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FOODBORNE S*TAPHYLOCOCCUS AUREUS*: IDENTIFICATION AND ENTEROTOXIN PRODUCTION IN MILK AND CHEESE

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

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SOUHRN

Onemocnění z potravin (alimentární onemocnění) vyvolaná bakteriemi jsou stále aktuálním tématem v celosvětovém měřítku. Abychom zajistili výrobu zdravotně nezávadných potravin, je potřeba nových poznatků o virulenci patogenů, které by doplnily již známé skutečnosti o jejich růstu a přežívání v potravinách. Také potřebujeme vyvíjet rychlé a citlivé metody na detekci těchto patogenů.

Dizertační práce popisuje metodu na detekci *S. aureus* v potravinách, která je založená na PCR v reálném čase ve spojení s namnožením v selektivním médium. Dále pojednává o vlivu environmentálních faktorů na růst *S. aureus* a tvorbu enterotoxinů v mléce a sýrech.

Vyvinuli jsme rychlou a citlivou metodu na detekci *S. aureus* v potravinách s použitím selektivního namnožení a PCR v reálném čase. Nově vyvinutá metoda umožnila detekci *S. aureus* na druhý den od přijetí vzorku. Tato metoda může být použita jako rychlejší, citlivější a vysoce specifická alternativní metoda ke konvenční mikrobiologické metodě.

Zkoumali jsme vliv tří různých teplot, 8°C, 12°C a 20°C na růst *S. aureus* a tvorbu enterotoxinu D v pasterizovaném mléce a na růst, expresi genu *sed* a tvorbu enterotoxinu D v tekutém médiu s extraktem z mozku a srdce (BHI). Experimenty byly prováděny v malých skleněných fermentorech po 6 dní. Genová exprese byla sledována pomocí qRT-PCR a tvorba enterotoxinu D byla měřena pomocí imunologické metody ELISA. Růstová křivka v BHI měla stejný průběh při 20°C a 12°C, ale v při 12°C začal růst se spožděním. Při 8°C nebyl pozorován žádný růst. Růst *S. aureus* v mléce byl ve srovnání s BHI menší. *sed* mRNA byla detekována při 20°C po 4 hodinách a při 12°C po 7 hodinách a produkce enterotoxinu se objevila v exponenciální fázi růstu. V mléce se produkce SED při 20°C a při 12°C objevila dříve, ale celkové množství vyprodukovaného SED bylo nižší než v BHI. Při 8°C nebyla pozorována žádná produkce SED stejně jako v BHI. Dále byl zkoumán společný vliv nízké teploty 12°C a přítomnosti kompetitivní doprovodné mikroflóry pocházející ze surového mléka na růst *S. aureus* a produkci enterotoxinu v pasterizovaném mléce. Byl pozorován inhibiční účinek na růst a produkci enterotoxinů a vliv kompetice byl výraznější než vliv nízké teploty. Produkce enterotoxinu byla nízká a odpovídala růstu. Snížením množství doprovodné mikroflóry a zvýšením inokula došlo pouze k nepatrnému zvýšení produkce enterotoxinu. V další fázi byly dva různé typy sýrů zaočkovány *S. aureus* za účelem simulace sekundární kontaminace při výrobě sýrů. Vzorky byly odebírány v průběhu 4 týdnů. Kritické faktory jako jsou kompetitivní mikrofóra nebo pH, které jsou zodpovědné za regulaci virulence *S. aureus* byly sledovány. Snažili jsem se rozlišit situace při kterých: (i) není pozorován růst, ale objevuje se produkce enterotoxinu a (ii) dochází k růstu ale bez produkce enterotoxinu.

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

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.

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

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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. Coast-to-coast and international distribution by mega-processing plants puts potential outbreaks on a national and international scale [43]. 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. The Czech Republic reported 23 food-borne outbreaks with 730 human cases, 60 hospitalizations and 3 deaths for 2008 [86]. 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%) [86].

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].

While the organism with the largest number of diagnosed cases may fluctuate from year to year and while food safety practices are improving, there remains a growing need for enhanced means of food pathogen detection. 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. Although no simple relationship can be established between *S. aureus* counts and SE production, data on the peak levels of *S. aureus* reached in a dairy product are considered critical in assessing the risk of SE production [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.

The work described in this dissertation covers two fields of interest. The first one concerns pathogen detection and deals with a complete PCR based method for *S. aureus* detection in food. Some drawbacks and assets of so called rapid methods are discussed and compared to ones of conventional methods. The results came out in the paper which is attached as Supplement I. In similar fashion, a method for detection of pathogenic *Yersinia enterocolitica* was developed and the results published in the paper attached as Supplement II. The second paper is not discussed in the dissertation. Both studies were carried out at Food Research Institute in Bratislava. The other field of interest relating to *Staphylococcus aureus* in the dairy chain was studied at Lund University in Sweden within the Erasmus project and represents the major part of the dissertation.

2. THEORETICAL PART

2.1 Staphylococcus aureus

2.1.1 The organism and its characteristics

The name staphylococcus, derived from the Greek nouns *staphyle* meaning a bunch of grapes and *coccus* meaning a grain or berry, was first used in 1882 by Sir Alexander Ogston to describe the cluster-forming cocci he observed in pyogenic abscesses in man [1]. 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]. *S. aureus* strains can be classified into biotypes according to their human or animal origin. Devriese (1984) developed a biotype scheme, including six different biotypes (human, non-β-hemolytic human, avian, bovine, ovine, and nonspecific), based on biochemical characteristics [4].

2.1.2 Characteristics of disease

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. However, once it contaminates a breach in the skin or mucous membranes, it can go on to infect any tissue of the body, causing diseases ranging in severity from minor skin infections to life threatening conditions, such as endocarditis and haemolytic pneumonia. In addition, *S. aureus* can cause toxin-mediated diseases, such as toxic shock syndrome, food poisoning and scalded skin syndrome [5].

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. It may be accompanied by other symptoms (nausea, diarrhea, abdominal pain) [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].

While staphylococcal food poisoning is almost always caused by *S. aureus*, enterotoxins also may be produced by some strains of *S. intermedius*, *S. hyicus* and *S. chromogenes*; these and some other coagulase-negative staphylococci may cause typical staphylococcal food poisoning [1].

2.1.3 Mechanism of pathogenicity

S. aureus produces a wide variety of exoproteins that contribute to its ability to colonize and cause disease in mammalian hosts. Nearly all strains secrete a group of enzymes and cytotoxins which includes four hemolysins (alpha, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase, and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth. Some strains produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins, the exfoliative toxins (ETA and ETB) and leukocidin. Each of these toxins is known to have potent effects on cells of the immune system, but many of them have other biological effects as well [10].

Staphylococcus aureus can produce slime, also called biofilm, which has several advantages: improves the ability to adhere to smooth surfaces, e.g. catheters, protects the bacteria from being discovered and eliminated by neutrophils, protects against antimicrobial agents, e.g. vancomycin [11].

2.1.3.1 The enterotoxins

To date, 21 staphylococcal enterotoxins (SEs) or enterotoxin-like proteins (SEls) have been identified and designated SEA to SElV [7], see **Table 2.1**. SEs are short, extracellular proteins that are soluble in water [1, 4, 14]. They are rich in lysine, aspartic acid, glutamic acid, and tyrosine residues. 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]. The overall shape of SE molecules is ellipsoid and they contain two unequal domains, A and B. The A domain is the larger one, and contains both the C and N terminal of the protein. The B domain often contains a cystein loop. This loop gives the protein the right conformation and is probably involved in generating the emesis [10]. 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 antigen is presented to T cell receptor in the form of processed peptides bound to molecules of the major histocompatibility complex (MHC), class I and II, which are membrane-bound proteins displayed on the surface of antigen-presenting cells (APC). This type of recognition is the key to the high specificity of the immmune response, where only few T-cells can recognize a specific antigen [15]. The superantigens, on the other hand, can interact with both the class II major histocompatibility complex on antigen presenting cells and with T cell receptors, outside the antigen-binding groove. In this way many more T cells, of different specificity, are activated [15, 18]. This mode of interaction triggers massive release of cytokines that can overwhelm the host regulatory network and thereby assist pathogen evasion of the adaptive immune response. The excessive and aberrant activation of T cells causes damage to tissues and organs which may result in disease and even death [6].

While SEs are the toxin with demonstrated emetic activity, related SEls either lack emetic activity or have not yet been tested for it. All possess superantigenic activity [7, 9]. 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]. The process of cell-to-cell signalling using small molecules was termed ‘Quorum sensing’ because it is a similar process to how decisions in a law court are made only when a threshold number of members (a quorum) are present. Many species of bacteria use quorum sensing to coordinate their gene expression according to the local density of their population [23]. The *agr* locus consists of five genes (agrA, agrC, agrD, agrB and hld), but is composed of two divergent transcripts, RNAII and RNAIII, which are under the control of two distinct promoters, P2 and P3. The P2 transcriptional unit (RNAII) encodes AgrB, AgrD, AgrC and AgrA, which are required for the transcription of P2 and for the activation of P3. AgrC and AgrA function as sensor and response regulator proteins, respectively, and have similar sequences to elements of other bacterial two-component signal

transduction systems. The partially translated P3 transcript, RNAIII, is the effector of the agr locus and also encodes the delta-hemolysin peptide which is not involved in regulation [12].
As the cell density increases in a growing culture, the intracellular level of RNAIII increases due to the activity of the agr system. The increased RNAIII level leads to increased transcription of many exotoxin genes and reductions of transcription of certain cell wall protein determinants and their associated protein genes. Inactivation of the agr system leads to a nonhemolytic and nonproteolytic phenotype, which is due to the failure of exoprotein induction [20]. The *seb*, *sec* and *sed* genes have been demonstrated to be *agr* dependent, whereas *sea agr* independent [4, 12].

Table 2.1 *The staphylococcal enterotoxins* [7]

Enterotoxin	Variant	ORF length (bp)	Mature length (aa)	Molecular weight (Da)	Genetic backbone
SEA		774	233	27 100	Prophage
	SEA_1	774	233	27 100	Prophage
	SEA_2	774	233	27 100	Prophage
SEB		801	239	28 336	SAPI
SEC					SAPI
	SEC_1	801	239	27 531	SAPI
	SEC_2	801	239	27 531	
	SEC_3	801	239	27 563	SAPI
	SEC_{bov}	816[1]	271[2]	27 618	SAPI
	SEC_{sheep}[3]			27 517	
SED		777	228	26 360	Plasmid
SEE		774	230	26 425	Prophage
SElG		777	233	27 043	egc
	$SElG_2$	729[1]	242[2]		Prophage
	$SElG_V$	777	233	26 985	egc
SElH		726	218	25 210	scc[4]
SEI		729	218	24 298	egc
	SEI_v	729[1]	242[2]		egc
SElJ		806	245	28 565	Plasmid
SElK		729	219	25 539	SAPI
	$SElK_2$	729[1]	242[2]		Prophage
SElL		723	215	24 593	SAPI
SElM		722	217	24 842	egc
SElN		720	227	26 067	egc
	$SElN_v$[3]				egc
SElO		783	232	26 777	egc
SElP		783[1]	260[5]	27 000	Prophage
SElQ		729[1]	242[5]	25 207	SAPI
SER		600[1]	259[5]	27 049	Plasmid
SES		774[1]	257[5]	26 217	Plasmid
SET		651[1]	216	22 614	Plasmid
SElU		786[1]	261[5]	27 100	egc
	$SElU_v$	771	256[5]		egc
SElV		720	239[2]		egc

ORF – open reading frame, SEX – staphylococcal enterotoxin X, SElX – staphylococcal enterotoxin-like protein X, SAPI – staphylococcal pathogenicity island, egc – enterotoxin gene cluster, scc – staphylococcal cassette chromosome

[1]ORF obtained from the National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov.ludwig.lub.lu.se/gene, March 25, 2010.

[2]precursor aa length from NCBI, http://www.ncbi.nlm.nih.gov.ludwig.lub.lu.se/sites/entrez?db=Protein&itool=toolbar, March 25, 2010.

[3]ORF length and aa sequence not found in NCBI.

[4]R. Cao, unpublished work.

[5]precursor aa length

2.1.4 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 2.2** and **Table 2.3**. *S. aureus* is considered an exigent bacterium in terms of nutritional requirements. It has been shown that four amino acids are frequently or almost always required for the growth: arginine, cysteine, proline and valine [24]. Valine is necessary for growth and arginine and cystine are necessary for both growth and SE production [25]. In contrast, eight other amino acids are rarely required (alanine, aspartate, histidine, isoleucine, lysine, serine, threonine and tryptophan) [24]. Glucose has been shown to have an inhibitory effect on SE production. This inhibitory effect has been attributed to a drop in pH, as a consequence of glucose metabolism. Glucose and low pH have an inhibitory effect on *agr* expression. Usually, SE production is inhibited in pH below 5. At a given pH, substances used to acidify the medium may have more or less effects. For instance, acetic acid has a greater inhibitory effect than lactic acid on SE production [1, 4]. High concentrations of sodium chloride increase the inhibitory effect of acidic pH. On the other hand, alkaline pH also decreases the production of SEB, SEC, and SED via decreased expression of *agr* [4]. *S. aureus* is quite sensitive to microbial competition. Genigeorgis demonstrated (1989) that the higher the concentration of competing microorganisms in milk the lower the rate of *S. aureus* growth and SE production [26]. Interactions between *S. aureus* and lactic acid bacteria have been reported by Le Loir [24]. The effect of lactic acid bacteria is mainly due to lactic acid production, lowering the pH, production of oxygen peroxide, competition for nutrients and sometimes due to the synthesis of antimicrobial substances, such as bacteriocins.

Table 2.2 *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 2.3 *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

2.1.5 Foods involved in staphylococcal poisoning

Many different foods can be a good growth medium for *S. aureus*, and have been implicated in SFP, including milk and cream, cream-filled pastries, butter, ham, cheeses, sausages, canned meat, salads, cooked meals and sandwich fillings [1, 4, 13]. The foods that are most involved in SFP differ widely from one country to another due to differences in the consumption and food habits [13]. In France, dairy products and especially raw milk cheeses are the most frequent vehicles [28], in the UK and Korea, meat and meat products are most involved [4, 27] and in the United States the most cases of SFP were reported because of red meat [13]. Contamination by *S. aureus* can come from raw material (e.g. mastitic milk), from the processing plant environment (e.g. biofilm on surfaces of processing plant) or from human activity (e.g. healthy carriage, sneeze, cough) [24].

2.1.5.1 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. When the udder is infected, *S. aureus* is excreted in the milk with large fluctuations in counts ranging from zero to 10^8 cfu/ml. *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. The pasteurization is a treatment combining time-temperature conditions to obtain an equivalent effect. At 50, 55, 60 and 65°C *S. aureus* has reported D-values of 10, 3.1, 0.9 and 0.2 minutes in whole milk. Staphylococcal enterotoxins have D-values from 8.3 to 34 min at 121°C [30]. Therefore SEs may be present in foods even when viable cells of *S. aureus* are absent [35, 36]. 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].

Production of cheese is a process of concentrating milk by the interaction of the milk, starter cultures and in most cases rennet. 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]. Until 2005, risk assessment relied on coagulase-positive staphylococci quantification in cheese at the time of production release. New European standards (EC regulation no. 1441/2007) require that control analyses be performed at the cheese-making stage where the *S. aureus* count is expected to be the highest. This change was based on the fact that SEs may be produced in cheese if sufficient levels of *S. aureus* were reached during the initial growth phase, even if the *S. aureus* levels decreased sharply during cheese ripening [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, 89].

2.1.6 Principles of detection

2.1.6.1 Detection of S. aureus

2.1.6.1.1 Microbiological methods

S. aureus forms fairly large colonies after 24 h on non-selective agar media, typically opaque, with smooth surface and entire edges, pigmented. The pigment ranges from white to yellow [3].

The agar media that may be used for the isolation and enumeration of *S. aureus* in foods can be divided into three broad types.

1. The first, and most simple of these media, uses NaCl as the selective agent and incorporates a metabolizable substrate such as manitol (production of acid), blood (hemolysis), or milk (zones of clearing/precipitation) as diagnostic agents. These media have substantial drawbacks: lack of specificity (there are many salt-tolerant organisms other than *S. aureus* in foods); use of substrate by other organisms; sensitivity of damaged cells (cells subjected to stress, e.g. heating, drying, freezing) towards NaCl.
2. The second group of media contains a combination of substances intended to achieve good selectivity, with egg yolk as the diagnostic agent. *S. aureus* uses the lipoprotein lipovitellenin, contained in egg yolk, resulting in the formation of clear zones around colonies of *S. aureus*; this may be followed, on further incubation, by the formation of

a white precipitate within the clear zones as a result of precipitation of calcium and magnesium salts of fatty acids released by the hydrolysis of fat. As selective agents, antibiotics (polymyxin, sulphamezathine) and inorganic salts (lithium chloride, potassium thiocyanate, and potassium tellurite) are used. A good example of the second group of media is Baird-Parker agar. The medium has been subjected to many international trials and even though it has been criticised for some shortcomings, for instance, inadequate selectivity, it remains the medium of choice for the enumeration of *S. aureus* in foodstuffs. 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.

3. A third group of media has been devised to overcome one of the principal drawbacks of the use of egg-yolk-containing media, namely that some strains from animal sources do not use lipovitellenin as a substrate and hence colonies do not show clearing on egg-yolk-containing agars. This problem is well-recognized when testing dairy products. Therefore, Bair-Parker medium containing pig plasma or rabbit plasma with bovine fibrinogen in place of egg yolk was formulated [1, 40]. Presumptive coagulase-producing staphylococci are grey or black, and surrounded by an opaque halo of precipitation which results from the coagulase reaction. When compared with BPA this medium was found to be less prone to overgrowth by competitive microflora coming from cheeses made from raw milk [42]. It also incorporates the coagulase reaction so avoiding the need for further confirmation of colonies. A disadvantage is that the enumeration is performed as a pour plate, rather than a spread plate, adding a complicating step to the procedure.

Selective media are 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. A major requirement of enrichment media for any organism is that they must be capable of resuscitating sub-lethally damaged cells present in many foods which have been subjected to relatively mild preservation processes. Early media formulations took advantage of the tolerance shown by *S. aureus* to concentrations of NaCl that inhibit accompanying organisms. Tryptone soya broth with added salt has been used for many years**.** Brain-Heart infusion broth with added salt has also been used. However, it has become apparent that high concentrations of NaCl (> 40g/l) are inhibitory to injured cells. Giolitti-Cantoni broth and Baird-Parker broth use different selective agents. 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, but as some other organisms that may also grow in the medium will do the same, the medium is not diagnostic. The broth must be streaked onto a staphylococcal isolation medium and identification tests carried out [40].

2.1.6.1.2 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]. A variety of rapid and convenient tests for the detection of staphylocoagulases are available commercially which besides clumping factor also detect protein A and capsular antigens. They are based on latex agglutination and they are more sensitive and specific than ordinary slide test [1]. The examples of such tests are Pastorex Staph Plus Kit (Bio-Rad), STAPHYTECT PLUS (Oxoid), Microgen Staph Latex (Microgen) or BBL™Staphyloslide™Latex Test Kit (BD Diagnostic Systems).

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].

The catalase test is important in distinguishing streptococci (catalase-negative) from staphylococci, which are vigorous catalase-producers. The test is performed by adding 3% hydrogen peroxide to a colony on an agar plate or slant. Calatase-positive cultures produce oxygen and bubble at once [41].

2.1.6.1.3 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. Several million of copies of the original DNA sequence can be produced by PCR in less than an hour. 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].

False-negative results, caused by inhibition of the PCR reaction, can be avoided by using an adequate sample preparation and by optimizing the PCR reaction. Rossen *et al*. contributed the most comprehensive study of PCR inhibition, identifying inhibitory factors in foods, bacterial culture media, and various chemical compounds. These inhibitory factors included organic and inorganic chemicals, detergents, antibiotics, buffers, enzymes, polysaccharides, fats, and proteins [48, 49]. Sample dilution is probably the easiest way to overcome inhibition; it also reduces PCR sensitivity. A short enrichment is also often used before PCR to enrich the bacteria and simultaneously dilute PCR inhibitors [48]. To check the amplifiability of the isolated DNA and thus to avoid false negative results, the amplification control is used. As an exogenous amplification control, various plasmids or λ bacteriophage DNA are used. These are added to the reaction mixture and are detected by their own primers and eventually by their own probe. Another alternative is represented by a mimic exogenous amplification control, which is a dedicated plasmid that is detected by the same primers and identified on the basis of a different molecular weight or using a second probe. A disadvantage of this approach is that new controls have to be prepared for each new primer.

An endogenous amplification control can be used as well, which is some universal DNA sequence present in any prokaryotic or eukaryotic DNA. Exogenous or endogenous amplification controls may be used externally, when PCR with the control and with the sample are carried out in separate tubes, or internally, when the control is analysed in the same tube as the sample using duplex PCR. The use of an internal amplification control is preferred because it facilitates identification of individual tubes in which PCR did not perform well and to correctly evaluate the PCR results [46].

Because of its very high sensitivity, PCR is especially susceptible to contamination. Therefore extreme care must be taken to avoid false-positive reactions. False positives can result from sample-to-sample contamination, but a more serious source is the carryover of DNA from a previous amplification of the same target. Anti-contamination measures involve the use of DNA-free disposable materials such as pipette tips with anti-aerosol filters, water and all solutions being kept in small aliquots, handling samples and reaction mixtures in different rooms, which are thoroughly decontaminated using UV-C light supported by simultaneous application of hypochlorite solution, and also a frequent decontamination of laboratory equipment should be applied [46, 48]. By following strict precautions, contamination problems can be avoided with 30-cycle PCR methods. When more sensitive PCR systems such as 35–40-cycle PCR or nested-PCR are applied, it is almost impossible to avoid occasional contamination, especially in routine laboratories.

Because PCR is based on the detection of intact nucleic acids rather than intact viable cells, positive reactions may arise from either dead cells or viable but nonculturable cells. This drawback may be overcome by an enrichment step to dilute out nonviable cells. An alternative is to use an RNA-based rather than a DNA-based detection [48]. RNA-based methods (e.g. reverse transcriptase RT-PCR) essentially rely on the fact that the bacterial mRNAs are short-lived and therefore the ability to amplify the target mRNA instead of DNA would indicate the presence of live organisms. However, the use of highly labile bacterial mRNA molecules as template and the multi-step nature of these reactions invariably make them lengthier, costlier and less sensitive compared to DNA-based amplification methods [50]. The safest way to detect viable bacteria is the use of a short enrichment step to dilute out dead cells, combined with a DNA-based detection method [48].

PCR has another limitation. If the reaction worked with perfect efficiency, there would be twice as much specific dsDNA after each cycle of PCR. In reality, reactions do not maintain perfect efficiency because reactants within the PCR are consumed after many cycles, and the reaction will reach a plateau. In addition, self-annealing of the accumulating product may also contribute to the “plateau effect” [51]. Thus, most PCR assays only allow qualitative statements, limiting their use to applications in which only the presence or absence of a specific DNA molecule must be determined. To compensate for limitations of end point measurements, researchers have developed a variety of quantitative PCR techniques [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. The quantitative information in PCR comes only from those few cycles in which the amount of DNA grows logarithmically from barely above the background to the plateau. Often only 4–5 cycles out of 30–40 will fall in this log linear portion of the curve [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. In the former case, the amount of all (non-specific) DNA in the amplified sample is monitored and after finishing PCR, melting curve analysis is carried out to detect the specific DNA fragment. 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 (**Figure 2.1**) [53]. Use of different probes labelled with different fluorescent dyes facilitates the monitoring of several specific DNA fragments in parallel.

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.

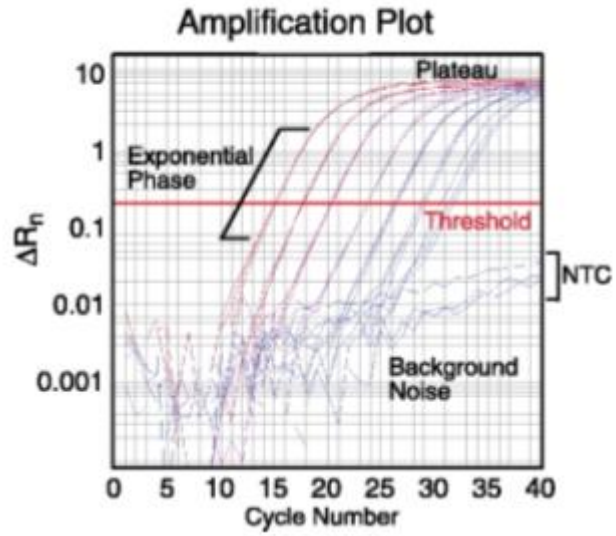


Figure 2.1 *Typical real-time PCR results. Amplification plot illustrating the increase in fluorescent reporter signal (y-axis) with each PCR cycle (x-axis). The y-axis units (ΔR_n) actually reflect the reporter signal normalized to a passive reference dye in the reaction buffer. The curves seen with a no-template control (NTC), which lacks added DNA, show that the primers alone do not generate a signal and that the reagents used in this assay showed no DNA contamination* [51].

Reverse Transcription PCR (RT-PCR)

Assessment of toxin genes expression has become crucial to understand the pathogenesis of staphylococcal infections [91]. 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 [92]. 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 [91].

Reference or housekeeping genes are present in all nucleated cell types since they are necessary for basis cell survival. The mRNA synthesis of these genes is considered to be stable and secure in various tissues, even under experimental treatments. However, numerous studies have already shown that the housekeeping genes are regulated and vary under experimental conditions. Therefore, if housekeeping genes are to be used, they must be validated for the specific experimental setup and it is probably necessary to choose more than one housekeeping gene for more accurate and reliable normalization of gene expression data. [90, 92, 28]. Derzelle *et al*. [45] investigated the expression stability of several reference genes in different environmental conditions and found out that *ftsZ, recA, pta* and *rpoB* were the four most stably expressed genes in *S. aureus* during growth in milk and *ftsZ, recA*, and

rpoB were found to be stably expressed in BHI broth. The use of 16S rRNA molecules as a standard is also a choice of many studies [94, 95].

Multiplex PCR

In multiplex PCR, several sets of primers and, eventually, probes are used in one tube, which facilitates a parallel detection of several DNA sequences. This configuration saves time and chemicals, but it often suffers from interference between individual reactions, from competition and from false negative results in case that individual template concentrations differ by one or more orders of magnitude. For practical purposes, use only two sets of primers and, eventually, probes in one tube is recommended (duplex PCR) [46].

Several PCR-based methods for food-borne *S. aureus* identification have been published. The predominant species-specific target was *nuc* gene used in either conventional PCR [54] or real-time PCR [55, 56]. Other popular targets were species-specific regions of the DNA coding for 16S or 23S rRNA or *coa* gene [57, 58, 59] and putative transcriptional regulator genes [60, 61]. These *S. aureus*-specific genes were used in many studies along with *se* (*ent*) genes coding for enterotoxins in multiplex conventional PCR [31, 62, 63, 64, 65].

There are also some commercial systems available, for example the BAX® System Real-Time PCR Assay for *S. aureus* (Dupont Qualicon, Inc.) that provides next-day results after 22-24 hours enrichment with less than 90 minutes of PCR processing time and with detection limit as low as 1 cfu/g in powdered infant formula [85].

2.1.6.2 Detection of staphylococcal enterotoxins

A number of techniques for the detection of staphylococcal enterotoxins are currently available. These techniques include radioimmunoassay (RIA) [66, 67], microslide double diffusion [72], enzyme immunoassay (EIA) [68], MS-based analysis [69, 70] or methods including biosensors [71]. Very recent press reports state that the USDA have produced a new sensitive method for the detection of SEA. The study [73] describes an *in vitro* splenocyte proliferation assay based on SEs superantigen activity as an alternative method for measuring the activity of staphylococcal enterotoxin A.

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, 62]. 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 [74].

The principal steps of sandwich ELISA includes five different steps: (1) Coating of microtiter plate wells with an antibody (primary antibody), (2) addition of the sample containing the antigen (the toxin to be measured) which binds to the antibodies, (3) addition of detection antibody (secondary antibody), (4) coupling of enzyme to detection antibody and (5) addition of substrate that is processed by the enzyme in order to determine the amount of antigen present in the sample. Quantification is based on a standard curve prepared using a serial dilution in appropriate matrix of highly purified staphylococcal enterotoxins. Absorbance values are plotted against toxin concentrations and unknown samples are determined from the linear regression equation [76].

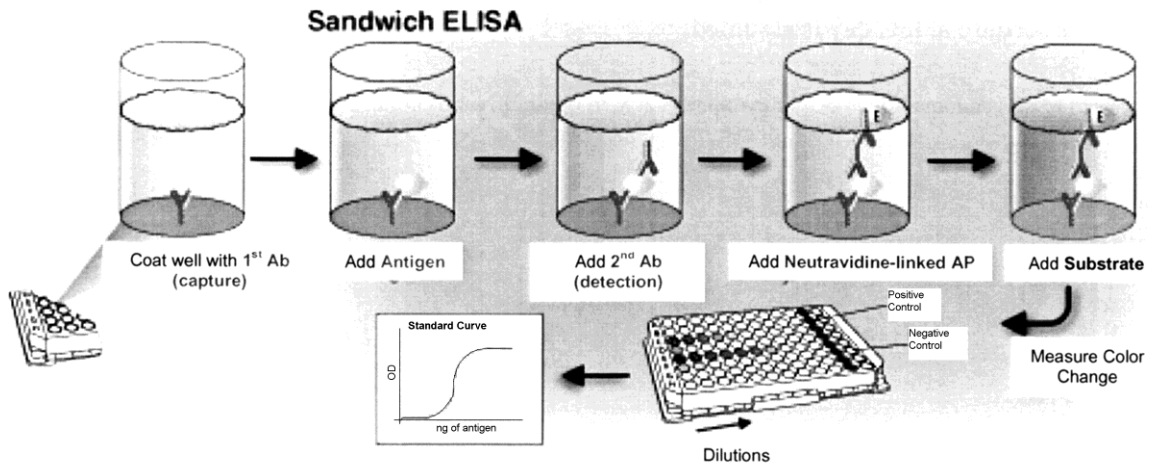


Figure 2.2 *Principle of the Sandwich ELISA* [75]

Methods of SE detection now are available commercially and have reduced preparation and detection time from several days to a few hours. The following is a description of the performance and use of most widely used, currently available test kits.

- TECRA kit (3M). It is an ELISA system which uses a polyvalent capture antibody against staphylococcal enterotoxin types A to E. It is widely used, having high sensitivity (1 ng/ml), speed (results in 4 h), and convenience. The method generally has good reliability but some false-positive results may arise from the presence of peroxides and non-enterotoxin metabolites in foods. Validated by AOAC [77].
- VIDAS SET2 kit (BioMérieux). It is a rapid (results in 80 min) and fully automated (uses VIDAS automated immunoanalyzer) kit employing the ELFA technique. It detects SE A-E without differentiation with sensitivity between 0.5 - 1 ng/g. The main difference between VIDAS^{TM} SET2 and VIDAS^{TM} SET is that the new generation of test uses two Fab fragments and no Fc fragment for constructing the conjugated antibody [79]. The kit

is described by AFSSA EU Community Reference Laboratory as a screening method to detect staphylococcal enterotoxins in milk and milk products, after an extraction step followed by dialysis concentration [80]. Validated by AOAC [78].

- TRANSIA PLATE Kit (Diffchamb). The kit is intended to be used for detection of staphylococcal enterotoxins A-E in food samples and in culture supernatants. The method is based on a sandwich-type ELISA. Sensitivity is similar to VIDAS kit [79]. The Transia Plate SE kit has been proved by the former EU CRL for milk and milk products to be one of the most suitable commercialised kits for performing the SE detection in milk products. As in the case of VIDAS kit, an extraction step followed by dialysis concentration is needed [81].
- SET RPLA (Oxoid). This is a test based on RPLA (reverse-passive latex agglutination) using monoclonal antibodies for SE capture and detects SE A-D. While it lacks the sensitivity, specificity, and speed of ELISA test kits, it is simple to use and does not require special equipment for carrying out the test [1, 82].
- RIDASCREEN (r-Biopharm). Visual immunoassay kit utilizing monovalent capture antibodies against SE types A to E; therefore, it simultaneously detects and identifies the enterotoxin types. The major advantages of the kit are (i) a high degree of specificity, (ii) excellent sensitivity (0.2 – 0.75 ng/ml extract, depending on the tested food sample), (iii) simplicity (direct assay of SEs in food extracts without the need for lengthy extraction or concentration procedures), (iv) rapidity (less than 3 h to complete the analysis) [83, 84].

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 which can range from 13 to 85% as a result of cross-reaction with unrelated antigens. Some kits my give misleading results when naturally occurring peroxidases in the food react with the colorogenic substrates used in the ELISA assay. False-positive results because of protein A, protein from *S. intermedius*, endogenous enzymes, or matrix proteins have been reported with EIA kits of SE [79].

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. MATERIALS AND METHODS

4.1 Materials and Methods used for the detection of *S. aureus*

Materials and methods used to develop a method for the detection of *S. aureus* in food by using selective enrichment and real-time PCR are well described in the article attached as Supplement I.

4.2 Materials and Methods used for milk and cheese experiments

4.2.1 Bacterial strains used in the milk experiments

For the performed milk experiments *Staphylococcus aureus* strains 45, 161:3 and 168:1 were used. For detailed information on strains, see 4.2.3.

4.2.2 Experimental set-up: milk

- *Pre-culture conditions*

Pre-cultures were prepared in 50 ml Brain Heart Infusion (BHI) growth medium (Bacto™, Becton Dickinson and Company, USA) and cultivated in 250 ml baffled Ehrlenmeyer flasks. The preculture was incubated aerobically O/N (~16 h) at 37°C and with rotation (~180 rpm) to an optical density (OD) at 620 nm of ~ 16.

- *Batch fermentation - principle*

S. aureus growth and SED production were studied in batch fermentations in small scale fermentors (**Figure 4.1**). These are made in glass with a rubber lid. On the top there are two filters to permit aeration and pipes connected with syringes in order to collect samples. Each fermentor contained an appropriate growth medium such as milk or BHI (Brain Heart Infusion) broth. As the fermentors can operate in parallel it is possible to make a comparison between a well characterized growth medium and an unknown matrix using the same strain and the same temperature. Strains were cultivated for several days and agitation was done by magnetic stirring (500 rpm/min). The temperature was controlled by liquid circulation in the jacket surrounding the fermentor. Samples were collected in sterile conditions at specified time points using syringes.

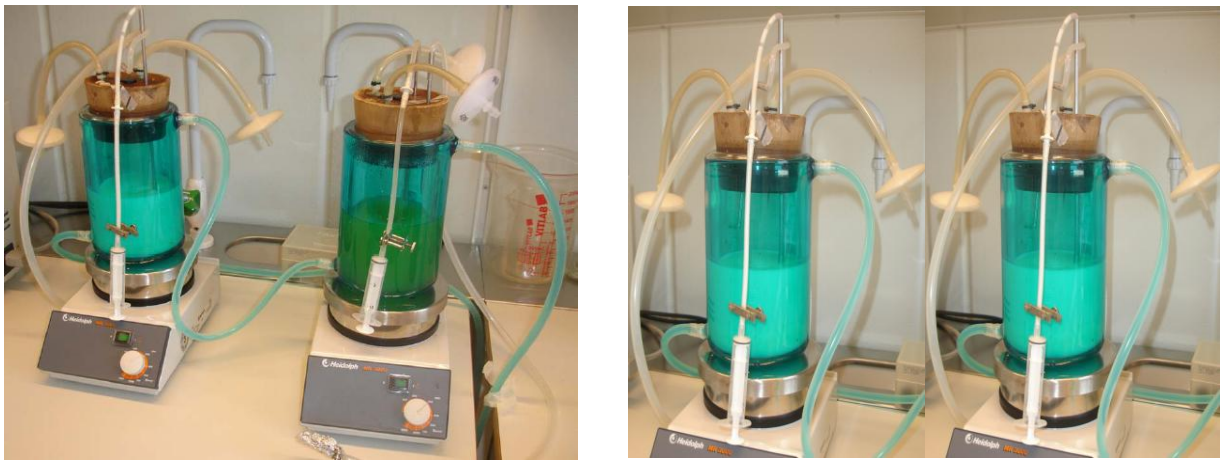


Figure 4.1 *Batch fermentation in milk and BHI to investigate the effect of temperature and two different matrices (left picture). Batch fermentation in milk to follow the combined influence of low temperature and background flora (right picture).*

Effect of three different temperatures, 8°C, 12°C and 20°C and two different matrices, BHI and milk

The day before the start of the experiment, fermentors were autoclaved with magnetic stirrers inside. One fermentor was autoclaved empty with tubing for the addition of milk after sterilization, while the second fermentor contained 500 ml of BHI. On the day of the experiment, 500 ml of pasteurized milk (1.5 % fat) was pumped under strerile conditions into the fermentor using a peristaltic pump. Fermentors were allowed to equilibrate to correct temperature before inoculation of bacteria.

- *Inoculation*

The OD_{620} value of the O/N culture was measured (spectrophotometer DigiLab, Hitachi, U-1800) and each fermentor was inoculated with a volume of O/N culture to reach a start OD_{620} of 0.1 (~10^7 cells) using syringe.

- *Washing of inoculum*

To avoid carry-over of enterotoxin that had been produced in the pre-culture, cells were washed under sterile conditions one time with 0.9% NaCl before inoculation. The cells were pelleted in a swing out rotor (Eppendorf Centrifuge 5810 R) at 3220 *g* for 10 minutes at 20°C and the supernatant was removed. An equal amount of 0.9% NaCl was added, the cells were resuspended followed by centrifugation like above. Finally, BHI was added to regenerate the native volume for inoculation to reach OD_{620} of 0.1.

- *Sampling*

Samples for growth and SED measurements were collected every hour between times 0 to 7 hr and at 24, 48, 72, 144 hr. For the cells cultivated in BHI, growth was followed during the experiments using optical density measurement at 620 nm. In addition, viable count was

performed on both cultures in BHI and milk to follow growth. Samples were collected and diluted (1/10 steps) with 0,9% NaCl to appropriate cell concentrations and a volume of 100 µl of three different dilutions was plated in duplicate onto BPA Agar (Merck, Darmstadt, Germany) plates. The plates were incubated O/N at 37 °C. To measure SED concentration samples were centrifuged at room temperature in a bench top centrifuge to pelet the cells and the supernatant was collected and kept at -20°C until analysis. Samples for RNA extraction were collected from cultures in BHI at time points 4, 7, 24, 48, 72 and 144 hr for experiments at 12°C and 20°C. For experiments at 8°C, RNA samples were collected at 24, 48, 72 and 144 hr. 20 ml of cells from each BHI culture (no RNA samples collected from milk) were collected at each sampling point. Cells were immediately pelleted in a swing out rotor and 3220 *g* for 10 min at 4°C. The supernatant was discarded and cells snap frozen in liquid nitrogen and stored in -80°C freezer awaiting RNA isolation.

Combined effect of low temperature (12°C) and the presence of background flora

These experiments were performed in pasteurized milk with 3% fat content. Both fermentors were autoclaved empty and 600 ml of milk was added to each fermentor afterwards like above. The background flora was introduced by the addition of defined volumes (5, 1, 0.5 and 0.1 % of the total volume) of raw milk to the pasteurized milk. The OD_{620} value of the O/N culture was measured and each fermentor was inoculated with a volume of O/N culture to reach different start concentration of cells varying between 10^3-10^6 cfu/ml. Cells were otherwise treated like above. The actual start concentration of cells was confirmed by viable plate counting. Samples for growth and SED protein measurements were collected every 24 hour between times 0 to 120 hr and during certain measurements also after 12 hr. Growth was followed by viable count like above. BGF was measured on plate count agar, PCA (Casein-peptone Dextrose Yeast Agar, Sigma-Aldrich, USA). Collection of samples for SED/SEA concentration determination using ELISA was performed as described above.

- *RNA extraction*

A modified method for total RNA extraction from *Bacillus* spp. with acidic phenol was used [93]. Cells harvested and stored as described above were resuspended in 500 µl ice-cold TES buffer, pH 7.5 (50 mM Tris, 5mM EDTA and 50 mM NaCl). The cell suspension was transferred to a 2 ml Bead Beater-tube containing: 1 ml (2.4 g) silica beads (0.1 mm), 600 µl acidic phenol (Aquaphenol; Saveen Biotech AB, Malmö, Sweden) and 100 µl of chloroform (Sigma Chemical Co.). The cells were disrupted with silica beads by using a bead miller (Mini-BeadBeater; BioSpec Products, Inc., Bartlesville, Okla) in the cold room during two minutes at "homogenize". The tubes were chilled on ice and then centrifuged in a fixed angle rotor at 2655 *g* for 5 min at 4°C (Eppendorf Centrifuge 5810 R). Total RNA was thus recovered by simultaneous disruption and extraction in acidic phenol and chloroform. After centrifugation the upper phase (470 µl) was collected and transferred to a clean tube. Two

additional extractions with phenol : chloroform (600 µl : 100 µl) were performed. Finally a chloroform extraction was performed to remove traces of phenol. RNA was precipitated with 0.1 volumes of 3 M NaOAc pH 4.8 and 2.5 volumes of 95% ice cold EtOH and incubated at -80°C O/N. After precipitation the RNA was pelleted at 4°C for 20 minutes at 20817 *g* and washed once with 70% EtOH. The RNA was resuspended in 50 – 100 µl of RNA stabilizing solution (Ambion, Austin, Texas, USA) and stored in -80°C freezer.

- *DNase treatment*

Before reverse transcription, contaminating DNA was degraded by treating 15 µl of each RNA sample with 15 U RNase-free DNase (Promega Co., Madison, USA) and 1× reaction buffer. The reaction mixture was incubated at 37°C for 45 min and the DNase was then inactivated by the addition of stop solution (Promega Co.) and incubation at 65°C for 10 min. Afterwards, a DNase treatment check using real-time PCR was achieved and the concentration and purity of total RNA was measured spectrophotometrically at 260 and 280 nm using a Biophotometer (Eppendorf, Hamburg, Germany). RNA samples were stored in -80°C freezer awaiting cDNA preparation.

- *cDNA synthesis (reverse transcription)*

First-strand cDNA was synthesized in two separate RT assays by using the reverse primers for *sed* and *rrn*. During the first reaction denaturation of RNA secondary structure and annealing of primers to RNA was achieved. The total volume of first reaction was 13 µl and contained 1 µl dNTP 10 mM (Roche Diagnostics GmbH, Mannheim, Germany), 0.1 – 1.0 µg of RNA, 1 µl rev primer (see sequence in paragraph below) 10 µM and DEPC-dH_2O <13 µl (the amount of water varied depending on the RNA concetration). The reaction mixture was incubated for 5 min at 65°C in a Gene Amp 9700 thermal cycler (Perkin-Elmer Cetus, Norwalk, USA) followed by chilling on ice. In the second reaction, 200 U of Superscript II RNase H^- reverse transcriptase (Life Technologies, Gaithersburg, USA), 20 U of RNasin RNase inhibitor (Life Technologies), 5 mM DTT (dithiothreitol, Life Technologies) and 1× first-strand buffer were added and incubated as above at 42°C for 50 min followed by incubation at 70°C for 15 minutes to inactivate the enzyme.

- *Quantitative PCR*

PCR reactions were performed using a Lightcycler instrument (Roche Diagnostics GmbH). The total volume of reaction was 20 µl. For amplification of *sed* the reaction contained 1× *Tth* PCR buffer (Roche), 2.75 mM MgCl (Roche), each dNTP (Roche) at a concentration of 0.2 mM, 0.5 µM SED forward primer (5'-CTAGTTTGGTAATATCTCCT-3'), 0.5 µM GSEDR-2 reverse primer (5'-ATTGGTATTTTTTTTCGTTC-3') (MWG Biotech AG, Ebersberg, Germany), 0.15 µM probe 1 (entD-FL;TACCCTATAAGATAT-AGCATTAATTGTT--FL), 0.15 µM probe 2 (entD-LC; LC Red640-TGGTGGTGAAATAGATAGGACTGCTTG--PH)

(TIB Molbiol GmbH, Berlin, Germany), 0.05 U *Tth* polymerase (Roche) and autoclaved Millipore-filtered water to a volume of 16 µl. 4 µl of template (cDNA) was added to each reaction. For amplification of *rrn* the reaction contained 1× *Tth* PCR buffer, 4.6 mM MgCl, 0.2 mM of each dNTP, 0.5 µM rRNA forward primer (5'-TGTCGTGAGATGTTGGG-3'), 0.5 µM rRNA reverse primer (5'-ACTAGCGATTCCAGCTT-3') (MWG Biotech AG), 0.15 µM probe 1 (16S; GGACAATACAAAGGGCAGCG--FL), 0.15 µM probe 2 (16S; LC Red705-ACCGCGAGGTCAAGCA--PH) (TIB Molbiol GmbH), 0.05 U *Tth* polymerase and water to a volume of 16 µl. 4 µl of template (cDNA) was added to each amplification. The Lightcycler amplification protocol that was used started with an initial denaturation at 95°C for 60 s, followed by 45 cycles of 95°C for 0 s (i.e. no hold at 95°C), annealing and fluorescence acquisition at 48°C for 5 s, and elongation at 72°C for 25 s. The temperature transition rate was 20°C/s. The crossing point (Cp) for each transcript was determined by using the second derivative maximum mathematical model in the Lightcycler software. In order to check for amplification of any contaminating DNA, a negative control was added to the PCR analysis. The negative control contained autoclaved Millipore-filtered water instead of template.

Total RNA was diluted 10-fold (in the range from 0.1 µg to 0.01 pg for *rrn*) and 2-fold (in the range from 1 µg to 0.03 µg for *sed*), reverse transcribed, and amplified with the Lightcler instrument to determine the amplification efficiency (E) and the log-linear range of amplification for each PCR assay. The amplification efficiency in the exponential phase was calculated as follows: $E = 10^{(-1/s)}$, where *s* is the slope of the log-linear range of amplification (the range of linear detection window).

Relative quantification was based on the level of mRNA of *sed* compared with the level of the reference gene, *rrn*, as described by Pfaffl [90]. To quantify the transcript levels of *sed* (i.e. the amounts of *sed* mRNA that accumulated at time points), the same amount of total RNA (0.5 µg) from a culture at each growth phase was used in the RT procedure. The relative expression (RE) was calculated from the amplification efficiencies for each PCR assay and the crossing point deviation (ΔC_p) of the unknown sample compared with a calibrator samples, as follows:

$$RE = \left((1+E_{sed})^{\Delta Cp_{sed}(calibrator-unknown\ sample)}\right) \Big/ \left((1+E_{rrn})^{\Delta Cp_{rrn}(calibrator-unknown\ sample)}\right)$$

4.2.3 Bacterial strains used in the cheese experiments

The following four strains of *Staphylococcus aureus* were used to post contaminate the cheese samples in all experiments:

Staphylococcus aureus (SA) 161:3. The strain produces SEA and SEH. It was originally isolated from fresh cheese (cow), made from pasteurized milk. The strain is kept as frozen stock in glycerol at -80°C.

Staphylococcus aureus (SA) 168:1. The strain produces SEA, SEG, SEI and TSST. The strain was originally isolated from fresh cheese (cow), made from non pasteurized milk. The strain is kept as frozen stock in glycerol at -80°C.

Staphylococcus aureus (SA) 45. The strain produces SEA and SED. It was originally isolated from boiled ham in a staphylococcal food poisoning outbreak. The strain is kept as frozen stock in glycerol at -80°C.

Staphylococcus aureus (SA) 564. The strain produces SED and TSST. The strain was originally isolated from a patient with toxic shock syndrome.

4.2.4 Experimental set-up: cheese

In all the experiments, 25 g of either cream cheese (ICA cream cheese natural, 21% fat) or semi-soft cheese (Wästgöta kloster, 31% fat, Arla) were inoculated with *S. aureus* (one of the four different strains) and incubated at 13 or 20°C. For each cheese sample, two biological replicas were made (A and B); for example: two cheese samples for day 3 were inoculated with the same strain, *S. aureus* 161:3, and were labeled "161:3 A, 3 days" and "161:3 B, 3 days". Samples were taken after 0 hours, 3 days, 6 days, 13 days, 20 days (or 19 days) and 27 days. Viable count of *S. aureus* was made by first homogenizing the cheese samples and then making appropriate dilutions and finally spreading onto plates. The count of total bacteria and lactic acid bacteria were also determined in this way. Enterotoxin level was determined by first performing extraction and dialysis to concentrate the proteins and then measured using ELISA.

- *Preparation of inoculum*

A fresh culture from each *S. aureus* strain was prepared for each experiment. Each strain was primarily plated from the frozen stock onto Brain Heart Infusion agar plates (Difco Laboratories, BD Diagnostic System, Le point de Claix, France) and incubated overnight at 37°C. The following day colonies of each strain were inoculated into baffled E-flasks containing 50 ml BHI broth (Difco Laboratories, BD Diagnostic System, Le point de Claix, France). In order to make two replicas of each strain, two different colonies of every strain were inoculated into separate flasks (**Figure 4.2**)**.** The cultures were incubated at 37°C with rotation at 160 rpm for 16 hours. Decimal dilutions of the cultures were then made in sterile 0.9% NaCl solution (1ml of culture + 9 ml of NaCl in 15 ml Falcon tube).

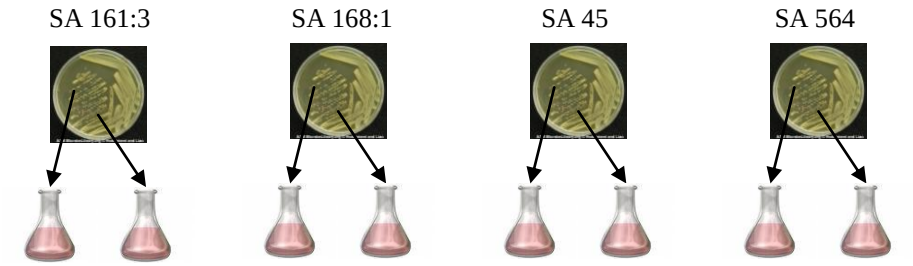


Figure 4.2 *Colonies of the four different S. aureus strains were inoculated into E-flasks. Two biological replicas (= two flasks) were made for each strain.*

- *Concentration of overnight culture*

In order to determine the concentration of *S. aureus* cells in the overnight cultures, 100 µl of the appropriate dilutions of the decimal dilution series prepared were spread on different agar plates. For each strain and each dilution two BHI agar plates (Difco Laboratories) and one Baird-Parker agar plate (Merck, Darmstadt, Germany) were used.

- *Washing of inoculum*

Before inoculating the cheese, the *S. aureus* cells (with appropriate concentration) were washed with sterile 0.9% NaCl to remove enterotoxins produced in the overnight culture (in order for the starting concentration of enterotoxin to be zero). For each strain, 2000 µl (6 x 250 µl + 500 µl in excess) was transferred to 15 ml Falcon tubes and centrifuged in a swing-out rotor (Eppendorf Centrifuge 5810 R) at 3220 *g* for 10 minutes at room temperature. The supernatant was discarded and 5 ml of sterile 0.9% NaCl was added. After careful mixing using a vortex, another 5 ml of sterile 0.9% NaCl was added. The samples were mixed like before and then centrifuged like above. After discarding the supernatant, the cell pellet was dissolved in 2000 µl of sterile 0.9% NaCl.

- *Preparation and inoculation of cheese*

To reach the desired inoculum concentration (10^4 – 10^6 cfu/ g of cheese) a certain dilution from the 10x dilution series of the cultures was used to inoculate the cheese samples. If for example the desired inoculum concentration is 10^6 cfu/g of cheese and the inoculum volume is 250 µl per 25 g of cheese (=10 µl /g), then assuming that the *S. aureus* cells have reached a concentration of 10^9 cfu/ml in the overnight culture, the first dilution (10^8 cfu/ml) is used to inoculate the samples.

For each time point and strain, with two biological replicas A and B, 25 grams of cheese was weighed and placed in Petri dishes under sterile conditions. There were six time points for sampling: 0 hours, 3 days, 6 days, 13 days, 20 days and 27 days. Four strains of *S. aureus* (SA 161:3, SA 168:1, SA 45 and SA 564) and two replicas for each strain, plus one control sample (inoculated with 0.9% NaCl instead of cells) for each sampling point resulted in a

total of 54 cheese samples for each experiment setup (6*(4*2+1) = 54). Each sample was labeled with strain, replica and time point, for example "168:1 A, 3 days".
The cheese samples were then sealed with parafilm and incubated at 13 or 20°C (depending on the experiment) for 3 days, 6 days, 13 days, 20 days or 27 days (depending on the sampling time point). The 0 hour samples were analyzed immediately after the startup of the experiment, according to the sampling described below. No enterotoxin measurements using ELISA were performed on the 0 hour samples.

- *Sampling*

At each time point in all experiments, 25g cheese samples were transferred into sterile stomacher bags (Somacher 400 Classic, Standard bags, Seward Limited, UK) and to each bag 40 ml of warm (30-40°C) distilled sterile water was added. This resulted in a 2.6 times dilution and was referred to as dilution -1 (25 g of cheese + 40 ml of H_2O → 65/25 = 2.6x dilution). The samples were homogenized in a Stomacher (Lab-Blender 400, Seward Medical Ltd. London, UK) for 2 minutes each and left at room temperature for 30 minutes to let the enterotoxins diffuse. 10x dilutions were then made (-2, -3, -4, etc.) in order to determine the concentration of cells by viable count.

- *Concentration of S. aureus in cheese samples*

100 µl of suitable dilutions of each homogenized sample was spread on Baird-Parker agar plates. For each dilution and samples two or three plates were used. The plates were incubated overnight at 37°C.

- *Concentration of microorganisms present in the cheese (total count)*

100 µl of suitable dilutions of each homogenized samples was spread on BHI agar, incubated overnight at 37°C then at 30°C for several days. For each dilution and sample two or three plates were used.

- *Concentration of lactic acid bacteria*

1 ml of suitable dilution of each homogenized samples was added on a Petri plate and then molten MRS agar (Merck, Darmstadt, Germany) was poured into the plate and mixed thoroughly. In parallel, 100 µl of the suitable dilutions was spread on MRS agar plates. The plates were incubated for 3 – 5 days at 37°C.

- *Calculation of cfu*

To calculate the amount of cfu/g of the samples, following equation was used.

$$n = 2.6*10^{(d-1)}*10*a$$

a is the average amount of colonies on the plates; 2.6 is the first dilution step; (d-1) is the dilution factor for the rest of the dilutions (the 10x dilutions) and it was multiplied by 10 to go from 100 µl (which was spread on each plate) to 1 ml (for the molten MRS agar plates, the amount of colonies were not multiplied by 10 since 1 ml of the samples was used).

4.2.5 Extraction and Dialysis

In order to be able to measure the enterotoxin level with ELISA, the samples had to be first concentrated, which was done by performing dialysis (**Figure 4.3**). Prior dialysis, extraction step was applied. Each sample from the stomacher bags (25 g of cheese + 40 ml water) was, after 1 ml was used for the cell concentration determination, transferred into two 50 ml Falcon tubes. The pH of each sample was measured. Thereafter the samples were acidified to pH 3.5±0.5 with 5M HCl in order to precipitate the casein of the cheese. The tubes were then centrifuged in a swing-out rotor at 3220 g at 4°C for 15 minutes (Eppendorf Centrifuge 5810 R) and the supernatant from the both tubes were transferred and pooled into one new tube. Then the supernatant was neutralized with 5M NaOH to pH 7.3±0.3 in order to keep the toxins stable and the tubes were once again centrifuged like above.

For each sample (except the control sample), about 30 cm of dialysis membrane (Spectra/Por®1, MWCO: 6000-8000 Daltons, flat width 23±2 mm, Spectrum) was cut and soaked in hot distilled water for at least five minutes in order to soften the membranes and make them easier to open. The inside and outside of the membranes were washed with distilled water and one end of each membrane was locked with a clamp (Closures, 1 = 35 mm, Spectra/Por®, Spectrum). Each neutralized sample was then transferred to a dialysis membrane using a funnel with glass fiber wool as filter, to remove any remaining cheese particle, and was then closed with a clamp in the other end. The dialysis membrane was put in a bowl with one liter of 30% PEG solution (Polyethylene glycol 20 000, Merck, Schuchardt OHG, Germany) and with a magnetic stirrer and was left to concentrate overnight at 5±3°C. For each strain, a separate bowl was used and the replicas were marked with “A” or “B” in each bowl.

The following day, the dialysis membrane was removed from the PEG solution and the outside was rinsed with distilled water in order to remove the PEG. Each concentrated extract was then recovered by adding 5 ml of PBS (phosphate buffered saline solution; $NaCl/Na_2HPO_4$: 145 mM/10 mM; pH 7.3±0.2) into membrane and dissolving the extract by massaging the membrane. The 5 ml extract was carefully transferred into 15 ml Falcon tubes, marked with strain + replica, sampling point, experiment number and date. The tubes were kept in freezer (-20°C) until analysis.

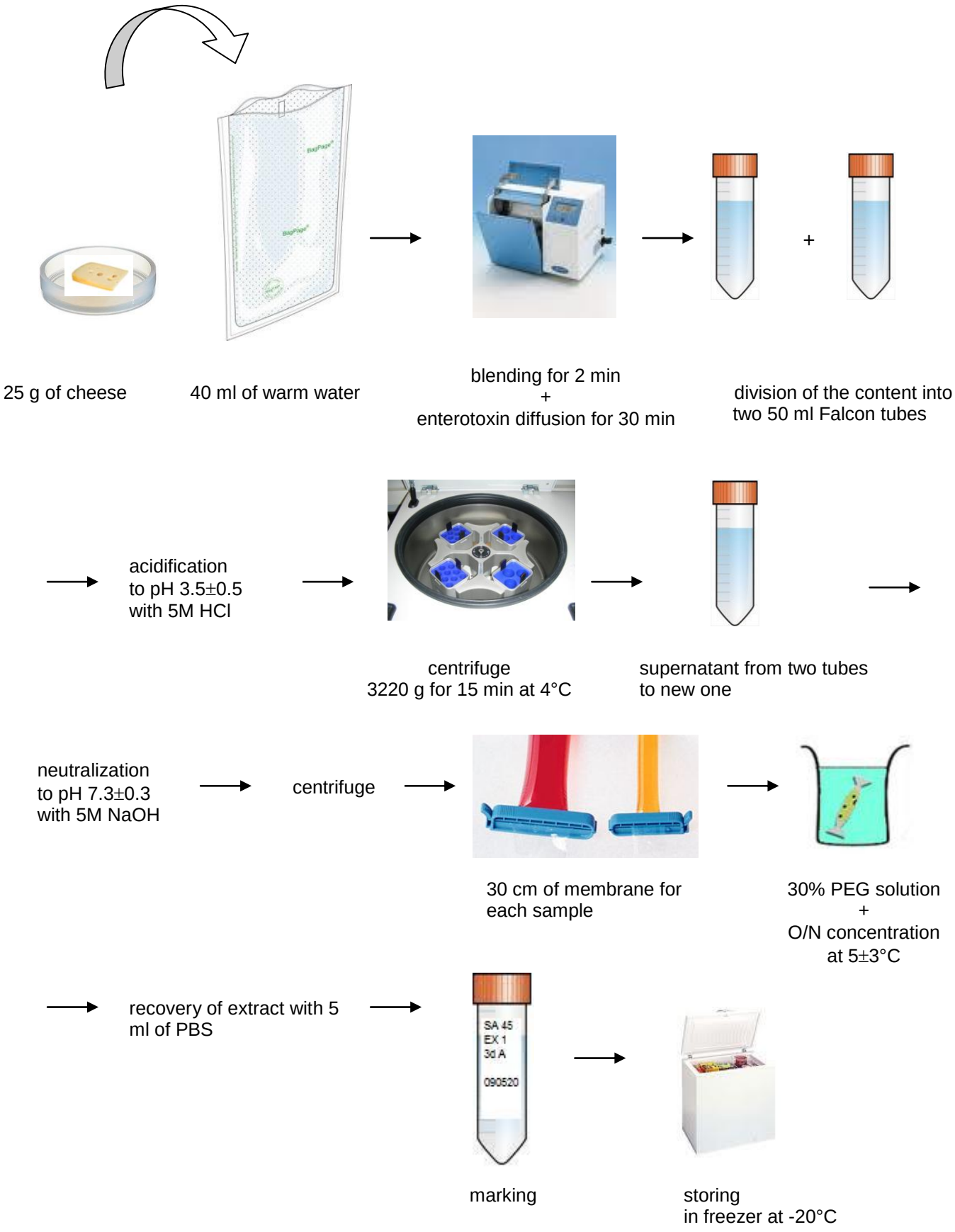


Figure 4.3 *Flow chart showing the procedure of extraction and dialysis*

4.2.6 ELISA

The concentration of staphylococcal enterotoxins was measured using sandwich ELISA (**Figure 2.2**). Five principal steps of ELISA are following:

1. *Coating with detection antibody*

The first step was to coat the microtiter plates (Immulon® 2HB polystyrene, Flat Bottom Microtiter® Plates, 96 wells solid) (one plate for each toxin to be measured) with affinity-purified antibodies (IgG), sheep anti SEA, SED or TSST IgG (Toxin Technology Inc. Sarasota, Fl, USA). 20 µl of the antibody (concentration 1 mg/ml) was added to 10 ml coating buffer (0.1 M Na_2CO_3, pH 9.6) in a 15ml Falcon tube and mixed gently to avoid air bubbles. This resulted in a concentration of 2 µg/ml. 100 µl of the solution was then dispensed into each well using a multi-channel pipette (8 channels). The plate was covered with parafilm and incubated at 4°C overnight.

- *Blocking of empty sites*

The following morning, the coating solution was discarded by firmly turning the plate upside down into the sink. The plate was tamped onto a thick layer of paper towels in order to remove the last droplets. Then 185 µl of blocking buffer (10 mM PBS [PBS 100 mM, pH 7.4: 80 g NaCl, 2 g KCl, 17.8 g $Na_2HPO_4.2H_2O$, 2.4 g KH_2PO_4, 1 l of dH_2O], 0.05% Tween 20, 5% milk powder) was added into each well using the multi-channel pipette. The plate was covered with parafilm and incubated at 37°C for on hour and then for 1 hour or longer at 4°C.

- *Preparation of standard curve, samples and positive control*

During the incubation, the standard curve, samples and positive controls were prepared. 1.5/2.0 ml sterile Eppendorf tubes were used. The samples were removed from the freezer and kept on ice while thawing. The samples were not diluted.

For preparation of standard curve, highly purified SEA, SED or TSST (Toxin Technology Inc., Sarasota, FL, USA) enterotoxin stock solution with a concentration of 100 µg/ml was brought from -80°C freezer and kept on ice.

The stock toxin was diluted from 100 µg/ml to 100 ng/ml in 10x dilution steps in sterile ddH_2O. To prepare the standard curve, the 100 ng/ml toxin was diluted in 2x steps in order to achieve the concentrations 10, 5, 2.5, 1.3, 0.63, …0.039 ng/ml in assay buffer. Three technical replicas of the standard curve were prepared for each plate. The dilutions were stored in Micro Rack at 4°C until dispensed.

For positive control for SEA and SED, *S. aureus* strain 45 supernatant of O/N culture (BHI growth medium, 37°C, rotation) was used and loaded into wells at both ends of the plate, in total 5 - 6 wells. For TSST, *S. aureus* strain 564 supernatant of O/N culture (TSB growth medium, 37°C, rotation) was used and loaded into wells at both ends of the plate, in total 5 – 6 wells. Assay buffer was added as negative control and loaded into 8 – 11 different wells throughout the plate.

150 µl of each dilution of standard curve samples, unknown samples and controls were then dispensed into 1.1 ml Micro Tubes (National Scientific Supply Co, Inc. Claremont CA) in a Micro Rack 8 x 12 holes (National Scientific Supply Co, Inc. Claremont CA) according to the sample outline for the microtiter plate specified in the experimental protocol. The filled Micro Rack was then stored at 4°C until the loading step.

- *Washing*

Washing buffer (10 mM PBS, pH 7.4, 0.05% Tween 20) was added to a multi-channel pipette reservoir. The blocking solution was discarded by turning the plate upside down and then tamped against some paper towels, as described above. 185 µl of washing buffer was added into each well using the multi-channel pipette. The washing solution was removed by turning the plate upside down and tamped to remove droplets. This quick washing step was repeated one more time.

185 µl of washing buffer was then once again added into each well and the plate was covered with parafilm and incubated for 5 minutes on a shaking table (250 min^{-1}). This longer washing step was repeated one more time.

2. *Loading of samples*

The unknown samples, standard curve samples, negative and positive controls were transferred from the Micro Rack into the microtiter plate. Eight samples (in one row) were transferred at a time using the multi-channel pipette and from left to right. The plate was covered with parafilm and incubated at 37°C for 1.5 h.

- *Preparation of antibody*

Detection IgG, biotinylated sheep anti SEA, SED or TSST IgG antibody (Toxin Technology Inc., Sarasota, FL, USA) was prepared right before the 1.5 hour incubation was finished. The antibody was diluted 2000 times by adding 5 µl of the antibody to 10 ml of assay buffer (50 mM PBS, pH 7.4, 0.01% Bovine Serum Albumin (BSA), 0.1% Tween 20) and mixed gently in order to avoid air bubbles. 0.1 g milk powder (1%) was added and once again mixed gently and then it was stored at 4°C until loading onto the microtiter plate.

- *Washing after incubation of samples*

Since there were high levels of toxin in the microtiter plate, the washing buffer for the two quick washing steps was discarded onto a large glass plate inside a fume hood. For the two five-minute washings it was discarded in the sink. Otherwise, this step was the same as the washing step described above.

3. *Loading of the detection antibody*

185 µl of the detection antibody solution stored at 4°C was loaded into each well of the microtiter plate using the multi-channel pipette. The microtiter plate was covered with parafilm and incubated at 37°C for 1 hour.

- *Washing after detection antibody incubation*

The detection antibody solution was discarded by turning the plate upside down and then tamped against some paper towels to remove remaining droplets. The washing was performed as above (washing solution always discarded in the sink).

4. *Dilution and loading of enzyme*

10 µl of neutravidin-linked alkaline phosphatase (0.9 µg/ml ImmunoPure NeutrAvidin™, Alkaline Phosphatase Conjugated, PIERCE in assay buffer) was added to 10 ml assay buffer in a 15 ml Falcon tube, and thereby diluted 1000 times. The tube was gently mixed to avoid air bubbles. The solution was added into a multi-channel pipette reservoir and then 100 µl was dispensed into each well of the plate. The plate was covered with parafilm and left to incubate at 37°C for 30 min.

- *Preparation of substrate*

During the incubation time, two tablets of substrate (SIGMA*FAST*™ p-Nitrophenyl phosphate tablets N2770, Sigma-Aldrich) were dissolved in 40 ml dH_2O in a 50 ml Falcon tube. The substrate is light sensitive and therefore aluminium foil was used to cover the tube. 11 ml was used for the microtiter plate and the remaining volume was stored in -20°C. The spectrophotometer was switched on in order for the lamp to warm up.

- *Washing*

Washing was performed as described above.

5. *Loading of substrate*

The substrate was transferred to a multi-channel pipette reservoir and then 100 µl was dispensed into each well of the microtiter plate. In this step it was important to work quickly and to avoid air bubbles which would disturb the signal. The plate was covered with both parafilm and aluminium foil and incubated in a dark cupboard at room temperature for 45 minutes.

- *Reading of microtiter plate*

Exactly after 45 minutes the microtiter plate was analyzed in a spectrophotometer at 405 nm (Multiskan Ascent, Thermo Electron Corporation). A standard curve was then constructed by plotting absorbance against toxin concentration. The linear regression equation of the standard curve was used to determine the toxin concentrations of the unknown samples. It was also controlled that the positive controls had equal values and that the blank samples had a low and equal absorbance.

5. RESULTS

5.1 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. The results and findings we obtained were published and are part of the dissertation as Supplement I. Here is just a brief synopsis of the outcome.

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.

5.2 Milk experiments

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

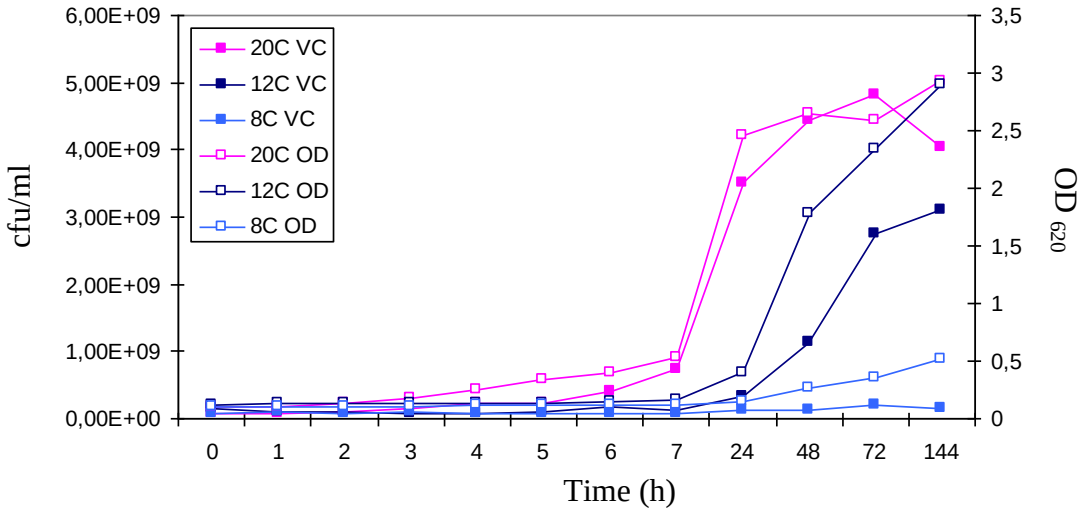
Growth of S. aureus in BHI and milk

To investigate the effect of three different temperatures, 8°C, 12°C and 20°C and two types of culture media, growth of *S. aureus* strain 45 in BHI medium and in fresh pasteurized milk was followed. The experiments were performed in small-scale fermentors. Samples were collected every hour between 0 and 7 h and at time points 24, 48, 72 and 144 h. Growth was measured with optical density at 620 nm and viable counts (VC) for samples in BHI and only viable counts for samples from milk. Two independent growth experiments were performed for each temperature and type of medium.

In BHI, growth, expressed as cfu/ml and OD_{620nm}, should be close to the ideal nutritional conditions as BHI is a rich culture medium (**Figure 5.1a**). At 20°C, the growth starts after 6 h and the exponential phase starts one hour later (at 7h) and continues until 24 h. After that, cells enter stationary phase and the growth is relatively stable until the end of the experiment at 144 h. At 12°C, the growth pattern is the same as for 20°C but delayed. The growth starts at 7 hour and exponential growth at 24 h. After 72 hours cells start to enter stationary phase of growth. At 8°C there is no growth observed. According to OD measurements there might be a small increase after 144 h but this is not supported by the VC data.

In milk, growth, expressed as cfu/ml, is more repressed compared to BHI (**Figure 5.1b**). At 20°C the exponential phase starts after 7 h and continues until 24 h. After that, cells enter stationary phase and growth is more or less stable. At 12°C, growth starts after 7 h in a non-exponential fashion and continues until the end of the experiment at 144 h. At 8°C, a possible increase in cfu/ml is observed after 72 h until 144 h. Comparison between growth in BHI and milk is described in more detail in **Figure 5.2**.

a)



b)

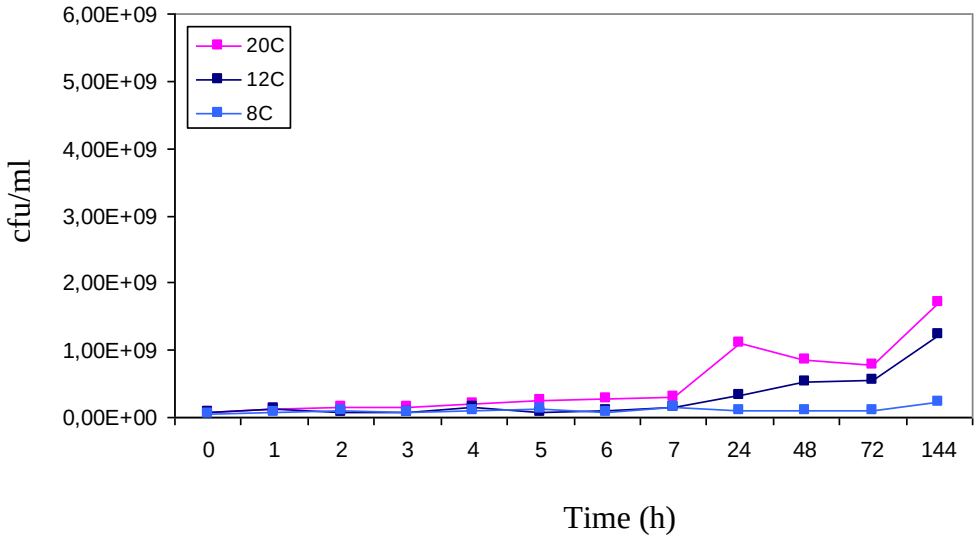
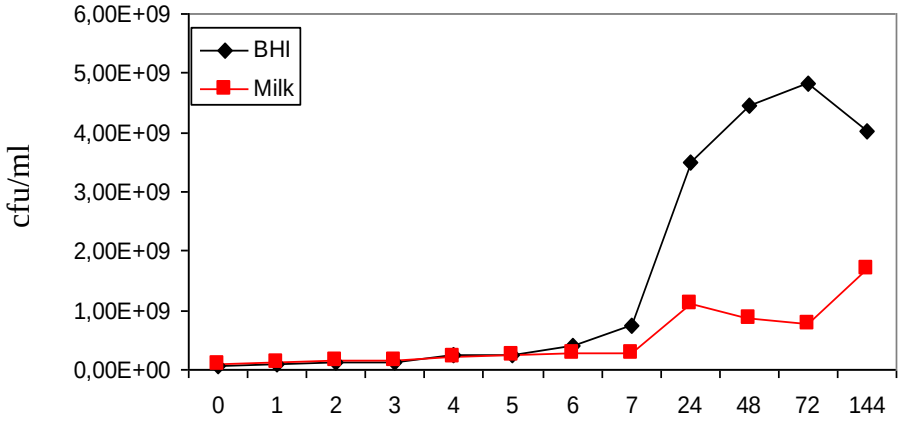


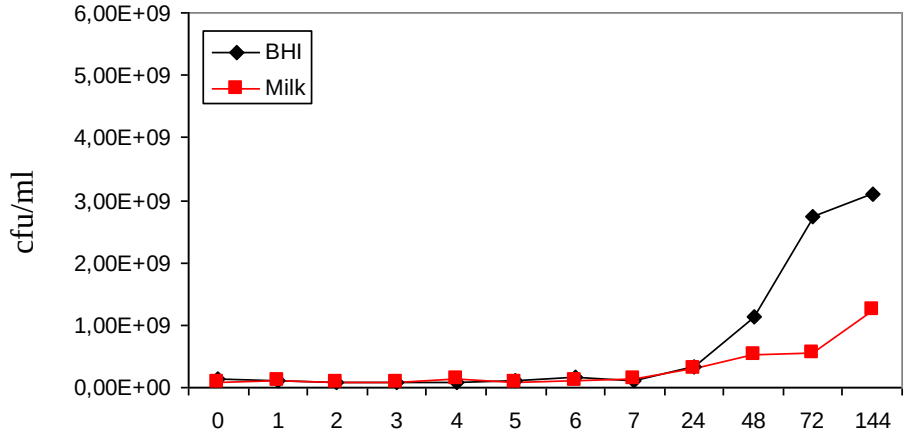
Figure 5.1 *S. aureus growth in BHI and milk at 8, 12 and 20°C. Average values of two replicas are shown. a) Growth in BHI is measured with* OD_{620nm} *(open squares) and viable count (VC, filled squares). b) Growth in milk is measured with VC (filled squares).*

a)



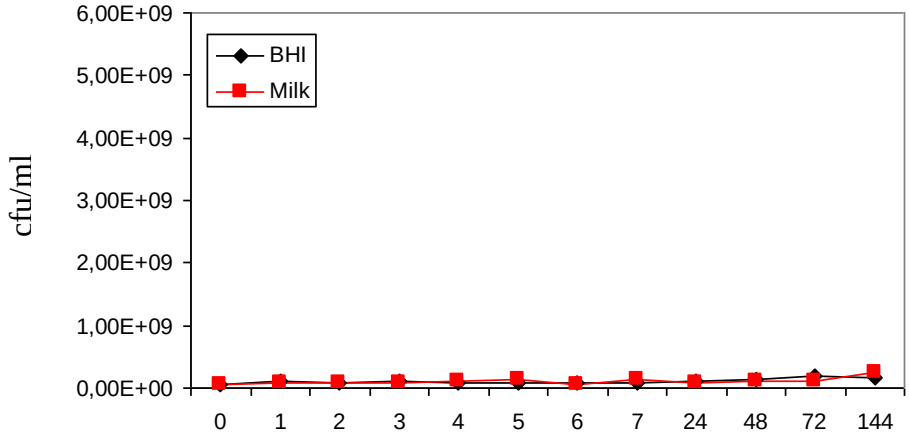
b)

b



c)

c



Time (h)

Figure 5.2 *Comparison between S. aureus growth in BHI and milk at different temperatures. a) 20°C; b) 12°C; c) 8°C. Average values of two replicas are shown.*

Relative expression of sed and SED formation

The growth, the level of *sed* mRNA and the extracellular SED concentration were determined from two independent growth experiments and are shown in **Figure 5.5**.

Total RNA was extracted from cell cultures during the incubation period in BHI. The same amount of total RNA (0.5 μg) from each cell culture was used for relative quantification of the transcript level of the mRNA of the *sed* gene. In order to correct for sample-to-sample variation, the *sed* transcript was normalized to the reference gene (the gene for 16S rRNA), *rrn*. The *rrn* gene was consistently expressed at all time points and very similar C_p values were obtained (**Figure 5.3.a**).

a) *b)*

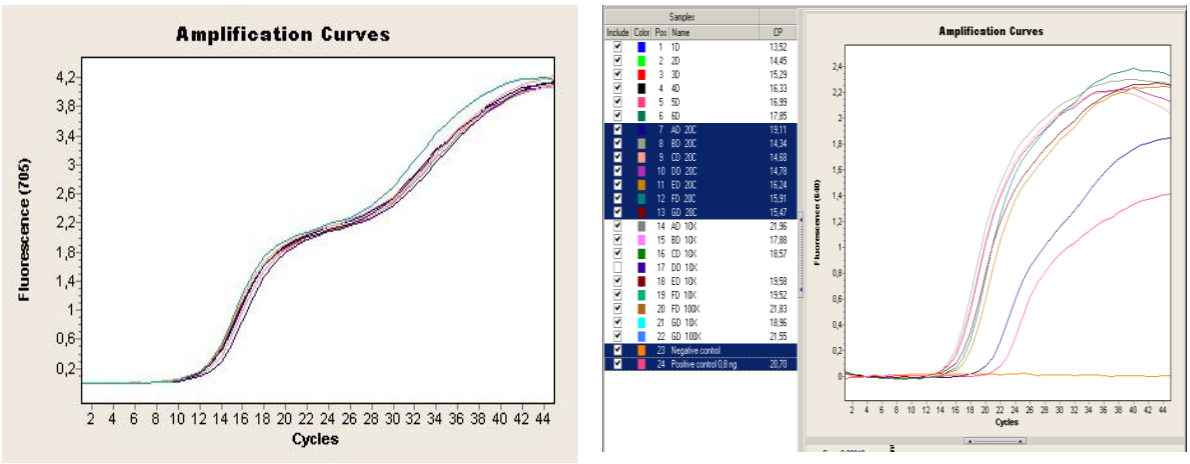


Figure 5.3 *Analysis of expression of reference gene and target gene using qRT-PCR. a) Amplification curves for rrn. b) Amplification curves for unknown samples for sed. Depending on time and growth conditions the amount of sed mRNA varies and thus different C_p values are obtained.*

a) *b)*

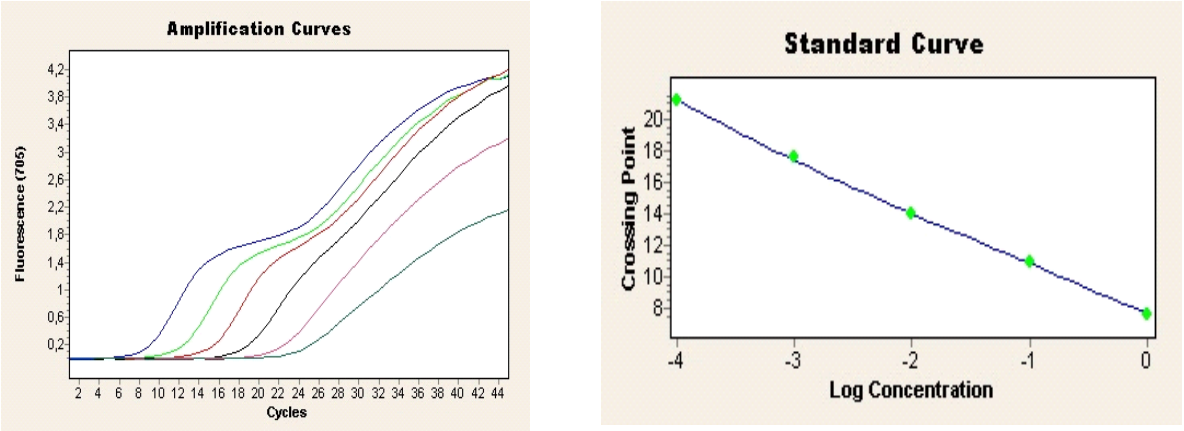
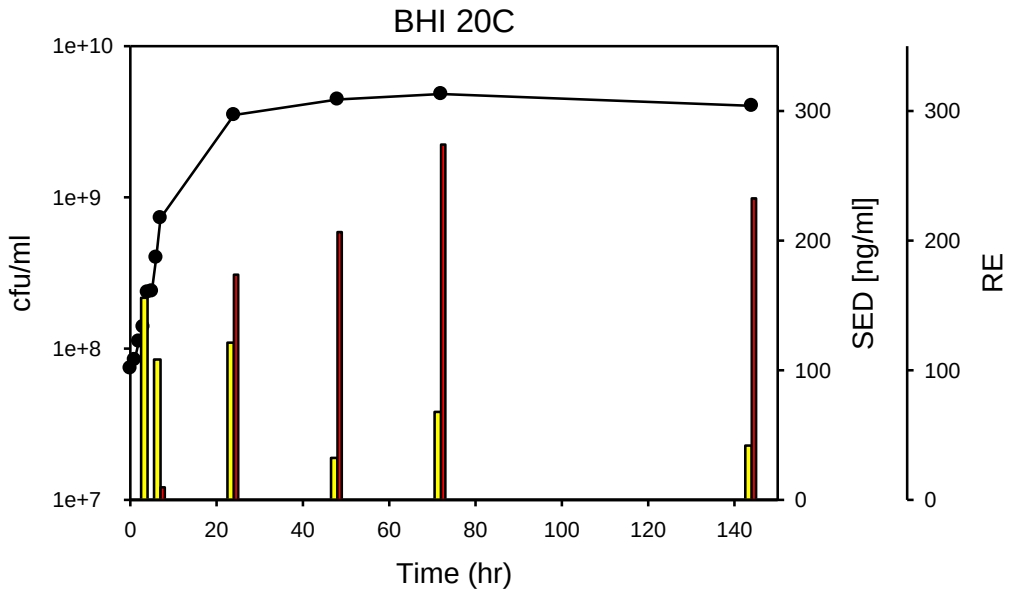


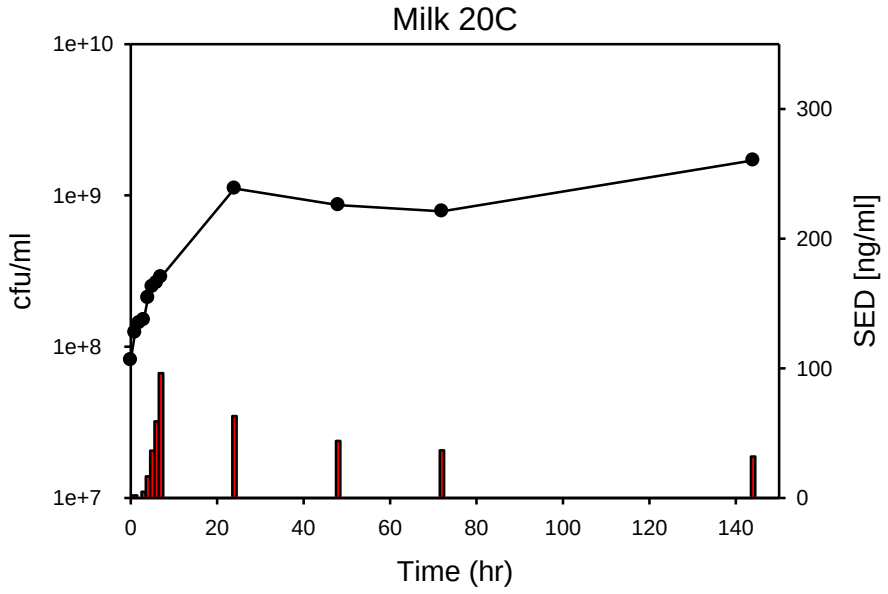
Figure 5.4 *Standard curve for rrn mRNA using qRT-PCR. a) Amplification curves. b) Standard curve constructed using obtained C_p values from the amplification curves.*

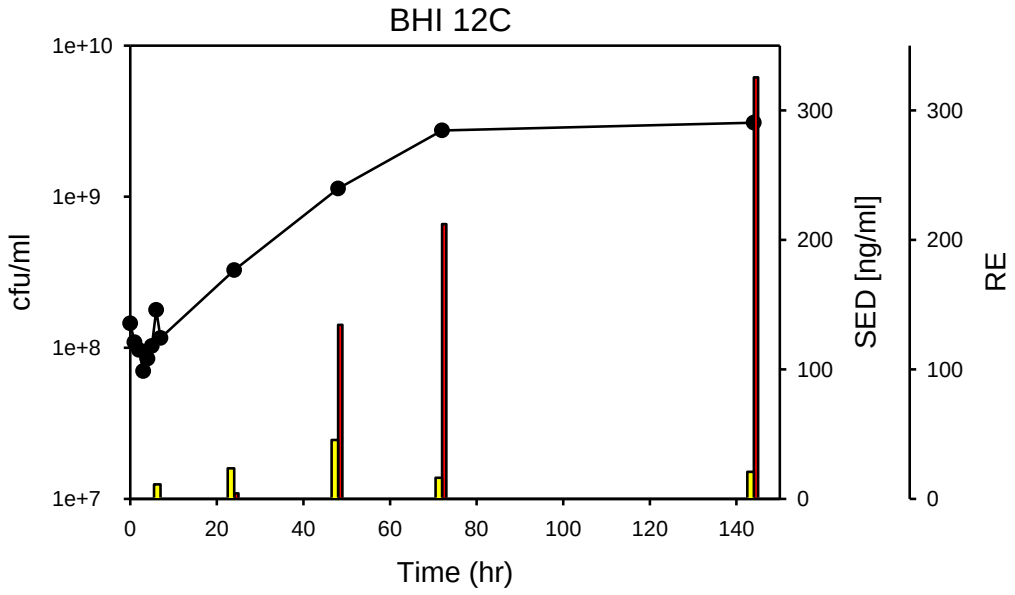
At 20°C, *sed* mRNA was detected at all RNA sampling time points. The highest level of *sed* mRNA was observed at 4 h and the first production of SED occurred during the exponential phase of growth about 3 hours later (**Figure 5.5** BHI 20C). The accumulated specific concentration of extracellular SED reached the highest level in the late exponential growth phase. After that the production stops and the level of mRNA decreases. The absolute amount of SED accumulates until 72 h where the highest concentration of ~ 280 ng/ml was measured. After that, the amount of SED remains stable. Specific production of SED (i.e. SED ng/ml per cfu) reaches a stable level after 24 hours and this correlates well with the growth and the absolute amount of SED. Data on specific SED production are not shown. The results indicate that there is no specific induction or repression of *sed* expression or SED formation and that the expression of *sed* is not affected under these conditions. In milk at 20°C, the SED production starts to increase already after 4 h and SED is accumulated until 7 h where a concentration of ~100 ng/ml is measured (**Figure 5.5** Milk 20C). The absolute amount then decreases steadily until 144 h indicating that SED might be somehow degraded or metabolized. Specific SED production in milk is significantly higher when compared to BHI and increases until 7 h. As the growth in milk is more repressed during this time than in BHI it is an indication that cells are more and more stressed and up-regulate the exterotoxin formation.

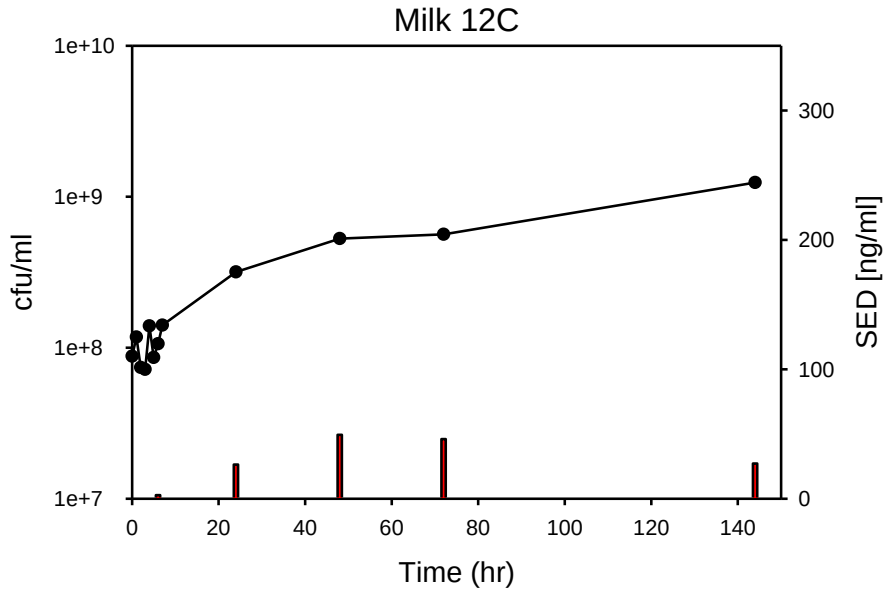
At 12°C *sed* mRNA appears after 7 h and the gene is actively expressed until 48 h, when cells are in exponential phase, followed by a decrease in expression as cells enter the stationary phase (**Figure 5.5** BHI 12C). The absolute amount of SED follows the same pattern as for 20°C but it is delayed about 20 h. The production occurs during exponential phase of growth and starts after 24 h. It continues until the end of the experiment at 144 h where the highest concentration of ~330 ng/ml is detected. In milk at 12°C, the first production of SED appears after 5 h and reaches the highest concentration of ~50 ng/ml at 48 h, followed by a decrease at 144 h what has been observed in milk at 20°C, but delayed (**Figure 5.5** Milk 12C).

At 8°C, there is no growth observed although there is a minute increase at 72 h when a high level of *sed* mRNA is observed (**Figure 5.5** BHI 8C). This is however not followed by protein synthesis as no active SED production was detected either in BHI or milk (**Figure 5.5** Milk 8C). The signal we got from the ELISA indicated there is a presence of enterotoxin but the level was stable throughout the experiment and the absolute amount of SED was less than 7 ng/ml.


BHI 20C
cfu/ml
1e+10
1e+9
1e+8
1e+7
SED [ng/ml]
RE
300
200
100
0
0
20
40
60
80
100
120
140
Time (hr)


Milk 20C
cfu/ml
1e+10
1e+9
1e+8
1e+7
SED [ng/ml]
300
200
100
0
0
20
40
60
80
100
120
140
Time (hr)


BHI 12C
cfu/ml
1e+10
1e+9
1e+8
1e+7
SED [ng/ml]
RE
300
200
100
0
0
20
40
60
80
100
120
140
Time (hr)


Milk 12C
cfu/ml
1e+10
1e+9
1e+8
1e+7
SED [ng/ml]
300
200
100
0
0
20
40
60
80
100
120
140
Time (hr)

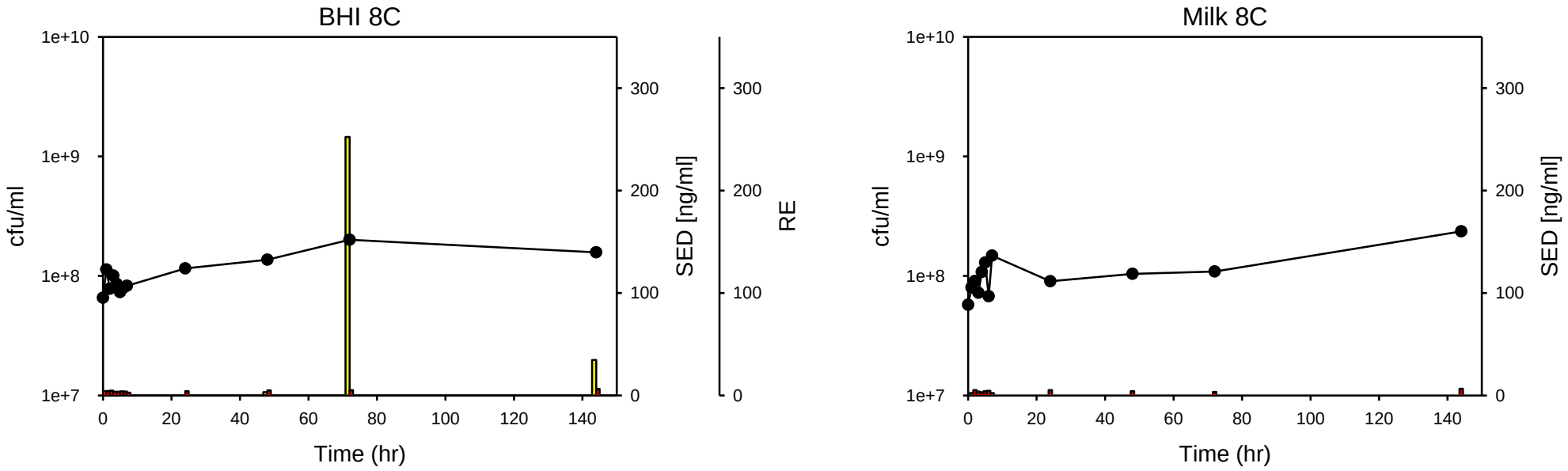


Figure 5.5 *Growth of S. aureus, relative sed levels in BHI and SED concentration when grown at 20, 12 and 8°C in BHI and milk. (•) Growth curve; (yellow bars) relative sed mRNA levels in BHI; (red bars) absolute SED concentration. The average of two independent growth experiments for each temperature and growth media are presented.*

5.2.2 The combined effect of 12°C and background flora

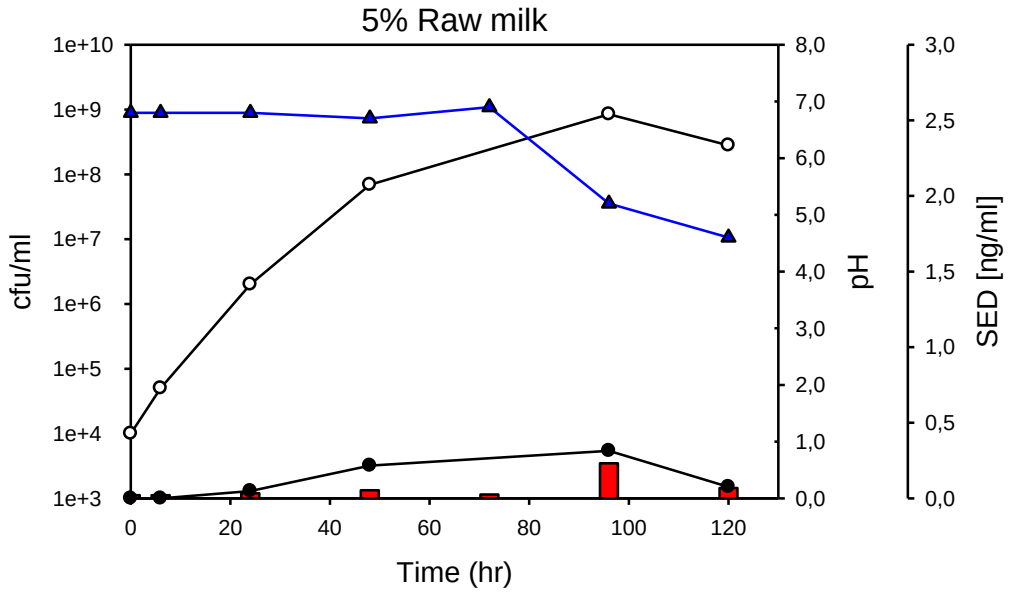
In these experiments, the combined influence of low temperature (12°C) and the presence of a competitor (BGF originating from raw milk provided by ARLA Foods) on *S. aureus* growth and enterotoxin A and D production in pasteurized milk with 3% fat content was investigated. The competitor (BGF) was introduced 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. 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. No raw milk was added to milk in the control experiments. (**Figure 5.6**) One growth experiment was performed for 0.5% raw milk experiment with strains SA 161:3 and SA 168:1 and two independent experiments for 0.1% raw milk experiment with the strain SA 161:3 (**Figure 5.7**). The parameters for the experiments in terms of starting concentration of *S. aureus* cells, the amount of BGF added and also types of *S. aureus* strains (SA45, SA 161:3 and 168:1) varied from run to run according to previous results and feedback from Biotracer partner. The total count of raw milk and pasteurized milk determined by PCA was between 1.5 - 6 $\times$ 10^4 cfu/ml and between 4.5 $\times$ 10^1 and 3 $\times$ 10^2 cfu/ml, respectively.

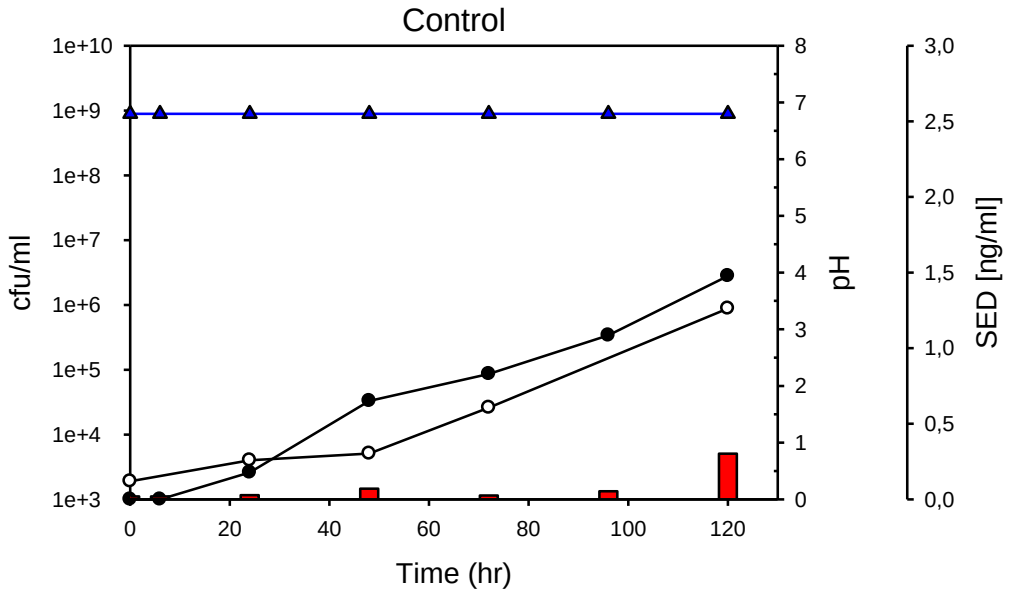
In all experiments with the strain SA 45, very mild growth of *S. aureus* was observed regardless the lower amount of BGF or higher starting concentration. If the starting concentration of *S. aureus* was between 10^3 and 10^4 cfu/ml, the population never reached the cell density (10^5 – 10^6 cfu/ml) 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 entrerotoxin D production correlated with the growth. In general, SED production was very low, between 0 and 3 ng/ml, more often close to 1 ng/ml. In one 0.5% raw milk experiment that had to be terminated after 3 days and is not included, the starting concentration was as high as 10^7 cfu/ml and without regard to slight growth, after 48 hours the enterotoxin production reached a concentration of ~ 5 ng/ml for SED and ~ 12 ng/ml for SEA. It follows that the higher the starting concentration the higher the enterotoxin concentration measured.

In order to test strain variation (*S. aureus* strains with different origins), the experiments with the *S. aureus* strains 161:3 and 168:1 were performed (**Figure 5.7**). The growth of both strains was similar to the one of SA 45, very mild. Neither of strains grew better than the other although the strain 168:1 started with lower starting concentration but caught up later in incubation period. However, the strain 161:3 produced more SEA, with the highest

concentration of ~3 ng/ml, than the strain 168:1 with the concentration of 0.5 ng/ml. The two independent experiments with the strain 161:3 and the lowest amount of BGF (0.1 % of raw milk) showed slightly better growth due to less competition but no production of SEA (the values obtained from ELISA were evaluated as blank sample values).

pH was measured in all experiments. The starting pH was ~6.8. Usually after 4 days when BGF reached a higher population and more acidic products were produced, a drop in pH was observed and milk started to coagulate. The bigger the amount of BGF the bigger and earlier in time the drop in pH. After 5 days milk was more or less coagulated and the experiments were terminated.


5% Raw milk
cfu/ml
pH
SED [ng/ml]
Time (hr)
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
8,0
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
20
40
60
80
100
120


Control
cfu/ml
pH
SED [ng/ml]
Time (hr)
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
8
7
6
5
4
3
2
1
0
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
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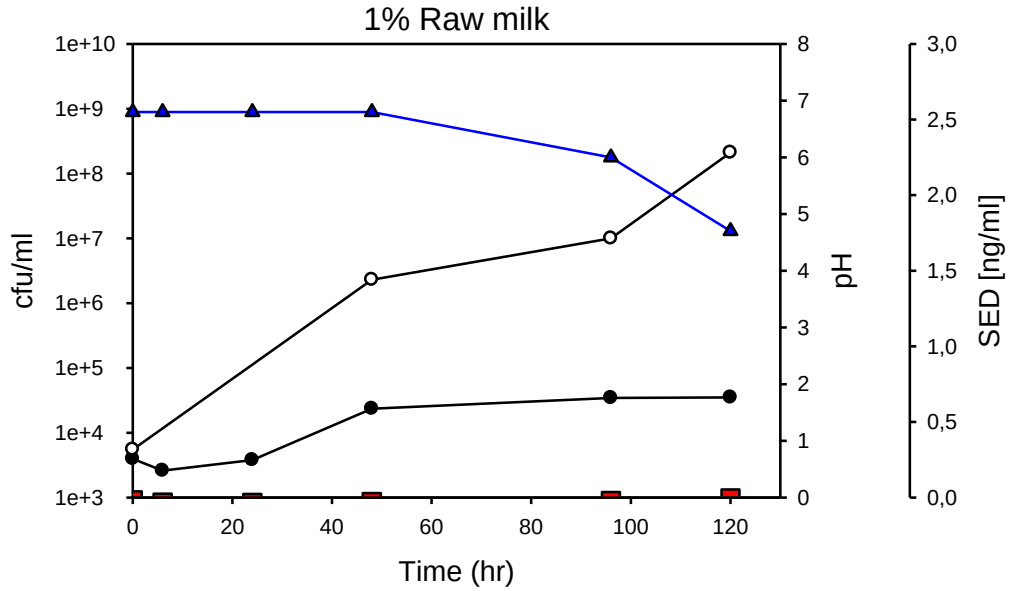
40
60
80
100
120


1% Raw milk
cfu/ml
pH
SED [ng/ml]
Time (hr)
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
8
7
6
5
4
3
2
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1,0
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0
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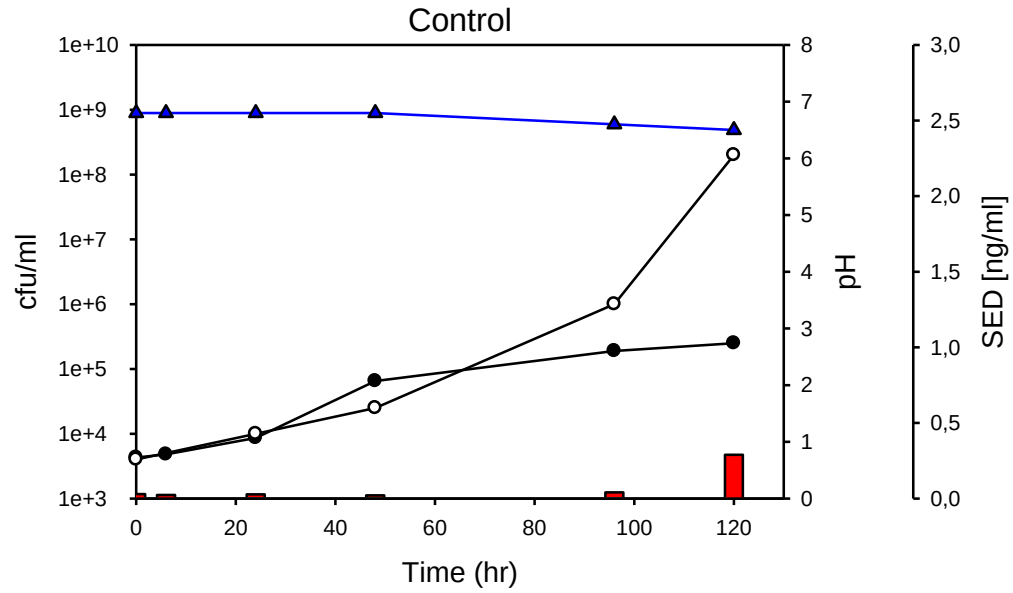
40
60
80
100
120


Control
cfu/ml
pH
SED [ng/ml]
Time (hr)
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1e+9
1e+8
1e+7
1e+6
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1e+4
1e+3
8
7
6
5
4
3
2
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3,0
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0,5
0,0
0
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40
60
80
100
120

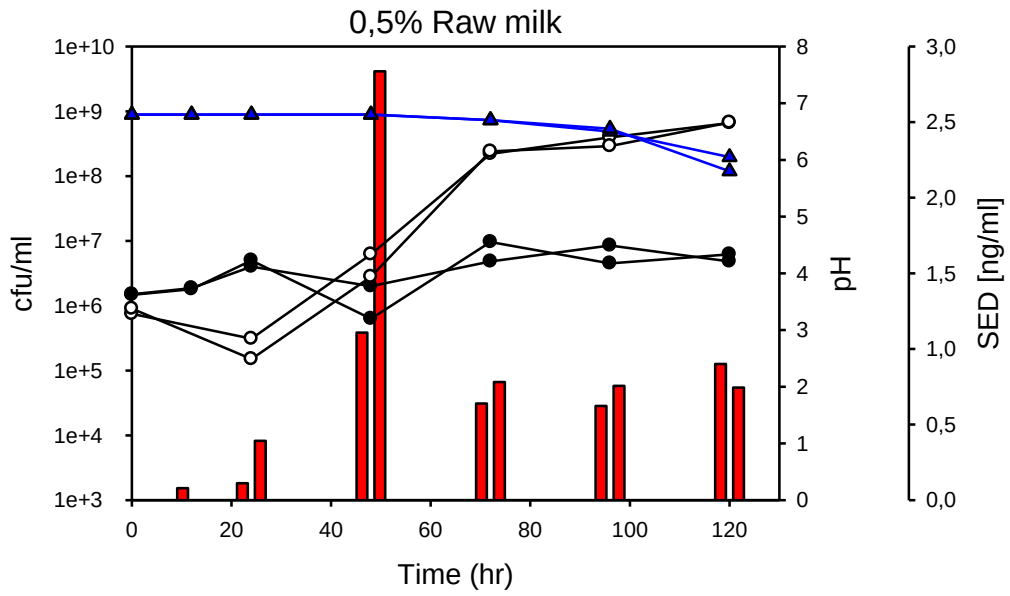
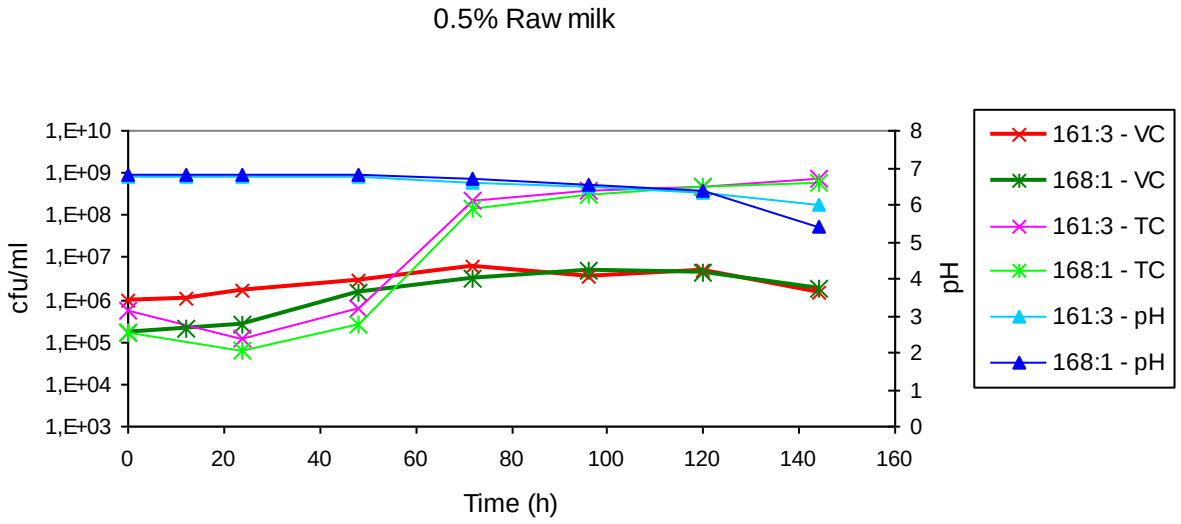
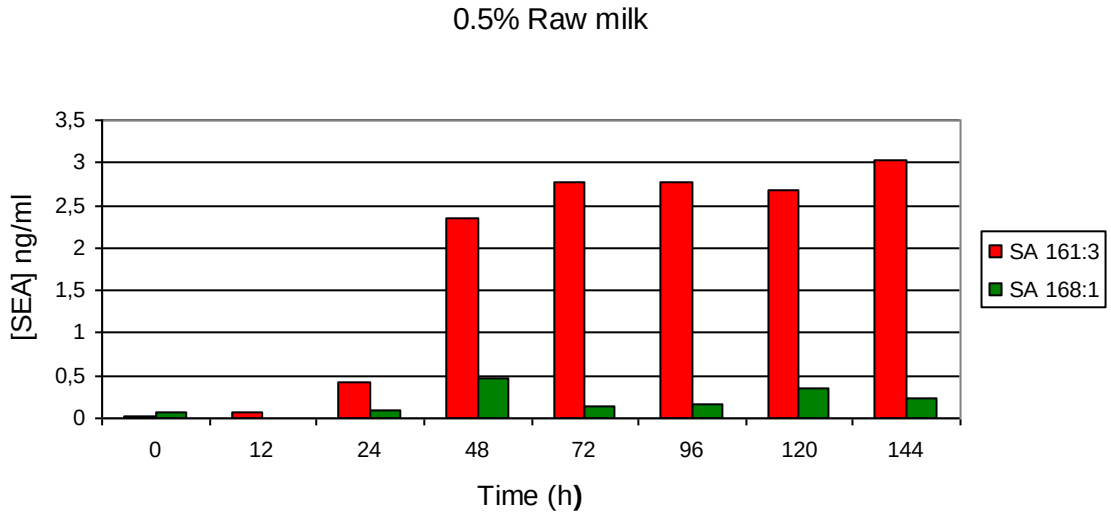


Figure 5.6 *(for all graphs) Growth of S. aureus (SA 45) and background flora coming from raw milk, pH and SED production in milk at 12°C. (•) Growth curve for S. aureus; (○) Growth curve for BGF; (▲) pH; (red bars) absolute SED concentration. No raw milk was added to milk in the control experiments. 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)



b)



c)

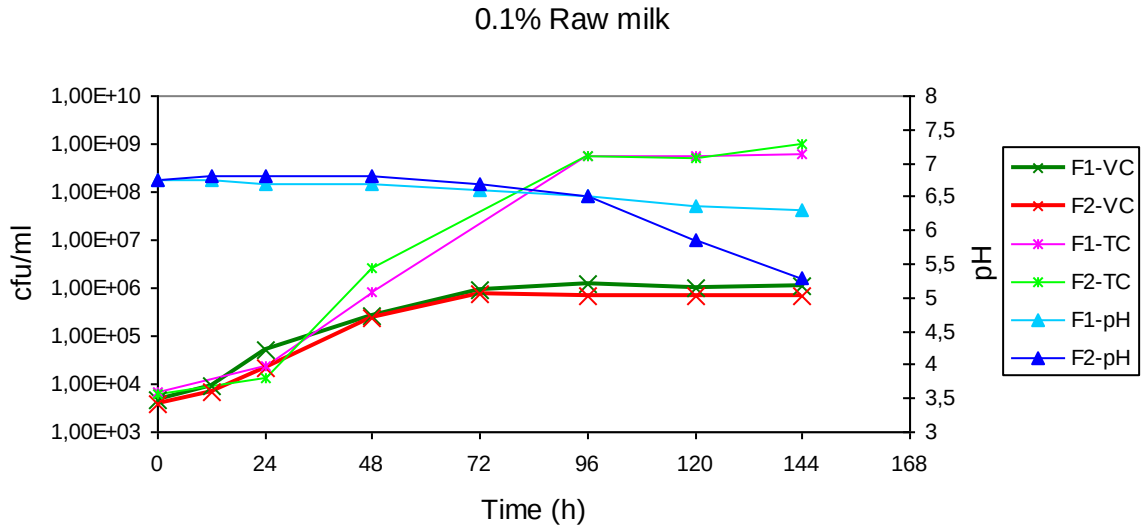


Figure 5.7 *Growth of S. aureus (SA 161:3 and SA 168:1) and background flora coming from raw milk, pH and SEA production in milk at 12°C. a) Growth of S. aureus described as viable count (VC), growth of BGF described as total count (TC), pH; b) absolute SEA concentration; c) Growth of S. aureus described as viable count (VC), growth of BGF described as total count (TC), pH. Two independent experiments with the strain 161:3.*

5.3 Cheese experiments

To study the growth of *S. aureus* in cheese, 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 5.8 *a) Cream cheese samples inoculated with different strains. b) Example of large unidentified colonies on BHI.*

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

25 g of semi soft cheese were inoculated with one of four strains of *S. aureus* (161:3, 168:1, 45 and 564) and incubated at 13°C. For each strain, two biological replicas were made (A and B). Samples were taken after 0 hours, and then after 3, 6, 13, 19 and 27 days. 54 samples of cheese were analyzed in total.

Growth

The growth of *S. aureus* strains is demonstrated in **Figure 5.9a**. The inoculum size, i.e. the number of viable *S. aureus* cells in the cheese at 0 hour sampling point, was between 2.7×10^3 and 2.4×10^5 cfu/g in the different cheese samples (counted on BPA). The growth of *S. aureus* 161:3 was slowly declining with time. At 27 days there were no viable cells detected

in replica A. The strain 168:1 kept surviving for the first 6 days, then we could not detect any cells at 13 days, we got high numbers at 19 days and no cells again at 27 days. The growth of *S. aureus* 45 shows slight decrease during the first 13 days. At 19 days there is an increase by 2 log units for both replicas and at 27 days, another increase by scant 2 log units for replica B. The growth of *S. aureus* 564 also decreased during the first 13 days. Replica B then starts to increase and gets up to 10^6 cells per gram of cheese at 27 days while no viable cells for replica A were detected after 13 days. In this experiment none of the cheese samples showed visible contamination with mold.

The total cell count and cell count of lactic acid bacteria presented in the different cheese samples are the averages of two independent replicas, A and B. The level of lactic acid bacteria corresponds to the total cell count and is more or less constant throughout the experiment (**Figure 5.9d**). In the control samples (inoculated with NaCl instead of cells) no *S. aureus* cells were detected and the total cell count and lactic acid bacteria are slightly increasing with time (**Figure 5.9e**).

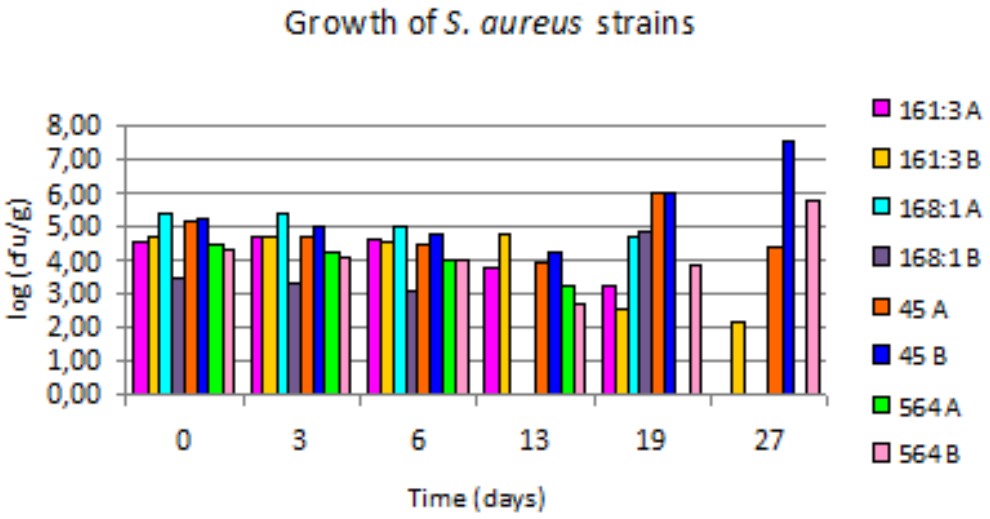
pH

The pH of the cheese samples was measured at each sampling point. The pH was around 5.6 – 5.7 at the very beginning and increased to 5.9 – 6.3 after 27 days for all samples, including the control samples.

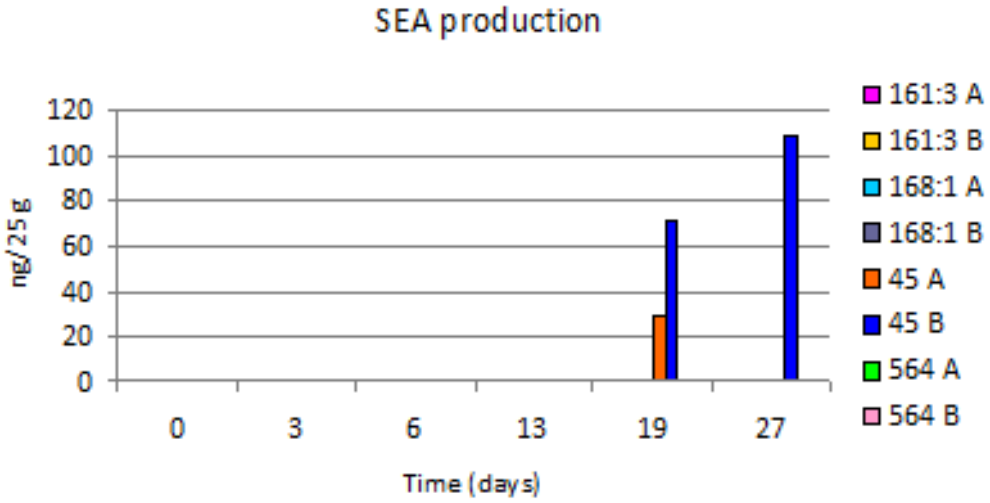
Enterotoxin production

The entrerotoxin level was measured in all samples (except the 0 hour samples and the control samples) using ELISA. *S. aureus* 161:3, 168:1 and 564 did not produce any detectable levels of enterotoxin A or D in any of the cheese samples. For both replicas of *S. aureus* 45, SEA and SED were detected at 19 days. At 27 days however, with the drop of growth of replica A, SEA and SED could only be detected in replica B (**Figure 5.9b,c**). None of the samples contained any detectable levels of TSST (data not shown).

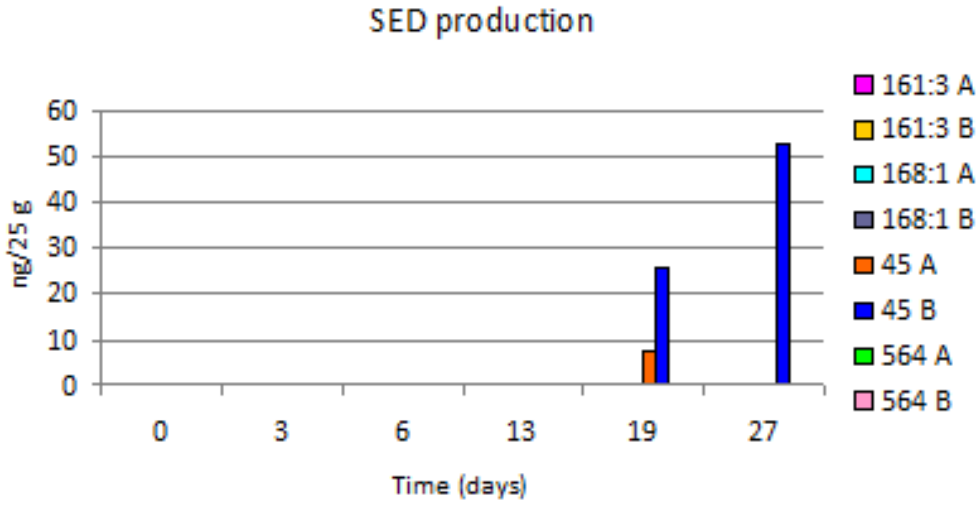
a)


Growth of S. aureus strains
log (cfu/g)
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
0
3
6
13
19
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

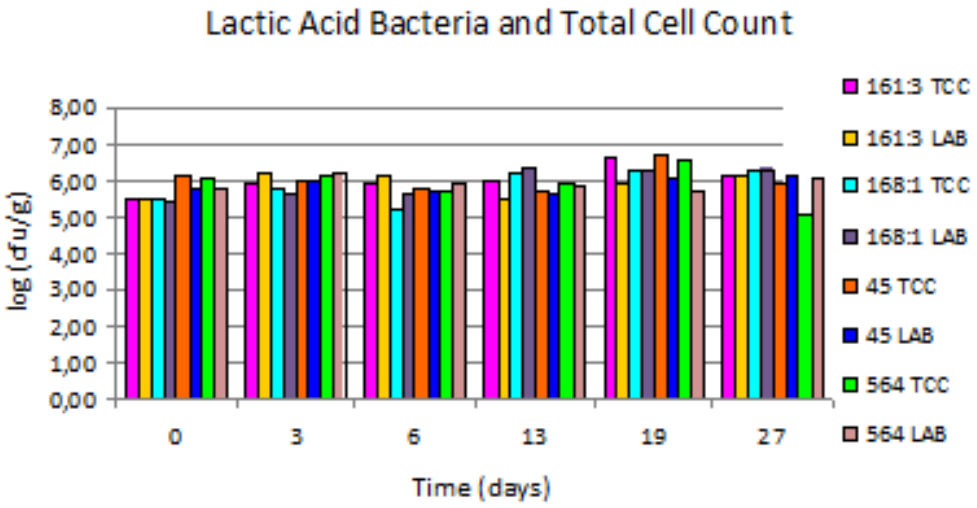
b)


SEA production
ng/25 g
120
100
80
60
40
20
0
0
3
6
13
19
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

c)


SED production
ng/25 g
60
50
40
30
20
10
0
0
3
6
13
19
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

d)



e)

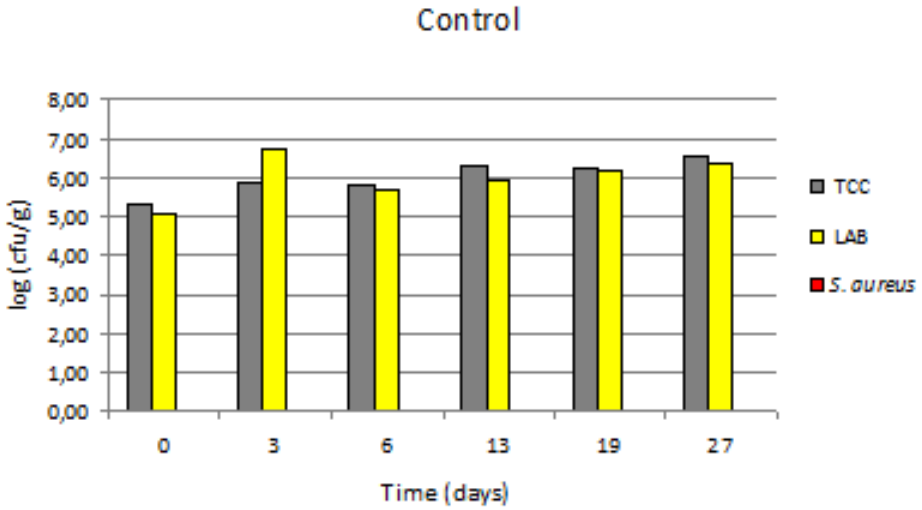


Figure 5.9 *a) Growth of S. aureus strains 161:3, 168:1, 45 and 564 in semi-soft cheese at 13°C. Two independent replicas, A and B. b) Total amount of enterotoxin A found in the 25 g of cheese samples, measured with ELISA. c) Total amount of enterotoxin D found in the 25 g of cheese samples, measured 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.*

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

25 g of semi soft cheese were inoculated with one of four strains of *S. aureus* (161:3, 168:1, 45 and 564) and incubated at 20°C. For each strain, two biological replicas were made (A and B). Samples were taken after 0 hours, and then after 3, 6, 13, 19 and 27 days. 54 samples of cheese were analyzed in total.

Growth

The inoculum size of *S. aureus* cells was between 5.2×10^3 and 1.3×10^5 cfu/g of cheese. The growth of the *S. aureus* strain 161:3 (replicas A and B) decreased steadily and no viable cells were present at 19 days for replica B. The strain 168:1, starting with lower inoculum size, kept surviving during first 6 days then we recorded increase of 1 log unit for replica A at 13 days, followed by decrease at 19 days and no viable cells for both replica at 27 days. A temporary increase in growth was recorded for replica B at 19 days where however mold was present on the cheese. The strain 45 maintained its cell level with slight increase in growth for replica A and slight decrease for replica B starting at 13 days. Furthermore, in the 19 days sample of strain 45 A mold was present and an increase in growth was observed. Also in the 27 days sample growth was increase even though no visible mold was detected. The growth of the strain 564 decreased with time with no viable cells for replica A at 19 days. The samples 564 A, 27 days and 564 B, 19 days both got contaminated with mold and showed an increase in growth. See **Figure 5.10a**.

The total cell count and lactic acid bacteria cell count (averages of independent replicas A and B) both increased with time (**Figure 5.10d**).

There was a slight increase in growth of lactic acid bacteria observed in control samples (**Figure 5.10e**).

pH

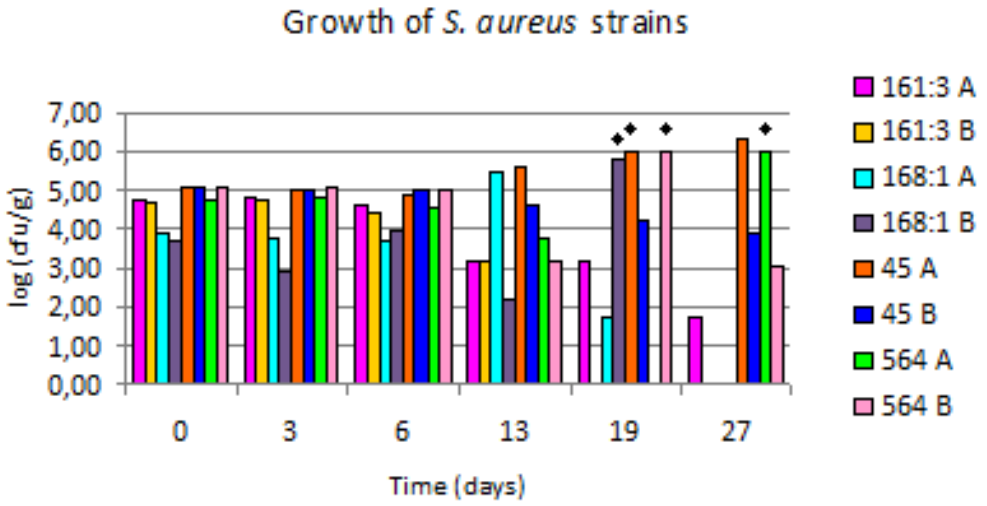
pH was slightly increasing with time. The pH was between 5.6 and 5.9 at 3 days and 6.3 and 6.6 after 27 days for all the samples (including the control samples), except one. For sample 564 A where mold was present, pH had increased to 7.3 after 27 days.

Enterotoxin production

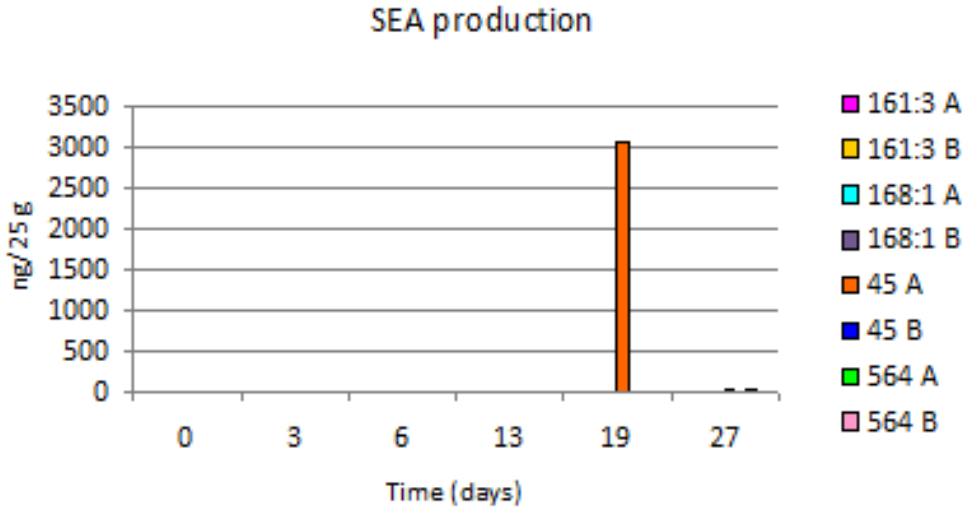
The enterotoxin level was measured with ELISA in all cheese samples, except the 0 hour samples and the control samples. There were no detectable levels of SEA or SED in any of the cheese samples inoculated with either *S. aureus* strain 161:3 or 168:1. The strain 45 A showed high levels of both SEA and SED in the 19 days cheese sample (contaminated with mold) and there was a detectable amount of SEA in the 27 days cheese sample (no mold). There was also a detectable amount of SEA in the 27 days cheese sample for the strain 45 B.

There was a high amount of SED present in the sample 564 A, 27 days (mold contamination) and no detectable amount of SED in the sample 564 B, 19 days (mold contamination) (**Figure 5.10b,c**). None of the samples contained any detectable levels of TSST (data not shown).

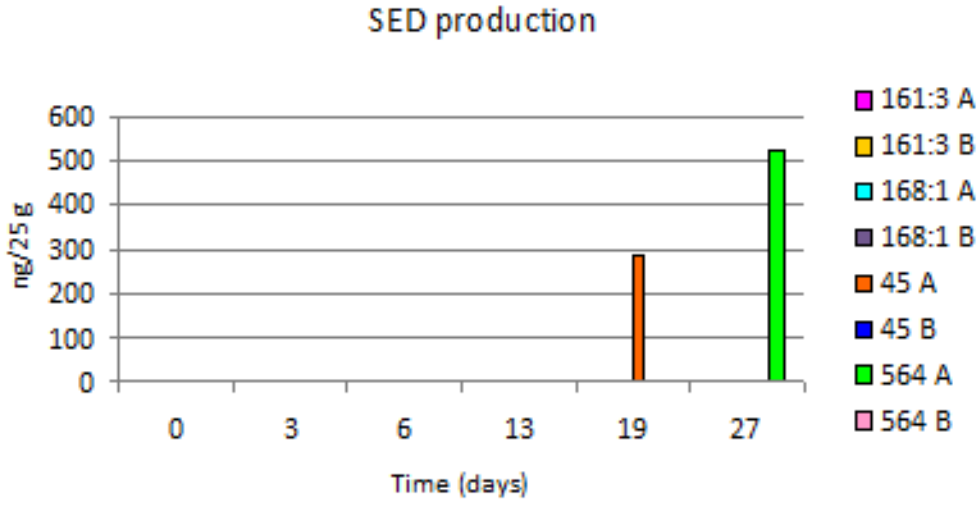
a)


Growth of S. aureus strains
log (cfu/g)
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
0
3
6
13
19
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

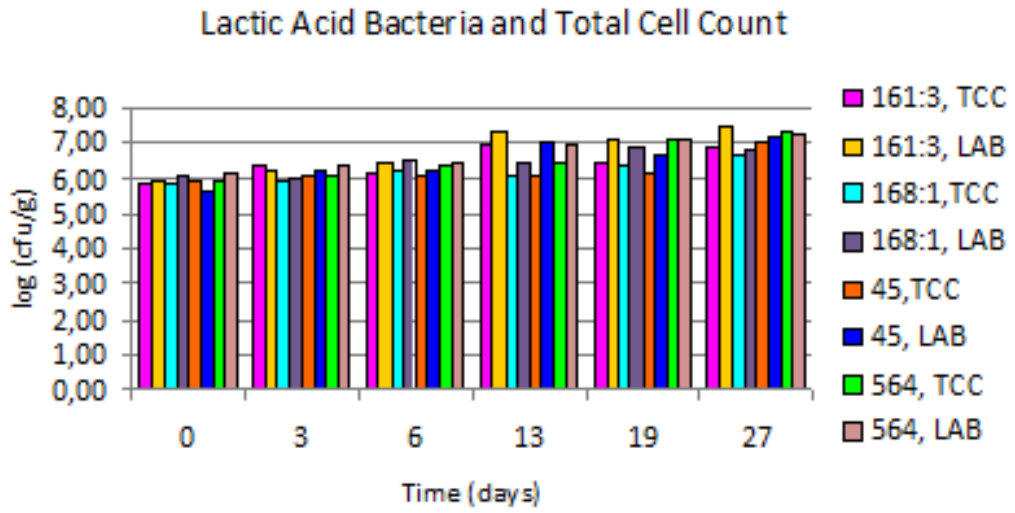
b)


SEA production
ng/25 g
3500
3000
2500
2000
1500
1000
500
0
0
3
6
13
19
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

c)


SED production
ng/25 g
600
500
400
300
200
100
0
0
3
6
13
19
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

d)



e)

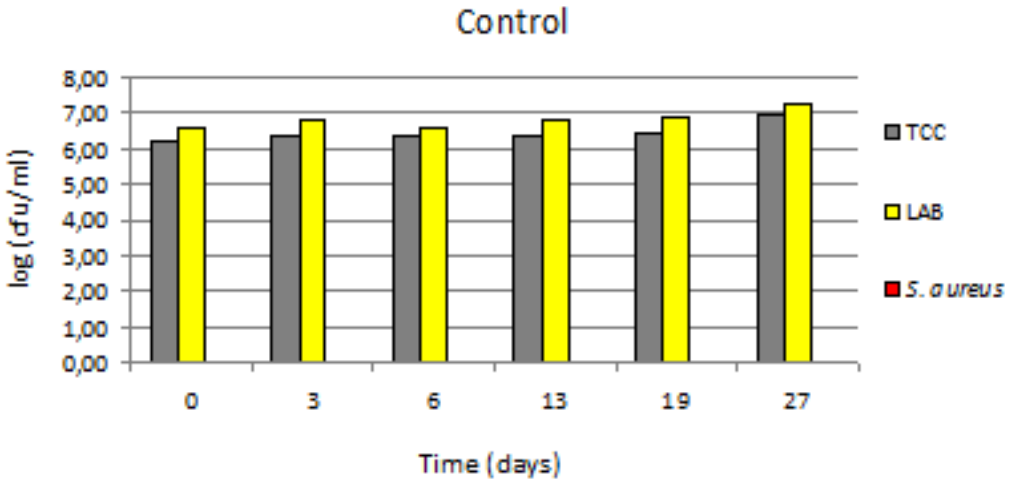


Figure 5.10 *a) Growth of S. aureus strains 161:3, 168:1, 45 and 564 in semi-soft cheese at 20°C. Two independent replicas, A and B. ♦ Samples contaminated with mould. b) Total amount of enterotoxin A found in the 25 g of cheese samples, measured with ELISA. c) Total amount of enterotoxin D found in 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.*

5.3.3 Experiment 3: Cream cheese, 13°C

25 g of cream cheese were inoculated with one of four strains of *S. aureus* (161:3, 168:1, 45 and 564) and incubated at 13°C. For each strain, two biological replicas were made (A and B). Samples were taken after 0 hours, and then after 3, 6, 13, 19 and 27 days. 54 samples of cheese were analyzed in total.

Growth

The higher inoculum level, between 4.1×10^6 and 7.7×10^6 cfu/g of cheese, was reached in this experiment. All strains maintained their high levels till 13th day of the experiment, some considerable changes happened afterwards. The strains 161:3 A and 564 A at 20 days and 161:3 A, 168:1 A and 45 A at 27 days were not detected at all. The growth of the strains 161:3 B and 168:1 B at 20 days and the strains 161:3 B and 564 A at 27 days was boosted by contamination with mold. The other strains were slowly dying off (**Figure 5.11a**).

The total count (averages of two independent replicas, A and B), seemed to correspond to the amount of *S. aureus* cells and was decreasing with time (**Figure 5.11d**). The MRS plates showed that there are no lactic acid bacteria present in the cream cheese samples in this experiment. Therefore, bars for lactic acid bacteria count were not included.

The control samples had no (or very low numbers of) cells that could be detected on MRS, BPA or BHI. Some of the BHI plates supported growth of large unidentified colonies of irregular shape and size that were impossible to count (**Figure 5.8b**).

pH

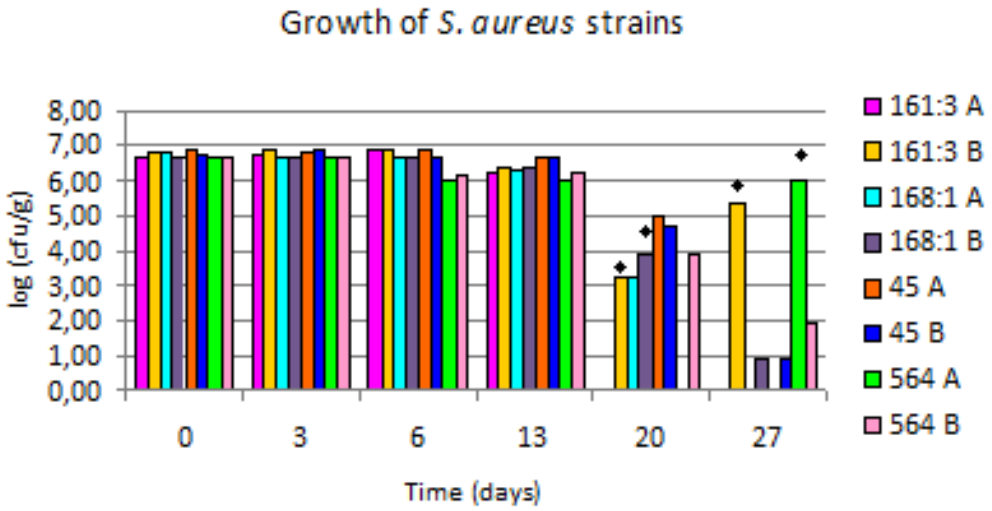
The pH was measured at all sampling points, starting with pH of 5.2 – 5.3 at 0 day and reaching pH values between 5.5 and 5.9 at the end of the experiment.

Enterotoxin production

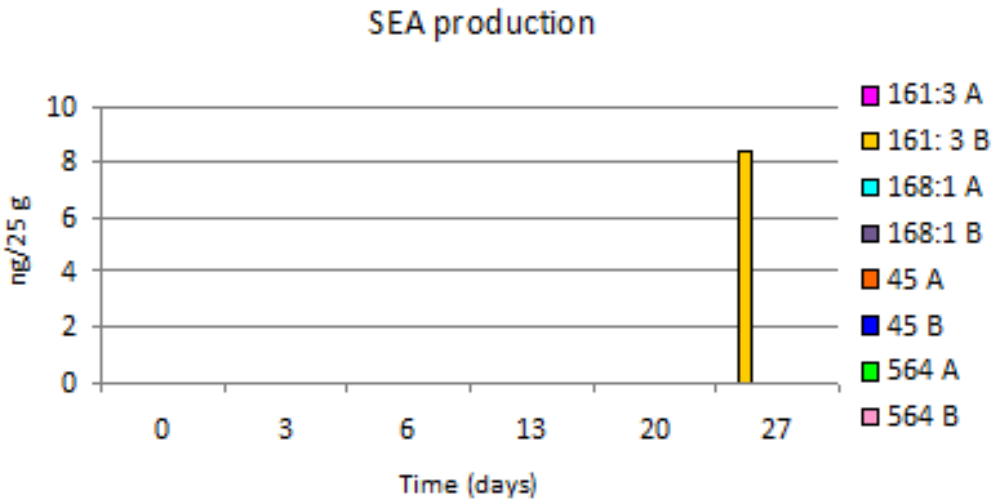
Four of the cream cheese samples contained low, but detectable amounts of enterotoxin.

Low production of SEA was observed in the sample 161:3 B, 27 days. Low production of SED was detected after 27 days in the samples 45 A, 564 A and 564 B (**Figure 5.11b,c**). None of the samples contained any detectable levels of TSST (data not shown).

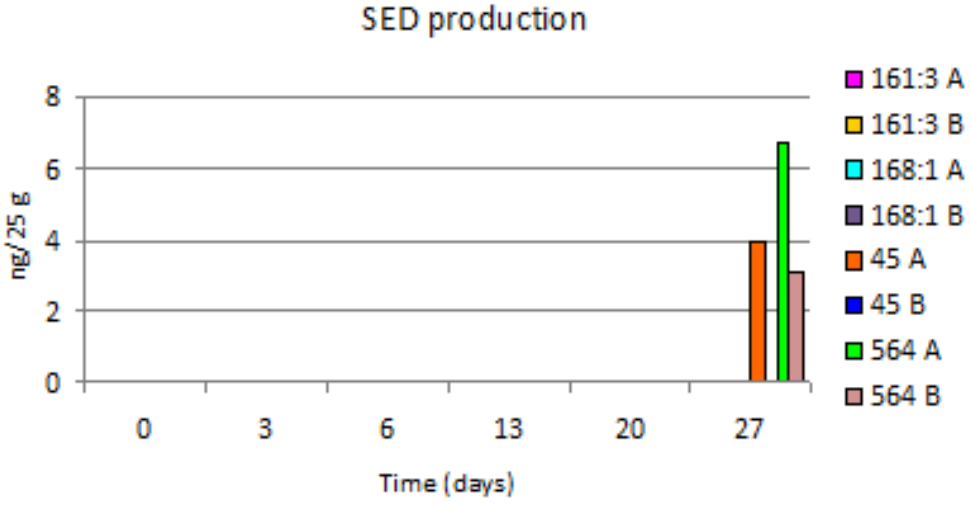
a)


Growth of S. aureus strains
log (cfu/g)
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
0
3
6
13
20
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

b)


SEA production
ng/25 g
10
8
6
4
2
0
0
3
6
13
20
27
Time (days)
161:3 A
161: 3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

c)


SED production
ng/25 g
8
6
4
2
0
0
3
6
13
20
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

d)

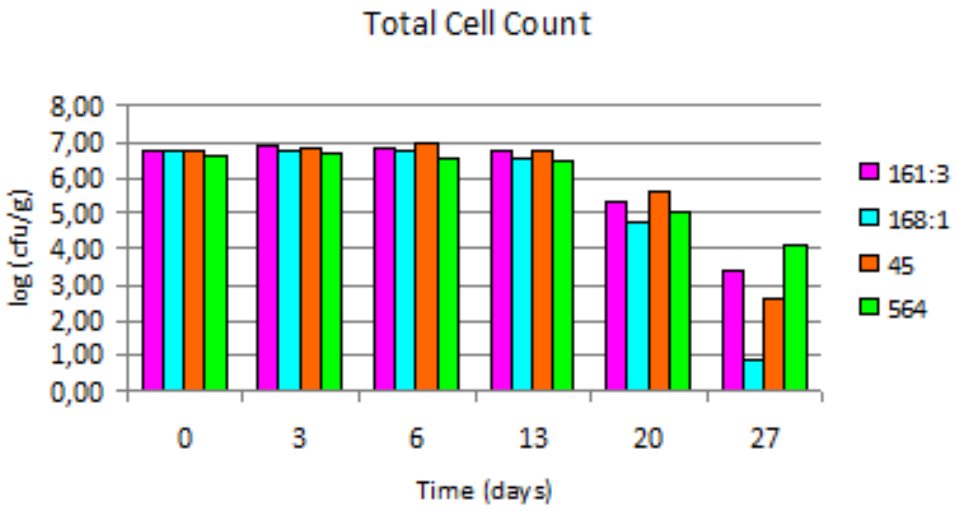


Figure 5.11 *a) Growth of S. aureus strains 161:3, 168:1, 45 and 564 in cream cheese at 13°C. Two independent replicas, A and B. ♦ Samples contaminated with mould. b) Total amount of enterotoxin A found in the 25 g cheese samples, measured with ELISA. c) Total amount of enterotoxin D found in the 25 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.*

5.3.4 Experiment 4: Cream cheese, 20°C

25 g of cream cheese were inoculated with one of four strains of *S. aureus* (161:3, 168:1, 45 and 564) and incubated at 20°C. For each strain, two biological replicas were made (A and B). Samples were taken after 0 hours, and then after 3, 6, 13, 19 and 27 days. 54 samples of cheese were analyzed in total.

Growth

The inoculum size at 0 hour sampling was between 2.2×10^2 and 3.3×10^3 cfu/g of cheese. During the course of the experiment the number of viable *S. aureus* cells was steadily decreasing for all of the four strains (**Figure 5.12a**). At 13 days, the strains 161:3 A and B and 564 A and B were no longer detected. At 20 days, only the strain 168:1 kept surviving. At 27 days, two samples (161:3 B and 45 A) were found with mould and the growth of *S. aureus* was markedly increased. The sample 564 B, 20 days had visible mould but no *S. aureus* could be detected on BPA

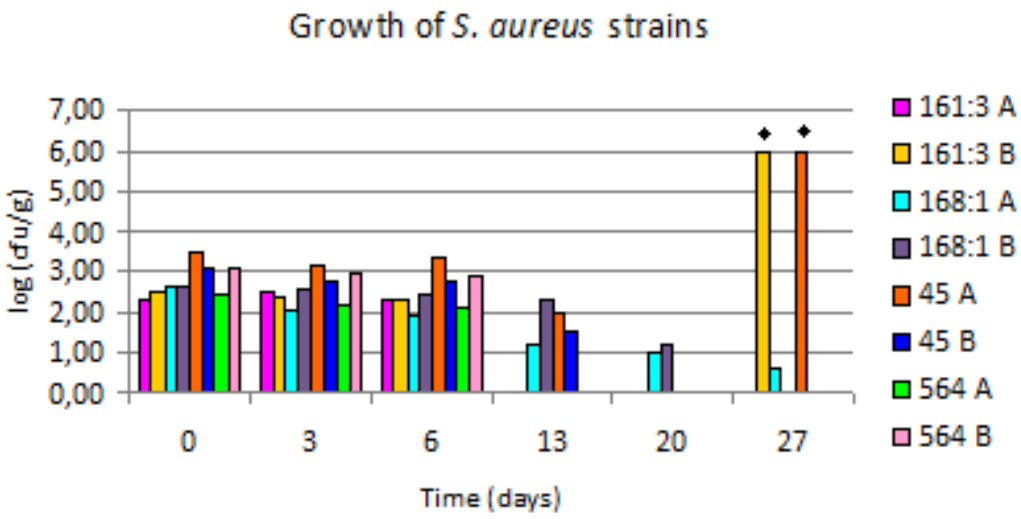
The total cell count was declining with time, except for the samples with mould contamination (**Figure 5.12d**). The presence of large unidentified colonies of irregular shape

and size on BHI or TSA plates made the counting difficult. No lactic acid bacteria were detected on the MRS plates.
The control samples contained no or almost no detectable levels of cells (on TSA, BPA or MRS). Some of the control samples also supported growth of the large unidentified colonies observed on TSA.

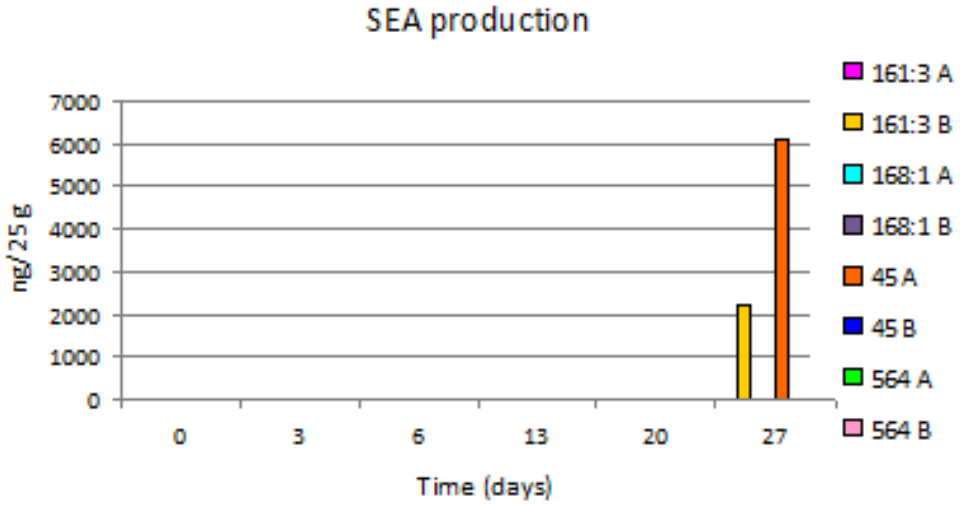
pH
The pH was between 5.2 and 5.3 at the beginning of the experiment and slightly increased to 5.4 – 5.6 after 27 days. Two of the samples contaminated with mould (161:3 B, 27 days and 45 A, 27 days) had a pH of 6.4 and 6.9 respectively.

Enterotoxin production
The two samples (161:3 B, 27 days and 45 A, 27 days) contaminated with mould and with a high number of *S. aureus* cells showed a significant production of enterotoxins (**Figure 5.12b,c**). None of the samples contained any detectable levels of TSST.

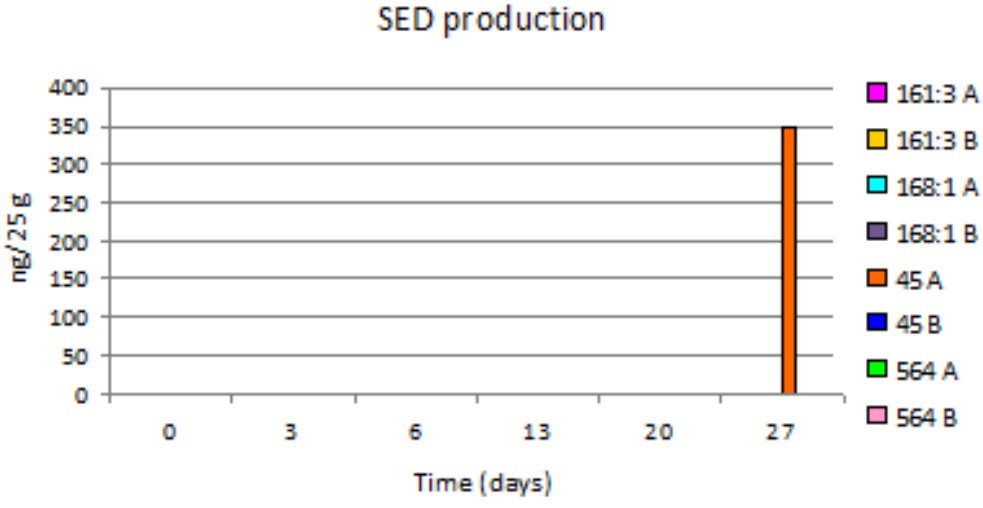
a)


Growth of S. aureus strains
log (cfu/g)
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
0
3
6
13
20
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

b)


SEA production
ng/25 g
7000
6000
5000
4000
3000
2000
1000
0
0
3
6
13
20
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

c)


SED production
ng/25 g
400
350
300
250
200
150
100
50
0
0
3
6
13
20
27
Time (days)
161:3 A
161:3 B
168:1 A
168:1 B
45 A
45 B
564 A
564 B

d)

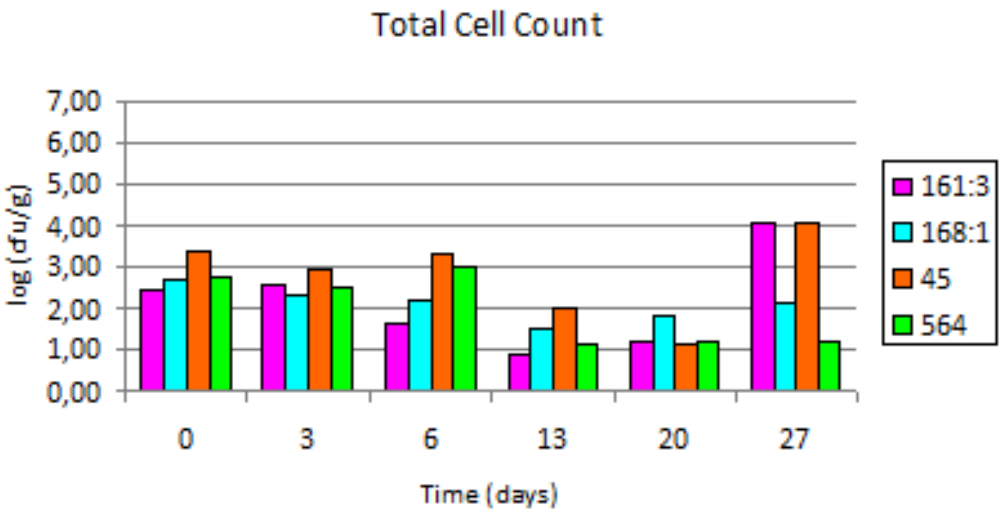


Figure 5.12 *a) Growth of S. aureus strains 161:3, 168:1, 45 and 564 in cream cheese at 20°C. Two independent replicas, A and B. ♦ Samples contaminated with mould. b) Total amount of enterotoxin A found in 25 g cheese samples, measured with ELISA. c) Total amount of enterotoxin D found in 25 g cheese samples, measured with ELISA. d) Total cell count in the cheese samples. Average of two independent replicas, A and B, for each sample.*

6. DISCUSSION

Despite the recent progress in food pathogen detection, there still exist many challenges and opportunities for the improvement of current methods. Traditional microbiological methods describe three basic protocols for the detection of pathogens: 1) direct plating on selective media; 2) selective enrichment and 3) pre-enrichment, followed by a selective enrichment, depending on the number of cells expected in a sample or the standards described by law or otherwise. Most often an enrichment procedure is required. The success of the protocols depends on the number and the state of the pathogen in the sample, incubation conditions (temperature, time, presence of oxygen) and the selectivity of the medium. The selectivity of the medium refers to the ease of distinguishing the target pathogen from competitive microflora and to a balance between inhibition of competitors and of the target. To finally confirm the pathogen, biochemical tests must be applied. The whole procedure of such detection is time-consuming, labour-intensive and may yield false-positive or false-negative results.

The standard method EN ISO 6888 used to evaluate risk coming from the presence of *S. aureus* in food consists of 3 parts. Part 1 and part 2 are based on plate-count direct enumeration and identification of selected typical and atypical colonies grown on selective agar medium (Baird-Parker agar or rabbit plasma fibrinogen agar) from sample dilutions. Theoretical detection limit of this procedure is 100 cfu/g for solid foodstuffs or 10 cfu/ml for liquid samples if one colony is detected. This can be improved by using more plates or plating a larger volume of food suspension. Part 3 refers to detection and MPN technique for low numbers of *S. aureus* in food and uses selective enrichment (Giolitti and Cantoni broth) with subsequent isolation of colonies on Baird-Parker agar. The confirmation of *S. aureus* is performed by coagulase test.

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. Since most rapid methods have not been validated and are generally used as screening techniques, they require confirmation by the appropriate official method which in many cases is based on microbiological cultivation.

In this work, the rapid, sensitive and reliable PCR-based method was developed and compared with the conventional cultural method. The designed method was highly specific for *S. aureus*. The specificity was determined using 146 bacterial strains and 100% inclusivity and 100% exclusivity was obtained. The sensitivity of the method was determined to be

approximately 6.8×10^1 cfu/ml which corresponds to approximately 1.7 cfu per reaction. The enrichment step was carried out in selective modified Giolitti and Cantoni medium at 37°C for 18 h under aerobic conditions. An easy, rapid and efficient DNA extraction method was applied. In comparison to the standard microbiological method, the developed method showed better sensitivity, better specificity and the results on the next day. Out of 112 food products, 61 were positive for *S. aureus* by the real-time PCR based method and 53 samples by the standard method. Furthermore, the newly developed method was able to detect 10^0 of artificially added *S. aureus* cells in 10 g of the sample in all investigated samples, whereas using the standard method, only three samples were found positive. Studies showing similar results have been published [55, 56]. The proposed method could be used as a screening method to check the presence of *S. aureus* in food production environment.

There are many environmental factors affecting *S. aureus* growth and SE production in foodstuffs. Dairy products have always been vehicles for staphylococcal food poisoning. A massive outbreak in Japan and recent incidents in Norway and Austria show that a considerable research effort is still required to ensure safe food for the consumer, as well as to minimize economic loss for the food producer. 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. Also, the combined effect of the unfavorable 12°C temperature and the presence of competing microflora derived from raw milk on growth and SED production in pasteurized milk was investigated. The amount of SED produced was correlated to bacterial growth to investigate if *S. aureus* produces an increased amount of SED under sub-optimal growth conditions. A comparison was made between the behaviour of *S. aureus* in an optimal bacterial growth medium (BHI) and a highly complex matrix, milk.

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. Although enterotoxins are described as very stable proteins resistant to degrading enzymes, we could observe a decrease in the absolute amount during the cultivation of *S. aureus* in milk at 20°C and 12°C. Recently, SEA and SED were found to decrease in boiled ham after a period of accumulation, and a number of earlier studies have reported the disappearance of SEA in broth, minced food and raw and pasteurized milk. The apparent decrease in enterotoxin levels could simply be an analytical artifact, such as loss of serological recognition using immuno-based methods such as ELISA, which is a technique commonly used to detect enterotoxins. However, it has also been proposed that either proteases produced by lactic acid bacteria cause the decrease in SEA levels, or that SEA becomes cell-associated and is, therefore, not detected [7].

S. aureus is sensitive to microbial competition. In the experiments with the presence of competing microflora from raw milk, an inhibitory effect on *S. aureus* growth and enterotoxin production was observed. Even though low temperature also created a stressful factor, it was found that the impact of competition on *S. aureus* growth was higher than low temperature. The enterotoxin production 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.

The cheese 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 cheeses at two different temperatures. Cheeses comprise a huge number of varieties with different technological parameters applied to each one. Thus, chemical and microbiological composition varies significantly.

The choice of two types of cheeses for the investigation was due to differences mentioned but also due to different texture. Cream cheese belongs to the types of cheese that are made by acid coagulation of caseins rather than rennet coagulation. Acid coagulation can be achieved naturally with the starter culture, or artificially with the addition of gluconodeltalactone [97]. Cream cheese is a fresh cheese and as the name implies is consumed fresh and has a shelf life of only 2 - 3 weeks. Semi-soft cheese was a working name for the cheese Wästgöta kloster (resembles the Czech Eidam but with small gas holes) which belongs to ripened cheeses with rennet and starter culture used to coagulate milk during production. The shelf life of such a cheese can be from weeks to months. Both Swedish cheeses were bought in a local supermarket.

Growth and subsequent toxin production can be prevented by storing "potentially hazardous" foods below 7°C and 10°C, respectively [7]. Results from milk experiments demonstrated no growth and enterotoxin production at 8°C and at 12°C *S.aureus* growth was poor and very low enterotoxin production was detected in the experiments with the presence of competing microflora. Therefore, we chose temperature 13°C to test temperature abuse (i.e. when a specific temperature should be kept, but it is not) and also to find out how high temperature can go before *S.aureus* starts to grow and produce toxin in the selected cheeses. The higher temperature, 20°C, was really as a challenge test to see the worst case scenario.

Lactic acid bacteria (LAB) are the principal organisms involved in the manufacture of cheese. In some fermented dairy products, additional bacteria, referred to as secondary microflora, are added to produce carbon dioxide, which influences the flavor and alters the texture of the final product. LAB comprise a heterogeneous bacterial group whose main common characteristic is the ability to ferment lactose into lactic acid which lowers the pH and in turn assists coagulation, helps prevent spoilage and pathogenic bacteria from growing, contributes to cheese texture, flavour and keeping quality. Generally, LAB that are important in the fermentation of dairy products include only certain species of the genera *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc* [24, 96, 97].

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 [87, 88]. 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.

7. 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.

8. FUTURE PERSPECTIVES

The findings of the studies performed on *S. aureus* behaviour in milk and cheese brought up several questions, as well as thoughts and suggestions for further investigation.

Here are some of them:

- To elucidate the decrease (disappearance) of enterotoxin in milk. Could *S. aureus* or other background flora break down or re-metabolize the enterotoxins? Or is it an analytical artifact in the ELISA method resulting in toxins being present but not detected?
- To find alternative ways of measuring enterotoxin concentrations other than just ELISA.
- To get a deeper understanding of the influence of other microorganisms present in food on *S. aureus* growth and enterotoxin production.
- To investigate the effect of mold on enterotoxin production.
- To understand the genetic regulatory machinery of enterotoxin expression in more detail. Which are the factors down- and up-regulating enterotoxin production and how could they be controlled to suppress virulence of *S. aureus* by either natural components in food or preservatives?
- To implement food-based data into QMRA (quantitative microbial risk assessment) in order to be able to predict different contamination scenarios using mathematical models.

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10. LIST OF ABBREVIATIONS

AcrB	acriflavin-resistant protein
Agr	accessory gene regulator
AOAC	association of analytical communities
BGF	background flora
BHI	brain heart infusion
BPA	Baird-Parker agar
cfu	colony forming unit
DEPC	diethylpyrocarbonate
egc	enterotoxin gene cluster
ELISA	enzyme-linked immunosorbent assay
EIA	enzyme immunoassay
EtOH	ethanol
FBD	foodborne disease
*fts*Z	cell division protein
LAB	lactic acid bacteria
MPN	most probable number
NaOAc	sodium acetate
OD	optical density
O/N	overnight
PCR	polymerase chain reaction
PEG	polyethylene glycol
pta	phosphate acetyltransferase
*rec*A	recombinase A
*rpo*B	RNA polymerase beta chain
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
USDA	United States Department of Agriculture
VC	viable count

11. LIST OF PUBLICATIONS

Papers

1. Trnčíková, T., Hrušková, V., Oravcová, K., Pangallo, D., Kaclíková, E. Rapid and sensitive detection of *Staphylococcus aureus* in food using selective enrichment and real-time PCR targeting a new gene marker. *Food Analytical Methods*, 2008, vol. 2, 241-250 p.
2. Hrušková, V., Kaclíková, E. Rapid and sensitive detection of pathogenic *Yersinia enterocolitica* strains in food using selective enrichment and real-time PCR. *Journal of Food and Nutrition Research*, vol 48, 2009, 100-108 p.

Abstracts – international congresses

3. Schelin, J., Bordignon, S., Hrušková, V., Rådström, P. Production of *S. aureus* enterotoxin D in pasteurized milk and Brain Heart Infusion at 8, 12 and 20°C. *3rd FEMS Congress of European Microbiologists*, Gothenburg, Sweden, Book of Abstracts, 2009.
4. Bordignon, S., Hrušková, V. Carlquist Wallin, N., Rådström, P., Schelin, J. Production of *Staphylococcus aureus* enterotoxin A and D in cheese. *22nd International IFCMH Symposium*, Food Micro, Copenhagen, Denmark, 2010.

12. SUPPLEMENTS

I. Trnčíková, T., Hrušková, V., Oravcová, K., Pangallo, D., Kaclíková, E. Rapid and sensitive detection of *Staphylococcus aureus* in food using selective enrichment and real-time PCR targeting a new gene marker. *Food Analytical Methods*, 2008, vol. 2, 241-250 p.

II. Hrušková, V., Kaclíková, E. Rapid and sensitive detection of pathogenic *Yersinia enterocolitica* strains in food using selective enrichment and real-time PCR. *Journal of Food and Nutrition Research*, vol 48, 2009, 100-108 p.

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Rapid and Sensitive Detection of *Staphylococcus aureus* in Food Using Selective Enrichment and Real-Time PCR Targeting a New Gene Marker

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Abstract *Staphylococcus aureus* is a bacterial pathogen considered a principal etiological agent of food poisoning. The aim of this study was to develop and evaluate a rapid and sensitive method for the detection of *S. aureus* in food by using selective enrichment and a new species-specific real-time polymerase chain reaction (PCR). Specific primers and a TaqMan probe targeted to specific *S. aureus* gene encoding for acriflavine resistance protein were designed. The real-time PCR was highly specific for *S. aureus* with 100% inclusivity and 100% exclusivity determined using 83 *S. aureus* strains and 64 non-*S.-aureus* strains. PCR detection limit of 6.8×10^1 and 3.4×10^1 CFU ml^{-1} were obtained with 100% and 70% detection probability, respectively. The single selective enrichment based on the study of different enrichment conditions was selected and a lysis by boiling was used to obtain bacterial DNA. Out of 112 food samples analyzed, 61 were positive by the PCR-based method and 53 by the standard method. Out of ten food matrices artificially contaminated at a level of 10° CFU g^{-1}, ten and six were positive by the respective methods. Moreover, 10° CFU 10 g^{-1} was detected in all ten artificially contaminated samples after a large-scale enrichment using PCR-based detection, in contrast to seven false negative by standard detection. The developed 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 microbiological method with the potential for providing of improved food-processing hygiene control.

Keywords *Staphylococcus aureus* · Real-time PCR · Acriflavine Resistance · Detection · Food

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Introduction

Staphylococcus aureus is the second most common pathogen associated with outbreaks of food poisoning, one of the most economically important food-borne diseases throughout the world. It has been found widespread in nature and also in warm-blooded animals, including humans (Alarcon et al. 2006), and so its presence in foods may often be an indicator of insufficient hygiene in the food production. As the bacterium is able to survive well in food-factory environments, it may become part of the microflora of the processing equipment and act as a source of contamination. Consequently, many foods may be contaminated by *S. aureus* representing hazard for human health.

According to the current European Union regulations on microbiological criteria (EC 2005), food safety criteria for cheese products placed on the market during shelf-life are defined by absence of staphylococcal enterotoxins. However, *S. aureus*' presence in raw milk is a major concern for the safety and the quality of traditionally produced cheeses (Delbes et al. 2006). Process hygiene criteria for fresh

cheeses from heat-treated milk or whey are limited by 10 CFU g^{-1} at the end of the manufacturing process. European Standard methods intended for this purpose, namely EN ISO 6888-1 (Anonymous 1999a) or EN ISO 6888-2 (Anonymous 1999b), are based on plate-count direct enumeration and identification of selected typical and atypical colonies grown on selective agar medium from sample dilutions. Detection limit of 10 CFU g^{-1} could be ensured if 1 ml of a decimally diluted sample is streaked on three Petri dishes containing agar media. However, in some circumstances, it should be reasonable to detect the lower numbers of *S. aureus* than this standard procedure is able to ensure. Suitable selective enrichment of the sample microflora proved to decrease the detection limit by several orders (Jaykus 2003; Ercolini et al. 2004). Currently available standard method for the detection of coagulase-positive staphylococci including *S. aureus* in food (based on the part of EN ISO 6888-3 (Anonymous 2003a)) is based on selective enrichment and subsequent isolation of colonies with characteristic morphology and identification by microbiological- and biochemical-based confirmations. This procedure is considerably labor-intensive and time-consuming. Moreover, it may not be sufficient for the reliable identification of the typical or atypical *S. aureus* colonies on Baird-Parker agar in the presence of high numbers of bacteria showing colony morphology closely similar to *S. aureus* or in the case of ambiguous results of biochemical confirmation. High percentage of atypical colonies on Baird-Parker agar has been identified as *S. aureus*, particularly from milk samples (Da Silva et al. 2000).

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 conventional methods (Nugen and Baeumner 2008). Several PCR-based methods for food-borne *S. aureus* identification have been published, targeting only a few different species-specific molecular markers. The predominant target was *nuc* gene used in either conventional PCR (Ercolini et al. 2004; Pinto et al. 2005; Ikeda et al. 2005; Cremonesi et al. 2005) or quantitative real-time PCR (Hein et al. 2005; Alarcon et al. 2006). Other popular targets were species-specific regions of the DNA coding for 16S or 23S rRNA or *coa* gene (Cremonesi et al. 2005; Baron et al. 2004; Sabet et al. 2006) and putative transcriptional regulator genes (Liu et al. 2005; Goto et al. 2007). These *S.-aureus*-specific genes were successfully amplified along with *se* (*ent*) genes coding for enterotoxins in multiplex conventional PCR (Ercolini et al. 2004; Pinto et al. 2005; Ikeda et al. 2005; Tamarapu et al. 2001; Cremonesi et al. 2007) or at simultaneous species identification and detection of methicillin resistance using multiplex real-time PCR assay (Baron et al. 2004).

Most of the PCR-based methods applied to foods do not contain the internal amplification control and are based on classic electrophoretic detection of PCR products.

Direct PCR detection of pathogen from food leads to poor detection limits in comparison with the use of preliminary culture enrichment. Moreover, separation technique optimized for one food system is not adaptable to others (Baruzzi et al. 2005). The use of optimal culture preparation of food sample microflora is considered the necessary step prior to PCR in order to ensure the detection limit of 10° CFU per sample, e.g., the detection of absence of target pathogenic bacteria. Two staphylococcus-enrichment broths are commercially available. The most frequently used and recommended by ISO standard is Giolitti–Cantoni enrichment broth, in which the growth of staphylococci is promoted by pyruvate, glycine, and a high concentration of mannitol. Gram-negative bacteria are inhibited by lithium chloride and gram-positive by tellurite. Anaerobiosis, which is recommended by ISO standard should be able to suppress micrococci to a certain degree. The composition of staphylococcus-enrichment broth acc. Baird is similar regarding the inhibitory components; mannitol is not included.

The methods based on real-time PCR demonstrated a great potential due to their higher specificity, higher sensitivity, and possibility to quantify target bacterial cells.

Only one published complex real-time PCR-based methods for a faster, highly sensitive, and less laborious detection of *S. aureus* in foods includes internal amplification control (Berrada et al. 2006). Nevertheless, the use of the internal amplification control in each reaction is considered necessary for diagnostic purposes (Rodriguez-Lazaro et al. 2004; Oikonomou et al. 2008) to eliminate of false-negative results.

Rapid, highly sensitive, and reliable detection method for *S. aureus* allowing the results of presence or absence should be of importance for the screening purposes in food processing to check the presence of *S. aureus* in raw materials or production environment. In this study, we describe a newly developed real-time PCR-based detection targeted to the gene sequence coding for acriflavine resistance family proteins (AcrB/AcrD/AcrF) specific for *S. aureus*. The ability of acriflavine resistance was successfully used in selective growth of *S. aureus* for the inhibition of background microflora including other *Staphylococcus* spp. (Davis et al. 2006) or for differentiating *S. aureus* isolates from non-*S.-aureus* spp. (Wallace et al. 1998). Rapid and sensitive *S. aureus* detection suitable for routine application has been applied to food samples and artificially contaminated food samples by comparing the real-time PCR with standard detection method. Different enrichment conditions and DNA extraction procedures have been used and compared to improve the sensitivity of the detection.

Materials and Methods

Bacterial Strains and Culture Conditions

S. aureus strains listed in Table 1 were obtained from Czech Collection of Microorganisms (CCM, Brno, Czech Republic) and identified clinical isolates were kindly provided by Dr. Juraj Hanzen (HPL, Laboratories of Clinical Microbiology, Bratislava, Slovakia). *S. aureus* strains used for the inclusivity determination listed in Table 2 were isolated from foods and throat swabs and identified in our laboratory. Bacterial strains other than *S. aureus* listed in Table 3 were obtained from culture collections or from reference laboratories. All strains were maintained at −18°C in 60% glycerol solution or freeze-dried for long-period storage. Working cultures were prepared by inoculation of Brain Heart broth (Merck, Darmstadt, Germany) and overnight incubation at 37°C with shaking. Decimal dilutions were prepared in 0.85% NaCl and the cell concentrations were determined by the plate-count method.

Table 1 *S. aureus* strains obtained from official collections and from a clinical laboratory and results of real-time PCR identification

No	Source and designation (origin)	PCR
1	CCM 1484 (NCTC 4163)	+
2	CCM 2107 (ATCC 9144)	+
3	CCM 2323	+
4	CCM 2351 (ATCC 10832)	+
5	CCM 2458 (CNCTC Mau 71/68)	+
6	CCM 2773 (ATCC 25904)	+
7	CCM 3958 (ATCC 25923)	+
8	CCM 4223 (ATCC 29213)	+
9	CCM 4516 (ATCC 6538)	+
10	CCM 4750 (ATCC 6538)	+
11	CCM 7097 (NCTC 8325)	+
12	CCM 7109	+
13	HPL 121/1	+
14	HPL 239/1	+
15	HPL 252/1	+
16	HPL 270/1	+
17	HPL 297/1	+
18	HPL 328/1	+
19	HPL 356/1	+
20	HPL 408/1	+
21	HPL 425/1	+
22	HPL 436/1	+
23	HPL 442/1	+
24	HPL 468/1	+
25	HPL 490/1	+
26	HPL 514/1	+
27	HPL 608/3	+
28	HPL 616/2	+
29	HPL 617/2	+
30	HPL 636/2	+
31	HPL 637/1	+
32	HPL 643/1	+
33	HPL 644/1	+
34	HPL 760/2	+
35	HPL 765/1	+
36	HPL 767/1	+
37	HPL 819/1	+
38	HPL 826/1	+
39	HPL 901/1	+
40	HPL 992/1	+

CCM Czech Collection of Microorganisms, Brno, Czech Republic, *CNCTC* Czech National Collection of Type Cultures, Praha, Czech Republic, *HPL* Laboratories of Clinical Microbiology, Bratislava, Slovakia

Isolation and Identification of *S. aureus* Strains

Strains of *S. aureus* were isolated from food products and throat swabs using the method based on EN ISO 6888-3:2003 in our laboratory from the selective enrichment in Modified Giolitti and Cantoni broth (Merck) with potassium tellurite under anaerobic conditions. Presumptive *S. aureus* colonies were isolated on Baird-Parker agar (Merck). Selected colonies were confirmed by the coagulase activity test using rabbit plasma (BioRad, Hercules, CA, USA). Identification was confirmed by profile determination using API Staph system (bioMérieux, Marcy l'Etoile, France).

Preparation of DNA Samples

For the PCR exclusivity and inclusivity evaluation process, DNA samples were prepared from overnight cultures using lysis by boiling. A volume of 1 ml of the bacterial suspension was centrifuged at 13,000×*g* for 5 min; the sediment was resuspended in 0.85% NaCl and centrifuged again at 13,000×*g* for 5 min. The washed sediment was resuspended in 100 μl of 1× PCR buffer (Qiagen, Hilden, Germany) and incubated at 95°C for 20 min. *S. aureus* CCM 3953 DNA used as PCR-positive control was extracted using QiaAmp DNA Mini Kit (Qiagen). To improve the PCR detection limit, three methods for the preparation of DNA samples were compared. Lysis by boiling in PCR buffer supplemented with 1% Triton X-100 (Merck) was performed as described above. InstaGene Matrix (BioRad, Hercules, CA, USA) and QiaAmp DNA Mini Kit (Qiagen) were used for DNA extraction according to manufacturers' instruction.

5′-Nuclease Real-Time PCR

Each reaction sample contained 300 nmol l^{-1} of primers: aurF (5′-tcaacatctttcgcatgattcaacac-3′), aurR (5′-ctagctttattt cagcaggtgacgat-3′), 200 nmol l^{-1} of the TaqMan probe aurP (5′-cttgctccgtttcaccaggcttcggtgt-3′; all from Qiagen Oper-

Table 2 *S. aureus* strains isolated and identified in our laboratory using coagulase test and API Staph identification system, and results of real-time PCR

No	Designation	Source	Coagulase activity	API Staph profile (% ident.)	PCR
1	170107/02	Sausage	+	6716153 (88.4)	+
2	240107/02	Sweet cream	+	6736113 (86.7)	+
3	240107/05	Cream dessert	+	6336153 (94.1)	+
4	310107/21	Bryndza cheese	+	6736113 (86.7)	+
5	140207/15	Cream dressing	+	6336153 (94.1)	+
6	240107/12	Pork meat	+	6736153 (97.8)	+
7	070307/18	Raw meat product	+	6736153 (97.8)	+
8	200307/19	Chicken liver	+	6736153 (97.8)	+
9	200307/33	Pork meat	+	6736153 (97.8)	+
10	280307/22	Pork meat	+	6736153 (97.8)	+
11	280307/26	Pork meat	+	6736153 (97.8)	+
12	280307/48	Chicken filets	+	6336151 (95.5)	+
13	280307/55	Mixed mincemeat	+	6736153 (97.8)	+
14	280307/64	Pork meat	+	6736153 (97.8)	+
15	280307/72	Pork slices	+	6736153 (97.8)	+
16	280307/77	Mixed mincemeat	+	6736113 (86.7)	+
17	280307/93	Meat product	+	6736153 (97.8)	+
18	280307/113	Meat product	+	6736153 (97.8)	+
19	280307/118	Sliced pork meat	+	6736153 (97.8)	+
20	030407/06	Pork meat	+	6736153 (97.8)	+
21	030407/17	Sliced bacon	+	6736153 (97.8)	+
22	040407/31	Mincemeat product	+	6336153 (94.1)	+
23	040407/37	Chicken sausage	+	6736113 (86.7)	+
24	040407/41	Eggs	+	6736153 (97.8)	+
25	150507/49	Bryndza cheese	+	6736153 (97.8)	+
26	230507/48	Cottage cheese	+	6736113 (86.7)	+
27	120607/22	Mexican dressing	+	6736113 (86.7)	+
28	VUP 418	Throat swab	+	6736153 (97.8)	+
29	VUP 419	Throat swab	+	6736153 (97.8)	+
30	VUP 420	Throat swab	+	6736153 (97.8)	+
31	VUP 421	Throat swab	+	6736113 (86.7)	+
32	VUP 422	Throat swab	+	6736113 (86.7)	+
33	VUP 423	Throat swab	+	6736153 (97.8)	+
34	VUP 424	Throat swab	+	6736113 (86.7)	+
35	VUP 425	Throat swab	+	6736153 (97.8)	+
36	VUP 426	Throat swab	+	6736153 (97.8)	+
37	VUP 427	Throat swab	+	6736153 (97.8)	+
38	VUP 518	Bryndza cheese	+	6736113 (86.7)	+
39	VUP 519	Fresh cheese	+	6736113 (86.7)	+
40	VUP 520	Camembert	+	6736153 (97.8)	+
41	VUP 521	Cheese salad	+	6736153 (97.8)	+
42	VUP 522	Ice-cream	+	6736113 (86.7)	+
43	VUP 523	Milk shake	+	6736153 (97.8)	+

Table 3 Non-*S. aureus* strains and results of real-time PCR

No	Strain	Source and designation	PCR
1	*Staphylococcus caprae*	CCM 3573^T (ATCC 35538)	–
2	*Staphylococcus chromogenes*	CCM 3387^T (ATCC 43764)	–
3	*Staphylococcus cohnii*	CCM 2736^T (ATCC 29974)	–
4	*Staphylococcus epidermidis*	CCM 2124^T (ATCC 14990)	–
5	*Staphylococcus epidermidis*	CCM 2446	–
6	*Staphylococcus epidermidis*	CCM 4418 (ATCC 12228)	–
7	*Staphylococcus haemolyticus*	CCM 2737^T (ATCC 29970)	–
8	*Staphylococcus hyicus*	CCM 2368^T (ATCC 11249)	–
9	*Staphylococcus intermedius*	CCM 5739^T (ATCC 29563)	–
10	*Staphylococcus sacharolyticus*	CCM 3539	–
11	*Staphylococcus saprophyticus*	VUP 218	–
12	*Staphylococcus sciuri*	CCM 4835^T (ATCC 700058)	–
13	*Staphylococcus xylosus*	CCM 2738^T (ATCC 29971)	–
14	*Bacillus cereus*	CCM 2010^T (ATCC 14579)	–
15	*Campylobacter coli*	CCM 7227 (ATCC 43478)	–
16	*Campylobacter jejuni*	CCM 6214^T (ATCC 33560)	–
17	*Citrobacter amalonaticus*	CCM 4706	–
18	*Citrobacter braakii*	CCM 3393	–
19	*Citrobacter freundii*	CCM 4475	–
20	*Citrobacter koseri*	CCM 2535	–
21	*Clostridium perfringens*	CCM 4435	–
22	*Edwardsiella tarda*	CCM 2238	–
23	*Enterobacter amnigenus*	CCM 3430	–
24	*Enterobacter cloacae*	CCM 1903	–
25	*Enterobacter intermedius*	CLO 427	–
26	*Enterobacter sakazakii*	CCM 5740^T (ATCC 29544)	–
27	*Enterococcus faecalis*	CCM 1875	–
28	*Enterococcus faecalis*	VUP 282	–
29	*Escherichia coli*	CCM 2024	–
30	*Escherichia coli*	CCM 3988	–
31	*Escherichia coli*	UMB 404	–
32	*Klebsiella pneumoniae*	SVU 137	–
33	*Lactobacillus brevis*	CCM 1815 (ATCC 8287)	–
34	*Lactobacillus buchneri*	CCM 1819^T (ATCC 4005)	–
35	*Lactobacillus casei*	CCM 7088^T (ATCC 393)	–
36	*Lactobacillus curvatus*	CCM 7271 (LMG 23109)	–
37	*Lactobacillus delbrueckii*	CCM 7191^T (ATCC 9649)	–
38	*Lactobacillus fermentum*	CCM 91 (ATCC 9338)	–
39	*Lactobacillus paracasei*	CCM 1752 (ATCC 25303)	–
40	*Listeria grayi*	CCM 4029	–
41	*Listeria grayi*	VUP 212	–
42	*Listeria innocua*	CCM 4030	–
43	*Listeria innocua*	VUP 214	–
44	*Listeria innocua*	VUP 217	–
45	*Listeria ivanovii*	CCM 5884	–
46	*Listeria ivanovii*	VUP 274	–
47	*Listeria monocytogenes*	NCTC 11994	–
48	*Listeria monocytogenes*	CAPM 5577	–
49	*Listeria monocytogenes*	CAPM 5580	–
50	*Listeria monocytogenes*	SZU 198	–
51	*Listeria monocytogenes*	SZU 244	–

Table 3 (continued)

No	Strain	Source and designation	PCR
52	*Listeria monocytogenes*	SZU 390	–
53	*Listeria seeligeri*	CCM 3970^T (35967)	–
54	*Listeria welshimeri*	CCM 3971^T (35897)	–
55	*Micrococcus luteus*	CCM 1048^T (7468)	–
56	*Proteus vulgaris*	CCM 1799	–
57	*Pseudomonas aeruginosa*	CCM 3955	–
58	*Rhodococcus equi*	CCM 3429	–
59	*Salmonella* Enteritidis	CCM 4420	–
60	*Salmonella* Typhimurium	CCM 4419	–
No	Strain	Source and designation	PCR
1	*Staphylococcus caprae*	CCM 3573^T (ATCC 35538)	–
2	*Staphylococcus chromogenes*	CCM 3387^T (ATCC 43764)	–
3	*Staphylococcus cohnii*	CCM 2736^T (ATCC 29974)	–
4	*Staphylococcus epidermidis*	CCM 2124^T (ATCC 14990)	–
5	*Staphylococcus epidermidis*	CCM 2446	–
6	*Staphylococcus epidermidis*	CCM 4418 (ATCC 12228)	–
7	*Staphylococcus haemolyticus*	CCM 2737^T (ATCC 29970)	–
8	*Staphylococcus hyicus*	CCM 2368^T (ATCC 11249)	–
9	*Staphylococcus intermedius*	CCM 5739^T (ATCC 29563)	–
10	*Staphylococcus sacharolyticus*	CCM 3539	–
11	*Staphylococcus saprophyticus*	VUP 218	–
12	*Staphylococcus sciuri*	CCM 4835^T (ATCC 700058)	–
13	*Staphylococcus xylosus*	CCM 2738^T (ATCC 29971)	–
14	*Bacillus cereus*	CCM 2010^T (ATCC 14579)	–
15	*Campylobacter coli*	CCM 7227 (ATCC 43478)	–
16	*Campylobacter jejuni*	CCM 6214^T (ATCC 33560)	–
17	*Citrobacter amalonaticus*	CCM 4706	–
18	*Citrobacter braakii*	CCM 3393	–
19	*Citrobacter freundii*	CCM 4475	–
20	*Citrobacter koseri*	CCM 2535	–
21	*Clostridium perfringens*	CCM 4435	–
22	*Edwardsiella tarda*	CCM 2238	–
23	*Enterobacter amnigenus*	CCM 3430	–
24	*Enterobacter cloacae*	CCM 1903	–
25	*Enterobacter intermedius*	CLO 427	–
26	*Enterobacter sakazakii*	CCM 5740^T (ATCC 29544)	–
27	*Enterococcus faecalis*	CCM 1875	–
28	*Enterococcus faecalis*	VUP 282	–
29	*Escherichia coli*	CCM 2024	–
30	*Escherichia coli*	CCM 3988	–
31	*Escherichia coli*	UMB 404	–
32	*Klebsiella pneumoniae*	SVU 137	–
33	*Lactobacillus brevis*	CCM 1815 (ATCC 8287)	–
34	*Lactobacillus buchneri*	CCM 1819^T (ATCC 4005)	–
35	*Lactobacillus casei*	CCM 7088^T (ATCC 393)	–
36	*Lactobacillus curvatus*	CCM 7271 (LMG 23109)	–
37	*Lactobacillus delbrueckii*	CCM 7191^T (ATCC 9649)	–
38	*Lactobacillus fermentum*	CCM 91 (ATCC 9338)	–
39	*Lactobacillus paracasei*	CCM 1752 (ATCC 25303)	–
40	*Listeria grayi*	CCM 4029	–
41	*Listeria grayi*	VUP 212	–

Table 3 (continued)

No	Strain	Source and designation	PCR
42	*Listeria innocua*	CCM 4030	–
43	*Listeria innocua*	VUP 214	–
44	*Listeria innocua*	VUP 217	–
45	*Listeria ivanovii*	CCM 5884	–
46	*Listeria ivanovii*	VUP 274	–
47	*Listeria monocytogenes*	NCTC 11994	–
48	*Listeria monocytogenes*	CAPM 5577	–
49	*Listeria monocytogenes*	CAPM 5580	–
50	*Listeria monocytogenes*	SZU 198	–
51	*Listeria monocytogenes*	SZU 244	–
52	*Listeria monocytogenes*	SZU 390	–
53	*Listeria seeligeri*	CCM 3970^T (35967)	–
54	*Listeria welshimeri*	CCM 3971^T (35897)	–
55	*Micrococcus luteus*	CCM 1048^T (7468)	–
56	*Proteus vulgaris*	CCM 1799	–
57	*Pseudomonas aeruginosa*	CCM 3955	–
58	*Rhodococcus equi*	CCM 3429	–
59	*Salmonella* Enteritidis	CCM 4420	–
60	*Salmonella* Typhimurium	CCM 4419	–
61	*Serratia marcescens*	CCM 303	–
62	*Yersinia bercovieri*	CCM 4205^T	–
63	*Yersinia enterocolitica*	CCM 5671	–
64	*Yersinia kristensenii*	CCM 3559	–

BF Faculty of Biotechnology, University of Ljubljana, Slovenia, *CCM* Czech Collection of Microorganisms, Brno, Czech Republic, *CLO* Agricultural Research Center, Melle, Belgium, *SVU* State Veterinary Institute, Bratislava, Slovakia, *SZU* State Institute of Public Health, Brno, Czech Republic, *UMB* Institute of Molecular Biology, Slovak Academy of Science, Bratislava, Slovakia, *UVZ* Institute of Public Health, Bratislava, Slovakia, *VUP* Food Research Institute, Bratislava, Slovakia

on), 500 μmol l^{-1} of each dNTP (Applied Biosystems Foster City, CA, USA), 1.5 U of HotStar Taq DNA polymerase (Qiagen), 1× concentrated PCR buffer and 4.5 mmol l^{-1} magnesium chloride, TaqMan Exogenous Internal Positive control VIC (Applied Biosystems), 2.5 μl of the DNA sample and water to make the total volume up to 25 μl. Real-time PCR was performed in a PTC-200 thermal cycler coupled to a Chromo 4 continuous fluorescence detector (MJ Research, Waltham, MA, USA) using a thermal program consisting of the initial denaturation of 15 min at 95°C, and 45 cycles of 15 s at 95°C and 60 s at 60°C. Three negative and two positive controls were included in each experiment. All data relating to the C_T values were expressed as mean value and standard deviation calculated by internal cycler software for minimally three parallel analyses.

Determination of PCR Detection Limit

In order to determine the theoretical PCR detection limit, DNA from the overnight culture of *S. aureus* CCM 3953

was isolated using QiaAmp DNA Mini Kit (Qiagen). Concentration of total extracted DNA was determined using fluorescence measurement in a Tecan Saphire2 plate reader (Salzburg, Austria) by using Quant-iT PicoGreen Assay (Invitrogen, Gaithersburg, MD, USA). Two parallel PCR analyses of *S. aureus* CCM 3953 DNA dilutions as templates were performed. For the determination of the practical PCR detection limit and PCR detection probability, two independent sets of three to ten parallel PCR analyses of suitable dilutions (ranging from 10^6 to 10^0 CFU ml^{-1}) of *S. aureus* strain CCM 3953 overnight culture were performed. Detection probability was calculated as the percentage of positive PCR results corresponding to particular concentrations of bacterial suspensions determined as colony-forming unit per milliliter by the plate-count method.

Enrichment Procedures

Two different media (Modified Giolitti and Cantoni broth and Baird broth, both from Merck) were used for *S. aureus* selective enrichment. Three different strains of *S. aureus* (CCM 3953, HPL 468/1, and VUP518) were inoculated at two initial concentrations of 10^0 and 10^1 CFU per 10 ml of each medium. Background microflora was prepared by homogenizing of cheese (25 g in 225 g of broth) in filter bag. The obtained natural cheese microflora free of *S. aureus* estimated as total counts of microorganisms was used as the background at suitable concentrations. Enrichment in aerobic and anaerobic conditions was performed. The anaerobiosis was obtained by pouring a plug of agar into the tube. *S. aureus* growth was followed by real-time PCR detection after 18, 24, 30, and 42 h of the respective enrichment and the results have been compared for *S. aureus* recovery.

Food Samples and Artificial Contamination

Food samples selected according to EN ISO 16140 (Anomynous 2003b) were obtained from retail markets in Slovakia. All food samples were analyzed for the presence of *S. aureus* using the microbiological method according to EN ISO 6888-3 and by the real-time PCR-based method. In all *S. aureus*-positive food samples, direct enumeration of *S. aureus* using decimally diluted samples was performed according to EN ISO 6888-1 using Baird-Parker selective agar. For artificial contamination, two concentration levels of approximately 4×10^0 or 4×10^1 CFU of three *S. aureus* strains (CCM 3953, HPL 468/1, and VUP 518) were added to the containers (tubes or plastic bags) together with the food samples and the enrichment broth in a (1:10) ratio to obtain the first decimal dilution. The samples used for artificial contamination were previously confirmed to be devoid of *S. aureus*.

Reference Method

The method according to EN ISO 6888-3 (Anonymous 2003a) utilizing the selective enrichment under anaerobic conditions (up to 48 h), followed by the isolation of presumptive typical and atypical *S. aureus* colonies on the Baird-Parker selective agar (Merck) and confirmation by coagulase reaction, was used as the reference one.

Results

Design of Oligonucleotides

Based on published *S. aureus* genome sequences and the results of the BLAST homology analysis, PCR primers and a probe were designed using Primer Express software (Applied Biosystems) to amplify a 103-bp DNA sequence of a gene coding for *S. aureus* acriflavine-resistant protein (AcrB) which belongs to cation/multidrag efflux pump proteins. No non-*S.-aureus* DNA sequences were found in the GenBank database to be homologous to the designed oligonucleotides.

Exclusivity and Inclusivity

The results on exclusivity and inclusivity of the developed real-time PCR system are presented in Tables 1, 2, and 3. All 40 *S. aureus* strains from official collections and clinical isolates identified in certified HPL Laboratories of Clinical Microbiology, as well as 42 *S. aureus* strains isolated from food and identified in our laboratory gave positive signals in the real-time PCR assay using DNA templates prepared by lysis of overnight cultures. All 64 non-*S.-aureus* strains, including 13 non-*S. aureus* species, gave negative PCR results with FAM fluorescence signal below the threshold along with positive signals for VIC-labeled internal amplification control (data not shown).

PCR Detection Limit and Detection Probability

PCR assay was performed by using 7.2 ng to 0.72 fg of extracted DNA from *S. aureus* CCM 3953 in duplicates. The DNA amount of ≥3.6 fg was detected. Based on the size of 2.7 to 2.9 Mbp for *S. aureus* complete genome (GeneBank), *S. aureus* genome weighs 2.9 to 3.1 fg. Therefore, the theoretical detection limit is equivalent to approximately one *S. aureus* genome copy. Practical PCR detection limit was estimated as the probability of *S. aureus*

detection determined for three different *S. aureus* strains (CCM 3953, HPL 468/1, and VUP518) using decimal dilutions of the respective overnight cultures and DNA samples prepared by lysis with Triton X-100 (Table 4). The detection probability of the cell suspension was 100% at a concentration of 6.8×10^{1} CFU ml^{-1} and at higher concentrations and 70% at a concentration of 3.4×10^{1} CFU ml^{-1}. Based on the obtained results, the concentration of more than 6.8×10^{1} CFU per milliliter of target *S. aureus* must be reached by enrichment to ensure the reliable detection of *S. aureus* in the food sample.

Enrichment

Results of *S. aureus* real-time PCR detection during the enrichment at 37°C in two different selective media at two levels of artificial contamination and various enrichment conditions are summarized in Table 5. These results evidently demonstrated that the best enrichment of *S. aureus* was achieved in modified Giolitti and Cantoni broth. Natural cheese background microflora of 1.2×10^{6} CFU ml^{-1} has been estimated as total bacterial counts in the medium. Despite of slightly more efficient inhibition of the present background microflora in anaerobic conditions in both media tested (data not shown), the PCR detection of target *S. aureus* cells was not affected in aerobic conditions. Based on resulting C_T values, the growth of *S. aureus* in anaerobic conditions was slightly reduced. The enrichment in selective modified Giolitti and Cantoni medium at 37°C for 18 h under aerobic conditions (as a less laborious procedure compared to anaerobiosis) was selected for further work on the evaluation of the complete PCR-based method for *S. aureus* detection in foods.

Table 4 Detection limit of real-time PCR using diluted cultures of *S. aureus* CCM 3953, HPL 468/1, and VUP518 strains

Concentration[a], CFU ml^{-1}	Number of reactions	Number of positive reactions	Detection probability [%]	C_T value± SD
6.8×10^{6}	18	18	100	20.27±0.20
6.8×10^{5}	18	18	100	23.49±0.19
6.8×10^{4}	18	18	100	26.78±0.24
6.8×10^{3}	18	18	100	30.03±0.27
6.8×10^{2}	18	18	100	33.26±0.32
6.8×10^{1}	60	60	100	37.34±0.39
3.4×10^{1}	60	42	70	37.91±0.42
6.8×10^{0}	60	0	0	nd

[a] Concentrations of individual strains were adjusted to be equal using optical density measurement and were subsequently determined from dilutions 5 a 6 (using 0.1 ml per plate in duplicate for each strain): mean±SD = $(6.8\pm0.2)\times10^{3}$

nd not detectable

Comparison of DNA Extraction Methods

Three protocols for DNA extraction were evaluated based on the quantitative real-time PCR of decimally diluted overnight cultures of three different *S. aureus* strains (CCM 3953, HPL 468/1, and VUP518). Based on the results obtained with model samples of the decimally diluted *S. aureus* cultures (data not shown), similar results regarding the efficiency of DNA extraction have been obtained using all three used procedures. Therefore, the lysis by boiling with Triton X -100, as the simplest and the least-expensive alternative, was selected for further experiments.

Detection of *S. aureus* in Food Samples

Subsequently, the procedure of 18-h enrichment in modified Giolitti–Cantoni selective broth and the developed real-time PCR detection was compared with the detection procedure based on the standard method according to EN ISO 6888-3 (Anonymous 2003a) for the detection of *S. aureus* presence by analysis of 112 food products. Sixty-one samples were found positive for *S. aureus* by the real-time PCR-based method and 53 samples were found positive for *S. aureus* by the standard detection method (Table 6). All culture-positive samples were identified as positive by PCR-based detection. Results of coagulase-positive staphylococci enumeration in PCR-positive samples according to EN ISO 6888-1 from 0.1 ml of the first sample dilutions showed that 47 samples contained less than 10^{2} CFU g^{-1}, 13 samples between 10^{2} and 10^{4} CFU g^{-1}, and one sample contained more than 10^{5} CFU g^{-1}. The identification of coagulase-positive colonies of presumptive *S. aureus* using Baird-Parker isolation agar following the enrichment was rather difficult and for part of *S. aureus* PCR-positive food samples completely failed. In the case of negative results by using standard method for seven food samples, no typical colonies were observed. All colony types of atypical morphology grown on Baird-Parker agar were isolated and identified as non-*S.-aureus*. The PCR detection of viable *S. aureus* cells in the sample and thereby elimination of false-positive PCR results for nonviable *S. aureus* cells was confirmed by lowered C_T values obtained after enrichment prolonged to 24 h.

Detection of *S. aureus* in Artificially Contaminated Food Samples

The efficacy of the developed real-time PCR-based detection method was further evaluated using ten food samples artificially contaminated with *S. aureus* at the levels of 4×10^{1} and 4×10^{0} CFU g^{-1}, respectively. All ten

Table 5 Results of the real-time PCR and standard detection of *S. aureus* strains at two inoculation levels during the selective enrichment at different conditions in the presence of 1.2×10^6 CFU ml^{-1} natural background cheese microflora

Strain	Enrichment [h]	Modified Giolitti and Cantoni broth								Baird-Parker broth							
		Aerobic				Anaerobic				Aerobic				Anaerobic			
		10^1 CFU g^{-1}			10^0 CFU g^{-1}			10^1 CFU g^{-1}		10^0 CFU g^{-1}		10^1 CFU g^{-1}			10^0 CFU g^{-1}		
		PCR	EN ISO	PCR	EN ISO	PCR	EN ISO	PCR	EN ISO	PCR	EN ISO	PCR	EN ISO	PCR	EN ISO	PCR	EN ISO
CCM	18	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–
3958	24	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–
	42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HPL	18	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
468/1	24	+	+	+	+	+	+	+	–	+	+	–	–	+	+	–	–
	42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VUP	18	+	+	+	+	+	–	–	–	+	–	+	–	–	–	–	–
518	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
	42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

samples were detected positive by the real-time PCR-based method. On the other hand, one and four samples produced negative results using reference method at the levels of 10^1 and 10^0 CFU g^{-1} artificial contaminations, respectively (Table 7). In these cases, no coagulase-positive, as well as PCR-positive colonies were identified among predominantly atypical colonies grown on Baird-Parker agar plates. Moreover, 10^0 CFU 10 g^{-1} was detected in all ten artificially contaminated samples by the developed PCR-based detection method, compared to only two positively detected using the standard detection. All false-negative results of *S. aureus* presence by the standard detection were obtained in samples with natural accompanying microflora higher than 10^4 CFU g^{-1}.

Table 6 Results of PCR and standard *S. aureus* detection in food samples

Food sample	Number	Positive samples		*S. aureus* CFU.g^{-1}		
		PCR	EN ISO	<10^{2a}	10^2–10^4	>10^5
Meat products						
Heat-processed	12	7	7	6	1	
Cured	14	6	6	6		
Fish products						
Smoked	10	8	7	6	2	
Dairy products						
Raw	15	13	11	6	6	1
Frozen	18	10	9	8	2	
Fermented	20	9	6	9		
Dry	10	1	1	1		
Delicatessen						
Dressing	6	4	3	2	2	
Creme	7	3	3	2	1	
Total	112	61	53	47	13	1

[a] No colony (typical or atypical) grown on Baird-Parker agar medium from 0.1 ml of the first decimal sample dilution was identified as *S. aureus*.

Discussion

At the present time, the standard method available for *S. aureus* detection in food is based on selective enrichment followed by microbiological and biochemical identification. These procedures are labor-intensive and time-consuming and providing not always accurate and reliable results. The selectivity of Baird-Parker agar medium is insufficient for the precise identification of typical and particularly atypical *S. aureus* colonies, which can be formed by coagulase-positive *S. aureus* contaminating, for example, dairy products or shrimps (Schoeller and Ingham 2001; Bennett and Lancette 1995; Anonymous 2003a). Moreover, the interpretation of the results of coagulase reaction could be misleading (Da Silva et al. 2000) and may lead to false identification of *S. aureus*. A high percentage of false-negative or false-positive results could occur when API 20E test kits are used in conjunction with conventional method, as the resulted color changes of biochemical reactions are not always exactly interpreted. In particular, if the decision on positive or negative result based on orange coloration depends on the results of other tests, the identification reliability could be inferior.

Generally, molecular-based methods have a well-grounded potential to overcome the insufficiencies of identification procedures associated with the results based on the biochemical characteristics. The aim of our study was to develop and optimize a highly sensitive method for the rapid and definite detection of *S. aureus* in food based

Table 7 Results of the PCR and standard *S. aureus* detection in food samples artificially contaminated by 10^1 and 10^0 CFU per 1 g of sample and by 10^0 per 10 g of sample, respectively

Food sample	Number of samples	Total count[a], CFU g^{-1}	Number of positive samples					
			10^1 CFU g^{-1}		10^0 CFU g^{-1}		10^0 CFU 10 g^{-1}	
			PCR	EN ISO	PCR	EN ISO	PCR	EN ISO
Meat products								
Heat-treated	1	6.5×10^2	1	1	1	1	1	1
Fish products								
Smoked	1	2.5×10^5	1	1	1	1	1	0
Dairy products								
Raw 1	2	6.0×10^6	2	1	2	0	2	0
Raw 2		4.7×10^6						
Frozen	1	5.4×10^4	1	1	1	1	1	0
Fermented 1	2	8.4×10^5	2	2	2	1	2	0
Fermented 2		1.2×10^7						
Dry	1	2.3×10^2	1	1	1	1	1	1
Delicatessen								
Dressing	1	1.8×10^5	1	1	1	0	1	0
Creme	1	7.4×10^2	1	1	1	1	1	1
Total	10		10	9	10	6	10	3

[a] Total counts of microorganisms were estimated according to EN ISO 4833:2003

on original species-specific real-time PCR performed after overnight selective enrichment.

DNA sequence encoding for the protein of acriflavine resistance was selected as a target and oligonucleotides were designed for amplification and real-time fluorescent identification system. The specific 103-bp DNA fragment was fluorescently detected with 100% inclusivity and 100% exclusivity determined using 146 bacterial strains, including 83 strains of *S. aureus* and 13 *Staphylococcus* spp. other than *S. aureus*. No false-positive and no false-negative results of PCR identification were obtained, which indicated that the developed method was highly specific for *S. aureus*.

Practical detection limit for the developed real-time PCR detection was determined to be approximately 6.8×10^1 CFU ml^{-1} with 100% detection probability, which corresponds to approximately 1.7 CFU per reaction. This very low limit of detection was supported by 70% detection probability of 3.4×10^1 CFU ml^{-1} which theoretically corresponds to less than one CFU per reaction. According to these results of Hein et al. (2005), *S. aureus* "cell equivalents"(corresponding to *S. aureus* genome copies) estimated by the real-time PCR method were higher than colony-forming units. This is probably caused by the fact that *S. aureus* individual CFU identified as a colony grown on agar could be formed by a clump of *S. aureus* cells.

The high number of contaminated food samples obtained from markets was not surprising, but the problem with providing of *S.-aureus*-free food samples, particularly of dairy products usable for artificial contamination, was unexpected. Most of the samples positive for *S. aureus* contained the level of total bacterial counts from 10^3 to 10^6 CFU g^{-1}. One sample with the presence of more than 10^4 CFU g^{-1} *S. aureus* (fresh cheese made from raw ewe's milk) contained total bacterial counts of 5×10^8 CFU g^{-1}. This high level of bacterial contamination including high numbers of estimated *S. aureus* probably originated from raw milk. Presence of *S. aureus* in most of the analyzed food products, particularly heat-treated, was probably caused by cross-contamination during the food processing.

To optimize the enrichment, the recovery of *S. aureus* low numbers using two commercially available selective broth and different culture conditions were compared. In order to check up the requirement of anaerobiosis recommended by the standard method, the enrichments were performed in parallel under anaerobic and aerobic conditions. Despite the fact that aerobic conditions allowed the better growth of potentially competitive background microflora estimated as total bacterial counts (data not shown), this had no effect on the ability of the real-time PCR to detect *S. aureus.*

The most impressive advantages of PCR-based detection method in comparison to the standard microbiological detection method are admittedly considered speed, sensitivity, and exactness of the obtained results. In this study, out of 112 food products, 61 samples were positive for *S. aureus* by the real-time PCR-based method and 53 samples by the standard detection method. Moreover, the proposed procedure allowed the detection of 10^0 of artificially added *S. aureus* CFU in 10 g of the sample on the next day in all

analyzed samples, whereas, using the standard method, only three samples were detected correctly. Similar results were presented by other authors. According to the results of Alarcón et al. (2006), out of 164 naturally contaminated foods tested for the presence of *S. aureus*, 74 were positive by PCR and 69 by traditional culture method. Problematic identification of *S. aureus* on Baird-Parker agar from the enrichments (detected PCR-positive for *S. aureus*) could be caused by a limited selectivity of Baird-Parker agar since only lipolytic and proteolytic *S. aureus* can be easily recognized (Baird and Lee 1995). Nonlipolytic strains with atypical morphology on Baird-Parker agar may be frequent in milk and dairy products (Bennett and Lancette 1995). Baird-Parker agar is often considered not sufficiently selective for the analysis of foods with a high level of contaminating flora (Zangerl 1999). Some of the microorganisms (*Staphylococcus* spp. other than *S. aureus*, *Bacillus* spp., *Proteus* spp., *Enterococcus* spp., or *Micrococcus* spp.) form colonies with a morphology similar to those of *S. aureus* (Isigidi et al. 1989).

The developed real-time PCR-based method, which involves overnight selective enrichment under aerobic conditions, facilitated sensitive next-day *S. aureus* detection. Moreover, the method was able to overcome the problematic colony identification using standard microbiological method, particularly in the case of atypical *S. aureus* colonies. Rapid and sensitive detection of *S. aureus* in food raw material, particularly in processing of dairy products should be crucial for microbiological food quality assurance.

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Rapid and sensitive detection of pathogenic *Yersinia enterocolitica* strains in food using selective enrichment and real-time PCR

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Summary

Yersinia enterocolitica is an important human enteroinvasive pathogen with a global distribution. Food contamination is associated with pigs considered a major reservoir of *Y. enterocolitica*. The aim of this study was to develop and evaluate a rapid and sensitive real-time PCR-based method for the detection of pathogenic *Y. enterocolitica* in food. A TaqMan probe-based real-time PCR detection system targeting a sequence of chromosomally located *ail* (attachment and invasive locus) gene was designed. The real-time PCR system was evaluated as 100% selective for pathogenic *Y. enterocolitica*, which was determined using 20 *Y. enterocolitica* strains, 16 non-enterocolitica *Yersinia* spp. and 30 other *Enterobacteriaceae* strains. The PCR detection limit was 1×10^2 CFU.ml^{-1}. Single selective enrichment based on the evaluation of various enrichment procedures was optimized. Out of 20 food samples analysed, four were positive by the PCR-based method and only one by the standard method. When 10 food matrices were artificially contaminated at a level of 10^0 CFU, ten and six sets of samples were positive by respective methods. The developed method may be used for the detection of pathogenic *Y. enterocolitica* as a specific and reliable alternative to the currently used microbiological method, furthermore providing a considerable reduction in the required analysis time.

Keywords

pathogenic *Yersinia enterocolitica*; real-time PCR; detection; food

Yersinia enterocolitica is a food-borne pathogen causing yersiniosis, which can range from a self-limiting gastroenteritis to a potentially fatal septicemia [1]. The primary route of infection of humans is most probably through ingestion of contaminated food. Healthy pigs have been identified as a major reservoir of the human pathogenic *Y. enterocolitica* strains and pork is therefore likely to be the most important vehicle for its transmission to humans [2].

Species *Y. enterocolitica* is divided into six biotypes: 1A, 1B, 2, 3, 4 and 5 [3]. Five biotypes (except for 1A) are considered pathogenic for humans. The strains belonging to biogroup 1A are regarded as avirulent, although they may be opportunistic pathogens [4, 5]. The serotype most frequently implicated in human infection is O:3 (almost all strains belonging to biogroup 4), then serotypes O:8 (biogroup 1B) and O:9, O:5,27 (biogroup 2 or 3) [6, 7]. Virulence of *Y. enterocolitica* isolates depends on the expression of several chromosomal and plasmid-encoded genes, and on a complex interplay between the secreted virulence factors [8]. First chromosomal factor required for the infection, *ail* (attachment invasion factor) gene, is a stable virulence marker limited to only pathogenic strains of *Y. enterocolitica*. The virulence plasmid pYV is not a good DNA target for the detection of pathogenic *Y. enterocolitica*, because of the possibility to obtain false negative results due to a potential loss of the plasmid during subculturing.

The currently available standard method for the detection of presumptive pathogenic *Yersinia enterocolitica*, EN ISO 10273 [9], is labour-intensive, time-consuming and does not provide the required selectivity and detection limit for reliable results. PCR-based methods are known to be a powerful tool for highly specific and sensitive identification of pathogenic bacteria in foods and are considered

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reliable alternatives to conventional methods [10]. The second generation of PCR methodologies, i.e. real-time PCR, is able to overcome several limitations of conventional PCR. The dominant target for the PCR-based detection of pathogenic *Y. enterocolitica* strains is *ail* gene, which has been used either in conventional PCR [11–13] or in real-time PCR [14–16].

In this study, a TaqMan real-time PCR-based detection targeted to *ail* gene is described. Being found rapid and sensitive, *Y. enterocolitica* PCR detection suitable for routine application has been applied to natural and artificially contaminated foods and evaluated by comparing real-time PCR with conventional detection. Different enrichment media and conditions have been used and compared to improve the sensitivity of the detection, in particular in the presence of high levels of the competing microflora.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Yersinia spp. used in the study (listed in Tab. 1) were obtained from Czech Collection of Microorganisms (CCM, Brno, Czech Republic), Spanish Type Culture Collection (CECT, Valencia, Spain), kindly provided by Prof. Naydenski (Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria), or isolated and identified in our laboratory (VÚP Food Research Institute, Bratislava, Slovakia). The strains from *Enterobacteriaceae* family other than *Yersinia* spp. were obtained from official collections. All strains were maintained at –18 °C in 60% (v/v) glycerol solution or freeze-dried for long-period storage. Working cultures were prepared in Brain Heart (BH) broth (Merck, Darmstadt, Germany) by overnight incubation at 37 °C with shaking. Decimal dilutions were prepared in 0.85% NaCl and cell concentrations were determined by the plate-count method.

Identification of *Yersinia enterocolitica* strains

Strains of *Yersinia* spp. were analysed by using published conventional PCR for selected chromosomal markers (*ail* and *ystB*), virulence plasmid pYV (*yadA*) and species-specific markers (Ye16S rDNA) described in Tab. 2. Growth and characteristic colony morphology of *Y. enterocolitica* strains was followed on a selective agar with cefsulodin, irgasan and novobiocin (CIN agar, Merck).

Preparation of DNA and 5'-nuclease PCR

DNA was prepared from overnight cultures using lysis by boiling, as previously described for other pathogenic bacteria [17]. As a positive control for PCR, DNA was extracted from *Y. enterocolitica* CCM 5671 using QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany). Each PCR reaction contained 300 nmol·l^{-1} of each of primers ailrtF and ailrtR, 200 nmol·l^{-1} of the TaqMan probe ailrtP (Qiagen Operon, Cologne, Germany), 500 μmol·l^{-1} of each dNTP (Applied Biosystems, Foster City, California, USA), 1.5 U of HotStar Taq DNA polymerase (Qiagen), 1× concentrated PCR buffer, 4.5 mmol·l^{-1} magnesium chloride, TaqMan exogenous internal positive control labelled with the dye VIC (Applied Biosystems), 2.5 μl of the DNA sample and water to make the total volume up to 25 μl. Real-time PCR was performed in a PTC-200 thermal cycler coupled to a Chromo 4 continuous fluorescence detector (MJ Research, Waltham, Massachusetts, USA) using a thermal programme consisting of the initial denaturation of 15 min at 95 °C, and 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Three negative and two positive controls were included in each experiment.

Tab. 1. Sequences of oligonucleotide used in the study.

Designation	Sequence and labelling	Product size	Reference
ailF/ailrtF	5´-TTAATGTGTACGCTGCGAGTG -3´	425 bp	[15]
ailR	5´-GGAGTATTCATATGAAGCGTC -3´		
ystBF	5´-TTGGACACCGCACAGCTTAT -3´	263 bp	this study
ystBR	5´-ACAGGCAGGATTGCAACATA -3´		
Ye16SF	5´-AATACCGCATAACGTCTTCG-3´	329 bp	[11]
Ye16SR	5´-CTTCTTCTGCGAGTAACGTC -3´		
yadAF	5´-AGATTCGGCAGTTACTTATGG-3´	308 bp	[15]
yadAR	5´-ATTGCGCGACATTCACT -3´		
ailrtR	5´-CAGGTTAAAACCTTTAGGGTTCA -3´	107 bp	this study
ailrtP	5´- FAM-TTAATGTGTACGCTGCGAGTG -TAMRA-3´		

Tab. 2. *Yersinia* spp. identification using PCR targeting different virulence markers.

No.	Strain and designation	PCR result				
		Ye16S (330 bp)	ail (425 bp)	ailrt (107 bp)	ystB (262 bp)	yadA (308 bp)
1	*Y. enterocolitica* VUP 25	+	–	–	+	–
2	*Y. enterocolitica* VUP 26	+	–	–	+	–
3	*Y. enterocolitica* VUP 27	+	–	–	+	–
4	*Y. enterocolitica* VUP 28	+	–	–	+	–
5	*Y. enterocolitica* VUP 29	+	–	–	+	–
6	*Y. enterocolitica* VUP 30	+	+	+	–	–
7	*Y. enterocolitica* VUP 31	+	+	+	–	–
8	*Y. enterocolitica* VUP 33	+	+	+	–	–
9	*Y. enterocolitica* VUP 54	+	+	+	–	–
10	*Y. enterocolitica* VUP 55	+	+	+	–	–
11	*Y. enterocolitica* VUP 61	+	+	+	–	–
12	*Y. enterocolitica* VUP 62	+	+	+	–	–
13	*Y. enterocolitica* VUP 64	+	–	–	+	–
14	*Y. enterocolitica* VUP 65	+	+	+	–	–
15	*Y. enterocolitica* VUP 155	+	+	+	–	–
16	*Y. enterocolitica* VUP 167	+	–	–	+	–
17	*Y. enterocolitica* CCM 5671	+	+	+	–	–
18	*Y. enterocolitica* CECT 4054	+	+	+	–	–
19	*Y. enterocolitica* CECT 4055	+	+	+	–	–
20	*Y. enterocolitica* CECT 559	+	+	+	–	–
21	*Y. bercovieri* CCM 4206	–	–	–	–	–
22	*Y. bercovieri* IP 22935	–	–	–	–	–
23	*Y. bercovieri* IP 22914	–	–	–	–	–
24	*Y. frederiksenii* CCM 3555	–	–	–	–	–
25	*Y. frederiksenii* IP 22775	–	–	–	–	–
26	*Y. intermedia* CCM 3558	–	–	–	–	–
27	*Y. intermedia* IP 22702	–	–	–	–	–
28	*Y. intermedia* IP 22803	–	–	–	–	–
29	*Y. kristensenii* CCM 3559	–	–	–	–	–
30	*Y. kristensenii* CCM 3561	–	–	–	–	–
31	*Y. kristensenii* IP 22468	(+)*	–	–	–	–
32	*Y. kristensenii* IP 22914	(+)	–	–	–	–
33	*Y. mollaretti* CCM 4208	–	–	–	–	–
34	*Y. pseudotuberculosis* CCM 5666	–	–	–	–	–
35	*Y. pseudotuberculosis* IP 32979	–	–	–	–	–
36	*Y. pseudotuberculosis* IP 32981	(+)	–	–	–	–
	Y. enterocolitica Y79**	+	+	+	–	+

* – weak PCR product, ** – DNA reference material obtained from Institute for Reference Materials and Measurements, Geel, Belgium.

Determination of PCR detection limit

DNA from the overnight culture of *Y. enterocolitica* CCM 5671 was isolated using QiaAmp DNA Mini Kit (Qiagen) and used to determine the DNA-based detection limit. Concentration of total extracted DNA was determined using Quant-iT PicoGreen Assay (Invitrogen, Gaithersburg, Maryland, USA) with fluorescence measured in a Tecan Saphire2 plate reader (Tecan, Salzburg, Austria). Two parallel PCR analyses of diluted DNA solutions were performed. For the determination of the practical PCR detection limit and PCR detection probability, two independent sets of three to ten parallel PCR analyses of suitable dilutions (ranging from 10^6 to 10^0 CFU·ml^{-1}) of *Y. enterocolitica* CCM 5671 overnight culture were performed.

Evaluation of enrichment procedures

Two selective broths and related enrichment procedures were used according to EN ISO 10273: enrichment in Peptone Sorbitol Bile (PSB) broth (Fluka, Buchs, Switzerland) at 25 °C for 2 to

3 days, and enrichment in Irgasan Ticarcillin Potassium Chlorate (ITC) broth (Fluka) at 25 °C for 48 h. Culture in a new Yersinia Selective Enrichment Broth (YSEB) according to Ossmer (Merck) at 30 °C for 24 h was also tested for comparison. For evaluation of different enrichment procedures, model *Enterobacteriaceae* microflora at levels of 10^8, 10^6 a 10^4 CFU per 100 ml of medium was used as the background. Real background microflora was prepared by homogenization of a raw meat sample in a bag with a filter insert. Total bacterial count of 6×10^8 CFU·ml^{-1} and *Enterobacteriaceae* count of 2×10^6 CFU·ml^{-1} were determined in this homogenate. *Y. enterocolitica* growth in individual enrichment cultures was followed by real-time PCR and the results were expressed in terms of *Y. enterocolitica* recovery.

Food samples and artificial contamination

Food samples were obtained from retail market in Slovakia. All food samples were analysed for the presence of *Y. enterocolitica* using a microbiological method according to EN ISO 10273 [9] and using the developed real-time PCR-based method. For artificial contamination, *Y. enterocolitica* CCM 5671 were inoculated at two initial concentrations of 10^0 and 10^1 CFU per 10 g sample and 90 ml of medium.

Reference method

The method according to EN ISO 10273 (Microbiology of food and animal feeding stuffs – Horizontal method for the detection of presumptive pathogenic *Yersinia enterocolitica*) [9] was used, simultaneously utilizing two selective enrichment media with incubations up to 5 days, followed by identification of characteristic *Y. enterocolitica* colonies on two selective agar media, and their subsequent confirmation using biochemical and serological tests.

RESULTS AND DISCUSSION

Evaluation of virulence markers application

Results of duplex PCR targeting *ail* gene for "more pathogenic" *Y. enterocolitica* strains (biotypes 1B, 2 to 5) and *ystB* gene for "less pathogenic" *Y. enterocolitica* strains (biotype 1A) clearly distinguished these two groups (Tab. 2, Fig. 1). Out of 20 analysed *Y. enterocolitica* strains, 13 were positive for *ail* gene marker and 7 for *ystB* gene marker. Marker of *yadA* gene detecting pYV plasmid was not identified for any *Y. enterocolitica* strain, the detection ability of PCR being verified using DNA reference material from Institute for Reference Materials and Measurements (Geel, Belgium). Species-specific marker targeting 16 S rDNA gave positive results for all *Y. enterocoolitica* analysed, but also false positive results for some other *Yersinia* spp. (Tab. 2). Our results confirmed successful application of *ail* gene, a chromosomally located marker. The *yadA* gene located on the virulence plasmid was considered unsuitable as a target for the detection because of plasmid instability during the laboratory treatment of strains.

Design of oligonucleotides and selectivity of real-time PCR

Based on published *Y. enterocolitica ail* gene sequence, a new reverse primer and a probe were designed using Primer Express software (Applied Biosystems) to amplify a 107 bp product; the forward primer previously designed for conventional PCR [15] was used. No non-*Y. enterocolitica* homological DNA sequences were found in the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA). Selectivity of the developed real-time PCR system was evaluated using 63 *Yersinia* spp. and other *Enterobacteriaceae* strains demonstrating a 100% inclusivity and 100% exclusivity.

PCR detection limit

The DNA-based detection limit, determined using dilutions of DNA isolated from *Y. entero-*

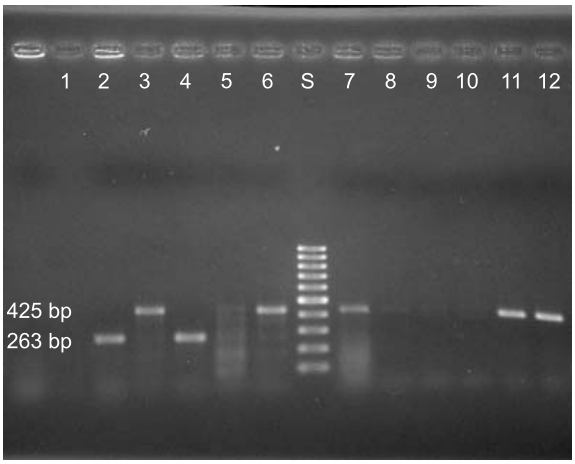

Fig. 1. Example of a duplex PCR targeting *ail* gene (425 bp) and *ystB* gene (263 bp).
1 – *Y. pseudotuberculosis* CCM 5666, 2 – *Y. enterocolitica* VUP25, 3 – *Y. enterocolitica* CCM 5671, 4 – *Y. enterocolitica* VUP27, 5 – *Y. intermedia* CCM 3558, 6 – *Y. enterocolitica* VUP31, 7 – *Y. enterocolitica* VUP62, 8 – *Citrobacter freundii* CCM 4475, 9 – *Serratia marcescens* CCM 303, 10 – *Y. frederiksenii* CCM 3555, 11 – *Y. enterocolitica* VUP65, 12 – *Y. enterocolitica* VUP155, S – molecular standard $n \times 100$ bp.

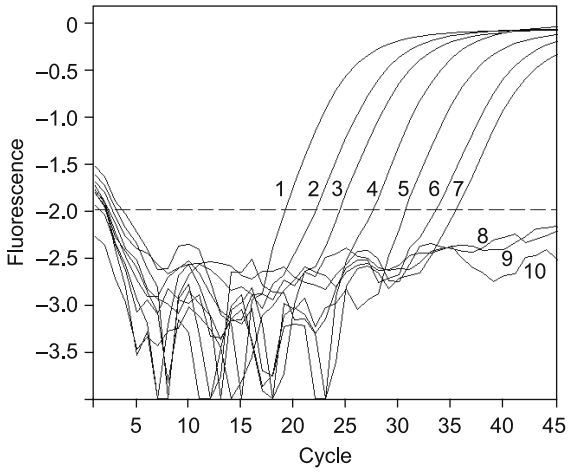

Fig. 2. Record of real-time PCR using *Y. enterocolitica* CCM 5671 culture dilutions.
1 – 6×10^7 CFU·ml^{-1}, 2 – 6×10^6 CFU·ml^{-1}, 3 – 6×10^5 CFU·ml^{-1}, 4 – 6×10^4 CFU·ml^{-1}, 5 – 6×10^3 CFU·ml^{-1}, 6 – 6×10^2 CFU·ml^{-1}, 7 – 1.2×10^2 CFU·ml^{-1}, 8 – 6×10^1 CFU·ml^{-1}, 9 – 1.2×10^1 CFU·ml^{-1}, 10 – 6×10^0 CFU·ml^{-1}.

colitica CCM 5671, was approximately 10 fg per PCR reaction (data not shown). This is equivalent to two genome copies, calculated on the basis of *Y. enterocolitica* genome size of 4.62 Mbp ≈ 5 fg, (BLAST database, AM286415, National Center for Biotechnology Information). The practical PCR detection limit was estimated using decimal dilutions of the overnight culture and DNA samples prepared by cell lysis (Fig. 2). The detection probability of the cell suspension was 100% at a concentration of 1.2×10^2 CFU·ml^{-1} and at higher concentrations, and 75% at a concentration of 6.0×10^1 CFU·ml^{-1} (data not shown). Comparable or higher detection limits were obtained by other researchers [14, 15]. Thisted-Lambertz et al. [16] showed in their study, that the probability of detecting the cell count level of 10 *Y. enterocolitica* cells per ml was 47% and that of cell count level of 100 *Y. enterocolitica* cells per ml was 97%. These results mean that a concentration of target *Y. enterocolitica* greater than 1.2×10^2 CFU·ml^{-1} must be reached by enrichment to ensure the reliable detection of *Y. enterocolitica* in the food sample using the developed real-time PCR method.

Evaluation of enrichment procedures

Y. enterocolitica CCM 5671 recovery was evaluated, using real-time PCR detection during the enrichment in three different selective media at two levels of artificial contamination and enrichment conditions according to manufacturer's instructions, and using model and real background microflora. PCR provided more sensitive detection in the case of high concentration of model *Enterobacteriaceae* background microflora, but at the extreme load of 10^8 CFU·ml^{-1}, the detection of 2×10^0 CFU of *Y. enterocolitica* per sample completely failed using both methods. On the other hand, using real microflora background (probably containing stressed cells) at a total bacterial count of 6×10^8 CFU·ml^{-1} with *Enterobacteriaceae* count of 2×10^6 CFU·ml^{-1}, the detection was 100% successful. Results for the detection at contamination levels 10^0 CFU, 10^1 CFU and 10^2 CFU per 100 ml culture in combinations with background microflora of 10^4, 10^6 and 10^8 total bacterial count are presented in Tab. 3 and Tab. 4. The obtained results demonstrate that the best results of positive samples recovery and most rapid PCR detection of *Y. enterocolitica* were obtained using Yersinia Selective Enrichment Broth (YSEB). Summarized results of enrichment recovery for positive samples are expressed in percentage in Tab. 5. Applicability of this medium was verified by model detection of 2×10^0 CFU of pathogenic *Y. enterocolitica* strains of different serotypes (CECT 4054 serotype O:8, CECT 4055 serotype O:3, VUP62 serotype O:5) and the same performance of the medium was observed (data not shown). Similar results were described by Thisted-Lambertz et al. [16], who determined the accuracy of the method according to ISO 10273 to be only 40%, compared to 95% of the real-time PCR, as determined using food samples artificially inoculated with low levels of *Y. enterocolitica* (10 to 300 CFU per 25 g sample).

Detection of pathogenic *Y. enterocolitica* in food samples

Subsequently, the enrichment for 24 to 48 h in Yersinia enrichment selective broth at 30 °C and the detection by the developed real-time detection was compared to the detection method according to EN ISO 10273 [9] for the detection of presumptive pathogenic *Y. enterocolitica* by analysis of 20 food products. Four samples were found positive for *Y. enterocolitica* by the real-time PCR-based method and only one sample was found positive by the standard detection method (Tab. 6).

The efficiency of the developed real-time PCR-based detection method was further evaluated using ten food samples artificially contaminated with *Y. enterocolitica* CCM 5671 at levels of 2×10^1 CFU per 10 g and 2×10^0 CFU per 10 g, respectively. For all 10 samples, positive detection results were obtained by the real-time PCR-based method. On the other hand, one and four samples produced negative results using the standard method at levels of 10^1 CFU per 10 g and 10^0 CFU

Tab. 3. Evaluation of different selective enrichment procedures by *ail*-targeted real-time PCR and standard method, using three levels of *Y. enterocolitica* contamination and three levels of model background microflora (*Enterobacteriaceae* strains).

Y. enterocolitica CCM 5671 [CFU]	Bacground microflora [CFU]	24 h		48 h		72 h	
		rtPCR	EN ISO	rtPCR	EN ISO	rtPCR	EN ISO
PSB medium, 25 °C, with shaking (EN ISO 10273)							
10^0	–	nd	nd	+	–	+	+
	10^4	nd	nd	+	–	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	–	–	–	–
10^1	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	–	–	–	–
10^2	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	+	–	+	+
ITC medium, 25 °C (EN ISO 10273)							
10^0	–	–	–	+	+	nd	nd
	10^4	–	–	+	+	nd	nd
	10^6	–	–	+	+	nd	nd
	10^8	–	–	–	–	nd	nd
10^1	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	–	–	+	+	nd	nd
	10^8	–	–	–	–	nd	nd
10^2	–	+	+	+	+	nd	nd
	10^4	+	–	+	+	nd	nd
	10^6	+	–	+	+	nd	nd
	10^8	–	–	+	+	nd	nd
YSEB medium, 30 °C (new)							
10^0	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	–	+	+	nd	nd
	10^8	–	–	+	–	nd	nd
10^1	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	–	+	+	nd	nd
	10^8	+	–	+	–	nd	nd
10^2	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	+	+	+	nd	nd
	10^8	+	–	+	+	nd	nd

nd – not determined.

Tab. 4. Evaluation of different selective enrichment procedures by real-time PCR and standard method using three levels of *Y. enterocolitica* contamination and three levels of real background microflora (raw meat filtered homogenate).

Y. enterocolitica CCM 5671 [CFU]	Background microflora [CFU]	24 h		48 h		72 h	
		rtPCR	EN ISO	rtPCR	EN ISO	rtPCR	EN ISO
PSB medium, 25 °C, with shaking (EN ISO 10273)							
10^0	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	–	–	–	–
10^1	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	–	–	+	–
10^2	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	+	+	+
	10^8	nd	nd	+	–	+	+
ITC medium, 25 °C (EN ISO 10273)							
10^0	–	–	–	+	+	nd	nd
	10^4	–	–	+	+	nd	nd
	10^6	–	–	+	+	nd	nd
	10^8	–	–	–	–	nd	nd
10^1	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	–	–	+	+	nd	nd
	10^8	–	–	+	+	nd	nd
10^2	–	+	+	+	+	nd	nd
	10^4	+	–	+	+	nd	nd
	10^6	+	–	+	+	nd	nd
	10^8	–	–	+	+	nd	nd
YSEB medium, 30 °C (new)							
10^0	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	+	+	+	nd	nd
	10^8	+	–	+	–	nd	nd
10^1	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	+	+	+	nd	nd
	10^8	+	–	+	–	nd	nd
10^2	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	+	+	+	nd	nd
	10^8	+	–	+	+	nd	nd

nd – not determined.

Tab. 5. Detection ability of *Y. enterocolitica* CCM 5671 at contamination levels of 10^0, 10^1 and 10^2 CFU with background microflora using three enrichment procedures.

	24 h		48 h		72 h	
	rtPCR	EN ISO	rtPCR	EN ISO	rtPCR	EN ISO
PSB medium, 25 °C (with shaking)	nd	nd	83%	46%	88%	79%
ITC medium, 25 °C	42%	25%	88%	88%	nd	nd
YSEB medium, 30 °C	96%	63%	100%	83%	nd	nd

nd – not determined.

Tab. 6. Results of the detection of pathogenic *Y. enterocolitica* in natural food samples.

Sample	Number of samples	Number of positive results	
		rtPCR detection	EN ISO detection
raw meat	6	2	1
bowels	3	0	0
fish	3	0	0
frozen vegetable	4	0	0
fresh chopped vegetable	4	2	0
Total	20	4	1

Tab. 7. Results of the detection of pathogenic *Y. enterocolitica* in food samples artificially contaminated at two levels.

Sample	Number of samples	Number of positive results			
		rtPCR detection		EN ISO detection	
		10^0 CFU	10^1 CFU	10^0 CFU	10^1 CFU
raw meat	2	2	2	1	2
bowels	2	2	2	2	2
fish	2	2	2	1	2
frozen vegetable	2	2	2	2	2
fresh chopped vegetable	2	2	2	2	2
Total	10	10	10	6	9

per 10 g, respectively (Tab. 7). Insufficient detection ability of the standard method was observed also by other investigators [11, 14, 16]. Fredriksson-Ahomaa and Korkeala [18] concluded that the reported low rate of pathogenic *Y. enterocolitica* strains isolated from foods is most probably due to a limited sensitivity of the traditional culture detection methods, which provided the detection limits between 10^3 to 10^6 CFU or more per g of pork products.

CONCLUSSION

The currently established standard method for the detection of pathogenic *Y. enterocolitica* in food (EN ISO 10273) is based on enrichment simultaneously in two selective broths followed by microbiological and biochemical identification. This method is labour-intensive, time-consuming and analytically insufficient [16]. Generally, molecular-based methods have a well-grounded potential to overcome the insufficiences of identification procedures associated with biochemical characteristics. The aim of our study was to develop and optimize a highly sensitive and reliable method for the rapid and definitive detection of *Y. enterocolitica* pathogenic biotypes in food, in particular in food products containing high levels of competing microflora. The method would be coupled to selective enrichment and ensure the required detection limit. The developed real-time PCR-based method, which involves an optimized, reduced selective enrichment, facilitates the detection of pathogenic *Y. enterocolitica* strains on the next day after the food sample reception. The developed

real-time PCR system targeting *ail* gene may be complemented by PCR targeting *ystB* gene, which would result in the detection of all *Y. enterocolitica* biotypes.

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