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Microbial biodiversity, quality and shelf life of microfiltered and pasteurized extended shelf life (ESL) milk from Germany, Austria and Switzerland

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ABSTRACT

Information on factors limiting the shelf life of extended shelf life (ESL) milk produced by microfiltration and subsequent pasteurization is very limited. In this study, three different batches of ESL milk were analyzed at different stages of the production process and during storage at 4 °C, 8 °C and 10 °C in order to evaluate the changes in bacterial cell counts, microbial diversity and enzymatic quality. Additionally, detailed biodiversity analyses of 250 retail ESL milk packages produced by five manufacturers in Germany, Austria and Switzerland were performed at the end of shelf life. It was observed that microfiltration decreased the microbial loads by 5–6 $\log_{10}$ units to lower than 1 CFU/mL. However, bacterial counts at the end of shelf life were extremely variable and ranged between <1 and 8 $\log_{10}$ CFU/mL. 8% of all samples showed spoilage indicated by cell counts higher than 6 $\log_{10}$ CFU/mL. The main spoilage groups of bacteria were Gram-negative post-process recontaminants (*Acinetobacter*, *Chryseobacterium*, *Psychrobacter*, *Sphingomonas*) and the spore formers *Paenibacillus* and *Bacillus cereus*, while other spore formers and *Microbacterium* spp. did not reach spoilage levels. *Paenibacillus* spp. and *B. cereus* apparently influenced enzymatic spoilage, as indicated by increased free fatty acid production, pH 4.6 soluble peptide fractions and off-flavors. In some cases, enzymatic spoilage was observed although microbial counts were well below 6 $\log_{10}$ CFU/mL. Thirteen *B. cereus* isolates were characterized for their toxin profiles and psychrotolerance. *Hbl*, *nhe*, and *cytK* toxin genes were detected in ten, thirteen, and four isolates, respectively, whereas the *ces* gene was always absent. Interestingly, only three of the thirteen isolates could be allocated to psychrotolerant genotypes, as indicated by the major cold shock *cspA* gene signature. Generally, large discrepancies in microbial loads and biodiversity were observed at the end of shelf life, even among packages of the same production batch. We suggest that such unexpected differences may be due to very low cell counts after ESL treatment, causing stochastic variations of initial species distributions in individual packages. This would result in the development of significantly different bacterial populations during cold storage, including the occasional development of high numbers of pathogenic species such as *B. cereus* or *Acinetobacter*.

1. Introduction

Consumers as well as the milk processing industry and distributors have a strong interest in an extended shelf life of products. While high temperature short time (HTST) pasteurized milk has a keeping quality of about 1 week at cold storage, ultra-high temperature (UHT) milk can be held at room temperature for several months. Yet, the production of this type of milk is accompanied by undesired thermally derived product alterations, i.e., cooked and rich caramelized flavors and off-flavors (Mehta, 1980; Shipe et al., 1978). Therefore, novel manufacturing techniques have been introduced for the production of Extended Shelf Life (ESL) milk with a taste like fresh milk, but a prolonged shelf life of up to 4 weeks in cold chain distribution. Besides high heat treatment at 123–127 °C for 1–5 s (Kaufmann et al., 2010; Mayr et al., 2004a,b) a combined processing of microfiltration and pasteurization (ESL) has also been established. First approaches of using microfiltration for the reduction of the microbial load have been undertaken for more than 25 years (Holm et al., 1986; Piot et al., 1987) and at this time the patented Bactocatch-procedure was introduced (Holm et al., 1986). By applying this method, raw milk is separated into skimmed milk and milk fat. The skimmed milk is microfiltered through ceramic membranes and subsequently pasteurized. The milk fat together with the germ-enriched retentate obtained after microfiltration of the skimmed milk is processed by ultra-high heat treatment and then reverted into the skimmed milk. To date, there is knowledge about the keeping quality of ESL milk in relation to different storage temperatures and other physico-chemical factors (Elwell and

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Barbano, 2006; Kaufmann et al., 2010, 2008; Kaufmann and Kulozik, 2006), but, to our knowledge, there is no information about which spoilage microorganisms influence the shelf life of ESL milk and which potentially pathogenic organisms belonging to risk group II according to German legislation (IfSG, 2000) are found in such products.

Of particular interest with regard to food quality and safety is the spore former *Bacillus cereus*, which is one of the major spoilage organisms of processed milk causing off-flavors, "sweet curdling" and "bitty cream" defects in milk due to proteinase, lipase and phospholipase activity. Some sporulated strains of *B. cereus* are able to germinate and multiply even under refrigerated conditions. Lechner et al. (1998) described *Bacillus weihenstephanensis* as the psychrotolerant species of the *B. cereus* complex, differing from *B. cereus* sensu strictu in its ability to grow at 4 °C, but not at 43 °C. *B. weihenstephanensis* can be detected by targeting the major cold shock protein *cspA* and a psychrotolerance signature in the 16S rDNA sequence (Lechner et al., 1998; von Stetten et al., 1998). Besides spoilage of processed milk, *B. cereus* is also capable of producing food poisoning toxins, i.e. the nonhemolytic enterotoxin complex NHE, the hemolytic enterotoxin complex HBL and the single protein cytotoxin and the emetic toxin cereulide, a dodecadepsipeptide (Ehling-Schulz et al., 2011). Intoxication with *B. cereus* toxins mostly has a moderate progression and is self-limiting (Stenfors Arnesen et al., 2008), but in some cases the emetic toxin has been reported to cause severe illness with fatal outcome due to fulminant liver failure and rhabdomyolysis (Mahler et al., 1997). Emetic toxins are mainly produced in starchy foods like rice, but low levels of cereulide have also been observed in milk (Agata et al., 2002). The occurrence of diarrheic toxins in milk is reported to be higher, as there are several case studies about intoxications with the diarrheic toxins after the consumption of contaminated milk (Granum and Lund, 1997).

This study is focused on the influence of microfiltration and pasteurization processes on the microbial populations of ESL milk after elaboration and at the end of the shelf life, as well as the evaluation of changes in lipolytic, proteolytic and sensory properties. Besides, the microbial status of ESL milk at retail from five different manufacturers was assessed at the end of the shelf life in order to isolate the most representative spoilage and pathogenic species belonging to risk group II.

2. Materials and methods

2.1. Processing of ESL milk and microbial analyses during storage at different temperatures

Three batches of ESL milk samples A, B, C (3.8% fat matter) and their precursor products raw milk and microfiltered milk (MF) were obtained from a German dairy over a period of 4 months. Milk was treated by the Bactocatch procedure. Raw milk was degreased to avoid blocking of the ceramic membranes by fat globules. Skimmed milk was microfiltered through ceramic membranes with a nominal pore size of 1.4 μm (Tami, France) and then pasteurized at 77 °C for 30 s. Cream was heated separately at 125 °C for 4 s and reverted to the pasteurized skimmed milk. Two raw milk samples, two MF samples and 2 carton packages (1.5 l) of ESL milk from each batch were analyzed at the day of production and a total of 51 carton packages were stored at 4 °C, 8 °C and 10 °C, respectively, for up to 29 days. Two packages were periodically analyzed after 7, 14, 16, 18, 20 and 29 days while after 22 days (end of shelf life), five packages of each storage temperature and each batch were analyzed. Milk was diluted with Ringer solution (Merck) and plated on Plate Count Agar supplemented with 0.1% skim milk powder (PC + MM, Merck) according to IDF standards and German legislation. Plates were incubated aerobically at 30 °C for 5 days and the total aerobic counts were determined. For analyzing the raw milk biodiversity, 100 isolates were randomly chosen. Due to the low microbial loads expected for the MF and ESL milk samples directly after production, an additional enrichment procedure was performed for these samples of each batch to ensure accurate cell count determination and a sufficiently high number of isolates for identification. 100 mL of milk were therefore divided into 1 mL aliquots, enriched with 9 mL of PC + MM broth and incubated at 30 °C for 5 days. After enrichment, one loopful of each sample was plated on PC + MM agar and plates were incubated at 30 °C for 120 h. Finally, all bacterial colonies grown in 100 mL enriched milk sample were chosen for species determination. For batch C, which had higher cell counts in the freshly produced ESL milk, 200 colonies were randomly selected and isolated from PC + MM agar after direct plating and due to their high spoilage potential additionally all spore formers were chosen from the enriched aliquots. From the refrigerated samples after 22 days of storage, representative colonies of each of the distinct morphologies were isolated from the agar plates. Additionally, during storage, representative colonies of *B. cereus* and spore formers dominating the microbiota were isolated and identified.

2.2. Analysis of retail ESL milk of different manufacturers

To obtain an overview of the microbiological quality of ESL milk available on the market and to determine which species are of particular relevance for spoilage, milk of five different manufacturers, three from Germany, one from Austria and one from Switzerland (manufacturer I to V), were analyzed for the microbiota in the fresh product and at the end of shelf life. From each manufacturer, five different batches and ten packages of each batch were analyzed. Apart from milk of manufacturer IV, which had more variable fat content, ranging from 0.5 to 3.8%, all other samples had 3.5 to 3.8%. Batches one and two from manufacturer V had been organically produced, which means that the cream separated before the microfiltration process was only treated at 90 °C for two to five seconds, while the cream of the conventional milks of this manufacturer was heated at 125 °C for the same time. Milk packages were either sent to our laboratory at refrigerated conditions by the manufacturer or they were directly collected from food retailing.

Out of the ten packages per batch, five were stored at 30 °C for 3 days to detect competitive mesophilic bacteria present in the milk, while the other five were stored at 8 °C (maximum storage temperature recommended by the manufacturers in Germany) till the end of shelf life as defined by the manufacturer in order to detect the spoilage flora at refrigerated temperatures. Samples were plated on PC + MM agar and aerobic counts were determined. Representative colonies having different morphologies were isolated from the agar plates and further identified by FT-IR spectroscopy.

2.3. Identification of isolates

All isolates were identified using FT-IR spectroscopy (Wenning et al., 2008). Spore formers were cultivated on TS agar (Oxoid) at 25 °C, lactic acid bacteria on All Purpose Tween (APT) agar (Merck) anaerobically at 34 °C according to Wenning et al. (2010) and all other bacteria on Tryptic Soy (TS) agar at 30 °C. Yeasts were incubated on Yeast Extract Glucose Chloramphenicol (YGC) agar (Merck) at 27 °C according to Kümmerle et al. (1998). Incubation time was in all cases for 24 ± 0.5 h. Few strains yielding a clumpy suspension were homogenized using the FastPrep instrument (MP Biomedicals) at 4.5 m/s for 10 s without the addition of beads in order to improve spectral quality. All spectra were recorded and evaluated according to the methods of Oberreuter et al. (2002) using an HTS-XT FT-IR spectrometer (Bruker Optics, Germany). To overcome the difficulties arising from baseline shifts and to improve the resolution of complex bands, the first (lactic acid bacteria, aerobic Gram-positive non spore formers) or second derivations (spore formers, yeasts, Gram-negative bacteria) of the digitized original spectra were used. Six different FT-