Indigenous enzymes and leukocyte in sheep milk are markers of health status and physiology of the mammary gland

Marzia Albenzio, Francesca D'Angelo,
Alessandra Marzano, Laura Schena, Agostino Sevi

Dipartimento di Scienze delle Produzioni, dell'Ingegneria, della Meccanica
e dell'Economia applicate ai Sistemi Agro-zootecnici (PrIME), Università di Foggia, Italy

Corresponding author: Marzia Albenzio. Dipartimento PrIME, Facoltà di Agraria, Università di Foggia. Via Napoli 25, 71100 Foggia, Tel. +39 0881 589327 - Fax: +39 0881 589331 - Email: m.albenzio@unifg.it

ABSTRACT - Plasmin, plasminogen and plasminogen activator in ewe bulk milk were not significantly affected by stage of lactation probably as a consequence of the good health of the ewe udders throughout lactation as indicated by SCC which never exceeded 600,000 cells/mL. Elastase content increased significantly during lactation whereas cathepsin showed the highest content in mid lactation. Changes in macrophages and neutrophyls levels in ewe bulk milk during lactation were also investigated. Macrophages minimally contributed to leukocyte cell count in milk and had the highest levels at the beginning of lactation. An opposite trend was recorded for polymorphonuclear leukocytes (PMNL) that increased throughout lactation showing the highest value in late lactation. The increase of PMNL percentage and elastase content in milk, in spite of relatively low SCC, suggests that PMNL and elastase underwent a physiological increase associated to the remodelling of mammary gland in late lactation.

Key words: Milk endogenous enzymes, Stage of lactation, Leukocyte differential count, Mammary gland.

Introduction - Several studies have focused on endogenous milk enzymes; in particular some proteolytic enzyme systems, such as plasmin and cathepsin D, have been well characterized in bovine milk for their origin and role in milk and cheese. Plasmin is the main native proteolytic enzyme; other endogenous proteolytic enzymes associated with somatic cell in milk are elastase, cathepsins, and collagenase (Kelly and McSweeney, 2002). Cathepsin D contributes to casein breakdown displaying a proteolytic activity similar to that of chymosin; the potential significance of elastase for proteolysis and quality of milk has been investigated and the cleavage specificity towards bovine αs1- and β-CN has been determined (Considine *et al.*, 2000). Many of the enzymes in milk originate from somatic cells; their presence in milk as active enzymes suggests that their leakage or secretion occurs depending on a number of physiological or external influences (i.e. stage of lactation or onset of intramammary infection). It is worth to investigate whether specific enzymatic activities could be associated with different somatic cell types in ewe bulk milk, because variations in the activity of milk enzymes are important not only for their physiological role but also as indicators of milk quality. The aim of this study was to determine the role of indigenous enzymes and leukocyte as markers of health status and physiology of the mammary gland.

Material and methods - The experiment was conducted from April to July 2007 in an intensively managed flock of Comisana ewes located in southern Italy. Ewes involved in the trial lambed in the winter of 2007. Ewes were housed on straw litter; they grazed and were supplemented with hay and concentrate. Ewes were healthy at the beginning of the trial and were checked for health by veteri-

narians throughout the experiment. Ewes showing any sign of clinical mastitis were excluded from milking. Ewes were milked using pipeline milking machines. Three sampling cycles were performed during early, mid and late lactation (less than 70d, from 110 to 130d, and more than 160d in lactation, respectively). For each sampling cycle, three samples were collected in triplicate on 3 consecutive days. Plasmin (PL), Plasminogen activator (PA) and Plasminogen (PG) activities were determined according to the method of Baldi *et al.* (1996). Cathepsin D activity was determined according to the Sigma enzymatic assay of cathepsin D (EC 3. 4. 23. 5) based on the methods of Perlmann and Lorand (1970) and Smith and Turk (1974). Elastase activity was determined according to the Sigma enzymatic assay of elastase (EC 3. 4. 21. 36) based on the method of Bieth *et al.* (1974). Separation of macrophages from milk somatic cells was performed according to Caroprese *et al.* (2008) by a magnetic positive separation (Do it yourself, EasySep, StemCell Tecnologies, Vancouver, Canada), using monoclonal mouse IgG anti-ovine macrophages (MCA919**,** Serotec, Oxford, UK), SC populations were enumerated using flow cytometry (Cell Lab Quanta SC™, Beckman Coulter Inc., Fullerton, CA). Data were processed by ANOVA, using the GLM procedure of SAS (1999). Bulk milk SCC and isolated macrophage and PMNL counts were transformed into logarithms to normalize their frequency distributions before performing statistical analysis. When significant effects were found (at $P<0.05$), the Student *t*-test was used to locate significant differences between means. Linear simple correlations between renneting parameters and milk constituents, and indogenous enzymes were also investigated.

Results and conclusions - Plasmin, plasminogen, and plasminogen activator in milk were not significantly affected by stage of lactation (Table 1). It has been reported that milk SC can convert plasminogen to plasmin through urokinase-plasminogen activator; stable levels of plasmin-plasminogen system throughout lactation could be partly explained by the lack of differences in SCC.

Table 1. Least squares means ±SEM of Plasmin, Plasminogen, Plasminogen/Plasmin, total Plasminogen Activator, elastase and cathepsin in sheep milk during lactation.

		Stage of Lactation			SEM	Effect stage of lactation
		Early	Mid	Late		
Plasmin	mg/L	0.84	1.32	1.48	0.18	NS
Plasminogen	mg/L	1.34	1.24	1.87	0.18	NS
Plasminogen/Plasmin		0.76	0.80	1.31	0.17	NS
Plasminogen Activator	mg/L	2.27	2.59	2.80	0.14	NS
Elastase	mg/L	0.10^{A}	0.19^{B}	0.95^{C}	0.09	**
Cathepsin	mg/L	2.41^{B}	3.4^{C}	2.04^{A}	0.06	***

$^{A-C}$=means within a row with different superscripts differ (P<0.05).
*NS= not significant; **P<0.01; ***P<0.001.*

Levels of elastase and cathepsin displayed a different trend throughout lactation; the content of elastase increased significantly, whereas cathepsin showed the highest content in mid lactation milk. The correlation between SCC and cathepsin D activity is controversial. O'Driscoll *et al.* (1999) suggested that cathepsin activity in milk is correlated with SCC but whether this reflects an increase amount of enzyme or increased activation of pro-cathepsin remains to be determined. Somers *et al.* (2003), instead, reported that cysteine protease activity was increased only in individual cow milk samples with more than 800,000 cells mL^{-1} and not in milk with lower SCC. The positive correlation

Table 2. Percentage of differential cell populations in sheep milk during lactation.

		Stage of Lactation			SEM	Effect Stage of lactation
		Early	Mid	Late		
Lymphocytes	%	43.02^{A}	40.57^{A}	26.15^{B}	3.52	***
PMNL	%	49.39^{B}	57.07^{B}	70.58^{A}	3.68	***
Macrophages	%	7.59^{B}	2.36^{A}	3.27^{A}	0.74	***

$^{A-B}$*=means within a row with different superscripts differ (P<0.05). ***P<0.001.*

between clotting time and cathepsin levels suggests that casein hydrolysis carried out by this enzyme can impair the rennet reactivity of sheep milk (data not shown). Percentages of the main leukocyte populations in milk, i.e. macrophages, PMNL, and lymphocytes, enumerated by flow cytometry were reported in table 2. Lymphocytes were the lowest at the end of lactation with the percentage found in late lactation being almost half the proportion observed in early and mid lactation; an opposite trend was recorded for PMNL that increased throughout lactation. Accordingly PMNL are known to increase physiologically during late lactation (Pillai *et al.*, 2001). In this study macrophages minimally contributed to total somatic cell count and this cell population was the highest in early lactation milk. It is worth to note that in this study both PMNL and elastase displayed the same behaviour during lactation supporting the hypothesis that both parameters could be related to the physiological state of the mammary gland. Thus, the elastase content in ewe bulk milk could be considered reliable indicator of mammary gland involution. The study of changes in endogenous proteolytic enzymes in sheep milk during lactation suggests that the plasmin-plasminogen system does not vary significantly when SCC remains relatively low throughout lactation. Differential cell count showed that PMNL cells increased physiologically with lactation, being largely predominant in late lactation milk. Changes in elastase levels in milk followed closely those found in PMNL suggesting that elastase concentration could be a reliable indicator of mammary gland involution in healthy udders.

REFERENCES - Baldi, A., Savoini, G., Cheli, F., Fantuz, F., Senatore, E., Bertocchi, L., Politis, I., 1996. Changes in plasmin-plasminogen activator system in milk from Italian Friesian herds. Int. Dairy J. 6:1045-1053. **Bieth,** J., Spiess, B., Wermuth, C., 1974. The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochem. Med. 11:350-357. **Caroprese**, M., Marzano, A., Schena, L., Sevi, A., 2008. Immunomagnetic procedure for positive selection of macrophages in ovine milk. J. Dairy Sci. 91:1908-1912. **Considine,** T., Healy, A., Kelly, A. L., McSweeney, P. L. H., 2000. Proteolytic specificity of elastase on bovine as1-casein. Food Chem. 69:19-26. **Kelly,** A. L., McSweeney P.L.H., 2002. Indigenous proteinases in milk. Adv. Dairy Chem. 1:494-519. **O'Driscoll,** B.M., Rattray, F.P., McSweeney, P. L. H., Kelly, A. L., 1999. Protease activities in raw milk determined using a synthetic heptapeptide substrate. J. Food Sci. 64:606-611. **Perlmann,** G. E., Lorand, L., 1970. Proteolytic enzymes. Pages 316-336 in Methods in enzymology. Vol. 19. S.P. Colowick and N.O. Kaplan, ed. Academic Press, New York, USA. **Pillai,** S.R., Kunze, E., Sordillo, L.M., Jayarao, B.M., 2001. Application of differential inflammatory cell count as a tool to monitor udder health. J. Dairy Sci. 84:1413-1420. **SAS** User's Guide: Statistics. Version 8.1 ed. SAS Inst. Inc., Cary, NC. **Smith, R.**, Turk, V., 1974. Cathepsin D: rapid isolation by affinity chromatography on haemoglobin-agarose resin. Eur. J. Biochem. 197. 48(1):245-254. **Somers,** J.M., O'Brien B., Meaney W. J., Kelly, A. L., 2003. Heterogeneity of proteolytic enzyme activities in milk samples of different somatic cell count. J. Dairy Res. 70: 45-50.