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***Staphylococcus aureus*: Characterisation and Quantitative Growth Description in Milk and Artisanal Raw Milk Cheese Production**

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Additional information is available at the end of the chapter

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1. Introduction

The safety and quality of fermented raw foods are generally determined by the presence of pathogenic and spoilage microorganisms, their interaction with lactic acid bacteria, intrinsic, extrinsic and technological factors [1]. This fact concerns also the short ripened ewes' lump cheese traditionally produced immediately after milking in Slovakian upland cottages. The cheese is curdled with rennet, fermented by native lactic acid bacteria and briefly ripened for 7 to 10 d. Then it is usually sent to a cheese factory for production of the soft Slovakian „Bryndza” cheese [2].

This chapter deals with the behaviour of coagulase-positive staphylococci as their populations belong to the ubiquitous microflora of ewes' milk. *S. aureus* is able to multiply rapidly, especially during the initial phase of preparation when natural lactic acid bacteria are in lag phase and a sufficient amount of lactic acid has not been produced. The initial period to reach pH 5.3 lasted on average up to 30 h in upland artisanal ewes' cheese production stations [3]. However, *S. aureus* is competitive in milk and dairy environments; it is quite sensitive to higher lactic acid concentration. The growth of *Staphylococcus aureus* and potential production of heat-stable enterotoxins 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. That is why the control of *S. aureus* growth during the fermentation of young raw milk cheese means prevention against staphylococcal enterotoxin production.

2. *Staphylococcus aureus* – general description

Staphylococcus aureus subsp. *aureus* (*S. aureus*) belongs to the genus *Staphylococcus* and to the family *Staphylococcaceae* [4]. It was firstly described by Sir Alexander Ogston in 1882 and 2

years later Rosenbach isolated it in a pure culture and introduced the name *Staphylococcus aureus*. The name of the organism is derived from Greek words *staphyle* (a bunch of grapes) and *coccus* (grain or berry) [5,6].

S. aureus is a Gram-positive, facultative anaerobic, catalase-positive, oxidase-negative, non-motile microorganism that does not form spores. It creates smooth, convex, lustrous, circular colonies reaching a size of 0.5-1.5 μm in diameter and growing in an irregular three-dimensional bunch of grapes-like clusters of cells. In dependence on growth conditions, the colony pigmentation varies from grey, grey-white with yellowish to orange shades with typical β -haemolysis on the blood agar [6-9].

For growth it requires B vitamins (thiamine and nicotinic acid), inorganic salts and amino acids as a nitrogen source, especially arginine, cysteine, proline and valine. Glutamic acid, leucine and tyrosine are not required for growth, but they are essential for enterotoxin production. Deprivation of any amino acid is much less responsive in SEA production than for SEB or SEC production. Arginine seems to be essential for enterotoxin B production [5,7,10].

S. aureus belongs among chemo-organotrophs with a respiratory and fermentative metabolism. Under aerobic conditions, acids are produced from glucose, lactose, maltose and mannitol, under anaerobic conditions acids are produced from many other sugars and alcoholic sugars [6,7].

Most strains hydrolyse native animal proteins (casein, gelatine, fibrin), lipids, phospholipoproteins and Tween. They also coagulate animal plasma with the assistance of a coagulase and the clumping factor. Besides that, the typical enzymatic activity of *S. aureus* includes production of coagulase, alkaline phosphatase, proteases, lipases, and esterases and some strains also produce lecithinase [5-7].

3. Production of enterotoxins and other virulent factors

S. aureus produces a wide range of virulence factors which can be divided into different groups. Due to the production of surface-associated factors like microbial surface components recognizing adhesive matrix molecules (MSCRAMM), protein A, polysaccharide A, peptidoglycan and a clumping factor, *S. aureus* is responsible for resistance to opsonophagocytosis, the formation of biofilm and adhesion to the host cell matrix [11,12]. Following colonization, *S. aureus* secretes various toxins and enzymes which are responsible for the lesions during the development of the infection. 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 [12].

The role of enzymes like coagulase, catalase, hyaluronidase, lipase, heat-resistant nuclease, staphylokinase and β -galactosidase is to disrupt cell structure, degrade cell lipids and hyaluronic acid, and to convert fibrinogen to fibrin. All those mechanisms promote *S. aureus* i) to affect leukocytes, sebaceous glands and subcutaneous tissues; ii) to increase propagation of infection and iii) to inactivate the effect of β -lactam antibiotics [9,11,13].

Toxins (leukocidins, haemolysins and epidermolytic toxin) possess haemolytic, cytotoxic, dermonecrotic and lethal activity. They are able to paralyse smooth and skeletal muscles, damage blood vessels, cause extensive lesions on the skin and reveal a moist glistening surface (called also Ritter's disease) and finally have a toxic effect on the central nervous system [5,11,14].

In addition to surface factors, enzymes and cytotoxins, strains of *S. aureus* are also equipped with superantigenic toxins, including shock syndrome toxin-1 (TSST-1) and enterotoxins. They not only modulate host immune response but are also able to cause food poisoning in human [11]. The release of TSST-1 into the bloodstream may give rise to a variety of severe clinical difficulties, such as toxic shock syndrome, sudden infant death syndrome and Kawasaki syndrome [15].

From the food point of view, the production of one or more staphylococcal enterotoxins (SEs) is crucial, because they are causative agents of staphylococcal food poisoning (SFP) outbreaks in human.

Staphylococcal enterotoxins are heat-stable exoproteins consisting from 236 to 296 aminoacids with a molecular mass of 25-35 kDa. Upon hydrolysis, 18 amino acids are present, mostly aspartic acid, glutamic acid, lysine and tyrosine. For the majority of these, an isoelectric point of pH 5.7-8.6 is considered. There are five different types of classical enterotoxins (SEA-SEE) which are distinct in antigen reaction. Recently, new types of enterotoxins and enterotoxin-like types (SEG-SEV) have been described in *S. aureus*. Classical enterotoxins are encoded by phage (SEA), chromosome (SEB and SEC) or by plasmid genes (SED). They are produced during all phases of growth (SEA and SED) or only as secondary metabolites in late exponentially or in stationary phase (SEB and SEC). Most strains are capable of producing one or more enterotoxins. Enterotoxins are resistant to proteolytic enzymes, such as trypsin, chymotrypsin, rennin and papain, but at pH of about 2, they are sensitive to pepsin [5,6,9,10,16,17].

The SFP is characterized as a relatively mild intoxication which occurs after ingestion of at least 20 ng of staphylococcal enterotoxins presented in the food. Although the numbers of outbreaks caused by bacterial toxins are generally underestimated, official EU data [18] reported 558 outbreaks in 2009 from which almost 53% were caused by *Staphylococcus* spp. Two cases were from a verified outbreak and one from a possible outbreak was fatal.

3.1. Resistance of *S. aureus* and its enterotoxins to environmental factors

3.1.1. Heat resistance (*D-values*)

S. aureus is a mesophilic organism with optimum growth temperature in the range from 37 °C to 40 °C [7-9,17]. The minimal temperature for growth is about 7.0 °C [5,8,10], but some strains do not even show growth at 8 °C [19]. *S. aureus* survives freezing, in meat at -18 °C it will survive for at least 6 months with no change in counts [6]. On the other hand, a temperature higher than 46 °C is not acceptable for the majority of strains, with some exceptions that do grow up to 50 °C [6,7,9,10]. Heating causes damage to the cell. A $D_{60^{\circ}\text{C}}$

value of 1-6 minutes in foods with high water activity or $D_{60^\circ\text{C}}$ of 1-2.5 minutes in phosphate buffer is expected. Cells heated in oil, fat or in low water activity environments showed higher D-values, e.g. $D_{60^\circ\text{C}}$ of 5.3 minutes in milk and $D_{60^\circ\text{C}}$ of 42.3 minutes in milk with 57% sucrose, $D_{60^\circ\text{C}}$ of 6 minutes in meat containing 3-4% of NaCl and $D_{60^\circ\text{C}}$ of 25 minutes at a salt content of 8%. Contrary to this, changes of pH value out of optimal values decrease heat resistance [6].

Enterotoxins are produced in a narrower range of temperature than the growth is noticed. In general, enterotoxins production is expected in a temperature range of 10-46 °C, with the optimum temperature for production in the range 40-45 °C [6,8,10,20]. Enterotoxins are heat-stable in milk. Their resistance to heating is represented by D-values at 121°C and 100°C ranging from 9.9-11.4 to 70.0 minutes, respectively [21,22]. Their heat-resistance decreases following SEC>SEB>SEA and is also significantly reduced in acidic conditions [10]. It should also be noticed that 99.6% of cells are destroyed by the pasteurisation of milk at 72 °C for 15s, and at 72 °C for 35s all cells are killed. Enterotoxins can resist both the process of milk pasteurisation or sterilisation of canned foods [6,7].

3.1.2. Acid tolerance

Regarding pH, *S. aureus* is able to grow in a range of pH 4.0-9.8, with an optimum of 6-7 pH [6,8-10]. The minimal values of pH for growth are influenced by other environmental factors. The growth of *S. aureus* is inhibited by 0.1% of acetic acid and also by the presence of lower (C₁-C₄) fatty acids [17]. Moreover, *S. aureus* is more sensitive to acidification when salt concentration is increased, although it is a halotolerant microorganism.

Fast acidification down to values unacceptable for growth is the most efficient way of *S. aureus* inhibition. Acids do not have the same inhibition capacity and for a given pH value, the impact on *S. aureus* physiology will vary with the nature of the acid used. Organic acids at pH values equivalent to those obtained by using inorganic acids are more effective against *S. aureus*. The effectiveness of organic acids generally depends on the concentration of their undissociated form, which is determined by the dissociation constant of organic acids. Thus, acetic acid and propionic acid with pK_a of 4.8 and 4.9 (pK_a is pH at which the ratio of dissociated to undissociated forms is 50:50) are more inhibitive than lactic acid whose pK_a is 3.9 [6,23].

In general, a tolerance of *S. aureus* to pH values higher than 5.5 is caused due to maintaining of the intracellular pH by the sequestering or releasing protons from cytoplasm and also by the expression of genes responsible for cytoplasm buffering. These genes include genes encoding intracellular chaperones, urease operon and genes involved in the metabolism and transport of amino acids (histidine, lysine, arginine), carbohydrates and phosphoric acid [23,24].

Complete inhibition of *S. aureus* is achieved at pH lower than 5.0. An acidic stress and the drop of intracellular pH alter the membrane structure and lead to a decrease in the activity of several enzymes which are pH-sensitive. Non-dissociated form of acid acts as uncouplers

of the respiratory chain. The protonated form diffuses into the cell at low pH and is followed by a dissociation of the proton. Bacterial growth is then strongly altered because most of the energy available in the cell is used for the de-acidification of the cytoplasm by generating a proton gradient across the cytoplasm membrane [24].

Similarly to temperature effect on enterotoxins production, the pH range allowing production of enterotoxins is also more limited than those for growth. The practical limit in acidic foods is pH 5.0, with an optimum of 7.0. The SEA is produced under a wider range of pH than SEB or SEC [6,20].

3.1.3. Salt resistance

A characteristic feature that distinguishes *S. aureus* from other pathogenic bacteria is its high tolerance to low water activity values and NaCl concentrations up to 20%. 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 [7,8,10,21]. Those values are dependent on the specific strain, the actual values of pH, temperature, humectants and atmospheric conditions. No growth of a mixture of *S. aureus* strains in BHI broth containing NaCl and sucrose was observed at 8 °C, pH 4.3 and a_w 0.85 (19% of NaCl) or at 12 °C, pH < 5.5 and a_w 0.9 (14% of NaCl) or at 12 °C, pH < 4.9 and a_w 0.96 (8% of NaCl) [10]. A single strain of *S. aureus* in PCA or BHI broth containing NaCl could not withstand concentrations of NaCl of 5% (a_w 0.97) at 12 °C, 13% (a_w 0.91) at 15 °C, 15% (a_w 0.89) at 18-21 °C and 18% (a_w 0.86) at temperatures in the range 25-30 °C. At optimal growth temperatures in the range from 35 °C to 37 °C it could multiply up to concentration of 20% of NaCl (a_w 0.84) [25].

The ability of *S. aureus* to grow at such high concentrations is related to its adaptive response to osmotic stress. It is due to the intracellular accumulation of compatible solutes including proline, betaine, choline, taurine which can occur by *de novo* synthesis or by transport from the growth medium. The transport systems appear to be constitutively synthesised and to be activated in a very specific way by osmotic stress. There are multiple transport systems for betaine and proline. There is probably a single specific system for each one and a less specific system which is strongly activated by osmotic stress and results in the accumulation of both proline and betaine. Compared to other pathogenic organisms, *S. aureus* does not accumulate sugars as compatible solutes and free peptides serve as a source of proline [26]. Besides the accumulation of compatible solutes to maintain turgor caused by the increased NaCl concentration, *S. aureus* also undergoes an extensive program of gene and protein expression in response to NaCl stress. One of them is probably an *ars* encoding the resistance to arsenate, arsenite and antimonite. However, mutation in the *ars* operon significantly decreases the ability of *S. aureus* to grow in the presence of NaCl, since the low expression of *ars* impedes the ability of *S. aureus* to rid itself of cytoplasmic Na⁺ in NaCl-stressed cells [27].

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 value is from 0.86 to 0.89 a_w . 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, SEB is produced only in the narrow range of water activity values 0.99-0.97 [10,28].

3.1.4. Tolerance of *S. aureus* against sanitizing agents and antibiotic resistance

In generally, *S. aureus* is sensitive to sorbic acid, peracetic acid and hydrogen peroxide. Unsaturated fatty acids and alkaline dyes also affect inhibitory. On the other hand, it is resistant to phenol, compounds of mercury, cadmium and arsenates. The ionization radiation kills cells with a D-value of 0.2-0.4 kGy in meat and fish products, but the enterotoxins are not affected even by a sterilization dosage of radiation [6]. The effect of ethanol is also not unique. Concentrations up to 7% may have an inhibitive effect, but concentrations higher than 9% act bactericidal.

The majority of disinfectants routinely used in the food industry (halogens, quarternary ammonium salts) will be effective when applied correctly. After inappropriate sanitation however, the cells can recover and become more resistant [8]. *S. aureus* has also a high degree of tolerance to compounds such as tellurite, mercuric chloride, neomycin, polymyxin and sodium azide, all of which have been used as selective agents in culture media [10].

Pathogenic *S. aureus* is regarded as a “superbug”, due to its amazing capacity to be resistant to a wide range of antibiotics. *S. aureus* strains resistant to methicillin (MRSA), vancomycin (VISA/VRSA), and to many other antibiotics represent an urgent problem in both community- and hospital-acquired infections. According to Girish et al. [29], the resistance results from *i*) surface protein modifications which promote colonization of host tissues, *ii*) biochemical variations which enhance survival in phagocytes and evasion of the host immune system, *iii*) enhanced release of toxins which lyse eukaryotic cell membranes and active efflux of antibiotics coupled with mutation events in target molecules.

The perspective targets for drugs in *S. aureus* may be the enzymes involved in lysine biosynthesis or genes encoding the activities essential for the life of the cell that have not been used for therapeutic intervention. In this context, the following antibiotics are used: *i*) linezolid by blocking the formation of the ribosomal initiation complex, *ii*) clarithromycin by the inhibition of the proteosynthesis, *iii*) phosphomycin by inhibition of the cell wall synthesis, *iv*) daptomycin by the insertion into the cell membrane, causing rapid depolarisation and the release of potassium ions, resulting in the inhibition of DNA, RNA and protein synthesis, *v*) tigecycline, erythromycins, tetracyclins, oxazolidinones and aminoglycosides by inhibition of the protein synthesis, *vi*) fluoroquinolones by inhibition of the DNA replication and repair [29-31].

3.2. Determination and identification of *S. aureus*

Staphylococci compete poorly with indigenous bacteria and are inhibited by the antagonistic activities of other organisms. Therefore the presence of *S. aureus* in foods must be considered in relation to the amount and types of the accompanying flora. Numerous

methods to isolate and identify *S. aureus* have been described and standardized by international and national organizations. The principal approach is to isolate it on solid agar media and subsequently identify it by the use of microbiological, biochemical and molecular methods.

3.2.1. Determination of *S. aureus* counts

Media for isolation and determination of *S. aureus* can be divided into three groups [6,7].

- In the first group are media such as tryptone soya broth, brain heart infusion broth, mannitol-salt agar, salt meat broth. They use sodium chloride as the selective agent and metabolizable substrates such as mannitol, blood or milk as diagnostic agents are incorporated. However, higher concentrations of salt and the lack of resuscitators in the media may inhibit injured or stressed cells (false negative results). Moreover, other microorganisms are salt-tolerant or can metabolize substrates, so the media are not specific enough.
- In the second group are media which contain combinations of selective and diagnostic agents. The list of selective agents which includes sodium azide, sodium chloride, lithium chloride, potassium tellurite, glycine and antibiotics (polymyxin or sulphametathine) is not large but provides many combinations. Media like tellurite-polymyxin agar, KRANEP agar, Giolitti-Cantoni broth, Baird-Parker agar and its modifications, and some other media are found in this group. The mode of diagnostic action is fermentation of mannitol, egg yolk reaction – clear zones around colonies, black colonies (reduction of tellurite to tellurium) and pigment production [5]. The problems of this media are that some animal strains of *S. aureus* do not use lipovitellenin from egg yolk, and competing microorganisms (spp. *Enterococcus*, *Proteus*, *Micrococcus*) are also able to reduce tellurite. In spite of this, some of them are widely used and are also recommended by the ISO, IDF or AOAC organisations.
- To correct the discrepancies of media in the previous groups, the addition of plasma (from rabbit, pig or rat) with bovine fibrinogen instead of egg yolk is used. These media allow the detection of coagulase directly on the plate due to the formation of fibrin zones around the colonies. Such media include Baird-Parker agar with plasma and Rabbit-plasma fibrinogen (RPF) medium. Because of the cost and variable performance of commercially available plasmas, they are not used in routine examinations.

Nowadays, there is also the possibility to use chromogenic media for detection of *S. aureus* [32]. To minimize false negative or positive false results further confirmatory tests are necessary.

3.2.2. Identification of *S. aureus*

The first step in the identification of suspected colonies is the Gram-staining, microscopic examination of the morphology, catalase test and also β -haemolysis surrounding colonies on the sheep-blood agar [16,17,33-37].

One of the preferred examinations is the coagulase test, either as a tube format for the presence of unbounded extracellular coagulase or as a slide coagulase test for the presence of a clumping factor - cell wall associated enzyme. There are commercially available rapid and convenient tests, and also laboratory procedures are permissible to detect the presence of coagulase. It should also be noted that the production of coagulase is not a property of only *S. aureus*, but also of some Gram-negative bacteria and other staphylococci. In addition to this, coagulase is not exclusively produced by *S. aureus* and coagulase-negative strains may be also enterotoxigenic. Also the test to detect nucleases (deoxyribonuclease - DNase and heat-stable endonuclease - thermonuclease) is useful by either the spectrophotometric method or by microbiological methods. The effect of lysostaphin on the cell wall destruction distinguishes staphylococci from micrococci, since staphylococci but not micrococci are lysed by an extracellular enzyme produced by *S. staphylolyticus* [13].

From among biochemical tests, the API-Staph system and the VITEK Gram-positive Identification Card are widely used. They are based on the reaction of microorganism with a set of specific substrates. There is also the possibility of fluorescence microscopy detection without previous growth of culture on selective media by the use of the VIT-Staphylococcus system. This is based on the penetration of a specific gene probe into the bacteria cell, marking the individual signature of the gene sequence with the dye and illuminating them. Subsequently, the samples are examined under fluorescence microscopy. Bacteria belonging to the genus *Staphylococcus* light up in green, bacteria belonging to the species *S. aureus* additionally light up in red [38].

However, the most reliable way to identify a suspicious colony as *S. aureus* is to investigate the presence of highly specific genes by the use of PCR technology. So from among the most employed genes, there is the possibility to detect the presence of 16S or 23S rRNA sequence, *tst* gene (encoding toxic shock syndrome), *coa* gene (encoding coagulase), *eta*, *etb* genes (encoding exfoliative toxin A and B), *clfA* and *clfB* genes (encoding clumping factors), *femA* gene (encoding resistance to methicilin), *cat* gene (encoding production of catalase), and *nuc* gene (encoding thermostable nuclease) [11,15,37,39-42]. Since the *nuc* gene is present in all *S. aureus* strains and is well conserved in this species at the nucleotide level but is either absent from or distinct in other bacterial species including coagulase-negative staphylococci it has been reliably used for *S. aureus* identification [43].

From the human health point of view, methods for the detection of staphylococcal enterotoxins are required. Firstly, the presence of genes encoding enterotoxins (*sea-sev*) are searched for by the use of PCR assays. Subsequently, the expression of the enterotoxin under the current conditions is investigated. One of the options is the use of immunological test system for routine use established in the ELISA procedure based on the monoclonal or polyclonal antibodies against enterotoxins detection. By using the reversed passive latex agglutination test (RPLA), enterotoxins antibodies are bounded to particles of latex, but the nonspecific agglutination is also possible. The immunofluorescence methodology has also been used to detect cell-associated enterotoxins, but this method has not been used to any

great extent. An alternative to the fluorescence method, radioimmunoassay can be employed by the radioactive iodine as a marker, but also it is not widely used. For scientific, not for routine examinations, other procedures including the electrophoresis, the electroimmuno-diffusion reversed immunoosmophoresis and the affinity chromatography methods may also be used [13,20].

4. *S. aureus* in milk and dairy products

S. aureus is a ubiquitous organism frequently isolated from raw milk manually drawn from individual animals, bulk raw milk and naturally, from milk of dairy cattle suffering from mastitis. In properly drawn milk, the typical counts of *S. aureus* are 100-200 CFU/ml. In the case of a contaminated udder, the counts may increase up to 10^4 CFU/ml [7].

4.1. Source of contamination and occurrence in the environment

The natural ecological niches of *S. aureus* are the nasal cavity and the skin of warm-blooded animals. The skin, mucosa membranes, teats and udders of milking animals are the most important reservoir of this contaminant. In the case of an infected udder, *S. aureus* can contaminate milk during milking in a density ranging from 10^1 - 10^8 CFU/ml, mostly about 10^4 CFU/ml [6,7,34]. It is responsible for approximately 30-40% of all mastitis cases in the world [35].

In primary production and the dairy environment, except for milk-producing animals, human beings and operational environment belong among the main sources of product contamination. One third of people are the asymptomatic carriers of *S. aureus*. It is frequently found on the skin, in nose, axilla, umbilicus, gastrointestinal and urogenital tracts of humans. The frequency of enterotoxigenic strains isolated from humans is high, varying between 40% and 60%. The organisms find their way into food through hands (infected wounds, skin lesions) or by coughing and sneezing [6,7,12,34,44].

According to references [34,45,46], the frequency of *S. aureus* occurrence varied from 6% to 28% in samples of raw milk. However, Rall et al. [37] found that *S. aureus* was present in 70.4% of raw milk samples. Although the density of *S. aureus* was not analysed, the prevalence of enterotoxigenic strains in these isolates ranged from 25.5% to more than 72%, with SEA and SEC as the predominant enterotoxins. It is assumed, that SEA together with SED were the most frequent agents in SFP outbreaks [6,17,47,48]. Furthermore, SEA is predominantly produced by human strains, so the contamination of food samples during manufacture is possible [33,48]. On the other hand, SEC is the most important cause of SFP associated with the consumption of dairy products [17].

In Slovakia, a similar incidence (4-9%) of *S. aureus* in raw cows' or ewes' milk was reported [49,50]. In our investigations of raw milk, we found that *S. aureus* was present in 20% of samples, with a density of 2.2 log CFU/ml. And, 33% of those isolates were enterotoxigenic, with *sea* as the only enterotoxin encoding gene found.

The lack of proper hygienic measures during food processing would also increase the counts of *S. aureus*, especially in manually prepared foods. Therefore, *S. aureus* can contaminate also heat-treated milk and can subsequently be present in cheeses prepared from both raw and pasteurized milk. In this connection, the presence of *S. aureus* in 46% of Slovakian cheeses (fresh lump cheese, “Bryndza” cheese) and even in 83% of whey after lump cheese manufacture was not surprising. Densities of 0.5, 1.6 and 4.5 log CFU/g or ml in “Bryndza” cheeses, whey and in lump cheeses were determined, respectively. 14% of those isolates possessed the gene for only one SE and the other 14% possessed the genes for two SEs. In the majority of the isolates, the gene for SEA was detected, in 11% of isolates the combination of *sea* and *sec* genes was found and *see* gene or *sea/see* genes combination occurred in one of the isolates. Neither *seb* nor *sed* genes were found throughout the collection of isolates.

In the study performed by Kousta et al. [51], 96% of both unpasteurized and pasteurized milk cheeses met the EU regulations for *S. aureus* either absent or present in very low numbers. The rest of them consistently had a density higher than 4 log counts but none of these tested positive for enterotoxin. By investigation of mostly dairy products including cheeses, whey, butter, but also some samples from meat, meat products, sausages and eggs, *S. aureus* was detected in 13-20% of samples [16,17,20,52,53] or 35-45% [33,46] and even in 70-80% [42,54]. The prevalence of enterotoxigenic strains was higher than 30% in all mentioned studies. The *sea* and *sec* genes were again the most frequent. But there were also found some strains with a presence of *seb*, *sed*, *see* genes or combinations of all of them.

The correlation between the presence of a respective gene and real enterotoxin production is about 70-80%, which might be explained by the incomplete expression of the enterotoxigenic genes. This is influenced by environmental conditions, such as temperature, pH and water activity which are important both for the growth and production of enterotoxins [20,42,52,54]. For this reason, it is necessary to know cardinal values of intrinsic and extrinsic factors preventing the growth of *S. aureus* in specific raw milk cheese production.

4.2. *S. aureus* in milk: Quantitative assessment of growth

S. aureus requires a complex organic source of energy. The main substrates used by this organism are sugars (glucose, fructose, galactose, mannose, ribose, maltose, sucrose, trehalose), alcohols (mannitol), organic acids (acetate), and in some conditions amino acids (glutamine, arginine). Genome sequence analysis revealed the presence of lactose phosphotransferase systems that enabled the growth of *S. aureus* in milk [55].

4.2.1. Effect of incubation temperature on the *S. aureus* growth in milk

The growth of various *S. aureus* strains is now well documented in databases of predictive microbiology tools such as Combase or Predictive Modelling Program [56]. As an example, the growth of two strains in relation to temperature is demonstrated in Fig. 1. The range in

which the SED was detected is also shown (Fig. 1a) as well as the average growth parameters in Table 1 [57]. According to references [17,58,59], SED is the second most common serotype of enterotoxins among staphylococcal strains isolated from dairy products associated with food poisoning. Fig. 1a indicates the fact that SED was already detected at the level of *S. aureus* of $10^{6.5}$ CFU/ml at the lower temperature of 12 °C. At the higher temperatures of 18 and 21 °C, the detectable amount of SED toxin was determined when *S. aureus* reached the density of 10^7 CFU/ml. Based on the previous literature data [7,47,58,60,61], the minimal concentration of *S. aureus* of 10^6 CFU/ml needed for enterotoxin production in food was confirmed.

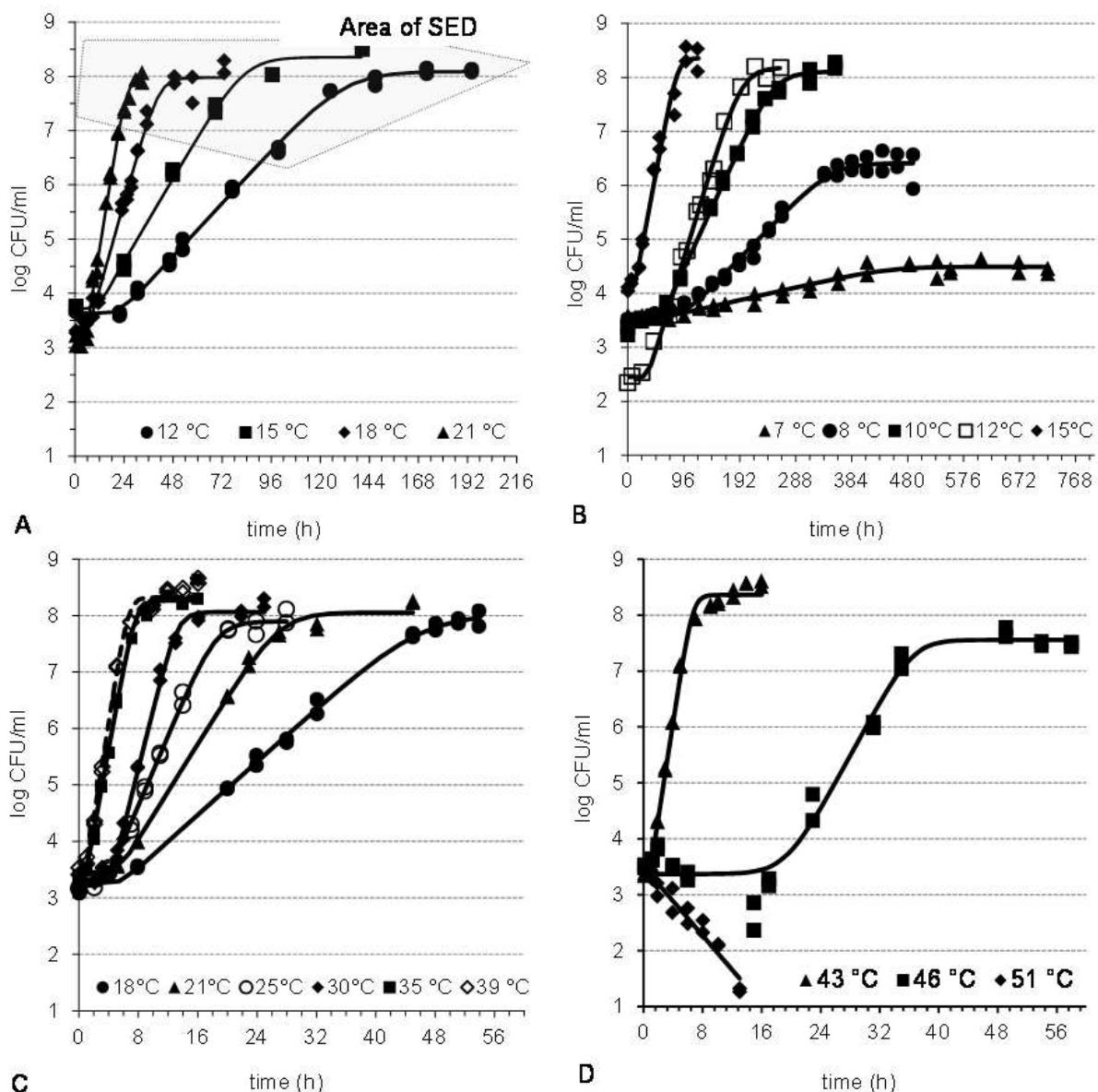


Figure 1. Growth of *S. aureus* strains D1 (a) and 2064 (b, c, d) in milk at temperatures from 7 °C to 51 °C. The growth data were fitted using DMFit tool [64]

T [°C]	D1		2064	
	μ [h ⁻¹]	t_d [h]	μ [h ⁻¹]	t_d [h]
7			0.006	120.3
8			0.026	27.0
10			0.055	12.7
12	0.103	6.8	0.082	8.5
15	0.148	4.7	0.145	4.8
18	0.313	2.2	0.264	2.6
21	0.545	1.3	0.484	1.4
25			0.711	1.0
30			1.215	0.6
35			1.664	0.4
39			1.931	0.4
43			1.903	0.4
46			0.562	1.2

Table 1. Specific growth rates and t_d of *S. aureus* strain D1 and 2064 in milk
 μ – specific growth rate, t_d – time to double

Despite the slow growth of the 2064 strain, the temperature of 7 °C can be considered as the minimal temperature for growth of *Staphylococcus aureus* 2064 as proposed by Tatini [62]. However, some authors [19,63] did not observe *S. aureus* growth at 8 °C even after 1 week of incubation. On the other hand, other literature sources mentioned the lowest *S. aureus* growth temperature of T_{min} 6.5-7.0 °C [6-10].

In order to know the variability of growth rates as calculated from the growth curves, we performed static cultivations of the 28 confirmed *S. aureus* isolates in duplicate at the same temperature (15 °C). The results of the descriptive statistics are summarised in Table 2. The highest variability among the growth parameters was associated with the lag phase duration, as the most variable parameter. It reflects the previous history of the inoculum, the physiological state of the cells, the time necessary for production of the biological components needed for replication and the period of adjustment to the new environment.

Comparing the determined parameters with values generated by the Combase Predictor ($\mu = 0.170 \text{ h}^{-1}$, lag = 14.3 h) or in the Pathogen Modeling Program ver. 7.0 ($\mu = 0.177 \text{ h}^{-1}$, lag = 8.9 h) [56,65], it can be concluded that all values are very close. The average values of growth rates of isolates were slightly lower than those predicted by world programmes and also, the lag phase duration of our isolates was longer. This difference may be attributed to the fact that both software programs processed data from growth experiments carried out in broth media, not in milk.

Taking into account that 12-37% of the bound of reliability during cultivation experiments is tolerable; these findings demonstrate that the duration of the lag phase and the growth rate

of *S. aureus* in milk can be predicted with a defined degree of reproducibility. Prediction of growth dynamic and effects of environmental factors on growth parameters, described further, resulting from analyzing the growth of the model *S. aureus* 2064 isolate in milk can be effectively and reliably used in food practice to reduce the risk of staphylococcal food poisoning outbreaks.

Parameter	μ	lag	N ₀	N _{max}	t _d
aver	0.163	13.8	2.93	8.17	4.3
sd	0.025	3.2	0.59	0.29	0.6
%v_c	15.3	23.0	20.0	3.6	14.0
min	0.104	4.4	0.78	7.19	2.2
max	0.318	21.4	3.72	9.02	6.6
med	0.159	14.3	3.13	8.19	4.4

Table 2. Growth parameters of *S. aureus* isolates in UHT milk at 15 °C (n = 28)
 μ [h⁻¹] – specific growth rate in exponential phase, lag [h] - duration of lag phase, N₀ [log CFU/ml] - initial concentration of *S. aureus*, N_{max} [log CFU/ml] - maximal concentration of *S. aureus* in stationary phase, t_d [h] - time to double, aver - average value, sd - standard deviation, v_c - coefficient of variation, min - minimal value, max - maximal value, med - median of the value

4.2.2. Effect of temperature on *S. aureus* growth parameters

Within quantitative predictive microbiology the secondary models are used to characterise the influence of intrinsic or extrinsic food factors on specific growth parameters. Among the temperature models, the Ratkowsky-type and cardinal temperature models are appreciated by users despite the basically empirical nature of the relationships [66].

The specific growth rates of three *S. aureus* strain determined in the suboptimal temperature range 7-39 °C were analyzed with Ratkowsky square root model and graphically compared with the Combase Predictor data [65]. The results presented in Fig. 2 showed high linearity with correlation coefficients R^2 from 0.962 to 0.995 when modelled with a square root model [64]. The following equations resulted from fitting the growth rates with the square root model in the temperature range from 7 to 39 °C for the strains 2064, D1 and B1, respectively:

$$\sqrt{\mu_{2064}} = -0.0883 + 0.042(T - T_{min}) \quad R^2 = 0.9948; \%V = 99.46$$

$$\sqrt{\mu_{D1}} = -0.0804 + 0.0455(T - T_{min}) \quad R^2 = 0.9784; \%V = 97.73$$

$$\sqrt{\mu_{B1}} = -0.0008 + 0.039(T - T_{min}) \quad R^2 = 0.9623; \%V = 95.85$$

Based on the testing of goodness of fit, the per cent of variance (%V) confirmed high correlation coefficients R^2 (above) for strains 2064, D1 and B1, respectively. Their model coefficients b ($\sqrt{\mu} = a + b(T - T_{min})$), except for the B1 strain, were very close not only to each other but also to the coefficient of Combase line $b_{Comb} = 0.048$ or $b = 0.0442$ found by Fujikawa and Morozumi [61].

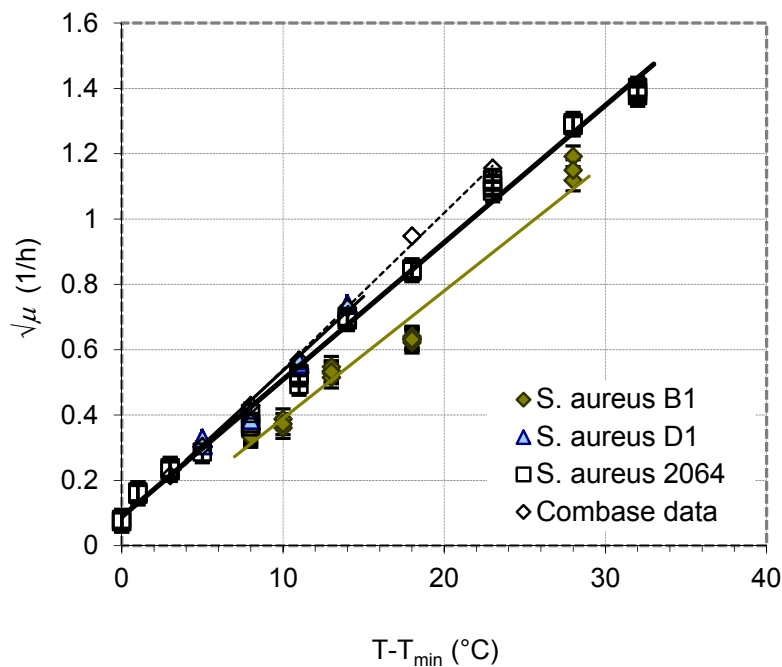


Figure 2. Comparison of the Ratkowsky model applied to the strains of *S. aureus* and selected data from the Combace Predictor within the sub-optimal growth temperatures

The effect of temperature in the whole range from 7 °C to 51 °C on the ability of *S. aureus* to grow in milk is depicted in Fig. 3a. *S. aureus* growth in milk was positively determined with the increasing of the incubation temperature, resulting in a shortening of the lag phase duration and more intensive growth in the exponential phase. Within an empirical approach, the extended model introduced by Ratkowsky [68] which includes data beyond the growth optimum, could be used for describing the impact of temperature on growth rate. The accuracy of the model was validated by comparison with accessible data for other *S. aureus* isolates. Since the data are very similar to each other, the prediction of *S. aureus* 2064 growth in milk can be reliably used for *S. aureus* generally.

According to the recommendation of Ratkowsky [68], maximal temperature for *S. aureus* 2064 of 47 °C was derived from data points in the high-temperature region. By use of this model, the optimal temperature for growth of *S. aureus* in milk of 38.5 °C was also calculated and validated by the use of the Gibson model. From the survival line, with the rate of -0.35 h⁻¹ a D-value of 6.7 h at 51 °C (Fig. 1d) was calculated.

From the food practice point of view, the model of Gibson et al. [69] is useful for the prediction of the time (t_3) to increase counts of *S. aureus* by 3 log, if the parameter of specific growth rate is replaced by the t_3 function. In the original equation, a useful $b_w = \sqrt{(1 - a_w)}$ transformation appears, in which the value of 1 represents maximal water activity. Analogically, in the case of temperature $T_w = \sqrt{(T_w - T)}$.

In the case of initial *S. aureus* counts in milk meant for cheese production of 10³ CFU/ml, *S. aureus* will increase its counts during fermentation at 18 °C in 10 h to the level ordered by European Commission Regulation 1441/2007 [70] and the enterotoxin production will occur

in 30 h at the same conditions. As is shown in Fig. 3b, in the case of optimal temperature, an increase of about 2 log or 4 log counts will occur in 2 h or 4 h, respectively.

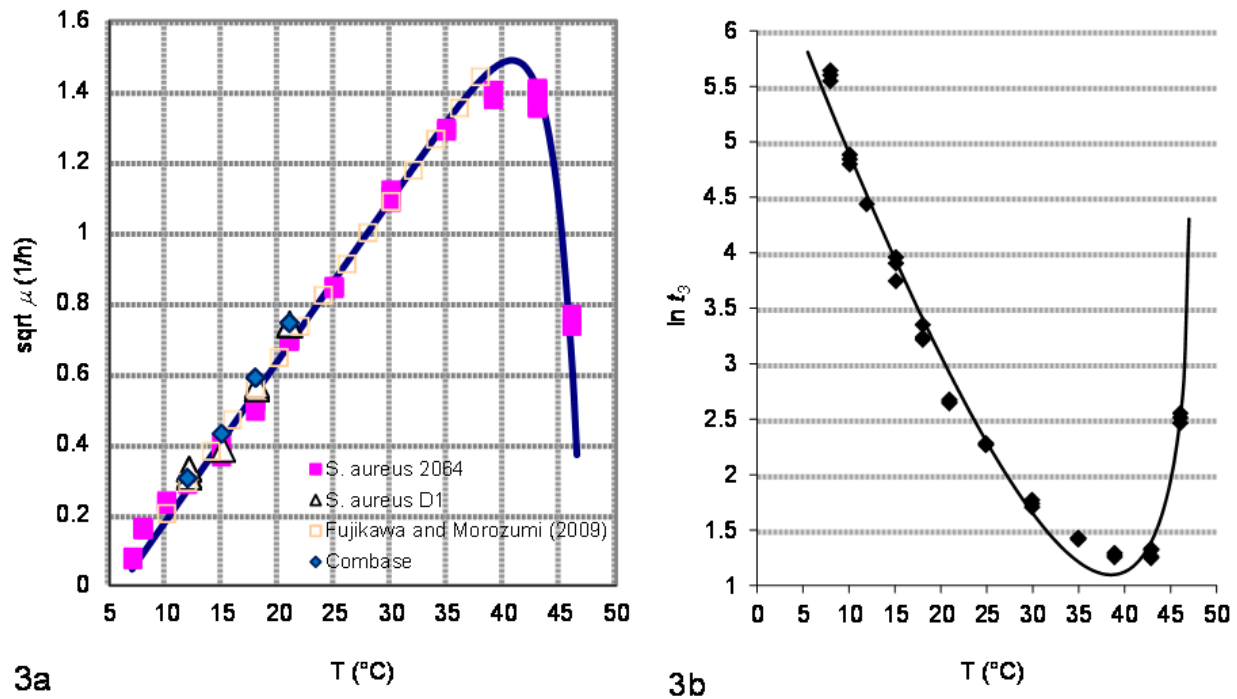


Figure 3. Plots of the square root of specific growth rates ($\sqrt{\mu}$) of *S. aureus* 2064 versus incubation temperature. Symbols indicate $\sqrt{\mu}$ calculated from growth curves at each incubation temperature. The continuous line indicates the fitted of $\sqrt{\mu}$ vs. T function, where $\sqrt{\mu} = 0.0456(T - T_{\min}) [1 - e^{0.447(T - T_{\max})}]$
b. Plots of the natural logarithm of time (t_3) necessary for an increase of *S. aureus* 2064 counts about 3 log counts against its initial numbers versus incubation temperature. Symbols indicate the t_3 calculated from growth curves at each incubation temperature. The continuous line indicates the fitted $\ln t_3 = 3/\mu$, where $\mu = \exp^{(-0.378T_w^2 + 2.202T_w - 2.371)}$ and $T_w = \sqrt{(T_{\max} - T)}$

As temperature was the only modifying environmental factor, lag phase was described by means of the model developed by Davey et al. [71] according to the following equation

$$(R^2 = 0.962) \text{ in the range from 8 to 43 } ^{\circ}\text{C: } \ln \ln \left(\frac{1}{\text{lag}} \right) = 1.973 - \frac{87.92}{T} + \frac{285.09}{T^2}.$$

4.3.2. Effect of pH value and LAB presence on *S. aureus* growth in milk

In dairy practice, 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 24h-old cheese. As described above, one of the most effective tools to inhibit the growth of *S. aureus* is to acidify the environment as soon as possible. This is performed by adding a sufficient amount of dairy starters, which are able to ferment lactose and to produce lactic acid very rapidly. As is obvious from Fig. 4, pH 6.0 and 5.5 influenced neither the growth dynamics nor the *S. aureus* counts in the stationary phase. However, pH 5.0 resulted in a decrease of growth rate for about 3.5-time and also in a reduction of total growth. If the pH of growth media is adjusted

to pH 4.5, a total diminution of *S. aureus* counts is observed. The same effect is achieved at pH 4.0 if inorganic acids are used.

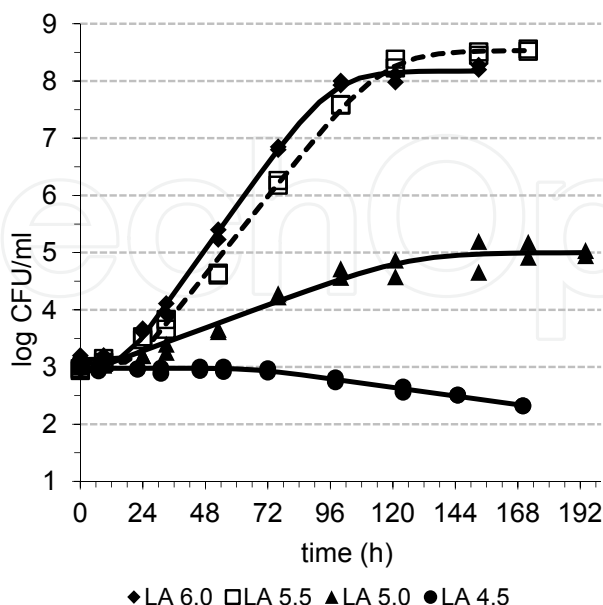


Figure 4. Growth dynamics of *S. aureus* 2064 in nutrient broth at 15 °C in dependence to initial pH value as adjusted with lactic acid to the values 6.0, 5.5, 5.0 and 4.5

Growth and fermentative metabolism of lactic acid bacteria, as a permanent component of raw milk microflora, are offered by a wide variety of fermented dairy products. Besides the most effective inhibitive activity against pathogen and spoilage microorganisms, which includes production of organic acids and subsequent pH decrease, they produce bacteriocins, H_2O_2 , and aromatic compounds and act as a strong competitor for nutritional factors (nicotineamide, biotine or niacine) [23,72,73].

If there is slow and insufficient acid production in the growth environment, no inhibitive effect is observed. This was the case of *Lactobacillus rhamnosus* GG and VT1 which did not produce the required amount of lactic acid in milk under aerobic conditions. Despite their inhibitive effect against *Candida maltosa* and *Geotrichum candidum* [74], no inhibition was achieved during the co-cultivation with *S. aureus* in milk. It was also found that the inhibition level of 7% of *Lactococcus lactis* strains was variable and ranged from bacteriostatic to no inhibitory effect on *S. aureus* growth, mainly due to low acidification ability [60]. As *S. aureus* is catalase-positive, we may also expect its resistance against hydrogen peroxide, approx. up to 8% [75].

Thus, it is interesting to select an appropriate starter culture of LAB which is able to efficiently inhibit *S. aureus* growth together with improving the sensorial quality of the final products. However, the strong acidification may limit the activities of other bacterial populations involved in the development of the sensorial properties of ewes' lump cheese [76].

The requirements assigned to a starter culture of LAB include fast growth and survival in dairy environment, rapid production of lactic acid resulting in pH value diminution and no production of toxic or other technologically and sensorially unacceptable metabolites.

The effectiveness of a starter culture of LAB is related to the rate at which it can produce sufficient amounts of lactic acid, predominantly in the first six hours of fermentation. It is connected with the phenomena of the pH lag phase. It is obvious in Fig. 5 that the higher the incubation temperature, the more intensive the metabolism of LAB, and the sooner a pH decrease will occur. The intensity of pH drop is determined by the initial counts of the starter culture, as was confirmed in our co-culture experiments with *S. aureus* 2064 and culture Fresco DVS 1010 (Christian Hansen, Hørsholm, Denmark). The following relation between the duration of pH lag phase resulted from the linear regression analysis shown in Fig. 5: $\ln \text{lag}_{\text{pH}} = 6.494 - 0.129 \times T - 0.230 \times N_{0 \text{ Fr}}$ ($R^2 = 0.970$) where T is temperature in °C, $N_{0 \text{ Fr}}$ are initial counts of lactic acid bacteria of the culture Fresco.

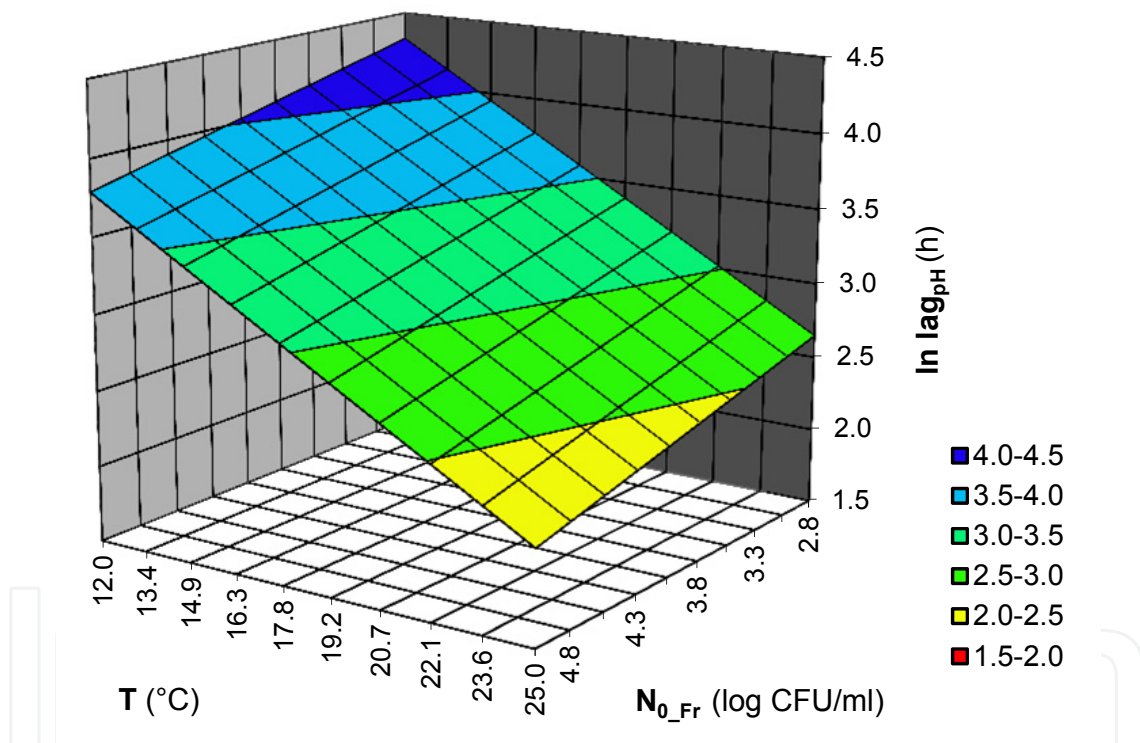


Figure 5. Period without any pH change (lag_{pH}) found during static cultivation of the starters Fresco 1010 (Christian Hansen, Hørsholm, Denmark) and *S. aureus* 2064 in co-culture, as dependent on temperature and initial LAB count ($N_{0 \text{ Fr}}$)

It was also observed that during co-cultivation of Fresco culture with *S. aureus* 2064 in milk, *S. aureus* was able to grow only during the pH lag phase. When the pH started to decrease, the growth of the pathogen stagnated or declined. This period was influenced by an appropriate amount of starter culture at a specific incubation temperature.

The ratio between the initial inoculum of *S. aureus* and LAB in a culture determines the efficiency of the inhibition as well. It was observed that when the *S. aureus* population was higher than that of *L. lactis*, *S. aureus* reached the counts as in a pure culture. On the other hand, for ratios of 1:1 or 1:10 for *L. lactis*, maximal *S. aureus* population reached counts about 4 to 5 log CFU/ml lower [23].

However, our data did not showed a direct relation between the inhibition of *S. aureus* counts in the stationary phase and the ratio of mesophilic culture Fresco DVS 1010 and *S. aureus* 2064. However, linear regression analysis (Fig. 6, 7) revealed strong relations between independent variables (as temperature, initial number of the starter) and specific growth rate of *S. aureus*, even between increases in the numbers of *S. aureus* during its growth ($N_{\max Sa}$).

Besides initial concentration of LAB, the applied temperature also has a strong effect on the microbial growth dynamics. With an increasing of the incubation temperature, the duration of pH and microbial lag phase is shortened. On the other hand, the higher the temperature, the higher the growth rates.

The combined effect of temperature and the initial Fresco culture is depicted in Fig. 7. From it, one is able to calculate the necessary addition of Fresco culture and thermal mode during milk or young cheese fermentation to ensure a minimal increase in the numbers of *S. aureus* ($N_{\max Sa} - N_0 Sa$). According to the EU regulation, the total *S. aureus* amounts in raw milk cheeses should not exceed 4 log CFU/g. Assuming properly drawn milk with 100 CFU/ml of *S. aureus*, to keep its increase in number at a level lower than 2.0 log CFU/ml, the initial Fresco density should be at least 4.0 or 2.5 log CFU/ml at 21 °C or 18 °C, respectively. Similarly, also the culture A, which contains *Lactobacillus acidophilus*, was able to inhibit growth of *S. aureus* 2064 or *S. aureus* D1 in milk co-cultures [74].

Alomar et al. [76] also found that *S. aureus* SA15 did not grow when *Lactococcus garvieae* was at a concentration of 7.8 log CFU/ml at temperature 22 °C or at 20 °C and initial concentration higher than 6.5 log CFU/ml. At an initial concentration of *L. garvieae* higher than 7.4 log CFU/ml and at temperatures between 22 °C and 34 °C, *S. aureus* growth was not negatively influenced. Higher temperatures favoured the growth of *S. aureus* and *L. garvieae* had no inhibitory effect regardless of concentration.

Although acidification plays an important role in *S. aureus* inhibition, other mechanisms of LAB inhibitive potential should not be excluded. If pH and LAB play only a minor role in the inhibition, it can still be hypothesized that the cessation of the growth is due to the accumulation of antistaphylococcal substances produced by the LAB [77]. Results from literature suggest that *S. aureus* is able to grow under stringent acid conditions (pH 5.25 at 15 °C and 4.48 at 30 °C). On the other hand, inhibition of *S. aureus* by some starter culture was observed at pH 6.8, which cannot be attributed to a drop in pH value. Indirect inhibitory effect may also be involved. The availability of nutrients may trigger other mechanisms, leading for instance to the secretion of metabolites, peptides or signalling molecules, which would in turn be responsible for the inhibitory effect of LAB [60].

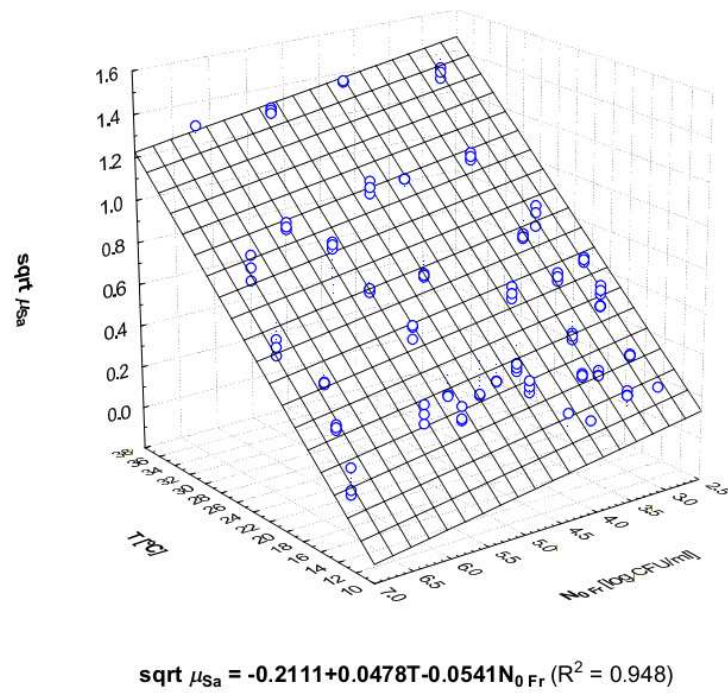


Figure 6. Dependence of specific growth rate (μ) of *S. aureus* 2064 on temperature and initial concentration of Fresco culture in milk

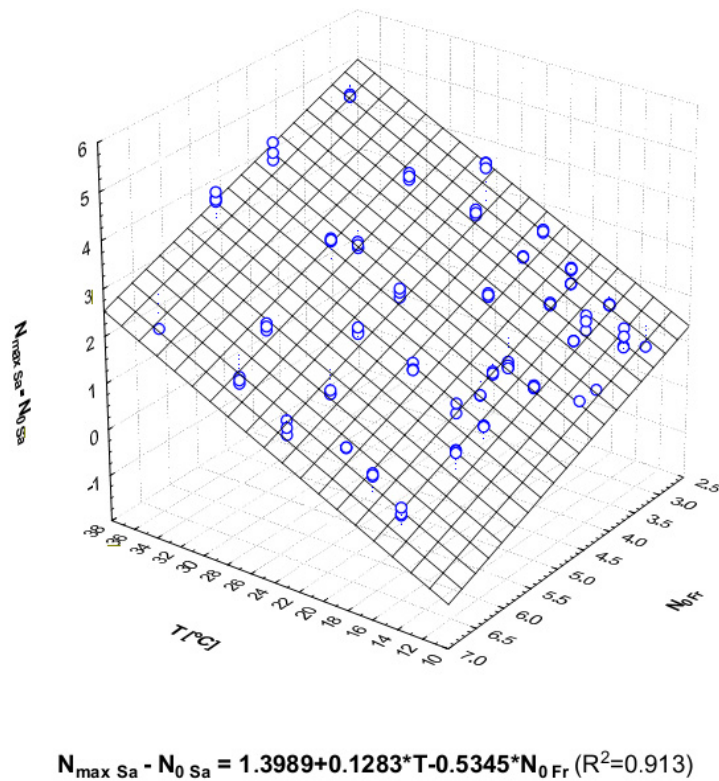


Figure 7. Dependence of increase in *S. aureus* counts ($N_{\text{max Sa}} - N_{0\text{Sa}}$) on temperature and initial concentration of Fresco culture (Fresco 1010, Christian Hansen, Hørsholm, Denmark) in milk in co-culture with lactic acid bacteria

5. Artisanal raw milk cheese production in Slovakia

5.1. Technology and microbiology: Description based on flow diagram

Original ewes’ lump cheese is an artisanal full-fat, soft rennet cheese from raw ewes’ milk manufactured on the farm level in Slovakian mountain areas according to the technological steps described and pictured in Fig. 8 and 9, respectively. After two weeks of ripening at temperatures from 18 °C to 21 °C, it is used for industrial production of the popular Slovakian “Bryndza” cheese [2]. Fermentation of the lump cheese relies on native mesophilic lactic acid bacteria (LAB) such as *Lactococcus lactis*, *Enterococcus faecalis*, *Lactobacillus casei*, *Lb. lactis* and *Lb. plantarum*. During ripening, the essential role is played by the milk mould *Geotrichum candidum* and oxidative yeasts of the genera *Torulopsis*, *Candida* and *Kluyveromyces* [1,3].

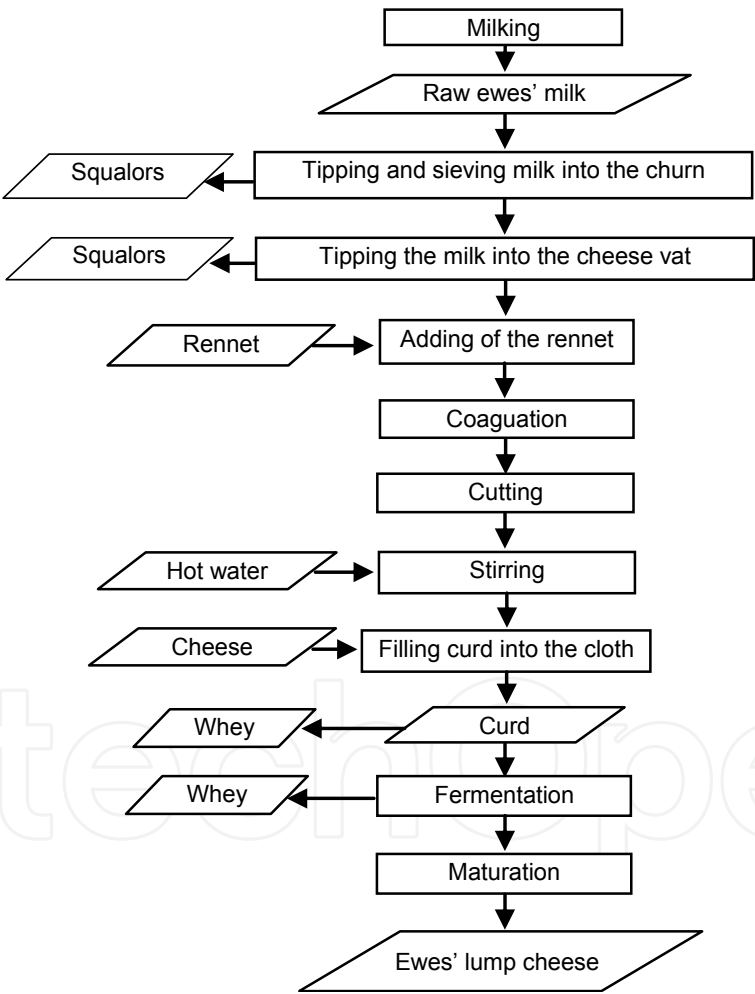


Figure 8. Flow diagram of artisanal production of ewes’ lump cheese

Generally, cheeses are considered as one of the safest foods currently consumed. However, pathogenic bacteria which can be transmitted by dairy products cannot be underestimated. Historically, there have been several outbreaks related to the consumption of cheeses. The predominantly responsible organisms *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp.

and *Staphylococcus aureus* have been reported. The sources of their contaminations were raw milk, inadequately pasteurized milk, or post-pasteurization contaminated milk [47,51,78,79]. In this context, microbiological specifications related to the finished cheeses made from raw milk defined by the Commission Regulation No. 1441/2007 [70] concern with food safety and process hygiene criteria. They comprise of absence *Salmonella* spp., *Listeria monocytogenes* and staphylococcal enterotoxins in 25 g and number of coagulase-positive staphylococci not exceeding $m = 10^4$ and $M = 10^5$ CFU/g at $c = 2$.



Figure 9. Photo-documentation illustrating the artisanal manufacture of ewes' lump cheese on the farm level in Slovakian mountain areas (Author: L. Valík)

Despite the raw milk origin and substantial proportion of raw milk cheeses containing enterotoxigenic *S. aureus*, ewes' lump cheese is also consumed as a fresh cheese at a regional level or it is used as a raw material for the production of original "Bryndza" cheese in Slovakia. The safety and quality of fermented original cheeses manufactured from raw milk at a primary level is generally determined by various specific hygienic, technological, and intrinsic and extrinsic environmental factors. The factors which contribute to the safety of cheeses with respect to pathogenic bacteria include milk quality, native lactic acid bacterial growth during cheese manufacture, pH, salt, environmental conditions and chemical changes during ripening. However, the most important role during fermentation is played by metabolism of the LAB participating in effective competition with pathogenic and spoilage microorganisms and subsequently in inhibition of undesirable microorganisms.

According to our investigations of eight products manufactured under upland farm conditions, the acidification of the curd started after a 10-20 h period and went on intensively for 20 h. Thus, a level of acidity equivalent to pH of 5.2-4.9 was usually reached in young cheese after 30-40 h. Such a fairly long time permits to the growth not only LAB but also of undesirable bacteria, including *S. aureus*. Within these field trials, the initial numbers of *S. aureus* in ewes' milk were about 2.2 log CFU/ml, but in cheeses after 3 days of fermentation they reached 6.2 log CFU/g.

The first 24 h of the process of making raw milk cheese appeared to be critical for *S. aureus* growth, with the most troublesome period taking place within the first 6 h, during which the exponential growth of *S. aureus* mainly occurs. In cheeses with relatively slow acidification during the first 6 h, pH has no effect on the initial growth phase of *S. aureus* before 6 h but may have a modulating effect on subsequent growth of up to 24 h. High pH value in the fresh cheese suggests a weak lactose fermentation ability by the non-starter LAB [7,58,76].

In order to prevent *S. aureus* from reaching the density of 10^6 CFU/g, it is necessary to shorten the pH lag phase by making the fermentative metabolism of LAB more effective under conditions related to lump cheese manufacture. As our previous experiments in model milk media confirmed, the Fresco culture is effective in the *S. aureus* inhibition. Verification of the microbial populations' behaviour under predicted safety conditions was analyzed in laboratory conditions during raw milk ewes' or cows' lump cheese fermentation at 18 °C with or without addition of 1% Fresco culture prior to coagulation.

As seen in Fig. 10a and 10b, a pH of 5.0, unacceptable for growth of *S. aureus*, was achieved after five hours of fermentation if Fresco culture was added. Such a short pH lag phase is crucial in pathogen growth inhibition during cheese manufacture, as has already been mentioned. Higher diminution of pH during the first 6 hours of fermentation means lower *S. aureus* increase in number. The *S. aureus* increase in ewes' lump cheese with the addition of Fresco culture was only 0.96 log counts, despite the growth rate in the exponential phase was more than twice as high as in milk mono-culture at 18 °C or in cheese prepared without Fresco. An increase in microbial counts in the first 24 h is a normal process in cheese making. This is partly due to the physical retention of microorganisms in the coagulum and

also due to the microbial multiplication during coagulation and whey drainage [7,80,81]. In contrast to cheese without the addition Fresco starter, the *S. aureus* increase in number was about 2.9 log counts and its maximal counts exceeded 6 log CFU/g. Consumption of such a cheese might represent a potential threat of food poisoning outbreak if the enterotoxigenic strains are present.

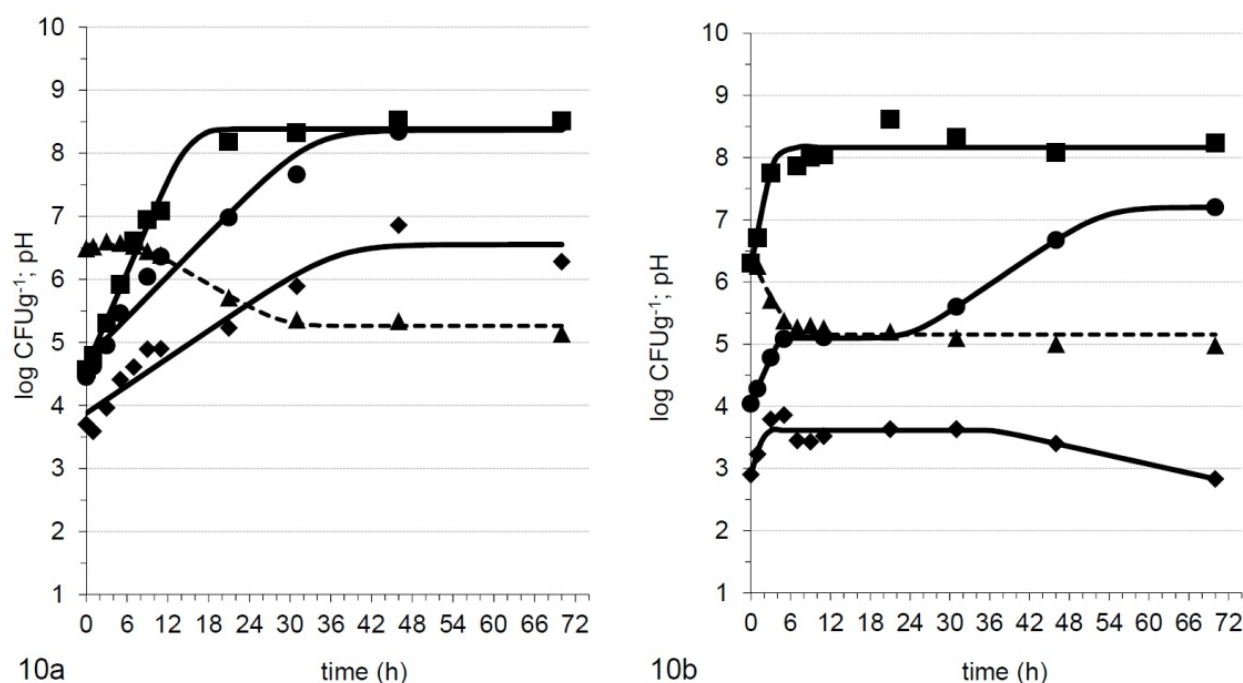


Figure 10. Growth dynamics and pH value changes during fermentation of ewes' lump cheese at 18 °C without starter culture (10a) and with addition of 1% Fresco culture (10b); ■ presumptive LAB on M17 agar, ● presumptive LAB on MRS agar, ◆ counts of *S. aureus*, ▲ pH value

In order to keep the numbers of *S. aureus* under the limit defined by the EU regulation No. 1441/2007 [70], the initial addition of Fresco culture into the raw milk should be higher than 10⁵ CFU/ml. These initial counts would be accompanied with the suitable timing of pH decrease down to pH 5.0. The addition of an appropriate amount of mixed mesophilic LAB culture, which produces inhibitory substances, provides opportunities to add additional barriers to the growth of bacterial pathogens. Moreover, it could be essential for the improvement of both the fermentation process and the quality of ewes' lump cheese.

This assumption was also confirmed by some other authors. Olarte et al. [82] observed differences in *S. aureus* growth in dependence on the addition of starter culture. In cheese without added starter culture, *S. aureus* exceeded concentrations higher than 5 log counts in 5 days. This was in contrast to the cheese prepared with starter culture, where decreases from counts higher than 4 log CFU/g to 2 log were observed within the fermentation and ripening process. The addition of starter culture of LAB during the manufacture of goats' milk cheese affected the pH value dynamics which after 5 days of fermentation decreased to pH 5.1 compared to 6.61 pH of cheese made without starter culture.

A rapid decrease in pH values from 6.7 to an average value of 5.24 was observed in 24 h old raw cows' milk cheeses [58]. From an initial average density of 1.89 log *S. aureus* grew rapidly during the first 6 h up to 5 log in average and then slowly up to 24 h, when the population reached a peak. In those cheeses *S. aureus* never reached 7 log CFU/g, but in 2 samples where SE production occurred, not only did they exceed 5 logs, but pH values of the cheeses at 6 h also exceeded 6.3. In Tenerife goats' raw milk cheese after 48 h pH reached value of 4.93, which led to a decrease in *S. aureus* counts from 3.14 log CFU/g in 2 days old cheeses to 1.62 log CFU/g in 30 day old cheeses [83]. Also in raw cows' milk cheeses counts of *S. aureus* in the end of 2-3 weeks fermentation were lower than 2 log CFU/g, mostly due to the rapid pH value decrease down to pH 5.09 [84]. During the ripening of Turkish White cheese made from raw cows' milk at 6 °C, pH was not changed and fluctuated from 4.63 to 5.06. Such acidic conditions contributed to the decrease in *S. aureus* counts from 5.03 log to 2.36 log CFU/g over 4 months [85].

In raw milk cheeses collected by Jakobsen et al. [80], a significant decline in pH values was observed after 5-6 h of fermentation and pH lower than 5.5 was achieved in all samples after 24 h. The highest *S. aureus* contamination was reached in 5-6 h old samples, in some samples higher than 4 log counts. Nevertheless, none of the sample exceeded counts higher than 5 logs and so it was concluded that *S. aureus* did not produce enterotoxins. A correlation between the contamination level of the milk and contamination level of 5-6 h old cheeses was noticed. The initial *S. aureus* level in raw milk greatly influences the level of staphylococci during this first period of cheese-making.

In raw goats' milk cheeses, the initial log counts of *S. aureus* were 4.86; 6.23 and 5.88 in winter, spring and summer cheeses. They were covered with brine (12%) for 10 days at 15 °C and then stored at 4 °C for 3 months. During the ripening, the counts of *S. aureus* decreased to 2.04-2.30 log in winter and spring cheeses or to 1.02 log counts in summer cheeses, respectively. During ripening of all 3 types of cheeses, pH was practically stable, reaching values in the range 5.23-6.06 [86]. In cheeses made from raw ewes' milk, *S. aureus* was not detected in either fresh or mature cheese. The pH of fresh or ripened cheese was 6.31 and 5.79 pH, respectively [87].

On the other hand, in raw milk Mexican cheese Fresco, pH decline from pH 6.7 to value 5.6 was achieved only after 10 days of ripening at 4 °C. Counts of *S. aureus* were close to the 10⁷ CFU/g level and did not undergo any noticeable change during cheeses storage. It may be due to the *S. aureus* capacity to withstand a wide range of temperatures, pH and water activity [81].

Based on these results and observations from literature, it is strongly recommended, to use the starter culture at least in artisanal cheese production. Rapid fermentation process prevents against the growth of *S. aureus* and other pathogenic and undesirable microorganisms. Even in mountain areas, this can be performed by the inoculation of LAB, e.g. in the form of fresh fermented milk. Moreover, the addition of adjunct starter culture

can improve flavour, reduced bitterness and increase the concentration of peptides, which impart desirable flavour, and of precursors of flavour volatiles [88]. It was also confirmed, that the Fresco culture addition had no negative effect on the sensorial descriptors of ewes' lump cheese and compared to the cheese made without starter culture it even achieved better sensorial acceptance. But it has been suggested that positive results for flavour and texture development are strongly strain-dependent [85], so the selection of appropriate starter culture is necessary.

The variation in the responses of *S. aureus* to pH value and its dynamic during cheese making process may be attributed to the variations in each dairy farm management of hygiene practices, environmental and personal diversities, process of manufacturing, herd characteristics, multiple sources of *S. aureus* contamination and geographical distribution of *S. aureus* strains [20,48,80]. Similarly, many factors are known to influence the SEs production, e.g. NaCl content, water activity value, pH, temperature, atmosphere, amino acid composition and competing microflora. For that reason, it is crucial to understand which factors control enterotoxin production in raw milk cheese, in order to be able to assess their safety and to prevent staphylococcal food poisoning.

Even if pasteurization kills *S. aureus* cells, a previous population reaching higher than 5 log counts may lead to enterotoxins production and once enterotoxins are produced they retain their activity. Besides this, pasteurization eliminates also enzymes and indigenous microflora, which are partly responsible for the development of the typical raw milk cheese flavour and texture [80]. Moreover, the raw milk contains a heat-labile lactoperoxidase system which has inhibitory effect to the growth of some pathogens [79]. The post-pasteurization addition of a starter culture may lead to losses in the unique organoleptic properties of the raw milk cheeses and to end products with uniform sensorial features [81,85,87]. Hence, there is today a renewed interest in traditionally produced raw milk cheeses due to consumer demands for increased varieties of cheese flavours and textures [80]. Consequently, as regards the safety of raw milk cheeses, potential pathogens associated with milk or milk products including *Staphylococcus aureus*, should still be of interest.

6. Conclusions

The inhibitory potential of LAB on *S. aureus* growth results from different factors described here and in the scientific sources used in this chapter. Taking into account the growth data obtained with a few *S. aureus* strains isolated from artisanal cheeses it was shown that they grew well in milk alone, in co-culture with LAB as well as in raw milk cheeses prepared in laboratory. As the specific strains isolated from raw ewes' milk and cheese were used in the study, the growth data may provide useful information for artisanal cheese practice. Taking into account that the initial *S. aureus* numbers in raw milk fluctuate about 3 log CFU/ml, artisanal ewes' lump cheese producers may apply our prediction of the time (t_3) directly as these t_3 times are in coincidence with the time to reach a critical density of 10^6 CFU/ml for possible enterotoxin presence.

Artisanal raw milk cheese production poses a few critical factors limiting its safety. With reference to the growth of *S. aureus*, many factors should be taken into consideration, such as its natural contamination in milk, quantitative growth data, cheese type, nature, activity and type of the starter culture and mutual relation between *S. aureus* and LAB populations. Factors that may prevent the reaching of *S. aureus* counts higher than 10^5 CFU/ml and production of enterotoxin are: low initial contamination of milk (less than 10^2 CFU/ml), high initial number of active LAB (higher than 10^5 CFU/ml), the capacity to pH during the first 6 h of fermentation and cease the growth of *S. aureus* as fast as possible within 24 h (in the best case within the first 6 h of young cheese fermentation). Inhibitory starters producing bacteriocins may also be used. Thus, the adding of a starter culture in artisanal cheese production is strongly recommended.

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