Aus der Tierklinik für Fortpflanzung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Novel approaches to diagnose, classify, and treat subclinical and clinical mastitis in dairy cows

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Luise Wollowski, geb. Hänicke
Tierärztin aus Potsdam

Berlin 2022
Journal-Nr.: 4343

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ABBREVIATIONS

AT	ambient temperature	ng	nanogram(s)
AUC	area under the curve	nm	nanometer(s)
°C	Celsius	NOD450	optical density measured at 450 nm
CATH	cathelicidin	NSAIDs	non-steroidal anti-inflammatory drugs
CM	clinical mastitis	OD450	normalized against internal controls
CMT	California Mastitis Test	*P*	probability
CNS	Coagulase-negative Streptococci	r	Pearson correlation coefficient
CV	coefficient of variation	RH	relative humidity
d	day(s)	ROC	receiver operating characteristic
D	examination day	SCC	somatic cell count
DIM	days in milk	SCM	subclinical mastitis
e.g.	exempli gratia	SD	standard deviation
ELISA	enzyme-linked immunosorbent assay	Se	sensitivity
et al.	et alii/aliae/alia	SE	standard error
h	hours(s)	SOP	standard operation procedures
HP	haptoglobin	Sp	specificity
i.e.	id est	Spp.	several species
IRT	infrared thermography	Staph.	Staphylococcus
kg	kilogram(s)	Strep.	Streptococcus
LSM	least square methods	THI	temperature-humidity index
MAA	milk amyloid A	UST	udder surface temperature
mg	milligram(s)	µg	microgram(s)
mL	milliliter	µl	microliter(s)
n	number of samples		

1. INTRODUCTION AND LITERATURE

The high and nutritional value (Guetouache et al. 2014, Garcia et al. 2019) of dairy milk has led to its substantial level of consumption worldwide (Handford at al. 2016). To fulfill the increasing demand on dairy milk and achieve farm profitability milk yield per cow (Thornton 2010) and herd size (Barkema et al. 2015) increased, and automatization of dairy cow husbandry advanced. At the same time people from all over the world request a high standard of food safety, ensuring safe and wholesome products (Garcia et al. 2019). Unfortunately, both requests are in contrast to each other, since milk yield and herd size were two out of several different factors significantly associated with mastitis in dairy cows (Abebe et al. 2016).

Mastitis is an infection of the udder tissue usually caused by various pathogens like *Staphylococci spp., Streptococci spp*., and *Enterobacteriaceae* (Gomes and Henriques 2016). But there are also mastitis causing pathogens, such as *Escherichia coli* or the less common mastitis causing pathogens like *Salmonella spp.* (Schonheyder 1980), *Listeria monocytogenes* (Bourry et al. 1995) and *Campylobacter jejuni* (Gudmundson and Chirino-Trejo 1993) that are some of the major causative bacteria for foodborne disease in, e.g., the US (Garcia et al. 2019).

There are two different types of mastitis to be differentiated, subclinical (**SCM**) and clinical mastitis (**CM**). Both types are associated with reduction of milk yield of the cow suffering from mastitis and overall productivity of a farm (Ruegg 2017). Milk of cows with SCM shows an elevated somatic cell count (**SCC**) but no clinical signs. Signs such as swelling, redness, and warming of the udder together with a change of milk quality characterize CM. Clinical mastitis can further be classified (Wenz et al. 2010; Pinzon-Sanchez and Ruegg 2011) as mild (abnormal appearance of milk), moderate (abnormal appearance of milk accompanied by swelling or redness of the mammary gland) and severe CM (in addition to signs of local inflammation systemic illness such as fever above 39.5°C).

Depending on the type of mastitis and mastitis causing pathogen, udder infections are commonly treated with antimicrobials (McDougall et al. 2016, Steeneveld et al. 2011) and non-steroidal anti-inflammatory drugs (**NSAID**s). Lately the extensive use of antimicrobials, not only in dairy cows, but also in other animals, and human medicine resulted in increased development of antimicrobial resistant bacteria (Garcia et al. 2019). Therefore, a prudent use of antimicrobials is required to fulfil the global food security needs of 2015 (EU 2015/C299/04).

One approach to support this goal might be an application of NSAIDs in cows with mastitis. Its application might reduce or even replace antimicrobial treatment (Suojala et al. 2010).

Besides the global request, there is an economic aspect (von Soest et al. 2016), which plays a major role for farmers when mastitis control programs are initiated. Financial losses caused by decreased milk production and additional cost for treatment were the two greatest costs associated with mastitis (Liang et al. 2017). Furthermore, farmers have to spend time and money for control and management of mastitis (von Soest et al. 2016).

Good management of mastitis, including optimization of disease detection (Barnouin et al. 2004; Green et al. 2007) can decrease the proportion of cows suffering from mastitis. It is also important to score the severity of CM consistently to assess treatment outcomes (Royster and Wagner 2015) and adapt treatment protocols accordingly (Roberson 2003). Effective diagnostics and efficient interventions can lead to faster and more efficient control and promote responsible use of antimicrobials (Krömker and Leimbach 2017) and at the same time lower the economic losses for the farmer.

Several studies have shown the positive effect of NSAIDs on mastitis therapy (Leslie et al. 2012). So far, the efficacy of the NSIADs flunixin (Yeiser et al. 2012), carprofen (Vangroenweg et al. 2005), and meloxicam (McDougall et al. 2009; McDougall et al. 2016) in mastitis therapy have been investigated. Results are promising. Specifically, flunixin reduced rectal temperatures and signs of inflammation significantly (Anderson et al. 1986). However, antimicrobial therapy is not always adequate. For example, for *Escherichia coli* mastitis science-based information is ambiguous (Shpigel et al. 1994; Suojala et al. 2010). Similar cure rates (e.g., bacteriological cure, clinical cure, and cow survival) were seen in cows with *Escherichia coli* mastitis, irrespective, if cows were treated with an antimicrobial (Enrofloxacin) in combination with a NSAID (Ketoprofen) or with a NSAID only (Suojala et al. 2010).

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 quarter (Swinkels et al. 2015). The widely used manual palpation, however, is subjective and yields only limited repeatability (Houe et al. 2002; Rees et al. 2014). Therefore, objective methods based on different tools or milk analysis become more important in CM diagnostic (Viguier et al. 2009).

Subclinical mastitis is more difficult to detect, because of a lack of obvious signs. Today, cow-individual somatic cell count (SCC) values are a well-accepted measure to diagnose SCM (Ruegg 2017). However, the widely used California Mastitis Test (**CMT**), described by Schalm and Noorland (1957), is a semi-quantitative measure. Interpretation can be subjective, leading to false positive and negative results (Viguier et al. 2009). The usability of SCC results is further limited, as levels usually remain elevated for several weeks after an intramammary infection, even after successful mastitis treatment (Pyorala 1988). It is additionally affected by various physiological (e.g., stage of lactation, age, and stress; Sharma et al. 2011) and environmental factors (e.g., heat stress, geographical region, and housing system; Rakib et al. 2020; Bielfeldt et al. 2004).

Therefore, objective parameters for the detection of SCM and CM (Ruegg 2017) are requested in times of increasing herd sizes (Barkema et al. 2015). The establishment of farm-specific protocols are essential to minimize human errors and ensure consistency of management practices (Barkema et al. 2015). Objective diagnostic tools could help to improve mastitis detection and minimize false positive errors which often lead to antimicrobial treatment. Furthermore, calculated thresholds can be implemented to standard operating procedures to make mastitis detection and treatment more efficient and reliable.

Earlier studies showed some evidence that increased udder firmness, one of the signs of CM, can be measured objectively using a dynamometer (Rees et al. 2017). Studies differentiating between severity scores or pathogens are, however, missing. The diagnostic value of this method to differentiate between healthy, SCM, and CM quarters has yet to be investigated.

Warming, another sign of udder inflammation associated with mastitis was measured using infrared thermography, earlier. Results showed that udder surface temperature (**UST**) increased in mastitis quarters with rising SCC (Sathiyabarathi et al. 2016b). Several researchers investigated the accuracy of diagnosing CM with an infrared camera (Metzner et al. 2014; Sathiyabarathi et al. 2016a) and SCM (Berry et al. 2003; Polat et al. 2010). While this technique measures the whole udder, I wanted to evaluate a smaller, less expensive device, such as a hand-held infrared thermometer. Such devices which measure only a small area, were, e.g., validated for measurements of ear surface temperature in cows with hypocalcemia (Venjakob et al.; 2016). An application in cows with mastitis might be feasible but has not yet been investigated.

Recent advances of proteomic techniques have led to the identification of several new neutrophil-produced proteins, involved in mastitis immune responses (Lippolis and Reinhardt 2005; Smolenski et al. 2007). Those proteins might be plausible biomarkers, usable for mastitis detection (Viguier et al. 2009; Ceciliani et al. 2012) by analyzing milk. Just recently, acute phase proteins like milk amyloid A (**MAA**) or haptoglobin (**HP**) have been discovered as mastitis markers (Jaeger et al. 2017; Sadek et al. 2017; Hussein et al. 2018). A study measuring MAA, showed that a more sensitive and specific identification of mastitis animals is possible compared to SCC (Jaeger et al. 2017). Furthermore, cathelicidin (**CATH**) in milk has been investigated in several research projects for mastitis diagnostics (Smolenski et al. 2011; Addis et al. 2016b; Addis et al. 2017). So far, available literature, however, focused on the applicability of one biomarker at a time. A recent study (Thomas et al. 2018) demonstrated promising results in diagnosing naturally occurring bovine mastitis by examining 3 acute phase proteins (HP, C-reactive protein, and mammary associated serum amyloid A3) simultaneously. Data on the comparability, their correlation, and the association with the health status of a given udder quarter (e.g., healthy, SCM, CM) or the severity are, however, lacking.

The presented studies evaluate different tools (Publication I) and biomarkers (Publication II) to diagnose and classify SCM and CM. They are aimed at improving mastitis management in dairy herds, reducing the number of mastitis cases and thus antimicrobial usage. Another approach to reduce antimicrobials and lower economic losses, could be the routine use of NSAIDs in mastitis therapy, not only in severe, but also in mild and moderate CM. A description of this study is provided in the section “additional unpublished data”.

The objectives of this thesis were 1) to utilizes a dynamometer and a hand-held infrared thermometer to diagnose and classify SCM and CM (Publication I), 2) to estimate the value of the biomarkers CATH, MAA, and HP to diagnose and classify SCM and CM (Publication II), and 3) to evaluate the benefits of an additional application of a NSAID (i.e., transdermal flunixin) in treatment of CM (additional unpublished data).

Publication I and II were published in the Journal of Dairy Science (current Impact Factor: 4.034) and are presented in the journal specific format.

2. PUBLICATION I

Short communication: Diagnosis and classification of clinical and subclinical mastitis utilizing a dynamometer and a handheld infrared thermometer

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2.1. Abstract

In times of ongoing automatization of dairy cow husbandry, objective and reliable tools for mastitis diagnostic are highly in demand. The objective of this study was to investigate the diagnostic value of a handheld dynamometer and an infrared thermometer to diagnose and score clinical and subclinical mastitis and to compare those values with results from palpation of the udder tissue. Overall, 218 cows with clinical mastitis (i.e., 46 mild, 106 moderate, and 66 severe cases), 142 with subclinical mastitis, and 68 healthy cows were enrolled. Our data provide evidence that the dynamometer is an accurate diagnostic tool to differentiate between healthy udder quarters, and those with subclinical and clinical mastitis. Furthermore, the severity score of clinical mastitis can be estimated by dynamometer. The firmness threshold for the detection of clinical mastitis was 1.002 kg. Using a threshold of 1.175 kg in clinical mastitis quarters, it was possible to differentiate between negative and positive bacteriological results. A differentiation between healthy and clinical mastitis quarters with the infrared thermometer was possible, albeit udder surface temperatures were highly influenced by ambient temperature. Udder surface temperature increased by 0.15 to 0.18°C for each degree of ambient temperature. In conclusion, the utility of an infrared thermometer to estimate the udder health status of dairy cows is limited, whereas the handheld dynamometer appeared to be an accurate and objective method.

2.2. Key words

udder firmness, udder surface temperature, dynamometer, mastitis diagnosis

2.3. Short Communication

Today's dairy industry with its increasing herd sizes (Barkema et al., 2015) and ongoing automatization of the milking process requires objective parameters for the detection of mastitis to reduce its economic impact (Ruegg, 2017). Effective diagnostic methods can lead to faster and more efficient control of mastitis and promote responsible use of antimicrobials (Kroemker and Leimbach, 2017). It is also important to reliably score the severity of clinical mastitis (**CM**) to predict treatment outcomes (Royster and Wagner, 2015) and adapt treatment protocols accordingly (Roberson, 2003). While most dairy farmers and veterinarians focus on detection, treatment and prevention of CM, management of subclinical mastitis (**SCM**) is hardly less important (Halasa et al., 2007) as it influences milk yield and overall productivity (Ruegg, 2017). Diagnostic methods for SCM are usually based on SCC. Unfortunately, those methods entail the collection of milk (e.g., California mastitis test, electrical conductivity) and thus limit their applicability (Ruegg, 2017), or lack in accuracy (e.g., N-acetyl-β-D-glucosaminidase,

lactate dehydrogenase, Nyman et al., 2016). Overall, there is a lack of easy to use, objective cow-side methods to diagnose SCM and CM which furthermore provide thresholds that can be used as relevant control points in standard operation procedures (**SOP**).

While there is some evidence that the measurement of udder firmness utilizing a dynamometer can be used to diagnose CM (Rees et al., 2017), studies differentiating different severity scores or pathogens are missing. The diagnostic value of this method to differentiate between healthy quarters and quarters with SCM and CM has yet to be investigated.

As udder surface temperature (**UST**) seems to rise in mastitis quarters just as SCC (Sathiyabarathi et al., 2016b), several researches investigated the accuracy of diagnosing CM (Metzner et al., 2014; Sathiyabarathi et al., 2016a) and SCM (Berry et al., 2003; Polat et al., 2010) using infrared thermography (**IRT**). While this technique measures the whole udder, we wanted to evaluate, whether a smaller, less expensive device, such as a hand-held infrared thermometer, which measures only a part of the udder, is also able to diagnose mastitis. Such devices were, e.g., validated for measurements of ear surface temperature in cows with hypocalcemia (Venjakob et al., 2016). An application in cows with mastitis might be feasible but has not yet been investigated.

Therefore, the overall objective of this study was to investigate the diagnostic value of two hand-held devices, i.e., an infrared thermometer and a dynamometer for udder health monitoring. Specifically, we set out 1) to determine the accuracy of each devices to differentiate between CM, SCM, and healthy udder quarters, 2) to determine the accuracy of each device to differentiate between mild, moderate and severe CM, 3) to determine the accuracy of each device to differentiate between gram-positive and gram-negative pathogens and no growth in CM cases, and 4) to compare surface temperature and udder firmness measured with those two devices with results acquired by palpation of the udder tissue.

The study was conducted between June 2016 and January 2017 on a commercial dairy farm in Brandenburg, Germany, housing approximately 2,500 dairy cows (305-d milk yield 9,839 ± 1,887 kg; mean ± SD). Overall, 251 Holstein Friesian dairy cows with CM, 150 with SCM, and 70 healthy cows were enrolled. Cows were between 1st and 9th lactation (2.9 ± 1.5), and on average 168.7 ± 113.7 DIM. They were housed in a free stall barn with slatted flooring and stall cubicles equipped with rubber mats and had ad libitum access to water. Cows were fed a balanced TMR twice daily and were managed according to the guidelines set by the International Cooperation on Harmonization of Technical Requirements for Registration of

Veterinary Medicinal Products (Hellmann and Radeloff, 2000). Fresh cows and high lactating cows were milked three times a day and late lactating cows two times a day in a 56-stall head-in rotary milking parlor, respectively. Special groups (i.e., hospital pen, colostrum and mastitis group) were milked twice daily in a 2 x 10 Herringbone milking parlor.

Cows with signs of CM (i.e., clotted milk, heat or swelling of the udder) that were identified by milking personnel during regular milking, were separated and examined by 1 out of 5 investigators. Before initiation of the study, the investigators were trained in the following manner. Based on information gathered from the scientific literature (i.e., palpation of firmness: Gleeson et al., 2007; measuring firmness with a dynamometer: Bertulat et al., 2012; palpation of UST: Hoeksma and Faber, 2000; measuring UST with a hand-held infrared thermometer: Sathiyabarathi et al., 2016b, Venjakob et al., 2016) a first version of a SOP was written that all investigator read and had to follow. Furthermore, several training sessions were carried out before the study started. When disagreement occurred between investigators during those sessions, the findings were discussed, definitions were reviewed, and the examinations were repeated until agreement was reached. The SOP was then finalized and implemented.

Healthy cows and those with SCM were preselected based on most recent DHIA results (i.e., SCC < 10,000 cells/mL and SCC > 1,000,000 cells/mL). Cows with CM within the last 30 d before enrollment, with signs of metabolic or infectious disease (e.g., ketosis or metritis), and cows that received any treatments (e.g., antimicrobials or anti-inflammatory drugs) were excluded from the study. Moreover, cows with teat lesions or lacerations of the udder surface, with CM in 2 or more quarters, and cows within 5 d after calving were not enrolled. Healthy quarters with a positive bacteriological result were retrospectively withdrawn.

After general examination (i.e., rectal temperature, pulse and respiration rate) of each cow, an examination of the udder was conducted including milk sampling and CMT (KerbaTEST, Albert Kerbl GmbH, Buchbach, Germany). Only one quarter per cow was included in the study. In case of CM, the mastitis quarter was chosen. In cows with SCM and in healthy cows, the quarter was chosen based in CMT results (e.g., healthy: 0, SCM: ++ or +++). If 2 or more quarters of a cow fulfilled the requirements for enrollment, the study quarter was selected based on convenience (e.g., best milkability, cleanliness). Sterile milk samples of each selected quarter were collected for bacteriological culturing and sent to accredited laboratories. Examinations of udder firmness and UST followed a previously established SOP to minimize the effect of investigator and ensure that all cows were examined and classified in

the same manner. Udder firmness was scored by palpation based on a scoring system validated by Gleeson et al. (2007). In brief, score 0 equals a soft udder, yielding significantly to gentle pressure from the finger and score 3 is used for very firm udders, not yielding to pressure. Udder skin temperature was scored on a 4-point scale similar to a scoring system that Hoeksma and Faber (2000) used to assessed skin temperature in leprosy patients (i.e., 0 = UST comparable to temperature of the flanks, 1 = mild, 2 = moderate, and 3 = strong increase compared to surface temperature of the flanks). Investigators determined firmness and UST independent from each other. Udder firmness was also measured with a dynamometer (Penefel DFT14; Agro Technologie, Forges-les-Eaux, France) according to the SOP described by Bertulat et al. (2012) and applied by Rees et al. (2017). Udder surface temperature was measured with a hand-held infrared thermometer (Fluke 568 IR Thermometer, Fluke Deutschland GmbH, Glottertal, Germany) at a distance of 1 m, resulting in a measured area of 2 cm in diameter on the udder surface (Sathiyabarathi et al., 2016b; Venjakob et al., 2016). The measuring point for both devices was located in the horizontal and vertical middle of the quarter. Forequarters were measured from the lateral side and hindquarters from the posterior side of the udder (Bertulat et al., 2012; Sathiyabarathi et al., 2016b).

All cases of CM were classified into mild (abnormal appearance of milk), moderate (abnormal appearance of milk accompanied by swelling or redness of the mammary gland) and severe CM (in addition to signs of local inflammation systemic illness such as fever above 39.5°C) according to Wenz et al. (2001) with slight modifications implemented by Pinzon-Sanchez and Ruegg (2011). After examinations, cows were treated according to the SOP implemented on the farm.

Climate loggers (Tinytag Plus II, Germini Loggers Ltd., Chichester, United Kingdom) were placed securely in the middle alley of the different pens collecting data on ambient temperature every hour.

Data were entered into Excel spreadsheets (Excel, version 2016; Microsoft, Redmond, WA) and statistical analyses were performed with SPSS for Windows (SPSS, version 24.0, IBM Deutschland GmbH, Ehningen, Germany). The statistical significance level was set at $P \leq 0.05$ and trends were discussed at $P < 0.10$. The bacteriological results were categorized based on gram staining characteristics (i.e., gram-negative, gram-positive, and no growth). Normality of distributions of continuous parameters (i.e., udder firmness, UST) was assessed by plotting the data, visual examination and calculating a Q-Q-plot. In order to calculate the

effect of udder health status (i.e., CM, SCM or healthy) on udder firmness measured by dynamometer or UST measured by infrared thermometer, linear models were used. All models were built according to the model building strategies published by Dohoo et al. (2009) and described by Bertulat et al. (2017). In brief, all independent parameters were initially tested with Spearman's correlation or Pearson's correlation for collinearity and analyzed in a univariate univariable model. Only parameters resulting in univariable models with $P \leq 0.2$ were included in the final multivariable univariate model. If 2 parameters showed a high, significant correlation ($r < -0.6$; $r > 0.6$), only the parameter with the smallest *P*-value was used in the final model. The final model was built in a conditional backward stepwise manner. Interactions were tested for all relevant parameters (e.g., DIM with milk yield, lactation number with total number of episodes with recurrent CM, and gram straining characteristics and severity score of clinical mastitis). Post hoc comparison was carried out applying LSD test. Validity of the final models was ensured by checking that the model assumptions were met, especially the normality of distribution of residues was verified using the Shapiro-Wilk and Kolmogorov–Smirnov-test, plotting the residues and calculating a Q-Q-plot. Concerning the effect of udder quarter health status, two separate models were calculated using either udder firmness or UST as outcome variable. Model building was repeated with severity score of CM (i.e., mild, moderate, severe) instead of udder health status. The following parameters were furthermore tested: lactation number, DIM, milk yield, total number of episodes with recurrent CM, time after milking, ambient temperature, investigator, quarter position, and gram straining characteristics. For ordinal variables (e.g. palpation of udder firmness and UST) a generalized linear model for ordinal variables was used. Model building was done in the same way as described before. A multinomial logistic regression was used in order to determine, if there were any confounders which could have had an influence on the udder health status or gram straining characteristics and thus would require a modification of the calculations of Se and Sp as described by Coughlin et al (1992). The effects of lactation number, DIM, milk yield, total number of episodes with recurrent CM, time after milking, ambient temperature, investigator and quarter position on the odds for udder quarters to develop a SCM or CM and for udder quarters having different bacteriological results were assessed. Receiver operating characteristic (**ROC**) curves were calculated to establish thresholds between cows with and without mastitis or with negative and positive bacteriological results. Interpretation of ROC curves was based on the area under the curve (**AUC**). The best thresholds were chosen based on the highest sum of sensitivity (**Se**) and specificity (**Sp**). In order to evaluate the relationship

between manually determined and measured values of UST and firmness, Spearman's correlation coefficients were calculated.

Overall, 218 cows with CM (i.e., 46 mild, 106 moderate, 66 severe), 142 with SCM and 68 healthy cows met all inclusion criteria. Thirty-four, 7, and 2 cows had to be excluded because of a CM within 30 d before enrollment, a positive bacteriological result, and signs of metabolic or infectious disease (e.g., metritis claw lesions, ketosis), respectively.

Milk samples categorized as gram-positive included *Streptococcus uberis* (35%), *Streptococcus agalactiae* (17%), *Staphylococcus aureus* (17%), coagulase-negative staphylococci (13%), *Streptococcus dysgalactiae* (7%), and other Streptococcus spp. (11%). In gram-negative samples coliform bacteria (56%), *Escherichia coli* (41%), and Klebsiella spp. (3%) were found. Distribution of different bacteria strains within different severity scores of CM are shown in table 1.

Table 1. Cross table of bacteriological results of quarter milk samples against clinical mastitis of different severity score (e.g., mild, moderate, and severe).

		Clinical mastitis			Sum
		Mild	Moderate	Severe	
Bacteria strain	Staph. aureus	4	6	4	14
	KNS	0	1	0	1
	Strep. uberis	3	13	11	27
	Strep. agalactiae	5	6	7	18
	Strep. dysagalactiae	3	5	0	8
	Other Strep.	2	10	6	18
	Coliform bacteria	3	5	15	23
	Escherichia coli	2	6	9	17
	Klebsiella spp.	0	0	1	1
	Trueperella pyogenes	0	0	1	1
	Culture-negative	23	41	8	72
Sum		45	93	62	200

The distribution of udder firmness values in quarters with different health status are shown in figure 1. Udder firmness measured by dynamometer in healthy quarters, quarters with SCM and CM averaged 1.054 ± 0.13 kg (LSM ± SE), 1.394 ± 0.09 kg and 1.974 ± 0.06 kg, respectively. Quarters of different udder health status differed in their udder firmness between each other ($P < 0.001$). There was no effect of lactation number, DIM, milk yield, total

number of episodes with recurrent CM, time after milking, ambient temperature, investigator, and quarter position on udder firmness ($P > 0.05$).

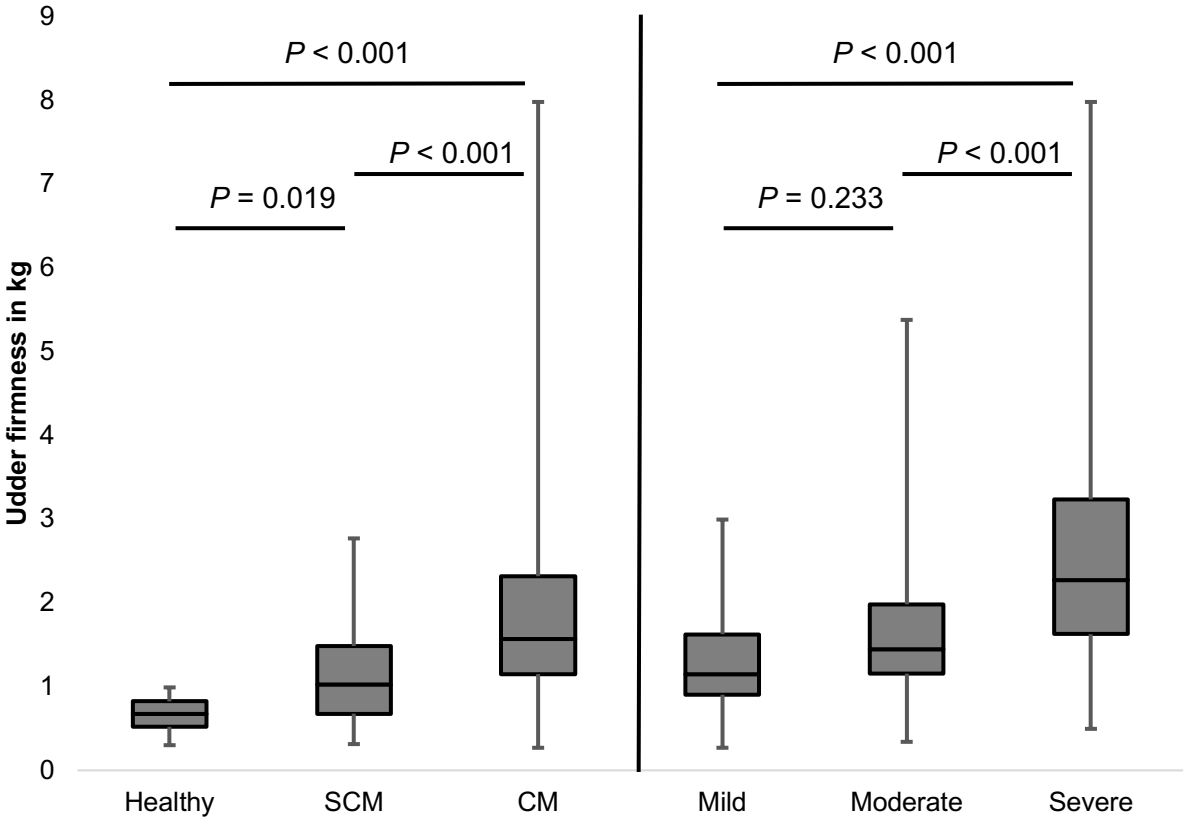

Figure 1. Boxplots, depicting udder firmness (kg) in udder quarters with different health status (left side - healthy: 68 healthy quarters, SCM: 142 quarters with subclinical mastitis, CM: 218 quarters with clinical mastitis and right side - clinical mastitis quarters with different severity scores: 46 cows with mild, 106 with moderate and 66 with severe clinical mastitis). 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.

Considering severity scores within CM quarters, udder firmness averaged 1.478 ± 0.17 kg, 1.726 ± 0.11 kg and 2.550 ± 0.13 kg, in mild, moderate, and severe cases, respectively. Within cows with CM, severity score and gram staining characteristics had a significant overall effect on udder firmness measured by dynamometer ($P < 0.001$). Measurement results of quarters of different severity scores of CM differed significantly between each other ($P < 0.001$). Gram-negative tested quarters (2.234 ± 0.17 kg) tended to be firmer compared to quarters infected with gram-positive strains (1.882 ± 0.11 kg; $P = 0.080$). A bacteriological

negative result was associated with lower firmness (1.638 ± 0.13 kg; $P = 0.007$) compared to gram-negative, but not to gram-positive mastitis pathogens ($P = 0.147$).

Based on the multinomial logistic regression, no effect of any of the tested confounders on the odds for an udder quarter being classified as SCM or CM instead of being healthy was found. These tested factors also did not influence the odds for having gram positive, negative or culture negative results.

The threshold providing the highest accuracy (Se = 0.73, Sp = 0.88) for differentiating between healthy and mastitis quarters was 1.002 kg (AUC = 0.832, $P < 0.001$). In CM quarters a threshold of 1.175 kg (Se = 0.70, Sp = 0.62, AUC = 0.704, $P < 0.001$) was established to differentiate between bacteriologically negative and positive. These thresholds do not meet the recommendations (e.g., Se = 0.80, Sp > 0.99) of the International Standard Organization (ISO/FDIS 20966) for diagnostic tests in automatic milking systems (ISO, 2007). Nevertheless, compared to electrical conductivity measurements (i.e., SCM Se = 0.194, Sp = 0.919; CM Se= 0.479, Sp = 0.919; Norberg et al., 2004), udder firmness is more accurate.

Our results support the assumptions of previous studies that udder firmness is a reliable indicator to identify CM (Swinkels et al., 2013; Fogsgaard et al., 2015). To evaluate a successful application of a dynamometer to diagnose and classify SCM and CM in the field further studies are warranted since the inclusion criteria for cows in our trial and the thorough diagnostic of all mastitis cases allowed us to generate a dataset that might differ from mastitis cases detected in day to day routine on commercial dairy farms. Nevertheless, our results confirm findings from Rees et al. (2017) that cows with CM had firmer udders compared to healthy cows. In contrast to the aforementioned study, we additionally were able to show that gram staining characteristics (i.e., gram positive, gram negative or no growth) affected udder firmness in mastitis quarters. While no growth and gram-negative quarters differed significantly, there was only a tendency for different udder firmness values in gram-positive and gram-negative quarters was detected. Interestingly, in our study udder firmness in cows with CM was approximately 80% higher compared to values reported by Rees et al. (2017), while values in healthy quarters were similar. While in our study front and hind quarters were measured, only hind quarters were included previously (Rees et al., 2017). These different types of quarters in both studies might be an explanation for differences in udder firmness, although in healthy cows, front quarters are less firm than hind quarters (Bertulat et al., 2012).

So far there are no data from CM cows, but it might be hypothesized that CM leads to higher firmness values in front quarters.

Considering palpation, an overall effect of different health status (i.e., CM, SCM, healthy; $P < 0.001$), gram straining characteristics ($P < 0.001$) and time after milking ($P = 0.022$) on udder firmness could be evaluated with no effect of further tested factors ($P > 0.05$). Most healthy quarters were scored 0 during palpation. Higher scores were found more frequently in CM and SCM quarters (Figure 2). Healthy quarters differed significantly from quarters with SCM ($P = 0.003$) and CM ($P < 0.001$). Additionally, SCM differed from CM quarters ($P < 0.001$). In CM cows, severity score ($P < 0.001$) and gram staining characteristic ($P = 0.018$) had an effect on the palpation score. With increasing severity score of CM, higher scores of udder firmness were given by palpation (Figure 2).

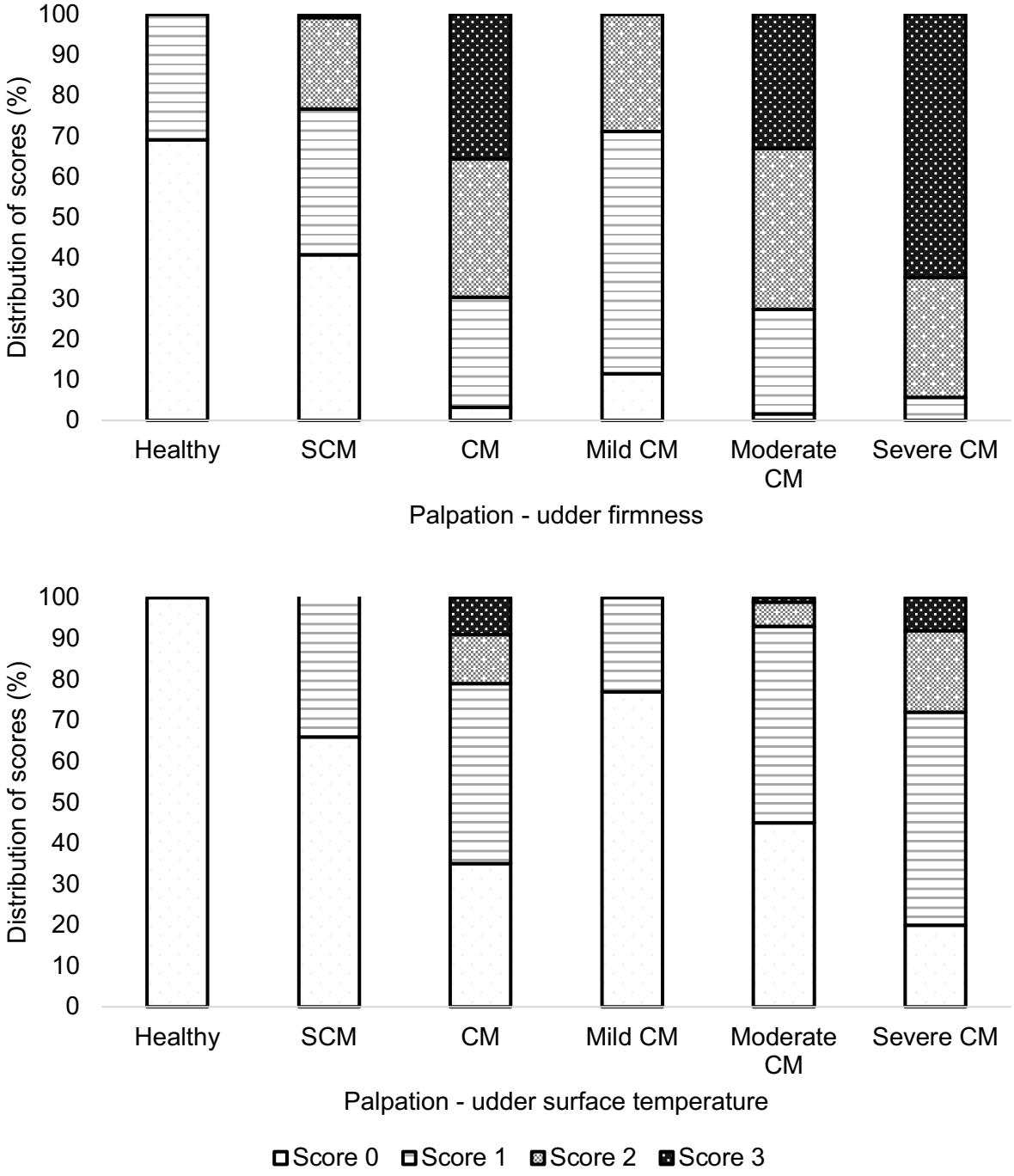

Figure 2. Distribution of palpation scores of udder firmness (top; i.e., 0 to 3; soft to very firm) and udder surface temperature (bottom; i.e., 0 to 3; no increasing to strong increase compared to body surface temperature of the flanks) in 68 healthy quarter, 142 quarters with subclinical mastitis and 218 with clinical mastitis of different severity scores (e.g., mild: 46, moderate: 106 and severe: 66).

In agreement with previous studies (Rees et al., 2014), a strong correlation between palpation of udder firmness and measurements by dynamometer was found (r = 0.749, $P < 0.001$). Considering our results, the differentiation between healthy quarters and those with CM and SCM and between different severity scores of CM by palpation is possible. Nevertheless, palpation is still a subjective parameter with limited repeatability when multiple observers are involved (Houe et al., 2002; Rees et al., 2014). Also in our study, the knowledge, that a given cow was healthy or had a CM before palpation could have had an influence on the estimation.

The UST measured by a hand-held infrared thermometer ranged from 25.0°C to 39.8°C. It was affected by the health status of a quarter and ambient temperature ($P < 0.001$, Table 2) but not by the other tested factors ($P > 0.05$). Udder quarters with CM (35.1 ± 0.1°C) had higher UST than those with SCM (33.7 ± 0.2°C; $P < 0.001$) and healthy quarters (34.4 ± 0.2°C; $P < 0.001$). Healthy quarters differed from CM and SCM quarters ($P < 0.001$). Within CM quarters, the UST depended on ambient temperature ($P < 0.001$) and severity score ($P = 0.037$), but not on the other tested factors ($P > 0.05$). Udder quarters with mild CM (35.1 ± 0.4°C) tended to be cooler than those with moderate (35.4 ± 0.4°C, $P = 0.088$) and severe CM (35.6 ± 0.4°C, $P = 0.010$). Moderate and severe CM quarters did not differ ($P = 0.223$). The correlation between palpation of UST and measurements by infrared thermometer was fair (r = 0.454, $P < 0.001$).

Table 2. Two separate generalized linear mixed models showing the influence of ambient temperature ($P < 0.001$) on udder surface temperature in cows with different udder health status ($P < 0.001$, model A) and in cows with different severity score of clinical mastitis ($P = 0.037$, model B) measured with a hand-held infrared thermometer. Data are based on 68 healthy quarters, 142 quarters with subclinical mastitis and 218 quarters with clinical mastitis (46 cows with mild, 106 with moderate and 66 with severe clinical mastitis).

Model	Variable	Level	Estimate	SE	*P*-value
A	Intercept		30.43	1.49	< 0.001
	Ambient temperature		0.18	0.16	< 0.001
	Udder health status	Healthy	Referent		
		Subclinical mastitis	- 0.89	0.19	< 0.001
		Clinical mastitis	0.51	0.15	0.001
B	Intercept		31.52	0.61	< 0.001
	Ambient temperature		0.15	0.02	< 0.001
	Clinical mastitis	Mild	Referent		
		Moderate	0.33	0.19	0.088
		Severe	0.53	0.20	0.010

The UST estimated by palpation (Figure 2) was scored 0 in all healthy quarters. Subclinical quarters were equally often diagnosed as 0 and 1. In CM cases, scores ranged between 0 and 3. Scores of SCM quarters differed from healthy ($P = 0.003$) and CM ($P < 0.001$) quarters. Within CM quarters, palpation results of UST differed between severity scores ($P < 0.001$). With increasing severity score, higher scores of UST were given by palpation (Figure 2).

In contrast to previous publications (Colak et al., 2008; Zaninelli et al., 2018), UST measured by IRT was lowest in SCM quarters. Our results of UST scores determined by palpation, however, had lowest scores in healthy quarters. During palpation all quarters were briefly palpated and sampled, making sure that only one quarter was affected. An unconscious comparison of quarters within a cow is likely and might have had an influence on scoring. Elevated UST observed in CM quarters may be explained by an increased blood flow (Dhondt et al., 1977). Bortolami et al. (2015) supports our findings and assumes that a deviation of blood flow during SCM could cause a decrease in UST.

Overall, it has to be considered, that the high impact of ambient temperature on UST compromised the diagnostic value of IRT in mastitis (Berry et al., 2003; Castro-Costa et al., 2014). Similar confounders have been described for other applications such as the measurement of ear temperature in cows with hypocalcemia (Venjakob et al., 2016) or claw temperature (Gloster et al., 2011) in lame cows. In our study UST increased by 0.15 to 0.18°C for each degree of ambient temperature (Table 2). In comparison, the effect of mastitis was negligible (i.e., 0.51°C in CM; -0.89 in SCM compared to healthy quarters). Considering these results, an application in the field seems to be limited and a calculation of thresholds was not reasonable.

In conclusion, it could be demonstrated that the measurement of udder firmness by using a hand-held dynamometer is an accurate and objective method to estimate the udder health status of dairy cows (e.g., CM, SCM, and healthy udder quarters). Within CM, severity scores and gram straining characteristics affected udder firmness. Severe CM quarters differed from mild and moderate CM ($P < 0.001$) and quarters with a bacteriological negative result differed significantly from gram-negative tested quarters ($P = 0.007$). Therefore, in times of continuing automatization, the dynamometer might be a valuable alternative in order to reliably diagnose mastitis. Udder firmness thresholds can help to preselect cows for further examination and might be used as critical control points in SOP for diagnostic classifications

and treatment decisions. Further studies, however, are warranted in order to confirm that our results can be transferred to other farms with different mastitis pathogens and different climatic conditions and that calculated threshold are valid on those farms as well. The diagnostic value of measuring UST with a hand-held thermometer in order to detect SCM and CM and differentiate between severity scores is highly limited since there is a strong impact of ambient temperature on UST. Nevertheless, a differentiation between quarters of different health status was possible. Differentiation between severity scores in CM, however, was not possible. The discrepancy between palpation results of UST and results gained by IRT should be further investigated.

2.4. Acknowledgements

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3. PUBLICATION II

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

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3.1. Abstract

Timely and objective diagnosis and classification of mastitis is crucial to ensure adequate management and therapeutic decisions. Analyzing specific biomarkers in milk could be advantageous compared to subjective or semiquantitative criteria such as palpation of the udder in clinical mastitis cases or evaluation of somatic cell count using cow side tests (e.g., California Mastitis Test) in subclinical mastitis quarters. The objective of this study was to investigate the diagnostic value of three biomarkers; i.e., cathelicidin, milk amyloid A, and haptoglobin for the diagnosis of subclinical and clinical mastitis. Furthermore, the suitability of these biomarkers to differentiate between mild, moderate, and severe clinical mastitis and the influence of different pathogens on biomarker levels was tested. A total of 67 healthy cows, 119 cows with subclinical, and 212 cows with clinical mastitis were enrolled in the study. While cathelicidin, haptoglobin, and milk amyloid A were measured in all samples from healthy cows and those with subclinical mastitis, haptoglobin, and cathelicidin results were only available from 121 out of 212 cows with clinical mastitis. Milk amyloid A was measured in all samples. In cows with clinical mastitis, the mastitic quarter and a second healthy quarter serving as a healthy in-cow-control quarter were sampled. It was possible to differentiate between healthy quarters, quarters with subclinical mastitis, and quarters with clinical mastitis, using all three biomarkers. Concerning cathelicidin, thresholds were 0.000 (Se = 0.83, Sp = 0.97) and 0.053 (Se = 0.98, Sp = 0.99) for normalized optical density at 450 nm (NOD450) for differentiating between healthy quarters and quarters with subclinical or clinical mastitis, respectively. Thresholds of 1.28 µg/mL (Se = 0.65, Sp = 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 = 0.94) and 5.40 µg/mL mL (Se = 0.96, Sp = 0.99) for haptoglobin were calculated, respectively. Healthy in-cow control quarters from cows with CM showed elevated milk amyloid A and haptoglobin levels compared with healthy quarters from healthy cows. Only the level of milk 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. 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.

3.2. Key words

milk amyloid A, cathelicidin, haptoglobin, mastitis diagnostic

3.3. 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 the prediction of treatment outcomes (Royster and Wagner, 2015). In the past, treatment decisions for CM were usually based on a farmer's or veterinarian's evaluation of clinical symptoms, such as 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 have 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 overall productivity of a farm (Ruegg, 2017). Due to a lack of clinical signs, diagnosis of SCM is based mostly on milk analysis.

Today, cow-individual SCC values are a well-accepted measure to diagnose SCM (Ruegg, 2017). The California Mastitis Test (**CMT**) described by Schalm and Noorland (1957) is widely used to evaluate SCC in milk. As it is a semiquantitative measure, however, the interpretation can be subjective, leading to false positive and negative results (Viguier et al., 2009). Even interpretation of CMT results by trained technicians may result in mediocre sensitivity (**Se**) and specificity (**Sp**) values (i.e., 82.4 and 80.6%; Dingwell et al., 2003). The interpretability of SCC results is further limited, as levels usually remain elevated for several weeks after an intramammary infection, even after successful mastitis treatment (Pyörälä, 1988). Additionally, SCC is affected by various physiological (e.g., stage of lactation, age, and stress; Sharma et al., 2011) and environmental factors (e.g., geographical zones and housing system; Bielfeldt et al., 2004).

Another method to identify SCM quarters is via changes in milk conductivity (Norberg et al., 2004), which can be monitored automatically and with a high Sp (ranging from 97.3 to

99.3% depending on algorithms). Sensitivity (ranging from 5.5 to 42.9% depending on algorithms), however, is not satisfying (Hovinen et al., 2006, as values do not meet the required 80% Se for diagnostic tests in automatic milking systems (ISO, 2007).

Recent advances of proteomic techniques have led to the identification of several new neutrophil-produced proteins involved in mastitis immune responses (Lippolis and Reinhardt, 2005; Smolenski et al., 2007). These proteins might be suitable biomarkers usable for mastitis diagnosis (Viguier et al., 2009; Ceciliani et al., 2012). One of the first proteins used to detect mastitis in milk was lactate dehydrogenase. Albeit the measurement of this protein lacked in accuracy (Nyman et al., 2016), it was demonstrated that in-line monitoring for lactate dehydrogenase is feasible, can be integrated in automated milking systems (Akerstedt et al., 2011), and achieves a Se of 80% in SCM (Hiss et al., 2007).

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

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

So far, available literature, however, focused on the applicability of one biomarker at a time. A recent study (Thomas et al., 2018) demonstrated promising results in diagnosing naturally occurring bovine mastitis by examining 3 acute phase proteins (HP, C-reactive protein, and mammary-associated serum amyloid A3) simultaneously. Data on the comparability, their correlation, and the association with the health status of a given udder quarter (e.g., healthy, SCM, or CM) or the severity of the inflammation, however, are lacking.

Therefore, the objective of this study was to investigate and compare the diagnostic value of CATH, MAA, and HP measured in milk to determine the udder health status. Specifically, we set out to investigate the accuracy of each biomarker to differentiate between: (1) CM, SCM, and healthy udder quarters; (2) mild, moderate, and severe CM; and (3) mastitis caused by different bacteria. Furthermore, the Se and Sp for the between CM quarters and healthy in-cow control quarters, and healthy quarters of healthy cows and healthy in-cow control quarters of cows with CM was determined.

3.4. Materials and Methods

Animals and Experimental Design

The study was conducted between June 2016 and January 2017 on a commercial dairy farm in Brandenburg, Germany, housing approximately 2,500 dairy cows (305-d milk yield of 9,839 ± 1,887 kg; mean ± SD). Seventy healthy Holstein Friesian dairy cows, 126 cows with SCM, and 251 cows with CM were initially included in the study. At the time of enrollment, cows were between first and ninth lactation (2.9 ± 1.5) and on average 168.7 ± 113.7 DIM. All cows were housed in a freestall barn with slatted flooring and stall cubicles equipped with rubber mats. Cows were managed according to the guidelines set by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH, 2000). They were fed a TMR consisting of corn, beet pulp, alfalfa, bruised grain and rape, straw, soybeans, and a concentrate mineral mix delivered on a conveyer belt system twice a day. Rations were formulated to meet or exceed the dietary requirements for dairy cows (NRC, 2001). All cows had ad libitum access to water. Fresh cows and high-lactating cows were milked 3 times/d, and late-lactating cows were milked 2 times/d in a 56-stall head-in rotary milking parlor. Special groups (i.e., hospital pen, colostrum, and mastitis group) were milked twice daily in a 2 x 10 herringbone milking parlor.

Cows with signs of CM (i.e., clotted milk, heat, or swelling) were identified by milking personnel during regular milking in the milking parlor, separated, and fixed in a cattle chute for

further examinations. Healthy cows and those with SCM were preselected based on most recent DHIA results (i.e., healthy: SCC <10,000 cells/mL, SCM: SCC >1,000,000 cells/mL). These thresholds were used to increase the probability to truly identify a quarter with SCM in a cow with high SCC in the composite sample. Thresholds were further used to find at least 1 healthy quarter in cows that had been healthy at the time of last DHI test day. After selection, cows were separated and examined. Cows with CM within the last 30 d before enrollment, cows with signs of metabolic or infectious disease (e.g., ketosis, hypocalcemia, fever), and those that received systemic or intramammary antibiotics or anti-inflammatory drugs were excluded from the study. Furthermore, cows with any teat lesion or lacerations of the udder surface, cows with mastitis in more than 1 quarter, and cows within 5 d after calving were not enrolled.

Finally, total of 67 healthy cows, 119 cows with SCM, and 212 cows with CM met the including criteria of the study and were enrolled. In healthy and SCM cows, CATH, HP, and MAA were measured in all samples. In CM cows, results of HP and CATH were only available from 121 out of 212. Milk amyloid A was measured in all samples.

After general examination (i.e., rectal temperature, pulse, and respiration rate) of each cow, an examination of the udder was conducted. Additionally, a CMT was done (KerbaTEST; Albert Kerbl GmbH, Buchbach, Germany), and 2 milk samples per quarter were collected (i.e., 1 sterile sample for bacteriological culturing and SCC, 1 unsterile sample for the analysis of biomarkers).

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

All CM cases were classified into mild (1: abnormal appearance of milk), moderate (2: abnormal appearance of milk accompanied by swelling or redness of the mammary gland), and severe CM (3: beside abnormal appearance of milk and swelling of the mammary gland, cow showed signs of systemic illness such as fever above 39.5°C) according to Wenz et al. (2001) and Pinzon- Sanchez and Ruegg (2011). After examinations and samplings, CM and

SCM cows were treated according to the standard operating procedures established on the farm.

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

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

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

Sterile milk samples were divided into 3 subsamples immediately after collection and sent to 3 different accredited laboratories for bacteriological culturing. In all 3 laboratories, bacteriological culturing was performed following the examination standards and regulations of the German Veterinarian Association guidelines (GVA, 2012) and the National Mastitis Council handbook (National Mastitis Council, 1999, 2004). In brief, 10 µL of a well-mixed sample were plated with a sterile loop onto an esculin sheep-blood agar plate (Oxoid, Wesel, Germany) and incubated for 48 h at 37°C under aerobic conditions. Examinations were carried out at 24 and 48 h after inoculation. Grown colonies were identified based on their colony morphology, Gram staining characteristics, hemolysis patterns, and their esculin hydrolysis. If necessary, other biochemical properties (e.g., activity of catalase, clumping factor test, Lancefield serotyping, activity of cytochrome oxidase C, and oxidation-fermentation of glucose) were considered for further identification.

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

Unsterile milk samples were divided into 3 subsamples and stored at – 20°C until analyses. One of those subsamples each was analyzed for MAA, CATH, and HP, respectively. Milk amyloid A and CATH measurements were carried out by Bioteck Lait (Pacé, France) and Porto Conte Ricerche S.r.l. (Alghero, Italy), respectively.

Cathelicidin was analyzed with a pan-cathelicidin ELISA based on 2 monoclonal antibodies developed against a pan-cathelicidin domain (Addis et al., 2016a, b). At the end of the assay, for each sample, the optical density measured at 450 nm (**OD450**) was normalized against internal controls (**NOD450**). Normalized values were generated by subtracting the average of 6 culture-negative samples with <50,000 cells/mL from each measured value. To assess CATH levels, each milk sample was measured in duplicate aliquots of 10 and 1 µL. When the results of the 10-µL aliquot provided a value above 2.5 NOD450, the value of the 1-µL aliquot was used multiplied by 10. The interassay CV for the CATH ELISA was calculated based on 2 internal standards loaded in each of the 22 total ELISA plates analyzed. The OD450 mean value of the 2 standards was 0.365 (± 0.04 SD), with an interassay CV of 11.16%. Measurements of MAA were performed by a dairy laboratory that is affiliated with the French DHI program (Oxygen Laboratoires d'Analyses, Maroeuil, France) as earlier described (Crosson et al., 2015) and validated (Gerardi et al., 2009). The concentration of MAA was determined using a commercial ELISA kit (Milk Amyloid A-MAA Assay Kit, cat. no. TP-807; Tridelta Development Ltd., Maynooth, Ireland) in accordance with the manufacturer's recommendations. Samples were added to microwells along with a biotinylated monoclonal antibody. After washing to remove unbound material, streptavidin-horseradish peroxidase was added and wells were incubated. Following the incubation, TMB substrate solution was added and the absorbance of each well was measured. Limit of detection of the ELISA was 0.4685 µg/mL stated by the manufacturer. The interassay CV for the MAA ELISA was 11.55%.

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

Statistical Analyses

Data were entered into Excel spreadsheets (version 2016; Microsoft Corp., Redmond, WA) and statistical analyses were performed with SPSS for Windows (version 24.0, IBM Deutschland GmbH, Ehningen, Germany). The statistical significance level was set at $P \leq 0.05$ and trends were discussed for $P \leq 0.10$. Normality of distributions of continuous parameters (i.e., level of CATH, MAA, HP) was assessed by plotting the data, visual examination, and calculating a Q-Q-plot.

Several different generalized linear mixed models were used to determine the effect of the health status of the udder quarter (e.g., healthy, SCM, CM, and healthy in-cow control), different severity scores (e.g., mild, moderate, and severe), and bacteriological results on CATH, MAA, and HP levels. Only bacteriological results with $n \geq 10$ were included into the model.

The statistical models were built according to the model-building strategies described by Dohoo et al., 2009 and Bertulat et al., 2017. In brief, all independent parameters were initially tested with Spearman's correlation (i.e., ordinal parameter) or Pearson correlation (i.e., scaled parameter) for colinearity and analyzed in a univariate univariable model. If 2 parameters showed a high, significant correlation, only the parameter resulting in the univariable model with the smallest *P*-value was used in the final multivariable model. Furthermore, only parameters resulting in univariable models with $P \leq 0.2$ were included in the final mixed model ANOVA. This final model was built in a conditional backward stepwise manner. Interactions were tested for all relevant parameters. Quarter within cow was included as random effect. Post-hoc comparison was carried out applying LSD test. Validity of the final models was ensured by checking that the model assumptions were met, especially the normality of distribution of residues was verified using the Shapiro-Wilk and Kolmogorov–Smirnov-test, plotting the residues and calculating a Q-Q-plot.

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

Receiver operating characteristic curves were generated and the area under the curve (**AUC**) was calculated to establish thresholds for CATH, MAA, and HP to differentiate between healthy quarters and healthy in-cow control quarters, quarters with SCM and CM, SCM and healthy quarters, CM and healthy quarters, and CM and healthy in-cow control quarters. Furthermore, thresholds between different severity scores of CM or thresholds to differentiate between different bacteria strains in either SCM or CM quarters were calculated. Thresholds were chosen based on the highest sum of Se and Sp. Pearson correlation coefficient was used to evaluate the relationship between levels of different biomarkers and between biomarker levels and SCC results.

3.5. Results

Study Population

Thirty-nine cows with CM, 7 with SCM, and 3 healthy cows had to be retrospectively withdrawn from analysis due to a CM within 30 d before enrollment, a positive bacteriological result in cows enrolled as healthy, or signs of metabolic or infectious disease (e.g., metritis, claw lesions, ketosis). Therefore, 212 cows with CM (i.e., 45 mild, 103 moderate, 64 severe), 119 with SCM, and 67 healthy cows met the inclusion criteria. For the final analysis data from 212 mastitis quarters, 212 healthy in-cow control quarters from CM cows, 119 SCM quarters, and 67 healthy quarters were used. Considering CM cows, MAA concentrations were available from 45 mild, 103 moderate, and 64 severe cases. For HP and CATH, however, concentrations from 20 cows with mild, 63 with moderate, and 38 with severe CM could be used for final analysis.

Bacteriological Results

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

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.

Cathelicidin

Cathelicidin levels in healthy, SCM, and CM quarters averaged 0.001 ± 0.008, 0.951 ± 0.046, and 2.420 ± 0.028 NOD450 (LSM ± SE; $P < 0.001$; Figure 1), respectively. Cathelicidin levels in healthy in-cow control quarters (0.045 ± 0.002 NOD450) were lower than in CM quarters ($P < 0.001$; Figure 1). Healthy quarters and healthy in-cow control quarters, however, did not differ ($P > 0.05$; Figure 1). Within CM cows, CATH levels were not influenced by severity score ($P > 0.05$) and bacteriological result ($P = 0.36$).

In contrast, CATH levels in SCM quarters infected with CNS, *Staph. aureus*, and *Strep. uberis* differed significantly ($P = 0.04$). Lowest levels of CATH were detected in samples positive for CNS samples (0.326 ± 0.070 NOD450) compared with samples positive for Staph. aureus (1.309 ± 0.185 NOD450; $P = 0.02$) and *Strep. uberis* (1.238 ± 0.119 NOD450; $P = 0.01$). Culture-negative samples (0.899 ± 0.074 NOD450) had numerically higher CATH values ($P = 0.06$) than CNS samples. Cathelicidin levels were lower in SCM compared with CM quarters of cows infected with *Staph. aureus* ($P = 0.04$), *Strep. uberis* ($P < 0.001$), and in culture-negative samples ($P < 0.001$), respectively. Descriptive values of measured levels of CATH depending on bacteriological result in milk are presented in Figure 3-2. There was no effect of any of the other tested factors (i.e., lactation number, DIM, milk yield, total number of episodes with recurrent CM, and THI) on CATH in any of the models ($P > 0.05$).

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

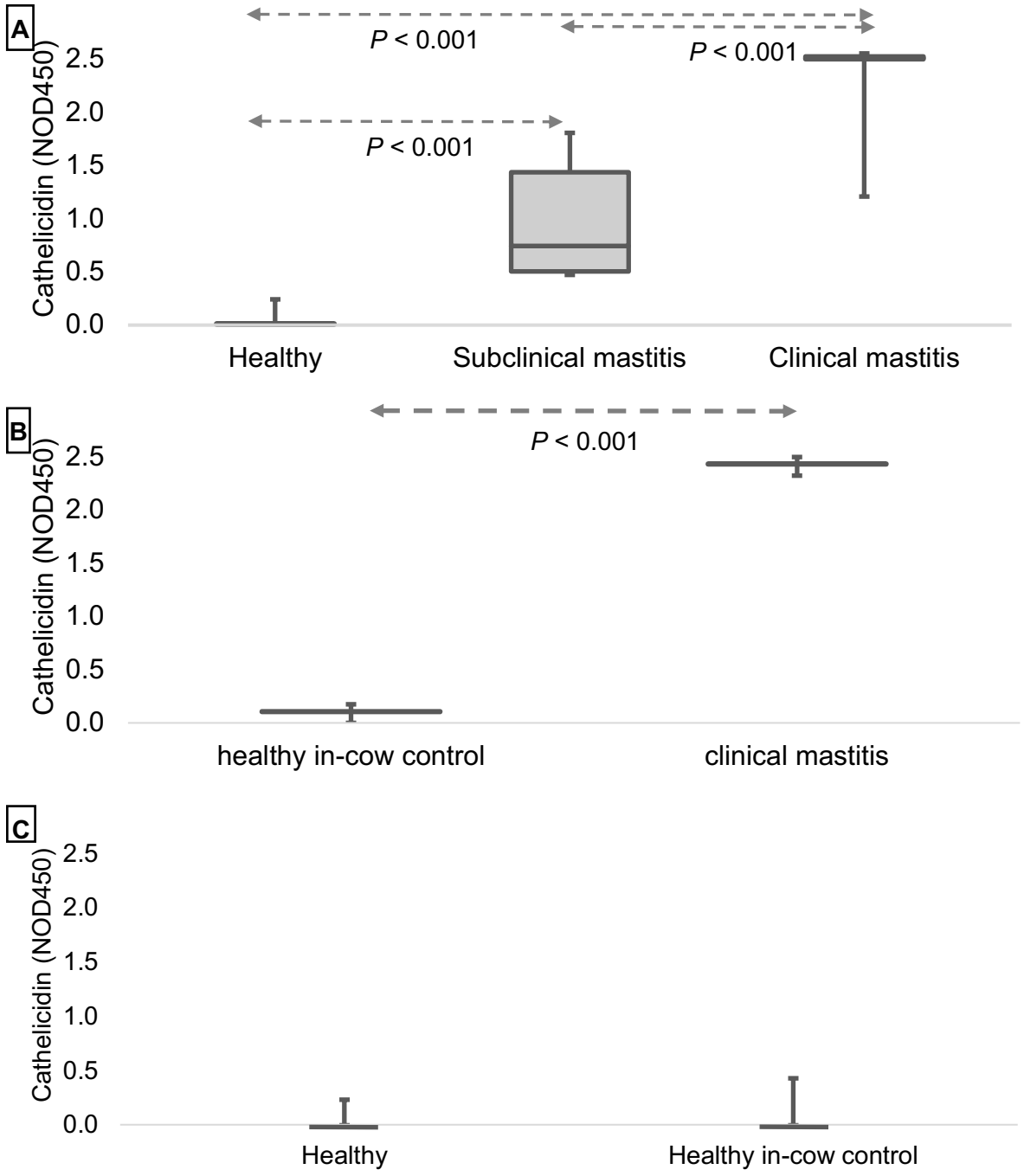

Figure 1. Comparison of cathelicidin levels (A) in healthy quarters (n = 67), quarters with subclinical mastitis (n = 119), and clinical mastitis quarters (n = 121), (B) in healthy in-cow control quarters (n = 121) and mastitis quarters (n = 121) of cows with one clinical mastitis quarter, and (C) in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

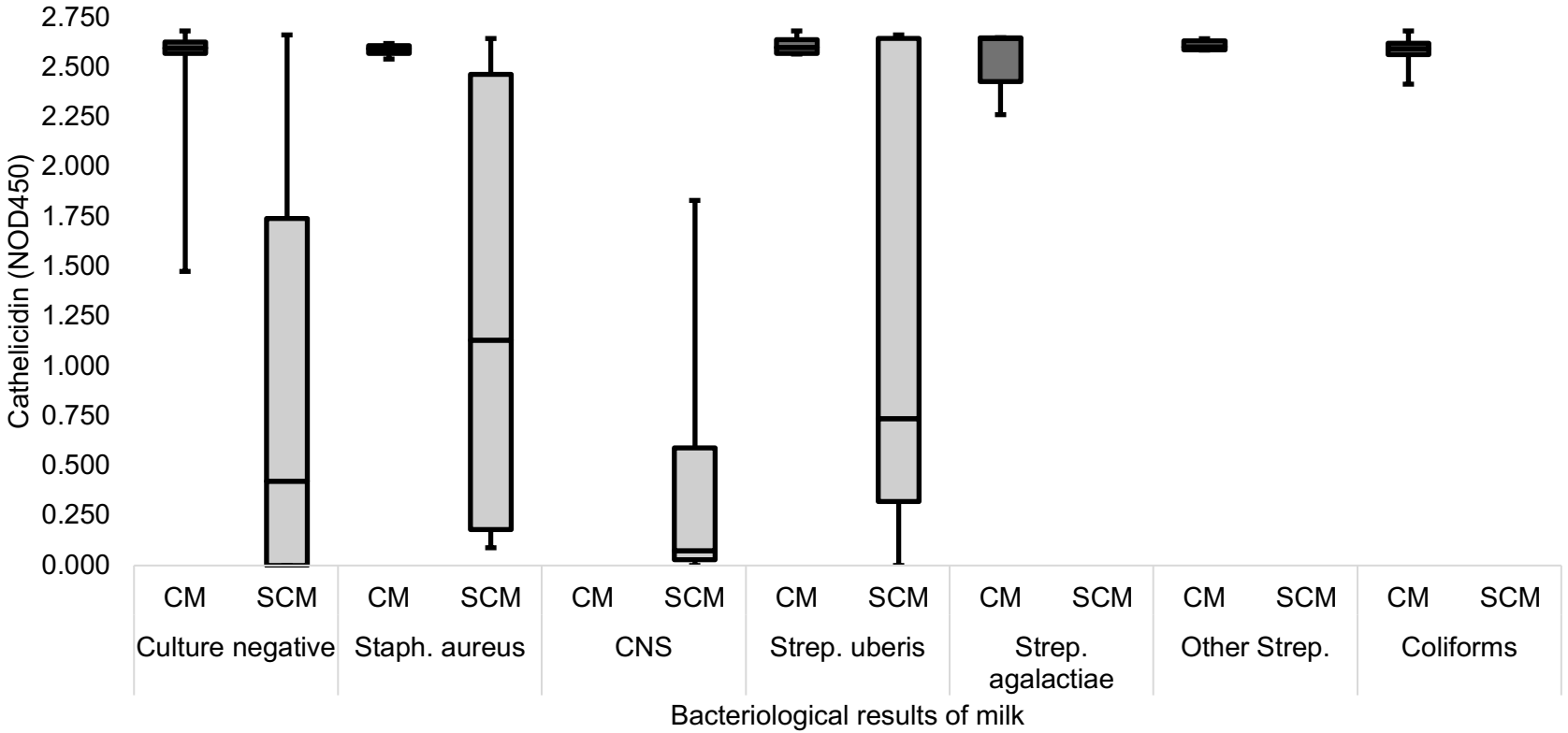

Figure 2. Cathelicidin levels in milk samples considering bacteriological results in subclinical mastitis (SCM; light grey; n = 107) and clinical mastitis quarters (CM; dark grey; n = 115). Values are expressed as normalized optical density at 450 nm (NOD450). The black line inside each box marks the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

Milk Amyloid A

Milk amyloid A in quarters of healthy cows, quarters with SCM, and quarters with CM averaged 1.06 ± 0.1 µg/mL (LSM ± SE), 2.62 ± 0.3 µg/mL, and 6.67 ± 0.2 µg/mL, respectively ($P < 0.001$; Figure 3). Levels of MAA in healthy in-cow control quarters (2.69 ± 0.2 µg/mL) were significantly lower compared with CM quarters (13.76 ± 0.1, $P < 0.001$; Figure 3). The latter (2.68 ± 0.1 µg/mL) exceeded values measured in healthy quarters of healthy cows (1.14 ± 0.1 µg/mL, P = 0.001; Figure 3).

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

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

Milk amyloid A level of SCM quarters were lower than in CM quarters in culture-negative samples ($P = 0.02$) and in samples positive for *Strep. uberis* ($P = 0.001$). Levels in quarters with a Staph. aureus infection did not differ between CM and SCM quarters ($P = 0.25$). Descriptive values of measured levels of MAA depending on bacteriological result in milk are presented in Figure 5. Similar to CATH, MAA levels were not affected by any of the other tested factors (e.g., lactation number, DIM, milk yield, total number of episodes with recurrent CM, and THI; $P > 0.05$). The thresholds providing the highest accuracy for differentiating between healthy and SCM quarters, healthy and CM, and SCM and CM were 1.28 µg/mL (Se = 0.65, Sp = 0.76, AUC = 0.755), 1.81 µg/mL (Se = 0.77, Sp = 0.83, AUC = 0.860), and 7.75 µg/mL (Se = 0.38, Sp = 0.92, AUC = 0.673), respectively (Table 2). A differentiation between healthy quarters and healthy in-cow control quarter was feasible using a threshold of 1.30 µg/mL (Se = 0.51; Sp = 0.76, AUC = 0.649). Furthermore, thresholds differentiating between CNS and culture-negative quarters, and CNS and *Staph. aureus* were calculated (Table 3).

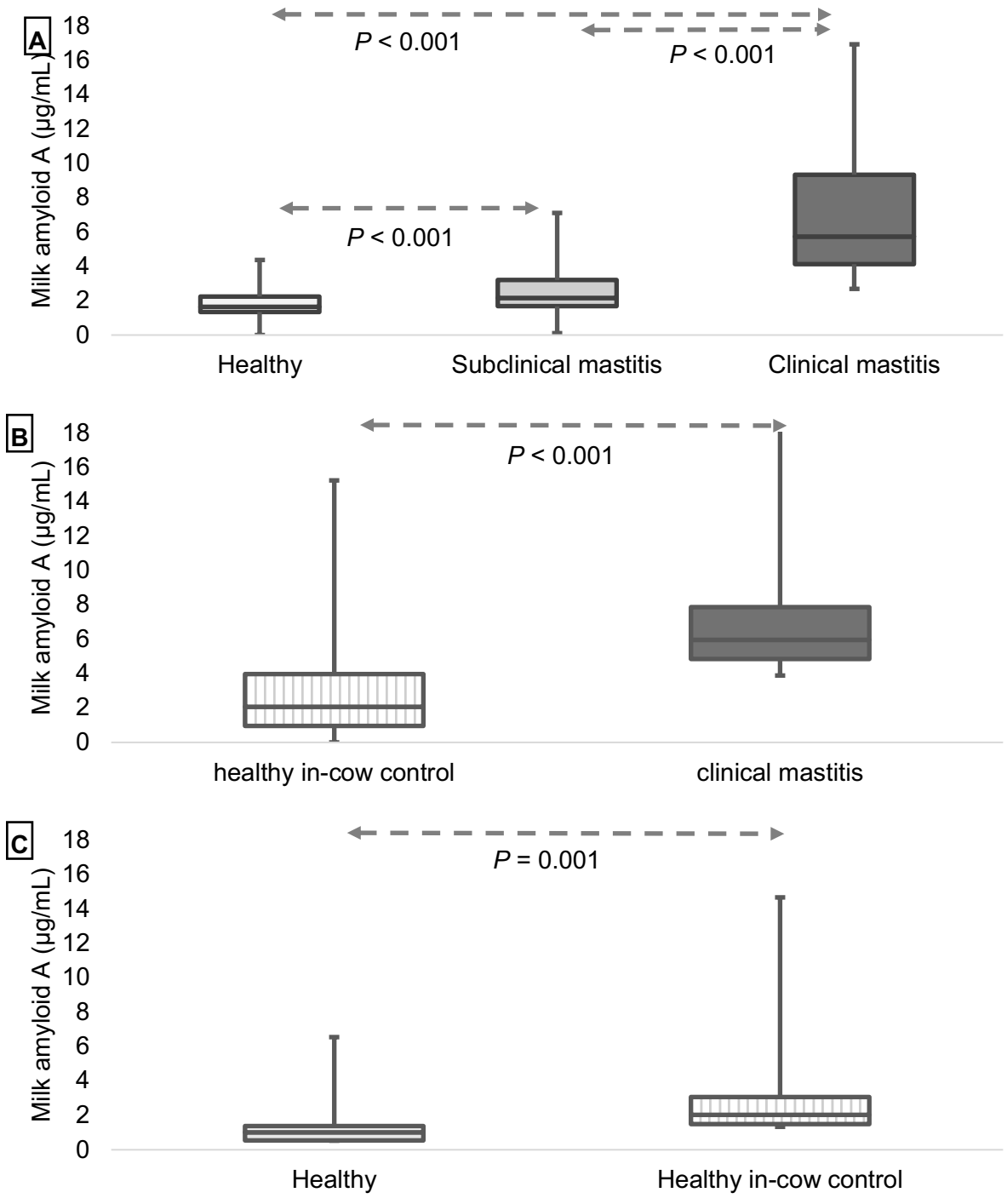

Figure 3. Comparison of milk amyloid A (µg/mL) levels (A) in healthy quarters (n = 67), quarters with subclinical mastitis (n = 119), and clinical mastitis quarter (n = 212), (B) in healthy in-cow control quarters (n = 212) and mastitis quarters (n = 212) of cows with one clinical mastitis quarter, and (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.

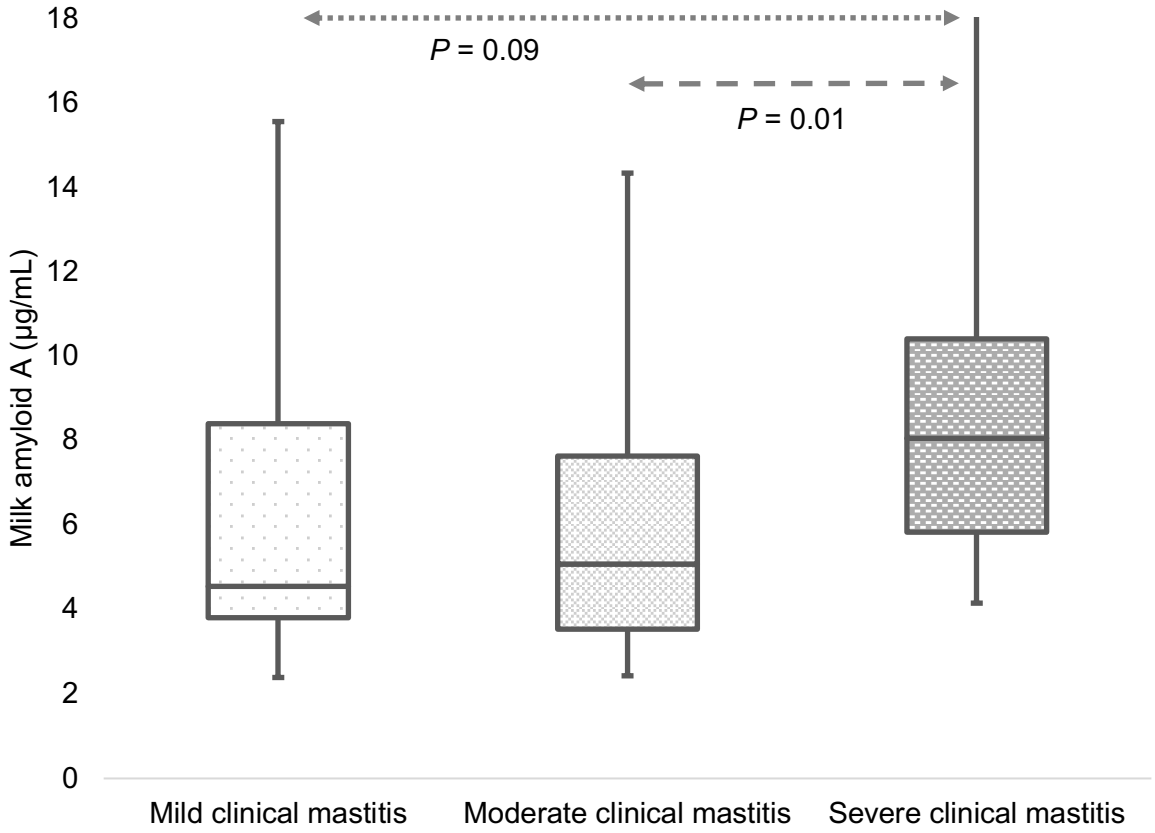

Figure 4. Comparison of milk amyloid A (µg/mL) levels in clinical mastitis quarters considering severity score. The black line inside each box marks the mean; the bottom and top of the boxes are the first and third quartiles; whiskers end at the smallest and largest statistical values. Data originate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

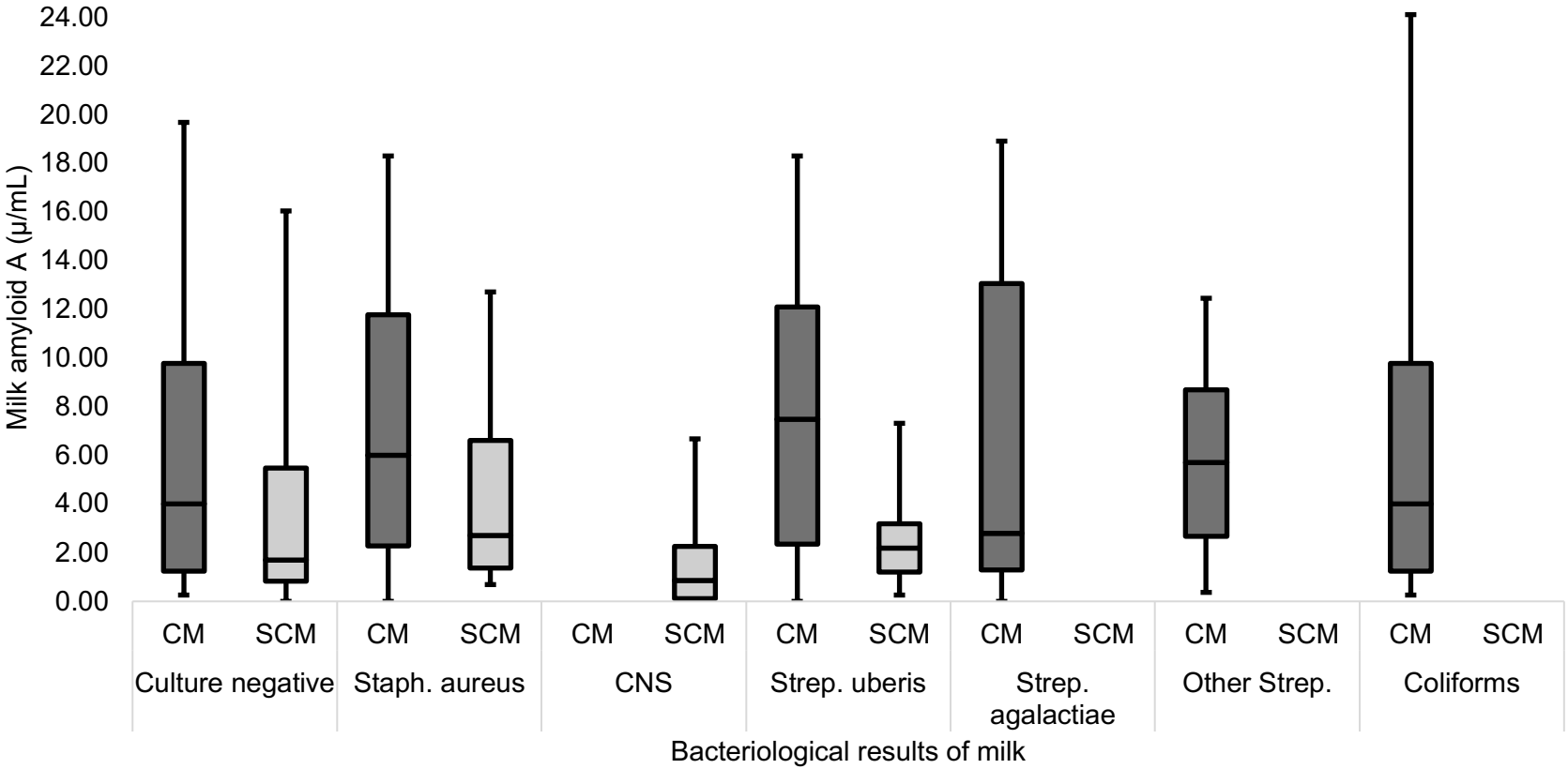

Figure 5. Milk amyloid A (µg/mL) levels in milk samples considering bacteriological results in subclinical mastitis (SCM; light grey; n = 107) and clinical mastitis quarters (CM; dark grey; n = 201). The black line inside each box marks the mean; the bottom and top of the boxes are the first and third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

Haptoglobin

In healthy, SCM, and CM quarters, HP level averaged 0.98 ± 0.1, 10.15 ± 0.2, 13.73 ± 0.1 µg/mL, respectively ($P < 0.001$; Figure 6). Haptoglobin levels in healthy in-cow control quarters (5.94 ± 0.1 µg/mL) were lower than in CM quarters (13.76 ± 0.1 µg/mL, $P < 0.001$; Figure 6). Healthy quarters (0.98 ± 0.1 µg/ mL) had lower HP level than healthy in-cow control quarters (5.99 ± 0.2 µg/mL, $P < 0.001$; Figure 6). In cows with CM, HP levels were not affected by severity ($P > 0.05$). Also, there was no effect of mastitis causing bacteria on HP levels in CM samples ($P = 0.63$).

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

Haptoglobin levels in SCM quarters with culturenegative samples ($P < 0.001$) and samples positive for *Strep. uberis* ($P = 0.002$) were lower than the respective quarters with CM. Descriptive values of measured levels of HP depending on bacteriological result in milk are presented in Figure 7. Overall, there was no effect of lactation number, DIM, milk yield, total number of episodes with recurrent CM, and THI ($P > 0.05$) on HP levels in any of the comparisons.

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

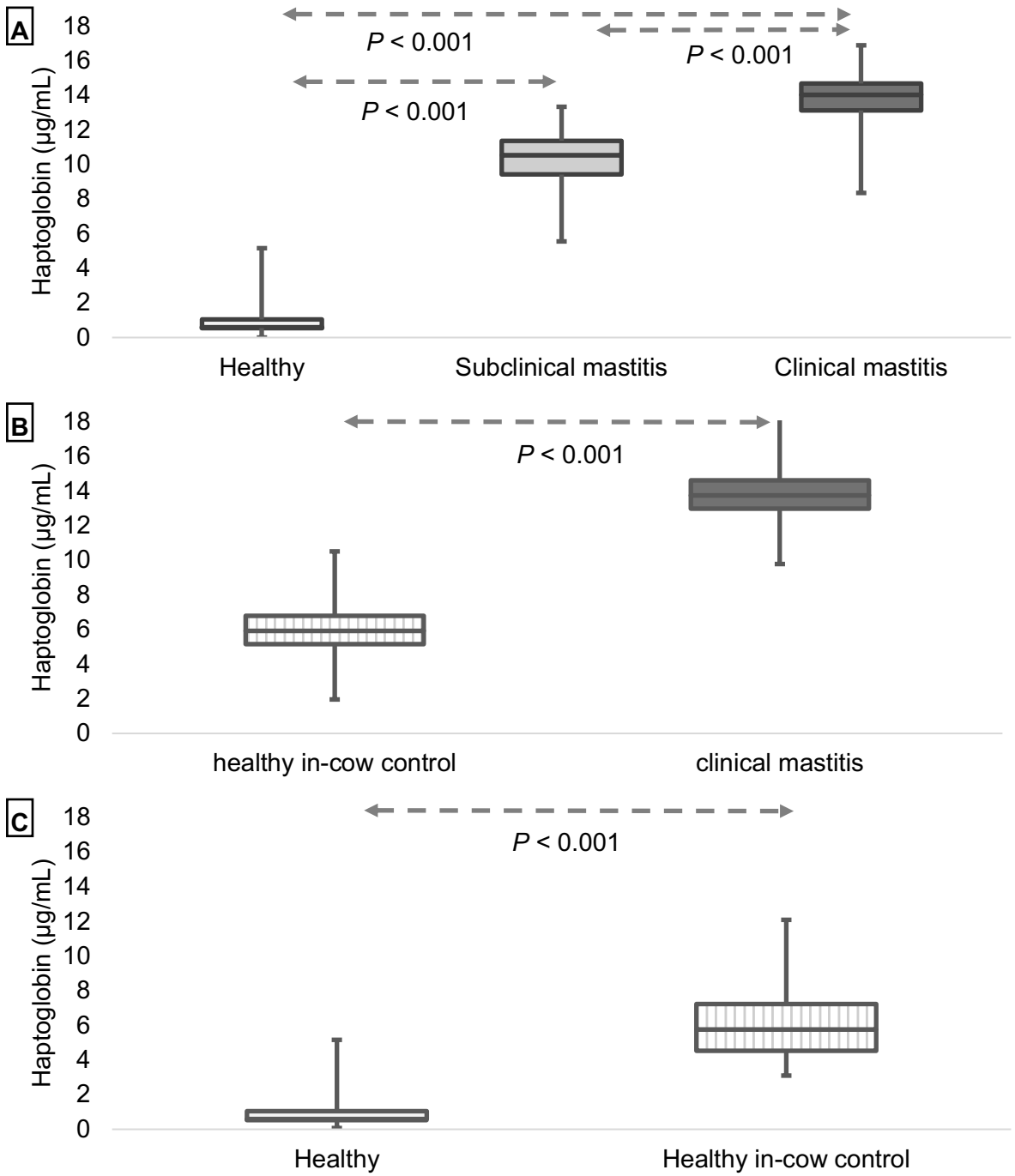

Figure 6. Comparison of haptoglobin (µg/mL) levels (A) in healthy quarters (n = 67), quarters with subclinical mastitis (n = 119), and clinical mastitis quarter (n = 121), (B) in healthy in-cow control quarters (n = 121) and mastitis quarters (n = 121) of cows with one clinical mastitis quarter, and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

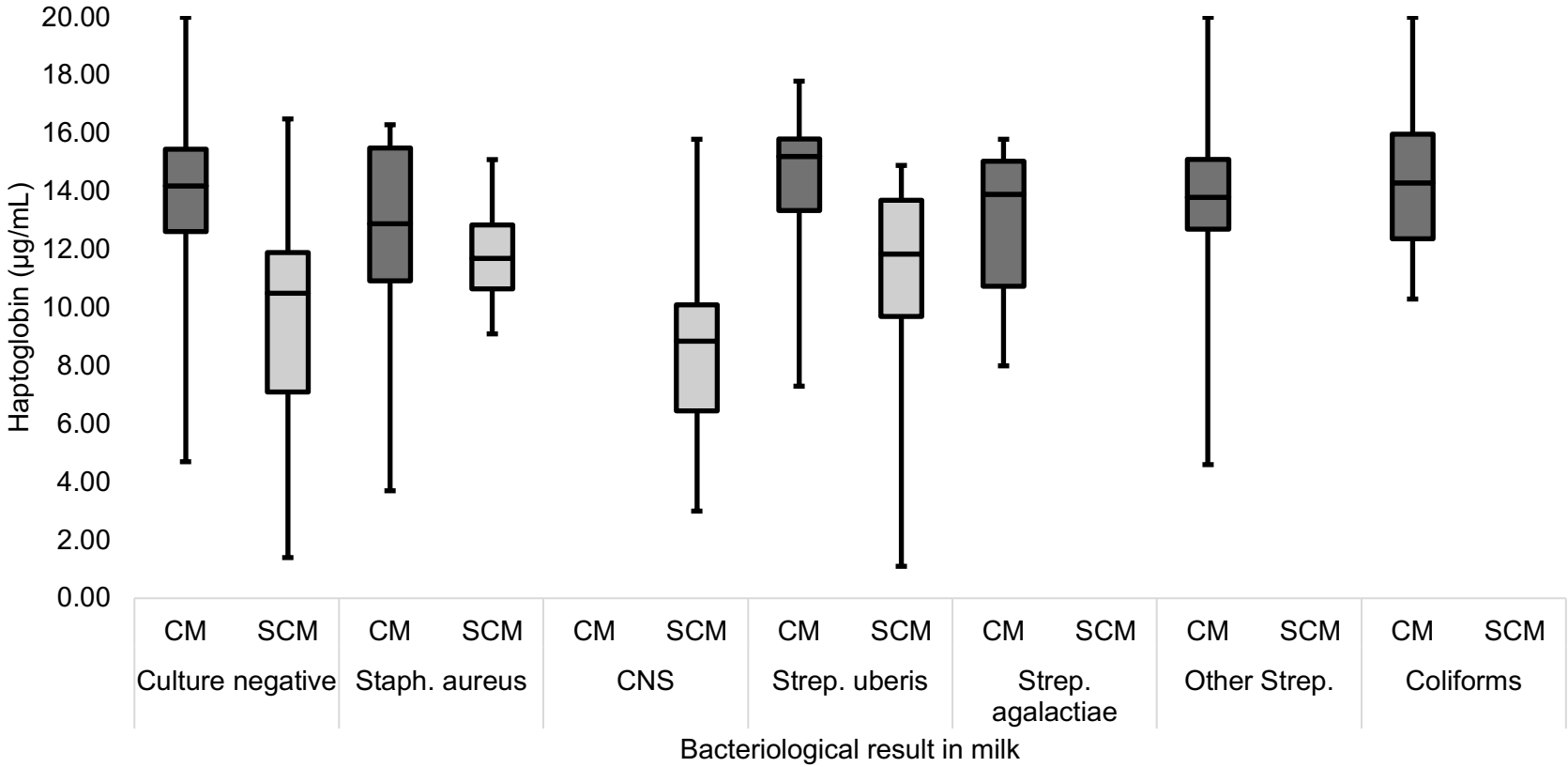

Figure 7. Haptoglobin (µg/mL) levels in milk samples considering bacteriological results in subclinical mastitis (SCM; light grey; n = 107) and clinical mastitis quarters (CM; dark grey; n = 115). The black line inside each box marks the mean; the bottom and top of the boxes are the first and third quartiles;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

Table 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[1].

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	1.30	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	5.40	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	0.99	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[1].

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*	11.00	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 ($P > 0.05$).

Relationship Between Different Biomarkers and SCC

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

3.6. Discussion

Our results support recent publications on CATH (Addis et al., 2016b; Pongthaisong et al., 2016; Addis et al., 2017), MAA (Jaeger et al., 2017; Hussein et al., 2018), and HP (Pedersen et al., 2003; Sadek et al., 2017; Thomas et al., 2018), which described the diagnostic value of these biomarkers for mastitis detection in milk. Measurement of these biomarkers achieved high accuracy for the detection of intramammary infections. Although most previous studies focused on either SCM or CM, our study directly compared both types of mastitis. Therefore, we were able to show not only that a differentiation between healthy quarters and infected quarters is possible, but also a differentiation between SCM and CM.

The levels of the 3 biomarkers in milk were quarter specific and increased in CM quarters. The inflammation process of the udder also affected healthy in-cow control quarters, indicated by slightly higher levels of MAA and HP in healthy in-cow control quarters compared with healthy cows. These elevated levels are most likely caused by diffusion of acute phase proteins during the initial stage of inflammation from the blood stream into the milk. This process, however, is probably not quarter specific. A production of acute phase proteins in the mammary gland cells occurs only at later stages of the inflammation (Eckersall et al., 2001; Hiss et al., 2004). In contrast, CATH levels in healthy in-cow control quarters were not elevated indicating a quarter specific mechanism. Therefore, elevated CATH levels in milk occurred only in infected quarters. Similar results were reported by Chromek et al. (2006) and Addis et al. (2011, 2013).

Not surprising, comparability with previous studies was best for CATH as measurements were conducted in the same laboratory (Addis et al., 2016a, b; Addis et al., 2017). Levels of MAA measured in our study was also similar to previous studies. In SCM experimentally induced with Staph. aureus, the MAA level averaged 5.6 ± 12 µg/mL (Eckersall et al., 2006), which is comparable with our level of 4.38 ± 0.6 µg/mL. In naturally occurring SCM samples (Gerardi et al., 2009), MAA values ranged from 9.8 ± 1.9 µg/mL to 5.5 µg/mL

± 1.0 depending on ELISA kit. In their study authors defined SCM as the presence of clots in milk and the absence of abnormalities, which we classified as mild CM. Considering those SCM quarters as mild CM (MAA = 6.17 ± 0.5 µg/mL) values are comparable.

In contrast, HP values reported in the literature are higher than our results. In SCM quarters, however, our results (11.15 ± 0.4 µg/mL) were higher than concentrations previously published for experimentally induced SCM (4.3 µg/mL; Eckersall et al., 2006). In CM quarters, our results (13.76 ± 0.1 µg/mL) were also much lower than previous findings (503 µg/mL: Wenz et al., 2010; 80.0 µg/mL: Pyörälä, 2011). Differences can be explained by different ELISA kits as shown by Geradi et al. (2009). Differences in the absolute concentrations of biomarkers for the detection of mastitic quarters might not be that important as healthy quarters showed similarly low levels (MAA = 0.1 ± 1.4 µg/mL; Gerardi et al., 2009; HP = 0.05 µg/ mL; Eckersall et al., 2006).

We could not confirm previous reports of high accuracy of HP and MAA (Wenz et al., 2010; Pyörälä et al., 2011; Kalmus et al., 2013) for the differentiation between severity scores of CM with lowest MAA levels in mild CM and significantly (HP: $P = 0.007$; MAA: $P < 0.001$) higher levels in moderate CM quarters (Kalmus et al., 2013). We assume that these differences might be caused by different mastitis pathogens. The previous study described CNS and Strep. uberis as main pathogens, whereas we observed twice as many culture-negative samples in moderate compared with mild CM quarters.

In contrast to previous reports on CATH (Addis et al., 2017) and HP (Wenz et al., 2010), we were not able to detect an effect of different pathogens on the concentration. One reason might be that the types of pathogens in the aforementioned studies differed from ours (e.g., more CNS in previous studies, more *E. coli* and *Staph. aureus* in our study). Albeit this was not specifically investigated in either of the studies, different genotypes might be linked to different levels of biomarkers.

It is noteworthy, however, that in SCM quarters the causative pathogen affected CATH and HP levels. The mean HP level in CNS samples (8.52 ± 0.4 µg/mL) was lower than in Staph. aureus (11.86 ± 0.3 µg/mL) samples. This relationship is in agreement with previous studies (Hiss et al., 2007; Pyörälä et al., 2011). Even though the absolute values in those earlier studies differed for CNS (3.1 µg/mL; 7.8 µg/ mL) and for *Staph. aureus* (39.6 µg/ mL; 33.0 µg/ mL) from results in our study (CNS = 8.52 ± 0.4 µg/mL; *Staph. aureus* = 11.86 ± 0.3 µg/mL), respectively.

Differences in HP levels between culture-negative and culture-positive SCM milk samples found by other authors (Hiss et al., 2007; Safi et al., 2009) were confirmed in our study. Differences were evident for *Staph. aureus* and *Strep. uberis* ($P \leq 0.05$).

The accuracy to differentiate between *Staph. aureus* and CNS was 100 and 85% for HP, and 100 and 57% for CATH using thresholds of 0.084 and 11.0 µg/mL, respectively. This is remarkable and might be interesting for eradication programs to reduce the incidence of *Staph. aureus* in herds (Barkema et al., 2009).

Interestingly, biomarker levels were higher in several culture-negative SCM samples compared with CNS positive samples. One explanation might be that the causative pathogen could either not be cultured or those culture-negative samples were actually false negatives. As the bacteriological examination is based on viable bacteria, encapsulated (e.g., *Staph. aureus*) or dead pathogens cannot be detected. In the udder, however, even encapsulated pathogens might cause an inflammatory response and cause elevated biomarker levels (Hill et al., 1983). Another explanation might be that immunological processes in the udder resulted in an elimination of the pathogen before sample collection. Inflammation processes, however, abate only slowly resulting in elevated levels of biomarkers even after bacteriological cure. This assumption is evidenced by an earlier publication which described elevated levels of HP and serum amyloid A in milk from quarters with SCM 21 to 35 d after an experimental infection with *Staph. aureus* (Gronlund et al., 2003).

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

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

Our proposed CATH threshold of 0.000 NOD450 to differentiate between healthy and SCM almost reached the recommended Sp of > 0.99 with a Se of 0.98. Overall, using the proposed thresholds to differentiate between healthy quarters and SCM, and SCM and CM quarters, accuracies of 97, 94, and 71% for CATH, HP, and MAA were calculated (Table 4). In previous studies test performances of MAA were affected by pathogens in SCM (Jaeger et al., 2017). This might be one reason why accuracy of MAA was lower than CATH and HP in our data set.

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

Indeed, based on our results utilizing CATH and HP measurements would lead to more accurate mastitis diagnoses compared with MAA (Table 3). The accuracies of CATH (71%), MAA (58%), and HP (67%) are furthermore superior to electrical conductivity measurements (i.e., SCM Se = 0.19, Sp = 0.92; CM Se = 0.48, Sp = 0.92) as reported by Norberg et al., 2004), but lower than for SCM diagnosis by measuring SCC (i.e., accuracy = 92%; Sharma at al., 2010).

Specificity of MAA (76%) and SCC (72%; Safi et al., 2009) are similar to differentiate healthy and SCM quarters. Both tests (MAA and SCC) can be used to detect healthy cows with a comparable reliability. Nevertheless, test method to detect MAA seems to detect slightly better false positive cows that could lead to a lower accuracy of the test. If using the CMT test to estimate SCC, Sp of up to 80.6% were described for the diagnosis of SCM quarters (Dingwell et al., 2003). Albeit the semiquantitative CMT may be cheaper, it has the disadvantage that only individual milk samples can be measured and appraisal of test results needs to be done in person. The MAA test, however, offers the possibility of automatization and integration in automatic milking systems and thus might be used for a timely and effective overview of the udder health status of all cows in a herd. In contrast to MAA, both CMT and measurement of SCC do not allow differentiation of the severity of mastitis, because SCC

increase, for example, is limited in severe, but short-term clinical infections that are typically induced by coliform bacteria (Rainard et al., 2018).

Milk amyloid A could be a valuable tool to preselect cows in a herd wide screening that need further examinations. The positive predictive value for the identification of healthy cows is quite high (detection of SCM = 76%; detection of CM = 83%), and the rate of false negatives is low (detection of SCM = 35%; detection of CM = 23%).

Comparing the performance of CATH and HP with results of SCC published in the literature, Sp of the detection of SCM using SCC (72%; Safi et al., 2009) was considerably lower than values calculated for CATH (97%) and HP (94%). Sensitivity (SCC = 90%; Safi et al., 2009) was higher for HP (92%).

Considering CM, Se values up to 97% and Sp up to 89% were found for SCC measurement (Sargeant et al., 2001). Cathelicidin (Se = 98%, Sp = 99% in CM; Se = 93%, Sp = 97% in SCM) and HP (Se = 92%, Sp = 94% in SCM; Se = 96%, Sp = 99% in CM) reached comparable, or slightly better results.

In contrast to SCC, the biomarkers investigated in our study were unaffected by DIM (Sargeant et al., 2001) or other physiological (e.g., stage of lactation, age, and stress; Sharma et al., 2011) and environmental factors (e.g., geographical area and housing system; Bielfeldt et al., 2004), which may cause an increase of SCC without an association to udder infections. The validity of SCC measurements on the other hand is limited in chronic subclinical infections and short-term clinical infections that are typically induced by coliform bacteria (Rainard et al., 2018). For example, Miltenburg et al. (1996) found that herds with low bulk milk SCC (<150,000 cells/mL) may have more CM cases than herds with higher SCC (>250,000 cells/mL). Though, high SCC in bulk tank milk can also be associated with high incidences of CM or a high proportion of chronically infected cows (Rainard et al., 2018). Mastitis pathogens play an important role for SCC. Although a high incidence of mastitis by coliform bacteria was linked to low bulk milk SCC herds (Hogan et al., 1989), CM caused by Streptococcus or Staphylococcus spp. leads to higher bulk milk SCC (Rainard et al., 2018). Overall, the investigated biomarkers showed a comparable or even better performance but remained unaffected by the most relevant influencing factors. In conclusion, accuracy, Se, and Sp for CATH, MAA, and HP allowed for the detection of SCM and CM. A routine measurement of those biomarkers whether in conventional milking parlors or in automatic milking systems might be a reliable and objective method to screen udder health. Especially for identification

of SCM the measurement of biomarkers is more sensitive and specific compared with SCC and lactate dehydrogenase (Se = 80%, Sp = 87%; Hiss et al., 2007). So far CATH and MAA can be measured only under laboratory conditions and further efforts are necessary to develop on-farm devices for measurements.

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4. ADDITIONAL UNPUBLISHED DATA

Effects of transdermal flunixin in therapy of clinical mastitis considering severity score in dairy cows

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4.1. Introduction

The type of clinical mastitis therapy depends on the causative pathogen and the severity score of the disease (Roberson 2012; Oliveira and Ruegg 2014). According to current industry recommendations, mild and moderate clinical mastitis should only be treated after bacteriological examination and with local antimicrobials (Roberson 2012; Mansion-de Vries et al. 2016; Kock et al. 2018). In severe clinical mastitis cases both antimicrobials and non-steroidal anti-inflammatory drugs (**NSAID**s) should be administered systemically (Roberson 2012, Mansion-de Vries et al. 2016, Kock et al. 2018). Unfortunately, antimicrobial treatment does not always appear to be appropriate. In case of *Escherichia coli* mastitis, for example, results are ambiguous (Shpigel et al. 1994; Suojala et al. 2010). Similar cure rates (e.g., bacteriological cure, clinical cure, and cow survival) were seen in cows with *Escherichia coli* mastitis, if cows were treated with an antimicrobial (Enrofloxacin) in combination with an NSAID (Ketoprofen) or with an NSAID only (Suojala et al. 2010).

Positive effects of NSAIDs in mastitis therapy are well documented (Vangroenweg et al. 2005; McDougall et al. 2009; Leslie et al. 2012; Yeiser et al. 2012; McDougall et al. 2016).

Non-steroidal anti-inflammatory drugs improved treatment outcomes in severe clinical mastitis and milder cases. Specifically, flunixin reduced rectal temperatures and signs of inflammation significantly during cure (Anderson et al. 1986). Recently, a transdermal formulation of flunixin was approved in the US (control of pyrexia associated with bovine respiratory disease and control of pain associated with foot rot; Bayer Animal Health 2018) and Europe (control of pyrexia associated with bovine severe clinical mastitis; Intervet Deutschland GmbH 2019). Its application increased clinical cure rates significantly in an earlier study (Kock et al. 2018) investigating the effect of transdermal flunixin in mastitis therapy following results of a rapid on-farm bacteriological test. Even though cows with mild, moderate, and severe clinical mastitis were included in this study, the effect of transdermal flunixin was not calculated separately for the 3 severities. Therefore, it remains unclear if efficacy of transdermal flunixin differs in different severity scores of clinical mastitis cows.

In order to fully validate the use of transdermal flunixin, its efficacy in different severities of clinical mastitis needs to be investigated. This requires study parameters, such as bacteriological cure, udder firmness, or levels of different biomarkers (e.g., cathelicidin, milk amyloid A) in milk, which were used earlier to diagnose and classify clinical mastitis (Wollowski et al. 2019, Wollowski et al. 2021).

Furthermore, if the efficacy of transdermal flunixin is comparable to injectable NSAIDs it could be a valuable alternative. A transdermal formulation can be applied by the farm personal as a pour-on treatment which is not invasive, painful, and may be conducted with less restraining of the cow. Furthermore, while the solution for injection needs to be administered repetitively over several days, the transdermal solution allows a single treatment. Therefore, the application of transdermal flunixin can be easily integrated into farm-based treatment and standard operating procedures during diagnosis and treatment of clinical mastitis.

Overall, the objective of this study was to determine, if transdermal flunixin effects parameters of cure considering mild, moderate, and severe clinical mastitis. Specifically, we investigated the effect of transdermal flunixin on udder firmness measured by dynamometer, concentrations of cathelicidin (**CATH**) and milk amyloid A (**MAA**) in milk, and bacteriological cure rates in milk.

4.2. Material and Methods

Between June 2016 and January 2017, 251 Holstein Friesian dairy cows with signs of clinical mastitis (i.e., clotted milk, heat or swelling of one udder quarter) identified by the milking

personnel were enrolled in the study. Cows were separated and fixed in a cattle chute for further examination. Cows with clinical mastitis within the last 30 d before enrolment, cows with signs of metabolic or infectious disease (e.g., ketosis, hypocalcaemia, metritis), and those that had received systemic or intramammary antibiotics or anti-inflammatory therapy were excluded from the study. Furthermore, cows with teat lesions or lacerations of the udder surface, with mastitis in more than 1 quarter and cows within 5 d after calving were not enrolled.

All cows were examined on the day of enrolment (**D1**) and daily for the next 4 days (**D2 - D5**). Furthermore, examinations on day seven (**D7**) and on day 21 (**D21**) followed. After general clinical examination (i.e., rectal temperature, attitude) of each cow, an examination of the udder was conducted on each of these days. Udder firmness was measured using the dynamometer as described previously by Wollowski et al. (2019). The healthy contralateral quarter was also measured for comparison. Unsterile milk samples were divided into 3 subsamples and stored at – 20°C until analyses analysis of biomarkers CATH and MAA as described earlier (Wollowski et al. 2021). In brief, CATH was analyzed with a pan-cathelicidin ELISA based on 2 monoclonal antibodies developed against a pan-cathelicidin domain (Addis et al. 2016a; b). At the end of the assay, for each sample, the optical density measured at 450 nm (**OD450**) was normalized against internal controls (**NOD450**). The concentration of MAA was determined using a commercial ELISA kit (Milk Amyloid A-MAA Assay Kit, cat. no. TP-807; Tridelta Development Ltd., Maynooth, Ireland) in accordance with the manufacturer's recommendations. Furthermore, a California Mastitis Test was conducted (KerbaTEST; Albert Kerbl GmbH, Buchbach, Germany). On D1, D5, and D21 an aseptic milk sample from the mastitis quarter was taken for bacteriological culturing and evaluation of SCC (Wollowski et al. 2021).

On D1 all clinical mastitis cases were classified into mild (1: abnormal appearance of milk), moderate (2: abnormal appearance of milk accompanied by swelling or redness of the mammary gland), or severe clinical mastitis (3: abnormal appearance of milk and swelling of the mammary gland, signs of systemic illness such as fever above 39.5°C) according to Wenz et al. (2010) and Pinzon-Sanchez and Ruegg (2011). Cows with mild or moderate clinical mastitis were treated with a placebo or with transdermal flunixin (Finadyne transdermal, Intervet Deutschland GmbH, 85716 Unterschleißheim, Germany). The placebo resembled transdermal flunixin in consistence and appearance ensuring treatment was blinded. Severe CM cases were alternately treated with transdermal flunixin or an injection of meloxicam (Metacam, Boehringer Ingelheim Vetmedica GmbH, 55216 Ingelheim am Rhein, Germany),

respectively. According to manufacture`s instruction, transdermal flunixin was given as a one-time pour-on application with a dosage of 50 mg/15 kg body weight. Metacam was given 20 mg/mL intravenously once a day for 3 consecutive days, respectively. Cows were allocated to treatment groups based on a randomization list created before initiation of the study.

Overall, 218 cows with clinical mastitis (i.e., 46 mild, 106 moderate, 66 severe) met all inclusion criteria (83.7%). Thirty-four cows had to be excluded due to a CM within 30 d before enrollment, signs of metabolic, or infectious disease (e.g., metritis, claw lesions, ketosis), respectively. A complete data set (i.e., D1-D5, D7, and D21) was available for 218 cows considering measurements by dynamometer. Concentrations of CATH and MAA were available for 121 cows (20 cows with mild, 63 with moderate, and 38 with severe clinical mastitis) for all examination days. For MAA, data from D1 - D5 and D21 of additional 24 cows (7 cows with mild, 7 with moderate, and 10 with severe clinical mastitis) could be used for the final analysis.

Data were entered into Excel spreadsheets (Excel, version 2016; Microsoft, Redmond, WA) and statistical analyses were performed with SPSS for Windows (SPSS, version 24.0, IBM Deutschland GmbH, Ehningen, Germany). The statistical significance level was set at $P \leq 0.05$ and trends were discussed at $P \leq 0.10$. Considering udder firmness, the difference between udder firmness in the mastitis quarter and the healthy contralateral quarter was calculated in order to account for inter cow variability caused e.g., by milk yield, resulting in a new variable “udder firmness difference”. Normality of distributions of continuous parameters (i.e., udder firmness difference, udder surface temperature) was assessed by plotting the data, visual examination and calculating a Q-Q-plot. An udder quarter was considered bacteriologically cured when the original causing pathogen of D1 was no longer detectable at D5 or D21, respectively. To calculate changes of udder firmness differences, the concentrations of CATH and MAA between days of examination, separate generalized linear mixed models were used. Bacteriological cure was evaluated using crosstable with χ^2 and t-Test. All models were built according to the model building strategies published by Dohoo et al. (2009). To evaluate the difference in type of treatment (i.e., mild or moderate CM: placebo vs. transdermal flunixin; in severe CM: meloxicam vs. transdermal flunixin) for each day (D1-D5, D7 and D21) a post hoc analysis (Bonferoni) was performed in clinical mastitis with different severity score, respectively. Before building the mixed models, each parameter was separately analyzed in a univariate model, including the parameter as fixed factor (i.e., categorical parameter: days of examination, type of clinical mastitis) or covariate (i.e.,

continuous parameter: lactation number, DIM, total number of episodes with recurrent clinical mastitis).

4.3. Results

Overall, 74 cows treated with placebo, 110 with transdermal flunixin, and 34 with meloxicam were included in the final analyses, respectively (Table 1).

Table 1. Distribution of treatment (transdermal flunixin, meloxicam, or placebo) considering severity score of clinical mastitis (mild, moderate, or severe).

		Treatment			Total
		Placebo	Transdermal flunixin	Meloxicam	
Severity score of mastitis	mild	19	27	0	46
	moderate	55	51	0	106
	severe	0	32	34	66
Total		74	110	34	218

Udder firmness difference and concentrations of CATH and MAA changed over time regardless of treatment. Treatment with transdermal flunixin had a positive effect on MAA concentration and bacteriological cure in mild or moderate clinical mastitis cases. In cows with severe clinical mastitis, bacteriological and clinical cure rates did not differ between transdermal flunixin and meloxicam treatment.

Specifically, MAA concentration in mild or moderate clinical mastitis was influenced by treatment ($P = 0.015$) and day of examination ($P < 0.001$). Concentration of MAA tended to be lower on D2 ($P = 0.1$) and was significantly lower on D3 ($P = 0.03$) in cows treated with transdermal flunixin compared to placebo (Figure 1). There was no effect of other tested variables (lactation number, DIM, total number of episodes with recurrent clinical mastitis).

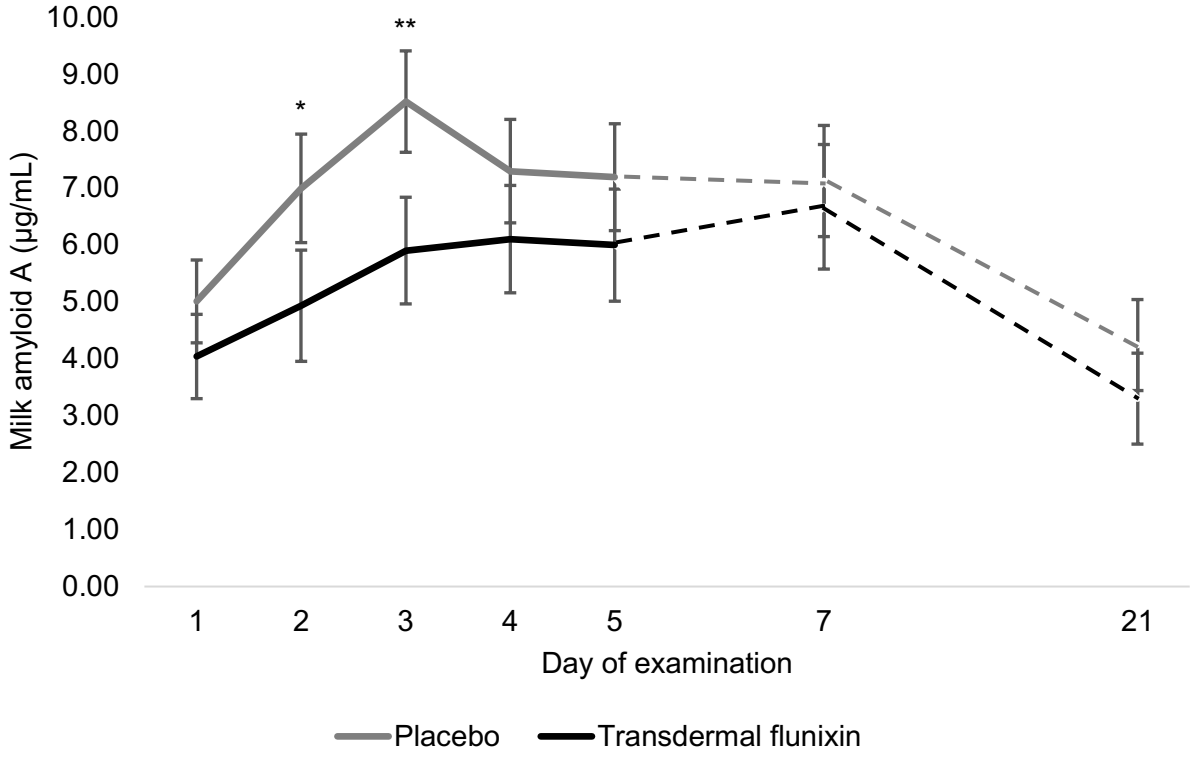

Figure 1. Concentrations of milk amyloid A (µg/mL) in milk over the study period from quarters with mild or moderate clinical mastitis (n = 97) treated with transdermal flunixin (n = 51) or a placebo (n = 46). (* $P \leq 0.1$; ** $P \leq 0.05$)

Cows with mild or moderate clinical mastitis treated with transdermal flunixin had higher bacteriological cure rates (Figure 2) than placebo treated cows on D5 (92.4% vs. 78%; $P = 0.002$) and D21 (100% vs. 88.9%; $P = 0.008$). On D21 all 72 cows treated with transdermal flunixin were bacteriologically negative.

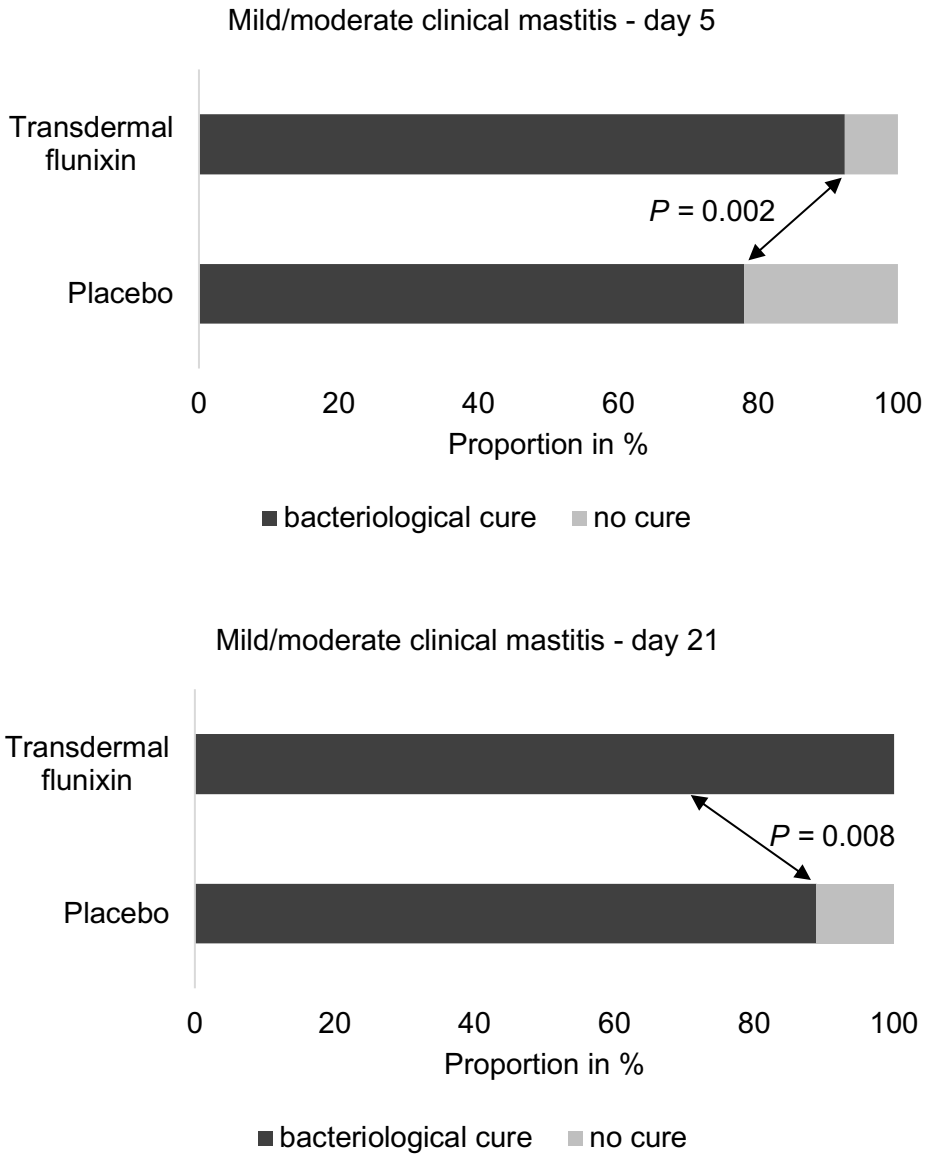

Figure 2. Effect of transdermal flunixin (D5: n = 78; D21: n = 69) or placebo (D5: n = 74; D21: n = 72) on bacteriological cure rates in cows with mild or moderate clinical mastitis on D5 and D21.

Udder firmness differences in mild or moderate clinical mastitis cows was affected by day of examination ($P < 0.001$) but not by treatment ($P = 0.870$). Nevertheless, from D4, differences of udder firmness were significantly lower compared to D1 irrespective type of treatment ($P = 0.03$; Figure 3).

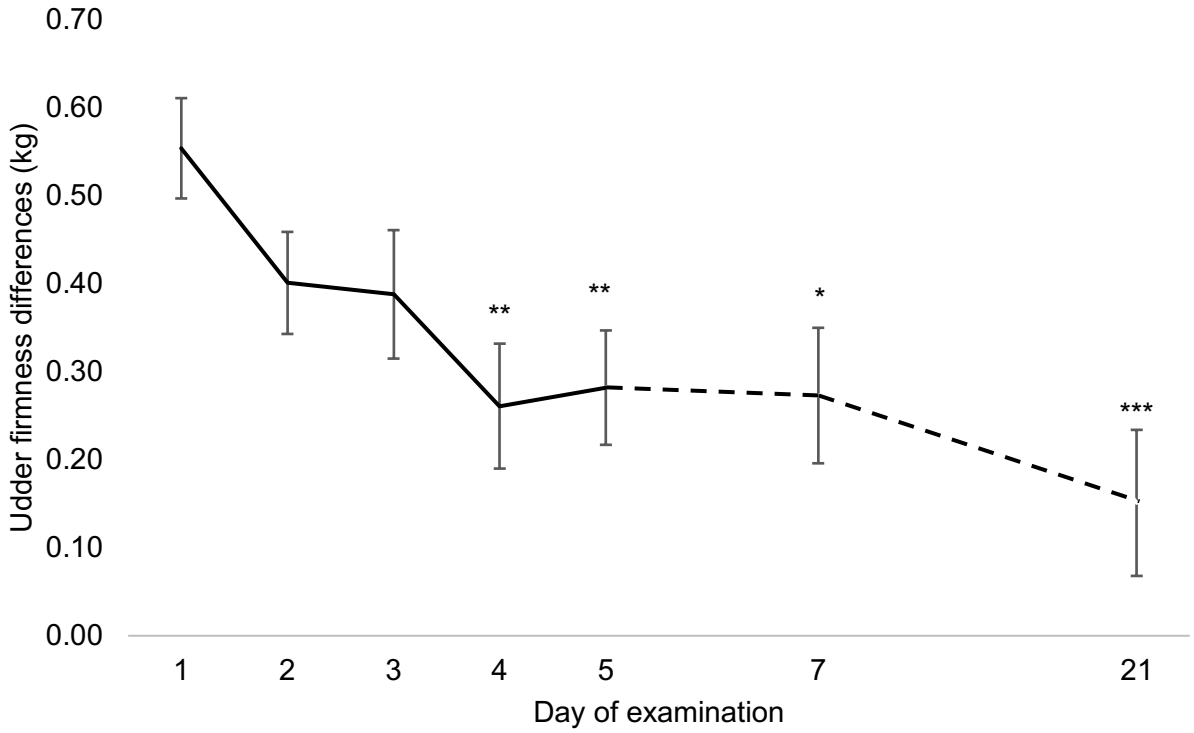

Figure 3. Effect of day of examination (irrespective type of treatment) on udder firmness difference in mild or moderate clinical mastitis (n = 152). (Difference to D1; * $P \leq 0.1$; ** $P \leq 0.05$; *** $P \leq 0.001$)

Concentration of CATH in mild or moderate mastitis cows was affected by day of examination ($P < 0.001$), but not by treatment ($P = 0.689$). Concentration of CATH decreased from D1 throughout the study period ($P = 0.002$; Figure 4). Concentration of CATH was significant lower on D3 compared to D2 ($P = 0.01$) and on D4 compared to D3 ($P = 0.01$). Level tended to be lower on D5 compared to D4 ($P = 0.1$) and on D7 compared to D21 ($P = 0.07$), respectively (Figure 4).

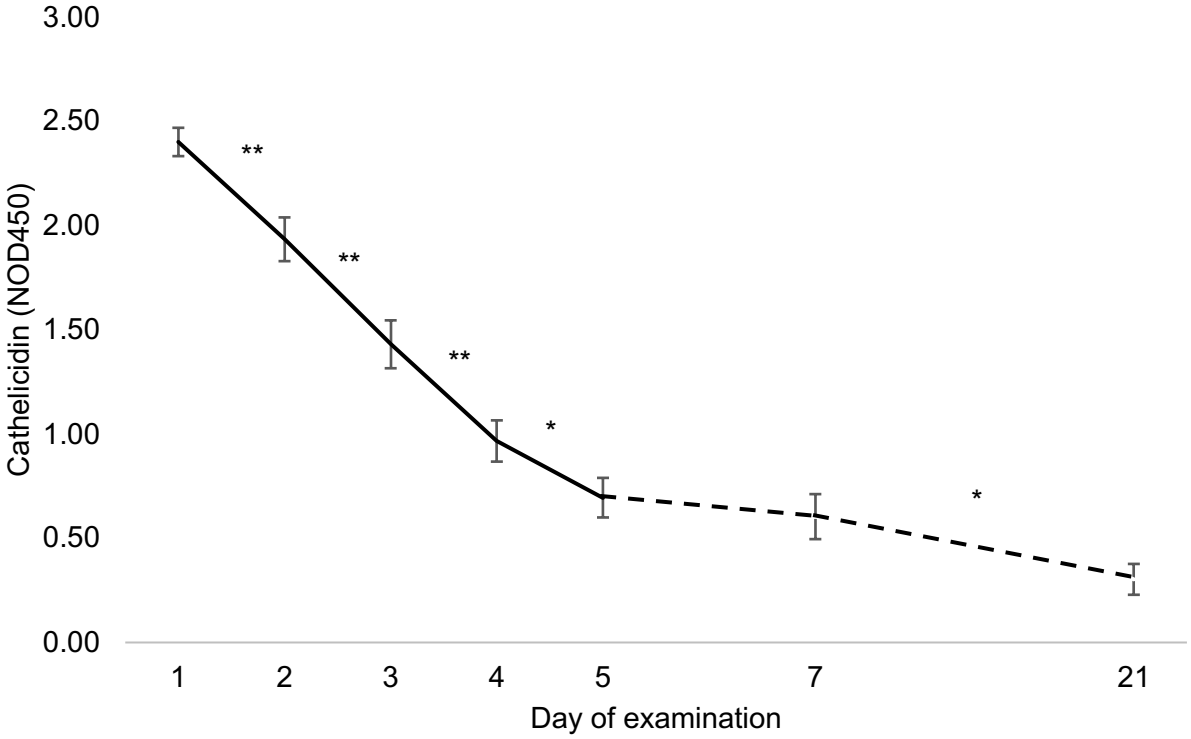

Figure 4. Concentrations of cathelicidin (NOD450) in milk over the study period from quarters with mild or moderate clinical mastitis (n = 83). (* $P \leq 0.1$; ** $P \leq 0.05$).

Albeit day of examination affected cure (udder firmness differences: $P < 0.001$; CATH: $P < 0.001$; MAA: $P = 0.039$) in cows with severe clinical mastitis, there was no effect of treatment (transdermal flunixin or meloxicam) on udder firmness differences ($P = 0.582$); concentration of CATH ($P = 0.441$) and MAA ($P = 0.116$). While differences of udder firmness and CATH continuously decreased throughout the study period starting on D5 ($P = 0.01$) and D3 ($P = 0.05$), respectively (Figure 5 and 6), concentration of MAA was lower only on D21 compared to D7 level ($P = 0.02$; Figure 7).

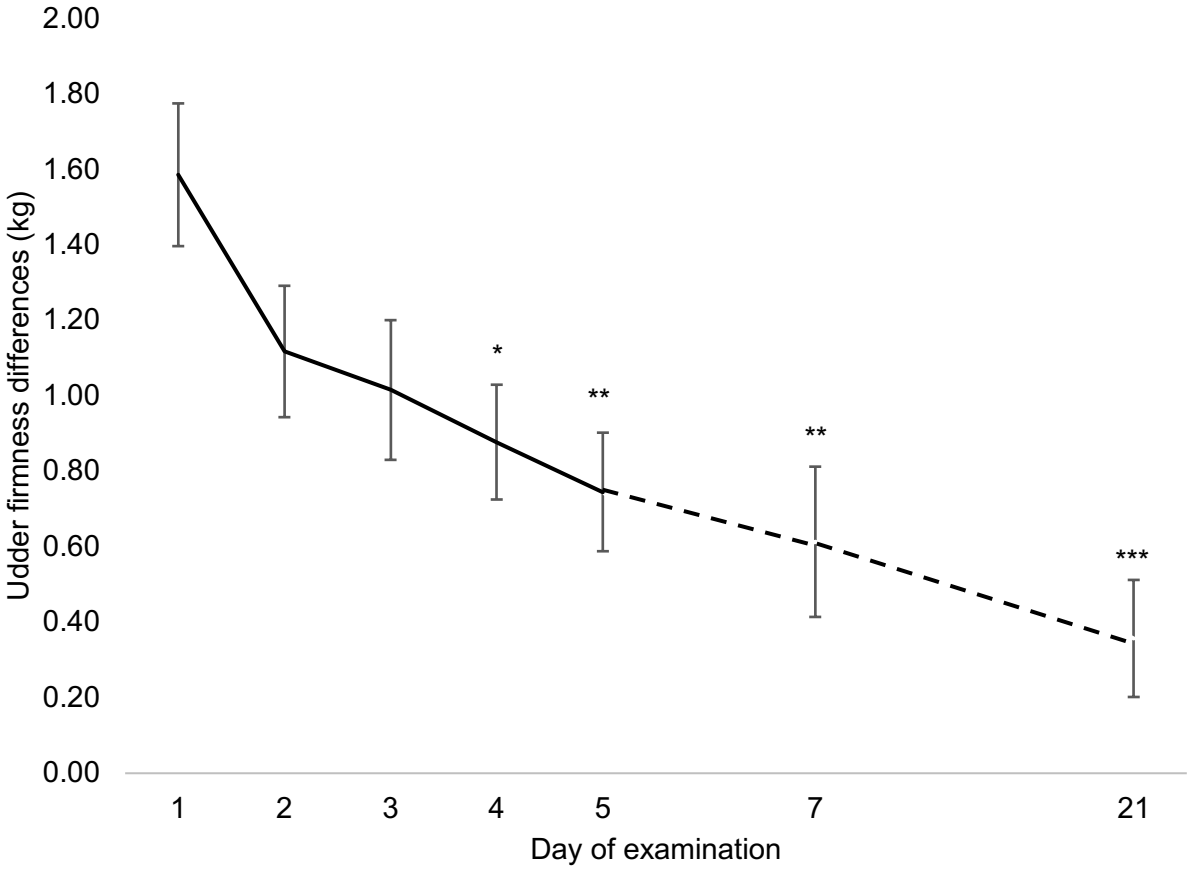

Figure 5. Udder firmness differences in milk over the study period from quarters with severe clinical mastitis (n = 66). (Difference to D1; * $P \leq 0.1$; ** $P \leq 0.05$; *** $P \leq 0.001$)
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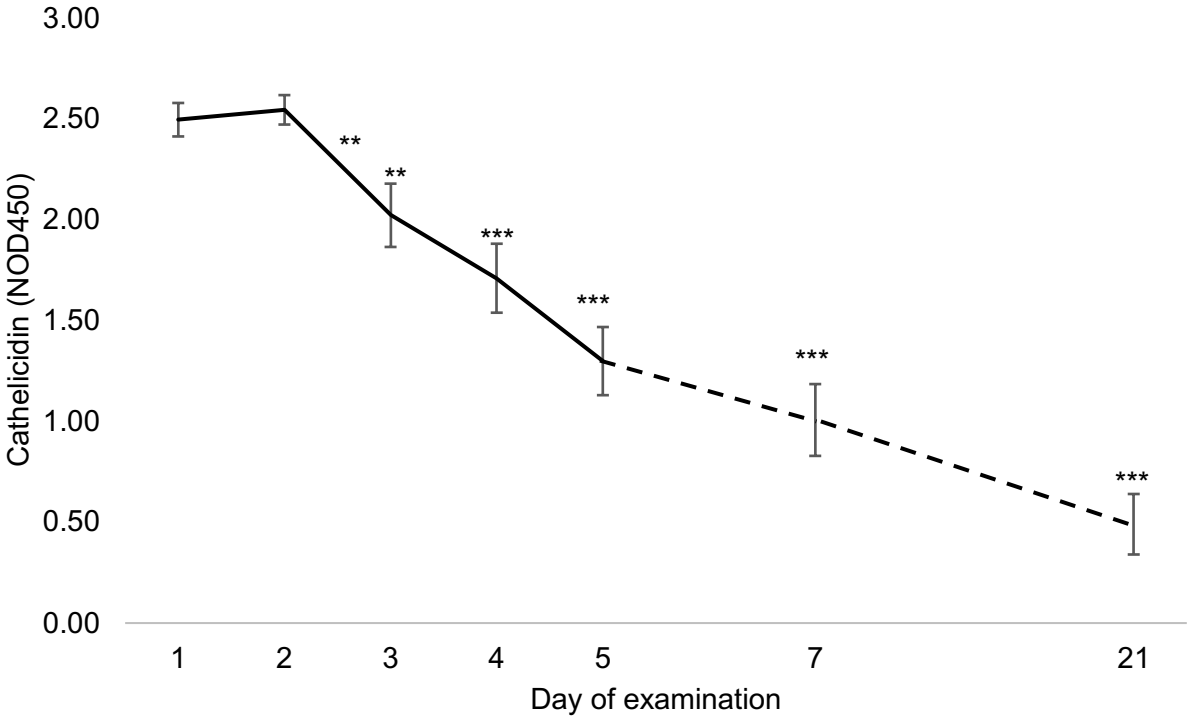

Figure 6. Concentration of cathelicidin (NOD450) in milk over the study period from quarters with severe clinical mastitis (n = 38). (* $P \leq 0.1$; ** $P \leq 0.05$; *** $P \leq 0.001$)

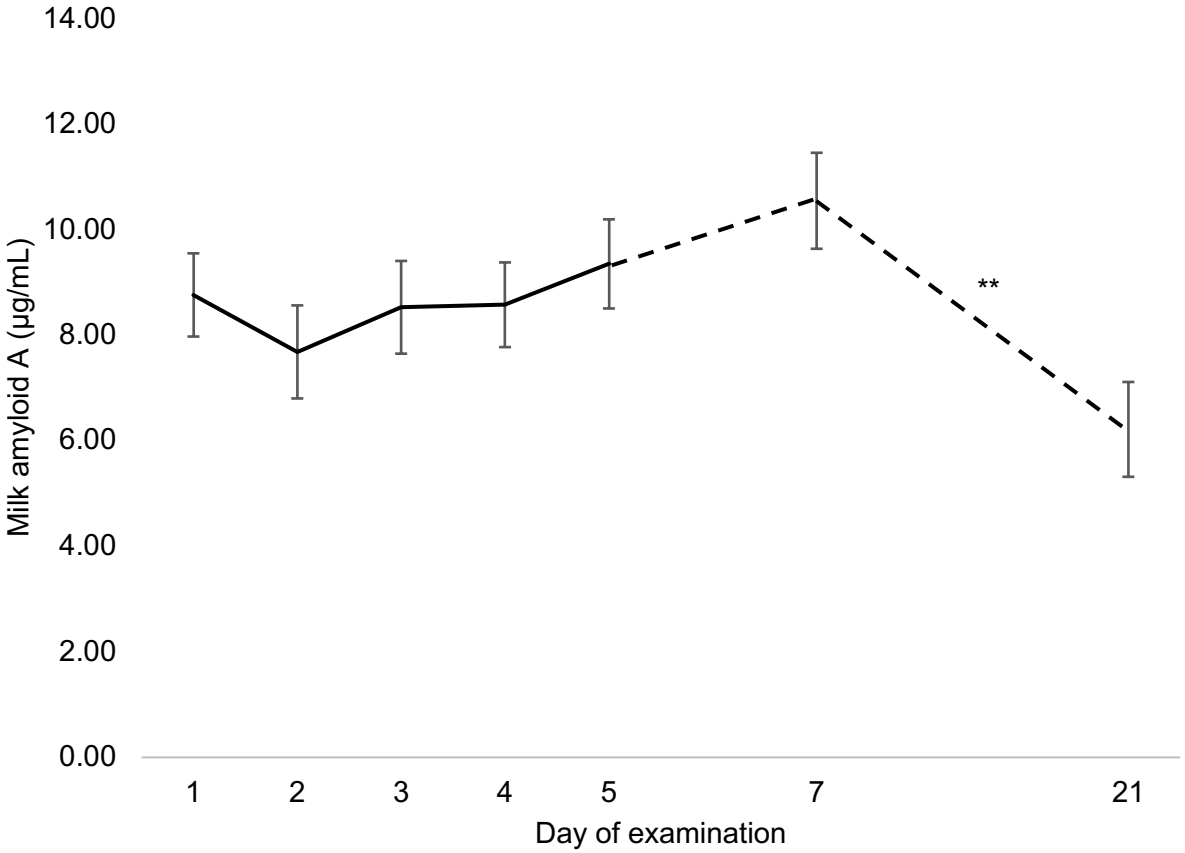

Figure 7. Concentration of milk amyloid A (µg/mL) in milk over the study period from quarters with severe clinical mastitis (n = 48). (** $P \leq 0.05$)

4.4. Discussion

This is the first report providing evidence that the administration of transdermal flunixin has a positive effect on clinical mastitis treatment, irrespective of severity score. Transdermal flunixin improved the bacteriological cure rate and decreased the concentration of an inflammation marker (MAA) compared to the control group in mild or moderate clinical mastitis cases. An earlier study was also able to show a positive effect on bacteriological cure on cows with mild or moderate clinical mastitis using injected meloxicam (McDougall et al. 2016). In my study efficacy of transdermal flunixin in severe clinical mastitis cows was comparable to meloxicam treatment, which is currently used as standard therapy.

Different biomarker, e.g., CATH (Pongthaisong et al., 2016; Addis et al., 2017), MAA (Jaeger et al., 2017; Hussein et al., 2018), and HP (Sadek et al., 2017; Thomas et al., 2018),

increase during inflammatory processes of clinical mastitis and NSAIDs have anti-inflammatory effects. This explains the faster decrease of MAA concentrations in cows with mild or moderate clinical mastitis treated with transdermal flunixin. No effect of transdermal flunixin or meloxicam was found in severe clinical mastitis cows. This agrees with an earlier study (Vangroenweghe et al. 2005), investigating other inflammatory parameters in milk, such as IL-8, complement component C5a, LBP, and soluble CD14, during hours and days of curing in milk of severe clinical mastitis cases treated with carprofen.

It is known that cows with experimentally induced severe clinical mastitis showed less severe udder edema (Fitzpatrick et al. 2013) and lower maximal swelling scores (Vangroenweghe et al. 2005), if treated with meloxicam or carprofen, respectively. We had not included an untreated control group for severe clinical mastitis cases due to animal welfare reasons. Therefore, we did not generate data on the effects on udder firmness. However, an earlier study (Rees et al. 2017) examining udder firmness during curing of clinical mastitis showed an initial increase of udder firmness within 14 days. Cows were treated with meloxicam or flunixin intravenously. Unlike this present study, mentioned study was not able to show a decrease of udder firmness on D4. Thus, transdermal flunixin might be more beneficial than flunixin or meloxicam administered intravenously. As we found no differences between meloxicam and transdermal flunixin in severe clinical mastitis cases, considering the investigated parameters, transdermal flunixin might offer an alternative to injected meloxicam. Further research is warranted to confirm this finding with a larger sample size. However, while a transdermal formulation has obvious benefits (e.g., easier application) there are also disadvantages (application only on dry skin), which should be mentioned. After application, it has to be ensured that the animal stays dry for at least 6 hours.

Altogether we evaluated only udder firmness, MAA, CATH, and bacteriological cure rates. Future studies should investigate other plausible parameters. Earlier studies, for example, demonstrated positive effects on dry matter intake, milk yield, SCC, pregnancy rates, and culling of mastitic cows treated with NSAIDs and antimicrobials (Yeiser et al. 2012; McDougall et al. 2009; McDougall et al. 2016). Therefore, further research on those factors in cows treated with naturally occurring clinical mastitis with transdermal flunixin is warranted. This study was conducted on only one dairy farm. Therefore, external validity is still unexplored. Also a larger study population in further studies, could certainly refine our current results.

4.5. Conclusion

This study was designed to determine, whether the application of transdermal flunixin influences curing of different severity score of clinical mastitis. Overall, we were able to show a faster bacteriological curing and a faster decrease of inflammatory reaction in mild or moderate clinical mastitis when cows were treated with transdermal flunixin instead of placebo. Transdermal flunixin showed similar results for therapy of severe clinical mastitis compared to meloxicam.

5. DISCUSSION

The overall objectives of this thesis are 1) to evaluate a dynamometer and a hand-held infrared thermometer to diagnose and classify subclinical (**SCM**) and clinical mastitis (**CM**), 2) to evaluate the biomarker cathelicidin (**CATH**), milk amyloid A (**MAA**), and haptoglobin (**HP**) to also diagnose and classify SCM and CM, and 3) to evaluate if there is an advantage of an additional application of transdermal flunixin in treatment of CM.

Such diagnostic methods might improve mastitis detection and scoring of severity of CM to enhance mastitis management, increase economics and overall productivity of a farm, and produce a high-quality product. In times of ongoing automatization of dairy cow husbandry, objective and reliable methods, are highly in demand. Furthermore, the request for prudent use of antimicrobials, therapy of CM with non-steroidal anti-inflammatory drugs (**NSAID**s), not only in severe, but also in mild and moderate CM is worth investigating.

In the first study of my thesis, data sets of 218 cows with CM (i.e., 46 mild, 106 moderate, 66 severe), 142 with SCM, and 68 healthy were analysed. Palpation results of the udder were compared with results of udder firmness measured by dynamometer and udder surface temperature (**UST**) using handheld infrared thermometer. Our results indicate that measuring udder firmness by using a handheld dynamometer is an accurate and objective method to estimate the udder health status of dairy cows (e.g., CM, SCM, and healthy udder quarters). The diagnostic value of measuring UST with a handheld thermometer to detect SCM and CM and differentiate between severity scores was limited, because ambient temperature biased UST.

In agreement with previous studies (Rees et al. 2014), a strong correlation between palpation of udder firmness and measurements by dynamometer was found ($r = 0.749$, $P < 0.001$). Our results support the earlier assumptions that udder firmness is a reliable indicator to identify CM (Swinkels et al. 2013; Fogsgaard et al. 2015). By using a dynamometer, it was possible to differentiate between CM, SCM, and healthy quarters objectively. No effect of lactation number, DIM, milk yield, total number of episodes with recurrent CM, time after milking, ambient temperature, investigator, and quarter position on udder firmness ($P > 0.05$) was observed. This is a major advantage over traditional methods of manual palpation or using the amount of SCC in milk, since those methods are subjective or influenced by different factors (Sharma et al. 2011; Bielfeldt et al. 2004). We were first to show that gram staining

characteristics (i.e., gram-positive, gram-negative, or no growth) was associated with udder firmness in mastitis quarters.

Even, if a differentiation of CM, SCM and healthy quarters was possible using UST, results disagreed with our palpation results, as SCM quarters were measured colder than healthy quarters ($P < 0.001$) or quarters with CM ($P < 0.001$). Lower temperature might be associated with a deviation of blood flow during SCM (Bortolami et al. 2015). Overall, results of UST measured by handheld infrared thermometer were significantly influenced by ambient temperature ($P < 0.001$). Other authors (Berry et al. 2003; Castro-Costa et al. 2014) found similar results, which compromise the overall diagnostic value of IRT in mastitis cows. Considering these results, an application in the field is not recommended and a calculation of thresholds was not reasonable.

The purpose of my second study to validate the biomarker CATH, MAA, and HP to diagnose and classify SCM and CM. Levels of the three different biomarkers were evaluated in 67 healthy cows, 119 cows with SCM, and 121 cows with CM. Additional 91 cows with CM were enrolled for MAA measurements. Our results support recent publications on CATH (Addis et al. 2016b; Pongthaisong et al. 2016; Addis et al. 2017), MAA (Jaeger et al. 2017; Hussein et al. 2018) and HP (Pedersen et al. 2003; Sadek et al. 2017; Thomas et al. 2018), which described the diagnostic value of these biomarkers for mastitis detection in milk. Comparing concentrations in milk of mastitis quarters between our and earlier publications, not surprisingly, highest comparability were seen in CATH, since measurements were conducted in the same laboratory (Addis et al. 2016a; Addis et al. 2016b; Addis et al. 2017). Levels of MAA, measured earlier in SCM, experimentally induced by inoculation with *Staphylococcus aureus*, averaged 5.6 ± 12 µg/mL (Eckersall et al. 2006), which is comparable with our level of 4.38 ± 0.6 µg/mL. In contrast, HP values reported in the literature differed from our results. Differences can be explained by different enzyme-linked immunosorbent assay kits as shown by Gerardi et al. (2009).

Measurement of the three biomarkers in our study achieved high accuracy for the detection of intramammary infections. While most previous studies focused on either SCM or CM, our study directly compared both types of mastitis. Therefore, we were able to show that not only a differentiation between healthy quarters and infected quarters is possible, but also a differentiation between SCM and CM.

The current studies could not confirm previous reports on high accuracy of HP and MAA for the differentiation between severity scores of CM (Wenz et al. 2010; Pyorala et al. 2011; Kalmus et al. 2013). We assume that these differences might be caused by different frequency distributions of the mastitis pathogens. Different types of pathogens might also explain, why we were, in contrast to previous reports on CATH (Addis et al. 2017) and HP (Wenz et al. 2010), not able to detect an effect of different pathogens on the concentration of CATH and HP. Also, different genotypes of pathogens might be associated with different levels of biomarkers. However, we were able to show that in SCM quarters the causative pathogen affected levels of CATH (P = 0.04) and HP (P = 0.05) significantly. Differences in HP levels between culture-negative and culture-positive SCM milk were also found by other authors (Hiss et al. 2007; Safi et al. 2009).

Comparing different level of biomarker with the SCC, we were able to show a good correlation of CATH, MAA, and HP with SCC, supporting previous calculations (Addis et al. 2017; Hussein et al. 2018). For HP and SCC Lai et al. (2009) describe a coefficient correlation of r = 0.742. However, the concentration of was stayed unaffected by lactation number, DIM, milk yield, total number of episodes with recurrent CM, and THI. For the identification of cows with SCM the measurement of biomarkers is more sensitive and specific compared to SCC and lactate dehydrogenase (Table 1, Se = 80 %, Sp = 87 %; Hiss et al. 2007), which both can already be measured automatically.

None of our diagnostic tests evaluated achieved the recommendations (e.g., Se = 0.80, Sp > 0.99) of the International Organization for Standardization (ISO/FDIS 20966). Neither calculated thresholds for udder firmness of 1.002 kg (Se = 0.73; Sp = 0.88) to differentiate between healthy and mastitis quarters nor 1.175 (Se = 0.70; Sp = 0.62) to differentiate between bacteriologically negative and positive CM cases. Nevertheless, compared (Table 1) for example with electrical conductivity measurements (i.e., SCM: Se = 0.194, Sp = 0.919; CM: Se = 0.479, Sp = 0.919; Norberg et al. 2004), udder firmness is more accurate. Results of biomarker thresholds were more accurate and sensitive then subjective parameters (i.e., palpation of the udder tissue; Houe et al. 2002; Rees et al. 2014) or semi-quantitative evaluation of SCC (CMT in SCM quarters; Safi et al. 2009; Viguier et al. 2009) in earlier studies. Our calculated thresholds of 0.053 NOD450 for CATH and 12.65 µg/mL for HP to differentiate between healthy and CM met the requirements proposed by the ISO (ISO/FDIS 20966) and showed better results than lactate dehydrogenase (Hiss et al. 2007).

Table 1. Table showing different publicized values for sensitivity and specificity in percentage for different parameter to diagnose either subclinical or clinical mastitis.

	Parameter	Sensitivity (%)	Specificity (%)	Reference
Subclinical mastitis	Lactate dehydrogenase	80	97	Hiss et al. 2007
	Electrical conductivity	19	92	Norberg et al. 2004
	Cathelicidin (NOD450)	83	97	Wollowski et al. 2021
	Milk amyloid A	65	76	Wollowski et al. 2021
	Haptoglobin	92	94	Wollowski et al. 2021
	Recommendations	73	88	ISO, 2007
Clinical masitits	Electrical conductivity	48	92	Norberg et al. 2004
	Udder firmness	73	88	Wollowski et al. 2019
	Haptoglobin	96	99	Wollowski et al. 2021
	Cathelicidin (NOD450)	98	99	Wollowski et al. 2021
	Milk amyloid A	77	83	Wollowski et al. 2021
	Recommendations	73	88	ISO, 2007

Therefore, using a dynamometer or measuring biomarkers (CATH, MAA, or HP) allows a reliable, objective mastitis detection and may improve mastitis management. Calculated thresholds could serve as critical control points for diagnostic classification and treatment decisions, not only in standard operation procedures (**SOP**s), but also in automatic milking systems, where our tested tools might be also integrated in. Especially in larger herds, the use of SOPs constantly increases to standardize and simplify working routines.

Implementing reliable thresholds by using udder firmness and biomarkers in working routine fulfills the need for good management practices of mastitis, including optimization of mastitis detection (Barnouin et al. 2004; Green et al. 2007) which can decrease the proportion of cows suffering from mastitis. Minimizing the mastitis incidence saves resources, reduces loss of milk, and allows a production of a high-quality product.

A safer product might also be reached by a more prudent use of antimicrobials in therapy of CM to reduce antimicrobial resistances. As my additional unpublished data could show, an additional administration of NSAIDs not only in severe, but also in mild and moderate CM is promising. So far, our results showed that the application of transdermal flunixin has a positive effect on cure rates of CM especially in mild and moderate cases.

Similar to a previous research project (McDougall et al., 2016), I was able to show faster bacteriological cure in mild or moderate CM (D5 and D21) for NSAIDs compared to a placebo. Furthermore, inflammation decreased faster (concentration of MAA on D2 and D3) if cows were treated with transdermal flunixin compared to a placebo treatment.

Positive effects of NSAIDs on udder firmness (Fitzpatrick et al. 2013), were not reproducible since cows with severe CM were not left untreated due to animal welfare reasons. Since cows with severe CM always received either transdermal flunixin or meloxicam and assuming that both NSAIDs have similar effects, it is plausible that results do not differ. Cows with severe CM treated with transdermal flunixin or meloxicam showed a decrease of udder firmness from D4. Such a decrease was not detected in an earlier study (Rees et al. 2017) examining udder firmness within 14 days after diagnosing CM and treatment with either meloxicam or flunixin (i.m or i.v.). Thus, transdermal flunixin might have a more positive effect on cure of mastitis at least considering udder firmness compared to systemically administered flunixin and meloxicam. However, while a transdermal formulation has obvious benefits (e.g., easier application) there are also disadvantages (application only on dry skin). After application, it must be ensured that the animal stays dry for at least 6 hours.

In conclusion, using a dynamometer or measuring biomarker (CATH, MAA, or HP) allows an objective mastitis detection to improve mastitis management and increase overall productivity of a farm. With udder firmness and concentrations of MAA and CATH in milk, identification of mastitis and cure rates becomes more objective. Moreover, it is possible to show a positive effect of NSAIDs on mastitic cows. These studies were conducted on only one dairy farm and with one kind of breed. Therefore, external validity is still unexplored. A larger study population in further studies, could certainly refine my current results.

6. SUMMARY

Novel approaches to diagnose, classify, and treat subclinical and clinical mastitis in dairy cows

The objectives of this thesis were (1) to utilizes a dynamometer and a handheld infrared thermometer to diagnose and classify clinical and subclinical mastitis (Publication I), (2) to evaluate the biomarker cathelicidin, milk amyloid A, and haptoglobin to diagnose and classify clinical and subclinical mastitis (Publication II), and (3) to study the advantage of an additional application of the non-steroidal anti-inflammatory drug flunixin in treatment of clinical mastitis using parameter of Publication I and II (Additional unpublished data).

In times of ongoing automatization of dairy cow husbandry, objective and reliable methods, are highly in demand. Analyzing specific biomarkers in milk or using tools such as the handheld dynamometer or infrared thermography (**IRT**) could be advantageous compared to subjective or semi-quantitative criteria such as palpation of the udder in clinical mastitis (**CM**) cases or evaluation of SSC using cow side tests (e.g., California Mastitis Test) in subclinical mastitis (**SCM**) quarters. To reduce antimicrobial resistance and economic losses, a treatment of cows with Non-steroidal anti-inflammatory drug (**NSAIDs**) not only in severe, but also in mild and moderate CM seems to be advantageous.

The first study investigated the diagnostic value of a handheld dynamometer and an infrared thermometer to diagnose and score CM and SCM and to compare those values with results from palpation of the udder tissue. Overall, data of 218 cows with CM (i.e., 46 mild, 106 moderate, and 66 severe cases), 142 with SCM, and 68 healthy cows were enrolled. Data provides evidence that the dynamometer is an accurate diagnostic tool to differentiate between healthy and mastitic udder quarters. The severity score of CM could be determined by using the dynamometer. Threshold for udder firmness to detect quarters with CM was 1.002 kg. Using a threshold of 1.175 kg in CM quarters, it was possible to differentiate between negative and positive bacteriological results. A differentiation between healthy and CM quarters with IRT was possible, albeit UST were highly influenced by ambient temperature. We were able to show, that UST increased up to 0.18°C for each degree of ambient temperature.

The objective of the second study was to investigate the diagnostic value of three biomarkers, i.e., cathelicidin (**CATH**), milk amyloid A (**MAA**), and haptoglobin (**HP**) for the diagnosis of SCM and CM. Furthermore, the accuracy of these biomarkers to differentiate

between mild, moderate, and severe CM and the influence of different pathogens on biomarker levels was tested. A total of 67 healthy cows, 119 cows with SCM, and 212 cows with CM were enrolled in the study. While CATH, MAA, and HP were measured in all samples from healthy cows and those with SCM, HP, and CATH results were only available from 121 cows with CM. Milk amyloid A was measured in all samples. In cows with CM, the mastitis quarter and a second healthy quarter, serving as a healthy in-cow-control quarter were sampled. Several different generalized linear mixed models were used to determine the effect of the health status of the udder quarter (e.g., healthy, SCM, CM and healthy in-cow control) different severity scores (e.g., mild, moderate, and severe) and bacteriological results on CATH, MAA, and HP levels. Furthermore, receiver operating characteristic curves were calculated to establish thresholds between different udder healthy status or different bacteria. A differentiation between healthy quarters, quarters with SCM, and CM was possible, using all three biomarkers. Thresholds for CATH to differentiate between healthy quarters and quarters with SCM or CM were 0.000 NOD450 (Se = 0.83, Sp = 0.97) and 0.053 NOD450 (Se = 0.98, Sp= 0.99), respectively. Thresholds of 1.28 µg/mL (Se = 0.65, Sp = 0.76) and 1.81 µg/mL (Se = 0.77, Sp = 0.83) for MAA and 3.65 µg/mL (Se = 0.92, Sp = 0.94) and 5.40 µg/mL mL (Se = 0.96, Sp = 0.99) for HP were also calculated, respectively. Healthy in-cow control quarters from cows with mastitis showed elevated MAA and HP levels compared to healthy quarters from healthy cows. Only the level of MAA was higher in severe CM cases compared to mild ones. In contrast to CM, CATH, and HP in SCM quarters were significantly influenced by different bacteriological results.

Additional unpublished data were presented in this thesis evaluating whether the application of transdermal flunixin influences cure rates of mild, moderate, or severe CM. Udder firmness measured by dynamometer, levels of the two different biomarker CATH and MAA in milk and bacteriological examination were used to evaluate curing. To be able to compare the curing success, we examined all animals after initially examination on day 1 (**D1**) the following 4 days (**D2** - **D5**), on day seven (**D7**) and on day 21 (**D21**). During these examinations, udder firmness and levels of biomarkers were measured. Two quarters per cow were examined, the mastitic quarter and a healthy in-cow control quarter. In addition, a bacteriological examination of the milk was carried out on D5 and D21 to determine bacteriological cure. Data of udder firmness was available for all enrolled 218 cows (i.e., 46 mild, 106 moderate, 66 severe) for all examination days. For the evaluation of level of biomarker CATH and MAA 121 cows (20 cows with mild, 63 with moderate, and 38 with severe

clinical mastitis) could be evaluate. For MAA, data from D1 - D5 and D21 of additional 24 cows (7 cows with mild, 7 with moderate, and 10 with severe clinical mastitis) were available. After examination on D1 mild or moderate CM were treated either with transdermal flunixin or with placebo, severe CM with transdermal flunixin or injected meloxicam, respectively. An udder quarter was considered cured, if the original mastitis causing pathogen of D1 was no longer detectable on D5 or D21, respectively. In mild or moderate CM, there was no significant difference of udder firmness differences and level of CATH between animals treated with transdermal flunixin and those treated with placebo throughout days of examination. Only on D2 and D3 level of biomarker MAA was lower in mastitis quarter in cows treated with transdermal flunixin instead of placebo. Cows, which were treated with transdermal flunixin showed a higher bacteriological cure rate than untreated control cows. Interestingly, all cows treated with transdermal flunixin instead of placebo had a negative bacteriological result on D21. In severe CM, transdermal flunixin and meloxicam treated animals showed similar results in udder firmness difference, level of MAA and CATH, and bacteriological curing.

Overall, this thesis demonstrates that the udder health status of dairy cows can be accurately and objectively classified using (1) a dynamometer that measures udder firmness at the udder or (2) biomarkers (CATH, MAA and HP) in the milk. Furthermore, some of investigated parameters (e.g., udder firmness, CATH, and MAA) can be used to objectively evaluate the cure of clinical mastitis.

7. ZUSAMMENFASSUNG

Neue Ansätze zur Diagnose, Klassifizierung und Behandlung subklinischer und klinischer Mastitis bei Milchkühen

Ziel dieser Arbeit war es, den Einsatz eines Dynamometers und eines Infrarotthermometers (Publikation I) sowie der Biomarker Cathelicidin, Milch-Amyloid A und Haptoglobin (Publikation II) zur Diagnose und Klassifizierung von subklinischen und klinischen Mastitiden unterschiedlichsten Schweregrades zu validieren. Zusätzlich sollte die ergänzende Anwendung des transdermal applizierten Nicht-steroiden Antiphlogistikums Flunixin bei der Behandlung von klinischen Mastitiden unterschiedlichen Schweregrades bewertet werden (Additional Unpublished Data).

In Zeiten der fortschreitenden Automatisierung in der Milchviehhaltung sind objektive und verlässliche Methoden sehr gefragt. Die Analyse spezifischer Biomarker in der Milch oder die Verwendung von Instrumenten wie dem Dynamometer oder dem Infrarotthermometer könnte, im Vergleich zu den bisher verwendeten subjektiven oder semi-quantitativen Methoden der Palpation des Euters bei klinischen Fällen, oder der Bewertung von somatischer Zellzahl (**SCC**) mittels California Mastitis Test (**CMT**) bei subklinischen Fällen von Mastitis, vorteilhaft sein. Um die Resistenzen von Antibiotika und die wirtschaftlichen Kosten zu reduzieren, scheint sich eine Behandlung der Mastitiskühe mit Nicht-steroidalen Antiphlogistika (**NSAID**s), nicht nur bei hochgradiger, sondern auch bei gering- und mittelgradigen Mastitiden, positiv auf die Heilung von Mastitiden auszuwirken.

In der ersten Studie wurde der diagnostische Wert eines portablen Dynamometers und eines Infrarotthermometers untersucht, um klinische und subklinische Mastitiden zu diagnostizieren und zu klassifizieren. Erhobene Werte wurden mit den Ergebnissen aus einer palpatorischen Untersuchung des Euters verglichen. Insgesamt wurden Daten von 218 Kühen mit klinischer Mastitis (46 gering-, 106 mittel- und 66 hochgradige), 142 mit subklinischer Mastitis und 68 gesunde Kühe ausgewertet. Unsere Daten zeigen, dass mithilfe eines Dynamometers zwischen gesunden Eutervierteln und solchen mit subklinischer oder klinischer Mastitis unterschieden werden kann. Die Einteilung des Schweregrads von klinischen Mastitiden ist mit einem Dynamometer ebenso möglich. Der Grenzwert für die Euterfestigkeit zur Erkennung von Vierteln mit klinischen Mastitiden lag bei 1,002 kg. Mit dem Grenzwert von 1,175 kg in Vierteln mit klinischer Mastitis war es möglich, zwischen Eutervierteln mit negativen und positiven bakteriologischen Befunden zu unterscheiden. Eine Unterscheidung zwischen gesunden Vierteln

und solchen mit klinischer Mastitis mithilfe des Infrarotthermometers war zwar möglich, allerdings hing die Euteroberflächentemperatur stark von der Umgebungstemperatur ab. Wir konnten zeigen, dass die Euteroberflächentemperatur mit jedem Grad steigender Umgebungstemperatur bis zu 0,18°C zunahm.

Ziel der zweiten Studie war es, den diagnostischen Wert der drei Biomarker Cathelicidin (**CATH**), Milch-Amyloid A (**MAA**) und Haptoglobin (**HP**) für die Diagnose von subklinischen und klinischen Mastitiden zu untersuchen. Darüber hinaus wurde die Eignung dieser Biomarker zur Unterscheidung zwischen gering-, mittel- und hochgradiger klinischer Mastitis und der Einfluss verschiedener Erreger auf ihre Konzentration getestet. Insgesamt wurden 67 gesunde Kühe, 119 Kühe mit subklinischer und 212 Kühe mit klinischer Mastitis in die Studie aufgenommen. Während CATH, MAA und HP in allen Proben von gesunden Kühen und Kühen mit subklinischer Mastitis gemessen wurde, lagen Ergebnisse von CATH und HP nur für 121 Kühe mit klinischer Mastitis vor. In allen Proben wurde MAA gemessen. Bei Kühen mit klinischer Mastitis wurde das Viertel mit Mastitis und ein zweites, gesundes Viertel, das als gesundes Kontrollviertel einer Kuh diente, beprobt. Verschiedene generalisierte lineare Modelle wurden berechnet, um den Einfluss des Gesundheitszustands eines Euterviertels (gesund, subklinische Mastitis, klinische Mastitis und gesundes Kontrollviertel), verschiedener Schweregrade klinischer Mastitiden (gering-, mittel- und hochgradig) und verschiedener bakteriologischer Erreger auf CATH-, MAA- und HP-Konzentration zu bestimmen. Darüber hinaus wurden Grenzwerte für Euter mit unterschiedlichem Eutergesundheitsstatus oder unterschiedlichem Mastitiserreger berechnet. Eine Unterscheidung zwischen gesunden Vierteln und Vierteln mit subklinischer oder klinischer Mastitis war unter Verwendung aller drei Biomarker möglich. Der Grenzwert für CATH zur Unterscheidung zwischen gesunden Vierteln und Vierteln mit subklinischer oder klinischer Mastitis lag bei 0,000 NOD450 (Se = 0,83, Sp = 0,97) beziehungsweise bei 0,053 NOD450 (Se = 0,98, Sp = 0,99). Die Grenzwerte für MAA lagen bei 1,28 µg/ml (Se = 0,65, Sp = 0,76) und 1,81 µg/ml (Se = 0,77, Sp = 0,83) und für HP bei 3,65 µg/ml (Se = 0,92, Sp = 0,94) und 5,40 µg/ml (Se = 0,96, Sp = 0,99). Gesunde Kontrollviertel von Kühen mit klinischer Mastitis wiesen im Vergleich zu gesunden Vierteln von gesunden Kühen erhöhte MAA- und HP-Konzentrationen auf. Nur die Konzentration von MAA war bei hochgradigen klinischen Mastitiden höher als bei geringgradigen Mastitiden. Im Gegensatz zu klinischen Mastitisvierteln, wurden CATH- und HP- Konzentrationen in subklinischen Vierteln durch unterschiedliche bakteriologische Befunde signifikant beeinflusst.

Die „Additional Unpublished Data“ dieser Arbeit zeigen Daten zur Behandlung von klinischen Mastitiden und bewerten, ob die Anwendung des transdermal applizierten Flunixins

einen Effekt auf die Heilung von gering-, mittel- oder hochgradigen klinischen Mastitiden hat. Hierzu wurden Entzündungszeichen des Euters und der Milch gemessen. Bei der Bestimmung der Euterfestigkeit kam ein Dynamometer zum Einsatz. In der Milch wurde die Konzentration verschiedener Biomarker (CATH und MAA) bestimmt. Des Weiteren fand eine bakteriologische Untersuchung der Milch statt. Um den Heilungserfolg vergleichen zu können, wurden alle Kühe nach der Aufnahmeuntersuchung am Tag 1 (D1), an den folgenden 4 Tagen (D2 - D5), am Tag 7 (D7) und am Tag 21 (D21) untersucht. Bei diesen Untersuchungen wurde die Euterfestigkeit gemessen und Milchproben zur späteren Untersuchung der Konzentration der Biomarker genommen. Pro Kuh wurden zwei Viertel untersucht, das erkrankte Viertel und ein gesundes Kontrollviertel. Darüber hinaus wurde eine bakteriologische Untersuchung der Milch an D5 und D21 durchgeführt, um die bakteriologische Heilung des Mastitisviertels zu bewerten. Daten zur Euterfestigkeit lagen für alle ausgewerteten 218 Kühe (46 Kühe mit gering-, 106 Kühe mit mittel- und 66 Kühe mit hochgradiger klinischer Mastitis) für alle Untersuchungstage vor. Für die Bewertung der Biomarker CATH und MAA konnten 121 Kühe (20 Kühe mit gering-, 63 Kühe mit mittel- und 38 Kühe mit hochgradiger klinischer Mastitis) ausgewertet werden. Für MAA lagen Daten von D1 - D5 und D21 von weiteren 24 Kühen (7 Kühe mit gering-, 7 Kühe mit mittel- und 10 Kühe mit hochgradiger klinischer Mastitis) vor. Nach der Untersuchung an D1 wurden Kühe mit gering- und mittelgradigen klinischen Mastitiden entweder mit transdermalen Flunixin oder mit einem Placebo behandelt. Kühe mit hochgradiger klinischer Mastitis bekamen transdermales Flunixin oder injiziertes Meloxicam. Ein Euterviertel galt als geheilt, wenn der ursprünglich verursachende Erreger von D1, an D5 oder D21 nicht mehr nachweisbar war. Bei gering- und mittelgradigen klinischen Mastitiden gab es innerhalb der gesamten Untersuchungstage keinen signifikanten Unterschied zwischen den mit transdermalen Flunixin und den mit Placebo behandelten Tieren hinsichtlich der Differenz der Euterfestigkeit und CATH-Konzentration. Nur an D2 und D3 war die Konzentration von MAA im Mastitisviertel bei den mit transdermalem Flunixin behandelten Kühen niedriger als bei Kühen, die mit Placebo behandelt wurden. Kühe, die mit transdermalem Flunixin im Gegensatz zum Placebo behandelt worden waren, wiesen darüber hinaus sowohl am D5 als auch am D21 eine höhere bakteriologische Heilungsrate auf. Interessanterweise hatten alle Kühe, die mit transdermalem Flunixin anstatt eines Placebos behandelt wurden, am D21 ein negatives bakteriologisches Ergebnis. Bei schweren klinischen Mastitiden zeigten die mit transdermalen Flunixin oder mit Meloxicam behandelten Tiere ähnliche Ergebnisse der Differenzen von Euterfestigkeit, der Konzentration von CATH und MAA und der bakteriologischen Heilung.

Insgesamt präsentiert diese Arbeit, dass der Eutergesundheitsstatus von Milchkühen mit Hilfe (1) eines Dynamometers, das die Euterfestigkeit am Euter misst, oder (2) den Biomarkern (CATH, MAA und HP) in der Milch, genau und objektiv klassifiziert werden kann. Außerdem lässt sich mit einigen der untersuchten Parameter (z.B. Euterfestigkeit, CATH und MAA) objektiv die Heilung von klinischen Mastitiden bewerten.

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9. PUBLICATIONS

Research articles

Wollowski L, Bertulat S, Kossatz A, Heuwieser W (2019):
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Oral presentations at conferences

Wollowski L, Bertulat S., Heuwieser W (2018):
Objektive Erfassung und Klassifizierung des Euter-Gesundheitsstatus unter Einsatz eines Dynamometers oder eines Infrarotthermometers.
Tagung der AG "Sachverständigenausschuss Subklinische Mastitis" zum Thema: Mastitisbekämpfung in Zeiten eines restriktiven Antibiotikaeinsatzes.
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11. SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit bestätige ich, Luise Wollowski, alle Studien selbstständig durchgeführt zu haben und die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Eigener Anteil[1] an den Forschungsprojekten der vorliegenden Dissertation:

	Studie 1[a]	Studie 2[b]	Studie3[c]
Studienplanung	++	++	++
Datenerhebung	+++	++	++
Datenanalyse	+++	+++	+++
Verfassen des Manuskripts	+++	+++	+++
Editieren des Manuskripts	++	++	++

[1]Legende: +++: >70%
++: 50-70%
+: <50%

[a] Short communication: Diagnosis and classification of clinical and subclinical mastitis utilizing a dynamometer and a handheld infrared thermometer

[b] The value of the biomarkers cathelicidin, milk amyloid A, and haptoglobin to diagnose and classify clinical and subclinical mastitis

[c] Effects of transdermal flunixin in therapy of clinical mastitis considering severity score in dairy cows

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