International Dairy Journal 89 (2019) 1–5

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Protease activity and protein profile in milk from healthy dairy cows and cows with different types of mastitis

N. Caggiano, A. Lorenzo Smirnoff, J.M. Bottini, E.A. De Simone*

Animal Physiology Chair, School of Veterinary Sciences, Buenos Aires University, Chorroarín 280 (C1427CWO), Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 20 March 2018
Received in revised form
30 July 2018
Accepted 31 July 2018
Available online 11 August 2018

ABSTRACT

Mastitis in dairy cattle has high morbidity and mortality rates, having serious consequences in milk production and quality. The aim of this work was to evaluate the activity of metalloproteinase-2 (MMP-2) and metalloproteinase-9 (MMP-9) in milk from healthy dairy cattle and from cows with signs of clinical and subclinical mastitis. The levels of total proteins and proteases with caseinolytic activity were also evaluated. The highest caseinolytic activity was found in milk samples obtained from animals with mastitis, in which lower levels of casein, especially the κ-casein fraction, were found, as compared with healthy animals.

1. Introduction

Mastitis is the inflammation of the mammary gland, usually caused by a primary bacterial infection (Edmonson & Bramley, 2004). Mastitis presents the greatest morbidity and mortality rates among dairy cows and it is known to affect milk production (Gröhn, Eicker, Ducrocq, & Hertil, 1998). In the United States, a country with high production standards, the morbidity, mortality and rejection rates due to mastitis were found to be 14.86%, 16.3% and 24.56%, respectively. In addition, in a study performed by Hertl, Schukken, Welcome, Tauer, and Gröhn (2014) 30% of cows with at least one episode of clinical mastitis was found (USDA, 2007). These percentages have remained relatively stable over the past years (Vakkamäki, Taponen, Heikkilä, & Pyörälä, 2017). A study carried out in France on dairy cows showed that the incidence rate of clinical mastitis was 15% (Govignon-Gion, Dassonneville, Baloche, & Ducrocq, 2015). In 2011, the Swedish Dairy Association reported incidence rates for clinical and subclinical mastitis of 17.3% and 66%, respectively (Nielsen & Emanuelson, 2013). Globally, the economic losses due to mastitis in dairy cows have a high impact, since they entail a reduction in milk production, an increase in the amount of discarded milk, increased veterinary costs and premature rejection of dairy animals (Bar et al., 2008). Each case of clinical mastitis is estimated to cost between US$547 and 581. Consequently, dairy herds with a high incidence of this pathology have a significant economic impact (Down, Green, & Hudson, 2013).

According to the clinical manifestations, mastitis can be classified into clinical and subclinical. The inflammatory process during mastitis results in the release of a variety of proteases, which are mainly secreted by polymorphonuclear (PMN) cells recruited from the blood (Prin-Mathieu et al., 2002). Mediators such as histamine, TNF-α, IFN-γ and acute phase proteins released during inflammation increase the permeability of the tight junctions in the mammary gland (Pyorala, 2003). The increased permeability allows proteases to reach the mammary gland (Nguyen & Neville, 1998). Many aetiological agents of mastitis are known to produce and secrete proteases (Bochniarz & Wawron, 2012).

In addition, the proteolytic activity in milk from cows with subclinical mastitis is due to the action of PMN cells recruited during inflammation (Napoli, Aiello, Di Donna, Prendushi, & Sindona, 2007). Matrix metalloproteinases (MMPs) are a family of calcium-dependent zinc-containing endopeptidases which participate in the normal remodelling process of the extracellular matrix in different tissues (Reynolds, 1996). During mastitis, PMN cells represent the first defence line; and their migration into the mammary ducts is favoured by MMPs (Nagahata et al., 2011). This family of proteases is divided into five subfamilies according to the substrate specificity, i.e., collagenases, gelatinases, stromelysins, membrane type MMPs, and a fifth group of enzymes that cannot be included in the previous groups (Burrage, Mix, & Brinckerhoff, 2006). The most widely studied MMPs are the gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B). As a rule, the damage to the extracellular matrix caused by the primary mastitis aetiologic

* Corresponding author. Tel.: +54 11 5287 2000.
E-mail address: edesimone@fvet.uba.ar (E.A. De Simone).

https://doi.org/10.1016/j.idairyj.2018.07.020
0958-6946/

agent is repaired and tissue functions and structure are restored. MMPs play an important role in the persistent inflammation occurring in the cases of recurrent mastitis, being the inhibition of these enzymes a possible therapeutic strategy (Haddadi, Moussaoui, Hebia, Laurent, & Le Roux, 2005). Inflammation can cause an imbalance in the physiological functions of MMPs, which results in a decreased milk production and quality (Mehrzad et al., 2005; Oliver & Calvinho, 1995). Mammary epithelial cells also express MMPs that are important for plasmin activation, which produces degradation of extracellular matrix proteins exerting a positive feedback on the protease activation process (Green & Lund, 2005). Increased levels of expression of MMP-9 have been found together with the activation of the apoptotic pathway in cases of mastitis caused by *Escherichia coli* (Long et al., 2001).

Literature data are scarce as regards the role of proteases in the pathophysiology of clinical and subclinical mastitis. The aim of this work was to evaluate the caseinolytic activity and the quality of protein components, mainly those relevant for the dairy industry, in milk samples obtained from clinically and subclinically infected cows. The importance of MMPs in the pathophysiology of mastitis and the possible relationship with either a clinical or a subclinical presentation was also investigated.

2. Materials and methods

2.1. Animals and samples

A total of 163 milk samples were collected from individual quarters of cows from 8 dairy farms located in the Province of Buenos Aires, Argentina. The cows were at different stages of lactation and their daily average milk production was 24 L. The procedures were in accordance with the UK Animals (scientific procedures) Act, 1986 and appended guidelines. Animals were divided into 3 groups: (i) healthy (H) [n = 68; cows without signs of mastitis and negative in the California Mastitis Test (CMT)], (ii) clinical mastitis (CM) (n = 44; animals with clinical signs of mastitis), and (iii) subclinical mastitis (SM) (n = 51; animals without clinical signs of mastitis but positive in the CMT).

Samples were taken in sterile flasks after the CMT. For each sample, an aliquot was used to perform the leucocyte count, while the remaining was frozen at −20 °C until used. The first milk flow was not included in the sample, but was used to investigate the presence of flocs, which indicated the occurrence of clinical mastitis.

2.2. California Mastitis Test

In animals without clinical mastitis, a positive CMT or a somatic cell count >200,000 cells mL^{-1} were used to detect the presence of subclinical mastitis (Schalm & Noorlander, 1957).Briefly, 5 mL of quarter milk samples were taken from each cow and mixed with 5 mL CMT reagent. Depending on the degree of gelling, samples were classified into four stages according to manufacturer's criteria. Stages ranged from 0 (negative) to 3 (serious) (Roy et al., 2009).

2.3. Somatic cell count

The somatic cell count (SCC) test is a main indicator of milk quality. The SCC was performed in duplicate using a Neubauer's haemocytometer. Samples were diluted 1:200 in diluent for white blood cell count (Biopur S.R.L., Rosario, Argentina). Counts were carried out under a light microscope at 40×.

2.4. Bacteriology

Briefly, 50 μL of sample were plated onto blood agar with 0.1% aesculin and incubated at 37 °C for 24–48 h. According to the National Mastitis Council, Inc., this selective culture medium favours the isolation and recognition of a variety of pathogens (Hogan et al., 1999).

2.5. Gelatin zymography

The gelatin zymography test for the determination of MMP-2/-9 activities was carried out according to Gruber et al. (1996). Briefly, 10% polyacrylamide gel electrophoresis (PAGE) was performed with the addition of 0.2% porcine gelatin. Polyacrylamide gels were prepared with porcine skin gelatin (Sigma–Aldrich, St. Louis, MO, USA) (2 mg mL^{-1} per gel) and 0.1% SDS. Before loading, 3 μL of each sample were dissolved by heating in sample buffer for 5 min at 95 °C. After electrophoresis, gels were washed twice with 50 mM Tris, 5 mM $CaCl_2$, 150 mM NaCl, and 2.5% Triton X-l00 (pH 7.5) at room temperature for 45 min. Gels were then incubated for 24 h at 37 °C with a solution containing 50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.1% Triton X-l00 (pH 7.5). Gels were stained with a 0.25% (w/v) Coomassie Brilliant Blue R-250 solution in methanol: water: acetic acid (5:1:5, by vol). The gel destaining was performed with 7.5% (v/v) acetic acid and 20% (v/v) methanol for 3 h. The gelatinolytic activity was detected through the presence of negative staining bands caused by the digestion of gelatin by the MMPs present in the milk sample. The gelatin digestion bands at 72 kDa and 92 kDa corresponded to MMP-2 and MMP-9, respectively.

The intensity of digestion bands were quantified by densitometry using the Image J software (National Institutes of Health, MD, USA). The MMP activity was expressed as percentage of proteolysis with respect to a control that saturated densitometry at 50%. As positive control, the supernatant of a chondrocytes cell line producing MMPs (kindly ceded by Dr Delpino MV, CONICET- Buenos Aires University, Argentina) was used. Data corresponding to different gels were standardized using internal controls (De Simone et al., 2015). The MMP-9 proteolytic activity was classified as follows: (−) no activity, (+) mild activity, (++) moderate activity, (+++) strong activity.

2.6. Casein zymography

The detection of the caseinolytic activity was carried out by 10% sodium dodecylsulphate- (SDS-) PAGE with the addition of 0.2% bovine casein. After electrophoresis, gels were washed as described for gelatin zymography, but with a final incubation period of 48 h (Mehrzad et al., 2005). The densitometric analysis was then performed as described above.

2.7. Milk protein profile analysis by SDS-PAGE

The Proti 2 commercial kit (Wiener Lab., Argentina) was used to determine the total protein concentration of each sample. Three μL of each milk sample were analysed by a 12% SDS-PAGE under reducing conditions and followed by protein staining with Coomassie Brilliant Blue R-250 (Urech, Puhan, & Schallibaum, 1999). The analysis was performed by densitometry using the Image J software (National Institutes of Health, MD, USA), the density of each individual band was calculated as a percentage of the total density, and then the percentages were referred to the total protein concentration. A molecular weight marker was used as reference (BioRad Laboratories, Inc., CA, USA).

2.8. Statistical analysis

The GraphPad Prism 6 software (GraphPad Software Inc, San Diego, CA, USA) was used to construct graphs and to perform the statistical study. Results were expressed as mean values ± SD. A one-way analysis of variance (ANOVA) was used to compare means among groups. When the distribution was not Gaussian, the Kruskal–Wallis test was performed. An effect was considered significant when $p \leq 0.05$. When significant differences were found, the means were assessed using the Tukey's test of multiple comparisons. The Chi-square test was used to analyse the statistical difference in the activity of MMP-9 between groups.

3. Results

Significant differences were found in the somatic cell counts among groups (Fig. 1). *Staphylococcus aureus*, *Streptococcus* spp. and *E. coli* were isolated in the CM group samples (Fig. 2A), whereas in the SM group samples, coagulase-negative staphylococci (43.8%), *S. aureus* (31.6%), *Streptococcus* spp (19.3%), and other pathogens (5,3%) were isolated (Fig. 2B).

As regards MMP-2 levels, no statistically significant differences were found between groups ($p > 0.05$) (Fig. 3). No MMP-9 activity was detected in any sample belonging to group H. The zymographic analysis of MMP-9 activity in milk samples belonging to the SM group (n = 51), showed that 31 samples did not display any activity, 10 samples had moderate activity, 6 samples showed high activity and only 4 samples showed mild activity. The CM group (n = 44) had the highest percentage of MMP-9 activity in milk, i.e., 39 samples had moderate to strong activity ($p < 0.0001$ versus H group and $p < 0.001$ versus SM group) (Fig. 4).

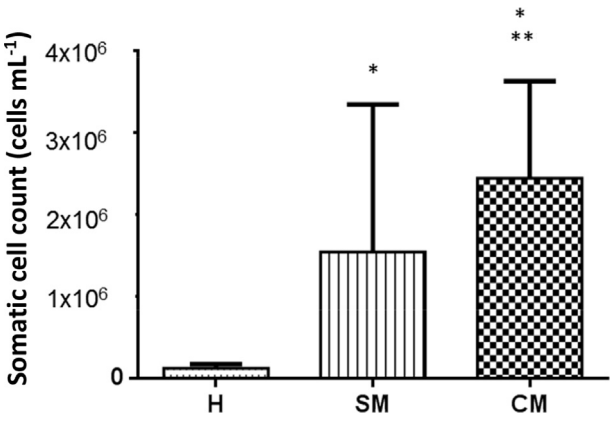

Fig. 1. Somatic cell count in milk samples of dairy cows from different groups: healthy (H), subclinical mastitis (SM) and clinical mastitis (CM). Significant differences were: * versus H ($p < 0.0001$); ** versus SM ($p < 0.001$); error bars indicate SD.

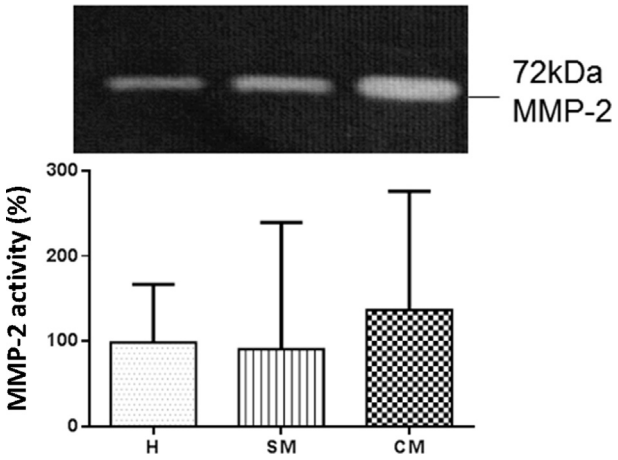

Fig. 3. MMP-2 activity in milk samples of dairy cows: healthy (H), subclinical mastitis (SM) and clinical mastitis (CM) groups. No significant differences were found ($p > 0.05$); error bars indicate SD.

	HEALTHY	SUBCLINICAL*	CLINICAL **,***
MMP-9 -	68/68 [100%]	31/51 [60,8%]	4/44 [9,1%]
MMP-9 +	0/68 [0%]	4/51 [7,8%]	11/44 [25%]
MMP-9 ++	0/68 [0%]	10/44 [19,6%]	18/44 [40,9%]
MMP-9 +++	0/68 [0%]	6/51 [11,8%]	11/44 [25%]

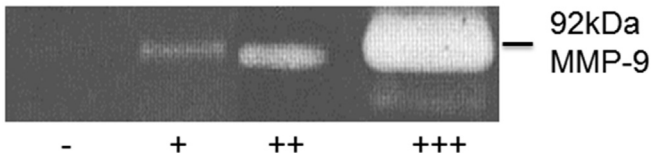

Fig. 4. MMP-9 activity in milk samples of dairy cows: healthy (H), subclinical mastitis (SM) and clinical mastitis (CM) groups.

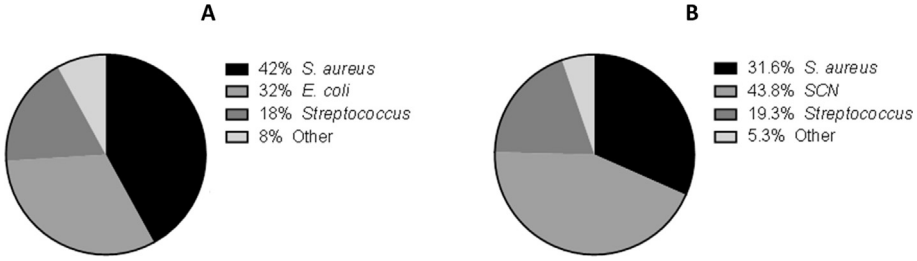

Fig. 2. Pathogens isolated in milk samples of dairy cows with clinical mastitis (A) and subclinical mastitis (B).

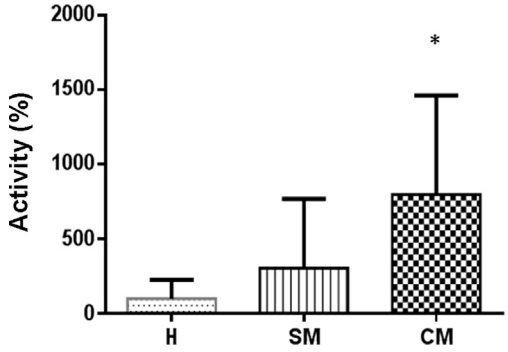

Fig. 5. Caseinolytic activity in milk samples of dairy cows: healthy (H), subclinical mastitis (SM) and clinical mastitis (CM) groups evaluated by casein zymography. An asterisk indicates CM significant difference versus H and SM ($p < 0.05$); error bars indicate SD.

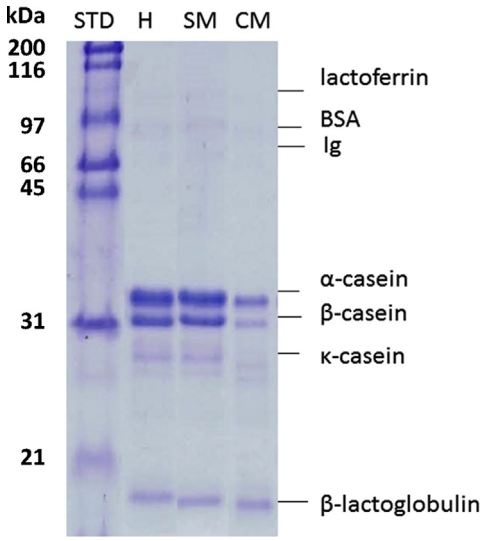

Fig. 6. SDS-PAGE of representative milk samples of dairy cows: healthy (H), subclinical mastitis (SM) and clinical mastitis (CM) groups; molecular mass marker was loaded in lane 1.

The total caseinolytic activity estimated by the casein zymography method indicated that the CM group had the highest activity when compared with the H and SM groups ($p < 0.05$) (Fig. 5).

The protein composition of milk samples was determined by SDS-PAGE (Fig. 6) followed by a densitometry analysis. No significant differences were found in the total protein concentration among the three groups (Fig. 7A). Nevertheless, a higher concentration of total casein was found in samples belonging to H and SM groups ($p < 0.001$ versus CM group) (Fig. 7B). In addition, significant differences were found in α_S- and κ-caseins contents, but not in β-casein. The levels of α_S-caseins were 1.17 ± 0.48 g dL^{-1} in the H group, 1.08 ± 0.59 g dL^{-1} in the SM group and 0.8 ± 0.68 g dL^{-1} in the CM group ($p < 0.001$, CM versus H and $p < 0.01$; CM versus SM). The results obtained for κ-casein were similar to those for α_S-caseins (Fig. 7C).

The proteins grouped together as high-molecular mass proteins included lactoferrin, lactoperoxidase and immunoglobulins; these proteins are associated with innate immunity. The levels of high-molecular mass milk proteins in the CM group were higher (1.04 ± 1.1 g dL^{-1}) than those of the other two groups, (0.57 ± 0.4 g dL^{-1} for H and 0.72 ± 0.55 g dL^{-1} for SM; Fig. 7D). Although no statistically significant differences were found ($p > 0.05$), a trend towards higher values was found in the SM group.

4. Discussion

S. aureus and *E. coli* were the most frequent pathogens isolated in cows with clinical mastitis. These findings are in agreement with those reported by Calvinho and Tirante (2005), and Rowbotham and Ruegg (2016). The predominance of coagulase-negative staphylococci followed by *S. aureus* in cows with subclinical mastitis has also been reported by Dieser et al. (2014).

No differences were found in the MMP-2 activity between groups. These results differ from those of Raulo et al. (2002), who have reported the occurrence of increased MMP-2 activity in cows with clinical mastitis caused by *E. coli*, as compared with animals infected with *S. aureus*. Further work should be carried out. including the aetiological agent and the stage of lactation as study variables (Lund et al., 1996; Weng et al., 2008).

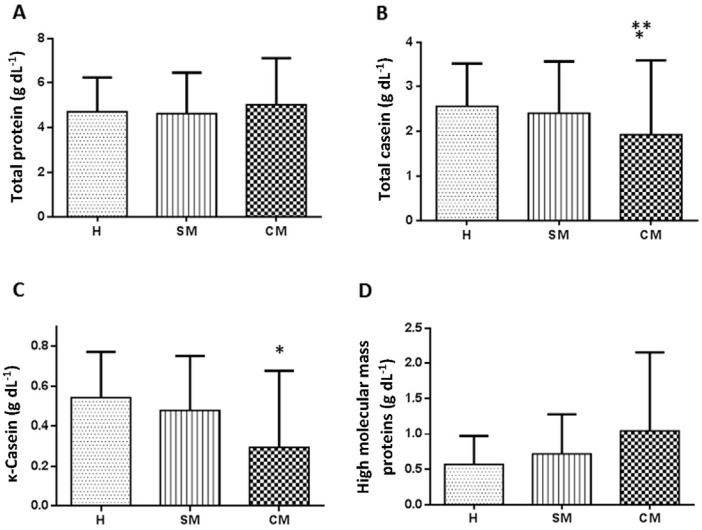

Fig. 7. Evaluation of milk samples of dairy cows in healthy (H), subclinical mastitis (SM) and clinical mastitis (CM) groups: A, total protein (no significant differences found; $p > 0.05$); B, total casein significant differences were * versus H ($p < 0.001$); ** versus SM ($p < 0.05$); C, κ-casein significant differences were * versus H and SM ($p < 0.001$); D, high molecular mass milk proteins (no significant differences; $p > 0.005$); error bars indicate SD.

Nagahata et al. (2011) and Raulo et al. (2002) have not observed any MMP-9 activity in healthy animals, which reinforces the idea that the activity of MMP-9 is increased in cows with mastitis.

In this study we have not characterised the enzymes involved in the increased caseinolytic activity observed in the CM group; however, plasmin and PMN-derived proteases would be responsible for such effect (Ismail & Nielsen, 2010). In any case, the increase in the caseinolytic activity directly affects the milk quality. We did not observe significant differences in the total milk protein concentration between groups. This is because the casein concentration is lower in the CM group, even though the serum proteins are increased due to the inflammatory process (Mudaliar et al., 2016). Moreover, the concentration of high molecular weight milk proteins trends to be higher in CM group, probably due to a higher vascular permeability (Ryman, Packiriswamy, & Sordillo, 2015). The total casein concentration was affected mainly in the CM group; this may be due to the action of different proteases, which are increased during the infectious process (Haddadi et al., 2006). Of the casein fractions, α- and κ-casein were the most affected; however, the reduction in the κ-casein fraction has more impact for the dairy processing.

5. Conclusions

Since decreased casein contents have a high impact in dairy industry, we analysed the effect of mastitis in milk quality. During the CM process, a marked increase in the gelatinolytic and caseinolytic activities was observed. However, a moderate protease activity was found in the SM group. Likewise, the activity of MMP-9 was higher than that of MMP-2 in all types of mastitis. Moreover, the increased activity of proteases correlated with a decreased concentration of κ-casein in CM milk samples, which is known to lead to greater economic losses. Further work evaluating the correlation between proteases activity and tissue damage would also be of interest, since such deleterious effect would also have a high impact on the dairy farm productive capacity.

Acknowledgement

The authors wish to thank the financial support from the University of Buenos Aires, Argentina (UBACyT 20020160100074BA).

References

Bar, D., Tauer, L. W., Bennett, G., González, R. N., Hertl, J. A., Schukken, Y. H., et al. (2008). The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming. *Journal of Dairy Science, 91*, 2205–2214.

Bochniarz, M., & Wawron, W. (2012). Haemolytic and proteolytic activity of coagulase-negative staphylococci isolated from mastitis cows. *Polish Journal of Veterinary Sciences, 15*, 61–65.

Burrage, P. S., Mix, K. S., & Brinckerhoff, C. E. (2006). Matrix metalloproteinases: Role in arthritis. *Frontiers in Bioscience, 11*, 529–543.

Calvinho, L. F., & Tirante, L. (2005). Prevalencia de microorganismos patógenos de mastitis bovina y evolución del estado de salud de la glándula mamaria en Argentina en los últimos 25 años. *Revista FAVE Sección Ciencias Veterinarias, 4*, 29–40.

De Simone, E. A., Perrone, G., Caggiano, N., Lastra, Y., Rubatino, F., Díaz, J., et al. (2015). Levels of cytokines and matrix metalloproteinases 2 and 9 in the synovial fluid of osteoarthritic horses treated with pamidronate. *Journal of Equine Veterinary Science, 35*, 577–583.

Dieser, S. A., Vissio, C., Lasagno, M. C., Bogni, C. I., Larriestra, A. J., & Odierno, L. M. (2014). Prevalence of pathogens causing subclinical mastitis in Argentinean dairy herds. *Pakistan Veterinary Journal, 34*, 124–126.

Down, P. M., Green, M. J., & Hudson, C. D. (2013). Rate of transmission: A major determinant of the cost of clinical mastitis. *Journal of Dairy Science, 96*, 6301–6314.

Edmondson, P. W., & Bramley, A. J. (2004). Mastitis. In A. H. Andrews, R. W. Blowey, H. Boyd, & R. G. Eddy (Eds.), *Bovine Medicine Diseases and Husbandry of Cattle* (2nd ed., Chapter 23). Oxford UK: Blackwell Science Ltd.

Govignon-Gion, A., Dassonneville, R., Baloche, G., & Ducrocq, V. (2015). Multiple trait genetic evaluation of clinical mastitis in three dairy cattle breeds. *Animal, 10*, 558–565.

Green, K. A., & Lund, L. R. (2005). ECM degrading proteases and tissue remodeling in the mammary gland. *Bioessays, 27*, 894–903.

Gröhn, Y. T., Eicker, S. W., Ducrocq, V., & Hertil, J. A. (1998). Effect of diseases on the culling of Holstein dairy cows in New York State. *Journal of Dairy Science, 81*, 966–978.

Gruber, B. L., Sorbi, D., French, D. L., Marchese, M. J., Nuovo, G. J., & Kew, R. R. (1996). Markedly elevated serum MMP-9 (gelatinase B) levels in rheumatoid arthritis: A potentially useful laboratory marker. *Clinical Immunology and Immunopathology, 78*, 161–171.

Haddadi, K., Moussaoui, F., Hebia, I., Laurent, F., & Le Roux, Y. (2005). *E. coli* proteolytic activity in milk and casein breakdown. *Reproduction Nutrition Development, 45*, 485–496.

Haddadi, K., Prin-Mathieu, C., Moussaoui, F., Faure, G., Vangroenweghe, F., Burvenich, C., et al. (2006). Polymorphonuclear neutrophils and Escherichia coli proteases involved in proteolysis of casein during experimental E. coli mastitis. *International Dairy Journal, 16*, 639–647.

Hertl, J. A., Schukken, Y. H., Welcome, F. L., Tauer, L. W., & Gröhn, Y. T. (2014). Effects of pathogen-specific clinical mastitis on probability of conception in Holstein dairy cows. *Journal of Dairy Science, 97*, 6942–6954.

Hogan, J. S., González, R. N., Harmon, R. J., Nickerson, S. C., Oliver, S. P., Pankey, J. W., et al. (1999). *Laboratory handbook on bovine mastitis* (1st ed.). Madison, WI, USA: National Mastitis Council Inc.

Ismail, B., & Nielsen, S. S. (2010). Plasmin protease in milk: Current knowledge and relevance to dairy industry. *Journal of Dairy Science, 93*, 4999–5009.

Long, E., Capuco, A. V., Wood, D. L., Sonstegard, T., Tomita, G., Paape, M. J., et al. (2001). *Escherichia coli* induces apoptosis and proliferation of mammary cells. *Cell Death and Differentation, 8*, 808–816.

Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., et al. (1996). Two distinct phases of apoptosis in mammary gland involution: Proteinase-independent and -dependent pathways. *Development, 122*, 181–193.

Mehrzad, J., Desrosiers, C., Lauzon, K., Robitaille, G., Zhao, X., & Lacasse, P. (2005). Proteases involved in mammary tissue damage during endotoxin-induced mastitis in dairy cows. *Journal of Dairy Science, 88*, 211–222.

Mudaliar, M., Tassi, R., Thomas, F. C., McNeilly, T., Weidt, S., McLaughlin, M., et al. (2016). Mastitomics, the integrated omics of bovine milk in an experimental model of *Streptococcus uberis* mastitis: 2. Label-free relative quantitative proteomics. *Molecular Biosystems, 12*, 2748–2761.

Nagahata, H., Kawai, H., Higuchi, H., Kawai, K., Yayou, K., & Chang, C. (2011). Altered leukocyte responsiveness in dairy cows with naturally occurring chronic Staphylococcus aureus mastitis. *Journal of Veterinary Medical Science, 73*, 885–894.

Napoli, A., Aiello, D., Di Donna, L., Prendushi, H., & Sindona, G. (2007). Exploitation of endogenous protease activity in raw mastitic milk by MALDI-TOF/TOF. *Analytical Chemistry, 79*, 5941–5948.

Nguyen, D. A. D., & Neville, M. C. (1998). Tight junction regulation in the mammary gland. *Journal of Mammary Gland Biology and Neoplasia, 3*, 233–246.

Nielsen, C., & Emanuelson, U. (2013). Mastitis control in Swedish dairy herds. *Journal of Dairy Science, 96*, 6883–6893.

Oliver, S. P., & Calvinho, L. F. (1995). Influence of inflammation on mammary gland metabolism and milk composition. *Journal of Animal Science, 73*, 18–33.

Prin-Mathieu, C., Le Roux, Y., Faure, G. C., Laurent, F., Béné, M. C., & Moussaoui, F. (2002). Enzymatic activities of bovine peripheral blood leukocytes and milk polymorphonuclear neutrophils during intramammary inflammation caused by lipopolysaccharide. *Clinical and Diagnostic Laboratory Immunology, 9*, 812–817.

Pyorala, S. (2003). Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research, 34*, 565–578.

Raulo, S. M., Sorsa, T., Tervahartiala, T., Latvanen, T., Pirilä, E., Hirvonen, J., et al. (2002). Increase in milk metalloproteinase activity and vascularpermeability in bovine endotoxin-induced and naturally occurring Escherichia coli mastitis. *Veterinary Immunology and Immunopathology, 85*, 137–145.

Reynolds, J. J. (1996). Collagenases and tissue inhibitors of metalloproteinases: A functional balance in tissue degradation. *Oral Diseases, 2*, 70–76.

Rowbotham, R. F., & Ruegg, P. L. (2016). Associations of selected bedding types with incidence rates of subclinical and clinical mastitis in primiparous Holstein dairy cows. *Journal of Dairy Science, 99*, 1–11.

Roy, J. P., Du Tremblay, D., Descoteaux, L., Messier, S., Scholl, D., & Bouchard, E. (2009). Evaluation of the California Mastitis Test as precalving treatment selection tool for Holstein heifers. *Veterinary Microbiology, 134*, 136–142.

Ryman, V., Packiriswamy, N., & Sordillo, L. M. (2015). Role of endothelial cells in bovine mammary gland health and disease. *Animal Health Research Reviews, 16*, 135–149.

Schalm, O. W., & Noorlander, D. O. (1957). Experiments and observations leading to development of the California Mastitis Test. *Journal of the American Veterinary Medicine Association, 130*, 199–207.

Urech, E., Puhan, Z., & Schallibaum, M. (1999). Changes in milk protein fraction as affected by subclinical mastitis. *Journal of Dairy Science, 82*, 2402–2411.

USDA. (2007). *Dairy 2007, Part II: Changes in the U.S. dairy cattle industry, 1991–2007.* Fort Collins, CO, USA: United States Department of Agriculture.

Vakkamäki, J., Taponen, S., Heikkilä, A. M., & Pyörälä, S. (2017). Bacteriological etiology and treatment of mastitis in Finnish dairy herds. *Acta Veterinaria Scandinavica, 59*. Article 33.

Weng, M. H., Yu, T. C., Chen, S. E., Peh, H. C., Liu, W. B., Chen, M. T., et al. (2008). Regional accretion of gelatinase B in mammary gland during gradual and acute involution of dairy animals. *Journal of Dairy Research, 75*, 202–210.