Variability of growth parameters of *Staphylococcus aureus* in milk

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Summary
Growth of 15 strains of *Staphylococcus aureus* in milk at 15 °C was studied in 87 experiments, in order to determine the reproducibility of the growth parameters measured. The average growth rate (*Gr*) at 15 °C was 0.067 log CFU.h^{-1}, ranging from 0.54 to 0.77 log CFU.h^{-1}, which was equivalent to a doubling time ranging from 12.8 h to 9.0 h. Using a standard inoculation of an 18 h culture, the initial counts in milk of 3.68 log CFU.ml^{-1} and the duration of the lag phase of 14.4 h were determined with the standard deviations of 0.2 log CFU.ml^{-1} and 2.4 h, respectively. Based on ANOVA tests, the growth rates of individual strains did not produce different variances at the statistically significant 5% level. The results show that despite the errors connected with cultivation methods of determination microbial counts, the coefficient of variation (*CV*) of the growth rates for all 15 strains was as low as 7.1%. When 6 growth curves for a single strain were studied in two independent experiments, a *CV* of 1.2% for the growth rate was determined.

Keywords
growth parameters; variability; *Staphylococcus aureus*; predictive microbiology

Staphylococcus aureus is a Gram-positive coccus, which forms grape-like clusters. This bacterium is facultatively anaerobic, mesophilic, catalase-positive and oxidase-negative. It grows within a temperature range from 7 °C to 48 °C and in a pH range from 4.2 to 9.3. Its distinctive feature is halotolerance equivalent to minimal water activity (a_w) values ranging from 0.83 to 0.85 [1]. Strains of these bacteria are characterized by production of heat-stable enterotoxins, which may be produced at a_w values as low as 0.86 under aerobic conditions.

S. aureus is generally associated with skin and mucous membranes of warm-blooded animals, including humans. BAIRD-PARKER [2] considered this organism to be transient and not usually becoming a part of the resident flora of the skin. Irrespective of this fact, growth of *S. aureus* is connected with artisanal bryndza cheese produced from raw ewes' milk in Slovakia. The level of milk contamination by these pathogenic bacteria may be increased when mastitis is present in the herd. During milking and primary treatment of milk on farms, *S. aureus* may colonize the parts of technological equipment that are difficult to clean [3]. For example, typical counts of *S. aureus* in properly drawn ewes' milk are between 100 and 200 CFU.ml^{-1} [4], and *S. aureus* is considered to be one of the ubiquitous contaminants. The presence of toxin-producing strains in ewes' lump cheese or bryndza cheese produced from raw milk is dependent on various factors that include animal health status and environmental, hygienic and technological conditions during production. On the other hand, fermentation and ripening of cheese reduce the probability of enterotoxin production. According to [5], increase in the bacterial population during initial 24 h of cheese production in normal conditions is about 1.5 to 3 log CFU.ml^{-1}. The maximum contents of *S. aureus* of $m = 10^4$ CFU.g^{-1} and $M = 10^5$ CFU.g^{-1}, ($n = 5, c = 2$; where n is number of units comprising the sample, c – number of sample units giving values greater than m or between m and M) is a criterion of process hygiene for cheeses produced from raw milk according to Regulation (EC) No. 2073/2005 [6].

When analysing a bacterial growth curve, lag time and the exponential growth phase are the most important for growth assessment. At present, the Baranyi model and Gompertz function are the most widely used primary models for the description of microbial growth [7, 8].

The growth of a culture of a particular microbial strain in foods is determined by its intrinsic

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properties and by the food environment [3, 9]. According to several authors [10, 11], the response of microbial populations to these factors is reproducible. This fact permits the definition of mathematical relations between the parameters calculated from the growth or mortality curves and factors of the food environment such as temperature, water activity (a_w), and pH. Such results are defined by secondary models [12]. Based on both primary and secondary models, the application software, databases and expert systems have been designed for microbiologists to predict the behaviour of microorganisms in food [13]. A primary goal of the predictive approach in food microbiology is to anticipate the growth of microorganisms based on known factors of the environment and to evaluate the implications of this process for the safety and durability of foods [14]. Its application was also incorporated as "ratio legis" of microbiological legislation [6].

Our study was aimed at statistical evaluation of the growth parameters of *S. aureus* strains isolated from ewes' milk in order to assess the precision of the determination of growth parameters. This information would indicate the precision and reliability of subsequent growth predictions related to the behaviour of *S. aureus* in other foods.

MATERIAL AND METHODS

Strains and culture condition

Fourteen strains of *Staphylococcus aureus* were isolated from ewes' milk and cheese by the State Veterinary and Food Institute (Prešov, Slovakia). One strain from a human maternal milk was provided by the Public Health Institute of Slovak Republic (Bratislava, Slovakia). The identity of the strains was additionally confirmed by the API system (BioMérieux, Marcy l'Etoile, France). The strains were maintained on slopes of glucose-tryptone-soya agar (GTSA; ImunaPharm, Šarišské Michaľany, Slovakia) at 5 °C. A standard stationary-phase inoculum (0.1 ml from 10^3 dilution of a 18 h culture of each strain in milk at 37 °C) was inoculated aseptically into 300 ml of pre-tempered ultra high temperature-treated cows' milk in order to reach as constant initial *S. aureus* counts in each sample as possible. The samples were incubated at (15 ± 0.5) °C without shaking and *S. aureus* counts were determined at predefined time intervals by ten-fold dilution and cultivation on Baird-Parker agar (ImunaPharm) according to the ISO 6888-1 standard procedure [15].

Primary modelling

S. aureus log counts and time were modelled and growth parameters estimated by Baranyi DMfit version 2.1 (Institute of Food Research, Norwich, United Kingdom) [16].

The growth parameters from individual parallel experiments were analysed by statistic tools of Microsoft Office 2003 (Microsoft, Redmond, Washington, USA) and Statistica data analysis software system, version 7.1 (Statistica, Tulsa, Oklahoma, USA) [17].

RESULTS AND DISCUSSION

Eighty-seven carefully done growth experiments with 15 staphylococcal strains were performed in milk in order to produce a database for statistical evaluation. The incubation temperature of 15 °C was selected for two reasons: first, for its correspondence with the ewes' cheese ripening temperature and, secondly, due to proper timing of *S. aureus* determination needed for the construction of the growth curves. Results are summarized in Table 1. The average initial counts of *S. aureus*, determined immediately after inoculation, showed a low standard deviation repre-

Tab. 1. Growth parameters of *S. aureus* in milk at 15 °C, based on 87 growth curves of 15 strains.

	Lag time	G_r	T_d	N_0	N_{max}
	[h]	[log CFU.h^{-1}]	[h]	[log CFU.ml^{-1}]	
Average value	14.4	0.067	4.5	3.68	8.17
Standard deviation	2.4	0.005	0.3	0.20	0.29
Median	14.8	0.068	4.4	3.71	8.21
Minimum	8.1	0.054	3.9	3.16	7.19
Maximum	18.7	0.077	5.5	4.14	8.71
n	87	87	87	87	87
CV [%]	16.7	7.1	7.3	5.5	3.5

Stationary 24 h cultures of each strain in milk at 37 °C were used as the inoculum. G_r - growth rate, T_d - time to double, N_0 - initial number of *S. aureus* after inoculation, N_{max} - maximal number of *S. aureus* in the stationary phase, *n* - number of growth data, *CV* - coefficient of variation.

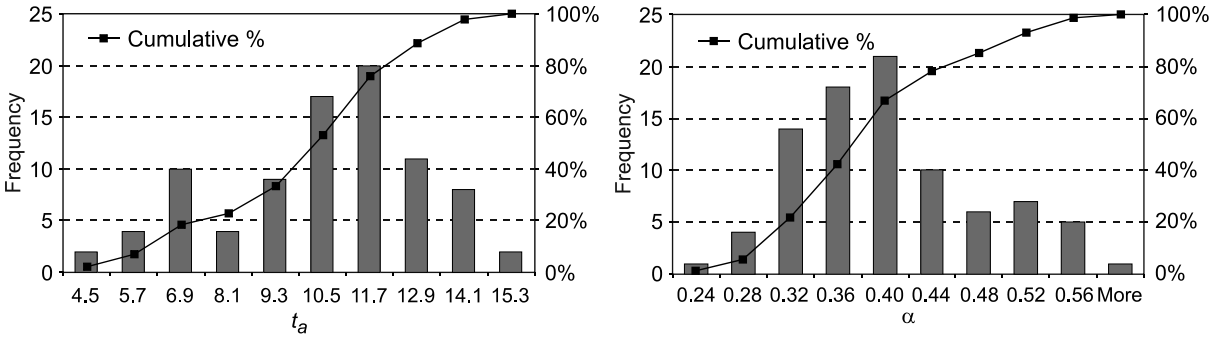

Fig. 1. Histograms of the time for adaptation (t_a) and the parameter of *S. aureus* inoculum physiological state α at pH 6.7 and 15 °C (n = 87).

sented by a coefficient of variation (CV) of 5.5% (log N_0 = 3.68 ± 0.20 CFU.ml^{-1}; where N_0 means initial number after inoculation). The counts in the stationary phase ranged between 7.2 and 8.7 log, (average log N_{max} = 8.17 ± 0.29 CFU.ml^{-1}; CV = 3.5%; where N_{max} means maximal number of *S. aureus* in the stationary phase).

Good standardization of the procedures prior to inoculation is demonstrated by the distribution of h_0 parameter, which ranged from 0.51 to 1.40 with a standard deviation of 0.20, with similar average and median values of 0.97 and 1.00, respectively. From this parameter introduced as "work to be done" by Baranyi, Roberts and McClure [18], the physiological state of our staphylococcal microbial population (α) was quantified by an average value of α = exp(-μ.λ) ≈ 0.37 to 0.38, where μ, λ are maximum specific growth rate and lag phase, respectively.

On the other hand, taking into account Buchanan three-phase linear model, the average period for adaptation of our stationary phase *S. aureus* inoculum was t_a = 9.9 ± 2.6 h. This model divides the population lag into two periods: a period for adaptation to the new environment (t_a) and the time for new generation to produce biological components for cell replication (t_m) [19]. The distribution of this adjustment period is shown in Fig. 1.

The highest variability among the growth data in Table 1 and Fig. 2 was associated with the lag phase duration. This fact is in agreement with the biological complexity of microbial growth included in various primary mathematical models [8]. Lag phase, as the most variable growth parameter, reflects the previous history of the stationary phase inoculum used, and the variability in the physiological state of the cells, the time for production of the biological components needed for replication and the adjustment period to the new environment. In unstressed populations, Robinson et al. [20] found the variation of lag times only about ± 2 doubling times, which was more than our lag time scatter of 5.76 h (equivalent to only about ± 1.3 doubling time).

The average growth rate (Gr) of *S. aureus* in milk at 15 °C was 0.067 ± 0.005 log CFU.h^{-1} (n = 87; CV = 7.1%), which corresponded to the doubling time (T_d) of 4.5 ± 0.3 h. The lag times

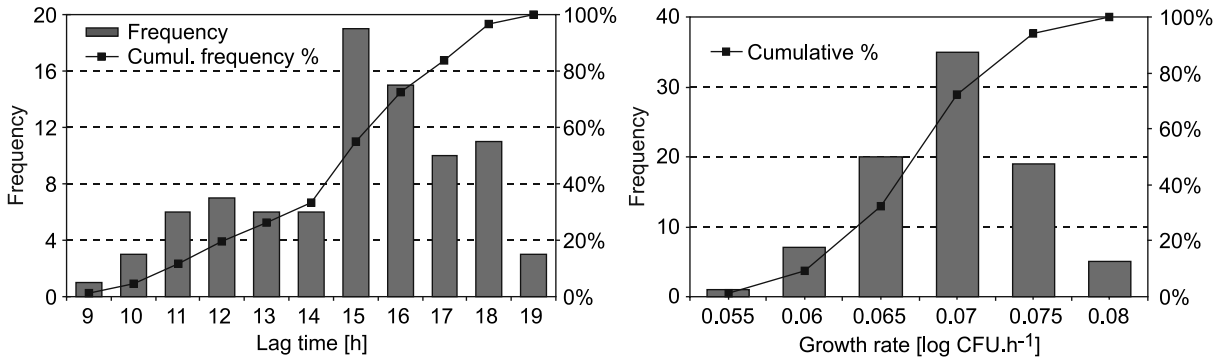

Fig. 2. Histogram of the values of lag time and growth rate of the 87 growth curves of the 15 *S. aureus* strains in milk at 15 °C.

Tab. 2. Average values and variances of growth rates determined for individual *S. aureus* strains and results of ANOVA test for group variability.

SUMMARY				
Strains	Count	Sum	Average	Variance
S. aureus 2064	6	0.385	0.064	5.96×10^{-5}
S. aureus 1744	6	0.412	0.069	7.34×10^{-7}
S. aureus 2006	6	0.405	0.067	5.38×10^{-6}
S. aureus 2610	6	0.392	0.065	2.22×10^{-5}
S. aureus 2070	6	0.415	0.069	9.87×10^{-6}
S. aureus 2609	9	0.616	0.068	2.28×10^{-5}

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*-value	*P*-value	*F crit*
Between Strains	0.00013	5	2.58×10^{-5}	1.268	0.301	2.503
Within Strains	0.00067	33	2.03×10^{-5}			
Total	0.0008	38				

SS - sum of squares, *df* - degree of freedom, *MS* - mean squares, *F*-value - the ratio of mean squares between the strain to mean squares within the strain, *P*-value - probability of observing a value of the *F*-statistic greater than *F* when population variances are of equal probability, *F crit* - the critical value *F* for a statistical level of $\alpha = 0.05$.

ranged from 8.1 h to 18.7 h with the average value of 14.4 h and standard deviation of 2.4 h ($n = 87$; $CV = 16.8\%$).

Anova test

To confirm the null hypothesis on the similarity of variances among growth rates belonging to the same strain and whole group of 15 strains, the strains 2064, 1744, 2006, 2610, 2070 and 2609 were selected. In these cases, six to nine growth rates were determined. The average values within the individual groups and the result of ANOVA test are shown in Table 2. First of all, the *F*-value of 1.27 showed that mean squares between the groups were just slightly higher than the mean square of the entire population tested within ANOVA test. As the *P*-value of 0.30 was higher than $\alpha = 0.05$, the previous hypothesis could not be rejected at a statistically significant 5% level. Thus, the variances among growth rates of various *S. aureus* strains showed mutual similarity.

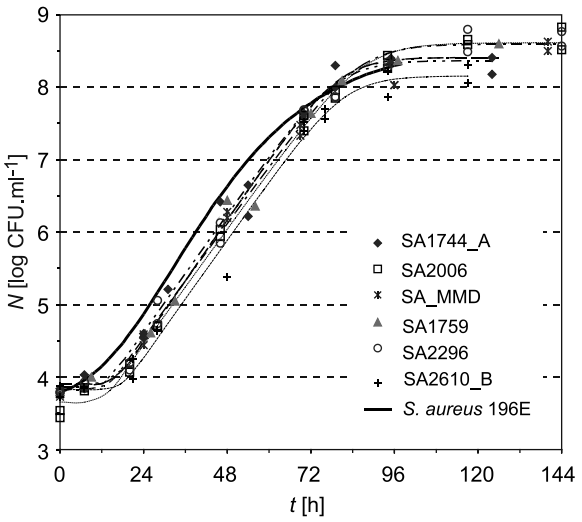

Fig. 3. Comparison of growth curves of 6 different strains of *S. aureus* in milk at 15 °C.

Validation of results

The growth curves shown in Fig. 3 represent three parallel independent trials with practically no difference between each other. A slight difference is shown in comparison with *S. aureus* 196E strain grown in broth (solid line; data from Pathogen Modeling Program – PMP, version 7, Wyndmoor, Pennsylvania, USA) [21].

The determined values of growth rate are very close to the values generated by PMP, and by the Combase Predictor (Institute of Food Research; Fig. 3 and Table 3). The average value of growth rates of the tested *S. aureus* strains was slightly lower than predicted by these programs, resulting in an increase in doubling time by 0.5 h. This difference may be attributed to the fact that both software programs processed data from growth experiments in which the growth medium was infusion broth, instead of milk used in our experiments.

CONCLUSION

The results demonstrate that the duration of the lag phase and the growth rate of *S. aureus* in milk can be determined with a high degree of re-

Tab. 3. Values of growth parameters of *S. aureus* at 15 °C generated by PMP software v. 7.0 and Combase Predictor.

	Lag time [h]	Growth rate [log CFU.h^{-1}]	Time to double [h]
PMP 7.0	8.9	0.077	3.9
Combase Predictor	14.3	0.074	4.1

producibility. This is a prerequisite for assessing risks associated with this bacterium in milk and dairy products by predictive microbiology.

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