J. Dairy Sci. 98:1–5
http://dx.doi.org/10.3168/jds.2015-9559

Short communication: Lipolytic activity on milk fat by *Staphylococcus aureus* and *Streptococcus agalactiae* strains commonly isolated in Swedish dairy herds

Janak K. Vidanarachchi,* Shengjie Li,† Åse Sternesjö Lundh,† and Monika Johansson†[1]
*Department of Animal Science, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka 20400
†Department of Food Science, Uppsala BioCenter, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden

ABSTRACT

The objective of this study was to determine the lipolytic activity on milk fat of 2 bovine mastitis pathogens, that is, *Staphylococcus aureus* and *Streptococcus agalactiae*. The lipolytic activity was determined by 2 different techniques, that is, thin-layer chromatography and an extraction-titration method, in an experimental model using the most commonly occurring field strains of the 2 mastitic bacteria isolated from Swedish dairy farms. The microorganisms were inoculated into bacteria-free control milk and incubated at 37°C to reflect physiological temperatures in the mammary gland. Levels of free fatty acids (FFA) were analyzed at time of inoculation ($t = 0$) and after 2 and 6 h of incubation, showing significant increase in FFA levels. After 2 h the FFA content had increased by approximately 40% in milk samples inoculated with *Staph. aureus* and *Strep. agalactiae*, and at 6 h the pathogens had increased FFA levels by 47% compared with the bacteria-free control milk. Changes in lipid composition compared with the bacteria-free control were investigated at 2 and 6 h of incubation. Diacylglycerols, triacylglycerols, and phospholipids increased significantly after 6 h incubation with the mastitis bacteria, whereas cholesterol and sterol esters decreased. Our results suggest that during mammary infections with *Staph. aureus* and *Strep. agalactiae*, the action of lipases originating from the mastitis pathogens will contribute significantly to milk fat lipolysis and thus to raw milk deterioration.
Key words: free fatty acids, bovine milk, mastitis bacteria, lipolysis, thin-layer chromatography

Short Communication

Lipases are enzymes catalyzing the hydrolysis of triglycerides (**TG**), the main lipid component of milk, resulting in free, nonesterified FA (Deeth, 2006). Elevated levels of FFA are known to affect the flavor of milk negatively, giving rise to rancid off-flavor and reducing the shelf life of milk and dairy products (Shipe et al., 1980). In the raw milk, the TG will be protected from action by lipases by the milk fat globule membrane. However, instability or mechanical disruption of the milk fat globule membrane will enhance the exposure of milk fat to degradation. In fresh milk, lipolysis is catalyzed by the endogenous lipoprotein lipase as well as by exogenous, microbial lipases (Jensen and Newburg, 1995; Deeth, 2006; Hanus et al., 2008). Whereas pasteurization of the milk will inactivate lipoprotein lipase, microbial lipases may be very heat resistant, remaining active after heat treatment (Sørhaug and Stepaniak, 1997). For instance, thermostable lipolytic and proteolytic enzymes originating from psychrotrophic bacteria have been shown to cause drastic reduction in shelf life of HTST and UHT pasteurized milk during storage (Teh et al., 2011).

Staphylococcus aureus and *Streptococcus agalactiae* are major causes of clinical and subclinical mastitis all over the world (Riffon et al., 2001). They are contagious bacteria, adhering to and multiplying in the mammary tissue, and are frequently found in bulk-tank milk samples (Keefe, 1997; Leitner et al., 2006). In connection with mastitis, protease activity hydrolyzing the technologically important caseins and also reducing shelf life and sensory attributes of dairy products is higher in milk from quarters infected with mastitis bacteria than in milk from healthy quarters (Politis et al., 1989). Haddadi et al. (2005) suggested that *Escherichia coli* proteases have a direct proteolytic effect on casein. Results in our previous studies showed the same for strains of *Staph. aureus* and *Strep. agalactiae* (Åkerstedt et al., 2012; Johansson et al., 2013a). In contrast, only a limited number of studies highlight the direct contribution of mastitis bacteria on milk fat degradation. Rosenstein and Götz (2000) characterized the staphylococcal lipases, and their significance has been related to bacterial lipid metabolism and patho-

Received March 11, 2015.
Accepted August 6, 2015.
[1]Corresponding author: monika.johansson@slu.se

genesis. Lu et al. (2012) showed that *Staph. aureus*, but also many other staphylococcal species, are able to produce FA-modifying enzymes, and some of these have also been shown to exhibit lipase activity. It has not yet been investigated to what extent lipase activity of *Staph. aureus* and *Strep. agalactiae* may affect the levels of FFA in milk and thus contribute to product deterioration. The aim of the current study was therefore to examine the lipolytic ability on milk fat of strains of the bovine mastitis pathogens *Staph. aureus* and *Strep. agalactiae*. The selected strains were a gift from the National Veterinary Institute, Uppsala, Sweden, from a nationwide survey aiming at characterization of strains of *Staph. aureus* and *Strep. agalactiae* isolated in Swedish dairy herds. The 2 strains used in this study were identified as the most commonly occurring strains of *Staph. aureus* and *Strep. agalactiae*, respectively.

Bacteriological examination was performed by accredited routine methods at the Mastitis Laboratory at National Veterinary Institute, Uppsala, Sweden, according to Hogan et al. (1999). Both strains were obtained as pure cultures initiated from single colonies in triplicates and cultured separately overnight in 5 mL of nutrient broth containing 10% horse serum and incubated at 37°C. The subcultured bacterial cultures (1.5 mL) were transferred to 28.5 mL of bacteria-free, UHT milk (1.5% fat) in triplicates according to the method described by Johansson et al. (2013a) and incubated at 37°C to reflect physiological temperatures in the udder. The milk samples were buffered with 0.1 *M* 3-morpholino-propanesulfonic acid to prevent acidification of the samples during incubation. Subsamples for analyses of FFA by the extraction-titration method were collected from the incubated UHT milk at 3 different time points (0, 2, and 6 h), whereas total bacteria count and pH were determined after 1, 2, 4, and 6 h as previously described by Åkerstedt et al. (2012). Lipid classes were determined at 2 and 6 h after inoculation using high-performance thin-layer chromatography (TLC).

For the extraction-titration method, based on Salih et al. (1977), the milk fat was extracted from 20 g of milk with a mixture of diethyl ether and hexane (80:20, vol/vol) in the presence of 26% NaCl and 4 droplets of 0.25% methyl orange indicator resolved in water. The samples were vortexed for 1 min in Teflon tubes. To obtain pH 2 to 3, H_2SO_4 (2%, vol/vol) was added, until the solution turned pink, followed by vortexing for 1 min. After centrifugation at 1,070 × *g* (Sorvall Super T21, Sorvall Products L.P., Newton, CT) for 5 min at 21°C, 40 mL of the supernatant was removed to a new tube. Four droplets of 0.1% α-naphtholphthalein indicator resolved in 99% ethanol were added. Free fatty acids were finally titrated with 0.02 *M* KOH resolved in 99% ethanol (EtOH) until the color turned blue-green, persisting for a few seconds. All chemicals, if not otherwise indicated, were from Sigma (Sigma-Aldrich Inc., Stockholm, Sweden).

Total milk lipids were methylated according to the procedure of Appelquist (1968) with slight modifications (Johansson et al., 2013b). Milk (10 mL) was mixed with 10 mL of ethanol and 1 mL of H_2SO_4 (2.5 mol/L). Extraction was carried out with 15 mL of ether:heptane (1:1, vol/vol) in screw-capped centrifuge tubes. After centrifugation at 740 × *g* (Sorvall Super T21) for 2 min at 25°C, the upper solvent layer was transferred to a new conical flask containing 1 g of anhydrous Na_2SO_4 to adsorb residual water. The extraction procedure was repeated twice with 10 mL of ether:heptane (1:1, vol/vol). The solvent was evaporated under nitrogen gas. The lipid content of the milk was determined from this total extracted lipid, which was dissolved in hexane and stored at −80°C until analysis. All samples were extracted in triplicate. Changes in the lipid classes during incubation (37°C) of UHT milk inoculated with the different bacterial species were evaluated using high-performance TLC coupled with the ATS4 Camag (Camag, Muttenz, Switzerland) lipid class analysis procedure. Total lipids were separated and quantified as described by Dutta and Appelqvist (1989). Thin-layer chromatography was performed using precoated glass silica gel TLC plates (20 × 20 cm; Silicagel 60; 0.20-mm layer, Merck, Darmstadt, Germany) and hexane:diethyl ether:acetic acid (85:15:1, vol/vol/vol) along with reference samples. The lipid samples (1 μg/μL of hexane) were applied to the TLC plate using Camag TLC sampler 4 (Camag) and separated in a 20 × 20 twin through chamber (Camag) using 25 mL of the mobile phase, hexane:diethyl ether:acetic acid (85:15:1, vol/vol/vol). The plates were removed from the chamber after developing 6.9 cm from the baseline. For derivatization, 20 g of phosphomolybdic acid in 200 mL of ethanol was applied for 10 min at 120°C. The plates were scanned using a Camag TLC scanner 3 (Camag) to identify the lipid classes. Phospholipids, diacylglycerols, triacylglycerols, cholesterol, sterol esters, and FFA were identified using external 16–1 A and C18–5 TLC standards (Nu-Chek Prep Inc., Elysian, MN).

For the statistical part, ANOVA was performed with SAS v. 9.3 (SAS Institute Inc., Cary, NC). Least squares means were calculated with the general linear model procedure. The model included the effect of treatment. The option PDIFF (probability difference) was used to estimate the differences among least squares means.

Changes in milk pH and total number of bacteria in the bacteria-free control milk and milk inoculated with *Staph. aureus* and *Strep. agalactiae*, respectively, were recorded at different time points. The pH was slightly