Improving udder health management in dairy herds with automatic milking systems

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Improving udder health management in dairy herds with automatic milking systems

Verbetering van uiergezondheidsmanagement op melkveebedrijven met een automatisch melksysteem (met een samenvatting in het Nederlands)

全自动挤奶机器人牧场奶牛乳房健康管理 (中文总结)

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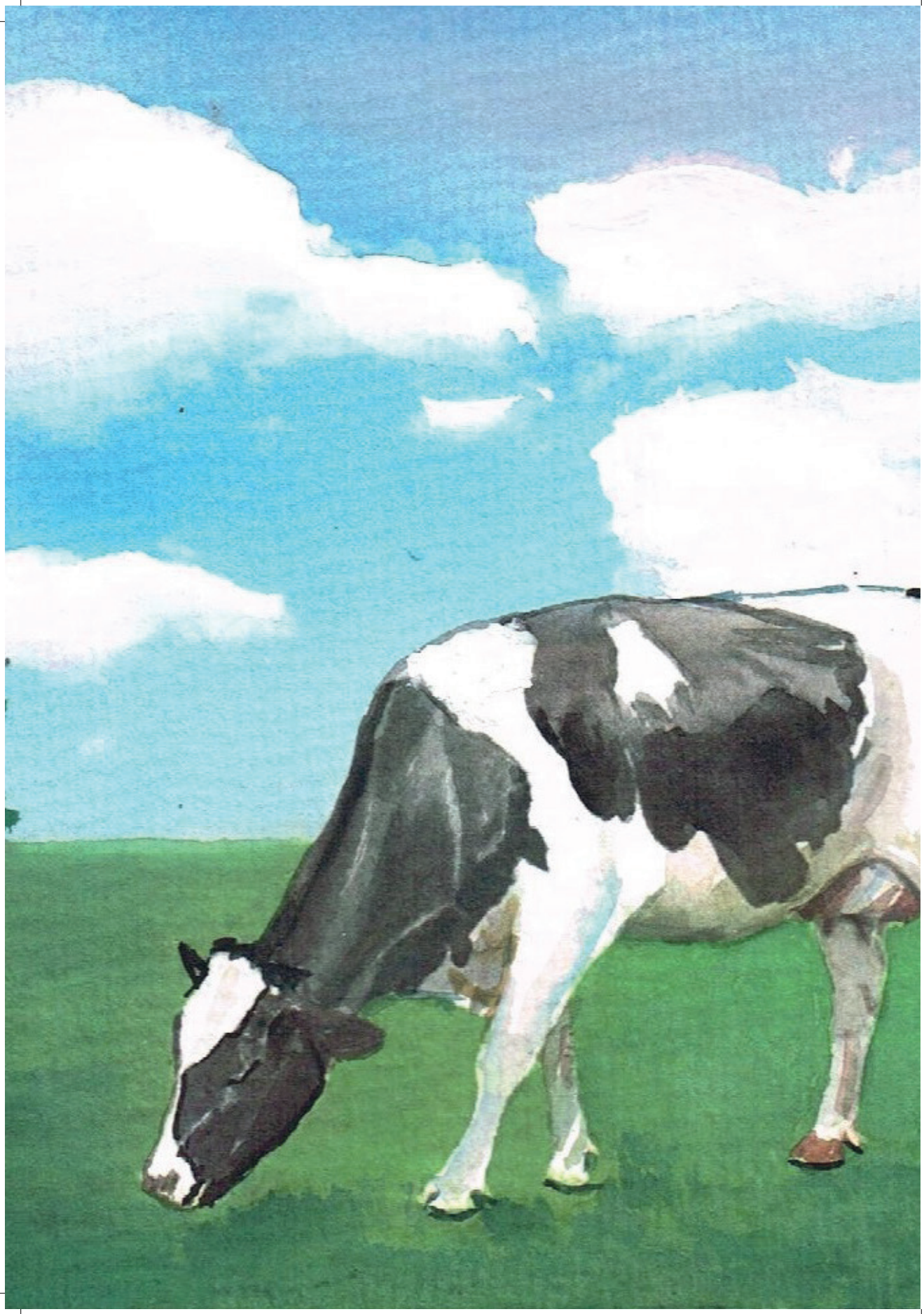

Chapter 1

General introduction

During the last decades, the dairy industry worldwide has profoundly changed. Scientific developments combined with managerial and technological advancements have led to practical applications that moved the dairy industry towards further improved milk production and milk quality (Barkema et al., 2015). More attention is given to aspects that may not be directly related to production such as animal welfare and antimicrobial use. Over the last century, herd size in the developed world has increased enormously and the milking of cows has evolved from hand milking to machine milking followed by robotic milking in recent decades (Jacobs and Siegford, 2012). Given the growing population worldwide and the increased need for animal proteins for human consumption, the worldwide growth in herd size, as well as technological developments in the dairy industry will likely continue. Alongside these technical developments, it is wise to study the consequences and possibilities these developments may have with respect to animal health, of which udder health is of primary importance.

BOVINE MASTITIS

Bovine mastitis is an inflammation of the mammary gland of dairy cows, which generally results from an intramammary infection **(IMI)** caused by pathogenic bacteria. Mastitis can be subdivided into subclinical and clinical mastitis based on the symptoms of the inflammation in the milk and the udder (Bradley, 2002). Clinical mastitis is defined as the presence of visible abnormalities of milk and/or udder, and can be found with the help of clinical diagnostics. To diagnose subclinical mastitis, further diagnostics are needed, like quantifying somatic cell counts (**SCC**) or bacteriological culturing. More than 150 pathogen species have been reported to be involved in bovine mastitis (Bradley, 2002). The most important pathogens with respect to udder health are the so-called major mastitis causing pathogens *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Escherichia coli*, and *Klebsiella* spp. and the minor pathogens *Corynebacterium* spp., non-aureus *Staphylococci* (**NAS**) and *Arcanobacterium pyogenes* (Bradley, 2002; Reyher et al., 2012). Mastitis causing pathogens are classified as "contagious" or "environmental" according to the epidemiological features of the pathogens (Bradley, 2002; Klaas and Zadoks, 2018). Contagious pathogens are considered to be transmitted from cow to cow (Keefe, 2012), while environmental pathogens are transmitting from an environmental reservoir to the cow (Klaas and Zadoks, 2018). The most important contagious mastitis pathogens are *Staph. aureus*, *Strep. agalactiae* and *Strep. dysgalactiae*, while *Enterobacteriaceae* (*E. coli* and *Klebsiella pneumoniae*) and *Strep. uberis* are the main environmental pathogens. In fact, contagious and environmental pathogens are not that clearly separated and most pathogens can behave according to both transmission routes (Fox and Gay, 1993; Barlow et al., 2013; Klaas and Zadoks, 2018).

The adoption of the "five-point" plan since the 1970s has limited the contagious transmission of pathogens drastically in dairy herds in developed countries (Green and Bradley, 2013). Therefore since

then, pathogens that generally behave more environmentally such as NAS, *E. coli* and *K. pneumoniae* are found relatively more frequently (Zadoks and Fitzpatrick, 2008; Persson Waller et al., 2011; Thomas et al., 2015). Although the prevalence of contagious pathogens may have decreased over the years, they still may play an important role in individual herds or in specific regions (Bradley et al., 2015; Bi et al., 2016; Ruegg, 2017) and can therefore not be neglected.

AUTOMATIC MILKING

Milking of cows has evolved from hand milking into machine milking in 1950s, and into automatic milking systems (**AMS**) in the 1990s. In 1992, the first commercial AMS was installed on a Dutch dairy farm. Since then, the adoption of AMS worldwide has steadily increased, especially in West and North Europe (Barkema et al., 2015). In 2017, more than 35,000 dairy farms worldwide milked their cows solely with AMS (Salfer et al., 2017). In the Netherlands, there were 4,325 AMS dairy farms in 2019 (ZuivelNL, 2021).

In AMS milking, cows are milked by a single milking cluster per AMS unit without a direct role of human labor. Cows initiate their own milking, hence the alternative name "voluntary milking system" for AMS. Most cows in AMS herds are milked 2.5-3 times per day (Erdman and Varner, 1995). The milk production can potentially increase by up to 12% with a decrease of labor to a maximum of 18% while at the same time animal welfare is improved as the cows are freely allowed to be milked (Jacobs and Siegford, 2012). The milking capacity of one AMS unit is approximately 60-80 cows (Hovinen, 2009). Although the number of AMS is quickly increasing on family farms in the EU, the high costs of AMS is still hampering a worldwide increase (Castro et al., 2012; Steeneveld et al., 2012; Salfer et al., 2017). Dissimilar to the family farms with small herd size in the EU, at this time AMS milking seems economically unattractive for many large dairy herds in North America and China (Houin, 2019; Melendez et al., 2019).

Nevertheless, the number of AMS installed in developing dairy industries such as China, is starting to increase (personal communication Xiurong Meng, 2021). Because also in China labor costs are constantly increasing, and because the population is growing older and the younger generation is less willing to work in agriculture, the number of AMS in the country is expected to further increase in the forthcoming decade. Also in other countries, important reasons for installing AMS are reduced availability of labor and increased requirements with respect to lifestyle of working people (Bijl et al., 2007; Salfer et al., 2017).

The use of AMS in dairy farms has a significant effect on the nature of the farmers' work. In a sense, their role changes from worker to manager and for these farmers it is therefore of big importance to

understand the information provided by the AMS in order to be able to make decisions in a data-driven way so they can optimize their management (de Koning, 2010; Jacobs and Siegford, 2012).

UDDER HEALTH MANAGEMENT IN AMS HERDS

Udder health at dairy farms in general has contracted much attention in the scientific literature and will likely continue to do this in the future. The number of publications on AMS also increased over the years (Dam Rasmussen et al., 2001; Hovinen and Pyörälä, 2011; Jacobs and Siegford, 2012; Penry, 2018), but the number of studies on udder health in AMS herds is still relatively limited. In Figure 1, data are presented that were retrieved from Scopus (accessed on 31st December 2020) using the keywords "bovine mastitis" OR "udder health" (Bovine mastitis), "automatic milking" OR "robotic milking" (Automatic milking), and "bovine mastitis" OR "udder health" AND "automatic milking" OR "robotic milking" (Bovine mastitis in automatic milking). As can be seen, the number of articles in the latter category has only slowly increased over the years.

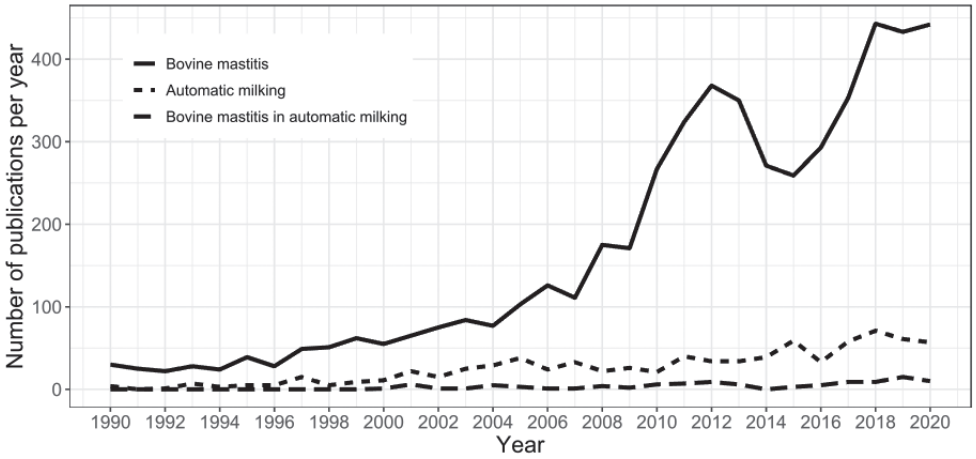

Figure 1. Number of publications per year related to bovine mastitis and automatic milking and both (retrieved from Scopus December 31, 2020).

In the studies on bovine mastitis in farms using AMS, both positive and negative effects of AMS on udder health have been reported. In some early studies, it was described that if the management was similar, no significant differences in udder health between herds using AMS and conventional milking systems (**CMS**) were found (Hamann et al., 2001; Berglund and Pettersson, 2002; Wirtz et al., 2004; Lopez-Benavides et al., 2006). Some small scale studies described a better udder health in AMS herds as compared to CMS herds (Berglund and Pettersson, 2002; Shoshani and Chaffer, 2002; Lopez-Benavides et al., 2006), while in some recent studies negative effects of using AMS, especially in the

first year after transitioning, were described (Hovinen et al., 2009). The adoption of AMS has been described as a risk factor for new IMI (Frössling et al., 2017) and a higher herd SCC (Svennersten-Sjaunja and Pettersson, 2008; Dufour et al., 2011). In another study, however, the adoption of AMS was not found to be a risk factor for a higher incidence rate of clinical mastitis (Tse et al., 2017). Obviously, different studies point in different directions, not leading to definite answers, and there is a need for more knowledge in this field.

In AMS herds, the visual checking of cows and udders, the pre-treatment before milking, attaching and removing liners as well as post-milking teat disinfection is fully automatically performed. The contact structure between cows and machines, as well as the milking process, are different between AMS and CMS herds. Therefore, the transmission of mastitis pathogens, specifically of contagious mastitis pathogens, may be different between AMS and CMS herds. The transmission of contagious pathogens is largely influenced by factors such as teat cleaning, pre-milking preparation, milking and post-milking disinfection (Lam et al., 1996; Elbers et al., 1998). Down et al. (2013) described that the transmission rate of mastitis pathogens is one of the most important factors associated with udder health and the most important factor associated with the costs of clinical mastitis in a herd. Even if the most important management measures related to transmission of contagious pathogens in AMS herds have been optimized in an AMS herd, still a large number of cows is milked in the same milking unit and contagious mastitis pathogens may be easily transmitted from one cow to another. Thus, because of the importance of pathogen transmission through milking units and the contact structure of AMS, this subject needs specific attention. The transmission dynamics of contagious pathogens in CMS herds have been extensively described (Zadoks et al., 2002; Leelahapongsathon et al., 2016; Kirkeby et al., 2019), while, to the best of our knowledge, scientific reports on the transmission dynamics of contagious pathogens in AMS herds is limited to one study (Dalen et al., 2019). Therefore, it is valuable to quantify the transmission rates of contagious mastitis pathogens in AMS herds. This information may be helpful in designing mastitis control programs in AMS herds.

Based on the generally increasing concern on antimicrobial resistance, awareness on prudent use of antimicrobials in mastitis also receives attention in AMS herds. Antimicrobial use as well as the determinants associated with antimicrobial usage in CMS herds have been studied worldwide (Thomas et al., 2015; Kuipers et al., 2016; Kayitsinga et al., 2017; More et al., 2017; Nobrega et al., 2018). There are, however, limited reports with this type of information specifically for AMS herds (Hovinen et al., 2009; Scherpenzeel et al., 2016; Vilar et al., 2018). Given the increasing number of AMS herds worldwide, there is a need for studies on mastitis causing pathogens and antimicrobial use specifically in AMS herds.

PRECISION TECHNOLOGY IN DAIRY FARMING

With the worldwide rapidly increasing herd size, human labor is more and more replaced by machines. A consequence of the larger herds is also that visual inspection of individual cows in a daily routine to screen for potential disorders is very difficult to be carried out. Because of this, combined with the advancement in precision technology, the reduction of available labor, and the seemingly attractive economic returns (de Koning, 2010), dairy farms tend to apply precision technologies in their farm management (Barkema et al., 2015). Among precision technologies in the dairy industry, sensors play a predominant role. According to Helwatkar et al. (2014), a sensor is defined as "a device that measures a physiological or behavioural parameter (related to health or estrus) of an individual cow and enables automated, on-farm detection of changes in this condition that is related to a health event (such as disease) and requires action on the part of the farmer (such as treatment)". In precision dairy farming, sensors in fact are a combination of the devices that measure parameters and the associated software that processes the data generated from those sensors (Rutten et al., 2013). Sensors can be categorized into two categories according to the type of installation: (1) attached sensors, these are the cow side sensors, such as sensors that are attached to or are planted inside a cow; (2) non-attached sensors, these are the off-cow sensors that measure parameters when the milk flows through the sensors or when cows approach the sensors. The non-attached sensors consist of two distinct forms: inline sensors that are measuring in a continuous flow of milk (such as a sensor measuring electrical conductivity) and online sensors that automatically take samples that are analysed by that same sensor (such as a milk SCC sensor) (Helwatkar et al., 2014). The development of dairy sensor systems, according to Rutten et al. (2013), can be described in 4 levels: (I) measuring parameters related to cows; (II) summarizing interpretations of changes in the data; (III) integrating information; (IV) making decisions. The majority of the sensors function at level I and II. A summary of sensors described in the literature that are available for dairy farming at this point in time is provided in Table 1 (Rutten et al., 2013; Helwatkar et al., 2014; Awasthi et al., 2016). Udder health related sensors are mostly in level II, and are used for mastitis diagnosis. A large number of sensors for mastitis diagnosis are currently available and this type of automatic measurements is developing rapidly. These sensors measure different aspects of mastitis, as will be described below, based on description in scientific literature and being commercially available.

Table 1. Overview of sensors available for disease detection in dairy farming (Rutten et al., 2013; Helwatkar et al., 2014; Awasthi et al., 2016).

Disease	Sensor	Location of sensor
Mastitis	Electrical conductivity	in-line
	Milk color	in-line
	Temperature	in-line
	Somatic cell count	on-line
	L-Lactate dehydrogenase	in-line
Estrus	Pedometer	leg
	Activity meter	neck collar
	Temperature	implant/reticulum
	Video camera	milking equipment
	Mounting activity	cow rump
	Biosensor	on-line
Locomotion	Accelerometer	leg/neck collar
	Pedometer	leg
	Weighting platform/Pressure plate or mat	floor/passageway
	Video camera	next to corridor
	Activometers	neck collar
Metabolic disorder	Electrode	rumen fistulated
	Telemetric bolus	rumen
	Temperature	rumen
	pH electrode	rumen
	Fiber optic spectrophotmeter	in-line
	Infrared spectroscopy	on-line
	Rumination sensor	rumen
	Pedometer	leg
	Thermal camera	above passageway after milking parlor
Ovarian Cysts	Accelerometer	neck
	Microphone	neck
	Temperature (Neck)	neck
	Electrical conductivity sensor	udder
Ketosis	Microphone	neck
	Accelerometer	neck
	Gáas sensor	nose
Milk Fever	Accelerometer	neck
	Microphone	neck
	Load sensors	under feet
	Temperature sensor	neck
	Heartbeat sensor	vein on neck
Heifer Diarrhea	Accelerometer	neck
	Temperature	neck
Heifer Pneumonia	Microphone	neck
	Temperature	neck

MEASURING SCC

Somatic cell count is a measure of the number of white blood cells (leukocytes) per mL of milk. In current mastitis control programs, SCC is a very important parameter to monitor udder health. Currently, three sensor systems are commercially available that measure SCC. Two systems are measuring SCC indirectly, either based on gel formation of the milk (comparable to the California Mastitis Test; Schalm and Noorlander, 1957) or physical measurements in the milk flow (GEA, 2021). Since these are indirect measurements, the outcomes of these sensors need to be transformed, either into SCC (in cells/ml) or into SCC classes. The third SCC sensor is a sensor that really counts cells, based on staining of a milk sample and optical counting of the number of cells (Dalen et al., 2019). The first type of sensor uses an online automated California Mastitis Testing (**O-CMT**). The O-CMT measurement makes it possible to determine the SCC of composite milk up to every milking, which enables a nearly continuous monitoring of individual cow udder health. These intensive measurements provide substantially more information on the SCC dynamics, than the SCC routinely measured every 4-6 weeks through sampling in a milk recording system, and thus can potentially identify SCC patterns that could not otherwise be found. This may provide a deeper understanding of IMI dynamics. These O-CMT sensors have been widely applied in herds milked with AMS (Jensen et al., 2018). However, the performance of these measurements is not clear yet. In order to optimally use the information that becomes available from these sensors, development of algorithms for mastitis detection and evaluation of the performance of the O-CMT measurements in the field are needed. There also are other types of sensors measuring electrical conductivity, color, or L-Lactate dehydrogenase for mastitis detection (Table 1, a detailed description of these sensors can be found in the recent publications of Rutten(2017) and Utriainen et al. (2019)). Other non-specific sensors to support mastitis detection are also applied in practice, such as behaviour sensors that measure cow activity (Van Hertem et al., 2016), location sensors that track the cows activity and location (Barker et al., 2018), rumination sensors that measure the rumination to indicate health problems (Grinter, 2019; Hamilton et al., 2019), rumen pH sensors (Doroodmand et al., 2016), temperature sensors (Kim et al., 2019), body condition scoring (Spoliansky et al., 2016; Mullins et al., 2019) and body weight sensors (Maltz, 1997; Berry et al., 2011).

AIM AND OUTLINE OF THIS THESIS

The overall goal of this thesis is to explore the potential use and benefit of using frequently measured data to optimize on-farm decision making in udder health management in AMS herds. The first objective is to identify the risk factors for mastitis on AMS farms and compare these risk factors to the existing studies of risk factors for mastitis on CMS farms. This will be described in Chapter 2 of this thesis. In Chapter 3 the second objective, to evaluate an online SCC measurement in comparison to SCC measured in laboratories, will be described. This will include an exploration of the added value of this

tool in individual cow udder health monitoring. The third objective is to explore possibilities of the new information coming available from frequently measured SCC. The continuously regularly fluctuating SCC pattern we found in our studies is described in Chapter 4. The fourth objective is to estimate the transmission rates, duration of IMI and the basic reproduction number of contagious pathogens (*Staph. aureus* and *Strep. agalactiae*) in a Dutch AMS herd in comparison to estimates in CMS herds (Chapter 5). The fifth and final objective of this thesis is to compare the antimicrobial use and the distribution of bovine mastitis causing pathogens between AMS and CMS farms. This objective will be described in Chapter 6.

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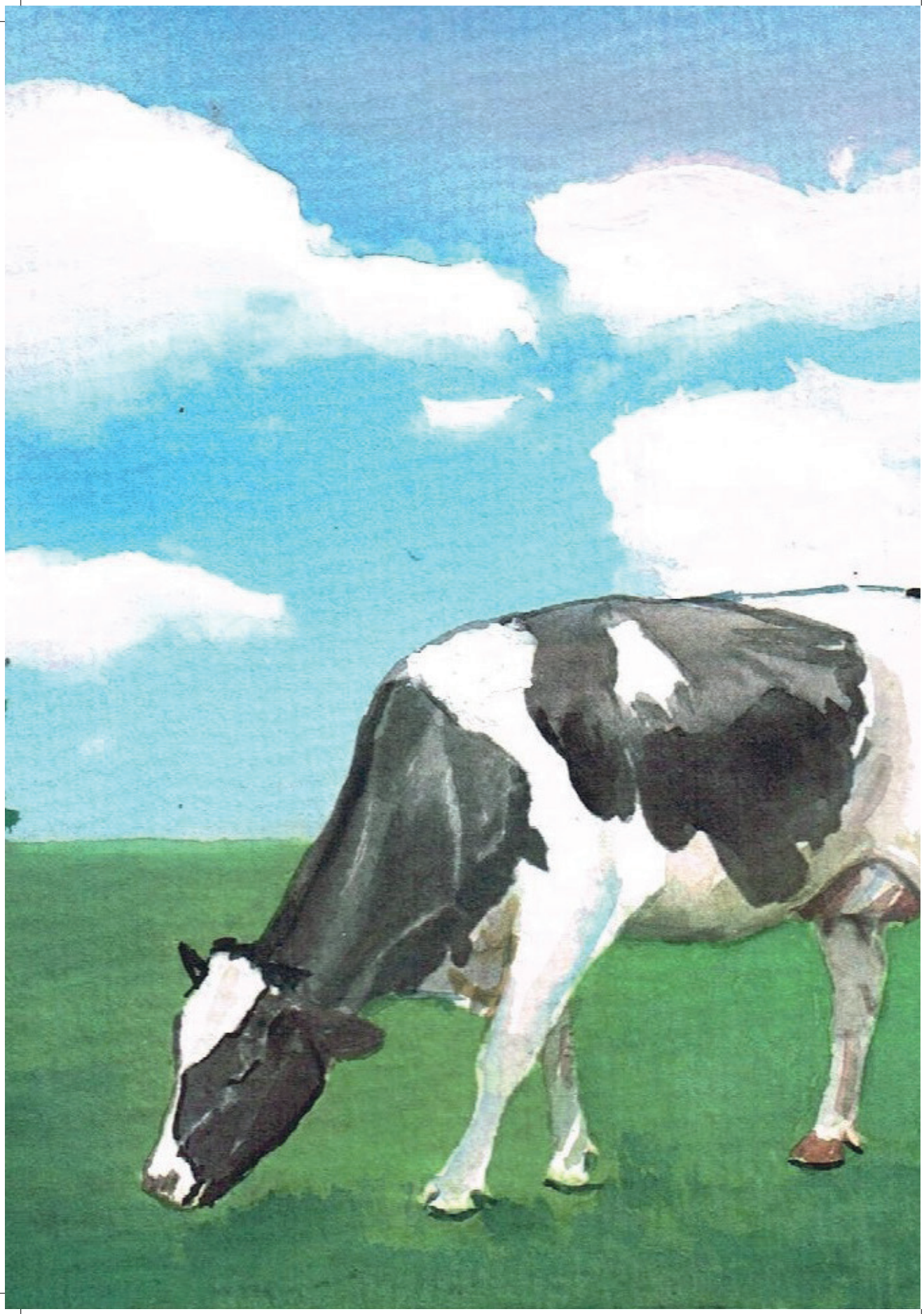

Chapter 2

Farm-level risk factors for bovine mastitis in Dutch automatically milking dairy herds

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ABSTRACT

Automatic milking systems (AMS) are installed on a growing number of dairy farms worldwide. Management to support good udder health might be different on farms with an AMS compared to farms milking with a conventional milking system (CMS), as risk factors for mastitis on farms using an AMS may differ. The aim of this study was to identify farm level factors associated with mastitis on Dutch dairy farms using an AMS.

In 2008, risk factor data were collected using a questionnaire combined with on-farm recordings of cow, stall and AMS hygiene on 135 farms. These risk factor data were linked to four udder health associated dependent variables: average herd somatic cell count (HeSCCav), variance of the average herd somatic cell count (SCC) on test days (HeSCCvar), the average proportion of new high SCC cases (NHiSCC) and the farmer-reported annual incidence rate of clinical mastitis (IRCM). We employed regression models using multiple imputation to deal with missing values. Due to the high dimensionality of the risk factor data, we also performed non-linear principal components analysis (NLPCA) and regressed the dependent variables on the principal components (PC).

Good hygiene of cows and of AMS were found to be related to a lower HeSCCav and less NHiSCC. Effective post-milking teat disinfection was associated with a lower NHiSCC. A higher bulk tank milk SCC threshold for farmers' action was related to more NHiSCC. Larger farm size was related to lower HeSCCvar but higher NHiSCC. Negative attitude of farmers to animal health, higher frequency of checking AMS and more time spent on viewing computer data were all positively related to higher IRCM.

An NLPCA with 3 PCs explained 16.3% of the variance in the risk factor variables. Only the first 2 PCs were associated with mastitis. The first PC reflected older and larger farms with poor cow hygiene and AMS hygiene, and was related to higher HeSCCav and NHiSCC, whereas the second PC reflected newly built smaller farms with poor cow hygiene and low milk production, and was associated with higher HeSCCvar and NHiSCC, but lower IRCM.

Our study suggests that many of the risk factors on CMS farms are applicable to AMS farms, specifically concerning hygiene of the cows and the milking machine, but on large AMS farms, udder health may need more attention than on smaller AMS farms. Multiple imputation is instrumental to deal with missing values and NLPCA is a useful technique to process high dimensional data in our study.

INTRODUCTION

Bovine mastitis is a multifactorial disease, and numerous papers have been published on management factors related to the disease (e.g., Dufour et al., 2012; Santman-Berends et al., 2016; Taponen et al., 2017). Risk factors for mastitis, according to Barkema et al. (1999), can be grouped into three categories: factors related to 1) exposure to causal pathogens, 2) host resistance to infection, and 3) cure of infection. On farms with conventional milking systems (**CMS**), frequently reported risk factors within the first category are, for instance, associated with hygiene of cow, milking machine and housing (Schukken et al., 1990; Schreiner and Ruegg, 2003; Breen et al., 2009), milking procedures (Barkema et al., 1999), post-milking teat disinfection (Lam et al., 1997; Santman-Berends et al., 2016) and milk leakage (Klaas et al., 2005). Risk factors related to host resistance to infection are, for example, breed (Heringstad et al., 2000), milk production level (Schukken et al., 1990), and nutrition (Hogan et al., 1993). Factors associated with cure of infection include cow, pathogen and treatment associated factors (Barkema et al., 2006). For instance, parity, strain type (van den Borne et al., 2010a) and the time of applying treatment (van den Borne et al., 2010b) are related to the cure rate of *Staphylococcus aureus* infection.

Since the first introduction of automatic milking systems (**AMS**) on commercial dairy farms in 1992, the use of AMS has rapidly increased in the Netherlands and many other countries (Barkema et al., 2015). Probably, many risk factors for mastitis on CMS farms are also applicable on AMS farms. However, because of differences in management between AMS and CMS farms (for example, on AMS farms, detection of clinical mastitis is based on a screening with sensors, whereas on CMS farms detection of clinical mastitis is done cow side by the farmer during milking. Moreover, udder preparation is done automatically on farms with an AMS. That means that the process is always carried out with the same intensity, while at a CMS farm, the milker can adjust the process to the circumstances (e.g., dirtiness of the udder)), some of the determinants of mastitis may differ on AMS farms. Additionally, risk factors that are present in both AMS and CMS farms may not have the same importance in both systems. Compared to the large number of studies on CMS farms, only a limited number of studies have been published on risk factors for mastitis on AMS farms. Persson Waller et al. (2003) reported that the incidence of milk leakage is higher on AMS farms than on CMS farms, which increases the risk of mastitis. Dohmen et al. (2010) indicated that on AMS farms, the hygiene of the cow and the milking system were related to udder health. Mollenhorst et al. (2011), in a cow-level study, reported that the milking interval was related to mastitis occurrence in cows milked by an AMS.

Because of differences in management between CMS and AMS farms, and because of the important role mastitis plays on AMS farms (Hovinen and Pyörälä, 2011), it is useful to specifically identify factors associated with udder health on AMS farms. This is generally done using questionnaires and multiple

regression analysis. This approach may result in inflated family-wise Type-I errors because of the high number of factors studied. Therefore, Principal Component Analysis (**PCA**) is an appropriate method that can be used to replace the large number of factors by a much smaller number of components. In questionnaire data, the presence of categorical variables and nonlinear relationships among factors is not rare. Therefore, nonlinear PCA (**NLPCA**), which is a nonlinear equivalent to PCA, was developed to deal with categorical variables and nonlinear relationships between variables (Linting et al., 2007a).

In this paper, we performed a comprehensive exploratory study of risk factors for mastitis on AMS farms. Due to the high dimensionality of the data resulting from the study design, we used NLPCA in addition to regression analysis. The aim of this study was to identify farm level risk factors for mastitis on Dutch dairy farms using AMS.

MATERIALS AND METHODS

Farm Selection and Data Collection

For this study, we used data from an extensive questionnaire about potential farm level risk factors for mastitis on AMS farms and on farm measurements on hygiene of cows, milking machines and barns, part of which was used by Dohmen et al. (2010) in a previous study.

There were about 2,400 (Reinemann, 2008) of the total 21,300 dairy farms (http://www.dutchfarmexperience.com/history-nl-dairy-farming/) using AMS in the Netherlands in 2007. Based on other published risk factor studies on mastitis (e.g., Schukken et al. (1990) with 125 farms, Elbers et al. (1998) with 171 farms and Santman-Berends et al. (2012) with 189 farms), a convenience sample of approximately 150 farms was aimed for. In order to reach that number and assuming a response rate of 25-35%, FrieslandCampina (Amersfoort, the Netherlands) approached 400 randomly selected farms with an AMS with the request to collaborate in this research in 2008. A total of 161 farms (40.3%) were willing to participate, of these 161 farms, 10 farms failed to meet the inclusion criteria below and so, 151 farms were visited. The following selection criteria were used: 1) the farm has had an AMS for more than one year and performed no conventional milking anymore; 2) the farm was participating in the Dutch milk production recording (**MPR**) system. Each farm was visited by a team of 2 trained veterinary master students." into "Data were collected by trained veterinary master students. In total, five students were involved in the data collection. All farms were visited by a team of two of these five students, in different combinations. The students collecting the data received a training session of one day. During that day, the questionnaire was explained to the students by the developers of the data collection protocol, including the background of the questions, the way the data should be retrieved from the various software systems and on the data collection in the barns (e.g., hygiene parameters). During the farm visit, questionnaires were completed by farmers with the assistance of the students

to clarify questions if needed. Additionally, hygiene scoring of the AMS and of the cows as well as an assessment of the functioning of the AMS were performed by pairs of students using standardized protocols. For data collection and hygiene scoring as well as for the evaluation of AMS units, protocols were developed in the following steps: as a first step, 10 Dutch mastitis experts drew up a list of potential risk factors. The list of risk factors was summarized in a number of questions and in data collection protocols, where existing scoring schemes (e.g., described by Schreiner and Ruegg (2003)) were as much as possible used. Protocols described how to retrieve data from the software systems of the various AMS manufacturers. Since the students followed the training together and visited the farms in pairs, we assumed that this would guarantee an as consistent as possible data collection procedure. Clinical mastitis data were collected based on farmers' reports and cow SCC data were based on MPR records. Milk production information, including cow identification, MPR test dates, milk yield and SCC on test dates, were extracted from the Dutch national milk production recording system (CRV, Arnhem, the Netherlands). Milk production data in 2008, from 106 to 296 days prior to the farm visit (with an average number of MPR test dates per farm of 8.5, ranging from 3 to 14) were used in the analysis. Data collection protocols, questionnaires and forms (in Dutch) are available from the last author.

Data Cleaning

Of the 151 farms that were visited, 135 farms were included in the final analysis. Of the 16 farms that were excluded from the analysis, 10 farms had no match of the farm identification with the questionnaire, MPR information, hygiene scores or the data on AMS functioning, 4 farms turned out to have been using an AMS for less than one year, and 1 farm was also using CMS, and 1 farm had invalid records (no clinical mastitis cases during the study period) with regard to the number of clinical mastitis cases.

Prior to analysis, records with test-day milk yield or SCC equal to zero (201,557 records), records from cows with an impossible parity (parity as 56 or 57; 27 records), records from MPR test days with ≤ 10 records (93 records) were excluded. Variables with more than 5% of missing values (29 variables) (Schafer, 1999) and binary variables with a category having less than 8 observations were discarded (24 variables) (Linting et al., 2007b), resulting in a total of 113 risk factor variables.

Explanatory Factors

The 113 risk factor variables that were captured using the questionnaire, hygiene scoring of the AMS and of the individual cows and of the barns, as well as from the assessment of the functioning of AMS were evaluated, of which 97 risk factors were derived from the questionnaire, 8 risk factors were about AMS hygiene, 3 about cow hygiene and 5 about AMS functioning. Hygiene scoring was performed on 8 AMS parts for each robot, scores ranging from 1 to 4 (1 = clean, 2 = slightly dirty, 3 = dirty, 4 = very dirty). The hygiene of udder, of thighs and of legs for at least 10 randomly selected milking

cows was scored (From the cows milked by the AMS, 25% were randomly selected, before entering the barn, based on the barn list, to be scored for hygiene, with a minimum of 10 cows per farm). The hygiene of teats before and after cleaning was scored during milking (1 = completely free of dirt or has very little dirt, 2 = slightly dirty, 3 = mostly covered in dirt, and 4 = completely covered, caked-on dirt) as described by Schreiner and Ruegg (2003). The functioning of the AMS was evaluated during 10 milkings on each farm during the farm visit by scoring 6 different procedures of the AMS, all with different scoring protocols. The scores for procedure and each farm were averaged for the 10 milkings (Dohmen et al., 2010).

Recoding of the hygiene-related variables was performed as follows:

1. for hygiene scores of the AMS units, the hygiene scores were transformed as dirty (average hygiene score of the units larger than 1 or clean (average score of 1).
2. The hygiene scores of individual cows were aggregated into 3 farm level variables denoting the proportion of dirty thighs, udders or legs per farm, with dirty defined as hygiene score > 2. The threshold for dirty was set as hygiene score > 2 since only less than 6% of the hygiene score equal to 1 in each of the variables.

Dependent Variables

The following dependent variables associated with udder health were defined: the average herd SCC (**HeSCCav**), the variance of the average herd SCC on each MPR test date (**HeSCCvar**), the average proportion of new high SCC cases (**NHiSCC**) and the farmer-reported incidence rate of clinical mastitis (**IRCM)**. To determine HeSCCav, first, for each farm and each test-day the arithmetic mean of SCC was calculated and then HeSCCav was calculated by taking the arithmetic mean of these test-day arithmetic means. Finally, it was $\log_{10}$ transformed. In the Netherlands, farms with a geometric mean BMSCC above 400,000 cells/mL in the last 3 months receive a penalty. The HeSCCvar was calculated for each farm by first calculating the farm average of SCC for each test day, then the variation of these test day farm-average SCC was calculated for each farm and then log10 transformed. New high SCC cows were defined as cows with a normal SCC on the previous test day and a high SCC (> 150,000 cells/mL for heifers and > 250,000 cells/mL for multiparous cows) on the current test day. The proportion of NHiSCC cows on each test day was calculated by dividing the number of NHiSCC cows by the number of cows with a SCC ≤ 150,000 cells/mL for heifers and ≤ 250,000 cells/mL for multiparous cows on the previous test day. Cows newly entered the farm or cows dried off during the study period were not considered in the calculation. The NHiSCC for each farm was calculated as the arithmetic mean of the proportion of NHiSCC cows of each of the test days. The annual average IRCM was calculated as the farmers' reported total number of cases of clinical mastitis in the past year, divided by the number of lactating cows in that year reported by the farmers. We calculated Pearson's correlation coefficient

between each of the 4 dependent variables to check for collinearity between these dependent variables and also knowing the correlations among the dependent variables may facilitate the interpretation of the beta-estimates of the same independent variable for different dependent variables (i.e., the beta-estimates to be similar or not).

Statistical Analysis

Risk factors were identified using two approaches: 1) linear and negative binomial regression models (after pre-screening for variable selection in univariable regression analysis) and 2) principal component regression analysis (PCR) (Massy, 1965). The PCR was performed by regressing the four udder health associated dependent variables to the principal components (PC) constructed by NLPCA on the evaluated risk factors.

Regression Analysis with Risk Factor Variables. In the regression analysis, the four udder health associated dependent variables were used as dependent variables in 4 different models, and the 113 risk factor variables were used as independent variables. Statistical significance was considered as $P < 0.1$ in univariable regression analysis, using one of the udder health variables as dependent variable and one of the risk factor variables as independent variable, and as $P < 0.05$ in multiple regression analysis. Because of missing values, and therefore differences in the number of observations between nested models, leading to incomparability of the model fit, we applied multiple imputation. Before imputing the missing values in the dataset, we used the aggr function from the VIM package version 4.7.0 (Kowarik and Templ, 2016) in R to check if the missing-values pattern fits the assumptions of multiple imputation. Multiple imputation with 10 imputations for 10 iterations, with other parameters set to default, was performed on all variables that were associated with one or more of the four udder health associated dependent variables in the univariable analysis together with the four udder health associated dependent variables by using the mice function from the MICE package version 2.46.0 (Buuren and Groothuis-Oudshoorn, 2011) in R. The default methods for imputation for different types of variables were: predictive mean matching for numeric data, logistic regression imputation for binary data or factor with 2 levels, polytomous regression imputation for unordered categorical data (factor >= 2 levels), proportional odds model for ordered factors with >= 2 levels. The imputed values for each variable with missing values were inspected by plotting the imputed and non-imputed values against iteration number. Convergence was evaluated by plotting the mean of the imputed values for each variable against the iteration number.

Collinearity was evaluated for continuous independent variables after the univariable analysis. If two continuous variables had a Pearson's correlation coefficient > 0.7, one of the variables was selected to be included in multivariable regression analysis, based on the biological interpretability of the variables. A stepwise forward method based on *P*-value was used for model selection for each dependent variable in

the multivariable regression analysis. Confounding was examined by evaluating the change in the beta-estimates of variables in the model when adding a new variable. A change in the beta-estimate of more than 20% was deemed indicative of confounding. Confounders were retained in the final model. To check robustness, the final models that were constructed using multiple imputation were rerun using the original dataset with missing values and the corresponding estimates were compared with the models constructed from the imputed dataset. We used linear regression models for HeSCCav, HeSCCvar and NHiSCC, and a negative binomial regression model for IRCM because of the negative binomial distribution of IRCM with overdispersion (Schukken et al., 1991). The linear and negative binomial regression models are described as follows:

$$Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_i X_i + \varepsilon_i \quad (1)$$

$$\log(CM) = \alpha + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_i X_i + offset(\log(herd\_size)) \quad (2)$$

Where Y = the continuous dependent variables (HeSCCav, HeSCCvar or NHiSCC), α = intercept, β_i = regression coefficient for each of the independent variables X_i, ε_i = residual random error, CM = the farmer-reported number of clinical mastitis cases last year, log (herd_size) = the offset term.

The model assumptions were evaluated as follows: for linear regression models, homoscedasticity was evaluated by plotting the standardized residuals against the predicted values, normality of residuals was examined by Q-Q plots and linearity was evaluated by plotting residuals against the fitted values for continuous variables; for the negative binomial regression model, residuals were evaluated by plotting standardized deviance residuals against predicted values.

Principal Component Regression Analysis. Because of the low variance explained in the dependent variables in ordinary regression analysis, and also in order to reduce the high dimensionality and to capture the underlying structure of the explanatory variables, we also did Principal Components Regression (Massy, 1965) for which we applied NLPCA on the 113 explanatory variables, with missing values imputed as the averages, from the Gifi package version 0.3.7 (Mair et al., 2017, https://rdrr.io/rforge/Gifi/) in R. In NLPCA, the categorical variables were transformed into numeric values through optimal scaling. These numeric values, referred to as category quantifications, for one variable together form that variable's transformation. Nominal or ordinal analysis levels were used for categorical variables and spline analysis levels for continuous variables. The number of principal components was determined by inspecting the scree plots of NLPCA solutions with different numbers of principal components, the interpretability of the principal components (Jolliffe, 2002; Linting et al., 2007b; Linting and Van der Kooij, 2012). We tested NLPCA with a 3, 4, 5, 6 and 7 dimensional solution. The scree plots for the solutions with 4 and 5 components showed a consistent elbow at the 3th principal component in NLPCA with different numbers of PCs, the eigen values of the first 4 PCs were all

larger than 1. We tried to interpret the PCs resulting from the NLPCA with different numbers of PCs, and the first 3 PCs were interpretable. We did not base our selection of the PCs on the cumulative variance accounted for (VAF) criterion, since with 113 explanatory variables even smaller cumulative VAF capture the variance that is present in a considerable number of variables. Outliers were defined as observations with object scores exceeding the range between -3.5 to 3.5 (Linting and Van der Kooij, 2012). NLPCA was repeated after deleting outliers until no outliers were present anymore. The 4 dependent udder health variables were then regressed on the first 3 principal components using negative binomial regression for IRCM and linear regression for the other 3 dependent variables, respectively. Model selection was performed as mentioned above. We did k-fold cross-validation with 10 folds for the 3 PCR linear regression models using the caret package version 6.0-80 in R. All statistical analyses were conducted in R version 3.4.4 (R Core Team, 2018).

RESULTS

Descriptive Statistics

In total, 135 farms were included in the analysis, of which 69 farms had complete records and 66 farms had missing values in at least one variable. The percentage of missing values on the 66 farms with missing values was on average 1.4%, ranging from 0.8% to 5.1%. Of the 113 explanatory variables, 70 variables had no missing values, while 43 variables did have missing values, on average 1.9%, ranging from 0.7% to 4.4%. Two farms did not have data on IRCM, but no missing values were present in other dependent variables.

The general farm characteristics of the 135 participating farms are provided in Table 1. The correlations among HeSCCav, HeSCCvar and NHiSCC ranged from 0.44 to 0.74, while the correlation between IRCM and other 3 dependent variables ranged from 0.097 to 0.17 (Figure 1).

Table 1. Descriptive statistics of 135 Dutch dairy farms using an automatic milking system (AMS)

Variable	Number	Mean	Min	Max
Number of cows	135	80	22	365
Number of AMS	131	1.6	1	6
Milk quota (×1,000 kg)	132	796	154	5,000
305-day milk yield (kg)	135	8,952	5,500	11,000
Average herd SCC (×1,000 cells/mL)[1]	135	271.8	83.4	505.3
Variance of average of herd SCC ($\times 10^7$)[2]	135	928	69	5,873
Average proportion of new high SCC cases (%)	135	13.4	4	29.3
Annual incidence rate of clinical mastitis (cases/100 cows/year)	133	27	1.4	135

[1]Arithmetic mean
[2]Variance of the average herd SCC between test days

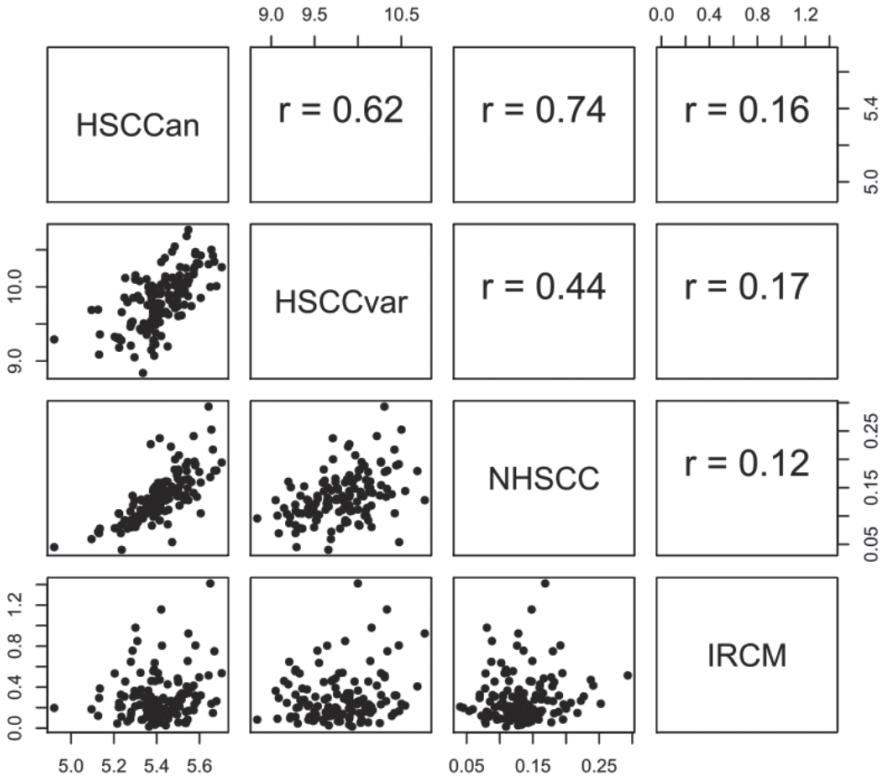

Figure 1. Pair-wise scatter plot with Pearson's correlation coefficient (*r*) between $\log_{10}$ transformed average herd SCC, $\log_{10}$ transformed variance of average herd SCC on each test day, average proportion of new high SCC cases and farmer-reported annual incidence rate of clinical mastitis per cow per farm, based on data collected on 135 Dutch dairy farms using an automatic milking system.

Regression Analysis with Risk Factor Variables

In the univariable regression analysis, a total of 48 risk factor variables were found to be associated with one or more of the dependent udder health variables (with $P < 0.1$), which are presented in supplemental Table S1 (https://doi.org/10.3168/jds.2018-15327). In Table 2, the results of the multiple regression models are given, showing that several risk factors were significantly associated with multiple dependent variables, namely participation in a *Salmonella* control program, the percentage of cows laying outside cubicles, including hay in the diet of dry cows, and the moment that the farmer stops antibiotic treatment of mastitis cases. None of the variables that remained significant in the IRCM model were present in any of the other models. The adjusted R-squared values of the regression models for HeSCCav, HeSCCvar, NHiSCC and IRCM based on the non-imputed dataset were 0.21, 0.18, 0.46 and 0.44, respectively. In general, the estimates of models using multiple imputation were very similar to the ones using non-imputed data.

Principal Component Regression Analysis

The three PCs in the NLPCA explained 16.3% of the variance in the risk factor variables. The component loadings for variables with loadings > 0.35 or < -0.35 (Hair, 2009) in 1 or more of the 3 PCs are presented in Table 3. Most of these variables were related to farm size, hygiene of the cows and hygiene of the AMS. The loadings of variables with loadings > 0.35 or < -0.35 are shown in Figure 2. The transformation plots, which display the shape of the relationship between the original variable and the transformed variable, are in Supplemental Figure S1 (https://doi.org/10.3168/jds.2018-15327) for the first PC and Figure S2 (https://doi.org/10.3168/jds.2018-15327) for the second PC. Many of the transformation plots showed linear relationships, but some of the transformation plots demonstrated nonlinear relationships.

Table 2. Multivariable regression models for 4 udder health variables, based on data collected on 4 dependent variables and 113 independent variables on 135 Dutch dairy farms using an automatic milking system. Models were constructed using the dataset with multiple imputation.

		HeSCCav[1]			HeSCCvar[2]			NHiSCC[3]			IRCM[4]		
	Variable	**β[5]**	**2.5%**	**97.5%**	**β**	**2.5%**	**97.5%**	**β**	**2.5%**	**97.5%**	**β**	**2.5%**	**97.5%**
	Intercept	5.22	5.09	5.34	9.96	9.71	10.21	-0.22	-0.52	0.09	-1.75	-1.99	-1.51
Farm size	Milk quota (×1,000 kg)				-0.01	-0.03	0.01						
	Number of AMS							0.01	0.00	0.02			
Hygiene	Percentage of cows laying outside cubicles	0.03	0.00	0.06				0.01	0.00	0.02			
	Percentage of teats completely not covered by spray							0.03	0.01	0.05			
	Farm average teat hygiene score before cleaning	0.10	0.05	0.16				0.02	0.00	0.03			
Animal health program	Participate in *Salmonella* program												
	No	Ref[6]			Ref			Ref					
	Yes	-0.08	-0.12	-0.03	-0.21	-0.36	-0.07	-0.02	-0.03	-0.00			
	Participate in BVD virus control program												
	No							Ref					
	Yes							-0.03	-0.05	-0.01			
	Participate in bacterial culture program												
	No										Ref		
	Yes										0.60	0.21	0.99
Nutrition	Including hay in dry cow diet												
	No	Ref						Ref					
	Yes	0.05	0.01	0.09				0.01	0.00	0.03			
	Leftovers from milking cow used as feed for dry cows												
	No							Ref					
	Yes							0.03	0.01	0.05			

		HeSCCav[1]			HeSCCvar[2]			NHiSCC[3]			IRCM[4]		
	Variable	**β[5]**	**2.5%**	**97.5%**	**β**	**2.5%**	**97.5%**	**β**	**2.5%**	**97.5%**	**β**	**2.5%**	**97.5%**
Other	305-day milk yield (×1,000 kg)							-0.01	-0.01	0.00			
	Time spent on viewing data in computer										0.47	0.01	0.94
	Time spent on non-dairy farming activities				-0.00	-0.00	0.000						
	Significant changes on farm recently												
	No				Ref								
	Yes				0.13	0.01	0.25						
	When do you stop antibiotic treatment for clinical mastitis cases												
	According to the prescription				Ref			Ref					
	Until no flakes present in milk				0.31	0.12	0.50	0.02	0.00	0.04			
	Other				0.13	-0.04	0.31	0.01	-0.01	0.02			
	Log_{10} (BMSCC)[7] that results in farmers' action							0.06	0.01	0.12			
	Check AMS in addition to regular check												
	No										Ref		
	Yes										0.37	0.09	0.65
	Ever checked AMS while milking												
	No										Ref		
	Yes										0.29	0.01	0.56

[1]Log_{10} transformed average herd SCC
[2]Log_{10} transformed variance of herd SCC on each test day
[3]Average proportion of new high SCC cows
[4]Farmer-reported annual incidence rate of clinical mastitis
[5]Estimate of regression coefficient
[6]Reference category
[7]Log_{10} transformed bulk tank milk SCC

Table 3. Variables with loadings > 0.35 or < -0.35 from nonlinear principal component analysis with 3 dimensions on 113 risk factor variables, based on data collected on 135 Dutch dairy farms using an automatic milking system.

			Dimension				
Category	**Abbreviation**	**Variable**	**1**	**2**	**3**	**Total VAF[1]**	**Sum**
Farm size	N_cow	Number of cows on farm	0.68	-0.54	-[2]	0.76	
	Milk_quota	Milk quota	0.59	-0.56	-	0.66	
	N_eat_place	Number of eating places	0.49	-	-	0.29	
	N_cubicle	Number of cubicles	0.57	-0.51	-	0.59	
	N_AMS	Number of AMS units	0.60	-0.41	-	0.53	
Hygiene of AMS	Camera_clean	Camera clean or dirty	0.40	-	0.40	0.37	
	Tube_clean	Tube clean or dirty	0.55	-	-	0.41	
	Cup_clean	Milk cups clean or dirty	0.51	-	-	0.32	
	Airintake_clean	Air-intake clean or dirty	0.51	-	-	0.35	
	Arm_clean	AMS arm clean or dirty	0.59	-	-	0.46	
	Manger_clean	Manger clean or dirty	0.47	-	-	0.28	
	Floor_clean	Floor clean or dirty	0.56	-	0.42	0.49	
	Brush_clean	Brush clean or dirty	0.50	-	-	0.35	
Hygiene of cow	Dirty_udder	Percentage of cows with udder hygiene score > 2	0.46	0.43	-0.48	0.62	
	Dirty_thigh	Percentage of cows with thigh hygiene score > 2	0.37	-	-0.43	0.43	
	Dirty_leg	Percentage of cows with leg hygiene score > 2	0.44	-	-0.48	0.45	
	Teat_clean_before	Average teat hygiene scores before cleaning	0.44	-	-	0.41	
	Dirty_teat_before	Percentage of teats with hygiene score > 2 before cleaning	0.40	-	-0.36	0.38	
	Dirty_teat_ after	Percentage of teats with hygiene score > 2 after cleaning	0.36	0.42	-0.41	0.47	
	Per_teat_not_covered_spray	Percentage of teats not spraying after milking	-	0.39	-	0.22	
Nutrition	Fresh_grass_milking	Fresh grass for milking cows	-	0.38	-	0.17	
	Maize_dry_cow	Maize for dry cows	-	-0.38	-	0.16	
	305_MY	305-d milk yield	-	-0.41	-	0.26	

Category	Abbreviation	Variable	Dimension 1	Dimension 2	Dimension 3	Total VAF[1]	Sum
Other	When_AMS_install	When did AMS install	-	-0.39	-	0.24	
	Feeding_system_milking	Feeding system for milking cows	-	-0.36	-	0.18	
	Type_liner	Type of liners	-	-0.36	0.37	0.27	
	Control_daily_work	Follow to daily work protocol	-	-	-0.35	0.27	
	Time_nondairy	Time spent on non-dairy farming	0.43	-	-	0.34	
	Use_checklist	Use checklist	-	-0.38	-	0.17	
	Fre_replace_filter	Frequency of replacing milk filter	-	-	-	0.10	
	When_replace_filter	When do you replace milk filter	-	-	0.38	0.19	
	Fre_manure_remove	Frequency of manure removal	-	-	-0.35	0.20	
	Additional_hand_manure	Remove manure by hand in addition to by robot	-	-0.43	-	0.28	
	N_cow_collect_wait	Number of cows collected in wait area	0.46	-	-	0.40	
	N_cow_connect_hand	Number of cows connected to robot by hand	-	-	-0.38	0.19	
	Time_monitoring_cow	Time spent on checking cows in barn	-	-0.35	-	0.16	
		Eigenvalue	7.38	6.36	4.66		18.4
		VAF (%)	6.53	5.63	4.12		16.28

[1]Cumulative variance accounted for in the first 3 principal components for each variable.
[2]Hyphen means the absolute value of the loading is > -0.35 and < 0.35.

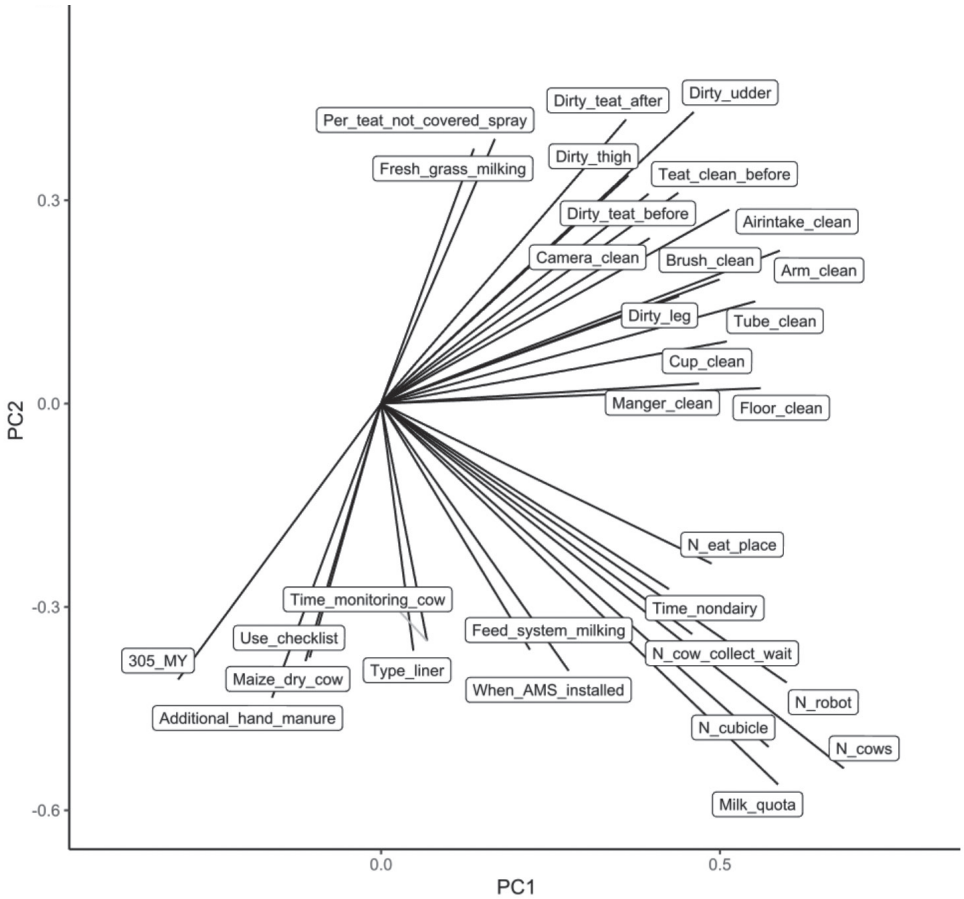

Figure 2. Loading plot for the first principal component (PC1) and the second principal component (PC2) of a non-linear principal component analysis on 113 risk factor variables based on data collected on 135 Dutch dairy farms using an automatic milking system, showing all variables with loadings > 0.35 or < -0.35 in PC1 or PC2 or both. The explanation of the variable names were given in table 3.

The variables that loaded high in the first PC were mostly related to farm size (5 variables), hygiene of the cows (8 variables) and of the AMS (8 variables). The variables that loaded high in the second PC were related to farm size (4 variables), hygiene of cows (3 variables) and some other variables. The variables that loaded high in the third PC were mostly related to hygiene of cows (7 variables). Based on the loadings and the corresponding transformation plots of these variables, the first PC represented larger farms with poor hygiene of cows and AMS, the second PC represented newly built smaller farms with poor cow hygiene and low milk production, and the third PC represented farms with good hygiene of cows. Regression model results of the PCs are presented in Table 4, showing that PC1 was positively associated with HeSCCav and NHiSCC, PC2 was positively associated with HeSCCvar,

NHiSCC, but negatively associated with IRCM. We plotted the PCs with 4 dependent variables and we did not find proof of nonlinear relationship. The adjusted R-squared for the regression models with PCs for HeSCCav, HeSCCvar, NHiSCC and IRCM were 0.041, 0.041, 0.086 and 0.089, respectively. The standard deviation of RMSE for PCR models with log10 (HeSCCav) was 0.0358, for log10 (HeSCCvar) was 0.0308 and for NHiSCC was 0.0078.

Table 4. Multivariable regression model results for 4 udder health variables with principal components (PC1 and PC2) from non-linear principal component analysis on the 113 risk factor variables, based on data collected on 134 Dutch dairy farms using an automatic milking system (after excluding one outlier farm).

				95% CI	
Dependent variable	**Independent variable**	**Number of farms**	**β[1]**	**2.5%**	**97.5%**
HeSCCav[2]	Intercept		5.416	5.394	5.438
	PC1	134	0.028	0.007	0.05
HeSCCvar[3]	Intercept		9.809	9.745	9.873
	PC2	134	0.081	0.017	0.144
NHiSCC[4]	Intercept		0.134	0.127	0.141
	PC1	134	0.011	0.004	0.018
	PC2	134	0.007	0.000	0.014
IRCM[5]	Intercept		-1.241	-1.362	-1.117
	PC2	134	-0.122	-0.24	-0.005

[1]Estimates of regression coefficient.
[2]Log_{10} transformed average herd SCC.
[3]Log_{10} transformed variance of average herd SCC on each test day.
[4]Average proportion of new high SCC cases.
[5]Farmer-reported annual incidence rate of clinical mastitis.

DISCUSSION

In this study, we explored farm level risk factors for mastitis on AMS farms by using regression analysis and NLPCA. Cow hygiene, as well as hygiene of the AMS were important factors related to various udder health parameters. In addition, we found several factors related to the farmers' behavior as well as herd size to be determinants of udder health. A previous study by Dohmen et al. (2010) was a first analysis on a part of the data that specifically aimed at hygiene variables. There might be other factors also playing a role in mastitis on AMS farms, so we carried out this study on many more potential risk factors. The results, however, confirmed that hygiene is important and, in addition, pinpoint that farm size is relevant to mastitis on AMS farms. The average farm size was 86 in our study, which was somewhat lower than 96 in 2002 and 105 in 2003 for dairy farms using AMS in Netherlands (Bijl et al., 2007). The udder health situation on a farm can be described by various parameters, including the 4 dependent variables used in our study, HeSCCav, HeSCCvar, NHiSCC and IRCM. Even though bulk

tank SCC is a widely used parameter for herd level udder health, the bulk tank SCC was only available as farmers' reported values; due to the high proportion of missing values in farmers' reported bulk tank SCC (14.8% missing) and also to have a more precise estimation of the herd SCC, we calculated the herd SCC using DHI data. These herd SCC were not weighted by milk production, even though it did not mimic the bulk tank milk SCC completely, the calculated average herd SCC still gives a good approximation of the BMSCC. The average HeSCCav was 271,828 cells/mL in our sample, which is higher than average HeSCCav of 214,000 cells/mL on Dutch AMS farms and 196,000 cells/mL on Dutch CMS farms (Steeneveld et al., 2015). We found NHiSCC in our study to be on average 13%, ranging from 4% to 29%, which is somewhat higher than the 9% (ranging from 0 to 18%) reported in a study on 1,903 Dutch CMS dairy farms (Mohd Nor et al., 2014). The IRCM was based on farmers' retrospectively reporting the number of clinical mastitis cases in the past year, which may well have been influenced by recall bias. Still, the average IRCM of 0.27 in our study is in line with the IRCM of 0.28 reported for Dutch CMS farms (Lam et al., 2013) and 0.29 for Dutch AMS farms (Huijps et al., 2008), although considerably higher than the IRCM of 0.20 reported by Mollenhorst et al. (2012) on Dutch AMS farms. These parameters, together with the general farm characteristics in Table 1, gave us the indications that the farms included in our study were representative for Dutch dairy farms using AMS.

We calculated HeSCCvar as the variance of the average herd SCC across test days, a parameter to our knowledge not previously studied. The HeSCCvar reflects temporal variation of herd SCC and thereby giving an estimate of the stability of the udder health situation. The three SCC based udder health variables were significantly associated with each other, but not to the IRCM (Figure 1). In our study, we did not find an association between IRCM and HeSCCav, which is in line with previous research (Barkema et al., 1998; Olde Riekerink et al., 2008).

The missing values in the data hampered regression modelling, as the number of observations differs between nested models, leading to incomparability of the model fit. To be able to run all models on the total sample, we therefore applied multiple imputation (Sterne et al., 2009). Although we did not find patterns in the missing values in our analysis, it is possible that such patterns were present. However, the effect of incorrect assumptions on patterns in missing values on the estimates is expected to be limited (Collins et al., 2001). A comparison of the regression models based on the imputed data with the models based on the non-imputed data showed that the differences between the estimates were negligible. The variables and the direction of the association between dependent variables and independent variables were the same, only the beta-estimate changed slightly (with a mean change of beta-estimate being 25%, with a 95% confidence interval of 14% to 36%). Moreover, with multiple imputation, we can use all farms in the model building process, which suggests that multiple imputation is a useful technique to facilitate the model building process, without substantially altering the model results. In the data

collection process, we did not evaluate the agreement between the 2 students in performing the interview or scoring. We had both of them trained and worked together, even though potential discrepancy in data collection between the 2 students might exist, we assume that this would guarantee a data collection process as consistent as possible. Since the questionnaire data were collected by the farmers' recall, lack of accuracy might exist. However, we had 28 numerical explanatory variables and 1 dependent variable (number of clinical mastitis cases last year) based on the farmers' recall. Most of these variables were related to farm size and frequency of cleaning AMS units (i.e., frequency of cleaning milk tube, frequency of cleaning teat cup, frequency of cleaning camera) , which were quite familiar to farmers. Farmers' recall on the number of clinical mastitis cases might have been problematic, however, not all farmers register clinical mastitis cases in Netherlands, so having all farmers recall the number of clinical mastitis cases provided us with an equal level of uncertainty for all farms. The final regression models contained a number of variables, several of which were hard to interpret in terms of causal associations. Some of these associations may be confounded, but given the high dimensionality of our data, a number of these associations may also have resulted from an inflated familywise Type-I error and expecting to explain more variance in the dependent variables, we also applied Principal Components Regression (Massy, 1965) with NLPCA, expecting the PCs could explain more variance in the dependent variables and avoid problems in the interpretation of the regression coefficients that are due to multicollinearity. However, the PCs in the regression models for 4 dependent variables only explained less than 1% of the variance in the dependent variables, and the variables that loaded high in each of the PCs were quite similar to the variables remained in the ordinary regression models, which suggested that NLPCA was useful in reducing the dimension of the dataset while it did not add much to the ordinary regression analysis in terms of explaining variance in the dependent variables. In the dataset, we had variables measured in different scales (units) and, without a rescaling to make sure that the all variables have zero mean and unit variance, PCA will be biased towards giving higher loadings to variables with larger variance. Reasons for this low cumulative VAF could be 1) randomness: to some extent, it is always existent in epidemiological studies; 2) resolution and accuracy of the measurements: we had 28 numerical variables based on the farmers' memory, which could contribute to a substantial amount of noise in the data, but on the other hand since these numerical variables were mostly related to farm size, frequency of cleaning AMS units, which were quite familiar to the farmers, we assumed that the accuracy of the measurements was acceptable. 3) unobserved factors: we had 10 Dutch mastitis experts working together during the study design, all obvious potential risk factors for mastitis on AMS farms were included in the questionnaire. But indeed, it is still possible that other factors outside our scope were still present and unmeasured in our study design. There are a number of potential reasons for the low amount of variance in the dependent variables explained by the PCs. First, the PCs extracted from the explanatory variables of course have not been strongly associated with the dependent variables (Hadi and Ling, 1998). In the ordinary multivariable analyses, however, there are a number of variables

that are present in the PCs that did show a strong association with the dependent variables (e.g., hygiene of udder and teats). It seems more likely that the strength of the causal association of the combined set of variables in a PC is lower than that of single variables. For instance, in the ordinary regression analysis, hygiene of the udder and the teats is strongly associated with udder health, but when these variables were combined in PC1 (21 variables with loadings > 0.35 or < -0.35, of which 6 variables were related to hygiene of cows) and PC2 (16 variables with loadings > 0.35 or < -0.35, of which 2 variables were related to hygiene of cows) with other hygiene-related variables, it may be that this causal relation is diluted by other, non-associated, variables. In addition, it seems that our analyses point out that there are likely to be other important drivers of udder health in AMS farms than the factors that we measured. Such an unobserved factor can lead to a low explained variance. Even though we collected data on a large number potential risk factors known to be related to udder health in CMS farms as well as risk factors that could potentially be related to udder health in AMS farms, it is possible that in AMS farms there are still factors that have not yet been identified in conventionally milking dairies by our expertise in the design of this study. This is intriguing and requires further research on factors that are specific for AMS farms.

Cleaner teats and less cows laying outside the cubicles were associated with lower HeSCCav in the regression model. The NLPCA results indicated that not only cow hygiene is associated with lower HeSCCav, but also good hygiene of the AMS is associated with lower HeSCCav as well as with less NHiSCC. It is known that good hygiene of cows is important. For instance, Schreiner and Ruegg (2003) and Sant'anna and Paranhos da Costa (2011) found good hygiene of cows to be associated with better udder health on CMS farms. A dirty udder and a dirty milking machine likely increase the chances of new intra-mammary infections (Cardozo et al., 2015), resulting in an overall increase in SCC and more NHiSCC. Hygiene of the AMS was positively associated with hygiene of the cows, suggesting that they have determinants in common, such as the farmers' attitude towards cleaning. Better and more frequent manure removal, a sufficient number of cubicles for the number of cows, effective and timely cleaning of the milking machine are likely to be effective interventions to improve udder health (Ruegg, 2006).

The percentage of teats not covered by spray at all was positively associated with more NHiSCC (Table 2). This is in line with previous work that showed a strong effect of post milking teat disinfection on transmission of mastitis pathogens (Lam et al., 1997). In our study, the average percentage of teats not covered by spray at all was 15.6%. On 13 out of 135 farms (9.6%), all teats that were inspected during the farm visit were completely missed by the spraying device. This clearly suggests that post milking teat disinfection deserves specific attention on AMS farms.

Participating in a *Salmonella* control program was found to be negatively associated with HeSCCav, HeSCCvar and NHiSCC, and participating in a bovine virus diarrhea (**BVD**) virus control program was negatively related to NHiSCC. Participation in an animal health program may have a direct beneficial

effect on udder health through a reduction of the total disease burden. Alternatively, participation in animal health programs may also suggest that the farmer is ambitious to improve animal health, including udder health, which merely reflects an attitude that is likely associated with a lower HeSCCav, lower HeSCCvar and less NHiSCC.

Other variables that reflect to some extent the farmers' attitude towards herd health, such as the use of bacteriological culturing of milk samples, checking the functioning of the AMS during milking on a regular basis and checking the AMS in addition to the regular checks, were surprisingly, positively associated with IRCM. It is unlikely that more frequent checking of the milking machine leads to more clinical mastitis. Rather, a more frequent checking of the milking machine may be the result of a high IRCM. On the other hand, the higher IRCM, to some extent reflects the accuracy of the farmer in diagnosing and recording CM, where farmers with a stronger motivation to improve udder health may actually report a higher IRCM. As farmers' attitude has been reported to be related to udder health on CMS farms (Jansen et al., 2009), interventions that contribute to a more positive attitude towards udder health likely are effective to improve udder health also on AMS farms.

We found that a larger milk quota, which essentially represents a larger herd size, was associated with a lower HeSCCvar, whereas the number of AMS, also a proxy for herd size, was positively related to more NHiSCC in the regression analysis. The same associations were also found with NLPCA. Herd size has been studied as a potential risk factor for clinical mastitis on CMS farms, but was not found to be significantly associated with IRCM (Schukken et al., 1990a; Nyman et al., 2007). To the best of our knowledge, this is the first time to find herd size related variables to be associated with lower HeSCCvar and more NHiSCC. On larger farms, the influence of temporary changes in SCC in some animals may be less visible because of the large farm size, resulting in a lower HeSCCvar. A larger number of animals seems to result in a HeSCCav that is less prone to fluctuations because of incidental effects on individual animals. Still, farmers with a large herd may have less time available per cow for udder health preventive measures, which may result in more frequent new infections, reflected in a higher NHiSCC. We did not find any herd size related variables to be associated with HeSCCav in the regression models. In the NLPCA, however, the first principal component summarized information on various herd size related factors, such as the number of cows, milk quota, number of feeding and lying places and number of AMS, and this PC was associated with a higher HeSCCav. Although these results are hard to interpret, our results suggest that udder health in larger AMS farms is more at risk than in smaller AMS farms. As currently, in many countries both the herd size and the proportion of farms with an AMS are increasing, further studies on dynamics of udder health in large AMS herds are needed.

The data that we used for this study are from 2008 and therefore relatively old. Naturally, since 2008 there have been changes in farm structure and mastitis management. The farm size of the farms in the current study was on average 86 cows. Since 2008 the farm size has increased but not considerably. A

study using data from 2013 found an average farm size of 103 (Steeneveld et al., 2015) and very recent data of 42 farms associated with the University Veterinary Practice (Utrecht, The Netherlands) showed an average farm size of 93 cows (unpublished data). Similarly, the udder health status, as represented by the average farm SCC did not change much since 2008. The farm average SCC of the farms in our study was 271,800 cells/mL, while this was 214,000 cells/mL in 2013 (Steeneveld et al., 2015) and 175,840 cells/mL (geometric mean) in 2018 (unpublished data). This means that the results of the current paper, based upon data from 2008 seem still to be valid for current AMS farms.

CONCLUSION

Multiple imputation is instrumental to deal with the missing values and NLPCA showed to be a useful technique to process high dimensional data in our study, which are common problems in questionnaire-based risk factor studies. Many of the risk factors identified in this study are comparable to those factors described in CMS farms, but farm size seems to be a factor that plays a specific role on AMS farms. Thus, our findings suggest that most of the management for mastitis control on CMS farms can be applied to AMS farms, while on large AMS farms, udder health may need more attention. Further studies on drivers of mastitis transmission in large AMS herds should aim to identify why larger AMS herds seem to have poorer udder health.

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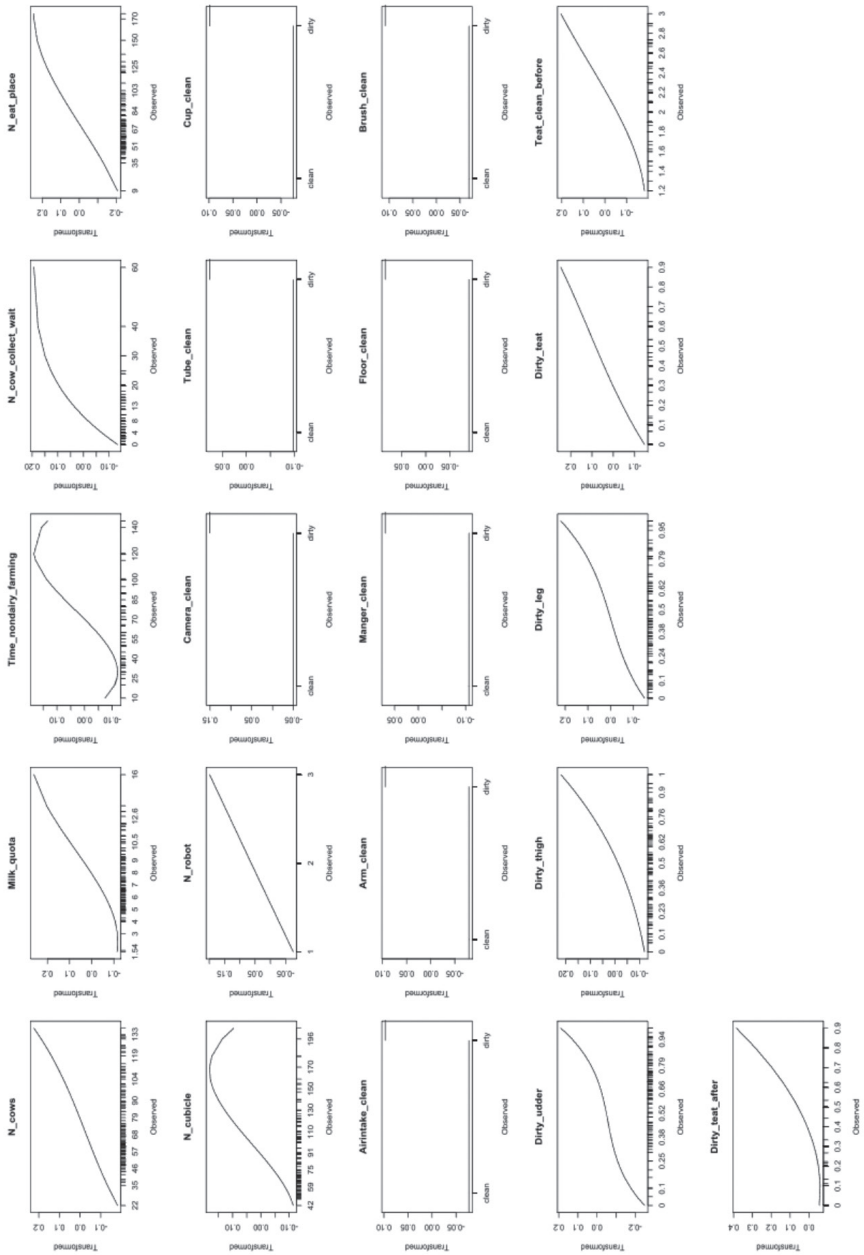
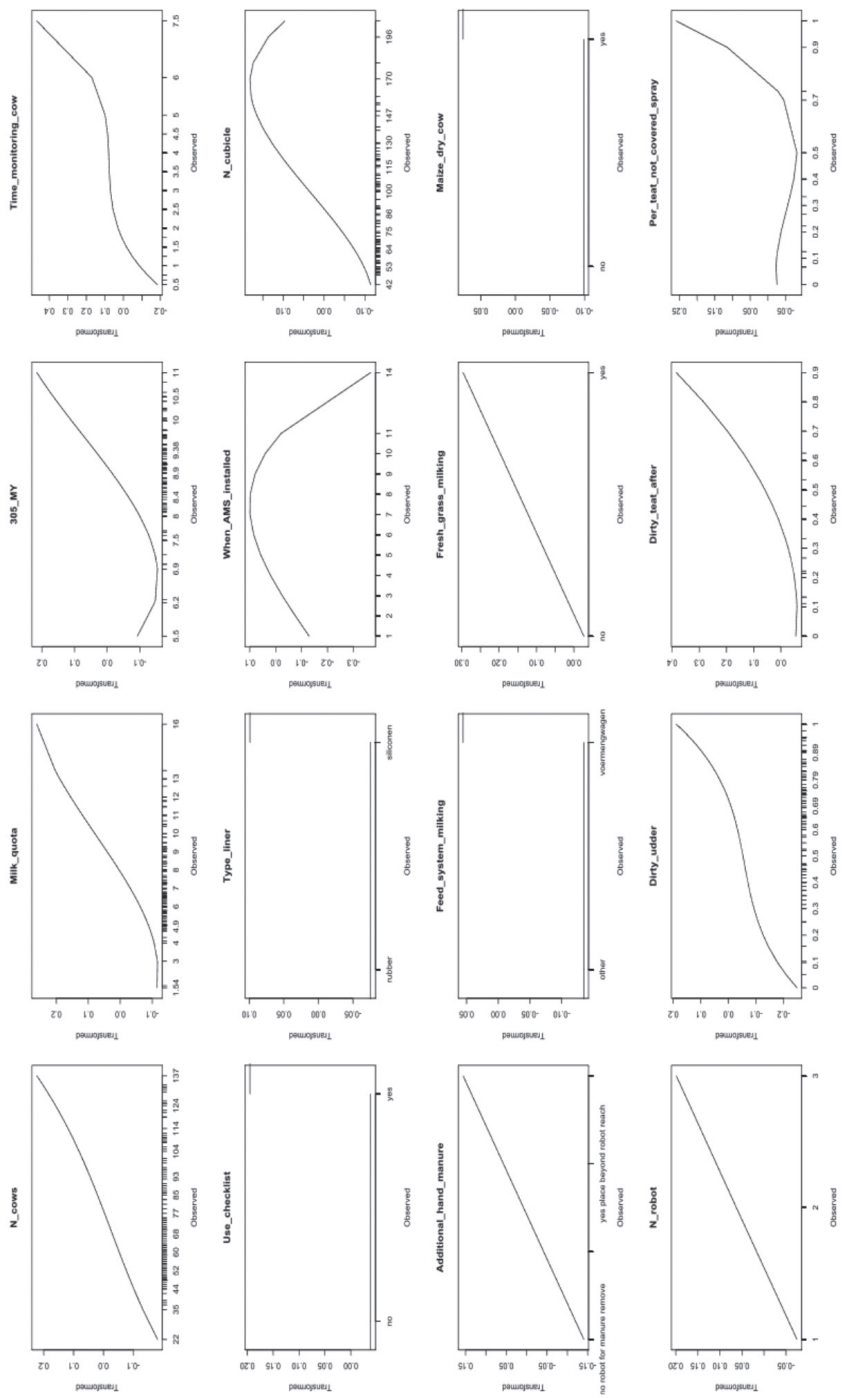
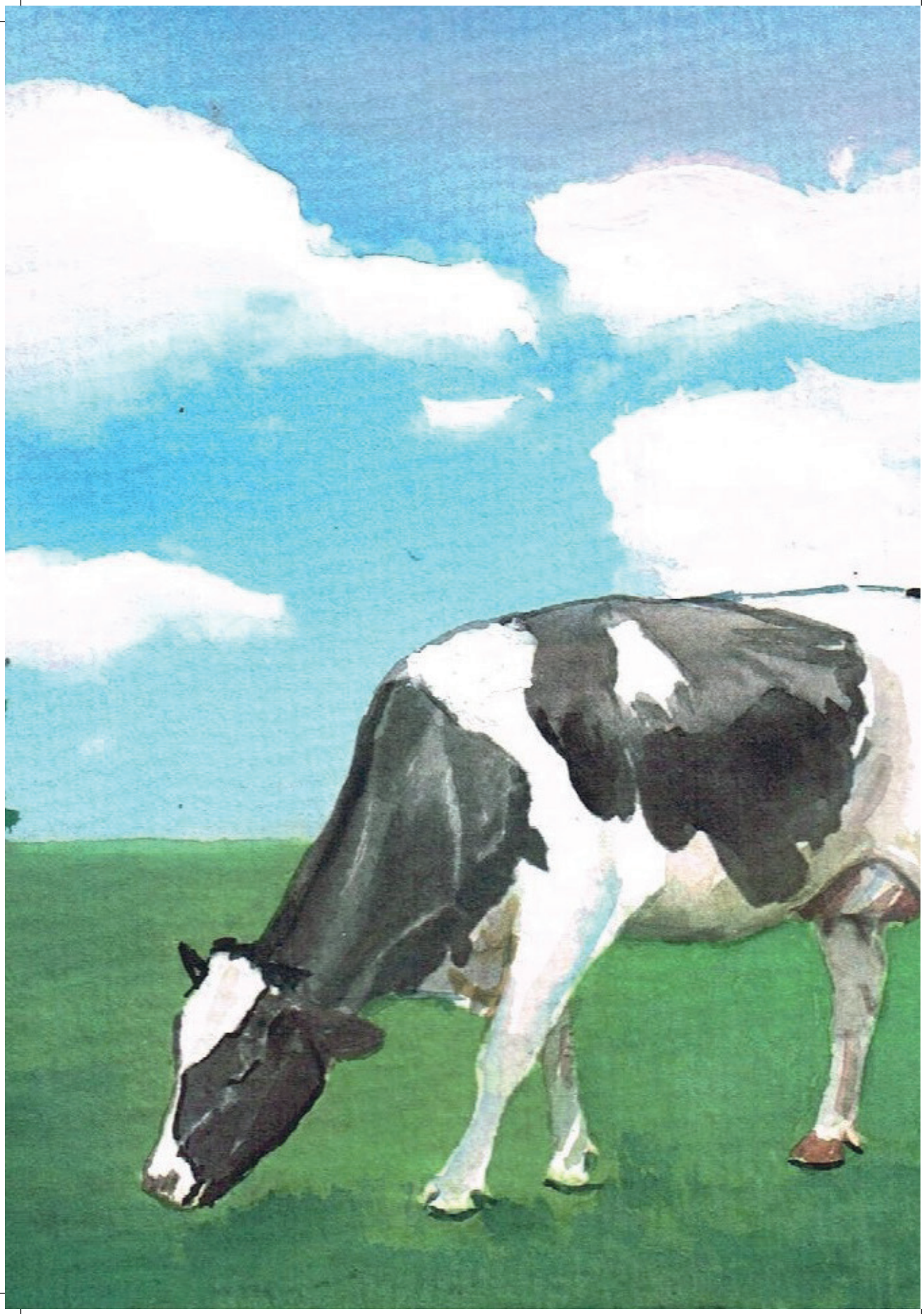


Supplemental Figure S1. Transformation plot showing the 590 observed values of each variable on the x-axis and the transformed values on the y-axis, for variables with loadings ≥ 0.35 or ≤ -0.35 in the first principal component from non-linear principal component analysis on the 113 risk factor variables based on data collected on 135 Dutch dairy farms using an automatic milking system.



Supplemental Figure S2. Transformation plot showing the observed values of each variable on x-axis and the transformed values on y-axis, for variables with loadings ≥ 0.35 or ≤ -0.35 in the second principal components from non-linear principal component analysis on the 113 risk factor variables based on data collected on 135 Dutch dairy farms using an automatic milking system.



Chapter 3

Performance of online somatic cell count estimation in automatic milking systems

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ABSTRACT

Somatic cell count (SCC) is one of the most important and widely used mastitis diagnostics. For detecting (sub)clinical mastitis, online SCC related measurements are more and more used in automatic milking systems (AMS). Sensors such as an automated online California Mastitis Test (O-CMT) allow for high frequency screening of high SCC cows within a herd, which makes it potentially powerful to identify episodes of mastitis. However, the performance of O-CMT measurements, as compared to SCC determined in the laboratory (L-SCC), has only scarcely been described. The aims of this study were (1) to assess the agreement between the O-CMT measurement averaged over different time windows and the corresponding L-SCC measurements; (2) to determine the optimal time window for averaging O-CMT as compared to L-SCC; (3) to explore the added value of time-series of frequent O-CMT measurements in individual cow udder health monitoring compared to L-SCC measurements.

Data were collected from 50 farms in 6 different countries that were equipped with AMS using O-CMT measurements and also performed regular L-SCC testing. We found that the overall concordance correlation coefficient (CCC) between O-CMT and L-SCC was 0.53 but differed substantially between farms. The CCC between O-CMT and L-SCC improved when averaging O-CMT over multiple milkings, with an optimal time-window of 24 hours. Exploration of time series of daily O-CMT recordings show that this is an effective screening tool to find episodes of high SCC. Altogether, we conclude that although O-CMT agrees moderately with L-SCC, because of its high measurement frequency, it is a promising on-farm tool for udder health monitoring.

INTRODUCTION

Mastitis is one of the main diseases in dairy cattle and leads to economic losses, usage of antimicrobials, and reduced animal welfare (Bradley, 2002; Hogeveen et al., 2011). Udder health monitoring programs including regularly measured somatic cell count (SCC) have been widely used as a first step to improve udder health (Mele et al. 2001). These monitoring programs create awareness of udder health problems which, combined with mastitis prevention plans, motivate farmers to change on-farm udder health management to decrease the incidence of mastitis (Green et al., 2007; Lam and De Vliegher, 2014).

The SCC of composite cow milk, as part of a dairy herd improvement (DHI) program, is a key indicator for udder health monitoring in current practice (Pyörälä, 2002) and is generally measured using flow cytometry-based laboratory techniques (Damm et al., 2017). This routinely measured SCC in the laboratory (L-SCC) has long been the standard for udder health monitoring (Schukken et al., 2003). The collection and shipping of samples for SCC measurement, however, is costly and time consuming and therefore generally DHI testing is done only every 3 - 6 wk. More frequent measurements would allow for earlier diagnosis but requires an on-farm test that can be performed at low per sample costs. The online California Mastitis Test (O-CMT) measurement is an automated sensor for mastitis monitoring in dairy farms with an automatic milking system (AMS).

The principle of the O-CMT sensor evaluated in our study is based on an automated CMT by taking a fixed volume of well mixed composite milk from cow milking. The milk is mixed with a fixed volume of reagent, after which the viscosity of the mixture is measured. The measured viscosity is transformed into a value, expressed in cells/mL, based on a calibration curve (Whyte et al., 2004). The O-CMT is not comparable to L-SCC in terms of test characteristics, missing data, calibration and quality control, but due to frequent measurements, it may serve as a useful on farm screening tool. Although a single O-CMT measurement may not be precise, averaging multiple O-CMT recordings within different time windows may be helpful in gaining precision. Thus, we assume frequently measured O-CMT averaged over a certain time window may yield a better correlation to L-SCC.

Until now, a number of comparisons between SCC measured on farms and L-SCC have been published (Casura et al., 1997; Leslie et al., 2007; Kamphuis et al., 2008; Mollenhorst et al., 2010; Neitzel et al., 2014; Sørensen et al., 2016). Due to the characteristics of the gelling process of the mixture, the agreement between O-CMT and L-SCC was found to be poorer in low SCC ranges (< 200,000 cells/mL; Whyte et al., 2004), while higher ranges of SCC (> 500,000 cells/mL) show a fair to good correlation (Kamphuis et al 2008). Hence, the performance of the O-CMT likely depends on the udder health situation of the farm. However, the performance of O-CMT in a large number of herds with varying udder health status is unknown and thus the practical value of this frequent O-CMT measurements in the field is unclear. Therefore, the aims of this study were (1) to assess the agreement between

O-CMT measurements in different time windows and L-SCC measurements under field conditions; (2) to determine the optimal time window for averaging O-CMT as compared to L-SCC; (3) to explore the added value of time-series of frequent O-CMT measurements in individual cow udder health monitoring compared to L-SCC measurements.

MATERIAL AND METHODS

Data Collection

Routinely collected O-CMT data from January 1st, 2015 to April 29th, 2016 from AMS farms having an O-CMT sensor system produced by Lely Industries N.V. (Maassluis, the Netherlands) and the DHI milk production recording data from the same farms over the same period were provided by Lely Industries N.V. Details of the data collection can be found in Jensen et al. (2017). The data consisted of the rough, non-validated data that farmers also use. In all datasets, country and farm identifications were transformed to non-traceable identifications by Lely because of privacy concerns. The AMS data consisted of country and farm identification, cow identification, parity, calving date, date and time of milking and O-CMT measurement. The default measurement frequency of O-CMT was every third milking. When a cow had a high SCC (>200,000 cells/mL), the measurement frequency became higher. Farmers were advised to calibrate the sensor twice per year using standardized cow milk samples provided by Lely. The DHI data consisted of farm identification, cow identification, DHI test date and L-SCC. The L-SCC were tested in different laboratories, depending from which country the farm was. Because of the position of those laboratories in the milk payment scheme, the laboratories were certified (ISO 13366-1) to ensure the quality of measurements. This study was carried out in accordance with the commitments contained in the Basel Declaration and adhered to the General Data Protection regulations of the European Union. As no animal experiments were performed, no ethical approval was required for this study.

Data Preparation

In the dataset we observed a small proportion (0.009%) of O-CMT being from milkings with an extremely low milk yield (< 1 kg). Incomplete milkings with O-CMT might occasionally be present in our dataset. The raw dataset contains 7,427,010 records and was cleaned as follows:

1. records (n = 95,669) with composite milk yield per milking < 1 kg were deleted;
2. records (n = 153,735) within 7 days after calving were deleted because of the confounding effect of early lactation on the SCC;
3. records with no O-CMT values (n = 4,527,244) or O-CMT = 1,000 cells/mL (n = 39,455) were deleted; The latter records were deleted, because the sensor automatically transforms all O-CMT ≤ 1,000 cells/mL to 1,000 cells/mL;

4. records from cows on L-SCC test dates when no L-SCC was available (n = 730) or with L-SCC ≤ 1,000 cells/mL (n = 377) were deleted;
5. records (n = 358,985) from cows with an L-SCC < 2, 000 cells/mL were deleted;
6. records (n = 2,693) from farms with ≤ 100 L-SCC measurements were deleted;
7. records from 7 days before until 7 days after the L-SCC test dates were selected for each cow. Within these 15 days (7 days before and after DHI test date plus the DHI test day) period, only records with valid O-CMT value for every day were selected, which resulted in 1,816,144 deleted records.

The resulting cleaned dataset used for further analyses. After cleaning the dataset, all SCC values were $\log_{10}$-transformed for the purpose of obtaining an approximate normal distribution.

Assessment of Repeatability of O-CMT

Before the evaluation of agreement between the two tests, we assessed the repeatability of the O-CMT measurements. We defined an episode as being the period 48h before and after an L-SCC test date. Consequently, the records within 48h before and after the L-SCC test dates for each cow were selected from the cleaned dataset. Only episodes with ≥ 1 O-CMT measurements for every day were selected. A linear mixed regression model was constructed using the O-CMT measurements as dependent variable and episode, herd and cow as random effects. That way we were able to estimate the variance in O-CMT within each episode for each cow from every herd. Consequently, the intraclass correlation coefficient (ICC), calculated from the four variance components (episode, cow, herd and the residual) extracted from this linear mixed model, represents the repeatability of O-CMT measurements (which equals to 1 – the underlying "true" variation and the measurement error of O-CMT measurements within the episode).

Agreement between O-CMT and L-SCC

Concordance correlation coefficient (CCC) between two continuous measurements is one of the most commonly used methods to evaluate the agreement between two tests (Carrasco et al., 2009). In this study, we calculated the CCC between O-CMT and L-SCC to evaluate the measurement performance of single O-CMT and its averages calculated over multiple time windows.

Single Comparison For the single comparison between O-CMT and L-SCC, all L-SCC and a randomly sampled O-CMT record per cow on the DHI test dates were selected. We first examined the CCC between the selected O-CMT records and the corresponding L-SCC records using the Bland-Altman plot (Bland and Altman, 1986) to display the relationship between O-CMT and L-SCC. Meanwhile, we calculated the CCC between these O-CMT and L-SCC records.

Because DHI test results only had a test date and no time stamp, for each DHI test date, there were possibly multiple O-CMT records. All of these O-CMT records were used in the CCC calculation with equal weight in determining the optimal time window that would result in the highest CCC between average of multiple O-CMT and L-SCC.

Time Window for Averaging Multiple O-CMT To determine which time window, using the average of O-CMT measured within, resulted in the highest correlation between the O-CMT and the L-SCC, 7 time windows centered around the DHI test dates were created. Time windows were constructed as multiples of 24h before and after the center of the DHI test date, leading to 7 time windows (spanning 24h, 48h, 72h, 96h, 120h, 144h and 168h). We first selected the records within the 168h time window (168h before and after the DHI test date) for each cow and each DHI test date from the dataset. The records within the 168h time window for each L-SCC record of each cow were regarded as an episode. For each episode, the number of O-CMT measurements per day was counted. Episodes were included when they were from farms that had at least 100 episodes with ≥ 1 O-CMT measurement(s) on every day within the episode. For each episode, the average of O-CMT for each of the 7 time windows was calculated.

To calculate the CCC, a linear mixed model was applied using the lme function in the nlme package (version 3.1-142; Pinheiro et al., 2019). To calculate the overall CCC of all farms, the test method (binary variable: O-CMT or L-SCC) was included in the model as fixed effect; random herd and random cow effect were also included in the model. To calculate the individual farm level CCC, test method and individual cow were used as fixed effect and random effect respectively, by using the epi.ccc function in epiR package (version 1.0-11; Stevenson, 2020). The CCC between the average of multiple O-CMT within different time windows and L-SCC were calculated for 3 different ranges of L-SCC (L-SCC within 1,000-9,999,000 cells/mL, 100,000-1,500,000 cells/mL (the performance range of L-SCC), 200,000-9,999,000 cells/mL).

In addition to identifying the optimal time window, we tried to find potential factors associated with the individual herd level CCC at the optimal time window using the available data. A linear regression model was built using the individual herd CCC as dependent variable and herd average parity, monthly herd geometric mean of L-SCC and monthly herd milk yield as independent variable. A full model, as well as a model using backward selection based on AIC, were fitted. All analyses were performed in R version 3.6.2 (R core team, 2018)

Case-wise Evaluation of O-CMT and L-SCC Measurements

The time window which resulted in the highest CCC in the previous analysis was used for calculating the moving averages for multiple O-CMT measurements over a longer time period. Four different

O-CMT24h patterns were selected, which were representative of SCC patterns observed in field data. These selected O-CMT patterns were plotted along with the L-SCC measurements in the same time frame. In this way, the practical relevance of frequent O-CMT measurements in detecting high SCC episodes due to (sub)clinical manifestations of mastitis was illustrated.

RESULTS

Descriptive Statistics

The descriptive statistics of the final dataset for the calculation of CCC between O-CMT and L-SCC are provided in Table 1. In total, 434,371 records from 4,829 cows at 50 farms in 6 countries were used in the analysis. Large differences in herd size were seen between farms and countries, with farms from country 2 and country 3 on average being larger than other farms. Overall, O-CMT values were higher than L-SCC values. All the herd average L-SCC values were below 200,000 cells/mL and only farms from country 2 had a herd average O-CMT higher than 200,000 cells/mL.

Table 1. General descriptive statistics of the farms in the cleaned dataset for SCC measured online (O-CMT) and SCC measured in the laboratory (L-SCC) and the data for calculating the correlation between both. A total of 2,361,484 records from 7,788 cows in 52 farms in 6 countries were included in the cleaned dataset. The numbers with parenthesis are averages and parenthesis are 2.5% to 97.5% quantile.

Country	Total Number of farms	Average number of cows per farm	Number of records	Farm geometric mean of L-SCC (×1,000 cells/mL)	Farm geometric mean of O-CMT on L-SCC test dates (×1,000cells/mL)	Farm average CV[1] of L-SCC	Farm average CV of O-CMT on L-SCC test dates per cow	Concordance correlation coefficient between L-SCC and single O-CMT one L-SCC test dates per farm
1	1	36	1,787	75	195	0.38	0.32	0.518
2	19	114 (12 to 475)	9,644 (485 to 34,644)	165 (49 to 336)	213 (83 to 339)	0.45 (0.37 to 0.53)	0.53 (0.46 to 0.61)	0.658 (0.241 to 0.851)
3	9	150 (69 to 278)	11,231 (4,302 to 29,613)	141 (75 to 262)	198.2 (132 to 313)	0.44 (0.36 to 0.52)	0.52 (0.43 to 0.62)	0.592 (0.437 to 0.778)
4	1	59	12,170	74	102	0.7	0.58	0.479
5	13	68 (17 to 161)	8,800 (579 to 28,623)	155 (39 to 315)	165 (79 to 309)	0.49 (0.37 to 0.61)	0.62 (0.51 to 0.72)	0.578 (0.341 to 0.759)
6	7	46 (28 to 62)	3,142 (1,955 to 4,959)	92 (45 to 141)	146 (97 to 230)	0.41 (0.34 to 0.49)	0.40 (0.35 to 0.46)	0.421 (0.136 to 0.533)
Total	50	97 (13 to 282)	8,693 (480 to 32,723)	145 (38 to 317)	186 (78 to 326)	0.46 (0.19 to 0.79)	0.53 (0.29 to 0.86)	0.525 (0.142 to 0.787)

[1]Coefficient of variation

Assessment of Repeatability of O-CMT

A total of 144,048 records from 14,504 episodes and 4,829 cows at 50 farms in 6 countries were used for the estimation of the repeatability of O-CMT measurements. The estimated ICC was 0.58, which suggests that 42% of the variance within the episode was due to the O-CMT measurement. However, it was not possible to distinguish the "true" variation between O-CMT measurements from measurement error of the O-CMT.

Concordance correlation coefficient between L-SCC and O-CMT

Single Comparison. In total, 29,008 O-CMT records of 4,829 cows in 50 farms from 6 countries could be linked to 29,008 valid L-SCC measurements on the same day.

Figure 1 shows the Bland-Altman plot of the log10-transformed single O-CMT compared with the L-SCC measurement. The Bland-Altman plot suggests that the correlation between O-CMT and L-SCC is non-linear. The difference between these two measurements decreases in the high SCC area.

Figure 2A displays a scatter plot of the L-SCC and the randomly selected O-CMT measurement on each DHI test date per cow and gives the CCC across several L-SCC ranges (1,000-9,999,000 cells/mL, 100,000-150,000 cells/mL, 200,000-9,999,000 cells/mL), showing that the agreement between L-SCC and O-CMT is better in the higher SCC regions but not necessarily with a higher CCC. The overall CCC between L-SCC and the average of O-CMT measurement within a 24h time window was 0.53 (95% CI: 0.14 to 0.79).

Time Window for Averaging Multiple O-CMT Figures 2B to 2I show that the CCC between averaged O-CMT within different time windows and L-SCC increased from Figure 2A to Figure 2B and to Figure 2C (the 24h time window) for all the 3 SCC ranges. The CCC in the 3 SCC ranges only increased marginally, when the time window was further expanded (Figure 2D to 2I). Therefore, we considered 24h as the optimal time window to average the multiple O-CMT measurements in this study.

We found substantial variation in CCC between O-CMT24h and L-SCC between farms. The farm-level CCC was positively related to the farm's geometric mean L-SCC (Table 3 and Figure 3).

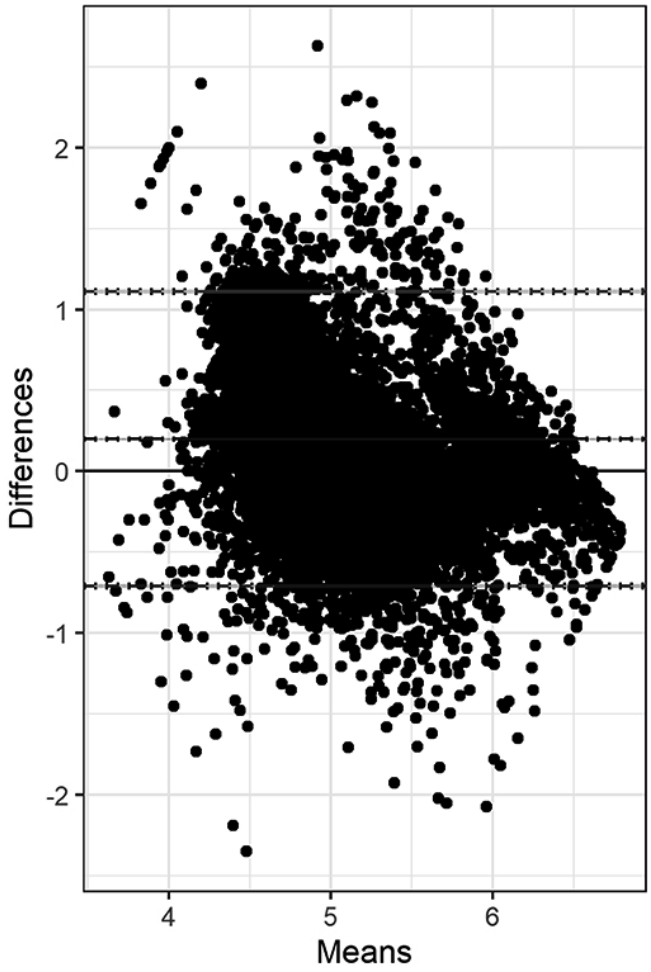

Figure 1. Bland-Altman plot displaying the difference between single log10-transformed online CMT (O-CMT) values and log10-transformed laboratory measured SCC (L-SCC) against the average of both measurements on the DHI test days. Most of the records are within the limits of agreement. Overall, the differences between the two measurements are decreasing.

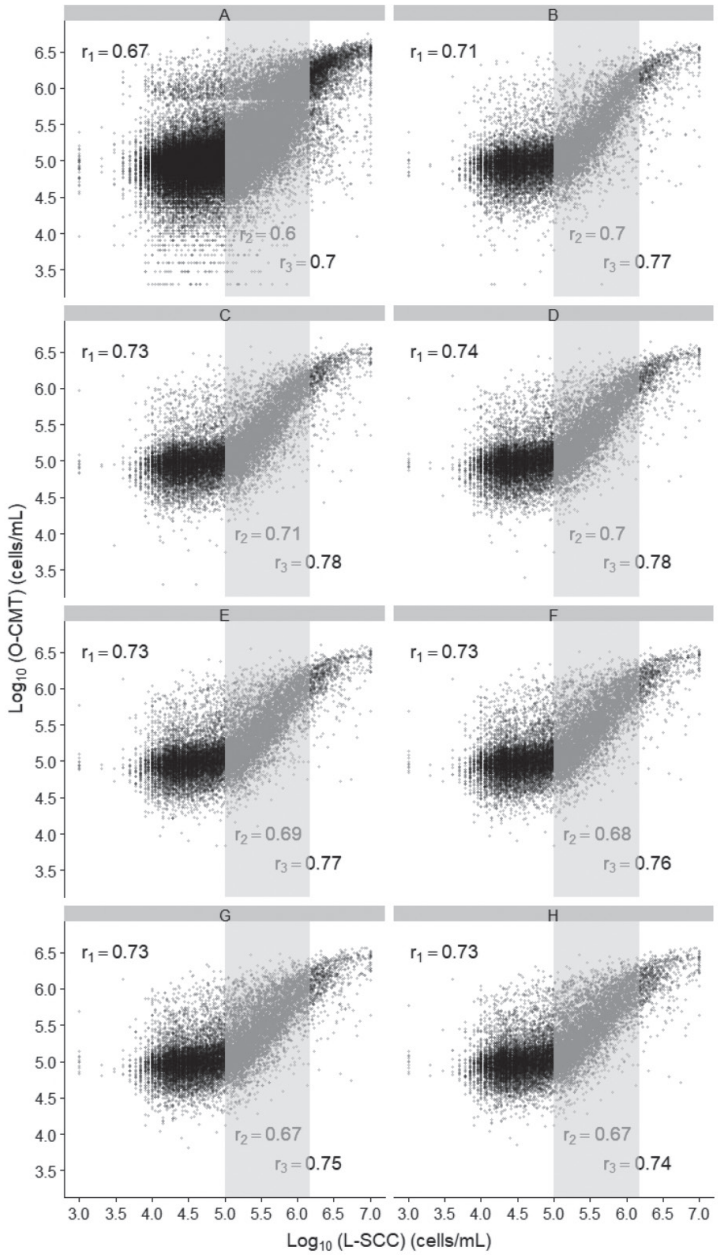

Figure 2. Scatter plot of the log10-transformed online CMT (O-CMT) values for randomly sampled one O-CMT records on DHI test dates against log10-transformed laboratory measured SCC (L-SCC) (A) and the average of multiple O-CMT within different time windows against L-SCC (B–I, corresponding to time windows from 0 to 168 h, increasing by steps of 24 h); ccc1 represents the overall concordance correlation coefficient between log10-transformed O-CMT and log10-transformed L-SCC, ccc2 is the concordance correlation coefficient with L-SCC within the range of 100,000–1,500,000 cells/mL and ccc3 is the concordance correlation coefficient with L-SCC in range of 200,000–9,999,000 cells/mL. Farms with ≥ 100 DHI tests with valid SCC results measured by O-CMT and L-SCC were included.

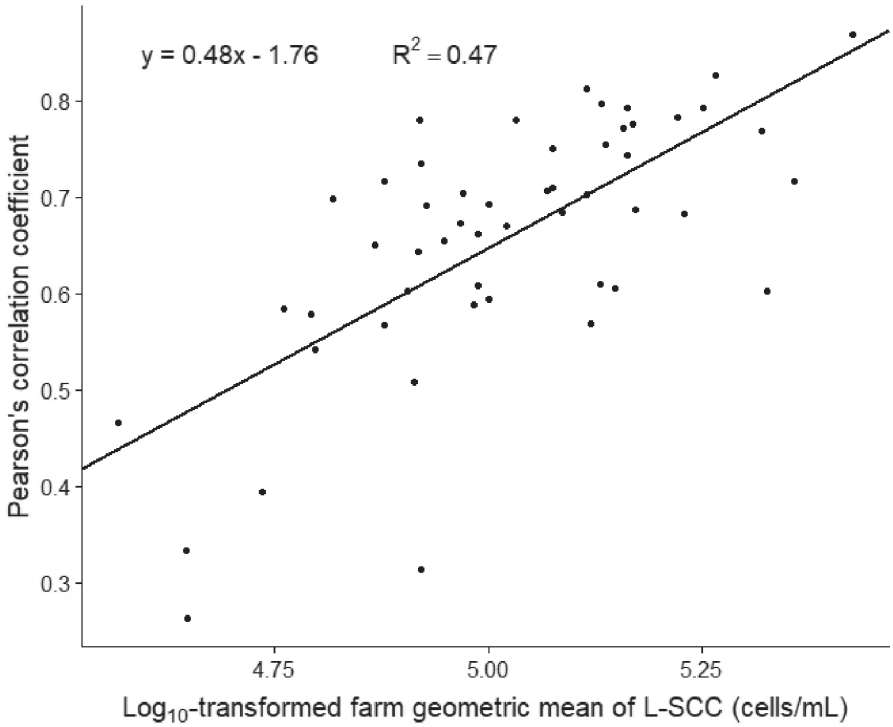

Figure 3. Scatter plot of concordance correlation coefficient between the average of multiple $\log_{10}$-transformed online measured CMT (O-CMT) values over a 24h time window and $\log_{10}$-transformed laboratory measured SCC (L-SCC) against the $\log_{10}$-transformed geometric mean herd SCC per farm on 50 farms. The regression line has a beta estimate of 0.54 and the R-squared is 0.64.

Figure 4 gives the number of O-CMT records per L-SCC record in different SCC ranges for the 7 time windows. It is obvious that the number of O-CMT measurements does increase with longer time windows. Moreover, it is also visible that more O-CMT measurements are made when O-CMT is higher (> 200,000 cells/mL). A 0h time window averages about 2 O-CMT values, whereas a 24h time window contains on average about 5 O-CMT records.

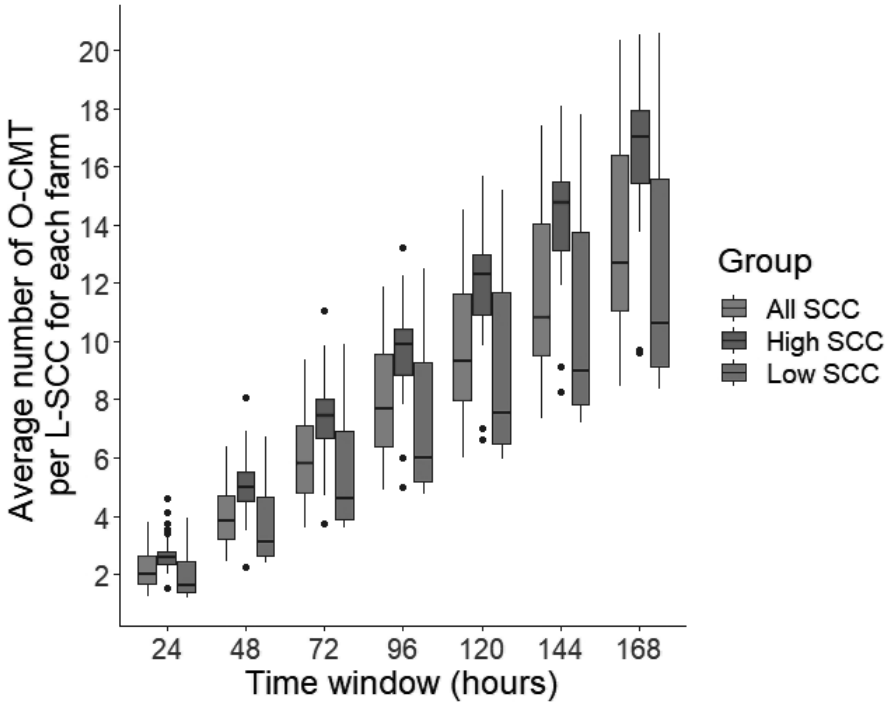

Figure 4. Farm average number of online CMT (O-CMT) values per SCC value measured in the laboratory (L-SCC) for all O-CMT and for L-SCC performance range (100,000 -1,500,000 cells/mL) as well as high SCC range (> 200,000 cells/mL) separately for different time windows. The first boxplot of each time window was all SCC group, the second boxplot of each time window was the high SCC group, and the last boxplot of each time window was the low SCC group.

Case-wise Comparison of O-CMT with L-SCC Measurements

Figure 5 displays 4 different SCC patterns from 4 different cows that were representative of our data. Overall, the O-CMT48h patterns were corresponding to the L-SCC patterns for each cow, Figure 5A shows a healthy udder before 130 DIM, with indication of two short (new) intramammary infection (IMI) occurring around 134 and 162 DIM, and of a chronic persistent IMI starting around 190 DIM; Figure 5B shows an IMI in early lactation that seemed to have cured between 64 to 180 DIM with indications of a new IMI in late lactation; Figure 5C presents an udder with a chronically persistent IMI with large variation in day-to-day O-CMT48h; Figure 5D indicates a healthy udder with a brief IMI in the late stage of lactation.

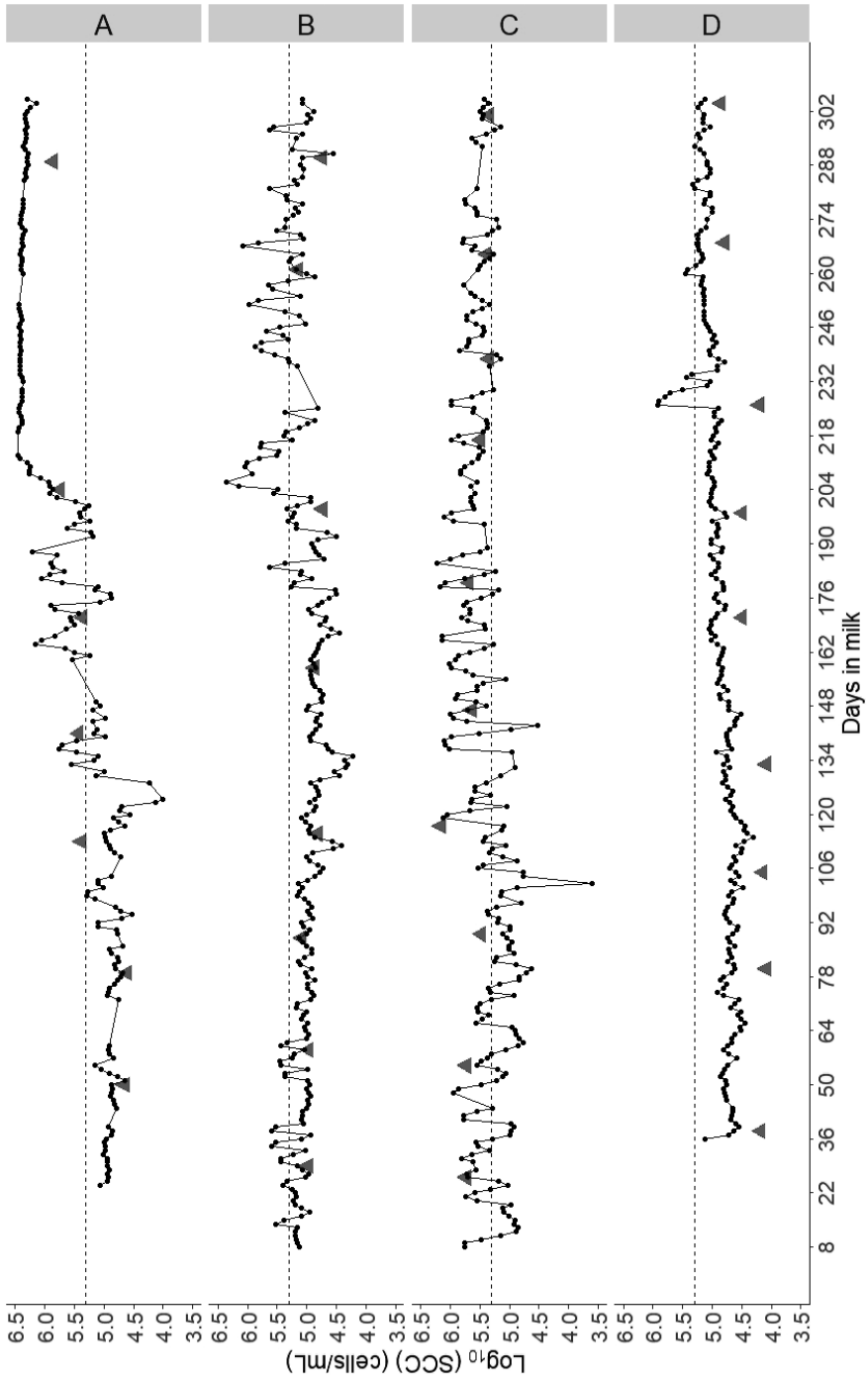

Figure 5. Four different SCC patterns to demonstrate the value of frequently measured online SCC in individual cow udder health monitoring. A indicates a chronic intramammary infection; B suggests an infected udder that is cured followed by a re-infection; C displays a cow likely with chronic IMI that shows a fluctuating SCC pattern and D probably is a healthy udder with one brief high SCC episode. The triangles represent laboratory measured SCC results and the dots connected by a line represent the online CMT measurements averaged over a 24h time window. The dashed horizontal line represents 200,000 cells/mL.

DISCUSSION

In this study, we aimed to evaluate the performance of O-CMT measurements in comparison to L-SCC. The value of O-CMT measurement is an estimation of SCC within ranges instead of an exact measurement of SCC (Whyte et al., 2004). Hence the O-CMT values should be interpreted with caution. The overall CCC between O-CMT within a 24h time window and L-SCC in 50 farms was 0.53 (95% CI: 0.14 to 0.79). The CCC increased most when averaging O-CMT over a 24h time window. Our results suggest that frequent O-CMT measurement is a valuable on-farm tool for monitoring udder health of individual cows, despite the fact that a single O-CMT measurement may be less accurate than a single L-SCC measurement.

The data we used in this study consisted of rough, non-validated data, representative of how the data arises in practice. The samples from the O-CMT differed from the samples for the L-SCC. Besides that, it is clear that there is a lower level of quality control for the O-CMT measurements, for instance by non-optimal calibration procedures, in comparison to the L-SCC measurements. This may jeopardize the agreement between the two tests. Therefore, a direct comparison between the measurement systems in order to establish the preciseness of the O-CMT measurement is impossible with our data. However, by comparing the O-CMT measurements with the L-SCC measurements on milk from the same cow on the same day, we were able to provide insight in the practical usability of the O-CMT measurements.

Prior to the correlation analysis, we evaluated the repeatability of the O-CMT measurements within a 48h time window assuming that the underlying SCC of a cow was stable within this 48h time window (5 days). The repeatability, as represented by the ICC, was 0.58. Since natural daily variation in SCC exists, we consider the repeatability of O-CMT measurement to be acceptable within the period of 5 days.

We found an overall CCC between O-CMT and L-SCC of 0.53, which is in line with previous studies, that found values somewhat higher or lower than our estimate (Table 2). Previous studies, however, only used a small number of farms to assess these correlations. In our data, we found a large variation in CCC between farms. This between-farm variation was largely explained by the farm level L-SCC (Figure 3), likely due to the fact that the correlation is higher in the higher SCC ranges. In other words, the CCC might depend on the prevalence of high SCC cows on farms. As displayed in Figure 1, the difference between O-CMT and L-SCC was deceasing as the herd average L-SCC increased. There are several other reasons for the fact that the CCC between O-CMT and L-SCC differs between farms. First, although the sensors are “factory calibrated” and farmers are advised to perform the calibration twice per year, not all farmers may actually have done this. Neitzel et al. (2014) reported a significant difference between sensor devices in measuring the O-CMT and showed that the Pearson’s correlation coefficient between O-CMT and L-SCC was higher after calibration. These differences in calibration between farms or sensors will likely have led to an underestimation of the true overall correlation between both SCC measurement methods relative to using well-calibrated sensors.

Table 2. Correlation between online SCC estimation and the SCC measured in the laboratory in different studies.

Study	SCC estimation method	Country	Number of farms	Number of AMS or SCC sensors[1]	Number of cows	Number of records	Correlation
Casura et al., 1997	CMT[2]	NA[3]	1	NA	298	2,331	0.57
Leslie et al., 2007	CMT	Canada	1	2	140	1,000	0.71
Kamphuis et al., 2008	CMT	New Zealand	1	2	200	456	0.76
Mollenhorst et al., 2010	CMT	Netherlands	3	6	191	3,191	0.47
Neitzel et al., 2014	CMT	Germany	1	7	165	1,357	0.2-0.57
Sørensen et al., 2016	Flow cytometry	Denmark	7	>16	2,325	713,326	0.93[4]
Current study	CMT	6 countries	50	113	4,829	434,671	0.53[5]

[1]Automatic milking system or online somatic cell count sensor.
[2]SCC estimated based on the California mastitis test principle.
[3]Not found.
[4]The square root of R squared from regression using log-transformed L-SCC as dependent variable and log-transformed O-CMT as independent variable.
[5]Concordance correlation coefficient between the average of online-SCC within a 24h time window and the SCC measured in the laboratory.

Table 3. Estimates from linear regression model using the herd level concordance correlation coefficient between online CMT and SCC measured in laboratory as dependent variable and the herd average of monthly geometric somatic cell count (SCC_{herd}), herd average parity ($Parity_{herd}$), as well as herd average monthly milk yield (Milk $yield_{herd}$) as independent variables. Backward selection using AIC was applied for model selection. The full model included all the independent variables and the final model only with the variable remained in the model after model selection.

	Estimate	
Variable	Full model	Backward selection model
Intercept	-2.33	-2.16
SCC_{herd}	0.55	0.54
$Parity_{herd}$	0.05	
Milk $yield_{herd}$	0	

Although CCC between O-CMT and L-SCC was rather not sufficient, we consider there are several reasons for this imperfect agreement between O-CMT and L-SCC. First, the O-CMT evaluated in our study uses a different technique, based on a CMT derived method to quantify the O-CMT whereas L-SCC actually counts the number of cells using flow cytometry. The online sensor has an algorithm that transforms the viscosity of the gel formed by DNA and test reagent, to an O-CMT value based on calibration against L-SCC. Thus, by definition, the indirectly measured single O-CMT is less accurate than a single L-SCC measurement. Second, the performance range of L-SCC (the range in which its accuracy is guaranteed) is 100,000 to 1,500,000 cells/mL (Foss, 2017) while we noticed that more than half of the L-SCC measurements in our dataset were less than 100,000 cells/mL. Measurements outside

the range in which the two tests perform well contributed substantially to the imperfect correlation between these two measurements (Figure 2A). The scatter plots in Figure 2 display weak S-shape, suggesting that the algorithm that transforms viscosity to an SCC value can be further optimized to better correlate to the L-SCC reference test. By adapting the transformation, the association between O-CMT and L-SCC can be made more linear, which should result in a higher (linear) correlation between the two. Lastly, although we did not evaluate that in this study, farmers may not re-fill the CMT reagent in time. Field experience learns this occurs and thus it may also affect the correlation between O-CMT and L-SCC.

With the availability of novel on-farm milk quality sensors, quality control of such measurements also has to be implemented on-farm. For decades, laboratories have calibrated their methods and compared their results, for instance by the use of ring trials. In contrast with these highly controlled laboratory systems, there is no systematic quality control system in place for automated on-line milk quality measurements. Since these on-farm milk quality systems become more and more important, it would be good if quality control programs for on-farm milk quality systems would be developed.

The L-SCC in our dataset were measured in different laboratories. Potentially there may be differences in L-SCC measurement between laboratories. However, data quality control in the laboratories for L-SCC measurements was assumed to be good because these laboratories are also involved in quality-based milk payment schemes and work under ISO certification (ISO13366-1). Meanwhile, by using a random herd effect in linear mixed models, potential laboratory effects were corrected for in the statistical modelling.

In Figure 2, we showed that the overall CCC between O-CMT and L-SCC in the range of 1,000 – 9,999,000 cells/mL, increased mostly at a 24h time window. The overall CCC between O-CMT and L-SCC was increasing only slightly with longer time windows. There seems to be an optimum time window for averaging O-CMT, and we suggest 24h as the optimal time window, in which the random error present in single measurements is strongly reduced, but the capacity to monitor infection dynamics over time is still acceptable.

The number of milkings with an O-CMT measurement per L-SCC measurement is substantially higher for high L-SCC (> 200,000 cells/mL) than for all SCC range, because of the algorithm that prescribes to measure O-CMT every milking after a high measurement is recorded, while the sensor only measures O-CMT every third milking in low SCC cows.

Figure 5 illustrates that the O-CMT measurements present the same trend as L-SCC, while giving more information on short high SCC episodes. This information is missed by L-SCC, given that DHI test is normally performed every 3-6 weeks, which limits the power of L-SCC in detecting high SCC

episodes. Thus, O-CMT seems more valuable in individual cow udder health monitoring. In addition, there may be pathogen species that cause specific SCC patterns. De Haas et al. (2004) found that clinical mastitis caused by Escherichia coli was significantly associated with a short peak in SCC while Staphylococcus aureus was significantly associated with longer increased SCC, whilst no clear patterns were found for Streptococcus dysgalactiae or Streptococcus uberis. Compared to traditional methods (e.g., bacteriological culturing), the use of frequent O-CMT measurements can serve as a cheap and fast on-farm screening method for mastitis. It is fully automated and can be executed for almost every milking. These characteristics make O-CMT and other on-line SCC measurement methods a suitable tool for on-farm individual udder health monitoring. The measurements may also be used to identify subclinical mastitis cases that warrant further diagnostics such as bacteriological culture to explicitly identify the mastitis-causing pathogens. Further research to link the O-CMT patterns to pathogen species would be useful and highly relevant to develop tailor-made treatment plans to further optimize treatment strategies and reduce antimicrobial usage. Our results show added value of O-CMT measurement, but to further quantify the added value of O-CMT in detecting high SCC episodes, more work is needed. Specifically, work should be carried out on algorithms to mine these intensively measured O-CMT for early detection of high SCC as well as to quantify long term udder health related effects (such as incidence rate of clinical mastitis, milk production, total antimicrobial usage) and the economic value of the use of O-CMT measurements.

CONCLUSION

The overall concordance correlation coefficient between O-CMT and L-SCC of all farms was 0.66, and increases when the farm level SCC is higher. The average of multiple O-CMT measurements over a 24h time window was found to provide an optimum correlation between O-CMT and L-SCC and the capacity to capture udder health dynamics. The O-CMT measurement shows to be a promising on-farm tool for individual cow udder health monitoring, specifically because of its high measurement frequency.

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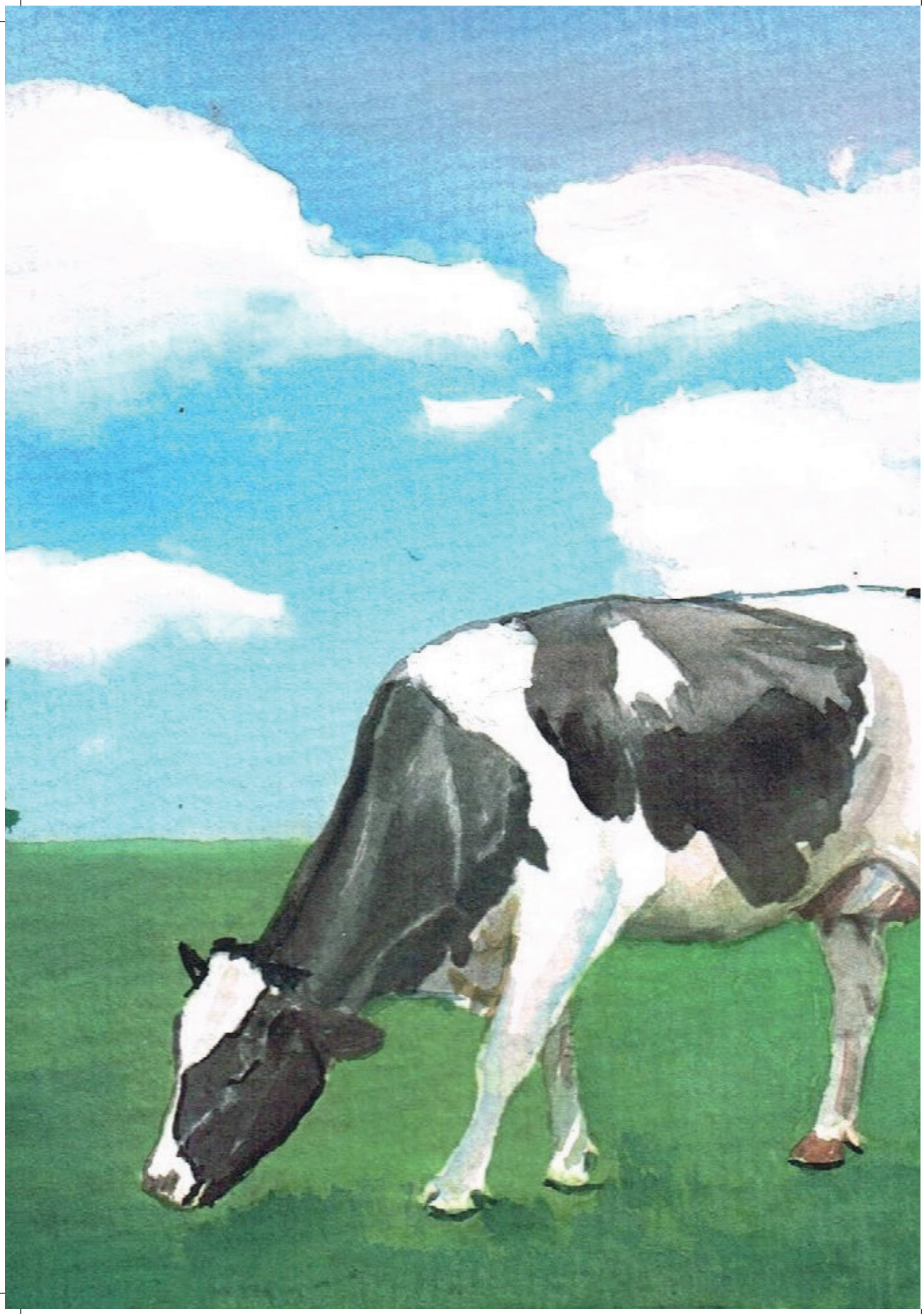

Chapter 4

Regularly fluctuating somatic cell count pattern in dairy herds

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ABSTRACT

Online somatic cell count (SCC) measurement is widely used in dairy herds milked with automatic milking systems (AMS) and gives the opportunity to closely monitor individual cow udder health. Using automated SCC data, we observed cows displaying a remarkably regularly fluctuating SCC (rfSCC) pattern, which is described in this study. We aimed to (1) estimate the prevalence of rfSCC in cows milked by AMS, (2) characterize the rfSCC pattern and (3) describe pathogens found in milk from cows displaying the rfSCC pattern. We analyzed 30 d episodes of composite SCC recordings of 1,000 cows from 55 dairy herds from 6 countries using an AMS with automated SCC measurement and identified the rfSCC pattern in 4.7% (95% CI: 3.5%-6.2%) of these episodes. The rfSCC episodes had a median SCC of 701 (2.5%-97.5% quantile: 539-1,162) × 1,000 cells/mL, a median amplitude of 552 (2.5%-97.5% quantile: 409-886) × 1,000 cells/mL and a median cycle length of 4.1 (2.5%-97.5% quantile: 3.7-4.9) days. Bacteriological culture data of quarter milk samples collected every 2 wk in one Dutch AMS herd were analyzed, yielding no clear association between pathogen species and the rfSCC pattern found in that herd. Altogether, we describe an intriguing phenomenon, present in almost 5% of the cows during a one-month study period. Further work is needed to quantify its importance in terms of udder health, but also to elucidate the mechanism leading to this remarkable SCC pattern.

INTRODUCTION

The somatic cell count (**SCC**) is the most often used diagnostic tool in udder health management of dairy cows (Schukken et al., 2003). Typically, SCC is measured by sampling milk and performing a laboratory test, often as part of a milk production recording scheme. In such systems, every 4-6 weeks SCC is measured for all cows in a herd. Following the development of automatic milking systems (**AMS**) and the developments in sensor technology, on-line devices have been constructed to enumerate or estimate SCC automatically (Sørensen et al., 2016; Deng et al., 2020). Such tools allow for highly frequent SCC measurements, up to every milking, enabling to study the temporal variation in SCC in detail over longer periods of time and in large numbers of animals and herds.

Recently, when we were working with daily SCC measurements, we noticed that several cows shared a similar SCC pattern that we termed a regularly fluctuating SCC (**rfSCC**). We described rfSCC as a string of SCC records of a cow that displays a regular cyclic fluctuating behavior, going up and down within several days. The patterns we observed were often stable and lasted for multiple weeks of time. The cyclicity and stability of the pattern were surprising and ask for a better understanding of this phenomenon.

At present it is unclear how often rfSCC occurs, what the amplitude and duration of the cyclic pattern is, and what causes it. It seems logical to expect an association with certain intramammary infections (**IMI**), but this needs to be investigated. Therefore, the aims of this study were to (1) determine the prevalence of rfSCC, (2) characterize the rfSCC pattern and (3) describe the pathogen species cultured from the milk of cows displaying rfSCC.

MATERIALS AND METHODS

In this research, data from 2 observational field studies were analyzed. Dataset 1 consists of routinely collected recordings of composite milk SCC measured by online-CMT (**O-CMT**), an online approximation of the SCC, in 55 AMS herds in 6 countries as described previously (Deng et al., 2020). This dataset was used to characterize the rfSCC pattern and estimate its occurrence. For dataset 2, we collected O-CMT data in one Dutch AMS herd (about 60 cows) for approximately 6 months and performed bacteriological culture every two weeks of quarter milk samples of each cow in the herd.

Identification of fSCC Patterns and Estimating the Prevalence

The raw data of dataset 1 consisted of farm identification, cow identification and the O-CMT measurements accompanied by the timestamp of the milking. The frequency of O-CMT measurements varied between farms between once every third milking up to every milking, dependent on AMS settings. We used O-CMT data of 11,219 lactating cows in 55 AMS herds which were available for

approximately 1 year (Deng et al., 2020). As a first step in the data preparation, the O-CMT recordings for each cow were cut into episodes of 30 consecutive days, resulting in 201,519 episodes of 11,219 cows. Only episodes with ≥ 1 O-CMT recordings per day on each of the 30 days were included, which we considered high frequent O-CMT recording, resulting in 9,756 episodes from 2,762 cows on 55 farms. As a next step, a total of 1,000 cows were randomly selected from the 2,762 cows with at least one episode with a high frequency of O-CMT recordings. Finally, for each selected cow, we randomly selected one episode. These 1,000 episodes were displayed as a graph with the date at the x-axis and the O-CMT reading at the y-axis. Black dots indicated the O-CMT measurements which were connected by lines. These graphs had a fixed lay-out, with the x-axis ranging from day 1 to day 30 and the y-axis ranging from 0 to 5,000,000 cells/mL. A dashed horizontal line was added to represent 200,000 cells/mL. The four authors of this paper, all active mastitis researchers, acted as raters and independently classified these 1,000 graphs into one of the following 8 categories: (1) *continuously regularly fluctuating* (the rfSCC pattern of interest), showing a cyclic fluctuation of O-CMT between high and low values and showing a regular pattern during the entire episode; (2) *partially continuously regularly fluctuating*, showing the rfSCC pattern mentioned under (1) during part of the episode; (3) *irregularly fluctuating*, showing fluctuation of O-CMT over time, but not showing a regular pattern; (4) *stable high*, showing a constantly high O-CMT throughout the episode; (5) stable low, showing a constantly low O-CMT throughout the episode; (6) *stable low with ≥1 peak(s)*, showing a constantly low O-CMT, with one or more brief peaks of single high O-CMT measurements; (7) *increasing*, showing a transition from a low O-CMT to a high O-CMT during the episode; (8) *decreasing*, showing a transition from a high O-CMT to a low O-CMT during the episode. In the descriptions above, low O-CMT was interpreted as up to approximately 200,000 cells/mL and high as above approximately 200,000 cells/mL. A typical example for each category is provided in Figure 1. Before the actual assessment was carried out, 30 test graphs were independently classified by the 4 raters, and discrepancies were discussed afterwards. Based on this discussion, the definitions were further adjusted, resulting in the definitions above. The graphs were presented to every rater in a random order and without any further information on the background of these episodes in an online questionnaire in which the raters could see the O-CMT graphs and a multiple-choice question representing the 8 categories of which 1 could be checked. The questionnaire can be accessed at *https://wj.qq.com/s2/6397417/2176/*.

The results of this questionnaire were cross tabulated, and all graphs that were categorized as pattern 1 (continuously regularly fluctuating SCC; n = 27) or pattern 2 (partially regularly fluctuating SCC; n = 64) by at least 1 of the 4, but not all 4 raters were discussed within the group, leading to a collective judgement for each of these graphs. The proportion of pattern 1 and pattern 2 was calculated as the number of graphs collectively identified as such divided by 1,000. In addition, we calculated the proportion using the number of episodes with the patterns of interest based on agreement of the initial

judgement of all 4 raters (the most conservative estimate) and based on ≥1 of the raters initially choosing this pattern (the most liberal estimate). Interrater agreement of the initial ratings was evaluated by Fleiss kappa which was calculated using the kappam.fleiss function in the *irr* package (Gamer et al., 2019) in R version 3.6.2 (R Core Team, 2019).

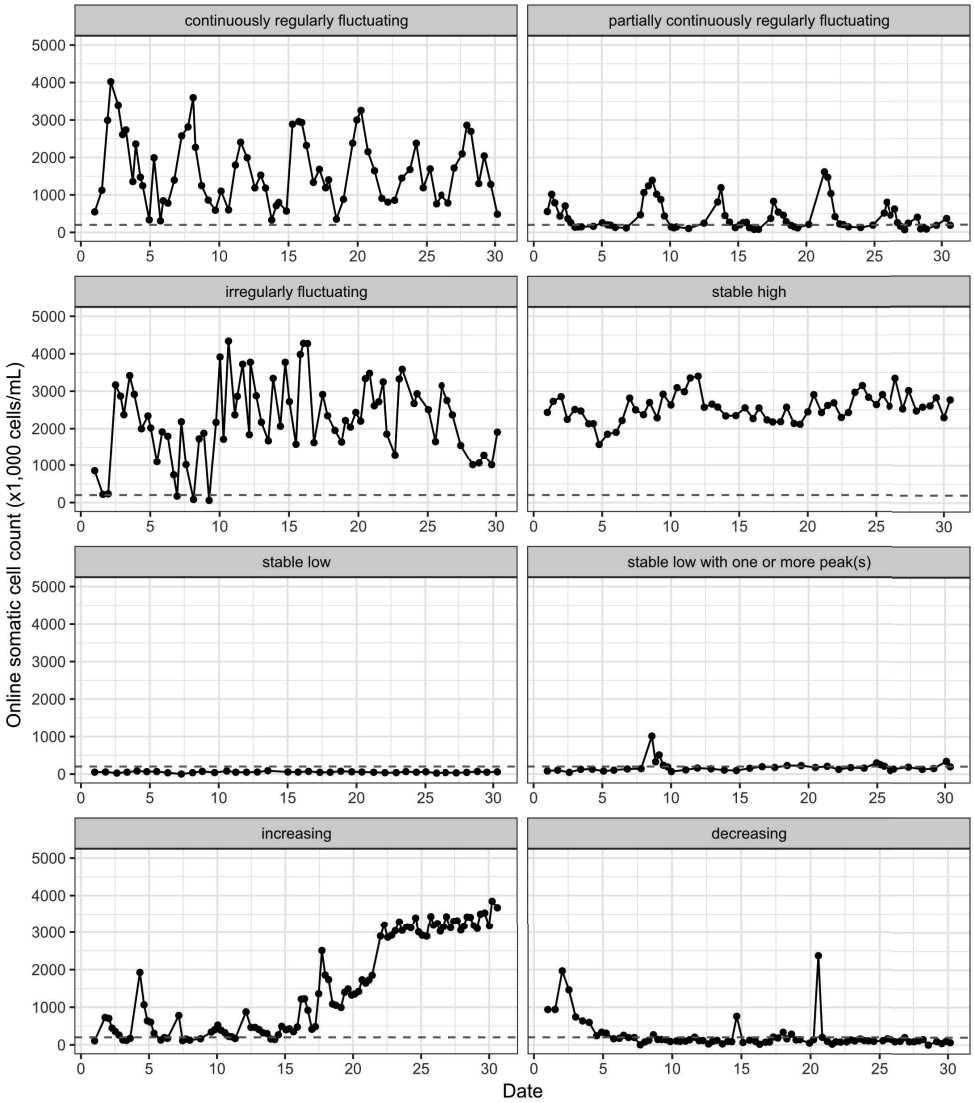

Figure 1. Examples of 30 days episodes of online SCC estimated by automated CMT for each pattern category. The black dots connected by lines represent the online SCC and the dashed horizontal line represents 200,000 cells/mL.

Characterization of the rfSCC pattern

The episodes from dataset 1 that were collectively identified as rfSCC pattern (pattern 1) were characterized by spectral analysis accounting for the unevenly spaced measurements using the spec. lomb function from the spectral package (Seilmayer, 2019) in R, to calculate the duration and amplitude of cycles as well as the average SCC within the 30-d episode.

Pathogen species associated with rfSCC pattern

Dataset 2 consisted of data from one group of cows in a Dutch AMS farm that were milked with an AMS equipped with an O-CMT sensor. This group of cows was prospectively studied from October 10th 2017 to February 27th 2018. Fresh cows were entering the group and cows were dried off or culled, resulting in a constant group size of around 60 cows during the study period. Every 2 wks, milk samples were collected from all quarters of all cows present as follows: the udder was cleaned with a paper towel and the first 2-3 squirts of milk were discarded in a plastic cup to check for clots and other abnormalities, followed by disinfection of teat ends using cotton wool soaked in 70% alcohol. Then, a milk sample of 1-2 mL was collected in a plastic sterile vial, which was stored on melting ice and transported to the laboratory to be cultured within the same day. Bacteriological culturing was performed according to NMC guidelines (NMC, 2017) and isolates were identified using MALDI-TOF (Bizzini and Greub, 2010). In short, 10 µL of quarter milk was inoculated on the sheep blood agar plate at 37 °C, results were read at 24 h and 48h. Contaminated samples were defined as samples with > 2 morphologically different colonies.

At every milking during the study period, the O-CMT in this group of cows was measured. This data of each cow was displayed as a graph similar to the graphs mentioned above, but with the x-axis spanning the entire period that the cow was part of the study. These graphs were judged by the 4 raters to identify episodes in which the rfSCC pattern was present. The raters were asked to record for every graph the start and end date(s) of the fluctuating pattern(s). Next, in a collective meeting, discrepancies were discussed resulting in consensus on the periods where the rfSCC pattern was present. Based on the quarter milk sample culture results, pathogen species found in the rfSCC patterns were described.

RESULTS

Prevalence of rfSCC

The numbers of episodes within each category classified by each of the 4 raters and the consensus about categories 1 and 2 (the rfSCC categories) after discussion are provided in Table 1. The estimated consensus proportion of pattern 1 (continuously regularly fluctuating) was 2.2% (22/1,000 episodes) and pattern 2 (partially continuously regularly fluctuating) was 2.5% (25/1,000 episodes). The total pro-

Table 1. Classification of 1,000 30 d episodes from individual cows in 55 dairy herds from 6 countries with highly frequent SCC measurement. Each episode consists of SCC values measured by a sensor system based on the California mastitis test. The episodes were classified into eight pattern categories. The categorization of four different raters are presented, as are the average and Fleiss' Kappa indicating the interrater agreement, the number of episodes chosen by at least 1 of the raters, all the raters collectively and the consensus after discussion (only for the first and the second pattern).

Pattern	Rater				Average	Kappa	Chosen by all 4 raters	Chosen by ≥ 1 rater	Consensus
	1	2	3	4					
Continuously regularly fluctuating (pattern 1)	36	16	18	12	21	0.64	11	38	22
Partially regularly fluctuating (pattern 2)	33	26	32	28	30	0.41	4	68	25
Irregularly fluctuating	128	169	198	117	153	0.55	43	255	-
Increasing	19	6	3	10	10	0.40	1	23	-
Decreasing	18	5	8	10	10	0.47	1	21	-
Stable high	36	54	28	27	36	0.45	4	74	-
Stable low	485	457	426	447	454	0.92	421	492	-
Stable low with one or few peaks	245	267	287	349	287	0.78	211	378	-

portion of episodes displaying the rfSCC pattern (including both pattern 1 and 2) was therefore estimated at 4.7% (95% CI: 3.5-6.2%). According to the conservative definition a proportion of 1.5% was classified as rfSCC, and according to the most liberal definition a proportion of 10.6% of all episodes was classified as such. The overall inter-rater agreement on 8 categories, according to Fleiss' kappa, was estimated to be 0.74, which was mainly driven by the high agreement between raters on the stable low pattern. Raters had moderate agreement on pattern 1 specifically (Table 1).

Characterization of rfSCC

The amplitude and duration of the rfSCC pattern are illustrated in Figure 2. All 22 episodes of Dataset I that were collectively identified as pattern 1 are displayed in Figure 3. Based on these patterns, the median of the average SCC of episodes was estimated as 701 (2.5%-97.5% quantile: 539-1,162) ×1,000 cells/mL, the median duration of the fluctuating cycles was estimated at 4.1 (2.5%-97.5% quantile: 3.7-4.9) days and the median amplitude at 552 (2.5%-97.5% quantile: 409-886) ×1,000 cells/mL, as displayed in Figure 3.

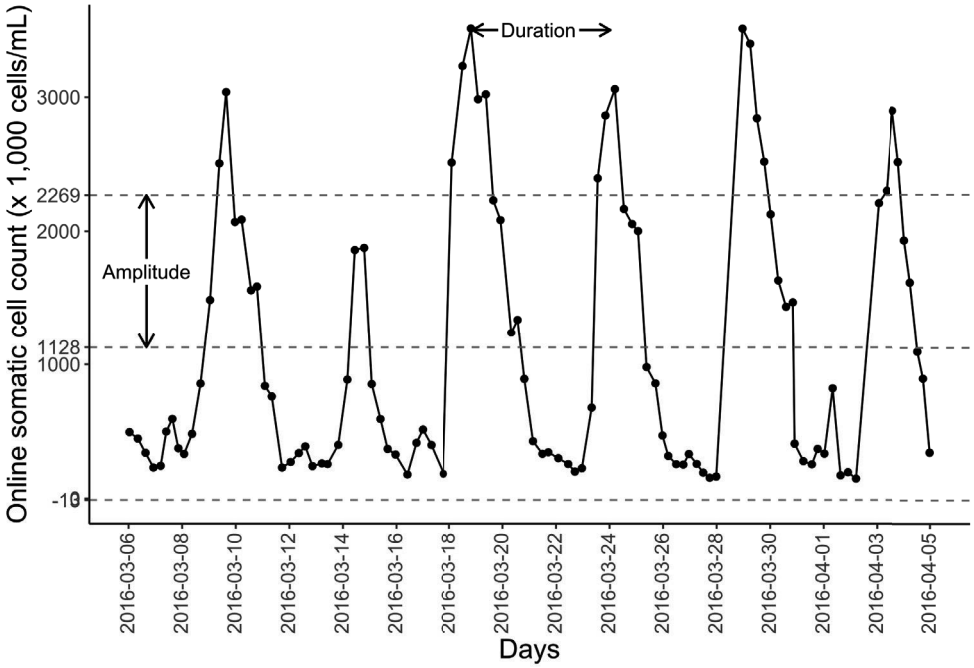

Figure 2. Example of a fluctuating SCC (estimated by automated CMT) pattern, to illustrate how amplitude (the distance between the middle and the outer dashed lines), the minimum and maximum of SCC and duration of the pattern were estimated by spectral analysis.

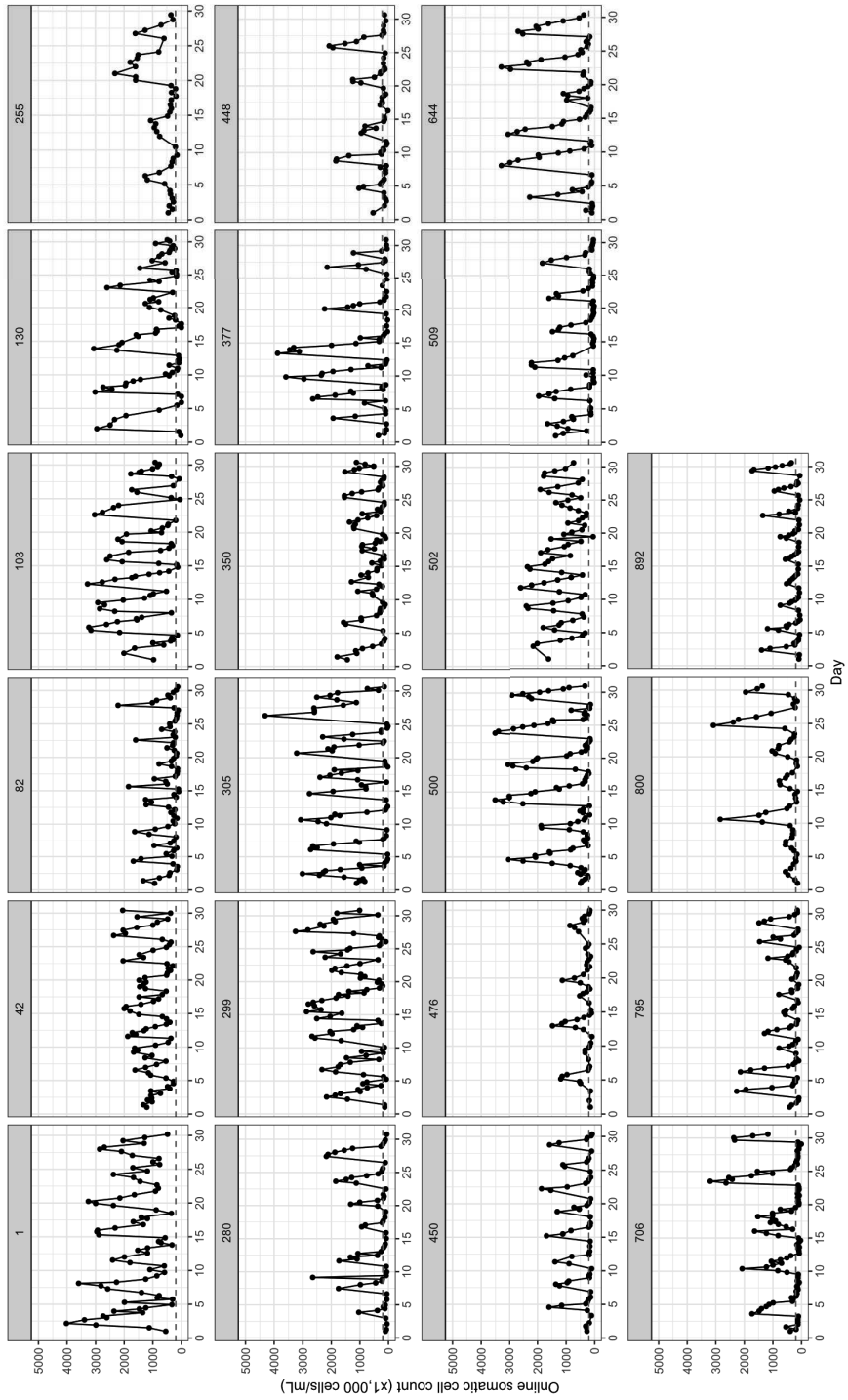

Figure 3. All 22 SCC episodes that were categorized as continuously regularly fluctuating by all 4 independent raters out of 1,000 episodes that were visually classified into 8 categories of SCC patterns. The dashed horizontal line represents 200,000 cells/mL.

Pathogen Species Associated with rfSCC

In Dataset 2, the rfSCC pattern was found in 6 out of 81 cows from September 26th 2017 to February 27th 2018. For each of these 6 cows, we identified a pathogen species that was found in the same quarter at ≥ 2 sampling moments during the period in which rfSCC was observed. For each of the 6 cows, in only one quarter the same bacterial species was found at ≥ 2 sampling moments, being *Strep. dysgalactiae* (n = 2), *Corynebacterium* species (n = 2), *Strep. agalactiae* (n = 1) and non-*aureus Staphylococcus* (n = 1). We observed no obvious differences in amplitude between the cows with different bacteriological culture results in dataset 2, but these numbers are too small to draw conclusions.

DISCUSSION

This study aimed to estimate the proportion of the newly described rfSCC pattern, to characterize it and to describe the pathogens found in quarters from cows showing the rfSCC pattern. To the best of our knowledge, this is the first report describing such a SCC pattern. This was based on data from a large number of dairy herds and cows with highly frequent SCC measurements. Previous studies that described SCC patterns were mostly executed in a small number of herds with relatively frequent measures of SCC (e.g., Sears et al., 1990; Schukken et al., 2003; Urioste et al., 2010) or in large numbers of herds with infrequently measured SCC (e.g., De Haas et al., 2004). Because of the setup of those studies, none of these was able to report the rfSCC pattern as we have defined it. It is the application of the automated, on-line SCC sensors (in our case O-CMT) that enables a close monitoring of individual cow udder health for a longer time in a large number of animals.

The frequently measured O-CMT enables the study of SCC dynamics on a real time basis, whereas when SCC is measured in DHI programs, an SCC record is provided generally every 4-6 wks. Substantially more information on the dynamics of SCC could be identified by using the frequent O-CMT measurements, and for instance changes in SCC that warrant intervention could be detected which would have gone unnoticed in normal DHI testing (Sørensen et al., 2016; Deng et al., 2020; Kirkeby et al., 2020).

About 4.7% of the episodes showed the rfSCC pattern. It seems plausible to interpret the rfSCC as a sign of subclinical mastitis and although we could not find a clear association with a specific pathogen species in the cows in dataset 2 that displayed rfSCC, most of these cows with the rfSCC pattern did have positive culture results in one or more quarters. If indeed rfSCC indicates subclinical mastitis, a proportion of 4.7% is substantial, given that the overall prevalence of subclinical mastitis at cow level has been estimated at 15.8% on Dutch dairy farms (Santman-Berends et al., 2016). In addition, rfSCC cows showed the pattern persistently and may therefore contribute substantially to a higher bulk tank SCC. It is also possible that the rfSCC is a physiological phenomenon, not actually representing disease,

linked to for instance fluctuations in milk yield or milking interval. Physiological variation has been described to account for a large proportion of the variation of composite milk SCC within individual dairy cows (Nørstebø et al., 2019; Deng et al., 2020). In dataset 2, we found *Strep. agalactiae*, *Strep. dysgalactiae* and *Corynebacterium* species in cows with the rfSCC pattern and, although the number of cows was too small to carry out any test for statistical significance, cows showing the rfSCC pattern were more often culture positive than cows that had persistently low SCC (results not shown).

The geometric mean of the average SCC within rfSCC episodes (774 × 1,000 cells/mL; 2.5%-97.5% quantile: 405-1,515 ×1,000 cells/mL) was higher than the geometric mean SCC of chronic IMI caused by non-*aureus Staphylococcus* IMI (Bexiga et al., 2014), but lower than the median SCC of chronic IMI caused by *E. coli* (Döpfer et al., 1999). This shows that the increase in SCC when the rfSCC pattern is present, is substantial. In addition, at the peaks of the fluctuations, SCC was often >2,000 × 1,000 cells/mL, although the estimated amplitude of the rfSCC pattern varied substantially between cows. This may reflect differences in host response between cows, but may also be caused by differences in pathogen species leading to the rfSCC. As the spectral analysis used to estimate the amplitude of the rfSCC pattern fits a curve to the data and thereby smoothens the peaks, the actual amplitude of the rfSCC may have been slightly underestimated as illustrated by Figure 2.

The median duration of cycles of around 4 d was shorter than the estimated duration of a short IMI caused by *E. coli* (6.7 days) or *Staph. aureus* (6.3 days, Bannerman et al., 2004). Therefore, and also because of the stability of the observed pattern, we consider it very unlikely that each peak represents a new intramammary infection, and we assume that the rfSCC patterns represent a continuation of the same IMI infection. As mentioned before, we are not sure that the rfSCC is caused by IMI. However, as an increase in SCC mainly reflects the immune response of a cow to an invading pathogen (Schukken et al., 2011; Bruckmaier and Wellnitz, 2017), fluctuations in SCC may reflect fluctuations in pathogen load. Regular fluctuations of SCC may be the result of a predator-prey cycle like system (Loor et al., 2011), in which the somatic cells suppress the infection, which then diminishes, enabling the pathogen to grow again and elicit a new SCC response. A cyclic shedding pattern of *Staph. aureus* has been described in the past in an experimental setting (Sears et al., 1990). However, in a later observational study, this phenomena was not described (Walker et al., 2011). In addition, the strong decrease in SCC that we saw, seems unlikely to happen within a few days after the stimulus of the bacteria has been removed or decreased (Schukken, 2003). The underlying mechanism of the rfSCC pattern is therefore unclear, and future research to identify the determinants of rfSCC is needed.

CONCLUSIONS

The availability of novel on-line sensor systems, allowing routine measurement of SCC on a very frequent (daily) basis enables detailed research in SCC patterns. In this study, we show that about 4.7% of the cows show a newly described rfSCC pattern, which is highly stable in nature and displays a repeated pattern of substantial increase and decrease in SCC within approximately 4 days. The mechanism behind rfSCC is unclear, and despite the fact that rfSCC was seen in a substantial proportion of the cows, the practical importance of this finding remains to be demonstrated.

ACKNOWLEDGEMENTS

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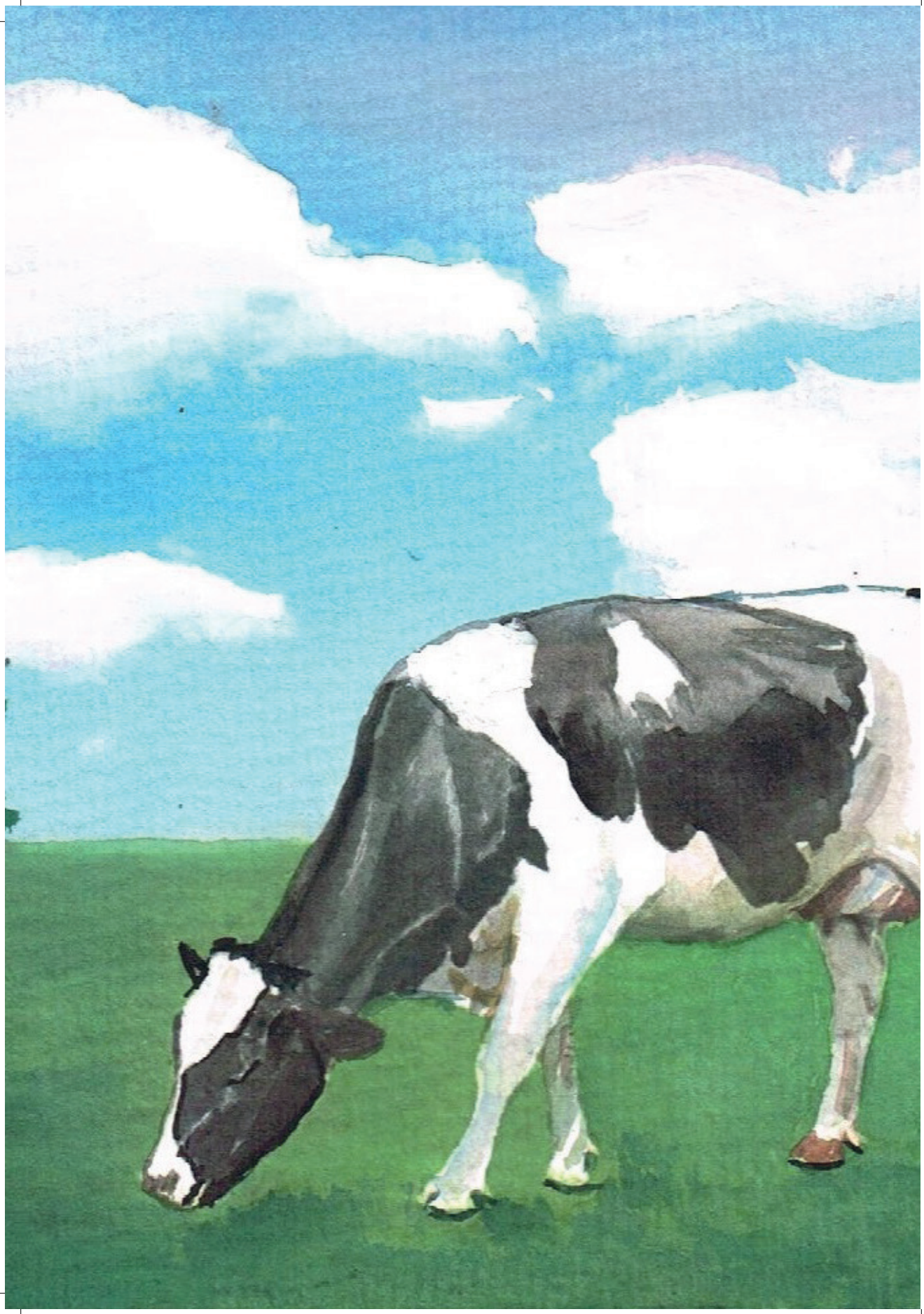

Chapter 5

Transmission dynamics of *Staphylococcus aureus* and *Streptococcus agalactiae* in a Dutch dairy herd using an automatic milking system

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ABSTRACT

Staphylococcus aureus and *Streptococcus agalactiae* are important contagious mastitis pathogens and are considered to mainly transmit between cows through the milking machine. Controlling contagious mastitis on dairy farms requires a reduction of the transmission rate or the duration of intramammary infections (IMI), or both. These parameters may differ in dairy herds milked with an automatic milking system (AMS) as compared to those milked with a conventional milking system (CMS). The aims of this prospective longitudinal study were to estimate the transmission rate, the median duration of IMI and the basic reproduction number (R_0) of *Staph. aureus* and *Strep. agalactiae* in a Dutch AMS herd. Bacteriological cultures of quarter milk samples were collected every 2 wks. Using 3 different definitions of IMI, we estimated the transmission rate for *Staph. aureus* to be within the range of 0.002 (95% CI: 0-0.005) quarter-day^{-1} to 0.019 (95% CI: 0.010-0.032) quarter-day^{-1}, and for *Strep. agalactiae* of 0.007 (95% CI: 0.005-0.010) quarter-day^{-1} to 0.019 (95% CI: 0.011-0.032) quarter-day^{-1}, the median duration of chronic IMI at 95 (95% CI: 72-125) days for *Staph. aureus* and at 86 (95% CI: 67-111) days for *Strep. agalactiae*, and the R_0 between 0.16 (95% CI: 0.05-0.27) and 0.34 (95% CI: 0.20-0.48) for *Staph. aureus*, and between 0.64 (95% CI: 0.41-0.87) and 0.68 (95% CI: 0.48-0.88) for *Strep. agalactiae*.

Transmission of these two contagious pathogens in this herd was limited and theoretically the IMI would not sustain, given that R_0 of both pathogens was lower than 1. The estimated transmission rate of *Staph. aureus* in this AMS herd was found to be comparable to those described for CMS herds, while for *Strep. agalactiae*, it was slightly higher than in CMS herds. The duration of *Staph. aureus* IMI was in line with results from CMS farms, while the duration of *Strep. agalactiae* was lower than what has been described in CMS herds. The R_0 of these contagious pathogens was found to be lower than the estimates in CMS herds. Our study suggests that the transmission rate of these two contagious pathogens in this AMS herd were comparable to what has been reported about well-performing CMS herds that have a low rate of transmission.

INTRODUCTION

Although contagious mastitis pathogens have long been the subject of research, and the five-point mastitis control plan has been shown to significantly reduce their prevalence (Green and Bradley, 2013), there still is much to gain in this respect in many farms worldwide (Bradley et al., 2015; Gao et al., 2017; Leuenberger et al., 2019). *Staphylococcus aureus* and *Streptococcus agalactiae* are still among the contagious pathogens that are most prevalent on dairy farms in many countries (Gao et al., 2017; Vakkamäki et al., 2017).

Clearly, the milking machine plays an important role in the transmission of mastitis causing pathogens (Mein, 2012). Contagious mastitis causing pathogens, such as *Staph. aureus* and *Strep. agalactiae*, have been reported to primarily spread from cow to cow during milking by a contaminated milking machine (Keefe, 2012). Although some evidence suggests that environmental infections also occur (Fox and Gay, 1993; Barlow et al., 2013), *Staph. aureus* is generally considered to be contagious and transmitted between cows, with the presence of a predominant genotype in a herd reflecting the epidemiological features of contagious mastitis pathogens (Sommerhäuser et al., 2003; Capurro et al., 2010; Klaas and Zadoks, 2018). The prevalence of *Strep. agalactiae* has been significantly reduced since the adoption of the five point mastitis control plan (Hillerton and Booth, 2018). However, recent research found its prevalence to be relatively high in herds using an automatic milking system (**AMS**) (Svennesen et al., 2019) and proved the existence of an environmental transmission route of *Strep. agalactiae* (Jørgensen et al., 2016). Transmission rates of these pathogens have been estimated in herds using conventional milking systems (**CMS**) (Zadoks et al., 2002; Leelahapongsathon et al., 2016; Kirkeby et al., 2019), but to the best of our knowledge, only once in an AMS herd, that evaluated data at the cow level rather than quarter level (Dalen et al., 2019).

In AMS herds, contacts between individual cows and interactions with the milking machine differ from those in CMS herds, which may have an effect on the transmission dynamics of contagious mastitis pathogens. For instance, automated cleaning of teats and automated post-milking teat disinfection may affect the transmission rate of these pathogens. Additionally, the use of sensors to identify infected animals may have an effect on the time until diagnosis of subclinical mastitis and therefore possibly on the time until interventions are implemented by herdsmen. The distinctive difference between AMS and CMS herds regarding the milking process is that cows are milked with a single milking unit per AMS. Hence, they may have a higher exposure to contaminated milking equipment, especially when cleaning and disinfection procedures are suboptimal. Intramammary infections, as indicated by a high SCC, seem to occur more frequently in AMS compared to CMS herds (Deng et al., 2019). Although reducing the transmission of IMI is one of the most important parts of mastitis control programs (Østerås and Sølverød, 2009; Down et al., 2013), the transmission rate of these pathogens in AMS herds is not fully clear. Therefore, the aim of this study was to estimate the transmission rate, the duration of IMI and

the basic reproduction number (**R_0**) of *Staph. aureus* and *Strep. agalactiae* in a Dutch dairy herd using an AMS and compare this to previous findings in CMS herds. Additionally, the effect of different definitions of IMI on transmission parameter estimates was evaluated.

MATERIALS AND METHODS

In a prospective longitudinal study, during 6 mo, bi-weekly quarter milk samples were collected for bacteriological culturing in a group of approximately 60 cows milked with an AMS. With the bacteriological culture data, we estimated transmission rate, median duration and R_0 of IMI for both *Staph. aureus* and *Strep. agalactiae.*

Farm and Cow Selection

A farm was selected based on the use of AMS, the availability of online SCC sensors (MQC-C, Lely Industries N.V., Maassluis, The Netherlands), the location of the farm relatively close to Utrecht University and the farmer's willingness to participate in this study. The selected dairy farm had four separate groups of cows, each milked with a single Lely Astronaut A4 AMS (Lely Industries N.V.). The AMS automatically performed cleaning of teats and post-milking teat disinfection by spraying the teats of each cow as well as rinsing and steaming of the milking unit between milkings. Data about the disease history of the cows in all the 4 groups were recorded by the farmer and were available. One of the four groups of cows was selected for quarter milk sampling based on the farmer's indication that this group of cows had the highest incidence of contagious mastitis among the 4 groups. The selected group consisted of approximately 60 lactating cows (24 cows in parity 2 and 21 cows in parity 3, the remaining cows were in parity 4-8). During the study period, there was an influx of fresh cows and an efflux of cows being dried off using selective dry cow therapy. Cows were fed automatically using a total mixed ration and had free access to feeding. Sawdust was used for bedding, and beddings were cleaned and refilled with sawdust every morning.

Data Collection

The milking robot in this group was equipped with an online sensor system (MQC-C, Lely Industries N.V., The Netherlands), which measures electrical conductivity in-line for each separate udder quarter, abnormalities in milk color in composite milk and composite milk SCC at the cow level. Electrical conductivity and color were measured every milking and SCC was measured almost every milking and expressed in cells/mL. Detection of clinical mastitis was based on the estimated SCC, in combination with electrical conductivity as well as the color of milk. Cows suspected to have clinical mastitis, based upon the system's algorithm, were listed on an alert list. Cows on the alert list were further examined by visually inspecting the appearance of milk, udder and cow by the farmer to confirm clinical mastitis. Cases of clinical mastitis were treated with intramammary antibiotics. History of mastitis was recorded

in the disease treatment recording system. Cows were milked on average 2.8 times/day with an average milk production of 12.3 kg/milking.

A pilot study was conducted to evaluate the sampling scheme and the processing of samples prior to the start of data collection for transmission modelling on September 26th, 2017. Every two weeks from October 10th, 2017 through February 27th, 2018 all cows in the study group were locked on the headlocks to collect quarter milk samples and were released immediately upon completion. Cows were sampled regardless of the time since their last milking.

Sampling and culturing of milk samples were performed according to NMC guidelines (NMC, 2017). In brief, first three squirts of milk were discarded, udders and teats were cleaned with paper and teat ends were subsequently disinfected with cotton soaked in 70% ethanol. For each quarter, 1-2 mL milk was collected aseptically, after which milk samples were cooled in an ice box and transported to the laboratory at Utrecht University to be processed immediately after arrival.

Laboratory Analysis

In the laboratory, milk samples were mixed by vortex of samples during 5s and 10 µL milk was inoculated on a sheep blood agar plate and incubated at 37 °C. Results were recorded at 24 and 48 h. A negative culture result was defined as a sample with no bacterial growth. Samples with ≥ 1 colony forming units (**CFU**) were considered culture positive, while samples with > 2 morphologically different colony types were considered to be contaminated. Colony forming units were counted for each colony type based on morphology in culture positive samples and were transformed into $\log_2$(CFU). One isolate of every morphologically different colony type of non-contaminated cultures was subjected to matrix-assisted laser desorption/ionization time-of-flight (**MALDI-TOF**) MS (Bizzini and Greub, 2010) in duplicate. Isolates with identification score ≥ 2.0 were considered as successful identifications and used in the analysis. Isolates of *Staph. aureus* were genotyped by sequencing the variable region of the surface protein A gene (Harmsen et al., 2003) in order to model the genotype specific transmission.

Bacteriological culture results from quarter milk samples of cows with mastitis within 7 d after calving or within 14 d after treatment for mastitis (which was indicated by farmer's records of treatment history for mastitis in conjunction with milk separation in the farm management system) were excluded from further analysis.

Data Analysis

Because different types of IMI (chronic and transient IMI) may have a different attribution to transmission of pathogens, we estimated the transmission rate, duration of IMI, and R_0 in 3 different scenarios. In each scenario, different definitions for a quarter having an (new) IMI, being infectious and being susceptible for new IMI, were applied. In general, if quarters were found to be culture positive

(≥1 CFU/0.01 mL) only once in a single isolated sample, these IMI were defined as transient IMI. If quarters were culture positive in subsequent samples for the same pathogen, these IMI were defined as chronic IMI. Definitions were specified for the different scenarios:

(1) scenario 1, using a strict definition of IMI: only chronic IMI (consecutive culture positive samples) are considered infectious and only new chronic IMI are considered as new IMI, transient IMI (single culture positive samples) are ignored and interpreted as culture negative in this scenario. In this scenario an IMI was thus defined as a quarter with ≥ 2 out of 3 consecutive samples being culture positive for the same pathogen species. A quarter was defined as susceptible for new IMI if ≥2 consecutive samplings were culture negative.

(2) scenario 2, using an intermediate definition of IMI: chronic and transient IMI are differentiated with respect to infectiousness, with only chronic IMI being considered as infectious. New chronic IMI as well as new transient IMI are, however, considered as new IMI. In this scenario transient IMI does not contribute to the infection pressure. A chronic IMI was defined as ≥ 2 out of 3 consecutive samples being culture positive for the same pathogen species. A transient IMI was defined as a single culture positive sample, not preceded or followed by a culture positive sample with the same pathogen and not being part of a chronic IMI. A quarter was defined as susceptible for new IMI, either chronic or transient, if ≥2 consecutive samplings were culture negative.

(3) scenario 3, using a lenient definition of IMI: no effect of type of IMI on infectivity is assumed and there is no differentiation between chronic IMI and transient IMI in this scenario. Any culture positive sample was considered as an IMI, being infected and infectious, until the quarter was found negative for that pathogen (Dohoo et al., 2011). A quarter was defined as susceptible for IMI if ≥1 consecutive samplings were culture negative.

Transmission Rate At any given timepoint during the study, quarters were defined to be either susceptible or infected. A stochastic Susceptible-Infectious-Susceptible (**SIS**) model as described by Zadoks et al. (2002) was used with the aforementioned definitions for IMI status for chronic and transient IMI in each of the three scenarios to estimate the transmission rate and R_0 of each pathogen. The stochastic SIS-model assumed homogeneous mixing, random contacts, equal infectivity of infectious quarters and equal susceptibility of susceptible quarters (Zadoks et al., 2002).

We estimated the transmission rate of the stochastic SIS model by a generalized linear model. Maximum likelihood estimation (equations are given in Supplementary Material S1) under the assumption of a binomial distribution of the number of new IMI occurring during a time step was used to estimate transmission rate (Van der Goot et al., 2003) by using the function mle2 from the bbmle package version 1.0.20 (Bolker, 2020). Because of the limited number of new IMI cases, the 95% confidence interval (**CI**) for estimates of transmission rate was calculated using the profile likelihood (Stryhn and

Christensen, 2003). For *Staph. aureus* in scenario 1, this method could not be used, because only 1 new IMI was observed, and the 95% CI was calculated assuming a normal distribution (estimate ± 1.96 × SE).

The number of new IMI during a sample interval Δt is given by:

$$E(C) = S\left(1 - \exp\left(-\beta \times \frac{I}{N} \times \Delta t\right)\right) \quad [1]$$

where **E(C)** is the expected number of new IMI, transmission rate is the transmission rate per day, *I* is the number of infectious quarters at the beginning of the sampling interval, *N* is the total number of quarters present at the beginning of the sampling interval (i.e. the total number of all infected and susceptible quarters at the beginning of each sampling interval), *S* is the number of susceptible quarters at the beginning of the sampling interval and Δt is the duration of the sampling interval (14 days).

Duration of IMI Duration of IMI was calculated using the midpoint between samplings (Zadoks et al., 2003). The start of the IMI was defined as the midpoint between the last sampling at which the quarter was considered susceptible and the first sampling at which it was considered infected. The end of the IMI was either one week after the last sampling moment of the whole study or when a quarter became susceptible again, at the midpoint between the last sampling at which the quarter was considered infected and the first sampling at which it was considered susceptible. We assumed that a quarter with a single missing culture result maintained its infection status from the previous sampling. The association between outcome of the infection (chronic or transient IMI) and $\log_2$(CFU) in samples with chronic and transient IMI at the first sampling for each pathogen was tested using Welch's *t*-test to correct for the unequal sample sizes in samples with chronic and transient IMI. The median duration of IMI was estimated by using the survreg function with Weibull distribution in the survival package version 2.44-1.1 in R (Therneau, 2020). Both left truncation and right censoring were ignored in the analysis due to the fact that the Surv function in the survival package of R could not model both left truncation and right censoring. For quarters with right censoring, the week after the last sampling was considered as the end of infection. Duration of transient IMI was considered as 14 d based on the definition described above. In addition to estimating the median duration of IMI, we tested the association (indicated by Pearson's correlation coefficient) between the observed duration of chronic IMI and $\log_2$(CFU) in chronic IMI of both pathogens.

Basic Reproduction Number (R_0) The R_0 is the average number of new IMI caused by one infectious quarter in a naïve susceptible population, and was calculated according to equation [2]:

$$R_0 = \beta \times (p_{chronic} \times D_{chronic} + (1 - p_{chronic}) \times D_{transient}) \quad [2]$$

where R_0 is the estimated basic reproduction number, $\mathbf{P}_{\mathbf{chronic}}$ is the proportion of new chronic IMI out of all new IMI across all sampling moments, transmission rateis the estimated transmission rate, $D_{chronic}$ is the estimated median duration of chronic IMI, $D_{transient}$ is the estimated duration of transient IMI (14 days). The 95% confidence interval for the estimator of $\mathbf{R_0}$ was constructed, assuming independence of the transmission coefficient and duration of the infectious period, such that we could calculate the standard deviation of the estimator: $\mathrm{SD}(\mathrm{R_0}) = \sqrt{\mathrm{Var}(\beta \times \mathrm{D})} = \sqrt{\mathrm{E}(\mathrm{D})^2 \times \mathrm{Var}\,(\beta) + \mathrm{E}(\beta)^2 \times \mathrm{Var}(\mathrm{D})}$ (Rao, 1973).

The analyses for *Staph. aureus* and *Strep. agalactiae* were performed separately, assuming independent infection dynamics. All analyses were performed in R version 3.6.2 (R Core Team, 2019).

RESULTS

Descriptive Statistics

During this study in total 317 quarters from 81 cows were sampled at ≥ 2 consecutive sampling moments. There were, on average, 61 (ranging from 57 to 67) cows and 240 (ranging from 222 to 261) quarters at each sampling moment, with 99 out of a total of 317 quarters being sampled at all the 11 sampling moments. The arithmetic average of monthly geometric mean SCC of the study group was 117,189 cells/mL (ranging from 73,644 to 145,911 cell/mL).

The summary of the culture results is given in Table 1. The non-*aureus staphylococci* (**NAS**) and *C. bovis* were the most prevalent groups of pathogens, followed by *Staphylococcus aureus* and *Strep. agalactiae*. There were 59 samples from 21 quarters of 15 cows culture positive for *Staph. aureus,* and *Strep. agalactiae* was cultured 60 times from 20 quarters of 14 cows. In total, 1 out of 11 (9%) new *Staph. aureus* IMI and 5 out of 13 (38%) new *Strep. agalactiae* IMI were chronic. Of the *Staph. aureus* isolates, 56/59 belonged to the same spa type, t529 (95% of the total number of isolates). The remaining 3/59 isolates belonged to t527 (5% of the total number of isolates), which is unrelated to t529 as it has a completely different repeated succession.

Table 1. The number of isolates at each sampling moment and the average number of isolates during the study for the indicated mastitis pathogens in one group of cows on a Dutch dairy farm using an automatic milking system. A total of 81 cows were included in this study. Pathogens in the 'Other pathogens' category were *Streptococcus* spp., *Enterococcus* spp., *Bacillus* species and *Trueperella pyogenes*.

Culture results	**2017-10-10**	**2017-10-24**	**2017-11-07**	**2017-11-21**	**2017-12-05**	**2017-12-19**	**2018-01-02**	**2018-01-16**	**2018-01-30**	**2018-02-13**	**2018-02-27**	**Average number of isolates (percentage)**
Non-*aureus Staphylococcus*	51	45	50	39	38	49	34	45	51	44	34	43.6 (16.8%)
Corynebacterium species	55	41	40	32	36	29	20	30	48	43	18	35.6 (13.7%)
Streptococcus agalactiae	4	4	5	5	5	7	6	5	6	4	6	5.2 (2.0%)
Staphylococcus aureus	5	4	4	5	6	6	4	5	7	7	2	5.0 (1.9%)
Streptococcus dysgalactiae	3	4	4	5	5	1	4	3	4	4	5	3.8 (1.5%)
Escherichia coli	0	1	1	1	1	3	1	1	0	2	2	1.4 (0.6%)
Streptococcus uberis	1	0	0	0	1	0	0	0	0	0	0	1.0 (0.4%)
Other pathogens	57	54	52	73	44	55	36	63	29	62	43	51.6 (19.9%)
Culture negative	59	100	97	81	99	98	142	85	106	73	135	97.7 (37.9%)
Contamination	24	22	12	19	15	19	5	18	7	18	2	14.6 (5.6%)
Total	259	275	265	260	250	267	252	255	258	257	247	26.0 (10.0%)

Transmission Dynamics

The number of infectious, susceptible and newly infected quarters in each infection status at every sampling moment for the 2 contagious pathogens in the 3 scenarios is provided in Table 2. The β estimates for *Staph. aureus* were largely similar to those for *Strep. agalactiae* within each scenario but estimates of β for both pathogens were substantially lower in scenario 1 compared to scenario's 2 and 3. The duration of chronic IMI was estimated at 95 (95% CI: 72-125) d for *Staph. aureus* and 86 (95% CI: 67-111) d for *Strep. agalactiae* in both scenario 1 and 2 when only the chronic IMI was considered contributing to the transmission process. The duration of IMI regardless of chronic or transient was 30 (95% CI: 20-45) d and 39 (95% CI: 27-57) d for *Staph. aureus* and *Strep. agalactiae*, respectively, in scenario 3 (Table 3).

The corresponding R_0 was estimated below 1 in all scenario's and ranged between 0.16 (95% CI: 0.05-0.27) (scenario 1) and 0.34 (95% CI: 0.20-0.48) (scenario 3) and between 0.64 (95% CI: 0.41-0.87) (scenario 1) and 0.68 (95% CI: 0.48-0.88) (scenario 3) for *Staph. aureus* and *Strep. agalactiae*, respectively (Table 3).

In chronic IMI, the duration of IMI was positively associated with the $\log_2$(CFU) in quarter milk samples during the whole study period for *Strep. agalactiae*, while for *Staph. aureus* no significant association between these two variables was found. For each of the pathogens, the $\log_2$(CFU) at the first culture positive sampling was not significantly associated with the type of IMI (transient or chronic).

Table 2. Number of quarters susceptible, infectious or newly infected, used to estimate transmission rate in 3 scenarios for *Staphylococcus aureus* and *Streptococcus agalactiae* on a Dutch dairy farm using an automatic milking system. In scenario 1, only chronic IMI were included in the analysis; in scenario 2, only chronic IMI were considered as infectious, while both, new chronic IMI and new transient IMI were defined as new IMI; in scenario 3, single sampling with bacteriological culture positive and negative were considered as infectious and susceptible, respectively.

Pathogen	Scenario	Infection status	10/10/2017	10/24/2017	11/7/2017	11/21/2017	12/5/2017	12/19/2017	1/2/2018	1/16/2018	1/30/2018	2/13/2018	2/27/2018
Staphylococcus aureus	1	Susceptible	121	231	233	235	234	232	216	220	222	216	210
		Infectious	2	4	4	5	6	5	4	4	5	4	2
		New IMI	NA[1]	0	0	0	0	0	0	0	1	0	0
	2	Susceptible	119	231	233	235	234	232	216	218	222	216	209
		Infectious	2	4	4	5	6	5	4	4	5	4	2
		New IMI	NA	1	0	0	0	2	2	2	3	1	0
	3	Susceptible	122	232	233	235	234	234	216	220	222	216	218
		Infectious	3	5	4	5	6	7	6	6	7	5	2
		New IMI	NA	1	0	0	0	2	2	2	3	1	0
Streptococcus agalactiae	1	Susceptible	209	232	234	238	238	235	217	222	224	218	211
		Infectious	3	5	5	5	5	6	5	4	6	4	2
		New IMI	NA	0	0	0	1	1	1	0	2	0	0
	2	Susceptible	208	232	234	238	238	235	217	222	224	218	211
		Infectious	3	5	5	5	5	6	6	4	6	4	2
		New IMI	NA	0	0	1	1	2	2	1	2	0	4
	3	Susceptible	213	233	234	238	238	236	217	222	224	218	220
		Infectious	4	5	5	6	5	7	6	5	6	4	6
		New IMI	NA	0	0	1	1	2	2	1	2	0	4

[1]Not applicable

Table 3. Transmission rate (number of transmission events per quarter-day), median duration of intramammary infection (IMI) that are considered as infectious and the corresponding basic reproduction number (R_0) estimated for *Staphylococcus aureus* and *Streptococcus agalactiae* on a Dutch dairy farm using an automatic milking system according to 3 different modelling scenarios. In scenario 1, only chronic IMI were included in the analysis; in scenario 2, only chronic IMI was considered as infectious and new chronic IMI and new transient IMI were defined as new IMI; in scenario 3, single sampling with bacteriological culture positive and negative were considered as infectious and susceptible, respectively. Only quarters with ≥ 2 consecutive samples were included. The 95% confidence intervals (95% **CI**) for estimates of transmission rates were profile likelihood based confidence intervals (except the 95% CI for transmission rate of *Staph. aureus* in scenario 1 which was calculated using the normal distribution because of only one new IMI case), while the 95% CI for median duration of IMI and R_0 were normal distribution confidence intervals.

	Scenario	**Transmission rate (cases/quarter-day, 95% CI)**	**Median duration of IMI (expressed as days, 95% CI)**	**R_0 (95% CI)**
Staph. aureus	Scenario 1	0.002 (0-0.005)	95 (72-125)	0.16 (0.05-0.27)
	Scenario 2	0.019 (0.010-0.032)	95 (72-125)	0.16 (0-0.32)
	Scenario 3	0.016 (0.008-0.027)	30 (20-45)	0.34 (0.20-0.48)
Strep. agalactiae	Scenario 1	0.007 (0.005-0.010)	86 (67-111)	0.64 (0.41-0.87)
	Scenario 2	0.019 (0.011-0.032)	86 (67-111)	0.64 (0.35-0.94)
	Scenario 3	0.016 (0.009-0.027)	39 (27-57)	0.68 (0.48-0.88)

DISCUSSION

With this study, it was our aim to get more insight in transmission of IMI in herds with an AMS by estimating the transmission rate, the duration of IMI and the R_0 of *Staph. aureus* and *Strep. agalactiae* in a Dutch dairy herd using an AMS. We found that the transmission rates of these two contagious pathogens were limited. The R_0 of both pathogens were <1, and the R_0 values were comparable to those in well-performing CMS herds (Zadoks et al., 2002; Kirkeby et al., 2019). Our results suggest that the different definitions of IMI are not largely affecting the R_0 even though the estimations of the transmission rates and median duration of IMI differed substantially in the different definition scenarios. By removing chronic IMI cows from the herd, the R_0 could be significantly decreased, which means that early intervention on mastitis cases infected by contagious pathogens would largely limit the transmission of contagious pathogens in the herd. Because of the existence of chronic IMI cases with both pathogens, spanning the whole study period, we were not able to distinguish the transmission between cow to cow and environment to cow transmission routes.

The number of farms using AMS worldwide is steadily increasing (Barkema et al., 2015) and as the contact dynamics between cows and milking machine in AMS herds are different from cows in CMS herds, contagious mastitis may behave differently in AMS herds as compared to CMS herds. In both systems, reduction of the transmission rate or duration of infection of contagious pathogens are key parts in a mastitis control program. Our study, therefore, aimed to estimate the transmission rate, duration of IMI and the corresponding R_0 of these two pathogens and we modelled possible differences in infectivity of transient and chronic IMI using 3 scenarios.

We found only 2 genotypes of *Staph. aureus* (the majority of isolates being *spa* type t529) in this herd, which is in line with other studies that found also only one or very few *Staph. aureus* genotypes in a herd (Anderson et al., 2012; Boss et al., 2016; Leuenberger et al., 2019). The genotyping of isolates enabled modelling of genotype specific transmission processes, therefore, the quarters solely infected with spa type t527 were treated as potential susceptibles for quarters infected with spa type t529 in the modelling. A highly predominant farm specific genotype of *Strep. agalactiae* has been reported in CMS herds (Radtke et al., 2012). Meanwhile, Holmøy et al. (2019) found all *Strep. agalactiae* isolates from the same herd belonged to the same multi-locus sequence types for both, AMS herds (n = 25) and CMS herds (n = 61) on 86 Norwegian dairy farms, which suggests that the *Strep. agalactiae* isolates in our study could be from the same genotype. Thus, we assumed in our analyses that the *Strep. agalactiae* isolates were of the same genotype when estimating the transmission rate in our study.

Transmission Dynamics

In our study, the average quarter prevalence of *Staph. aureus* per sampling for chronic IMI was 1.9% (ranging from 0.94% to 2.5%). The estimated transmission rate was comparable to results from previous

studies in CMS herds that also had a low *Staph. aureus* prevalence. Zadoks et al. (2002) described β in 3 herds, being 0.014 (0.008-0.023) for naïve susceptible quarters, while excluding quarters that could be considered more susceptible after being recovered from an IMI. Kirkeby et al. (2019) described β to be varying from 0.007 (95% CI:0-0.0175) to 0.009 (95% CI: 0.006-0.015) in a herd with a low *Staph. aureus* prevalence. In the only comparable study on transmission dynamics of *Staph. aureus* in an AMS herd (Dalen et al., 2019), transmission rate was found to be 0.009 (95% CI: 0.006-0.014)). All these studies did not differentiate between IMI with different duration and considered transient IMI as "true IMI" as long as they were from a single sample with clinical mastitis and/or the CFU in the sample exceeded a certain threshold. The estimated transmission rates from these studies were in the range of our estimates, which suggests that the transmission of *Staph. aureus* is comparable in AMS and CMS herds. However, our estimates for R_0 were lower than in previous studies in CMS herds that had a comparable prevalence of *Staph. aureus*. Zadoks et al. (2002) described that untreated subclinical *Staph. aureus* mastitis with a mean duration of 64 d had an R_0 of 0.42 (95% CI: 0.24-0.68). Kirkeby et al. (2019) described that *Staph. aureus* IMI with a median duration of infection of 64 d had an R_0 varying from 0.48 (95% CI: 0-1.23) to 0.59 (95% CI: 0.35 to 0.94) in their herd with a low *Staph. aureus* prevalence. Dalen et al. (2019) described *Staph. aureus* IMI with a mean duration of 128 days in an AMS herd to have an R_0 of 0.76 (95% CI: 0.41 to 1.42). To estimate the duration in an unbiased way was not possible, because we were dealing with data with both left truncation and right censoring, essentially leading to insufficient information to make a good estimation of duration of infection. As a consequence, it is likely that we underestimated the duration of IMI and therefore, of R_0, by ignoring the left truncation and right censoring in our study. This may be an explanation of the somewhat lower R_0 we found in an AMS herd in comparison to studies in CMS herds that have been carried out so far.

In CMS herds with a higher prevalence of *Staph. aureus*, both the estimated transmission rate and R_0 were higher than our estimates. Barlow et al. (2013), using the definition of IMI from Zadoks et al. (2002), estimated transmission rate at 0.008 (95% CI: 0.006-0.012) with the corresponding R_0 as 1.08 (95% CI: 0.81-1.62) for *Staph. aureus* IMI in a group of cows without antimicrobial treatment. Schukken et al. (2014) calculated the transmission rate ranging from 0.008 (95% CI: 0.007-0.010) to 0.010 (95% CI: 0.008-0.012) and the corresponding R_0 as 1.72 (95% CI: 1.06-3.17) for unvaccinated cows while van den Borne et al. (2017) reported transmission rate as 0.023 (95% CI: 0.020-0.027) in Swiss dairy herds. Finally, Kirkeby et al. (2019) reported transmission rate as 0.013 (95% CI: 0.010-0.016) and the R_0 as 1.16 (95% CI: 0.93-1.45) in a herd with a high *Staph. aureus* prevalence. In these herds with a higher prevalence of *Staph. aureus*, all R_0 were higher than 1 and outbreaks of *Staph. aureus* mastitis were highly likely or occuring. The AMS herd of our study could maintain $R_0 < 1$, which is comparable to well-performing CMS herds, and is therefore able to prevent transmission and outbreaks of contagious pathogens. This is likely a result of the AMS functioning well in terms of teat cleaning, teat disinfection as well as steaming of milking clusters in between cows.

In all scenarios evaluated, we found the estimated transmission rate for *Strep. agalactiae* to be no less than 0.007 (95% CI: 0.001-0.060), which was estimated in a CMS herd by Leelahapongsathon et al. (2016), which indicates *Strep. agalactiae* may transmit easier in this AMS herd. The estimated R_0 in our study herd, however, was lower than the 1.86 (95% CI: 0.21-16.61) estimated by Leelahapongsathon et al. (2016). Our study, however, only lasted for 6 mo, which again potentially underestimated the duration of IMI, as compared to the 10 mo study of Leelahapongsathon et al. (2016) and likely resulted in an underestimation of R_0 in our study. In addition, the homogeneity assumption for susceptibility may have been violated, which may also result in an underestimation of the transmission rate of both pathogens. Zadoks et al. (2002) found that quarters recovered from infection (regardless of being the result of treatment or natural cure) seemed to be more susceptible to new IMI than naïve susceptible quarters, which would contribute to higher estimates of transmission rate. In our study, we did not distinguish between the susceptibility of naïve susceptible quarters and recovered quarters, which might have resulted in an underestimation of transmission rate.

The estimated median duration of IMI for chronic IMI of *Staph. aureus* and *Strep. agalactiae* were in-between the estimates from previous studies. Previous studies estimated the duration of IMI for *Staph. aureus* at 135.8-177.9 days (mean duration of IMI; Lam et al., 1996), 29 days (mean duration of IMI; Zadoks et al., 2002), and 64-91 days (median duration of IMI; Kirkeby et al., 2019). There are two factors contributing to the differences between duration of IMI in these studies and our current study: the definition of IMI and the length of the study period that could capture the potential full length of the duration of IMI. Lam et al. (1996) excluded short subclinical infections by the definition of IMI, while both Zadoks et al. (2002) and Kirkeby et al. (2019) included transient IMI in their analysis. By taking transient IMI into account, the estimated average duration of IMI obviously is shortened, and in addition a shorter study period might have contributed to a shorter duration of IMI and thus an underestimation of R_0 in our study.

The duration of chronic IMI was significantly and positively associated with the $\log_2$(CFU) for *Strep. agalactiae*, which suggests that higher bacterial loads in milk samples are likely to be persistent infections for IMI caused by *Strep. agalactiae*. However, the duration of chronic IMI caused by *Staph. aureus* could not be forecasted by the bacterial load in quarter milk samples. We did not find a significant association between the $\log_2$(CFU) at the first positive sampling and whether this infection became chronic or transient. This suggests that the bacterial load in the first culture positive sample does not predict the outcome of the infection.

Staphylococcus aureus and *Strep. agalactiae* are generally considered to be contagious pathogens that transmit between cows during milking. However, there are several studies that describe environmental transmission of *Staph. aureus* (Fox and Gay, 1993; Barlow et al. 2013; Cobo-Ángel et al., 2018) and

Strep. agalactiae (Jørgensen et al., 2016 ; Klaas and Zadoks, 2018). The contribution of environmental transmission may be underestimated in low *Staph. aureus* IMI prevalence herds (Barlow et al., 2013). We were unable to estimate the transmission rate of environmental transmission of both pathogens in this study because there were chronic IMI spanning the whole study period, and new IMI resulting from environmental transmission could not be distinguished from those derived from infectious quarters. Isolates of spa type t527 have been found in bulk tank milk samples (Boss et al., 2016; Patel, 2018), but we are not aware of reports of environmental sources of spa type t527. Further studies are needed to elucidate the role of AMS and of the environment in the transmission of these contagious pathogens.

The estimated R_0 for both pathogens was below 1 in all scenarios, which theoretically would lead to fading out of the infection in this herd. However, both pathogens seemed to be persistently present in the herd during our study. This has been seen in CMS herds as well (Zadoks, et al., 2002; Leelahapongsathon et al., 2016). Possible explanations could be more heterogeneity in infectivity and susceptibility of IMI in the population than we were able to evaluate, with a subgroup of cows maintaining the IMI throughout the course of our study. Zadoks et al., (2002) found that quarters recovered from IMI were more susceptible to new IMI than naïve susceptible quarters. When recovered quarters are more susceptible than naïve quarters this might lead to an endemic situation even when R_0 is below the threshold of 1 (Greenhalgh et al., 2000), thus eradication of the infection will take more effort than prevention of entrance of new infected animals into the herd (Zadoks et al., 2002).

CONCLUSION

Transmission of *Staph. aureus* and *Strep. agalactiae* between quarters in this AMS herd is limited and R_0 of both pathogens was estimated to be below 1, although some underestimation of R_0 is likely. The estimated transmission rate of *Staph. aureus* in the AMS herd studied was comparable to findings described on well-performing CMS herds, while it was slightly higher for *Strep. agalactiae*. The estimated R_0 of these contagious pathogens was lower than the estimates on CMS farms. Future studies are needed to quantify transmission in more AMS herds and get more insight in the transmission routes of these pathogens in AMS herds to better facilitate further prevention of transmission.

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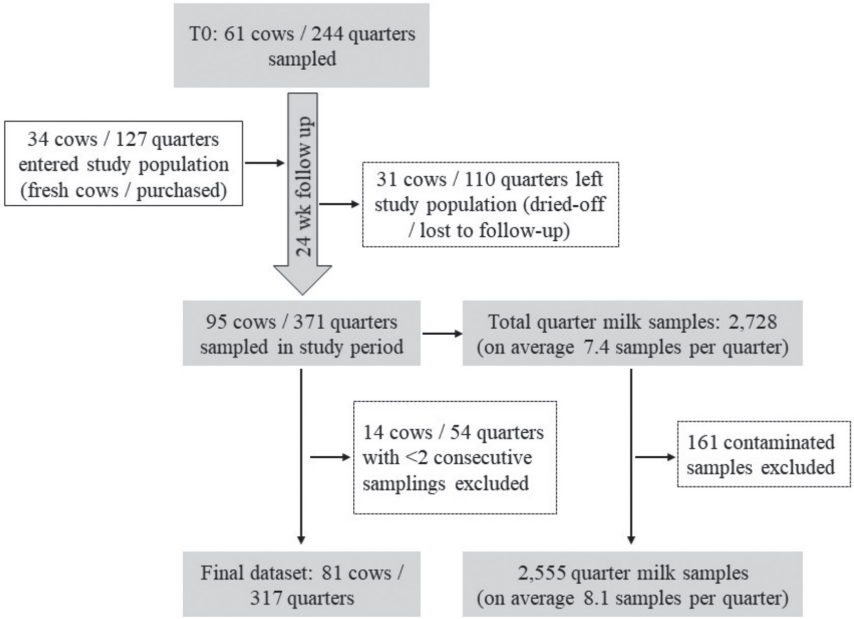
SUPPLEMENTARY MATERIAL S1

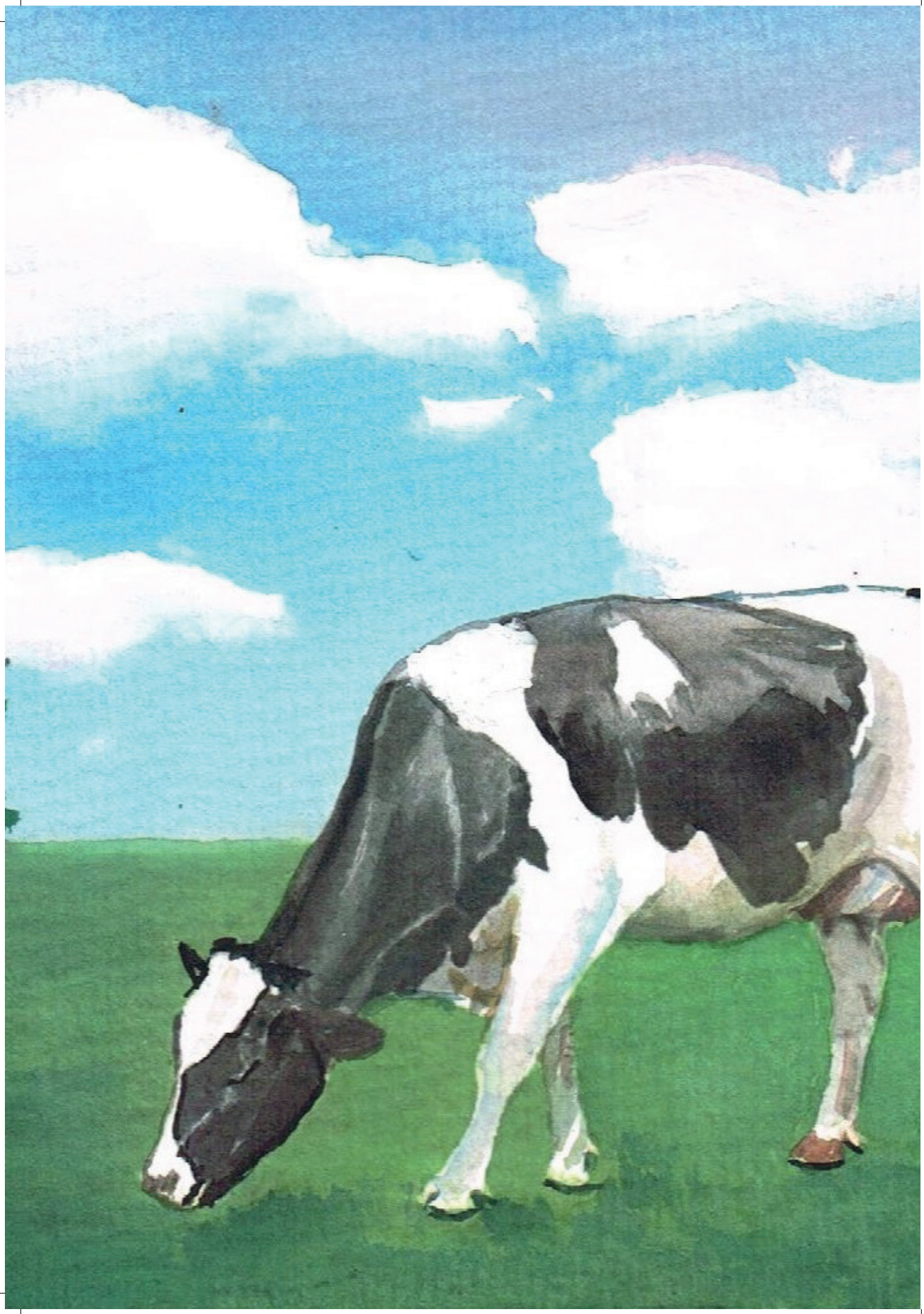
Maximum likelihood estimation of transmission rate for *Staph. aureus* and *Strep. agalactiae*:

```
# function to calculate the log likelihood

# β is the transmission rate, C is the number of new intramammary infection (IMI), S is the number of susceptible quarters, I is the number of infectious quarters and N is the total number of quarters according to the definitions in each scenario.

min.ll <− function(β, C, S, I, N, deltat){


 # functions to calculate force of infection

  foi <− β * I/N   # force of infection due to chronic

  # probability of infection during sampling interval

  p <−  (1 - exp(-(foi ) * deltat))


  # log likelihood for each sampling interval

  lprob <− dbinom(x = C, size = S, prob = p, log = TRUE)

# return log likelihood over all sampling intervals.

  -sum(lprob)

}


# fitting the model

fit <−  mle2(minuslogl = min.ll, start = list(β = (0.00001)),

                data = list(C = C, S = S, I = I,  N = N, deltat = deltat),

                method = "Nelder-Mead",

control = list(maxit = 5000000,
```

```
type = 1,

trace = 1,

reltol = 10^(8)*.Machine$double.eps))

coef(summary(fit))  # estimate of transmission rate
```



Supplementary Figure S1. Flow diagram of the number of cows and quarters enrolled in the study, and the number quarter milk samples collected during the study period.



Chapter 6

Antimicrobial use and farmers' attitude towards mastitis treatment in dairy farms with an automatic and a conventional milking system

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ABSTRACT

Mastitis is one of the major causes for antimicrobial use on dairy cattle farms. On farms with an automatic milking system (AMS), diagnostics differ from those with a conventional milking system (CMS), with potentially a different attitude towards mastitis treatment. This may result in differences in antimicrobial usage (AMU) between these two types of farms. The aims of this study were (1) to compare AMU between AMS and CMS farms, (2) to identify variables associated with AMU in both types of herds and (3) to describe the distribution of mastitis-causing pathogens and their antimicrobial resistance patterns.

Data on AMU was collected for 42 AMS and 254 CMS farms in the Netherlands and was expressed as animal defined daily dose (ADDD). The ADDD variables were total usage ($ADDD_{TOTAL}$), intramammary usage during lactation ($ADDD_{IMM}$), usage for dry cow therapy ($ADDD_{DCT}$) and usage by injection ($ADDD_{INJ}$).

Eighteen AMS farms and 24 CMS farms participated in a survey on factors potentially related to AMU. These farmers collected five quarter milk samples from clinical mastitis or high somatic cell count quarters, which were subjected to bacteriological culture and antimicrobial susceptibility testing. In addition, routinely collected udder health data of these farms was used in the analysis. Nonlinear principal component analysis (NLPCA) was used to explore associations between AMU, udder health and questionnaire variables.

The $ADDD_{TOTAL}$ and $ADDD_{DCT}$ were comparable between AMS and CMS farms, while $ADDD_{IMM}$ tended to be lower and $ADDD_{INJ}$ to be higher on AMS farms than on CMS farms. The NLPCA yielded three principal components (PCs) that explained 48% of the variation in all these variables. The AMS farms were not distinguished from CMS farms in the principal component space. The 3 PC's represented different aspects of udder health, $ADDD_{TOTAL}$, and treatment strategy. Differences in treatment strategy were unrelated to total antimicrobial usage or overall udder health. The distribution of mastitis-causing pathogens and their antimicrobial resistance was comparable between AMS and CMS farms.

In conclusion, our study shows that AMU on AMS farms was similar to CMS farms, but AMS farmers tend to apply more injectable and less intramammary treatments during lactation than CMS farmers. Across both farm types, the farmers' attitude towards udder health in general and towards mastitis treatment are associated with AMU.

INTRODUCTION

Antimicrobial usage (**AMU**) in food producing animals is one of the drivers of antimicrobial resistance (**AMR**) (Van Boeckel et al., 2014; Tang et al., 2017). For that reason, various policies to reduce AMU in livestock have been proposed and implemented (e.g. Levy, 2014; Speksnijder et al., 2014; Mathew et al., 2007). Most AMU in dairy cattle is related to udder health (Mitchell et al., 1998). In the Netherlands, for instance, 22% of AMU on dairy farms was related to clinical mastitis (**CM**) and 44% to dry cow treatment (Kuipers et al., 2016) and comparable proportions have been described in Canada (Nobrega et al., 2018) and Ireland (More et al., 2017). Controlling AMU is important as, for instance, the emergence of AMR in *Staphylococcus aureus* was reported to be associated with herd level AMU of certain antimicrobials (Saini et al., 2012), as was the prevalence of multi-drug resistant non-aureus *Staphylococcus* (**NAS**) isolates (Nobrega et al., 2018).

Diagnosis is an important first step in the decision making process that leads to use of antimicrobials related to mastitis. On farms with an automatic milking system (**AMS**), mastitis is diagnosed differently than on farms with a conventional milking system (**CMS**), because in AMS farms, mastitis diagnostics relies primarily on sensors (Jacobs and Siegford, 2012; Viguier et al., 2009), which may affect AMU. For instance, the use of electrical conductivity for mastitis detection was found to be positively related to AMU for treatment of both subclinical mastitis (**SCM**) (Biggadike et al., 2002) as well as CM (Kayitsinga et al., 2017). Later in the mastitis treatment process, AMS farmers may also differ from CMS farmers in terms of attitude towards treatment for mastitis. Vilar et al. (2018) reported that Finnish AMS farmers tend to more frequently use blanket dry cow therapy (**DCT**) than CMS farmers. Differences in diagnostics and in attitude towards treatment of mastitis may result in differences in AMU between AMS and CMS farms. In addition, the milking system may also affect udder health in general (Lam et al., 2013; Nor et al., 2014; Deng et al., 2019) and the distribution of mastitis-causing pathogens in a herd. It is important to understand the drivers of AMU and to know how AMS farmers differ in their approach to mastitis treatment and AMU from CMS farmers in order to tailor programs to further reduce AMU to prevent further development of AMR in dairy herds. To our knowledge, no studies have compared AMU between AMS and CMS farms and investigated the diagnostic and treatment approach of the farmers associated with AMU. In this study, we use AMU data that has been recorded for a large number of AMS and CMS farms as well as data from a telephone interview to obtain more in-depth information on potential drivers of AMU in a selection of these farms. Given the large number of variables obtained in this approach, we use principal component analysis (**PCA**) to analyze our data. PCA is a technique that can be used to reduce the dimension of data and to identify structure in the relationships between variables. As heterogeneous variable types, such as categorical and numerical variables, and nonlinear relationships among variables are common in questionnaire data, nonlinear PCA (**NLPCA**) is employed, in which categorical variables are transformed into

numeric quantifications. After this quantification, the subsequent analysis steps are the same as in linear PCA (Linting et al, 2007).

The aims of this study were (1) to compare AMU between AMS and CMS farms, (2) to determine variables associated with AMU in both types of herds, including the farmers' attitude towards mastitis detection and treatment and (3) to describe the distribution of mastitis-causing pathogens and their antimicrobial resistance patterns in both types of herds.

MATERIALS AND METHODS

Study Outline

For this study, we used routinely collected AMU data on all 296 dairy farms (42 AMS and 254 CMS farms) served by the University Farm Animal Practice (ULP, Harmelen, the Netherlands) which gave a written consent to use their data. All 42 AMS farmers and an equal number of randomly selected CMS farmers were invited by email to participate in a more detailed study. This resulted in a subset of 42 farmers (18 AMS and 24 CMS farms) which were willing to participate, on which we also collected udder health data and administered a telephone interview about the approach of farmers towards diagnosis and treatment of mastitis. Farmers who participated in the interview were also invited to submit 5 milk samples from CM cases (if needed, supplemented with SCM cases) to describe mastitis-causing pathogens and the corresponding AMR profile.

Antimicrobial Usage Data

The AMU data of 42 AMS farms and 254 CMS farms served by the ULP were used for analysis of the AMU data that were extracted from the Dutch national database for registration of AMU in cattle (Medirund). The AMU data was expressed as animal defined daily dosage (**ADDD**) as described by Santman-Berends et al. (2016), and included total usage (**$ADDD_{TOTAL}$**), intramammary usage during lactation (**$ADDD_{IMM}$**), usage for dry cow therapy (**$ADDD_{DCT}$**) and usage by injection (**$ADDD_{INJ}$**). There was no information on the indications for antimicrobial use available in the dataset. Thus, we could not determine if the $ADDD_{INJ}$ was associated with mastitis treatment. In addition to the comparison of the absolute values of AMU variables for each application route, the proportion for each application route of the $ADDD_{TOTAL}$ was also compared between AMS and CMS farms. The median of all AMU variables in the small dataset were compared to the corresponding median of the same AMU variables in the large dataset on farms with the same milking system. The median of the same AMU variables in both datasets were compared between AMS and CMS farms using the Wilcoxon rank-sum test.

Questionnaire Data

Questionnaire questions were designed for this study and were not based on a pre-existing theoretical framework. During the telephone interviews, farmers were asked open questions, or closed questions which were followed up by open questions, by a trained master student. The 42 farmers (18 AMS and 24 CMS) were questioned on their definition of mastitis, the methods they used to diagnose mastitis, their approach towards treatment of (sub)clinical mastitis and towards antimicrobial use. The questionnaire was pretested in 3 farms that were not otherwise involved in the research, and subsequently several questions were changed to improve understanding by the farmers. The questionnaire was administered in Dutch and consisted of 31 questions, of which 19 were open questions and the interviews were recorded. The responses were categorized into inductively created themes after finalization of the interviews to accommodate answer categories given by the farmers as follows: one of the authors translated the questionnaire into English and this translation was reviewed by another author of the paper. Then, for open questions, answer categories were designed based on the various answers given by the farmers. This recoding of the open questions was checked by a second author independently and in case of disagreement, the coding of the answers was discussed among the authors. Variables with more than 8 missing records were presented in the descriptive statistics but were excluded from further statistical analyses. Fisher's exact test was used to compare the distribution of answer categories in the questionnaire results between the two types of farms.

Udder Health Data

For the 42 farms that participated in the telephone interview, during the year preceding the administration of the questionnaire (November 2016 to November 2017) rolling yearly averages were collected for DHI parameters related to udder health and other farm characteristics, and included: total number of cows, annual geometric mean bulk milk somatic cell count (**BMSCC**), annual geometric mean of herd SCC, average percentage of high SCC cases, average percentage of new high SCC cases, average percentage of new high SCC cases in the dry period and the average percentage of cured high SCC cases during the dry period. The threshold for high SCC was ≥ 250,000 cells/mL for multiparous cows and ≥ 150,000 cells/mL for heifers (de Haas et al., 2008). The percentage of new high SCC cases during the dry period was calculated as the percentage of cows with a high SCC at the first milk recording after calving out of the total number of cows with a low SCC at drying off. The percentage of cured cases during the dry period was calculated as the percentage of cows with a low SCC at the first recording after calving out of the number of cows with a high SCC at the last recording before drying off (Vanhoudt et al., 2018).

Milk Samples

Around the time of the interview (November 2017), the farmers were provided with 5 labeled milk tubes and were invited to collect quarter milk samples of cases of CM from 5 different cows until January 2018. If less than 5 cases of CM were seen in this time period, the farmer could supplement these by samples of SCM cases (according to the farmers' own definition of SCM) to a total of 5 quarter milk samples. The farmers were asked to store the milk samples at -20 °C until all 5 samples were collected. They filled out a form for each milk sample to record sampling date, cow identification, parity, most recent calving date, affected quarter, clinical signs and whether the cow had received treatment.

Bacteriological Culture and Antimicrobial Resistance Testing

Milk Samples were subjected to bacteriological culture according to the NMC guidelines (NMC, 2017). In brief, 10µL milk was plated on a blood agar plate, and was incubated at 37 °C. Results were read at approximately 24 and 48h incubation. Bacteria were identified with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using the MALDI Biotyper Microflex LT (Bruker Daltonics GmbH, Germany) (Barreiro et al., 2010). Isolates with identification score ≥ 2.0 were considered as successfully identified. Culture results were recorded as culture negative if no bacteria were cultured on the blood agar plate and as culture positive if 1 or 2 morphologically different colony types with ≥ 1 colonies were found. Samples yielding ≥ 3 phenotypically distinct colonies were considered as contaminated. Of all available *Staph. aureus*, *Strep. uberis*, *Strep. dysgalactiae* and NAS isolates from non-contaminated samples, antimicrobial susceptibility was tested with the Micronaut-S Mastitis 3 plate (Merlin Diagnostics, Berlin, Germany). Inoculum preparation, broth composition and incubation conditions were performed according to the manufacturer's guidelines. The antimicrobials tested were amoxicillin/clavulanic acid, ampicillin, cefazolin, cefoperazone, cefquinome, erythromycin, kanamycin/cephalexin, marbofloxacin, oxacillin, penicillin G and pirlimycin. The plates were read after incubation with a photometer (Skan, Merlin Diagnostika, Germany). We used *Staph. aureus* ATCC 29213 as quality control strain. Human breakpoints for each antimicrobial were used as clinical breakpoints in antimicrobial resistance test.

Statistical Analysis

The ADDD for various purposes, farm characteristics and udder health variables were compared between AMS and CMS farms and differences were tested using Wilcoxon rank-sum test for variables with a non-normal distribution or Students *t*-test for normally distributed variables (normality was checked by visual inspection of the quantile-quantile plot for each variable). In order to explore the possible factors associated with AMU variables, we performed a nonlinear principal component analysis (**NLPCA**) on the questionnaire variables, udder health data and the AMU data using the *princals* function from Gifi

package version 0.3-7 (Mair et al., 2017; https://rdrr.io/rforge/Gifi/) in R version 3.5.1 (R Core Team, 2018). We tested NLPCA with 3, 4, 5 dimensional solutions, and the number of principle components (**PC**) was determined by scree plot and the interpretability of the PCs (Linting et al., 2007). Of all 42 farms, there were 3 farms with a missing value in 1 variable and 1 farm with missing values in 9 variables. These 12 missing values were imputed as the mean of all other farms for the same variable to enable running the NLPCA using all farms and variables.

RESULTS

Descriptive Statistics

Antimicrobial Usage and Udder Health The median $ADDD_{TOTAL}$ was 2.12 and was similar between AMS and CMS farms (Table 1). On average, 39% of the $ADDD_{TOTAL}$ could be attributed to dry cow therapy and this too was very similar between the 2 farm types.The $ADDD_{IMM}$ tended to be lower while the $ADDD_{INJ}$ tended to be higher on AMS farms compared to CMS farms. The average proportion of $ADDD_{IMM}$ of $ADDD_{TOTAL}$ was significantly lower ($P < 0.05$) on AMS farms and the average proportion of $ADDD_{INJ}$ of $ADDD_{TOTAL}$ was significantly higher ($P < 0.05$) on AMS farms compared to CMS farms. The subset of 42 farms that participated in the questionnaire had higher AMU than the non-participating farms in each type of farms, but the comparison between AMS and CMS farms in AMU gave similar results as compared to what was found in the larger dataset (Table 2) (none of the comparisons was significant by Wilcoxon rank-sum test). The AMS herds were significantly ($P < 0.05$) larger than CMS herds and AMS herds tended to have a higher average 305d milk yield (Table 2). Udder health parameters were similar between the AMS and CMS farms, except for the proportion new high SCC (p-value: 0.06) and the proportion of new high SCC during the dry period (p-value: 0.10), which tended to be slightly higher in AMS herds (Table 2).

Table 1. Total antimicrobial usage expressed as animal defined daily dosage per year (ADDD) and antimicrobial usage for intramammary mastitis treatments during lactation, dry cow therapy and injectables and their proportions of the total ADDD on farms with an automatic milking system (AMS) or a conventional milking system (CMS). ADDD values and proportions are presented as median and interquartile range (IQR). Differences between medians for AMS and CMS farms are tested using Wilcoxon rank-sum test.

	AMS		CMS		
Item	**N (farms)**	**Median (IQR)**	**N (farms)**	**Median (IQR)**	***P*-value**
Total antimicrobial usage ($ADDD_{TOTAL}$)	42	2.18 (1.39 to 2.46)	254	2.11 (1.97 to 2.25)	0.73
Intramammary mastitis treatments ($ADDD_{IMM}$)	42	0.48 (0.37 to 0.62)	254	0.62 (0.55 to 0.69)	0.08
Dry cow therapy ($ADDD_{DCT}$)	42	0.86 (0.59 to 1.07)	254	0.88 (0.68 to 1.00)	0.94
Injectables ($ADDD_{INJ}$)	42	0.56 (0.45 to 0.61)	254	0.47 (0.41 to 0.51)	0.11
Proportion intramammary treatments of total ADDD ($ADDD_{IMM}$/ $ADDD_{TOTAL}$)	42	0.24 (0.20 to 0.28)	251	0.32 (0.27 to 0.34)	0.02
Proportion dry cow therapy of total ADDD ($ADDD_{DCT}$/$ADDD_{TOTAL}$)	42	0.39 (0.34 to 0.44)	251	0.39 (0.35 to 0.40)	0.69
Proportion injectables of total ADDD ($ADDD_{INJ}$/$ADDD_{TOTAL}$)	42	0.29 (0.24 to 0.38)	251	0.23 (0.22 to 0.26)	0.01

Table 2. Descriptive statistics of the farm characteristics, udder health parameters and antimicrobial usage (animal defined daily dose (ADDD)) for 18 automatic milking system (AMS) farms and 24 conventional milking system (CMS) farms that participated in an in-depth study in November 2017. Variables were compared between AMS and CMS farms using Wilcoxon rank-sum test. Within square brackets, the abbreviation of the variable is mentioned for variables with strong loadings in the nonlinear principal component analysis (NLPCA).

Item [abbreviation in NLPCA]	Mean or median (95% CI or IQR)[2]		*P*-value
	AMS (n = 18)	CMS (n = 24)	
Total number of cows [N_cow]	117 (56 to 178)	72 (56 to 88)	0.02
305-day milk yield per cow (× 1,000 kg)	9.1 (8.3 to 10.0)	8.3 (7.8 to 8.8)	0.08
Annual geometric mean herd SCC (× 1,000 cells/mL) [Herd_SCC]	171 (125 to 190)	165 (131 to 182)	0.96
Annual geometric mean of BMSCC [1] (× 1,000 cells/mL) [BMSCC]	175.6 (144.1 to 207.1)	165.5 (142.6 to 188.4)	0.53
Annual percentage of cows with new high SCC during dry period (%) [Percentage_new_high_SCC_dry_period]	15.5 (12.4 to 18.6)	13.4 (9.7 to 17.1)	0.10
Annual percentage of cured high SCC during dry period (%) [Percentage_cure_dry_period]	0.8 (0.69 to 0.86)	0.8 (0.70 to 0.83)	0.96
Annual percentage of cows with high SCC (%) [Percentage_high_SCC]	16.3 (13.2 to 19.4)	15.5 (13.1 to 18.0)	0.60
Annual percentage of cows with new high SCC (%) [Percentage_new_high_SCC]	8.7 (7.2 to 10.2)	7.2 (6.1 to 8.3)	0.06
Total antimicrobial usage [$ADDD_{TOTAL}$]	2.77 (1.89 to 3.31)	2.29 (1.93 to 3.24)	0.50
Intramammary mastitis treatment [$ADDD_{IMM}$]	0.59 (0.41 to 0.86)	0.81 (0.51 to 1.06)	0.27
Dry cow treatments [$ADDD_{DCT}$]	1.03 (0.81 to 1.56)	1.11 (0.37 to 1.59)	0.95
Injectables treatments [$ADDD_{INJ}$]	0.79 (0.52 to 1.03)	0.53 (0.17 to 0.80)	0.06
Proportion of antimicrobial usage for intramammary treatment [$ADDD_{IMM}/ADDD_{TOTAL}$]	0.24 (0.19 to 0.28)	0.32 (0.25 to 0.42)	0.03
Proportion of antimicrobial usage for dry cow therapy [$ADDD_{DCT}/ADDD_{TOTAL}$]	0.42 (0.32 to 0.49)	0.43 (0.28 to 0.54)	0.84
Proportion of antimicrobial usage for injectable use [$ADDD_{INJ}/ADDD_{TOTAL}$]	0.32 (0.23 to 0.43)	0.22 (0.13 to 0.33)	0.06

[1]bulk tank milk somatic cell count [2]Values reported for antimicrobial usage were medians and interquartile range (IQR), other values were mean and 95% confidence interval.

Questionnaire

The first open question was about what farmers consider to be 'clinical mastitis'. Some farmers indicated 'when a cow has abnormal milk', but most farmers added that the udder should also show symptoms such as clots in milk, abnormalities in the udder, or that the cow should have systemic symptoms of disease. Interestingly, AMS farmers significantly ($P < 0.05$) more frequently mentioned systemic symptoms, whereas CMS farmers more often talked about symptoms of the udder (Table 3). In a follow-up question, farmers were asked about what grades of mastitis they discern. Most farmers distinguished mild and severe cases. The interviewer then asked about the farmer's definition of mild and severe. Some farmers grouped cows with an abnormal udder under mild mastitis, whereas others categorized this as severe. About one third of the farmers mentioned the three grades of mastitis as defined by Pinzón-Sánchez and Ruegg (2011). The approach toward treatment was very similar between AMS and CMS farmers. The majority of farmers does not treat all CM cases with antimicrobials, but selects cows for treatment based on severity of the mastitis case, or only treats after a non-antimicrobial treatment failed. Around 45% of the farmers report to never treat high SCC cows with antimicrobials. Bacteriological culture is sometimes done by most farmers, mostly to better understand the mastitis problem on the farm, or to tailor the treatment for a specific cow. The majority of farmers reported to follow up on treatments, which CMS farmers mostly based on clinical inspections of the cow or the milk, whereas AMS farmers primarily used milk quality data like electrical conductivity or SCC.

Table 3. Categorized results of a telephone interview on mastitis definition and treatment approach on 18 farms using an automatic milking system (AMS) and 24 farms using a conventional milking system (CMS). Within square brackets, the abbreviation of the variable is mentioned for variables with strong loadings in the nonlinear principal component analysis (NLPCA). The association between answers given to each question and AMS versus CMS farms was tested by Fisher's exact test.

Question [abbreviation in NLPCA]	Answer categories	Frequency	AMS (%)	CMS (%)	*P*-value
When would you say a cow has clinical mastitis? [Definition_CM]	Mentions abnormal milk	6	4 (22%)	2 (8%)	0.006
	Mentions abnormal milk and abnormal udder	21	4 (22%)	17 (71%)	
	Mentions abnormal milk and abnormal udder and systemic signs	15	10 (56%)	5 (21%)	
Do you distinguish clinical mastitis based on severity?[1] [CM_grading]	Clusters grade 1 and 2 as mild, grade 3 as severe	13	6 (33%)	7 (29%)	0.6
	grade 1 mild, grade 2 moderate, grade 3 severe	14	5 (28%)	9 (38%)	
	Clusters grade 1 as mild, grade 2 and 3 as severe	13	7 (39%)	6 (25%)	
	Makes no distinction	2	0 (0%)	2 (8%)	
Do you treat all clinical mastitis cases with antimicrobials? [Treats_all_CM]	Yes	14	5 (28%)	9 (38%)	0.7
	No	28	13 (72%)	15 (63%)	
Which clinical mastitis cases do you treat with antimicrobials? [Select_CM_for_treatment]	All clinical mastitis cases	14	5 (28%)	9 (38%)	0.3
	Only the severe cases	20	11 (61%)	9 (38%)	
	Cases in which treatment with non-antimicrobials has failed	8	2 (11%)	6 (25%)	
What is your standard approach for clinical mastitis treatment?	Mentions intramammary treatment	37	16 (89%)	21 (88%)	1
	Mentions intramammary and systemic treatment	5	2 (11%)	3 (13%)	

Question [abbreviation in NLPCA]	Answer categories	Frequency	AMS (%)	CMS (%)	*P*-value
Do you do bacteriological culture in case of clinical mastitis treatment? [Uses_BC_CM]	Sometimes	31	13 (72%)	18 (75%)	1
	Never	11	5 (28%)	6 (25%)	
What follow up do you do in case of clinical mastitis?	Clinical inspection of the cow	19	4 (22%)	15 (63%)	0.02
	Inspection of milk quality data	17	11 (61%)	6 (25%)	
	I do no follow up	6	3 (17%)	3 (13%)	
Do you ever treat subclinical mastitis cases with antimicrobials? [Treats_high_SCC]	Sometimes	23	10 (56%)	13 (54%)	1
	Never	19	8 (44%)	11 (46%)	
What is your standard treatment approach towards subclinical mastitis? [Standard_treatment_SCM]	Mentions antimicrobial treatment	16	6 (33%)	10 (42%)	0.7
	Mentions no treatment or treatment without antimicrobials	19	8 (44%)	11 (46%)	
	Mentions diagnostic testing (CMT or bacteriological culture)	7	4 (22%)	3 (13%)	

[1] The verbal explanations of the farmers were interpreted as grade 1, 2 or 3 mastitis as defined by Pinzón-Sánchez and Ruegg (2011).

Table 4. Bacteriological culture results of 138 quarter milk samples from cases of clinical or subclinical mastitis from 15 farms with an automatic milking system (AMS) and 23 farms with a conventional milking system (CMS) that submitted milk samples.

Culture result	Clinical mastitis		Subclinical mastitis		NA[1]		Total	
	AMS	CMS	AMS	CMS	AMS	CMS	AMS	CMS
Staphylococcus aureus	3 (8%)	11 (15%)	1 (11%)	8 (32%)	3 (30%)	0 (0%)	7 (12%)	19 (19%)
Streptococcus uberis	10 (26%)	7 (9%)	0 (0%)	3 (12%)	2 (20%)	0 (0%)	12 (21%)	10 (10%)
Escherichia coli	6 (16%)	13 (18%)	1 (11%)	1 (4%)	0 (0%)	0 (0%)	7 (12%)	14 (14%)
Non-*aureus Staphylococcus*	4 (11%)	10 (13%)	2 (22%)	4 (16%)	1 (10%)	0 (0%)	7 (12%)	14 (14%)
Streptococcus dysgalactiae	5 (13%)	7 (9%)	1 (11%)	2 (8%)	2 (20%)	0 (0%)	8 (14%)	8 (8%)
Other pathogens	3 (8%)	8 (11%)	3 (34%)	1 (4%)	1 (10%)	0 (0%)	7 (12%)	8 (8%)
Contamination	4 (10%)	7 (9%)	0 (0%)	5 (20%)	0 (0%)	0 (0%)	4 (7%)	12 (12%)
Culture negative	3 (8%)	12 (16%)	1 (11%)	1 (4%)	1 (10%)	0 (0%)	5 (9%)	13 (13%)
Total	38 (100%)	75 (100%)	9 (100%)	25 (100%)	10 (100%)	0 (0%)	57 (100%)	100 (100%)

[1]The farmer did not record whether the sample was from a clinical or a subclinical mastitis case.

Table 5. Number (n) and percentage (%) of isolates resistant to 11 antimicrobials for the five most prevalent mastitis pathogens isolated from quarter milk samples from 15 farms with an automatic milking system (AMS) and 23 farms with a conventional milking system (CMS) that provided milk samples of clinical or subclinical mastitis cases or both. Antimicrobial resistance data for 1 *Strep. uberis* and 2 non-aureus *Staphylococcus* isolates from AMS farms were missing.

	Staph. aureus		***E. coli***		***Strep. uberis***		***Strep. dysglactiae***		**Non-aureus *Staphylococcus***	
	AMS (n =7)	**CMS (n = 19)**	**AMS (n = 7)**	**CMS (n = 14)**	**AMS (n= 11)**	**CMS (n = 10)**	**AMS (n =8)**	**CMS (n= 9)**	**AMS (n = 5)**	**CMS (n = 14)**
AMC[1]	0 (0%)	1 (5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (25%)
AMP[2]	0 (0%)	0 (0%)	0 (0%)	2 (14%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
CEQ[3]	0 (0%)	2 (11%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (12%)	2 (25%)
CEZ[4]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
CPZ[5]	2 (29%)	1 (5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (25%)	1 (12%)
ERY[6]	0 (0%)	1 (5%)	NA[12]	NA	0 (0%)	1 (10%)	0 (0%)	3 (34%)	1 (13%)	1 (12%)
K/C[7]	1 (14%)	5 (26%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	6 (75%)
MAF[8]	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (25%)
OXA[9]	0 (0%)	0 (0%)	NA	NA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (12%)
PEN[10]	2 (29%)	4 (21%)	NA	NA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (38%)	3 (38%)
PIR[11]	5 (71%)	6 (32%)	NA	NA	4 (40%)	2 (20%)	1 (12%)	2 (22%)	4 (50%)	7 (88%)

[1]Amoxicillin-clavulanic acid

[2]Ampicillin

[3]Cefquinome

[4]Cefazolin

[5]Cefoperazon

[6]Erythromycin

[7]Kanamycin/Cephalexin

[8]Marbofloxacin

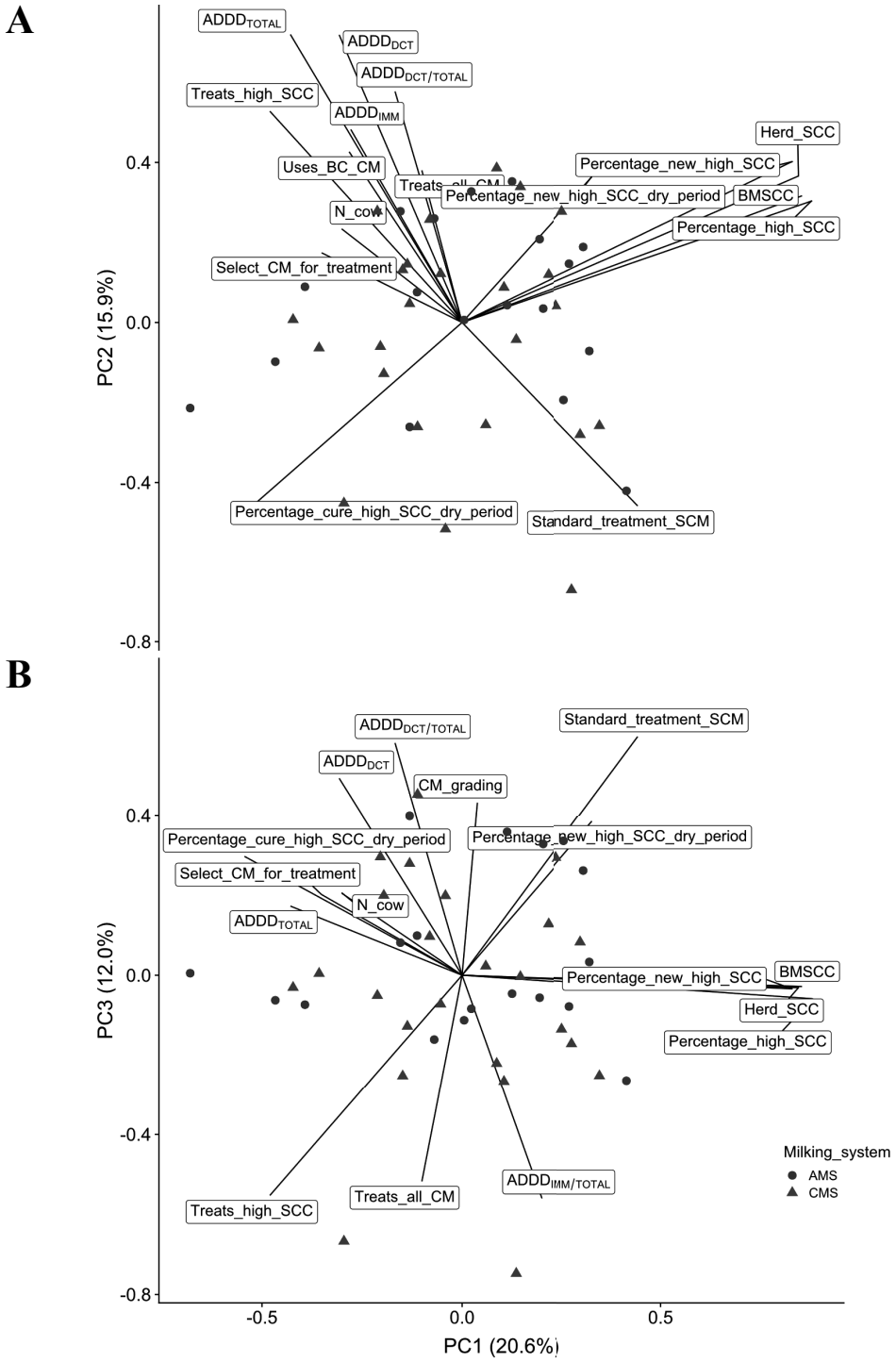
[9]Oxacillin

Bacteriological Culture and Antimicrobial Resistance

CMS farmers submitted more milk samples per farm to us than AMS farmers did (on average 3.8 and 3.4 samples per farm, respectively). Of the 42 participating farms, 3 AMS farms and 1 CMS farm did not provide any milk samples. Table 4 shows that *Staph. aureus, Strep. uberis, E. coli,* NAS (*Staph. chromogenes*, *Staph. epidermidis*, *Staph. equorum*, *Staph. haemolyticus*, *Staph. hominis*, *Staph. sciuri*, *Staph. simulans*, *Staph. succinus*, *Staph. vitulinus*, *Staph. xylosus*) and *Strep. dysgalactiae* were the most prevalent pathogens in these samples and their distribution was similar in AMS and CMS farms. Most of the isolates were susceptible to the antimicrobials tested (Table 5). Most resistance was observed in *Staphylococcus* spp. but no significant differences in the proportions of resistant isolates were seen between isolates from AMS and CMS farms.

Non-Linear PCA

The NLPCA with three dimensions explained in total 48% of the variation in AMU variables, udder health variables, questionnaire information and other farm characteristics such as herd size and milk yield. Figure 1 shows biplots of the variables that are most influential in the NLPCA and the location of the AMS and CMS farms in principle component space. Transformation plots, which are needed for the interpretation of these results and show how the original variables were transformed into numerical variables, are given in Figure 2 for variables with loadings > 0.30 or < -0.30 in the NLPCA. The abbreviations used in the biplots are given in Table 2 and Table 3. Clearly, the two farm types could not be distinguished by the combination of the 3 PCs as their localization in the graphs were overlapping. The first PC primarily distinguishes farms based on udder health parameters and antimicrobial usage variables. A high score on this PC represents poorer udder health, exemplified by high BMSCC and more (new) high SCC, but less antimicrobial usage. The $ADDD_{TOTAL}$ and $ADDD_{DCT}$ both had negative loadings in this PC, suggesting that better udder health was associated with more AMU in general and less AMU at drying off. Farms scoring high on PC1 have farmers that are less inclined to treat clinical or subclinical mastitis with antimicrobials. The second PC had strong positive loadings for $ADDD_{TOTAL}$, $ADDD_{DCT}$ and $ADDD_{IMM}$, suggesting that this PC represents higher usage of antimicrobials in general. Interestingly, this was associated with low loadings for cure of high SCC in the dry period. Farmers scoring high on PC2 were more inclined to treat CM and SCM with antimicrobials. The third PC distinguishes farmers that tend to use antimicrobials at drying off (loading positive on this axis) versus during the lactation (loading negative on this axis): $ADDD_{DCT}$ and the proportion of $ADDD_{DCT}$ of $ADDD_{TOTAL}$ loaded positively on this axis, whereas the proportion $ADDD_{IMM}$ of $ADDD_{TOTAL}$ and variables indicating that farmers would treat all cases of CM and cases of SCM with antimicrobials had a negative loading on PC3.


A
ADDD$_{TOTAL}$
ADDD$_{DCT}$
ADDD$_{DCT/TOTAL}$
Treats_high_SCC
ADDD$_{IMM}$
Herd_SCC
Uses_BC_CM
Percentage_new_high_SCC
Treats_all_CM
Percentage_new_high_SCC_dry_period
BMSCC
N_cow
Percentage_high_SCC
Select_CM_for_treatment
Percentage_cure_high_SCC_dry_period
Standard_treatment_SCM
PC2 (15.9%)
0.4
0.0
-0.4
-0.8
B
ADDD$_{DCT/TOTAL}$
Standard_treatment_SCM
ADDD$_{DCT}$
CM_grading
Percentage_cure_high_SCC_dry_period
Percentage_new_high_SCC_dry_period
Select_CM_for_treatment
N_cow
ADDD$_{TOTAL}$
Percentage_new_high_SCC
BMSCC
Herd_SCC
Percentage_high_SCC
Milking_system
AMS
CMS
ADDD$_{IMM/TOTAL}$
Treats_all_CM
Treats_high_SCC
PC3 (12.0%)
0.4
0.0
-0.4
-0.8
-0.5
0.0
0.5
PC1 (20.6%)

◄**Figure 1.** Biplots based on a non-linear principal component analysis (NLPCA) of 18 AMS farms and 24 CMS farms. For all farms, questionnaire information, udder health data and antimicrobial usage data was collected and analyzed by NLPCA. The biplots present the location of farms in the principal component space and the variance explained by each variable on the principal component axes. (A) Biplot of the second against the first principal component and (B) the third against the first principal component. The loadings of variables with loadings > 0.3 or < -0.3 are shown as lines. Object scores were scaled to 0.25 of the raw value and depicted as circles for farms with an automatic milking system (AMS) and as triangles for farms with a conventional milking system (CMS). Explanations of the variable abbreviations can be found in Tables 2 and 3.

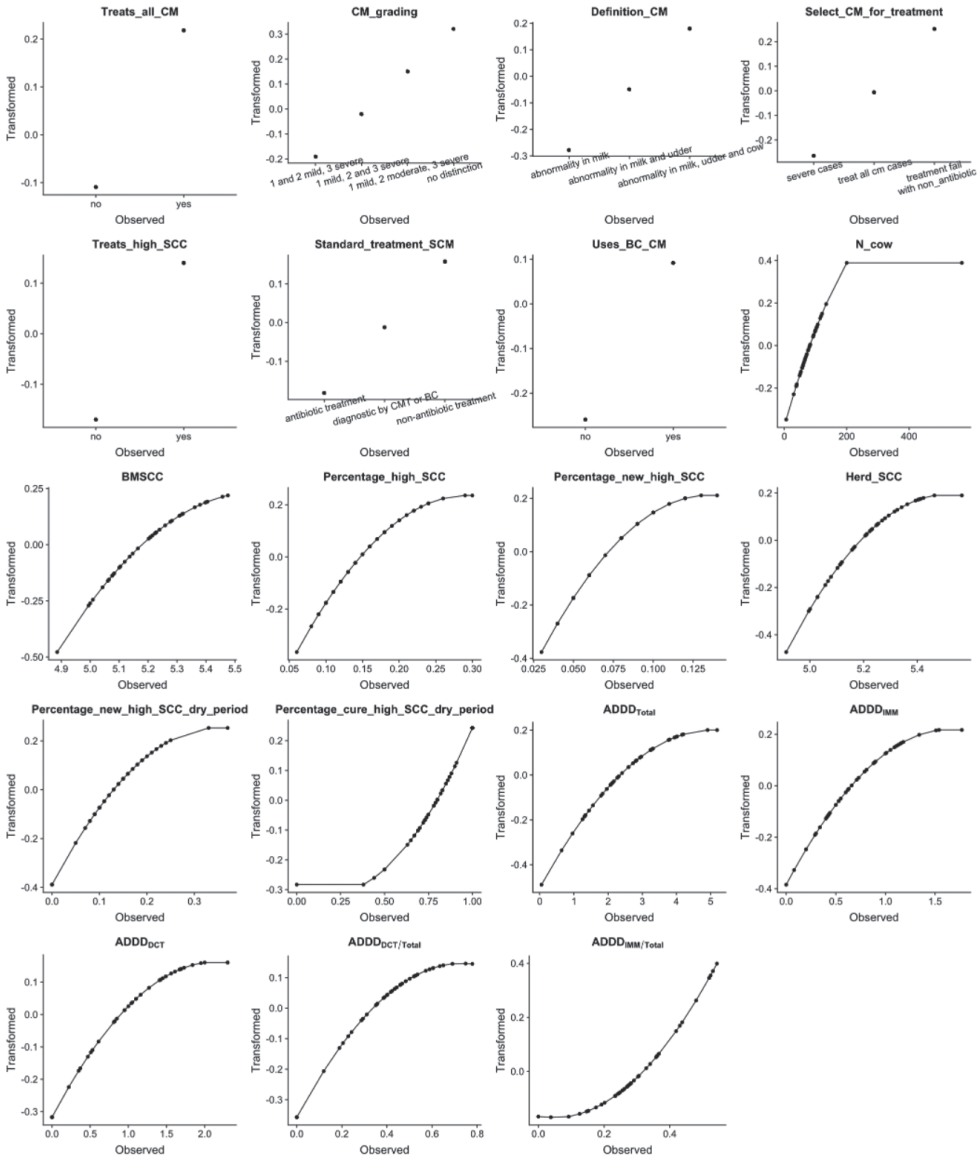


Figure 2. Transformation plots of the variables with variable loadings > 0.3 or < -0.3 used in a three-dimensional non-linear principal component analysis on variables from a telephone interview, antimicrobial usage data and udder health data.

DISCUSSION

In this study, we aimed to compare the antimicrobial use between AMS and CMS farmers, and explore factors potentially associated with AMU. AMS farmers tended to use less intramammary treatments and more injectables than CMS farmers, but the total AMU was the same. Although AMS farmers more often mentioned severe symptoms than CMS farmers when asked about their description of CM, the two farm types could not be distinguished in an NLPCA based on their definition and treatment approaches toward mastitis and AMU.

Antimicrobial Usage

In comparison of AMU, we not only compared the absolute value of AMU variables, but also the proportions of AMU for each specific purpose between AMS and CMS farms in order to identify the treatment strategies on these two different types of farms. The median $ADDD_{TOTAL}$ and $ADDD_{IMM}$ in our study were comparable to the median usage of Dutch dairy farms, which were 2.1 and 0.5, respectively, in 2017 (SDa, 2018). However, $ADDD_{DCT}$ in our sample (median: 0.88) was lower than the national median (1.1). The subset of farms that participated in the interviews had higher $ADDD_{TOTAL}$, but their $ADDD_{DCT}$ was in line with the national data. In terms of herd size, AMS farms were larger and CMS farms smaller than the Dutch average herd size (regardless of AMS or CMS) of 97 cows at the time of the study (Van der Peet et al., 2018). The udder health parameters of these 42 herds were comparable to what was reported for Dutch dairy farms in previous work (Nor et al., 2014; Steeneveld et al., 2015). The average 305 d milk yield was higher in both farm types compared to what was previously reported by Steeneveld and Hogeveen (2015). Thus, the farms included in our study were reasonably representative for the Dutch dairy farms using AMS and CMS.

Few studies have investigated differences in AMU between AMS and CMS herds. Vilar et al. (2018) reported that AMS farms were more likely to apply blanket DCT than CMS farms. Blanket DCT, however, is not allowed in the Netherlands (Scherpenzeel et al., 2016). In our study, AMS farmers used equal amounts of antimicrobials during the lactation and at drying off as CMS farmers, but we found that AMS farmers use less intramammary treatments, at the expense of more injectables. It should, however, be noted that the higher $ADDD_{INJ}$ in AMS herds is not necessarily related to mastitis treatments. The injectables may also have been used for reasons other than udder health, which we could not evaluate because the indication for the use of the antimicrobials is not recorded in the national antimicrobial registration system. The lower $ADDD_{IMM}$ and higher $ADDD_{INJ}$ in AMS herds may be explained by the fact that it is simply more challenging to apply intramammary treatments outside a milking parlor. In a conventional milking parlor, administering intramammary treatments is convenient and can be implemented easily in the daily milking routine. For farmers with an AMS, however, injectable treatments may be more convenient. Still, according to our questionnaire data, most

AMS and CMS farmers considered intramammary treatments as the standard for treatment of CM. The Dutch formulary for treatment of mastitis leaves the veterinarian free to decide whether treatment is intramammarily or (also) parenterally. Only 5 farmers added systemic treatments to intramammary treatments in the description of their approach and these 5 were equally distributed over AMS and CMS farms. This suggests that AMS farmers more often deviate from their primary approach, which may be for convenience reasons. Another explanation for the lower $ADDD_{IMM}$ may be that the AMS herds had a lower incidence rate of CM therefore used less intramammary treatments. The udder health parameters recorded in this study were generally similar for AMS and CMS herds, but the incidence rate of CM was not specifically recorded. Hovinen et al. (2009) reported a lower number of mastitis treatments in herds after transitioning from CMS to AMS. Comparing the incidence rate of CM between AMS and CMS farms would be necessary to better understand why AMS farmers use less intramammary treatments, but such comparisons are greatly hampered by the different diagnostic approaches that can be used on both farm types, which would result in misclassification bias. As Mollenhorst et al. (2012) reported, AMS farmers prefer mastitis alerts that emphasize on more severe cases, it is likely the cases found by farmers using AMS, are more severe, resulting in more mastitis treatments by injectables.

Approach Toward Mastitis

Farmers were assured of anonymity in order to reduce bias towards socially acceptable responses as much as possible. In addition, interviews were performed by a student rather than an experienced veterinarian or scientist which likely encouraged farmers to give honest answers. The data from these interviews suggests that AMS farmers in our study have a different concept of CM than the CMS farmers, and more often mentioned severe clinical signs as characteristics of mastitis. This may be related to the fact that AMS farmers are not manually involved in the milking process and therefore do not fore-strip their cows. Cases of mastitis in an AMS herd are detected through the sensors in the milking machine and checked by the farmers to confirm. However, most AMS farmers prefer sensors to emphasize on more severe cases (Mollenhorst et al., 2012) and tend to check only a limited proportion of the alerts (Hogeveen et al., 2013). This process may result in a large proportion of mastitis cases missed and cases that are found, are likely to be more severe on AMS farms. Still, despite this difference, AMS farms could not be distinguished from CMS farmers in the 3 dimensional NLPCA we performed. The AMS herds in our study had lower $ADDD_{IMM}$ and the AMS farmers have a different perception of CM, but this does not seem to result in an overall better or worse udder health situation, as is reflected in Table 2. Still, across both farm types, the NLPCA yielded that parameters related to SCC were positive on the first PC axis (Figure 1A), and loaded opposite to $ADDD_{TOTAL}$, showing that more AMU in general was related to better udder health. This may reflect that higher usage of antimicrobials is positively associated with better udder health, or may show a tendency of farmers who are capable of maintaining good udder health to use antimicrobials more often. However, the second

and third PCs show that different strategies of using antimicrobials are employed by different groups of farmers. Farms scoring high on PC2 had higher $ADDD_{TOTAL}$, $ADDD_{DCT}$ and $ADDD_{IMM}$, but loaded relatively high on SCC variables at the same time. It seems that these are farms that use antimicrobials fairly indiscriminately, resulting in limited improvement of udder health and low cure of high SCC over the dry period. The third PC contrasts farms that use antimicrobials at drying off versus farms that use antimicrobials mainly during the lactation (Figure 1B). Farmers who indicated to treat cases of SCM and to treat all cases of CM scored low on this axis. Both percentage of high SCC cases cured during the dry period and percentage of new high SCC cases during the dry period loaded positive on this axis, but the other udder health variables had small loadings on this axis, suggesting that both strategies have a similar effect on overall udder health. Altogether, this analysis suggests that the farmer's attitude towards mastitis strongly influences how much antimicrobials are used, but that the usage in itself is not directly linked to the outcome in terms of udder health.

Bacteriological Culture of Milk Samples and Antimicrobial Resistance of Mastitis-Causing Pathogens

Although the CMS farmers sent in more mastitis samples than the AMS farmers, the differences in culture results were small and any differences in distribution of pathogens cultured may well be the result of chance, given the limited sample size of our study. Bacteriological results may be slightly biased by the fact that quarters were selected for sampling by the farmers themselves, based on the criteria they use for CM and SCM in their daily work. A slightly higher percentage of clinical mastitis cases provided by AMS farmers than CMS farmers was observed. This might again reflect that AMS farmers considered more severe cases to be clinical mastitis than CMS farmers did, which consequently may have contributed to a lower $ADDD_{IMM}$ but a higher $ADDD_{INJ}$ for AMS farmers. Nevertheless, it seems clear that the AMR levels of the pathogens grown are generally low. Most resistance was found in staphylococci, and was primarily seen against penicillin G and pirlimycin, which are frequently used antimicrobials for mastitis in the Netherlands. Penicillin G resistance was higher than the 14% reported in a previous study for the Netherlands (Thomas et al., 2015). We found no MRSA, but we did identify one NAS isolate resistant to both oxacillin and Penicillin G. *Streptococcus* species were largely free of antimicrobial resistance, in line with Thomas et al. (2015), but showed some resistance against erythromycin and pirlimycin.

CONCLUSION

This study shows that AMS farmers use an equivalent total amount of antimicrobials as CMS farms, but tend to use more injectable and less intramammary antimicrobial treatments. The signs of CM described by an AMS farmer are on average more severe than the signs mentioned in this description by a CMS farmer. Across AMS and CMS farms, AMU was mainly associated with the farmer's tendency

to treat during lactation or at drying off and differences in this strategy did not correlate to better udder health in general. Larger studies are needed to better characterize differences between AMS and CMS farmers in how they treat mastitis and to elucidate reasons for a higher use of injectables on AMS farms.

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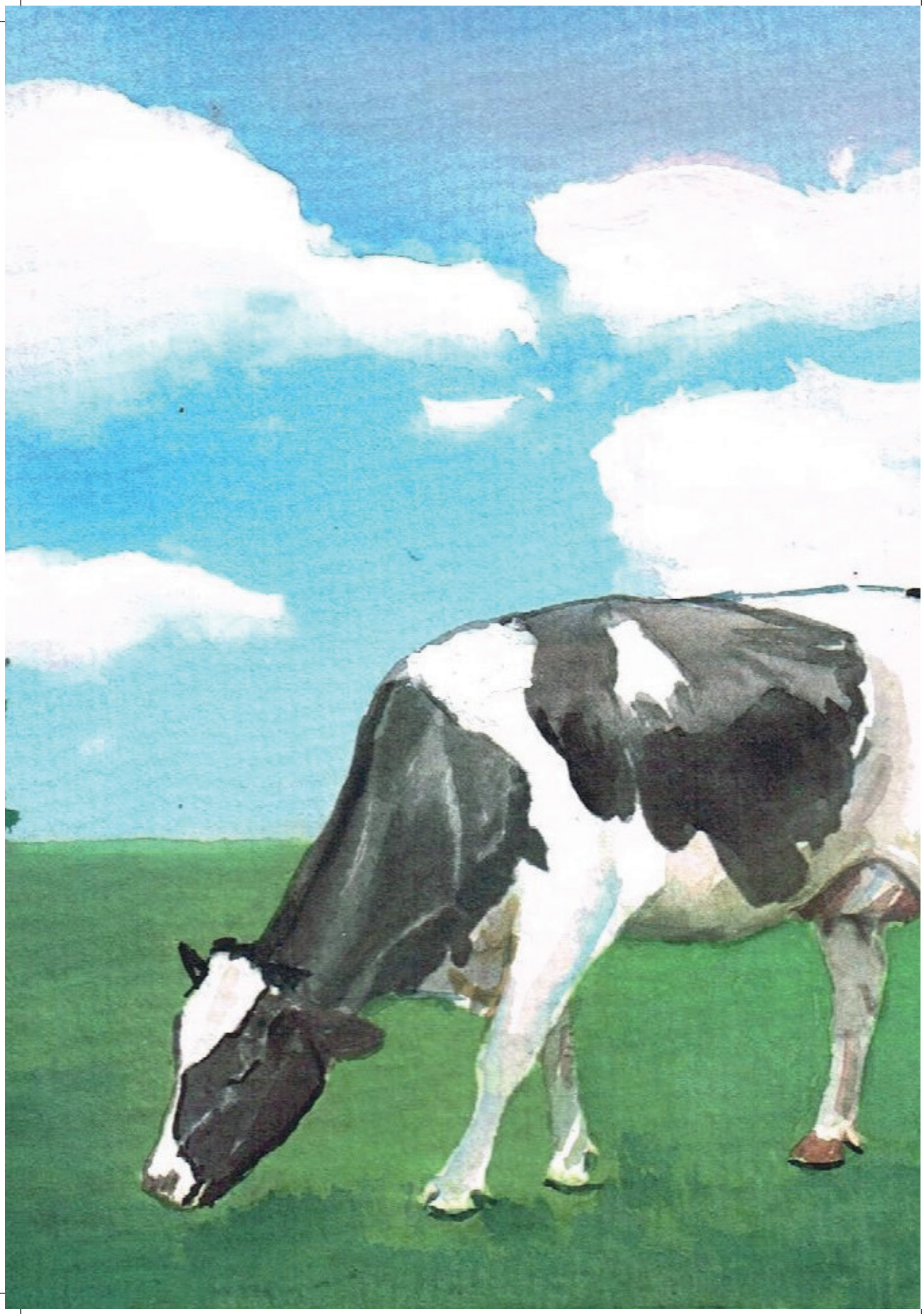

Chapter 7

General discussion

The overall objective of this thesis was to explore the potential use and benefits of using frequently measured data to optimize on-farm decision making in udder health management in herds using an automatic milking system (**AMS**). This was done by identifying risk factors for bovine mastitis in AMS herds (chapter 2), evaluating the potential benefits of using existing on-farm data in AMS herds to optimize decision making in udder health management (chapter 3), trying to find new information on udder health using these data such as specific somatic cell count (**SCC**) patterns (chapter 4), and by quantifying transmission dynamics in AMS herds and comparing these to herds with a conventional milking system (**CMS**) (chapter 5). Finally, antimicrobial use was compared between AMS and CMS herds (chapter 6).

The introduction of the AMS was the biggest change in decades in the dairy industry. Firstly, it led to a totally different organization of labor regarding the time consuming process of milking, including the detection and treatment of mastitis. Secondly, through precision dairy farming, it led to many new opportunities in dairy farm management in general, in udder health management and in mastitis research. However, using this type of technology also causes various concerns. After briefly summarizing the main findings of the studies described in this thesis, these opportunities and concerns will be discussed.

Main Findings

As described in chapter 2, the herd average SCC in AMS herds in the Netherlands was higher than in CMS herds, as was the annual average herd new high SCC rate. The annual incidence rate of clinical mastitis, however, was found to be more or less comparable between these two types of herds. In chapter 6, we found that the overall antimicrobial usage was comparable between AMS and CMS herds. All these findings together indicate udder health in AMS herds is comparable or maybe slightly worse as compared to CMS herds. Similar findings were also found in Nordic countries (Hovinen and Pyörälä, 2011).

There is an extensive body of research on risk factors for mastitis in CMS herds, while studies on risk factors for mastitis in AMS herds have been published only sporadically (Persson Waller et al., 2003; Dohmen et al., 2010). Although in some studies the use of the AMS itself was found to be a risk factor for new mastitis cases (Frössling et al., 2017), we found in chapter 2 that within AMS herds, most risk factors for mastitis are similar to those in CMS herds. Despite the differences in general management between AMS and CMS herds, measures to control mastitis in CMS herds seem to be also applicable in AMS herds: good hygiene of cows and participating in animal disease control programs were associated with a lower risk of new intramammary infections (**IMI**) in both type of herds. Remarkably, herd size was found to be a risk factor for the incidence rate of clinical mastitis in AMS herds (Deng et al., 2019a), but not in CMS herds. Herd size was found to be negatively associated with the variance of herd SCC on test days and positively associated with the annual average proportion of new high SCC cases.

This indicates that in larger AMS herds, less fluctuation in herd SCC occurs while there is a higher rate of transmission of IMI than in small AMS herds. On larger farms, the influence of temporary changes in SCC in a limited number of cows may be less visible because of the large farm size, resulting in a lower variation in herd SCC. A larger number of cows seems to result in a herd SCC that is less prone to fluctuations because of incidental effects on limited number of cows. Still, farmers with a large herd may have less time available per cow for udder health preventive measures, which may result in more frequent new IMI, reflected in a higher new high SCC cases. As the herd size is increasing worldwide and AMS are also frequently used in large herds (e.g., Houin, 2019; Melendez et al., 2019), the adoption of AMS may be a risk factor for mastitis in larger dairy herds and asks for specific attention.

Nevertheless, udder health and risk factors for mastitis are largely comparable between AMS and CMS herds. There are, however, a number of important differences between AMS and CMS herds related to mastitis management (Jacobs and Siegford, 2012). A first important difference is the way cases of clinical mastitis are detected. In AMS herds, cows with clinical mastitis are detected using sensor systems. Algorithms using the data collected by on-line or in-line sensors provide alerts that need to be interpreted and followed-up by the farmer in order to treat cows. Sensors measure indicators for mastitis, such as electronical conductivity, SCC, and the color of the milk. The first and most used sensors used for detection of mastitis are based upon electrical conductivity (Rutten et al., 2013). In recent years sensor systems based upon measurement of SCC became increasingly available. There is now a growing number of herds using sensors that measure or estimate composite milk SCC, for instance by a device that uses the gel forming methodology of the California Mastitis Test (Schalm and Noorlander, 1957) online in an automated way (**O-CMT**), or by on-line flowcytometry that directly enumerates the SCC (**O-SCC**) (Sørensen et al.) or by inline electrical permittivity threshold technology that measures the SCC (Gea, 2018). However, despite the commercial availability and increased use of O-CMT sensors, the performance of this type of sensors has not been evaluated in the field. In chapter 3, we found the overall agreement between the O-CMT measurements and the SCC measured in the laboratory (**L-SCC**), indicated by concordance correlation coefficient (**CCC**), to be 0.53 across all the 55 AMS herds. The highest agreement between the average of multiple O-CMT measurements and the corresponding single L-SCC measurement was found with the average of O-CMT measurements within 24 hours before and after the L-SCC test date. Still, the agreement between O-CMT measurements and L-SCC is far from perfect. The agreement between the two measurements, as indicated by the CCC was positively associated with herd SCC, which suggests the O-CMT is a better indication for L-SCC in higher SCC ranges.

Although the L-SCC has a higher precision than O-CMT, it often is only measured once every 4-6 weeks on a composite milk sample. Therefore, L-SCC is not an ideal indicator of the actual udder health situation. A Bayesian latent class analysis showed that the test characteristics of the average of multiple

O-CMT measurements within a time window of 24 hours outperformed a single L-SCC measured once every 4-6 weeks in detecting the intramammary infection status (Deng et al., 2019b). Therefore, the frequently measured O-CMT holds great potential in individual cow udder health monitoring.

Because of the high frequency of measuring, O-CMT enables the collection of much more detailed information on SCC dynamics than the infrequent L-SCC measurement. This opens a new range of research opportunities because in the past, frequent measurement of SCC was only possible in very time consuming and expensive data collection protocols where daily milk samples need to be collected manually and analyzed in a laboratory. In chapter 4, we analyzed SCC patterns using the frequently measured O-CMT measurements. In this highly exploratory study, we discovered a new SCC pattern that we termed as "continuously regularly fluctuating SCC". A proportion of 4.7% of the cows displayed this continuously regularly fluctuating SCC pattern in the study herds and this study demonstrated the frequently measured O-CMT measurements in capturing new SCC patterns.

An important difference in mastitis management between farms with an AMS and a CMS is the contact structure of cows during milking. The different contact structure may theoretically have an effect on the transmission of mastitis pathogens. In an AMS, cows are milked by less milking clusters, which increases the risk of transmission via the milking cluster. Moreover, milking with an AMS is carried out 24 hours per day, which is in contrast to the fixed milking times when a CMS is used. That means in an AMS herd, it is hard to change the management in such a way that cows are kept standing after milking for some time, to prevent infection when cows are lying down with open teats shortly after milking. On the other hand, in an AMS the milking cluster is rinsed after each milking and it is possible to steam-clean or otherwise disinfect the milking cluster after each milking in order to reduce the transmission of mastitis pathogens during milking. Additionally, clusters are taken off per quarter, which may decrease the risk of overmilking. Recent studies found that the so-called contagious pathogens might not exclusively transmit during milking, but environmental transmission of these pathogens also occurs. Therefore, studies to elucidate the transmission of contagious pathogens in AMS herds are warranted. In chapter 5, we estimated the R_0 of *Staphylococcus aureus* to be in the range of 0.16 to 0.34, and for *Streptococcus agalactiae* 0.64 to 0.68. These results suggest that transmission of these two contagious pathogens in the study herd was limited. In theory, with such transmission parameters, these two contagious pathogens will not sustain, because the R_0 of both pathogens was less than 1. The estimates of transmission parameters as found in this study are comparable to transmission parameters estimated for well-performing CMS herds (Lam et al., 1996; Zadoks et al., 2002; Kirkeby et al., 2019). In this study, the data were insufficient to enable us to study a potential transmission from an environmental reservoir, but it would be interesting to further identify the transmission routes of these IMI in AMS herds. Using the same data, we did quantify the probability of a cow getting infected by contagious mastitis pathogens after being milked directly after an infectious cow (data not shown). We did not

find a statistically significant (P<0.05) association between being milked directly after an infectious cow and the acquisition of IMI. Although the number of cows and new IMI by contagious mastitis pathogens was relatively low, this suggests that other routes of transmission than via the milking cluster play a role in the transmission of contagious mastitis pathogens in herds with an AMS.

Finally, because antimicrobial therapy is probably the most widely adopted strategy in treatment of clinical mastitis, differences in occurrence of mastitis and transmission of mastitis between CMS and AMS herds should also be reflected in the usage of antimicrobials. In chapter 6, we investigated the antimicrobial use and the factors associated with the antimicrobial usage (**AMU**) as well as the mastitis causing pathogens in AMS and CMS herds. We found the overall AMU and the AMU for dry cow therapy in AMS herds were comparable to those in CMS herds, while the AMU for intramammary usage tended to be lower and the AMU by injection was higher in AMS farms compared to CMS farms. The AMS herds could not be distinguished from CMS herds in the principal component space. The treatment strategy for mastitis was unrelated to the overall udder health status or the total AMU. The distribution of mastitis causing pathogens was comparable in AMS and CMS herds. Because in chapter 2, we found the risk factors in AMS herds to be comparable to CMS herds and in chapter 5 we found the transmission of contagious pathogens to be limited and comparable to well-performing CMS herds, AMU on AMS farms was expected to not deviate too much from that on CMS farms.

Opportunities for Precision Technology and Big Data in Dairy Farm Management

The research described in this thesis is based on the use of sensor systems and automation in dairy farming and is linked to important developments in dairy cattle husbandry. Worldwide, the number of dairy farms is decreasing while the herd size and intensity of farming is increasing. Automation, such as the use of AMS, enables farmers to decrease the need for labor to manage dairy cows. Nevertheless, with the increasing intensity of dairy farming, cows may need to receive even more individual attention and care to secure their health and welfare. On large, intensive dairy farms, day to day sufficient visual inspection of cows to safeguard their health and wellbeing is challenging. Precision technology to manage animal health therefore seems to become increasingly important (Awasthi et al., 2016). Precision technology that aims at monitoring animal health is widely implemented and the number of dairy farms using this type of technology is worldwide continuously growing (Drewry et al., 2019; Groher et al., 2020).

The process of adopting precision technology by dairy farmers is influenced by multiple factors which vary between farming system, type of technology (Rutten et al., 2018; Konrad et al., 2019), country (Barnes et al., 2019) and the level of integration of these technologies in milking parlors (Groher et al., 2020). Although precision farming technology mostly is implemented on farms with an AMS

(Steeneveld and Hogeveen, 2015), CMS farms are also expected to adopt more and more precision dairy farming technologies (Rutten et al., 2013). This indicates that the technique is expected to be widely adopted in the future. Factors that affect that adoption are the uncertainty on the performance of a sensor system as well as the expected future improvement of sensor systems and their associated decision support systems (Rutten et al., 2018; Eastwood and Renwick; 2020). Because of the link to AMS, the adoption of AMS is also an important driving force of the application of sensors and precision farming systems in dairy farming.

The sensor systems present in an AMS, in conjunction with other sensors implemented in dairy herds can compose a real-time surveillance framework for data-driven animal health management (King and Devries, 2018; Eckelkamp, 2019). Such an approach generates large amounts of data from different sources and at different levels. The development of sensor systems to support mastitis management fits in the technological developments such as the "internet of things" and big data analytics applied in precision dairy farming (Sundmaeker et al., 2016; Wolfert et al., 2017; Lokhorst et al., 2019). The increased use of big data in dairy farming gives the opportunity to better understand the complex dairy farming system, to improve animal care and consequently to improve the economic return. This way, the application of precision technology can lead to more sustainable dairy farming systems (Walter et al., 2017; Eastwood et al., 2019).

To release the potential of big data-based farm management support, it is important that various sources of data (animals, groups, farms, environment) are integrated and are analyzed simultaneously (Lokhorst et al., 2019). Big data generated from sensors as well as data collected routinely on-farm are important sources of data. Several attempts have been done to integrate data from multiple sensors and to apply cutting edge data analytics, such as machine learning and deep learning approaches, to aid the decision making in farm management (Khatun et al., 2018; Cabrera et al., 2020; Ferris et al., 2020; Neethirajan, 2020; Newton et al., 2020). An interesting approach was proposed by Cabrera et al. (2020) who reported the "Dairy Brain" project initialized by University of Wisconsin that mimics actual farm management using machine learning approaches to support real-time farm management decisions. By integrating data from genetic, management and dairy herd information programs, they constructed a prediction model to identify first-lactation cows that were at risk of contracting clinical mastitis. Similar projects were also established in EU, such as the "D4Dariy" project (https://d4dairy.com/en/#start) with data integration as the first area of focus in order to identify early predictors to improve animal health and the "SmartCow" project (https://www.smartcow.eu/) which integrates data from different sensors using a multivariate approach for animal health.

Opportunities for Precison Technology in Udder Health Management

The currently most widely used sensors in AMS herds are aimed at detection of clinical mastitis. Every AMS contains a sensor system measuring electronical conductivity (Rutten et al., 2013; Sørensen et al., 2016) and a growing number of AMS herds also has an SCC-based sensor system. Hitherto, the debate about sensor systems for mastitis management generally is mainly about the detection performance of clinical mastitis (Hogeveen et al., 2010; Sørensen et al., 2016). The results shown in this thesis, however, show that SCC-based sensor systems may be used in a much broader way. If it is possible to analyze SCC patterns automatically, it may become possible to categorize cases of clinical as well as subclinical mastitis for different types of intervention (e.g. treatment).

Machine learning and deep learning approaches are being implemented to mine the massive amounts of data becoming available with the current precision technologies. When these data can be combined with other on farm data, such as data from the dairy herd information program, this approach can be used as a basis for herd as well as cow-specific decision support. The currently mostly used data analytic methods are supervised learning (87%) and unsupervised learning methods (12%) (Lokhorst et al., 2019). Time series data were used in 61% of the studies of which the majority was measured at animal level. Numerous applications of big data in dairy farming have been published in terms of disease management such as hyperketonemia (Pralle and White, 2020), mastitis (Cabrera et al., 2020), lameness (Taneja et al., 2020; Warner et al., 2020). Hyde et al. (2020) applied a random forest algorithm to predict contagious and environmental mastitis cases and Esener et al. (2018) used supervised machine learning methods to automatically classify the *Streptococcus uberis* isolates from contagious and environmental mastitis cases to aid the decision making in mastitis treatment. In chapter 3, we illustrated 4 distinctive SCC patterns of which we were unable to link them to specific mastitis pathogens. To be able to do that, we likely need pattern-recognition algorithms, such as a neural network, in order to automatically identify certain SCC patterns in dairy cows. When bacteriological culture data and other farm data are combined, pathogen specific SCC patterns can potentially be automatically identified. In our own research, we visually identified those continuously regularly fluctuating SCC patterns in Chapter 4, using pre-defined patterns but unsupervised machine learning methods may be able to identify even more SCC patterns which potentially can be associated with various mastitis pathogens.

The current practice in udder health management is mostly focusing on clinical mastitis, however, the prevalence and the costs of subclinical mastitis (48% of the total costs are attributed to subclinical mastitis) are much higher than clinical mastitis (34% of the total costs) (Aghamohammadi et al., 2018). Herd specific interventions against both clinical and subclinical mastitis are more cost-effective than interventions solely targeted at clinical mastitis (Gussmann et al., 2019). Therefore, measures to control both clinical and subclinical mastitis are important in order to maximize the profit of dairy farming. To design such programs, data from different aspects regarding animal health are needed. With the data

generated in AMS and the sensors, more detailed information at individual cow and herd level with different aspects becomes available.

In the future, even farm-specific risk factors for occurrence of mastitis may be determined automatically, as we did manually in chapter 2. By using sensor data in combination with other farm and environmental data, we may be able to automate the process of data collection for udder health parameters and risk factors and utilize data analysis pipelines to produce nearly real-time identification of farm specific risk factors for udder health.

Veterinarians are important advisors of farmers regarding mastitis management. Typically, veterinarians advise about treatment protocols and may even be consulted by a farmer in case of severe clinical mastitis. Moreover, the knowledge and experience of veterinarians are important as part of herd health programs, aimed at safeguarding animal health and welfare and prevention of production diseases such as mastitis. Data used by veterinarians typically consist of overviews of dairy herd information programs. These overviews are generated every 4-6 weeks. However, with the nearly real-time continuously measured sensor data, data are becoming available at a day-to-day basis. It is therefore important that veterinarians will become familiar with this type of data and the reports that may be generated by them in order to be sufficiently able to interpret the results. The consulting role of veterinarians will become more data-based, for instance because farm-specific risk factors or farm-specific transmission estimates become available. This seems challenging to the current education systems in veterinary medicine. Current educational schemes in veterinary schools may need to provide training for veterinary students with more (advanced) data analytic tools and interpreting the results generated from these smart systems. Such skills, in combination with the strong knowledge veterinarians have about the pathophysiological aspects of mastitis will increase the value of veterinarians as consultants in supporting the farmers with their mastitis management.

Opportunities for Mastitis Research

Transmission of mastitis causing pathogens is believed to take place either during the milking process (contagious mastitis pathogens) or by contact with the environment, mainly the cubicles (environmental pathogens). Worldwide, only a limited number of studies have been carried out on quantifying the contribution of different transmission routes to the transmission of mastitis (Zadoks et al., 2002; van den Borne et al., 2017; Esener et al., 2018). Because of the high costs of this type of transmission studies, it is interesting to explore whether sensor system data can be used to quantify transmission routes of mastitis pathogens, for instance by combining data on contact between cows in an AMS with the infection status (based on an SCC sensor). Dalen et al. (2019) investigated the transmission dynamics of mastitis pathogens using SCC patterns that were captured by an SCC sensor. Location sensors that measure the position of a cow in the barn, may be used to identify the moment a cow was

in a particular cubicle. In that way, cows that followed each other in cubicles may be studied in relation to the infection status of cows. In that way the transmission routes (contagious transmission during the milking process and environmental transmission in cubicles) might be quantified. We planned to carry out such an analysis in the transmission study described in chapter 5 but found out the location sensor at that stage was not yet precise enough. We were not able to determine the position of cows in sufficient detail to identify the cubicles cows were in.

Concerns on Precision Dairy Farming

Although there are many opportunities for precision technologies to improve animal health management on dairy farms, there are several concerns about the use of big data in the application of big data analytics in dairy farming. These issues include, but are not limited to, the ownership of the data, the data quality, and the ethics and societal acceptance of modern technology in animal husbandry.

Currently, laws and legal frameworks seem to be lacking to regulate data ownership and the use of data in dairy farming. In the discussion about precision dairy farming, farmers' concerns are often neglected, however, it is pivotal to include the farmers in this cooperation. Recent studies suggest that there is a lack of trust between farmers and the third parties who collect data. This lack of trust is the core concern that contributes to the reluctance of farmers to share their data (van der Burg et al., 2019; Wiseman et al., 2019). Other factors such as privacy, security and the sharing of benefits of digitalization and data collection also influence the farmers' attitude towards precision farming (Wolfert et al., 2017; Fleming et al., 2018; Kosior, 2018; Drewry et al., 2019). Therefore, legislation that takes all the stakeholders into consideration is needed in order to delineate the rights in ownership, use and sharing of data.

Typically, the data collected by precision technology are rough data, where often datapoints are lacking or not precise enough (Ferris et al., 2020). Inaccurate measurements and missing data are not rare in practice and are possibly due to the sampling process and factors such as the failure to supplement reagent in time, even when calibration of sensor systems is performed at regular basis at some farms (Ferris et al., 2020; Pralle and White, 2020). Detailed description of statistical methods for treatment of missing values that arise from different forms (unit level and variable level) can be found in a paper of Dong and Peng (2013).

Societal acceptance is also a concern in adopting precision dairy farming technology (Boogaard et al., 2011). Mastitis is painful for cows and reduces animal welfare (Bradley, 2002; Ruegg, 2017). Improving udder health improves animal welfare and also reduces AMU. Naturalness and humane treatment are the core components in good animal welfare (Clark et al., 2016). Although precision farming technology may help to improve animal welfare by improving the udder health status of farms, it also presents challenges in this respect. The use of precision technology may drive farmers

to be more concerned about data than about the actual animals and animal welfare (Blok and Long, 2016). Furthermore, due to the use of AMS and sensors in combination with increasing herd sizes, interactions between farmers and animals in precision dairy farming is largely reduced in comparison to smaller CMS dairy farms where direct contact between humans and animals is made on a daily basis during milking. That way, deviations in animal behavior and health status are harder to be noticed by farmers. These ethical issues should also be taken into consideration along with the development of precision dairy farming, such as monitoring and improving animal welfare while adopting these precision technologies on farms. Meanwhile, the opinion of the general public on adoption of precision technology especially with respect to animal welfare issues, also has an impact on what happens on farms (Pfeiffer et al., 2020).

Although extensive research in bovine mastitis has been published and udder health has improved dramatically, bovine mastitis is still considered as one of the most costly diseases in dairy farming. In chapter 2, we found that a positive attitude of farmers towards udder health is associated with lower incidence of clinical mastitis. Meanwhile, communication seems of great importance in bridging the gap between research and farmers' actions in improving udder health (Jansen et al., 2010a; Jansen et al., 2010b; Jansen and Lam, 2012). However, communication with farmers about the opportunities and benefits of adopting precision technologies alone is insufficient to motivate the farmers to take actions, and efforts to bridge the gap between research findings and the farmers are still needed.

In addition, apart from proper communication of the opportunities of precision technology and automation to farmers, it is also important to communicate these to society. Communication potential and benefits of precision technology to the society may help to make a proper judgement and weigh positive aspects against the potential drawbacks of the use of this technology as was described above.

CONCLUSIONS

The research described in this thesis aimed to explore the potential use and benefit of using frequently measured data to optimize on-farm decision making in udder health management in AMS herds. Based on the findings in this thesis, we conclude that the frequently measured sensor data in AMS herds holds great potential to support data-driven mastitis management decision making. This can be done for instance by monitoring individual cow udder health, identifying herd specific risk factors automatically, capturing patterns of IMI dynamics and quantifying the transmission process of infectious mastitis pathogens. Concerns that now limit the implementation of sensor technologies must be addressed. Further research to integrate the data from different sources, and algorithms to turn the data into interpretable information that can be used by the farmer and their advisor are needed.

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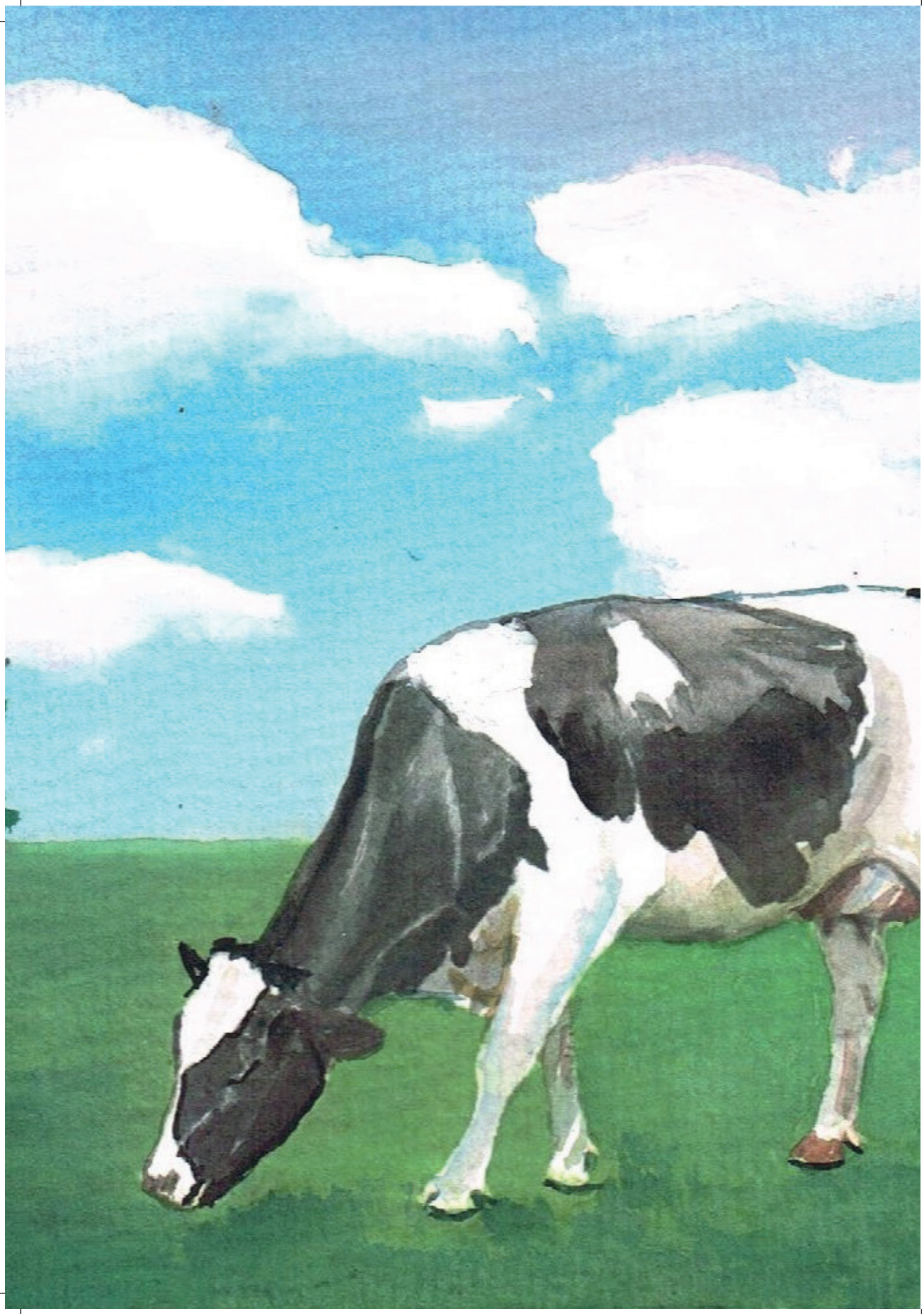


Summary

Over time, the dairy industry worldwide has changed from a local small holder activity to a highly technological driven industry that uses wealth of science and knowledge. Milk production has increased and milk quality has improved enormously over time, as has attention for aspects such as animal welfare and antimicrobial use. This helps to meet the demand for animal proteins for human consumption due to the continuously increasing world population. Worldwide the herd size of dairy herds is growing, with increasing application of technological developments. Despite numerous publications on mastitis research and large amounts of data generated in dairy herd management, farmers still need adequate decision support systems for udder health management. The overall goal of this thesis therefore was to explore the potential use and benefit of using frequently measured data to optimize on-farm decision making in udder health management in herds using an automatic milking system (**AMS**).

Mastitis is a multifactorial problem and identification of risk factors for mastitis is important for farmers to better control risks and improve udder health management. Contrary to farms using an AMS, risk factors for bovine mastitis have extensively been studied on dairy farms with a conventional milking system (CMS). In chapter 2, the risk factors for bovine mastitis in Dutch AMS herds were described. Data on possible risk factors for mastitis were collected by questionnaire and recordings of cow, stall and milking machine hygiene in 135 AMS herds. Four indicators for udder health were investigated, being the average herd somatic cell count (**SCC**), the variance of the average herd SCC on test days, the average proportion of new high SCC cases (derived from the milk recording), and the farmer-reported annual incidence rate of clinical mastitis (IRCM). To identify risk factors for mastitis in AMS herds, potential risk factors were regressed against these four udder health indicators, with multiple imputation to deal with missing values. The results indicated that mastitis control measurements as advised in CMS herds generally are also applicable in AMS herds, while specifically in larger herds, extra attention should be given to hygiene of cows and of the AMS.

In an increasing number of dairy herds, online tools are implemented to monitor individual cow udder health. In chapter 3, we compared the results of an online automated California Mastitis Test sensor (**O-CMT**) to estimate the SCC, with the SCC as measured in a milk quality laboratory (**L-SCC**). The potential added value of the O-CMT in individual cow udder health monitoring was explored. Data of O-CMT and L-SCC were collected from 50 AMS farms in 6 different countries. The repeatability of the O-CMT measurements for each cow within 5 days episodes was assessed. The concordance correlation coefficient between O-CMT and L-SCC was calculated to evaluate the agreement between O-CMT and L-SCC measurements. Different time windows to aggregate multiple O-CMT measurements as compared to L-SCC measurements were also evaluated. The intraclass correlation coefficient was estimated at 0.58, which suggests that 42% of the variance in O-CMT results within each episode can be attributed to the O-CMT measurement. The overall concordance correlation coefficient between O-CMT and L-SCC was 0.53, with substantial variation between farms. The optimal time window for

aggregating multiple O-CMT measurements was found to be 24h. We also found that the agreement between O-CMT and L-SCC was positively associated with herd SCC. We concluded that the O-CMT moderately agreed with the L-SCC and that frequently measured O-CMT can be a useful on-farm tool for individual cow udder health monitoring.

By utilizing frequently measured O-CMT measurements, we found some cows displaying a specific SCC pattern we described as continuously regularly fluctuating SCC (**rfSCC**). In chapter 4, we described the SCC pattern based on O-CMT in 1,000 cows from 55 dairy herds using AMS in 6 different countries. From these cows, we randomly selected 30 d episodes of O-CMT measurements, and evaluated these with respect to rfSCC. The rfSCC patterns were characterized by estimating the average of SCC and amplitude as well as the duration of cycles. The 1,000 episodes were manually rated by 4 independent mastitis researchers and classified into 8 different SCC pattern categories. We identified the rfSCC pattern in 4.7% (95% CI: 3.5%-6.2%) of these episodes. The rfSCC episodes had a median SCC of 701 (2.5%-97.5% quantile: 539-1,162) × 1,000 cells/mL, a median amplitude of 552 (2.5%-97.5% quantile: 409-886) × 1,000 cells/mL and a median cycle length of 4.1 (2.5%-97.5% quantile: 3.7-4.9) days. Bacteriological culture data of quarter milk samples collected every 2 wk in one Dutch AMS herd were analyzed comparing dairy cows with a rfSCC pattern with other cows. No clear association between pathogen species and the rfSCC pattern was found in that herd. This study shows that the frequent O-CMT measurements is useful in identifying specific SCC patterns, such as the rfSCC pattern. The rfSCC pattern itself is an intriguing phenomenon that requires further study.

In AMS herds, cows are milked with a single milking cluster. Therefore, the contact structure between cows differs between AMS and CMS farms, and transmission of contagious mastitis pathogens may be different between these types of farms. In chapter 5, the transmission rates, duration of intramammary infections (**IMI**) and the basic reproduction number of *Staphylococcus aureus* and *Streptococcus agalactiae* in a Dutch AMS herd were estimated and compared with those of CMS farms. Longitudinal bacteriological culture data were collected every two weeks for half a year in one AMS herd and a Susceptible-Infected model was employed. The transmission rate for *Staph. aureus* was estimated to be within the range of 0.002 (95% CI: 0-0.005) quarter-day^{-1} to 0.019 (95% CI: 0.010-0.032) quarter-day^{-1}, and for *Strep. agalactiae* of 0.007 (95% CI: 0.005-0.010) quarter-day^{-1} to 0.019 (95% CI: 0.011-0.032) quarter-day^{-1}. The median duration of chronic IMI was estimated at 95 (95% CI: 72-125) days for *Staph. aureus* and at 86 (95% CI: 67-111) days for *Strep. agalactiae*, and the R_0 between 0.16 (95% CI: 0.05-0.27) and 0.34 (95% CI: 0.20-0.48) for *Staph. aureus*, and between 0.64 (95% CI: 0.41-0.87) and 0.68 (95% CI: 0.48-0.88) for *Strep. agalactiae*. Our study suggested that transmission of these two contagious pathogens in this herd was limited and comparable to that of well-performing CMS herds.

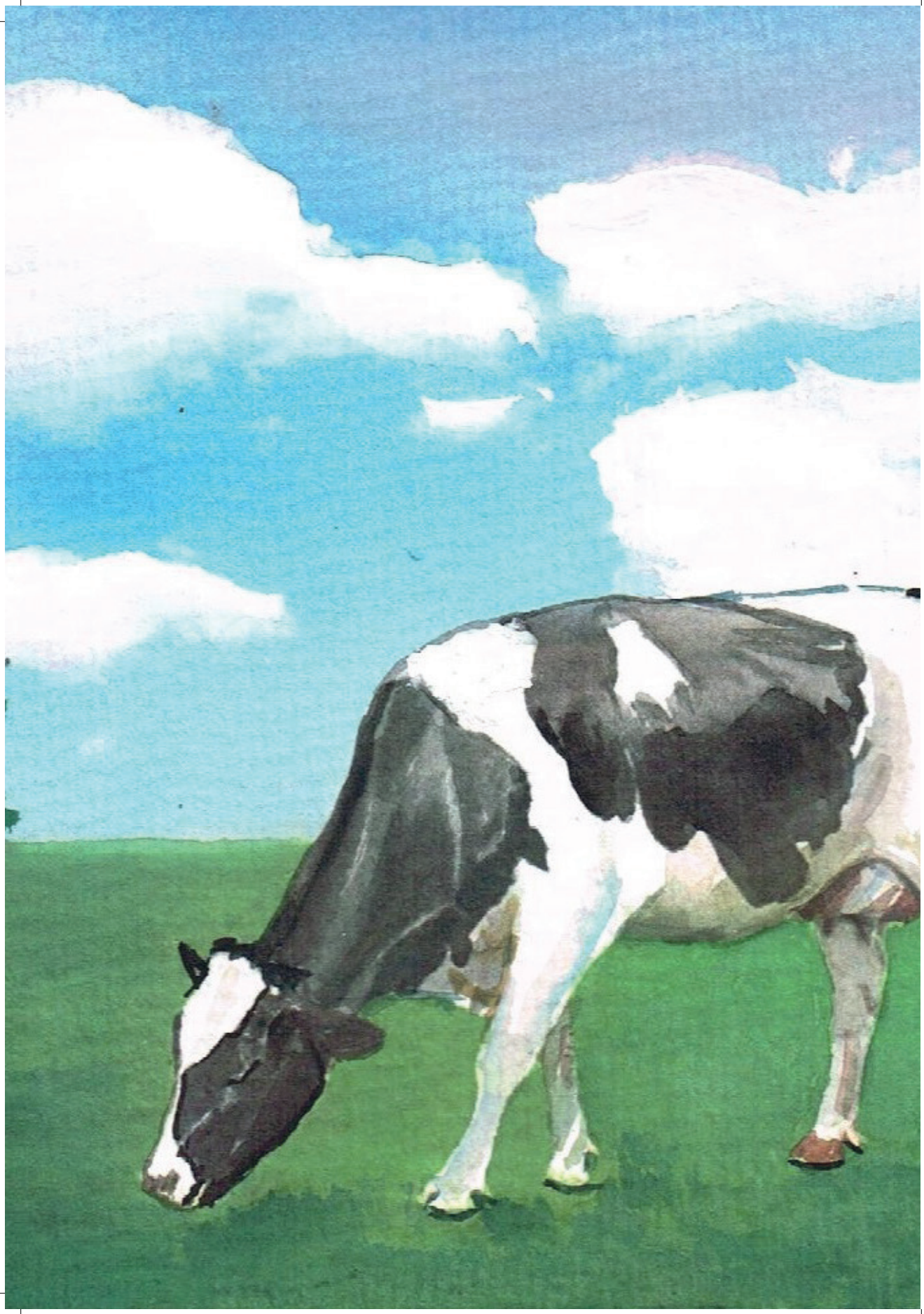
In the current epoch, the dairy industry as any other sector in animal husbandry pays attention to antimicrobial use. So far, however, scarce studies investigated the antimicrobial use on AMS farms. In

chapter 6, we compared the antimicrobial usage (**AMU**) and the distribution of bovine mastitis causing pathogens between AMS and CMS farms. Data on AMU was expressed as animal-defined daily dose (ADDD). The ADDD variables evaluated were total usage (**$ADDD_{TOTAL}$**), intramammary usage during lactation (**$ADDD_{IMM}$**), usage for dry cow therapy (**$ADDD_{DCT}$**), and usage by injection (**$ADDD_{INJ}$**). These parameters were collected on 42 AMS and 254 CMS farms in the Netherlands. A subset of 18 AMS farms and 24 CMS farms were included in a survey to identify factors associated with AMU. In these herds a total of 5 quarter milk samples of cows with clinical or subclinical mastitis were collected to investigate the distribution of mastitis pathogens as related to AMU. Nonlinear principal component analysis was used to explore the associations between udder health variables, AMU and the variables in the survey.

The $ADDD_{TOTAL}$ and $ADDD_{DCT}$ were comparable between AMS and CMS farms, whereas $ADDD_{IMM}$ tended to be lower and $ADDD_{INJ}$ was higher on AMS farms than on CMS farms. The AMS farms were not distinguished from CMS farms in the principal component space. The distribution of mastitis pathogens and their antimicrobial resistance were comparable between AMS and CMS farms. These results suggest AMU is comparable between AMS and CMS farms, but AMS farms tend to use more injectables and less intramammary treatments during lactation. Farmers' attitudes toward udder health and toward mastitis treatment were associated with AMU in both AMS and CMS herds.

CONCLUSIONS

Based on the findings in this thesis, we conclude that the frequently measured data in AMS herds holds great potential to support data-driven mastitis management decision making. This can be done for instance by monitoring individual cow udder health, identifying herd specific risk factors automatically, capturing patterns of IMI dynamics and quantifying the transmission process of infectious mastitis pathogens. Concerns that now limit the implementation of sensor technologies must be addressed. Further research to integrate the data from different sources, and algorithms to turn the data into interpretable information that can be used by the farmer and his advisor are needed.



Nederlandse samenvatting

De zuivelindustrie is wereldwijd in de loop der tijd veranderd van een lokale activiteit van kleine boeren naar een hoogtechnologische industrie die gebruikmaakt van een schat aan wetenschap en kennis. De melkproductie is gestegen en de melkkwaliteit is in de loop van de tijd enorm verbeterd, en er is een toegenomen aandacht voor aspecten zoals dierenwelzijn en antibioticumgebruik. Deze ontwikkelingen helpen bij het voldoen aan de wereldwijd stijgende vraag naar dierlijke eiwitten voor menselijke consumptie vanwege de steeds groter wordende wereldbevolking. Wereldwijd worden melkveebedrijven steeds groter, waarbij steeds meer technologische ontwikkelingen worden toegepast. Ondanks talrijke publicaties over mastitisonderzoek en grote hoeveelheden gegevens die worden gegenereerd in het management van melkveebedrijven, hebben boeren nog steeds behoefte aan adequate beslissingsondersteunende systemen voor uiergezondheid. Het overkoepelende doel van dit proefschrift was daarom om het potentieel en de voordelen van het gebruik van frequent gemeten gegevens op het melkveebedrijf te onderzoeken, om daarmee bij te dragen aan optimalisatie van beslissingen over uiergezondheidsmanagement op melkveebedrijven met een automatisch melksysteem (**AMS**).

Mastitis is een multifactorieel probleem en de identificatie van risicofactoren voor mastitis is belangrijk voor melkveehouders om uiergezondheidsrisico's te kunnen beheersen en de uiergezondheid te verbeteren. Op melkveebedrijven met een conventioneel melksysteem (**CMS**) zijn, in tegenstelling tot bedrijven met een AMS, de risicofactoren voor mastitis uitgebreid onderzocht. In hoofdstuk 2 zijn de risicofactoren voor mastitis in Nederlandse melkveebedrijven met een AMS beschreven. Hierbij werden gegevens over mogelijke risicofactoren voor mastitis verzameld middels een enquête en het beoordelen van de hygiëne van koeien, stallen en melkmachines op 135 melkveebedrijven met een AMS. Er zijn vier uiergezondheidsindicatoren onderzocht, te weten het gemiddelde celgetal (somatic cell count, **SCC**) van alle koeien (het bedrijfs-SCC), de variantie van het bedrijfs-SCC tijdens de melkcontrole, het gemiddelde aandeel nieuwe koeien met een hoog SCC (afgeleid van de melkcontrole) en de door de veehouder gerapporteerde jaarlijkse incidentie van klinische mastitis. Om risicofactoren voor mastitis op AMS bedrijven vast te stellen, werden potentiële risicofactoren vergeleken met deze vier uiergezondheidsindicatoren, waarbij imputatie werd toegepast om met de ontbrekende waarden om te gaan. De resultaten lieten zien dat mastitismaatregelen zoals die bij CMS bedrijven worden geadviseerd, in het algemeen ook effectief zijn op AMS bedrijven, waarbij met name op grotere bedrijven extra aandacht moet worden besteed aan de hygiëne van koeien en van het melksysteem.

Op steeds meer melkveebedrijven worden online tools gebruikt om de uiergezondheid van koeien te monitoren. In hoofdstuk 3 vergeleken we de resultaten van een online California Mastitis Test sensor (**O-CMT**) om SCC vast te stellen, met het SCC zoals dat wordt gemeten in een laboratorium (**L-SCC**). Hierbij werd de mogelijke meerwaarde van de O-CMT bij het monitoren van uiergezondheid op koe niveau verkend. Hiervoor werden O-CMT en L-SCC gegevens van 50 AMS bedrijven uit zes landen verzameld. De herhaalbaarheid van de O-CMT-metingen op koe niveau werd binnen episodes van 5

dagen beoordeeld. De concordantie correlatie coëfficiënt tussen O-CMT en L-SCC werd berekend om de overeenkomst tussen O-CMT- en L-SCC bepalingen vast te stellen. Hierbij werden verschillende tijdvensters geëvalueerd waarbij meerdere O-CMT en L-SCC metingen werden verzameld en vergeleken. De intraclass-correlatiecoëfficiënt werd geschat op 0.58, wat suggereert dat 42% van de variantie in O-CMT-resultaten binnen elke episode kan worden toegeschreven aan de O-CMT-meting. De totale concordantie correlatie coëfficiënt tussen O-CMT en L-SCC was 0.53, met substantiële variatie tussen bedrijven. Het optimale tijdvenster voor het samenvoegen van meerdere O-CMT-metingen bleek 24 uur te zijn. We ontdekten ook dat de overeenkomst tussen O-CMT en L-SCC positief geassocieerd was met bedrijfs-SCC. We concludeerden dat de O-CMT redelijk overeenkwam met de L-SCC en dat frequent gemeten O-CMT een nuttig instrument kan zijn voor het monitoren van de uiergezondheid op koe niveau.

Door gebruik te maken van frequente O-CMT-metingen, ontdekten we dat sommige koeien een specifiek SCC patroon vertoonden dat we beschreven als een continu regelmatig fluctuerende SCC (**rfSCC**). In hoofdstuk 4 hebben we dat SCC-patroon beschreven op basis van O-CMT waarden bij 1.000 koeien van 55 melkveebedrijven met een AMS uit 6 verschillende landen. Van elk van deze koeien hebben we een willekeurige episode van 30 d gekozen waarin de O-CMT metingen zijn geëvalueerd op het voorkomen van rfSCC. De rfSCC-patronen werden gekarakteriseerd door het gemiddelde van SCC en amplitude te schatten, evenals de duur van cycli. Elk van de 1000 episodes werd beoordeeld door 4 onafhankelijke mastitisonderzoekers en werd ingedeeld in een van 8 van te voren vastgestelde verschillende categorieën van SCC-patronen. We vonden dat het rfSCC-patroon in 4.7% (95% BI: 3.5% - 6.2%) van deze episodes voorkwam. De rfSCC-episodes hadden een mediane SCC van 701 (2.5% - 97.5% kwantiel: 539 - 1162) x 1000 cellen/ml, een mediane amplitude van 552 (2.5% -97.5% kwantiel: 409-886) x 1000 cellen/ml en een mediane cyclusduur van 4.1 (2.5% -97.5% kwantiel: 3.7-4.9) dagen. Het rfSCC patroon werd verder onderzocht met behulp van resultaten van bacteriologisch onderzoek van de kwartiermelkmonsters die elke 2 weken werden verzameld op een Nederlands AMS bedrijf. Bacteriologische resultaten van koeien met het rfSCC patroon werden vergeleken met de resultaten van andere koeien. In het onderzochte bedrijf werd geen duidelijk verband gevonden tussen het rfSCC-patroon en bepaalde pathogene bacteriesoorten. Dit onderzoek laat zien dat frequente O-CMT-metingen nuttig kunnen zijn bij het identificeren van specifieke SCC-patronen, zoals het rfSCC-patroon. Het rfSCC-patroon is een intrigerend fenomeen dat verder bestudeerd moet worden.

In een melkveebedrijf met een AMS worden alle koeien gemolken met één melkstel, waardoor de contactstructuur tussen individuele koeien en de overdracht van besmettelijke mastitispathogenen anders is dan op CMS bedrijven. In hoofdstuk 5 werden transmissie parameters, de duur van intramammaire infecties (**IMI**) en het basale reproductiegetal van *Staphylococcus aureus* en *Streptococcus agalactiae* op een Nederlands AMS bedrijf vastgesteld en vergeleken met die van CMS bedrijven. Op dat

AMS bedrijf werd gedurende een half jaar elke twee weken bacteriologisch onderzoek gedaan van melkmonsters van de koeien en verwerkt in een SI (Susceptible – Infected) model. De transmissie snelheid van *Staph. aureus* lag in de range van 0.002 (95% BI 0 - 0.005) kwartierdag^{-1} tot 0.019 (95% BI: 0.010 - 0.032) kwartiedag^{-1}, en voor *Strep. agalactiae* lag dit tussen 0.007 (95%BI 0.005 - 0.010) kwartierdag^{-1} tot 0.019 (95% BI 0.011 - 0.032) kwartierdag^{-1}. De mediane duur van chronische IMI werd geschat op 95 dagen (95% BI 72 - 125) voor *Staph. aureus* en op 86 dagen (95% BI 67 - 111) voor *Strep. agalactiae.* De R_0 lag tussen 0.16 (95% BI 0.05 - 0.27) en 0.34 (95% BI 0.20 - 0.48) voor *Staph. aureus*, en tussen 0.64 (95% BI 0.41 - 0.87) en 0.68 (95% BI 0.48 - 0.88) voor *Strep. agalactiae*. Ons onderzoek liet zien dat de overdracht van deze twee besmettelijke pathogenen in dit bedrijf beperkt was en vergelijkbaar met die op een goed presterend melkveebedrijf met een CMS.

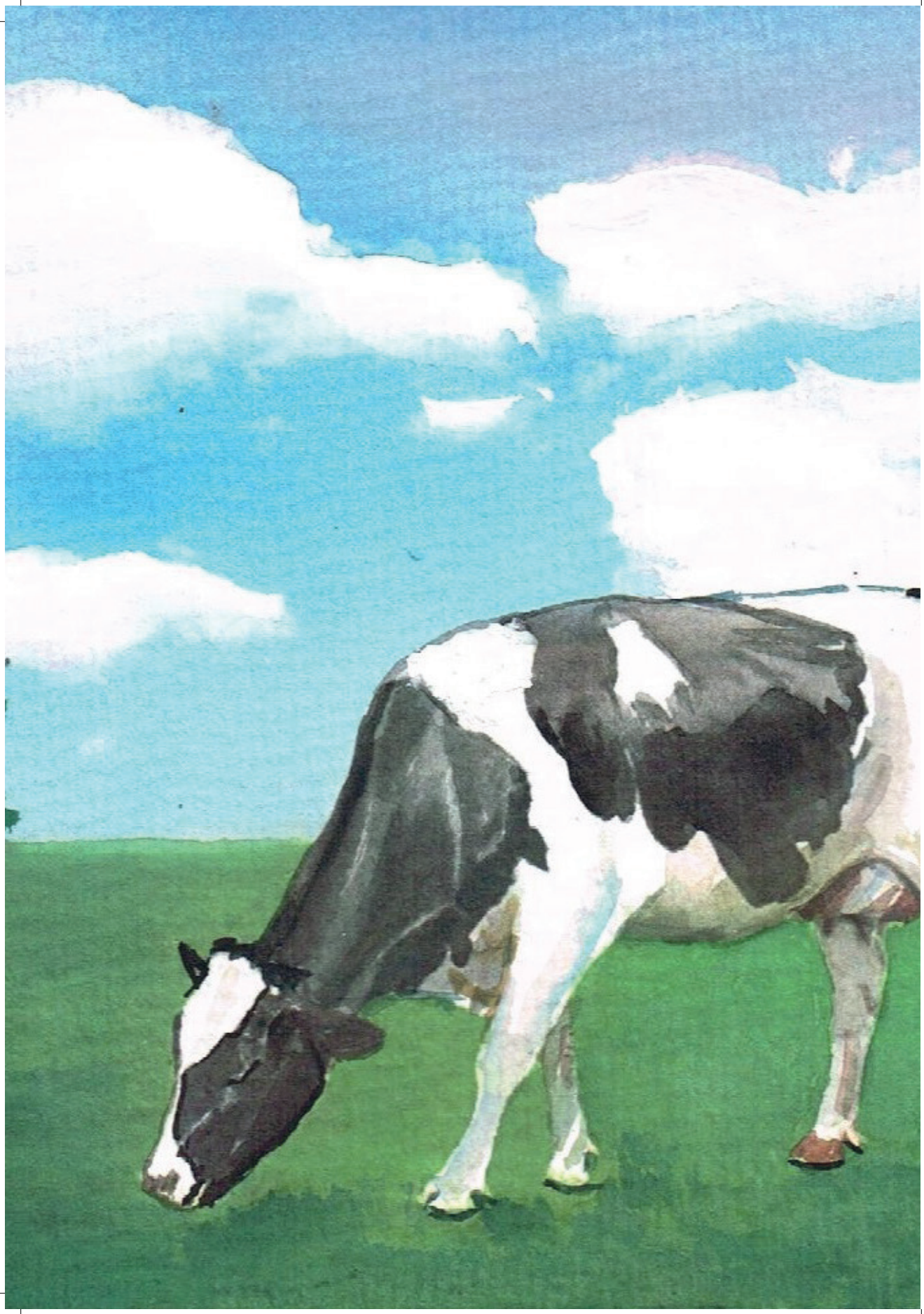
In de huidige tijd wordt in de melkveehouderij , net zoals in andere sectoren, veel aandacht besteed aan het gebruik van antibiotica. Antibioticumgebruik wordt gekwantificeerd als dierdagdosering (Animal Defined Daily Dose: **ADDD**). Deze is gespecificeerd als totaal antibioticumgebruik (**$ADDD_{TOTAL}$**), intramammair gebruik tijdens de lactatie (**$ADDD_{IMM}$**), gebruik voor droogstandsbehandeling (**$ADDD_{DCT}$**) en gebruik per injectie (**$ADDD_{INJ}$**). Deze gegevens zijn verzameld op 42 AMS en 254 CMS bedrijven in Nederland. In een subgroep van deze bedrijven (18 AMS en 24 CMS bedrijven) is tevens een enquête afgenomen om factoren die geassocieerd zijn met het antibioticumgebruik op deze bedrijven in beeld te brengen. Op elk van deze bedrijven zijn 5 kwartier melkmonsters verzameld van koeien met klinische of subklinische mastitis, om de voorkomende mastitispathogenen in beeld te brengen. Vervolgens is via een niet-lineaire principale componentenanalyse de associatie tussen de uiergezondheidsvariabelen, het antibioticumgebruik en de factoren uit de enquête geëvalueerd.

De $ADDD_{TOTAL}$ en $ADDD_{DCT}$ waren vergelijkbaar tussen AMS en CMS bedrijven, terwijl $ADDD_{IMM}$ lager en $ADDD_{INJ}$ hoger was op AMS dan op CMS bedrijven. In de principale componenten ruimte konden AMS bedrijven niet onderscheiden worden van CMS bedrijven. Het voorkomen van mastitispathogenen en hun antimicrobiële resistentie waren vergelijkbaar tussen AMS en CMS bedrijven. Deze resultaten suggereren dat het antibioticumgebruik tijdens de lactatie vergelijkbaar is in AMS en CMS bedrijven, hoewel AMS bedrijven meer behandelingen per injectie en minder via uierinjectoren toepassen. De attitude van melkveehouders ten opzichte van uiergezondheid en de behandeling van mastitis bleken geassocieerd te zijn met het antibioticumgebruik in zowel AMS als CMS bedrijven.

CONCLUSIES

Op basis van de bevindingen in dit proefschrift concluderen we dat het frequent meten van data in AMS bedrijven veel potentie heeft om de besluitvorming in het mastitis management op melkveebedrijven te ondersteunen. Dit kan worden gedaan door de uiergezondheid van individuele koeien te monitoren, door automatisch bedrijfsspecifieke risicofactoren te identificeren, door patronen in de dynamiek van

IMI vast te leggen en door de overdracht van infectieuze mastitispathogenen te kwantificeren. Dat neemt niet weg dat er nog verschillende stappen genomen zulle moeten worden om het volledige potentieel van sensortechnologieën tot zijn recht te doen komen. Er is verder onderzoek nodig, waarin de gegevens uit verschillende bronnen geïntegreerd worden en waarin algoritmen worden ontwikkeld die de beschikbare resultaten omzetten in voor de boer en zijn adviseurs interpreteerbare en bruikbare informatie.



中文总结

中文总结

随着技术变革和知识更新，奶牛养殖业逐渐从地方性小农经济发展成为高科技驱动的产业。最近几十年，奶产量和牛奶品质得到了显著提升，同时动物福利和抗生素的使用也越来越多的引起大众的关注。这些都有助于满足日益增长的人口对动物性蛋白消费的需求。世界范围内，在牧场规模不断增加的同时，牧场对高新科技的应用也在逐渐增多。尽管有很多奶牛乳房炎的相关研究被发表，同时牧场日常生产产生大量数据，牧场主仍然需要从基于数据驱动的决策系统获得足够的支持来辅助牧场日常生产管理。本课题的主要目标是探索高频率测量的（大）数据在全自动挤奶机器人(automatic milking system, **AMS**) 牧场优化奶牛乳房健康管理决策中的使用和收益。

奶牛乳房炎的发病涉及众多因素，鉴定奶牛乳房炎的相关风险因素对牧场控制奶牛乳房炎发病和提升乳房健康至关重要。与AMS牧场相比，传统机械挤奶 (conventional milking system, **CMS)** 牧场奶牛乳房炎相关风险因素的研究已经较为透彻。第二章主要描述在AMS牧场奶牛乳房炎相关风险因素的分析。通过调查问卷和牧场日常生产记录收集荷兰135个AMS牧场奶牛乳房炎潜在相关风险因素的数据。根据DHI数据，我们计算得到4个奶牛乳房炎相关指标：牛群年均体细胞数， 牛群年均DHI测试日体细胞数的方差，牛群年均新发高体细胞病例所占比例，牧场口头汇报的临床乳房炎年发病率。通过将潜在的风险因素作为自变量，以上4个乳房炎相关指标分别作为因变量，同时通过多重插补缺失值，建立回归模型从而鉴定乳房炎相关风险因素。结果显示大部分在CMS牧场的乳房炎控制措施都适用于AMS牧场乳房炎管理，在规模比较大的AMS牧场，牛体和AMS卫生状况对控制奶牛乳房炎尤为重要。

在线传感器越来越多的被用于监测个体奶牛乳房健康。我们比较了运用在线传感器(automated California Mastitis Test, **O-CMT**) 测得的4个乳区混合奶样体细胞数和在DHI实验室测得的体细胞数 (**L-SCC**)，同时探索了O-CMT在个体奶牛乳房健康监测方面的应用和增益。我们收集了50个AMS牧场O-CMT和L-SCC数据，评估了O-CMT在连续5天内的可重复性 (intraclass correlation coefficient, **ICC**), 同时计算了O-CMT和L-SCC之间的一致性相关系数 (concordance correlation coefficient, **CCC**),另外也评估了O-CMT在不同时间窗口的平均值与L-SCC的一致性。O-CMT的可重复性参数ICC为0.58，意味着42%的方差来源于O-CMT测量本身。O-CMT与L-SCC之间总体的一致性参数CCC为0.53，各个牧场间一致性参数CCC变化较大，牧场牛群体细胞数越高，O-CMT与L-SCC的一致性参数CCC越高。计算O-CMT平均值与L-SCC一致性的最佳的时间窗口为24小时。因此，O-CMT与L-SCC之间一致性在可以接受范围，高频率测量的O-CMT可以用作牧场端个体奶牛乳房健康监测。

通过应用高频率测量的O-CMT，我们发现有些牛表现出特定的SCC动力学模式：连续有规律的波动模式(regularly fluctuating SCC, **rfSCC**). 我们在第四章通过分析随机采样得到的1000头来自6个国家50个AMS牧场O-CMT数据描述了rfSCC模式，对这1000头牛，我们随机选取了连续30天O-CMT的数据记录片段，然后通过4个奶牛乳房炎专家判定这1000个O-CMT片段属于8

种SCC动力学模式的哪一种。我们计算了rfSCC模式的平均SCC，振幅，持续时长。此外，我们每隔两周收集了另一个牧场奶牛各个乳区奶样细菌培养数据，用来比较rfSCC和非rfSCC模式细菌培养结果是否不同。结果显示有4.7% (95% CI: 3.5%-6.2%) 的O-CMT片段属于rfSCC模式，rfSCC模式的平均SCC的中位数为701 (2.5%-97.5% quantile: 539-1,162) × 1,000 cells/mL, 振幅的中位数为552 (2.5%-97.5% quantile: 409-886) × 1,000 cells/mL， 每个周期持续时长的中位数为 4.1 (2.5%-97.5% quantile: 3.7-4.9) 天。我们没有发现rfSCC和非rfSCC模式之间细菌培养结果存在明显的差别。本试验结果显示高频率测量的O-CMT在鉴定特定SCC模式，例如rfSCC模式，方面具有重要作用。全自动识别rfSCC模式算法的开发以及rfSCC模式形成的相关机制仍需深入研究。

在AMS牧场，奶牛都是通过同一个挤奶单元进行挤奶。因此，在AMS牧场奶牛之间的接触模式与CMS牧场奶牛之间的接触模式具有较大差异，这可能会导致传染性病原在AMS牧场的传播与在CMS牧场的传播出现差异。我们通过连续收集一个牧场半年乳区奶样细菌培养数据，运用流行病学SIS模型估算了金黄色葡萄球菌(*Staphylococcus aureus*)和无乳链球菌(*Streptococcus agalactiae*)的传播动力学参数 (transmission rate)，感染持续时长 (duration of IMI)和基本再生数 (basic reproduction number)。金黄色葡萄球菌的传播动力学参数介于0.002 (95% CI: 0-0.005) quarter-day^{-1} 和 0.019 (95% CI: 0.010-0.032) quarter-day^{-1}， 无乳链球菌的传播动力学参数介于0.007 (95% CI: 0.005-0.010) quarter-day^{-1} 和 0.019 (95% CI: 0.011-0.032) quarter-day^{-1}之间。金黄色葡萄球菌和无乳链球菌的感染持续时长中位数分别为95 (95% CI: 72-125) 天和 86 (95% CI: 67-111) 天，金黄色葡萄球菌和无乳链球菌基本再生数分别介于0.16 (95% CI: 0.05-0.27) 和 0.34 (95% CI: 0.20-0.48) 之间以及0.64 (95% CI: 0.41-0.87) 和 0.68 (95% CI: 0.48-0.88) 之间。 本实验结果显示这两种传染性病原在这个AMS牧场的传播有限，这两种病原的传播动力学与管理良好的CMS牧场传播动力学相似。

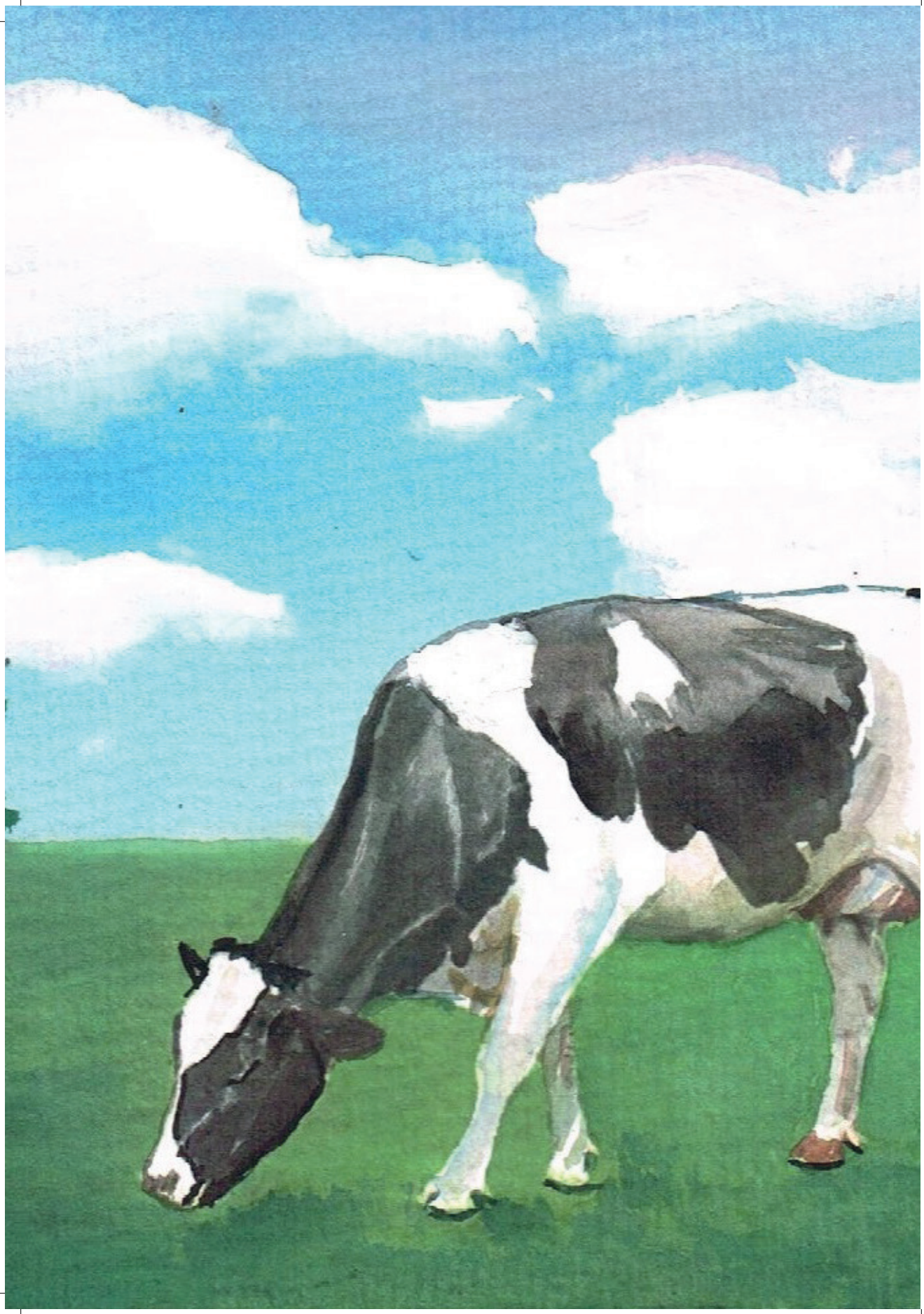
奶牛产业同其他养殖业一样越来越重视抗生素的使用。但是目前为止，对AMS牧场抗生素使用的研究非常少。我们在第六章比较了AMS和CMS牧场之间抗生素的使用 (antimicrobial use, **AMU**) 以及奶牛乳房炎病原的分布的情况。抗生素使用量包括以下四个变量：抗生素年使用总量 (**$ADDD_{TOTAL}$**)，抗生素年泌乳期使用量 (**$ADDD_{IMM}$**), 抗生素年干奶期使用量 (**$ADDD_{DCT}$**)，抗生素年（肌肉）注射使用量 (**$ADDD_{INJ}$**)。我们收集了荷兰42个AMS和254个CMS牧场抗生素使用量数据。同时收集了这些牧场中18个AMS牧场和24个CMS牧场临床型乳房炎乳区奶样 (每个牧场收集5个临床型乳房炎乳区奶样，临床型乳房炎奶样不够5个的用隐性乳房炎奶样补充)用来分离鉴定相关病原。我们通过使用非线性主成分分析来分析乳房健康相关变量，抗生素使用量相关变量和调查问卷里的变量之间的相关性。

$ADDD_{TOTAL}$在AMS和CMS牧场之间相似，$ADDD_{IMM}$ 在AMS牧场较低而$ADDD_{INJ}$ 在 AMS牧场比CMS 牧场高。AMS和CMS牧场在这些变量组成的主成分空间重合在一起。奶牛乳房炎相关病原的分布和耐药分布在AMS和CMS牧场之间相似。本实验结果显示抗生素使用量在AMS和

CMS牧场之间大致相同，但是AMS牧场使用更多注射用抗生素而更少使用泌乳期抗生素。在AMS和CMS牧场，牧场主对奶牛乳房健康和奶牛乳房炎在治疗的态度都与抗生素使用相关。

结论

基于本课题的发现，我们认为高频率测量的数据在基于数据驱动的奶牛乳房炎管理应用方面具有非常大的潜力。这些潜力可以通过持续监测每个奶牛乳房健康状态，全自动鉴定每个特定牧场奶牛乳房炎的相关风险因素，捕捉新的乳区感染动力学模式以及估算传染性乳房炎病原在奶牛群体内的传播率等得到发掘和应用。为了释放这些潜力，我们应该解决上面提及的限制传感器和大数据在奶牛养殖业应用的问题。未来研究应该尝试融合不同来源的传感器数据和开发将这些不同来源的数据转换成牧场和兽医能够解读的报告的算法。



Acknowledgements

The rain must fall-Yanni.

Although it was not an enjoyable journey, often rather struggling for most of the people switching from a researcher in laboratory work with pathogens to a more data scientist perspective that applies data analytics in the population health management practice. It was those struggling times that built my strength and laid the way to my future development. There were a lot of colleagues, friends and enthusiastic strangers that came into my assistance, without which I would have never arrived at the destination of my PhD study.

I am extremely lucky to pursue PhD degree with my supervisor team, each of them etched their own personalities and tastes in research on my self-development.

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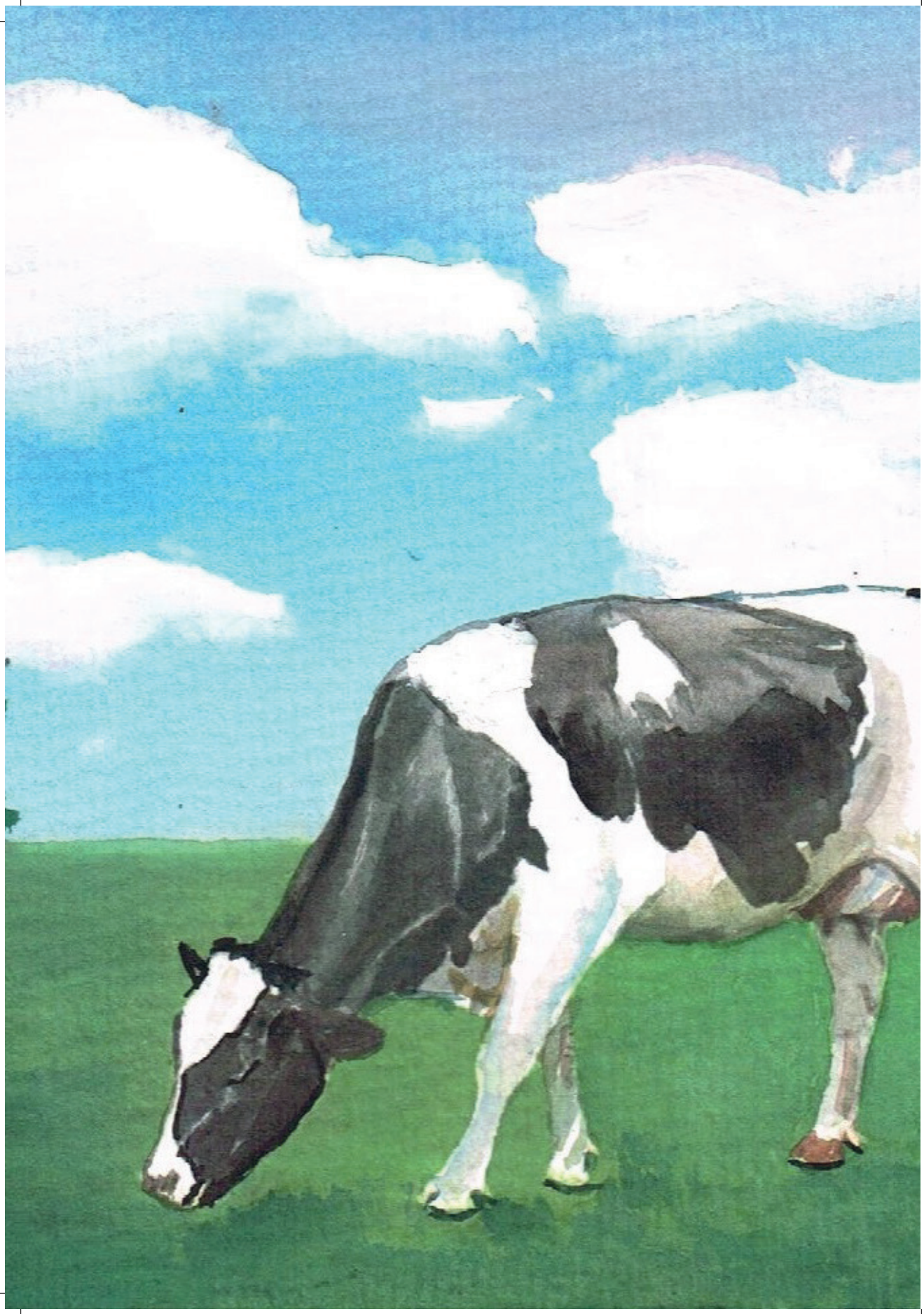
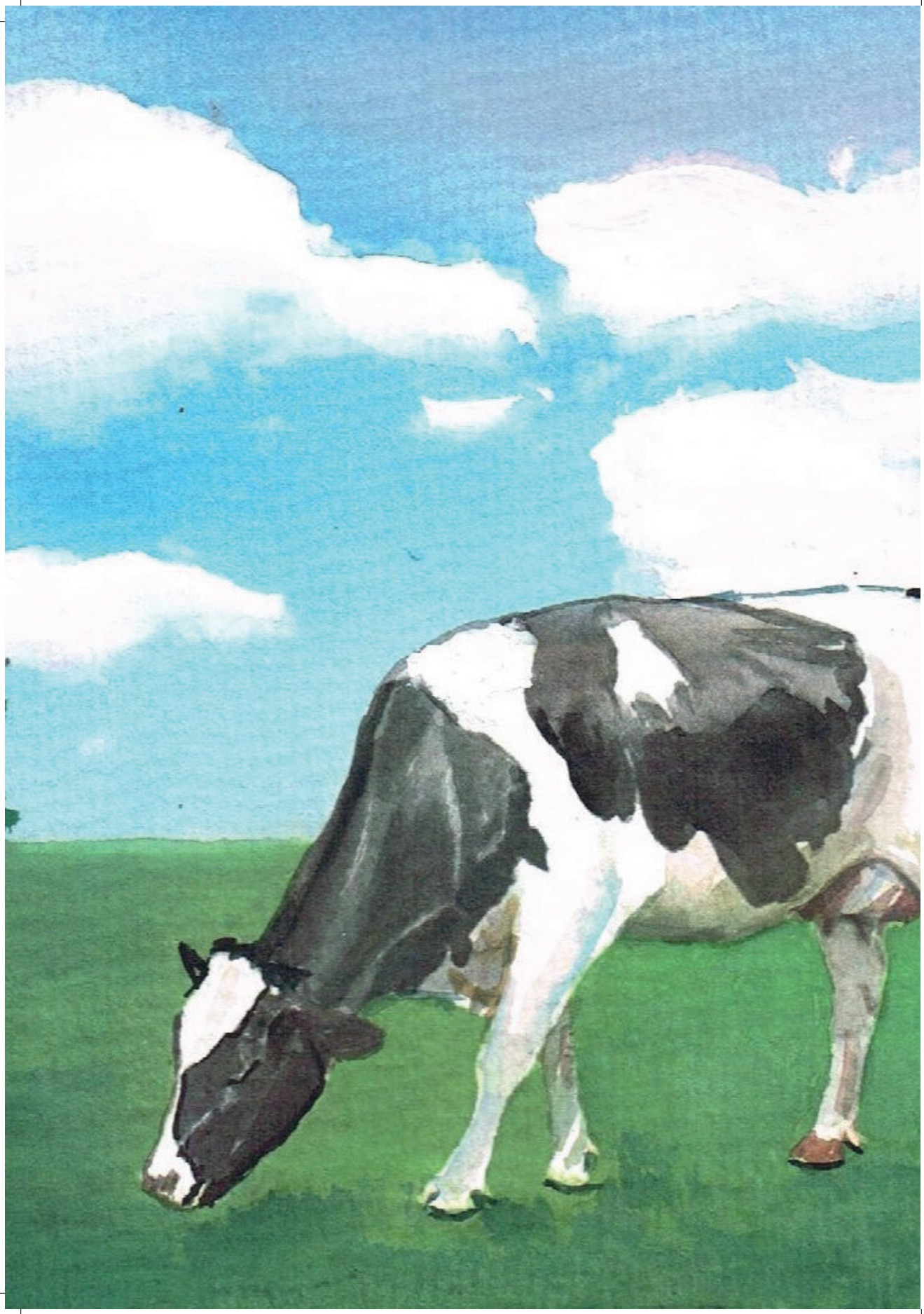


Curriculum vitae

CURRICULUM VITAE

Zhaoju Deng was born on Dec. 19th, 1991 in Qianjiang, Hubei province, China. He finished his DVM in Huazhong Agricultural University after 5 years study and continued his master research in clinical veterinary medicine in China Agricultural University, in which he started research in bovine mastitis. It is during the time of master research, he visited several mega dairy farms around Beijing, in which he decided to dedicate himself in the research in population health of cattle. In 2015, he was approved to pursue his PhD degree in bovine mastitis epidemiology in dairy herds milked with automatic milking systems in Utrecht University under the supervision of Prof. Theo Lam, Prof. Henk Hogeveen and Dr. Gerrit Koop. In August 2020, Zhaoju started to work informally in the large animal group at China Agricultural University doing research in population health in Chinese dairy herds in addition to teaching master students with "Study design and data analysis using R programming in veterinary research".

In search of his inner peace, he followed Shakuhachi course in Rotterdam supervised by Hélène Codjo for six months in 2017.



List of publications

LIST OF PUBLICATIONS

1. **Deng, Z**., G. Koop, T. J. G. M. Lam, I. A. van der Lans, J. C. M. Vernooij, and H. Hogeveen. 2019. Farm-level risk factors for bovine mastitis in Dutch automatic milking dairy herds. ***J. Dairy Sci.*** 102(5):4522-4535. doi:10.3168/jds.2018-15327.

2. **Deng, Z.**, T. J. G. M. Lam, H. Hogeveen, M. Spaninks, N. Heij, M. Postema, T. van Werven, and G. Koop. 2020. Antimicrobial use and farmers' attitude toward mastitis treatment on dairy farms with automatic or conventional milking systems. ***J. Dairy Sci***. 103(8):7302-7314. doi:10.3168/jds.2019-17960.

3. **Deng, Z.**, H. Hogeveen, T. J. G. M. Lam, R. van der Tol, and G. Koop. 2020. Performance of online somatic cell count estimation in automatic milking systems. ***Front. Vet. Sci.*** 7:221. doi:10.3389/fvets.2020.00221.

4. **Deng, Z**., T. J. G. M. Lam, H. Hogeveen, E. A. J. Fischer, B. H. P. van den Borne, R. van der Tol, G. Koop. 2021. Transmission dynamics of *Staphylococcus aureus* and *Streptococcus agalactiae* in a Dutch dairy herd using an automatic milking system. ***Prev. Vet. Med.*** 192:105384. https://doi. org/10.1016/j.prevetmed.2021.105384.

5. **Deng, Z.**, T. J. G. M. Lam, H. Hogeveen, G. Koop. Regularly fluctuating somatic cell count pattern in dairy herds.(minor revision in ***J. Dairy Sci.***)

6. **Deng Z**., M. Shahid, L. Zhang, J. Gao, X. Gu, S. Zhang, J. Zou, S. Fanning, B. Han. 2016. An investigation of the innate immune response in bovine mammary epithelial cells challenged by *Prototheca zopfii*. ***Mycopathologia***. 181:823–832. doi:10.1007/s11046-016-0053-0.

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