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Staphylococcus aureus Enterotoxin Production in Relation to Environmental Factors

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Abstract

Staphylococcal enterotoxins (SEs) and SE-like toxins (SEIs) are the most notable virulence factors associated with *Staphylococcus aureus*. They are involved in food poisoning, toxic shock syndrome and staphylococcal infectious diseases in human. In dairy practise, the initial numbers of *S. aureus* play an important role especially at the beginning of the milk fermentation within the first 6 h or in 24-h-old cheese. As we presented in our previous works, one of the most effective tools to inhibit *S. aureus* growth is by adding a sufficient amount of active dairy starters, which are able to produce lactic acid very rapidly. Thus, by inhibiting the growth of *S. aureus* the production of SEs may be reached. Based on this study focusing on the effect of temperature, pH, water activity and initial numbers of lactic acid bacteria on the growth and the ability of *S. aureus* 14733 to produce SED, we consider it as a strong SED producer. The SED production was not limited with the incubation temperatures and the NaCl addition related to traditional cheese manufacture. As this isolate comes originally from such an artisanal cheese production, we can expect that other strong SE producer could be present in milk or environment. Besides strict prerequisites approach in production hygiene, it is necessary to add the starters ensuring the initial dominance of lactic acid bacteria (LAB) and supporting the growth of the natural LAB present in raw milk.

Keywords: staphylococcal enterotoxins, growth inhibition, water activity, lactic acid bacteria, predictive microbiology

1. Introduction

In Slovakia, the manufacture of “Bryndza” cheese from ewes’ lump cheese is of great importance to preserve the national gastronomic heritage. In the traditional way of production, it is

produced immediately after milking from raw milk in upland cottages. The cheese is curdled with rennet, fermented by native lactic acid bacteria (LAB) and ripened for 7–10 days. Then, it is usually sent to a cheese factory, where the next technology processes (including salting) take part resulting in the production of the final soft “Bryndza” cheese [1, 2].

As coagulase-positive staphylococci are ubiquitous in milk, the control of *Staphylococcus aureus* growth during the fermentation of young raw milk cheese means prevention against staphylococcal enterotoxins (SEs) production. During milk fermentation and cheese production, *S. aureus* is exposed to growth competition with LAB and the effect of their metabolites during artisanal raw milk cheese manufacture. However, *S. aureus* is competitive in milk and dairy products; it is quite sensitive to higher lactic acid concentration.

The growth of *S. aureus* and potential production of heat-stable staphylococcal enterotoxins (SEs) with respect to the food matrices and conditions of food preparation represent a potential, even actual threat of a public health menace residing in food-poisoning outbreaks. From the food point of view, the production of one or more SEs is the most crucial, because they are causative agents of staphylococcal food-poisoning outbreaks.

As a pathogen, *S. aureus* disposes of remarkable wide range of virulent factors causing different infectious and food-borne outbreaks. Due to the production of surface-associated factors, *S. aureus* can avoid opsonophagocytosis, form biofilm and adhere to the host cell matrix [3, 4]. Following colonization, *S. aureus* secretes various toxins and enzymes that are responsible for the lesions. Once *S. aureus* penetrates the subcutaneous tissues and reaches the blood stream, it can infect almost any organ, most notably bone tissue and cardiac valves [4]. The role of enzymes is to disrupt cell structure, degrade cell lipids and hyaluronic acid, and to convert fibrinogen to fibrin. All those activities promote *S. aureus* to affect leukocytes, sebaceous glands, subcutaneous tissues and to increase propagation of infection [3, 5]. On the other hand, toxins (leukocidins, haemolysins and epidermolytic toxin) are able to paralyse smooth and skeletal muscles, damage blood vessels, cause extensive lesions on the skin and reveal a moist glistening surface and finally have a toxic effect on the central nervous system [3, 6, 7].

In addition, some strains of *S. aureus* are also equipped with superantigenic toxins, including shock syndrome toxin-1 (TSST-1) and SEs. They not only modulate host immune response but are also able to cause food poisoning in human [3]. The release of TSST-1 into a bloodstream may give rise to a variety of severe clinical difficulties, such as toxic shock syndrome and sudden infant death syndrome [8]. Besides *S. aureus*, also some other staphylococcal species, including *S. intermedius*, *S. hyicus*, *S. xylosus* and *S. epidermidis*, are able to produce SEs [9].

1.1. Biological characteristics of staphylococcal enterotoxins

To date, 23 SEs and enterotoxin-like (SEIs) types have been described based on their antigenicity. They have sequentially been assigned a letter of the alphabet in order of their discovery (SEA, SEB,, SEIX) [10–12]. SEA and SEB were the first one SEs characterized by Casman and Bergdoll in 1959 and 1960. From the late 1990s, new toxins were discovered one after another by sequencing the entire genome of *S. aureus*, genomic DNA library screening and genetic analysis of plasmids and pathogenicity islands [10]. Besides classical enterotoxins

with emetic activity (SEA to SEE, SEG to SEI and SER to SET), there are also staphylococcal-like (SEI) proteins that exhibits vomiting activity in oral primate model (SEIL and SEIQ) or SEIJ, SEIK, SEIM-SEIP, SEIU-SEIX that have not been tested yet [10, 12–15].

SEs and SEIs proteins are globular, single peptides with molecular weights ranging from 19 to 29 kDa [16, 19]. Their molecular composition is characterized by containing only two residues of half cystine and one or two residues of tryptophane [20]. They are rich in lysine, aspartic and glutamic acids and tyrosine. Most of them possess a cysteine loop required for proper conformation and which is probably involved in the emetic activity [21]. Overall, 15% of amino acid residues are entirely conserved in SEs and occurred in four stretches of primary sequence located either centrally or at the C-terminus [9].

As it is seen in **Table 1**, all genes for SEs and SEIs are located on mobile genetic elements, including plasmids, transposons, prophages, *S. aureus* pathogenicity islands (SaPI), variable genomic region vSa β , or next to the staphylococcal cassette chromosome (SCC) elements. Only the staphylococcal gene cluster *egc* is organized as an operon. Most of these are mobile elements, thus horizontal transfer between strains is not rare [15, 16, 18, 22].

Distribution of superantigens (SAG) gene is strain dependent. As reported by Jin and Yamada [17], 80% of human isolates contain at least one of these genes, including 50% which contain the *egc* cluster. In animal isolates, 57% contain at least one of the SAG genes and the *egc* cluster was detected in 30% of isolates [21]. No single SAG is encoded by more than 50% of strains and that some strains may not have superantigenic capacity at all [15]. Moreover, the production of specific SEs may depend on the host environment [17].

Staphylococcal enterotoxins (SEs) and SE-like toxins (SEIs) are the most notable virulence factors associated with *S. aureus* and they are involved in food poisoning, toxic shock syndrome and staphylococcal infectious diseases in human. They belong to the broad family of pyrogenic toxin superantigens that stimulate non-specific T-cell proliferation. As superantigens, SEs bind directly to the outside of the major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and cross-link it to T-cell antigen receptor variable β (V β)-chain, which initiates non-specific activation of the T-cell without proteolytic processing in antigen-presenting cells. Thanks to it, the massive release of chemokines and pro-inflammatory cytokine is followed after T-cell proliferation, resulting in systemic shock [12, 15, 19, 23, 24]. The interaction activates as many as one in five T-cells, whereas the conventional antigen presentation activates on in 10,000 T-cells [21].

Besides superantigen activity, SEs (but not SEIs) act also as a potent gastrointestinal toxins causing emesis. SEs can penetrate the epithelium, accumulate in the submucosa, enter the blood stream and circulate through the body allowing activation of local and systemic immune response by their interaction with antigen-presenting- and T-cells [16, 25]. SEA binds in submucosa to the submucosal mast cells or directly to neuron cells [10, 26]. The binding of SEA to an unidentified receptor expressed on the surface of these cells induces the degranulation, resulting in the release of 5-hydroxytryptamine (5-HT). This stimulates 5-HT receptor on adjacent vagal afferent nerves in the intestine resulting in depolarization of the vagal nerves and stimulation of the vomiting centre in the brain [10, 16]. The release

Toxin	Molecular weight (kDa)	Emetic activity	Super-antigenic activity	gene	Genetic element	Accessory genetic element
SEA	27.1	+	+	<i>sea</i>	prophage	ΦSa3ms; ΦSa3mw; Φ252B; ΦNM3; ΦMu50a
SEB	28.4	+	+	<i>seb</i>	chromosome, SaPI, plasmid	pZA10; SaPI3
SEC1-SEC3	27.5-27.6	+	+	<i>sec</i>	SaPI	SaPI _n 1; SaPI _m 1; SaPI _m w2; SaPI _b ov1
SED	26.9	+	+	<i>sed</i>	plasmid (pIB485)	pIB485-like
SEE	26.4	+	+	<i>see</i>	prophage	ΦSa
SEG	27.0	+	+	<i>seg</i>	<i>egc</i> , chromosome	<i>egc</i> 1(<i>vSa</i> β I); <i>egc</i> 2(<i>vSa</i> β III); <i>egc</i> 3; <i>egc</i> 4
SEH	25.1	+	+	<i>seh</i>	transposon	MGE _m w2/ <i>mssa</i> 476 <i>seh</i> /Δ <i>seo</i>
SEI	24.9	+	+	<i>sei</i>	<i>egc</i> , chromosome	<i>egc</i> 1(<i>vSa</i> β I); <i>egc</i> 2(<i>vSa</i> β III); <i>egc</i> 3
SEIJ	28.6	n _d	+	<i>selj</i>	plasmid (pIB485, pF5)	pIB485-like; pF5
SEIK	25.3	n _d	+	<i>selk</i>	SaPI	ΦSa3ms; ΦSa3mw; SaPI1; SaPI3; SaPI _b ov1; SaPI5
SEIL	24.7	-	+	<i>sell</i>	SaPI	SaPI _n 1; SaPI _m 1; SaPI _m w2; SaPI _b ov1
SEIM	24.8	n _d	+	<i>selm</i>	<i>egc</i> , chromosome	<i>egc</i> 1(<i>vSa</i> β I); <i>egc</i> 2(<i>vSa</i> β III)
SEIN	26.1	n _d	+	<i>seln</i>	<i>egc</i> , chromosome	<i>egc</i> 1(<i>vSa</i> β I); <i>egc</i> 2(<i>vSa</i> β III); <i>egc</i> 3; <i>egc</i> 4
SEIO	26.8	n _d	+	<i>selo</i>	<i>egc</i> , chromosome	<i>egc</i> 1(<i>vSa</i> β I); <i>egc</i> 2(<i>vSa</i> β III); <i>egc</i> 3; <i>egc</i> 4; MGE _m w2/ <i>mssa</i> 476 <i>seh</i> /Δ <i>seo</i>
SEIP	26.7	n _d	+	<i>selp</i>	prophage (Sa3n)	ΦN315; ΦMu3A
SEIQ	25.2	-	+	<i>selq</i>	SaPI	ΦSa3ms; ΦSa3mw; SaPI1; SaPI3; SaPI5
SER	27.0	+	+	<i>ser</i>	plasmid (pIB485, pF5)	pIB485-like; pF5
SES	26.2	+	+	<i>ses</i>	plasmid (pF5)	pF5
SET	22.6	+	+	<i>set</i>	plasmid (pF5)	pF5
SEIU	27.2	n _d	+	<i>selu</i>	<i>egc</i> , chromosome	<i>egc</i> 2(<i>vSa</i> β III); <i>egc</i> 3
SEIV	27.6	n _d	+	<i>selv</i>	<i>egc</i> , chromosome	<i>egc</i> 4
SEIW	23.2	n _d	n _d		chromosome	
SEIX	19.3	n _d	+	<i>selx</i>	chromosome	<i>oriC</i>

Source: Argudín et al. [15], Hu et al. [10], Omoe et al. [16], Jin and Yamada [17].

Table 1. General properties of SEs and SEIs and genomic location of the encoding genes.

of 5-HT can be direct after interaction of SEA with enterochromaffin cells or neurons or indirect through the release of pro-inflammatory molecules or free-radical formation [13, 26]. It appears that besides 5-HT, also the serotonin pathway is involved in emesis, since serotonin is an important signalling mediator in the gastrointestinal tract and can activate enteric neurons, stimulate muscle responses and enhance secretion [23]. Release of inflammatory mediators (histamine, leukotrienes and neuroenteric peptide substance P) is responsible for local damage of gastrointestinal tract. The most severe lesions appear in the stomach and the upper part of the small intestine. Due to the inhibition of water and electrolyte reabsorption in small intestine, diarrhoea may occur [16, 25]. The dose of SEs inducing emetic activity in monkeys after oral administration ranged from 5 to 600 µg/animal [10]. The minimal dose required for intoxication in human is 144 ± 50 ng/humans for SEA and 0.4 µg/humans for SEB. All the SEs that were tested induced emetic reaction in monkeys at a dose of 100 µg/kg [11].

Although emetic and superantigenic activities are two separate functions localized on separate domains of the proteins, there is a high correlation between these activities and in most cases a loss of superantigen activity results in loss of emetic activity as [11, 18]. However, the role of SEs in human food-poisoning outbreaks currently remains unclear [12].

1.2. Prevalence of staphylococcal enterotoxins in humans and animals

Approximately 20–60% of humans are permanent or intermittent carriers of *S. aureus*, which harbours SE genes in one- to two-thirds of cases [17]. Among SEs, SEA and SED are the most frequent agents in food-borne intoxications [27, 28]. The regulation of production of SE is SE-dependent, as well as strain- and environment-dependent. Under the same conditions, different strains may produce different amounts of SE and in different growth phases. This reflects also in considerable variability in amounts and types of SEs produced by *S. aureus* growing under optimal conditions.

For SEB and SEC, the amounts may exceed 100 µg/ml, compared with 1–10 µg/ml for SEA and SED. Some indications exist that low amounts of SEB are produced already in early exponential growth phase and it can appear in cultures as early as 4–6 h. However, SEA and SED are produced in foods under a wider range of pH, redox potential (E_h) and water activity (a_w) than are the other SEs, which explain why SEA and SED are principal toxins involved in staphylococcal food poisoning [11]. SEA is expressed from the mid-exponential growth phase, but is not regulated by the accessory gene regulator *agr*, unlike *seb*, *sec* and *sed*, which require a functional *agr* for maximal expression [18].

The *sea* gene was the most predominant (41%) among isolates from raw and pasteurized milk studied by Rall et al. [29]. In food samples analysed by Aydin et al. [30], the SEA was found in 38%. Also, SEA and also SEB were presented in bovine isolates in 5–19%, in ewes' and goats' isolates in about 2–11% [21]. SEA is predominantly produced by human strains, so the connection with food contamination during the manufacture is possible [31]. On the other hand, SEC is considered the most important cause of staphylococcal food poisoning associated with the consumption of dairy products [27]. In dairy goat herds, the most prevalent was SEC (71%), with overall 72% prevalence of enterotoxinogenic isolates [32]. Also in 152 *S. aureus*

strains isolated from cheese samples, the SEC was detected in 44% [28]. In food samples, the SEC was the most prevalent (52%) [30]. In bovine isolates, the SEC occurred in 1–27%, 22–42% in ewes' isolates and 23% in caprine isolates. SED was produced by 6–35% of bovine isolates, by 2–35% of ewes' milk isolates and by 2% of caprine isolates [20].

1.3. Resistance of staphylococcal enterotoxins to environmental factors

SEs are highly stable, resist most proteolytic enzymes (pepsin or trypsin) thus keeping their activity in the digestive tract after ingestion. They are also resisting chymotrypsin, rennin and papain. Based on the poor ability of proteolytic enzymes to affect the biological activity of SEs, it is not surprising that SE levels are unaffected by proteolytic or enteric bacteria. Lactic acid bacteria (LAB), however, do decrease SE concentrations. It could not be accounted for the addition of lactic acid alone, suggesting the involvement of specific enzymes of other metabolites. Alternatively, selective physical adsorption of toxin to LAB may have occurred during removal of cells to obtain supernatants from toxin assays [9].

1.3.1. Heat resistance

SEs are in general produced in a temperature range of 10–46°C, with the optimum at 40–45°C. Their production is substantially reduced at 20–25°C and it is unlikely that they are produced at temperatures below 10°C [19, 33, 34]. They can resist both the process of milk pasteurization and sterilization of canned foods [20, 36]. The heat stability of SEs is not the same for all of them and depends on the food matrix and toxin concentration. It decreases in the order SEC>SEB>SEA and significantly reduces in acidic conditions [3].

The thermal inactivation can generally be described by *D*- and *z*-values representing the time (at certain temperature) and the increase of temperature responsible for decimal reduction of their activity, respectively. They are for SEs as follows: $D_{121^{\circ}\text{C}}$ ranges from 8.3 to 34 min or $D_{100^{\circ}\text{C}}$ is about 70 min and *z*-value is 25–33°C, with some differences among specific SEs [9, 36, 37]. The biological activity of SEB retains after heating at 60°C for 16 h and pH 7.3. Heating of SEC for 30 min at 60°C did not result in any change in serological reactions. However, the loss of serological reaction of SEA was noticed after its heating for 3 min at 80°C or for 1 min at 100°C. It should also be mentioned that even after SEs lose serological activity in detection of immunological assay, they can remain biologically active [11]. Heat stability seems to be dependent on the media the toxin is in, the pH, salt concentration and other environmental factors related to the level of toxin denaturation [18].

1.3.2. Acid tolerance

The pH range allowing the production of SEs is limited in higher degree as the growth of a producing strain. Optimum enterotoxin production occurs at pH 6–7 and it is influenced by environmental conditions, carbon and nitrogen source and salt level [33]. Already pH 5.0 is generally considered as a lower limit pH value. The SEA is produced under a wider range of pH than SEB or SEC [19, 38]. SEB can be destroyed by pepsin digestion at pH 2 but it is resistant at higher pHs, which are normal conditions in the stomach after food ingestion [9].

1.3.3. Salt resistance

A characteristic feature that distinguishes *S. aureus* from other pathogenic bacteria is its high tolerance to NaCl concentrations up to 20% which means high osmotic pressure and low water activity values. Generally, it is reported that the minimal water activity for the *S. aureus* growth is in the range of a_w from 0.83 to 0.86 [33–36].

With respect to enterotoxins production requirements, values of water activity for their production are mostly in the same range as for the growth of the producer. In food with decreased water activity and at aerobic conditions, the enterotoxins can be produced even if the a_w value is from 0.86 to 0.89 (at 22–17% NaCl). The production of SEB appears to be more sensitive to reduced water activity than SEA production. Whereas SEA is produced up to a_w 0.87–0.89 (20–17% NaCl), SEB is produced only in the narrow range of 2–5% NaCl (a_w 0.99–0.97) [34, 39].

2. Effect of intrinsic and extrinsic factors on the growth dynamics of *S. aureus* and enterotoxin D production

Many intrinsic and extrinsic factors affect not only the growth of food-borne microbial pathogens but also metabolism and production of toxins. As SEs are extremely heat-stable and cannot be inactivated by measures such as heating of food, it is crucial to prevent their formation by preventing *S. aureus* growth in food matrix. In this term, NaCl addition (expressed as water activity) and LAB are suggested as the most frequent, not only during the production of cheese. The inhibition of staphylococci by LAB is related to the poor competition of *S. aureus* with antagonistic activities of indigenous bacteria in raw milk.

2.1. Effect of temperature and water activity on the growth dynamics of *S. aureus* and enterotoxin D production

The growth of *S. aureus* 14733 isolate in nutrient broth in dependence on mutual effect of temperature and water activity in the range from 1.0 to 0.84 (adjusted by NaCl addition) is demonstrated in **Figure 1**. Corresponding growth parameters were calculated using DMFit tool [40] and further analysed by secondary models (**Figure 2**, **Table 2**). Characterization of *S. aureus* ability to grow and to produce SEs at such low water activity values is important in respect to cases when the competitive microbiota is inhibited by a_w down to 0.92 and the monoculture *S. aureus* growth may still occur following SEs production.

In general, a decrease of water activity prolonged the lag-phase duration and slowed down growth rate, until the minimal water activity was reached. At 18°C and $a_w = 0.869$ (18.17% NaCl), there was no upgrowth observed and even more, the slow reduction ($\mu = -0.007 \text{ h}^{-1}$) of *S. aureus* counts was noticed. Similar effect was observed also at 21°C as the isolate could not withstand the same a_w value of 0.869 and started to decline with specific rate $\mu = -0.023 \text{ h}^{-1}$. On the contrary, the growth of *S. aureus* at $a_w = 0.867$ and 19°C was noticed with the high probability that highlights the differences between strains [41]. At 37°C, *S. aureus* was able to grow up to almost 20% of NaCl ($a_w = 0.860$) in the nutrient media. Only if the water activity reached 0.855, the decline of *S. aureus*

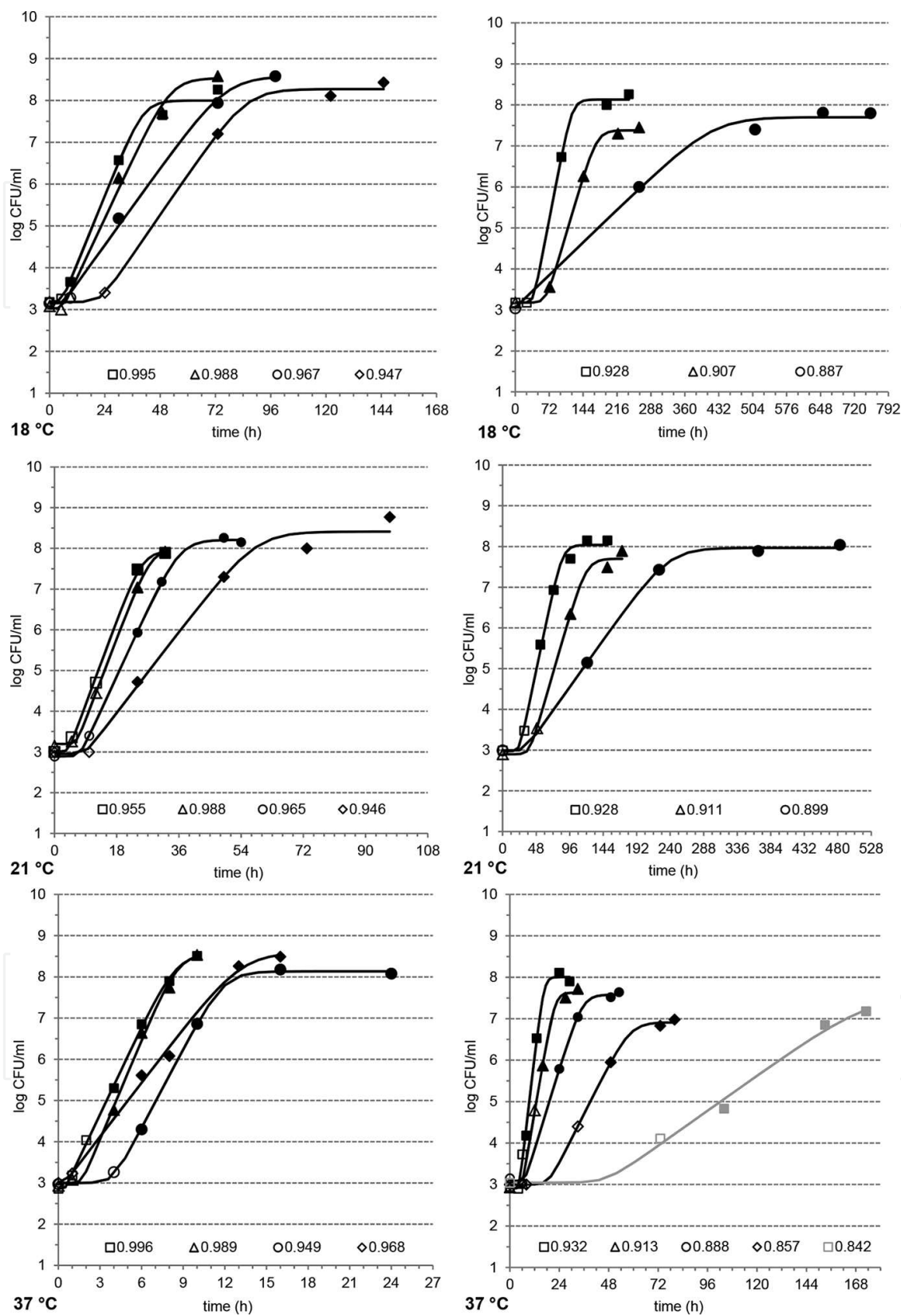


Figure 1. Growth dynamics of *S. aureus* 14733 in nutrient broth at 18, 21 and 37°C in dependence on water activity (♦, ■, ▲, ● counts of *S. aureus* 14733, no SED; ◇, □, △, ○ counts of *S. aureus* 14733, SED detected).

was observed with rate $\mu = -0.025 \text{ h}^{-1}$. It grew at 37°C much faster compared to strain ATCC 13565 in BHI broth [42]. Specific growth rates of isolate 14733 and strain ATCC 13565 at $a_w = 0.997$ were 1.796 h^{-1} and 0.970 h^{-1} , respectively. At $a_w = 0.960$, the following specific growth rates were calculated, 1.558 h^{-1} and 0.240 h^{-1} , respectively. On the other hand, the growth rates of 14733 isolate in nutrient broth at 18, 21 and 37°C were comparable with isolates 2064 and D1 grown in both milk and nutrient broth [43] and also with the isolate used by Fujikawa and Morozumi [44]. The average values of growth rates of *S. aureus* 14733 were slightly lower than those predicted by Combase Predictor or Pathogen Modelling Program [45, 46].

It was also noticed that except for cases when *S. aureus* 14733 population was inhibited by high salt amounts, it reached 7 log counts in stationary phase. At all studied temperatures, counts higher than 7 log were reached up to a_w 0.890 (approx. 13% NaCl). Thirty per cent glucose in LB broth resulted in a decreased maximal cell densities in stationary phase of about 0.5–1 log [47].

The range in which the SED was (full markers) or was not (empty markers) detected during the growth of *S. aureus* 14733 in nutrient broth at 18, 21 and 37°C is also shown in **Figure 1**. It is assumed that the minimal concentration of *S. aureus* of 10^5 CFU/ml is needed for SEs production [35, 48, 49]. However, in our case, *S. aureus* 14733 was able to produce SED also at lower cell counts. At 18°C , the SED was detected at a_w 0.995 after only 9 h, even if the *S. aureus* concentration was $4.6 \times 10^3 \text{ log CFU/ml}$. Surprisingly, also at such a low water activity value as a_w 0.907 (13.05% NaCl), the SED was detected after 73 h if the *S. aureus* concentration was only $3.6 \times 10^3 \text{ log CFU/ml}$. Although the isolate was able to grow at a_w 0.887 with the specific growth rate of $\mu = 0.025 \text{ h}^{-1}$, the SED was detected only after 11 days of incubation and counts higher than 6 log CFU/ml. There were also some evidences of lack of the SEs production at counts higher than 10^5 CFU/ml [11].

At 21°C , the SED was not produced as soon as after 24 h of incubation and even not at almost optimal water activity value (a_w 0.988) and *S. aureus* densities of $2.8 \times 10^4 \text{ log CFU/ml}$. On the other hand, at a_w 0.946 the SED was detected in 24 h at $1.4 \times 10^4 \text{ log CFU/ml}$ and also at $1.4 \times 10^5 \text{ log CFU/ml}$ at a_w 0.899 after 121 h of incubation. Production of SEA seems to be more dependent on *S. aureus* counts [50]. They detected SEA in tryptone soy (TSB) broth at 20°C after 30 h of incubation and if *S. aureus* counts were higher than 6.04 log CFU/ml.

The most rapid SED production was naturally observed at 37°C . At the higher water activity values, a_w 0.996 and 0.989, the SED was produced after only 4 h of incubation. The *S. aureus* counts needed for the SED production reached concentrations of $2.0 \times 10^5 \text{ log CFU/ml}$ and $5.9 \times 10^4 \text{ log CFU/ml}$, in order. Also at $a_w = 0.949, 0.932$ and 0.913, the SED was produced when *S. aureus* 14733 reached minimal counts of 4 log CFU/ml. Moreover, at 37°C the SED was also detected at such low water activity values as a_w 0.857 or 0.842, when *S. aureus* counts were only 5 log CFU/ml. Higher minimal *S. aureus* counts (5.65 log CFU/ml) were needed for SEA production in TSB broth that was detected after 9 h of incubation [50].

Further, the Gibson's model secondary model Gibson et al. [51] was used to characterise the influence of water activity and temperature on the specific growth rate of *S. aureus* 14733. Growth of *S. aureus* 14733 in nutrient broth was positively determined with the increasing

value of water activity, resulting in shortening of the lag phase duration and more intensive growth in exponential phase. The growth of *S. aureus* in dependence on water activity at 18, 21 and 37 °C can be characterised by equations summarised in **Table 2** and it is depicted in **Figure 2a**. The lag phase was described by means of the model by Daughtry et al. [52] according to equation summarised in **Table 2** which graphical representation is shown in **Figure 2b**.

For the Gibson's model, the discrepancy factors ranged from 9.6% to 17.5%, so the model can be considered as very consistent. This model can be also used for the determination of optimal water activity value at each temperature. So, the optimal growth of *S. aureus* 14733 in nutrient broth at 18 °C can be expected at $a_w = 0.994$, at 21°C at $a_w = 0.980$ and at 37°C at water activity value of 0.986. The prediction of lag phase duration would be estimated with 22-33 % error according to Davey's model. Taking into account that 12-37% of the bound of reliability during cultivation methods is tolerable; these finding demonstrate that the duration of lag phase and also the growth rate of *S. aureus* can be predicted with a defined degree of reproducibility.

With regard to the EU Commission Regulation 1441/2007 [53], the total *S. aureus* numbers in raw milk cheese should not exceed the process hygiene criterion of 4 log CFU/g. Based on a total of 23 examinations of SED presence in nutrient broth with different NaCl concentration at temperatures 18, 21 and 37°C, in 8.7% of cases *S. aureus* 14733 was able to produce SED even if the cell concentration was lower than 4 log CFU/ml. In further 22% of cases, the SED was detected if the *S. aureus* 14733 counts were lower than 5 log CFU/ml, considered as a safe limit of mentioned EU regulation. It is well documented in **Figure 3**, where the pro-

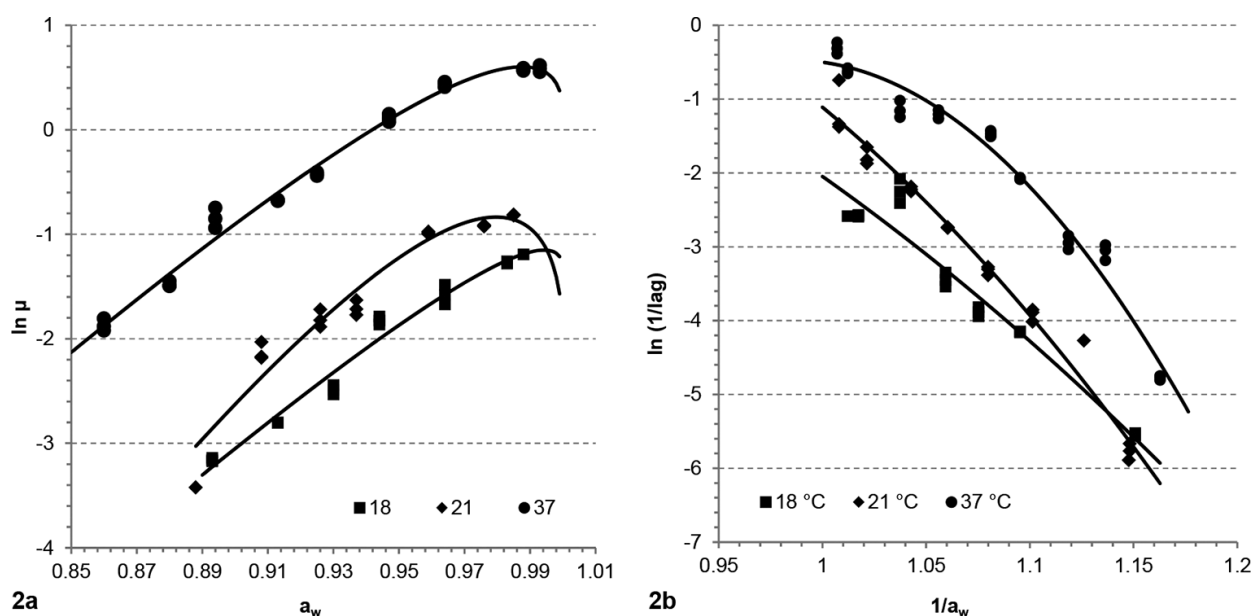


Figure 2. Plots of natural logarithm of specific growth rate (2a; $\ln \mu$) and reciprocal lag phase (2b; $\ln 1/\text{lag}$) of *S. aureus* in nutrient broth at 18 (■), 21 (♦) and 37°C (●) in dependence on water activity. Symbols indicate values calculated from growth curves at each incubation conditions. The continuous lines indicate the fitted of $\ln \mu$ (or $\ln 1/\text{lag}$) versus a_w function, where $\ln \mu = A \cdot b_w^2 + B \cdot b_w + C$, $\ln 1/\text{lag} = A/b_w^2 + B/b_w + C$ and $b_w = \sqrt{1 - a_w}$; A, B and C are the estimated parameters.

Model equation/validation coefficients							
A_f	B_f	$\%D_f$	R^2	$\%V$	RSS	RMSE	$\%SEP$
$\ln \mu_{18} = -32.829 \cdot b_w^2 + 4.961 \cdot b_w - 1.338$							
1.117	1.001	11.7	0.974	96.9	0.0038	0.0142	9.2
$\ln \mu_{21} = -59.491 \cdot b_w^2 + 16.983 \cdot b_w - 2.048$							
1.175	1.000	17.5	0.942	93.0	0.0121	0.0252	12.8
$\ln \mu_{37} = -35.936 \cdot b_w^2 + 8.010 \cdot b_w + 0.159$							
1.096	0.999	9.6	0.988	98.6	0.0782	0.0538	8.2
$\ln(1/la_{g_{18}}) = -24.835 \cdot a_w^2 + 29.858 \cdot a_w - 7.068$							
1.327	1.003	32.7	0.921	90.3	1.4436	0.2832	0.8
$\ln(1/la_{g_{21}}) = -49.760 \cdot a_w^2 + 76.322 \cdot a_w - 27.672$							
1.217	0.999	21.7	0.980	97.7	0.8456	0.1961	1.3
$\ln(1/la_{g_{37}}) = -129.915 \cdot a_w^2 + 255.970 \cdot a_w - 126.570$							
1.246	0.999	24.6	0.973	96.9	1.3102	0.2203	5.1

Table 2. Result of validation of Gibson’s model describing the effect of water activity on specific growth rate and Davey’s model describing lag phase duration of *S. aureus* 14733 in nutrient broth.

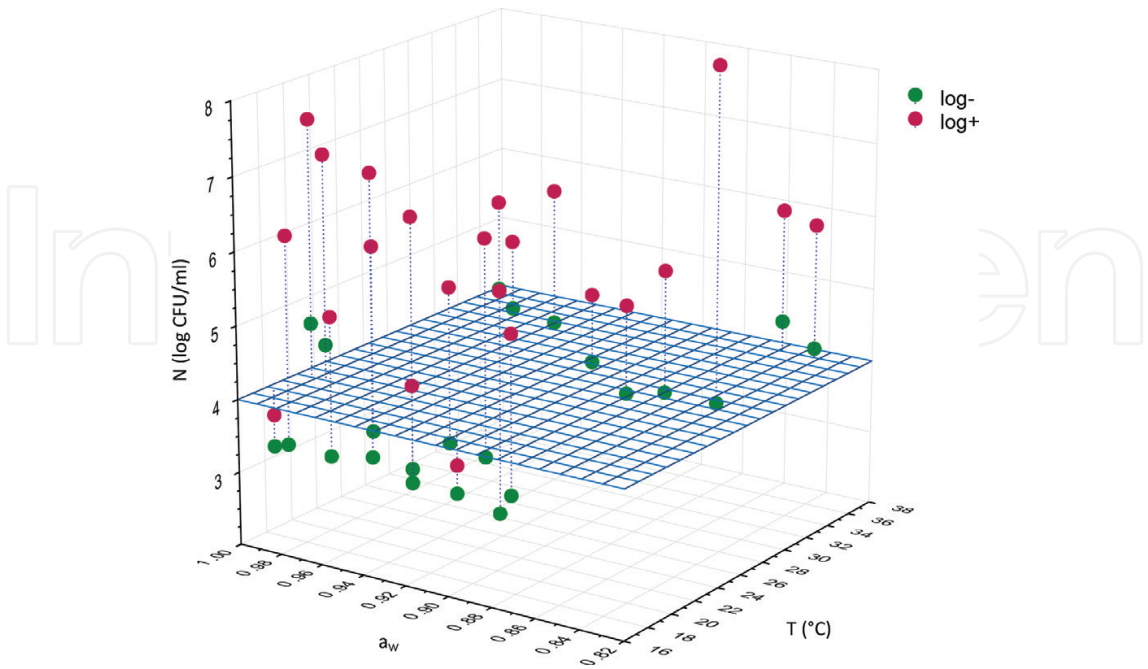


Figure 3. Mutual effect of water activity and temperature on the production of SED in nutrient broth in dependence to *S. aureus* 14733 counts. The green dots represent samples negative for SED presence and blue dots represent positive SED samples.

cess hygiene criteria are depicted with the red net, green dots represent samples negative for SED presence and blue dots represent positive SED samples at each water activity and temperature.

Based on the results, *S. aureus* 14733 can be considered as a strong SED producer, and the SED production is not limited with the incubation temperature and the NaCl addition (up to 15% at 18 and 21°C and up to 20% at 37°C). This complies with the study of Sihto et al. [47] who also observed that glucose stress (in 30% addition) had no statistically significant effect on *sed* expression during all phases of *S. aureus* growth in LB broth. Moreover, Valihrach et al. found that the fat content of milk, origin of milk (cow, goat and sheep), had only an insignificant effect on the SEC production by different *S. aureus* strains [54]. On the other hand, some strain-dependent differences were observed and the higher SEC production was achieved by strains originated from ewes' milk indicating a better adaptation of such a strain on the host environment. Naturally, the highest amounts of SEs are produced at 37°C, as an optimal temperature, compared to lower temperatures. However, SEs production is significantly decreased in milk compared to synthetic nutrient medium. It is worth mentioning also the faster growth of *S. aureus* in liquid culture compared to solid food matrices indicating that the higher growth rate may stimulate enterotoxin gene expression. On food matrix, *S. aureus* cells must attach, adapt, and substantially grow under food condition, while in the nutrient broth the bacteria are planktonic and produce enterotoxin earlier than on solid medium [50]. Also, the expression of SEs is coordinated by many regulatory elements, including *agr* regulatory system. The *agr* positively regulates expression not only of SEs and this expression increases simultaneously with increasing cell density [55].

2.2. Effect of temperature, pH value and water activity on the growth dynamics of *S. aureus* and enterotoxin D production

As it was mentioned above, the traditional artisanal production of "Bryndza" cheese includes fermentation in the presence of LAB, ripening at temperatures from 18 to 21°C and salting with 2–5% NaCl resulting in final soft cheese [1, 2]. In this context, the growth and the production of SED by *S. aureus* 14733 in nutrient broth at 18 and 21°C and optimal temperature of 37°C were observed in relation to pH value (adjusted by lactic acid to values 6.0, 5.5, 5.0 and 4.5). Subsequently, the mutual effect of lactic acid (pH 6.0, 5.5 and 5.0) and water activity ($a_w = 0.99$; 1.7% NaCl and $a_w = 0.97$; 5% NaCl) at the same temperatures on the *S. aureus* growth and the SED production in the nutrient broth was analysed.

As it is shown in **Figure 4**, the combination of reduced pH value (to values 6.0 and 5.5) and water activity value (0.99 and 0.97) did not inhibit the growth dynamic of *S. aureus* 14733 almost at all. However, further decrease of pH value down to 5.0 led to the lag-phase prolongation, slowing of the growth rate in exponential phase and to the decrease of the maximal cell concentration in stationary phase at 18 and 21°C. In accordance to these results, it can be supposing that during cheese ripening at 18–21°C, the *S. aureus* growth inhibition can be expected only if the rapid decrease of pH is achieved. But in the case of optimal temperature, even the pH 5.0 is not sufficient to slow down its growth dynamic.

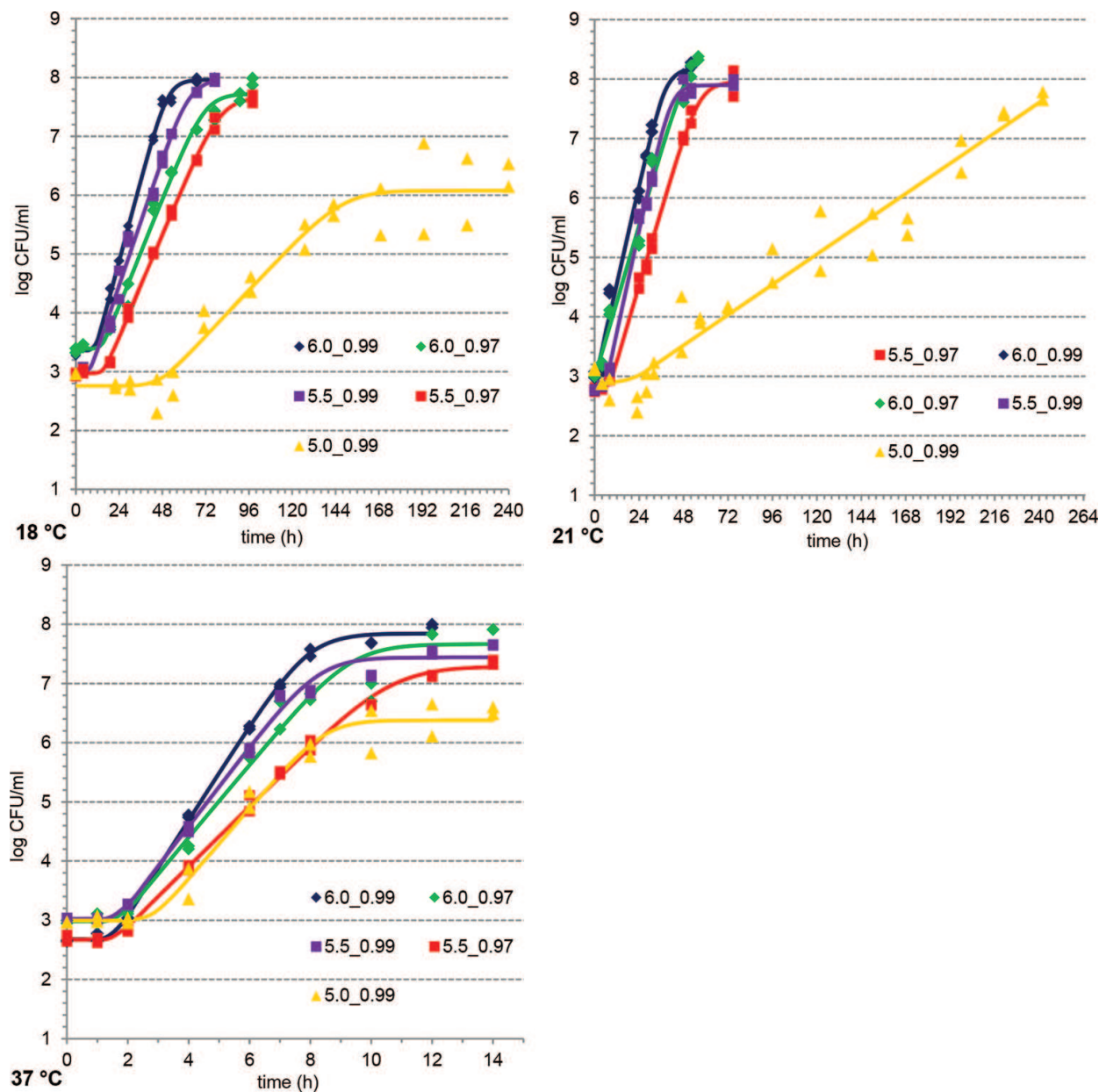


Figure 4. Growth dynamics of *S. aureus* 14733 in nutrient broth at 18, 21 and 37°C in dependence on water activity and pH value (♦ value 6.0, water activity 0.99, ■ pH value 5.5, water activity 0.99, ♦ pH value 6.0, water activity 0.97, ■ pH value 5.5, water activity 0.97, ▲ pH value 5.0, and water activity 0.99).

Taking into account the SED production by *S. aureus* 14733 in dependence to pH and water activity, the strong inhibitive effect of pH down to 4.5 or the combination of pH 5.5 and a_w 0.97 was observed (Table 3). The SED was not detected at any temperature if the pH of 4.5 was adjusted by the lactic acid. Compared to the experiments when no NaCl was added to the nutrient broth at pH 6.5, the minimal *S. aureus* counts for SED production of 4.6×10^3 CFU/ml (at 18°C) were needed. In the case of pH decreased to 6.0, the minimal *S. aureus* counts increased to 6.3×10^5 CFU/ml. Also, the prolongation of the time when the SED was detected from 9 to 28 h was observed at 18°C. And even the minimal *S. aureus* counts did not increase

<i>T</i> (°C)	pH	<i>a_w</i>	<i>μ</i> (h ⁻¹)	(+) log CFU/ml
18	6.0	–	0.221	5.8 (28 h)
	5.5	–	0.320	5.7 (42 h)
	5.0	–	0.119	4.9 (42 h)
	4.5	–	–0.016	–
	6.0	0.99	0.246	5.5 (28 h)
	6.0	0.97	0.239	4.9 (28 h)
	5.5	0.99	0.364	5.4 (28 h)
	5.5	0.97	0.216	–
21	6.0	–	0.392	6.2 (22 h)
	5.5	–	0.375	6.3 (22 h)
	5.0	–	0.306	6.1 (32 h)
	4.5	–	0.007	–
	6.0	0.99	0.378	6.1 (22 h)
	6.0	0.97	0.295	6.5 (32 h)
	5.5	0.99	0.389	6.0 (22 h)
	5.5	0.97	0.258	6.2 (32 h)
37	6.0	–	2.287	5.2 (4 h)
	5.5	–	2.057	3.9 (4 h)
	5.0	–	1.064	3.3 (6 h)
	4.5	–	0.039	–
	6.0	0.99	1.847	4.8 (4 h)
	6.0	0.97	1.534	3.6 (4 h)
	5.5	0.99	1.403	3.9 (4 h)
	5.5	0.97	1.073	4.9 (6 h)

μ, specific growth rate of *S. aureus* 14733; (+), SED detected in specific time of incubation.

Table 3. Effect of pH value and water activity on the growth parameters and production of SED by *S. aureus* 14733 in nutrient broth at 18, 21 and 37°C.

with the decreasing pH value; the time of positive SED determination extended to 42 h at pH 5.5 and 5.0. The longer time required for SED production in the presence of only lactic acid, compared to combination of lactic acid and NaCl presence, was observed also at 21 and 37°C, although that at 37°C the SED was detected at pH 5.0 after 6 h of incubation.

In the term of SEs production inhibition during cheese manufacture, a rapid decrease in pH value down to pH 5.0 as fast as possible within first 6 h of cheese production is strongly recommended. This was also emphasized by Delbes et al. [48]. They observed that the critical phase of exponential phase of staphylococci occurs mainly within the first 6 h and the rapid pH decrease within this phase significantly contributed to the inhibition of staphylococci in

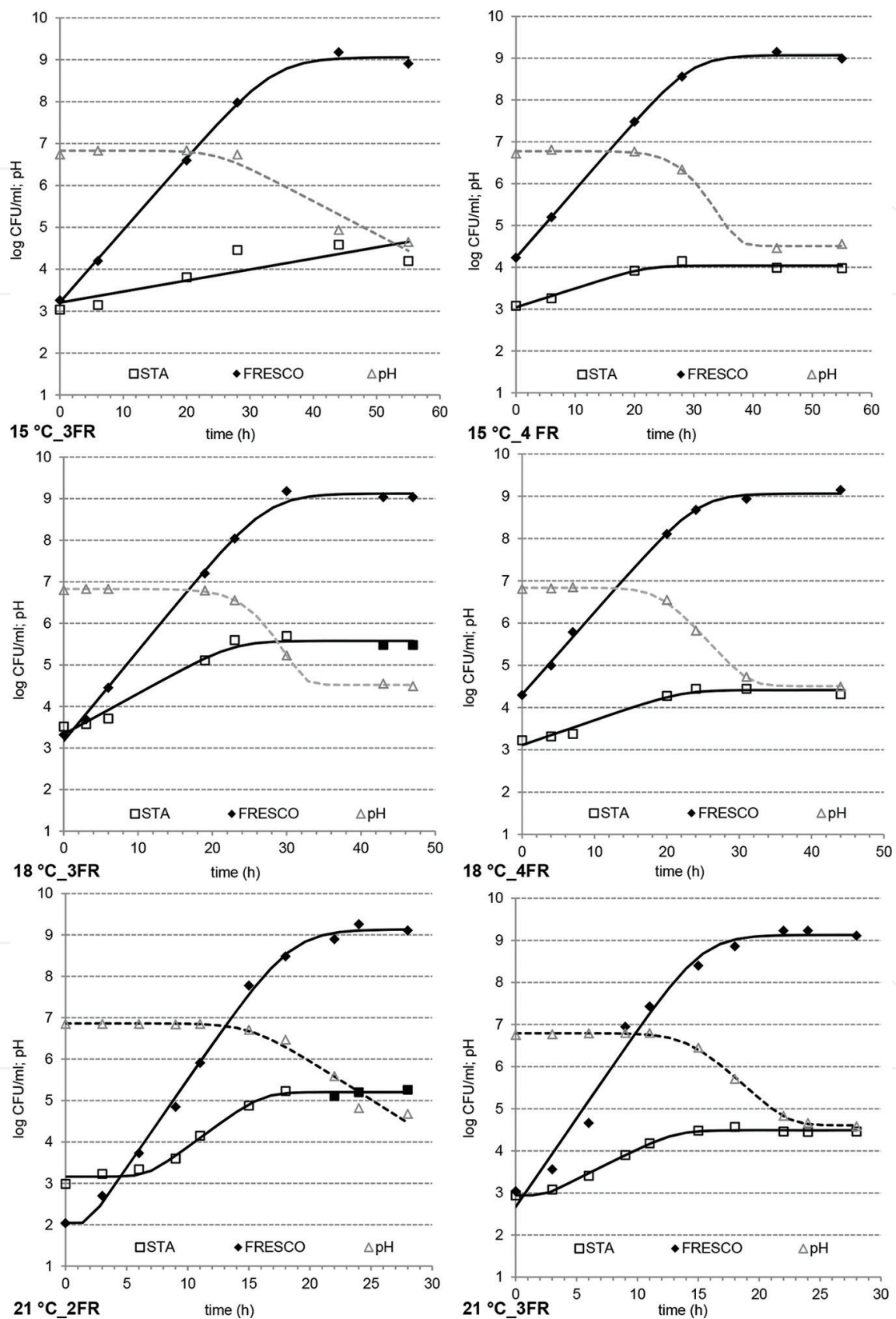


Figure 5. Growth dynamics and pH value changes during co-cultivation of *S. aureus* 14733 and Fresco culture in milk at 15, 18 and 21°C. ♦ presumptive LAB on M17 agar, □ counts of *S. aureus* 14733, no SED, ■ counts of *S. aureus* 14733, SED detected, Δ pH value.

young cheese. Moreover, if pH exceeded 6.3 within the first 6 h, also the SEs production was detected in cell concentration higher than 5 log CFU/g.

With regard to the study of Valihrach et al. [54], based on a total of 24 examinations of SED presence in the nutrient broth in dependence to mutual effect of water activity (a_w 0.99 and 0.97) and pH value (6.0 and 5.5) or pH value (6.0, 5.5, 5.0 and 4.5) at temperatures 18, 21 and 37°C, in 17% of cases *S. aureus* 14733 was able to produce SED even if the cell concentration was lower than 4 log CFU/ml. In further 17% of cases, the SED was detected if the *S. aureus* 14733 counts were lower than 5 log CFU/ml.

2.3. Effect of lactic acid bacteria addition and temperature on the growth dynamics of *S. aureus* and enterotoxin D production

In dairy practise, the initial numbers of *S. aureus* play an important role especially at the beginning of the milk fermentation within the first 6 h or in 24-h-old cheese. As we presented in our previous works [43, 56], one of the most effective tools to inhibit *S. aureus* growth is to add a sufficient amount of active dairy starters, which are able to produce lactic acid very rapidly. Thus, inhibiting the growth of *S. aureus* also, the production of SEs may be inhibited. This effect can be also seen in **Figure 5**, where the growth of *S. aureus* 14733 in the presence of two different Fresco starter culture additions was studied at 15, 18, and 21°C in milk. Those temperatures represent temperatures during ripening of original ewes’ lump cheese from raw milk and 15°C is the minimal temperature for proper fermentation process.

During co-cultivation of *S. aureus* 14733 with Fresco culture in milk, the cessation of *S. aureus* growth was observed before the drop of pH observed could significantly affect the growth of *S. aureus* (**Table 4**). In the experiment at 18°C, inhibition occurred at pH values of 6.55–6.55. At 21°C, cessation of growth was observed for pH values around 6.45. As it was shown previously, *S. aureus* 14733 was able to grow under much more acidic stress. When only lactic acid was present in the nutrient media, the specific growth rate of *S. aureus* 14733 of 0.119 h⁻¹ was calculated at pH 5.0 and 18°C. At 21°C, the growth at pH 4.5 with very low specific growth rate (0.007 h⁻¹) was noticed, but at pH 5.0, specific growth rate 0.306 h⁻¹ was reached by *S. aureus* 14733. Results also showed that *S. aureus* could grow at much lower pH values

T (°C)	N _{0, FR} (log CFU/ml)	pH _{lag} (h)	N _{max, STA} (log CFU/ml)	N _{max, STA} - N _{0, STA} (log CFU/ml)	μ _{STA} (h ⁻¹)	μ _{FR} (h ⁻¹)
15	3.26	24.7	4.20	1.16	0.061	0.397
	4.23	28.6	3.98	0.90	0.104	0.375
18	3.32	25.7	5.48	1.56	0.226	0.500
	4.30	19.0	4.32	1.09	0.135	0.447
21	2.04	15.2	5.26	2.27	0.215	0.426
	3.04	14.2	4.46	1.52	0.144	0.421

Table 4. Growth parameters of *S. aureus* 14733 and the pH lag phase in dependence to Fresco culture addition and incubation temperature.

in liquid media (pH 5.25 and 4.48 at 15 and 30°C, respectively) [36]. On the other hand, they did not observe its growth during co-culture with starter cultures at pH 6.8, assuming that the *S. aureus* growth inhibition cannot be attributed only to a drop in pH.

So, even pH and lactic acid play only a minor role in growth inhibition, we may suppose that the expression of genes responsible for SEs production may be influenced negatively. In addition, the *S. aureus* growth inhibition may be accounted to the accumulation of antistaphylococcal substances produced by the LAB present in the Fresco culture [56]. As reported by Charlier et al. [49], several parameters were proposed as involved in *S. aureus* inhibition by LAB, including bacteriocin and hydrogen peroxide production, and competition for nutrients.

During co-cultivation of 14733 isolate with Fresco culture, the SED was produced only after reaching *S. aureus* late stationary phase, however, only if its concentration was higher than 5 log CFU/ml. The minimal starter culture addition needed for *S. aureus* growth and SEs production inhibition at temperatures related to raw milk cheese manufacture should be at least 4 log CFU/ml. Taking into account Commission Regulation 1441/2007 [53], the presence of active starter culture of LAB is able to not only inhibit the growth of *S. aureus* but mainly prevent from SEs production. On top of that, the addition of starter culture can support the growth of natural LAB present in raw milk. Their inhibitory potential, involving not only acidification, can be used actively in safe cheese preparation. Moreover, the starter cultures not only effectively inhibit the growth of *S. aureus* during the fermentation of milk and raw milk cheese but also improve the sensorial properties of prepared cheese [57]. Certainly, these findings do not mean to overestimate the role of LAB and on the other hand to underestimate hygiene control. Minimizing the initial *S. aureus* numbers in raw milk down to 10² CFU/ml is of utmost importance in preventing from *S. aureus* population to reach densities necessary for SEs production during cheese manufacture. Our experiments were not focused on the inhibition of staphylococcal isolates with higher NaCl concentrations as salt generally inhibits LAB and support undesirable salt-tolerant bacteria, including staphylococci.

3. Conclusion

Based on this study focusing on the effect of temperature, pH, water activity and initial numbers of lactic acid bacteria on the growth and the ability of *S. aureus* 14733 to produce SED, we may consider it as a strong SED producer. The SED production was not limited with the incubation temperatures and the NaCl addition related to traditional cheese manufacture. As this isolate comes originally from such an artisanal cheese production, we can expect that also other enterotoxin-producing *S. aureus* strains could be able to produce SEs within the population under 10⁴ CFU/ml or g in practice. It is absolutely necessary to apply strict prerequisites approach in hygiene to reach the initial number of *S. aureus* as low as possible.

In terms of SEs production inhibition during cheese manufacture, a rapid decrease in pH value down to pH 5.0 as fast as possible within the first 6 h of cheese production is strongly

recommended. The minimal starter culture addition needed for *S. aureus* growth and SEs production inhibition at temperatures related to raw milk cheese manufacture should be at least 4 log CFU/ml.

Artisanal raw milk cheese production poses a few critical factors limiting its safety. With reference to the growth of *S. aureus*, commonly present in raw milk, many factors should be taken into consideration. From them, quantitative growth data, cheese type, NaCl content, nature, activity and type of the starter culture and mutual relation between *S. aureus* and lactic acid bacteria populations are predominant. Inhibitory starters producing bacteriocins may also be used. Thus, adding of a starter culture in artisanal cheese production is strongly recommended. This can be the only capable way of assuring the initial dominance of lactic acid bacteria and also supporting the growth of the natural LAB present in raw milk in competition with other undesirable bacteria.

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