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Modulation der Erregerausscheidung infizierter Milchdrüsenviertel beim Rind

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Inhaltsverzeichnis

1 Einleitung

Eutererkrankungen gehören neben Fruchtbarkeitsstörungen sowie Klauen- und Gliedmaßenerkrankungen zu den drei häufigsten Abgangsgründen für Milchkühe in Deutschland (ADR, 2017). Mastitiden sind Entzündungen des Milchdrüsengewebes eines oder mehrerer Euterviertel, die in den meisten Fällen durch infektiöse Pathogene verursacht werden. Am häufigsten sind Staphylokokken, Streptokokken und coliforme Spezies ursächlich (Ruegg, 2017). Es handelt sich um eine Faktorenerkrankung, da neben dem Vorhandensein dieser Erreger endogene und exogene Faktoren für die Entstehung und Ausprägung einer Mastitis bedeutsam sind. In der praktischen Diagnostik werden die Infektionen in klinische und subklinische Fälle eingeteilt. Bei ersteren sind äußerlich erkennbare Symptome, wie Rötung, Schwellung, Schmerzen, erhöhte Temperatur oder makroskopische Sekretveränderungen (Flocken) festzustellen. Subklinische Mastitiden dagegen sind äußerlich nicht erkennbar. Ein somatischer Zellgehalt von > 100.000/ml weist jedoch auf eine entzündliche Reaktion hin (Krömker, 2007). Durch Mastitiden werden hohe wirtschaftliche Verluste verursacht, die zum einen durch direkte Kosten, wie Diagnostik, tierärztliche Dienstleistungen, Medikamente, Verwerfen von Milch oder Mehrarbeit in der Tierbetreuung entstehen. Zum anderen wirken sich indirekte Kosten nachteilig auf die Ökonomie der Milchviehhaltung aus. Dazu gehören eine geringere zukünftige Milchproduktion von erkrankten Tieren, eine verminderte Fruchtbarkeit, das Auftreten anderer assoziierter Krankheiten sowie vorzeitige Abgänge und damit verbundene höhere Remontierungsraten (Goncalves et al., 2018, Heikkilä et al., 2018). Der größte Anteil (ca. 70 %) der wirtschaftlichen Schäden wird durch geringere Milchleistungen verursacht. Subklinische Mastitiden verursachen insgesamt betrachtet höhere ökonomische Verluste als die klinischen Fälle (Huijps et al., 2008).

Mastitiserreger werden aus infizierten Milchdrüsenvierteln ausgeschieden. In ~ 25 % der Milchproben aus klinischen und ~ 30 % aus subklinischen Mastitiden ist jedoch keine Kultivierung von Erregern möglich (Scherpenzeel und Schukken, 2018). Mögliche Begründungen dafür sind, dass der verursachende Erreger zum Zeitpunkt der Probenentnahme nicht mehr in der Milch vorhanden ist (bei toxinbildenden

Mikroorganismen, wie z.B. *Escherichia coli* (*E. coli*)), dass aufgrund antibiotisch wirkender Substanzen in der Milch der Erreger nicht kultiviert werden kann oder dass die Erregerkonzentration aufgrund der köpereigenen Abwehr zu niedrig für die Kultivierung ist (Krömker, 2007). Erreger-ausscheidende Euterviertel stellen eine Infektionsquelle für andere Tiere in einer Milchviehherde dar (White et al., 2006, Barlow et al., 2009). Von praktischer Relevanz ist die Erregerausscheidung auch in Hinblick auf die zu erwartende Heilungsrate. Es konnte nachgewiesen werden, dass die Wahrscheinlichkeit für eine bakteriologische Heilung von klinischen Mastitiden höher ist, wenn vor der antibiotischen Behandlung eine niedrigere Erregerkonzentration in der Milch vorhanden war (Ziesch und Krömker, 2016). Untersuchungen von Deluyker et al. (2005) kamen zu ähnlichen Ergebnissen für die bakteriologische Heilungsrate von subklinischen Mastitiden. Die Ausscheidungsintensität von Mastitiserregern ist Spezies-spezifisch. So sind die Erregerkonzentrationen in der Milch aus Eutervierteln, die mit *Staphylococcus aureus* (*S. aureus*) infiziert sind, niedriger als bei Infektionen mit *Streptococcus uberis* (*S. uberis*) oder *E. coli* (Sears et al., 1990, Schukken et al., 2011). Auch ein Einfluss des Stammes konnte gezeigt werden, zum Beispiel bei *S. uberis*-Infektionen (Tassi et al., 2013). Bei den Angaben in der Literatur steht die Ausscheidungsintensität jedoch häufig nicht im Mittelpunkt der Untersuchungen. Zudem gibt es keine Studien, welche die Ausscheidung von Mastitiserregern mit statistischen Verfahren vergleichend betrachten. Neben dem Erreger sind Tier-individuelle Faktoren für die Ausprägung und Schwere von Mastitiden bedeutsam, wie etwa die Energieversorgung, der Immunstatus, die Parität oder das Laktationsstadium (Keane, 2019). Beobachtungen aus der praktischen Milchviehhaltung weisen darauf hin, dass auch Veränderungen in der Umwelt Einfluss auf das Auftreten und die Ausprägung von Mastitiden haben. Kehrli et al. (2009) nehmen an, dass ungünstige Fütterungsbedingungen und die Aufnahme giftiger Stoffe sowie Tiertransporte, sozialer Stress oder bestimmte Arzneimittel (Glukokortikoide) zu schlechterer Eutergesundheit beitragen. Dass Stressereignisse auch Einfluss auf die Ausscheidungsintensität von Mastitiserregern haben können, wurde in einer Studie von Krömker et al. (2008) gezeigt. Dabei konnte der Nachweis von *S. aureus*-infizierten Eutervierteln durch die Probenentnahme nach

Stress-verursachenden Ereignissen, wie Klauenpflege, erhöht werden, da die Ausscheidungsintensität des Erregers erhöht war. Es ist bekannt, dass auch klimatische Bedingungen Einfluss auf Milchkühe und die Milchproduktion haben. So führt Hitzestress zu geringeren Futteraufnahmen und Milchleistungen (West et al., 2003, Gorniak et al., 2014) sowie erhöhten Gehalten an somatischen Zellen in der Milch (Nasr und El-Tarabany, 2017). Welchen Einfluss Hitzestress auf die Ausscheidungsintensität von Mastitiserregern hat, ist bislang nicht wissenschaftlich untersucht. Eine Hypothese dieser Arbeit besteht darin, dass Stressoren zu einer Exazerbation von Mastitiden und somit zu einer Erhöhung der Ausscheidung von Mastitiserregern führen können. Ein besseres Verständnis über die Exkretion von Mastitiserregern könnte zu einer Verbesserung der Eutergesundheit beitragen. Ziel des ersten Teiles dieser Arbeit war es, die Ausscheidung verschiedener Mastitiserreger aus infizierten Eutervierteln und beeinflussende Faktoren zu untersuchen.

Nicht-*S. aureus*-Staphylokokken (NAS) gehören zu den am häufigsten in Milchproben nachgewiesenen Bakterien (Thorberg et al., 2009, DVG, 2015). Diese Bakteriengruppe umfasst ca. 50 Spezies, von welchen ca. 20 regelmäßig in Milch vorkommen. Die Bedeutung der NAS für die Eutergesundheit ist nicht vollständig geklärt. Infektionen verlaufen zumeist subklinisch und können über Monate persistieren (Taponen et al., 2006, Gillespie et al., 2009, Mørk et al., 2012). Einige Untersuchungen kamen zu dem Ergebnis, dass NAS-Infektionen die Milchleistung erhöhen können (Piepers et al., 2008, Schukken et al., 2009). Andere Studien dagegen beschreiben nachteilige Effekte, wie geringere Milchleistung (Heikkilä et al., 2018) oder erhöhte somatische Zellgehalte in der Milch aus infizierten Eutern (Supré et al., 2011, Fry et al., 2014). Ohne eine Spezies-Differenzierung ist die Beurteilung des Effektes der NAS auf die Eutergesundheit nicht möglich (Supré et al., 2011). Da bedeutende Unterschiede hinsichtlich der Eigenschaften der NAS-Spezies bestehen, wurde von der Betrachtung als Bakteriengruppe in der jüngeren Forschung Abstand genommen (De Visscher et al., 2015). Dieses wurde auch durch die Entwicklung geeigneter diagnostischer Methoden möglich. Anfänglich wurden häufig phänotypische Methoden zur Differenzierung von NAS-Spezies in Kuhmilch eingesetzt, wie zum Beispiel das

kommerzielle Testkit API Staph ID 32 (bioMérieux, France). Diese Tests wurden ursprünglich für den Nachweis von Staphylokokken in der Humanmedizin entwickelt. Ein Anteil an NAS-Spezies wird von den phänotypischen Tests nicht erfasst, und die Sensitivität und Spezifität sind für wissenschaftliche Fragestellungen nicht zufriedenstellend, weshalb diese Methode als unbrauchbar für die Differenzierung von NAS-Spezies in Kuhmilch beurteilt wurde (Sampimon et al., 2009). Eine verlässlichere Differenzierung konnte durch die Entwicklung molekularbiologischer Methoden erreicht werden (Vanderhaeghen et al., 2015). Mittels MALDI-TOF MS (Matrix assisted laser desorption ionization - time of flight mass spectrometry) ist die Identifizierung von NAS-Spezies mit hoher Genauigkeit und Zuverlässigkeit möglich (Cameron et al., 2017). NAS kommen auch auf der Körperoberfläche (von Mensch und Tier) und in der Umwelt (Liegeboxen, Spaltenboden, Luft) häufig vor (De Visscher et al., 2014, Adkins et al., 2018). Daraus ergibt sich die Hypothese, dass NAS in Milchproben auch als Kontaminanten vorkommen und es somit zu falsch-positiven Annahmen intramammärer NAS-Infektionen kommt. Im zweiten Teil dieser Arbeit wurde die Bedeutung der NAS als Ursache von Infektionen bzw. Kontamination von Milchproben sowie die Spezies-spezifische Erregerausscheidung der NAS untersucht.

2 Manuskript 1

BACTERIA SHEDDING FROM MAMMARY QUARTERS

Heat stress and cow factors affect bacteria shedding pattern from naturally infected mammary gland quarters in dairy cattle

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Interpretive summary:

Bacteria shedding from infected mammary gland quarters in dairy cattle, Hamel

Mastitis-causing pathogens are shed from infected mammary gland quarters and lead to further infections in a herd. There are pathogen-specific properties of shedding as well as several factors that influence the shedding characteristics. A better understanding of the characteristics of pathogen shedding in milk and the influencing factors will help in controlling mastitis. The current study has shown that pathogen shedding with milk is greater with a higher milk somatic cell count, lower parity and higher temperature-humidity index. Consequently, the prevention and mitigation of heat stress in the German temperate climate can have a positive influence on udder health.

2.1 Abstract

Mastitis-causing pathogens are shed from infected mammary gland quarters and thus contribute to an increased risk of new intramammary infections (**IMI**). The objective of the current study was to investigate the shedding characteristics of various mastitis-causing pathogens and associated animal-specific (somatic cell score (**SCS**) and parity) and environmental (heat stress) factors. In a longitudinal study, infected udder quarters were sampled consecutively on five dairy farms in Germany. To capture climatic factors, temperature-humidity index (**THI**) was calculated. In the laboratory analysis, the pathogens and their counts in the milk samples were determined. A general linear mixed model with Gamma-link was used to evaluate the factors influencing pathogen shedding characteristics. The variables SCS, pathogen, parity and THI had significant influence on the pathogen shedding. Staphylococci were shed in lower values than streptococci. The pathogen shedding from mammary gland quarters with IMI was higher in the first and second lactation than in higher lactations. Exceeding the THI threshold 60 resulted in higher pathogen counts on the same day. This was only caused by the pathogens yeasts and *Strep. uberis*. Possible mechanisms causing differences in pathogen shedding are changes in the counts due to influenced milk quantities, better growth conditions at higher temperatures or altered immunological reactions. The mechanisms often remain speculative and require further investigations. The study underlines the contribution of cows with high somatic cell counts regarding the transmission of mastitis pathogens within a herd. Furthermore, it becomes clear that heat stress in Germany influences udder health and that prevention measures are useful.

Keywords: intramammary infection, bacteria shedding, somatic cell count, heat stress

2.2 Introduction

IMI are one of the main causes of economic losses in dairy farming (Heikkilä et al., 2018). The causative udder pathogens are predominantly staphylococci, streptococci and coliform species (Ruegg, 2017). There are pathogen-specific characteristics associated with the bacterial counts in milk. Compared to other common mastitis-causing pathogens, *Staphylococcus aureus* is shed at relatively low counts (Sears et al., 1990, Schukken et al., 2011, Walker et al., 2011). Higher shedding intensities are described for streptococci, like *Streptococcus uberis* (Tassi et al., 2013) or *Streptococcus dysgalactiae* (Maye et al., 2017). *Escherichia coli* is also shed in high bacterial counts from infected mammary quarters (Schukken et al., 2011). In addition to the species-specific shedding intensity, strain-specific properties are also known, for example for *Strep. uberis* (Tassi et al., 2013). However, there are no studies that compare the shedding intensity of mastitis-causing pathogens from naturally occurring IMI. The shedding characteristics of bacteria from infected mammary quarters are not only determined by the pathogens and their characteristics. Hosts factors such as innate immune resistance, energy balance, immune status, parity or stage of lactation can also be crucial for the severity and outcome of IMI (Keane, 2019). Basically, an increasing number of lactations is detrimental for the udder health, which is reflected in higher somatic cell counts (Hagnestam-Nielsen et al., 2009), a higher risk of IMI (Hertl et al., 2011) or lower bacteriological cure rates (Deluyker et al., 2005). Observations of dairy farmers support the hypothesis that changes in the husbandry environment affect the udder health and the occurrence of clinical mastitis. Kehrli et al. (2009) postulated that factors such as nutritional deficits and toxicities as well as animal transport and commingling or the influence of drugs like glucocorticoids can lead to poorer udder health in dairy cows. It has previously been observed that the detection rate of *Staph. aureus*-infected udder quarters can be improved by sampling after stress-causing events like claw trimming because of a higher bacteria shedding intensity (Krömker et al., 2008). Another possible environmental stress-causing factor on bacteria shedding is the climate or the change in weather conditions. A considerable number of studies have been published on the impact of heat stress on dairy cows. It is described that there is a negative influence of increasing temperature-humidity index

(**THI**) on dry matter intake and milk yield (West et al., 2003, Gorniak et al., 2014). Also, the somatic cell count increases at higher THI levels (Nasr and El-Tarabany, 2017). Heat stress therefore seems to have an influence on udder health and probably also on the shedding intensity of mastitis-causing pathogens. However, there are no studies on the influence of the aforementioned environmental factors on the species-specific shedding intensity. In a study conducted by Deluyker et al. (2005), it was shown that the cure rate of subclinical mastitis (**SCM**) was higher for lower numbers of colonies in the pre-treatment culture. In a study by Ziesch and Krömker (2016), the probability of bacteriological cure for clinical mastitis cases with a low shedding intensity of pathogens pretreatment was also significantly ($P = 0.01$) higher than for cases with a high shedding rate (odds ratio = 2.535). Bacteria-shedding udder quarters are of practical relevance as they are an important source of infection for other cows in the herd (White et al., 2006, Barlow et al., 2009). A greater understanding of the shedding characteristics of mastitis-causing pathogens and the influencing factors thereof could contribute to improving the udder health in dairy farming.

The aim of the present study was (1) to investigate the shedding intensity of various mastitis-causing pathogens from mammary quarters with naturally occurring cases of IMI and (2) to evaluate the influence of associated factors under farm conditions in Germany.

2.3 Materials and Methods

2.3.1 Herds and Sample Collection

During the period from May to November 2018, a longitudinal study was conducted in five conventional dairy herds (convenience sample) in northwestern Germany (Table 1). Selection criteria for the study were participation in the monthly German DHI program, the occurrence of SCM, a proper milking system for manual milk sampling (no automatic milking system) and a high level of cooperation from the employees. Based on the latest DHI report, cows with SCC ≥ 200,000 cells/mL were selected for individual quarter foremilk sampling as this value is an internationally used threshold for detecting SCM cows (Schepers et al., 1997). Animals with clinical mastitis or chronic IMI (≥ 700,000 cells/mL (Østerås, 2006)) in the previous three months) were deliberately not included in the study. After foremilking and cleaning by the milker, mammary quarter milk samples were collected twice weekly (modal period between samplings: three days) by the same investigator prior to milking. Mammary quarters with SCC ≥ 200,000 cells/mL or culture positive results in the first three sampling dates were included in the study until the end of the collection period (at least four and up to 17 times per herd). In the herds III and IV, unexpectedly few pathogen-shedding udder quarters were found. Therefore, the periods were kept short and sampling was continued on other farms. Milk samples were collected before evening milking (exception herd I: morning milking) in 10 mL sterile plastic vials (containing boric acid, Ly20), following procedures recommended by the German Veterinary Association (DVG, 2018). For microbiological examination, samples were transported under cooled conditions to the laboratory of the University of Applied Sciences and Arts Hannover, Germany. The sampling periods on the farms were carried out one after the other. A total of 5,736 milk samples from 180 cows were collected. Cows of all parities (1 to 7) and lactation stages were included in the study. To investigate the influence of parity in statistical analysis, cows were divided into two lactation groups (**LGR**): LGR1 (first and second lactation) and LGR2 (> second lactation).

Table 1: Herds and sampling characteristics of five German dairy farms involved in a longitudinal field study on the shedding intensity of mastitis-causing pathogens.

	Herd				
	I	II	III	IV	V
Number of cows	48	125	238	140	376
Sampled cows (No.)	29	51	26	20	54
Sampled cows (%)	60	41	11	14	14
Average milk yield (kg/d) DHI	30.7	30.8	31.3	25.6	27.7
Average SCC (x10 ^{3} cells/mL) DHI	454	253	316	197	218
Milking system	herringbone	herringbone	herringbone	rotary	rotary
Times visited	12	17	6	4	14
Duration in weeks	6	9	3	2	8
Number of milk samples	585	2,128	604	316	2,103

2.3.2 Laboratory Analysis

Cultivation of the quarter milk samples and bacterial identification were carried out in accordance with the guidelines of the German Veterinary Association (DVG, 2018). Ten microliters of each milk sample were mixed with 90 µL Ringer's solution (Merck KGaA, Darmstadt, Germany) and plated on one separate esculin blood agar plate (Oxoid Deutschland GmbH, Wesel, Germany). The agar plates were incubated aerobically at 37 °C and evaluated after 24 and 48 h. In the presence of at least one colony, the samples were considered culture positive. If more than two pathogens were present, the sample was classified as contaminated. In pure and mixed cultures (maximum two different pathogens), the number of colonies was counted from one up to 300 (area of detection: 100 to 30,000 cfu/mL), and the pathogens were differentiated on the basis of the DVG guidelines (2018). Additionally, a clumping factor test (DiaMondiaL, Staph Kit, VIROTECH Diagnostics GmbH, Vienna, Austria) was performed to differentiate *Staph. aureus* from NAS. To distinguish the Lancefield

grouping of esculin-negative streptococci, a streptococcal grouping latex kit (DiaMondiaL, Strep Kit, VIROTECH Diagnostics GmbH, Vienna, Austria) was used. Milk SCC was determined by flow cytometry (SomaScope Smart, Delta Instruments, B.V., Drachten, The Netherlands), and for statistical analysis transformed to the SCS using the formula:

SCS = Log2 (SCC / 100,000) + 3

2.3.3 Meteorological Data

Meteorological data were obtained from the Deutscher Wetterdienst / DWD (German Meteorological Service, Offenbach). The measurements were made at three different weather stations with an average distance to the farm of 11 km (maximum distance 14 km). Mean hourly records of temperature (°C) and relative humidity (%) were used to calculate hourly THI in Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA) based on the following equation (Bohmanova et al., 2007):

$$THI = (1.8 \times T + 32) - (0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)$$

where T is the mean ambient temperature (°C) and RH is the mean relative humidity (%). For statistical analysis, the THI was calculated for the current day as well as one and two days earlier, as studies showed the influence of the previous weather (West et al., 2003). The applied THI formula is appropriate for temperate climate zones (Bohmanova et al., 2007) and has already been used by other authors in Germany (Brügemann et al., 2012). Brügemann et al. (2012) considered a THI of 60 units as a general threshold for the upper point of thermoneutral zone for dairy cows in the German temperate climate. It was therefore calculated for each sampling day and up to two days previously whether the mean THI was less than or equal to 60 or greater than 60 (encoded as 0 or 1). This variable is referred to as THI 60 d-x, where x is the time interval in days.

2.3.4 Statistical Analysis

The collection and processing of data was carried out with Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA) and statistical analysis with SPSS 26.0 (IBM SPSS 25.0.0.0., IBM Inc., Armonk, NY, USA). Pathogen-shedding udder quarters were

each considered as subject. The mastitis pathogens studied were *Staph. aureus,* NAS, yeasts*, Strep. uberis* and *Strep. dysgalactiae.* In cases of mixed infections (two pathogens in one udder quarter), the respective udder quarter was considered as two subjects, one for each pathogen. To approximate the normal distribution, a logarithmic transformation (Log10) of the bacterial numbers was performed. The shedding intensity (in Log10 cfu/mL) was regarded as a dependent variable in all calculations. For the risk factor analyses, a generalized linear mixed model with a Gamma-link was used. The model allowed for gaps in the bacterial contents of consecutive milk samples, as non-evaluable samples may occur (e.g., due to contamination) or the bacteria concentrations may be below the detection level. Preliminary univariable analyses were done to select explanatory variables for the final analysis. Variables with $p \leq 0.1$ were retained for inclusion in the multivariable model. Farm, cow within farm and udder quarter in a cow were included as (nested) random effects. Factors thought to affect the bacterial counts in milk, like the pathogen (**PATH**), SCS, LGR, THI 60 d-x, maximum temperature as well as numerous combinations of these, were tested. The multivariable analysis was performed using a backward stepwise selection and elimination procedure. After each run, the variable with the highest p-value was excluded from the model until all variables had $p \leq 0.05$. The most optimal model was evaluated using the Akaike information criterion (AIC), where an AIC with the lowest value indicated the best model. Confounding was monitored by the change in the coefficient of a variable after removing another variable from the model. If the change of the estimates exceeded 25%, the removed variable was considered a potential confounder and was included again in the model. In the final model, all biologically plausible two-way interactions were tested. Model fit was evaluated by checking normality of the residuals. The random effects were kept as design variables even though they were not significant in the models. Least square means from the model were calculated. A p-value < 0.05 was considered indicative of a statistically significant difference.

2.4 Results

The total number of measured values of bacteria (and yeasts) from shedding udder quarters included in the analysis was 2,133 (*Strep. dysgalactiae,* n=73; yeasts, n=89; *Staph. aureus*, n=201; *Strep. uberis*, n=311; NAS, n=1,459), shed from 703 subjects (means an udder quarter, which sheds a pathogen several times). The maximum number of measurements per subject was 16. In the generalized mixed model, the following factors were found to have a significant ($P < 0.01$) influence on the target variable: SCS, PATH, LGR, THI threshold 60 d0 (Table 2) and their interactions. The examined staphylococci (*Staph. aureus* and NAS) were shed at relatively low concentrations (3.41 vs. 3.55 Log10 cfu/mL), but with significant ($P < 0.01$) differences between them (Table 3). Yeasts were shed at the same counts (3.62 Log10 cfu/mL) as NAS. Significantly ($P < 0.01$) highest were the counts of *Strep. uberis* and *Strep. dysgalactiae* (4.03 vs. 4.04 Log10 cfu/mL), which did not differ between the two species. The mean shedding intensity of the considered pathogens was higher in the first and second lactation than in higher lactations (3.80 vs. 3.65 Log10 cfu/mL). Exceeding the THI threshold 60 led to an increase in pathogen shedding on the same day. Figure 1 presents the influence of THI on the individual species shedding. Yeasts and *Strep. uberis* were shed at higher counts when the THI threshold 60 was exceeded.

Table 2: Final generalized linear mixed model with variables significantly affecting pathogen shedding intensity from udder quarters with IMI.

					95% Confidence interval	
Variable	Coefficient	SE	T-statistics	P-value	Lower limit	Upper limit
SCS	0.012	0.002	6.375	0.001	0.008	0.015
Pathogen						
Strep. dysgalactiae	-0.020	0.033	-0.620	0.536	-0.084	0.044
Yeasts	-0.048	0.024	-2.036	0.042	-0.094	-0.002
NAS	-0.165	0.013	-12.496	0.001	-0.191	-0.139
Staph. aureus	-0.197	0.022	-8.936	0.001	-0.240	-0.153
Strep. uberis	0*					
Paritiy[1]						
LGR1	0.041	0.012	3.414	0.001	0.017	0.064
LGR2	0*					
THI threshold d0						
THI ≤ 60	-0.073	0.023	-3.117	0.002	-0.119	-0.027
THI > 60	0*					

[1] LGR1 = first and second lactation, LGR2 = third and higher lactations

* This coefficient is set at zero because it is redundant.

Table 3: Least square means of the Log-transformed bacterial shedding depending on the pathogen, the parity and the THI threshold 60 of the same day.

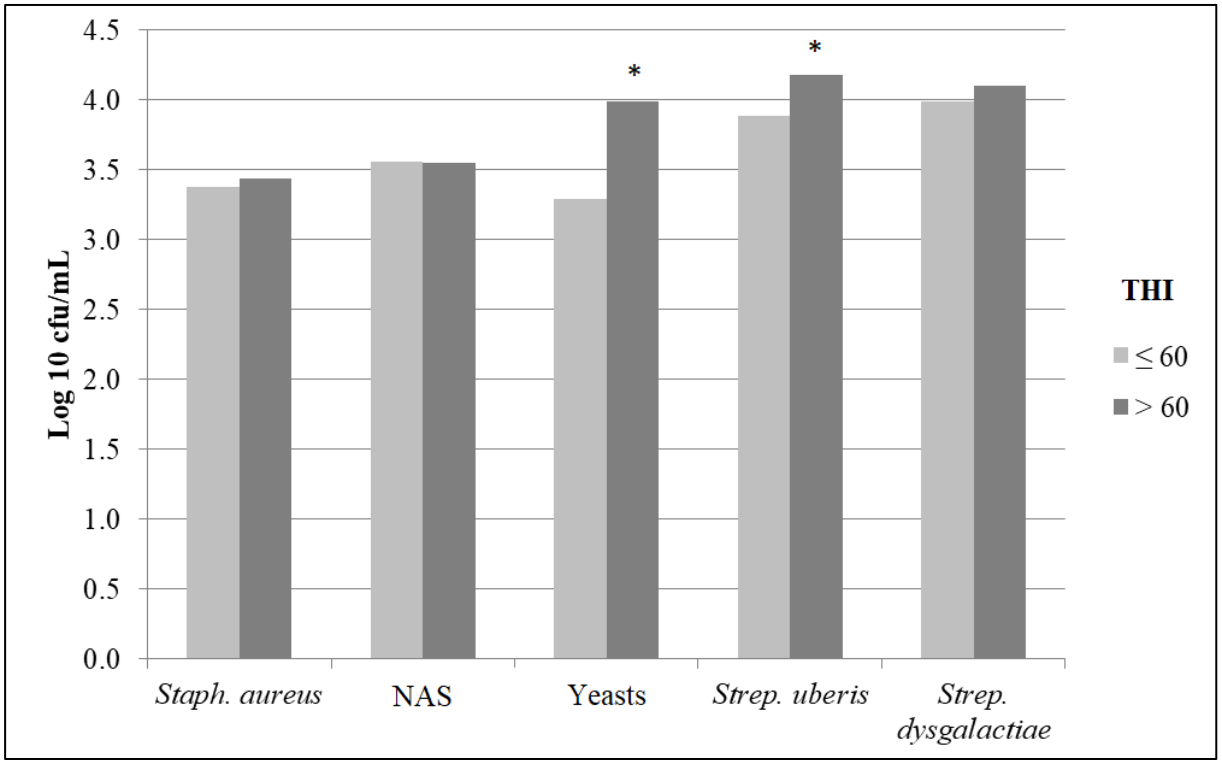
			95% Confidence interval	
	Log10 cfu/mL	SE	Lower limit	Upper limit
Pathogen				
Staph. aureus	3.41[a]	0.11	3.20	3.63
NAS	3.55[bc]	0.11	3.35	3.77
Yeasts	3.62[c]	0.13	3.37	3.90
Strep. uberis	4.03[d]	0.13	3.78	4.30
Strep. dysgalactiae	4.04[d]	0.15	3.76	4.34
Paritiy[1,2]				
LGR1	3.80[a]	0.12	3.57	4.05
LGR2	3.65[b]	0.11	3.44	3.87
THI threshold d0[2]				
THI ≤ 60	3.61[a]	0.12	3.39	3.85
THI > 60	3.84[b]	0.12	3.61	4.08

[a–d] Means within a category with different superscripts differ ($p < 0.05$).

[1] LGR1 = first and second lactation, LGR2 = third and higher lactations

[2] The values for parity and temperature-humidity-index (THI) threshold d 0 contain all pathogens.

Figure 1: Pathogen shedding intensity from infected mammary gland quarters and the influence of THI threshold 60 of the same day. Symbol (*) above bars indicates significant difference ($p < 0.05$).



2.5 Discussion

In the present study, the shedding intensity of five mastitis-causing pathogens from mammary quarters with IMI and the influence of associated factors were investigated under farm herd conditions in Germany. To evaluate this over time, the longitudinal study design was chosen and individual udder quarters were sampled twice a week at relatively short intervals (modal: three days). After the described selection of udder quarters at the beginning of the sampling period, only few udder quarters were eliminated. Thus, the panel mortality had almost no relevance in this longitudinal study. It is critical to note that pathogens with a relatively short duration of infection, such as *E. coli* (Schukken et al. 2011), could nevertheless not be adequately depicted and were therefore not included in the present study. The SCS was positively associated with the shedding intensity of the mastitis-causing pathogens being studied. This indicates that cows with a high somatic cell count endanger the udder health of a herd (White et al., 2006, Barlow et al., 2009), as it can be assumed that a higher shedding intensity also increases the risk of infections of other cows. The shedding intensity differed between the pathogens. The lowest counts of bacteria in milk were found for *Staph. aureus* compared to the other studied pathogens. In a frequently cited study of Sears et al. (1990), a differentiation was made between low and high shedders. Experimentally infected udder quarters with a mean of < 1,000 cfu/mL were considered to be low shedders, and those with a mean of ≥ 2,000 cfu/mL as high shedders (corresponding to 3.0 and 3.3 Log10 cfu/mL, respectively). The *Staph. aureus* infections of the current study would thus be categorized as “high shedders”. It should be noted that these are natural and not experimental infections, and that no clinical signs were found. Walker et al. (2011) found a mean shedding of 242.9 cfu/0.01 mL (corresponding to 4.4 Log10 cfu/mL) from naturally infected udder quarters. In a study by Pellegrino et al. (2010), the shedding from experimentally infected udder quarters was 4.1 Log10 cfu/mL. Investigations by Mørk et al. (2012) indicated that *Staph. aureus* infections with a bacterial shedding intensity of ≥ 500 cfu/mL (corresponding to 2.7 Log10 cfu/mL) are more likely to persist than those with lower shedding intensities. The results overall confirm that *Staph. aureus* is shed at low levels. NAS were shed at a significantly ($P < 0.01$) higher intensity than *Staph. aureus*

in the present investigation. In a study by Wald et al. (2019), the median NAS shedding intensity in clinical and subclinical mastitis was in the range of 3 to 4 Log10 cfu/mL. Although this corresponds in the broader sense to the NAS bacteria shedding estimated in our study (3.55 Log10 cfu/mL), the range indicated is very wide. Furthermore, since NAS are an inhomogeneous group of staphylococci, it is no longer recommended to make statements about them as a group (De Visscher et al., 2015). Recent research has shown that there are differences in the shedding intensity of NAS species (Hamel et al., 2020). In the present study, yeasts were shed at the same level as NAS. In the laboratory analyses, mainly Candida species were found, which were not further differentiated. These are considered to be partly responsible for mastitis, but also occur as contaminants (Williamson and di Menna, 2007). There are no previous studies on the shedding intensity of the group "yeasts" or individual species. Further research is therefore needed in this respect. Significantly ($P < 0.01$) higher shedding intensities were estimated for *Strep. uberis* and *Strep. dysgalactiae*. *Strep. uberis* has become a pathogen of growing interest in recent years, that is considered to be environment-associated. However, persistence in the udder tissue is still possible and a contagious pattern of transmission is also assumed (Krömker et al., 2014). Tassi et al. (2013) demonstrated strain-specific pathogenicity, which was also reflected in the shedding intensity. A putatively host-adapted *Strep. uberis* strain reached a bacterial concentration of 7.67 Log10 cfu/mL 36 hours after the artificial infection. After 96 h, the concentration decreased to 3.49 Log10 cfu/mL and remained at this level until the end of the study (312 h). In contrast, the unadapted strain under investigation only reached a maximum shedding intensity of 2.91 Log10 cfu/mL (24 h post challenge). In the present study, naturally occurring cases of IMI caused by *Strep. uberis* were investigated, and it was found that the average shedding intensity was 4.03 Log10 cfu/mL. This can be compared with the host-adapted strain by Tassi et al. (2013). The shedding of *Strep. dysgalactiae* occurs in similar or even higher values. In a study conducted by Maye et al. (2017), bacterial counts peaked at 8.7 – 9.3 Log10 cfu/mL. It is critical to note that in the current study, the detection range at the upper limit only included bacteria concentrations up to 30,000 cfu/mL (corresponding to 4.48 Log10 cfu/mL). About 52% of the values of *Strep. dysgalactiae*

and 36% of *Strep. uberis* were at the upper limit. Therefore, higher detection limits should be used in further studies to investigate the shedding characteristics of streptococci. Finally, it does not change the central statement that streptococci are shed in higher concentrations than staphylococci, which is in agreement with the statement of Schukken et al. (2011). In further investigations into the shedding characteristics of streptococci, the detection range should be extended.

In the current study, the shedding intensity of mastitis-causing pathogens was higher in the first and second lactation compared to higher parities. This seems contradictory, as cows of higher parities tend to have poorer udder health, which is reflected, for example, in higher levels of somatic cells in milk (Hagnestam-Nielsen et al., 2009). The risk of contracting an IMI and also the lactational incidence risk of second and third cases are higher in multiparous cows (Hertl et al., 2011). Furthermore, the bacteriological cure rate is lower in higher parities (Deluyker et al., 2005). Nevertheless, the question remains why cows in the current study in the first and second lactation shed the considered pathogens in higher concentrations than older cows. A possible justification could be the culling regime of the herds under study. Hertl et al. (2011) showed that both primipara and multipara with clinical mastitis in the first ten months of lactation had a higher risk of being sold. Perhaps cows with mastitis in the higher lactations of the studied herds were more consistently culled than in the first two lactations. Another justification could be a dilution effect. The milk yield increases from lactation to lactation. Assuming that the reproductive intensity of the pathogen is independent of the host's parity, this could result in lower pathogen shedding intensities in older cows with higher milk yields. Furthermore, the immunological status of older cows could be relevant. The immune responses to mastitis pathogens are very species-specific and not fully understood. Petzl et al. (2018) assume that the induction of innate immune mechanisms in the udder by any (mastitis) pathogen leads to a better immune response (cross-protection). Herry et al. (2017) examined the effect of local immunization against *E. coli* and found an overall positive effect by limiting inflammation and accelerating bacterial clearance. In the current study, it can be assumed that multipara cows had already had contact with mastitis-causing pathogens in previous lactations, which did not necessarily lead to elevated SCC or clinical signs,

but stimulated the local immunological processes and finally led to the lower shedding intensities of pathogens from udder quarters with IMI at the time of our investigations. Indeed, causal factors leading to higher pathogen shedding from cows in first and second lactation remain speculative and require further research.

It has long been known that heat stress adversely affects dairy cows (Kadzere et al., 2002, West et al., 2003, Nasr and El-Tarabany, 2017). In the temperate climate in Germany, cows are affected by heat stress in the warmest months of the year (Gorniak et al., 2014). THI takes into account ambient temperature and relative humidity and is often-used to investigate the effect of heat stress. The choice of the appropriate THI depends primarily on the climate zone (Bohmanova et al., 2007). The THI used in the current study is suitable for the humid climate. Brügemann et al. (2012) viewed the threshold value of THI=60 as the upper point of the thermoneutral zone for dairy cows in Germany. This threshold may appear low. It should be noted that the THI values with the formula applied in the temperate climate in Germany cannot be compared with those in hotter climates, which are often considerably higher. In the current study, significant ($P < 0.01$) effects on shedding intensity were found at this threshold value. The increased bacterial count in repeated detection of a pathogen from one udder quarter led to this result. It should be critically noted that the repeated detection of a pathogen could also be caused by a new infection with the same pathogen or with another strain of it (Wente et al. 2020). However, the sampling intervals in this longitudinal study were relatively short (modal: three days), which should minimize any adverse effects in this context. The difference in the shedding intensity when THI rises above 60 was finally only caused by the pathogens yeasts and *Strep. uberis*. Little is known about the direct influence of heat stress on mastitis and the mechanisms behind it. One reason for the higher shedding intensity of these mastitis-causing pathogens could be a concentration of the pathogens at lower milk yield due to heat stress. Despite the fact that the milk yield was not measured in the current study, it can be assumed that high THI led to a lower milk production as is frequently described (West et al., 2003, Gorniak et al., 2014, Zeinhom et al., 2016, Nasr and El-Tarabany, 2017). Assuming a constant growth rate of the pathogens, a concentration of the pathogens in milk could have been caused by a lower milk yield alone. However, what speaks

against this assumption of concentration is that the THI of the previous days had no influence on the pathogen shedding. In a study by West et al. (2003), the THI two days earlier had the greatest impact on milk yield. Another reason for higher pathogen counts could be that the pathogens have better conditions for reproduction in the mammary gland, due to, for example, higher milk temperatures, which have been shown to be caused by high ambient temperatures (West et al., 2003). A further reason for the higher shedding intensity of pathogens at high THI could be an inadequate immune function. It is known that the mammary gland function is influenced by heat stress. The mechanisms behind this are manifold, with systemic reactions of the immune system as well as affected local immune functions in the mammary gland (Tao et al., 2018). Finally, the question arises why yeasts and *Strep. uberis* were shed at higher intensities when the THI threshold 60 was exceeded, but the other pathogens investigated were not. For *Strep. dysgalactiae*, it should again be noted that the shedding intensity was largely above the detection range. Consequently, the effect of the THI could therefore not be established and further investigations should be carried out. *Staph. aureus* is regarded as a mastitis pathogen well adapted to the mammary gland, partly because it has developed means to evade immune system recognition (Schukken et al., 2011, Keane, 2019). The situation is probably similar for NAS, which show some similarities to *Staph. aureus* (Taponen and Pyorala, 2009). It is therefore possible that the THI had no influence on their shedding intensity in our investigations. Finally, this study confirms that udder health in the temperate German climate is influenced by heat stress. Consequently, preventive mitigating measures should be taken.

Seasonal effects on the incidence rate of clinical mastitis caused by different pathogens have been reported. For example, incidence rate of clinical mastitis for *Strep. uberis* was highest in summer (August, Northern Hemisphere), while other pathogens, like *Staph. aureus, E. coli* and *Strep. dysgalactiae,* more likely appear in winter (December and January) (Olde Riekerink et al., 2007). However, the findings are not uniform. Zeinhom et al. (2016) reported a higher isolation rate for *Staph. aureus* and *E. coli* at high THI levels. It can be assumed that pathogen shedding influences the detection of certain mastitis pathogens. Thus, it is plausible that existing cases of

IMI only become easier to detect through heat stress, as the pathogen shedding increases and is therefore easier to identify in the microbiological examination. Furthermore, it can be assumed that clinical mastitis may become more frequent due to the exacerbation of subclinical cases that progress to a clinical state.

Overall, stress appears to have a considerable effect on the shedding intensity of mastitis-causing pathogens, which was demonstrated in the present study for heat stress. Different theories exist in the literature regarding the mechanisms by which stress causes an inadequate immune response. Elvinger et al. (1992) observed a reduced migration of leukocytes to the mammary gland due to heat stress as well as increased plasma cortisol concentrations. It has been demonstrated that the experimental injection of glucocorticoids (cortisol or dexamethasone) can increase the shedding intensity of *Staph. aureus* (Burton and Kehrli, 1995). It is therefore assumed that the host's response to a mastitis-causing pathogen is influenced by several stressors that lead to an increase in plasma cortisol concentration, which can influence the bacteria shedding intensity. Based on these stress mechanisms and the influence of THI shown in this study, we assume that stress events can exacerbate existing IMI. Further studies should investigate the influence of possible stress-causing events in the environment of dairy cows under more controlled conditions.

2.6 Conclusions

The shedding intensity of mastitis-causing pathogens differs. Staphylococci are shed at lower levels than streptococci. Higher pathogen counts occur with a higher somatic cell content, lower parity and higher THI. This underlines that cows with high somatic cell counts are a source of mastitis pathogens in a herd. The higher shedding intensities in the first two lactations compared to higher parities could not be conclusively clarified. This study confirms that dairy cows in the German temperate climate are affected by heat stress and that this is also specifically detrimental to udder health. Even if the causes cannot be conclusively explained, it can be assumed that the prevention and mitigation of heat stress are beneficial to udder health.

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3 Manuskript 2

Non-*S. aureus* staphylococci (NAS) in milk samples: Infection or contamination?

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Highlights

Whether NAS are contaminants or cause IMI is often unclear.

NAS species occur unnoticed as contaminants in milk samples.

S. simulans, *S. chromogenes* and *S. epidermidis* are less common contaminants.

NAS species more important for IMI appear to be shed in higher concentrations.

3.1 Abstract

Non-*S. aureus* staphylococci (NAS) are the most frequently isolated pathogens from bovine milk and can cause intramammary infections (IMI). They can also be found in teat canals, on bovine skin and in cows' environment, which may lead to unnoticed contamination of milk samples. The aim of this study was to investigate the role of NAS species as mastitis-causing pathogens or contaminants, and to identify possible differences between NAS species. A longitudinal study was conducted with consecutive milk sampling in five German dairy herds. Species identification was performed using matrix-assisted laser desorption ionization-time of flight mass spectrometry. Infections were distinguished from contaminations using two different definitions based on the repeated detection of an NAS species. Of 15 NAS species found, eight and ten, respectively, were associated with an IMI. *Staphylococcus simulans* and *S. chromogenes* were associated with IMI in more than 90 % of the findings. *S. warneri, S. xylosus, S. microti, S. haemolyticus* and *S. succinus* seem to be frequent causes of IMI as well as contaminants. If a species-differentiation is available after cultivating NAS, the findings should be interpreted in consideration of the observations made in this study, whether it is more likely a question of a contaminant or a cause of intramammary infection. The bacteria shedding intensity of the NAS species with a more substantially adverse effect on udder health seems to be higher than that of the less important NAS pathogens.

Keywords: Intramammary infection, Non-*S. aureus* staphylococci, Bacteria counts, Contamination, Milk samples

3.2 Introduction

Mastitis is one of the most common diseases in dairy cows and adversely affects the economy of dairy farming, mainly through milk losses (Heikkilä et al., 2018). Non-*S. aureus* staphylococci (NAS) are the most frequently isolated pathogens from bovine milk (Pitkälä et al., 2004; Tenhagen et al., 2006) as well as from subclinical mastitis (Sampimon et al., 2009a) in many countries. The epidemiology and role of NAS as mastitis pathogens are not fully understood. The intramammary infections (IMI) caused by a species of this group of bacteria usually remain subclinical (Taponen et al., 2006) and can persist for months (Gillespie et al., 2009; Mørk et al., 2012). Several studies have revealed that NAS may increase milk yield (Piepers et al., 2008; Schukken et al., 2009) or not cause milk losses (Hertl et al., 2014). Other studies described adverse effects like lower milk production (Heikkilä et al., 2018) and elevated somatic cell counts (Fry et al., 2014; Supré et al., 2011). Due to the fact that there are species-specific differences between NAS which affect udder health and epidemiology, it is no longer recommended to study them as a group (De Visscher et al., 2015). Indeed, some research was conducted at species-level. *S. chromogenes, S. simulans, S. epidermidis, S. haemolyticus* and *S. xylosus* are commonly isolated bacteria from IMI (Mørk et al., 2012; Supré et al., 2011). In addition, NAS species are not only isolated from milk samples, NAS are also colonizing the teat canal (Traversari et al., 2019). De Visscher et al. (2014) isolated at least one NAS species from almost 60 % of teat apex samples and 68 % of the milker's skin or gloves. At other body sites of cattle, such as the inguinal skin, muzzle or perineum from heifers, isolation proves easy (Adkins et al., 2018). Furthermore, NAS species can be abundantly isolated from cows' environment, for example from the stall air, slatted floor or fresh and used litter (Piessens et al., 2011). Some species seem to occur only in the udder or the environment, others can be detected in both environmental and milk samples. It is therefore reasonable to assume that there is a risk of milk samples being contaminated by NAS that do not cause an intramammary infection. This leads to the hypothesis that contaminants are often unrecognized, often resulting in a false diagnosis of IMI. The occurrence of NAS as contaminants is probably underestimated. Especially in cases of single milk samples, that are usually taken in agricultural and veterinary practice within the scope

of udder health management, false positive interpretations of IMI due to contamination of milk samples are common. Many scientific studies based the NAS infection status of udder quarters on single quarter milk samples without species differentiation. Repeated sampling increases the reliability of the diagnosis. Three consecutive samples were regarded as the gold standard for evaluating the infection status of udder quarters (Andersen et al., 2010). With regard to the NAS, the diagnosis is actually only reliable if species differentiation is carried out. The differentiation methods initially used for NAS were phenotypic commercial kits with relatively limited typability and accuracy (Sampimon et al., 2009b). Through the development and improvement of molecular biological methods, a more reliable differentiation has become possible (Vanderhaeghen et al., 2015). Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry analysis enables the identification of NAS isolates at species level with high accuracy (Cameron et al., 2017).

The objective of the present study was to investigate the role of NAS species as mastitis-causing pathogens or contaminants in five dairy herds. Repeated detection of an NAS species was used to distinguish contamination from infection. Furthermore, the study analyzed which species occur more frequently as infectious agents and which as contaminants. The intensity of pathogen excretion of the NAS species and the influence on the somatic cell count were investigated.

3.3 Materials and methods

3.3.1 Study design

The design of the study consisted of repeated sampling of milk from NAS-positive udder quarters. Isolates from NAS-positive milk samples were stored. After the collection period, udder quarters with at least three NAS findings were considered "presumably NAS positive". The associated isolates were selected for species differentiation, which was performed using MALDI-TOF mass spectrometry. Finally, the infection status was assessed by applying two different definitions as described below. Repeated detection of an NAS species was used to distinguish between infection of udder quarters and contamination of milk samples.

3.3.2 Herds and sample collection

A longitudinal study was conducted from May to November 2018 on five conventional dairy farms in northwestern Germany in succession (Table 1). Herd size varied from 48 to 376 cows, which were housed in free-stall barns equipped with a lying area with straw (herd I) or cubicles (herds II-IV). Cows of all parities and lactation stages were involved. Three lactation groups (LGR) were defined for statistical analysis (LGR1 = first lactation, LGR2 = second lactation, LGR3 ≥ third lactation). Based on the monthly results of the German Dairy Herd Improvement (DHI) program investigations, cows with composite somatic cell counts (SCC) ≥ 200,000 cells/mL were selected for individual mammary quarter foremilk sample collection. Not included were cows with clinical mastitis or chronic IMI (≥ 700,000 cells/mL in the last three months). Quarter milk samples were collected prior to milking aseptically twice weekly by the same investigator (modal period between samplings: three days), based on the procedures recommended by the German Veterinary Association (DVG, 2018). After foremilking and pre-cleaning by the farmer or milker, the teats and teat ends were each disinfected with a disposable cotton wipe soaked in 70 % methylated alcohol and the milk sample was collected. After three samplings, udder quarters with SCC < 200,000 cells/mL or no detection of mastitis-causing pathogens were excluded. The remaining quarters were sampled until the end of the collection period. Cows were excluded from sampling immediately after antibiotic treatment, drying or culling. Samples were transported under cooled conditions to the laboratory of the University of Applied Sciences and Arts Hannover, Germany.

Table 1: Herds and sampling characteristics

	Herd				
	I	II	III	IV	V
Number of cows	48	125	238	140	376
Sampled cows (No.)	29	51	26	20	54
Sampled cows (%)	60	41	11	14	14
Ø milk yield (kg/d) DHI	30.7	30.8	31.3	25.6	27.7
Ø SCC ($x10^{-3}$ cells/ml) DHI	454	253	316	197	218
Milking system	herringbone	herringbone	herringbone	rotary	rotary
Times visited	12	17	6	4	14
Duration in weeks	6	9	3	2	8
Number of milk samples	585	2,128	604	316	2,103

DHI = Dairy Herd Improvement

SCC = somatic cell count

3.3.3 SCC and bacterial identification

Quarter milk SCC measurement was performed by flow cytometry (SomaScope Smart, Delta instruments, Drachten, The Netherlands). The bacteriological culturing was carried out in accordance with the Guidelines of the German Veterinary Association (DVG, 2018) which are similar to the National Mastitis Council Recommendations (NMC, 1999). Milk samples (inoculum volume of 10 µL) were streaked onto sheep blood agar (Oxoid Inc., Wesel, Germany) containing 0.1 % esculin. Agar plates were incubated aerobically at 37 °C and bacterial growth was examined after 24 and 48 hours. Samples were considered culture positive in the presence of at least one colony (i.e. 100 cfu/mL), according to Dohoo et al. (2011). The number of colonies was counted to a maximum of 300 for the quantification of pathogen shedding. In the presence of more than 300 colonies, this value was taken as the upper limit. Preliminary identification was done by colony morphology features, hemolytic characteristics, gram-staining and catalase reaction. NAS were differentiated according to the following rules: gram-positive cocci, catalase-positive, ß-hemolysis

negative. In cases of positive ß-hemolysis, a commercial test kit (Staph Plus Latex kit, DiaMondiaL, Vienna, Austria) was used to examine the clumping factor. If the clumping factor was negative, the colony was also considered as NAS. In the presence of two pathogens, the culture was classified as mixed growth. When more than two pathogens grew, it was considered as contaminated. Isolates from pure NAS cultures or from samples of mixed identification (except cases with two NAS species) were stored at -80 °C for further investigation.

3.3.4 MALDI-TOF mass spectrometry

After the sampling periods, udder quarters showing at least three NAS-positive findings (considered as "presumably NAS positive") were selected. The related isolates were streaked on sheep blood agar (Oxoid Inc., Wesel, Germany), containing 0.1 % esculin, and incubated at 37 °C for up to 24 hours. Identification at species level was performed with MALDI-TOF using an MTB smart device (Bruker Daltonics Inc., Bremen Germany). Colony material was placed on a target field and allowed to dry. The formic acid extraction method was performed by adding 1 µL of ≥ 98 % formic acid (Karl Roth Inc., Karlsruhe, Germany). After drying, 1 µL of α-cyano-4-hydroxyciannamic acid (HCCA)-matrix (Bruker Daltonics Inc., Bremen, Germany) was added. Again, after drying, the classifications were carried out using the MBT Compass Library (Version 8, ECCMID 2018) containing 7,854 MSPs (reference spectra), covering 2,748 species. The manufacturer's cut-off for the species-level identification of 2.0 was reduced to ≥ 1.7 according to Cameron et al. (2017). A score of < 1.7 was considered to be no reliable identification. In cases where identification was not possible, a new cultivation of the isolate was performed and an analysis with MALDI-TOF was performed up to two times.

3.3.5 Definitions of NAS IMI

Udder quarters with at least three NAS findings during the sampling period were considered as "presumably NAS positive". Before applying the definitions for IMI, it was first verified whether they could lead to a clear result (infection or contamination). Several udder quarters were excluded because crucial NAS findings could not be identified by MALDI-TOF or because the time intervals between species detection

were too long. The following two definitions were used to assess the infection status: in definition 1 (DEF1), a mammary quarter was defined as having an IMI if a given NAS species was identified in at least three samples. The maximum allowed interval between findings was three farm visits (about two weeks apart). Definition 2 (DEF2) considered an udder quarter infected if the same species was cultured from at least two of three consecutive samples, which is similar to the approach used by Andersen et al. (2010). Additional findings of the same species during the sampling period were considered associated with the infection up to an interval of two sampling dates (about 1.5 weeks). For both definitions, the NAS findings associated with an IMI were marked for further evaluation (1 = NAS finding is related to IMI; 2 = no association to IMI). Overlapping of infections over time was possible when species alternated with one another. Repeated infection with the same or a different NAS species in a trial period was also possible.

3.3.6 Descriptive and statistical analysis

Processing of the data and evaluations for infection status were carried out with Microsoft excel (Microsoft Corp., 2010). Statistical analyses of bacterial counts and SCC from infected quarters were performed with SPSS 25.0 (IBM SPSS 25.0.0.0., Armonk, USA). Logarithmic transformations of cfu (log10) and SCC were applied to approximate normal distribution. A linear mixed model was used to investigate the bacteria shedding with log10 cfu/mL as outcome variable:

$$y = \mu + h(i) + l(j) + p(k) + s(l) + lp(jk) + ls(jl) + sp\,(lk) + lps\,(jkl) + e\,(ijkl)$$

where μ is the overall mean, h is the random effect of the herd (i=1-5), l is the fixed effect of lactation group (j=1-3), p is the fixed effect of the NAS species, s is the fixed effect of SCC (log) and e is the residual error. A mammary quarter was the unit of analysis and the sampling dates were considered as repeated measurements. Finally, the least square means were estimated for the NAS species associated with IMI according to DEF1 using SCC as covariates. A p-value < 0.05 was considered indicative of a statistically significant difference in pairwise comparisons.

Moreover, the possible effect of the NAS species on the SCC was investigated using a mixed model:

$$y = \mu + p(i) + q(j) + r(k) + pq(ij) + pr(ik) + qr(jk) + pqr(ijk) + e\ (ijk)$$

where y is the outcome variable Log10 SCC, μ is the overall mean, p is the fixed effect of NAS species, q is the fixed effect of DEF1 (j = 1 or 2) and r is the fixed effect of DEF2 (k = 1 or 2), and e is the residual error.

3.4 Results

3.4.1 Descriptive results

The number of cows per herd selected for individual mammary quarter sampling varied from 20 to 54. There were at least four to a maximum of 17 sampling dates per herd in a period of two to nine weeks (Table 1). Due to at least three NAS findings during the sampling period, a total of 178 udder quarters were classified as “presumably NAS positive”.

3.4.2 MALDI-TOF analysis

The 178 presumably NAS positive udder quarters included 853 isolates, of which 843 were identified as NAS at species level (98.8 %). Nine isolates were not further considered as they contained microorganisms other than NAS (*Rhodococcus fascians*, n=3; *Pantoea agglomerans*, n=3; *Candida krusei*, n=1; *Citrobacter koseri*, n=1; *Kocuria carniphia*, n=1, respectively). A total of 15 NAS species were differentiated, ranging from two to 11 species per herd (Table 2). *S. haemolyticus, S. microti* and *S. chromogenes* were the most frequently analyzed NAS species, accounting for more than two thirds of the samples (68.4 %). *S. haemolyticus* was the most common NAS species (36 % of all isolates), but was not detected in herds I and IV. *S. microti* was detected most frequently in herd V and only a few times in Herd III. The only species isolated in each of the five herds was *S. chromogenes* (12.4 % of all isolates).

Table 2: Species distribution of NAS isolates from quarter milk samples of the 178 presumably NAS positive udder quarters

NAS species	No. of Isolates (percent)					
	All herds	I	II	III	IV	V
S. haemolyticus	300 (36.0)		109 (39.4)	22 (27.9)		169 (42.7)
S. microti	167 (20.0)			7 (8.9)		160 (40.4)
S. chromogenes	103 (12.4)	11 (39.3)	45 (16.3)	10 (12.7)	11 (20.4)	26 (6.6)
S. succinus	66 (7.9)	3 (10.7)	54 (19.5)	1 (1.3)		8 (2.0)
S. epidermidis	51 (6.1)		1 (0.4)	7 (8.9)	43 (79.6)	
S. xylosus	41 (4.9)		40 (14.4)			1 (0.3)
S. warneri	33 (4.0)			32 (40.5)		1 (0.3)
S. simulans	30 (3.6)	13 (46.4)				17 (4.3)
S. sciuri	22 (2.6)	1 (3.6)	18 (6.5)			3 (0.8)
S. gallinarum	8 (1.0)		6 (2.2)			2 (0.5)
S. vitulinus	7 (0.8)					7 (1.8)
S. equorum	2 (0.2)					2 (0.5)
S. auricularis	2 (0.2)		2 (0.7)			
S. cohnii	1 (0.1)		1 (0.4)			
S. hominis	1 (0.1)		1 (0.4)			
Total	**834 (100)**	**28 (100)**	**277 (100)**	**79 (100)**	**54 (100)**	**396 (100)**
No. of species	15	4	10	6	2	11

3.4.3 NAS IMI

DEF1 could be applied to 144 and DEF2 to 163 of the 178 initially selected presumably NAS positive udder quarters (Table 3). The 144 quarters that fulfilled the conditions for DEF1 could also meet those of DEF2. DEF2 was applicable to 19 further udder quarters. The proportion of udder quarters affected by intramammary infections differed between the two definitions (Table 4). According to DEF1, 110 of 144 were infected, with one quarter having two different NAS species. In the remaining 34 quarters (23.6 %), no IMI could be confirmed, which is why the NAS findings could be considered as contamination. DEF2 on the other hand lead to the result that 90.8 % of

the presumably NAS positive mammary quarters were infected at least once during the sampling period. No infection was found in 15 of the 163 udder quarters (9.2 %) considered contaminated in this definition. According to both definitions, the NAS *S. haemolyticus, S. microti, S. chromogenes* and *S. epidermidis* were the causative pathogens for approximately 85 % of IMI (data not shown).

Table 3: Applicability of the definitions to the presumably positive udder quarters

	Mammary quarters (No.)	Isolates (No.)
Presumably NAS pos.	178	834
DEF1 applicable	144	745
DEF1 not applicable	34	89
DEF2 applicable	163	804
DEF2 not applicable	15	30

Table 4: NAS infection and contamination status of udder quarters according to DEF1 and DEF2 (No. of quarters, %)

	DEF1	DEF2
1 NAS infection	109 (75.7)	125 (76.7)
2 NAS infections	1 (0.7)	22 (13.5)
3 NAS infections		1 (0.6)
No association to IMI	34 (23.6)	15 (9.2)
Total[a]	144	163

[a] Number of udder quarters to which the respective definition was applicable from the 178 presumably NAS positive udder quarters

3.4.4 Classification of NAS findings in infection or contamination

According to DEF1, eight of the 15 NAS species identified by MALDI-TOF were associated with IMI. According to DEF2, there were ten NAS species (Table 5). Both definitions lead to the results that in more than 90 % of the findings of *S. simulans* and *S. chromogenes*, an IMI was present; according to DEF2, also *S. epidermidis*. Associations with IMI could rarely be established (in less than 37 % of the isolates) for

the pathogens *S. succinus* according to DEF1 as well as for *S. gallinarum* and *S. sciuri* (both only in DEF2). The NAS species *S. cohnii, S. vitulinus, S. equorum, S. auricularis* and *S. hominis* were never associated with infections in this study.

Table 5: Findings of NAS species in association with intramammary infections according to DEF1 and DEF2

	DEF1			DEF2		
NAS species	Isolates Total (No.)[a]	Isolates infection (No.)	Isolates infection (%)	Isolates Total (No.)[a]	Isolates infection (No.)	Isolates infection (%)
S. simulans	30	30	100.0	30	30	100.0
S. chromogenes	99	93	93.9	103	97	94.2
S. epidermidis	47	40	85.1	51	49	96.1
S. warneri	32	25	78.1	32	27	84.4
S. xylosus	35	27	77.1	38	28	73.7
S. microti	164	122	74.4	167	150	89.8
S. haemolyticus	263	189	71.9	289	221	76.5
S. succinus	46	7	15.2	58	21	36.2
S. sciuri	13			17	2	11.8
S. gallinarum	5			6	2	33.3
S. vitulinus	6			7		
S. equorum	2			2		
S. auricularis	2			2		
S. hominis	1			1		
S. cohnii				1		
Total	745	533	71.5	804	627	78.0

[a] Number of isolates belonging to the 144 (DEF1) or 163 (DEF2) udder quarters to which the definitions were applicable

3.4.5 Milk bacterial counts and SCC of NAS species

There were significant differences in the shedding intensity of the NAS species. The Log 10 bacterial counts of the pathogens that caused infection according to the stricter definition (DEF1) are shown in Table 6. *S. succinus, S. haemolyticus* and *S. microti* were excreted in significantly lower concentrations than *S. simulans*, *S. chromogenes* and *S. epidermidis*. Parity (LGR) had no significant effect on NAS bacterial counts.

Table 6: Significance of differences between the least square means of Log10 bacterial counts for NAS findings in association with IMI according to DEF1

NAS species	Isolates infection (No.)	LSM	SE	CI
S. succinus	7	3.28 [a]	0.21	2.87 – 3.69
S. warneri	25	3.39 [*]	0.57	2.27 – 4.51
S. haemolyticus	189	3.50 [a]	0.12	3.25 – 3.75
S. microti	122	3.67 [a]	0.12	3.42 – 3.92
S. xylosus	27	3.75 [*]	0.29	3.17 – 4.32
S. simulans	30	4.03 [b]	0.11	3.81 – 4.26
S. chromogenes	93	4.07 [b]	0.08	3.89 – 4.25
S. epidermidis	40	4.17 [b]	0.12	3.92 – 4.43

LSM = least square means, SE = standard error, CI = confidence interval (95 %)

[*] = No differences to any other Staph. spp.

a, b = different letters between rows indicate a significant difference ($p < 0.05$)

The least square means of the Log10 SCC of the NAS species found in at least 30 isolates are presented in Table 7. The highest Log10 SCC were calculated for *S. chromogenes, S. xylosus* and *S. microti*, the lowest for *S. warneri* and *S. succinus*. There were no significant differences between the NAS species. Furthermore, no differences were identified between the findings, classified as infection or contamination according to DEF1 or DEF2.

Table 7: Least square means of mammary quarter Log10 SCC

NAS species	Isolates (No.)	LSM	SE	CI
S. warneri	32	5.08	0.16	4.78 - 5.39
S. succinus	46	5.30	0.10	5.11 - 5.49
S. epidermidis	47	5.30	0.25	4.81 - 5.79
S. haemolyticus	263	5.31	0.06	5.18 - 5.43
S. simulans	30	5.40	0.10	5.20 - 5.60
S. microti	164	5.46	0.08	5.29 - 5.63
S. xylosus	35	5.47	0.14	5.18 - 5.77
S. chromogenes	99	5.54	0.19	5.17 - 5.91

LSM = least square means, SE = standard error, CI = confidence interval (95 %)

3.5 Discussion

In this longitudinal field study, consecutive milk samples were collected from five conventional dairy herds. In order to maximize the sensitivity, all bacteriological cultures were considered positive when ≥ 1 colony was detected (equivalent to 100 cfu/mL), in accordance with the study of Dohoo et al. (2011). This relatively low threshold seemed appropriate due to the small intervals between samplings (modal: three days) and the relatively high frequency of samplings, which are rarely found in other studies. The definitions used to assess the infection status of udder quarters differed in frequency of detection and time period. DEF1 was stricter because an NAS species had to be detected at least three times, whereby a maximal interval of three farm visits for sampling (the equivalent of two weeks) was allowed. The advantage here is that infections are taken into account despite gaps in consecutive detection. For example, missing samples or those with bacterial content below the detection limit do not lead to an inappropriate classification of the infected udder quarters as contamination due to the fact that the infection is not confirmed. It is therefore suitable for NAS species, which can cause persistent infections such as *S. chromogenes, S. simulans, S epidermidis, S. haemolyticus* or *S. warneri* (Mørk et al., 2012). However, infections lasting only a few days may not be taken into account. DEF2 is applied to take account of shorter infections. Furthermore, less frequently occurring findings of NAS species because of shorter sampling periods (e.g., herds III and herd IV) should not be underestimated. However, in cases of repeated contamination of milk samples, which may not have been considered as contamination, false positive results of IMI may occur. There is still no standard definition for NAS infections, only generally for IMI, where no differentiation between NAS species was made (Andersen et al., 2010). In investigations by Dohoo et al. (2011), the sensitivity of a single sample for detecting NAS was 91 % and the specificity thereof 87 % (≥ 1 cfu/mL, mixed growth allowing no minimum SCC). For comparison, a gold standard (three samples taken weekly, at least two of them positive) was used. The relatively low specificity in NAS (compared to 99.8 % for *S. aureus*) could possibly be explained by the fact that no NAS differentiation was performed and a whole group of staphylococci was behind the finding "NAS" (possibly also unnoticed contaminations). Whether the so-called gold

standard with regard to NAS is suitable for the investigations must therefore be critically questioned. Which of the definitions of this study is more applicable depends on the specific question. DEF2 is probably more practicable, since even the simple repeated detection of an NAS species indicates an IMI.

By using MALDI-TOF mass spectrometry, fifteen NAS species were differentiated, two to 11 per herd. This is comparable to other studies, for example Piessens et al. (2011), who isolated 13 NAS species from six dairy herds, with five to seven different species per herd. The pathogens *S. haemolyticus, S. chromogenes, S. epidermidis* and *S. xylosus* belong to the six most frequently detected NAS in this study, which is in agreement with other investigations (Condas et al., 2017; Nyman et al., 2018; Piessens et al., 2011). *S. simulans* was rarely found in the isolates in our study (3.6 %), although it accounted for a larger percentage in other investigations (Nyman et al., 2018). Nevertheless, other European studies also found similar small percentages for *S. simulans* (De Visscher et al., 2016; Dolder et al., 2017; Supré et al., 2011). The herds differ considerably in their pathogen patterns. Herd-specific patterns of NAS have already been frequently described (Dolder et al., 2017; Mahmmod et al., 2018; Supré et al., 2011). However, it should be noted that comparability with other studies is limited in this respect due to the relatively small number of herds (n=5) in which NAS-shedding quarters in particular were repeatedly sampled in unequal numbers of samples per mammary quarter.

S. simulans and *S. chromogenes* have been shown to adversely affect udder health (Supré et al., 2011; Vanderhaeghen et al., 2014). We can confirm their ability to colonize the mammary gland, as these pathogens were associated with IMI in more than 90 % of our findings according to both applied definitions. These two NAS species can also cause persistent IMI (Mørk et al., 2012; Piessens et al., 2011), which may explain the reliable consecutive detection over the sampling intervals in the present study. These species seem to be well adapted to the udder, allowing successful colonization and proliferation in the mammary gland. This is in agreement with Piessens et al. (2011), who classified *S. chromogenes* and *S. epidermidis* as more host-adapted. The present study found *S. epidermidis* associated with IMI (according to DEF2) in 96.1 % of the findings. The current results, however, contradict those of

Piessens et al. (2011) that the environment should be the reservoir of *S. simulans*. We did not examine samples from the environment but NAS findings were instead classified as environmental contamination if there was no association with an IMI. Even though only 30 *S. simulans* isolates were found in the study, it is remarkable that all of them were associated with an IMI, which confirms the relevance of this NAS species for IMI. This should be verified in further investigations with more *S. simulans*-positive milk samples. NAS species with a greater impact on udder health have been rarely found in the environment (parlor-associated niches), according to De Visscher et al. (2014). This is confirmed indirectly in this study, since *S. simulans, S. chromogenes* and *S. epidermidis* were not or were rarely classified as contaminants.

In the current study, *S. microti* was found to be the cause of subclinical IMI in 74.4 % and 89.8 % of the findings, respectively. *S. microti* was first described by Nováková et al. (2010) when it was isolated from organs of common voles (*Microtus arvalis*). In 2016, the first case report on mastitis with the pathogen was published (Król et al., 2016). The authors suspected a host-adapted nature. However, little is known about the characteristics thereof as a mastitis-causing pathogen. According to DEF2, *S. microti* was more often classified as responsible for an IMI, suggesting a shorter duration of infection. Nevertheless, since not all findings could be assigned to IMI, the pathogen also appears to occur in the environment and contaminate milk samples unnoticed. Further research should be conducted on the characteristics of *S. microti*.

The most frequently found NAS species *S. haemolyticus* was the cause of IMI in 71.9 % and 76.5 % of the findings, respectively (DEF1 vs. DEF2). Consequently, almost 30 % of the findings were contaminations of milk samples which are not classified as such in the microbiological examination as there were no more than two different types of colonies on the agar plate. A study by Piessens et al. (2011) showed that the environment is a possible reservoir of *S. haemolyticus*. In addition, it was detected in about 40 % of stall air isolates. This is in line with our assumption that the presence of *S. haemolyticus* as a contaminant is probably common. An odds ratio (OR) of 3.3 for an IMI with *S. haemolyticus* was analyzed in the study by Dolder et al. (2017) by coinfection with “other NAS”, which included *S. equorum*, *S. auricularis, S. sciuri, S. hominis* and *S. vitulinus*. The definition of an IMI was based on single milk samples.

Similarly, another study showed that the risk of NAS infections after parturition with the species *S. cohnii, S. equorum, S. saprophyticus* or *S. sciuri* was higher (OR=6.4) if the teat apices were dirty before calving, the findings also based on single milk samples (De Visscher et al., 2016). In the current study, the NAS species mentioned were not or were rarely the cause of IMI, and were found only sporadically in samples from various animals. From our point of view, the simultaneous occurrence of several NAS species is more likely to be a sign of contamination of milk samples than that of a coinfection. NAS species from the teat canal can also cause contamination of milk samples. In investigations carried out by Traversari et al. (2019), *S. equorum*, *S. vitulinus* and *S. succinus* were found as colonizers of the teat canal. The detection of this NAS species in milk samples is presumably possible even in the absence of IMI. Therefore, previous studies should be interpreted with caution, especially when diagnoses are based on single milk samples.

Bacterial concentrations in milk are species-specific. Staphylococci are generally excreted in lower concentrations than for example *Streptococcus uberis* or *Escherichia coli* (Schukken et al., 2011). According to Wald et al. (2019), the number of colony-forming units in subclinical and clinical NAS infections is 10^3-10^4 /mL (3-4 Log 10 cfu/mL). In the present study, significantly higher milk bacterial counts were found for *S. simulans*, *S. chromogenes* and *S. epidermidis*, which are generally regarded as being more relevant for udder health (Vanderhaeghen et al., 2015). The findings of the three pathogens were furthermore most frequently associated with IMI in the current study (in both definitions used).

To evaluate the cellular response of the udder to the NAS species, the Log10 SCC was calculated for species detected at least 30 times. No significant differences were found between NAS species, which is in line with Supré et al. (2011). However, the highest values were found for NAS species considered to be particularly important for udder health. Furthermore, no differences were found between the findings, classified as infection or contamination according to DEF1 or DEF2. This raises doubts concerning the definitions used to distinguish between infection and contamination, as higher SCC are generally expected in infected quarters. However, it should be noted that the increase in SCC in NAS infections is generally moderate (Djabri et al., 2002).

Furthermore, increased SCC was a selection criterion in this study for the udder quarters and a control group is missing. To assess the influence of the NAS species on the SCC, more differentiated investigations are necessary.

3.6 Conclusions

NAS species occur as contamination in milk samples, and this is often not considered as such in microbiological examination. For scientific investigations on NAS species, milk samples should always be taken consecutively. The interval between samplings should be no more than one week to take into account NAS species with shorter infection periods. Finally, the choice of definitions when assessing the infection status depends on the case and the species of interest. The current study provided new understanding regarding which pathogens among NAS are more likely to cause IMI (*S. simulans, S. chromogenes, S. epidermidis*), and which pathogens may cause IMI or contaminate milk samples (*S. warneri, S. xylosus, S. microti, S. haemolyticus, S. succinus*). These findings can be used in dairy herd management after NAS species differentiation, and may provide additional justification for differentiation. NAS species that seems to be more important for udder health appear to shed at higher bacterial concentrations. This is probably due to virulence factors and a better adaptation of the relevant NAS species to the udder tissue.

Conflict of interest

The authors declare no conflicts of interest.

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4 Übergreifende Diskussion

In der vorliegenden Arbeit wurden infizierte Euterviertel wiederholt beprobt, um die Ausscheidung von Mastitiserregern sowie die Bedeutung von NAS als Ursache von intramammären Infektionen oder von Kontaminationen zu untersuchen. Von Mai bis November 2018 wurden dazu in fünf konventionellen Milchviehbetrieben in Niedersachsen und Nordrhein-Westfahlen zweimal pro Woche Milchproben entnommen. Auf Grundlage der Ergebnisse der Milchleistungsprüfung wurden Kühe mit einem Gehalt somatischer Zellen im Gesamtgemelk von ≥ 200.000/ml ausgewählt. An drei Probenentnahme-Terminen wurden von diesen Tieren Viertelanfangsgemelksproben aller Euterviertel entnommen. Nach drei Probenentnahme-Terminen wurden Euterviertel von der weiteren Probenentnahme ausgeschlossen, wenn der Gehalt somatischer Zellen < 200.000/ml war oder kein Erreger identifiziert wurde. Die Probenentnahmen erfolgten über einen Zeitraum von zwei bis neun Wochen, wobei die Betriebe nacheinander in die Studie einbezogen wurden. Die verschiedenen Häufigkeiten der Probenentnahmen sind mit dem unterschiedlichen Vorkommen von Mastitiserregern zu begründen. So war die Anzahl an infizierten Eutervierteln in den Betrieben III und IV niedriger als erwartet, weshalb die Probenentnahmen auf diesen Betrieben nach sechs bzw. vier Besuchen nicht weiter fortgesetzt wurden. Die Panel-Mortalität war für diese longitudinale Studie nicht von Bedeutung, da kaum Euterviertel während des Probenentnahmezeitraumes, etwa durch Trockenstellen, antibiotische Behandlung oder Merzung von Tieren, ausschieden.

4.1 Ausscheidung von Mastitiserregern

Im ersten Teil der Arbeit wurden die Ausscheidungsintensität von fünf Mastitis-verursachenden Erregern sowie beeinflussende Faktoren untersucht. Bei der statistischen Auswertung kam ein gemischtes verallgemeinertes lineares Modell mit Gamma-verknüpfung zur Anwendung. Es wurden Unterschiede in der Ausscheidungsintensität zwischen den Erregern nachgewiesen. Am niedrigsten wurde *S. aureus* ausgeschieden (3,41 Log10 KbE/ml). Sears et al. (1990) führten Untersuchungen zur *S. aureus*-Ausscheidung aus natürlich vorkommenden und

experimentell erzeugten Infektionen durch. Die Autoren kamen in der vielfach zitierten Studie zu dem Ergebnis, dass niedrig ausscheidende (low shedding, ≤ 3 Log10 KbE/ml, in Originalquellen nicht logarithmiert) und hoch ausscheidende (high shedding, ≥ 3,3 Log10 KbE/ml) Infektionen vorkommen (Sears et al., 1990). Die *S. aureus*-Infektionen der aktuellen Untersuchung müssten somit den „high shedders“ zugeordnet werden. Andere Autoren geben ebenfalls relativ hohe Mittelwerte um 4,1 Log10 KbE/ml (Pellegrino et al., 2010) oder 4,3 Log10 KbE/ml an (Walker et al., 2011).

Auf signifikant höherem Niveau wurden in der aktuellen Studie NAS ausgeschieden (3,55 Log10 KbE/ml). Wald et al. (2019) geben einen Bereich von 3 bis 4 Log10 KbE/ml für die NAS-Ausscheidung an. Auch wenn der Wert der aktuellen Untersuchung in diesem Bereich liegt, ist anzumerken, dass die angegebene Spanne sehr weit ist (entspricht 1.000 bis 10.000 KbE/ml). Des Weiteren wird von der Betrachtung der NAS als Gruppe in wissenschaftlichen Untersuchungen abgeraten, da es sich um eine inhomogene Gruppe von Staphylokokken handelt (De Visscher et al., 2015). Im zweiten Abschnitt dieser Arbeit wird näher auf die Spezies-spezifische Ausscheidung der NAS eingegangen.

Die Ausscheidungsintensität von Hefen aus infizierten Milchdrüsenvierteln betrug in der aktuellen Untersuchung 3,62 Log10 KbE/ml und unterschied sich nicht von der der NAS. In der Literatur sind keine Vergleichswerte für diese eher seltenen Mastitiserreger zu finden. Hefen können Mastitiden bei Milchkühen hervorrufen und auch als Kontaminanten in Milchproben vorkommen (Williamson und di Menna, 2007). Sie gelten als seltene Mastitiserreger (Krömker, 2007), wurden in der aktuellen Studie jedoch relativ häufig nachgewiesen (vor allem in Betrieb II). Eine nähere Begründung kann dafür nicht gegeben werden. Durch das gehäufte Vorkommen konnte ein least sqare mean für diese Pathogene geschätzt werden, was eine gute Voraussetzung für weitere Untersuchungen ist. Diese sollten auch Spezies-Differenzierungen beinhalten.

Die Ausscheidungsintensitäten von *S. uberis* und *Streptococcus dysgalactiae* (*S. dysgalactiae*) waren signifikant höher als die der anderen untersuchten Erreger, unterschieden sich jedoch nicht voneinander (4,03 vs. 4,04 Log10 KbE/ml). Tassi et

al. (2013) führten Untersuchungen zur Stamm-spezifischen Pathogenität von *S. uberis* durch. Ein Stamm mit einer höheren Pathogenität führte 36 Stunden nach der Infektion zu einem *S. uberis*-Gehalt von 7,67 Log10 KbE/ml. Im weiteren Verlauf sank die Erregerkonzentration auf 3,49 Log10 KbE/ml und verblieb bis zum Ende der Studie (312 Stunden) auf diesem Niveau. Ein anderer Stamm dagegen erreichte 24 Stunden nach der Infektion nur einen maximalen Wert von 2,91 Log10 KbE/ml. Die *S. uberis*-Infektionen der aktuellen Studie sind somit eher mit denen des stärker pathogenen Stammes aus der Studie von Tassi et al. (2013) vergleichbar. Für die Ausscheidungsintensität von *S. dysgalactiae* gaben Maye et al. (2017) Werte von 8,7 bis 9,3 Log10 KbE/ml an, was weit über den angegebenen Ergebnissen der aktuellen Untersuchung liegt. Dabei ist kritisch anzumerken, dass der Detektionsbereich der vorliegenden Untersuchungen im Bereich von 100 bis 30.000 KbE/ml liegt. Die obere Grenze des Detektionsbereiches wurde in etwa 36 % der *S. uberis*- und 52 % *S. dysgalactiae*- positiven Proben erreicht. Dieses schränkt die Vergleichbarkeit mit anderen Studien ein. Auch ist nicht auszuschließen, dass es einen Unterschied zwischen der Ausscheidung der beiden betrachteten Streptokokken-Spezies gab, welcher jedoch durch die Grenze des Detektionsbereiches nicht dargestellt wurde. Jedoch bleibt eine wesentliche Erkenntnis der Studie von dieser methodischen Einschränkung unberührt: Staphylokokken werden in niedrigeren Konzentrationen aus infizierten Eutervierteln ausgeschieden als Streptokokken. Dieses steht auch in Übereinstimmung mit Schukken et al. (2011). Bei weiterführenden Untersuchungen zur Ausscheidungsintensität von Streptokokken sollte ein größerer Detektionsbereich angewandt werden.

Der Gehalt somatischer Zellen in der Milch war positiv mit der Ausscheidungsintensität der betrachteten Erreger assoziiert. Dieses bestätigt, dass Kühe mit erhöhten Gehalten an somatischen Zellen die Eutergesundheit einer Herde negativ beeinflussen können, da ein höheres Infektionsrisiko für andere Kühe der Herde besteht (White et al., 2006, Barlow et al., 2009). Schlussfolgerungen zur Kausalität können jedoch nicht erfolgen, da der Zusammenhang zwischen Ursache und Wirkung unklar bleibt. So stellt sich die Frage, ob der somatische Zellgehalt die Erregerausscheidung bedingt oder umgekehrt. Die Parität hatte in der vorliegenden Studie einen Einfluss auf die

Erregerausscheidung. So war die Ausscheidungsintensität in der Laktationsgruppe 1 (LGR1, erste und zweite Laktation) höher als in der LGR2 (≥ dritte Laktation), was widersprüchlich erscheint, da sich mit steigender Anzahl an Laktationen die Eutergesundheit eher verschlechtert. Dieses wird zum Beispiel durch höhere Gehalte somatischer Zellen in der Milch (Hagnestam-Nielsen et al., 2009), ein höheres Risiko für Mastitiden (Hertl et al., 2011) sowie geringere bakteriologische Heilungsraten (Deluyker et al., 2005) mit steigender Anzahl an Laktationen deutlich. Die niedrigere Ausscheidungsintensität in den höheren Laktationen in der aktuellen Studie könnte darauf zurückzuführen sein, dass die Betriebe möglicherwiese Tiere mit schlechterer Eutergesundheit konsequent merzen. Dieses stünde in Übereinstimmung mit Hertl et al. (2011), welche ein höheres Abgangsrisiko für Kühe mit Mastitiden berechneten. Eine weitere Begründung könnte ein Verdünnungseffekt sein, da die Milchleistung von Laktation zu Laktation ansteigt. Selbst wenn die Vermehrungsrate der betrachteten Pathogene unabhängig von der Parität wäre, könnten höhere Milchleistungen zu geringen Erregerkonzentrationen mit steigender Anzahl an Laktationen führen. Auch der immunologische Status älterer Kühe kann für die Erregerausscheidung von Bedeutung sein. Untersuchungen von Herry et al. (2017) haben gezeigt, dass die lokale Immunisierung gegen *E. coli* zu einer milderen Entzündungsreaktion sowie einer schnelleren Elimination des Erregers bei einer Infektion führt. Da davon auszugehen ist, dass Kühe höherer Paritäten in der vorliegenden Studie bereits Kontakt zu zahlreichen Mastitiserregern hatten, die nicht zwangsläufig zu einer klinischen Ausprägung, jedoch zu einer immunologischen Reaktion führten, könnte dieses eine Begründung für geringere Erregerausscheidungen von Kühen in der LGR2 sein. Letztlich bleiben die Gründe jedoch unklar. Es sollten daher weitere Untersuchungen erfolgen.

Um den möglichen Einfluss der vorherrschenden klimatischen Bedingungen in die Auswertungen einzubeziehen, wurde ein THI (temperature-humidity index) berechnet. Der verwendete THI ist für das gemäßigte Klima geeignet (Bohmanova et al., 2007) und fand bereits in anderen deutschen Studien Anwendung (Brügemann et al., 2012). Die Messwerte der Temperatur (°C) und relativen Luftfeuchte (%) wurden in drei Wetterstationen des Deutschen Wetterdienstes gemessen, welche sich in einem

durchschnittlichen Abstand von 11 km zum Probenentnahmebetrieb befanden (maximal 14 km). Die Untersuchungen ergaben, dass es bei Überschreiten des THI-Wertes von 60 zu einer signifikant höheren Ausscheidungsintensität der betrachteten Erreger kam. Dieser Wert wurde von Brügemann et al. (2012) als Grenzwert der thermoneutralen Zone für Milchkühe in Deutschland angesehen. Im Vergleich zu Studien aus anderen Ländern bzw. anderen Klimazonen wirkt dieser Wert zunächst niedrig. Jedoch ist anzumerken, dass die Vergleichbarkeit verschiedener THIs nicht immer gegeben ist (Bohmanova et al., 2007). In der aktuellen Studie waren die Pathogene – Hefen und *S. uberis* – für die höhere Ausscheidung bei Überschreitung der THI-Schwelle 60 ursächlich, während bei den NAS, *S. aureus* sowie *S. dysgalactiae* keine Wirkung auf die Ausscheidungsintensität bei Überschreiten des THI von 60 festgestellt wurde. *S. aureus* ist sehr gut an die Milchdrüse angepasst und ist in der Lage, sich der immunologischen Abwehr zu entziehen (Schukken et al., 2011, Keane, 2019). Ähnliches gilt für die NAS, von welchen einige Spezies eine hohe Ähnlichkeit zu *S. aureus* aufweisen (Taponen und Pyorala, 2009). Vermutlich begründet die gute Angepasstheit dieser Erreger, dass Hitzestress keinen Einfluss auf deren Ausscheidungsintensität hatte. Bezüglich *S. dysgalactiae* ist erneut auf die obere Grenze des Detektionsbereiches hinzuweisen. Es ist nicht auszuschließen, dass es einen Einfluss auf die Ausscheidungsintensität gab, welche aufgrund des relativ hohen Ausscheidungsniveaus dieses Erregers nicht dargestellt werden konnte. In Untersuchungen von West et al. (2003) hatte der THI zwei Tage zuvor den größten vermindernden Einfluss auf die Milchleistung und die Futteraufnahme. In der vorliegenden Studie konnte eine sogenannte „lag time“ in Bezug auf die Ausscheidungsintensität der Mastitiserreger nicht festgestellt werden. Die Untersuchungen bestätigen, dass die Eutergesundheit von Milchkühen in Deutschland durch Hitzestress beeinflusst wird. Folglich sind Maßnahmen zur Verminderung zu empfehlen.

Während der Probenentnahme-Zeiträume erfolgte die Dokumentation von Veränderungen in der Umwelt bzw. möglichen Stressereignissen (Beispiele: Klauenpflegeaktion, anderes Melkpersonal, mehrere Tiere brünstig, Verfütterung neu berechneter Rationen oder Silage aus neu geöffneten Futterstöcken). In der

statistischen Auswertung konnte kein Zusammenhang zwischen diesen sogenannten Events und der Ausscheidungsintensität der untersuchten Erreger festgestellt werden. Dabei ist darauf hinzuweisen, dass es in praktischen Milchviehbetrieben viele potentielle Stressereignisse gibt, die in Feldversuchen nur schwer vollständig erfasst werden können und dass die Beurteilung solcher Ereignisse durch das Betriebspersonal sehr unterschiedlich und subjektiv ist. Dennoch kann weiterhin davon ausgegangen werden, dass Stressereignisse die Erregerausscheidung erhöhen bzw. beeinflussen oder zur Exazerbation bestehender subklinischer Mastitiden hin zu klinischen Fällen führen können. Es sollten weitere Untersuchungen unter strenger kontrollierten Bedingungen erfolgen.

In der vorliegenden Studie konnte nachgewiesen werden, dass die Ausscheidungsintensität von Mastitiserregern durch Hitzestress beeinflusst wird, und es besteht die Annahme, dass auch andere Stressoren diese beeinflussen können. Welche Mechanismen für eine Erhöhung der Erregerausscheidung eine Rolle spielen, bleibt jedoch unklar. Tao et al. (2018) gehen davon aus, dass es durch Hitzestress zu einer Beeinflussung der Immunfunktion der Milchdrüse kommt. Es ist beschrieben, dass Hitzestress eine geringere Migration von Leukozyten in die Milchdrüse sowie höhere Konzentrationen an Cortisol im Blutplasma verursachen kann (Elvinger et al., 1992). Möglicherweise führt Ersteres zu einer weniger erfolgreichen Bekämpfung der Mastitiserreger und somit zu einer höheren Ausscheidung. Burton und Kehrli (1995) wiesen eine höhere Ausscheidung von *S. aureus* nach der parenteralen Injektion von Glukokortikoiden (Kortison/Dexamethason) nach. Folglich kann davon ausgegangen werden, dass jedes Stress-verursachende Ereignis auch Einfluss auf die Eutergesundheit haben kann.

4.2 NAS

Bei der mikrobiologischen Untersuchung von Milchproben werden NAS am häufigsten nachgewiesen (DVG, 2015). Der Befund wirft jedoch häufig die Frage auf, welche Bedeutung für die Eutergesundheit besteht und ob tatsächlich eine Infektion vorliegt. Für die Interpretation von NAS-positiven Laborergebnissen liegen bislang keine Studien vor, welche das Vorhandensein einer Infektion auf Spezies-Ebene untersuchen. In der aktuellen Studie wurden anhand des wiederholten Nachweises einer NAS-Spezies Infektionen von Kontaminationen unterschieden. Euterviertel, die über den Versuchszeitraum mindestens drei Mal NAS-positiv waren, wurden als „vermutlich NAS-infiziert“ eingestuft (n=178) und die dazugehörigen 847 Isolate mittels MALDI-TOF differenziert. Fünfzehn unterschiedliche NAS-Spezies wurden analysiert. Um zu beurteilen, ob diese mit einer Infektion in Zusammenhang standen, wurden zwei Definitionen angewandt. Nach Definition 1 (DEF1) wurde das Vorliegen einer Infektion angenommen, wenn die gleiche Spezies aus mindestens drei Milchproben kultiviert wurde, wobei der Abstand zwischen diesen Befunden höchstens drei Probenentnahme-Termine betragen durfte (entspricht ca. zwei Wochen). Eine weitere aufgestellte Definition (DEF2) dagegen bestätigt das Vorliegen einer Infektion, wenn eine Spezies aus mindestens zwei von drei Milchproben isolierte wurde (maximal zulässiger Abstand zwischen den Nachweisen von einem Probenentnahme-Termin). Diese Definition wurde in Anlehnung an Andersen et al. (2010) entwickelt, welche ebenfalls von einer Infektion ausgingen, wenn zwei von drei Proben Erreger-positiv waren. Weitere Befunde derselben Spezies wurden der Infektion zugeordnet, wenn der Abstand max. drei bzw. zwei (DEF1 vs. DEF2) Probenentnahme-Termine betrug.

Nach DEF1 konnten in 76,4 %, nach DEF2 in 90,8 %, der vermutlich NAS-positiven Euterviertel das Vorliegen mindestens einer NAS-Infektion bestätigt werden. Folglich waren in 23,4 % bzw. 9,2 % der untersuchten Euterviertel Kontaminationen von Milchproben, welche nicht als solche erkannt wurden, die Ursache für positive NAS-Befunde. Die Unterschiede zwischen den Definitionen ergeben sich daraus, dass eine NAS-Spezies nach DEF1 mindestens dreimal nachgewiesen werden musste, während nach der weniger strengen DEF2 bereits der wiederholte Nachweis ausreichte, dass das Vorliegen einer Infektion angenommen wurde. In der aktuellen Arbeit wurde die

strengere DEF1 aufgestellt, um mit möglichst hoher Sicherheit nicht infizierte Euterviertel auszuschließen. Ein Vorteil von DEF2 besteht darin, dass auch kürzere Infektionen berücksichtigt werden können. Bei NAS-Infektionen handelt es sich nach Angaben der Literatur häufig um persistierende Infektionen (Taponen et al., 2006, Gillespie et al., 2009, Mørk et al., 2012). Dabei stellt sich die Frage, inwiefern es Unterschiede zwischen den Spezies gibt und ob auch kürzere Infektionsdauern möglich sind. Diese könnten durch DEF2 besser dargestellt werden. Die Wahl der Definition hängt letztlich von der jeweiligen Fragestellung ab.

Durch Anwendung der Definitionen konnte untersucht werden, in welcher Häufigkeit eine NAS-Spezies im Zusammenhang mit Infektionen nachgewiesen wurde. Von den 15 gefundenen NAS-Spezies wurden nach DEF1 acht mit intramammären Infektionen in Verbindung gebracht (*S. simulans, S. chromogenes, S. epidermidis, S. warneri, S. xylosus, S. microti, S. haemolyticus, S. succinus*), während dieses nach DEF2 auf 10 Spezies zutraf (zusätzlich *S. sciuri* und *S. gallinarum*). Fünf der nachgewiesenen NAS-Spezies wurden bei keiner der angewandten Definitionen mit Infektionen in Verbindung gebracht (*S. vitulinus, S. equorum, S. auricularis, S. hominis, S. cohnii*). Dabei ist kritisch anzumerken, dass *S. hominis* und *S. cohnii* aus insgesamt nur zwei Milchproben isoliert wurden, wodurch bei Anwendung der Definitionen die Diagnose „Infektion“ gar nicht möglich ist. Sowohl das in dieser Studie angewandte enge Probenentnahme-Intervall (zweimal je Woche) als auch die relativ häufige Beprobung der Euterviertel (4 bis 17 Besuche je Betrieb) hätten Infektionen mit den genannten Erregern sichtbar machen können.

In über 90 % der Nachweise von *S. simulans* und *S. chromogenes* wurde unter Anwendung der Definitionen eine Infektion angenommen. Dieses bestätigt, dass diese Vertreter die Milchdrüse besiedeln und mit hoher Sicherheit wiederholt nachgewiesen werden können. In Untersuchungen von De Visscher et al. (2014) wurde nachgewiesen, dass die NAS-Spezies, die für die Eutergesundheit von größerer Bedeutung sind, seltener aus der Umwelt isoliert wurden. In der aktuellen Studie wurden zwar keine Proben aus der Umwelt untersucht. Jedoch wird dieser Sachverhalt bestätigt, da die genannten Erreger nicht bzw. selten als Kontaminanten vorkamen.

Der am häufigsten nachgewiesene Erreger war *S. haemolyticus*. In 71,9 % bzw. 76,5 % (DEF1 vs. DEF2) der Nachweise wurde ein Zusammenhang zu einer Infektion hergestellt. Folglich handelte es sich bei ~30 % der *S. haemolyticus*-positiven Milchproben um Kontaminationen. Es ist bekannt, dass dieser Erreger einfach aus der Umwelt isoliert werden kann. In Untersuchungen von Piessens et al. (2011) wurde er in 40 % der Stallluft-Proben gefunden. Dolder et al. (2017) kamen zu dem Ergebnis, dass *S. haemolyticus*-Infektionen häufig zusammen mit “anderen NAS“ auftreten (Odds ratio 3,3). Zu diesen gehörten *S. equorum*, *S. auricularis, S. sciuri, S. hominis* und *S. vitulinus.* Die Diagnose einer intramammären Infektion basierte in der Untersuchung auf dem einmaligen Nachweis (keine wiederholten Beprobungen für Bestätigung einer Infektion). Dabei wurden bis zu drei Spezies in einer Kultur akzeptiert. In der aktuellen Studie konnten die genannten “anderen NAS“ nicht bzw. kaum (*S. sciuri* in DEF2) mit intramammären Infektionen in Verbindung gebracht werden. Folglich ist die oben genannte Studie kritisch zu betrachten, da das gleichzeitige Vorliegen mehrerer NAS-Spezies eher auf eine Kontamination als auf das Vorliegen einer Infektion hindeutet.

S. microti war der am zweithäufigsten nachgewiesene Erreger in Herde V (40,4 % der Nachweise) und wurde auch in Herde III gefunden (8,9 %). Es handelt sich um einen Vertreter der NAS, der 2010 entdeckt wurde. Die Isolation erfolgte aus den Organen einer Waldmaus (*Microtus arvalis*) (Nováková et al., 2010). 2016 wurde *S. microti* erstmals mit intramammären Infektionen bei Milchkühen in Verbindung gebracht (Król et al., 2016). Die aktuelle Studie bestätigt das Vermögen von *S. microti*, die Milchdrüse zu besiedeln, da der wiederholte Nachweis aus konsekutiv entnommenen Milchproben

zuverlässig gelang und 74,4 % bzw. 89,9 % (DEF1 vs. DEF2) der Nachweise mit Infektionen in Verbindung gebracht werden konnten. Der relativ große Unterschied zwischen den Definitionen von 15,5 Prozentpunkten deutet auf eher kurze Infektionsdauern hin, da die weniger strenge DEF2 bereits beim wiederholten Nachweis vom Vorhandensein einer Infektion ausgeht. Weitere Studien sind notwendig, um die Eigenschaften und die Verbreitung der relativ neuen NAS-Spezies zu untersuchen.

Zur Spezies-spezifischen Ausscheidungsintensität der NAS liegen in der Literatur bislang keine Untersuchungen vor. Es ist bekannt, dass Staphylokokken in eher niedrigen Konzentrationen ausgeschieden werden (Schukken et al., 2011). Laut Wald et al. (2019) liegen die Bakterienkonzentrationen der NAS im Bereich von 10^3-10^4 /ml (entspricht 3-4 Log 10 KbE/ml), was eine recht weit gefasste Angabe ist. In der aktuellen Studie wurden die Bakteriengehalte der Erreger unter Einbeziehung der Milchproben, die laut DEF1 mit Infektionen in Verbindung standen, berechnet. Die NAS-Spezies *S. epidermidis*, *S. chromogenes* und *S. simulans* wurden in signifikant höheren Konzentrationen ausgeschieden als *S. microti, S. haemolyticus* und *S. succinus*. Dieses zeigt, dass die Spezies, die für die Eutergesundheit eher von Bedeutung sind (Vanderhaeghen et al., 2015), in signifikant höheren Konzentrationen ausgeschieden werden.

Welchen Einfluss die NAS-Spezies auf den Gehalt somatischer Zellen in der Milch haben, wurde für die Spezies untersucht, die in mindestens 30 Milchproben gefunden wurden, wobei nur Proben einbezogen wurden, die nach DEF1 mit Infektionen in Verbindung standen. Die Werte lagen im Bereich von 5,08-5,54 Log10/ml (entspricht ca. 120.000-347.000 somatische Zellen/ml), wobei es keine Unterschiede zwischen den NAS-Spezies gab. Dies ist in Übereinstimmung mit Untersuchungen von Supré et al. (2011), die ebenfalls keine Unterschiede zwischen den Spezies feststellten. Die numerischen Unterschiede in der aktuellen Studie weisen jedoch darauf hin, dass Spezies, die eher bedeutsam für die Eutergesundheit sind, tendenziell höhere Gehalte an somatischen Zellen hervorrufen. Grundsätzlich verursachen NAS moderate Erhöhungen der somatischen Zellzahl (Djabri et al., 2002). Schließlich ist darauf hinzuweisen, dass der Gehalt somatischer Zellen in der Milch zwar ein sehr wichtiger

Parameter in der Mastitisdiagnostik ist, jedoch letztlich eine Reaktion des Organismus auf Pathogene ist. Vor allem bei gut angepassten Erregern, wie den NAS, ist eine Besiedlung des Euters auch ohne eine dauerhafte Erhöhung der somatischen Zellzahl denkbar, da die Erreger in der Lage sind, sich den Mechanismen der Immunabwehr erfolgreich zu entziehen. Schließlich sind weitere Untersuchungen zur Spezies-spezifischen Beeinflussung der somatischen Zellzahl notwendig.

Die aktuelle Studie bestätigt, dass NAS in Milchproben als Kontaminanten vorkommen, jedoch teilweise nicht als solche erkannt werden und somit zu falsch-positiven Annahmen von NAS Infektionen führen können. In Betrieben mit wiederholt hohem Anteil NAS-positiver Milchproben sollten zunächst die Qualität der Probenentnahme und die Sauberkeit der Euter geprüft und verbessert werden, da diese einen wesentlichen Einfluss auf den Kontaminationsgrad von Milchproben haben. Schließlich kann eine Spezies-Differenzierung das NAS-Muster des Betriebes darstellen. Die aktuelle Studie liefert Entscheidungshilfen zur Beurteilung, welche Erreger eher Ursache von intramammären Infektionen sind oder eher als Kontaminanten in Milchproben vorkommen.

5 Zusammenfassung

Johannes Hamel

Modulation der Erregerausscheidung infizierter Milchdrüsenviertel beim Rind

Mastitiden verursachen in der Milchviehhaltung hohe wirtschaftliche Verluste. Infizierte Euterviertel scheiden Mastitiserreger aus, welche für die Infektion weiterer Tiere in der Herde von Bedeutung sind. Im ersten Teil der vorliegenden Arbeit wurden die Spezies-spezifische Ausscheidung von fünf Mastitiserregern sowie der mögliche Einfluss Tier-spezifischer Faktoren (Parität, somatische Zellzahl) sowie Umwelteinflüsse (Hitzestress, Veränderungen im Management) näher untersucht. Von Mai bis November 2018 wurden in fünf Milchviehbetrieben in Niedersachsen und Nordrhein-Westfalen infizierte Euterviertel über einen Zeitraum von zwei bis neun Wochen wiederholt beprobt (etwa zwei Mal wöchentlich). Während der Probenentnahme-Zeiträume erfolgte die Dokumentation von Veränderungen in der Umwelt bzw. möglichen Stressereignissen (Beispiele: Klauenpflegeaktion, anderes Melkpersonal, mehrere Tiere brünstig, Verfütterung neu berechneter Rationen oder Silage aus neu geöffneten Futterstöcken). Um den möglichen Einfluss der vorherrschenden klimatischen Bedingungen in die Auswertungen einzubeziehen, wurde ein THI (temperature-humidity index) berechnet. In der statistischen Auswertung hatten folgende Variablen einen signifikanten Einfluss auf die Ausscheidungsintensität: nachgewiesene Spezies, Parität, somatische Zellzahl sowie Überschreiten der THI-Schwelle 60. *Staphylococcus aureus* (*S. aureus*) und nicht-*S. aureus*-Staphylokokken (NAS) wurden in niedrigeren Werten als *Streptococcus uberis* (*S. uberis*) und *Streptococcus dysgalactiae* (*S. dysgalactiae*) ausgeschieden. In der ersten und zweiten Laktation war die Ausscheidungsintensität höher als bei Kühen mit drei oder mehr Kalbungen. Das Überschreiten der THI-Schwelle 60 führte zu einer höheren Erregerausscheidung. Mögliche Begründungen für diesen Effekt sind eine Konzentrierung der Erreger aufgrund einer niedrigeren Milchmenge durch Hitzestress oder bessere Vermehrungsbedingungen der Pathogene durch höhere Temperaturen im Euter. Auch könnte die höhere Ausscheidungsintensität durch nicht adäquate immunologische Reaktionen der Milchdrüse unter Hitzestress begründet werden. Die

Mechanismen bleiben jedoch spekulativ und erfordern weiterführende Untersuchungen. Die beobachteten potenziellen Stressereignisse in den Milchviehbetrieben hatten in der vorliegenden Untersuchung keinen Einfluss auf die Ausscheidungsintensität. Jedoch wird davon ausgegangen, dass auch diese – ähnlich wie Hitzestress – einen Einfluss haben können, welcher unter Feldbedingungen jedoch nicht dargestellt werden konnte. Weitere Untersuchungen sollten unter standardisierten Bedingungen erfolgen. Die Studie unterstreicht die Bedeutung von Kühen mit hohen somatischen Zellzahlen hinsichtlich der potentiellen Übertragung von Mastitis-Erregern innerhalb einer Herde, da eine Assoziation zwischen somatischer Zellzahl und der Erregerausscheidung nachgewiesen wurde. Darüber hinaus wird deutlich, dass Hitzestress in Deutschland die Eutergesundheit beeinflusst und Präventionsmaßnahmen in der Milchviehhaltung sinnvoll sind.

Im zweiten Teil der vorliegenden Arbeit wurde die Bedeutung der NAS als Ursache intramammärer Infektionen bzw. als Kontamination von Milchproben näher untersucht. Es handelt sich um die am häufigsten nachgewiesenen Bakterien in Milchproben. Die Gruppe der NAS umfasst ca. 50 Spezies, von denen ca. 20 regelmäßig in Milch vorkommen. Auch auf der Haut (von Mensch und Tier) sowie in der Umgebung (Spaltenboden, Einstreu, Luftproben) sind NAS nachweisbar. Daraus ergibt sich die Hypothese, dass Milchproben regelmäßig durch NAS kontaminiert sind, jedoch nicht als kontaminiert erkannt werden, wodurch es zu falsch-positiven Annahmen intramammärer NAS-Infektionen kommt. Wiederholte Probenentnahmen sowie die Differenzierung der NAS-Spezies können die Sicherheit der Diagnose erhöhen. Anhand des wiederholten Nachweises einer NAS-Spezies wurden in der aktuellen Studie Kontaminationen von Infektionen unterschieden. Dabei wurde auch untersucht, ob es Unterschiede zwischen den Spezies gibt hinsichtlich des Vorkommens als Kontamination in Milchproben bzw. als Ursache von Infektionen. Die untersuchten Proben stammen aus den oben beschriebenen Probenentnahmen. Von NAS-positiven Kulturen wurden Isolate für die weitere Differenzierung gesammelt. Euterviertel, die über den Versuchszeitraum mindestens drei NAS-Nachweise aufwiesen, wurden als „vermutlich NAS-infiziert“ eingestuft (n=178) und die dazugehörigen 847 Isolate mittels MALDI-TOF (Matrix assisted laser desorption ionization - time of flight mass

spectrometry) differenziert. Fünfzehn unterschiedliche NAS-Spezies wurden analysiert. Nach Definition 1 (DEF1) wurde vom Vorliegen einer NAS-Infektion ausgegangen, wenn die gleiche Spezies aus mindestens drei Milchproben kultiviert wurde. Der Abstand zwischen diesen Befunden durfte dabei höchstens drei Probenentnahme-Termine betragen (entspricht ca. 2 Wochen). Eine weitere aufgestellte Definition (DEF2) dagegen führte zu der Diagnose „NAS-Infektion“, wenn eine Spezies aus mindestens zwei von drei Milchproben isoliert wurde, wobei ein maximaler Abstand zwischen den Nachweisen von einem Probenentnahme-Termin zulässig war. Nach DEF1 lag bei 76 % der „vermutlich NAS-infizierten“ Euterviertel eine Infektion vor, nach DEF2 dagegen in 91 %. Dieses zeigt, dass der wiederholte Nachweis von NAS ohne Spezies-Differenzierung eine Unsicherheit birgt und dass die Wahl der Definition einen erheblichen Einfluss auf die Diagnose hat. Die NAS-Spezies *S. simulans, S. chromogenes, S. epidermidis, S. warneri, S. xylosus, S. microti* und *S. haemolyticus* standen in über 70 % der Nachweise im Zusammenhang mit Infektionen (nach DEF1). Für die Spezies *S. succinus, S. sciuri, S. gallinarum, S. vitulinus, S. equorum, S. auricularis, S. cohnii* und *S. hominis* dagegen konnte dieser Zusammenhang nicht bzw. seltener hergestellt werden. Folglich wird deren Vorkommen in Milchproben als Kontamination interpretiert. Wie die aktuelle Studie bestätigt, kommen NAS in Milchproben als Kontaminanten vor, werden jedoch teilweise nicht als solche erkannt, wodurch es zu falsch-positiven Annahmen von NAS-Infektionen kommen kann. Bei wiederholt hohem Anteil NAS-positiver Milchproben sollten zunächst die Qualität der Probenentnahme und die Sauberkeit der Euter geprüft und verbessert werden, da diese für die Kontamination von Milchproben bedeutsam sind. Schließlich kann durch eine Spezies-Differenzierung das NAS-Muster des Betriebes dargestellt werden. Durch die Untersuchungen aktuelle Studie ist anschließend eine zuverlässigere Beurteilung von NAS-Befunden in Bezug auf die Bedeutung für die Eutergesundheit bzw. das Vorliegen von Kontaminanten möglich.

6 Summary

Johannes Hamel

Modulation of pathogen shedding from infected mammary gland quarters in cattle

Mastitis causes high economic losses in dairy farming. From infected udder quarters mastitis pathogens are shed, which contribute to the infection of other animals in the herd. In the first part of the present study, the species-specific shedding of five mastitis pathogens and the possible influence of animal-specific (parity, somatic cell count) as well as environmental factors (heat stress, changes in management) were investigated. From May to November 2018, milk samples were collected from infected udder quarters on five dairy farms in Lower Saxony and North Rhine-Westphalia over a period of two to nine weeks (about twice-weekly). During the sampling periods, changes in the environment of the cows or possible stress events were documented (examples: claw trimming, different milking personnel, several animals in heat, feeding of a recalculated total mixed ration or the use of grass silage from other sources). To investigate the possible influence of the climatic conditions, a THI (temperature-humidity index) was calculated. In the statistical evaluation, the following variables had a significant influence on the shedding intensity: species, parity, somatic cell count and exceeding THI threshold 60. *Staphylococcus aureus* (*S. aureus*) and non-*S. aureus* staphylococci (NAS) were excreted in lower levels than *Streptococcus uberis* (*S. uberis*) and *Streptococcus dysgalactiae* (*S. dysgalactiae*). In the first and second lactation the shedding intensity was higher than in higher lactations. Exceeding the THI threshold 60 led to a higher pathogen shedding. Possible reasons for this effect are a concentration of the pathogens due to a lower milk quantity caused by heat stress or better reproduction conditions of the pathogens caused by higher temperatures in the udder. The higher shedding intensity could also be caused by inadequate immunological reactions of the mammary gland under heat stress. However, the mechanisms remain speculative and require further investigation. The observed potential stress-causing events in the dairy farms had no influence on the shedding intensity in the present study. However, it is assumed that these can also have an

influence - similar to heat stress - which could not be represented under field conditions. Further investigations should be carried out under more standardized conditions. The study underlines the importance of cows with high somatic cell counts for the potential transmission of mastitis pathogens within a herd, as an association between somatic cell count and pathogen excretion was found. Furthermore, the study shows that heat stress in Germany influences udder health and that preventive measures in dairy farming are useful.

In the second part of the present study, the importance of NAS as a cause of intramammary infections or as contamination of milk samples was investigated in more detail. NAS are the most commonly detected bacteria in milk samples. The group of bacteria includes about 50 species, of which about 20 occur frequently in milk. NAS can also be detected on the skin (of humans and animals) and in the environment (slatted floor, litter, stall air). This leads to the hypothesis that milk samples are regularly contaminated by NAS but are not recognized as contaminated. This can lead to false positive assumptions of intramammary NAS infections. Repeated sampling and differentiation of NAS species can increase the reliability of the diagnosis. Based on the repeated detection of a NAS species, contamination was distinguished from infection in the current study. It was also investigated whether there are differences between the species with regard to their occurrence as contamination in milk samples or cause of infections. The samples examined were taken from the sample collection described above. Isolates were collected from NAS-positive cultures for further differentiation. Udder quarters with at least three NAS findings over the test period were classified as „presumably NAS infected“ (n=178) and the corresponding 847 isolates were differentiated by MALDI-TOF (Matrix assisted laser desorption ionization - time of flight mass spectrometry). Fifteen different NAS species were identified. According to definition 1 (DEF1), the presence of NAS infection was assumed if the same species was cultured from at least three milk samples. The maximum interval between these findings was allowed to be three sample collection dates (corresponds to approx. 2 weeks). Another established definition (DEF2), on the other hand, led to the diagnosis of "NAS infection” when a species was isolated from at least two of three milk samples, with a maximum interval between findings of one sample collection date.

According to DEF1, 76% of the "presumably NAS-infected" udder quarters had an infection, according to DEF2, however, in 91%. This shows that the repeated detection of NAS without species differentiation is unreliable and that the choice of definition has a significant impact on the diagnosis. The NAS species *S. simulans*, *S. chromogenes*, *S. epidermidis*, *S. warneri*, *S. xylosus*, *S. microti* and *S. haemolyticus* were associated with infections in more than 70 % of the findings (according to DEF1). On the other hand, in the case of *S. succinus*, *S. sciuri*, *S. gallinarum*, *S. vitulinus*, *S. equorum*, *S. auricularis*, *S. cohnii* and *S. hominis*, this correlation could not be established or was less frequent. Consequently, their presence in milk samples is interpreted as contamination. As the current study confirms, NAS occur as contaminants in milk samples and are in some cases not recognized as such, which can lead to false positive assumptions of NAS infections. On farms with a repeatedly high proportion of NAS-positive milk samples, the quality of sampling and the cleanliness of the udders should first be inspected and improved, as these have a considerable influence on the contamination of milk samples. Finally, the NAS pattern of the farm can be represented by a species differentiation. The current study provides information to help decide which NAS species are more likely to cause intramammary infections or are more likely to be present as contaminants in milk samples.

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