Staphylococcus aureus 2064 growth as affected by temperature and reduced water activity

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Abstract

Based on 247 growth data, the growth of *S. aureus* 2064 in dependence on temperatures (8-50°C) and a_w values (0.999-0.83) was described. Optimal values of a_w at all studied temperatures were determined by using Gibson model. Its compatibility was confirmed by several statistical indices, *e.g.* root mean square errors (*RMSE* 0.003-0.138), standard errors of prediction (*%SEP* 0.6-17.5). Cardinal values for *S. aureus* growth (T_{min}=7.7°C, T_{opt}=40.6°C, T_{max}=46.7°C, a_{wmin}=0.808, a_{wopt}=0.994, μ_{opt}=1.97 1/h) were determined by using CM model with indices *RMSE*=0.071, *SEP*=17.5%. Our findings can provide relevant growth information that can be used in *S. aureus* exposure assessment or in validation of other data regarding the growth of this opportunistic pathogen in foods.

Introduction

In the traditional way of production of some cheese's varieties, raw milk is used. Thanks to the ubiquitous presence of coagulase-positive staphylococci in raw milk, also *Staphylococcus aureus* may be present. Its growth and potential production of 23 different types of heat-stable enterotoxins (SEs) with respect to food matrices and environmental conditions represent a potential risk to public health resulting in foodborne staphylococcal outbreaks. Besides SEs, *S. aureus* also disposes of other pathogenic factors – surface associated factors, enzymes and toxins (Asperger and Zangerl, 2003) that all contribute to the remarkable potential of *S. aureus* as a pathogen of human and animals. Moreover, this potential is multiplied by frequent occurrence of *S. aureus* in environment. In primary production and dairy environments, animals, people and operational environments are the main sources of a product's contamination by *S. aureus*. One third of human population is asymptomatic carrier of *S. aureus*, so this microorganism can contaminate foods through food handlers' cross-contamination (infected wounds, skin lesions) or by coughing and sneezing (Baird-Parker, 2000). Following contamination of food matrices, further destiny of microorganisms is dependent on the actual environmental factors. The knowledge of their effects on the pathogens growth is crucial to control their behavior (McCann *et al*., 2003) and consecutively to ensure microbiological safety of foods. A scientific basis of the HACCP concept and quantitative microbial risk assessment provides predictive microbiology that is based on a mathematical relationship between microbial growth responses and environmental factors (Mellefont *et al*., 2003). Such a knowledge may help to estimate the growth of microorganisms i) in the context of food safety (Ingham *et al*., 2009) or *e.g. Listeria monocytogenes* (Cornu *et el*., 2011) or ii) in the context of food spoilage (Pin *et al*., 1999) and extended shelf-life (Gougouli *et al*., 2011).

The nutritional requirements of *S. aureus* are complex and vary from strain to strain (Asperger and Zangerl, 2003). A characteristic feature which distinguishes *S. aureus* from other pathogenic bacteria is its high tolerance to low water activity (a_w) values and NaCl concentrations of up to 20%. Generally, minimal a_w required for *S. aureus* growth is 0.83-0.86 (Medveďová and Valík, 2012). Ability of *S. aureus* to grow at such high salt concentrations is related to its adaptive response to osmotic stress. This is due to the intracellular accumulation of compatible solutes, including proline, betaine, choline, taurine, or by their transport from a growth medium (O'Byrne and Booth, 2002). There is a variety of transport systems, activated/induced by NaCl, which are responsible for entry of osmoprotectants into the cell. Besides the accumulation of compatible solutes (*e.g.* proline, betaine, choline, taurine) to maintain intracellular turgor pressure in response to high osmolarity environments, *S. aureus* also responds to NaCl stress by altering specific gene and protein expression (Scybert *et al.,* 2003).

Several *S. aureus* growth data determined in both artificial media and in foods are present in databases. However, the majority of them do not provide *S. aureus* growth parameters in the whole growth range of selected environmental factors or a combination of several environmental factors. Uniformity of specific growth rates (0.163±0.025 1/h) of 64 different *S. aureus* strains all isolated from raw milk cheeses was confirmed by coefficient of variance v_c=15.5% in Medveďová *et al.* (2014) supposing uniformity of whole species responses to environmental factors. Also, the similarity of growth rates between isolate 2064 and enterotoxinogenic *S. aureus* 14733 isolated from milk vending machine biofilm was confirmed (Medveďová *et al.*, 2017). Based on that, the main objective of this study was to provide additional data of *S. aureus* 2064 isolated from specific artisanal raw milk cheese that can be used in the validation of *S. aureus* growth already determined. The preliminary results of salt addition effect at only 15 and 18°C were published by Medveďová *et al.* (2009). So, the main objective of this work is to characterize the growth of *S. aureus* isolate in dependence on a_w (adjusted by NaCl, as the main food preservative) and in whole temperature range, and subsequently to determine cardinal environmental factors. In this connection, these data may be taken into consideration in prediction of additional growth studies of various isolates from specific foods and food environment that may contribute to reliable assessing of the variability of *S. aureus* growth dynamic under specific conditions.

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Materials and Methods

Microorganism

S. aureus 2064 isolate was selected for this study. It was isolated from ewes' lump cheese by MVDr. Hanzélyová (State Veterinary and Food Institution, Prešov, Slovakia) in 2009 by using cultivation method on selective Baird-Parker agar according to EN ISO 6888-1. Its identity was confirmed by Medveďová *et al.* (2009), however the isolate does not produce enterotoxins.

Media

The isolate was maintained between experiments on Plate Count Agar slopes (PCA; Sigma-Aldrich, St. Louis, USA) at 6±1°C and stored for months at -40°C. A standard *S. aureus* 2064 inoculum (0.3 mL from 10^3 dilution of an 18h culture grown on PCA agar at 37°C) was inoculated aseptically into 300 mL of pre-tempered PCA broth (Difco; Le Pont de Claix, France) to reach initial *S. aureus* counts as close as possible to 10^3 CFU/mL. The samples were incubated in three parallels and repetitions at static aerobic conditions at 8, 10, 12, 15, 18, 21, 25, 30, 35, 37, 39, 43, 46 and 50°C±0.5°C in order to study *S. aureus* 2064 growth in dependence on temperature and a_w.

The effect of water activity on *S. aureus* 2064 growth

The effect of a_w on *S. aureus* 2064 growth was studied in three parallels and repetitions of PCA broth with adjusted a_w value at temperatures from 8 to 50°C at static aerobic conditions. The a_w values of broth were set by the NaCl addition (Sigma-Aldrich, Buchs, Switzerland) and controlled by an a_w-meter (Aw-Sprint TH500, Novasina, Lachen, Switzerland).

Counts of *S. aureus* 2064 in growth media

At chosen time intervals, depending on actual temperature, numbers of the isolate were determined according to ISO 4833-1:2013 on PCA agar.

Fitting the growth curves and primary modeling

S. aureus 2064 growth parameters were analyzed, fitted and calculated using DMFit Excel Add-in package version 3.5 (ComBase managed by United States Department of Agriculture-Agricultural Research Service, Washington, USA and University of Tasmania Food Safety Centre Hobart, Australia) that incorporates the mechanistic model of Baranyi and Roberts (1994). The actual counts were plotted against time and fitted to a model for the estimation of μ (specific growth rate), ΔN (*S. aureus* 2064 growth increase in stationary phase against initial density). Growth parameters from the individual parallel experiments were further analyzed by the secondary models using the Microsoft Office, version 2007 (Microsoft, Redmond, USA) and the Statistica data analysis software system, version 8.0 (Statsoft, Inc., Tulsa, USA).

Secondary modeling

The specific growth rate was firstly modeled as a function of a_w according to Gibson *et al.* (1994). Using the model described by the following formula

$$ln\mu = C_{0+}C_1 b_w + C_2 b^2{}_w$$

the optimum a_w value at each temperature for the maximum growth rate can be calculated as follows:

$$a_{wopt} = 1 - \left(\frac{C_1}{2C_2}\right)^2$$

Indices C_0, C_1 and C_2 where estimated by linear regression and a_{wopt} is the value of a_w at which the maximum specific growth rate equals its optimal value μ_{opt}. The parameter b_w is calculated as:

$$b_w = \sqrt{1 - a_w}$$

Cardinal model CM was used to describe the cumulative influence of temperature and a_w on the microbial growth rate. The specific growth rate was subjected to secondary modelling in relation to the incubation temperature (Rosso *et al*., 1993). The combined effect of temperature and a_w was determined according to the gamma concept (Zwietering *et al*., 1991), based on individual cardinal models (Rosso *et al*., 1993):

$$\mu_{max}(T, a_w) = CM(T, a_w) = \mu_{opt} \cdot \tau(T) \cdot \gamma(a_w) \quad (1)$$

where

$$\tau(T) = \frac{(T-T_{max})(T-T_{min})^2}{(T_{opt}-T_{min})[(T_{opt}-T_{min})(T-T_{opt}) - (T_{opt}-T_{max})(T_{opt}+T_{min}-2T)]} \quad (2)$$

and

$$\gamma(a_w) = \frac{(a_w-a_{w,max})(a_w-a_{w,min})^2}{(a_{w,opt}-a_{w,min})[(a_{w,opt}-a_{w,min})(a_w-a_{w,opt}) - (a_{w,opt}-a_{w,max})(a_{w,opt}+a_{w,min}-2a_w)]} \quad (3)$$

where T_{opt} is the temperature at which the maximum specific growth rate equals its optimal value μ_{opt}. The cardinal values of temperature and a_w were subsequently defined by nonlinear regression as the equation parameters.

Validation of the growth parameters

To evaluate goodness of fit of the mathematical equations describing *S. aureus* responses to the various temperature and a_w conditions, several mathematical and statistical indices were used. The regression coefficient (R^2) and the root mean square error (*RMSE*; as the average deviation between observed and predicted values) was calculated according to Garcia *et al*. (2011), the per cent variance (*%V*; as a measure of the goodness of the model fit) was used as was given by Daughtry *et al.* (1997). Finally, the bias (B_f) and discrepancy ($\%D_f$) factors as defined by Baranyi *et al*. (1999) were used. The indices were calculated according equations:

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(\mu_{obs}-\mu_{pred})^2}{n}} \quad (4)$$

$$\%V = \left[1 - \frac{(1-R^2)(n-1)}{(n-N_T-1)}\right] \times 100 \quad (5)$$

$$Bf = exp^{\frac{\sum_{i=1}^{n}(ln\mu_{pred}-ln\mu_{obs})}{n}} \quad (6)$$

$$\%Df = (Af-1) \times 100 = exp\sqrt{\frac{\sum_{i=1}^{n}(ln\mu_{pred}-ln\mu_{obs})^2}{n}} \quad (7)$$

where μ_{obs} and μ_{pred} are observed and predicted values of the specific growth rates, n is number of the data points, N_T is number of model terms, Af is accuracy indices.

Results and Discussion

Study of a_w and incubation temperature effect on *S. aureus* 2064 growth dynamic

The effect of the a_w and incubation temperature (at gradually ranked temperatures from 8°C to 50°C) on the *S. aureus* 2064 growth dynamic was described in model media (PCA broth). The a_w values of the tested media were adjusted by NaCl, using its addition in the range from 0% up to 21%, and subsequently expressed as the actual a_w value. The initial *S. aureus* 2064 concentration in all experiments (n=307) was 3.20±0.23 log CFU/mL with c_v=7.35% – where coefficient of variance is dependent on standard deviation and average specific growth rate value as

$$c_v = {}^{s_d}/_{\bar{\mu}} \times 100\%$$

– which was necessary for comparing the growth ability of the tested isolate under changing environmental conditions. On the other hand, the counts in stationary phase were strongly dependent on the incubation temperature and/or salt addition.

The growth parameters obtained from primary growth curves are summarized in Table 1. Generally, growth of isolate 2064 was faster as more optimal incubation temperature and a_w values were reached. Also, by increasing the incubation temperature, *S. aureus* 2064 tolerated more drastic conditions as expressed by lower minimal a_w values at which it grew. Since isolate 2064 was incubated at 8°C, it could not resist either the NaCl addition or the temperature of 8°C itself. These findings are in accordance with Normanno *et al.* (2005) who also did not observe growth of *S. aureus* at 8°C. However, according Valero *et al.* (2009), *S. aureus* grew at 8°C, a_w=0.989 (2% of NaCl) and *pH*<6 in 50% of cases, while at a_w levels below 0.983, its growth was only observed at *pH* values 6.0-7.0. Also, Valík and Görner (1993) reported that the ability of *S. aureus* to resist high salt concentration is strain dependent, since the minimal a_w value for growth in their experiments ranged from 0.93 to 0.86. At 10°C and 12°C, *S. aureus* 2064 was able to grow until the NaCl concentration of 5% (a_w=0.968) and 8% (a_w=0.951), in order. However, its dying at these temperatures was observed at NaCl additions higher than 8% (a_w=0.951) or 10% (a_w=0.928). Contrary, no growth at 10°C and a_w=0.971 was reported by Castillejo-Rodríguez *et al.* (2002) and at 12°C and a_w=0.950 as reported Buchanan *et al.* (1993). Also, Lee *at al.* (1977) observed that *S. aureus* did not grow at 12°C and a_w=0.915 (13% of NaCl). As reported Valero *et al.* (2009), resistance of *S. aureus* to low a_w values at 19°C was shown, since the growth was detected at *pH* 7.0 and a_w=0.867. In our case, *S. aureus* 2064 was

Table 1. *S. aureus* 2064 growth parameters in relation to the incubation temperature and a_w.

T	a_w	μ	Δ_N	T	a_w	μ	Δ_N	T	a_w	μ	Δ_N
8	0.998	-0.004*	-1.66*	21	0.890	0.026	4.35	37	0.913	0.509	4.15
8	0.989	-0.0004*	-0.24*	21	0.871	-0.023*	-1.62*	37	0.894	0.427	3.60
8	0.972	-0.003*	-0.77*	25	0.988	0.737	5.67	37	0.880	0.224	3.76
10	0.997	0.044	4.27	25	0.977	0.622	5.27	37	0.860	0.152	3.71
10	0.989	0.029	3.46	25	0.955	0.547	5.16	37	0.855	-0.025*	-2.15*
10	0.968	0.035	5.04	25	0.941	0.534	4.26	39	0.998	1.867	5.75
10	0.951	-0.002*	-0.98*	25	0.917	0.319	3.77	39	0.994	1.863	5.75
12	0.992	0.064	4.33	25	0.894	0.138	4.75	39	0.966	1.502	5.24
12	0.988	0.092	4.59	25	0.879	0.092	4.20	39	0.947	1.132	4.97
12	0.969	0.063	4.50	25	0.865	0.037	3.79	39	0.930	1.014	4.68
12	0.955	0.048	5.02	25	0.859	-0.261*	-2.19*	39	0.909	0.627	4.24
12	0.928	-0.005*	-0.95*	30	0.987	0.968	5.00	39	0.894	0.554	3.75
12	0.909	-0.006*	-1.28*	30	0.983	1.062	4.89	39	0.862	0.223	3.63
15	0.998	0.155	5.76	30	0.969	0.923	5.15	39	0.842	-0.003*	-1.48*
15	0.992	0.162	5.79	30	0.953	0.732	5.04	43	0.997	1.744	5.38
15	0.966	0.114	5.09	30	0.930	0.510	4.73	43	0.985	1.801	5.07
15	0.945	0.073	5.13	30	0.896	0.221	4.74	43	0.965	1.274	4.49
15	0.923	0.052	4.37	30	0.883	0.219	4.14	43	0.945	1.114	3.80
15	0.904	0.012	4.10	30	0.868	0.087	3.82	43	0.925	0.714	3.68
15	0.888	-0.005*	-0.84*	30	0.856	-0.004*	-2.19*	43	0.913	0.521	3.19
15	0.865	-0.005*	-0.87*	35	0.993	1.632	5.58	43	0.889	0.449	2.98
18	0.988	0.304	4.69	35	0.997	1.602	5.11	43	0.860	0.089	2.92
18	0.983	0.280	4.87	35	0.966	1.236	5.05	43	0.840	-0.020*	-2.02*
18	0.964	0.206	4.97	35	0.947	0.965	4.02	46	0.997	-0.831	-2.32
18	0.944	0.161	5.21	35	0.927	0.965	4.47	46	0.991	0.770	2.56
18	0.930	0.083	4.76	35	0.913	0.601	3.96	46	0.972	0.668	3.26
18	0.913	0.061	4.00	35	0.886	0.325	4.35	46	0.954	0.395	2.90
18	0.893	0.007	4.12	35	0.870	0.226	4.08	46	0.929	0.170	2.76
18	0.869	-0.007*	-0.84*	35	0.863	0.059	3.30	46	0.909	0.088	2.20
21	0.992	0.423	4.65	35	0.855	-0.228*	-2.25*	46	0.891	-0.055*	-2.06*
21	0.979	0.431	4.42	37	0.993	1.796	5.38	50	0.998	-1.013*	-2.96*
21	0.959	0.374	5.22	37	0.988	1.784	5.05	50	0.989	-0.873*	-2.42*
21	0.943	0.237	4.97	37	0.964	1.558	4.83	50	0.971	-0.486*	-1.70*
21	0.926	0.162	5.37	37	0.947	1.117	4.88				
21	0.908	0.114	4.45	37	0.925	0.654	4.51				

T - incubation temperature (°C), a_w - actual aw value of media, μ – specific growth rate (1/h), Δ_N – *S. aureus* 2064 growth increment in stationary phase against initial density (log CFU/mL), *decreasing of counts

not able to grow at 18°C if the NaCl addition was higher than 15% (a_w=0.893).

At higher temperatures (39 and 46°C) a similar effect of salt presence in the growth media on *S. aureus* growth was observed. The isolate confirmed its high salt tolerance even at these temperatures. The ability of bacteria to grow at high salt conditions is related to their adaptive response to osmotic stress by the intracellular accumulation of compatible solutes or by their transport from the growth medium. It was mentioned by Vaaomonde *et al*. (1984) that *S. aureus* cells died relatively rapidly in the presence of NaCl. However, a variety of transport systems, *e.g.* betaine (BPI and BPII) or proline (PutP) transport systems (O´Byrne and Booth (2002) are activated or induced by NaCl and are responsible for entry of osmoprotectants (betaine, proline etc.) into the cell. Besides that, *S. aureus* may also undergo an extensive program of gene and proteins expression in response to NaCl stress (Scybert *et al*., 2003) or temperature stress (Rigoulay *et al*., 2004). It was shown that HtrA proteins (heat shock-induced envelope-associated serine proteases) are essential for bacteria to survive at high temperatures (Lipinska *et al*., 1990) and that they are responsible for degradation of denatured proteins produced at high temperatures and or under osmotic stress (Rigoulay *et al*., 2004).

Interestingly, isolate 2064 was not able to grow at 46°C in media without NaCl. But 2% NaCl addition (a_w=0.991) into PCA broth caused that *S. aureus* 2064 started to grow and it grew only till 5 log counts. The final NaCl concentration at which the microorganism was able to multiply at 46°C was 13% NaCl (a_w=0.909). Studies have shown that in most cases a reduced a_w leads to an increase in thermotolerance (O´Byrne and Booth, 2002). Shebuski *et al.* (2000) observed the similar protective effect when *S. aureus* was heat treated in the presence of increasing salt concentrations. Santoro *et al.* (1992) reported that activation energy of water molecules is higher in presence of heat and so they have a potent ability to interact with proteins and accelerate their duration. O´Byrne and Booth (2002) have also found that increased proteins thermostability is frequently observed when the a_w value of the solvent is reduced, and that compatible solutes enhance thermostability. Cebrián *et al.* (2010) observed that 48°C was the last most protective heat-shock temperature for *S. aureus* in terms of developed thermotolerance. In our case, at 50°C no growth of *S. aureus* 2064 was detected at all.

Secondary modeling

Firstly, to describe the a_w effect on *S. aureus* 2064 specific growth rate at each incubation temperature in PCA broth, the model of Gibson *et al*. (1994) was used. Equations for *S. aureus* 2064 growth dynamic responses as a function of a_w values at all incubation temperatures are summarized in Table 2 with graphical representations depicted in Figure 1. By using this model, the optimal a_w values for *S. aureus* 2064 in PCA broth at each single incubation temperature can be calculated as it is summarized in Table 2. The optimal a_w values were strongly dependent on the incubation temperature. It was also interesting that at extreme temperatures *S. aureus* required some NaCl in the media, as is expressed by the lower optimal a_w values in comparison to the almost optimal incubation temperature. It can be assumed that the halophilic character of *S. aureus* is manifested in the need for some NaCl amount in the media to initiate the metabolism under less favorable conditions. However, in such a condition the a_w range allowing the staphylococcal growth is more limited, as it is expressed by the higher minimal a_w values in contrast to the higher or more optimal incubation temperature conditions.

Due the fact that the growth rate was simultaneously influenced by a_w and incubation temperature, we used the complete gamma concept. In this case, the gamma factors for studied environmental factors were calculated to establish the cardinal model (CM, Figure 2) that was adequate to describe the effect of incubation temperature and a_w on *S. aureus* 2064 specific growth rate. As one can see, with increases of the incubation temperature up to 40°C, the specific growth rate increased with a constant slope. At temperatures higher than 40°C, the growth of *S. aureus* slows down. Therefore, the curvature is observed in the optimal region, *i.e.* 35–40°C. According to CM model and combining a global fit, seven parameters of CM model, *i.e.,* cardinal temperatures (T_{min}=7.72±0.03°C, T_{opt}=40.63±0.04°C, T_{max}=46.73±0.02°C), cardinal a_w values (a_{wmin}=0.808±0.0023,

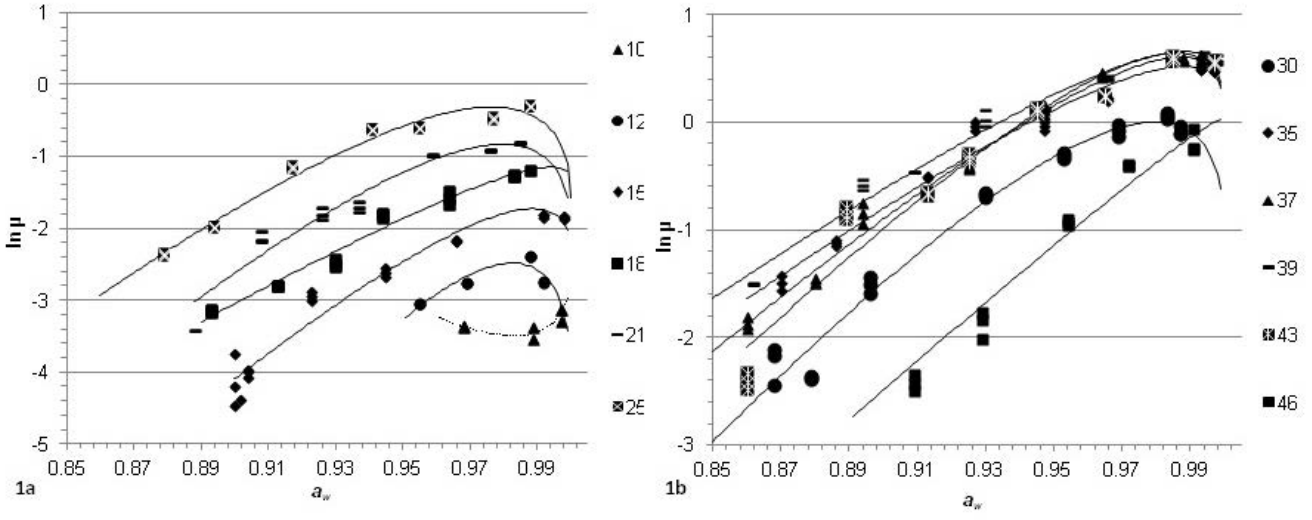

Figure 1. Plots of the natural logarithm of specific growth rates (*ln μ*) *versus* aw for *S. aureus* 2064. The symbols indicate the natural logarithm of the specific growth rate calculated from the growth curves at each a_w and incubation temperature. The continuous lines indicate fitted *ln* *vs.* a_w functions, where $b_w = \sqrt{(1 - a_w)}, \mu = exp^{(c_0 \cdot b_w^2 + c_1 \cdot b_w + c_2)}$, (1/h) and the actual equations for each individual temperature are summarized in Table 2.

a_{wopt}=0.994±0.0004, a_{wmax} fixed to 1) and the optimal growth rate (μ_{opt}=1.97 1/h) were identified. The optimal temperature of T_{opt}=40.63°C seems to be high, however, according to experimental data the isolate grew with the maximal specific growth rate at 39°C and the model only supported this observation. Also Sutherland *et al*. (1994) reported temperature about 40°C as optimal and Taub *et al*. (2003) published that *S. aureus* grew in bread with the highest specific growth rate also at 40°C. Knowing of cardinal values may increase the stability of products with increase *S. aureus* prevalence as they can help to set conditions during manufacture (heat treatment conditions), maximal allowed storage temperature ensuring no increase of *S. aureus* or maximal addition of salt leading to *S. aureus* growth inhibition. Further, the results can be also used in exposure assessment of *S. aureus* in raw milk cheeses manufactured traditionally in Slovakia as was earlier published by Ačai *et al*. (2014a) and Ačai *et al*. (2014b).

Validation

Several mathematical and statistical indices were used to validate mathematical equations describing *S. aureus* 2064 responses to environmental conditions (Table 2). In view of R^2 or more stringent term *%V*, the worst fit (*%V*<93%) of the Gibson model to the isolate growth rate at 10 and 12°C was achieved. This might be accounted for the worse *S. aureus* ability to adapt to the low incubation temperature, which was also noticed during the primary growth parameters analysis. However, it is worthy of note that *%V* of the CM model and of the majority of Gibson models, were higher than 98%. Taking into account that the smaller the *RMSE*, the better is the fit, the highest error is estimated by using the CM model. On the other hand, the lowest error of predictions (formulated as *%SEP*) was calculated for the Gibson model at 39°C although the highest error is expected at 46°C. Further, bias factor *Bf*<1 indicates slower predicted growth than the observed one, and *Bf*>1 indicates faster predicted growth than observed. In our case, faster growth in real media can be expected only if using Gibson model at 15°C and 18°C and in using CM model. Moreover, according to recommendation of Ross (1999) all models can be considered as acceptable since *Bf* is in range 0.90-1.05.

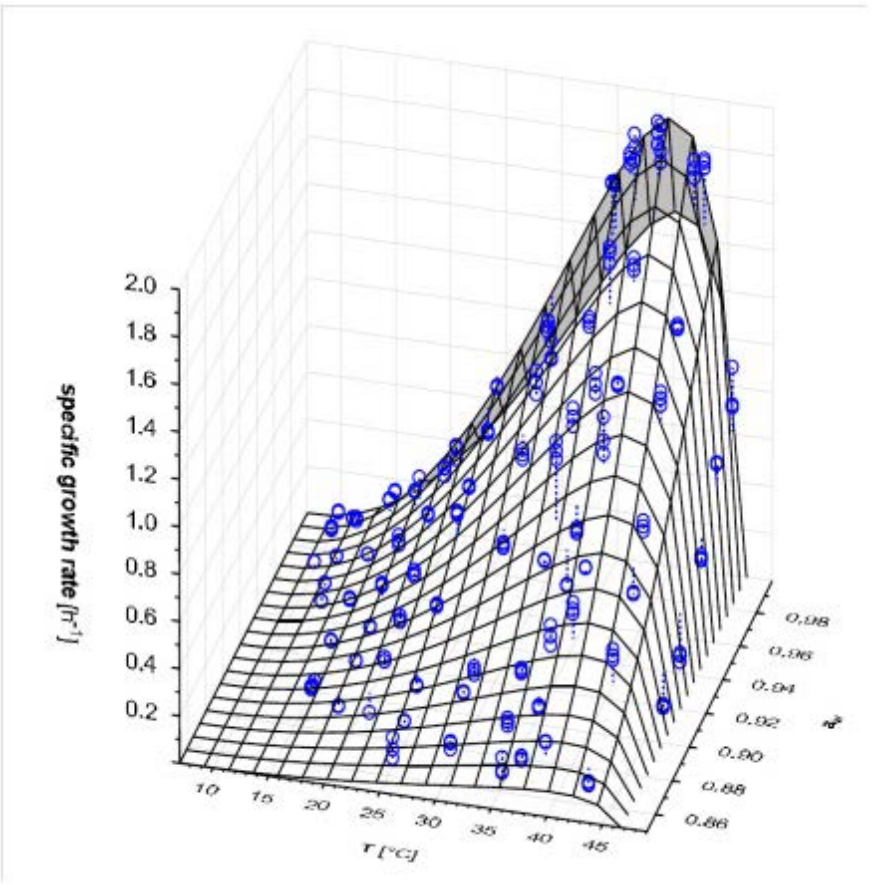

Figure 2. Graphical representation of specific growth rate responses of *S. aureus* 2064 in PCA broth as a function of temperature and aw (fitted with CM model).

Table 2. The Gibson model equations at each incubation temperature with optimal aw values or the CM model coefficients and the related indices of validation.

Equation	n	a_{wopt}	B_f	$\%D_f$	R^2	*%V*	*RMSE*	*%SEP*
$ln\mu_{10} = 53.972b_w^2 - 14.087b_w - 2.577$	9	0.982	0.999	6.9	0.755	51.0	0.0025	7.2
$ln\mu_{12} = -93.792 + 24.711b_w - 4.112$	12	0.983	0.999	10.6	0.814	74.4	0.0077	12.2
$ln\mu_{15} = -53.802b_w^2 + 11.471b_w - 2.342$	21	0.989	1.001	22.3	0.954	94.6	0.0105	14.8
$ln\mu_{18} = -32.829b_w^2 + 4.961b_w - 1.338$	21	0.994	1.001	11.7	0.974	96.9	0.0142	9.2
$ln\mu_{21} = -59.491b_w^2 + 16.983b_w - 2.048$	21	0.980	1.000	17.5	0.942	93.0	0.0252	12.8
$ln\mu_{25} = -53.522b_w^2 + 16.387b_w - 1.569$	21	0.977	0.999	11.9	0.977	97.3	0.0616	11.5
$ln\mu_{30} = -49.647b_w^2 + 14.186b_w - 1.013$	27	0.979	0.999	15.3	0.977	97.4	0.0422	6.8
$ln\mu_{35} = -30.633b_w^2 + 6.652b_w - 0.155$	24	0.988	0.999	12.2	0.972	96.8	0.1113	11.5
$ln\mu_{37} = -35.936b_w^2 + 8.010b_w - 0.159$	30	0.988	0.999	9.6	0.988	98.6	0.0538	8.2
$ln\mu_{39} = -29.074b_w^2 + 6.191b_w - 0.325$	24	0.989	1.000	9.7	0.981	97.8	0.0067	0.6
$ln\mu_{43} = -42.346b_w^2 + 10.144b_w - 0.035$	27	0.986	0.999	23.7	0.949	94.1	0.1381	12.7
$ln\mu_{46} = -29.621b_w^2 + 1.486b_w - 0.0025$	21	0.999	0.931	22.1	0.976	96.9	0.0692	17.5
STA 2064: CM model	27	0.0711	1.026	32.3	0.982	98.2	0.0711	17.5

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