

Levels of milk glycosaminoglycans in Holstein cows with clinical and subclinical mastitis

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Abstract: Mastitis is an inflammation of the mammary gland whose diagnosis is based on clinical criteria and the determination of inflammatory markers. The disease is mainly caused by bacteria causing intra-mammary infections. The etiological agent/s are detected by milk culture or by molecular biology approaches. However, these methodologies are unpractical for on-farm diagnosis, where rapid decisions must be made when infected animals are detected. For this reason, the levels of glycosaminoglycans (GAGs) in milk were evaluated as a tool for the diagnosis of mastitis. GAGs levels were determined by the dimethylmethylene blue assay (DMMB) method in milk from healthy animals and in milk from animals with mastitis caused by different etiological agents. Low levels of GAGs (1.93 ± 0.40 mg) were found in milk samples obtained from healthy animals (H), while intermediate (2.4 ± 0.61 mg) and high levels (2.87 ± 0.7 mg) were found in milk obtained from cows with subclinical (SM) and clinical mastitis (CM), respectively. Statistically significant differences were found between the SM and H groups ($p < 0.001$) and between CM and the other groups ($p < 0.0001$). The highest levels of GAGs were detected in the CM group, in which coagulase negative *Staphylococcus* (CNS) and *Streptococcus* spp. were isolated. Animals infected by *S. aureus* had the highest levels of GAGs in the SM group. The sensitivity (Se) and specificity (Sp) for the assessment of GAGs levels for CM diagnosis were 89.7 % and 81.6 %, respectively. For the diagnosis of SM, Se and Sp were 73.2 % and 95 %, respectively. The area under the curve for the diagnosis of CM was 0.902 ($p < 0.0001$), while that for SM was 0.833 ($p < 0.0001$). Results suggest that the measurement of GAGs levels in milk can be used as an indicator of udder health in dairy cattle, since increased levels of GAGs were associated with mastitis.

Key words: Bovine mastitis, glycosaminoglycans, milk

1. Introduction

Bovine mastitis is a disease with high morbidity within dairy herds, reducing the profitability of milk production [1]. Not only does mastitis decrease the production of the affected animals, but it also increases the expenditure due to veterinary treatments, the high amounts of milk that have to be discarded, and the premature rejection of dairy cows from the herd, causing an economic loss of about 70 %. Two types of mastitis have been described, i.e., clinical and subclinical [2]. The incidence of subclinical mastitis is high in dairy herds, varying from 10 % to 50 %, generating great economic losses [3]. A study performed by Dieser et al. (2013) indicated that the prevalence of cows with subclinical mastitis in herds from Córdoba, Argentina is 53.9 % [4]. The premature rejection of dairy cows is due to the irreversible mammary tissue damage caused by subclinical mastitis [5].

The etiology of bovine mastitis is mainly bacterial, being *Staphylococcus aureus* (*S. aureus*), *Streptococcus*

spp., and *Escherichia coli* (*E. coli*) the most frequent microorganisms found in clinical samples [6]. Coagulase negative staphylococci (CNS) are mostly associated with subclinical mastitis [7].

The early detection of mastitis is crucial to keep the herd healthy by isolating and treating infected animals [8]. The reference method for the detection of the main bacterial mastitis agents in milk samples is the bacteriological culture. This method, however, is time-consuming and difficult to apply as a routine monitoring strategy. One of the most common methods for the diagnosis of mastitis is the California Mastitis Test (CMT), which is based on the semiquantification of DNA from somatic cells in milk samples by the addition of a detergent. CMT is suitable for the detection of the inflammation caused by most mastitis pathogens, having a sensitivity of 66.7 % for larger pathogens such as *S. aureus*, while, for minor pathogens, the sensitivity is 49.5 %. The highest sensitivity of CMT is observed for environmental streptococci (84

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%) [9]. An alternative to on-farm SCC tests is the somatic cells count to make early management decisions [10]. Modern methods evaluate the enzymatic activity of lactate dehydrogenase (LDH) during the milking procedure. A rapid test performed in the field would accelerate the diagnosis of mastitis decreasing the negative economic impact [11]. In this sense, glycosaminoglycans (GAGs) levels could be used as a marker.

GAGs are long unbranched polysaccharides that are hydrophilic in nature due to the presence of carboxyl groups and sulfates in their structure [12]. In the extracellular matrix, GAGs were found as a gel, giving support to different tissues [13]. Most GAGs are covalently linked to core proteins forming proteoglycans [14]. The most important GAG in the mammary gland is the hyaluronic acid. This GAG has been shown to perform important biological functions during embryogenesis, morphogenesis, tissue turnover and inflammation of this organ [15]. Versican is a GAG controlling the activity of stem cells in mammary tissue remodeling [16]. Small leucine-rich proteoglycans (decorin and lumican) regulate cell growth [17]. Not only are GAGs structural components of the extracellular matrix, but they also play a role in regulating the cellular activity of the tissues [18]. GAGs are also present in milk, where they play an immunoregulatory role in the mammary gland [19]. The composition of GAGs in processed and packaged milk has previously been described [20]. However, the behavior of GAGs in fresh unpasteurized milk from dairy cows with clinical and subclinical mastitis remains to be determined. The aim of this study was to quantify the levels of total GAGs in milk from dairy cows with clinical and subclinical mastitis caused by different etiological agents. The quantification of GAGs could be an alternative diagnostic tool for mammary affections in dairy cows.

2. Material and methods

2.1. Animals and experimental model

Individual milk samples were collected from 480 cows at 8 dairy farms located in Buenos Aires, Argentina. Details of herd sizes are presented in Table 1. The assumptions used to calculate the sample size of cows in every dairy farm were: 95 % confidence and a 10 % maximum allowable error. This allowed us to compare differences between mean values. Animals were divided into 3 groups: healthy (H) (n = 250), clinical mastitis (CM) (n = 80) and subclinical mastitis (SM) (n = 150). Group H included animals without clinical or subclinical mastitis and with a bacteriological culture with no growth. At the moment of sampling, animals included in the CM group presented a certain degree of affection. Clinical mastitis was classified into three grades. Grade I: abnormal milk secretion. Grade II: abnormal milk secretion and swelling of the affected quarter. Grade III: abnormal milk secretion accompanied by swelling of the affected quarter and systemic signs, like fever [21]. The SM group included animals with subclinical mastitis as determined by the CMT and the somatic cells count. Cows from the SM group were subdivided into three grades according to the CMT results (Grades I-III) [10]. After performing the CMT, samples were collected according to the National Mastitis Council, Inc. [22]. 10 mL per sample were collected from individual quarters. Briefly, a first milk jet was done to visualize the presence of flocs. This sample was then discarded. Aliquots from subsequent jets were kept at 6 °C for the leukocyte count and at -20 °C for the other determinations. Productive data of all sampled animals were collected to determine expected variations throughout lactation in the H group. Animals belonging to the H group were divided into three groups according to their daily production levels: low level (< 20 L), medium level (20–30 L), and high level (>

Table 1. Animal sampled per group in dairy farms and GAGs level in healthy cows and cows with subclinical and clinical mastitis.

Dairy farm	N° of lactating cow	N° of animals sampled per group			GAGs level (mg/dL)		
		H	SM	CM	H	SM	CM
1	593	31	23	3	1.81 ± 0.06 [95% CI=1.6–1.9]	2.55 ± 0.36 [95% CI= 2.34–2.95]	3.22 ± 0.8 [95%CI= 2.85–3.84]
2	151	34	23	6	2.15 ± 0.45 [95% CI= 1.68–2.52]	2.22 ± 0.68 [95% CI=1.83–2.34]	3.02 ± 0.92 [95% CI=2.06–4.12]
3	105	17	20	8	1.96 ± 0.30 [95% CI=1.85–1.97]	2.62 ± 0.36 [95% CI=2.42–2.73]	3.64 ± 0.45 [95% CI=3.26–4.06]
4	700	30	23	20	1.85 ± 0.14 [95% CI=1.85–1.97]	2.67 ± 0.35 [95% CI=2.49–2.84]	2.74 ± 0.52 [95% CI=2.41–3.16]
5	330	38	12	10	1.80 ± 0.13 [95% CI=1.74–1.97]	2.58 ± 0.33 [95% CI=2.39–2.96]	3.63 ± 0.29 [95% CI=3.23–3.99]
6	718	47	11	16	1.73 ± 0.16 [95% CI=1.8–1.91]	2.19 ± 0.58 [95% CI=1.92–2.52]	2.73 ± 0.59 [95% CI=1.96–3.58]
7	202	20	23	10	2.17 ± 0.55 [95% CI=1.6–2.76]	2.59 ± 0.34 [95% CI=2.39–2.96]	3.61 ± 0.46 [95% CI=3.23–4.06]
8	145	33	15	7	1.98 ± 0.13 [95% CI=1.88–2.04]	2.69 ± 0.25 [95% CI=2.29–20.96]	3.16 ± 0.66 [95% CI=2.66–3.71]

30 L). The H group was also divided into three subgroups according to the stage of lactation. The first subgroup corresponded to dairy cows from day 0 to day 100 of lactation ($n = 80$), the second were cows from day 100 to 200 of lactation ($n = 100$) and in the third subgroup the animals between days 200 and 305 ($n = 70$). Experimental procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL, University of Buenos Aires, Argentina) and all herd managers agreed to participate in the study.

2.2. California Mastitis Test

The CMT was performed in animals showing no evidence of clinical mastitis. This test allowed detecting cows with subclinical mastitis. A positive CMT correlates with a somatic cell count greater than 200,000 cells/mL [23]. The CMT was performed according to the manufacturer's recommendations (Pampafarma, Argentina). Briefly, 5 ml of sample were mixed with an equal volume of CMT reagent in each cup of a paddle. According to the degree of gelification, samples were classified as negative (no gelification), positive grade I (low gelification and 200,000–1,200,000 cells/mL), positive grade II (intermediate gelification and 1,200,000–5,000,000 cells/mL) and positive grade III (high gelification > 5,000,000 cells/mL) [24].

2.3. Somatic cell count

The somatic cells count was performed in duplicate in a Neubauer's hemocytometer under a light microscope at 40X. Samples were diluted 1:200 in diluent for white blood cell counting (Biopur S.R.L., Rosario, Argentina).

2.4. Bacteriological studies

Briefly, 50 μ L of milk were plated onto blood agar containing 0.1 % aesculin and incubated at 37 °C during 24–48 h. According to the National Mastitis Council, Inc., this selective culture medium is used to isolate a variety of pathogens known to cause mastitis [22]. Samples with three or more pathogens were considered contaminated and were excluded from the study.

2.5. Determination of GAGs levels

GAGs levels were determined by the dimethyl methylene blue (DMMB) method with little modifications. Briefly, 200 μ L of a solution containing 16 μ g/mL DMMB were mixed with 500 μ L of sample diluted 1:10 in distilled water [25]. The absorbance of the mixture was read at 525 nm (Metrolab 1000, Argentina). A standard curve was generated using known concentrations of chondroitin sulfate (Sigma-Aldrich, USA).

2.6. Statistical analysis

A one-way analysis of variance (ANOVA) was used to compare means among groups. The GraphPad Prism 6.0 statistical package (GraphPad Software Inc, San Diego, CA, USA) was used. Results were expressed as mean values \pm SD

and the corresponding confidence interval (CI). When the distribution was not Gaussian, the Kruskal–Wallis test was performed. A difference was considered significant when $p < 0.05$. When significant differences were found, means were compared by Tukey's test for multiple comparisons.

The statistical analysis to determine the area under the receiver operating characteristic (ROC) curve, sensitivity (Se), specificity (Sp), the area under the curve (AUC), the Youden index, and positive and negative predictive values were performed with the XLSTAT 2020.2.2 software (Addinsoft, USA) [26].

Values of GAGs obtained from animals with CM and SM were used to determine the sensitivity and specificity of the DMMB test. In CM group, we used clinical diagnosis, described in animals and model experiment, as the gold standard test for clinical mastitis diagnosis. Meanwhile, in SM group, we used CMT as the gold standard test for the diagnosis of subclinical mastitis. The point on the ROC curve closest to the upper left corner that optimizes prevalence-independent summary measures of Se and Sp, assessed as the Youden index ($J = Se + Sp - 1$) was selected as a potential cut-off [27]. Three different scenarios of prevalence (low, 10 %; medium, 20 % and high 30 %) were evaluated to calculate predictive values for subclinical mastitis. The prevalence of the scenarios selected was chosen according to Dieser et al. [4].

3. Results

The comparison of GAGs levels among H, SM, and CM groups are shown in Figure 1. Healthy animals had significantly lower GAGs levels, the SM group had an intermediate level, while the CM group had the highest GAGs levels. Statistically significant differences were found between the SM and H groups ($p < 0.001$) and between CM and the other groups ($p < 0.0001$).

During lactation, GAGs levels remained constant in the three subgroups of H cows. Cows from day 0 to day 100 of lactation presented levels of 1.82 ± 0.10 mg (95 % CI=1.63–2.04); cows from day 100 to 200 of lactation presented levels of 1.91 ± 0.13 mg (95 % CI=1.79–2.10); and animals analyzed between days 200 and 305 of lactation displayed levels of 1.93 ± 0.12 mg (95 % CI= 1.66–1.97) ($p > 0.05$). No significant differences were found in the GAGs levels among productivity groups. Cows with low production levels had 1.81 ± 0.14 mg (95 % CI=1.72–1.90), animals with medium production levels had 1.84 ± 0.10 mg (95 % CI= 1.73–1.85) and animals with high production levels had 1.91 ± 0.15 mg (95 % CI= 1.70–2.03) ($p > 0.05$). The number of sampled animals on each dairy farm in each group (H, SM and M) and GAGs levels for each dairy herd are shown in Table 1.

The GAGs mean levels in animals with different grades of CM are exposed in Figure 2A. We found no significant

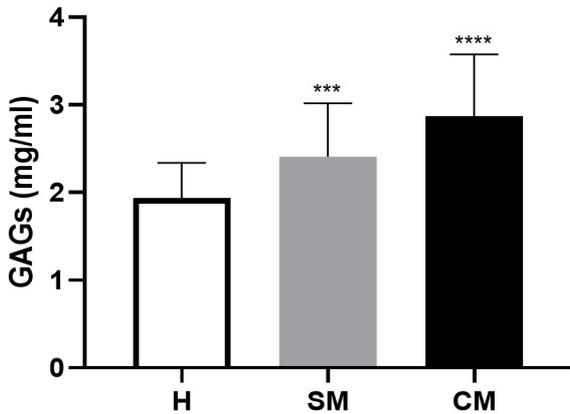


Figure 1. Levels of GAGs in healthy animals and animals with clinical and subclinical mastitis *** SM vs. H ($p < 0.001$), **** CM vs. H and SM ($p < 0.0001$).

differences between different degrees of clinical mastitis. Animals belonging to the CM group (grades I, II and III) had significantly higher GAGs levels than those of the H group (grade I vs. H, $p < 0.001$; grade II vs. H, $p < 0.01$; and grade III vs. H, $p < 0.05$). When the etiological agents of CM were considered in the analysis, it was found animals infected with SCN ($n = 10$) *Streptococcus* spp. ($n = 11$) *S. aureus* ($n = 30$) and with *E. coli* ($n = 15$). We also had samples with no growth ($n = 14$). No significant differences were found in the GAGs levels among the groups of animals infected with different pathogens. Upon comparing the GAGs levels between animals with CM and those from the H group, it was found that those infected with *S. aureus*, SCN and *Streptococcus* spp. showed the highest statistical difference ($p < 0.0001$), as compared to

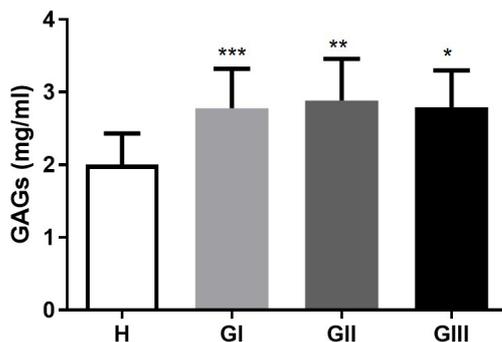


Figure 2A. Levels of GAGs in animals with different grades of clinical mastitis *** GI vs. H ($p < 0.001$), ** GII vs. H ($p < 0.01$) and *GIII vs. H ($p < 0.05$). **FIGURE 2B.** Levels of GAGs in animals with different etiological agents of clinical mastitis *** *S. aureus*, SCN and *Streptococcus* spp. H ($p < 0.0001$), *** *E. coli* vs. H ($p < 0.001$) and negative vs. H ($p < 0.01$).

H vs. *E. coli* ($p < 0.001$) and H vs. animals with negative culture ($p < 0.01$) (Figure 2B).

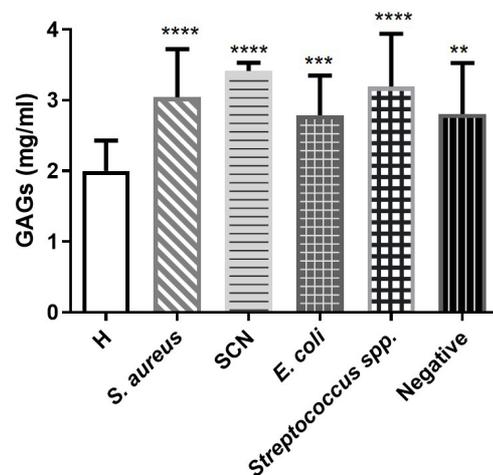
In Figure 3A, we can visualize values of GAGs in SM animals diagnosed by CMT. No significant differences were found among the different grades. Significant differences were found for SM grade I vs. the H group ($p < 0.001$).

The pathogens isolated from the milk of dairy cows with SM were *S. aureus* ($n = 70$), *Streptococcus* spp. ($n = 20$), SCN ($n = 40$), and gram negative bacteria ($n = 10$) such as *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. The remaining animals were negative ($n = 10$). Moreover, it was observed that the highest levels of GAGs were found in the animals infected with *S. aureus*. Significant differences were found for *S. aureus* ($p < 0.0001$), SCN and *Streptococcus* spp. ($p < 0.001$) when compared to the H group (Figure 3B).

The ROC analysis for GAGs in cows with clinical and subclinical mastitis compare with healthy animals is available in Table 2. There we can observe a GAGs better performance in animals with clinical mastitis. Then, we compared the GAGs performance with SCC. The results are in Table 3, and, again, we can observe a better performance of GAGs in animals with clinical mastitis than in those with subclinical mastitis. Although GAGs in animal with subclinical mastitis had an acceptable performance compared to SCC, the determination of them could be useful for early detection of subclinical mastitis in dairy cows. PPV and NPV for SM in different prevalence scenarios are shown in Table 4.

4. Discussion

In this study, we determined the levels of GAGs in different types of bovine mastitis, also analyzed the usefulness of the DMBB test for the diagnosis of mastitis. According to



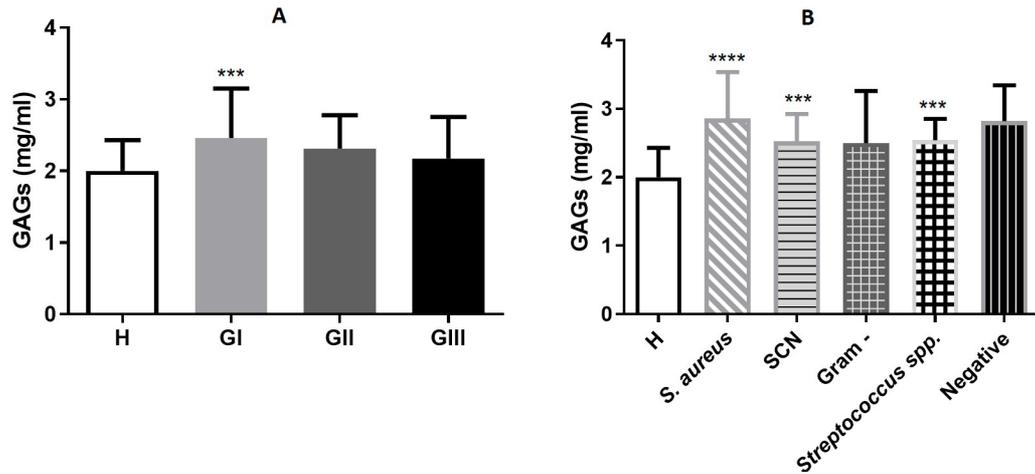


Figure 3A. Levels of GAGs in animals with different grades of subclinical mastitis *** GI vs. H ($p < 0.001$). **FIGURE 3B.** Levels of GAGs in animals with different etiological agents of subclinical mastitis. **** *S. aureus*, vs. H ($p < 0.0001$), *** SCN and *Streptococcus spp.* vs. H ($p < 0.001$).

Table 2. ROC analysis of GAG in cows diagnosed with clinical and subclinical mastitis compared with healthy ones.

Predictive values	Clinical Mastitis	Subclinical Mastitis
Area under ROC curve (95% CI)	0.902 [0.83–0.97]	0.833 [0.79–0.96]
p value	< 0.0001	< 0.0001
Sensitivity (95% CI)	89.7 [72.6–97.1]	73.2 [57.9–84.4]
Specificity (95% CI)	81.6 [68.3–90.2]	95 [74.3–1]
Positive predictive value	74.3 [57.6–86.9]	96.8 [84.5–1]
Negative predictive value	93 [85.1–1]	63.3 [62.8–93]

Table 3. ROC curve and sensitivity/specificity of the GAGs test compared with SCC.

Predictive values	Clinical Mastitis		Subclinical Mastitis	
	GAGs	SCC	GAGs	SCC
Area under ROC curve (95% CI)	0.977 [0.93–1]	0.833 [0.54–1.00]	0.867 [0.657–1.00]	0.924 [0.845–0.981]
P value	< 0.0001	< 0.01	< 0.001	< 0.0001
Sensitivity (95% CI)	93 [76–98.8]	89.7 [72.6–97.1]	93.1 [77.1–89.1]	93.3 [77.4–99.1]
Specificity (95% CI)	88.3 [78.4–99.4]	79.6 [66.1–88.6]	65 [43.1–81.19]	75 [52.6–89]
Positive Predictive Value	87.3 [78.5–96.1]	72.2 [57.6–86.9]	80 [66.7–93.3]	84.8 [72.6–97.1]
Negative Predictive Value	77.8 [68.2–87.4]	92.9 [85.1–1.00]	86.7 [69.5–1.00]	88.2 [72.9–1.00]

our results, GAGs levels in milk samples could be used to distinguish between healthy animals and animals affected by subclinical and clinical mastitis. Inflammatory tissue damage is known to lead to an increase in the levels of GAGs [28]. In this regard, the results obtained in milk samples from dairy cows with clinical mastitis are in agreement with those found in other inflammatory diseases such as

hepatic dysfunction, systemic lupus erythematosus, and osteoarthritis [29,30].

No significant differences were found in the GAGs levels among the different grades of clinical mastitis, but significant differences were found among the different grades and healthy cows. The same pattern, although with slight differences, was observed for the different

Table 4. Predictive values (95% IC) of GAGs test for subclinical mastitis in different prevalence scenarios.

Predictives values GAGs	PREVALENCE		
	10%	30%	50%
PPV	32.8 [18.9–36.8]	53.3 [36.8–69.9]	72.7 [58–87.5]
NPV	98.9 [93.5–1.00]	95.8 [85.6–1.00]	90.7 [76.01–1.00]

grades of subclinical mastitis. It was also found that GAGs levels were increased in cows with grade I SM. The latter finding is of relevance, since the CMT is rather deficient for the diagnosis of this type of mastitis [31,32]. The uneven distribution of GAGs among the different grades of clinical mastitis could be attributed to the variety of etiological agents and different stages of the inflammatory process [33]. This could be associated, in part, with the increased activity of MMP-2 and MMP-9 during clinical and subclinical mastitis [34].

As mentioned above, differences between grades of clinical or subclinical mastitis could be due to the etiological agents. In this sense, it is known that bacterial proteases from *S. aureus* and *Streptococcus pneumoniae* can degrade the protein component of the proteoglycan, thus increasing GAGs levels in body fluids [35]. In addition, it has been demonstrated that bacteria belonging to the genus *Streptococcus*, *Escherichia*, and *Pasteurella* can synthesize GAGs [36]. Therefore, both the degradative activity of bacterial proteases on extracellular matrix proteoglycans and/or the bacterial synthesis would explain the increase of GAGs levels in infectious diseases of the mammary gland. Increased GAGs levels have also been found in noninfectious inflammatory conditions, which would explain the increased GAGs levels observed in culture negative mastitis [37]. It is also possible that, in these animals, the bacteriological methods employed were not suitable to isolate the etiological agent of mastitis.

In our work, the highest levels of GAGs were detected in subclinical mastitis and clinical mastitis caused by *S. aureus*. This may be due to the high levels of proinflammatory cytokines that are secreted in response to the infection with *S. aureus* [38]. This finding is of relevance, considering that *S. aureus* is a major pathogen in bovine mastitis and is associated with important economic losses [39]. No correlation was found between the levels of GAGs and the bacterial species causing mastitis, probably due to the fact that GAGs are released into the milk during inflammatory responses other than mastitis. Further studies will be carried out to determine the composition of GAGs in milk from cows with mastitis of different stages of chronicity. Determining the type/s of GAG involved in mastitis could help in the identification of the bacteria involved in the infectious process [40].

Although bacterial culture is the gold standard diagnostic test in mastitis, this method is not always successful [27]. Another option is the determination of SCC that is spread in the productive context. SCC is still considered the most suitable and accurate parameter for detecting the presence of IMI. Although SCC allows separating infected cows from healthy ones, it is far to be a perfect method [41].

GAGs determination can clearly distinguish healthy animals from ill ones with clinical or subclinical mastitis presentations. The GAGs measurement could display a suitable value of AUC of ROC curves in subclinical mastitis; moreover, for clinical mastitis, this value was higher. This is a relevant finding considering robotic milking systems where the technicians must diagnose cows with clinical mastitis with the information supported by the software of the milking system. The information supported by these systems is poorly specific for clinical mastitis [42]. For this reason, GAGs determination could be important, since milk levels could be only influenced by local tissues inflammation. Therefore, the determination of GAGs levels could be standardized for the automated processing of samples. Moreover, commercial kits are available for GAGs quantification on-farm in only 40 min [43]. This could be an option for dairy farms with not robotic milking machine with online analysis for subclinical mastitis diagnosis. Moreover, GAGs determination had a similar performance to SCC online determination and flow cytometry method [41,44].

The comparison of the predictive values for the detection of GAGs levels in different prevalence scenarios allowed concluding that this test is more efficient when prevalence values are high. Although the technique seems to be suitable for a semiextensive productive system compared with SCC, further studies in herds with a low mastitis prevalence must be carried out to evaluate the usefulness of the GAGs test with different cut-off points [45,46]. But when we observe the predictive values of SCC by online determination, we observe a low NPV. So, from the mix of two methods like SCC online determination and the GAGs evaluation we can obtain very reliable results [41].

In summary, the assessment of GAGs levels proved to be a useful tool to detect the presence of pathological

processes in mammary gland and to predict the productive performance of dairy cows. Results are more consistent for clinical mastitis, and this is very important for robotic dairy farms; in subclinical mastitis, results are similar to those obtained with an SCC, but the diagnosis could be more reliable with the combination of both evaluations, especially in dairy herds with a low prevalence of subclinical mastitis.

5. Conclusion

This study suggests that GAGs levels can be used as a marker of mammary health, since they remain constant in

healthy animals and rise up even during mild pathological processes. Since this method is simple and not time-consuming, it can be considered in the future as an alternative for on-farm technique or online determinations included in robotic milking systems for the detection of clinical mastitis, or the method can be combined with SCC for the detection of subclinical mastitis.

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