

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

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

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## 30 **Summary**

31 This research communication reports the evaluation of cathelicidin in dairy goat milk for its  
32 relationship with the somatic cell count (**SCC**) and microbial culture results. Considering the limited  
33 performances of SCC for mastitis monitoring in goats, there is interest in evaluating alternative  
34 diagnostic tools. Cathelicidin is an antimicrobial protein involved in innate immunity of the mammary  
35 gland. In this work, half-udder milk was sampled bimonthly from a herd of 37 Alpine goats along an  
36 entire lactation and tested with the cathelicidin ELISA together with SCC and bacterial culture.  
37 Cathelicidin and SCC showed a strong correlation ( $r = 0.72$ ;  $n = 360$  milk samples). This was highest  
38 in mid-lactation ( $r = 0.83$ ) and lowest in late lactation ( $r = 0.61$ ), and was higher in primiparous (0.80,  
39  $n = 130$ ) than in multiparous goats (0.71,  $n = 230$ ). Both markers increased with time of lactation, but  
40 cathelicidin increased significantly less than SCC. In addition, peak level in late lactation was lower  
41 for cathelicidin (5.05-fold increase) than for SCC (7.64-fold increase). Twenty-one (5.8%) samples  
42 were positive to bacteriological culture, 20 for coagulase-negative staphylococci and one for  
43 *Streptococcus* spp.; 18 of them were positive to the cathelicidin ELISA (85.71% sensitivity).  
44 Sensitivity of SCC > 500,000 and of SCC > 1,000,000 cells/mL was lower (71.43% and 23.81%,  
45 respectively). Therefore, the high correlation of cathelicidin with SCC during the entire lactation,  
46 along with its lower increase in late lactation and good sensitivity in detecting intramammary  
47 infection (**IMI**), indicate a potential for monitoring subclinical mastitis in dairy goats. However,  
48 based on this preliminary assessment, specificity should be improved (40.41% for cathelicidin vs  
49 54.57% and 67.85% for SCC > 500,000 and > 1,000,000 cells/mL, respectively). Therefore, the  
50 application of cathelicidin for detecting goat IMI will require further investigation and optimization,  
51 especially concerning the definition of diagnostic thresholds.

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

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

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

88

## 89 **Materials & Methods**

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### 91 *Herd description*

92           The herd was composed of 37 Alpine goats (24 multiparous, 13 primiparous) and was certified  
93 free of brucellosis, tuberculosis, mycoplasmosis and caprine arthritis-encephalitis. The farm was  
94 located in Lombardy, Italy, on the Orobic Alps foothills (Latitude 45° 50' 18'' and Longitude 9° 43'  
95 59''). Housed animals were fed hay and feed concentrate with ad-lib water. From June to September,  
96 animals grazed freely during the day. The farm practiced seasonal milking and kidding occurred  
97 between February and March 2016. Kids nursed from their mothers. One day post conducting the  
98 Dairy Herd Improvement test in March, May, July, September, and November months, goats were  
99 clinically examined, and milk samples were collected from both half-udders for a total of 360 samples  
100 (10 were missed). The mean SCC for the bulk tank milk in the year was 1,027,000 cells/mL, with a  
101 mean daily production per animal of 1.6 Kg.

102

### 103 *Milk sampling for bacteriological analyses and determination of somatic cell counts*

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

107

### 108 *Milk cathelicidin ELISA*

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

114

### 115 *Statistics*

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

122 formula  $\log_2 (\text{SCC}/100,000) + 3$  (Kirk et al. 1984) did not lead to normalization of the data. The  
123 correlation coefficient ( $r$ ) was calculated as NOD450/LS and plotted with Microsoft Excel (Microsoft  
124 Corp., Richmond, VA). Since data had a repeated measurement nature, the influence of sampling  
125 time and parity (Fixed effects) on SCC and normalized cathelicidin levels (outcome variables) was  
126 also assessed using a GEE (Generalized Estimating Equation). To enable analysis of negative values,  
127 NOD450 was adjusted by adding 0.1 to each measurement (Addis *et al.* 2017). The threshold for  
128 statistical significance was  $p < 0.05$ . The diagnostic performance of SCC and cathelicidin ELISA in  
129 identifying culture-positive and culture-negative samples was assessed with a 2x2 diagnostic table.  
130 The selected thresholds were 0.014 NOD450 for cathelicidin ELISA (Addis et al., 2016a) and  
131 500,000 cells/mL plus 1,000,000 cells/mL for SCC (Souza *et al.*, 2012).

132

## 133 **Results**

134

### 135 *Relationship between SCC and cathelicidin*

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

143

### 144 *Somatic cell counts and cathelicidin trends along the lactation*

145 Trends of milk cathelicidin and SCC levels along the lactation length are represented in Fig.  
146 2A, while median, interquartile range (IQR), mean and standard deviation values for all time points  
147 and animal groups are provided in Supplementary Table 1. SCC and cathelicidin levels increased  
148 constantly from March to September and peaked in November. However, the increase at the end of  
149 lactation was less pronounced for cathelicidin than for SCC. Specifically, the September to November  
150 increase in the median value was 7.64-fold for SCC vs 5.03-fold for cathelicidin. Primiparous goats  
151 (Fig. 2B) showed lower levels of both markers than multiparous goats (Fig. 2C) for all sampling  
152 points. The influence of sampling time and parity on SCC and cathelicidin levels was statistically  
153 significant ( $p < 0.05$ ) along the lactation. Concerning the differences among samplings (March, May,  
154 July, September, and November) for each marker, cathelicidin levels were similar only between July

155 and September, while SCC levels were similar only between March and May (Supplementary Table  
156 2).

157

### 158 *Microbiologic culture results and cathelicidin*

159 All the enrolled animals remained clinically healthy during the study period. Out of 360  
160 samples, only 21 (5.8%) were bacteriologically positive (colony forming units-CFU/mL  $\geq$  500), 20  
161 for coagulase-negative staphylococci (CNS) and 1 for *Streptococcus* spp. Even in positive samples,  
162 bacterial load was generally low: the median value was 900 CFU/mL. Keeping in mind these  
163 limitations, a 2x2 diagnostic table based on bacterial culture as the gold standard was elaborated to  
164 preliminarily assess the ability of cathelicidin vs SCC in detecting goat mammary glands positive to  
165 minor pathogens. Details are reported in Supplementary Table 3. According to ROC analysis, the  
166 diagnostic performance of cathelicidin was comparable to SCC > 500,000 cells/mL in terms of AUC  
167 (0.631 vs 0.630, respectively) although with different Se and Sp characteristics. For cathelicidin  
168 ELISA at 0.014 NOD450, Se was 85.71% and Sp was 40.41%. For SCC > 500,000 cells/mL, Se was  
169 71.43% and Sp was 54.57%. For SCC > 1,000,000, Se was 23.81% and Sp was 67.85%. Nevertheless,  
170 the very low IMI prevalence and bacterial load in the study herd, combined with the limited sensitivity  
171 of the gold standard bacterial culture, may have negatively influenced Sp estimates.

172

### 173 **Discussion**

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

181 In goats, the influence of factors other than IMI on the milk SCC is well-known (Stuhr & Aulrich  
182 2010; Souza *et al.*, 2012), and the availability of an alternative inflammation marker with a dedicated  
183 assay might enable a more reliable and robust goat herd screening (Bagnicka *et al.*, 2011). A pre-  
184 requisite, however, would be for this alternative marker to be less influenced by the same factors  
185 affecting SCC. Here, a strong correlation was observed between goat milk cathelicidin levels and  
186 SCC. Median and IQR of both parameters increased gradually along lactation, especially in  
187 multiparous goats. The increase in SCC along lactation, its peaking in late lactation, and the higher  
188 SCC in multiparous vs primiparous goats is well-known and it has been described by several authors

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

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

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

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

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267 **Figure Legends**

268

269 **Figure 1:**

270 Correlation coefficients ( $r$ ) observed for milk SCC and cathelicidin in the different sampling months.

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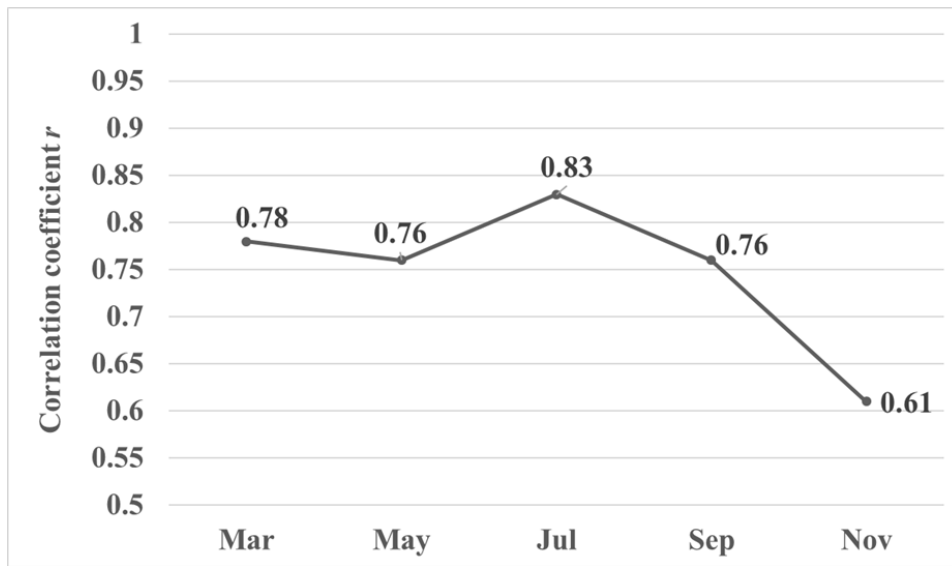
272 **Figure 2:**

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

277

278 **Figure 1:**

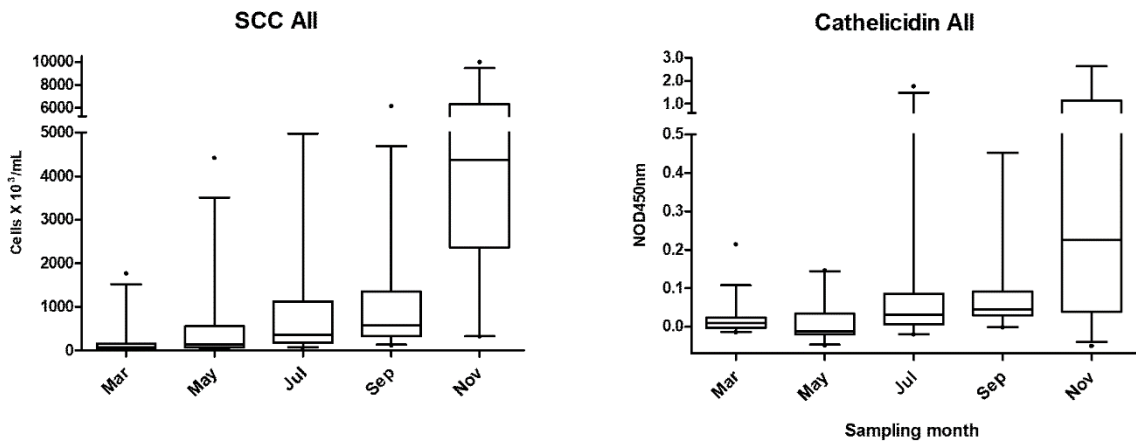
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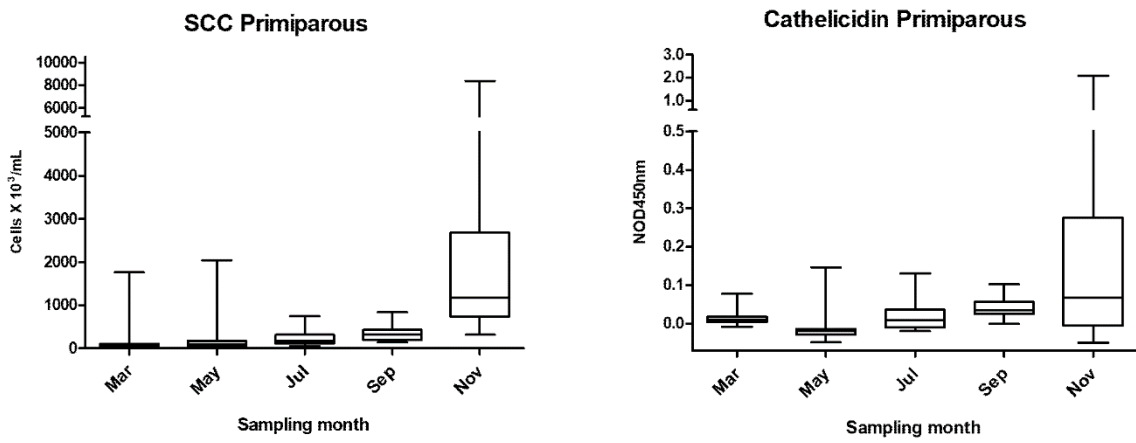
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281

**A**



**B**



**C**

