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Milk powder risk assessment with *staphylococcus aureus* toxigenic strains

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ABSTRACT

Staphylococcus aureus is the causative agent of staphylococcal enterotoxicosis foodborne illness. Milk powder and whey powder are at risk of contamination by coagulase-positive staphylococci, which reflects the requirement for microbiological examination of foods listed in Regulation (EC) 2073/2005, as amended. Microbiological criteria for coagulase-positive staphylococci are up to 10^1–10^2 cfu g^{-1}. This study evaluates the possibility of survival and growth of *S. aureus* in milk powder after its reconstitution. Powdered milk was inoculated with 10^2 and 10^5 cfu g^{-1} of toxigenic strains of *S. aureus* and then stored as reconstituted milk for 48 h at 4, 15, and 25 °C. Staphylococcal growth and production of staphylococcal enterotoxins A, B, and C (SEA, SEB, and SEC) was regularly detected during the 48 h storage period. With inoculation of *S. aureus* high counts at 25 °C, the production of staphylococcal enterotoxins (SEs) was detected as early as after 7 (SEB) or 8 (SEA) hours of storage; at 15 °C as early as after 48 h (SEA, SEB). With inoculation of low counts of *S. aureus* (complying with legislative requirements) only at 25 °C, SEs production was detected after 24 (SEA, SEB) or 48 (SEA, SEB, SEC) hours. Model experiments evaluated SEs consumer risk resulting from extended storage of reconstituted milk at improper temperatures.

1. Introduction

Modern food production chains are evolving into very complex systems that provide greater opportunities for contamination and growth of pathogens. Coast-to-coast and international distribution by mega-processing plants puts potential outbreaks on a national and international scale (Nugen and Baeumner, 2008). As a direct consequence, preventing foodborne diseases (FBDs) becomes a difficult task. FBDs are defined by the World Health Organization as "diseases of infectious or toxic nature caused by or thought to be caused by the consumption of food or water". More than 250 different FBDs have been described, and bacteria are the causative agents of two thirds of FBD outbreaks (Le Loir, Baron, & Gautier, 2003). Staphylococcal food poisoning is one of the most common food-borne diseases worldwide (Hennekinne et al., 2009), resulting from the ingestion of staphylococcal enterotoxins preformed in food by enterotoxigenic strains of coagulase-positive staphylococci, mainly *Staphylococcus aureus*. The growth of *S. aureus* in foods presents a potential public health hazard, because many strains of *S. aureus* produce enterotoxins which are the causative agents of staphylococcal food poisoning (Peles et al., 2007).

Staphylococcus aureus is a coagulase-positive staphylococcus that causes a range of infections in both humans and animals. It is one of the major etiological agents of mastitis in dairy cattle (Rabello et al., 2007). These infections lead to considerable economic losses as a result of reduced milk production and poor quality of milk and are a source of contamination of milk and dairy products (Spanu et al., 2013). Up to 50–70% of *S. aureus* strains are able to produce – under suitable conditions – extracellular heat-stable staphylococcal enterotoxins (SEs). These toxins are classified into the large family of staphylococcal and streptococcal

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pyrogenic enterotoxins responsible for food poisoning (staphylococcal enterotoxicosis) and some allergic diseases (Balaban & Rasooly, 2000; Sharma, Rees, & Dodd, 2000). However, the presence of an enterotoxigenic gene does not always imply the production of the respective enterotoxin (Sharma et al., 2000). Staphylococcal enterotoxin A (SEA), either alone or along with other classical SEs (SEB, SEC, SED, and SEE), is considered as the main cause of staphylococcal food poisoning throughout the world (Dinges, Orwin, & Schlievert, 2000). Intoxications by *S. aureus* are most frequently identified as the cause of human outbreaks due to the consumption of raw milk or products made thereof (Claeys et al., 2013; Yamashita et al., 2003). In *S. aureus* strains isolated from goat milk powder, the most common genes encoding the production of classical SEs were *sec* (23.2%) and *seb* (12.6%) (Xing et al., 2016).

Powdered milk is not sterile and could be contaminated with various bacteria including certain pathogens. It is a high risk food in terms of the presence of coagulase-positive staphylococci. Their maximum count in this commodity is restricted by European legislation to 10 cfu g^{-1}, and in two of five samples, their count can reach even 100 cfu g^{-1} (Regulation (EC) 2073/2005). The limit of 10^5 cfu g^{-1} is considered as a high-risk count of *S. aureus* in food which enables the production of enterotoxins (Necidová, Janštová, & Karpíšková, 2012; Regulation (EC) 2073/2005). Wang et al. (2012) analyzed samples of powdered infant formula milk, and 11.2% of them were positive for *S. aureus* carrying genes for classical staphylococcal enterotoxins *sec* (18.5%), *sea* (7.4%), *seb* (7.4%), *sed* (5.6%), and *see* (5.6%). If infant foods contaminated with enterotoxigenic *S. aureus* are reconstituted at a low temperature and stored at room temperature for a long period, they still could become a potential hazard to infant health. An extensive outbreak in Japan caused by the consumption of reconstituted milk contaminated with staphylococcal enterotoxin A was reported by Asao et al. (2003).

A chilled temperature of 4 °C does not permit the growth of most pathogenic bacteria, with the exception of psychrotrophic organisms. *S. aureus* replicates at temperatures between 7 °C and 48 °C. The limits for the production of SEs do not overlap with those for the replication of *S. aureus*; in general, SEs are produced in food at temperatures ranging from 10 °C to 46 °C (Bhunia, 2008; Schelin et al., 2011). *S. aureus* is relatively resistant to drying, the minimum water activity value for its proliferation is 0.86 (Bhunia, 2008; Fernandes, 2009) or even 0.83 (Schelin et al., 2011). *S. aureus* survival in powdered milk is common, and SEs are produced right from a value of 0.83 (Schelin et al., 2011).

The aims of this study were to examine *S. aureus* growth dynamics and production of staphylococcal enterotoxins A, B, and C in reconstituted milk powder and to evaluate the potential for the production of SEA, SEB, and SEC in reconstituted milk powder which will or will not meet the parameters required by the European legislation (100 cfu g^{-1}) and will (4 °C) or will not (15 °C, 25 °C) be stored properly.

2. Material and methods

2.1. Staphylococcus aureus

The *S. aureus* strains used in our present study were recovered from 228 raw cow's milk samples collected from 40 farms in the Czech Republic in 2012–2014. Colonies from the plates were examined with a plasma coagulase test (Staphylo LA Seiken DENKA SEIKEN Co. Ltd., Japan), and suspected *S. aureus* strain confirmation was determined by polymerase chain reaction (PCR) based on detection of the species specific genomic fragment SA442 (Martineau, Picard, Roy, Ouellette, & Bergeron, 1998). For our study purposes, three strains carrying the *sea* gene (SA 393, SA 562, SA 650), three strains carrying the *seb* gene (SA 536, SA 652, SA 879), and three strains carrying the *sec* gene (SA 289, SA 315, SA 360) were selected. All nine strains were screened by PCR to detect the presence of genes encoding enterotoxins *sea, seb,* or *sec*. For the detection of the genes for enterotoxins SEA–SEE and SEH, multiplex PCR according to Lovseth et al. (2004) and Monday and Bohach (1999) was used. All primers used in the study are listed in Appendix 1. The ability of the strains to produce enterotoxins was tested by enzyme-linked fluorescence assay (ELFA) after enrichment in both pasteurized milk and the laboratory Brain Heart Infusion medium (Oxoid, UK) at 37 °C.

2.2. Samples

Powdered milk samples (Hami infant formula, Nutricia Inc.) were contaminated with 1 ml suspension of *S. aureus* developer with the McFarland standard (the first equivalence corresponds to the number of 3×10^8 bacteria/ml) and mixed thoroughly. The inoculated milk powder was reconstituted with boiled water cooled to 40 °C at the ratio recommended by the manufacturer (13.5 g of infant formula +90 ml of water). This situation created a reconstituted milk model relating to a consumer perspective. Lower and higher *S. aureus* counts were pre-designed using a mathematical calculation. The real and exact number of *S. aureus* in the reconstituted milk was determined by the method specified in each sample (9 for low count and 9 for the higher). These milk samples were also inoculated with two dilutions – low counts (5.0×10^0–2.7×10^1 cfu g^{-1}) or high counts (1.3×10^4–2.0×10^4 cfu g^{-1}) of one of nine *S. aureus* toxigenic strains.

All 18 inoculated samples with accurately calculated *S. aureus* number were divided into 3 sub-samples, which were then stored at 4 °C, 15 °C, and 25 °C to simulate both suitable and improper storage conditions. Samples from each temperature were individually analyzed. Experiments were run without replications, resulting in total 54 samples being analyzed (9 strains of *S. aureus*/3 different temperature/two dilutions). *S. aureus* was enumerated in all samples at regular intervals. Sampling took place at hourly intervals during the first 12 h of storage and then again after 24 and 48 h. Our samples were examined for *S. aureus* count and screened for the presence of SEA, SEB, and SEC. In parallel, control samples of reconstituted milk not inoculated with *S. aureus* were analyzed.

2.3. Quantitative detection of S. aureus

Individual samples were examined, and coagulase-positive staphylococci were enumerated according to CSN EN ISO 6888-1 (1991) by a technique using Baird-Parker agar medium supplemented with egg yolk-tellurite emulsion (Bio-Rad, France).

2.4. SEs detection

Staphylococcal enterotoxins were detected by an enzyme-linked fluorescence assay (ELFA) using the MiniVIDAS analyzer (BioMérieux, Marcy l'Étoile, France), capable of detecting the sum of enterotoxins SEA–SEE, with detection limits of 0.5 ng g^{-1} or per ml of food for SEA and SEB and of 1.0 ng g^{-1} or per ml of food for SEC–SEE. Since the ELFA technique does not record quantitative detection of SEs, results are expressed as either positive or negative. To monitor SEs production dynamic during the reconstituted milk incubation process, Relative Fluorescence Values (RFVs) and Test Values (TVs) were determined. For each sample a test value TV < 0.13 is considered negative and a TV ≥ 0.13 is considered positive. RFV is calculated by subtracting the background reading