VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
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Foodborne *Staphylococcus aureus*: Identification and enterotoxin production in milk and cheese

Zkrácená verze dizertační práce

Key words
Staphylococcus aureus, real-time PCR, enterotoxin production, milk, cheese

Klíčová slova
Staphylococcus aureus, PCR v reálném čase, produkce enterotoxinu, mléko, sýr

Místo uložení práce
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CONTENTS

1. INTRODUCTION

Modern food production chains are evolving to very complex systems that provide greater opportunities for contamination and growth of pathogens. As a direct consequence, preventing foodborne diseases (FBDs) becomes a difficult task. FBDs are defined by the World Health Organization as "diseases of infectious or toxic nature caused by, or thought to be caused by the consumption of food or water". More than 250 different FBDs have been described and bacteria are the causative agents of two thirds of FBD outbreaks [4].

In 2008, a total of 5,332 food-borne outbreaks were reported within the EU. 45,622 human cases, 6,230 hospitalizations and 32 deaths were related to the reported outbreaks [25]. In the USA, it is estimated that FBDs affect 6 million to 80 million people each year, causing up to 9000 deaths, and cost about 5 billion US dollars [35]. The largest number of reported food-borne outbreaks in the EU was caused by *Salmonella (*35.4% of all outbreaks), followed by viruses (13.1%), bacterial toxins (9.8%) and *Campylobacter* (9.2%). The most important food vehicles in the outbreaks were eggs and egg products (23.1%), pig meat and products thereof (10.2%) and mixed or buffet meals (9.2%) [25].

Staphylococcus aureus is considered the third most important cause of disease in the world among the reported FBDs. The growth of *S. aureus* in foods presents a potential public health hazard because many strains of *S. aureus* produce enterotoxins (SEs) which are the causative agents of staphylococcal food poisoning (SFP) [35].

Rapid methods of pathogen testing have been gaining increasing interest in the food industry [43]. Polymerase chain reaction (PCR)-based methods provide a powerful tool for highly specific and sensitive identification of pathogenic bacteria in foods and are considered reliable alternatives to traditional microbiological methods.

Milk and cheeses are foods that have frequently been associated with staphylococcal food poisoning. When enterotoxigenic strains of *S. aureus* replicate to numbers exceeding 10^5 cfu/ml, they may produce staphylococcal enterotoxins. Above this threshold (10^5 cfu/ml or g of a dairy product), there is an obligation to screen for SEs. If SEs are detected, products have to be destroyed, recalled or withdrawn from the market [38].

To improve the production of microbiologically safe food, data about food-borne pathogen virulence is required to complement already existing knowledge about the growth and survival ability of pathogenic bacteria. Recent research has shown that there are significant differences in the behaviour of bacteria in laboratories, i.e. in a controlled environment and in actual food products. New knowledge about relation between bacterial growth and virulence expression under adverse environmental conditions will give rise to new approaches in the prevention of foodborne diseases and enable the advancement of quantitative risk assessments.

2. THEORETICAL PART

2.1 Staphylococcus aureus

2.1.1 The organism and staphylococcal food poisoning

Currently, 49 different species of staphylococci exist, out of them 9 are coagulase positive. *Staphylococcus aureus* remains to be the predominant pathogen [2].

Staphylococcus aureus is a Gram-positive, non-motile, oxidase negative, catalase and coagulase positive coccus about 1 μm in diameter dividing in more than one plane to form irregular three-dimensional clusters of cells [1, 3, 4]. *S. aureus* is a facultative anaerobe and uses glucose by glycolysis and use of the hexose monophosphate pathway. It usually ferments mannitol and, in the presence of air, uses a wide range of hexoses, pentoses, and sugar alcohols; lactic acid and acetoin are the main end products of glucose metabolism [1]. The cell wall of *S. aureus* contatins three main components: peptidoglycan, teichoic acid and protein A [1, 3]. *Staphylococcus aureus* is found in the nostrils, and on the skin and hair of warm-blooded animals [4]. It lives as a commensal of the human nose in 30–70% of the population.

Staphylococcal food poisoning (SPF) is caused by staphylococci (principally *S. aureus*) growing in food and forming enterotoxins as a product of their metabolism [1]. SFP presents as a self-limiting gastrointestinal illness with emesis following a short incubation period (ca. 4 h) after ingestion of food containing preformed enterotoxin(s). Vomiting is the hallmark symptom of SFP [6]. The severity of the illness depends on the amount of food ingested, the amount of toxin in the ingested food and the general health status of the victim [7]. The intake of as little as 20 – 100 ng of enterotoxin can cause intoxication [8]. Ingested bacteria do not produce toxin, therefore the symptoms normally wear off within 24 h [7].

2.1.2 The enterotoxins

To date, 21 staphylococcal enterotoxins (SEs) or enterotoxin-like proteins (SEls) have been identified and designated SEA to SElV [7]. SEs are short, extracellular proteins that are soluble in water [1, 4, 14]. They are heat resistant (depending on the SE type, SE concentration and food matrix) and highly stable to proteolytic enzymes, such as pepsin, trypsin chymotrypsine, rennin and papain [1, 4, 15, 16]. Enterotoxins stimulate the relase of serotonin in the gut. The serotonin acts on neuron receptores in the gut, stimulating the vomiting center in the brain via the vagus nerve [17].

The SEs belong to a family of the so-called pyrogenic toxins. Pyrogenic toxins include SEs, TSST, exfoliatins A and B and streptococcus pyrogenic toxins. These toxins share some structure, function and sequence similarities [15]. Although pyrogenic toxins are involved in distinct pathologies, they have common biological activities, i.e. pyrogenicity, immune

suppression, and nonspecific T-cell proliferation. These activities are referred to as superantigen activity [4].

The enterotoxins are located on and spread by different mobile genetic elements, i.e. pathogenicity islands (SAPIs), prophages, plasmids, enterotoxin gene cluster (*egc*) and staphylococcal cassette chromosome (*scc*) [4, 5, 6, 9, 15, 19, 20]. Most of reported SFP outbreaks are associated with the classical enterotoxins, SEA-SEE [7].

SEA is the most common toxin implicated in SFP. The *sea* gene is carried by a temperate bacteriophage [15] and the expression peaks at late exponential phase of *S. aureus* growth [21]. It has not yet been fully understood how sea expression is regulated. The second most common enterotoxin associated with SFP is SED. The *sed* gene is located on a 27.6-kb penicillinase plasmid designated pIB485 [15]. The expression of *sed* peaks in the late exponential growth phase, a consequence of the regulation by the Agr system [22].

As for *Staphylococcus aureus*, several global regulators have been reported to regulate the production of virulence-associated exoproteins and cell wall components. Among these regulatory systems, the accessory gene regulator (agr) system has been the best characterized. The agr system is a quorum-sensing system and a two-component regulatory system which responds to an autoinducer peptide [20].

2.1.3 Environmental factors

Environmental factors such as temperature, nutrients, pH, presence or absence of oxygen and water activity, disturb homeostasis of a cell and affect the cell's growth and toxin production. The most important environmental factors affecting the growth and enterotoxin production of *S. aureus* are shown in **Table 2.1** and **Table 2.2**.

Table 2.1 *Environmental factors affecting the growth of S. aureus* [11]

	Minimum	Optimal	Maximum
Temperature (^{o}C)	7	35-37	48
pH	4.0	6.0-7.0	9.8
NaCl (%)	0	0.5-4.0	20
Water activity	0.83	0.98->0.99	>0.99

Table 2.2 *Environmental factors affecting enterotoxin production* [11]

	Minimum	Optimal	Maximum
Temperature (^{o}C)	10	35-40	45
pH	4.8	5.3-6.8 (Ent. A) 6-7 (Others)	9.0
NaCl (%)	0	0.5	20
Water activity	0.86	>0.99	>0.99

2.1.4 Milk and cheeses

Milk and milk products have always been vehicles for staphylococcal food poisoning [30]. In 2000, a mass outbreak of SFP caused by consumption of reconstituted milk occurred in Japan, and more than 10,000 cases were reported [31]. Other recently reported outbreaks were associated with mashed potatoes made with raw milk in Norway [32], pasteurized milk products in Austria [33], and Minas cheese in Brazil [34].

S. aureus can gain access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling and processing of raw milk. *S. aureus* is responsible for approximately 30% to 40% of all mastitis cases [35]. There are many other possible sources of *S. aureus* contamination for processed milk including humans themselves. The potentially adverse effects of *S. aureus* on human health are reduced through pasteurization. It is widely believed that the majority of food poisoning cases associated with pasteurized milk are due to improper pasteurization or post pasteurization contamination [30].

S. aureus is a poor competitor against the normal microflora in unpasteurized dairy products. However liquid milk, raw or pasteurized, is a good substrate for growth of *S. aureus* and for enterotoxin production if it is held at temperatures higher than 10°C and the natural bacterial flora is low. In fact pasteurized milk is a better substrate than raw milk for the growth of *S. aureus* and for enterotoxin production because the competitive natural flora has been partially eliminated [14, 30].

Cheeses comprise a huge number of varieties and thus the composition also varies [37]. For each cheese variety, different technological parameters such as starter culture, milk and curd heating temperature or stirring conditions, are applied throughout the cheese-making process and induce different environmental conditions (i.e. pH, temperature, NaCl concentration or oxygen disponibility) that affect *S. aureus* growth and enterotoxin production [37, 38]. Above a threshold of 10^5 coagulase-positive staphylococci per gram of a dairy product, European legislation stipulates the obligation of testing for the presence of enterotoxins [39]. If SEs are detected, products have to be destroyed, recalled or withdrawn from the market resulting in substantial economic loss. It has been demonstrated that *S. aureus* growth occurs mainly during the first 24 hours of cheese-making process [29, 38]. However, SEs are not always detected in the final product even if the population of *S. aureus* reaches a value above 10^5 cfu/g of cheese [38, 13].

2.1.5 Principles of detection

2.1.5.1 Detection of S. aureus

2.1.5.1.1 Microbiological methods

There are three broad types of agar media that may be used for the isolation and enumeration of *S. aureus* in foods from which Baird-Parker agar remains the medium of choice (EN ISO 6888-1). The good performance of this medium depends on: a balance of selective agents (lithium chloride, glycine and potassium tellurite) to inhibit Gram-negative and Gram-positive bacteria; the inclusion of sodium pyruvate to protect *S. aureus* damaged cells and the presence of egg yolk, which, in addition to providing one of the main diagnostic features of the medium, also assists in the recovery of damaged cells. Potassium tellurite is metabolized by *S. aureus* with the release of tellurium, resulting in the formation of black colonies, which is a further useful diagnostic feature [1].

Giolitti-Cantoni broth and Baird-Parker broth are selective media used in Most Probable Number (MPN) techniques for detection of small numbers of *S. aureus* and when necessary, for selective enrichment before subculture to agar media. The growth of Gram-negative lactose-fermenting bacilli is inhibited by lithium chloride and Gram-positive bacilli are inhibited by potassium tellurite in combination with glycine. Anaerobic cultivation inhibits the growth of micrococci. The growth of *S. aureus* causes blackening [40].

2.1.5.1.2 Biochemical methods

The principal method used to confirm that an isolate is *S. aureus* is to apply the coagulase test. The literature now commonly refers to two forms of coagulase: free coagulase which is detected in the tube test and bound coagulase (which may be given an alternative name clumping factor) which is detected in the slide test. Free coagulase is an extracellular protein that is produced when the organism is cultured in broth. Bound coagulase remains attached to the cell wall of the organism. The test is positive if clotting of plasma occurs [3, 40].

Thermonuclease test has a useful role in food microbiology because it will survive conditions, e.g. pasteurization, that would destroy *S. aureus* but not the enterotoxins. Thus it can be used as a screening test for possible presence of enterotoxins [1].

2.1.5.1.3 Rapid methods

Rapid methods of pathogen testing have been gaining increasing interest in the food industry. These methods include antibody-based assays, genetic amplification methods and newer sensor development. Traditional plating methods following enrichment can take days to yield results, while newer rapid methods require hours. Genetic amplification methods such as the polymerase chain reaction (PCR) have made it possible to significantly reduce assay times while maintaining a high level of sensitivity and specificity. These methods are also able to distinguish closely related species which most antibody tests could not [43]. Nevertheless, rapid methods are currently used as screening techniques, with negative results

accepted as is, but positive results requiring confirmation by the appropriate official method, which, in many instances, is cultural [44].

Polymerase chain reaction

PCR is a method for enzymatic amplification (multiplication) of a specific DNA fragment in vitro. PCR consists of repeated cycles, each involving the following steps taking place at a defined temperature:

1. Denaturation of DNA, i. e. conversion of the double-stranded DNA to single-stranded DNA at 94–95°C,
2. Annealing of the oligonucleotide primers to homological DNA sequences at 37–70°C,
3. Polymerization of the second DNA strand from nucleotides by the action of a thermostable DNA polymerase at 60–72°C.

PCR is carried out in a programmable thermocycler. In a conventional version, the PCR product is analysed by agarose gel electrophoresis and presence/absence of the DNA fragment of a given molecular weight is evaluated [46]. The most commonly used stain for detecting DNA is Ethidium Bromide (EtBr). EtBr is a DNA intercalator, inserting itself into the spaces between base pairs of the double helix and forming a complex that emits fluorescence under UV light. The major drawback to EtBr is that it is a potent mutagen [47].

The main application of PCR is a rapid (15 min – 2 h), highly sensitive (limit of detection $\geq 10^0$ molecules) and highly selective detection of a defined DNA fragment, which is detectable even at an excess (of up to at least 5 orders of magnitude) of other DNA fragments [46].

In summary, several problems that can be encountered when PCR-based methods are used to detect food pathogens are inhibition of the reaction, the presence of false-positive reactions caused by contamination, detection of dead bacteria and results that are not quantitative. However, these problems can be overcome [48].

Real-time PCR

In real-time PCR, the PCR products are detected as they accumulate. In contrast to end point analysis in which only the plateau phase of the PCR can be detected, real-time PCR allows monitoring of the exponential phase [48]. The presence of amplified DNA fragments is detected by continuous monitoring of fluorescence. As a source of fluorescence, an intercalation complex of DNA with a dye SYBR Green I is used, or various types of probes or primers labelled with fluorescent dyes. When labelled probes or primers are to be used, various possibilities are available. Out of them, 5′-nuclease PCR has become the most widely used, thanks to its robustness [46]. This method utilises so called TaqMan probes. TaqMan probes are oligonucleotides longer than the primers (20 – 30 bases long with a T_m value of 10°C higher) that contain a fluorescent dye usually on the 5′ base, and a quenching dye (usually TAMRA) typically on the 3′ base. When irradiated, the excited fluorescent dye

transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET = Förster or fluorescence resonance energy transfer). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5′ exonuclease activity cleaves the probe. This ends the activity of quencher (no FRET) and the reporter dye starts to emit fluorescence [52]. When a sufficient amount of probe has been cleaved, the intensity of reporter fluorescence emission increases. A threshold level of emission above the base line is selected and the point at which the amplification plot crosses the threshold is defined as C_T and is reported as the number of cycles at which the log phase of product accumulation is initiated [53].

The main advantages of real-time PCR are minimization of the laboratory contamination, because the entire process is carried out in closed microtubes, and a potential for quantification of the specific DNA fragment based on amplification curves [46]. Furthermore, real-time PCR is quicker, less laborious, more sensitive and more specific when compared to conventional PCR.

Reverse Transcription PCR (RT-PCR)

Assessment of toxin genes expression has become crucial to understand the pathogenesis of staphylococcal infections [10]. Reverse-transcription polymerase chain reaction remains the most sensitive technique for the detection of often-rare mRNA targets, and its application in a real-time setting has become the most popular method to quantitate steady-state mRNA levels [12]. Generally two quantification types in real-time RT-PCR are possible: absolute and relative. The absolute RT-PCR relates the PCR signal to input copy numbers using a calibration curve, and neither comparisons nor references are needed. The relative RT-PCR determines the expression level in comparison with a reference sample. It is based on the relative expression of a target gene versus a reference gene. Normalization of target gene expression is useful in order to compensate for sample-to-sample and run-to-tun variations and to ensure the experimental reliability [10].

2.1.5.2 Detection of staphylococcal enterotoxins

A number of techniques for the detection of staphylococcal enterotoxins are currently available. These techniques include radioimmunoassay (RIA) [50, 51], microslide double diffusion [36], enzyme immunoassay (EIA) [49], MS-based analysis [45, 42] or methods including biosensors [41].

Out of enzyme immunoassay methods, enzyme-linked immunosorbent assay (ELISA), enzyme-linked fluorescent assay (ELFA) and reverse passive latex agglutination (RPLA) are widely used [1, 54]. Several ELISA methods have been proposed for the identification of enterotoxins in foods, but, except for a polyvalent ELISA and ELFA, their specificity has not

been studied extensively. Among ELISA methods, the "double antibody sandwich" ELISA is the method of choice, because reagents are commercially available in polyvalent and monovalent formats for both toxin screening and serotype specific identification. An automated enzyme-linked fluorescent immunoassay (ELFA) has been developed and is commercially available. This method has undergone specificity and sensitivity evaluations and has proven to be an effective serological system for the identification of staphylococcal enterotoxin in a wide variety of foods [28].

Methods of SE detection now are available commercially and have reduced preparation and detection time from several days to a few hours. Among most widely used, currently available test kits belong TECRA kit (3M), VIDAS SET2 kit (BioMérieux), TRANSIA PLATE Kit (Diffchamb), SET RPLA (Oxoid), RIDASCREEN (r-Biopharm).

In many cases, purification and concentrations techniques are needed prior to testing to produce a sample able to be analysed. With regard to dairy products, concentration technique based on trichloroacetic acid precipitation and dialysis concentration method against 30% polyethylene glycol are used.

It is well known that one of the main drawbacks associated with EIA kits designed for detecting SE, is the high frequency of false-positive results depending on the type of food assayed as a result of cross-reaction with unrelated antigens [26].

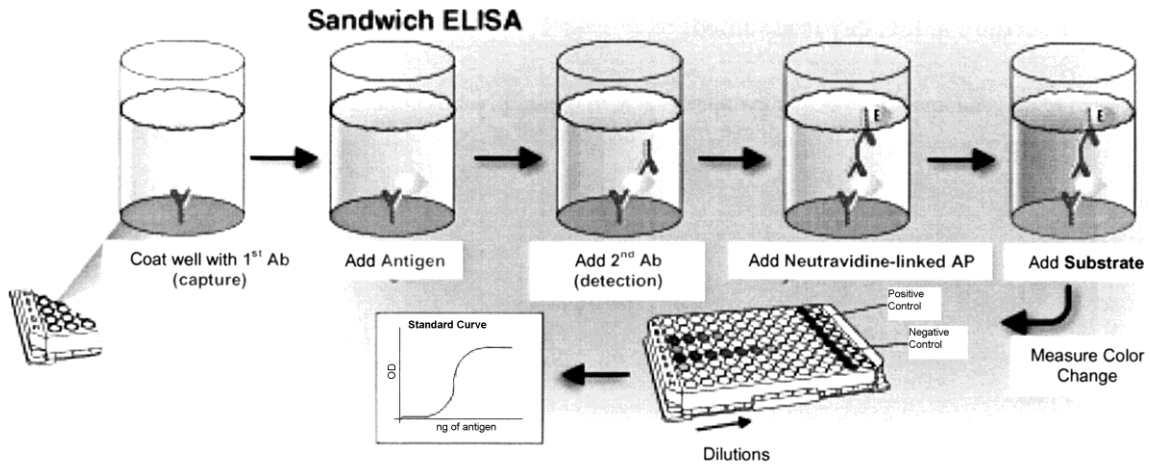


Figure 2.1 *Principle of the Sandwich ELISA* [27]

3. THE AIMS

The dissertation focuses on two main goals. The first one is to present a newly developed real-time PCR-based method for the detection of *Staphylococcus aureus* in food and the other one is to investigate and bring about more information on the behavior of *S. aureus* in the dairy chain. In order to improve our understanding about *S. aureus* virulence in dairy products we investigated:

- The effect of temperature on *S. aureus* enterotoxin D production in pasteurized milk and brain heart infusion
- The combined influence of low temperature and background flora on *S. aureus* enterotoxin D production in pasteurized milk
- The growth of *S. aureus* and enterotoxin A and D production in two types of cheese

4. RESULTS AND DISCUSSION

4.1 Detection of *S. aureus* in food by PCR

A rapid and sensitive method for the detection of *S. aureus* in food based on real-time polymerase chain reaction was developed. Specific primers and a TaqMan probe targeting a 103-bp DNA sequence of a gene coding for *S. aureus* acriflavin-resistant protein (AcrB) were designed using Primer Express software. 83 *S. aureus* strains from either clinical collections or as clinical or food isolates gave positive signals in PCR assay (100% inclusivity) and 64 non-*S. aureus* strains gave negative PCR results confirming 100% exclusivity of the method. PCR detection limit determined using decimal dilutions of overnight cultures was 6.8×10^1 cfu/ml and 3.4×10^1 cfu/ml with 100% and 70% detection probability, respectively. Two different selective media, modified Giolitti and Cantoni broth and Baird-Parker broth, at two levels of artificial contamination and oxygen conditions were investigated to find out which one gives better results. Giolitti and Cantoni medium at 37°C for 18 h under aerobic conditions was selected for further work on the evaluation of the complete method for foodborne *S. aureus* identification. Three DNA extraction methods were compared and since all gave similar results the simplest and the least expensive one, the lysis by boiling with Triton X-100, was the method of our choice. Finally, the developed method of selective enrichment and real-time PCR detection was compared with the standard method (EN ISO 6888-3) by analysis of 112 food samples. 61 samples were found positive for *S. aureus* by our method and 53 samples were found positive for *S. aureus* by the standard method. Ten food samples were artificially contaminated with *S. aureus* at the levels of 4×10^1 and 4×10^0 cfu/g to evaluate the efficiency of the developed method. All ten samples were detected positive by our method whereas one and four samples produced negative results using the standard method at the levels of 10^1 and 10^0 cfu/g, respectively. Moreover, 10^0 cfu/10 g was detected in all ten artificially contaminated samples by PCR-based method in contrast to seven false negative by the standard method. All false negative results of *S. aureus* presence by the standard detection were obtained in samples with natural background flora higher than 10^4 cfu/g.

The standard method for the detection of *S. aureus* in food (EN ISO 6888-3) is based on selective enrichment and subsequent isolation of colonies with characteristic morphology and identification by microbiological- and biochemical-based confirmations. This procedure is time-consuming, labor-intenstive and may yield false-positive or false-negative results.

The methods based on real-time PCR referred to as rapid methods have shown a great potential to overcome limitations of standard methods relating to sensitivity, specificity and speed. Almost all methods used to detect specific pathogens in foods require some cultural enrichment which prolongs assay speed. On the other hand, the enrichment step provides a benefit in terms of diluting the effects of inhibitors coming from food matrix as well as differentiation of viable from non-viable cells. The disadvantages that have to be considered

if PCR-based methods are to be applied to laboratories are the cost, molecular biology skills and the implementation of excellent laboratory procedures to prevent cross contamination.

The proposed method can be used for *S. aureus* detection as a faster, more highly specific, and more sensitive alternative to the microbiological method with its potential for providing improved food-processing hygiene control.

4.2 Milk experiments

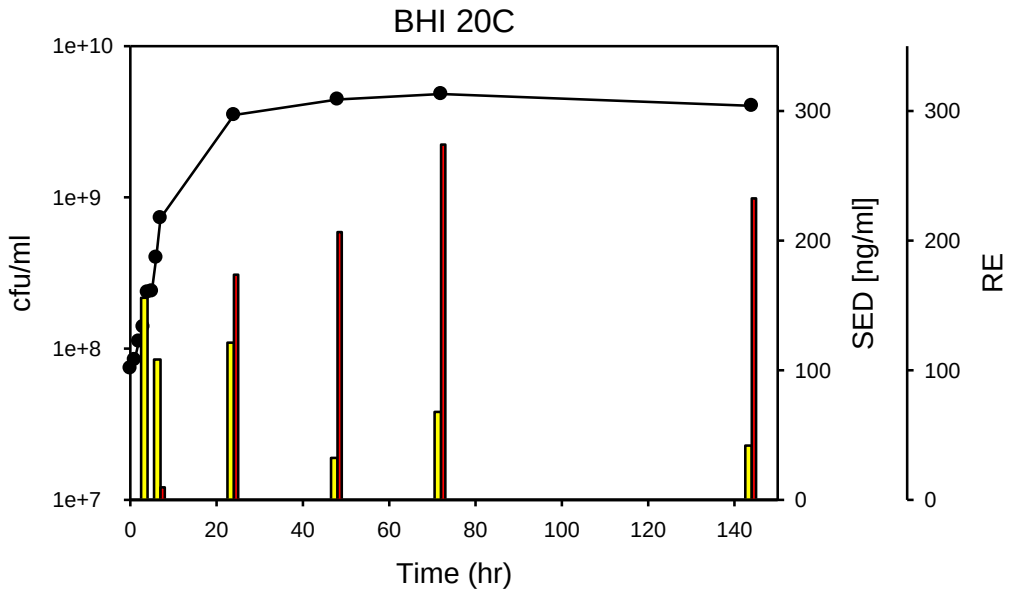
4.2.1 The effect of temperature on *S. aureus* enterotoxin D production in milk and BHI

The effect of three different temperatures, 8°C, 12°C and 20°C on *S. aureus* growth and SED production in pasteurized milk and on growth, *sed* gene expression and SED production in a rich chemically defined medium, Brain heart infusion, was investigated (**Figure 4.1**). The amount of SED produced was correlated to bacterial growth to investigate if S. arueus produces an increase amount of SED under sub-optimal growth conditions. A comparison was made between the behaviour of S. aureus in a optimal bacgterial growth medium (BHI) and a highly complex matrix, milk. The experiments were performed in small-scale fermentors. Two independent growth experiments were performed for each temperature and type of medium.

The growth pattern in BHI was the same at 20°C and 12°C but delayed in the latter. At 8°C there was no growth observed. The growth in milk was lower compared to BHI at all temperatures and the lag phase of the growth increased gradually with decreasing temperatures. Expression profile of staphylococcal enterotoxin D studied in BHI showed that transcription and translation patterns were correlated at 20°C and 12°C. *sed* mRNA was detected at 20°C and 12°C after 4 and 7 hours respectively. The production of SED occurred 3 and 17 hours later and during the exponential phase of growth which contradicts the generally quoted *agr*-dependent expression of *sed* gene induced during the transition from the exponential to the stationary phase of growth. At 8°C, a high level of *sed* expression was detected at 72 h but no protein synthesis occurred in response. In milk, SED production at 20°C and 12°C occurred earlier in growth but a lower total amount was produced compared to BHI. At 8°C there was no SED production.

Research has been performed to identify key parameters that prevent or stimulate enterotoxin production in laboratory media and in different food products and it was found out that a multifaceted network of environmental and genetic factors is likely to be responsible for the regulation of enterotoxin production [7].

Temperature is one of the identified environmental factors with impact on production of staphylococcal enterotoxins. The minimal temperature needed to induce enterotoxin production is 10°C and temperature seems to affect enterotoxin production more than growth. Our investigation supports the knowledge about the behaviour of *S. aureus* under low temperatures since no growth or enterotoxin D production was observed at 8°C.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1e+9
1e+8
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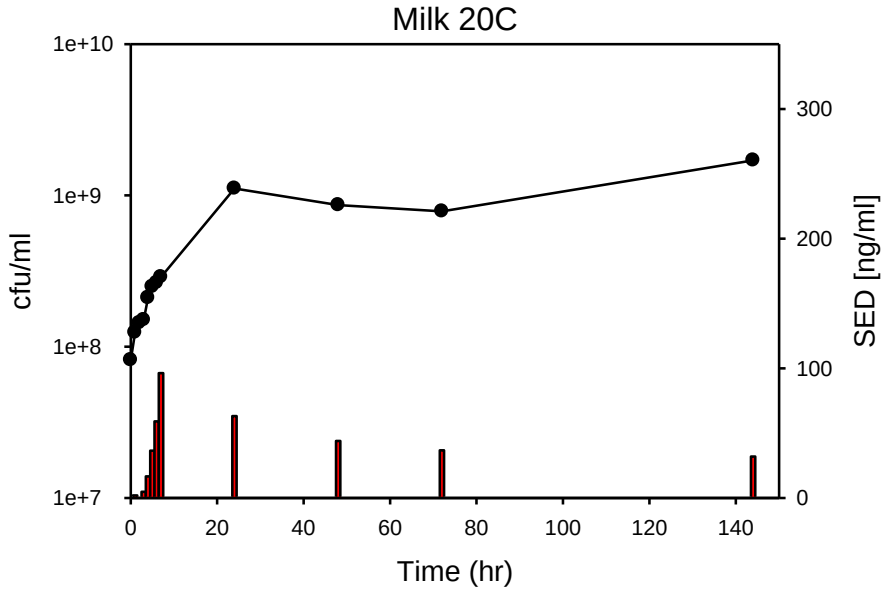
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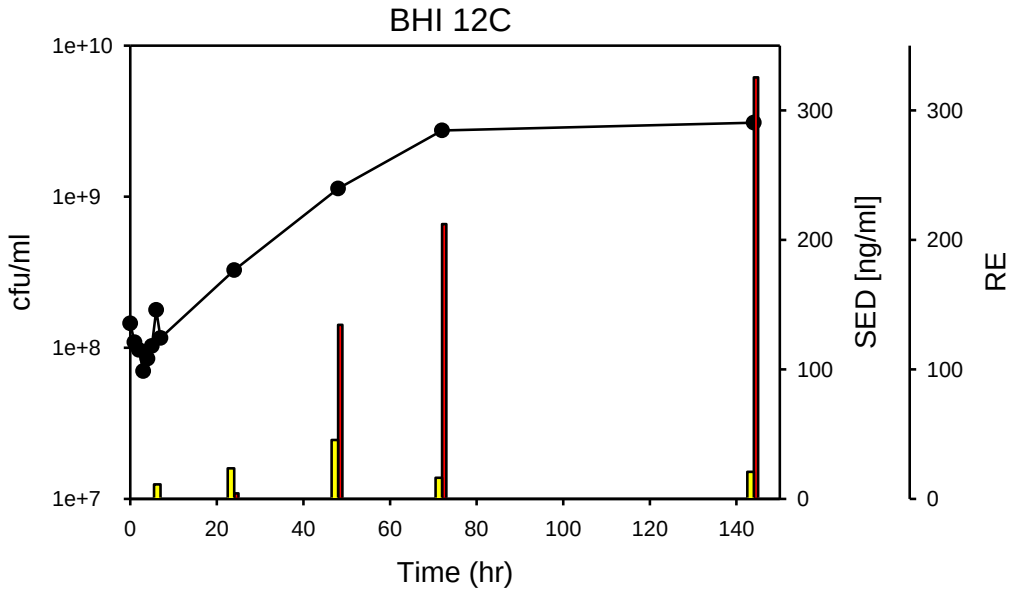
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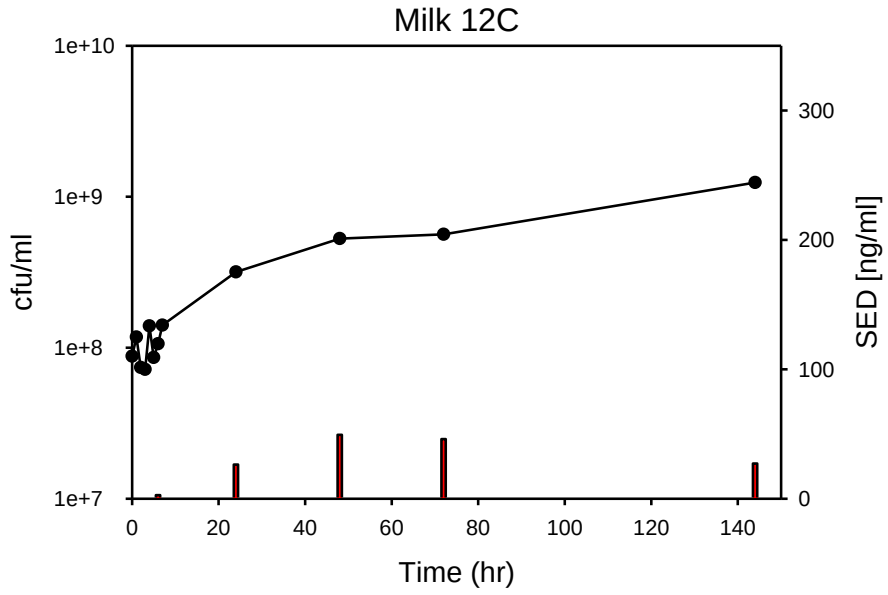
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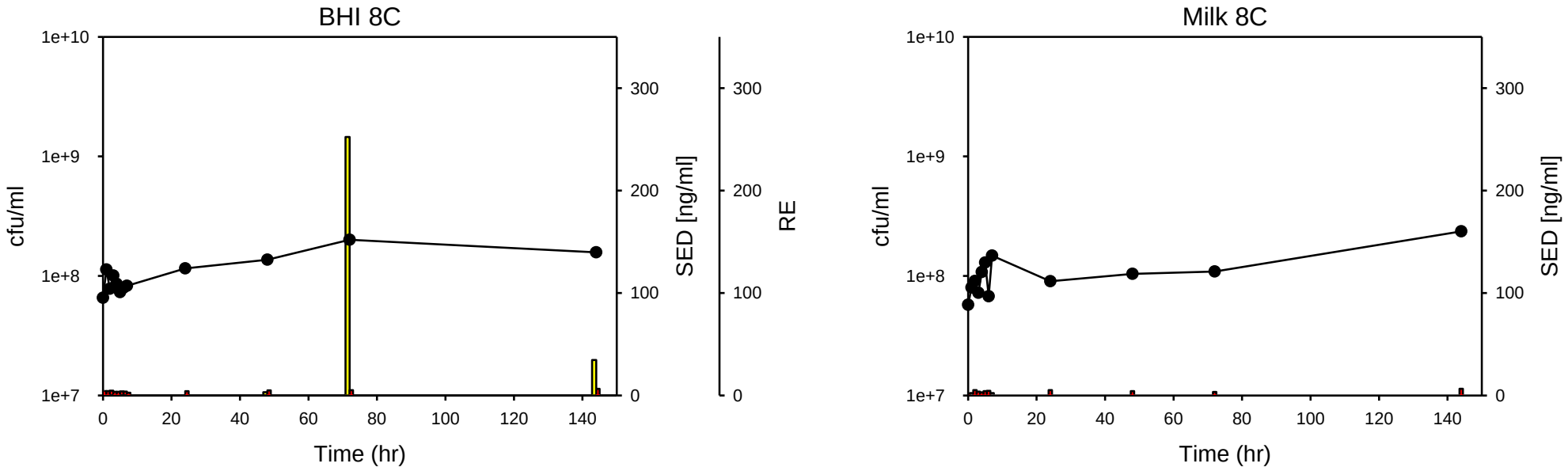


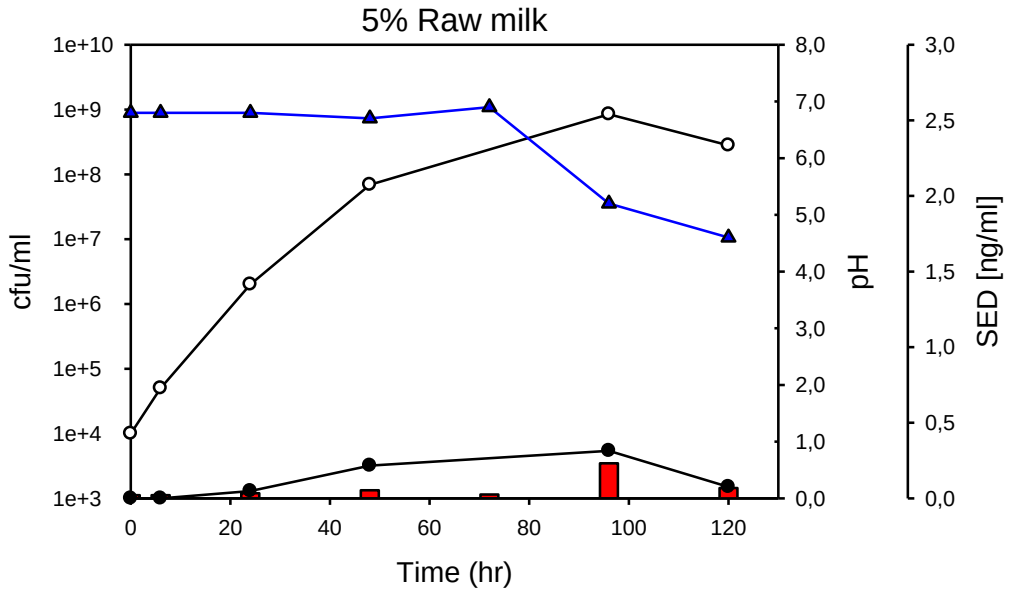
Figure 4.1 *Growth of S. aureus, relative sed levels in BHI and SED concentration when grown at 20, 12 and 8°C in BHI and milk. (•) Growth curve; (yellow bars) relative sed mRNA levels in BHI; (red bars) absolute SED concentration. The average of two independent growth experiments for each temperature and growth media are presented.*

4.2.2 The combined effect of 12°C and background flora

In these experiments, the combined influence of low temperature (12°C) and the presence of a competitor (BGF originating from raw milk provided by ARLA Foods) on *S. aureus* growth and enterotoxin A and D production in pasteurized milk with 3% fat content was investigated. The competitor (BGF) was introduced by the addition of defined volumes (5, 1, 0.5 and 0.1 % of the total volume; 600 ml) of raw milk to the pasteurized milk. In total, 5 runs of experiments were carried out with two fermentors in parallel in each run, in length of 5 days for each experiment.

In all experiments with the strain SA 45, very mild growth of *S. aureus* was observed regardless the lower amount of BGF or higher starting concentration. If the starting concentration of *S. aureus* was between 10^3 and 10^4 cfu/ml, the population never reached the cell density (10^5 – 10^6 cfu/ml) to be considered hazardous for enterotoxin production. If the starting concentration was higher, ~ 10^6 cfu/ml, the growth was slight and within the same order of magnitude. *S. aureus* is a well-known poor competitor. An inhibitory effect of BGF on *S. aureus* growth and enterotoxin production was observed in all experiments. Despite the low temperature as a stressful factor, the control experiments without BGF demonstrated that the impact of a competitor is higher. The production of enterotoxin D was low and correlated with the growth. By lowering the amount of competing microflora and increasing inoculation level of *S. aureus*, only a slight increase in enterotoxin production occurred. No major strain variation was observed when testing three different strains with different origin. pH was measured in these experiments. The bigger the amount of competing microflora the bigger and earlier in time the drop in pH due to accumulation of acidic products.

S. aureus and/or enterotoxins can enter the pasteurized milk chain on the farm and can continue to be introduced or exacerbated at any point in the dairy chain until the milk reaches the consumer. A probabilistic model for the representation of the risks that arise from the presence of *S. aureus*, and particularly staphylococcal enterotoxins, in the pasteurized milk chain, has been developed by the Institute of Food Research, Norwich, United Kingdom within the EU-funded IP project BIOTRACER. The probabilistic analysis has been implemented using a Bayesian belief network (BBN) technique. BBNs are a type of expert system that integrates a graphical representation of a hazard domain with a probabilistic model of the events. The obtained experimental data set described above, especially the correlation between *S. aureus* growth and enterotoxin production kinetics, was incorporated in the Bayesian belief network in order to update the model.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SED [ng/ml]
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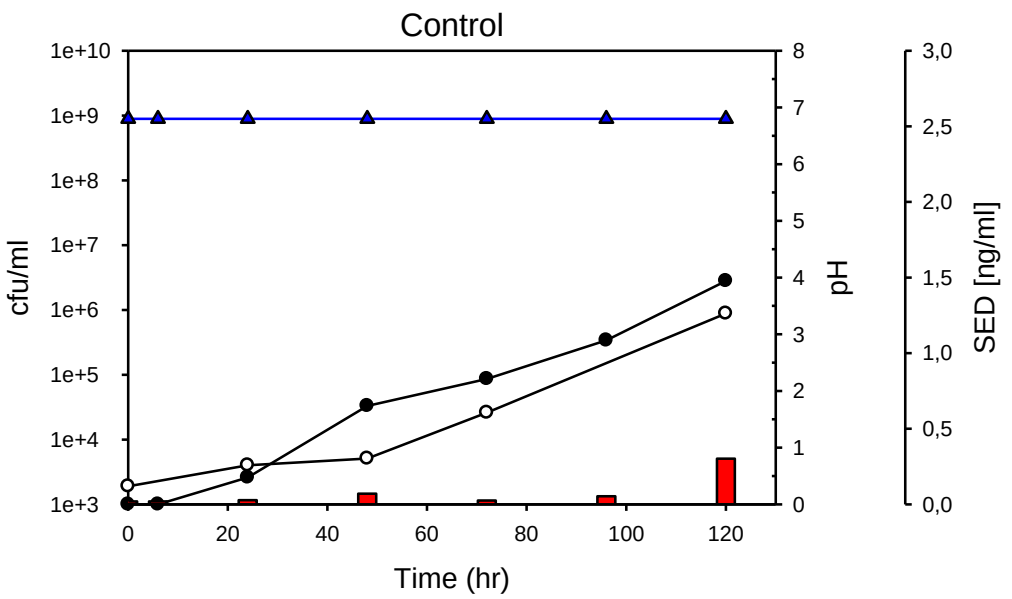
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SED [ng/ml]
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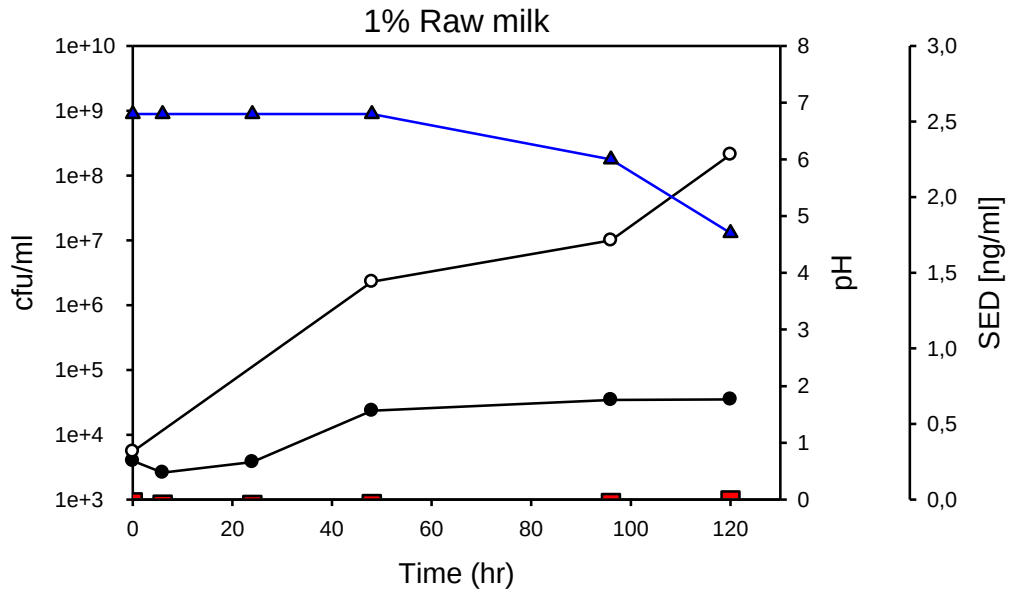
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pH
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Time (hr)
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7
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4
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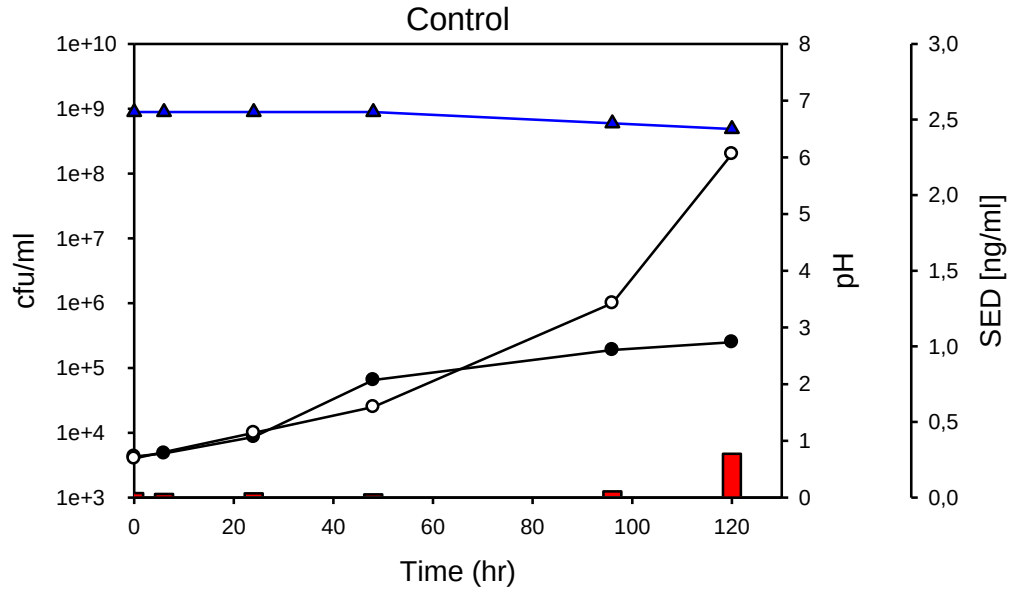
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pH
SED [ng/ml]
Time (hr)
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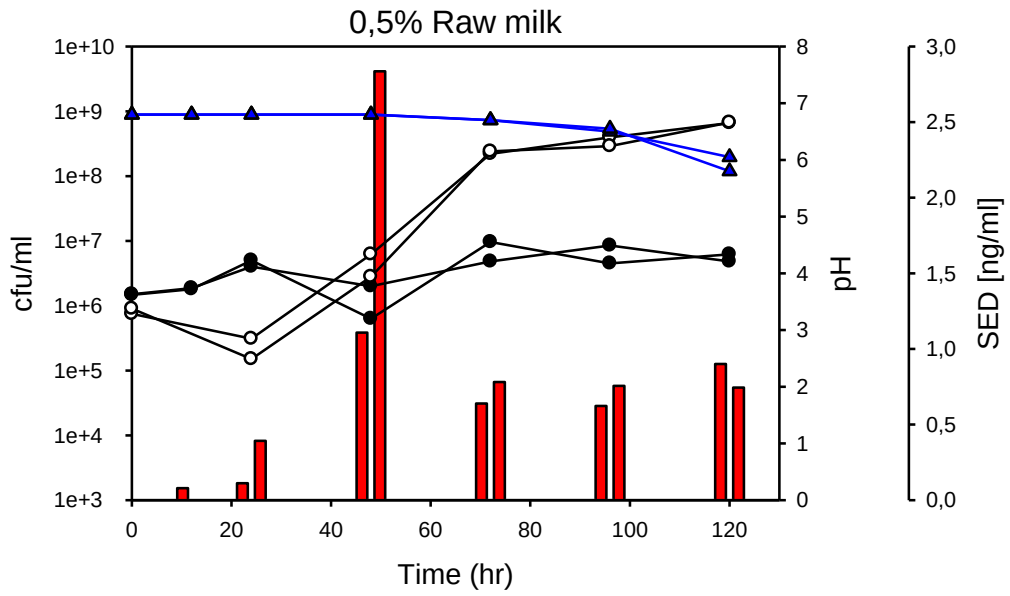
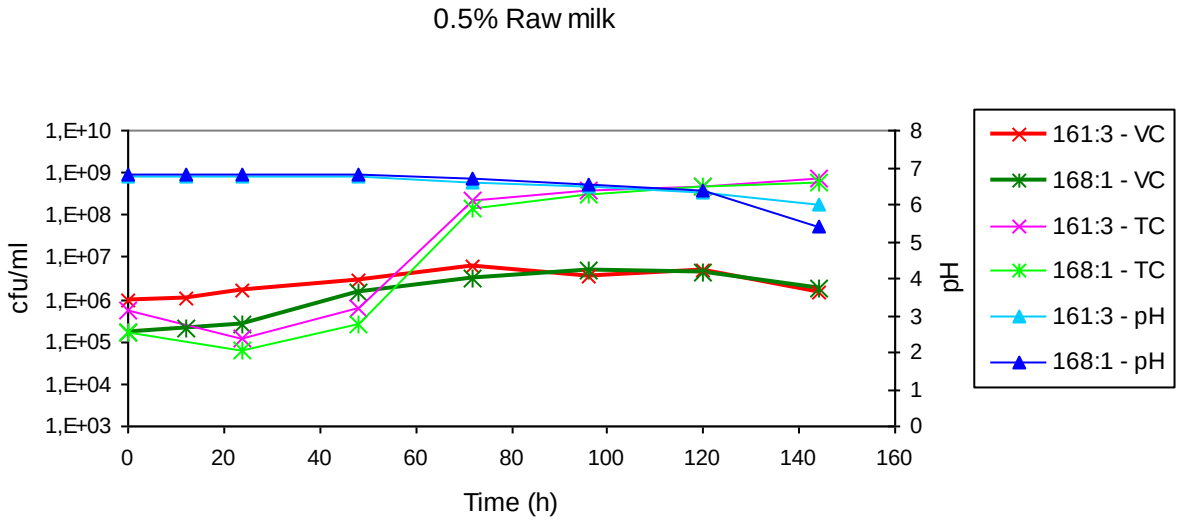


Figure 4.2 *(for all graphs) Growth of S. aureus (SA 45) and background flora coming from raw milk, pH and SED production in milk at 12°C. (•) Growth curve for S. aureus; (○) Growth curve for BGF; (▲) pH; (red bars) absolute SED concentration. No raw milk was added to milk in the control experiments. One growth experiment was performed for 5% and 1% raw milk and corresponding control experiments. Two independent growth experiments were performed for 0.5% raw milk experiment.*

a)



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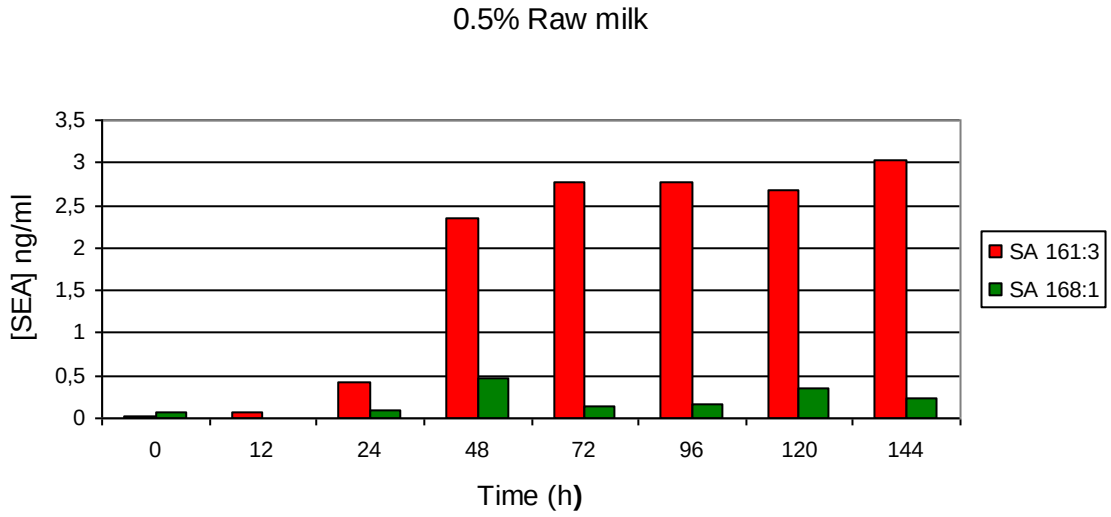


c)

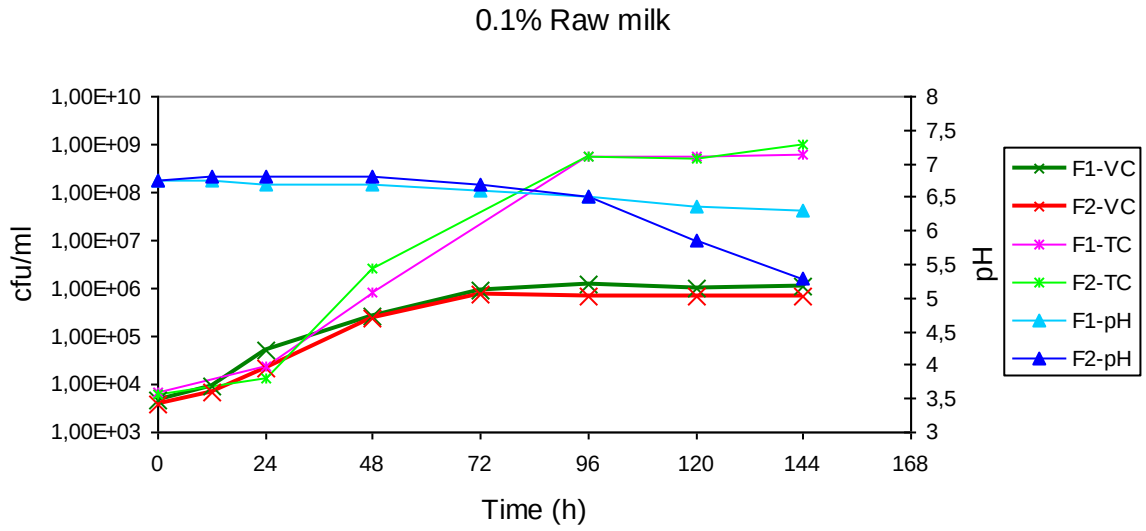


Figure 4.3 *Growth of S. aureus (SA 161:3 and SA 168:1) and background flora coming from raw milk, pH and SEA production in milk at 12°C. a) Growth of S. aureus described as viable count (VC), growth of BGF described as total count (TC), pH; b) absolute SEA concentration; c) Growth of S. aureus described as viable count (VC), growth of BGF described as total count (TC), pH. Two independent experiments with the strain 161:3.*

4.3 Cheese experiments

These experiments were set up to simulate a post-contamination scenario during cheese production. The growth and enterotoxin A and D production of four *S. aureus* strains with different origins were investigated in two types of cheese at two different temperatures.

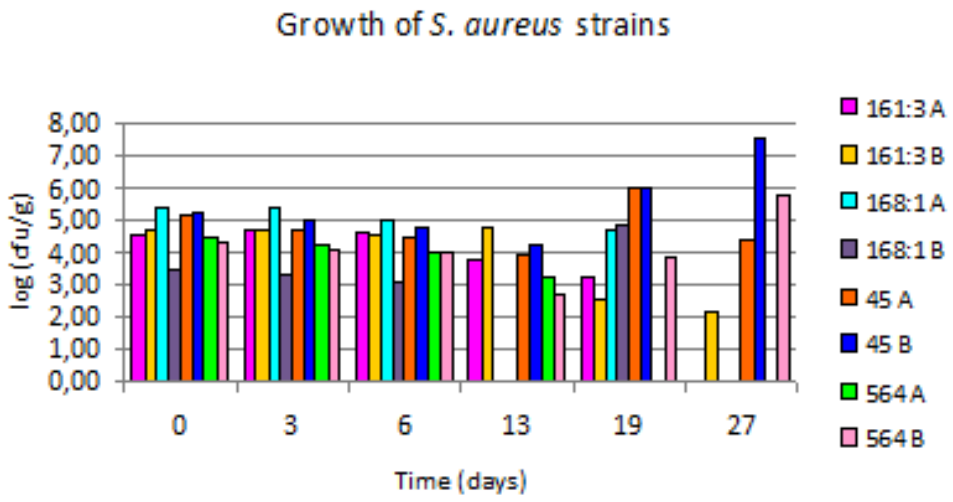
Four different experiments were performed. 25 g of semi soft cheese or cream cheese were inoculated with one of four stains of *S. aureus* and incubated at 13°C or 20°C. Two biological replicas were made (A+B) for each of the four *S. aureus* strains tested. In total, 54 cheese samples were used in each experiment. Samples were collected after 0 hour, 3 days, 6 days, 13 days, 20 days (or 19 days) and 27 days. Growth of *S. aureus* was followed by viable count measurements on Baird-Parker agar plates, BHI or TSA plates were used for total cell count and MRS plates for cell count of lactic acid bacteria. The enterotoxins were extracted and concentrated using dialysis, and finally the enterotoxin levels were measured with ELISA.

a) *b)*

Figure 4.4 *a) Cream cheese samples inoculated with different strains. b) Example of large unidentified colonies on BHI.*

4.3.1 Experiment 1: Semi-soft cheese, 13°C

a)



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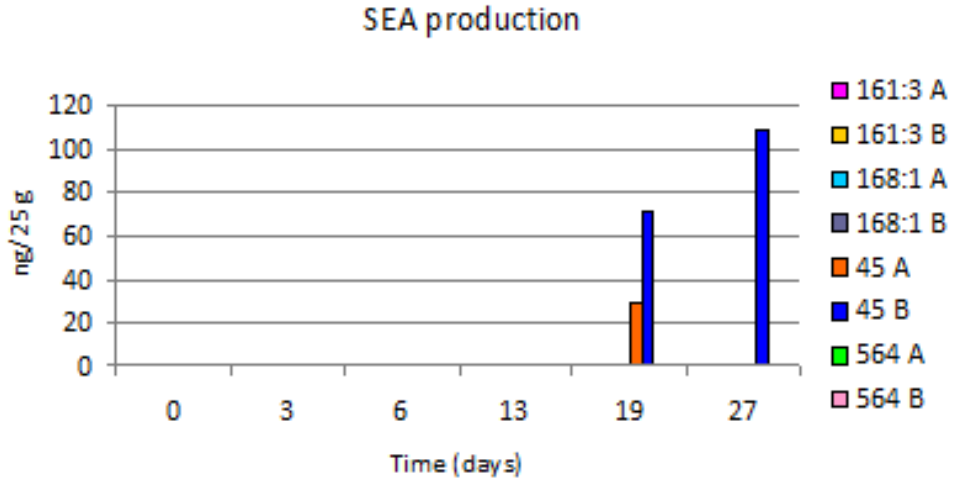

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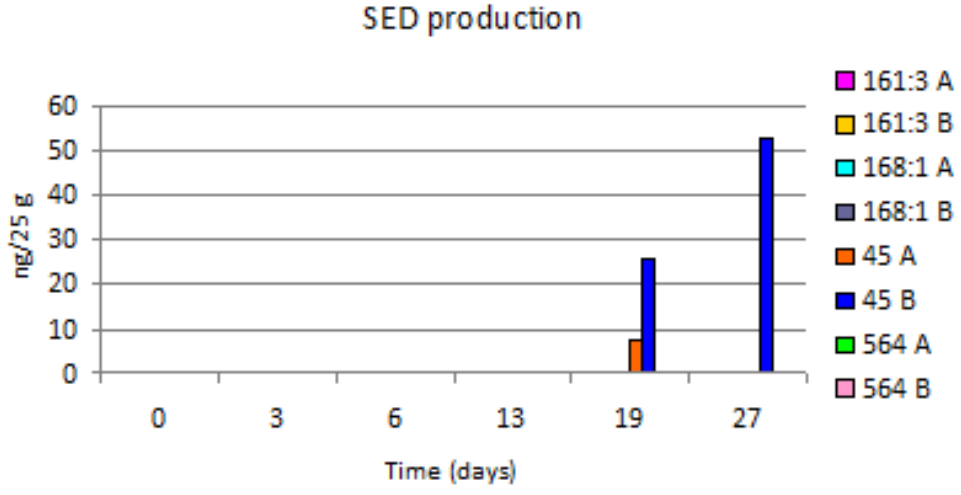
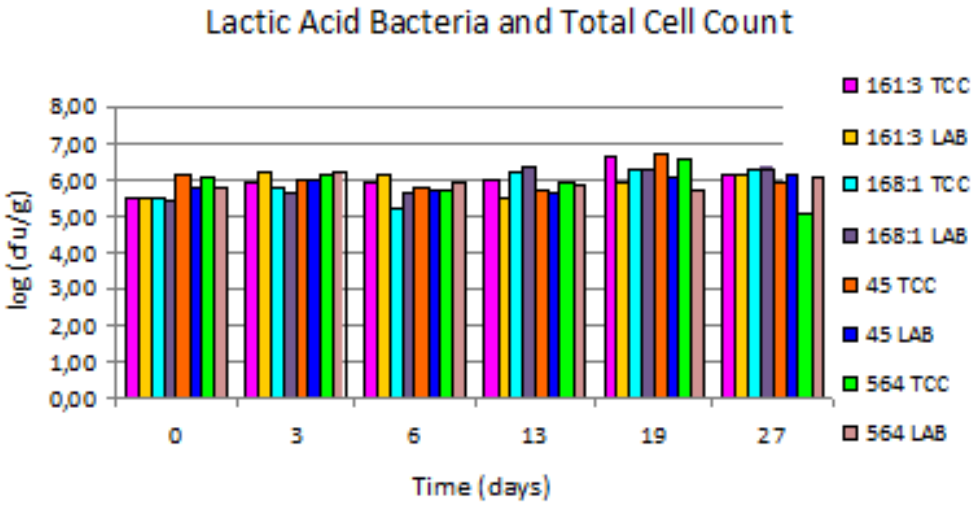


d)



e)

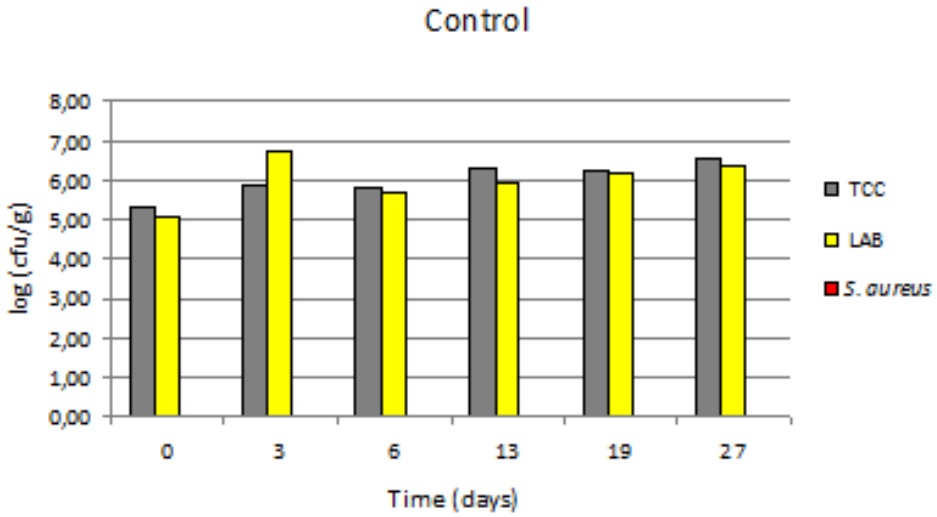
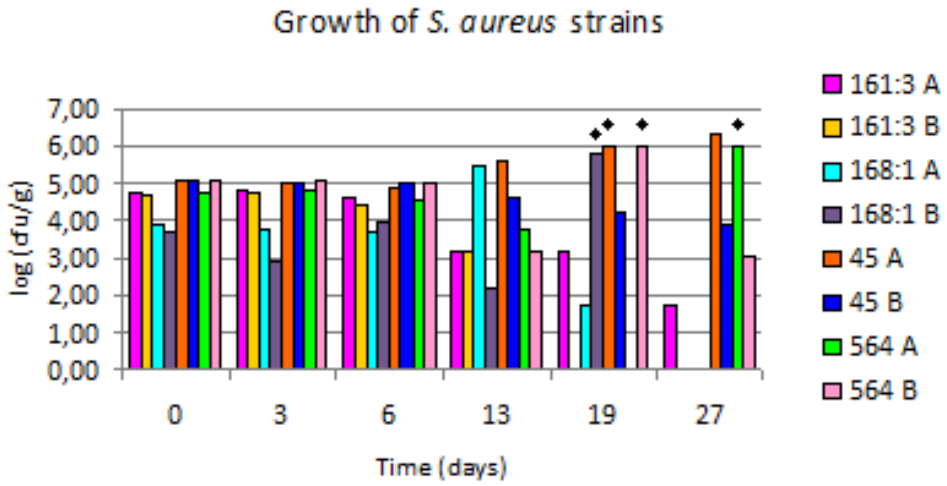


Figure 4.5 *a) Growth of S. aureus strains 161:3, 168:1, 45 and 564 in semi-soft cheese at 13°C. Two independent replicas, A and B. b) Total amount of enterotoxin A found in the 25 g of cheese samples, measured with ELISA. c) Total amount of enterotoxin D found in the 25 g of cheese samples, measured with ELISA. d) Total cell count (TCC) and the cell count of lactic acid bacteria (LAB) in the cheese samples. Average of two independent replicas, A and B, for each sample. e) Total cell count (TCC), lactic acid bacteria (LAB) cell count and S. aureus cell count in the control samples.*

4.3.2 Experiment 2: Semi-soft cheese, 20°C

a)



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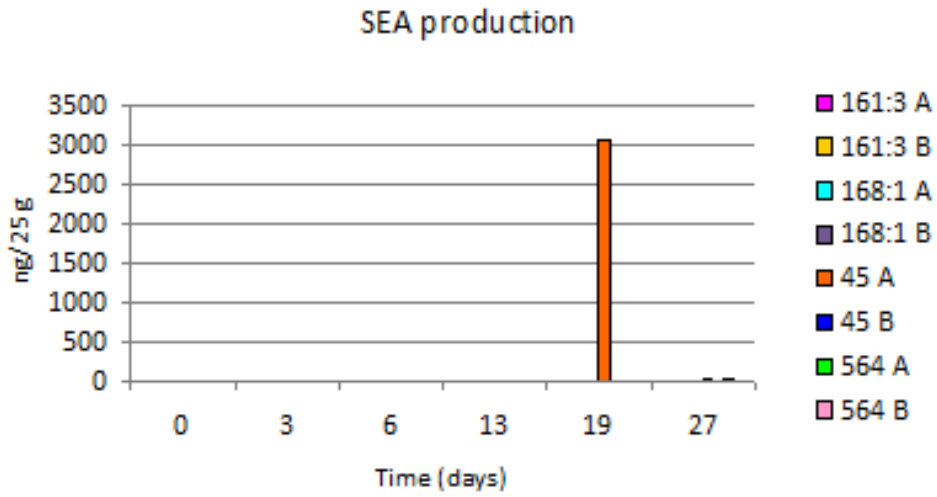

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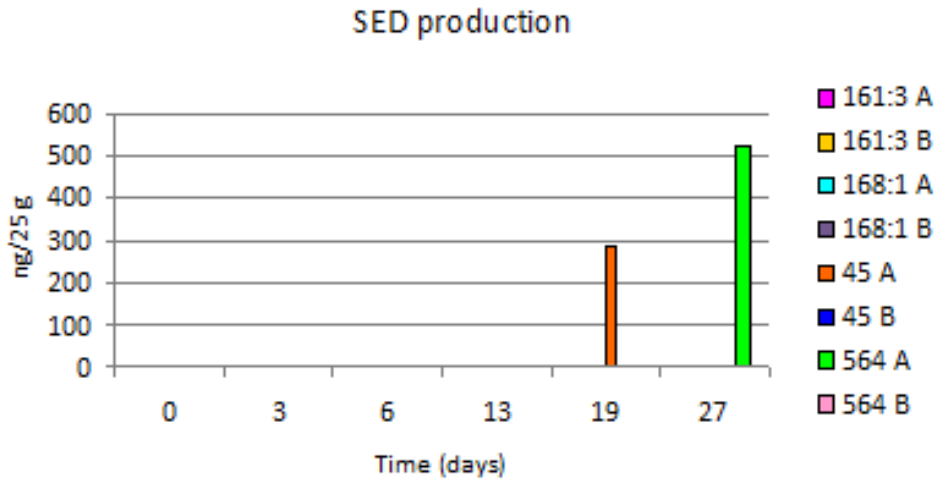

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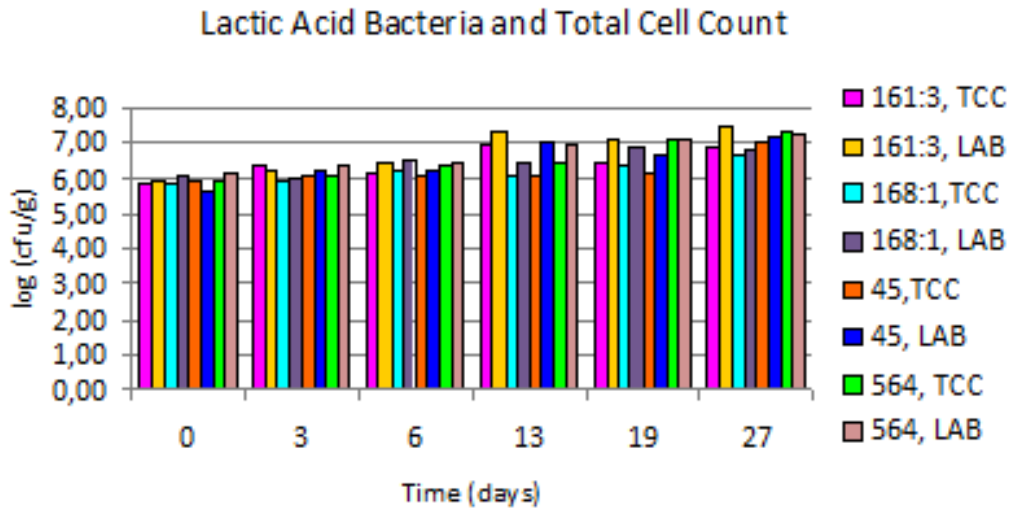

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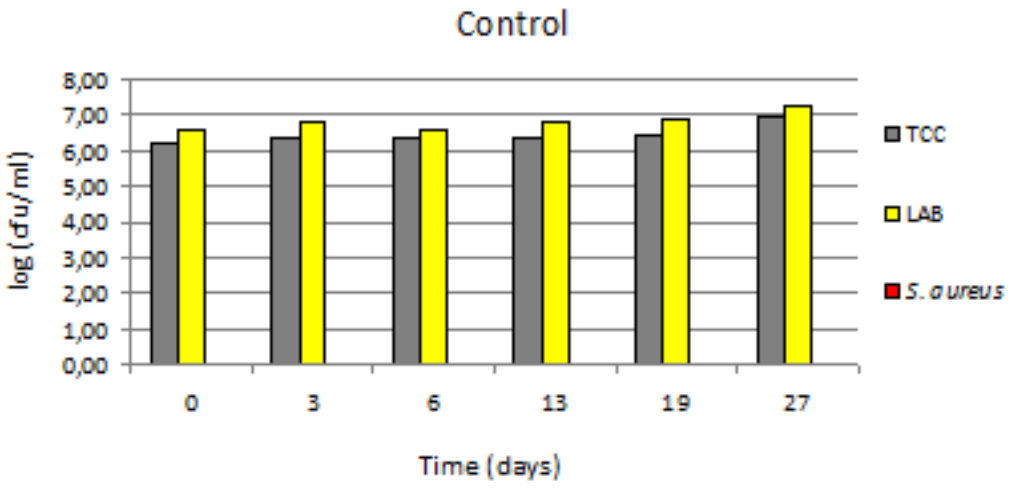
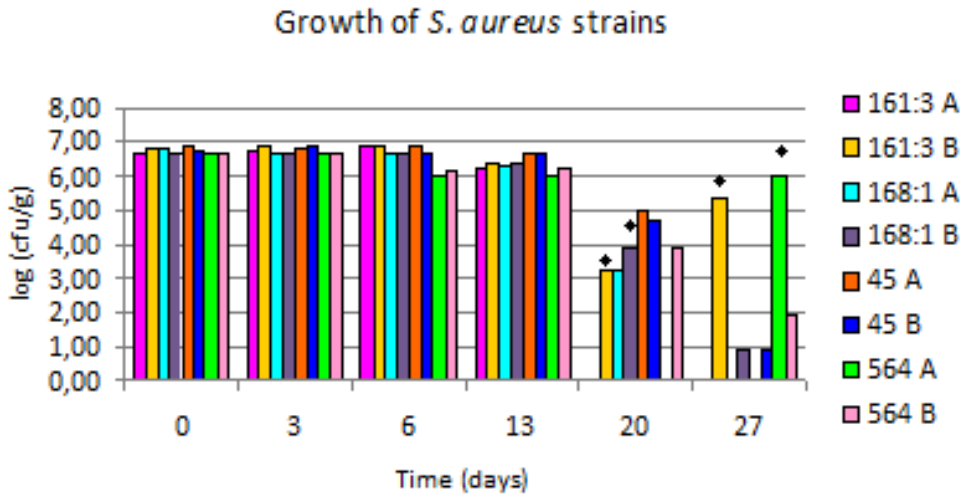


Figure 4.6 *a) Growth of S. aureus strains 161:3, 168:1, 45 and 564 in semi-soft cheese at 20°C. Two independent replicas, A and B. ♦ Samples contaminated with mould. b) Total amount of enterotoxin A found in the 25 g of cheese samples, measured with ELISA. c) Total amount of enterotoxin D found in the 25 g of cheese samples, measured with ELISA. d) Total cell count (TCC) and the cell count of lactic acid bacteria (LAB) in the cheese samples. Average of two independent replicas, A and B, for each sample. e) Total cell count (TCC), lactic acid bacteria (LAB) cell count and S. aureus cell count in the control samples.*

4.3.3 Experiment 3: Cream cheese, 13°C

a)



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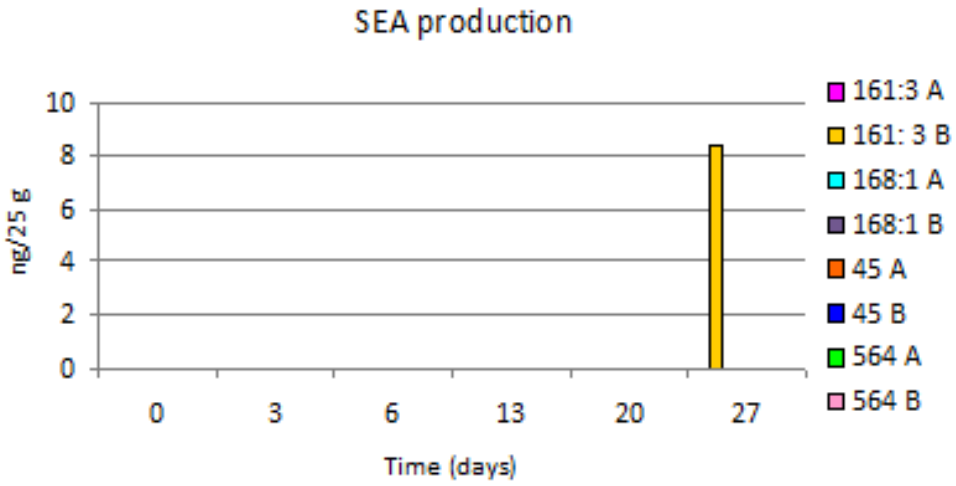

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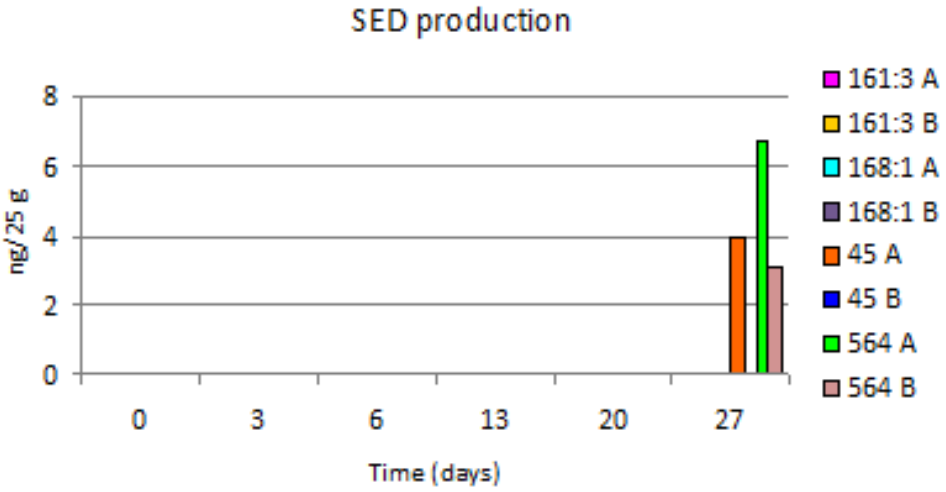


d)

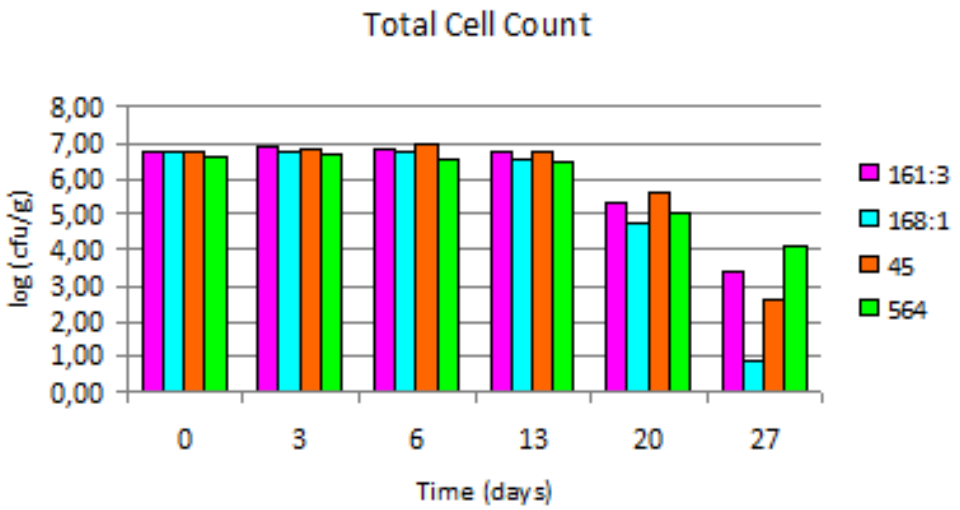
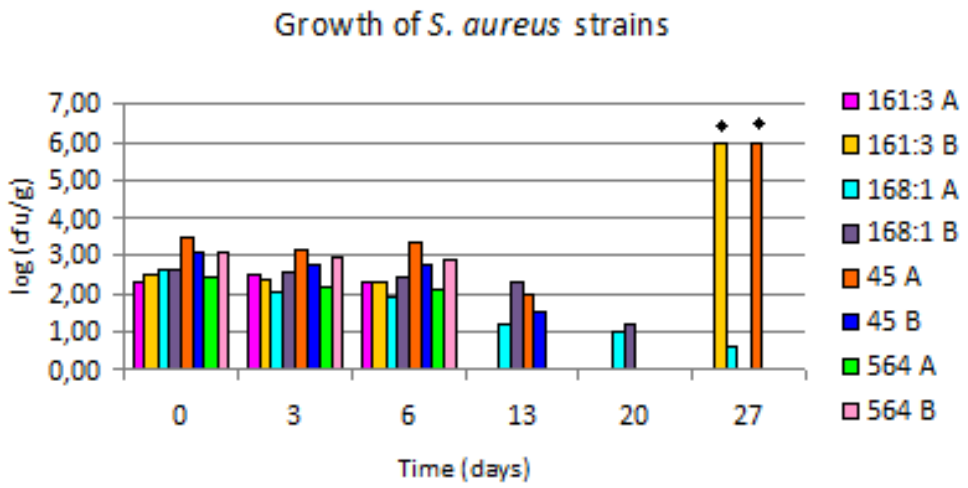


Figure 4.7 *a) Growth of S. aureus strains 161:3, 168:1, 45 and 564 in cream cheese at 13°C. Two independent replicas, A and B. ♦ Samples contaminated with mould. b) Total amount of enterotoxin A found in the 25 g cheese samples, measured with ELISA. c) Total amount of enterotoxin D found in the 25 g cheese samples, measured with ELISA. d) Total cell count in the cheese samples. Average of two independent replicas, A and B, for each sample.*

4.3.4 Experiment 4: Cream cheese, 20°C

a)



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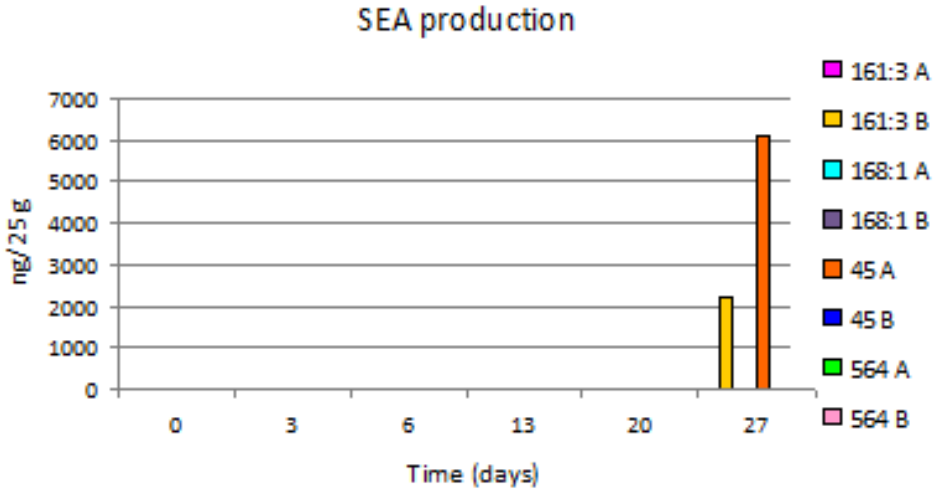

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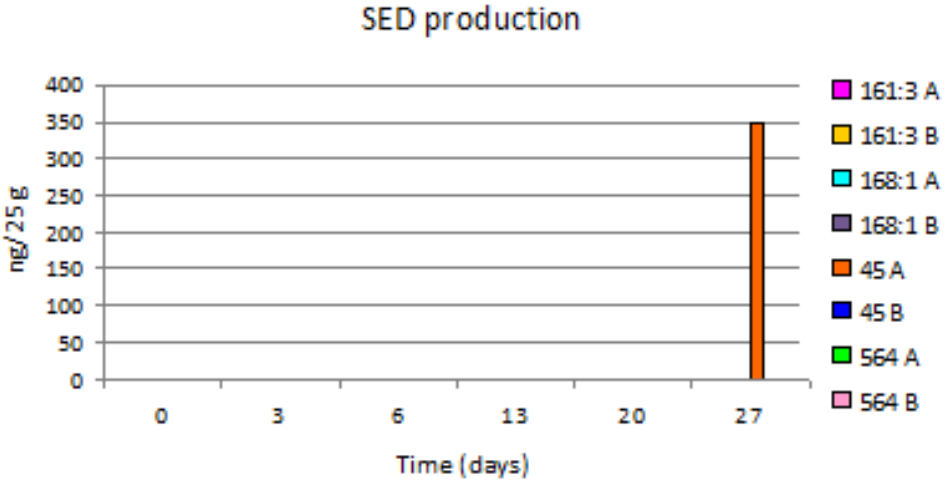

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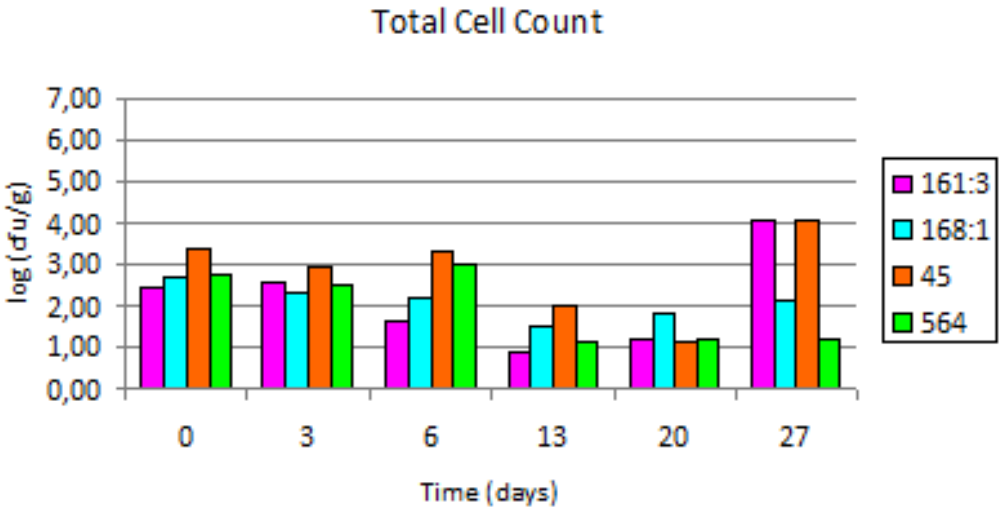

Figure 4.8 *a) Growth of S. aureus strains 161:3, 168:1, 45 and 564 in cream cheese at 20°C. Two independent replicas, A and B. ♦ Samples contaminated with mould. b) Total amount of enterotoxin A found in 25 g cheese samples, measured with ELISA. c) Total amount of enterotoxin D found in 25 g cheese samples, measured with ELISA. d) Total cell count in the cheese samples. Average of two independent replicas, A and B, for each sample.*

The semi-soft cheese used in the experiments contained a high level of background flora (LAB). Several studies have demonstrated that the ratio between the inoculation level of *S. aureus* and the number of LAB determines the efficiency of the inhibition. For example, with ratios of 1:1 or 1:10 (*S. aureus* : *L. lactis*) which approximately corresponds to ratios (*S. aureus* : LAB) in the experiment 1 and 2, respectively, the maximal level which *S. aureus* can reach is 10^5 – 10^6 cfu/ml, compared to 10^{10} cfu/ml in a control culture [24]. This finding can be supported by our results with one exception, when population of *S. aureus* 45 replica B crossed 10^7 cfu/g of cheese in the experiment 1. Even in the samples contaminated with mold, which frequently boosted *S. aureus* growth, the population of *S. aureus* did not grow higher than 10^6 cfu/g. LAB, similarly to *S. aureus*, can grow in a wide range of temperature but prefer higher temperature, above 30°C. The growth of LAB was better at 20°C (exp. 2). The number of LAB also tended to be higher than the total count at 20°C. The reason could be that LAB due to limited biosynthetic ability and thus requirements for special nutrients like amino acids or vitamins do not grow on BHI agar as well as on MRS which was designed to recover LAB from various food products. In general, no overall increase in growth of any strain during 27 days in semi-soft cheese was observed. *S. aureus* 45 despite being isolated from ham was the best-growing strain, always present over time in higher or lower counts compared to inoculum in both replicas. Out of strains isolated from dairy products, the strain

161:3 had rather the tendency to die off, the strain 168:1 seemed to have a greater ability to compete. The behaviour of the strain 564 varied, with at least one replica always survived till 27 days.

The cream cheese seemed to lack background flora. No colonies were observed on MRS agar plates when analyzing control samples showing that the cream cheese is void of LAB (or at least those species of LAB that can be recovered on MRS agar). In both experiments 3 and 4, the total cell count more or less corresponded to the cell count of *S. aureus*. The growth of large unidentifed colonies of irregular shape and size on BHI or TSA agar plates used for total cell count made the counting difficult and less accurate. In the experiment 3, the population of all *S. aureus* strains started with high numbers and kept surviving for 13 days and then declined except in the contaminated samples. In the experiment 4, starting with lower inoculum of *S. aureus* strains, despite the higher temperature the situation was similar, although some strains were not detected even at 13 days. The technology applied to make the cream cheese seemed to create an unfavourable environment for *S. aureus* since no growth was observed despite the absence of competing microflora.

According to commission regulation (EC) No 1441/2007 on microbiological criteria for foodstuffs, 25 g of cheese must be void of staphylococcal enterotoxins and there is an obligation to screen for enterotoxins if *S. aureus* concentration exceeds 10^5 cfu/g in any food.
In all experiments, SEs could not be detected before 19 days. In the experiment 1 (semi-soft cheese, 13°C), *S. aureus* 45 produced quite a high amount of enterotoxins, more SEA than SED, in response to high cell counts (10^6 cfu/g in one sample and above 10^7 cfu/g in the other). The counts of other *S. aureus* strains were not high enough (or due to other conditions) to trigger SE production. In all the other experiments, we encountered a contamination by mold which created an interesting phenomenon and which is discussed below.
In the experiment 2 (semi-soft cheese, 20°C), two contaminated samples with 10^6 cell counts of strains 45 and 564 contained a huge amount of enterotoxins. On the contrary, 2 contaminated samples with the same cell counts (of the strain 564 in one sample) showed no enterotoxins. In addition, enterotoxin A and D could be detected in two replica samples with no contamination and with counts of ~10^6 and ~10^4 cfu/g of *S. aureus* strain 45. The possible explanation of the last could be a mistake during the analysis. The extraction of enterotoxin consists of many steps performed manually, such as acidification followed by neutralization and finally dialysis where especially the recovering of toxins from the dialysis membrane can result in inaccuracy. The explanation of the detection of enterotoxin in the sample with lower counts (10^4 cfu/g) of *S. aureus* could be that some toxin was formed and stayed but the number of cells declined over time. In the experiments with cream cheese, enterotoxin could only be detected after 27 days. In the experiment 3 (cream cheese, 13°C) with high inoculum of all *S. aureus* strains, enterotoxin was detected in two contaminated samples, in the sample with low cell counts (~10^2 cfu/g) and in the sample with no viable cells. Detection of enterotoxin D in the sample with no viable cells of *S. aureus* 45 could be a mistake since the

strain 45 produces both enterotoxins, A and D and we could detect only D. In the experiment 4 (cream cheese, 20°C) a massive enterotoxin production occurred in two contaminated samples with high cell counts (10^6 cfu/g) increased from low initial inoculum level.
TSST was not detected in any of the samples with TSST producing strains, 168:1 and 564. This might be due to unfavorable experimental conditions for TSST production.

Cheese represents one of the more challenging matrices to work with. In the experiments with semi-soft cheese, the independent replicas A and B seem to differ slightly more in the number of *S. aureus* cells than replicas in the cream cheese experiments. This could be due to semi-soft cheese being a less homogenous food matrix (with holes, different composition in different parts of the cheese, etc.) and/or due to the fact that the cheese samples did not obtain the same amount of inoculum. Despite great effort to reach the same initial inoculum, some drops might have ended up on the bottom of the plates containing the cheese samples (holes in cheese). Furthermore, there are several steps in the analysis that might result in loss of cells. For instance, when transferring the diluted sample from the stomacher bag into new tubes, some cells might still remain inside the bag.

Many cheese samples got contaminated by mould. The contamination was probably caused by spores from the environment since moulds are ubiquitous, possibly during preparation and inoculation of samples. Visible mould was never detected before 19 days. The phenomenon observed in most contaminated samples was that mould stimulated the growth of *S. aureus* and triggered a significant enterotoxin production. A huge enterotoxin production was accompanied by an increase of the pH to 7.3 in one semi-soft cheese sample and to 5.9 and 6.4 in two cream cheese samples. Moulds have a complex enzyme system and as they proliferated on the cheese samples they might have consumed lactic acid and have formed alkaline metabolites due to proteolysis which led to an increase in pH [18, 23]. The other factors coming into play are what type and when mould appeared (was visible) and how it interacted with other microorganisms present in the cheese including different strains of *S. aureus*. More research would be needed to elucidate the encountered phenomenon but it would have little impact on food safety since a consumer would not eat mouldy cheese. However in some circumstances, very rare probably, a block of cheese gets contaminated with *S. aureus*, is kept under inadequate temperature and mold develops on the surface. The moldy area is cut away, cheese sold and possible outbreak born. The mould with its high metabolism was not the only reason contributing to the increase of pH. The overall change in pH was bigger in semi-soft cheese as a consequence of the biochemical changes occurring during ripening and continuing over time in the experiments.

5. CONCLUSIONS

We are confronted with the ongoing challenge to control foodborne diseases caused by bacteria. Although we know a great deal about these bacteria, they are still causing significant problems in the food industry. The development of rapid methods for detecting foodborne pathogens and the improvement of our understanding of pathogenic virulence is critical to ensure the food safety.

The work described in this dissertation has provided a new method for rapid and sensitive detection of *S. aureus* in food and new information about *S. aureus* and its enterotoxin formation in the dairy chain. Here is a brief summary of the conclusions from:

Food pathogen detection

- The developed real-time PCR based method involving overnight selective enrichment under aerobic conditions facilitated sensitive next-day *S. aureus* detection. The method was able to overcome the problematic colony identification on Baird-Parker agar, particularly in the case of atypical *S. aureus* colonies.

Milk experiments

- The effect of temperatures 20, 12 and 8°C on *S. aureus* growth and enterotoxin D production in milk and BHI showed that growth was lower in milk compared to BHI. SED production in milk at 20°C and 12°C occurred earlier in growth but a lower total amount was produced compared to BHI. At 8°C, there was no growth and SED production.
- The presence of competing microflora frow raw milk created an inhibitory effect on *S. aureus* growth and enterotoxin production. The impact of competition was higher than low temperature (12°C). Low SED production at *S. aureus* inoculation levels from 10^3 to 10^6 cfu/ml was observed. By lowering the amount of competing microflora and increasing inoculation level of *S. aureus*, only a slight increase in enterotoxin production occurred. No major strain variation was observed when testing three different strains with different origin.
- Studies performed in laboratory media do not necessarily reflect the situation in a real food product. Food represent a more complex matrix with different types of microorganisms often present, which interact with each other and the matrix.

Cheese experiments

- The different origin of the strains did not influence the ability to survive, grow or produce enterotoxin in semi-soft or cream cheese.
- No overall increase in growth of any strain during 27 days was observed.

- Enterotoxin production was never detected before 19 and 27 days in semi-soft cheese and cream cheese, respectively. The amount of enterotoxin produced was sufficient to cause food poisoning.
- Mould contamination often caused increase in pH and promoted growth of *S. aureus* with concomitant enterotoxin production.
- No generalizations should be made about the behaviour of *S. aureus* in the types of cheese other than those studied.

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7. LIST OF ABBREVIATIONS

AcrB	acriflavin-resistant protein
Agr	accessory gene regulator
BGF	background flora
BHI	brain heart infusion
BPA	Baird-Parker agar
cfu	colony forming unit
egc	enterotoxin gene cluster
ELISA	enzyme-linked immunosorbent assay
EIA	enzyme immunoassay
FBD	foodborne disease
LAB	lactic acid bacteria
MPN	most probable number
PCR	polymerase chain reaction
RT-PCR	reverse-transcription polymerase chain reaction
SAPI	*Staphylococcus aureus* pathogenicity island
scc	staphylococcal cassette chromosome
SE	staphylococcal enterotoxin
SEl	staphylococcal enterotoxin-like proteins
SEA	staphylococcal enterotoxin A
SED	staphylococcal enterotoxin D
SFP	staphylococcal food poisoning
TCC	total cell count
TSA	tryptic soy agar
TSST	toxic shock syndrome toxin
VC	viable count

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9. ABSTRACT

Foodborne diseases caused by bacteria are an actual issue worldwide. To produce food, which is safe for human consumption, data about food-borne pathogen virulence is required to complement the already existing knowledge about the bacterial growth and survival in food. There is also a growing need for rapid and sensitive methods to detect these pathogens.

In this dissertation, the real-time PCR-based method for the detection of *S. aureus* in food using selective enrichment and the impact of environmental factors on *S. aureus* growth and enterotoxin production in milk and cheese are described.

We developed a rapid and sensitive method for the detection of *S. aureus* in food using selective enrichment and a new species-specific real-time PCR. The method facilitated the detection of *S. aureus* on the next day after the sample reception. This method can be used for *S. aureus* detection as a faster, highly specific, and more sensitive alternative to the microbiological method.

We investigated the effect of three different temperatures, 8°C, 12°C and 20°C on *S. aureus* growth and SED production in pasteurized milk and on growth, *sed* gene expression and SED production in Brain heart infusion. The experiments were performed in small-scale fermentors for six days and gene expression was followed by qRT-PCR. SED production was measured using Enzyme-Linked ImmunoSorbent Assay (ELISA). In BHI the growth pattern was the same at 20°C and 12°C but delayed in the latter. At 8°C there was no growth. In milk, growth was lower compared to BHI. *sed* mRNA was detected at 20°C and 12°C after 4 and 7 hours respectively in BHI and the production occurred during the exponential phase of growth. In milk the SED production at 20°C and 12°C occurred earlier in growth but a lower total amount was produced compared to BHI. At 8°C, there was no SED production like in BHI. The combined effect of low temperature, 12°C, and the presence of competing background microflora derived from raw milk on the growth of *S. aureus* and SED production in pasteurized milk was further investigated. An inhibitory effect on *S. aureus* growth and enterotoxin production was observed and the impact of competition was greater than the impact of low temperature. The enterotoxin production was low and correlated with the growth. By lowering the amount of competing microflora and increasing the inoculation level of *S. aureus*, only a slight increase in enterotoxin production occurred. In the next stage, two different cheese matrices were inoculated with *S. aureus* to simulate a post-contamination scenario in cheese manufacture. Samples were collected over period of 4 weeks. Critical food factors, like competing microflora and pH, which are responsible for down- and up-regulation of the virulence of *S. aureus*, were monitored. We tried to indentify if there are situations in which: (i) no growth but enterotoxin formation is observed, and (ii) growth and no enterotoxin formation occurs.