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Transcripts and protein levels of *CSN1S1* and *CSN3* genes in dairy cattle mammary gland secretory tissue during chronic staphylococcal infection

Ewelina Kawecka-Grochocka[1,2], Magdalena Zalewska[3], Aleksandra Kapusta[1], Tomasz Ząbek[4], Magdalena Rzewuska[2], Sławomir Petrykowski[5] and Emilia Bagnicka[1]

[1]Department of Biotechnology and Nutrigenomics, Institute of Genetics and Biotechnology, Polish Academy of Sciences, 36A Postepu St., Jastrzębiec, Poland; [2]Preclinical Sciences, Institute of Veterinary Medicine, Warsaw University of Life Sciences, 8 Ciszewskiego St., Warsaw, Poland; [3]Department of Applied Microbiology, Faculty of Biology, University of Warsaw, Institute of Microbiology, 1 Miecznikowa St., Warsaw, Poland; [4]Department of Animal Molecular Biology, The National Research Institute of Animal Production, 1 Krakowska St., Balice near Krakow, Poland and [5]Experimental Farm, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 36A Postepu St., Jastrzębiec, Poland

Research Article

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Author for correspondence:
Emilia Bagnicka,
Email: e.bagnicka@igbzpan.pl

Abstract

Our objective was to determine the influence of chronic coagulase-positive staphylococci (CoPS) or coagulase-negative staphylococci (CoNS) infection on the mRNA and protein levels of two main milk proteins responsible for cheese curd quantity and quality, alpha-S1-casein (CSN1S1) and kappa-casein (CSN3). Measurements were made in cow mammary parenchyma with a prevalence of secretory tissue (MGST). Samples of MGST were collected from the separate quarters and divided into CoPS, CoNS and bacteria-free (H) groups according to the microbiological status of the quarter milk. No differences in CSN1S1 and CSN3 mRNA level were found between groups, however, CSN1S1 protein level was significantly higher in the H group than the CoNS group, and CSN3 protein level was significantly higher in H than CoPS group. Hence, while the *CSN1S1* and *CSN3* genes appear to be constitutively expressed at the mRNA level in dairy cow MGST during mastitis, CoNS infection negatively affected CSN1S1 protein level, and CoPS infection negatively affected CSN3 protein level. The lack of change at the mRNA level suggests that staphylococcal infection may affect the post-transcriptional or post-translational modifications.

In cows, the mammary gland frequently demonstrates varying degrees of inflammation, resulting in a range of physical, chemical and microbiological changes in the milk (Alnakip *et al.*, 2014), usually with a deterioration in its composition and technological properties (Kalińska *et al.*, 2018). During subclinical mastitis, the bacteria isolated most commonly from milk are staphylococci (Taponen and Pyörälä, 2009). Both coagulase-positive (CoPS) and coagulase-negative staphylococci (CoNS) can be found, and both can cause negative changes in the quantity and quality of milk proteins (Marsilio *et al.*, 2018). While CoNS act as bacterial commensals and opportunistic pathogens and have been isolated from the skin and mucous membranes of mammals, CoPS such as *Staphylococcus aureus* are considered as major pathogens, and can pose a serious threat for animals and humans (Rigarlsford, 2006).

Milk proteins are typically divided into two main groups: whey proteins and caseins. Caseins, which constitute 80% of all milk proteins (Alim *et al.*, 2014), consist of four fractions: alpha-S1-casein (CSN1S1), alpha-S2-casein (CSN1S2), beta-casein (CSN2), and kappa-casein (CSN3). CSN1S1 represents almost 40% of all caseins content in milk, while CSN3 represents only about 12%. Casein micelles are composed of CSN1S1 (40%), CSN1S2 (10%), CSN2 (35%), and CSN3 (15%) (Broyard and Gaucheron, 2015). CSN3 plays key roles in maintaining the structure and stability of casein micelles (Volkandari *et al.*, 2017) and affects milk coagulation and cheese quality. Therefore, as CSN1S1 and CSN3 are arguably the most important proteins for cheese production determining curd yield and quality, respectively (Comin *et al.*, 2008), the present study focuses on their response to infection.

The objective of the study was to determine changes in *CSN1S1* and *CSN3* gene expression at the mRNA and protein levels in bovine mammary gland secretory tissue (MGST) infected with CoPS or CoNS compared to samples taken from healthy, bacteria-free (H) udder quarters.

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Materials and methods

The experiment was carried out on 40 Polish Holstein-Friesian dairy cows of the black and white variety kept on the Experimental Farm at the Institute of Genetics and Animal Biotechnology in Jastrzębiec, near Warsaw, Poland. All were between their first and fourth lactation. The owner of the herd gave written permission for sample collection. Animal husbandry and feeding conditions have been described previously by Kościuczuk *et al.* (2014). The animals were culled in the last stage of lactation (286 d, SD = 27 d) due to chronic inflammation after several unsuccessful antimicrobial therapies; these were included in experimental groups CoPS or CoNS, depending on the type of bacteria present. Others culled due to reproduction or hoof problems, i.e. without any udder problems were added to the control group (H) according to the herd management system. All cows were slaughtered in a certified slaughterhouse at least one month after the last therapy with antimicrobials.

Milk microbiological analysis

Foremilk samples were taken manually from each udder quarter aseptically two days before the slaughter of animals, just before evening milking, to determine the udder health status. The microbiological analysis of the milk samples was described by Kościuczuk *et al.* (2014). Briefly, 100 µl of the mixed milk samples were streaked on a Columbia Agar supplemented with 5% sheep blood and on Mannitol Salt Agar (bioMerieux, Craponne, France). The plates were then incubated for 24–48 h at 37 °C. The bacterial colonies growing on the media were differentiated based on morphology of the colony. Isolates were differentiated using catalase and coagulase production ability tests. Accurate species identification was performed with the API® Staph biochemical test (bioMerieux, Craponne, France). Bacteria which demonstrated coagulase production were additionally subjected to the SlidexStaph-Kit test (bioMérieux, Craponne, France) to confirm identification of *Staphylococcus aureus*.

Tissue samples

Dairy cattle mammary gland samples (1 × 1 × 5 cm) were taken from the deep layers of the secretory part of the udder (mammary gland parenchyma) demonstrating a predominance of secretory tissue (MGST). One sample was taken per udder quarter just after slaughter, and no more than two udder samples were taken per animal. To remove the remaining milk and blood, the samples were washed in ice-cold phosphate-buffered saline, rapidly frozen in liquid nitrogen, then stored at −80°C for further analysis.

Based on microbiological analysis results, 54 samples were selected for the study. These were divided into three groups according to the health status of the quarter. The first was a healthy group (H) consisting of samples ($N = 13$) taken from cows without pathogenic bacteria in their milk in all four quarters, ie no quarter infected with any bacterial pathogens and median somatic cell count (SCC) in milk of 6×10^4/ml (minimum 1.2×10^4/ml and maximum 1.64×10^5/ml). The second was a CoPS group consisting of samples collected from cows with coagulase-positive staphylococci (mainly *S. aureus*) in the milk ($N = 27$ samples) and a median SCC of 2.44×10^6/ml (minimum 6.5×10^4/ml and maximum 4.26×10^6/ml). The third was the CoNS group, including samples infected with coagulase-negative staphylococci (e.g. *S. epidermidis, S. vitulinus, S. hyicus, S. sciuri, S. xylosus,* and *S. saprophiticus)* ($N = 14$ samples) and a median SCC value of 4.61×10^5/ml (minimum value 3.7×10^4/ml and maximum 3.58×10^6/ml). All cows from the experimental groups had elevated SCC in milk during the last lactation, however, to eliminate the samples from the udders with acute phase response and ensure that the experimental groups only included samples derived from animals suffering from subclinical mastitis, all animals with clinical signs of mastitis were excluded from the study. It should be stressed that to eliminate any possible effects of infection, none of the uninfected quarters included in the control group were adjacent to infected ones.

RNA isolation and assessment

Briefly, 30 mg of MGST was homogenized for 20 s at 4 m/s, 24 × 2 cycles in the Fast-Prep 24 Homogenizer (MP Biomedicals, California, USA) using the tubes with silica beads (A&A Biotechnology, Gdynia, Poland). RNA extraction and purification was performed with RNeasy Mini kit (Qiagen, Hilden, Germany) with the addition of β-mercaptoethanol to the lysis buffer (Merck, Darmstadt, Germany), and ssDNA/RNA Clean & Concentrator (Zymo Research, Irvine, USA) according to the manufacturer's recommendations.

Only RNA samples with a RNA integrity number higher than seven (RIN)>7 were selected for further analysis, this was established using a Bioanalyser 2100 (Agilent, Santa Clara, USA) with the RNA 6000 Nano LabChipKit (Agilent, Santa Clara, USA).

Gene-expression analysis

cDNA was obtained using the A Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) with a negative control (i.e. without template) according to the manufacturer's protocol. The samples of cDNA were diluted to 50 ng/µl.

RT-qPCR analysis was performed with LightCycler480 equipment (Roche, Basel, Switzerland) according to the 'LightCycler®480 SYBR Green I Master' protocol. Two housekeeping genes with M-values below 0.5 according to NormFinder software (Andersen *et al.*, 2004) and geNORM algorithm (Vandesompele *et al.*, 2002) were used to normalize mRNA levels between samples: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*). The primer sequences for the analyzed and reference genes, the length of the amplicons, and the GenBank accession numbers are listed in online Supplementary Table S1. Amplification was carried out in three repeats with SYBR Green (Roche, Basel, Switzerland) technology, using optical, transparent 96-well plates. The presence of any amplicons of interests was confirmed by electrophoretic analysis in 2% agarose gel stained with 0.5 µg/ml ethidium bromide (final concentration) (Sigma, Kawasaki, Japan). The relative gene expression estimations were developed based on Pfaffl's (2001), model adapted by Kościuczuk *et al.* (2014).

Enzyme-linked immunosorbent assay (ELISA)

To determine the concentrations of the two proteins of interest, ELISA tests were conducted according to the protocols: CSN1S1 according to EIAab (Wuhan, Hubei, China) and CSN3 according to Fine Biotech (Wuhan, Hubei, China). The tissue samples were homogenized in 700 µl of PBS using tubes with silica beads (A&A Biotechnology, Gdynia, Poland).

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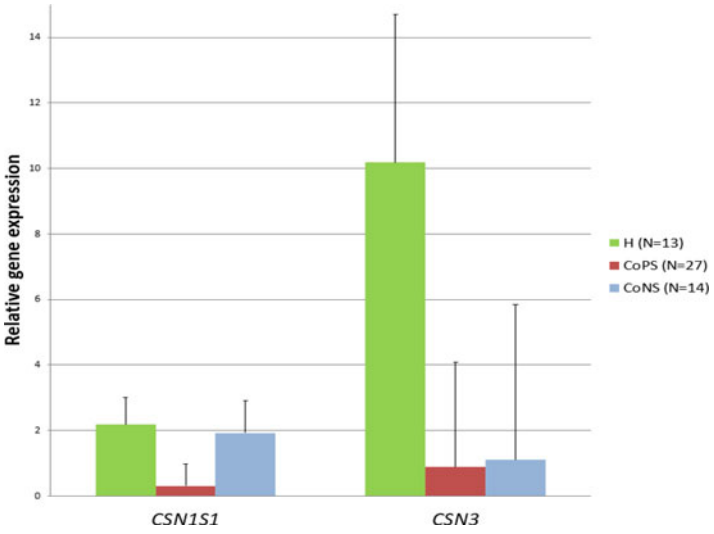

Fig. 1. The relative expression of the *CSN1S1* and *CSN3* genes in mammary gland secretory tissue determined by RT-qPCR. Green: H – healthy tissue, free from bacteria. Red: CoPS, tissue infected with coagulase-positive staphylococci. Blue: CoNS, tissue infected with coagulase-negative staphylococci. *CSN1S1* – alpha-S1-casein, *CSN3* – kappa-casein. The values within the same gene do not differ significantly at $P > 0.05$.

Relative gene expressions and statistical analysis

To determine the differences between the analyzed groups, analyses of variance of gene expression were performed at the mRNA and protein levels. Both the level of transcripts and protein levels were checked for normality of distribution, and the values for the mRNA level were transformed into a natural logarithmic scale (ln). The GLM procedure was used with the Tukey –Kramer multiple range test using SAS software (SAS/STAT, 2002–2012). The preliminary analysis indicated that parity had no impact on the transcript and protein levels, therefore, this factor was not included in the final model. Finally, the one-way ANOVA was conducted, with the type of pathogenic bacteria found in the milk as the fixed effect.

Results

No differences in *CSN1S1* and *CSN3* gene expression were observed between groups at the mRNA level. Although *CSN3* expression was 10-fold higher in the H group than in the other two, this was not significant due to high intra group variability (Fig. 1). Regarding protein levels, whilst CSN1S1 concentration was found to be two-fold higher in the H group than in the CoNS group ($P < 0.05$), no differences were found between CoPS and CoNS (Fig. 2, left panel). Moreover, while CSN3 concentration was three-fold higher in the H group than in the CoPS group ($P < 0.01$) no difference was observed between the CoPS and CoNS (Fig. 2, right panel).

Discussion

Little is known of casein gene expression in secretory epithelial cells, especially at the mRNA level. Moreover, most studies on the topic were conducted on artificial infection models. An RT-qPCR study by Lutzow *et al.* (2008), did not find differences in *CSN1S1* and *CSN2* expression in udder secretory tissue 16 h after *S. aureus* infusion, however, microarray analysis found *CSN1S1* to be downregulated during inflammation. In addition, the tissue appeared to remain undamaged during the early stage of infection, suggesting that physiological function also remained unaltered. In another study, Vanselow *et al.* (2006) found experimental infection of mammary gland tissue with *S. aureus* strain to cause subclinical rather than acute mastitis, and that infection decreased milk yield and lowered CSN1S1 protein synthesis by 30%, even after prolonged periods of up to 84 h post-infection.

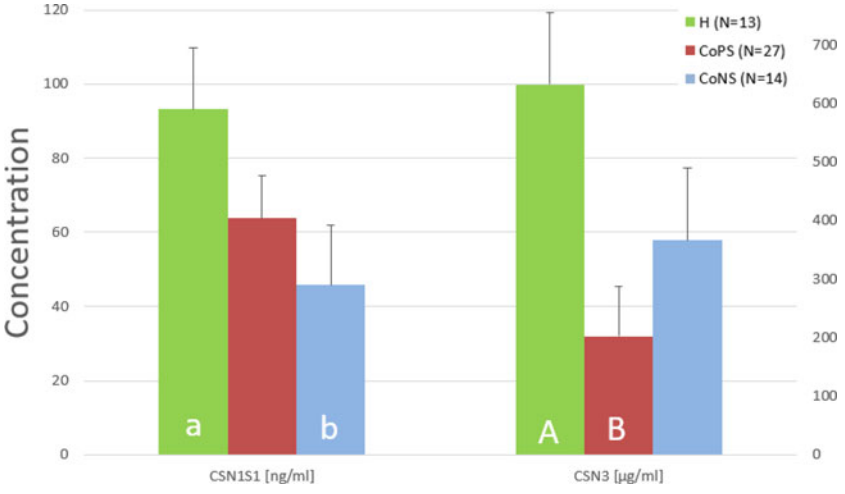

Fig. 2. The concentration of CSN1S1 and CSN3 in mammary gland secretory tissue, as determined by ELISA. Green: H – healthy tissue, free from bacteria. Red: CoPS, tissue infected with coagulase-positive staphylococci. Blue: CoNS, tissue infected with coagulase-negative staphylococci. *CSN1S1* – alpha-S1-casein, *CSN3* – kappa-casein. a, b – the values with different letters differ significantly at $P \leq 0.05$. A, B – the values with different letters differ significantly at $P \leq 0.01$.

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Although these study models differ from ours, our results are in agreement, insofar that mastitis caused by CoPS did not affect CSN1S1 protein concentration.

Johansson *et al.* (2013) report that the concentrations of CSN1S1 and CSN3 proteins were significantly reduced in milk inoculated with *S. aureus* six hours previously compared to bacterial-free milk. It is a well-known phenomenon that casein is partially digested in milk derived from healthy udders, while whey proteins are more resistant to the proteolytic activity of the plasmin (Dallas *et al.*, 2015). However, protease activity has been found to increase during mastitis (Haddadi *et al.*, 2006). Moreover, milk obtained from cows' udder quarters infected with staphylococci is known to contain more total protein due to higher whey protein concentration, but lower casein content, particularly the CS1NS1 and CSN2 fractions, compared to milk from healthy quarters (Malek dos Reis *et al.*, 2013). Johansson *et al.* (2013) suggest that, in addition to naturally occurring proteolysis, *S. aureus* also secretes extracellular proteases such as serine protease and aureolysin with proteolytic activity against caseins. This hypothesis is in line with previous observation that staphylococcal mastitis is associated with elevated casein proteolysis (Leitner *et al.*, 2006), and greater micelle damage (Coulon *et al.*, 2002). A study of casein concentration using microfluidic chip electrophoresis identified a reduction in casein levels in cows' milk infected with mesophilic bacteria such as *S. aureus* and also showed that increased SCC was associated with a decrease in CSN2 and CSN3 concentrations (Ramos *et al.*, 2015). What is more, Bobbo *et al.* (2017) report that udder infection with both Gram-positive (mainly *S. aureus*) and Gram-negative (e.g., *E. coli*) bacteria affects casein content in milk (CS1NS1, CS1NS2, CNS2) irrespective of pathogen type. In addition, factors that cause casein proteolysis causes have been found to inhibit mammary gland secretion (Leitner *et al.*, 2006), however, it is unknown whether casein proteolysis begins when staphylococci enter the secretory tissue (alveoli) or if it occurs only in infected milk as a result of staphylococcal infection. Hence, it can be concluded that concentrations of the analyzed proteins in milk do not reflect that of their genes in epithelial cells.

In the past, CoNS bacteria were considered minor opportunistic pathogens, and since CoNS mastitis demonstrated a high spontaneous cure rate (16–70%) it was usually left untreated (Zalewska *et al.*, 2020). However, it is now known that many CoNS, such as *S. hemolyticus*, *S. epidermidis*, *S. chromogenes*, *S. warneri*, or *S. xylosus* also produce endotoxins similar to *S. aureus*, which is major pathogenic bacteria (de Freitas *et al.*, 2013), and the mastitis caused by CoNS can persist throughout lactation (Taponen *et al.*, 2007).

CoPS infection results in a slightly different form of mastitis to CoNS. In addition to changes in SCC levels in milk (Kalińska *et al.*, 2018), our present findings indicate that CoPS negatively influences CSN3 production in MGST while CoNS reduces CSN1S1 production. It is well known that, unlike the CSN3 gene, the promoter of CSN1S1 has highly conserved regulatory motifs (Bionaz *et al.*, 2012), thus, the two genes probably employ different regulatory processes since these motifs control the activation of particular genes depending on conditions.

Interestingly, although differences were observed in protein expression between the two types of infection, no such differences were observed at the mRNA level. This may be evidence of epigenetic phenomena occurring during inflammation or that the two pathogens exert different influences on expression. Vanselow *et al.* (2006) report that, unlike all other mammal tissues, only mammary gland tissue demonstrate casein genes with hypomethylated promoter regions, and that the methylation status of these gene promoters is affected by mastitis. This may mean that CoPS and CoNS influence the casein gene promoters to different degrees, resulting in the observed changes in protein expression. Alternatively, several studies have proposed that various changes in gene expression during mastitis may be associated with miRNA (Ju *et al.*, 2018; Luoreng *et al.*, 2018). However, such studies have tended to focus on the genes involved in immunity and very few, if any, have examined the particular miRNAs targeting bovine casein genes. An *in silico* analysis by Zidi *et al.* (2010) found that mutations in the 3′UTR region of goat casein genes may destroy a potential miRNA target site. In addition, Zhang *et al.* (2016) found that circular RNAs (circRNAs) are also formed during bovine casein gene splicing, and that they may regulate the expression of their paternal genes. We can only speculate that miRNAs or circRNAs, previously considered as molecular flukes or as aberrations formed during RNA splicing, may regulate casein gene expression, and that their profile during bovine mammary gland infection may be influenced to varying degrees depending on the type of infection.

In conclusion, infection by contagious pathogens does appear to influence milk protein gene expression, resulting in lower casein levels in milk, and thus lower recoveries of protein and fat in cheese (Leitner *et al.*, 2006). Therefore, chronic mastitis caused by staphylococcal infection results in economic losses in the dairy industry. Although the mean concentrations of *CNS3* gene transcripts were higher in H than in CoPS and CoNS groups, they were not statistically confirmed because of the high variability within groups. Therefore, further research on larger experimental groups is needed. However, the concentrations of the CSN1S1 and CSN3 proteins in MGST were observed to decrease during chronic mastitis caused by staphylococci. A moderate fall in CSN1S1 concentration was recorded during CoPS infection, and a stronger fall in CSN3 during CoNS mastitis. However, the CoPS and CoNS groups demonstrated no differences in studied gene expression at either mRNA and protein levels. It could be assumed that expression at the mRNA and protein levels may be influenced by some epigenetic phenomena such as post-transcriptional and post-translational changes associated with pathogenic bacteria, and further study is needed to explain this phenomenon.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000145

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