The Effect of Temperature and Water Activity on the Growth of *Staphylococcus aureus*

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Abstract: The growth responses of *Staphylococcus aureus* 2064 as affected by water activity and incubation temperature were studied in two different laboratory media. Growth parameters at temperatures from 7 to 51°C and a_w in the range from 1.0 to 0.86 were fitted using Ratkowsky models. The effect of temperature within its whole range on the specific growth rate was modelled by the extended model under the following equation: $\sqrt{\mu} = 0.0456\,(T - T_{min})\,[1 - e^{0.447(T - T_{max})}]$. The water activity values of tested media were adjusted by sodium chloride in the range from a_w = 1.0 to 0.86 and experiments were conducted at 15 and 18°C. The growth responses of *S. aureus* on water activity at 15°C and 18°C in PCA broth and BHI broth was described by simplified Ratkowsky model in the form: $\sqrt{\mu} = b \times a_w$. Validation of the found relationships confirmed sound fitting of the data and thus the referred results of the isolate originated from ewes' cheese can be used in the growth prediction of *S. aureus*, reliably.

Keywords: *Staphylococcus aureus*; temperature; water activity; predictive microbiology

Many intrinsic and extrinsic factors affect the growth and metabolism of foodborne microbial pathogens. The study of environmental conditions on the growth of pathogens is crucial to controlling and limiting their potential risk (McCann *et al.* 2003) and evaluation of microbiological safety and quality of foods (McMeekin *et al.* 2002). According to classical definition of Monod from 1949, "the growth of bacterial cultures, despite the immense complexity of the phenomena to which it testifies, generally obeys relatively simple laws", the responses of population of microorganisms to environmental factors are reproducible and are basis of predictive microbiology (Ross & McMeekin 1994).

Staphylococcus aureus, mainly its growth and staphylococcal enterotoxins production with respect to food conditions, symbolises potential even real threat of public health menace lying on food poisoning outbreaks. According to Akineden *et al.* (2008) and Kérouanton *et al.* (2007), *S. aureus* represents the second cause of foodborne diseases after spp. *Salmonella*. A characteristic feature that distinguishes *S. aureus* from other pathogenic bacteria is its high tolerance of low water activity values and NaCl concentrations up to 20% (Sutherland *et al.* 1994). Generally it is reported, that the minimal water activity for the *S. aureus* growth is in the range from 0.83 to 0.86 a_w (Troller & Stinson 1975), but those values dependent on the pH, temperature, humectants and atmospheric conditions (Sutherland *et al.* 1994; Baird-Parker 2000). With respect to enterotoxins production requirements, values of water activity for their production are mostly in the same range as for the growth of producer. In food with decreased water activity and at aerobic conditions, the enterotoxins can be produced even if the value is from 0.86 to 0.89 a_w (Ewald & Notermans 1998).

The aim of this work was to describe growth dynamic of *S. aureus* in dependence on temperature

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in milk and water activity in two different model media with the use of predictive microbiology approach.

MATERIAL AND METHODS

Microorganisms. The isolate of *Staphylococcus aureus* 2064 originated from the ewes' lump cheese was isolated by MVDr. Hanzélyová from the State Veterinary and Food Institution (Prešov, Slovakia). The identity of *S. aureus* was confirmed by the API system (BioMérieux, Marcy l'Etoile, France). Additionally, gram staining and catalase tests were performed.

Effect of temperature on** S. aureus* 2064 ***growth. The effect of temperature on the *S. aureus* 2064 growth was studied in two parallels of ultra high temperature-treated cows' milk (Rajo, Bratislava, SR) at temperature range from 7 to 51°C ± 0.5°C at static aerobic conditions.

Effect of water activity on** S. aureus* 2064 ***growth. The effect of water activity on the *S. aureus* 2064 growth was studied in two parallels of PCA broth (Imuna, Šarišské Michaľany, Slovak Republic) or BHI broth (Merck, Darmstadt, Germany) with adjusted water activity value at temperatures 15 and 18°C ± 0.5°C at static aerobic conditions. The water activity value of broths was set by NaCl (Sigma-Aldrich, Buchs, Switzerland) according to Rödel *et al.* (1979) and controlled by a_w-meter (Aw-sprint TH500, Nowasina, Lachen, Switzerland).

Inoculation and cultivation conditions. The isolate of *S. aureus* 2064 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 18 h culture grown on the PCA agar at 37°C. This staphylococcal suspension was inoculated aseptically into the 300 ml of pre-tempered milk, PCA broth or BHI broth 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). The static aerobic incubation of inoculated milk samples was performed at temperatures.

Number of** S. aureus **in milk. The actual numbers of *S. aureus* in samples inoculated with a constant concentration of the individual 2064 isolate were determined at predefined time intervals by ten-fold dilution and cultivation on the Baird-Parker Agar according to the STN ISO 6888-1 standard procedure (1999) in order to gain the growth curves.

Fitting the growth curves and calculating the growth parameters. The growth data, curves and parameters of the isolate under study were analysed, fitted and calculated, respectively, using the mechanistic modelling technique of Baranyi *et al.* (1993) which is incorporated in the DMF it tools kindly provided by Dr. J. Baranyi (IFR Norwich, UK). The growth parameters (lag phase duration, growth rate and others) from each individual growth curve were analysed in the secondary phase of modelling by statistic tools of the Microsoft Office 2007 (Microsoft, Redmond, Washington, USA) and the Statistica data analysis software system, version 7.1 (StatSoft, Inc., Tulsa, USA).

Secondary models. The maximal specific growth rate (μ) was modelled as a function of the incubation temperature. For the growth dependence on the incubation temperature the Ratkowsky extended model was used and is described by Equation:

$$\sqrt{\mu} = b(T - T_{min})\,[1 - e^{c(T - T_{max})}] \qquad (1)$$

where:
T – actual temperature
T_{min} – minimal growth temperature
T_{max} – maximal growth temperature
b, c – coefficients

The coefficients were preliminary determined as proposed Ratkowsky *et al.* (1983) and then optimised with the Microsoft Excel tool Solver.

For the purpose of the study of water activity effect on the growth rate, McMeekin *et al.* (1987) included into Equation (1) the parameter of water activity (a_w) and theoretical minimal water activity for the growth ($a_{w,\,min}$), that resulted in Equation 2:

$$\sqrt{\mu} = b\,(T - T_{min})\,\sqrt{(a_w - a_{w,\,min})} \qquad (2)$$

As only two temperatures were used in our experiments, the second term in the equation ($T - T_{min}$) was disregarded. Also the member of minimal water activity was ignored as the exact minimal value at optimal growth condition was not found for this strain. With respect to this approaches, the dependence of *S. aureus* growth in relation to water activity was by linear regression with the following modification of Equation 3:

$$\sqrt{\mu} = b\,\sqrt{a_w} \qquad (3)$$

Validation of the growth parameters. To validate the mathematical equations describing *S. aureus* responses to the various temperature and water activity conditions, several mathematical and statistical indices were used including the accuracy (A_f), bias (B_f) and discrepancy (% D_f) factors and regression coefficient (R^2). The accuracy (A_f), bias (B_f) and discrepancy (% D_f) factors were calculated as defined by BARANYI *et al.* (1999):

$$A_f = \left(\sqrt{\frac{\exp \Sigma_{k=1}^{m} [\ln f(\mu^k) - \ln \mu^k]^2}{m}}\right) \quad (4)$$

$$B_f = \left(\sqrt{\frac{\exp \Sigma_{k=1}^{m} [\ln f(\mu^k) - \ln \mu^k]^2}{m}}\right) \quad (5)$$

$$\%D_f = (A_f - 1) \times 100 \quad (6)$$

where:
μ – growth rate obtained from the growth curves
$f(\mu^k)$ – growth rate calculated from the equations describing experimental values
m – number of measurements

RESULTS AND DISCUSSION

Study of temperature influence on the *Staphylococcus aureus* 2064 growth dynamic

The growth of *Staphylococcus aureus* 2064 in relation to the incubation temperature from 7 to 51°C was studied in our previous work (MEDVEĎOVÁ *et al.* 2009). This work is focused on using of Ratkowsky extended model that describes growth rate dependence on temperature in whole temperature range (ROSSO *et al.* 1993; MCMEEKIN *et al.* 1993). The model prerequisite parameters are minimum and maximum temperatures that should be determined for the growth of organism under study. Both these cardinal values, T_{min} and T_{max} were determined as the temperature at which specific growth rate was zero from the data below and above temperature optimum, respectively.

Previous growth parameters obtained from primary growth curves are summarised in Table 1. Based on data, we can see that increase of incubation temperature positively influenced the rate of multiplication. In accordance with observation of

Table 1. Growth parameters of *S. aureus* 2064 in milk in relation to the incubation temperature

T (°C)	μ (h^{-1})	t_d (h)	λ (h)	N_0 (log CFU/ml)	N_{max} (log CFU/ml)
7	0.005	123.0	66.1	3.56	4.49
8	0.025	27.2	97.9	3.61	6.41
10	0.055	12.70	53.6	3.44	8.11
12	0.081	8.60	33.6	2.45	8.18
15	0.131	5.28	11.6	4.14	8.36
18	0.272	2.55	5.8	3.26	8.03
21	0.484	1.43	5.2	3.45	8.05
25	0.712	0.97	3.4	3.22	7.90
30	1.214	0.57	4.3	3.32	8.06
35	1.662	0.42	0.9	3.39	8.27
39	1.930	0.36	0.8	3.46	8.32
43	1.969	0.35	0.8	3.35	8.36
46	0.560	1.24	19.3	3.37	7.56
51	–0.136	–	–	3.49	–

T – the incubation temperature; μ – specific growth rate; t_d – time to double; λ – lag phase; N_0 – initial counts of *S. aureus*; N_{max} – numbers of *S. aureus* in the stationary phase

Burdová and Lauková (2001) and also Tatini (1973), the slowest growth was observed at 7°C. The duplication of staphylococcal population occurred after 123 h ($\mu = 0.005\ h^{-1}$). Despite studies of Dengremont and Membré (1995) and Notermans and Heuvelman (1983), who did not contemplated growth of *S. aureus* at 8°C even after one month of incubation, the isolate 2064 started to growth after 98 h (4 days) and the growth rate was about 5.5 times higher than at 7°C. Next increase of temperature to 10°C caused that growth rate was about 50% higher in contrast to previous value. The most intensive growth was observed at 43°C. The staphylococcal population duplicated every 21 minute with specific growth rate $\mu = 1.969\ h^{-1}$. At 46°C, the slowing of growth dynamic occurred and the specific growth rate was reduced about 3.5 times to the value of $\mu = 0.560\ h^{-1}$.

In keeping with above observation, the minimal and maximal growth temperature for the model development of 6°C and 47°C were applied. For the growth rate dependence of *S. aureus* 2064 on the temperature (Figure 1a), the model acquired the shape that is described by Equation (7):

$$\sqrt{\mu} = 0.0456\ (T - T_{min})\ [1 - e^{0.447(T - T_{max})}] \qquad R^2 = 0.993 \qquad (7)$$

The validation is inevitable part of mathematical prediction of microorganism growth in specific condition. Firstly, the graphical confrontation of observed and predicted values of growth rate is depicted with high agreement, expressed by the correlation coefficient of $R^2 = 0.993$. Secondly, the mathematical validation according to Equations 4 to 6 was carried out. The bias ($B_f = 0.976$) and accuracy ($A_f = 1.205$) factors indicate a good fit between the predictions with 21% of discrepancy. As a result of bias factor lower than 1, the Ratkowsky model will predict faster growth of pathogen microorganism that is acceptable especially in the case of pathogen growth predictions.

In addition, we compared the values predicted by Ratkowsky model with ComBase Predictor database, data reported by Fujikawa and Morozumi (2006) and also with isolate D1 as is shown in Figure 1a. Since, the origin of D1 isolate was maternal milk and data in ComBase database originated from synthetic media, the discrepancy between our data and specific growth rates for D1 isolate or data generated by ComaBase ranged from 21.5% to 22.4% ($B_f = 0.854$, $A_f = 1.224$ for ComBase data and $B_f = 0.888$, $A_f = 1.215$ for D1 isolate). Based on bias factors, the specific growth values were smaller than the model predicted. In other words, *S. aureus* D1 grew slower than it was assumed, as it was also in the case of data from ComBase database. Therefore, we can conclude that the Ratkowsky model provided realistic and safe estimates of growth dynamic of pathogenic microorganism *S. aureus*.

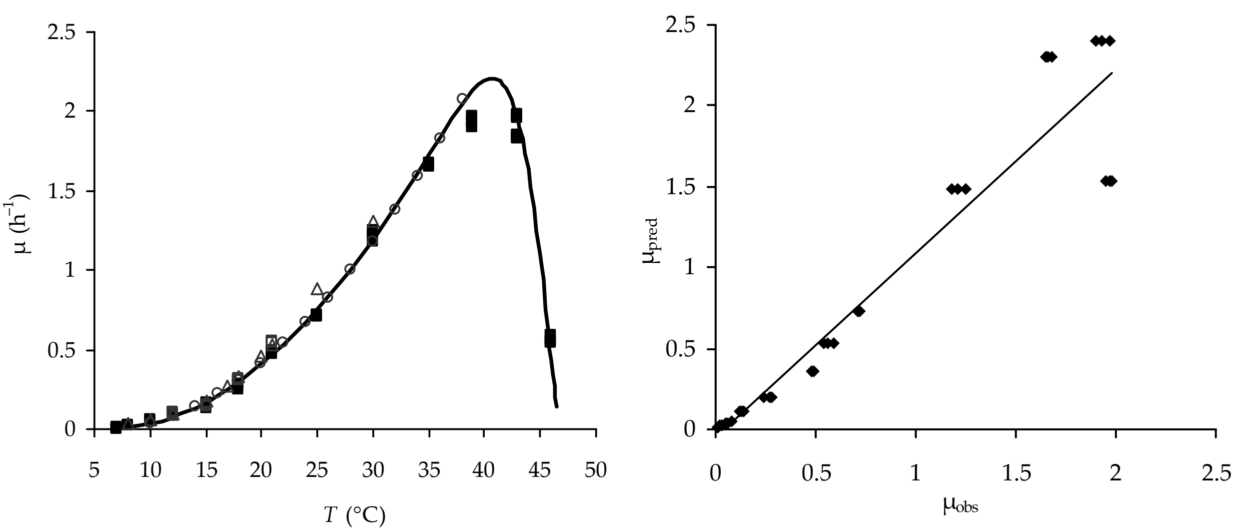

Figure 1a and 1b. Plots of the natural of specific growth rates (μ) versus T for *S. aureus* 2064 (a). Symbols (■) indicate the specific growth rate calculated from growth curves at each incubation temperature. The continuous line indicates the fitted μ vs. T function, where $\sqrt{\mu} = 0.0456\ (T - T_{min})\ [1 - e^{0.447(T - T_{max})}]$. The values of specific growth rates were compared with values reported by Fujikawa and Morozumi (2006) (○), ComBase Predictor (Δ), with isolate D1 from maternal milk (□). The comparison of observed and predicted values according to Ratkowsky model is shown in 1b with $R^2 = 0.993$

Study of water activity effect on the *Staphylococcus aureus* 2064 growth dynamic

Another important factor affecting the growth of microorganisms is the availability of the water for the growth and metabolic processes, termed as the water activity value. The effect of water activity on the growth of *S. aureus* 2064 was tested at two temperatures, 15 and 18°C.

More likely due to different media composition, the lowest or none addition of NaCl caused dissimilar a_w values of BHI and PCA broths. As a result, also the growth parameters deviated from each other about up to 20%. As there was no salt in culture media, *S. aureus* grew about 10% than in UHT milk in both PCA and BHI broth and at both temperatures. The increasing amount of NaCl reflected in prolonging of the lag phase duration and growth rate slowing. At 15°C and 13% of NaCl in media, there was no up growth observed in BHI broth in contrast to PCA broth were *S. aureus* still grew with the growth rate of μ = 0.012 h^{-1}. NaCl addition higher than 13% led to the inhibition of *S. aureus* 2064 in both synthetic media. Based on higher growth rates and shorter lag phases at all NaCl concentration, the PCA broth seemed to be more favourable substrate for the pathogen growth.

Similar effect of salt occurrence was observed also at 18°C. As the NaCl addition increased, the lag phase duration lengthened and the growth rate decreased. *S. aureus* 2064 confirmed high salt tolerance. As reported Valík and Görner (1993), the ability to withstand high concentration of salt is also strain dependent, since the minimal value of water activity for growth in their experiments ranged from 0.93 to 0.86 a_w. According to Baird-Parker (2000), minimal value of water activity in BHI broth adjusted by NaCl at 30°C was 0.86 (18% of NaCl). Jay (2000) observed, that no growth occurred at 8°C and pH = 4.3 and a_w = 0.85 (19% of NaCl), or at 12°C and pH below 5.5

Table 2. Growth parameters of *S. aureus* 2064 in PCA and BHI broth at 15°C and 18°C

PCA broth			%NaCl	BHI broth		
μ (h^{-1})	λ (h)	a_w		μ (h^{-1})	λ (h)	a_w
At 15°C						
0.155	0	0.998	0	0.164	19.4	0.984
0.162	11.1	0.992	1.72	0.108	27.0	0.979
0.114	31.5	0.966	5.00	0.096	39.5	0.957
0.073	65.2	0.945	7.95	0.074	71.3	0.940
0.053	104.8	0.923	10.6	0.042	150.1	0.923
0.012	285.8	0.902	13.05	–0.004	–	0.895
–0.0053	444.0	0.888	15.25	–0.005	171.2	0.881
–0.0048	412.0	0.865	18.17	–0.006	63.0	0.862
At 18°C						
0.304	13.2	0.988	0	0.273	11.6	0.985
0.280	13.1	0.983	1.72	0.268	10.5	0.978
0.206	9.5	0.964	5.00	0.169	15.6	0.955
0.161	31.5	0.944	7.95	0.143	38.0	0.936
0.083	48.6	0.930	10.6	0.067	68.5	0.923
0.060	63.4	0.913	13.05	0.053	95.2	0.900
0.007	408.8	0.893	15.25	0.007	303.2	0.884
–0.007	259.7	0.869	18.17	–0.006	140.1	0.865

μ – specific growth rate; λ – lag phase; a_w – value of water activity; % NaCl – addition of NaCl

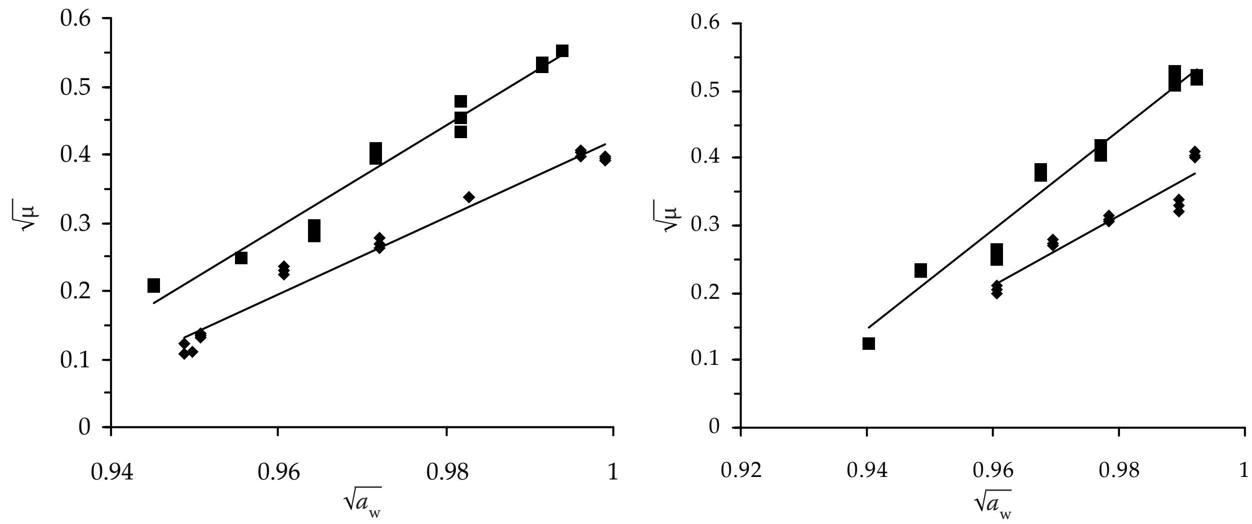

Figure 2a and 2b. Plots of the square of specific growth rates (μ) versus square of a_w for *S. aureus* 2064 at 15°C (◆) and at 18°C (■) in PCA (a) and BHI (b) broth. Symbols indicate the specific growth rate calculated from growth curves at each incubation temperature. The continuous line indicates the fitted μ vs. a_w function, where $\sqrt{\mu} = b\sqrt{a_w}$

and a_w = 0.9 (14% of NaCl) or when pH < 4.9 and a_w = 0.96 (8% of NaCl). In our case, the isolate 2064 showed negative growth at 15°C at a_w in the area from 0.888 to 0.895 (addition of NaCl about 13 to 15%) or at 18°C at a_w from 0.865 to 0.869 (addition of NaCl about 18%).

It was also noticed that except of cases when the *S. aureus* population was inhibited by high amounts of salt, *S. aureus* grew till 7 log counts at 15°C in PCA broth at NaCl concentration up to 13% or in BHI broth up to 11% of NaCl. Counts higher than 7 log were reached also in PCA broth at 18°C till 15% of NaCl and in BHI broth at 18°C up to 13% of salt in media.

With the aim to use Ratkowsky modified model, the negative values of specific growth rate representing inhibition of the microbial population were ignored. In contrast to observations of Dengremont and Membré (1995), the square of specific growth rate of *S. aureus* 2064 decreased linearly with the increasing of NaCl content in both broths. Naturally, the higher the incubation temperature was the higher the specific growth rates. The equations obtained by fitting the linear Ratkowsky model to the experimental data for the considered temperature and model media are reported in Table 3. The higher the value of R^2, as an overall measure of the fit attained, the better is the fit of the data to the model (Sutherland *et al*. 1994). With this connection, the model describing the growth responses in BHI broth at 15°C is the only one with correlation coefficient

Table 3. The indices of the internal performance of the Ratkowsky extended and Ratkowsky modified models for the *S. aureus* 2064 in liquid media

Model	A_f	B_f	% D_f	R^2
$\sqrt{\mu} = 0.0456(T - T_{min})[1 - e^{0.447(T - T_{max})}]$	1.205	0.976	20.5	0.993
$\sqrt{\mu_{PCA,15°C}} = 5.624\sqrt{a_w} - 5.203$	1.209	1.026	20.9	0.972
$\sqrt{\mu_{PCA,18°C}} = 7.387\sqrt{a_w} - 6.797$	1.168	1.001	16.8	0.967
$\sqrt{\mu_{BHI,15°C}} = 5.280\sqrt{a_w} - 4.859$	1.141	1.006	14.1	0.888
$\sqrt{\mu_{BHI,18°C}} = 7.344\sqrt{a_w} - 6.758$	1.199	1.006	19.9	0.961

$\sqrt{\mu}$ – square of specific growth rate; T – incubation temperature; T_{min} – minimal growth temperature; T_{max} – maximal growth temperature; a_w – value of water activity; A_f – accuracy factor; B_f – bias factor; % D_f – discrepancy factor; R^2 – correlation coefficient

Table 4. Published specific growth rates for *S. aureus* in various media

Predicted values of *S. aureus* 2064				Sutherland *et al.* (1994)		
T (°C)	NaCl (%)	μ_{BHI} (h^{-1})	μ_{PCA} (h^{-1})	T (°C)	NaCl (%)	μ (h^{-1})
15	0.5	0.175	0.175	15.5	0.5	0.150
15	4.0	0.128	0.125	15.5	4.0	0.121
15	8.0	0.083	0.078	15.5	8.0	0.089
18	0.5	0.339	0.343	19	0.5	0.392

T – incubation temperature; NaCl – addition of NaCl; μ_{BHI} – specific growth rate of *S. aureus* in BHI broth; μ_{PCA} – specific growth rate of *S. aureus* in PCA broth; μ – specific growth rate of *S. aureus*

below 0.900. However, the discrepancies between observed and predicted values of specific growth rates from all models give only up to 20% deviation. Thus, these four models can be considered as very consistent. As might be expected from bias factors, models were slightly slower than real values observed in laboratory media that were considered as more optimal for microorganism growth (Baranyi *et al.* 1999) and as was also mentioned above.

Furthermore, the comparison of the assumed behaviour of isolate 2064 with published data was performed and summarised in Table 4. The growth parameters predicted at 15°C in PCA broth and at 18°C in both media agreed relatively well with data predicted by Sutherland *et al.* (1994) obtained in tryptone soya broth at 15.5°C or at 19°C.

CONCLUSION

In this work, the temperature and water activity influence on the growth of *S. aureus* in milk was studied. The influence of incubation temperature in range from 7°C to 51°C was satisfactory described by Ratkowsky extended model. The results of validation showed that Ratkowsky extended model is useful tool for pathogen growth prediction in milk. The simple modification of Ratkowsky model with incorporated water activity parameter was also used for the prediction of *S. aureus* growth under changing salt conditions. Data obtained from modified Ratkowsky model were discussed in connection to apposite published data. However, further investigations are needed to study the growth responses of the pathogen on the water activity conditions at higher temperatures to classify combinations ensuring that critical densities of dangerous microorganism will not reach, e.g. in ewes' cheese which the strain under study come from.

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