INTERPRETIVE SUMMARY

The availability of sensitive and specific tools for monitoring dairy ruminant mastitis is paramount for an effective control of the disease. Currently, mastitis monitoring is based on the somatic cell count, but novel immunoassay-based systems may represent valuable, sensitive, and flexible alternatives. A sensitive and specific ELISA based on the inflammatory protein cathelicidin has recently been developed for mastitis monitoring and detection. Here, the authors analyze how its abundance in milk is influenced by the causative microorganism and how this correlates to the somatic cell count, and discuss the implications of these findings for understanding and diagnosing mastitis.

Running Head: Cathelicidin in clinical bovine mastitis

Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis

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ABSTRACT

The availability of reliable tools enabling a sensitive and specific detection of mastitis in dairy cows can significantly aid control strategies and promote a more rational use of antibiotics. We have recently developed a milk cathelicidin ELISA showing elevated sensitivity and specificity for dairy cow mastitis based on the latent class analysis approach. Here, we investigated the impact of the microbial agent on cathelicidin abundance in the milk of cows with clinical mastitis.

A total of 535 quarter milk samples, of which 435 collected from quarters showing signs of clinical mastitis and 100 collected from healthy quarters as a control, were subjected to milk cathelicidin ELISA, somatic cell count (**SCC**), and microbiologic culture. Of the 435 clinical mastitis samples, 431 (99.08%) were positive for cathelicidin ELISA, 424 (97.47%) had SCC > 200,000 cells/mL, and 376 (86.44%) were positive to culture. Of the 59 clinical culture-negative samples, 58 (98.30%) were positive for cathelicidin and 55 (93.22%) had SCC > 200,000 cells/mL. The abundance of cathelicidin and the extent of SCC increase changed depending on the causative agent, with *Streptococcus agalactiae* and coagulase-negative staphylococci showing the highest and the lowest changes, respectively. We did also observe differences in behavior between the two markers depending on the isolated pathogen; *Streptococcus agalactiae* induced the highest cathelicidin abundance, while *Serratia* spp. induced the highest SCC. Nevertheless, the different ability of the microorganism to induce cathelicidin release in the milk did not compromise its value as a mastitis marker, also in consideration of the higher Se observed in comparison to SCC or to microbiologic culture. All the 100 negative control samples, collected from healthy quarters with SCC < 100,000 cells/mL and negative to culture, were also negative to the cathelicidin ELISA, corresponding to a 100% specificity in the evaluated sample cohort.

In conclusion, this study confirms the value of the milk cathelicidin ELISA for detection of bovine mastitis and highlights an influence of the mastitis-causing microorganism on its abundance. Such influence does not compromise diagnostic performance, but it may instead provide a better ability to reflect disease severity and evolution when compared to SCC.

Keywords: clinical mastitis; cathelicidin; dairy cow; ELISA; intramammary infection.

INTRODUCTION

The constant search for sensitive and specific tools enabling mastitis detection in dairy cows is a requirement for reducing the economic impact of mastitis. The availability of effective diagnostic methods can aid a faster and more efficient control of mastitis in dairy ruminants and promote a more responsible use of antibiotic therapy. Currently, mastitis monitoring is mainly based on the SCC, but immunologic detection of inflammation markers can represent a valuable alternative providing practical benefits and enabling improvement of diagnostic performances (Viguier et al., 2009; Ceciliani et al., 2012). One of the most promising inflammation marker is cathelicidin, a term that includes a class of proteins with antimicrobial activity and potent pro-inflammatory and chemotactic functions (Zanetti, 2004, 2005). Cathelicidin is released in milk first by epithelial cells upon contact with an invading pathogen and then by activated neutrophil polymorphonuclear leukocytes (**PMN**) retrieved into milk, both by degranulation and through formation of neutrophil extracellular traps (**NETs**) (Addis et al., 2013; Pisanu et al., 2015).

We have recently developed a highly performing milk pan-cathelicidin ELISA that showed elevated sensitivity (**Se**) and specificity (**Sp**) for dairy cow mastitis relative to SCC and microbial culture (Addis et al., 2016b). Assay performance was estimated with a latent class analysis approach in a Bayesian framework, that is, without referring to a gold standard for the true disease status. The release of cathelicidin in milk had been observed also by other authors in natural and experimental infections (Smolenski et al., 2011; Wheeler et al., 2012; Reinhardt et al., 2013). However, in their pioneering study Smolenski and coworkers (2011) had reported a lack of cathelicidin reactivity in about 25% of mastitis milk samples by immunoblotting analysis. The authors hypothesized, among other causes, that different microbial infections could induce different levels of cathelicidin release, thereby compromising its diagnostic sensitivity. Nevertheless, the analytical technique and the

antibodies used by these authors may also have influenced the sensitivity and specificity of pan-cathelicidin detection.

In view of these reports, there was a need to assess the influence of the microbial agent on cathelicidin abundance as measured by our pan-cathelicidin ELISA, and to evaluate the impact on its diagnostic value in comparison to SCC. To this aim, we investigated the relationships existing among the inflammation marker cathelicidin, the somatic cell count and culture results, by considering the presence of clearly detectable clinical signs (clinical mastitis) as the gold standard for the true disease status.

MATERIALS AND METHODS

Milk sample collection, microbiologic culture and somatic cell count

Milk samples were obtained from 16 dairy herds between March and May 2016. The farms enrolled were required to have trained technicians able to detect clinical mastitis, to use an adequate milking routine including fore-stripping for detection of abnormal milk, and to sample any case of clinical mastitis and to freeze quarter milk immediately. Clinical mastitis was defined as presence of udder or quarter swelling, heat, hardness, redness, or pain, or of visible alterations in the aspect of milk, such as watery appearance, flakes, clots, or pus. In the farms, sample collection was carried out after careful cleaning and disinfection of teat ends with chlorhexidine-embedded disposable towels. Approximately 10 mL of milk were collected into sterile vials after discharging the first streams. Samples were stored at -20°C and sent to the laboratory weekly for microbiological analyses and SCC determination. A total of 535 quarter milk samples were included in the sample cohort: 435 from cows with clinical mastitis and 100 from clinically healthy cows with negative microbiologic culture and with SCC < 100,000 cells/mL.

At the laboratory of the Department of Veterinary Medicine, University of Milan, milk samples were thawed at room temperature and 100 µL of each sample was spread onto blood agar plates (5%

defibrinated sheep blood). Plates were incubated aerobically at 37°C and evaluated after 24 and 48 h. Microbiologic culture was carried out following the National Mastitis Council guidelines (NMC, 1999), with the exception of a 10x increase in inoculum volume. Provisional identification of colonies was based on Gram stain, morphology, and hemolysis patterns. Representative colonies were sub-cultured on blood agar plates and incubated again at 37°C for 24 h. Gram-positive, catalase positive cocci were tested by coagulase tube test to differentiate *Staphylococcus aureus* from other staphylococci. Gram-positive, catalase negative cocci were identified as streptococci and differentiated by further biochemical tests (growth in 6.5% NaCl broth, esculin hydrolysis, fermentation of ribose, sorbitol, sucrose, and inulin) and by the Christie, Atkins, and Munch-Petersen (CAMP) test. Gram-negative bacteria were identified by Gram staining characteristics, oxidase reaction, and colony morphology on MacConkey's agar (Oxoid, Bakingstoke, UK) and Eosin Methylene Blue agar (Laboratorios Conda, Madrid, Spain). Microorganisms other than bacteria were confirmed by microscopic appearance. When growth of two different microorganisms was detected (25 samples out of 376, 6.65%), the case was classified as mixed infection.Samples with growth of 3 or more pathogens were considered contaminated and were not included in the study. The somatic cell count was determined with an automated somatic cell counter (Bentley Somacount 150, Bentley Instrument, Chaska, MN, USA).

Pan-cathelicidin ELISA

Cathelicidin abundance in milk was assessed at the Porto Conte Ricerche laboratories with a pan-cathelicidin sandwich ELISA based on two monoclonal antibodies developed against a pan-cathelicidin domain, as previously described (Addis et al., 2016a; Addis et al., 2016b). At the end of the assay, the OD450 value of all samples was normalized against internal controls. To this aim, 6 culture-negative samples with < 50,000 cells/mL were included in all ELISA plates, and their average OD450 + 3 SD was subtracted from all OD450 values to obtain the normalized OD450 value (NOD450). For assessing cathelicidin abundance, each milk sample was tested in duplicate aliquots

of 10 µL and 1 µL. When the results of the 10 µL aliquot provided a NOD450 higher than 2.5, the NOD of the 1 µL aliquot multiplied by 10 was considered. Finally, for enabling comparison and logarithmic visualization, a correction factor of 0.1 was added to all NOD450 values to obtain the adjusted OD450 value (AOD450).

Statistics

The Shapiro-Wilk normality test indicated that the data followed a non-normal distribution. Therefore, statistical significance of the differences among result distributions was evaluated using the Kruskall-Wallis test with the Dunns post-test correction. Statistical analysis and descriptive statistics (medians and interquartile range [**IQR**] values) were carried out using GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA). For the assessment of diagnostic performance, a threshold of 0.115 AOD450 was applied for the cathelicidin ELISA (Addis et al., 2016b), and a threshold of 200,000 cells/mL was applied for SCC (Dohoo and Leslie, 1991; Schukken et al., 2003; Bradley and Green, 2005), respectively. For evaluation of Se and Sp, the presence of clinical signs was considered as the gold standard for the true disease status.

RESULTS

Cathelicidin ELISA, SCC, and culture results

The 535 quarter milk samples were evaluated by cathelicidin ELISA, SCC, and culture, and diagnostic performance was assessed based on the presence of clinical mastitis as the true disease status. The results obtained on the 435 clinical mastitis samples are summarized in Table 1; 431 out of 435 were positive to the cathelicidin ELISA (Se 99.08%), 424 out of 435 had SCC > 200,000 cells/mL (Se 97.47%), and 376 out of 435 were culture-positive (Se 86.44%). *Streptococcus uberis*, *Escherichia coli*, *Streptococcus agalactiae*, and coagulase-negative staphylococci (**CNS**) were the most commonly isolated microorganisms. Of the 59 clinical, culture-negative samples, 58 (98.30%)

were positive for cathelicidin and 55 (93.22%) had SCC > 200,000 cells/mL, with one sample being negative for both markers. All 100 milk samples from healthy mammary quarters (selected for being culture-negative and for having SCC < 100,000 cells/mL) were negative to the cathelicidin ELISA, resulting in a Sp of 100%.

Cathelicidin abundance and SCC in clinical and healthy mammary quarters

To estimate cathelicidin abundance in milk, aliquots of 1 and 10 µL were tested by ELISA for calculating AOD450. Figure 1 illustrates the result distribution obtained for cathelicidin and SCC in all samples, classified by healthy, all clinical, clinical positive to culture (**C+**), and clinical negative to culture (**C-**). Healthy quarters were significantly different from clinical quarters ($P \leq 0.001$), both as a group and when considered separately for being C+ or C-. The median AOD450 value was slightly higher in clinical C+ than in clinical C-, but this difference was not statistically significant. All clinical mastitis samples but 4 in the case of cathelicidin (3 clinical C+ and 1 clinical C-) and 11 (7 clinical C+ and 4 clinical C-) in the case of SCC were above the respective positivity thresholds. The median and **IQR** values of cathelicidin AOD450 and SCC in cells/mL measured in all samples are reported in Table 2.

Cathelicidin abundance and SCC according to the pathogen group

First, we analyzed the distribution of cathelicidin and SCC results obtained on clinical milk samples based on relevance of the isolated microorganisms as IMI agents (Harmon, 1994). Specifically, the following groups were considered for comparing cathelicidin and SCC results: contagious pathogens (*Streptococcus agalactiae* and *Staphylococcus aureus*), environmental pathogens (*Streptococcus* spp. and Gram-negative), CNS, other microorganisms, mixed infections, and clinical C- samples (Figure 2). According to the Kruskal-Wallis test, result distributions were significantly different for both cathelicidin and SCC, with a more pronounced effect on the former. In the pairwise comparisons, cathelicidin abundance in IMI by *Streptococcus agalactiae* was significantly different from all other

189 groups with the exception of *Staphylococcus aureus* and mixed infections. Cathelicidin abundance in
190 IMI by CNS was also significantly different from the groups *Streptococcus* spp. and Gram negative
191 (Figure 2A). In the case of SCC (Figure 2B), we observed a statistically significant difference only
192 in IMI by *Streptococcus agalactiae* when compared to IMI by CNS. Statistically significant
193 differences and their respective P value classes are outlined in Figure 2C for cathelicidin and in Figure
194 2D for SCC. Table 3 reports the descriptive statistics for the different microorganism classes, listed
195 in decreasing order of median abundance for cathelicidin and SCC, respectively.
196
197 ***Cathelicidin abundance and SCC according to the isolated microorganism***
198 To gain further detail on the differences in the ability to induce cathelicidin release by the various
199 microorganisms, we also analyzed cathelicidin abundance and SCC by considering separately all the
200 IMI agents that were identified in a minimum of 6 clinical milk samples (Figure 3). The
201 microorganisms that did not satisfy such criteria were grouped as other Gram-positive or other Gram-
202 negative.
203 In the case of cathelicidin (Figure 3A and 3C), we observed statistically significant differences for
204 cathelicidin abundance in IMI by *Streptococcus agalactiae* when compared to 6 out of 15
205 microorganism groups, and in IMI by CNS when compared to 3 out of 15 microorganism groups.
206 SCC did not show any statistically significant difference among groups (Figure 3B and 3D).
207 Table 4 reports the medians and IQRs of cathelicidin (AOD450) and of SCC (cells/mL) according to
208 the respective decreasing order of abundance in IMI by the different microorganisms. We observed
209 several differences among the two considered parameters. The microorganism inducing the highest
210 cathelicidin abundance (median AOD450 of 27.480) was *Streptococcus agalactiae*, while *Serratia*
211 spp. induced the lowest cathelicidin abundance (median AOD450 of 2.474). On the other hand,
212 *Enterococcus faecalis* induced the highest SCC increase (median SCC of 8,184,000 cells/mL),
213 followed by *Serratia* spp. (median SCC of 7,330,000)with an opposite behavior to cathelicidin.

214 *Streptococcus agalactiae* ranked only fourth (median SCC of 6,362,000 cells/mL). CNS induced the
215 lowest SCC increase (median SCC of 2,808,000 cells/mL).
216 Concerning the microbial load in milk, we did not observe any statistically significant correlation of
217 the CFU with cathelicidin abundance or SCC increase, neither collectively nor according to the
218 microorganism or microorganism group.
219

DISCUSSION

Cathelicidins are a class of potent antimicrobial and proinflammatory proteins involved in innate immune response to infection that are released quickly and abundantly following an IMI, and have therefore potential as early and sensitive markers of mastitis (Addis et al., 2013; Smolenski et al., 2014). We have recently developed a pan-cathelicidin ELISA with higher Se and comparable Sp to SCC (Addis et al., 2016a; Addis et al., 2016b). Previous studies had suggested that different microorganisms might induce different levels of cathelicidin release in milk (Smolenski et al., 2011). To assess the extent of these differences and their possible impact on diagnostic performance, this study investigated cathelicidin abundance and SCC in mammary quarters with clinical mastitis due to different microorganisms.

As a first observation, almost all clinical samples examined were positive to cathelicidin ELISA, showing a Se higher than SCC > 200,000 cells/mL (99.08% *vs* 97.47%, respectively). In our previous article we had reported sensitivities of 80.6%, 74.4%, and 38.8%, and specificities of 94.9%, 96.3%, and 92.8%, for cathelicidin ELISA, SCC > 200,000 cells/mL, and microbiologic culture, respectively (Addis et al., 2016b). In that case, the evaluation of diagnostic performances had been carried out on a population including healthy, clinical, and subclinical mastitis quarters, and the latent class analysis approach was therefore implemented in consideration of the lack of a gold standard for the true disease status (Hui and Walter, 1980; Koop et al., 2011; Van Smeden et al., 2014). Accordingly, we had also noted that cathelicidin Sp might be underestimated by the LCA approach in consideration of its higher Se (Addis et al., 2016a). Here, by selecting true positives (clinical mastitis samples) and reliable negatives (healthy, very low SCC, culture-negative samples) as the gold standards for the true diseased and healthy status, we confirmed the superior sensitivity of cathelicidin ELISA, and we highlighted also its elevated specificity.

Culture-negative mastitis remains an issue. In fact, a percentage ranging from 10 to 40% of clinical mastitis cases yield a negative result by culture, and their number seems to be increasing (Makovec and Ruegg, 2003). This is due to numerous and complex factors, including fastidiousness or inability

of some microorganisms to grow in culture, presence of microbes below the detection thresholds, antibiotic treatment or presence of host-produced antimicrobial molecules, mastitis caused by non-bacterial microorganisms, or mastitis due to dysbiosis (Oikonomou et al., 2014). Here, we observed a slightly lower median for clinical C- samples when compared to clinical C+ samples, but the difference was not statistically significant. Therefore, negativity to culture should not be expected to impact significantly on the diagnostic performance of cathelicidin.

When examining the results of clinical C+ samples according to the pathogen group or to the microorganism, milk cathelicidin abundance was significantly influenced by the mastitis agent, but its levels did always remain well above the diagnostic threshold. Therefore, the observations of Smolenski and coworkers (2011) on a differential release of cathelicidin in milk according to the pathogen were confirmed by this study. However, these authors had reported lack of reactivity in about 25% of culture-positive clinical mastitis samples, while we observed a sensitivity close to 100%. This might be due to a poorer detection performance of the antibodies or of the technique they employed for detection, the western immunoblotting. Accordingly, we have observed a slightly lower sensitivity of western immunoblotting *vs* ELISA also with the same anti-pan-cathelicidin monoclonal antibodies used for this study (data not shown).

The microorganisms displaying the most pronounced differences in cathelicidin abundance were *Streptococcus agalactiae* and CNS. These produced the highest and the lowest AOD450 values, respectively. In the case of cathelicidin, *Streptococcus agalactiae* and CNS did also differ significantly from almost all other pathogen groups, in agreement with their respective roles as mastitis pathogens. *Streptococcus agalactiae* is a major contagious mastitis agent together with *Staphylococcus aureus*; accordingly, the latter induced the second highest increase in cathelicidin abundance and the differences with the former were not statistically significant. On the other hand, CNS were among the lowest inducers, in agreement with their lesser potential as acute udder pathogens (Schukken et al., 2009).

When looking at the microorganisms taken separately, several differences were observed, although it should be considered that the methods used to make isolate identifications to the genus and species level may include some misclassification error, particularly for the streptococcal organisms, as phenotypic methods can be inadequate. A striking observation was the lower ability of *Serratia* spp. and *Corynebacterium* spp. to induce cathelicidin increase as opposite to SCC increase. The reason for this is yet to be investigated, but among other causes this might be due to a different ability of these microorganisms to recall PMNs versus other cells in milk. On the other hand, the factors leading to a higher abundance of cathelicidin in milk can be numerous and complex, and may include a more efficient PMN recruitment, PMN degranulation, or NET formation (Lu et al., 2012), as well as ability of the pathogen to mediate PMN lysis (Oliver et al., 1998; Kobayashi et al., 2010; Le Maréchal et al., 2013). Adding to these factors, microbial resistance to lysis by antimicrobial peptides (**AMPs**) with mechanisms including repulsion, digestion, sequestration, or excretion (Yeaman and Yount, 2003; Kraus and Peschel, 2006; Joo and Otto, 2015) may also contribute to the observed differences in cathelicidin abundance. According to one report (Wheeler et al., 2012), milk cathelicidin-derived AMPs display different growth suppression activities on different mastitis-causing microorganisms. The authors reported a MIC_{50} of 8 µg/mL for a cathelicidin extract against different Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*) *vs* a MIC_{50} of 33 µg/mL against *Streptococcus uberis*. Chaneton and coworkers (2008) observed that the ability of *Streptococcus uberis* to resist to the antimicrobial agent lactoferrin was probably responsible for the very high abundance of this protein in the milk of animals suffering *Streptococcus uberis* mastitis. This would suggest that microbial species that are associated with higher concentrations of antimicrobial molecules might be less susceptible to their activity when compared to more sensitive microorganisms. That is, the mammary gland may respond to microbial infection by increasing secretion of antimicrobial proteins in a pathogen-specific manner, and pathogenic agents that trigger a greater response may have adapted to these conditions by becoming increasingly resistant to their antimicrobial activity. In addition, when considering that phagocytosis and other PMN defense

297 mechanisms are inhibited by milk (Lippolis et al., 2006), the role of AMPs in the biology of mastitis
298 becomes even more relevant (Pisanu et al., 2015; Cacciotto et al., 2016).
299 The advantages of a sensitive and specific mastitis test based on protein markers linked to innate
300 immunity as opposite to somatic cells are numerous (Viguier et al., 2009) and have been discussed in
301 depth in our recent articles describing the cathelicidin ELISA (Addis et al., 2016a; Addis et al.,
302 2016b). Among these, the ability to be measured with immunoassays suitable for the laboratory, the
303 field, and in-line systems can be mentioned, as well as their closer correlation with presence of
304 inflammation. Due to its elevated Se and Sp, as demonstrated also in this work, the cathelicidin
305 ELISA may find application for improving detection of subclinical mastitis, as well as of clinical
306 mastitis in automated milking systems, and for helping the identification of mammary quarters
307 eligible for selective dry cow therapy.
308 When considering that the diagnostic performance is maintained, the correlation of cathelicidin
309 abundance with the mastitis pathogen may actually provide several advantages. In fact, cathelicidin
310 may reflect different aspects of the disease biology and evolution when compared to SCC, being more
311 closely related to the number of activated PMNs inside the mammary gland. As such, it may enable
312 to monitor the success of therapy more efficiently (Kawai et al., 2015) and it may find utility as a
313 research tool in mastitis, such as for investigating ability of a pathogen to resist lysis by AMPs or to
314 induce inflammation and PMN recruitment, activation, and killing. On the other hand, the influence
315 of the mastitis pathogen on cathelicidin levels does not possess enough discriminatory power to guide
316 treatment decisions, and culture will still be needed for selecting the correct therapeutic interventions.
317

318 **Conclusion**

319 Cathelicidin abundance in milk is increasingly demonstrating its potential as mastitis marker, and its
320 elevated sensitivity and specificity have been confirmed by this study. Although different pathogens
321 are able to induce cathelicidin release to a different extent, its diagnostic value is not compromised.
322 Instead, such differences may be a better reflection of microbial pathogenicity and may more closely

represent disease severity, finding utility for disease classification, for investigating ability of different pathogens to cause mastitis, for monitoring disease recovery, for providing indications on the success of mastitis treatment, or for guiding selection of mammary quarters eligible for selective dry cow treatments.

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TABLES

Table 1. Summary of the results obtained on the 435 clinical mastitis samples.

Item	N	%
Cathelicidin ELISA	431/435	99.08
SCC > 200,000 cells/mL	424/435	97.47
Microbiologic culture	376/435	86.44
Streptococcus uberis	*66*	*15.17*
Escherichia coli	*63*	*14.48*
Streptococcus agalactiae	*59*	*13.56*
CNS	*41*	*9.43*
Streptococcus dysgalactiae	*26*	*5.98*
Mixed infection	*25*	*5.75*
Klebsiella spp.	*17*	*3.91*
Staphylococcus aureus	*14*	*3.22*
Enterococcus faecalis	*10*	*2.30*
Lactococcus lactis	*9*	*2.07*
Serratia spp.	*7*	*1.61*
Corynebacterium spp.	*7*	*1.61*
Yeast	*6*	*1.38*
Streptococcus bovis	*5*	*1.15*
Other G-	*5*	*1.15*
Enterobacter spp.	*3*	*0.69*
Pasteurella spp.	*3*	*0.69*
Aerococcus viridans	*3*	*0.69*
Streptococcus spp.	*2*	*0.46*
Pseudomonas spp.	*2*	*0.46*
Citrobacter spp.	*2*	*0.46*
Trueperella pyogenes	*1*	*0.23*

335

336 **Table 2**

337 Median and interquartile ranges for cathelicidin and SCC.

Item	Sample class	Median	IQR
Cathelicidin (AOD450)			
	Healthy	0.089	0.084/0.094
	Clinical, All	11.850	3.090/27.120
	Clinical, C+	12.260	3.170/27.495
	Clinical, C-	10.620	2.803/18.920
SCC (cells/mL)			
	Healthy	7,500	3,000/21,000
	Clinical, All	5,588,000	2,540,000/7,814,000
	Clinical, C+	5,692,000	2,778,000/7,752,000
	Clinical, C-	5,049,000	1,106,000/7,992,000

338

339 **Table 3.** Median and interquartile ranges of cathelicidin and SCC observed for the different
340 microorganism groups. Groups are reported in decreasing order (rank) according to the respective
341 median value.

Cathelicidin (AOD450)				SCC (cells x 10^3/mL)			
R	Sample class (N)	Median	IQR	R	Sample class (N)	Median	IQR
1	*Strep. agalactiae* (59)	27.480	12.290/30.100	1	Mixed infection (25)	6,543	4,316/8,409
2	*Staph. aureus* (14)	16.165	7.071/22.302	2	*Strep. agalactiae* (59)	6,362	4,586/7,992
3	*Streptococcus* spp. (99)	13.020	3.727/30.000	3	Other (36)	5,818	3,748/8,665
4	Gram negative (102)	11.640	4.805/22.302	4	*Staph. aureus* (14)	5,513	3,108/7,368
5	Culture negative (59)	10.620	2.803/18.920	5	Gram negative (102)	5,522	2,762/7,521
6	Mixed infection (25)	8.970	3.023/30.100	6	*Streptococcus* spp. (99)	5,405	2,461/7,394
7	Other (36)	6.420	2.454/20.827	7	Culture negative (59)	5,049	1,106/7,992
8	CNS (41)	3.120	0.866/9.055	8	CNS (41)	3,037	1,078/6,146

342 R: rank; N: number of samples in the class; IQR, interquartile range. Other: *Enterococcus faecalis*,
343 *Lactococcus lactis*, *Corynebacterium* spp., Yeast, *Aerococcus viridans*, *Trueperella pyogenes*.
344

345 **Table 4.** Median and interquartile ranges of cathelicidin and SCC observed for the different
346 microorganisms in the 435 clinical samples. Microorganism classes are reported in decreasing order
347 (rank) according to the respective median value.

Cathelicidin (AOD450)				SCC (cells x 10^3/mL)			
R	Sample class (N)	Median	IQR	R	Sample class (N)	Median	IQR
1	*Streptococcus agalactiae* (59)	27.480	12.290/30.000	1	*Enterococcus faecalis* (10)	8,184	4,248/9,259
2	*Enterococcus faecalis* (10)	20.065	9.719/27.452	2	*Serratia* spp. (7)	7,330	1,075/9,663
3	*Streptococcus dysgalactiae* (26)	16.260	2.970/30.000	3	Mixed infection (25)	6,543	4,316/8,409
4	*Staphylococcus aureus* (14)	16.165	7.071/22.302	4	*Streptococcus agalactiae* (59)	6,362	4,586/7,992
5	*Klebsiella* spp. (17)	15.350	11.795/30.000	5	*Corynebacterium* spp. (7)	6,029	2,859/9,483
6	*Streptococcus uberis* (66)	14.350	3.962/30.000	6	*Streptococcus uberis* (66)	5,685	2,335/7,777
7	*Escherichia coli* (63)	11.670	5.080/22.160	7	*Escherichia coli* (63)	5,536	3,945/7,103
8	Culture negative (59)	10.620	2.803/18.920	8	*Klebsiella* spp. (17)	5,526	1,719/7,572
9	Mixed infection (25)	8.970	3.023/30.000	9	*Staphylococcus aureus* (14)	5,513	3,108/7,368
10	Other Gram-positive (11)	5.800	1.962/12.420	10	*Streptococcus dysgalactiae* (26)	5,341	2,349/7,171
11	*Lactococcus lactis* (9)	5.620	2.888/23.355	11	Yeast (6)	5,318	600/8,894
12	Other Gram-negative (15)	5.280	0.543/27.190	12	*Lactococcus lactis* (9)	5,209	3,751/7,275
13	Yeast (6)	4.869	0.364/17.062	13	Culture negative (59)	5,049	1,106/7,992
14	CNS (41)	3.120	0.866/9.055	14	Other Gram-negative (15)	4,469	2,556/8,059
15	*Corynebacterium* spp. (7)	3.040	1.275/7.310	15	Other Gram-positive (11)	4,336	3,406/7,016
16	*Serratia* spp. (7)	2.947	1.962/9.070	16	CNS (41)	3,037	1,078/6,146

348 R: rank; N: number of samples in the class; IQR, interquartile range. Other Gram-negative: Gram-negative
349 bacilli, *Enterobacter* spp., *Pasteurella* spp., *Pseudomonas* spp., *Citrobacter* spp. Other Gram-positive:
350 *Aerococcus viridans*, *Streptococcus bovis*, *Steptococcus* spp., *Trueperella pyogenes.*
351

FIGURES

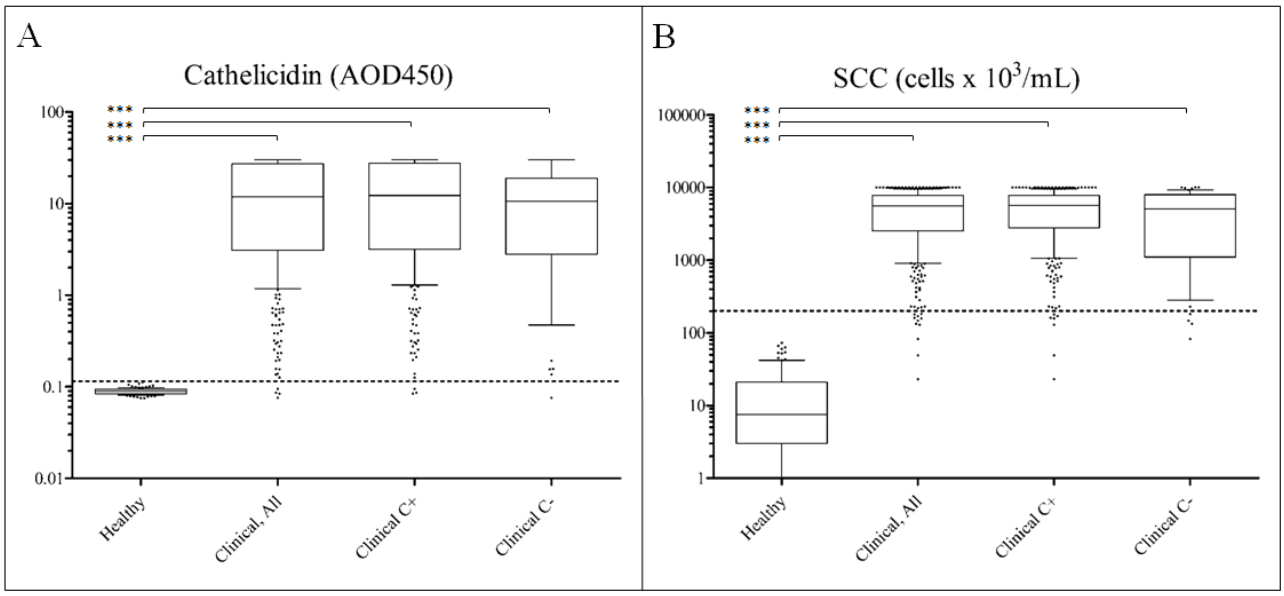

Figure 1. Distribution on a $\log_{10}$ scale of cathelicidin (A) and SCC (B) values measured in the 535 samples, plotted according to the clinical condition and to culture results. The boxes indicate values falling within the 25th and 75th percentiles, with the central line indicating the median value. Whiskers indicate values falling within the 10th and 90th percentiles, and the individual dots represent values falling outside the whiskers. The dashed lines indicate the positivity threshold for each plot (0.115 AOD450 for cathelicidin, A, and 200,000 cells/mL for SCC, B). Statistically significant differences (***, $P \leq 0.001$) among classes according to the Kruskall-Wallis test with Dunns post-test correction are indicated by a continuous line.

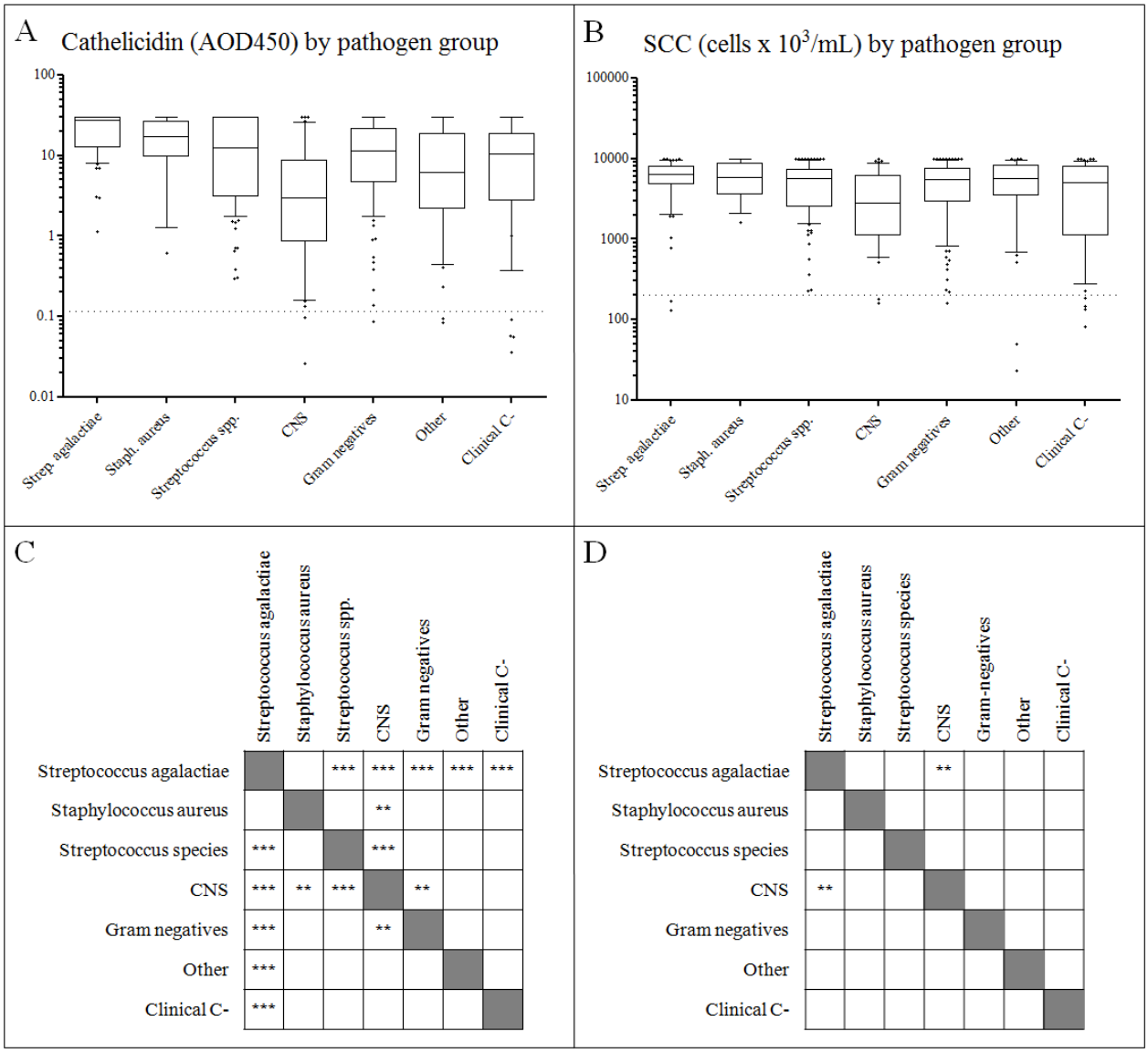

Figure 2. Top. Distribution on a $\log_{10}$ scale of cathelicidin (A) and SCC (B) values measured in the 435 clinical milk samples according to the pathogen group. The box indicates values falling within the 25th and 75th percentiles, with the central line indicating the median value. Whiskers indicate values falling within the 10th and 90th percentiles, and the individual dots represent values falling outside the whiskers. The dashed lines indicate the positivity thresholds for each plot (0.115 AOD450 for cathelicidin, A, and 200 cells x 10^3/mL for SCC, B). Bottom. Matrix tables summarizing statistical significance of the differences for cathelicidin (C) and SCC (D), respectively. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

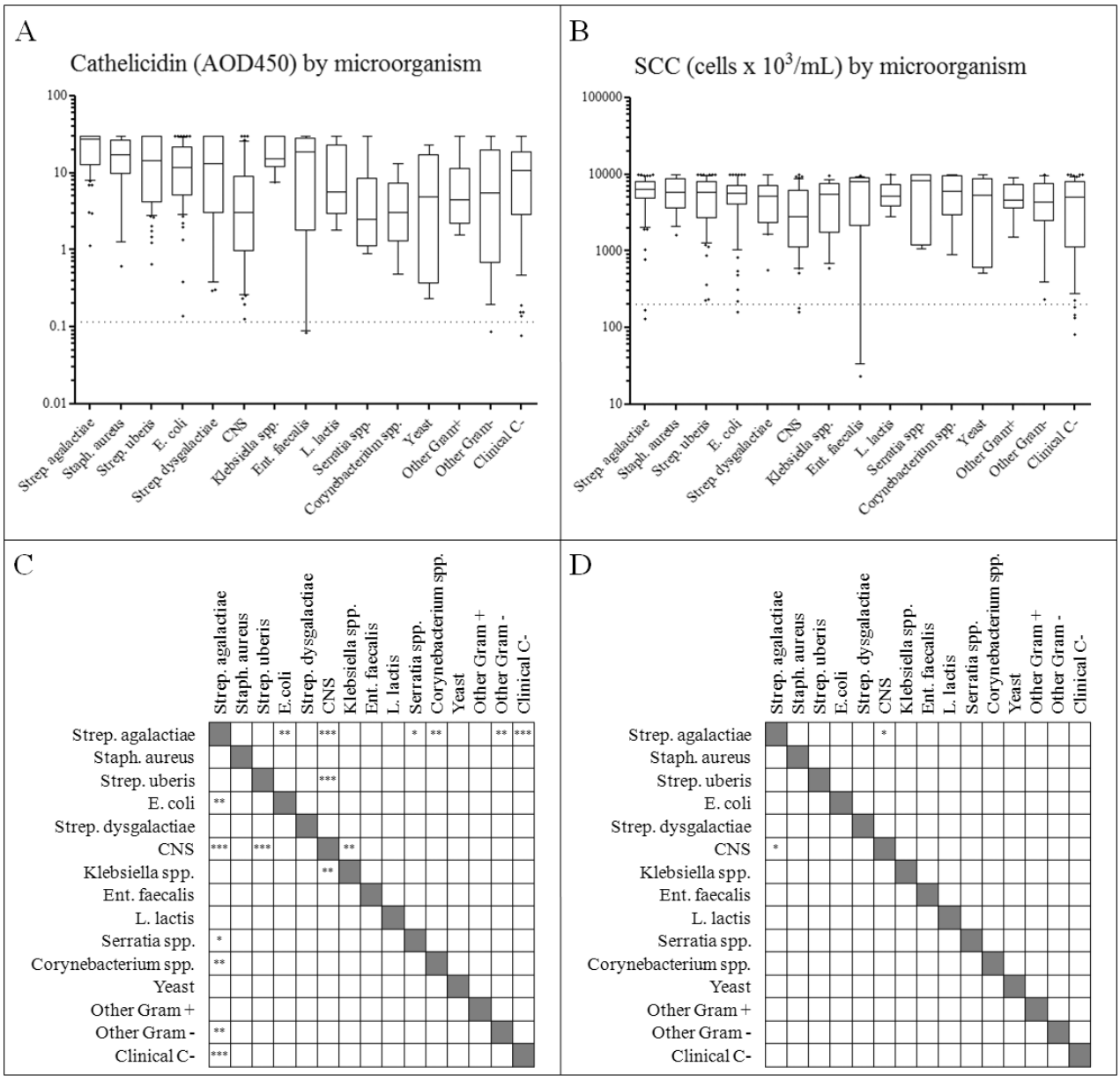

Figure 3. Top. Distribution on a $\log_{10}$ scale of cathelicidin (A) and SCC (B) values measured in the 435 clinical milk samples according to the microbiologic culture results. The box indicates values falling within the 25th and 75th percentiles, with the central line indicating the median value. Whiskers indicate values falling within the 10th and 90th percentiles, and the individual dots represent values falling outside the whiskers. The dashed lines indicate the positivity thresholds for each plot (0.115 OD450 for cathelicidin, A, and 200 cells x 10^3/mL for SCC, B). Bottom. Matrix tables summarizing statistical significance of the differences for cathelicidin (C) and SCC (D), respectively. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

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