Microbiological Food Safety of Cheese Produced in Swedish Small-scale Dairies

Characteristics, growth and enterotoxin production of *Staphylococcus aureus*

Åsa Rosengren
Faculty of Natural Resources and Agricultural Sciences
Department of Microbiology
Uppsala

Licentiate Thesis
Swedish University of Agricultural Sciences
Uppsala 2012

Cover: ‘Waiting for better times to come’
(photos: Flemming Lund and Susanne Sylvén, edited by Åsa Rosengren)

ISSN 1652-6880
ISBN 978-91-576-9074-6

Print: SLU Service/Repro, Uppsala 2012

Microbiological Food Safety of Cheese Produced in Swedish Small-scale Dairies

Characteristics, growth and enterotoxin production of *Staphylococcus aureus*

Abstract

The number of small-scale dairies in Sweden has increased during the past decade. Current agricultural policy encourages such small-scale production and various ways to help producers have been proposed. Combining traditional cheese making and good product safety is a challenge, since several human pathogens pose a hazard in the products. *Staphylococcus aureus* is an important foodborne pathogen that can be transmitted via milk to cheese. Some strains can produce staphylococcal enterotoxin (SET) during growth in foods and cause food poisoning. Environmental conditions during the initial phase of cheese making are close to optimal for growth of *S. aureus*.

This study examined small-scale artisan cheese production and relevant pathogens, with the aim of formulating advice on microbiologically safe production. A survey of fresh and short-time ripened cheeses produced in Swedish small-scale dairies investigated the occurrence and levels of *S. aureus*, *Listeria monocytogenes* and *Escherichia coli*. Information about production practices was collected and *S. aureus* isolates were characterised with emphasis on enterotoxin genes, antibiotic resistance, biotyping and genetic variation. *Staphylococcus aureus* isolates from cheeses were investigated in broth cultures for their potential for growth and production of staphylococcal enterotoxin A (SEA) in typical cheese making conditions as regards temperature, pH, lactic acid/undissociated lactic acid and water activity.

Detected levels of the three pathogens were reasonable in most cheese samples tested. *Listeria monocytogenes* and SET were not found, but *E. coli* and enterotoxigenic *S. aureus* were frequently found in raw milk cheeses, sometimes at high levels. The *S. aureus* isolates were mainly of animal biotype and two-thirds were enterotoxigenic. The isolates tested were able to grow and produce SEA in the presence of moderate concentrations of undissociated lactic acid. SEA was produced continuously during incubation and high concentrations were found long after *S. aureus* levels had peaked. In practice, further storage of cheese samples prior to SET analysis may be beneficial when high staphylococcal levels are detected. Increased awareness of the importance of hygiene barriers is needed. Possible improvements in process control include *e.g.* controlling raw milk quality, using active starter cultures and monitoring acidification.

Keywords: Survey, Food Safety, Cheese, Small-scale dairies, Lactic acid, *Staphylococcus aureus*, growth rate, lag time, staphylococcal enterotoxin

Author's address: Åsa Rosengren, National Food Agency,
P.O. Box 622, 751 26 Uppsala, Sweden
E-mail: asa.rosengren@ slv.se

Mikrobiologisk livsmedelssäkerhet i ostar som tillverkats på svenska småskaliga mejerier

Egenskaper, tillväxt och enterotoxinproduktion av *Staphylococcus aureus*

Sammanfattning

Antalet småskaliga mejerier i Sverige har ökat under det senaste årtiondet. Dagens jordbrukspolitik uppmuntrar denna livsmedelproduktion och olika förslag till stöd och hjälp har lags fram. Kombinationen av traditionell osttillverkning och god livsmedelssäkerhet är en utmaning då det finns ett antal livsmedelsburna patogener som kan utgöra en fara i produkterna. *Staphylococcus aureus* är en viktig livsmedelsburen patogen, som bland annat kan förorena osten via mjölkråvaran. Vissa stammar av *S. aureus* har förmågan att producera stafylokockenterotoxin (SET) när de växer i livsmedel och kan därigenom orsaka matförgiftning. De miljöbetingelser som råder under osttillverkningens första fas gynnar tillväxt av *S. aureus*.

Denna studie undersökte småskalig osttillverkning och relevanta patogener i syfte att formulera råd till en mikrobiologiskt säker ostproduktion. Färska och korttidslagrade ostar, tillverkade på svenska småskaliga mejerier, analyserades med avseende på förekomst och halter av *S. aureus*, *Escherichia coli* och *Listeria monocytogenes*. Information om tillverkningsmetoder samlades in och *S. aureus*-isolat karaktäriserades med avseende på enterotoxingener, antibiotikaresistens, biotypning och genetisk variation. *Staphylococcus aureus*-isolat från de analyserade ostarna undersöktes i buljongkulturer för deras förmåga att tillväxa och producera stafylokockenterotoxin A (SEA) under förhållanden som liknar osttillverkning avseende temperatur, pH, mjölksyra/odissocierad mjölksyra och vattenaktivitet.

Halterna av de tre patogenerna var rimliga i de flesta analyserade ostproven. *Listeria monocytogenes* och SET detekterades inte i något prov, men *E. coli* och *S. aureus* påvisades ofta i ostar gjord av opastöriserad mjölk, ibland i höga halter. Isolaten av *S. aureus* var huvudsakligen av animaliska biotyper och två tredjedelar bar på minst en av de analyserade enterotoxingenerna. I måttliga koncentrationer odissocierad mjölksyra kunde de undersökta isolaten både tillväxa och producera SEA. SEA producerades kontinuerligt under inkubering och höga koncentrationer påvisades långt efter det att cellhalten nått sitt maximum.

När höga halter av stafylokocker påvisats i ost kan det i praktiken vara fördelaktigt att förvara ostprovet en tid innan det ska analyseras för SET. Det finns behov av en ökad medvetenhet om hygienbarriärernas betydelse och möjliga förbättringar i produktionskontrollen inkluderar mjölkråvarans kvalitet, användandet av aktiv starterkultur och övervakning av syrning

Age is something that doesn't matter, unless you are a cheese

Billie Burke

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Åsa Rosengren, Ane Fabricius, Bengt Guss, Susanne Sylvén and Roland Lindqvist (2010) Occurrence of foodborne pathogens and characterization of *Staphylococcus aureus* in cheese produced on farm-dairies. *Int J Food Microbiol*. 144 (2010) 263-269.

II Åsa Rosengren, Mats Lindblad and Roland Lindqvist. The effect of undissociated lactic acid on *Staphylococcus aureus* growth and enterotoxin A production. *Submitted.*

Paper I is reproduced with the permission of the publisher.

My contribution to the papers included in this thesis was as follows:

I – II Planned the studies with co-authors, performed most laboratory work and had main responsibility for writing and revising manuscripts.

Abbreviations

a_w	Water activity
CMT	California Mastitis Test
CPS	Coagulase positive staphylococci
ELISA	Enzyme linked immuno sorbent assay
FBO	Food business operator
GNG	Growth/no growth
HACCP	Hazard analysis critical control points
HLac	Undissociated lactic acid
LAB	Lactic acid bacteria
Lac_{tot}	Total concentration of lactic acid
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PM	Predictive food microbiology
SAgs	Superantigens
SCC	Somatic cell count
SE	Emetic staphylococcal enterotoxin
SEA	Staphylococcal enterotoxin A
SEC	Staphylococcal enterotoxin C
SE*l*	Staphylococcal enterotoxin-*like*
SET	Staphylococcal enterotoxin
SFP	Staphylococcal food poisoning
SFPO	Staphylococcal food poisoning outbreak
TSS	Toxic shock syndrome
TTG	Time-to-growth

1 Introduction

1.1 Small-scale cheese production in Sweden

During the last decade, there has been increased interest in, and political encouragement of, small-scale production of *e.g.* cheese and other milk products. Government authorities within the food and agricultural area have issued a number of suggestions to facilitate and support small-scale manufacturers. The suggestions include stimulation of networks between businesses within an area, education in food hygiene and food safety, and establishment of centres for small-scale food production for guidance and knowledge dissemination (Kemi & Sylvén, 2005).

In the Swedish small-scale dairy sector, the number of dairies increased by 50%, from 78 to 117, during the period 2005-2011 (Eldrimner, 2011; National Food Agency, 2011; Sveriges gårdsmejerister, 2011). Some of the incentives for starting up a small-scale dairy have been reported to be value added in the form of local or ecological regional food production in close contact with consumers, good husbandry, increased farm profits, the challenge and the fact that the production contributes to rural livelihoods and increased employment (Johansson, 1998). There is an increasing trend for using unpasteurised milk, especially among recently started producers and those using milk from their own animals[1]. The increased production of raw milk cheeses may be a result of the current food legislation. In contrast to earlier, the legislation now allows Swedish raw milk cheeses ripened <60 days to be sold on the common market, and not only directly to the consumer (EC, 2004b).

A typical feature of small-scale production of artisan cheese is that the milk used comes from one or a few sources. Compared with larger dairies, the

[1]. Birgitta Sundin, Eldrimner, pers. communication 2011-12-19

small-scale food businesses face different challenges in meeting increased consumer demands, and many have expressed difficulties in complying with food hygiene and safety legislation (Anonymous, 2005).

The majority of Swedish small-scale dairies are small enterprises consisting of a few persons. Among the producers, there is a wide range of experience, from those recently started to those with long experience. This is also reflected in the design of the dairy premises (Fig. 1). Limited numbers of non-academic cheese making courses are available, and since artisan cheese making is based on traditional production techniques, knowledge is often transferred orally among cheese making colleagues. Hence, there is reason to expect a large variation in knowledge and awareness of the microbial safety of the products. However, to assess the microbial safety of the products, data regarding production practices, properties and virulence potential of bacterial hazards, and the impact of pasteurisation and starter cultures are needed (Lindqvist *et al.*, 2002).

Figure 1. Examples of exteriors and interiors of Swedish small-scale dairies (photo: Åsa Rosengren)

1.2 Milk and cheese microbiology

1.2.1 Raw milk microbiology

Raw milk contains a mixed microflora that comes from several sources. In the mammary gland, milk does not contain bacteria unless the animal suffers from udder or systemic disease. However, as milk is excreted it may become contaminated with saprophytic bacteria of *e.g.* the genera *Micrococcus*, *Streptococcus, Bacillus* and *Staphylococcus* living in the epithelium of the teat canal. Thus, even in a healthy animal, the milk may contain many bacteria when it leaves the animal (Lejeune & Rajala-Schultz, 2009). In addition, the animals, dairy farm environment and milking equipment are important reservoirs for many foodborne pathogens. Soil, bedding materials and faeces are important contamination sources of *e.g. Campylobacter* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* spp. and verocytotoxigenic *Escherichia coli* (ICMSF, 1998; Jayarao & Henning, 2001; Oliver *et al.*, 2005; Jayarao *et al.*, 2006).

Staphylococcus aureus is often found in milk, and has also been reported to be isolated from skin of udders and teats, wounds and mucosa, milking equipment and shelves, floor, door knobs, *etc*. (Jorgensen *et al.*, 2005b). This bacterium is the most important species causing mastitis, which is an infection of the mammary tissue. Mastitis is caused when bacteria migrate from the teat canal to the udder cistern and the mammary glands. The disease can be clinical or sub-clinical. Clinical symptoms vary from being mild to severe, but in sub-clinical mastitis no signs of disease are observed. The number of microorganisms shed into the milk during mastitis ranges from <4 $\log_{10}$ colony forming units (CFU) per mL for sub-clinical mastitis to >7 $\log_{10}$ CFU per mL for clinical mastitis (Chambers, 2002).

To detect sub-clinical mastitis, the milk must be tested for the presence of mastitis bacteria or increased somatic cell count (SCC) (Thorberg, 2008). SCC measures the number of leukocytes (white blood cells) in the milk and is an indication of udder health (Walstra *et al.*, 2006). An increase in SCC is related to increased levels of *S. aureus* and/or other bacteria causing mastitis (Jayarao *et al.*, 2004). The leukocytes produce heat-stable proteins and fat degradation enzymes, which affect the quality and shelf-life of milk and milk products regardless of whether the milk is pasteurised or not (Ma *et al.*, 2000; Barbano *et al.*, 2006). SCC can be measured directly using an automated cell counter, either using a portable cell counter at the farm or sending samples to a laboratory (Persson & Olofsson, 2011). An alternative method is the California

Mastitis Test (CMT), which is an indirect method that measures the quantity of DNA in the milk and hence, the number of SCC (Quinn *et al.*, 1994b) (Fig. 2). This method is fast and easy to use on the farm and can be used to test milk of both cows and goats. However, the results for these are interpreted differently (Sargeant *et al.*, 2001; Persson & Olofsson, 2011). Healthy goats normally have higher SCC in the milk compared with cows, and the number increases during the lactation and with parity (Paape & Capuco, 1997). SCC in goat's milk also varies between individuals and herds (Schaeren & Maurer, 2006).

a. b. c.

Figure 2. Indirect test for somatic cell count (SCC) in cow's raw milk using the California Mastitis Test (CMT); a) A small amount of milk from each udder quarter is added to a tray with four cups. b) CMT liquid is added to each cup and mixed with the milk. c) A check is made for gel formation in the milk-CMT liquid mix (photo: Åsa Rosengren).

1.2.2 Cheese microbiology

Milk makes an excellent substrate for many microorganisms, including many foodborne pathogens, and it is believed that cheese making was first developed as an attempt to store milk in an appropriate way for a long time. During cheese making, highly perishable milk is converted into a less perishable product by acidification with starter culture, rennet coagulation, followed by dehydration and salting (Fox, 1993). The microbiological status of cheese depends on the quality of the milk, possible contamination during processing and cheese type. Raw milk can contain spoilage organisms as well as pathogens, and therefore many dairies prefer to pasteurise the milk before cheese making.

Pasteurisation kills vegetative pathogenic microorganisms such as *Mycobacterium tuberculosis*, *Salmonella* spp., *E. coli*, thermotolerant *Campylobacter* spp., *L. monocytogenes* and *S. aureus*. Most spoilage microorganisms are also killed, which reduces problems with off-flavours and gas formation. However, thermoresistant microorganisms and spores of *Bacillus* spp. and *Clos-*

tridium spp. survive and may cause quality defects in the cheese (Walstra *et al.*, 2006). In addition, heat-resistant staphylococcal enterotoxin (SET) produced by *S. aureus* is not inactivated by pasteurisation (Jablonski & Bohach, 1997). Heat-treated milk is sensitive to recontamination by microorganisms from air, water, equipment, people, utensils, starter cultures, rennet and packaging (ICMSF, 1998; Boor & Murphy, 2002).

Lactic starter cultures containing lactic acid bacteria (LAB) are most often added to cheese milk for both safety and sensory purposes (ICMSF, 1998). The main task of starter cultures is to ferment lactose to lactic acid and thereby decrease pH in the curd, but aroma components, carbon dioxide and sometimes proteolytic enzymes are also produced (Fig. 3). Acidification, which continues throughout the initial phases of cheese making and the early stage of ripening, is important for suppression of the growth of undesirable microorganisms in the cheese curd (Bylund, 1995). Mesophilic and/or thermophilic starters are used depending upon cheese type, and curd temperature during fermentation is ≤30 °C and 42-45 °C, respectively (Cogan & Hill, 1993). Hence, fermentation temperature is critical since a sub-optimal temperature may cause slow or no acidification (ICMSF, 1998; Robinson *et al.*, 2002). Starter cultures are sensitive to adverse conditions and should be properly handled and stored. Unsuccessful acidification may also be due to antibiotics or detergent residues in the milk, bacteriophages, small inoculum or detrimental activity due to old age of the starter culture (Stadhouders *et al.*, 1978; Bylund, 1995). To avoid the risk of inconsistent or undesirable results when using natural indigenous flora, well-defined commercially available starters are commonly used today (ICMSF, 1998). Bacteriophage infection in starter culture results in slow acidification and some starter strains are more sensitive than others. The use of mixed cultures is preferable because if one strain becomes infected, the others in the culture continue to produce acid. Other precautions are exchanging starters regularly or using phage-resistant strains (ICMSF, 1998; Tamine, 2002).

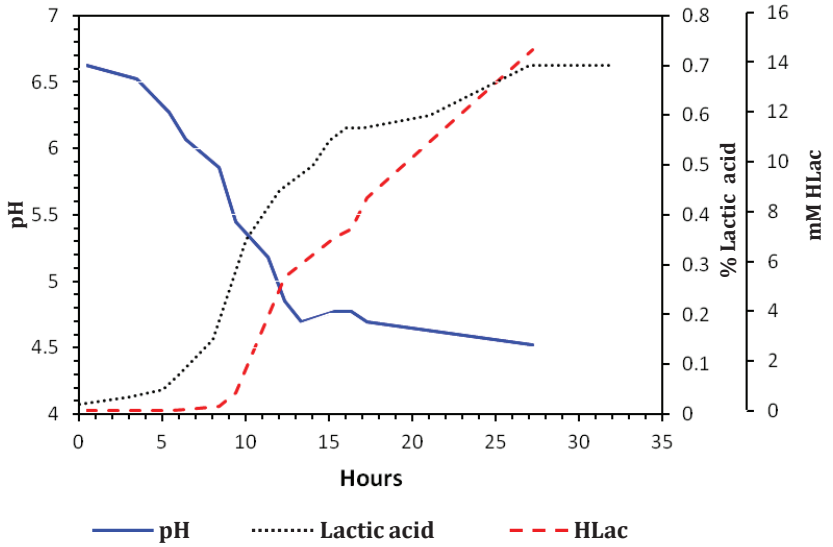

Figure 3. Changes in the concentrations of total lactic acid and undissociated lactic acid (HLac) and in pH when a mesophilic starter culture ferments lactose in milk at 21 °C (based on information in Cogan and Hill (1993))

Even if cheese making is a preservation method, pathogenic bacteria may be present and cause foodborne disease. Their ability to grow and survive depends on heat, acid and salt tolerance, initial numbers and individual characteristics of the species or strain in question. Cheese type also plays a major role, with soft cheese with a high moisture content providing a more favourable environment for microbial growth than hard cheese. The most relevant foodborne hazards in cheese are *S. aureus, Salmonella, L. monocytogenes* and pathogenic *E. coli* (ICMSF, 1998; De Buyser *et al.*, 2001). If pathogens are present, they are expected to lose viability during ripening. However, inactivation rates vary considerably between cheese types, species and strains (Bachmann & Spahr, 1995) (Fig. 4) and in soft cheeses with high moisture content *e.g.* Camembert, *L. monocytogenes* grows during ripening (Ramsaran *et al.*, 1998).

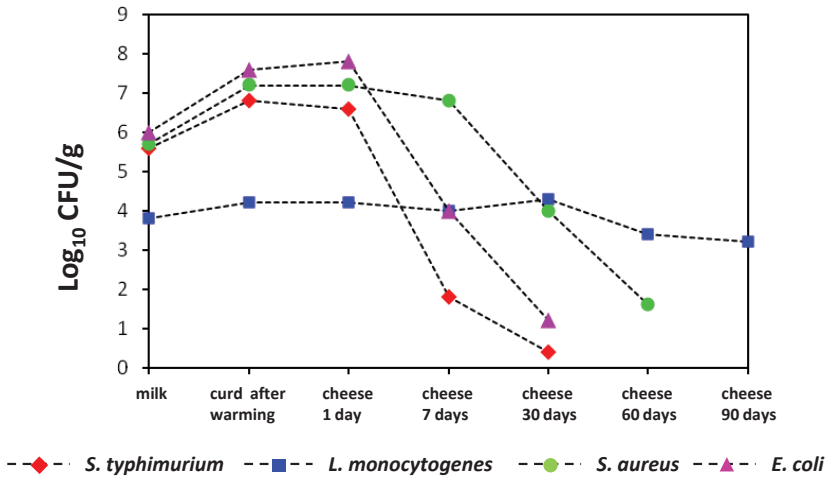

Figure 4. Changes in population numbers of *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* during cheese making and ripening of semi-hard raw milk cheese (Tilsiter) (modified from Bachmann & Spahr (1995))

1.3 The cheese making process

When milk is made into cheese, casein and fats are concentrated approximately 10-fold. Other milk components, mostly water and water-soluble proteins, are removed with the whey by dehydration. Dehydration is achieved by combining cutting, stirring, pressing, salting and ripening. By altering parts of the dehydration steps in a variety of ways, different pH, salt content, moisture and microflora determine the flavour, aroma and texture of different cheese varieties (ICMSF, 1998). However, some stages are shared by almost all cheese types (Walstra *et al.*, 2006):

1. *Clotting of milk:* Clotting or gelation is achieved by the rennet proteins chymosin and pepsin or low pH (or a combination of both). Rennet enzymes change κ (kappa)-casein to aggregating paracasein micelles, while acid neutralises electrical charges on micelles, resulting in gel-like aggregation.
2. *Dehydration by whey removal:*Gel aggregation leads to spontaneous expulsion of whey, a process called syneresis. The removal of whey is enhanced when the gel is cut into pieces, followed by stirring and warming.

3. *Acid production during manufacture:* Lactic acid bacteria convert lactose to lactic acid causing a decrease in pH. Acidification influences the firmness of the curd, syneresis, ripening and microbiological safety.
4. *Salting:* NaCl is added generally to a concentration of 1 to 4%, and affects shelf-life, flavour and consistency in particular.
5. *Fusion of curd grains:* This is done by pressing grains together in a mould, resulting in a coherent loaf and further expulsion of whey. Pressing can be done either with or without weights.
6. *Curing/ripening:* To achieve the desired flavour, texture and water content of a given cheese type, it needs to be ripened under appropriate storage conditions in terms of *e.g.* temperature, humidity and time.

Thus, cheese making is a complex interaction of physical, biochemical and biological processes and involves many process steps and conditions, as illustrated in Figure 5. Each process step or condition may not alone ensure a microbiologically safe cheese, but a high degree of safety can be achieved when many sub-optimal environmental factors collectively contribute to the preservative characteristic. This phenomenon is described as the 'hurdle effect', and each factor is seen as a 'hurdle to be leaped over' by the microorganism. Important hurdles in cheese are temperature, acidity (pH), water activity (a_w), undissociated lactic acid (HLac) and competitive LAB (Leistner, 2000).

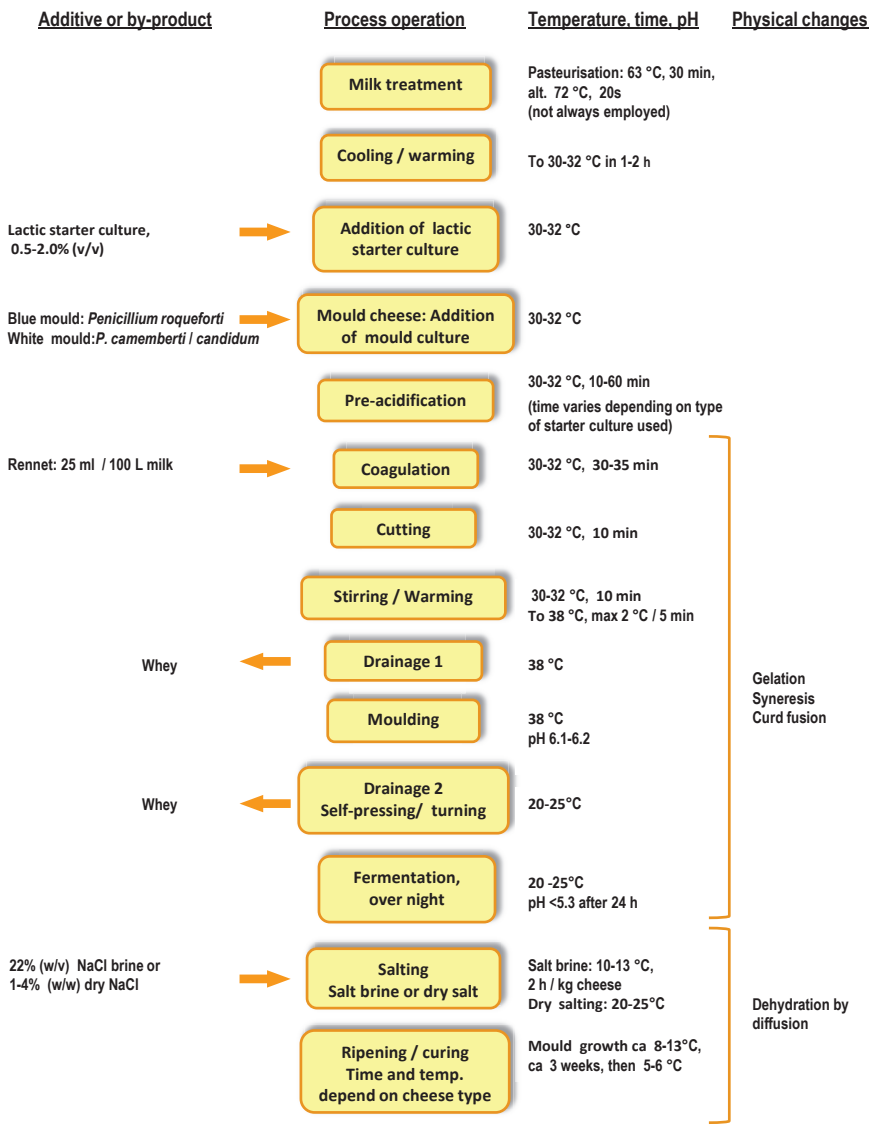

Figure 5. General flow chart for production of small-scale semi-soft rennet mould cheese with mesophilic starter culture (based on Bylund (1995), Walstra (2006), Olofsson (2010) and K. Jürss. pers.communication (2012-03-02)).

1.3.1 Process control

Cheese production is regulated within the European Union (EU) and all food business operators (FBOs) (*i.e.* producers), large and small, are currently regulated by a number of EU Food Hygiene Regulations (EC, 2002, 2004a, b, 2005). The FBOs are obliged to establish, carry through and maintain an internal system ensuring safe and hygienic production based on numerous general and specific requirements (pre-requisites) prescribed in Regulations (EC) no. 852/2004 and 853/2004. Examples of pre-requisites include compliance with the microbiological criteria for foodstuffs (EC, 2005), maintenance of the cold chain, sanitation procedures, water supply, premises, education/training and personal hygiene.

In addition to the pre-requisites, cheese making FBOs must implement and maintain procedures based on the principles of Hazard Analysis Critical Control Point (HACCP), which is a control system focusing on preventive measures (FAO/WHO, 2003; EC, 2004b). HACCP is widely recognised and must be adopted for each product before it is released on the market and when any modification is made to the product.

The HACCP system involves the following steps:

1. Identification of hazards that must be prevented eliminated or reduced to an acceptable level.
2. Identification and establishment of Critical Control Points (CCPs) in the process where control is necessary for elimination or reduction of the hazards to an acceptable level.
3. Establishment of critical limits of CCPs that distinguish acceptable from unacceptable levels of hazards.
4. Establishment of monitoring procedures for the CCPs.
5. Establishment of corrective measures when monitoring exceeds critical limits.
6. Verification of the HACCP plan to ensure it is working properly.
7. Documentation of records to demonstrate monitoring and measures are effective.

The HACCP methodology is flexible, prescribes the principles and can be adapted to all types of food production. Detailed national guidelines have been developed by many food business sectors according to the requirements in Regulation (EC) no. 852/2004 (Articles 7-8). The guidelines are voluntary and allow producers to describe in detail how to comply with food legislation. Swedish guidelines for the production of different types of small-scale artisan cheeses have been published (Eldrimner, 2009).

Process control in cheese production depends on cheese type and whether milk is heat treated or not. When making raw milk cheese, high-quality milk must be used. Milk quality is controlled by ensuring good animal health, good quality of animal feeding, adequate housing and hygienic milking conditions (ICMSF, 1998; Olofsson, 2010). Regular cleaning of equipment and facilities, heat treatment of brine and overall hygiene are some preventive measures to be taken (Arvanitoyannis & Mavropoulos, 2000).

To control the growth, survival and inactivation of possible microbiological hazards, factors such as time, pH and temperature must be checked throughout the cheese making process. Therefore, the procedures for the initial phases of artisan cheese making include several control points to monitor the process in general and a few CCPs to assure that critical control point limits are not exceeded. For example, time and temperature are measured during most process steps. Pasteurisation is a CCP where time-temperature combinations, either 63 °C for 30 min or 72 °C for 15 s (EC, 2004b), must be carefully controlled (FAO/WHO, 2003). Starter culture activity is considered a CCP in small-scale dairies, and pH is checked during fermentation up to approx. 24 h (Olofsson, 2010). The pH of milk is about 6.7 and for most cheese types, successful acidification results in a pH below 6 after 5-6 h and around 5 or lower after 24 h (Walstra *et al.*, 2006) (Figs. 3 and 5). For safety and quality reasons, most cheeses, with the exception of fresh cheese, require ripening of variable duration depending on type, and during ripening time and humidity are checked. Water is lost during ripening and biochemical reactions favour texture and flavour development, but also make conditions unfavourable for most vegetative foodborne pathogens (ICMSF, 1998) (Fig. 4).

1.4 Staphylococcus aureus

1.4.1 Characteristics of the organism

Staphylococcus aureus is a Gram-positive coccus that may occur as single, paired or irregular grapelike clusters of cells (*Staphylé* is Greek for 'bunch of grapes'). The species derives its name from its carotenoid pigment, which results in golden-yellow colonies (*aureus* meaning golden). However, many strains lack the pigment and thus form white or grey colonies on blood agar. *Staphylococcus aureus* is coagulase- and catalase-positive, oxidase-negative, non-motile, does not form spores and has both respiratory and fermentatory metabolism. It is typically mesophilic, with a growth optimum at 37 °C. Some strains are able to produce staphylococcal enterotoxin (SET) during growth in foods. SET is the causative agent of staphylococcal food poisoning (SFP) (ICMSF, 1996). The bacterium is killed by pasteurisation, but SETs are very heat-resistant. Thus, the bacteria may be killed, but if preformed in food, SET may remain and cause SFP (Le Loir *et al.*, 2003).

Staphylococcus aureus is ubiquitous and can be found in the nostrils and on the skin and hair of warm-blooded animals. Up to 30-50% of the human population are carriers. Hence, important contamination sources for foods are the hands and noses of food handlers, which contaminate foods via manual contact and respiratory secretions (Le Loir *et al.*, 2003; Lai *et al.*, 2011). *Staphylococcus aureus* can grow in many food types and, in combination with its ecological niche, its incidence in processed, manipulated and fermented foods makes it an important foodborne pathogen. Other contamination sources of *S. aureus* may be the farm or the food processing environment (Borch *et al.*, 1996; Jorgensen *et al.*, 2005b). It is an udder pathogen and is often isolated in mastitis milk from cows, sheep and goats (Bergonier *et al.*, 2003; Jorgensen *et al.*, 2005b; Jakobsen *et al.*, 2011).

In common with the other coagulase-positive staphylococci (CPS), *S. intermedius* and *S. hyicus*, *S. aureus* is isolated and directly confirmed for coagulase reaction on Rabbit Plasma Fibrinogen Agar (RPFA) after aerobic incubation 48 h at 37 °C (NMKL, 2003) (Fig. 6). Biochemically, *S. aureus* is differentiated from the other CPS species by its ability to rapidly ferment maltose (Quinn *et al.*, 1994a). There are both biochemical and molecular methods to subtype *S. aureus* strains and these can be useful when investigating staphylococcal food poisoning outbreaks (SFPOs). By analysing a number of biochemical characteristics, *S. aureus* strains can be divided into different biotypes based on their human, animal or non-host-specific (NHS) origin,

namely: human, non-β-haemolytic human, poultry, bovine, ovine, NHS and abattoir (Devriese, 1984; Hennekinne *et al.*, 2003). However, these biotypes are not host-specific in a strict sense. Human biotypes can be isolated on animals and *vice versa*, but the biotype designation indicates the host of origin (Jablonski & Bohach, 1997). Molecular typing methods such as pulsed-field gel electrophoresis (PFGE), *spa*-typing, multi locus sequence typing (MLST) and microarray are also used to subtype strains (Wei & Chiou, 2002; Feil *et al.*, 2003; Concepcion Porrero *et al.*, 2011; Wattinger *et al.*, 2011).

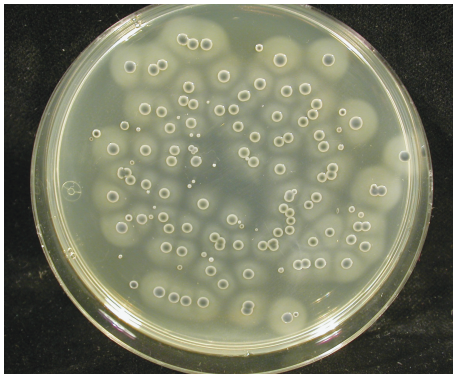

Figure 6. Staphylococcus aureus isolated from raw milk fresh cheese on Rabbit Plasma Fibrinogen Agar (RPFA) after 48 h at 37 °C. Typical colonies are grey and surrounded by an opaque halo due to coagulase activity (Photo: Flemming Lund).

The presence of *S. aureus* in raw milk and the risk of re-contamination after heat treatment make it a food safety concern in cheese production, since conditions during the initial processes favour growth of the organism (Fig. 4). Several studies have shown that *S. aureus* grows during the early stages of cheese making, even if the bacterium is present in low levels in milk (Meyrand *et al.*, 1998; Vernozy-Rozand *et al.*, 1998; Delbes *et al.*, 2006). European food legislation prescribes cheese making FBOs to analyse for CPS in several dairy product processes. If CPS are present at levels >5log$_{10}$ CFU/g, there is an obligation to test for the presence of SETs (EC, 2005).

Staphylococcus aureus is also a clinical human pathogen of major concern and is a common agent in hospital-acquired (nosocomial) infections. Disease is caused by combinations of many virulence factors such as exotoxin production, antibiotic resistance and invasiveness. Infection is enabled if the skin barrier is breached, and the bacterium may give rise to a wide spectrum of symptoms, from pimples and boils to toxic shock syndrome (TSS) and sepsis (Le Loir *et al.*, 2003).

1.4.2 Staphylococcal enterotoxins

Staphylococcal enterotoxins are short, single-chain polypeptides of approx. 600 to 800 amino acids, forming two unequal domains, A and B. Domain A is larger, and is proposed to be involved in binding to T-cell receptor sites. It contains both the carboxyl and amino termini (Dinges *et al.*, 2000; Argudin *et al.*, 2010). Domain B contains a cystein loop, which is suggested to stabilise a necessary conformation for the emetic (vomiting) property (Hovde *et al.*, 1994). While many efforts have been made to identify the amino acids and domains involved in emesis, the picture is not complete. There are no specific cells or receptors in the intestines linked to SFP, but in the review by Argudin *et al.* (2010), it is suggested that SETs stimulate neurons leading to the vagus nerve, which transmits the signal to the vomiting centre in the brain.

SETs that have been shown to induce emesis in primates are called SEs, whereas related toxins that either lack emetic activity or have not been tested are called staphylococcal enterotoxin-*like* (SE*l*) (Lina *et al.*, 2004). To date, 21 SEs or SE*l*s proteins have been identified and are designated A, B, C… and so on up to V (Schlievert & Case, 2007; Thomas *et al.*, 2007; Schelin *et al.*, 2011). SEA-SEE are called the classical enterotoxins, SEG- SEI and SER- SET the new enterotoxins, and SE*l*J- SE*l*Q and SE*l*U- SE*l*V are designated SE*l*s.

SETs are heat-resistant, and may retain biological activity after 28 min at 121 °C. However, heat stability has been reported to vary between different food matrices and is also affected by the purity of the toxin, initial amount, pH, salt concentration and detection method (Balaban & Rasooly, 2000; Ikeda *et al.*, 2005). The ability of SETs to resist proteolytic enzymes such as pepsin and trypsin, helps them to keep activity in the digestive tract after ingestion (Genigeorgis, 1989).

SETs are considered to be superantigens (SAgs), which means that they interact non-specifically with molecules of the major histocompability complex (MHC) class II on the surface of antigen presenting cells (Dinges *et al.*, 2000). The non-specific interaction leads to activation of a large number of T-cells followed by proliferation of immuno cells and massive cytokine release that may lead to life-threatening symptoms such as TSS (Balaban & Rasooly, 2000). The superantigen activity and the emetic activity are two separate functions on the SET-molecule. However, there is a correlation between the activities, since genetic mutations with no superantigen activity in most cases also lose emetic activity (Harris *et al.*, 1993). Despite being typical SAgs, it has

been proposed that SETs be called enterotoxins because of their emetic activities (Lina *et al.*, 2004).

Enterotoxigenic *S. aureus* strains have different genes encoding for SET (*se*-genes) and sometimes they may carry many types of these. The *se*-genes are encoded on different mobile genetic elements such as plasmids, prophages, enterotoxin genetic clusters (*egc*), *S. aureus* pathogenicity islands (SaPIs) and staphylococcal cassette chromosomes (Noto *et al.*, 2008; Schelin *et al.*, 2011). Hence, regulation of *se*-gene expression varies. Among the classical SETs (SEA-E), *sea* and *see* are carried on prophages, which are bacteriophages incorporated into the bacterial chromosome where they behave like the rest of the genome (Betley & Mekalanos, 1985). The *seb*-gene and the five *sec* variants, are situated on different SaPIs (Schelin *et al.*, 2011) and *sed* is located on a plasmid (Bayles & Iandolo, 1989). SaPIs are mobile pathogenicity islands and are very common in *S. aureus*. They occupy specific sites in the chromosome and carry the genes for many SAgs, including *se*-genes and the toxic shock syndrome (*tst*)-gene (Novick & Subedi, 2007). The *seb*-, *sec*- and *sed*-genes are regulated by the accessory gene regulator (Agr) system. The Agr system is a quorum sensing system that controls the expression of a number of genes connected to virulence. Quorum sensing is a cell density-dependent system for extracellular signalling between bacteria, and may explain why high bacterial levels are needed before SET is produced (Novick & Geisinger, 2008; Thoendel *et al.*, 2011).

SETs are commonly detected and quantified by antibody-based enzyme-linked immunosorbent assay (ELISA). Detection of classical SEA-SEE in foods is performed according to the European Reference laboratory (EU-RL) screening method. This method comprises an extraction step followed by dialysis concentration and immune-enzymatic detection either by using Vidas SET2 kit or Ridascreen SET total (ANSES, 2010). Capture sandwich ELISAs using standard curves with known concentrations of the tested SET are used when separating and quantifying SETs (Hennekinne *et al.*, 2007; Wallin-Carlquist *et al.*, 2010a) (Fig. 7). At present, there are no immunoassay kits or reliable antibodies commercially available for detection of new SE or SE*l*s. Therefore, several methods based on polymerase chain reaction (PCR)-techniques are used to detect and quantify *se*-genes (Chen *et al.*, 2004; Chiang *et al.*, 2006). In addition, a mass spectrophotometry method for detection and quantification of SETs has recently been developed (Hennekinne *et al.*, 2009).

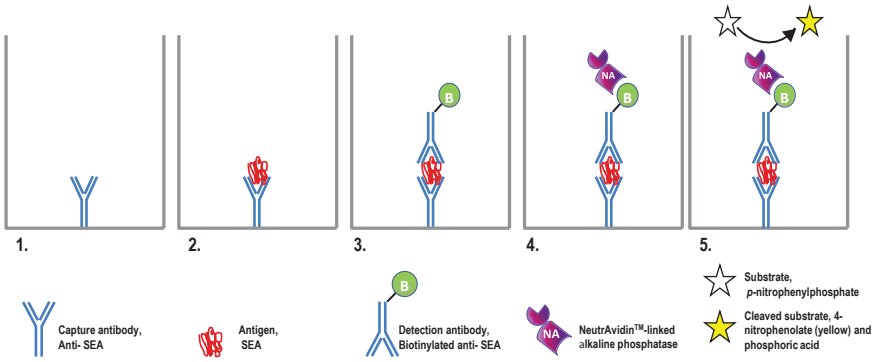

Figure 7. Analytical principle of capture sandwich enzyme-linked immunosorbent assay (ELISA) detecting staphylococcal enterotoxin A (SEA) in a microtitre plate well. 1) Microtitre wells are coated with capture antibody against SEA (anti-SEA). 2) Sample is added and if SEA (antigen) is present, it binds to the capture antibody. 3) A detection antibody, biotinylated anti-SEA, is added and binds to the captured SEA-antigen. 4) NeutrAvidin™-linked alkaline phosphatase is added and binds to the biotin part of the detection antibody. 5) Substrate *p*-nitrophenylphosphate is added and cleaved by the alkaline phosphatase into phosphoric acid and *p*-nitrophenolate, which forms a yellow colour. The intensity of the colour formation is measured at 405 nm after 45 min in darkness (based on Wallin-Carlqvist et al (2010a) and (University of Bergen, 2012))

1.4.3 Staphylococcal food poisoning

Staphylococcal food poisoning (SFP) is an intoxication that occurs when foods containing sufficient amounts of preformed SETs are consumed. SETs produced by *S. aureus* are the only virulence factors directly involved in SFP, and they are produced when foods are improperly handled or stored (Genigeorgis, 1989). SFP is characterised by rapid and acute onset (normally 2-4 h) followed by copious vomiting, abdominal cramps, nausea, retching and prostration. Diarrhoea is often reported, but never occurs alone (ICMSF, 1996). The symptoms can also be more severe, with headache, muscle cramping and transient changes in blood pressure and pulse rate (FDA, 2011). Symptoms vary depending on individual susceptibility, SET type, amount of consumed food and amount of SETs in the food (Jablonski & Bohach, 1997). The disease is usually self-limiting within two days, but depending on the severity of the symptoms complete recovery may sometimes take longer. Occasionally, in cases of dehydration, collapse or pallor, hospitalisation may be needed (Argudin *et al.*, 2010; FDA, 2011). Fatalities are rare, but deaths have been reported among the elderly, infants and severely weakened persons (Mead *et al.*, 1999; EFSA, 2009).

As little as 20-100 ng SET may be enough to cause SFP (Asao *et al.*, 2003). In 2008, SFPs constituted 5.5% of the total number of reported foodborne outbreaks within the EU (EFSA, 2009). In Sweden, 111 cases and 30 SFPOs were reported in the period 2003-2009, representing 1% and 2% of the total reported cases and outbreaks, respectively (Lindqvist *et al.*, 2004, 2005, 2006; Lindblad *et al.*, 2008, 2009, 2010). However, the reported number is most likely a small proportion of the total number of SFPOs. Underreporting can be caused by improper sample collection, misdiagnosis and difficulties in laboratory examination of SETs. Foodborne diseases are generally underreported, especially those that are self-limiting with short duration and not officially notifiable (Mead *et al.*, 1999; Chiang *et al.*, 2006; Argudin *et al.*, 2010; SMI, 2011). Two studies have been conducted in Sweden to estimate the annual number of food poisoning cases and the proportion of cases reported. In 1994, the National Food Agency (NFA) conducted a survey regarding food poisoning and estimated that 500 000 persons were food-poisoned annually in Sweden (Norling, 1994). This can be compared with the total number of reported cases which is 1300-2900 per year (Lindqvist *et al.*, 2004, 2005, 2006; Lindblad *et al.*, 2008, 2009, 2010). Similarly, a one-year in-depth study of food poisoning cases in Uppsala, Sweden, estimated the total number of cases to be 10- to 67-fold higher than reported (Lindqvist *et al.*, 2001).

SEA is considered to be the most important cause of SFP, but SFPOs caused by SEB-SEE have also been reported (Wieneke *et al.*, 1993; Cha *et al.*, 2006; Kerouanton *et al.*, 2007). Of the new SETs, only SEH has been reported to be responsible of SFPO (Ikeda *et al.*, 2005; Jorgensen *et al.*, 2005a). Swedish reports of SFPOs caused by consumption of milk and milk products are rare (Lindqvist *et al.*, 2004, 2005, 2006; Lindblad *et al.*, 2008, 2009, 2010; SMI, 2011), but a number of SFPOs involving raw and pasteurised milk and milk products have been reported in other countries (De Buyser *et al.*, 1984; Evenson *et al.*, 1988; De Buyser *et al.*, 2001; Schønberg & Wåltorp, 2001; Asao *et al.*, 2003; Schmid *et al.*, 2009; Ostyn *et al.*, 2010).

1.5 Predictive food microbiology

Predictive food microbiology (PM) attempts to describe the behaviour of microorganisms in foods using mathematical models. It is based on the premise that the responses of microbial populations to environmental conditions are reproducible and that a limited number of factors determine the growth, death and survival of microorganisms in foods. These factors include temperature, water activity, pH (acidity), preservatives, redox potential, atmosphere and combined effects of such factors (Bassett *et al.*, 2012). Predictive models can be used as tools when for example there is a need to predict the possible increase in *S. aureus* during the first hours of fermentation in cheese making or to estimate growth limiting conditions during ripening. By characterising environmental factors under defined and controlled conditions, microbial responses to new situations and scenarios can be predicted even though experimental data are lacking (Ross & McMeekin, 1994; McMeekin *et al.*, 2002).

The use of PM can enable:

* Prediction of safety, spoilage and shelf-life of products, when a product formulation is changed, or when new processes are being developed.
* Objective evaluation of processing operations in the HACCP-plan.
* Objective evaluation of consequences of mistakes during production.

Predictive models are available for many foodborne bacterial pathogens, specific spoilage bacteria and microfungi. The models describe growth and inactivation rates (kinetic models) and limits for growth under stress (probability and boundary models) (McKellar & Lu, 2004; Ross & Dalgaard, 2004). A commonly used classification of predictive models is as *primary*, *secondary* and *tertiary* models (Whiting & Buchanan, 1993):

Primary models describe the growth, survival or death of a microorganism as a function of time under a specified set of culture conditions. Primary growth models can describe changes in numbers, absorbance, concentrations, *etc.* and can be used for estimation of specific growth rates or lag times. A typical microbial growth curve (as log numbers) has a sigmoid shape, starting with an

initial lag phase (no increase in numbers), followed by an exponential phase (log-linear increase in numbers), and finally, a stationary phase when the maximum number has been reached (Fig 8). A variety of models have been used to fit the growth curve, *e.g.* the modified logistic model, the modified Gompertz model, the Baranyi model, the Hills model, the McKellar model and the Buchanan model (McKellar & Lu, 2004; Bassett *et al.*, 2012). Primary survival models are either linear or non-linear. A well-known example of a linear model is the first-order survival curve, which is used to estimate the *D*-value, the time for one-log reduction in microbial numbers at a given temperature. Non-linear survival models describe more complex inactivation kinetics and are generally divided into shoulder and tail models (McKellar & Lu, 2004).

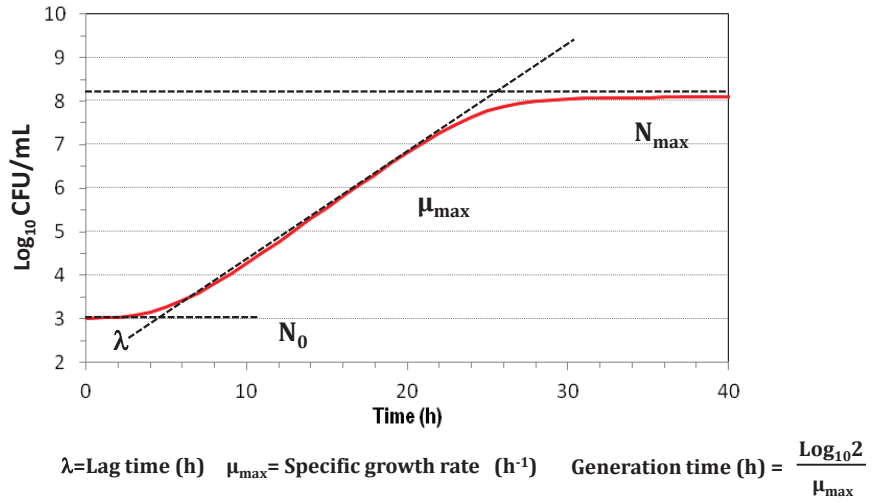

Figure 8. Example of a typical microbial growth curve. N_0 and N_{max} are the initial and maximum number of cells, respectively. If N is expressed as natural logarithm (ln), the expression of the generation time is also changed to ln.

Secondary models describe how parameters of the primary models (*i.e.* lag time, specific growth rate, *D*-value, *etc.*) change when environmental conditions change, *e.g.* the effect of temperature on growth rate, pH on survival, water activity on lag time, *etc.* (Whiting & Buchanan, 1993). Commonly used secondary models are the square-root-type model, polynomial models, the gamma concept and cardinal parameter models and models based on the Arrhenius equation. Secondary probability models deal with the likelihood of *e.g.* growth, toxin production or spore germination of a microorganism of con-

cern occurring in different environmental conditions. Models that define the environmental conditions that prevent growth are called growth/no growth (GNG) interface (Fig. 9), time-to-growth (TTG), growth boundary or growth limit models (Ross & Dalgaard, 2004).

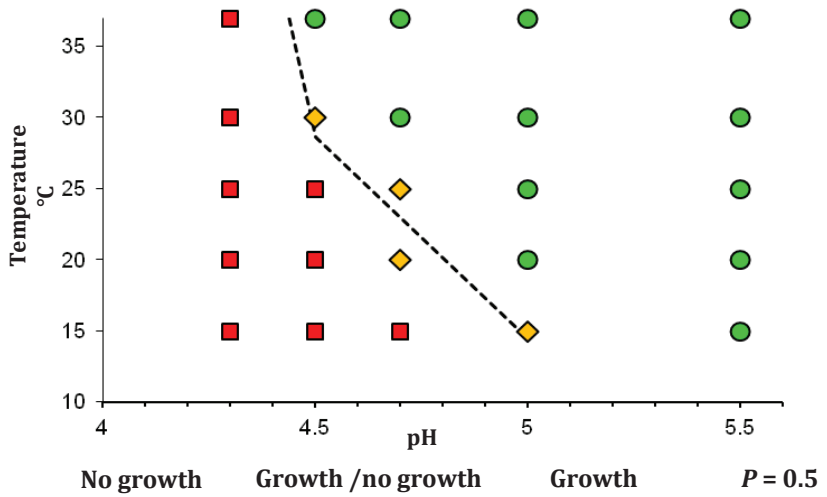

Figure 9. Example of a growth/no growth (GNG) diagram for predicting growth limits of *S. aureus* after incubation for 24 h at water activity (a_w) values 0.95-0.99. The dashed line indicates 50% probability of growth (modified from Fabricius (2006)).

Tertiary models are user-friendly computer applications that make predictions based on primary or secondary models (Whiting & Buchanan, 1993). Tertiary models for microbial predictions with user-friendly interfaces have increased the use of microbial models for many applications of food safety and quality (McKellar & Lu, 2004). Examples of two widely used applications freely available are the downloadable and/or internet-based Pathogen Modelling Program (PMP) (PMP 7.0, 2007) and the internet-based ComBase (ComBase, 2011).

PMP includes models describing the growth, survival, inactivation and toxin production of several foodborne pathogens. All PMP models are built on scientific publications. The majority are based on experiments carried out in defined laboratory media without competition from other microorganisms, but a few are based on experiments in food matrices such as beef, chicken, turkey or fish (PMP 7.0, 2007).

ComBase consists of a database and a modelling toolbox for predictive models. Users can add data from their experiments to the database. The ComBase modelling toolbox includes: (1) ComBase Predictor, enabling growth, inactivation, and survival predictions; (2) Perfringens Predictor, a special application for predicting the response of *Clostridium perfringens* in bulked meat during cooling; and (3) DMfit. DMfit allows microbiologists to fit growth models to their data for estimation of parameters such as specific growth/inactivation rate and lag time/shoulder based on the models of Baranyi & Roberts (1994), the trilinear model, biphasic models or linear models (ComBase, 2011).

1.5.1 Environmental factors affecting growth and toxin production

Adaptation to various environments is crucial for survival, and is often a pre-requisite for virulence in pathogenic bacteria. *Staphylococcus aureus* can grow and produce SET over a wide range of temperature, pH, NaCl concentration and a_w values (ICMSF, 1996) (Table 1). As a general rule, ranges of environmental factors for *S. aureus* growth are usually wider than the SET production ranges. The robustness of *S. aureus,* tolerance to NaCl and reduced a_w permits growth and SET production in many food types. Examples of foods that have been involved in outbreaks are cured, cooked, salty, protein rich foods such as meat, fish, poultry and dairy products (Genigeorgis, 1989; Wieneke *et al.*, 1993; De Buyser *et al.*, 2001; Kerouanton *et al.*, 2007).

In the presence of weak organic acids, the pH limit for growth and SET production is higher than presented in Table 1. Weak organic acids such as lactic- and acetic acid stress cells, in that pH homeostasis in bacteria is disturbed. Weak organic acids are lipophilic in the undissociated form, which enables acid molecules to freely diffuse across the bacterial cell membrane. Once inside, the acid dissociates and releases protons that acidify the cytoplasm. Much of the cell energy is expended to maintain internal pH and growth is reduced or inhibited (Shelef, 1994). Among weak acids commonly used in food production, acetic acid is more inhibiting than lactic acid (Genigeorgis, 1989; ICMSF, 1996). The a_w limits for growth and SET production change when humectants other than NaCl, *e.g.* sucrose, glycerol or fructose, are used (Stewart *et al.*, 2002).

Table 1. *Limits for growth and enterotoxin production for* S. aureus *grown under otherwise optimal conditions and HCl as acidulant (after ICMSF, 1996)*

	Growth		SET production	
	Optimum	**Range**	**Optimum**	**Range**
Temperature (°C)	37	7-48	40-45	10-48
pH	6-7	4-10	7-8	4.5[†]-9.6
Water activity (a_w)	0.98	0.83->0.99[**]	0.98	0.87->0.99 [††]
NaCl (%)[*]	0.5-4.0	0 -20	0.5	0-20
Atmosphere	Aerobic	Anaerobic-aerobic	Aerobic (5-20 % dissolved O_2)	Anaerobic-aerobic

[*]Adams & Moss 1996
[**] Aerobic (anaerobic aw 0.90->0.99)
[†]Aerobic (anaerobic pH 5.0)
[††]Aerobic (anaerobic aw 0.92->0.99)

1.5.2 Predictive models for *Staphylococcus aureus*

There are several published *S. aureus* models not included in tertiary software models. These include boundary models such as growth/no growth (GNG) models (Lanciotti *et al.*, 2001; Stewart *et al.*, 2002; Valero *et al.*, 2009) and kinetic models describing growth and survival as a function of temperature, NaCl, a_w, pH with HCl as acidulant, nitrite, and/or LAB (Sutherland *et al.*, 1994; Fujikawa & Morozumi, 2006; Le Marc *et al.*, 2009). A model for predicting SEA in milk has been developed by (Fujikawa & Morozumi, 2006).

Tertiary *S. aureus* models are available in both PMP and ComBase Predictor. PMP provides models for growth and survival whereas ComBase Predictor includes an *S. aureus* growth model. In both applications, the user enters the specified environmental variables, *e.g.* atmosphere, pH, NaCl content, temperature, a_w, and the model outputs include generation time, lag time *etc.* shown in tables or charts (PMP 7.0, 2007; ComBase, 2011).

2 Aims

Small-scale production of artisan cheese is increasing and previous studies have indicated a potential risk of staphylococcal food poisoning from these products. The overall aim of this licentiate thesis was to provide more knowledge about small-scale artisan cheese production and relevant pathogens so that advice for microbiologically safe production can be formulated. The specific objectives of the studies reported in papers I-II were to:

* Describe production practices on Swedish small-scale dairies (Paper I).
* Investigate the occurrence and levels of the potential foodborne pathogens *S. aureus, E. coli* and *L. monocytogenes* in cheese from Swedish small-scale artisan dairies (Paper I).
* Describe the characteristics, origin, diversity and pathogenic potential of *S. aureus* isolates from cheese (Paper I).
* Investigate factors affecting microbial growth and enterotoxin A production in conditions relevant for cheese making (Paper II).

3 Present investigations

3.1 Survey of small-scale artisan produced cheeses (Paper I)

A microbiological survey was carried out to investigate the occurrence and levels of CPS (mainly *S. aureus*), *E. coli* and *L. monocytogenes* in fresh and short-term ripened (<60 days) artisan cheeses from Swedish small-scale dairies. Efforts in recruiting participants resulted in 83% (43/52) of known dairies making these cheese types sending cheese samples for analysis. Participation was voluntary and, due to practical limitations, random sampling was not possible. Most dairies used pasteurised milk, but to increase the numbers of raw milk cheeses, dairies making raw milk cheese were asked to provide samples on two occasions.

In total, 151 cheese samples were analysed, of which 62% (94/151) were made from goat's milk and 32% (49/151) from cow's milk. The few remaining cheese samples were made from sheep's milk or a mix of cow's/goat's milk (Table 1 in Paper I). Pasteurised milk was used for 64% (96/151) of the cheeses and starter culture was added when making 89% (134/151) of the cheeses (Tables 3-4 in Paper I). The most common cheese types analysed in the survey were fresh cheese (52%) followed by Camembert (24%) and Chevre (15%) (Table 3 in Paper I). Most cheeses had a_w values in the range 0.96-0.99 and pH in the range 4.3-7.7 (Table 4 in Paper I).

3.2 Description of producers, animals and production practices (Paper I)

To understand and describe strengths and weaknesses as regards the food safety of Swedish small-scale artisan cheese production, all participants were asked to fill in a questionnaire regarding their cheese production and, if applicable, their farm and milk-producing animals (Table 2 in Paper I). The dairy questions included personnel, education, dairy premises, utensils, production volume, hygiene barriers, control measures, water supply, pasteurisation conditions and procedures for handling starter cultures. The questions about the farm and animals included animal species, herd size, mastitis problems, silage feeding and milking procedures.

Eighty-three per cent of dairies (35/42) made cheese using milk from their own animals. Among the dairies, there was wide variation regarding size of milk-producing herd and, hence, the production volumes (<50 L to 1500 L milk per batch). The goat farms ranged from 20 to 200 goats, and most had less than 60 animals. The cow farms were small, almost half had up to 5 cows, and only one farm had more than 20 cows. The three sheep farms had between 30 and 100 animals (Table 2 in Paper I). Thus, the impact of the cheeses from the different dairies on consumers varies.

Workforces were generally small, usually consisting of three persons or less. Most had education/training from non-academic cheese making courses combined with experience, study trips and learning from colleagues. Of those using pasteurised milk, low pasteurisation in a closed vat at 63-68 °C for 20-45 min was reported to be the dominant pasteurisation procedure. Surprisingly, less than 50% of the dairies surveyed reported using a timer and thermometer to check pasteurisation.

Three of the dairies did not add lactic starter cultures and relied exclusively on the natural flora of the raw milk. The handling routine for the starter cultures varied. Of those dairies responding to the questions regarding starter culture routines, 67% (23/34) used an overnight (so-called mother-daughter) starter culture, 12% (4/34) added freeze dried grains with mixes of different LAB directly to the cheese milk, 9 % (3/34) added commercially fermented milk (*filmjölk*) directly to the cheese milk, and 12% (4/34) did not specify their routine. Mesophilic starter cultures were added at 20 to 40 °C, where approx. 60% of the dairies added the starter culture below 30 °C. In all, 78% (29/37), of

respondents used a pH-meter to check acidification; 8% (3/37) used pH sticks, 5% (2/37) relied on smell and/or vision, 3% (1/37) had no pH check and 5% (2/37) did not specify.

Ten percent of respondents reported unsatisfactory water quality due to indicator bacteria during the previous year. The water supply for dairies with water quality problems was private wells, drilled or dug. A number of measures were taken by the cheese making FBOs to avoid contamination by microorganisms in the dairy, but only 38% (14/38) reported change of clothing and 42% (16/38) physical separation of animal house and dairy as hygiene barriers (Table 2 in Paper I).

Mastitis was a common problem in the herds, with 60% (21/35) of those cheese producers who replied stating that they had experienced mastitis in their herd during the previous year. Mastitis prevention measures reported by the producers included breeding, milking hygiene and culling of chronically infected animals. Monitoring udder health with CMT was only reported sporadically. Mastitis treatment differed between animal species; cows were more often treated with antibiotics whereas affected goats and sheep were culled (Table 2 in Paper I).

3.3 Microbial hazards in cheese (Paper I)

Staphylococcus aureus (the most common species in foods among the CPS), *E. coli* and *L. monocytogenes* are important foodborne bacteria in milk and milk products, including cheese. In the survey, CPS were detected in 69% (38/55) of the raw milk cheese whereas the occurrence was 6% (6/96) in cheeses made from pasteurised milk. CPS levels were higher in raw milk cheeses, especially in those made without the addition of starter culture (Fig. 10) (Table 3 in Paper I). Levels >5 $\log_{10}$ CFU/g were found in 16% (6/39) of the raw milk cheeses, and the highest level, 6.56 $\log_{10}$ CFU/g, was detected in a raw milk cheese with no starter culture added (Fig. 10) (Table 3 in Paper I). However, SET (SEA-E) was not detected in any cheese samples.

Listeria monocytogenes was not detected in any of the cheese samples and, based on the 151 samples, occurrence was estimated to be lower than 2% with 95% probability. *Escherichia coli* was more frequently found in raw milk cheese than in cheeses from pasteurised milk, 34% (19/55) and 3% (3/96) respectively (Table 3 in Paper I).

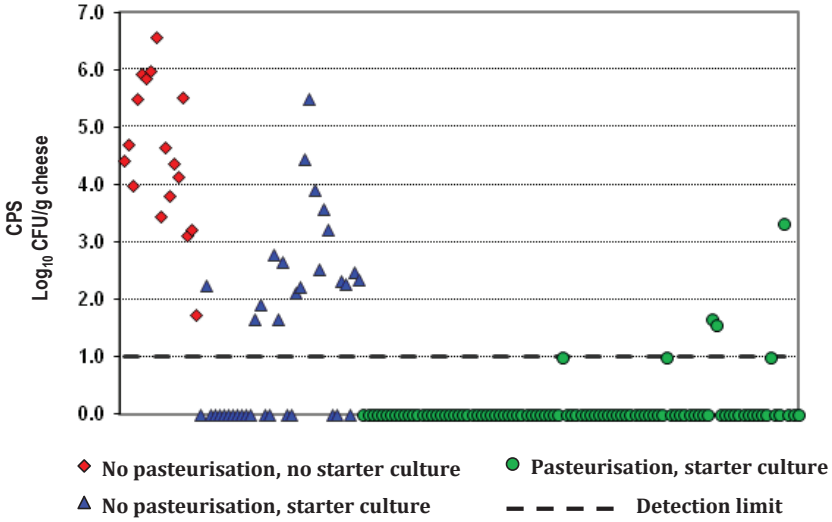

Figure 10. Relationship between levels of coagulase-positive staphylococci (CPS) and production conditions for cheeses in the survey (modified after Fabricius (2006))

Of the 156 CPS strains isolated from cheeses, 97% (152) were identified as *S. aureus* and further characterised. Screening of the *se*-genes *sea* to *sej* was done using the PCR-technique and, among isolates, *sec* was most frequent and found in 44% (67/152) of isolates (Fig. 1 in Paper I). The *sec*-gene was always detected alone, and all were of the ovine biotype. In contrast, isolates with other *se*-gene profiles (26%; 40/152) carried combinations of *sea, seg* and *sei* or *sea* and *seh* representing human and non-host (NHS) specific biotypes, respectively. Of the isolates, 29% (45/152) did not carry any of the *se*-genes tested and these isolates represented many different biotypes (Fig. 1 in Paper I). When PFGE was used to investigate genetic variation among isolates, the same PFGE-types were observed on geographically separated dairies and different PFGE-types were observed within the same dairy (Fig. 2 in Paper I). These results indicate different contamination sources, a wide geographical spread of some strains, and/or possible transmission of strains via trade in live animals.

3.4 Growth and enterotoxin A production potential of *S. aureus* under cheese making conditions (Paper II)

SET-producing *S. aureus* were commonly found in the cheese samples examined in the survey, but despite high levels, no SET was detected in the cheese samples (Paper I). To estimate ranges and magnitudes where *S. aureus* growth and SET production can occur during the initial steps of cheese making, a selected number of cheese isolates from the survey in Paper I were investigated for their potential to grow and produce SEA under conditions typical of fresh cheese or cheese curd during the initial stages of production. These experiments were carried out using pre-defined laboratory media in the presence of 0.5% (55 mM) lactic acid (Lac_{tot}) (representative of the Lac_{tot} range during the initial phase of cheese making, Fig. 3), low pH (4.3 to 6.5, adjusted to different set concentrations of HLac), temperatures between 15 and 37 °C, and a_w between 0.95 and 0.99 (Tables 1-3 in Paper II). The selected isolates in the growth experiments came from cheeses with high *S. aureus* levels, representing different biotypes and *se*-gene profiles. For the toxin potential experiment, SEA-producing isolates were chosen.

A predictive model for the time-to-growth (TTG) was developed based on absorbance data from an automated turbidity reader. The model predicted TTG as a function of HLac, temperature and a_w (eqn. 1 in Paper II). With a Lac_{tot} of 0.5% (55 mM), the TTG model predicted *S. aureus* growth within the first 18 h in the temperature range 20-25 °C and for HLac concentrations between 1.2 and 2.7 mM, corresponding approximately to pH 5.3-4.9. Growth-limiting concentrations were predicted to be in the range 7-9 mM HLac, corresponding approximately to pH 4.5-4.3 (Fig. 1 and Table 1 in Paper II). At intermediate HLac concentrations, TTG was >18 h. In comparison, lactic acid produced during successful fermentation decreases pH in cheese curd from approx. 6.7 to approx. 5.3-4.6 depending on the buffering capacity of curd and type of starter culture (Cogan & Hill, 1993) (Fig. 3).

Specific growth rates and lag times of *S. aureus* strains were estimated after two pre-treatments in skim milk at three HLac concentrations and two incubation temperatures. The pre-treatments were designed to simulate start of cheese production directly after milking, and refrigerated storage at 4 °C for 24 h before cheese making. Detection times of serial dilutions using optical turbidity data were analysed using a method developed by Baranyi & Pin (1999). Lag time was dependent on both pre-treatment and temperature, and the growth potential of *S. aureus* appeared to be greater when milk was used directly after

milking rather than being held at 4 °C for one day after milking (Table 2 in Paper II).

Production of SEA in combination with specific growth and inactivation rates was investigated in conditions representing cheese making as regards Lac_{tot}, HLac and temperature. Growth rates were slightly slower compared with predictions made in PMP, which does not include the effect of lactic acid (Fig. 11). The slower growth rate in our experiments may suggest an effect of HLac, despite its low concentrations. SEA was detected at cell concentrations greater than 6.8 $\log_{10}$ CFU/mL and the concentration increased linearly during the first days of incubation (Fig. 2 in Paper II). The SEA production rate increased with increasing temperature and decreasing HLac concentration (Fig. 2 in Paper II). After two weeks, final SEA concentrations tended to be higher in samples with a higher initial HLac concentration, but then initial pH had changed (Table 4 in Paper II). This change in the chemical properties of growth medium in un-controlled batch experiments highlights a general limitation in this experimental approach (Paper II).

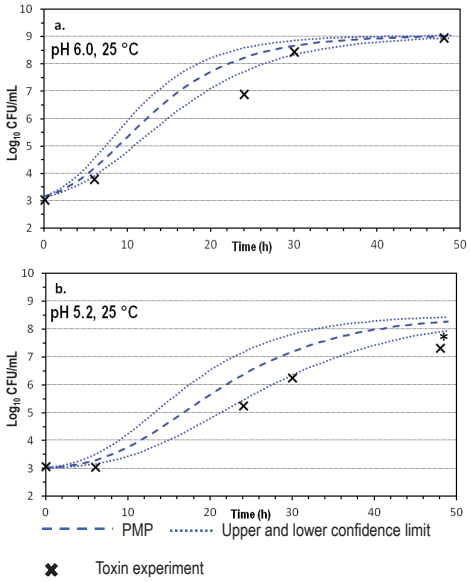

Figure 11. Predicted *S. aureus* growth curves produced by the Pathogen Modelling Program (PMP) at 25 °C at pH values 6.0 (a.) and 5.2 (b.) compared with growth data from the toxin experiment (Table 3 in Paper II) performed at the same temperatures and pH. The thin dotted lines represent the upper and lower 95% confidence limit for the PMP prediction. The PMP model does not include lactic acid, but the toxin experiment included 0.5% (55 mM) lactic acid, corresponding to 0.2 and 1.6 mM undissociated lactic acid (HLac) at pH 6.0 and 5.2, respectively.

* Underestimated data point due to overgrown plates

3.5 Process control considerations (Paper I and II)

The results presented in Papers I and II generated a number of considerations regarding process control in the production of small-scale artisan cheeses. First, the factors and activities identified as contributing to microbiologically safe cheese based on the results of the questionnaire in Paper I were summarised in a mind map (Fig. 12). In the mind-map, sanitary procedures, water supply and hygiene barriers represent pre-requisite factors, whereas raw milk quality, starter culture, pasteurisation and acidification are factors to be monitored in a HACCP-plan. Education and experience provide the essential basis for all activities. Second, steps in the artisan cheese production process important for improving microbiological safety were identified.

3.5.1 Raw milk quality

Staphylococcus aureus of animal biotype was frequently found in the cheese samples, highlighting raw milk as an important possible contamination source (Fig. 1 in Paper I). Therefore, producers using milk from their own animals should regularly monitor raw milk for increased levels of SCC by using CMT or, if available, other cell counters.

The species *E. coli* consists of both non-pathogenic and pathogenic strains and both types have been isolated from cows, goats and sheep. *Escherichia coli* was found in about one-third of the raw milk cheeses, showing the high probability of faecal contamination of milk during milking (Table 3 in Paper I). The contamination decreases with good milking hygiene, but it cannot be eliminated unless the milk is treated, *e.g.* by pasteurisation.

Decreased susceptibility to penicillin was most common in *S.aureus* strains isolated from cheese made of cow's milk and may be a result of the more frequent use of antibiotics in cow herds (Table 2 in Paper I). Therefore, the risk of antibiotic residues in raw milk may be greater in cow's milk compared with goat's and sheep's milk.

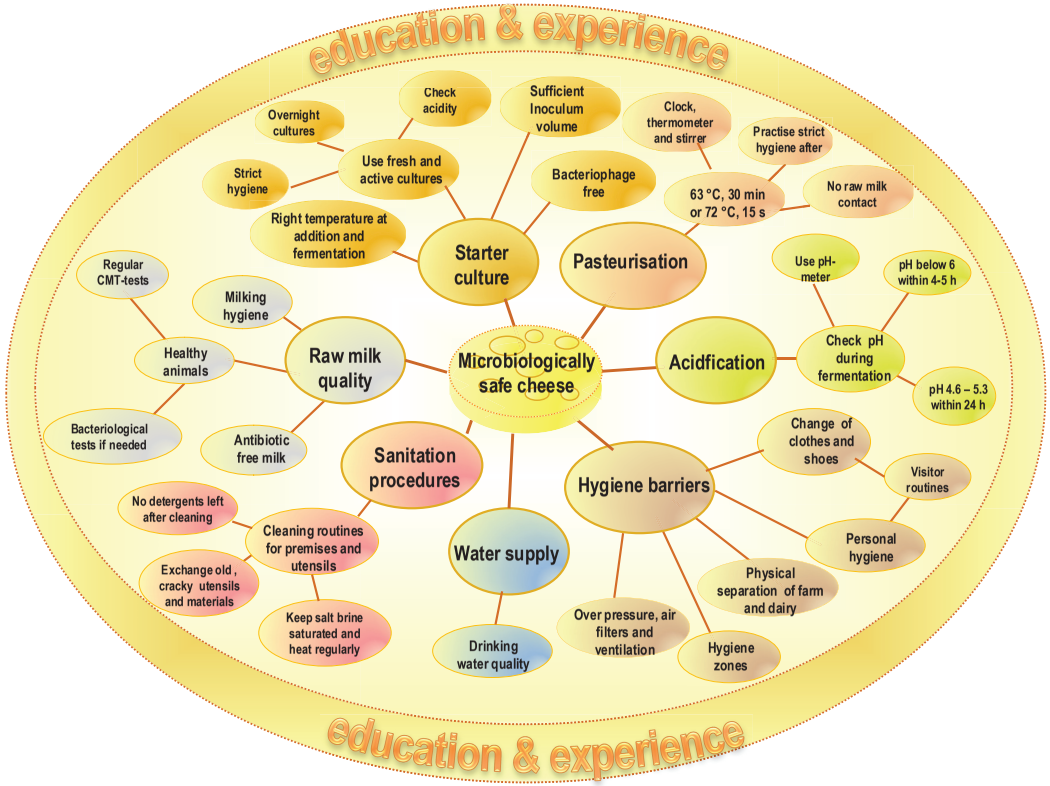

Figure 12. Summary of factors identified in Papers I and II, as contributing to the microbiological safety of small-scale produced cheese. Sanitation procedures, water supply and hygiene barriers constitute pre-requisites; raw milk quality, starter culture, pasteurisation and acidification are control points in a HACCP-plan.

3.5.2 Handling of starter cultures

The responses by the producers indicated a need for enhanced awareness of the importance of proper handling of starter cultures (Table 2 in Paper I). An active starter culture at an appropriate temperature starts acidification quickly and minimises the time to growth of pathogens in the cheese milk. Starter activity can be tested by measuring pH or titratable acidity (Bylund, 1995).

Fresh overnight cultures made from heat-treated milk and defined lactic acid bacteria, bought as freeze-dried grains, are recommended because the culture is well-defined and fit-for-purpose, grows exponentially and is adapted to the milk environment and to room temperature (if mesophilic). When preparing overnight cultures, good hygiene is important to prevent contamination by airborne fungi or bacteriophages and, if possible, cultures should be prepared in a separate space (Bylund, 1995). Direct addition of commercially fermented milk (*e.g. filmjölk*) to milk should be avoided because of the risk of acidification failure. Starter activity declines with age (Stadhouders *et al.*, 1978) and the age of a fermented milk product is difficult to assess. In addition, fermented milks are usually kept refrigerated, which prolongs the lag time of the starter culture. Even freeze-dried grains are not adapted to the milk environment, and if added directly to milk they have longer lag time, which requires longer pre-acidification (up to 1 h)[2] compared with overnight cultures (Olofsson, 2010).

3.5.3 Monitoring of acidification

Milk is an excellent growth substrate for microorganisms and environmental conditions at the start of cheese making favour microbial growth. Hence, rapid start of acidification is necessary to reduce growth of *S. aureus* and other possible foodborne pathogens (Paper II). Acidification can be monitored by measuring pH with a pH-meter during the initial phases of cheese making. However, acidification rates differ depending on cheese type and type of starter culture used. To identify acidification disturbances, a pH control chart for each cheese type can be made. Such a chart can be constructed by the producer from pH data measured at close intervals (*e.g.* every hour) during the first 7-8 h and after ~ 24-25 h of 5 to 10 successful cheese makings. Once constructed, it can be used as a 'cheese-type pH standard' in every cheese making batch. Then, pH can be measured less frequently at defined time intervals and the results plotted in the chart (Fig. 13).

[2]. Kerstin Jürss. Pers. communication 2012-03-02

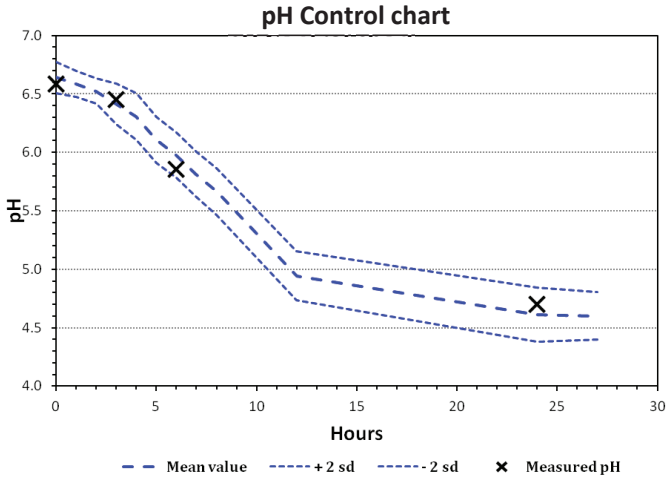

Figure 13. Example of how acidification during the initial phase of cheese making can be plotted in a pH control chart and compared against a 'cheese-type pH standard'. The dashed and dotted lines, showing mean value and standard deviation (sd), are based on repeated pH measurements from eight (in this fictitious example) successful cheese makings. The upper and lower 95% confidence limits are shown as +2 sd and -2sd, respectively.

3.5.4 Sampling for analysis

The growth of *S. aureus* and other foodborne pathogenic bacteria occurs mainly during the first 24 h of cheese making (Fig. 4). However, in the defined laboratory media used in this study, the highest SEA concentration occurred later than the peak *S. aureus* levels and it gradually increased with time (Fig. 2 in Paper II). According to EC-regulation on microbiological criteria in foods (EC, 2005), CPS sampling in cheeses should be done when CPS levels are expected to be at their highest, which is during the first day of cheese making (Fig. 4). Furthermore, if the levels exceed 5 $\log_{10}$ CFU/g, screening for the presence of SET should be carried out. Based on the results of the broth experiments in Paper II, the probability of detecting SET in a cheese may increase if the SET analysis is postponed for a few days (Fig. 2 in Paper II). In addition, cheese samples should be kept at the appropriate ripening temperature for the cheese type in question and not routinely kept refrigerated while awaiting SET analysis. For fresh cheeses, however, the appropriate storage is at refrigeration temperatures.

4 Summary and concluding remarks

The main findings of this thesis are that:

* Levels of the investigated pathogens were reasonable in most cheese samples analysed and no staphylococcal enterotoxin (SEA-E) was detected. However, high levels (5-6.5 $\log_{10}$ CFU/g) of staphylococcal enterotoxin C producing *Staphylococcus aureus* were found in several samples of raw milk fresh cheese, especially those without starter culture. These results suggest an increased risk of staphylococcal food poisoning in those cheeses.

* *Escherichia coli,* indicating faecal contamination, was found 10-fold more frequently in the raw milk cheeses than in cheeses made from pasteurised milk. Such contamination can be reduced with good milking hygiene, but *E. coli* cannot be eliminated unless the milk has been treated, for example by pasteurisation.

* *Listeria monocytogenes* was not detected in any of the 151 cheeses analysed, indicating that the occurrence was less than 2% in these products.

* To improve the food safety of small-scale artisan cheeses, there is a need to raise awareness of the importance of some pre-requisites and process control points. For example, the handling of the starter cultures was a concern, since active cultures were not always used and fermentation temperatures varied considerably when using *e.g.* mesophilic starter cultures.

* A majority of *S. aureus* cheese isolates were enterotoxigenic and carried one or more of the *se*-genes tested. The *sec*-gene, followed by *sea* in combination with *seg/sei,* were the most common genes detected. *Staphylococcus aureus* strains with different properties were found in cheeses from the same dairy and strains with the same PFGE-pattern were found on geographically distant dairies. These results suggest multiple contamination sources and routes.

* In defined broth cultures, *S. aureus* could grow and produce staphylococcal enterotoxin A (SEA) at HLac concentrations, temperatures and a_w typical of the initial phases of cheese making. Growth was delayed if milk was refrigerated at 4 °C rather than being used immediately after milking. The SEA production rate increased with increasing temperature and decreasing HLac concentration. High levels of SEA were detected after prolonged incubation, suggesting that staphylococcal enterotoxin (SET) production proceeds for a long period after the bacterial stationary phase has been reached. Therefore, further storage of cheese samples prior to SET analysis may be beneficial when high levels of coagulase-positive staphylococci are detected during the first day of cheese production.

* Staphylococcal enterotoxin A was produced when *S. aureus* levels exceeded 6.8 $\log_{10}$ CFU/mL. Hence, to reduce the risk of high *S. aureus* levels followed by SET production in the cheese, initial levels of *S. aureus* and the time taken to reach growth-inhibiting conditions must be minimised. This emphasises the importance of starter cultures, which affect the growth of *S. aureus* by both acidification and other mechanisms.

5 Future research

Based on the studies presented in this thesis, the following research needs were identified:

Follow-up study
Based on some of the issues highlighted in Paper I, a follow-up questionnaire is needed to investigate whether and how the production practices of small-scale artisan cheese have changed since 2005. Many new small-scale dairies have started, a number of educational measures have been made and food hygiene legislation has been changed. An increase in raw milk cheese production is expected because current legislation allows the production of Swedish raw milk cheese ripened <60 days to be sold not only directly to the consumer as before, but also on the common market.

Growth and enterotoxin production rates of SEC-producing strains
The toxin SEA was chosen in the SET production experiments since it is the most common SET involved in staphylococcal food poisoning cases. In our survey, most of the enterotoxigenic isolates produced SEC. The genes coding for SEA and SEC are located on different sites in the genome and expression of the genes is regulated by different mechanisms, so there is reason to believe that SEC production differs from SEA production under the conditions tested here.

Milk and cheese matrices
The modelling experiments on growth and enterotoxin production were carried out in well-defined laboratory media as a first step to investigate the growth and SET-producing potential of *S. aureus* cheese isolates. Future research should include experiments in milk and cheese systems to further resemble the dynamics during cheese making. However, milk and cheese are complex matrices and several methodological difficulties with this approach are foreseen.

Growth and enterotoxin production at constant conditions

Enterotoxin production was modelled in batch experiments where the conditions in the growth medium changed during incubation. In order to study SET production under constant conditions, further studies are needed in continuous cultures where each parameter can be varied in a controlled way and studied in detail.

References

Anonymous (2005). *Småskalig livsmedelsförädling*. Stockholm: Regeringskansliet. (Departementserien. ISSN Ds 2005:22. (*In Swedish*)

ANSES (2010). Detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices. European screening method of the EU-RL for 2 coagulase positive staphylococci, including *Staphylococcus aureus*. In.

Argudin, M.A., Mendoza, M.C. & Rodicio, M.R. (2010). Food Poisoning and *Staphylococcus aureus* Enterotoxins. *Toxins (Basel)* 2(7), 1751-73.

Arvanitoyannis, I.S. & Mavropoulos, A.A. (2000). Implementation of the hazard analysis critical control point (HACCP) system to Kasseri/Kefalotiri and Anevato cheese production lines. *Food Control* 11(1), 31-40.

Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., Nakazawa, H. & Kozaki, S. (2003). An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiology and Infection* 130(1), 33-40.

Bachmann, H.P. & Spahr, U. (1995). The fate of potentially pathogenic bacteria in Swiss hard and semihard cheeses made from raw milk. *Journal of Dairy Science* 78(3), 476-83.

Balaban, N. & Rasooly, A. (2000). Staphylococcal enterotoxins. *International Journal of Food Microbiology* 61(1), 1-10.

Baranyi, J. & Pin, C. (1999). Estimating bacterial growth parameters by means of detection times. *Applied and Environmental Microbiology* 65(2), 732-6.

Baranyi, J. & Roberts, T.A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology* 23(3-4), 277-94.

Barbano, D.M., Ma, Y. & Santos, M.V. (2006). Influence of Raw Milk Quality on Fluid Milk Shelf Life1,2. *Journal of Dairy Science* 89, E15-E19.

Bassett, J., Nauta, M., Lindqvist, R. & Zwietering, M. (2012). *Tools for microbiological risk assessment*. Brussels, BE: ILSI Europe Risk Analysis in Food Microbiology Task Force. (ILSI Europe Report Series.

Bayles, K.W. & Iandolo, J.J. (1989). Genetic and molecular analyses of the gene encoding staphylococcal enterotoxin D. *Journal of Bacteriology* 171(9), 4799-806.

Bergonier, D., de Cremoux, R., Rupp, R., Lagriffoul, G. & Berthelot, X. (2003). Mastitis of dairy small ruminants. *Veterinary Research* 34(5), 689-716.

Betley, M.J. & Mekalanos, J.J. (1985). Staphylococcal enterotoxin A is encoded by phage. *Science* 229(4709), 185-7.

Boor, K.J. & Murphy, S.C. (2002). Microbiology of market milks. In: Roberson, J.R. (Ed.) *Dairy Microbiology Handbook*. 3. ed. pp. 91-122. New York, N.Y.: John Wiley and Sons Inc. ISBN 0 471 38596 4.

Borch, E., Nesbakken, T. & Christensen, H. (1996). Hazard identification in swine slaughter with respect to foodborne bacteria. *International Journal of Food Microbiology* 30(1-2), 9-25.

Bylund, G. (1995). *Dairy processing handbook*. Lund: Tetra Pak Processing Sytems AB.

Cha, J.O., Lee, J.K., Jung, Y.H., Yoo, J.I., Park, Y.K., Kim, B.S. & Lee, Y.S. (2006). Molecular analysis of *Staphylococcus aureus* isolates associated with staphylococcal food poisoning in South Korea. *Journal of Applied Microbiology* 101(4), 864-871.

Chambers, J.V. (2002). The microbiology of raw milk. In: Robinson, R.K. (Ed.) *Dairy microbiology handbook,* third. ed. pp. 56-57. New York: Wiley and Sons Inc. ISBN 0-471-38596-4.

Chen, T.R., Chiou, C.S. & Tsen, H.Y. (2004). Use of novel PCR primers specific to the genes of staphylococcal enterotoxin G, H, I for the survey of *Staphylococcus aureus* strains isolated from food-poisoning cases and food samples in Taiwan. *International Journal of Food Microbiology* 92(2), 189-97.

Chiang, Y.C., Chang, L.T., Lin, C.W., Yang, C.Y. & Tsen, H.Y. (2006). PCR primers for the detection of staphylococcal enterotoxins K, L, and M and survey of staphylococcal enterotoxin types in *Staphylococcus aureus* isolates from food poisoning cases in Taiwan. *Journal of food protection* 69(5), 1072-9.

Cogan, T.M. & Hill, C. (1993). Cheese starter cultures. In: Fox, P.F. (Ed.) *Cheese: Chemstry, physics and microbiology, General aspects*. 2. ed. pp. 193-256. London, U.K.: Chapman& Hall; 1). ISBN 0 412 53500 9.

ComBase *ComBase Modelling Toolbox*. [online] Available from: http://modelling.combase.cc/.

Concepcion Porrero, M., Hasman, H., Vela, A.I., Fernandez-Garayzabal, J.F., Dominguez, L. & Aarestrup, F.M. (2011). Clonal diversity of *Staphylococcus aureus* originating from the small ruminants goats and sheep. *Veterinary Microbiology*.

De Buyser, M.L., Dufour, B., Maire, M. & Lafarge, V. (2001). Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology* 67(1-2), 1-17.

De Buyser, M.L., Janin, F. & Dilasser, F. Contamination of ewe cheese with *Staphylococcus aureus*: Study of an outbreak of food poisoning. In: Jeljaszewicz, J. (Ed.) *Proceedings of The staphylococci: proceedings of V international symposium on staphylococci and I staphylococcal infections, Warsawa, June 26-30 1984*, Warsaw1984. p. 705: Stuttgart; Fisher. ISBN 3437109405 and 0895742160.

Delbes, C., Alomar, J., Chougui, N., Martin, J.F. & Montel, M.C. (2006). *Staphylococcus aureus* growth and enterotoxin production during the manufacture of uncooked, semihard cheese from cows' raw milk. *Journal of Food Protection* 69(9), 2161-7.

Devriese, L.A. (1984). A simplified system for biotyping *Staphylococcus aureus* strains isolated from animal species. *Journal of Applied Bacteriology* 56(2), 215-20.

Dinges, M.M., Orwin, P.M. & Schlievert, P.M. (2000). Exotoxins of *Staphylococcus aureus*. *Clinical Microbiology Reviews* 13(1), 16-34, table of contents.

EC, European Parliament (2002). Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety

EC, European Parliament (2004a). Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs

EC, European Parliament (2004b). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin

EC, European Commission (2005). Commission Regulation no. 2073 of 15 November 2005 on microbiological criteria for foodstuffs.

EFSA (2009). *European Food Safety Authority. The Community summary report on Food-borne outbreaks in the European Union in 2007, The EFSA Journal (2009), 271*. Parma, Italy.

Eldrimner (2009). *Branschriktlinjer för gårdsmejerier*. Ås, Östersund. (*In Swedish*)

Eldrimner *Hitta mathantverkare*. [online] Available from: http://www.eldrimner.com/16281.hitta_mathantverkare.html. (*In Swedish*)

Evenson, M.L., Hinds, M.W., Bernstein, R.S. & Bergdoll, M.S. (1988). Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. *International Journal of Food Microbiology* 7(4), 311-6.

Fabricius, A. (2006). *Staphylococcus in cheeses produced on Swedish farm dairies-Evaluation of growth using a combination of survey data and predictive microbiology*. Master thesis. Copenhagen: Department of Food Science, Food Microbiology.

FAO/WHO, F.S. (2003). General Principles of Food Hygiene. In. Codex alimentarius. p. 31.

FDA *Food and Drug Administration. Bad Bug Book:Foodborne Pathogenic Microorganisms and Natural Toxins Handbook. Staphylococcus aureus*. [online] Available from: http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllness FoodbornePathogensNaturalToxins/BadBugBook.

Feil, E.J., Cooper, J.E., Grundmann, H., Robinson, D.A., Enright, M.C., Berendt, T., Peacock, S.J., Smith, J.M., Murphy, M., Spratt, B.G., Moore, C.E. & Day, N.P. (2003). How clonal is *Staphylococcus aureus*? *Journal of Bacteriology* 185(11), 3307-16.

Fox, P.F. (1993). Cheese: An overview. In: Fox, P.F. (Ed.) *Cheese: Chemistry, physics and microbiology, General aspects*. pp. 1-36. London: Chapman & Hall; 1). ISBN 0 412 53500 9.

Fujikawa, H. & Morozumi, S. (2006). Modelling *Staphylococcus aureus* growth and enterotoxin production in milk. *Food Microbiology* 23(3), 260-7.

Genigeorgis, C.A. (1989). Present state of knowledge on staphylococcal intoxication. *International Journal of Food Microbiology* 9(4), 327-60.

Harris, T.O., Grossman, D., Kappler, J.W., Marrack, P., Rich, R.R. & Betley, M.J. (1993). Lack of complete correlation between emetic and T-cell-stimulatory activities of staphylococcal enterotoxins. *Infection and Immunity* 61(8), 3175-83.

Hennekinne, J.A., Brun, V., De Buyser, M.L., Dupuis, A., Ostyn, A. & Dragacci, S. (2009). Innovative application of mass spectrometry for the characterization of staphylococcal enterotoxins involved in food poisoning outbreaks. *Applied and Environmental Microbiology* 75(3), 882-4.

Hennekinne, J.A., Guillier, F., Perelle, S., De Buyser, M.L., Dragacci, S., Krys, S. & Lombard, B. (2007). Intralaboratory validation according to the EN ISO 16 140 Standard of the Vidas SET2 detection kit for use in official controls of staphylococcal enterotoxins in milk products. *Journal of Applied Microbiology* 102(5), 1261-72.

Hennekinne, J.A., Kerouanton, A., Brisabois, A. & De Buyser, M.L. (2003). Discrimination of *Staphylococcus aureus* biotypes by pulsed-field gel electrophoresis of DNA macro-restriction fragments. *Journal of Applied Microbiology* 94(2), 321-9.

Hovde, C.J., Marr, J.C., Hoffmann, M.L., Hackett, S.P., Chi, Y.I., Crum, K.K., Stevens, D.L., Stauffacher, C.V. & Bohach, G.A. (1994). Investigation of the role of the disulphide bond in the activity and structure of staphylococcal enterotoxin C1. *Molecular Microbiology* 13(5), 897-909.

ICMSF (1996). *Staphylococcus aureus*. In: Roberts, T.A*., et al.* (Eds.) *Microorganisms in Foods 5: Microbiological specification of food pathogens.* pp. 299-333. London, UK: Blackie Academic & Professional. ISBN 0 412 47350 X.

ICMSF (1998). Milk and dairy products, XI Cheese. In: Roberts, T.A*., et al.* (Eds.) *Microorganisms in foods ; Microbial ecology of food commodities*. pp.

549-558. London: Blackie Academic and professional; 6). ISBN 0-7514-0430-6.

Ikeda, T., Tamate, N., Yamaguchi, K. & Makino, S. (2005). Mass outbreak of food poisoning disease caused by small amounts of staphylococcal enterotoxins A and H. *Applied and Environmental Microbiology* 71(5), 2793-5.

Jablonski, L.M. & Bohach, G.A. (1997). *Staphylococcus aureus*. In: Doyle, M.P.*, et al.* (Eds.) *Food Microbiology, Fundamentals and frontiers*. pp. 353-375. Washington, D.C.: American Society for Microbiology (ASM) Press. ISBN 1-55581-117-5.

Jakobsen, R.A., Heggebo, R., Sunde, E.B. & Skjervheim, M. (2011). *Staphylococcus aureus* and *Listeria monocytogenes* in Norwegian raw milk cheese production. *Food Microbiology* 28(3), 492-6.

Jayarao, B.M., Donaldson, S.C., Straley, B.A., Sawant, A.A., Hegde, N.V. & Brown, J.L. (2006). A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in pennsylvania. *Journal of dairy science* 89(7), 2451-8.

Jayarao, B.M. & Henning, D.R. (2001). Prevalence of foodborne pathogens in bulk tank milk. *Journal of Dairy Science* 84(10), 2157-62.

Jayarao, B.M., Pillai, S.R., Sawant, A.A., Wolfgang, D.R. & Hegde, N.V. (2004). Guidelines for Monitoring Bulk Tank Milk Somatic Cell and Bacterial Counts. *Journal of Dairy Science* 87(10), 3561-3573.

Johansson, N. (1998). *Går det att få lönsamhet på ett gårdsmejeri?* Alnarp: Instutitionen för jordbrukets biosystem och teknologi.

Jorgensen, H.J., Mathisen, T., Lovseth, A., Omoe, K., Qvale, K.S. & Loncarevic, S. (2005a). An outbreak of staphylococcal food poisoning caused by enterotoxin H in mashed potato made with raw milk. *FEMS Microbiology Letters* 252(2), 267-72.

Jorgensen, H.J., Mork, T. & Rorvik, L.M. (2005b). The occurrence of *Staphylococcus aureus* on a farm with small-scale production of raw milk cheese. *Journal of Dairy Science* 88(11), 3810-7.

Kemi, P. & Sylvén, S. (2005). *Åtgärder för att främja och lätta för småskalig livsmedelsförädling*: Swedish Board of Agriculture. ISSN 2005:10. (*In Swedish*)

Kerouanton, A., Hennekinne, J.A., Letertre, C., Petit, L., Chesneau, O., Brisabois, A. & De Buyser, M.L. (2007). Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. *International Journal of Food Microbiology* 115(3), 369-75.

Lai, C.F., Liao, C.H., Pai, M.F., Chu, F.Y., Hsu, S.P., Chen, H.Y., Yang, J.Y., Chiu, Y.L., Peng, Y.S., Chang, S.C., Hung, K.Y., Tsai, T.J. & Wu, K.D. (2011). Nasal carriage of methicillin-resistant *Staphylococcus aureus* is associated with higher all-cause mortality in hemodialysis patients. *Clinical Journal of the American Society of Nephrology* 6(1), 167-74.

Lanciotti, R., Sinigaglia, M., Gardini, F., Vannini, L. & Guerzoni, M.E. (2001). Growth/no growth interfaces of *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella enteritidis* in model systems based on water activity, pH,

temperature and ethanol concentration. *Food Microbiology* 18(6), 659-668.
Le Loir, Y., Baron, F. & Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research* 2(1), 63-76.
Le Marc, Y., Valik, L. & Medvedova, A. (2009). Modelling the effect of the starter culture on the growth of *Staphylococcus aureus* in milk. *International Journal of Food Microbiology* 129(3), 306-11.
Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology* 55(1–3), 181-186.
Lejeune, J.T. & Rajala-Schultz, P.J. (2009). Food safety: unpasteurized milk: a continued public health threat. *Clinical Infectious Diseases* 48(1), 93-100.
Lina, G., Bohach, G.A., Nair, S.P., Hiramatsu, K., Jouvin-Marche, E. & Mariuzza, R. (2004). Standard nomenclature for the superantigens expressed by *Staphylococcus*. *Journal of Infectious Diseases* 189(12), 2334-6.
Lindblad, M., Karnehed, N., Lindqvist, R. & Hjertqvist, M. (2010). *Rapporterade misstänkta matförgiftningar 2009*: National Food Agency. (*In Swedish*)
Lindblad, M., Westöö, A., Lindqvist, R., Hjertqvist, M. & Andersson, Y. (2008). *Rapporterade misstänkta matförgiftningar 2007*: National Food Agency. (*In Swedish*)
Lindblad, M., Westöö, A., Lindqvist, R., Hjertqvist, M. & Andersson, Y. (2009). *Rapporterade misstänkta matförgiftningar 2008*: National Food Agency. (*In Swedish*)
Lindqvist, R., Andersson, Y., Lindback, J., Wegscheider, M., Eriksson, Y., Tidestrom, L., Lagerqvist-Widh, A., Hedlund, K.O., Lofdahl, S., Svensson, L. & Norinder, A. (2001). A one-year study of foodborne illnesses in the municipality of Uppsala, Sweden. *Emerging Infectious Diseases* 7(3 Suppl), 588-92.
Lindqvist, R., Sylven, S. & Vagsholm, I. (2002). Quantitative microbial risk assessment exemplified by *Staphylococcus aureus* in unripened cheese made from raw milk. *International Journal of Food Microbiology* 78(1-2), 155-70.
Lindqvist, R., Westöö, A., Hjertqvist, M. & Andersson, Y. (2004). *Rapporterade misstänkta matförgiftningar 2003*: National Food Agency. (*In Swedish*)
Lindqvist, R., Westöö, A., Hjertqvist, M. & Andersson, Y. (2005). *Rapporterade misstänkta matförgiftningar 2004*: National Food Agency. (*In Swedish*)
Lindqvist, R., Westöö, A., Hjertqvist, M. & Andersson, Y. (2006). *Rapporterade misstänkta matförgiftningar 2005*: National Food Agency. (*In Swedish*)
Ma, Y., Ryan, C., Barbano, D.M., Galton, D.M., Rudan, M.A. & Boor, K.J. (2000). Effects of Somatic Cell Count on Quality and Shelf-Life of Pasteurized Fluid Milk1. *Journal of Dairy Science* 83(2), 264-274.
McKellar, R.C. & Lu, X. (2004). Primary Models. In: X., M.R.C.a.L. (Ed.) *Modelling microbial responses in food*. pp. 21-62. Boca Raton, FL: CRC Press LLC. ISBN 0-8493-1237-X.

McMeekin, T.A., Olley, J., Ratkowsky, D.A. & Ross, T. (2002). Predictive microbiology: towards the interface and beyond. *International Journal of Food Microbiology* 73(2–3), 395-407.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. & Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases* 5(5), 607-25.

Meyrand, A., Boutrand-Loei, S., Ray-Gueniot, S., Mazuy, C., Gaspard, C.E., Jaubert, G., Perrin, G., Lapeyre, C. & Vernozy-Rozand, C. (1998). Growth and enterotoxin production of *Staphylococcus aureus* during the manufacture and ripening of Camembert-type cheeses from raw goats' milk. *Journal of Applied Microbiology* 85(3), 537-44.

National Food Agency *List of Approved Swedish Establishments for raw milk and dairy products according to Regulation (EC) No 853/2004*. [online] Available from: http://www.slv.se.

NMKL (2003). Nordic Committee on Food Analysis. Method no. 66: *Staphylococcus aureus.* Determination in foods.

Norling, B. (1994). *Matförgiftningar i Sverige - resultat av en intervju-undersökning.* . Uppsala: National Food Agency. ISSN Report no 41/94. (*In Swedish*)

Noto, M.J., Kreiswirth, B.N., Monk, A.B. & Archer, G.L. (2008). Gene acquisition at the insertion site for SCCmec, the genomic island conferring methicillin resistance in *Staphylococcus aureus*. *Journal of Bacteriology* 190(4), 1276-83.

Novick, R.P. & Geisinger, E. (2008). Quorum sensing in staphylococci. *Annual Review of Genetics* 42, 541-64.

Novick, R.P. & Subedi, A. (2007). The SaPIs: mobile pathogenicity islands of *Staphylococcus*. *Chemical Immunology and Allergy* 93, 42-57.

Oliver, S.P., Jayarao, B.M. & Almeida, R.A. (2005). Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodborne Pathogens and Disease* 2(2), 115-29.

Olofsson, I. (2010). *Guidelines for food safety control of artisan cheese-making* Copenhagen. (TemaNord 2010:596.

Ostyn, A., De Buyser, M.L., Guillier, F., Groult, J., Felix, B., Salah, S., Delmas, G. & Hennekinne, J.A. (2010). First evidence of a food poisoning outbreak due to staphylococcal enterotoxin type E, France, 2009. *Eurosurveillance* 15(13).

Paape, M.J. & Capuco, A.V. (1997). Cellular defense mechanisms in the udder and lactation of goats. *Journal of Animal Science* 75(2), 556-65.

Persson, Y. & Olofsson, I. (2011). Direct and indirect measurement of somatic cell count as indicator of intramammary infection in dairy goats. *Acta Veterinaria Scandinavica* 53, 15.

PMP 7.0 (2007). *Pathogen Modelling Program. USDA ARS. http://ars.usda.gov/Services/docs.htm?docid=11550*.

Quinn, P.J., Carter, M.E., Markey, B. & Carter, G.R. (1994a). *Staphylococcus* species. In: *Clinical veterinary microbiology,* 1. ed. pp. 118-126 Mosby International Limited. ISBN 0723417113.

Quinn, P.J., Carter, M.E., Markey, B.K. & Carter, G.R. (1994b). Mastitis. In: *Clinical veterinary microbiology*. 1. ed. pp. 327-344. ISBN 0 7234 1711 3.

Ramsaran, H., Chen, J., Brunke, B., Hill, A. & Griffiths, M.W. (1998). Survival of bioluminescent *Listeria monocytogenes* and *Escherichia coli* O157:H7 in soft cheeses. *Journal of Dairy Science* 81(7), 1810-7.

Robinson, R.K., Tamine, A.Y. & Wszolek, M. (2002). Microbiology of fermented milks. In: Robinson, R.K. (Ed.) *Dairy Microbiology Handbook*. 3. ed. New York: John Wiley and Sons Inc. ISBN 0-471-38596-4.

Ross, T. & Dalgaard, P. (2004). Secondary Models. In: McKellar, R.C.a.L., X. (Ed.) *Modelling microbial responses in food*. pp. 63-150. Boca Raton, FL: CRC press LLC. ISBN 0-8493-1237-X.

Ross, T. & McMeekin, T.A. (1994). Predictive microbiology. *International Journal of Food Microbiology* 23(3-4), 241-64.

Sargeant, J.M., Leslie, K.E., Shirley, J.E., Pulkrabek, B.J. & Lim, G.H. (2001). Sensitivity and Specificity of Somatic Cell Count and California Mastitis Test for Identifying Intramammary Infection in Early Lactation1. *Journal of Dairy Science* 84(9), 2018-2024.

Schaeren, W. & Maurer, J. (2006). Prevalence of subclinical udder infections and individual somatic cell counts in three dairy goat herds during a full lactation. *Schweizer Archiv für Tierheilkunde* 148(12), 641-8.

Schelin, J., Wallin-Carlquist, N., Thorup Cohn, M., Lindqvist, R. & Barker, G.C. (2011). The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. *Virulence* 2(6), 580-92.

Schlievert, P.M. & Case, L.C. (2007). Molecular analysis of staphylococcal superantigens. *Methods in Molecular Biology* 391, 113-26.

Schmid, D., Fretz, R., Winter, P., Mann, M., Hoger, G., Stoger, A., Ruppitsch, W., Ladstatter, J., Mayer, N., de Martin, A. & Allerberger, F. (2009). Outbreak of staphylococcal food intoxication after consumption of pasteurized milk products, June 2007, Austria. *Wiener Klinische Wochenschrift* 121(3-4), 125-31.

Schønberg, K.C. & Wåltorp, M. (2001). *Stafylokokkmatforgifting etter inntak av kvit geitost (In Norwegian)*: The Norwegian Institute of Public Health (MSIS-rapport , 2001

Shelef, L.A. (1994). Antimicrobial Effects of Lactates: A Review. *Journal of Food Protection* 57(5), 445-450.

SMI *Swedish Institute for Communicable Disease Control*. [online] Available from: http://www.smittskyddsinstitutet.se/in-english/statistics/.

Stadhouders, J., Cordes, M.M. & van Schouwenburg-van Foeken, A.W.J. (1978). The effect of manufacturing conditions on the development of staphylococci in cheese. Their inhibition by starter bacteria. *Netherlands Milk and Dairy Journal* 32, 198-203.

Stewart, C.M., Cole, M.B., Legan, J.D., Slade, L., Vandeven, M.H. & Schaffner, D.W. (2002). *Staphylococcus aureus* growth boundaries: moving towards mechanistic predictive models based on solute-specific effects. *Applied and Environmental Microbiology* 68(4), 1864-71.

Sutherland, J.P., Bayliss, A.J. & Roberts, T.A. (1994). Predictive modelling of growth of *Staphylococcus aureus*: the effects of temperature, pH and sodium chloride. *International Journal of Food Microbiology* 21(3), 217-36.

Sveriges gårdsmejerister *Medlemmar*. [online] Available from: http://www.sverigesgardsmejerister.se. (*In Swedish*)

Tamine, A. (2002). Microbiology of starter cultures. In: Roberson, J.R. (Ed.) *Dairy Microbiology Handbook*. 3. ed. pp. 261-430. New York, NY: John Wiley and Sons Inc. ISBN 0-471-38596-4.

Thoendel, M., Kavanaugh, J.S., Flack, C.E. & Horswill, A.R. (2011). Peptide signaling in the staphylococci. *Chemical Reviews* 111(1), 117-51.

Thomas, D., Chou, S., Dauwalder, O. & Lina, G. (2007). Diversity in *Staphylococcus aureus* enterotoxins. *Chemical Immunology and Allergy* 93, 24-41.

Thorberg, B.-M. (2008). *Coagulase-negative staphylococci in bovine sub-clinical mastitis*. Diss. Uppsala:Swedish University of Agricultural Sciences.

University of Bergen *ELISA / Multiplex protein analyses*. [online] Available from: http://www.uib.no/rg/cardio/research/methods/elisa-multiplex-protein-analyses.

Valero, A., Perez-Rodriguez, F., Carrasco, E., Fuentes-Alventosa, J.M., Garcia-Gimeno, R.M. & Zurera, G. (2009). Modelling the growth boundaries of *Staphylococcus aureus*: Effect of temperature, pH and water activity. *International Journal of Food Microbiology* 133(1-2), 186-94.

Vernozy-Rozand, C., Meyrand, A., Mazuy, C., Delignette-Muller, M.L., Jaubert, G., Perrin, G., Lapeyre, C. & Richard, Y. (1998). Behaviour and enterotoxin production by *Staphylococcus aureus* during the manufacture and ripening of raw goats' milk lactic cheeses. *Journal of Dairy Research* 65(2), 273-81.

Wallin-Carlquist, N., Cao, R., Marta, D., da Silva, A.S., Schelin, J. & Radstrom, P. (2010a). Acetic acid increases the phage-encoded enterotoxin A expression in *Staphylococcus aureus*. *BMC Microbiology* 10, 147.

Walstra, P., Wouters, J.T.M. & Geurts, T.J. (2006). *Milk Dairy science and technology* 2. ed. Boca Raton, FL, U.S.A.: CRC Press, Taylor & Francis group. ISBN 0-8247-2763-0.

Wattinger, L., Stephan, R., Layer, F. & Johler, S. (2011). Comparison of *Staphylococcus aureus* isolates associated with food intoxication with isolates from human nasal carriers and human infections. *European Journal of Clinical Microbiology & Infectious Diseases*

Wei, H.L. & Chiou, C.S. (2002). Molecular subtyping of *Staphylococcus aureus* from an outbreak associated with a food handler. *Epidemiology and Infection* 128(1), 15-20.

Whiting, R.C. & Buchanan, R.L. (1993). Letter to the Editor. *Food Microbiology* 10(2), 175-177.
Wieneke, A.A., Roberts, D. & Gilbert, R.J. (1993). Staphylococcal food poisoning in the United Kingdom, 1969-90. *Epidemiology and Infection* 110(3), 519-31.

Acknowledgements

This work was carried out at the Microbiology Division at National Food Agency, Uppsala, Sweden. The project was partly supported by the Ivar and Elsa Sandberg Foundation.

I would like to send my special thanks to:

My kind and skilful supervisors:

My main supervisor Bengt Guss for giving me the opportunity of being his external and first (!) *licentiate* student, for sharing expert knowledge in the huge field of *Staphylococcus* and for all the inspiring educational discussions.

My supervisor Roland Lindqvist for everyday guidance, coaching mixed with support, encouragement, asking challenging questions, all new 'interesting and creative' ideas, for always taking the time to talk despite loads of other work, and for the endless patience when teaching me predictive modelling.

My supervisor Susanne Sylvén for introducing me to the world of small dairies, delicious cheeses, cows, goats and sheep, control programmes, critical thinking and for teaching me the art of converting EC-regulations into practice.

~❀~

My colleagues at the National Food Agency:

Mats Lindblad for enthusiastic, skilful and invaluable statistical support, for many fun cheese chats and for the important contributions to the manuscripts.

Monica Olsen, former Head of the Microbiology Division, for supporting the idea and giving me the opportunity to initiate the study.

Hans Lindmark, current Head of the Microbiology Division for introducing me to real-time PCR, for support and encouragement and for having the patience of letting me complete the study.

All past and present colleagues at the Microbiology Division for the good co-operation, for creating a cheerful and 'microbiological' atmosphere in the laboratory and for all fun, educational and thoughtful topics around the coffee table. Special thanks to Jimmy Sahlin and Jane Karlsdotter for supporting me with loads of laboratory media; Ingela Tillander, for teaching me how to extract, concentrate and detect those darn enterotoxins; Mia Egervärn, for teaching me valuable PCR- and antibiotic susceptibility skills; Anna Westöö, for donating all her cheese books on retirement; and Susanne Thisted Lambertz for sharing experiences from her time as an external post-graduate student.

The service-minded librarians at the National Food Agency; Mikaela Bachmann, for all the help with tracing and ordering articles, books and reports, and Jannes Engqvist for valuable EndNote support.

~❀~

Students:

Ane Fabricius, for the excellent laboratory work and good cooperation during the survey and the growth experiments.

Maria Karlsson, for the excellent laboratory work and the important contribution of enabling quantification of the enterotoxins at our laboratory.

~❀~

SLU-friends:

The staff of the Department of Microbiology for hospitality and helpfulness during my not too frequent 'expeditions' to Ultuna.

~❀~

'Cheese and *Staphylococcus* people':

All the Swedish small-scale dairies that participated in the survey and whose contribution with cheeses was invaluable for the study.

Ida Olofsson, my cheese making teacher in Jämtland, for teaching me how to make an edible cheese, for all enthusiasm and for sharing practical experiences from 'the field'.

Kerstin Jürss, for being an experienced and wise dairy woman willing to share her expertise in cheese making with me.

Jenny Schelin, for being a really nice person and for inviting me to her department in Lund and teaching me how to quantify enterotoxins.

~❁~

My dear family:
My loving mother and father, sister and brother with families far up north (too far), for warmth and care when we go ‘home’, and for supporting and encouraging me in whatever I’m up to.

Lars, for all love and care, for making me happy and for being such an ace in algebra.

Elias, Agnes and Ingrid, the sunshine of my life.

~❁~