Milk cathelicidin and somatic cell counts in dairy goats along the course of lactation

Vittorio Tedde[1], Valerio Bronzo[2], Giulia Maria Grazia Puggioni[1], Claudia Pollera[2], Antonio Casula[2], Giulio Curone[2], Paolo Moroni[2,3], Sergio Uzzau[1,4], and Maria Filippa Addis[1,2]*

[1]Porto Conte Ricerche, Alghero, Italy
[2]Università degli Studi di Milano, Dipartimento di Medicina Veterinaria, Milan, Italy
[3]Cornell University, Animal Health Diagnostic Center, Ithaca, NY, USA
[4]Università degli Studi di Sassari, Dipartimento di Scienze Biomediche, Sassari, Italy

Short title: **Cathelicidin and SCC in goat milk**

*Correspondence: Maria Filippa Addis
Dipartimento di Medicina Veterinaria
Università degli Studi di Milano
Via Celoria 10
20133 Milano
Italy
Phone: +39-02-503-18076
FAX +41-02-503-1870
E-mail: filippa.addis@unimi.it

Summary

This research communication reports the evaluation of cathelicidin in dairy goat milk for its relationship with the somatic cell count (**SCC**) and microbial culture results. Considering the limited performances of SCC for mastitis monitoring in goats, there is interest in evaluating alternative diagnostic tools. Cathelicidin is an antimicrobial protein involved in innate immunity of the mammary gland. In this work, half-udder milk was sampled bimonthly from a herd of 37 Alpine goats along an entire lactation and tested with the cathelicidin ELISA together with SCC and bacterial culture. Cathelicidin and SCC showed a strong correlation (r = 0.72; n = 360 milk samples). This was highest in mid-lactation (r = 0.83) and lowest in late lactation (r = 0.61), and was higher in primiparous (0.80, n = 130) than in multiparous goats (0.71, n = 230). Both markers increased with time of lactation, but cathelicidin increased significantly less than SCC. In addition, peak level in late lactation was lower for cathelicidin (5.05-fold increase) than for SCC (7.64-fold increase). Twenty-one (5.8%) samples were positive to bacteriological culture, 20 for coagulase-negative staphylococci and one for *Streptococcus* spp.; 18 of them were positive to the cathelicidin ELISA (85.71% sensitivity). Sensitivity of SCC > 500,000 and of SCC > 1,000,000 cells/mL was lower (71.43% and 23.81%, respectively). Therefore, the high correlation of cathelicidin with SCC during the entire lactation, along with its lower increase in late lactation and good sensitivity in detecting intramammary infection (**IMI**), indicate a potential for monitoring subclinical mastitis in dairy goats. However, based on this preliminary assessment, specificity should be improved (40.41% for cathelicidin vs 54.57% and 67.85% for SCC > 500,000 and > 1,000,000 cells/mL, respectively). Therefore, the application of cathelicidin for detecting goat IMI will require further investigation and optimization, especially concerning the definition of diagnostic thresholds.

Keywords: cathelicidin, ELISA, dairy goats, subclinical mastitis, somatic cell counts.

Subclinical mastitis (**SCM**) causes significant economic losses in dairy goat farming due to its detrimental effects on milk production, hygienic status and processing properties. In fact, a great part of intramammary infections (**IMI**) in this dairy species do not produce clinical signs of disease, making the implementation of sensitive and specific SCM detection strategies a priority (Stuhr & Aulrich 2010). Currently, the gold standard is the microbial culture of milk, but the detection of inflammation parameters rather than bacteria provides a more rapid SCM screening test. The somatic cell count (**SCC**) is the standard parameter for monitoring mammary gland inflammation in cows. However, its reliability for SCM detection in goats is strongly limited by the influence of physiological factors and management variables including among others breed, parity, lactation stage, estrus, milking frequency and machine or hand milking (Stuhr & Aulrich 2010). SCC does also increase considerably in late lactation and some uncertainties remain in the exact dynamics, physiology and timing, as well as the changes in the milk cell relative ratios (Souza *et al.* 2012). Considering these factors, further efforts are needed to improve SCM monitoring and diagnosis strategies.

Inflammation-related protein biomarkers can represent a valuable alternative with advantages in terms of diagnostic and outcome performance (Viguier *et al.* 2009). Cathelicidins are a family of small proteins involved in the innate immune response of epithelial and mucosal tissues, often referred together as cathelicidin. These proteins exhibit both direct anti-microbial activity as well as chemotactic and regulatory functions and are believed to play a relevant role in immunity of dairy ruminants, as indicated by the unusually high number of genes present in their genomes. For instance, cows have 10 known cathelicidin genes, sheep have 7 genes and goats have at least 5 genes, while humans and mice have only one gene (Zanetti 2005). Although produced by mammary epithelial cells also upon exposure to a microbial pathogen (Cubeddu *et al.* 2017), cathelicidins are mainly associated to polymorphonuclear neutrophils (**PMNs**) in which they are stored pre-formed within intracellular granules (Borregaard *et al.* 2007). Following an infective stimulus, PMNs are recalled into the mammary gland and release massive amounts of cathelicidin both by degranulation and by formation of neutrophil extracellular traps (**NETs**) (Pisanu *et al.* 2015). As a result, cathelicidin concentration increases significantly in the milk of animals with IMI caused by many different etiologic agents (Addis *et al.* 2017; Cubeddu *et al.* 2017). Due to the specific and consistent release in association with inflammation, several authors have proposed its use for mastitis diagnosis. A cathelicidin ELISA was recently developed and validated in ewes and in cows (Addis *et al.* 2016a, 2016b), showing promising diagnostic performances and a strong correlation with SCC. In this study, we evaluated cathelicidin ELISA in goat milk and assessed its correlation with SCC and microbial culture by monitoring a whole herd for an entire lactation length.

Materials & Methods

Herd description

The herd was composed of 37 Alpine goats (24 multiparous, 13 primiparous) and was certified free of brucellosis, tuberculosis, mycoplasmosis and caprine arthritis-encephalitis. The farm was located in Lombardy, Italy, on the Orobic Alps foothills (Latitude 45° 50' 18'' and Longitude 9° 43' 59''). Housed animals were fed hay and feed concentrate with ad-lib water. From June to September, animals grazed freely during the day. The farm practiced seasonal milking and kidding occurred between February and March 2016. Kids nursed from their mothers. One day post conducting the Dairy Herd Improvement test in March, May, July, September, and November months, goats were clinically examined, and milk samples were collected from both half-udders for a total of 360 samples (10 were missed). The mean SCC for the bulk tank milk in the year was 1,027,000 cells/mL, with a mean daily production per animal of 1.6 Kg.

Milk sampling for bacteriological analyses and determination of somatic cell counts

Milk sampling and bacteriological analyses were performed as recommended by the National Mastitis Council (National Mastitis Council 2017). The SCC was determined for each milk sample on an automated somatic-cell counter (Bentley Somacount 150, Bentley Instrument, USA).

Milk cathelicidin ELISA

Milk cathelicidin ELISA was carried out as described previously (Addis *et al.* 2016a, 2016b). For absorbance normalization, six culture-negative goat milk samples with less than 50,000 cells/mL were included in all ELISA plates. All OD450 values were then subtracted of the average OD450+3SD of the six internal normalization samples for obtaining the normalized OD450 values (**NOD450**). Intra-assay and inter-assay CV were <11.5% and <15.1%, respectively.

Statistics

Statistical analysis was carried out using GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA, USA) for descriptive statistics and column statistics; MedCalc Statistical Software version 16.2.1 (MedCalc Software bvba, Ostend, Belgium) for receiver-operator characteristics (ROC), area under the curve (AUC), sensitivity (Se) and specificity (Sp) evaluations; and IBM SPSS software for Windows 25.0 (IBM SPSS Software, Armonk, NY, USA) for multi factorial analysis. Transformation of somatic cell counts into Linear Score values (LS) with the

formula $\log_2$ (SCC/100,000) + 3 (Kirk et al. 1984) did not lead to normalization of the data. The correlation coefficient (*r*) was calculated as NOD450/LS and plotted with Microsoft Excel (Microsoft Corp., Richmond, VA). Since data had a repeated measurement nature, the influence of sampling time and parity (Fixed effects) on SCC and normalized cathelicidin levels (outcome variables) was also assessed using a GEE (Generalized Estimating Equation). To enable analysis of negative values, NOD450 was adjusted by adding 0.1 to each measurement (Addis *et al.* 2017). The threshold for statistical significance was $p<0.05$. The diagnostic performance of SCC and cathelicidin ELISA in identifying culture-positive and culture-negative samples was assessed with a 2x2 diagnostic table. The selected thresholds were 0.014 NOD450 for cathelicidin ELISA (Addis et al., 2016a) and 500,000 cells/mL plus 1,000,000 cells/mL for SCC (Souza *et al.*, 2012).

Results

Relationship between SCC and cathelicidin

SCC and cathelicidin were measured in all 360 samples, and their correlation coefficients (*r*) were calculated both for the entire lactation as well as separately for each sampling time. The *r* for all milk samples collected along the study was 0.72 (n = 360), showing a good general agreement between the two markers. The *r* value was 0.80 for primiparous (n = 130) and 0.71 for multiparous goats (n = 230). Fig. 1 represents the *r* values calculated separately for each sampling time. The highest correlation was observed in July ($r = 0.83$), in mid-lactation, and the lowest correlation was observed in November, at the end of lactation ($r = 0.61$).

Somatic cell counts and cathelicidin trends along the lactation

Trends of milk cathelicidin and SCC levels along the lactation length are represented in Fig. 2A, while median, interquartile range (IQR), mean and standard deviation values for all time points and animal groups are provided in Supplementary Table 1. SCC and cathelicidin levels increased constantly from March to September and peaked in November. However, the increase at the end of lactation was less pronounced for cathelicidin than for SCC. Specifically, the September to November increase in the median value was 7.64-fold for SCC *vs* 5.03-fold for cathelicidin. Primiparous goats (Fig. 2B) showed lower levels of both markers than multiparous goats (Fig. 2C) for all sampling points. The influence of sampling time and parity on SCC and cathelicidin levels was statistically significant ($p<0.05$) along the lactation. Concerning the differences among samplings (March, May, July, September, and November) for each marker, cathelicidin levels were similar only between July

and September, while SCC levels were similar only between March and May (Supplementary Table 2).

Microbiologic culture results and cathelicidin

All the enrolled animals remained clinically healthy during the study period. Out of 360 samples, only 21 (5.8%) were bacteriologically positive (colony forming units-CFU/mL ≥ 500), 20 for coagulase-negative staphylococci (CNS) and 1 for *Streptococcus* spp. Even in positive samples, bacterial load was generally low: the median value was 900 CFU/mL. Keeping in mind these limitations, a 2x2 diagnostic table based on bacterial culture as the gold standard was elaborated to preliminarily assess the ability of cathelicidin vs SCC in detecting goat mammary glands positive to minor pathogens. Details are reported in Supplementary Table 3. According to ROC analysis, the diagnostic performance of cathelicidin was comparable to SCC > 500,000 cells/mL in terms of AUC (0.631 vs 0.630, respectively) although with different Se and Sp characteristics. For cathelicidin ELISA at 0.014 NOD450, Se was 85.71% and Sp was 40.41%. For SCC > 500,000 cells/mL, Se was 71.43% and Sp was 54.57%. For SCC > 1,000,000, Se was 23.81% and Sp was 67.85%. Nevertheless, the very low IMI prevalence and bacterial load in the study herd, combined with the limited sensitivity of the gold standard bacterial culture, may have negatively influenced Sp estimates.

Discussion

In view of its important role in innate immunity of the mammary gland, the antimicrobial and chemotactic protein cathelicidin possesses a significant potential as a sensitive and specific mastitis marker. Recently, an ELISA was developed for its detection in sheep milk and in cow milk (Addis *et al.* 2016a, 2016b). In these dairy species, the test showed a higher sensitivity of IMI detection relative to SCC and microbial culture while maintaining high specificity. Here, the cathelicidin ELISA was demonstrated to detect also goat milk cathelicidins, opening interesting perspectives for its application in goat herd screening.

In goats, the influence of factors other than IMI on the milk SCC is well-known (Stuhr & Aulrich 2010; Souza *et al*., 2012), and the availability of an alternative inflammation marker with a dedicated assay might enable a more reliable and robust goat herd screening (Bagnicka *et al., 2011*). A pre-requisite, however, would be for this alternative marker to be less influenced by the same factors affecting SCC. Here, a strong correlation was observed between goat milk cathelicidin levels and SCC. Median and IQR of both parameters increased gradually along lactation, especially in multiparous goats. The increase in SCC along lactation, its peaking in late lactation, and the higher SCC in multiparous vs primiparous goats is well-known and it has been described by several authors

in different goat breeds (Stuhr & Aulrich, 2010; Souza *et al*., 2011). Apparently, cathelicidin levels followed similar trends; however, cathelicidin increased less than SCC along lactation, and its late lactation peaking was less intense. Among other potential causes, this may originate from changes in cell type abundance ratios (Goncalves *et al.*, 2017). PMN are the main cell type in goat milk in non-infectious conditions but undergo a further increase upon infection (Haenlein 2002). On the other hand, milk cell types different than PMN including macrophages and desquamated epithelial cells increase in physiological conditions, such as in late lactation (Paape *et al.* 2007). Being mainly associated with neutrophils, where it is abundantly stored inside cytoplasmic granules (Borregaard *et al.* 2007), cathelicidin might reflect PMN increase better than total SCC and therefore act as a more reliable inflammation marker, especially at the end of lactation.

The preliminary comparison of cathelicidin with SCC in terms of diagnostic performances was promising in terms of improved sensitivity, while specificity was not satisfactory. However, signs of clinical mastitis were never observed in the study herd, and the prevalence of bacteriologically positive milk samples was very low. In addition, only minor pathogens were detected, with generally low CFU values. Therefore, further studies with a higher number of bacteriologically-positive samples will be needed to confirm the exact role/indication of increased cathelicidin levels for goat udder health. Thresholds and diagnostic algorithms will have to be defined for a reliable implementation of the cathelicidin ELISA in the dairy goat production systems.

The successful application of a cathelicidin ELISA might provide a convenient alternative to SCC and to differential cell counting also for its outcome performance in terms of cost and ease of use (Flatland *et al.* 2014). One advantage is the reduced cost and widespread diffusion of ELISA readers and other related instrumentation and devices as opposed to the investment required for the acquisition and maintenance of differential cell counting instrumentation. The availability of a reliable ELISA would also enable frozen storage of small volumes of milk samples for later testing in batch or for assay repetitions, instead of the short-term refrigerated storage of larger milk aliquots as required for somatic cell counting.

In conclusion, the milk cathelicidin ELISA developed for cows and ewes showed good detection performances in goats. The cathelicidin levels measured were strongly correlated to SCC but underwent a lower increase along lactation with less intense peak values in late lactation. Most of the bacterial culture-positive samples were also positive to the cathelicidin ELISA. Considering the practical advantages of an ELISA compared to cell counting, cathelicidin might hold potential for udder health monitoring in dairy goats. However, further investigations will be required to comparatively assess its diagnostic performance over SCC in detecting goat mastitis, especially for what concerns specificity.

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Figure Legends

Figure 1:
Correlation coefficients (*r*) observed for milk SCC and cathelicidin in the different sampling months.

Figure 2:
SCC and cathelicidin levels in milk along lactation. A, all goat samples (n = 360); B, primiparous goat samples (n = 130); C, multiparous goat samples (n = 230). Boxes indicate values within the 25th and 75th percentiles, and the central line indicates the median value. Whiskers indicate values within the 2.5th and 97.5th percentiles, and individual dots represent values outside the whiskers.

278 **Figure 1:**

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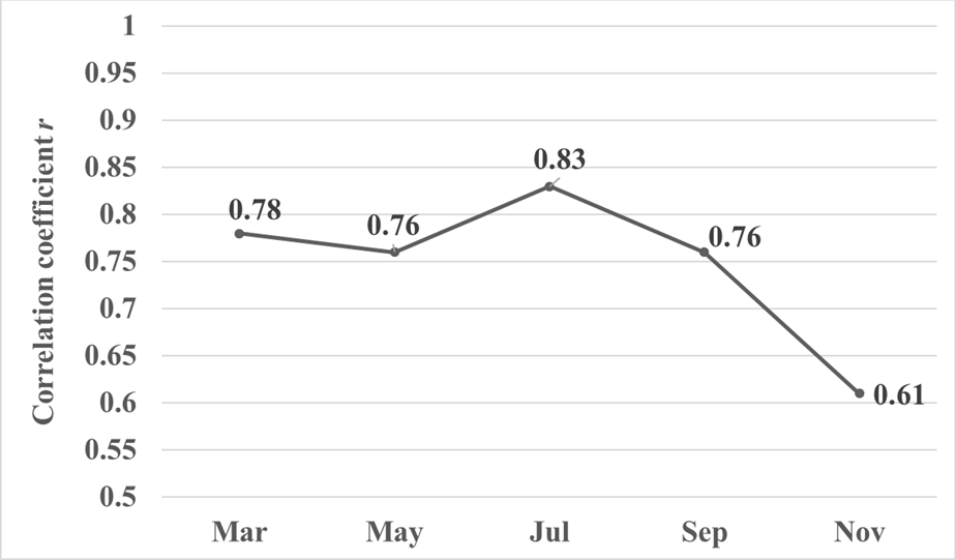

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282 **Figure 2:**

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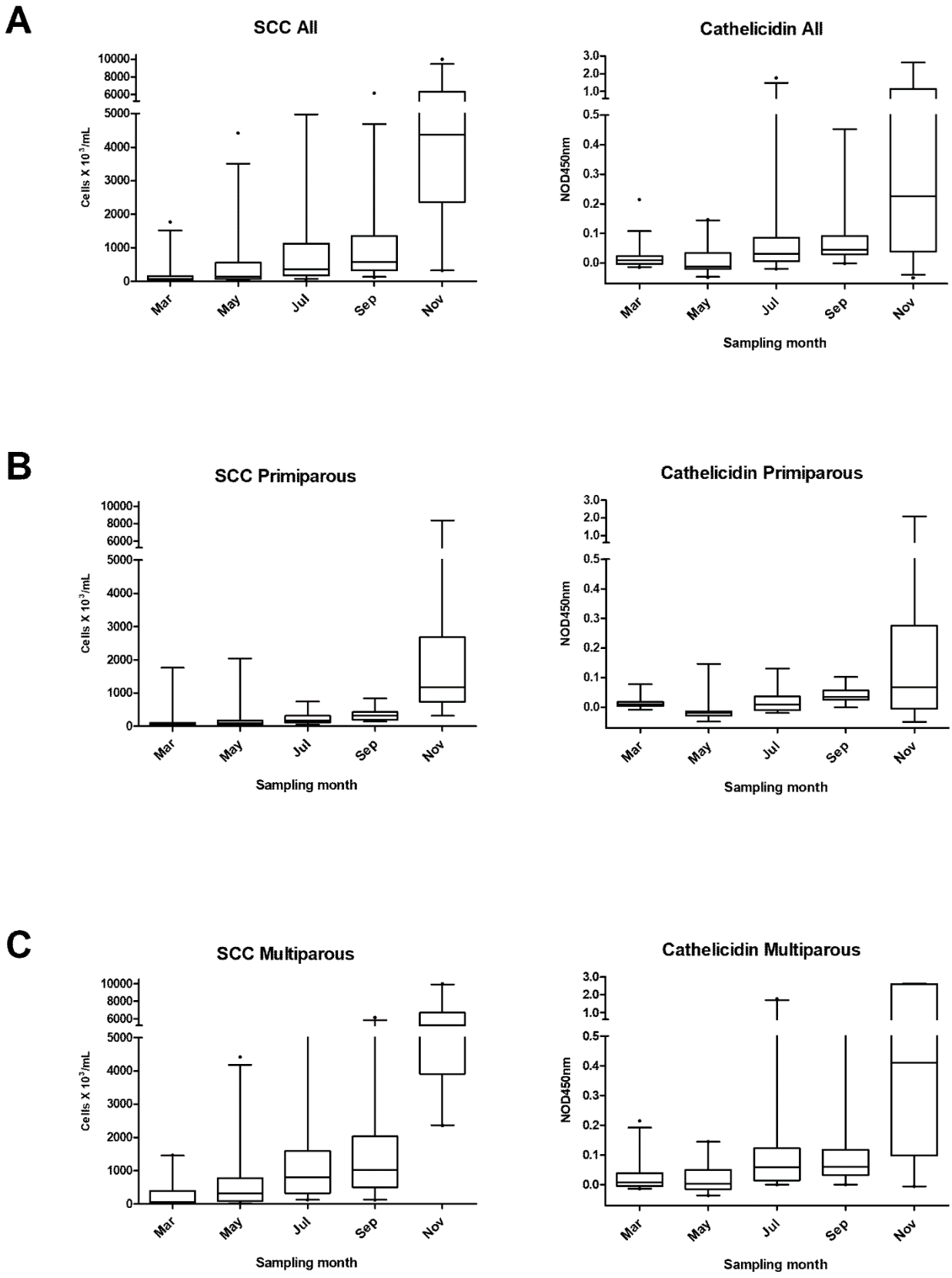

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