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Milk maturation temperature and time are key technological parameters to limit staphylococcal enterotoxin production during uncooked semi-hard cheese manufacture

Manon Duquenne [a, b], Sylviane Derzelle [b], Isabelle Fleurot [a], Marina Aigle [a], Claire Darrigo [a], Jacques-Antoine Hennekinne [b], Isabelle Mutel [b], Marielle Bouix [c], Véronique Deperrois-Lafarge [b], Agnès Delacroix-Buchet [a, *]

[a] INRA, UMR1319 Micalis, 78350 Jouy en Josas, France
[b] Université Paris-Est, Anses, Laboratoire de sécurité des aliments, Unité SBCL, 94700 Maisons-Alfort, France
[c] AgroParisTech, UMR782 Génie et Microbiologie des Procédés Alimentaires, 78850 Thiverval-Grignon, France

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ABSTRACT

The influence of five technological parameters selected amongst uncooked semi-hard cheese-making practices, i. e. milk maturation temperature and time, stirring during maturation, curd stirring time and pressing time was examined on *Staphylococcus aureus* growth, enterotoxin gene expression and enterotoxin (SE) production during cheese manufacture. A fractional factorial experimental design was applied to perform 32 cheese batches independently inoculated at 10^3 cfu/ml of milk using four strains producing SEA, SEB, SEC or SED.

The *S. aureus* population was found to exceed 10^5 cfu/g of cheese four hours after molding. SED was the only enterotoxin detected. It was produced in very low quantities that varied with the parameters studied. Early *sed* gene expression during cheese processing was correlated with SED detection in curd and cheese. Milk maturation temperature and time emerge as key technological parameters that control SED production. A response surface methodology was then carried out to further characterize the relationships between both factors and SE production in cheese and whey. Two SED-producing strains were used to perform two sets of ten cheese batches based on a central composite design of experiments at five levels. Predictive mathematical models were established.

Increasing the temperature at the beginning of the cheese-making process was shown to increase SED production. Furthermore, we determined that the proportion of SED drained after molding from the curd in the whey depended only on the technological parameters. The two SED-producing strains showed similar trends of behavior but specific level of gene expression and enterotoxin production in response to the same set of milk maturation parameters.

1. Introduction

Staphylococcus aureus is a worldwide cause of food-borne diseases. This bacterium is the causative agent of intoxication characterized by symptoms including nausea, vomiting, abdominal cramps and diarrhea a few hours after the ingestion of food containing preformed staphylococcal enterotoxins (SEs) (Balaban & Rasooly, 2000). In Europe, 777 outbreaks in 2012 were caused by bacterial toxins produced by *Bacillus* spp., *Clostridium* spp., and coagulase-positive staphylococci (CPS); the latter correspond to the second most common causative agent in food-borne outbreaks. Among them, 346 were due to SEs, of which 20% correlated with cheese as food vehicle (Anonymous, 2014). Since 2005, criteria on CPS and SEs in various food categories have been introduced in the EU (Anonymous, 2005, 2007). In this EU regulation, SE detection must be performed when CPS counts are higher than 10^5 cfu/g at the stage where they are expected to be the highest. If SEs are detected in a 25-g test portion, the food is considered as unsafe, and food operators have to withdraw it. It is therefore of major interest

* Corresponding author. INRA, UMR1313 Génétique Animale et Biologie Intégrative, 78350 Jouy-en-Josas, France.
E-mail address: agnes.delacroix-buchet@jouy.inra.fr (A. Delacroix-Buchet).

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to determine the peak level of *S. aureus* and limit the production of SEs during the cheese-making process.

To date, 23 or so SEs have been described but only SEH and the five classical SEs, SEA to SEE, have been clearly involved in staphylococcal food-poisoning outbreaks. Commercial detection kits are available for SEA to SEE (Hennekinne, De Buyser, & Dragacci, 2012). SE genes encoding these toxins are found in various genetic supports and can be carried by mobile genetic elements such as plasmids (*seb*, *sed*), phages (*sea*, *see*), pathogenicity islands (*seb*, *sec*) or by genomic islands (*seh*). Some but not all of these SEs are controlled by, the accessory gene regulator (*agr*) quorum-sensing system, one of the main regulatory system controlling virulence expression in *S. aureus*. The *seb*, *sec* and *sed* genes have been demonstrated to be *agr* dependant whereas *sea* is *agr* independent (Balaban & Rasooly, 2000; Hennekinne et al., 2012; Le Loir, Baron, & Gautier, 2003). Many studies have investigated the environmental conditions (i.e. pH, temperature or oxygenation) that affect *S. aureus* development and impact SEs gene expression and production in laboratory media or in foodstuffs, including a few concerning the cheese-making process (see Cretenet, Even, & Le Loir, 2011 for review).

It is now accepted that, to be understood and controlled, the behavior of *S. aureus* must be analyzed directly *in situ*, in the food matrix (Schelin et al., 2011). The impact of technological parameters on enterotoxin gene expression and production during cheese manufacturing is an important question. We developed a rapid and efficient toolbox to extract and monitor bacterial mRNA in cheese (Duquenne et al., 2010). Until now molecular technologies have been applied to model strains in simplified conditions. The objective of the present study was to identify, in conditions encountered in real cheese-making processes, key technological parameters relevant to SE production. We chose to manufacture semi-hard cheeses due to their economic importance and because the limited acidification which characterized them is more permissive to *S. aureus* growth. We used realistic levels of *S. aureus* contamination. Questions often raised by data presented in studies where pathogen levels used are at extremely high level. In practice, these levels would raise concerns about raw milk quality (Donnelly, 2001) and, the initial inoculum level of bacteria in milk was recently shown to strongly influence the spatial distribution of bacterial colonies in cheese (Jeanson et al., 2011). From a survey conducted on industrial practices amongst uncooked semi-hard cheese French manufacturers, five technological parameters that may affect SE production, and may thus be crucial to control the cheese-making process were chosen. A fractional factorial design was applied to investigate these five parameters and to identify the most important ones. A central composite experimental design was next implemented to further examine the effect of the two key parameters.

2. Materials and methods

2.1. Bacterial strains

Five SE-producing *S. aureus* strains isolated from cheese were used throughout this study. Strains CIM479 (*seb, seg, sei, sem, seo, ser*), CIM441 (*sec, seg, sei, sel, sem, sen, seo*) and CIM433 (*sed, sej, ser*) are from the ARILAIT collection (La Roche-sur-Foron, France). Strains 432G (*sea*) and 361F (*sea, sed, sej, ser*) have been isolated at ANSES (Maisons-Alfort, France) from raw ewe milk cheese implicated in food-borne outbreaks. The industrial freeze-dried starter culture (Ezal MA400 and MY800, Danisco, Dangé-Saint-Romain, France), used for cheese-making was stored at −20 °C. It includes *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis biovar diacetylactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lb. delbrueckii* subsp. *bulgaricus*.

2.2. Inoculum preparation for cheese manufacture

Milk contamination with *S. aureus* was performed using an exponential growth phase culture grown on brain heart infusion broth (BHI, Oxoid, France) at 37 °C. The lactic acid bacterial starter culture was prepared according to the manufacturer's instructions and suspended, just before used, in pasteurized milk from the cheese vat to ensure a satisfactory homogenization (see below).

2.3. Cheese manufacture

Raw bulk milk cooled at 4 °C was collected from a local 300 dairy cow's farm (Jouy-en-Josas, France). Before milk pasteurization (30 s at 72 °C), milk fat and protein composition were standardized so that fat content was equal to 95% of the total nitrogen content. Uncooked semi-hard cheeses were prepared in four automated 20-L tanks in a P2-level experimental plant at INRA (Jouy-en-Josas, France). Simultaneous manufacturing cycles with four distinct *S. aureus* strains (432G, CIM479, CIM441 or CIM433) or two distinct *S. aureus* strains (CIM433 or 361F) were performed respectively, for the first (E1) and second (E2) experimental designs. Cheeses were manufactured using different combinations of the five selected technological parameters (E1) (Table 1) or combinations of set temperature and given time of maturation (E2) (Table 2). Following the addition of $CaCl_2$ (15.7 g/100 l of milk), 15 l of pasteurized milk (pH 6.5) pre-heated at the maturation temperature were inoculated with *S. aureus* (to a final concentration of 10^3 cfu/ml) and with starter culture (to a final level of 10^6 cfu/ml). According to the experimental design, milk maturation was then performed for given times, with or without stirring before addition of filtered rennet extracts (520 mg of chymosin/l, Berthelot, France) at quantities depending on the set temperature. Coagulation then proceeded for about 45 min before the curd was cut into small cubes and, after slow stirring, pre-pressed in whey for 30 min. The curd was finally cut into eight equal parts, poured into molds and pressed. Whey draining was continued for 4 or 24 h (E1) or for only 4 h (E2) while temperature slowly decreased to 16 °C (over 4 h). For E2, the volume of whey extracted and cheese weights were determined *i*-from the draining, *ii*-after pre-pressing, *iii*- 10 min after molding and *iv*- 4 h after molding. After de-molding, cheeses were salted for 5 h in sterile brine (pH 5, 10 °C), dried for 24 h and conditioned in plastic bags under vacuum (for E1) or dried at 16 °C overnight (for E2). Model cheeses weighted approximately 250 g each. Mean values for moisture on a fat free basis, fat in dry matter, salt content and curd pH of one-day-old cheeses were respectively 67.2 ± 1.9%, 47.5 ± 2.4%, 2.0 ± 0.4% and 5.03 ± 0.10. These values were in accordance with the physico–chemical parameters of uncooked semi-hard cheeses highlighting that the processed cheeses mimicked real cheeses. A flow chart of the uncooked semi-hard cheese process summarizing the different sampling steps and analyses performed at each step is presented on Fig. 1.

2.4. Bacterial enumeration in cheeses

The absence of *S. aureus* was checked in all pasteurized milk samples before inoculation. *S. aureus* and starter bacteria in cheeses were estimated over the first three days (E1) or the first 24 h (E2) of the cheese-making procedure by plating as described (Duquenne et al., 2010).