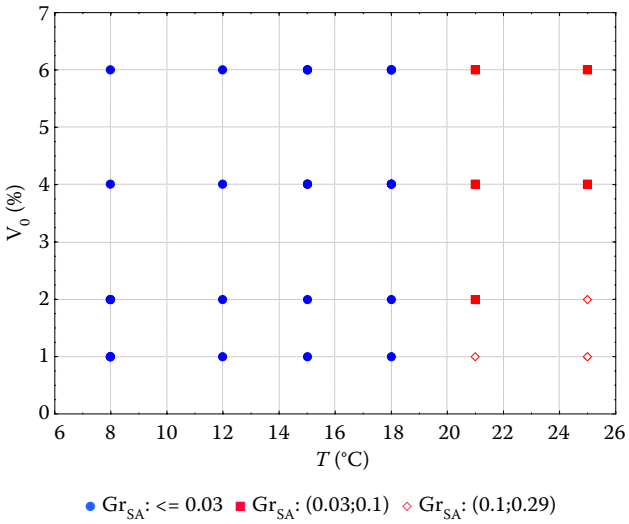


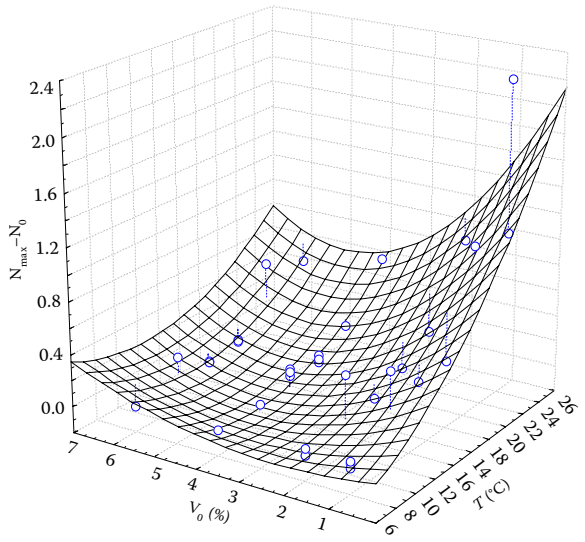
Figure 3. The effect of temperature and inoculum size of LAB on distribution of *S. aureus* growth rate (Gr_{SA}) in milk (the symbols represent the intervals of the Gr_{SA} values in log CFU/ml/h)

Effect of temperature and LAB inoculum size on the growth parameters of *S. aureus* D1

The surface methodology was also used to describe to which extent the growth parameters of *S. aureus* D1 were affected by the factors mentioned in milk fermented by mixed culture A consisting of mesophilic lactococci and *L. acidophilus.* Multiple linear regression analysis with the factors interaction showed that the growth rate of *S. aureus* (Gr_{SA}; log CFU/ml/h) in co-culture with culture A was determined by the temperature, volume of the culture A inoculum, and lag pH under the following equation: $Gr_{SA} = -0.465 + 0.0209 \times T - 0.0006 \times T \times V_0 + 0.0013 \times T \times \text{lag pH} - 0.0072 \times \text{lag pH}$. Good fitting of experimental data and using it in a practical argumentation can be demonstrated by the high correlation coefficient $R^2 = 0.922$ and low residual mean square error $RMSE = 0.037$. Additionally, the effect of the LAB inoculum and temperature on the growth rate of *S. aureus* can be presented in the response profile (Figure 3) showing the area of its maximal rates at lowest LAB inoculation and the highest temperature used in the experiments (empty red symbols). The growth rates from 0.1 to 0.29 log CFU/ml/h are equivalent approximately to the values of time to double (t_d) from 3 to 1 h. On the other hand, the conditions under which the lowest *S. aureus* rates were observed provided some arguments how to control microbial contaminants in artisanal cheese production. For example, the rates lower than 0.03 log CFU/ml/h ($t_d > 10$ h) were observed at 18°C and below any LAB inoculum volumes.

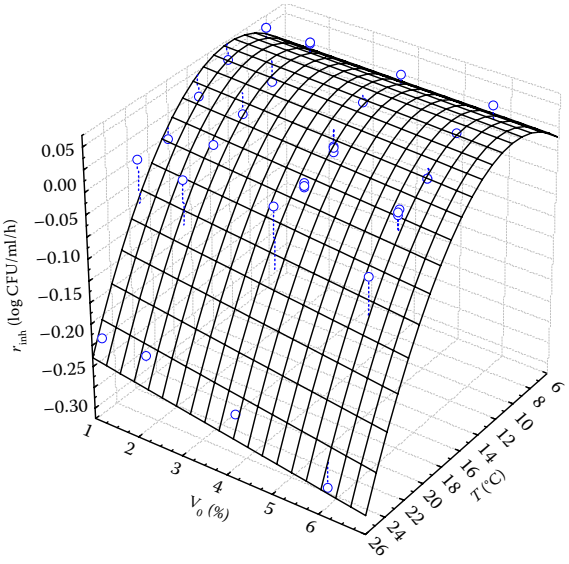
Other results in Figures 3 and 4 refer to total increase of *S. aureus* density (the difference between maximal density reached in the stationary phase and initial numbers, $N_{max}–N_0$) and the rate of inhibition estimated in *S. aureus* death phase (r_{inh}), respectively. Both responses affected by LAB growth and metabolism were recognised easily in the primary modelling and are described above in Figure 1. The surface methodology enabled to find the optimal range of the factors studied such as the initial LAB inoculum volume (V_0) and temperature (T) for the lowest *S. aureus* increase ($N_{max}–N_0$; Figure 4) or the highest decrease after reaching the stationary phase (Figure 5). For example, the intersection of 1 log increase during the growth and the inhibition of *S. aureus* in the death phase faster than –0.03 log CFU/ml/h (equivalent to the decrease in 1 log for 33.3 h) was achieved in the area of 18°C to 21°C and LAB culture initial size of 1–6 % v/v except, however, of the combination of 1% v/v inoculum and 21°C.

Coagulase-positive staphylococci represent a population consisting of real *S. aureus* strains able to produce heat-stable staphylococcal enterotoxins (SEs) causing food intoxication (Jay 2000). Kousta *et al.* (2010) presented their investigation of *S. ureus*



$N_{max}–N_0 = -0.02991 + 0.106 \times V_0 - 0.02852 \times T \times V_0 + 0.00322 \times T^2 + 0.00242 \times T \times V_0^2$

Figure 4. Total increase of *S. aureus* D1 in milk vs. temperature and inoculum size of mesophilic LAB culture ($R = 0.887$)



$r_{inh} = -0.1302 + 0.02325 \times T - 0.000975 \times T^2 - 0.00001 \times T^2 \times V_0$

Figure 5. Inhibition rates of *S. aureus* D1 in milk vs. temperature and inoculum size of mesophilic LAB culture ($R = 0.952$)

levels in both unpasteurised and pasteurised milk cheeses from different geographic regions of Ireland. Their results showed that 336 out of 351 cheese samples (96%) met the EU regulations for *S. aureus*. Twelve samples collected from the same producer revealed *S. aureus* counts higher than 10^4 CFU/ml but none of them was positive as to enterotoxin. Jakobsen *et al.* (2011) reported the mean numbers of *S. aureus* in raw milk cheese production. The highest levels were recorded after 5–6 h (after first pressing) and the limit just mentioned above was exceeded rather in caprine than bovine young cheeses. In coincidence also with our previous works (Valík *et al*. 2004a,b) and that if Delbes *et al.* (2006) studying raw milk cheeses ripening including semihard, soft, or traditional ones, the first 24 h of the fermentation appeared to be critical for *S. aureus* growth. LeMarc *et al*. (2009) also found starter LAB culture Fresco 1100 (Chr. Hansen, Hørsholm, Denmark) that effectively inhibited the growth of *S. aureus* during milk fermentation. When this culture was inoculated at the levels ranging approx. from 10^3 CFU/ml to 10^5 CFU/ml, the inhibition occurred at a relatively constant concentration of the lactic acid bacteria. They also developed a model taking this critical LAB population density into account.

The inhibitory potential of LAB on *S. aureus* involving acidification, bacteriocin production and H_2O_2 is well documented. It may act at more than one levels. Firstly it involves with hampering its growth physiology thus disturbing its growth rate and/or survival and secondly with interfering with the modulation of the expression of the virulence factors, thus impairing its pathogenic potential Charlier *et al.* (2009).

In this work, we report that the competition and inhibitory effect of LAB on *S. aureus* was determined by the initial LAB inoculum size and temperature. The combinations of these factors were optimised by response surface methodology providing the range in which the growth rate and total increase of the toxigenic strain D1 were minimal and the rate of inhibition maximal. Except of other hygiene measures preventing from contamination these are probably the only factors that can be used actively in safe cheese preparation in upland farm conditions.

CONCLUSION

The LAB may allow *S. aureus* to grow to some extent depending mostly on their prevalence (inoculum size) and temperature during milk fermentation. *S. aureus* is able to reach maximal density for a limited period until acids or other metabolites have achieved inhibitory levels. After reaching maximal density in the stationary phase, *S. aureus* is strongly inhibited. Similarly to the first order chemical reaction, the inhibition is linear in relation to time while the rate of inhibition is more dependent on the temperature than on the inoculum size. The increase of the *S. aureus* numbers (N_{max}–N_0) during fermentation was dependent on the temperature and initial dominance of the LAB.

Based on the results of this study and in compliance with Adams and Nicolaides (1997), the use of starters is strongly recommended in artisanal ewes' lump cheese production because only this step can assure the initial dominance of LAB and also support the growth of natural LAB present in raw milk.

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