1 **INTERPRETIVE SUMMARY**

2 **The value of the biomarkers cathelicidin, milk amyloid A and haptoglobin to diagnose**
3 **and classify clinical and subclinical mastitis.** By L. Wollowski et al.

4 Mastitis is the inflammation caused by an infection of the udder and one of the most
5 common diseases in dairy cows. A correct and timely diagnosis allows informed therapeutic
6 decisions and reduces economic losses. In our study we tested three different biomarkers
7 (cathelicidin, milk amyloid A, and haptoglobin) for their diagnostic value.

8 By measuring those biomarkers in milk, it was possible to reliably detect cows with
9 mastitis and differentiate between different types of mastitis. Our results encourage further
10 research, as the measurement of biomarkers is objective.

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13 **The value of the biomarkers cathelicidin, milk amyloid A and haptoglobin to diagnose**
14 **and classify clinical and subclinical mastitis**

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25

26 **ABSTRACT**

27 Timely and objective diagnosis and classification of mastitis is crucial to ensure
28 adequate management and therapeutic decisions. Analyzing specific biomarkers in milk could
29 be advantageous compared to subjective or semi-quantitative criteria such as palpation of the
30 udder in clinical mastitis cases or evaluation of somatic cell count using cow side tests (e.g.,
31 California Mastitis Test) in subclinical mastitis quarters. The objective of this study was to
32 investigate the diagnostic value of three biomarkers; i.e., cathelicidin, milk amyloid A, and
33 haptoglobin for the diagnosis of subclinical and clinical mastitis. Furthermore, the suitability of
34 these biomarkers to differentiate between mild, moderate and severe clinical mastitis and the
35 influence of different pathogens on biomarker levels was tested. A total of 67 healthy cows,
36 119 cows with subclinical, and 212 cows with clinical mastitis were enrolled in the study. While
37 cathelicidin, haptoglobin and milk amyloid A were measured in all samples from healthy cows
38 and those with subclinical mastitis, haptoglobin and cathelicidin results were only available
39 from 121 out of 212 cows with clinical mastitis. Milk amyloid A was measured in all samples.
40 In cows with clinical mastitis, the mastitic quarter and a second healthy quarter serving as a
41 healthy in-cow-control quarter were sampled. It was possible to differentiate between healthy
42 quarters, quarters with subclinical mastitis, and quarters with clinical mastitis, using all three
43 biomarkers. Concerning cathelicidin, thresholds were 0.000 NOD450 (Se= 0.83, Sp = 0.97) and
44 0.053 NOD450 (Se= 0.98, Sp = 0.99) for differentiating between healthy quarters and quarters
45 with subclinical or clinical mastitis, respectively. Thresholds of 1.28 µg/mL (Se= 0.65, Sp =
46 0.76) and 1.81 µg/mL (Se=0.77, Sp = 0.83) for milk amyloid A and 3.65 µg/mL (Se= 0.92, Sp
47 = 0.94) and 5.40 µg/mL mL (Se= 0.96, Sp = 0.99) for haptoglobin were calculated, respectively.
48 Healthy in-cow control quarters from healthy cows showed elevated milk amyloid A and
49 haptoglobin levels compared to healthy quarters from healthy cows. Only the level of milk
50 amyloid A was higher in severe clinical mastitis cases compared to mild ones. In contrast to

clinical mastitis, cathelicidin and haptoglobin in subclinical mastitis quarters were significantly influenced by different bacteriological results.

In conclusion, the measurement of cathelicidin, milk amyloid A, and haptoglobin in milk proved to be a reliable method to detect quarters with subclinical or clinical mastitis.

Key words: milk amyloid A, cathelicidin, haptoglobin, mastitis diagnostic

INTRODUCTION

Today's dairy industry with its increasing herd sizes (Barkema et al., 2015) and ongoing automatization of the milking process requires a reliable identification and classification of clinical mastitis (**CM**) cases to ensure adequate management and therapeutic decisions (Roberson, 2012). Effective diagnostic methods can lead to more efficient control of mastitis and promote a more responsible use of antimicrobial therapy (Krömker and Leimbach, 2017). A correct and constant scoring of the severity of CM cases allows for the prediction of treatment outcomes (Royster and Wagner, 2015).

In the past, treatment decisions for CM were usually based on farmer's or veterinarian's evaluation of clinical symptoms, e.g., changes of milk characteristics and clinical signs of the infected udder quarter (Swinkels et al., 2015). Manual palpation, however, is subjective with limited repeatability when multiple observers are involved (Houe et al., 2002; Rees et al., 2014) and its practicability is limited by herd size. Therefore, objective methods based on milk analysis become more important in CM diagnostic (Viguier et al., 2009).

Most dairy farmers and veterinarians focus on detection and treatment of CM complemented by prevention strategies. Management of subclinical mastitis (**SCM**), however, is hardly less important (Halasa et al., 2007) as SCM influences product quality, milk yield, and

75 overall productivity of a farm (Ruegg, 2017). Due to a lack of clinical signs, diagnosis of SCM
76 is mostly based on milk analysis.
77 Today, cow-individual somatic cell count (**SCC**) values are a well-accepted measure to
78 diagnose SCM (Ruegg, 2017). The California Mastitis Test (**CMT**), described by Schalm and
79 Noorland (1957) is widely used to evaluate SCC in milk. As it is a semi-quantitative measure,
80 however, the interpretation can be subjective, leading to false positive and negative results
81 (Viguier et al., 2009). Even interpreting CMT results by trained technicians may result in
82 mediocre sensitivity (**Se**) and specificity (**Sp**) values (i.e., 82.4% and 80.6%; Dingwell et al.,
83 2003). The interpretability of SCC results is further limited, as levels usually remain elevated
84 for several weeks after an intramammary infection, even after successful mastitis treatment
85 (Pyorala, 1988). Additionally SCC is affected by various physiological (e.g., stage of lactation,
86 age, and stress; Sharma et al., 2011) and environmental factors (e.g., geographical zones and
87 housing system; Bielfeldt et al., 2004).
88 Another method to identify subclinical mastitis quarters is via changes in milk
89 conductivity (Norberg et al., 2004), which can be monitored automatically and with a high Sp
90 (ranging from 97.3 to 99.3% depending on algorithms). Sensitivity (ranging from 5.5 to 42.9%
91 depending on algorithms), however, is not satisfying (Hovinen et al., 2006) as values do not
92 meet the required 80% Se for diagnostic tests in automatic milking systems (ISO, 2007).
93 Recent advances of proteomic techniques have led to the identification of several new
94 neutrophil-produced proteins, involved in mastitis immune responses (Lippolis and Reinhardt,
95 2005; Smolenski et al., 2007). These proteins might be suitable biomarkers usable for mastitis
96 diagnostic (Viguier et al., 2009; Ceciliani et al., 2012). One of the first proteins used to detect
97 mastitis in milk was lactate dehydrogenase. Albeit the measurement of this protein lacked in
98 accuracy (Nyman et al., 2016) it was demonstrated that in-line monitoring for lactate
99 dehydrogenase is feasible, can be integrated in automated milking systems (Åkerstedt et al.,

100 2011), and achieves a Se of 80% in SCM (Hiss et al., 2007). Just recently, acute phase proteins
101 like milk amyloid A (**MAA**) or haptoglobin (**HP**) have also been discovered as mastitis markers
102 (Jaeger et al., 2017; Sadek et al., 2017; Hussein et al., 2018). A study measuring milk amyloid
103 A showed that a more sensitive and specific identification of mastitis cows is possible compared
104 to SCC (Jaeger et al., 2017). Acute phase proteins like MAA or HP are part of the inflammatory
105 process following bacteriological infections and seem to be most promising for mastitis
106 diagnosis in ruminants (Eckersall et al, 2006, Tothova et al., 2014). In cases of inflammation of
107 the udder acute phase proteins diffuse from the blood into the milk but they also originate
108 directly from the mammary gland cells (Eckersall et al., 2001, Hiss et al., 2004). Recent studies
109 showed the diagnostic value of MAA and HP to diagnose SCM (Safi et al., 2009; Hussein et
110 al., 2018) and CM (Gronlund et al., 2003; Kalmus et al., 2013) in naturally occurring and
111 experimentally induced mastitis. Haptoglobin (Nielsen et al. 2004), as well as MAA can be
112 measured in milk (Eckersall et al., 2001).

113 Furthermore, cathelicidin (**CATH**) has been investigated in several research projects on
114 mastitis diagnostics in milk (Smolenski et al., 2011; Addis et al., 2016b; Addis et al., 2017).
115 Cathelicidins are peptides with proinflammatory and chemotactic functions (Zanetti, 2005) and
116 an antimicrobial activity in the immune defense (Smolenski et al., 2007; Smolenski et al., 2011;
117 Zhang et al., 2015). They initially originate from epithelial cells (Chromek et al., 2006; Addis
118 et al., 2011; Addis et al., 2013) and are later degranulated by migrated neutrophils (Reinhardt
119 et al., 2013, Pisanu et al., 2015). Consequently, the level of CATH in milk increases during
120 mastitis (Addis et al., 2016b; Pongthaisong et al., 2016). Elevated CATH levels were associated
121 with positive bacteriological results and increased SCC in CM (Addis et al., 2017).

122 So far, available literature, however, focused on the applicability of one biomarker at a
123 time. A recent study (Thomas et al., 2018) demonstrated promising results in diagnosing
124 naturally occurring bovine mastitis by examining 3 acute phase proteins (HP, C-reactive

125 protein, and mammary associated serum amyloid A3) simultaneously. Data on the
126 comparability, their correlation, and the association with the health status of a given udder
127 quarter (e.g., healthy, SCM, or CM) or the severity of the inflammation are, however, lacking.
128 Therefore, the objective of this study was to investigate and compare the diagnostic
129 value of CATH, MAA, and HP measured in milk to determine the udder health status.
130 Specifically, we set out to investigate the accuracy of each biomarker to differentiate between
131 1) CM, SCM and healthy udder quarters, 2) mild, moderate and severe CM, and 3) mastitis
132 caused by different bacteria. Furthermore, the Se and Sp for the differentiation between CM
133 quarters and healthy in-cow control quarters and healthy quarters of healthy cows and healthy
134 in-cow control quarters of cows with CM was determined.
135

136 **MATERIALS AND METHODS**

137 ***Animals and Experimental Design***

138 The study was conducted between June 2016 and January 2017 on a commercial dairy
139 farm in Brandenburg, Germany, housing approximately 2,500 dairy cows (305-d milk yield of
140 9,839 ± 1,887 kg; mean ± SD). Two hundred fifty-one, Holstein Friesian dairy cows with
141 clinical mastitis, 126 cows with subclinical mastitis and 70 healthy cows were initially included
142 in the study, respectively. At the time of enrollment, cows were between 1st and 9th lactation
143 (2.9 ± 1.5) and on average 168.7 ± 113.7 DIM. All cows were housed in a free stall barn with
144 slatted flooring and stall cubicles equipped with rubber mats. Cows were managed according
145 to the guidelines set by the International Cooperation on Harmonization of Technical
146 Requirements for Registration of Veterinary Medicinal Products (Hellmann and Radeloff,
147 2000). They were fed a TMR consisting of corn, beet pulp, alfalfa, bruised grain and rape, straw,
148 soybeans, and a concentrate mineral mix delivered on a conveyer belt system two times per
149 day. Rations were formulated to meet or exceed the dietary requirements for dairy cows (NRC,

150 2001). All cows had ad libitum access to water. Fresh cows and high lactating cows were milked
151 three times, late lactating cows two times a day in a 56-stall head-in rotary milking parlor.
152 Special groups (i.e., hospital pen, colostrum and mastitis group) were milked twice daily in a 2
153 x 10 herringbone milking parlor.
154 Cows with signs of CM (i.e., clotted milk, heat or swelling) were identified by milking
155 personnel during regular milking in the milking parlor, separated and fixed in a cattle chute for
156 further examinations. Healthy cows and those with SCM were preselected based on most recent
157 DHIA results (i.e., healthy: SCC < 10,000 cells/mL, SCM: SCC > 1,000,000 cells/mL). These
158 thresholds were used to increase the probability to truly identify a quarter with SCM in a cow
159 with high SCC in the composite sample. Thresholds were further used to find at least 1 healthy
160 quarter in cows, which have been healthy at the time of last DHI test day. After selection, cows
161 were separated and examined. Cows with CM within the last 30 d before enrolment, cows with
162 signs of metabolic or infectious disease (e.g., ketosis, hypocalcaemia, fever), and those that
163 received systemic or intramammary antibiotics or anti-inflammatory drugs were excluded from
164 the study. Furthermore, cows with any teat lesion or lacerations of the udder surface, cows with
165 mastitis in more than 1 quarter and cows within 5 d after calving were not enrolled.
166 Finally, total of 67 healthy cows, 119 cows with subclinical, and 212 cows with CM met
167 the including criteria of the study and were enrolled. In healthy cows and those with SCM
168 CATH, HP and MAA were measured in all samples. In CM cows results of HP and CATH
169 were only available from 121 out of 212. Milk amyloid A was measured in all samples.
170 After general examination (i.e., rectal temperature, pulse and respiration rate) of each
171 cow, an examination of the udder was conducted. Additionally, a CMT was done (KerbaTEST;
172 Albert Kerbl GmbH, Buchbach, Germany) and 2 milk samples per quarter were collected (i.e.,
173 1 sterile sample for bacteriological culturing and SCC, 1 unsterile sample for the analysis of
174 biomarkers).

175 In healthy cows and cows with SCM, the quarter was chosen based on CMT results
176 (e.g., SCM: ++ or +++, healthy: 0). In CM cows, however, milk samples from the mastitis
177 quarter as well as 1 healthy in-cow control quarter were analyzed. Healthy quarters and healthy
178 in-cow control quarters were preselected based on a CMT result 0 and confirmed by a negative
179 bacteriological result. Subclinical mastitis quarters were selected based on a CMT result of at
180 least ++.

181 All CM cases were classified into mild (1: abnormal appearance of milk), moderate (2:
182 abnormal appearance of milk accompanied by swelling or redness of the mammary gland), and
183 severe CM (3: beside abnormal appearance of milk and swelling of the mammary gland, cow
184 showed signs of systemic illness such as fever above 39.5°C) according to Wenz et al. (2001)
185 and Pinzon-Sanchez and Ruegg (2011). After examinations and samplings, CM and SCM cows
186 were treated according to the standard operation procedures established on the farm.

187 Climate loggers (Tinytag Plus II, Germini Loggers Ltd., Chichester, United Kingdom)
188 were secured in the middle alley of the different pens at beams 3 m from the ground, collecting
189 temperature and humidity data every full hour. Measured ambient temperature (AT) and
190 relative humidity (RH) data were used to calculate the temperature-humidity index (THI)
191 according to the equation reported by Kendall and Webster (2009):

192 $$THI = (1.8 \times AT + 32) - ((0.55 - 0.0055 \times RH) \times (1.8 \times AT - 26)).$$

193

194 ***Milk Samples: Measurement of Biomarkers, SCC, and Bacteriological Culturing***

195 Sterile milk samples were divided into 3 subsamples immediately after collection and
196 sent to 3 different accredited laboratories for bacteriological culturing. In all three laboratories,
197 bacteriological culturing was performed following the examination standards and regulations
198 of the GVA guideline (GVG, 2012) and the National Mastitis Council handbook (National
199 Mastitis Council, 1999; 2004). In brief, ten microliters of a well-mixed sample were plated with

200 a sterile loop onto an aesculin sheep-blood agar plate (Oxoid, Wesel, Germany) and incubated
201 for 48 hours at 37°C under aerobic conditions. Examinations were carried out twice - 24 and
202 48 hours after inoculation. Grown colonies were identified based on their colony morphology,
203 gram staining characteristics, hemolysis patterns, and their aesculin hydrolysis. If necessary,
204 other biochemical properties (e.g., activity of catalase, clumping factor test, Lancefield
205 serotyping, activity of cytochrome oxidase C, and oxidation-fermentation of glucose) were
206 considered for further identification.

207 Healthy quarters were retrospectively withdrawn, if any bacterial growth was found by
208 1 or more laboratories. For CM and SCM, at least 2 out of 3 labs had to identify the same
209 pathogen in order to obtain a high accuracy of the results. Somatic cell counts were determined
210 using 2.5 mL of milk and were measured by somatic cell counter (Fossomatic FC 5000, Foss
211 Electric, Hillerød, Denmark).

212 Unsterile milk samples were divided into 3 subsamples and stored at – 20°C until
213 analyses. One of those subsamples each was analyzed for MAA, CATH and HP, respectively.

214 Milk amyloid A and CATH measurements were carried out by Bioteck Lait (Pacé,
215 France) and Porto Conte Ricerche S.r.l. (Alghero, Italy), respectively.

216 Cathelicidin was analyzed with a pan-cathelicidin ELISA based on 2 monoclonal
217 antibodies developed against a pan-cathelicidin domain (Addis et al., 2016a, b). At the end of
218 the assay, for each sample, the optical density measured at 450 nm (OD450) was normalized
219 against internal controls (NOD450). Normalized values were generated by subtracting the
220 average of 6 culture-negative samples with <50,000 cells/mL from each measured value. To
221 assess CATH levels, each milk sample was measured in duplicate aliquots of 10 and 1 μL.
222 When the results of the 10 μL aliquot provided a value above 2.5 NOD450, the value of the 1
223 μL aliquot was used multiplied by 10. The inter-assay CV for the CATH ELISA was calculated
224 based on two internal standards loaded in each of the 22 total ELISA plates analyzed. The

225 OD450 mean value of the two standards was 0.365 (±0.04 SD), with an inter-assay CV of
226 11.16%

227 Measurements of MAA were performed by a dairy laboratory that is affiliated with the
228 French DHI program (Oxygen Laboratoires d'Analyses, Maroeuil, France) as earlier described
229 (Crosson et al., 2015) and validated (Gerardi et al., 2009). The concentration of MAA was
230 determined using a commercial ELISA kit (Milk Amyloid A-MAA Assay Kit, cat. no. TP-807;
231 Tridelta Development Ltd, Maynooth, Ireland) in accordance with the manufacturer's
232 recommendations. Samples were added to microwells along with a biotinylated monoclonal
233 antibody. After washing in order to remove unbound material, streptavidin-horseradish
234 peroxidase was added and wells were incubated. Following the incubation, TMB substrate
235 solution was added and the absorbance of each well was measured. Limit of detection of the
236 ELISA was 0.4685 µg/mL stated by the manufacturer. The inter-assay CV for the MAA ELISA
237 was 11.55%.

238 Level of HP in milk was measured using the eProCheck 2.0 (FrimTec GmbH,
239 Oberostendorf, Germany), an automatic portable ELISA. As standard solution, a bovine
240 haptoglobin originating form bovine serum (purity >90 %) was used (Pedersen et al., 2003;
241 Nielsen et al., 2004). No cross-reactivities are known by measuring HP in bovine milk. The
242 inter-assay CV for the HP ELISA considering 3 standards were 6% (918.58 ng/mL ± 56.64 SD,
243 n = 8) 3% (476.77 ng/mL ± 13.68 SD, n = 8), and 5% (106.93 ng/mL ± 5.06 SD, n = 8),
244 respectively. The accuracy of test was depending on dilution between 80% and 100%. The limit
245 of detection was 0.1 µg/mL. Measurements were carried out according to the standard operation
246 procedure provided by the manufacturer. In brief, 50 µl of each sample were pipetted onto a
247 well plate. Once, wells were loaded, they were inserted into the device, and the following
248 procedures were conducted automatically: addition of conjugate, incubation, washing, addition

249 of enzyme-substrate complex, incubation, photometric measuring, and results output. The
250 results of HP level were given in µg/mL.
251
252 ***Statistical Analyses***
253 Data were entered into Excel spreadsheets (version 2016; Microsoft Corp., Redmond,
254 WA) and statistical analyses were performed with SPSS for Windows (version 24.0, IBM
255 Deutschland GmbH, Ehningen, Germany).
256 The statistical significance level was set at $P \leq 0.05$ and trends were discussed for $P \leq$
257 0.10.
258 Normality of distributions of continuous parameters (i.e., level of CATH, MAA, HP)
259 was assessed by plotting the data, visual examination and calculating a Q-Q-plot.
260 Several different generalized linear mixed models were used to determine the effect of
261 the health status of the udder quarter (e.g., healthy, SCM, CM and healthy in-cow control)
262 different severity scores (e.g., mild, moderate, and severe) and bacteriological results on CATH,
263 MAA, and HP levels. Only bacteriological results with $n \geq 10$ were included into the model.
264 The statistical models were built according to the model-building strategies described
265 by Dohoo et al., 2009 and Bertulat et al., 2017. In brief, all independent parameters were
266 initially tested with Spearman's correlation (i.e., ordinal parameter) or Pearson correlation (i.e.,
267 scaled parameter) for colinearity and analyzed in a univariate univariable model. If 2 parameters
268 showed a high, significant correlation, only the parameter resulting in the univariable model
269 with the smallest *P*-value was used in the final multivariable model. Furthermore, only
270 parameters resulting in univariable models with $P \leq 0.2$ were included in the final mixed model
271 ANOVA. This final model was built in a conditional backward stepwise manner. Interactions
272 were tested for all relevant parameters. Quarter within cow was included as random effect. Post-
273 hoc comparison was carried out applying LSD test. Validity of the final models was ensured by

274 checking that the model assumptions were met, especially the normality of distribution of
275 residues was verified using the Shapiro-Wilk and Kolmogorov–Smirnov-test, plotting the
276 residues and calculating a Q-Q-plot.

277 The following factors were tested depending on the target variable (i.e., CATH , MAA,
278 and HP), the study population (i.e., only CM; only SCM; healthy and healthy in-cow controls,
279 or healthy, SCM and CM without healthy in-cow controls) and the major independent variables
280 (i.e., different udder health conditions, mastitis score or bacteriological result), bacteriological
281 result, lactation number (categorized, i.e., 1, 2, and 3 or higher), DIM (continuous), milk yield
282 (continuous), total number of episodes with recurrent clinical mastitis (categorical), time after
283 milking (continuous) and THI (continuous).

284 Receiver operating characteristic (ROC) curves were generated and the area under the
285 curve (AUC) was calculated in order to establish thresholds for CATH, MAA, and HP to
286 differentiate between healthy quarters and healthy in-cow control quarters, quarters with SCM
287 and CM, SCM and healthy quarters, CM and healthy quarters, CM and healthy in-cow control
288 quarters. Furthermore, thresholds between different severity scores of CM or thresholds to
289 differentiate between different bacteria strains in either SCM or CM quarters were calculated.
290 Thresholds were chosen based on the highest sum of Se and Sp.

291 Pearson correlation coefficient was used in order to evaluate the relationship between
292 levels of different biomarkers and between biomarker levels and SCC results.

293

294 RESULTS

295 *Study Population*

296 Thirty-nine, 7, and 3 CM, SCM and healthy cows, respectively, had to be retrospectively
297 withdrawn from analysis due to a CM within 30 d before enrollment, a positive bacteriological
298 result in cows enrolled as healthy, or signs of metabolic or infectious disease (e.g., metritis,

299 claw lesions, ketosis). Therefore, 212 cows with CM (i.e., 45 mild, 103 moderate, 64 severe),
300 119 with SCM and 67 healthy cows met the inclusion criteria.

301 For the final analysis data from 212 mastitis quarters, 212 healthy in cow-control
302 quarters from mastitis CM cows, 119 SCM and 67 healthy quarters were used. Considering CM
303 cows, MAA concentrations were available from 45 mild, 103 moderate and 64 severe cases.
304 For HP and CATH, however, concentrations from 20 cows with mild, 63 with moderate and 38
305 with severe CM could be used for final analysis.

306 ***Bacteriological Results***

307 Predominantly found bacteria strains were coliforms (e.g., *Escherichia coli*, Klebsiella
308 spp.), *Streptococcus uberis* and other Streptococcus spp. in CM quarters and *Staphylococcus*
309 *aureus*, CNS and *Streptococcus uberis* in SCM quarters (Table 1). While more than 38% of
310 mild and moderate CM quarters were culture-negative, the most common pathogens found in
311 severe CM cases were coliforms (up to 41%).

312

313 ***Cathelicidin***

314 Cathelicidin levels in healthy, SCM, and CM quarters averaged 0.001 ± 0.008, 0.951 ±
315 0.046, and 2.420 ± 0.028 NOD450 (LSM ± SE; $P < 0.001$; Figure 1), respectively.

316 Cathelicidin levels in healthy in-cow control quarters (0.045 ± 0.002 NOD450) were
317 lower than in CM quarters ($P < 0.001$; Figure 1). Healthy quarters and healthy in-cow control
318 quarters, however, did not differ ($P > 0.05$).

319 Within CM cows, CATH levels were not influenced by severity score ($P > 0.05$) and
320 bacteriological result ($P = 0.36$).

321 In contrast, CATH levels in SCM quarters infected with CNS, *Staph. aureus* and *Strep.*
322 *uberis* differed significantly ($P = 0.04$). Lowest levels of CATH were detected in samples
323 positive for CNS samples (0.326 ± 0.070 NOD450) compared to samples positive for *Staph.*

324 *aureus* (1.309 ± 0.185 NOD450; *P* = 0.02) and *Strep. uberis* (1.238 ± 0.119 NOD450; *P* = 0.01).
325 Culture-negative samples (0.899 ± 0.074 NOD450) had numerically higher CATH values (*P* =
326 0.06) than CNS samples. Cathelicidin levels were lower in SCM compared to CM quarters of
327 cows infected with *Staph. aureus* (*P* = 0.04), *Strep. uberis* (*P* < 0.001) and in culture-negative
328 samples (*P* < 0.001), respectively. Descriptive values of measured levels of CATH depending
329 on bacteriological result in milk are presented in Figure 2.

330 There was no effect of any of the other tested factors (i.e., lactation number, DIM, milk
331 yield, total number of episodes with recurrent CM, and THI) on CATH in any of the models (*P*
332 > 0.05).

333 The thresholds to differentiate between the health status (healthy, SCM, and CM
334 quarters) are presented in Table 2. The optimal threshold to differentiate between healthy and
335 CM quarters was 0.053 NOD450 (Se = 0.98, Sp = 0.99, AUC = 0.991, Table 2). With a
336 threshold of 0.000 NOD450 healthy and SCM quarters could be differentiated (AUC = 0.908)
337 with a Se of 0.83 and a Sp of 0.97. A threshold for healthy and healthy in-cow control quarter
338 was not calculated as there was no difference between CATH values in both groups. Thresholds
339 for relevant bacteriological results are presented in Table 3.

340 ***Milk Amyloid A***

341 Milk amyloid A in quarters of healthy cows, quarters with SCM and CM averaged 1.06
342 ± 0.1 µg/mL (LSM ± SE), 2.62 ± 0.3 µg/mL, and 6.67 ± 0.2 µg/mL, respectively (*P* < 0.001;
343 Figure 3).

344 Levels of MAA in healthy in-cow control quarters (2.69 ± 0.2 µg/mL) were significantly
345 lower compared to CM quarters (13.76 ± 0.1, *P* < 0.001; Figure 3). The latter (2.68 ± 0.1 µg/mL)
346 exceeded values measured in healthy quarters of healthy cows (1.14 ± 0.1 µg/mL, *P* = 0.001;
347 Figure 3).

348 Levels of MAA in CM quarters were influenced by severity score (*P* = 0.029, Figure 4).
349 Average levels were 6.17 ± 0.5 µg/mL, 5.69 ± 0.3 µg/mL, and 8.63 ± 0.4 µg/mL in mild,
350 moderate, and severe CM quarters, respectively. Levels in severe CM quarters differed
351 significantly from moderate (*P* = 0.01) and tended to be different from mild CM (*P* = 0.09).

352 In CM samples, there was no effect of the causative mastitis pathogen on MAA levels
353 (*P* = 0.55) while values measured in SCM samples tended to be affected by different
354 bacteriological result (*P* = 0.1). Levels were lowest in CNS samples (1.57 ± 0.3 µg/mL)
355 compared to culture negative results (3.88 ± 0.3 µg/mL; *P* = 0.04) and SCM quarters with *Staph.*
356 *aureus* (4.38 ± 0.6 µg/mL; *P* = 0.08).

357 Milk amyloid A level of SCM quarters were lower than in CM quarters in culture
358 negative samples (*P* = 0.02) and in samples positive for *Strep. uberis* (*P* = 0.001). Levels in
359 quarters with a *Staph. aureus infection* did not differ between CM and SCM quarters (*P* = 0.25).
360 Descriptive values of measured levels of MAA depending on bacteriological result in milk are
361 presented in Figure 5.

362 Similar to CATH, MAA levels were not affected by any of the other tested factors (e.g.,
363 lactation number, DIM, milk yield, total number of episodes with recurrent CM, and THI (*P* >
364 0.05).

365 The thresholds providing the highest accuracy for differentiating between healthy and
366 SCM quarters, healthy and CM, and SCM and CM were 1.28 µg/mL (Se = 0.65, Sp = 0.76,
367 AUC = 0.755), 1.81 µg/mL (Se = 0.77, Sp = 0.83, AUC = 0.860), and 7.75 µg/mL (Se = 0.38,
368 Sp = 0.92, AUC = 0.673), respectively (Table 2). A differentiation between healthy quarters
369 and healthy in-cow control quarter was feasible using a threshold of 1.30 µg/mL (Se = 0.51; Sp
370 = 0.76, AUC = 0.649). Furthermore, thresholds differentiating between CNS and culture
371 negative quarters, and CNS and *Staph. aureus* were calculated (Table 3).

372

373 ***Haptoglobin***

374 In healthy, SCM, and CM quarters HP level averaged 0.98 ± 0.1, 10.15 ± 0.2, 13.73 ±
375 0.1 µg/mL, respectively (*P* < 0.001; Figure 6). Haptoglobin levels in healthy in-cow control
376 quarters (5.94 ± 0.1 µg/mL) were lower than in CM quarters (13.76 ± 0.1 µg/mL, *P* < 0.001;
377 Figure 6). Healthy quarters (0.98 ± 0.1 µg/mL) had lower HP level than healthy in-cow control
378 quarters (5.99 ± 0.2 µg/mL; *P* < 0.001, Figure 6).

379 In cows with CM, HP levels were not affected by severity (*P* > 0.05). Also, there was
380 no effect of mastitis causing bacteria on HP levels in CM samples (*P* = 0.63).

381 In SCM, however, HP level was influence by bacteriological results (*P* = 0.05). Samples
382 positive for CNS (8.52 ± 0.4 µg/mL) had lower HP levels than quarters infected with *Strep.*
383 *uberis* (11.1 ± 0.4 µg/mL; *P* = 0.04) and *Staph. aureus* (11.86 ± 0.3 µg/mL; *P* = 0.032). Culture-
384 negative samples (9.46 ± 0.3 µg/mL) and samples from cows with CNS (*P* = 0.40) or *Staph.*
385 *aureus* (*P* = 0.07) did not differ, respectively.

386 Haptoglobin levels in SCM quarters with culture-negative samples (*P* < 0.001) and
387 samples positive for Strep. uberis (*P* = 0.002) were lower than the respective quarters with CM.
388 Descriptive values of measured levels of HP depending on bacteriological result in milk are
389 presented in Figure 7.

390 Overall, there was no effect of lactation number, DIM, milk yield, total number of
391 episodes with recurrent CM, and THI (*P* > 0.05) on HP levels in any of the comparisons.

392 The optimal threshold to differentiate between healthy and CM quarters was 5.40 µg/mL
393 (Se = 0.96, Sp = 0.99, AUC = 0.997, Table 2). With a threshold of 3.65 µg/mL, a Se of 0.92
394 and a Sp of 0.94 healthy and SCM quarters could be differentiated (AUC = 0.980). The
395 threshold between healthy and healthy in-cow control quarter could be set at 1.55 µg/mL (Se =
396 0.91, Sp = 0.80, AUC = 0.929). Furthermore, thresholds differentiating between CNS and
397 *Staph. aureus* and between CNS and *Strep. uberis* were calculated (Table 3).

398

399 ***Relationships between Different Biomarkers and SCC***

400 Considering health status, there was a moderate correlation between MAA and CATH
401 (r = 0.55, $P < 0.001$) as well as between MAA and HP (r = 0.48, $P < 0.001$). Cathelicidin and
402 HP were strongly correlated (r = 0.83, $P < 0.001$). The correlation between biomarkers and SCC
403 results was also strong for CATH (r = 0.75, $P < 0.001$) and HP (r = 0.73, $P < 0.001$) and
404 moderate for MAA (r = 0.41, $P < 0.001$), respectively.

405 **DISCUSSION**

406 Our results support recent publications on CATH (Addis et al., 2016b; Pongthaisong et
407 al., 2016; Addis et al., 2017), MAA (Jaeger et al., 2017; Hussein et al., 2018) and HP (Pedersen
408 et al., 2006; Sadek et al., 2017; Thomas et al., 2018), which described the diagnostic value of
409 these biomarkers for mastitis detection in milk. Measurement of these biomarkers achieved
410 high accuracy for the detection of intramammary infections. While most previous studies
411 focused on either SCM or CM, our study directly compared both types of mastitis. Therefore,
412 we were able to show that not only a differentiation between healthy quarters and infected
413 quarters is possible, but also a differentiation between subclinical and clinical mastitis.

414 The levels of the 3 biomarkers in milk were quarter specific and increased in CM
415 quarters. The inflammation process of the udder affected also healthy in-cow control quarters
416 indicated by slightly higher levels of MAA and HP in healthy in-cow control quarters compared
417 to healthy cows. These elevated levels are most likely caused by diffusion of acute phase
418 proteins during the initial stage of inflammation from the blood stream into the milk. This
419 process, however, is probably not quarter specific. A production of acute phase proteins in the
420 mammary gland cells occurs only at later stages of the inflammation (Eckersall et al., 2001;
421 Hiss et al., 2004). In contrast, CATH levels in healthy in-cow control quarters were not elevated
422 indicating a quarter specific mechanism. Therefore, elevated CATH levels in milk occurred

423 only in infected quarters. Similar results were reported by Chromek et al. (2006) and Addis et
424 al. (2011; 2013).
425 Not surprising, comparability with previous studies was best for CATH as
426 measurements were conducted in the same laboratory (Addis et al., 2016a, b; Addis et al.,
427 2017). Levels of MAA measured in our study was also similar to previous studies. In SCM
428 experimentally induced with *Staph. aureus*, the MAA level averaged 5.6 ± 12 µg/mL (Eckersall
429 et al., 2006), which is comparable with our level of 4.38 ± 0.6 µg/mL. In naturally occurring
430 SCM samples (Gerardi et al., 2009), MAA values ranged from 9.8 ± 1.9 µg/mL to 5.5 µg/mL
431 ± 1.0 depending on ELISA kit. In their study, authors defined SCM as the presence of clots in
432 milk and the absence of abnormalities, which we classified as mild CM. Considering those
433 SCM quarters as mild CM (MAA = 6.17 ± 0.5 µg/mL) values are comparable.
434 In contrast, HP values reported in the literature are higher than our results. In SCM
435 quarters, however, our results (11.15 ± 0.4 µg/mL) were higher than concentrations previously
436 published for experimentally induced SCM (4.3 µg/mL: Eckersall et al., 2006).
437 In CM quarters, our results (13.76 ± 0.1 µg/mL) were also much lower than previous
438 findings (503 µg/mL: Wenz et al., 2010; 80.0 µg/mL: Pyorala, 2011). Differences can be
439 explained by different ELISA kits as shown by Geradi et al. (2009).
440 Differences in the absolute concentrations of biomarkers for the detection of mastitic
441 quarters might not be that important as healthy quarters showed similarly low levels (MAA =
442 0.1 ± 1.4 µg/ml; Gerardi et al., 2009; HP = 0.05 µg/mL; Eckersall et al., 2006).
443 We could not confirm previous reports of high accuracy of HP and MAA (Wenz et al.,
444 2010; Pyorala et al., 2011, Kalmus et al., 2013) for the differentiation between severity scores
445 of CM with lowest MAA levels in mild CM and significantly higher levels in moderate CM
446 quarters (Kalmus et al., 2013). We assume that these differences might be caused by different
447 mastitis pathogens. The previous study described CNS and *Strep. uberis* as main pathogens

448 while we observed twice as many culture negative samples in moderate compared to mild CM
449 quarters.
450 In contrast to previous reports on CATH (Addis et al., 2017) and HP (Wenz et al., 2010)
451 we were not able to detect an effect of different pathogens on the concentration. One reason
452 might be that the types of pathogens in the aforementioned studies differed from ours (e.g.,
453 more CNS in previous studies, more *E. coli* and *Staph. aureus* in our study). Albeit this was not
454 specifically investigated in either of the studies, different genotypes might be linked to different
455 levels of biomarkers.
456 It is noteworthy, however, that in SCM quarters the causative pathogen affected CATH
457 and HP levels. The mean HP level in CNS samples (8.52 ± 0.4 μg/mL) was lower than in *Staph.*
458 *aureus* (11.86 ± 0.3 μg/mL) samples. This relationship is in agreement with previous studies
459 (Hiss et al., 2007; Pyorala et al., 2011). Even though the absolute values in those earlier studies
460 differed for CNS (3.1 μg/mL; 7.8 μg/ mL) and for *Staph. aureus* (39.6 μg/ mL; 33.0 μg/ mL)
461 from results in our study (CNS = 8.52 ± 0.4 μg/mL; *Staph. aureus* = 11.86 ± 0.3 μg/mL),
462 respectively.
463 Differences in HP levels between culture-negative and culture-positive SCM milk
464 samples found by other authors (Hiss et al., 2007; Safi et al., 2009) were confirmed in our study.
465 Differences were evident for *Staph. aureus* and *Strep. uberis* ($P \leq 0.05$).
466 The accuracy to differentiate between *Staph. aureus* and CNS was 100% and 85% for
467 HP and 100% and 57% for CATH using thresholds of 0.084 and 11.0 μg/mL, respectively.
468 This is remarkable and might be interesting for eradication programs to reduce the
469 incidence of *Staph. aureus* in herds (Barkema et al., 2006).
470 Interestingly, biomarker levels were higher in several culture-negative SCM samples
471 compared to CNS positive samples. One explanation might be that the causative pathogen could
472 either not be cultured or those culture-negative samples were actually false negatives. As the

473 bacteriological examination is based on viable bacteria, encapsulated (e.g., *Staph. aureus*) or
474 dead pathogens cannot be detected. In the udder, however, even encapsulated pathogens might
475 cause an inflammatory response and cause elevated biomarker levels (Hill et al., 1983). Another
476 explanation might be that immunological processes in the udder resulted in an elimination of
477 the pathogen prior to sample collection. Inflammation processes, however, abate only slowly
478 resulting in elevated levels of biomarkers even after bacteriological cure. This assumption is
479 evidenced by an earlier publication which described elevated levels of HP and serum amyloid
480 A in milk from quarters with SCM 21 to 35 days after an experimental infection with *Staph.*
481 *aureus* (Gronlund et al., 2003).

482 In agreement with previous authors (Addis et al., 2017; Hussein et al., 2018), we were
483 able to show a good correlation of CATH, MAA, and HP with SCC. Lai et al. (2009) mentioned
484 coefficient of correlation between HP and SCC of $r = 0.742$.

485 Based on our results, it was possible to calculate reliable thresholds to differentiate
486 healthy quarters from SCM and CM quarters. Furthermore, thresholds could be determined for
487 the identification of CM quarters compared to SCM. Our results were more accurate and
488 sensitive compared to diagnostic methods such as subjective parameters (i.e., palpation of the
489 udder tissue; Houe et al., 2002; Rees et al., 2014) or semi-quantitative evaluation of SCC (CMT
490 in SCM quarters; Safi et al., 2009; Viguier et al., 2009). Our calculated thresholds of 0.053
491 NOD450 for CATH and 12.65 µg/mL for HP to differentiate between healthy and CM met the
492 requirements proposed by the ISO and showed better results than lactate dehydrogenase (Hiss
493 et al., 2007).

494 Our proposed CATH threshold of 0.000 NOD450 to differentiate between healthy and
495 SCM almost reached the recommended Sp of > 0.99 with a Se of 0.98. Overall, using the
496 proposed thresholds to differentiate between healthy quarters and SCM and SCM and CM
497 quarters, accuracies of 97%, 94% and 70% for CATH, HP, and MAA were calculated (Table

498 4). In previous studies test performances of MAA were affected by pathogens in SCM (Jaeger
499 et al., 2017). This might be one reason, why accuracy of MAA was lower than CATH and HP
500 in our data set.

501 Indeed, based on our results utilizing CATH and HP measurements would lead to more
502 accurate mastitis diagnoses compared to MAA (Table 4).The accuracies of CATH (70%), MAA
503 (58%), and HP (67%) are furthermore superior to electrical conductivity measurements (i.e.,
504 SCM Se = 0.19, Sp = 0.92; CM Se= 0.48, Sp = 0.92) as reported by Norberg et al., 2004), but
505 lower than for SCM diagnosis by measuring SCC (i.e., accuracy = 92%; Sharma et al., 2010;
506 Sharma and Pendey, 2010).

507 Specificity of MAA (76 %) and SCC (72%; Safi et al., 2009) are similar to differentiate
508 healthy and SCM quarters. Both tests (MAA and SCC) can be used to detect healthy cows with
509 a comparable reliability. Nevertheless, test method to detect MAA seems to detect slightly
510 better false positive cows that could lead to a lower accuracy of the test. If using the CMT test
511 to estimate SCC, Sp of up to 80.6% were described for the diagnosis of SCM quarters (Dingwell
512 et al., 2003). Albeit the semi-quantitative CMT may be cheaper, it has the disadvantage that
513 only individual milk samples can be measured and appraisal of test results needs to be done in
514 person. The MAA test, however, offers the possibility of automatization and integration in
515 automatic milking systems and thus might be used for a timely and effective overview of the
516 udder health status of all cows in a herd.

517 In contrast to MAA, both CMT and measurement of SCC do not allow differentiation
518 of the severity of mastitis, since SCC increase for example is limited in severe, but short-term
519 clinical infections that are typically induced by coliform bacteria (Rainard et al., 2018).

520 In conclusion, milk amyloid A could be a valuable tool to preselect cows in a herd wide
521 screening that need further examinations. The positive predictive value for the identification of

522 healthy cows is quite high (detection of SCM = 76%; detection of CM = 83%) and the rate of
523 false negatives low (detection of SCM = 35%; detection of CM = 23%).
524 Comparing the performance of CATH and HP with results of SCC published in the
525 literature, Sp of the detection of SCM using SCC (72%; Safi et al., 2009) was considerably
526 lower than values calculated for CATH (97%) and HP (94%). Sensitivity (SCC = 90%; Safi et
527 al., 2009) was higher for HP (92%).
528 Considering CM, Se values up to 97% and Sp up to 89% were found for SCC
529 measurement (Sargeant et al., 2001). Cathelicidin (Se = 98%, Sp = 99% in CM; Se = 93%, Sp
530 = 97% in SCM) and HP (Se = 92 %, Sp = 94% in SCM; Se = 96 %, Sp = 99 % in CM) reached
531 comparable, or slightly better results.
532 In contrast to SCC, the biomarkers investigated in our study, however, were unaffected
533 by DIM (Sargeant et al., 2001) or other physiological (e.g., stage of lactation, age, and stress;
534 Sharma et al., 2011) and environmental factors (e.g., geographical area and housing system;
535 Bielfeldt et al., 2004) which may cause an increase of SCC without an association to udder
536 infections. The validity of SCC measurements on the other hand is limited in chronic subclinical
537 infections and short-term clinical infections that are typically induced by coliform bacteria
538 (Rainard et al., 2018). For example, Miltenburg et al. (1996) found that herds with low bulk
539 milk SCC (<150,000 cells/mL) may have more CM cases than herds with higher SCC (>
540 250,000 cells/mL). Though, high SCC in bulk tank milk can also be associated with high
541 incidences of CM or a high proportion of chronically infected cows (Rainard et al., 2018).
542 Especially mastitis pathogens play an important role for SCC. While a high incidence of
543 mastitis by coliform bacteria was linked to low bulk milk SCC herds (Hogan et al., 1998), CM
544 caused by Streptococcus or Staphylococcus spp. leads to higher bulk milk SCC (Rainard et al.,
545 2018). Overall, the investigated biomarkers showed a comparable or even better performance
546 but remained unaffected by the most relevant influencing factors.

547 In conclusion, accuracy, Se, and Sp for CATH, MAA, and HP allowed for the detection
548 of SCM and CM. A routine measurement of those biomarkers whether in conventional milking
549 parlors or in automatic milking systems might be a reliable and objective method to screen
550 udder health. Especially for identification of SCM the measurement of biomarkers is more
551 sensitive and specific compared to SCC and lactate dehydrogenase (Se = 80 %, Sp = 87 %; Hiss
552 et al., 2007). So far CATH and MAA can be measured only under laboratory conditions and
553 further efforts are necessary to develop on-farm devices for measurements.
554

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TABELS AND FIGURES

Table 1. Summary of bacteriological results consistently identified by 2 out of 3 labs considering type of mastitis and severity score.

	No. of samples							
	Healthy	Subclinical mastitis[1]	Clinical mastitis[2]					
			Mild		Moderate		Severe	
Bacteriological result	Milk amyloid A/ cathelicidin/ haptoglobin	Milk amyloid A/ cathelicidin/ haptoglobin	Milk amyloid A	Cathelicidin/ haptoglobin	Milk amyloid A	Cathelicidin/ haptoglobin	Milk amyloid A	Cathelicidin/ haptoglobin
Culture-negative	67	45	20	14	41	21	8	4
Staphylococcus aureus	0	12	4	3	6	5	4	1
CNS	0	14	0	0	3	2	0	0
Streptococcus uberis	0	22	3	0	13	10	11	10
Streptococcus agalactiae	0	8	5	2	6	2	7	1
Streptococcus dysgalactiae	0	6	3	2	5	2	0	0
Other Streptococcus spp.	0	0	2	1	10	6	6	6
Coliforms	0	0	8	2	11	7	25	14
Total	67	107	45	24	95	55	61	36

[1] In subclinical mastitis quarters, 107 out of 119 met the inclusion criterion of 2 consistent findings.
[2] In clinical mastitis quarters, 115 out of 121 met the inclusion criterion of 2 consistent findings.

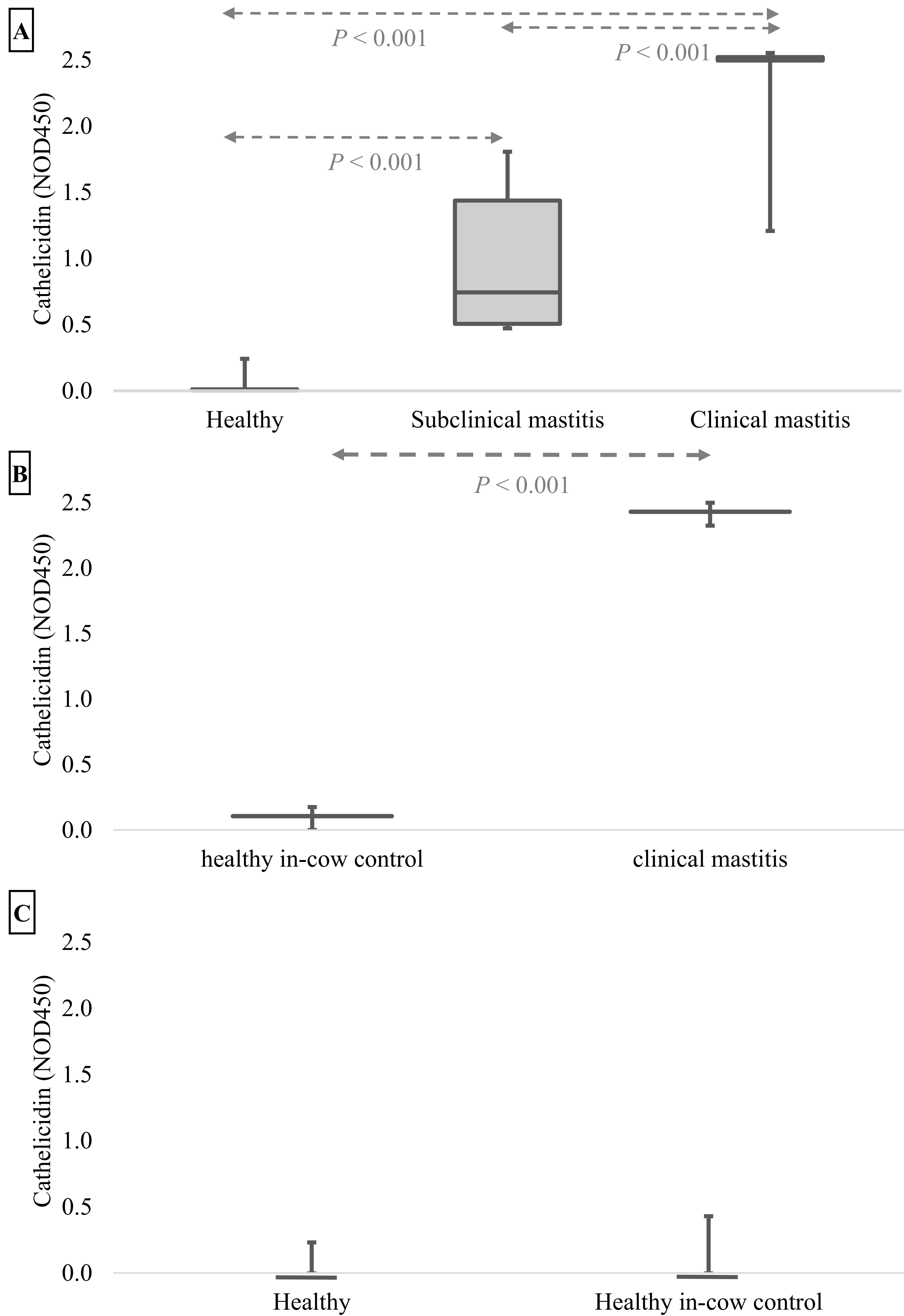

Figure 1. Comparison of cathelicidin levels (A) in healthy quarters (n = 67), quarters with subclinical mastitis (n = 119), and clinical mastitis quarters (n = 121), (B) in healthy in-cow

control quarters (n = 121) and mastitis quarters (n = 121) of cows with one clinical mastitis quarter, and (C) in healthy quarters of healthy cows (n = 67) and healthy in-cow control quarters (n = 121) of cows with one clinical mastitis quarter. Values are expressed as normalized optical density at 450 nm (NOD450). The black line inside each box marks the mean; the bottom and top of the boxes are the first and third quartiles; whiskers end at the smallest and largest statistical values.

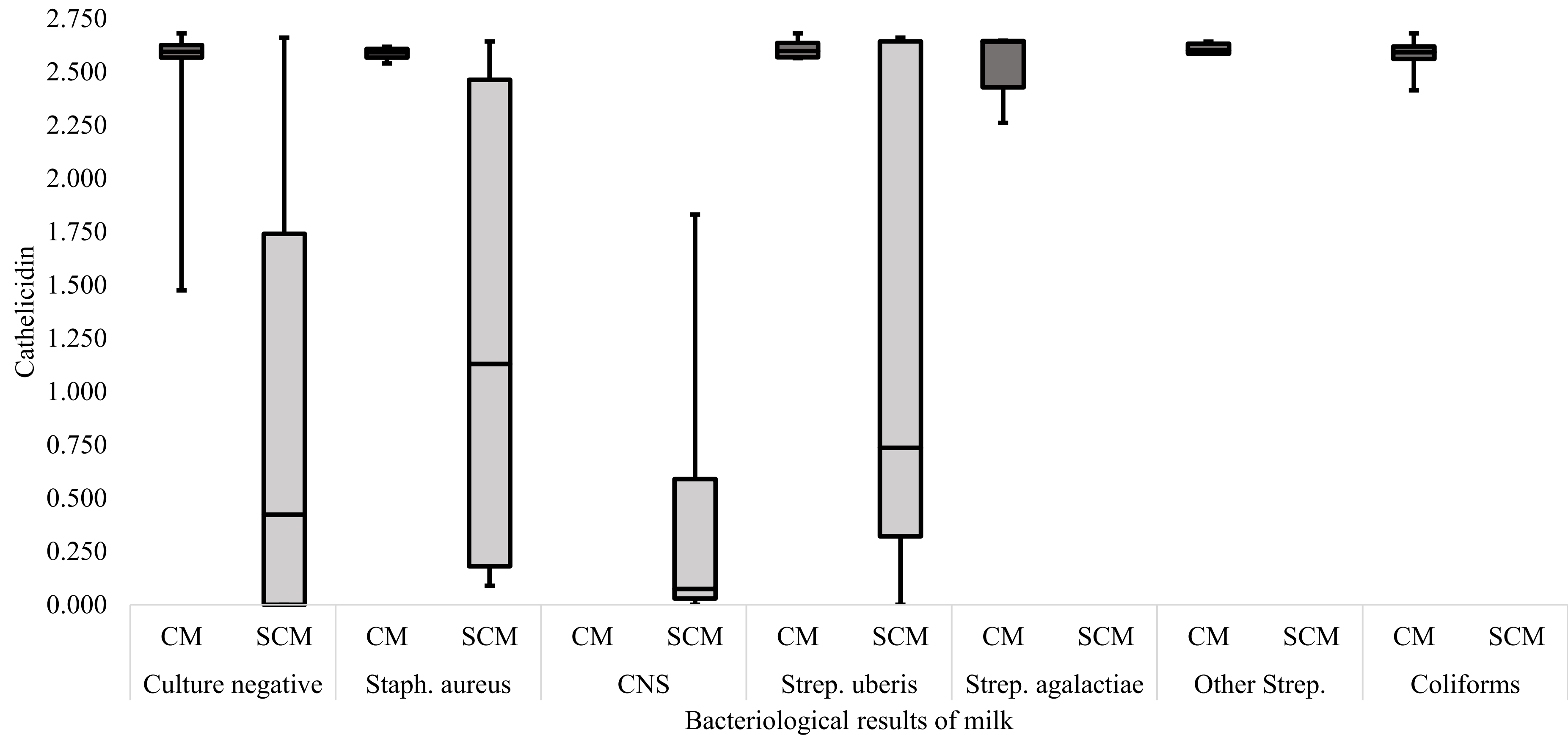

Figure 2. Cathelicidin levels in milk samples considering bacteriological results in subclinical mastitis (SCM; light grey; n = 107) and clinical mastitis quarters (CM; dark grey; n = 115). Values are expressed as normalized optical density at 450 nm (NOD450). The black line inside each box marks the mean; the bottom and top of the boxes are the first and third quartiles; whiskers end at the smallest and largest statistical values.

Table 2. Threshold values used to differentiate between healthy, subclinical and clinical mastitis quarters by measuring milk amyloid A (µg/mL), cathelicidin (normalized optical density at 450 nm, NOD450) and haptoglobin (µg/mL) considering the highest sum of sensitivity and specificity.

Biomarker	Distinction of udder health status		Thresholds	Sensitivity	Specificity	Area under the curve	*P*-value
Cathelicidin (NOD450)	Healthy	Subclinical mastitis	0.000	0.83	0.97	0.908	< 0.001
	Healthy	Clinical mastitis	0.053	0.98	0.99	0.991	< 0.001
	Subclinical mastitis	Clinical mastitis	2.361	0.88	0.82	0.810	< 0.001
	Healthy	Healthy in-cow control	- [1]	- [1]	- [1]	- [1]	- [1]
	Healthy	Mastitis [2]	0.000	1	1	0.950	< 0.001
	Moderate clinical mastitis	Severe clinical mastitis	- [1]	- [1]	- [1]	- [1]	- [1]
Milk amyloid A (µg/mL)	Healthy	Subclinical mastitis	1.28	0.65	0.76	0.755	< 0.001
	Healthy	Clinical mastitis	1.81	0.77	0.83	0.860	< 0.001
	Subclinical mastitis	Clinical mastitis	7.75	0.38	0.92	0.673	< 0.001
	Healthy	Healthy in-cow control	1.3	0.51	0.76	0.649	< 0.001
	Healthy	Mastitis [2]	1.28	0.75	0.76	0.820	< 0.001
	Moderate clinical mastitis	Severe clinical mastitis	3.64	0.48	0.64	0.560	0.08
Haptoglobin (µg/mL)	Healthy	Subclinical mastitis	3.65	0.92	0.94	0.980	< 0.001
	Healthy	Clinical mastitis	5.4	0.96	0.99	0.997	< 0.001
	Subclinical mastitis	Clinical mastitis	12.65	0.74	0.75	0.796	< 0.001
	Healthy	Healthy in-cow control	1.55	0.91	0.80	0.929	< 0.001
	Healthy	Mastitis [2]	5.05	0.91	99	0.989	< 0.001
	Moderate clinical mastitis	Severe clinical mastitis	- [1]	- [1]	- [1]	- [1]	- [1]

[1] was not calculated, since quarter did not differ from each other in generalized linear mixed models ($P > 0.05$).

[2] subclinical and clinical mastitis

Table 3. Threshold values to differentiate between SCM quarters of different bacteriological result by measuring milk amyloid A (µg/mL), cathelicidin (normalized optical density at 450 nm, NOD450) and haptoglobin (µg/mL) considering the highest sum of sensitivity and specificity.

Biomarker	Distinction of pathogens		Thresholds	Sensitivity	Specificity	Area under the curve	*P*-value
Cathelicidin (NOD450)	CNS	Staph. aureus	0.084	1.00	0.57	0.849	0.01
	CNS	Strep. uberis	0.220	0.81	0.71	0.773	0.01
	CNS	Culture negative	0.019	0.86	0.32	0.404	0.28
Milk amyloid A (µg/mL)	CNS	Staph. aureus	1.030	0.89	0.57	0.778	0.03
	CNS	Strep. uberis	-[1]	-[1]	-[1]	-[1]	-[1]
	CNS	Culture negative	0.91	0.74	0.57	0.677	0.05
Haptoglobin (µg/mL)	CNS	Staph. aureus	11	0.78	0.86	0.841	0.01
	CNS	Strep. uberis	10.45	0.68	0.85	0.756	0.01
	CNS	Culture negative	-[1]	-[1]	-[1]	-[1]	-[1]

[1] was not calculated, since quarter did not differ from each other in generalized linear mixed models ($P > 0.05$).

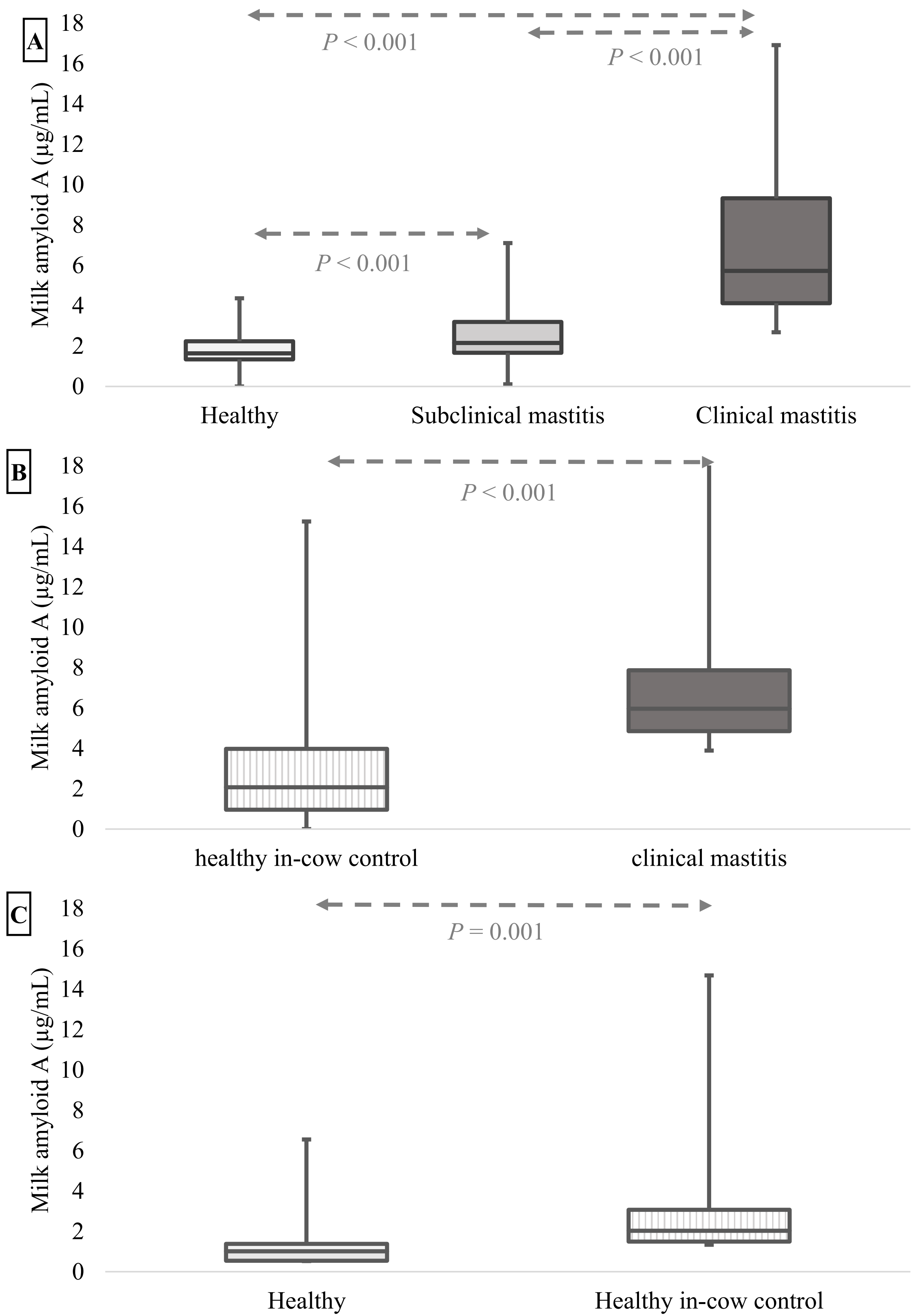
A
Milk amyloid A (µg/mL)
18
16
14
12
10
8
6
4
2
0
P < 0.001
P < 0.001
P < 0.001
Healthy
Subclinical mastitis
Clinical mastitis
B
Milk amyloid A (µg/mL)
P < 0.001
healthy in-cow control
clinical mastitis
C
Milk amyloid A (µg/mL)
P = 0.001
Healthy
Healthy in-cow control

Figure 3. Comparison of milk amyloid A (µg/mL) levels (A) in healthy quarters (n = 67), quarters with subclinical mastitis (n = 119), and clinical mastitis quarter (n = 212), (B) in healthy in-cow control quarters (n = 212) and mastitis quarters (n = 212) of cows with one clinical mastitis quarter, and (C) in healthy quarters of healthy cows (n = 67) and healthy in-cow control quarters (n = 212) of cows with one clinical mastitis quarter. The black line inside each box marks the mean; the bottom and top of the boxes are the first and third quartiles; whiskers end at the smallest and largest statistical values.

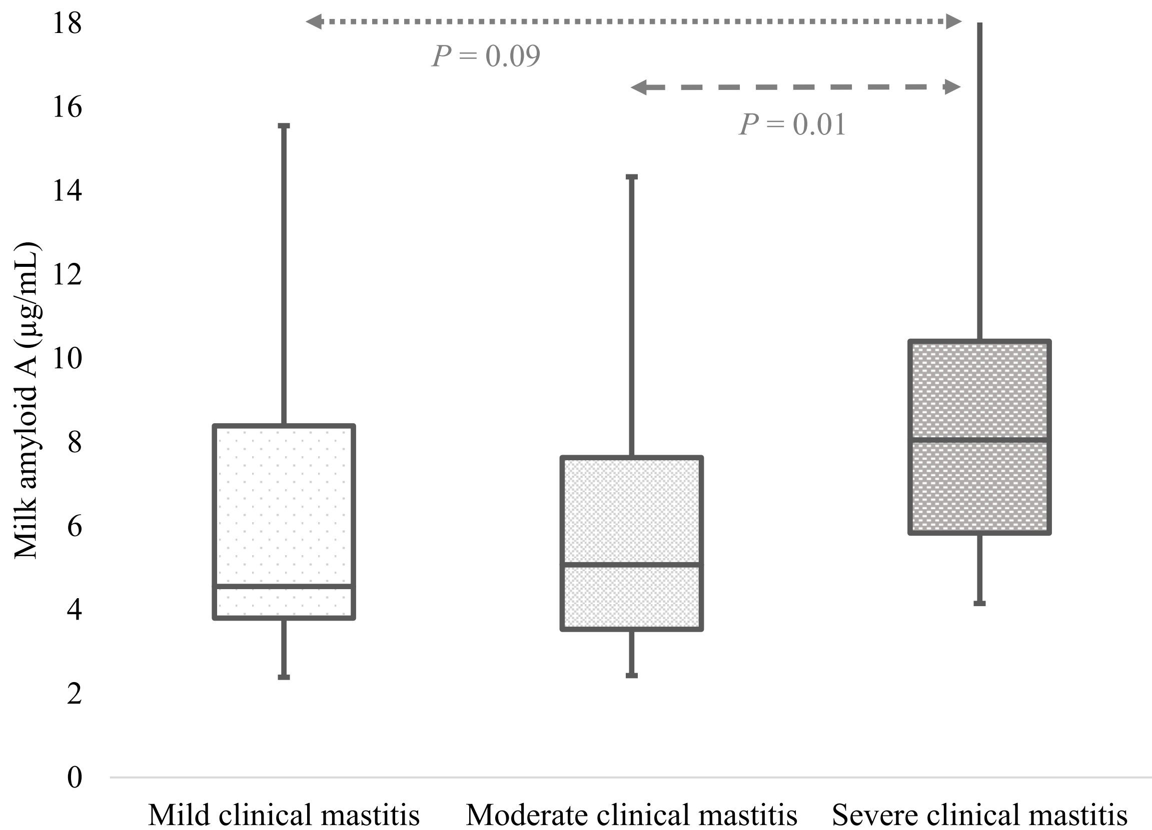

Figure 4. Comparison of milk amyloid A (µg/mL) levels in clinical mastitis quarters considering severity score. The black line inside each box marks the mean; the bottom and top of the boxes are the first and third quartiles; whiskers end at the smallest and largest statistical values. Data originate from 212 clinical mastitis quarters (45 mild, 103 moderate, 64 severe clinical mastitis quarters.

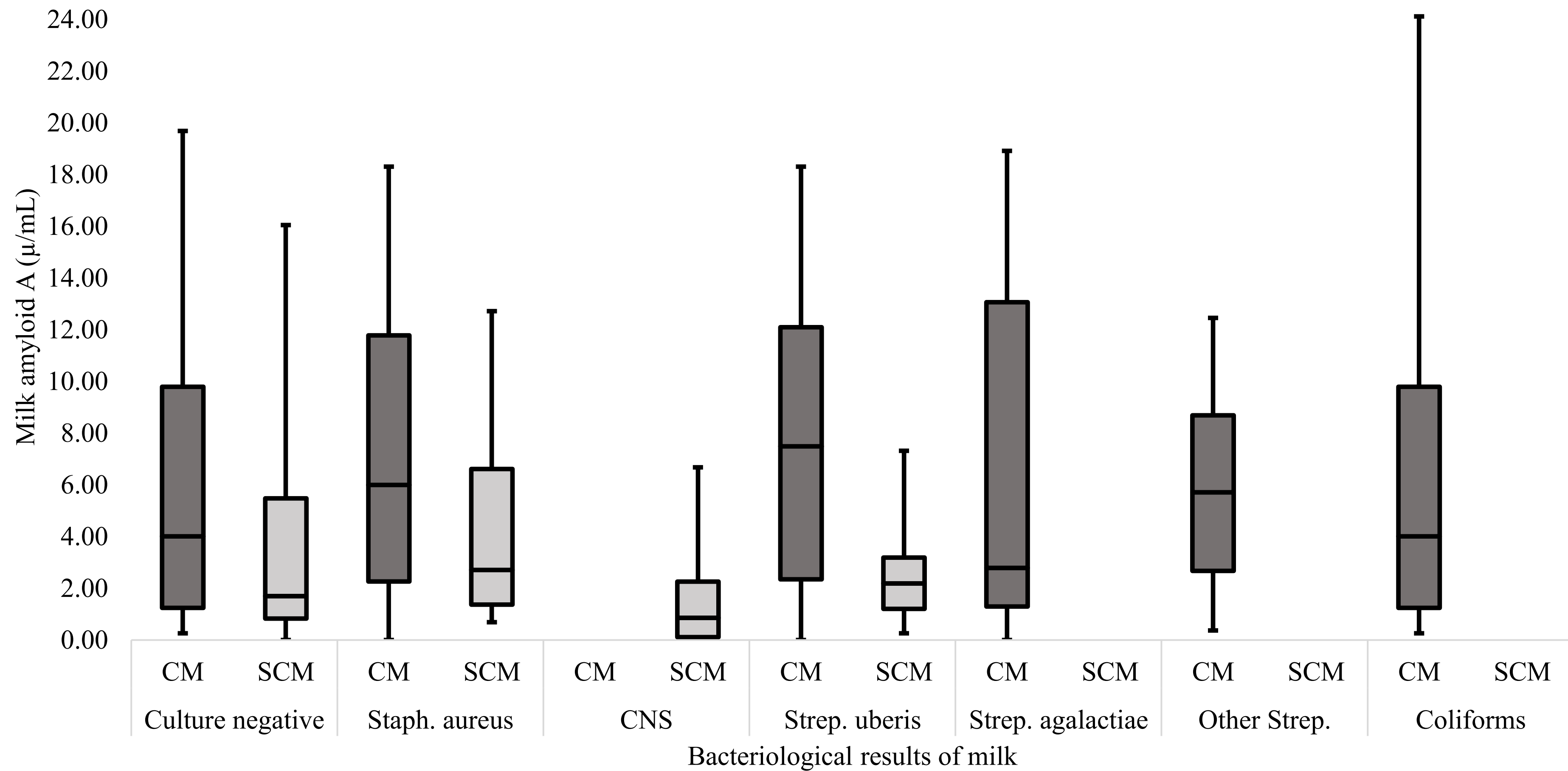

Figure 5. Milk amyloid A (µg/mL) levels in milk samples considering bacteriological results in subclinical mastitis (SCM; light grey; n = 107) and clinical mastitis quarters (CM; dark grey; n = 201). The black line inside each box marks the mean; the bottom and top of the boxes are the first and third quartiles; whiskers end at the smallest and largest statistical values.

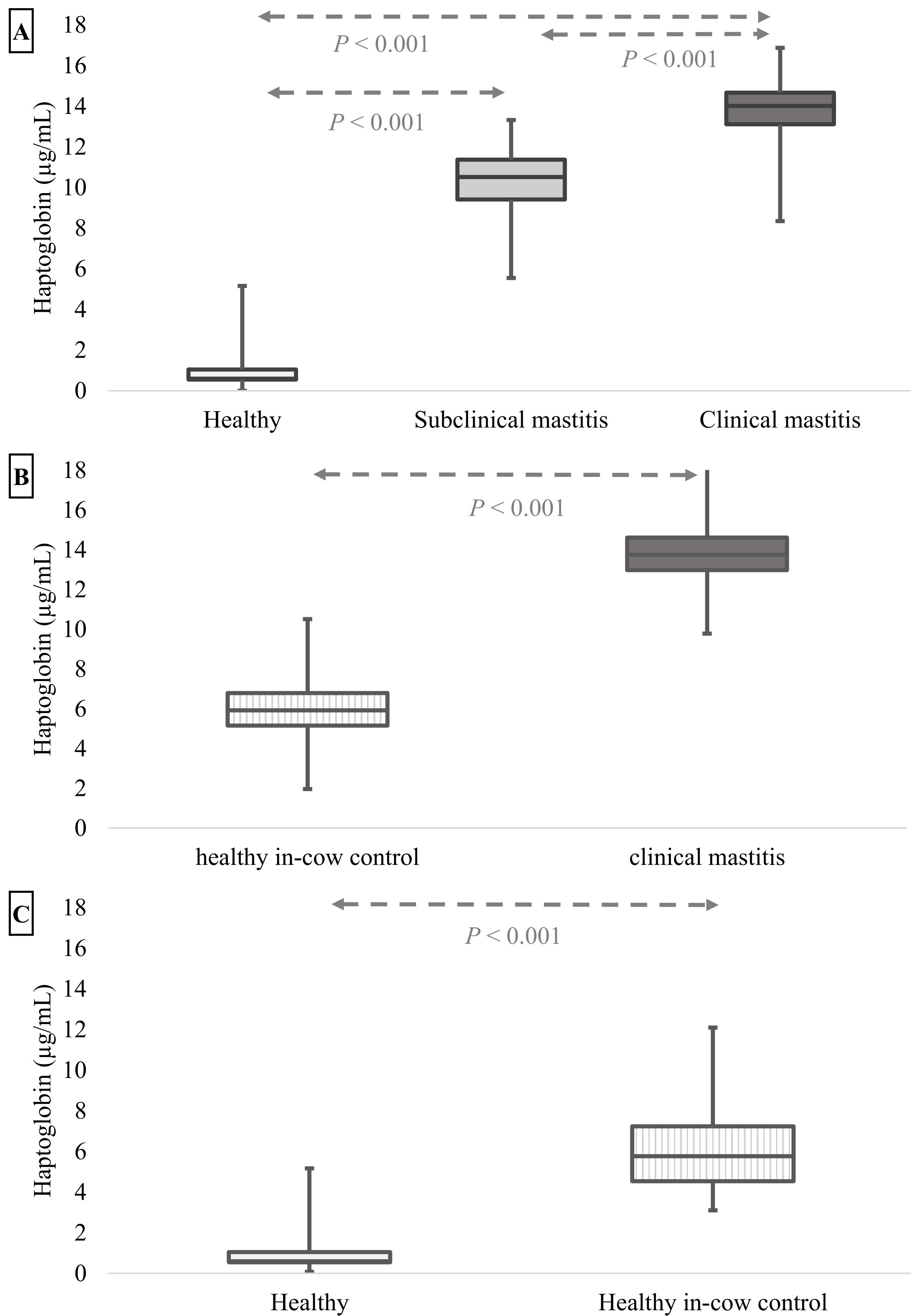

Figure 6. Comparison of haptoglobin (µg/mL) levels (A) in healthy quarters (n = 67), quarters with subclinical mastitis (n = 119), and clinical mastitis quarter (n = 121), (B) in

healthy in-cow control quarters (n = 121) and mastitis quarters (n = 121) of cows with one clinical mastitis quarter, and (C) in healthy quarters of healthy cows (n = 67) and healthy in-cow control quarters (n = 121) of cows with one clinical mastitis quarter. The black line inside each box marks the mean; the bottom and top of the boxes are the first and third quartiles; whiskers end at the smallest and largest statistical values.

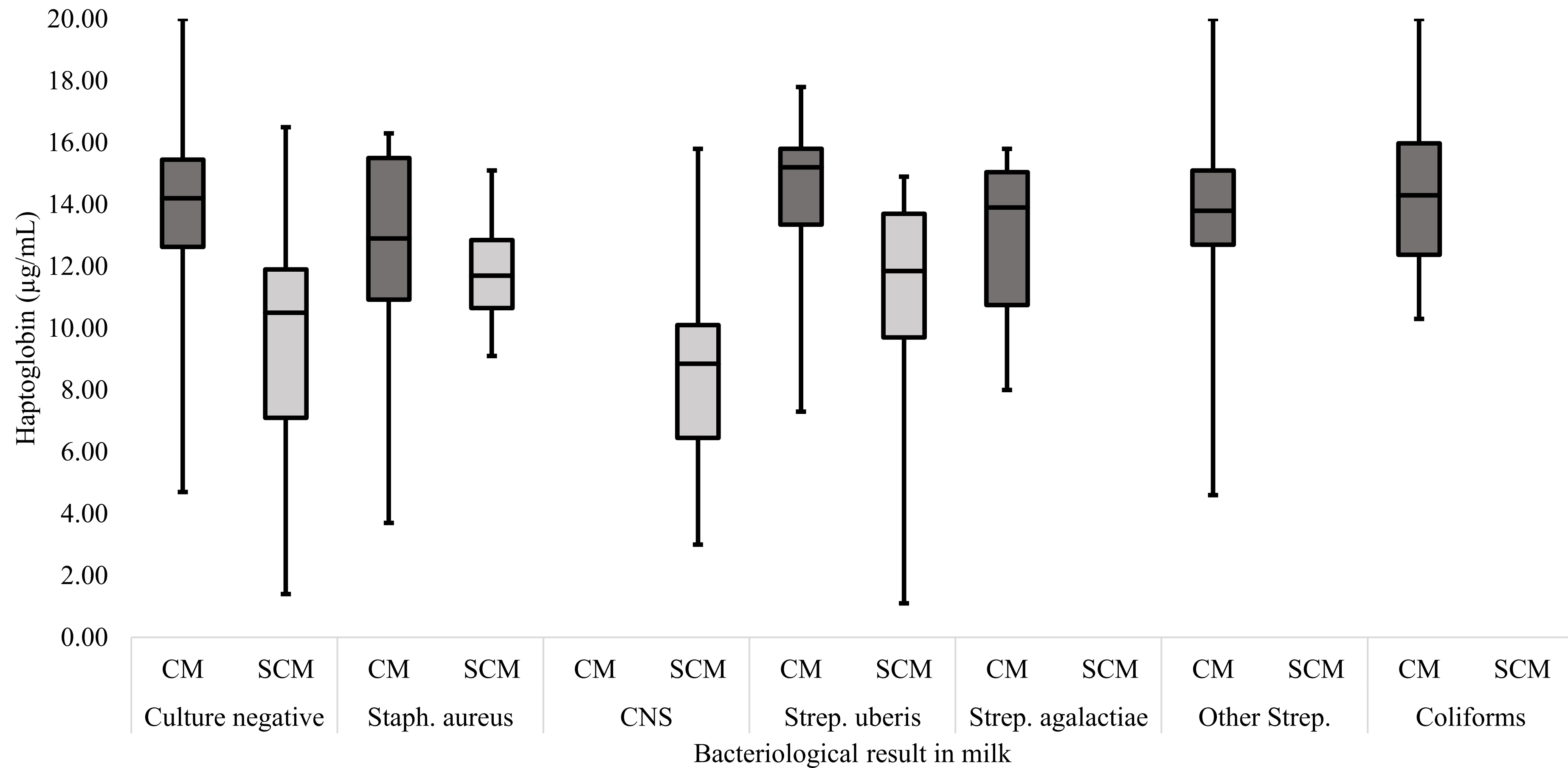

Figure 7. Haptoglobin (µg/mL) levels in milk samples considering bacteriological results in subclinical mastitis (SCM; light grey; n = 107) and clinical mastitis quarters (CM; dark grey; n = 115). The black line inside each box marks the mean; the bottom and top of the boxes are the first and third quartiles; whiskers end at the smallest and largest statistical values.

Table 4. Accuracy of cathelicidin, milk amyloid A and haptoglobin for the correct classification of health status (i.e., healthy quarters, quarters with subclinical mastitis and clinical mastitis) considering thresholds of 0.000 and 2.361 NOD450, 1.28 µg/mL and 7.75 µg/mL, and 3.65 µg/mL and 12.65 µg/mL for cathelicidin, milk amyloid A, and haptoglobin, respectively.

	Percentage of correctly classified quarters		
Biomarker	Healthy	Subclinical mastitis	Clinical mastitis
Cathelicidin (AOD450)	97.0	70.7	89.9
Milk amyloid A	69.8	58.3	44.1
Haptoglobin	94.0	67.2	74.2