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Advanced statistical modeling of complex traits related to subacute ruminal acidosis in dairy cows

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List of papers and conference contributions

The following papers form the main part of the dissertation:

1. **Mensching, A.**, J. Hummel, and A.R. Sharifi. 2020. Statistical modeling of ruminal pH parameters from dairy cows based on a meta-analysis. J. Dairy Sci. 103:750–767. http://dx.doi.org/10.3168/jds.2019-16802.
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3. **Mensching, A.**, M. Zschiesche, J. Hummel, A.O. Schmitt, and A.R. Sharifi. 2020. An innovative concept for a multivariate plausibility assessment of synchronously recorded data. Animals 10:1412. http://dx.doi.org/10.3390/ani10081412.
4. **Mensching, A.,** M. Zschiesche, J. Hummel, C. Grelet, N. Gengler, S. Dänicke, and A.R. Sharifi. 2021. Development of a SARA risk score and its prediction using milk mid-infrared spectra in early-lactation cows. J. Dairy Sci. 104: 4615–4634. http://dx.doi.org/10.3168/jds.2020-19516.

In addition, the following non-peer-reviewed articles were published in the scope of the dissertation. The results were also presented in conference talks:

1. **Mensching, A.**, M. Zschiesche, J. Hummel, and A.R. Sharifi. 2018. Subakute Pansenazidose bei der Milchkuh – Vorhersage ruminaler pH Parameter basierend auf einer Metaanalyse. In: 8. cattle workshop (Uelzen, Germany, 2018), DGfZ publication series 75:176-187.
2. **Mensching, A.**, M. Zschiesche, H.M. Jansen, J. Hummel, K. Bünemann, U. Meyer, D.v. Soosten, S. Dänicke, D. Albers, E. Bohlsen, H. Hammami, N. Gengler, F. Dehareng, and A.R. Sharifi. 2019. Entwicklung innovativer Indikatoren für die subakute Pansenazidose laktierender Kühe. In: Proceedings of the 2nd Goettingen Animal Welfare Conference (Goettingen, Germany, 2019):41-46. http://dx.doi.org/10.3249/ugoe-publ-3.

Furthermore, results were presented in the following conferences talks:

1. **Mensching, A.**, M. Zschiesche, J. Hummel, and A.R. Sharifi. 2018. Derivation of the metabolic status in dairy cows – prediction of daily mean ruminal pH. In: Book of Abstracts of the 69th Annual Meeting of the European Federation of Animal Science (Dubrovnik, Croatia, 2018), Wageningen, Netherlands: Wageningen Academic Publishers:190.
2. **Mensching, A.**, M. Zschiesche, J. Hummel, and A.R. Sharifi. 2018. Mathematisch-statistische Modellierung ruminaler pH-Parameter mittels metanalytischer Methoden. In: Book of Abstracts of the Annual Symposium of the DGfZ and GfT (Bonn, Germany, 2018):A9.
3. **Mensching, A.**, K. Bünemann, U. Meyer, D. v. Soosten, S. Dänicke, J. Hummel, and A.R. Sharifi. 2019. Prediction of reticular and ruminal pH progressions with transponder based feed intake. In: Book of Abstracts of the 70th Annual Meeting of the European Federation of Animal Science (Ghent, Belgium, 2019), Wageningen, Netherlands: Wageningen Academic Publishers:534.
4. **Mensching, A.**, K. Bünemann, U. Meyer, D. v. Soosten, S. Dänicke, J. Hummel, and A.R. Sharifi. 2019. pH-Entwicklung im ventralen Pansensack und Retikulum in Abhängigkeit des Futter- und Wasseraufnahme- sowie Wiederkauverhaltens. In: Book of Abstracts of the Annual Symposium of the DGfZ and GfT (Gießen, Germany, 2019):A12.
5. **Mensching, A.**, M. Zschiesche, J. Hummel, C. Grelet, N. Gengler, and A. R. Sharifi. 2020. Using milk mid-infrared spectra to predict the SARA risk of dairy cows. In: Book of Abstracts of the 71st Annual Meeting of the European Federation of Animal Science (Virtual Meeting, 2020), Wageningen, Netherlands: Wageningen Academic Publishers:223.

List of abbreviations

α_{PMR}, α_C, α_W	Decreasing rates for the intake of partial mixed ration, concentrate and water
ΔpH	Daily ventral ruminal pH range
Δt	Time lag
$\hat{\sigma}$	Empirically estimated standard deviation
Σ.Et, Σ.Lt, Σ.Rt	Daily duration of eating, lying and rumination
ΣPMR, ΣC, ΣW	Daily total consumed amount of partial mixed ration, concentrate and water
τ	Time lag in the cross-correlation analysis
ADF	Acid detergent fiber
AICc	Second-order Akaike information criterion
aNDFom	Neutral detergent fiber after amylase treatment and ashing
ANOVA	Analysis of variance
BCFA	Branched-chain fatty acids
BHB	β-hydroxybutyrate
BW	Body weight
C_{35}, C_{60}	Feeding groups with 35 or 60% concentrate in the diet
CF	Crude fiber
CL	Crude fat
CP	Crude protein
DFF	Daily feeding frequency
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake [kg/d]
ECM	Energy corrected milk yield
EE	Ether extract
F:C	Forage to concentrate ratio
FA	Fatty acid
FM	Fresh matter
FPR	Milk fat to milk protein ratio
GH	Standardized Mahalanobis distance, Global H-value
GLU	Glucose

I^2, I^2_{bs}	Overall heterogeneity and between-study heterogeneity
LCFA	Long-chain fatty acids
LF	Lactoferrin
LMM	Linear mixed model
LPS	Lipopolysaccharide
LSmeans	Estimated least-squares means
LV	Latent variable
MCFA	Mid-chain fatty acids
MIR	Mid-infrared
MPA	Multivariate plausibility assessment
MUN	Milk urea nitrogen
MY	Milk yield
NDF	Neutral detergent fiber
NEFA	Non-esterified fatty acids
NE_L	Net energy lactation
NFC	Nonfiber carbohydrates
NfE	Nitrogen-free extract
OFA	Odd-chain fatty acids
PC	Principal component
PCA	Principal component analysis
peNDF	Physically effective neutral detergent fiber
peNDF>1.18, peNDF>8	Physically effective neutral detergent fiber defined as the proportion of the total mixed ration or forage that retained on a 1.18- or 8-mm sieve multiplied with the dietary content of neutral detergent fiber
$\overline{pH}$	Ventral ruminal daily mean pH
PLS	Partial least squares
PMR	Partial mixed ration
PSPS	Penn State Particle Separator (Nasco, Fort Atkinson, Wisconsin)
r.ΔpH	Daily reticular pH range
$r.\overline{pH}$	Reticular daily mean pH

$r.T_{med}$	Median of the reticular temperature
R^2	Coefficient of determination
R_c^2 , R_m^2	Conditional and marginal coefficient of determination for linear mixed models
R_{cal}^2 , R_{cv}^2	Coefficient of determination of the calibration model and of cross-validation
RB	Rumination behavior
$\overline{RCf}$	Daily mean rumination chewing frequency
RMSE	Root mean square error
$RMSE_{cal}$, $RMSE_{cv}$	Root mean square error of calibration and of cross-validation
RPD_{cv}	Ratio between standard deviation and the root mean square error of cross-validation
rPMR, rC, rW	Intake rates of partial mixed ration, concentrate and water
RSE	Residual standard error
$s.\overline{T}$	Daily mean temperature in the barn
SARA	Subacute ruminal acidosis
SCC	Somatic cell count
SCFA	Short chain fatty acids
SCS	Somatic cell score
SD	Standard deviation
SED	Standard error of the differences
SEM	Standard error of the mean
SMA	Standardized major axis regression
SRS	Subacute ruminal acidosis risk score
$T[rPMR]$, $T[rC]$, $T[rW]$	Transformed intake rates of partial mixed ration, concentrate and water
TMR	Total mixed ration
TpH<5.8	Daily duration with a ventral ruminal pH < 5.8
VFA	Volatile fatty acids
WN	Number of wavenumbers
$\overline{x}$	Arithmetic mean

Summary

Adequate feeding of lactating cows is particularly challenging for high-performing animals. In this respect, subacute ruminal acidosis (**SARA**) is considered the most important nutritional disease. Its emergence is promoted by an imbalanced design of the diet, where both an excess of easily fermentable carbohydrates and a deficiency in the physical structure of the feed can be critical. The pH parameters derived from continuous ruminal pH measurements are considered the gold standard for the diagnosis of SARA, but they cannot be measured on a large scale in agricultural practice. Since SARA corresponds to a subacute stage, no specific clinical signs can be detected at the animal level. However, associations with clinical signs at herd level are described, such as reduced feed intake, lower milk production efficiency and a higher risk of loose stools and claw diseases. Consequently, there is a need to identify indicators that allow for a comprehensive and precise monitoring of SARA to minimize economic losses and in particular to ensure animal welfare.

The objective of this dissertation was therefore to investigate the associations between data that were measured *in vivo* in the reticulorumen, properties of the diet, various behavioral and blood parameters and the composition of the milk using different statistical methods, in order to contribute to the development of better indicators for SARA.

First, the associations between ruminal pH parameters, feed properties and particularly the main milk components were investigated in a meta-analysis. To create a data basis, the results of 32 studies with continuous ruminal pH measurements in dairy cows were gathered. The main part of the analysis consisted of a systematic examination of potential predictors for ruminal pH parameters using mixed multi-level meta-regression models. Significant associations between ruminal pH parameters and the protein and fat content of milk as well as the milk fat to milk protein ratio were confirmed. However, the associations can only be classified as a trend, since most of the observed variability of pH parameters is due to a high level of heterogeneity both within and between the individual studies which means that only a small proportion of the variance was explained by the predictor variables.

In a further analysis, the ruminal pH development both in the reticulum and in the ventral rumen in the course of the day was examined based on data collected in an experimental station. The aim was to model the pH development using sensor-based records of the feed intake, water intake and rumination behavior. An extensive data preparation was crucial, whereby all available data were transformed into a uniform 1-minute resolution. In addition, signal transformation methods were used to model feed and water intake events over time. For the statistical evaluation, all data were analyzed in the form of high-resolution time series using linear mixed regression models. It was shown that the daily pH development is highly associated with the animal's individual feed intake and rumination behavior in the course of the day.

Based on the previously obtained knowledge, a procedure was developed to predict the risk of suffering from SARA using milk mid-infrared (**MIR**) spectral data. The data were collected on 10 commercial farms and a total of 100 cows. This included reticular pH measurements, behavioral data, feed analysis data, performance and blood data. Furthermore, MIR spectral data as well as information on the main components of the milk and the fatty acid composition of the milk fat were available. Since sensitive sensor systems were used, which are prone to technically caused errors under the environmental conditions occurring in agricultural practice, a multivariate plausibility check was first developed to prepare the data set for downstream analyses. The aim was to develop a procedure to classify the individual observations of several simultaneously recorded sensor, blood and milk data into ‘physiologically normal’, ‘physiologically extreme’ and ‘implausible’. In the final analysis, the association between potentially SARA-indicating traits and the composition of the milk was examined using the processed data set. First, an innovative SARA phenotype (‘SARA risk score’, **SRS**) in the form of an index trait was developed. This SRS is based on information from intra-ruminal pH and temperature measurements, rumination and feed intake behavior as well as the milk performance. Using partial least squares regression models, a MIR-based prediction model with a moderate prediction quality could be established for the SRS. In addition, significant associations between the fatty acid profile of milk and the SRS were determined.

On the one hand, well-known relationships between ruminal fermentation, animal behavior and milk constituents were verified using innovative statistical methods. On the other hand, it was shown that the MIR spectral data of milk, which are already routinely collected, offer an enormous potential for the characterization of the health status of lactating cows. The obtained findings in this work thus provide the basis for the development of a routine and comprehensive SARA monitoring which can be applied in agricultural practice.

Zusammenfassung

Die wiederkäuergerechte Fütterung von laktierenden Kühen ist insbesondere bei hochleistenden Tieren eine große Herausforderung. In dieser Hinsicht gilt die subakute Pansenazidose (**SARA**) als wichtigste ernährungsbedingte Krankheit. Ihre Entstehung wird durch eine unausgewogene Zusammensetzung der Ration begünstigt, wobei sowohl ein Überschuss an leicht fermentierbaren Kohlenhydraten als auch ein Mangel der physikalischen Struktur des Futters kritisch sein können. Die von einer kontinuierlichen ruminalen pH-Messung abgeleiteten pH-Parameter gelten zwar als Goldstandard zur Diagnose einer SARA, können aber nicht flächendeckend in der landwirtschaftlichen Praxis erfasst werden. Da es sich bei der SARA um ein subakutes Stadium handelt, sind keine spezifischen klinischen Symptome auf tierindividueller Ebene erfassbar. Allerdings können Assoziationen mit klinischen Symptomen auf Herdenebene festgestellt werden, wie z.B. eine reduzierte Futteraufnahme, eine geringere Effizienz der Milchproduktion und ein höheres Risiko für Durchfall- und Klauenerkrankungen. Demzufolge besteht die Notwendigkeit innovative Indikatoren zu identifizieren, mit denen ein möglichst umfassendes und präzises SARA Monitoring zur Prävention ermöglicht werden kann, um wirtschaftliche Einbußen zu minimieren und insbesondere zur Sicherstellung des Tierwohls beizutragen.

Ziel dieser Dissertation war es daher, in unterschiedlichen statistischen Verfahren die Assoziationen zwischen ruminal gemessenen Daten und Eigenschaften der Ration, verschiedener Verhaltens- und Blutparameter sowie der Zusammensetzung der Milch zu untersuchen, um mit den dabei gewonnen Kenntnissen die Entwicklung besserer Indikatoren für SARA zu fördern.

Zunächst wurden in einer Meta-Analyse die Assoziationen zwischen ruminalen pH-Paramatern und erfassten Eigenschaften der Ration sowie den Hauptinhaltsstoffen der Milch untersucht. Die Datengrundlage hierfür waren die Ergebnisse von 32 Studien, in denen bei laktierenden Kühen der ruminale pH-Wert kontinuierlich aufgezeichnet wurde. Der Kern der Analyse bestand aus einer systematischen Prüfung potentieller Prädiktoren zur Vorhersage der ruminalen pH-Parameter unter Verwendung von gemischten Multi-Level Meta-Regressionsmodellen. Dabei wurden signifikante Assoziationen zwischen ruminalen pH-Parametern und dem Protein- und Fettgehalt der Milch sowie auch dem Fett-Protein-Quotient bestätigt. Allerdings sind die Assoziationen nur als Trend einzustufen, da der größte Teil der beobachteten Variabilität der pH-Paramater auf eine hohe Heterogenität sowohl innerhalb als

auch zwischen den einzelnen Studien zurückzuführen ist und daher nur ein geringer Anteil der Varianz mit den unabhängigen Variablen erklärt werden konnte.

In einem weiteren Analyseverfahren wurde anhand von Daten, die in einer Versuchsstation erhoben wurden, die ruminale pH-Entwicklung sowohl im Retikulum als auch im ventralen Pansen im Tagesverlauf untersucht. Ziel war es dabei, die pH-Entwicklung mit Hilfe des sensortechnisch erfassten Futteraufnahme-, Wasseraufnahme- und Wiederkauverhaltens zu modellieren. Entscheidend dafür war eine umfangreiche Datenvorbereitung, wobei alle verfügbaren Messgrößen in eine einheitliche 1-minütige Auflösung transformiert wurden. Zudem wurden Methoden zur Signaltransformation verwendet, um Futter- und Wasseraufnahmeereignisse im Zeitverlauf zu modellieren. Bei der statistischen Auswertung wurden alle vorliegenden Daten in Form von hochauflösenden Zeitreihen in linearen gemischten Regressionsmodellen analysiert. Dabei konnte gezeigt werden, dass die tägliche pH-Entwicklung in hohem Maße mit dem tierindividuellen Futteraufnahme- und dem Wiederkauverhalten im Tagesverlauf assoziiert ist.

Aufbauend auf den zuvor gewonnen Kenntnissen erfolgte die Entwicklung eines Verfahrens, bei dem mit Hilfe von Mittelinfrarot-Spektraldaten (**MIR**) der Milch das Risiko an einer SARA zu leiden geschätzt werden kann. Die dafür verwendeten Daten wurden in 10 Praxisbetrieben und an insgesamt 100 Kühen erhoben. Dies umfasste retikulär gemessene pH-Daten, sensortechnisch erfasste Verhaltensdaten, Daten über die Rationsgestaltung und Futterinhaltsstoffe sowie Leistungs- und Blutparameter. Desweitern standen MIR-Spektraldaten sowie Informationen über die Hauptinhaltsstoffe der Milch als auch die Fettsäurezusammensetzung des Milchfetts zur Verfügung. Da sensible Sensorsysteme verwendet wurden, die unter den in der landwirtschaftlichen Praxis vorkommenden Umweltbedingungen zu technisch bedingten Fehlern neigen, erfolgte zunächst die Entwicklung eines multivariaten Plausibilisierungsverfahrens zur Vorbereitung des Datensatzes für nachfolgende Analysen. Ziel war es dabei, unter Verwendung statistischer Methoden die einzelnen Beobachtungen mehrerer simultan erfasster Sensor-, Blut- und Milchdaten in ‚physiologisch normal', ‚physiologisch extrem' und ‚unplausibel' zu klassifizieren. In der finalen Analyse wurde anhand des aufbereiten Datensatzes die Assoziation zwischen potentiell SARA indizierenden Merkmalen und der Zusammensetzung der Milch untersucht. Dafür wurde ein innovativer SARA Phänotyp (‚SARA risk score', **SRS**) in Form eines Index-Merkmals entwickelt. Dieser SRS basiert auf Informationen aus intra-ruminalen pH- und Temperaturmessungen, Wiederkau- und Futteraufnahmeverhalten sowie der Milchleistung. Unter Verwendung von Partial-Least-Squares Regressionsmodellen konnte für den SRS ein MIR basiertes Vorhersagemodell mit einer moderaten Vorhersagegüte etabliert werden. Darüber hinaus konnten signifikante Assoziationen zwischen dem Fettsäureprofil der Milch und dem SRS festgestellt werden.

In dieser Arbeit wurden zum einen bekannte Zusammenhänge zwischen der ruminalen Fermentation, dem Verhalten der Tiere sowie den Inhaltsstoffen der Milch mit innovativen statistischen Methoden belegt. Zum anderen wurde aufgezeigt, dass die bereits routinemäßig erhobenen MIR-Spektraldaten der Milch ein enormes Potential für die Charakterisierung des Gesundheitsstatus laktierender Kühe bieten können. Die in dieser Arbeit gewonnenen Erkenntnisse bilden somit die Grundlage für die Entwicklung eines routinemäßigen und umfassenden SARA-Monitorings, das in der landwirtschaftlichen Praxis angewendet werden kann.

Chapter 1

General introduction

Prologue

In recent years, technological progress has facilitated the collection, management and analysis of 'Big Data' and built the basis of agricultural research today. Besides creating new opportunities for working on complex research topics, new tasks and challenges emerged, which have to be met with advanced statistical and bioinformatics procedures. In animal sciences, the increasing use of genotyping based on high density single-nucleotide polymorphism markers or even sequencing of the entire genome, high-throughput analysis methods, e.g. for feed or milk samples, or the use of automated and computerized data acquisition systems for the continuous recording of animal-individual traits can be given as examples. These methods offer the possibility to develop strategies in order to solve complex tasks, which go beyond the mere increase of the performance. In today’s agriculture, the focus is put increasingly on approaches that improve resource efficiency of agricultural production and promote animal welfare. This includes in particular the work on research topics, such as the behavioral disorders ‘tail biting’ in pigs or ‘feather pecking’ and cannibalism in poultry or metabolic and digestive disorders in dairy cows. Disorders like these are difficult to detect on a large scale and show high inter-animal variance. However, the use of new technologies as well as comprehensive statistical methods offers the possibility to develop innovative detection strategies. This allows for a deeper understanding of the causality of these disorders and reveals ways to prevent their occurrence. With this knowledge, cost-effective monitoring systems can be developed to improve the management in commercial farming. Furthermore, the comprehensive generation of phenotypes would facilitate the investigation of the genetic background of these disorders in order to take this into account in the design of future breeding schemes.

In the following, an introduction to the digestive disorder bovine acidosis and its subacute form, the subacute ruminal acidosis (**SARA**), in lactating cows is presented. After highlighting the impact of SARA on animal welfare and the economic efficiency in dairy farming, insights into the milk composition-based monitoring are given. Afterwards, the objectives and methods developed in this dissertation are summarized.

Defining subacute ruminal acidosis

SARA is a digestive disorder of ruminants and is characterized by a temporarily altered rumen fermentation with an imbalance between production and absorption of fermentation end products as well as changes in the composition of the ruminal microbiome (Nordlund and Garrett, 1994). The emergence is mainly promoted by an inadequate diet, where the chemical composition and the physical structure of the diet as well as the degree of adaptation of the animal to the diet are important factors (Nocek, 1997). The acute acidosis and SARA differ particularly in their severity, with specific signs being observable in acute acidosis and affected animals appearing significantly sick, whereas this is not the case for SARA (Owens et al., 1998). While SARA can be found in high yielding dairy herds, its acute form is primarily found in feedlot cattle during fattening (Owens et al., 1998; Enemark, 2008). An attempt is made to divide the state of health, which in terms of these disorders represents a continuum with a smooth transition between healthy and sick, into 3 discrete stages. The distinction between 'physiologically normal', 'SARA' and 'acute clinical acidosis' is made based on biochemical and fermentation-specific characteristics, with the ruminal pH being in the focus of most investigations (Enemark et al., 2002). However, the delimitations and strategies for diagnosis of SARA are controversial and numerous threshold-based definitions can be found in the literature.

Etiology and risk factors

SARA is caused by an increased accumulation of volatile fatty acids (**VFA**) in the rumen that lead to an acidotic milieu and consequently to a decrease of the ruminal pH (Oetzel, 2017). Besides the fermentative release of VFA, the absorption of VFA via the rumen mucosa as well as the neutralizing effect of bicarbonate and phosphate from saliva are important processes in pH regulation (Enemark et al., 2002). Furthermore, it is assumed that bicarbonate secreted by the ruminal epithelium contributes similarly to the buffering in high performing cows (Aschenbach et al., 2011). Thus, the ruminal system is subject to a number of interacting processes, which together determine the ruminal pH development. However, some of these processes can be influenced by a variety of factors, the most important of which are summarized in the following.

From the nutritional point of view, the properties of the feed play a major role. These include the chemical composition as well as the physical structure of the feed. One factor is the total amount of easily fermentable carbohydrates, which is typically attributable to high proportions of concentrate and low proportions of roughage in the diet. Jiang et al. (2017), for example, observed a decrease in the ruminal daily mean pH accompanied by an increased concentration of ruminal VFA as consequence of the gradual change of the diet’s concentrate proportion from 30 to 60%. This relationship can also be quantified *in vitro* using a Hohenheim gas test, in which the cumulative gas production during fermentation is measured from a feed sample inoculated with rumen fluid (Williams, 2011). In this test, the amount and rate of released gas allow for conclusions about the microbial fermentation activity and thus the release of VFA. Zicarelli et al. (2011), for example, showed that the higher the concentrate content of the diet, the higher is the maximal gas production rate and the total gas production. High concentrations of rapidly fermentable carbohydrates as well as low contents of physically effective structure are not only typical of diets containing concentrated feed. Lush and sugar-rich pastures in grazing-systems can also promote the development of SARA (O'Grady et al., 2008).

Another important factor is the chemical composition of the carbohydrate fraction as the fermentation rates of various carbohydrate fractions differ. For example, the fermentation rate is highest for glucose, medium for starch and rather low for cellulose, where in particular the rapidly fermentable carbohydrates can lead to a short-term increased accumulation of VFA (Noike et al., 1985). In this regard, ruminal availability also depends on the cereal species, with starch of maize being available more slowly than of wheat, barley or oats (Herrera-Saldana et al., 1990). In addition, the intensity of grain processing is decisive. A more intensive mechanical treatment of the grains can increase the availability of starch in the rumen and thus the fermentation activity, which in turn can result in a lower ruminal pH (Yang et al., 2001).

As mentioned above, ruminant saliva with its pH of about 8.2 and containing bicarbonate and phosphate plays an important role in ruminal pH development (Aschenbach et al., 2011). Saliva is produced while eating, ruminating and resting, with the highest quantities being produced during rumination (Maekawa et al., 2002a). In general, the more fiber in the diet, the higher is the rumination activity, which in turn leads to increased saliva production and thus increased buffering of the rumen (Mertens, 1997). However, it is not only the fiber content that is decisive for stimulating rumination activity, but also the physical structure of the diet as well as the particle size distribution. There are various systems for assessing and quantifying the structure of the diet. A common method is the determination of physically effective neutral detergent fiber (**peNDF**) with a Penn State Particle Separator (Nasco, Fort Atkinson, Wisconsin) as introduced by Lammers et al. (1996). Thereby, the peNDF>1.18 and peNDF>8 of forage or total mixed ration (**TMR**) are defined as the proportion of material retained on a 1.18-, and 8-mm sieve multiplied with the neutral detergent fiber (**NDF**) content of the diet. In this context, it is also relevant to what extent the feed properties let cows sort against different particle sizes. Since cows obviously prefer concentrated feed to roughage (e.g., Lawrence et al., 2015), it is recommended to provide the ration in the form of a TMR, which results in a more even feed intake in the course of the day and a reduced sorting against long particles and thus decreases the risk of SARA (DeVries and Von Keyserlingk, 2009; Humer et al., 2018). However, management-related interventions are also reflected in daily pH development, with higher feeding frequencies leading to less fluctuation of the pH in the course of the day (French and Kennelly, 1990; Le Liboux and Peyraud, 1999).

According to Nordlund et al. (1995), a lactation stage based distinction between 2 SARA risk groups can be made. The first group consists of the periparturient cows and the second includes cows at the peak of lactation. For both groups, there is an increased risk of SARA, if the formulation of the diet is not adequate. However, the risk is particularly increased in the early postpartum phase, when the feed is changed to the lactation diet, which is typically high in easily fermentable carbohydrates and energy and has only a moderate level of physical effective fiber (Kleen et al., 2003). Besides, shifts in the composition of the microbiome and the adaptation of the rumen mucosa play an important role (Nordlund et al., 1995). The decisive factor here is the absorption capacity of the rumen mucosa, which is reduced during the periparturient period in heifers and multiparous cows (Liebich et al., 1987). Promoted by ruminal butyrate and propionate formation, which are released during the microbial fermentation of carbohydrates, the mucosa is able to adapt to the diet to a certain extent by an increased mucosal proliferation and a consequent higher absorption capacity (Liebich et al., 1987). Therefore, the absorption capacity increases as soon as feed is changed to the lactation diet with a higher content of concentrate. Since the morphological adaptation of the rumen mucosa is time consuming, a too fast increase of concentrate in the diet after parturition can increase the risk of SARA (Liebich et al., 1987). Nordlund et al. (1995) assumed that the adaptation from a diet rich in roughage to a high-energy lactation diet takes between 3 and 5 weeks.

The amount of feed consumed influences the ruminal pH development to a certain extent. On the one hand, this can be deduced from the results of Krause and Oetzel (2005), who made a one-day feed restriction to 50% of the baseline amount of feed previously to a SARA challenge and observed a considerable increase in ruminal pH. On the other hand, this is in line with the results of Dado and Allen (1995), who artificially reduced the amount of digesta in the rumen by using rumen-inert bulks and observed lower VFA concentrations and higher pH values. This is also confirmed by the meta-analysis of Zebeli et al. (2008), where the covariable dry matter intake (**DMI**) had a negative regression coefficient in a multiple regression model with pH as response variable. However, it can be assumed that the relationship between pH and DMI is not strictly linear, but shows a rather curvilinear relation. In the previously mentioned study of Jiang et al. (2017), both a higher DMI and an increased milk yield as well as reduced ruminal pH were observed with increasing concentrate contents in the diet.

However, it can be assumed that if the concentrate content had been increased even further, SARA or even acidosis would have occurred, which in turn would have led to a decreased DMI. Krajcarski-Hunt et al. (2002), for example, observed a reduced DMI as a result of increasing the concentrate content from 60.3 to 70.2% in a SARA induction experiment.

The last decisive factor is the individual animal itself. In general, a high inter-animal variance is described, with some animals being more prone to SARA than others (Humer et al., 2015; Jing et al., 2018; Schmitz et al., 2018a). The results of certain studies indicate that some of the observed variation with regard to the ruminal pH development is attributable to the animal's individual feed intake behavior. Macmillan et al. (2017), for example, determined that cows which are more prone to SARA showed a more uneven feed intake in the course of the day. Furthermore, Coon et al. (2019) observed that cows classified in high and low risk by an acidosis index based on reticuloruminal pH measurements differed in their sorting behavior. After all, low SARA risk cows sorted less feed and thus ate a more balanced diet than cows classified as high risk SARA cows. Interestingly, DeVries et al. (2008) noted that severe acidosis cows in turn increasingly sorted for long particles, possibly counteracting the acidosis-causing effect of the diet. In addition, the regulation of dry matter intake can be mentioned in this context. If the DMI is reduced due to SARA, this leads to a lower production of rumen acids and thus facilitates the return to a physiologically normal pH value. For this reason Oetzel (2017) described SARA as ‘self-limiting’.

It is still to be clarified to which extent genetics are responsible for the development of SARA. Golder et al. (2018), who investigated the interaction between host and metabolome as well as between host and microbiome, provided a first approach to answer this. Despite a relatively small sample size, the authors identified genetic markers and candidate genes for ruminal phenotypes such as the ratio of acetate to propionate in a genome wide association study.

Pathophysiological changes

The ruminal accumulation of VFA and resulting decrease in pH can trigger a cascade of pathophysiological changes that initially affect the microbial ecosystem in the reticulorumen and subsequently also the cow as the host. In the following an overview of the most important pathophysiological changes is given.

The pH value as an important abiotic factor influences the growth and the composition of the ruminal microbiome, which is attributable to different acid tolerances of the ruminal microorganisms (Allison et al., 1997). In this regard, cellulolytic bacteria are more sensitive to acid than amylolytic bacteria, so that a decrease in ruminal pH promotes a shift towards the amylolytic bacteria. At ruminal pH values below 6, a continuous decrease of fiber and total organic matter digestibility can be observed (Hoover, 1986). In addition, diets rich in rapidly fermentable carbohydrates also provide a competitive advantage for amylolytic bacteria over cellulolytic bacteria in terms of the available substrate (Russell and Dombrowski, 1980). Not only can a shift in the composition but also a decrease of the microbial dry matter production per kilogram feed be observed with decreasing pH (Hoover, 1986). Furthermore, the pH affects also the ruminal protein metabolism. On the one hand, a lower fiber digestibility can lead to a reduced access of proteolytic microorganisms to their substrate (Allison et al., 1997). On the other hand, protein degradation and the activity of the microorganisms is pH dependent and has its optimum in the range between pH 5.5 to 7.0 (Allison et al., 1997). Reduced protein degradation in turn means that the supply of ammonia nitrogen, which is a key substrate for microbial protein synthesis in the rumen, is also reduced and lowers microbial growth (Allison et al., 1997; Fuentes et al., 2011).

Accompanied by the altered ruminal milieu and modified microbial composition, changes of the ruminal VFA profile occur. Sutton et al. (2003), for example, observed that lower pH values were accompanied by lower acetate and especially higher propionate concentrations by reducing the forage to concentrate ratio of the diet. Since acetate is an important precursor for the *de novo* synthesis of fatty acids (**FA**) up to a chain length of 16 carbon atoms in the mammary gland (Rook and Balch, 1961), low ratios of acetate to propionate are associated with an overall lower milk fat synthesis (Mertens, 1997). In the case of acute acidosis, even lactate accumulation occurs and has a high influence on the pH due to its strong acidity (Nocek, 1997; Enemark, 2008).

As consequence of SARA inducing diets, a modification of unsaturated FA caused by microbial hydrogenation can be observed, which affects not only the ruminal FA composition, but also the amount of milk fat and the milk FA profile (Bauman and Griinari, 2001). As a cause for milk fat depression the accumulation of C18:1 *trans*-10 (oleic acid) and its rumen precursor C18:2 *trans*-10 *cis*-12 (conjugated linoleic acid) were identified (‘biohydrogenation theory’).

A change in the microbiome is accompanied by a change in the ruminal FA profile, since the individual bacteria differ in their composition. For example, cellulolytic bacteria contain more iso-FA and amylolytic bacteria tend to contain less branched-chain FA and relatively more linear odd-chain FA (Vlaeminck et al., 2006a). This can influence the cow's metabolism up to the synthesis of milk fat and be reflected in a modified milk FA profile (Vlaeminck et al., 2006b; Jing et al., 2018).

As a consequence of the reduced digestibility of fiber and organic matter, the increased appearance of visibly undigested particles in the feces as well as an increased hindgut fermentation is likely (Hall, 2002). Excessive hindgut fermentation can lead to a damage of the epithelium of the large intestine, resulting in mucin and fibrin residues in the feces (Hall, 2002). Consequently, feces with a smoother consistency or even diarrhea are related with SARA (Nordlund et al., 1995).

The previously mentioned VFA accumulation promotes the proliferation of the mucosal tissue of the rumen that can lead to a thickening of the stratum cornea of the rumen mucosa, which is called parakeratosis (Enemark, 2008). Neubauer et al. (2018b), for example, were able to observe an increase of the rumen mucosa thickness as a consequence of an increased proportion of concentrated feed in the diet using transabdominal rumen ultrasound. Parakeratosis can even lead to a reduced absorption of the fermentation end products. In this regard, Krehbiel et al. (1995) observed a reduced ruminal absorption capacity of VFA in lambs up to 6 months after an acidosis induction, which therefore explains delayed consequences of SARA.

Depending on the severity of the acidotic conditions, inflammations of the rumen epithelium (rumenitis) and mucosal lesions can occur. The latter may be the gateway for bacterial endotoxins in form of lipopolysaccharides (**LPS**) and bacteria to the bloodstream (Enemark, 2008; Oetzel, 2017). The LPS originate from gram-negative bacteria and their ruminal enrichment is also promoted by SARA inducing diets that are rich in concentrate (Gozho et al., 2005; Emmanuel et al., 2008). It is assumed that the development mechanism of secondary diseases caused by SARA such as laminitis is mainly based on increased levels of ruminal LPS (Plaizier et al., 2012). In this context, also the rumenitis-hepatic-abscess complex and the caudal vena cava syndrome are mentioned as potential secondary diseases (Nordlund et al., 1995). At higher levels of LPS, the risk for both local and systemic inflammation increases and consequently affects productivity and longevity of cows (Khiaosa-ard and Zebeli, 2018).

Signs of subacute ruminal acidosis

With regard to SARA, a variety of different signs are described, which result from the pathophysiological changes described above and which are recommended as indicators for monitoring purposes. The most important indications can be summarized as follows (Nordlund et al., 1995; Nocek, 1997; Kleen et al., 2003; Enemark, 2008; Oetzel, 2017):

- Increased prevalence of laminitis in the herd
- Altered properties of the feces; loose feces up to diarrhea
- Reduced dry matter intake and inconsistent ingestion behavior
- Reduced body condition although energy intake is high
- Higher culling rate
- Reduced production efficiency
- A decreased milk fat content and thus a reduced milk fat to milk protein ratio

Since SARA only covers a subacute, respectively subclinical condition, the signs are generally weak and occur particularly time-delayed (Nordlund et al., 1995; Nocek, 1997). Since there is a high degree of animal individuality and the signs are more likely to be observed in a group of animals than in individuals, SARA is considered a herd problem (Enemark, 2008). Some signs such as the increased prevalence of laminitis or a higher culling rate are by design herd parameters and are derived by statistical aggregation of the data of all animals in a herd.

The frequency with which specific signs are mentioned in the literature sometimes suggests a higher relevance than can actually be found in empirical studies. An example is the frequently cited symptom ‘diarrhea’. In this regard, it is striking that many citations can be traced back to the work of Nordlund et al. (1995), e.g. in the review articles by Nocek (1997), Kleen et al. (2003) and Enemark (2008). Interestingly, in a later article by Nordlund et al. (2004) it was stated that the evaluation of feces has only a limited value for SARA monitoring. Generally, there are only few studies in the current literature that have examined this aspect in detail. Ireland-Perry and Stallings (1993), for example, were able to determine a significant higher feces score on a scale from 1 = liquid to 4 = firm as a result of an increase in the dietary acid detergent fiber (**ADF**) content from 17 to 25%, but at the same time they found a lower DM content in the feces in the feeding group with the higher ADF content. In line with the former, Schmitz et al. (2018b) also observed significantly higher feces scores on a scale of 1 = liquid to 5 = firm, when feeding a diet in which the energy content of the roughage was reduced by adding wheat straw. In contrast, in a field study in which 27.6% of a total of 196 cows were diagnosed with SARA by the application of rumenocentesis, Tajik et al., (2009) found no association between SARA and the consistency of the feces.

All in all, the signs mentioned are generally unspecific and can be the sequel of various causes. For example, a temporary reduction in feed intake and performance and a consequently overall higher culling rate is not attributable to SARA only but can be a symptom of clinical mastitis (Halasa et al., 2007; Fogsgaard et al., 2012). Also diarrhea is not a specific symptom, as is can be a consequence of spoiled or moldy feed (Hall, 2002). Another example is the fat to protein ratio of the milk. In Germany, a milk fat to milk protein ratio (**FPR**) < 1.0 is regarded as an indicator for a non-sufficient supply of fiber and for SARA (KTBL, 2016). Enemark et al. (2002) and Enemark (2009) have also specified FPR < 1.0 as indicator for SARA. The FPR can be influenced by a variety of factors. On the one hand, the FPR is dependent on the lactation stage and parity, with a higher variance being observed especially in early lactation (Buttchereit et al., 2010). On the other hand, the fat content of the milk and thus the FPR are also feed-dependent and can, for example, be lowered by feeding diets rich in polyunsaturated FA (Harvatine and Allen, 2006a).

Strategies for diagnosis and *in vivo* pH measurements

The diagnosis of SARA is generally difficult due to its subacute state and possible time delayed effects (Enemark, 2008). With regard to diagnosis under field conditions, it is recommended to consider SARA in the differential diagnosis, if at least one of the previously described signs is observed (Nordlund and Garrett, 1994; Enemark, 2008).

In agricultural sciences as well as in veterinary medicine various approaches for diagnosis based on pH measurements in the reticulorumen have been established. The methods differ in the type of measurement (spot sample vs. continuous measurement), the measurement location (reticulum vs. rumen vs. different locations within the rumen), the field of application (field conditions vs. experimental conditions) and also in the choice of thresholds used to define SARA. However, the *in vivo* pH measurement is challenging for several reasons. The pH measurement itself is challenging because the measuring instruments are susceptible to failure and measured values tend to drift randomly after a certain period of time (Penner et al., 2006). Calibration with standardized buffer solution is therefore fundamental and helps to limit measurement errors.

The daily reticuloruminal pH is a measure that is highly variable in the course of the day, where pH fluctuations up to 1.5 pH units can be observed (e.g., Macmillan et al., 2017). Additionally, both farm-specific and animal-individual pH curves can be found, which can be attributed to management-related events such as milking and feeding and to animal-individual variability (Denwood et al., 2018). Furthermore, the pH measurement location plays a decisive role. Generally, a strong association between measurements in the reticulum and the ventral rumen with differences in the daily mean of 0.24 to 0.54 pH units are described (Sato et al., 2012; Falk et al., 2016; Neubauer et al., 2018a). It is assumed that the higher pH in the reticulum is caused by increased mixing of the reticular content with saliva as well as fresh and less fermented feed (Falk et al., 2016). Sato et al., (2012) observed larger pH differences in animals challenged with a SARA inducing diet, with temporary differences up to 0.7 pH units. But even within the rumen, Duffield et al. (2004) determined average deviations up to 0.3 pH, when taking rumen samples in cannulated cows at 4 different locations, differentiating between the cranial-ventral, caudal-ventral, central, and cranial-dorsal rumen. The sampling technique shows an impact on the measured pH as well. Duffield et al. (2004) observed this by comparing spot samples of rumen liquid taken with oro-ruminal tubes according to Geishauser (1993) and samples obtained with rumenocentesis as described by Nordlund and Garrett (1994). The higher pH values of approx. 0.4 pH units for oro-ruminal tubes were attributed to saliva contamination.

One of the first systematic procedures for herd based diagnosis of SARA under field conditions was described by Nordlund and Garrett (1994) and is based on spot samples collected by rumenocentesis. In this method, sampling in a subgroup of the herd is carried out at the time of the expected daily minimum pH, which occurs about 5-8 h post feeding in TMR fed herds. If more than 30% of the samples show pH values ≤ 5.5, the group should be classified as abnormal. This concept has been revised by Garrett et al. (1999), who stated that more than 25% of cases with pH ≤ 5.5 in a sample of 12 animals could indicate whether a herd is at risk or not.

Under experimental conditions, continuous pH measurements with indwelling data loggers in the ventral sac of the rumen are usually conducted as described by Dado and Allen (1993) and Penner et al. (2006). However, this method requires ruminally fistulated animals and therefore cannot be applied under field conditions. The values obtained by continuous pH measurement correspond to complex curves per cow and day, and are most often aggregated to values on a daily basis. These include daily mean pH, daily minimum and maximum pH, daily pH range calculated as difference between maximum and minimum, the standard deviation, the duration where the pH is below a threshold or the area between the pH curve and a threshold (e.g., Keunen et al., 2002; Maekawa et al., 2002; Taylor and Allen, 2005). Further, Alzahal et al. (2007) described a procedure, in which a logistic curve is fitted on the cumulated time with the respective pH below a corresponding pH value, resulting in 2 parameters that reflect the daily mean pH and the daily pH range.

Regarding studies with continuous pH measurements, different thresholds were used. Beauchemin et al. (2003), for example, used pH 5.8 and the daily duration with a pH below 5.8 and the area under the pH curve below 5.8. In comparison, Gozho et al. (2005) and Khafipour et al. (2009) described SARA as the condition when the pH was below 5.6 for more than 180 min/d. Based on studies using continuous pH measurement and experimentally induction of SARA in case-control experiments, Zebeli et al. (2008) derived recommendations for SARA thresholds based on a meta-analysis. They state that in order to minimize the risk of SARA, the daily mean pH in the ventral rumen should not be less than 6.16 and the time with a pH < 5.8 should no longer be than 5.24 h/d.

Due to the association between reticular and ruminal pH values, attempts have been made to define a threshold value for SARA that is applicable to intra-reticular pH measurements with boluses. In this regard, Neubauer et al. (2018a) proposed the threshold 6.0 as indicator for SARA, that corresponds to a pH of 5.8 in the free rumen liquid. However, Falk et al. (2016) concluded that no fixed conversion between reticular and ventral ruminal pH measurements can be determined due to variation between the 2 measurement locations over time.

Impact on economy and animal welfare

The economic impact of SARA is difficult to evaluate. The quantification requires accurate estimates of the prevalence and of the financial loss caused by a SARA case, which are both hard to assess due to the subacute stage and the resulting complexity in diagnosis.

Only few estimates for the economic impact of SARA can be found in the literature. As an example, the frequently quoted, but not peer-reviewed results of Stone (1999) can be referred to. In a case study, the author compared measured pH values and the performance before and after an adjustment of the feeding regime on a dairy farm with 500 cows, which was previously diagnosed as SARA affected using rumenocentesis. After the change in feed, an increase of the pH, an increase of the milk yield by 2.7 kg/d and increase of the milk fat and protein contents by 0.3 and 0.1 percentage points respectively were observed within one month. Based on this, Stone (1999) estimated the apparent cost in form of loss of production profit caused by SARA at about $40 per cow and month. Other consequences, such as laminitis, were not considered so that the loss could be even higher. In another non-peer reviewed publication, van Laarhoven (2012) estimated the costs per cow and year at 210 €.

The number of studies that estimated the prevalence of SARA is also limited. In most studies, pH spot samples were collected using rumenocentesis, with animals diagnosed as SARA affected when the pH was ≤ 5.5 at the time of sampling. In an US field study with a total of 15 farms with Holstein cows, Garrett et al. (1997) determined a prevalence of 19% for cows in the early lactation and 26% for cows in the peak lactation. In another field study with a total of 26 German farms, Kleen et al. (2013) estimated a prevalence of 20%. In both studies the cows received TMR at the time of sampling. In comparison, O’Grady et al. (2008) examined the prevalence of Irish grazing cows and found a prevalence of 11% with a pH ≤ 5.5.

Not only is the economic impact of SARA difficult to quantify, but also the influence of SARA on the well-being and health of the animals. However, it can be assumed that in particular the increased risk and more frequent occurrence of secondary diseases such as laminitis, the rumenitis-hepatic-abscess complex and the caudal vena cava syndrome, would lead to undesirable impairment of health and will compromise animal welfare (Nordlund et al., 1995; Oetzel, 2017). That inadequate feeding leads to obvious short-term health impairments can be deduced from a SARA induction experiment conducted by Colman et al. (2010). In their experiment, in which SARA was induced in 12 cows by gradually increasing the concentrate, the induction had to be stopped before the actual end of the trial, as the animals showed obvious signs of sickness.

Milk composition-based monitoring

The composition of milk and its properties are already considered in routine health monitoring of lactating cows. An important trait is the somatic cell count of the milk, which is considered an auxiliary trait for udder health in routine milk performance testing and has been integrated in the breeding value estimation (vit, 2020). In addition, a reduced lactose content of milk is also discussed as an indicator of mastitis. Berning and Shook (1992), for example, could detect a slight decrease in lactose content with increasing severity of the udder inflammation. Furthermore, an increased electrical conductivity is used as an auxiliary trait to detect mastitis in automatic milking systems (De Mol and Ouweltjes, 2001). The milk fat and the FPR are considered indicators for the metabolic status with regard to the energy balance as well as an undersupply of crude fiber and thus SARA (KTBL, 2016). To assess the energy and protein supply of lactating cows, the protein and urea content of the milk are evaluated (König et al., 2008).

In addition to the main components of milk, the precise composition of milk fat has been increasingly investigated in recent decades. Gross et al. (2011), for example, observed that the energy balance is reflected in the FA profile, where an increasing negative energy balance resulted in lower contents of short- and medium-chain FA with up to 16 carbon atoms and higher contents of long-chain FA and especially C18:1 *cis*-9. With regard to SARA, in particular odd- and branched-chain FA in milk are considered as promising biomarkers, which can be traced back to SARA-related changes in the composition of the ruminal microbiome (Vlaeminck et al., 2006b; Fievez et al., 2012). Van Nespen et al. (2005), for example, determined a changed FA profile of the milk with increased iso C17:0, C15:0, C18:2 *cis*-7 *trans*-11, C18:1 *trans*-10 + C18:1 *trans*-11, and lower contents of iso C14:0 as consequence of an increased starch content of the diet. In individual cows, the authors observed that the changes in the FPR were not as large as for the FA profile, which led them to suggest that SARA is more rapidly reflected in the FA profile than in the FPR. Analyzing the results of 10 different experiments, Vlaeminck et al. (2006) determined in a regression analysis that the content of ruminal propionate is significantly associated with increased C15:0 and C17:0 + C17:1 *cis*-9 as well as lower contents of iso C14:0 and iso C15:0. Interestingly, the authors found an inverse relationship when modeling the acetate content. This is of great importance with regard to SARA, as higher ruminal propionate and lower acetate contents can also be observed in SARA typical diets with a low roughage to concentrate ratio and high amounts of rapidly fermentable carbohydrates (e.g., Sutton et al., 2003). Furthermore, an altered microbial biohydrogenation of long-chain unsaturated FA caused by low pH values can influence not only the milk fat content but also the milk fat composition (Bauman and Griinari, 2001; Dann et al., 2015). The aforementioned studies have in common that the analysis of milk FA was performed using a gas chromatography-based analysis, which is the reference method to measure the milk FA content and is known to provide accurate and detailed information about the milk composition (De Marchi et al., 2014). However, gas chromatography is a costly and labor-intensive method and is not suitable for the analysis of samples on a large scale (Soyeurt et al., 2011).

In the last decades, mid-infrared (**MIR**) spectrometry was established in the dairy industry as routine method for the analysis of milk samples, which is a fast and cost-effective high-throughput phenotyping method (De Marchi et al., 2014). MIR spectrometry is based on the fact that when matter is exposed to electromagnetic light with different wavelengths in the MIR range (2,500-25,000 nm), part of the energy is absorbed and thus the bonds of the molecules start to move. If the amount of energy emitted and the amount of energy absorbed for each wavelength is known, the spectrum resulting from the values of the different wavelengths can be used to draw conclusions about the chemical composition of the sample and correlated traits (De Marchi et al., 2014). As already formulated by Gengler et al. (2016), the MIR spectrum of the milk can therefore be seen as a ‘fingerprint’ of the milk composition. In this respect, milk fat plays a major role as it is the most variable component of the milk, both in terms of concentration and composition (Palmquist, 2006). In addition to the MIR-based prediction of the milk main components (fat, protein, lactose, urea) and individual FA (e.g., Soyeurt et al., 2011), the prediction of complex traits, which do not correspond to direct milk components, but are associated with the milk composition, got into the focus of actual research (Gengler et al., 2016). Examples are the MIR-based prediction of body condition score changes, energy intake, energy balance, the body weight and the methane emission (McParland et al., 2014a; Vanlierde et al., 2015). Gengler et al. (2016) suggested that the direct consideration of the MIR spectrum as predictor for the traits of interest might be advantageous compared to the use of MIR-predicted FA, since this avoids an intermediate step and thus further prediction errors.

Research project and objectives of this dissertation

The present work was done within the project 'Evaluation of Animal Welfare in Dairy Farming – Indicators for the Metabolism and Feeding' (IndiKuh). The project focused on metabolic and digestive disorders in lactating cows and aimed both to validate existing and to develop novel indicators. Furthermore, new monitoring methods and procedures should be developed, which at best can be integrated into livestock evaluation systems and can be applied in agricultural practice on a large scale. The investigations targeted the disorders subclinical ketosis and SARA, which both can impair animal welfare and economy, particularly in early lactating cows.

Due to the complexity and scope of the research questions, the project was carried out in cooperation with several project partners: the Ruminant Nutrition group and Animal Breeding and Genetics group of the Department of Animal Sciences at the University of Goettingen (Germany), the Institute for Animal Nutrition of the Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health, Brunswick, Germany), the Chamber of Agriculture of Lower Saxony (Oldenburg, Germany), the Vereinigte Informationssysteme Tierhaltung w.V. (vit, Verden, Germany) and the Landeskontrollverband Weser-Ems e.V. (Leer, Germany). Additionally, a working group of the Agriculture, Bio-Engineering and Chemistry Department (Gembloux Agro-Bio Tech, University of Liège, Gembloux, Belgium) and the Walloon Agricultural Research Center (CRA-W, Valorisation of Agricultural Products Department, Gembloux, Belgium) were involved.

Within the framework of the research project, 2 comprehensive data sets were collected: one was compiled at the experimental station of the Friedrich-Loeffler-Institut (FLI) in Brunswick, Germany and the other data set was collected under field conditions on 10 commercial farms in the northwest of Lower Saxony, Germany. Besides the recording of feed, blood and milk samples, in particular advanced automated sensor based data-acquisition systems were used in both data collections. This included, for example, the continuous *in vivo* measurement of the reticular pH and of chewing behavior with noseband sensor halters. The project stands out due to the evaluation of milk MIR spectral data and MIR predicted FA profiles of the milk as indicator for subclinical ketosis and SARA.

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This dissertation focuses on the statistical modeling of SARA-associated traits. The resulting papers can be found in the next 4 chapters and the objectives can be summarized as follows:

Chapter 2: The aim is to investigate the associations between pH parameters of the ventral rumen with various milk and diet parameters in an explorative meta-analysis. Further, the considered parameters are examined both individually and together in multiple multi-level meta-regression models for their suitability as indicator for ruminal pH traits.

Chapter 3: In order to gain deeper insights in the underlying causality of daily pH development, high-resolution sensor data of the animal-individual feed and water intake as well as the rumination behavior are used to model continuously recorded pH data both in the reticulum and in the ventral rumen with time series analysis methods.

Chapter 4: Based on the data set collected in 10 commercial farms, a statistical procedure for a multivariate plausibility assessment is developed to ensure the data quality and to prepare the data set for downstream analyses. It is aimed to distinguish simultaneously recorded data between 'physiologically normal', 'physiologically extreme' and 'implausible' observations in an automated way.

Chapter 5: Using the plausibility checked data set, a novel indicator trait for SARA is developed based on a combination of several traits from different data acquisition systems – the ‘SARA risk score’ (**SRS**). After examination of associations to the feed composition, blood metabolites, performance data and the fine milk composition, milk MIR spectra-based partial least squares regression models are established for the SRS and the other traits, which are derived from sensor data. Finally, the MIR-based prediction equation for the SRS is applied to test day records of the 10 herds in order to study the association between the MIR predicted SRS and the milk FA profile.

In the last chapter of this work (**Chapter 6**), the achieved results are discussed from various perspectives and are partly supplemented with further results. Finally, an outlook on future research is given and the main conclusions are summarized.

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Chapter 2

Statistical modeling of ruminal pH parameters from dairy cows based on a meta-analysis

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ABSTRACT

Adequate feeding of high-performance dairy cows is extremely important to avoid the digestive disorder subacute ruminal acidosis (**SARA**). SARA is defined as a status with a below-average ruminal pH that does not cause direct clinical symptoms at the individual level but is relevant for animal welfare due to a higher risk of secondary health problems at the herd level. The main objective of this study was to apply meta-analytical methods in an exploratory approach to investigate the association between pH parameters of the ventral rumen with milk and diet parameters. Data from 32 studies using continuous pH measurement in the ventral rumen of lactating cows were included in the meta-analysis. Available information extracted from all studies was categorized into management, cow, diet, milk and pH associated parameters. The statistical analysis was divided into 4 sections. First, a multiple imputation procedure based on a principal component model was applied, since approximately 19% of the data set consisted of missing values due to heterogeneity in provided information between the studies included in the analysis. In a second step, all potential predictors for the pH parameters, including the daily mean pH, the time with a pH below 5.8 and the pH range, were examined for their prediction suitability using multi-level mixed effects meta-regression models. These analyses were performed on the raw and on the imputed data. Since the results of both approaches were consistent, the imputing procedure was considered to be appropriate. Thirdly, automated variable selection was applied to all 3 pH parameters separately for the predictor groups milk and diet using the imputed data set. Thereby, multi-model inference was used to estimate the relative importance of the selected variables. Finally, a functional relationship between the 3 pH parameters was established. The fat to protein ratio of milk, milk fat, and milk protein showed significant associations in meta-regression analysis for all 3 pH parameters when used as a single predictor. Out of the group of diet specific variables, the ADF, NDF, NFC, starch content, as well as the forage to concentrate ratio showed the highest significance in the models. In particular, the multi-model inference showed that the protein, fat and lactose content of the milk can best quantify the association to the daily mean pH and the time with a pH below 5.8 in a multiple regression model.

Key words: Ruminal pH, SARA, meta-regression, statistical modeling.

INTRODUCTION

Adequate nutrition is a major challenge in milk production, where a compromise between sufficient energy and fiber supply is essential. High-performance dairy cows require high energy feed, typically based on carbohydrates from grain. However, this increases the risk for the digestive disorder SARA, which is often found in high yielding herds (Nocek, 1997). The risk for the clinical manifestation of this digestive disorder is increased especially in the first month of lactation (Gröhn and Bruss, 1990). The subacute form of ruminal acidosis shows no or no distinctive individual clinical signs and symptoms on the individual level, but impairs animal welfare in the long run and can cause economic losses (Nocek, 1997). The main reasons are associations with various clinical symptoms at the herd level, including reduced feed intake, lower milk production efficiency, a higher risk for diarrhea and hoof diseases, as well as an overall higher culling rate (Nocek, 1997; Kleen et al., 2003). Therefore, Enemark (2008) claimed SARA to be the most important nutritional disorder of dairy cattle. Denwood et al. (2018) found that reticular pH profiles are farm as well as animal specific and follow a predictable pattern in the course of the day; deviations from these specific profiles could be associated with reduced productivity. Bramley et al. (2008), who clustered observations from a total of 100 farms into 3 categories based on rumenocentesis pH, VFA and ammonia concentrations, could not detect a decrease in milk yield in the category with the lowest pH values, but identified significant lower milk fat contents and lower milk fat to milk protein ratios (**FPR**).

The direct indicator for SARA is generally the ruminal pH, which is associated with fermentation properties (Enemark et al., 2002). A drop in pH is primarily the result of an accumulation of short chain fatty acids (**SCFA**) due to a high amount of fermentable carbohydrates (Nocek, 1997; NRC, 2001). Associations between changes in the feeding regime and the ruminal pH have been known for a long time and were already determined by Johnson and Sutton (1968) using a continuous pH measurement in the ventral rumen of dairy cows. In addition, Fulton et al. (1979) observed adapting feeding behavior and subsequent pH changes on steers depending on alterations in diet composition.

Genetics and thus animal individuality can also play an important role in an animal's susceptibility to SARA. For example Golder et al. (2018), who worked with acidosis challenged dairy heifers, observed large between-animal variance with regards to clinical signs of acidosis, rumen characteristics and the ruminal microbiome. In a genome-wide association study, the authors were able to identify genetic markers and candidate genes for ruminal phenotypes such as the ratio of acetate to propionate. Therefore, one could hypothesize that the individual ruminal absorption capacity of fermentation products can vary between animals when regulated by genetic make-up and thus influence ruminal pH development.

The major challenge in diagnosing this digestive disorder is to identify a precise phenotype for SARA. In previous studies, pH thresholds were defined as an indication of SARA, but these thresholds vary among studies. Zebeli et al. (2008) stated that it seems to be a “matter of individual choice” (page 2047) to define a pH threshold for SARA. Consequently, there is no widely accepted description of a phenotype for SARA.

In a meta-analysis, Zebeli et al. (2008) related the ruminal daily mean pH ($\overline{\mathbf{pH}}$) to the squared physically effective NDF retained on a 1.18 mm sieve (**peNDF>1.18**) and negatively to ruminally degradable starch from grain and DMI. Additionally, they derived thresholds for $\overline{\mathrm{pH}}$ and the average daily time with pH<5.8 (**TpH<5.8**) from studies of cattle challenged with SARA. It was concluded that $\overline{\mathrm{pH}}$ should be higher than 6.16 and TpH<5.8 should not exceed 5.24 h/d in order to decrease the incidence probability of SARA. These thresholds are predominantly used in agricultural science, due to the fact that the measurement of ventral ruminal pH requires expensive technical equipment and ruminally fistulated cows. Other studies investigating the prevalence of SARA on a farm-level have used a pH of 5.5 as threshold to define SARA by applying a non-continuously pH measurement via rumenocentesis (Nordlund et al., 1995; Garrett et al., 1999). In agricultural practice, the FPR with values <1.0 is also commonly used as an indicator for SARA (Enemark et al., 2002). The protein and fat content of milk depend on lactation stage (Stanton et al., 1992), which influences the FPR (Buttchereit et al., 2010); thus limiting the use of a fixed FPR threshold as an indicator for SARA at the individual level. Further, milk fat content can be significantly reduced by dietary intake of PUFA independently of any effect on ruminal pH (Harvatine and Allen, 2006a; b).

The main objective of this study was to investigate the association between ruminal pH parameters of the ventral rumen of fistulated cows, and milk and diet specific parameters through an exploratory meta-analysis approach. The focus was on milk specific traits in order to determine better indicators for SARA.

MATERIALS AND METHODS

Search for Published Literature

A comprehensive literature search was carried out to identify studies characterized by automated and continuous data acquisition of the pH in the ventral rumen of lactating dairy cows. Studies were of relevance if either different diets were fed or if the frequency of feed offered was varied using the same diet.

Within the database query the search terms ‘ruminal pH’, ‘continuous measurement’, ‘acquisition system’, ‘subacute ruminal acidosis’, ‘SARA’, ‘lactating cows’, ‘dairy cows’ as well as combinations of these were used. The literature search was conducted using 2 search engines, Google Scholar (http://scholar.google.com/) and ISI Web of Science (http://apps.webofknowledge.com). Furthermore, the search function on the websites of *Journal of Dairy Science* (http://www.journalofdairyscience.org/) and *Animal* (https://www.cambridge.org/core/journals/animal) were used. The references of other meta-analyses that were carried out in the same research field in the past were another important source, for example the studies of Zebeli et al. (2008) and White et al. (2017a; b). Journal articles already identified and selected for meta-analysis were examined for further references if titles indicated the same research topic. Only studies published in English were considered. The process of data collection is visualized in **Figure 2.1** using a PRISMA flow chart according to Moher et al. (2009). The flow chart was created using the yEd graph editor (yWorks GmbH, 2019).

Inclusion and Exclusion Criteria

The selection of studies was based on pre-defined criteria. In total, 148 studies were identified. First, they were screened to see if the continuous measurement of pH and dairy cows was the subject of investigation. Studies with discontinuous pH measurement were excluded because a very broad spectrum of methods is available, consisting of various sampling techniques, sample times and frequencies or different sample locations in the rumen. These diverse methods could undoubtedly have an effect on the measured pH values, as already described by Lane et al. (1968) in the case of different sampling locations in the rumen. Previous meta-analyses, for example by Zebeli et al. (2008) and White et al. (2017a; b), considered both continuous and discontinuous pH measurements. Due to the aforementioned heterogeneity of the methods used for discontinuous measurements, this approach bears the risk of distorting the results. Therefore, the focus in this study was exclusively on studies with continuous pH measurements for at least 24 h in the ventral rumen of fistulated lactating cows. Additionally, studies were excluded if other species (sheep, goats) or males (steers) were examined or if the pH was measured in the reticulum. After the initial screening, 43 remaining studies were assessed for eligibility.

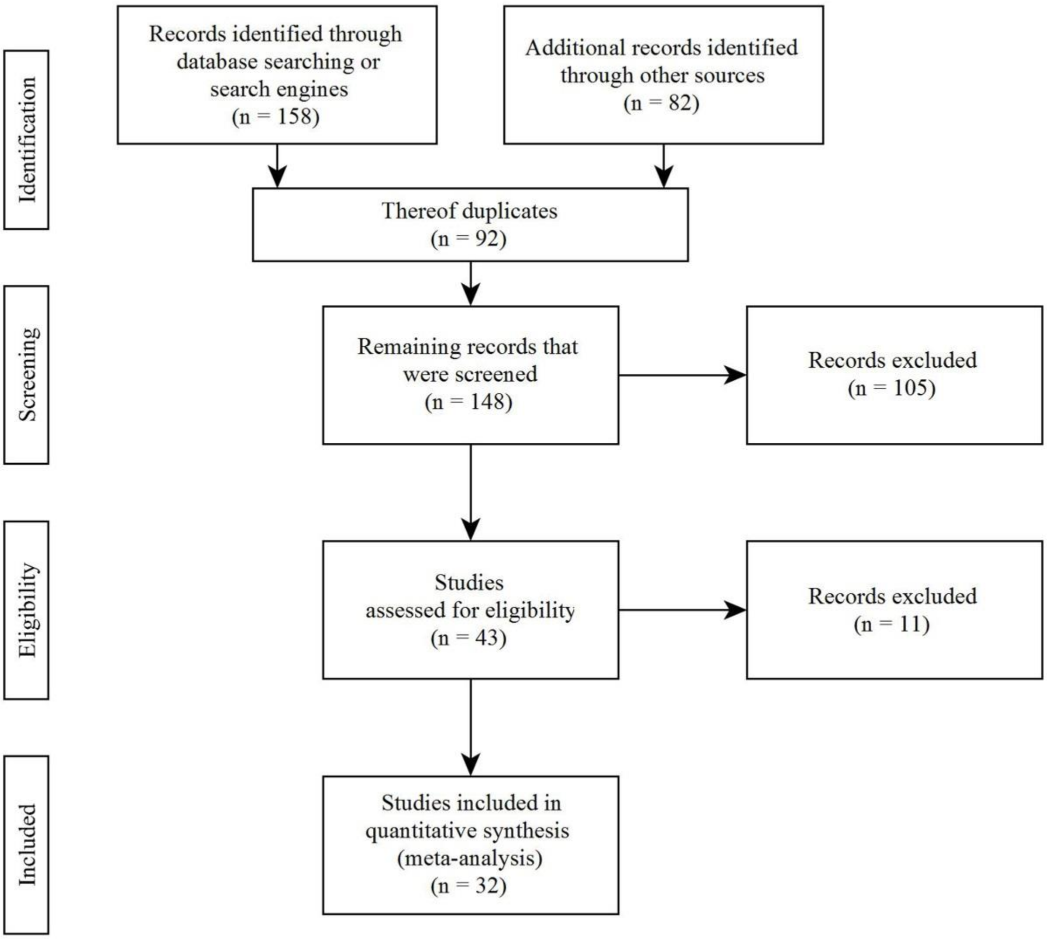

Figure 2.1: PRISMA flow chart according to Moher et al. (2009) describing the systematic search for published literature.

In the following step, studies were excluded from further analysis if the diet was supplemented with PUFA due to the fact that such treatments have been shown to cause a significant milk fat depression which could create a statistic bias (Bauman and Griinari, 2003; Harvatine and Allen, 2006a). Another exclusion criterion was the use of fermentation modifiers such as Monensin and Fermenten or the dietary supplementation of essential oils, fibrolytic enzymes and sodium bicarbonate in at least one treatment of a study in order to reduce potential bias. Furthermore, studies were excluded if the statistical analysis was contestable or the statistics relevant for the meta-analysis were not reported. The study by Yang et al. (2001) can be cited as an example, since the analysis was not based on a mixed effect model and results were only reported on a main effect basis. The final data set contains data from 32 experiments published between 2000 and 2017 (**Table 2.1**).

The experimental design of most included studies can be characterized by a single (n = 4) or replicated (n = 21) Latin squares design. Additionally, 3 cross-over and 4 case control studies were considered. Holstein cows were used in all experiments.

Table 2.1: Studies used in the meta-analysis

AlZahal et al., 2007
AlZahal et al., 2009
Beauchemin et al., 2003
Beauchemin and Yang, 2005
Bhandari et al., 2008
Chibisa et al., 2015
Dann et al., 2014
Dann et al., 2015
Farmer et al., 2014
Hassanat et al., 2013
Jiang et al., 2017
Krause et al., 2002a; b
Krause and Combs, 2003
Krause et al., 2003
Longuski et al., 2009
Macmillan et al., 2017
Maekawa et al., 2002a; b
Oba and Allen, 2000a; b
Penner and Oba, 2009
Rustomo et al., 2006a
Rustomo et al., 2006b
Silveira et al., 2007
Sullivan et al., 2012
Sun and Oba, 2014
Taylor and Allen, 2005a; b; c
Voelker and Allen, 2003a; b
Yang and Beauchemin, 2006a; b
Yang and Beauchemin, 2006c
Yang and Beauchemin, 2007a; b
Yang and Beauchemin, 2009
Zhang et al., 2010b
Zhang et al., 2010a

Variables Extracted for Meta-Analysis

In addition to the usual reported $\overline{\text{pH}}$ in studies with continuous pH measurement, TpH<5.8, the minimum and maximum pH, as well as the pH range (**ΔpH**) were collected. The minimum and maximum pH, however, were used to estimate ΔpH if it was not provided directly. The standard error of the mean (**SEM)** was recorded for all of the pH variables. The SEM is needed to determine the sample variance of the treatment means in order to weigh the treatments within the statistical modeling, as customary in a meta-analysis (see chapter Multi-Level Meta-Regression).

Furthermore, information on the different treatments, which in most studies corresponds to different diets, was collected. This includes energy content, forage and concentrate content, as well as other results of feed analyses, such as DM, CP, ether extract (**EE**), starch, NDF, ADF, peNDF>1.18, peNDF>8, and NFC from the offered diets. Daily feeding frequency (**DFF**) was also captured as a management characteristic. In addition, information about the milk yield and the milk ingredients fat, protein, lactose, and MUN was collected from the studies.

The DMI was recorded as a cow specific parameter. Other animal specific variables like body weight (BW) and the lactation stage represented by days in milk (DIM) were initially discussed. However, the estimated treatment means refer to the entire experimental period lasting approximately 3 months. Such a wide range of the lactation period makes DIM unsuitable for meta-analytical approaches. Even though the lactation stage is very important and significantly influences the DMI, milk yield, milk protein, milk fat, and FPR, its applicability in a meta-analytical approach is limited. Since the BW is currently not routinely measured on commercial farms, it was not taken into account.

Data Preparation and Calculations

During data extraction, it became apparent that reported information was heterogeneous and that not all studies reported the same parameters. This also includes the fact that some studies reported the same variables in different measurement units which therefore had to be standardized. For example, some reported TpH<5.8 in h/d (e.g., AlZahal et al., 2008), others in min/d (e.g., Jiang et al., 2017). This was unified by converting TpH<5.8 and its SEM to h/d. If studies provided average daily time with pH below 5.6 and 6.0, TpH<5.8 and its SEM was approximated by averaging these values (e.g., Bhandari et al., 2008). The difference between the minimum and maximum pH (ΔpH) was also calculated (e.g., Beauchemin and Yang, 2005a). In such cases, the SEM was estimated as the mean of the SEM from the minimum and maximum pH.

Information on diet composition was used to calculate % forage content, if it was not reported directly (e.g., Zhang et al., 2010a). The forage to concentrate ratio (**F:C**) was calculated based on the percentage of forage and concentrate in the diet by dividing the forage by the concentrate content. If peNDF>1.18 or peNDF>8 were not reported directly, they were computed by multiplying the proportions of dry matter >1.18 and >8 mm and the NDF content of the offered diet. In such cases, the particle size distributions were measured with a Penn State Particle Separator (e.g., Silveira et al., 2007) or a Analysette 3 (Fritsch, Oberstein, Germany) vertical oscillating sieving machine (Beauchemin et al., 2003).

In studies in which only the NE_L intake per day was given instead of the NE_L content of the diet, the NE_L content was calculated by dividing the total energy intake by the dry matter intake (e.g., Voelker and Allen, 2003a; b).

Another variable of interest was the FPR, which was calculated by dividing the milk fat content by the milk protein content. Additionally, the energy corrected milk yield (**ECM**) was calculated after imputation using the formula from (Kirchgessner, 1987)

$$\mathrm{ECM}\left[\mathrm{kg/d}\right] = \mathrm{milk[kg/d]} \times \frac{0.38 \cdot \mathrm{fat}\left[\%\right] + 0.21 \times \mathrm{protein}\left[\%\right] + 1.05}{3.28} \qquad [2.1]$$

A special case was the study of AlZahal et al. (2009), where 2 treatments were used in a case-control study, but the results were given on a weekly basis over a period of 4 weeks. For this reason, the mean value of all observations was determined for each treatment, so that only 2 instead of 8 observations were considered from this study. Due to this subsequent averaging, the SEM had to be divided by $\sqrt{4}$ because of the quadruplication of n.

Further, Rustomo et al. (2006a), Krause et al. (2002a; b, 2003) and Krause and Combs (2003) provided the standard error of the differences (**SED**) instead of the SEM for the estimated treatment means of the pH parameters. Therefore, the SEM was back-calculated by dividing the SED by $\sqrt{2}$ (Roman-Garcia et al., 2016).

Three further variables had to be generated in order to consider the hierarchical data structure in the meta-analytical evaluation: a variable that records the overall affiliation of the individual treatments (study), a variable for a sub-cluster structure (sub-study) if the animals were divided into 2 groups in case control studies and a unique identifier (id) for each individual treatment mean.

After data collection, all metric variables were first graphically examined using box plots to identify potential extreme values or outliers. In this respect, 2 studies showed conspicuous values. One of these is the study by Krause et al. (2002a; b), where non-physiological lactose contents between 2.0 and 2.1% were reported. In the second study, diets with EE > 7% DM were fed (Hassanat et al., 2013). In order to minimize the probability of bias, the values of the respective affected variable were removed in both studies.

In total, 23 variables were captured from the studies, which can be categorized into 5 groups: Management, cow, diet, milk, and pH parameters. **Table 2.2** gives an overview with a statistical description of all groups and the variables contained.

Statistical Analysis

Multiple Imputation Based on a Principal Component Analysis Model. Due to the fact that the metabolism of dairy cows is a complex system, it can be assumed that several variables are required to build a statistical model capable to predict complex parameters such as $\overline{\mathrm{pH}}$, TpH<5.8, and ΔpH. Missing values considerably restrict the analysis when using multiple regression models, since these models only work with complete observations of all variables in the model. An overview of missing values of the present data set for all 23 variables is given in **Figure 2.2** by showing a modified matrix plot, which was created with the *matrixplot* function from the VIM (Templ et al., 2019) package. Even though the selection of variables focused on those that were provided by most of the studies, about 19% of the data across the 23 variables was missing. Variables without missing values were the frequency of daily feed offers, DMI, F:C, CP, NDF and $\overline{\mathrm{pH}}$. White et al. (2017a; b) reported on the problem of missing values in their meta-analysis and solved it by assuming tabular values reported by the National Animal Nutrition Programme for unavailable information on chemical components of the diet before calculating the nutrient composition of the entire diet. In contrast to their approach, a multiple imputing procedure based on a multivariate principal component analysis (**PCA**) model using the *MIPCA* function from the missMDA (Husson et al., 2019) package in R (R Core Team, 2018) was used in this study. The theory behind it has been described in detail by Josse and Husson (2016). Before the multiple imputation was performed, the number of main components had to be determined via cross validation using the *estim_ncpPCA* function from the same package. In the MIPCA procedure 100 imputed data sets were created using the bootstrap method. After imputing, the FPR and the ECM were recalculated for the imputed observations.

Table 2.2: Statistical description of the data set

	Statistics[2]						
Variable[1]	k	n	$\overline{x}$	SD	x_{min}	x_{max}	NA [%]
Management							
DFF, 1/d	32	112	1.87	0.78	1	3	-
Cow							
DMI, kg DM/d	32	112	23.2	2.9	16.7	29.8	-
Diet							
NE_L, Mcal/kg DM	24	85	1.63	0.08	1.48	1.83	24.1
F:C	32	112	0.93	0.44	0.42	2.88	-
DM, %	30	105	54.3	9.4	35.4	77.7	6.3
CP, % DM	32	112	17.9	1.4	15.2	21.8	-
EE, % DM	15	48	3.5	1.0	1.8	4.8	57.1
starch, % DM	30	106	27.1	6.6	12.6	41.2	5.4
NDF, % DM	32	112	32.2	5.1	23.2	44.0	-
ADF, % DM	22	78	19.9	4.4	11.5	30.3	30.4
peNDF>1.18, % DM	11	40	27.9	5.6	18.6	41.2	64.3
peNDF>8, % DM	14	49	15.6	5.3	7.9	26.6	56.3
NFC, % DM	18	57	37.0	4.8	27.9	48.6	49.1
Milk							
milk, kg/d	30	107	35.3	6.6	21.8	51.6	4.5
ECM, kg/d	30	107	32.9	5.6	21.6	48.6	4.5
fat, %	30	107	3.55	0.36	2.43	4.42	4.5
protein, %	30	107	3.19	0.21	2.77	3.89	4.5
FPR, %/%	30	107	1.11	0.10	0.86	1.39	4.5
lactose, %	28	99	4.70	0.19	4.30	5.16	11.6
MUN, mg/dL	13	43	14.2	2.4	10.2	21.3	61.6
pH							
$\overline{pH}$	32	112	6.02	0.21	5.49	6.56	-
TpH<5.8, h/d	28	97	7.37	4.18	0.76	17.60	13.4
ΔpH	24	80	1.21	0.20	0.78	1.78	28.6

[1] DFF = daily feeding frequency; F:C = forage to concentrate ratio; EE = ether extract; FPR = fat to protein ratio; peNDF>1.18 and peNDF>8 = physically effective NDF retained on a 1.18- and 8 mm sieve, respectively; $\overline{pH}$ = daily mean ruminal pH; TpH<5.8 = average daily time with pH<5.8; ΔpH = pH range.

[2] NA = not available, missing values; k = number of studies; n = number of treatment means; SD = standard deviation; $\overline{x}$ = arithmetic mean; x_{min} = minimum; x_{max} = maximum.

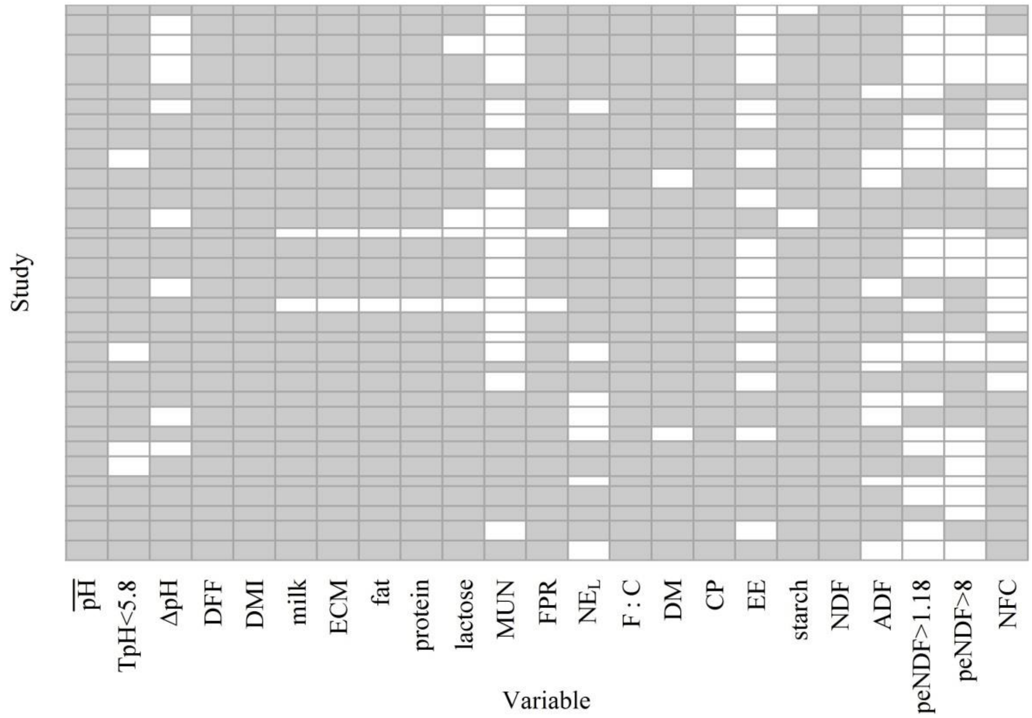

Figure 2.2: Visualization of data availability with a matrix plot (Templ et al., 2019). Gray rectangles show available data and white rectangles indicate missing values. The height of each rectangle is proportional to the number of treatment means of the respective study. DFF = daily feeding frequency; EE = ether extract, F:C = forage to concentrate ratio; FPR = fat to protein ratio; peNDF>1.18 and peNDF>8 = physically effective NDF retained on a 1.18- and 8 mm sieve, respectively; $\overline{pH}$ = daily mean ruminal pH; TpH<5.8 = average daily time with pH<5.8; ΔpH = pH range.

The uncertainty of prediction can be derived based on the variance between the imputed values. In addition, the suitability of the imputations was examined in 2 ways. Firstly, the distributions of the continuous variables before and after imputing were compared visually and with a 2-sample Kolmogorov-Smirnov test using the *ks.test* function in R (R Core Team, 2018). Thereby, a test was carried out to determine whether each of the 2 samples are subject to the same continuous distribution. Secondly, the associations between the 3 pH parameters and all individual potential predictors were analyzed using a multi-level meta-regression model for the raw incomplete data set as well as for the imputed data set. Then, the estimates of the regression coefficients of all models were compared for the 2 data sets.

Since the TpH<5.8 and ΔpH consisted of 13.4 and 28.6% of missing values, their SEM were estimated using the arithmetic mean of the recorded SEM of non-missing observations.

Multi-level Meta-Regression. The analysis with meta-regression models was performed using the *rma.mv* function of the metafor (Viechtbauer, 2019a) package in R (R Core Team, 2018). The multi-level mixed effect meta-regression models can be described as

$$
\begin{aligned}
y_{ijk} = \beta_0 + \beta_1 x_{ijk,\,1} + \beta_2 x_{ijk,\,2} + \ldots + \text{study}_k + \left(\text{study} \times \text{sub-study}\right)_{jk} \\
+ \left(\text{study} \times \text{sub-study} \times \text{id}\right)_{ijk} + e_{ijk}
\end{aligned}
\qquad [2.2]
$$

where y_{ijk} is the effect size of the i-th treatment id, hierarchically nested in a sub-study j which itself is nested in study k. This nesting structure is taken into account via the interaction terms $\left(\text{study} \times \text{sub-study}\right)_{jk}$ and $\left(\text{study} \times \text{sub-study} \times \text{id}\right)_{ijk}$. Concerning the fixed regression coefficients, β_0 corresponds to the intercept and $\beta_1, \ldots, \beta_m$ to the regression coefficients of the explanatory variables $x_{ijk,\,1}, \ldots, x_{ijk,\,m}$. It was assumed that the random effects are independent and identically distributed to a normal distribution within $\text{study}_k \sim N\left(0,\ \sigma_1^2\right)$, $\left(\text{study} \times \text{sub-study}\right)_{jk} \sim N\left(0,\ \sigma_2^2\right)$, $\left(\text{study} \times \text{sub-study} \times \text{id}\right)_{ijk} \sim N\left(0,\ \sigma_3^2\right)$ and $e_{ijk} \sim N\left(0,\ v_i\right)$. In this meta-analytical approach, the sampling variance v_i is assumed to be known and corresponds to the squared SEM of each effect size, i.e. the treatment mean of the pH parameter. The σ_1^2, σ_2^2 and σ_3^2 correspond to the variances of the random effects, which have to be estimated. In meta-analysis, the individual observations are usually weighted by their reciprocal of the sampling variance (Borenstein et al., 2009; Schwarzer et al., 2015; Viechtbauer, 2019a). Thus, treatments with a lower SEM get a higher weight in the regression analysis, resulting in a higher robustness of estimated regression coefficients.

After fitting the models, robust cluster-based estimated values for the SE as well as the *P*-values of the regression coefficients were estimated using the *robust* function from the metafor (Viechtbauer, 2019a) package. Hence, the respective study affiliation was considered for the clusters.

Furthermore, the estimated variance components were used to quantify how much of the variance not captured by the fixed effects is due to heterogeneity. This was done by calculating the generalized overall heterogeneity I^2 as well as considering the between-study heterogeneity I_{bs}^2 according to Viechtbauer (2019b) as follows:

$$
I^2[\%] = 100 \times \frac{\sigma_1^2 + \sigma_2^2 + \sigma_3^2}{\sigma_1^2 + \sigma_2^2 + \sigma_3^2 + \dfrac{k - p}{\operatorname{tr}\left(P\right)}} \quad \text{and} \quad I_{bs}^2[\%] = 100 \cdot \frac{\sigma_1^2}{\sigma_1^2 + \sigma_2^2 + \sigma_3^2 + \dfrac{k - p}{\operatorname{tr}\left(P\right)}} \qquad [2.3]
$$

Here, σ_1^2, σ_2^2 and σ_3^2 are variance components corresponding to the model in **Equation 2.2**, k is the number of treatment means, p is the number of fixed effects in the model and $\mathrm{tr}(\mathrm{P})$ the trace of P. Thus, P had to be determined using the following equation:

$$\mathrm{P} = \mathbf{W} - \mathbf{WX}(\mathbf{X'WX})^{-1}\mathbf{X'W} \qquad [2.4]$$

where **W** is a diagonal matrix with the reciprocal sampling variances ($1/v_\mathrm{i}$) on the diagonal and **X** is the model matrix of fixed effects. I^2 can range from 0 to 100%, where I^2 = 25% corresponds to a low, I^2 = 50% to a moderate and I^2 = 75% to a high heterogeneity (Higgins et al., 2003).

Variable Selection and Multi-Model Inferenc**e.** To identify the subset of variables that best quantifies the pH parameters, automated model selection was performed using the *dredge* function from the MuMIn (Barton, 2019) package in combination with the *rma.mv* function from the metafor (Viechtbauer, 2019a) package separately on milk and diet parameters. Since the *dredge* function by default is not able to handle rma.mv objects, the cooperation of these 2 packages had to be activated as described by Viechtbauer (2019c). When using the *dredge* function, sub-models are selected in the form of combinations of the fixed effects terms of the full model. This model selection can be restricted by optional rules. Besides the intercept, it was decided to limit the number of parameters to a maximum of 4 regression coefficients to achieve a reasonable and robust model. This is in accordance with Borenstein et al. (2009), who recommended one covariate per 10 studies in meta-regression. In addition to limiting the maximum number of parameters, dependency chains were used in the *dredge* function to ensure that only subset models containing both fat and protein were considered whenever FPR is included in a model.

In a further step, multi-model inference was used to determine the relative importance of the variables used for variable selection. This approach is based on the assumption that not only one specific model can describe the relationships of interest, but that all models found in the course of variable selection are suitable with a certain probability. This probability is determined by the Akaike weights, which are derived from the fit statistics of the entire set of models (Burnham and Anderson, 2002). As a fit statistic, the second-order Akaike information criterion (**AICc**) for small samples was used as recommended by Burnham and Anderson (2002) if n/K < 40, where n is the number of observations and K is the number of parameters. The resulting importance of the individual variables was calculated using the *importance* function from the MuMIn (Barton, 2019) package. In automated variable selection only linear regression effects were considered, given that the full model contained always the DFF as well as the DMI.

Relationship between $\overline{pH}$, TpH<5.8 and ΔpH. The relationship between $\overline{\mathrm{pH}}$, TpH<5.8, and ΔpH, which are all dependent on the daily pH development, was also studied. In the literature, observed pH curves are characterized by a serrated sine-curve progression (Johnson and Sutton, 1968; Maekawa et al., 2002a). On the one hand, the daily pH development can be associated with the individual feed intake behavior and the DFF. Le Liboux and Peyraud (1999), for example, observed a significantly ($P < 0.05$) smaller ΔpH if the DFF was increased from 2 to 6. On the other hand, $\overline{\mathrm{pH}}$ and TpH<5.8 can be associated with the dietary roughage level, and thus the energy content as well as the content of easily fermentable carbohydrates, as shown by Jiang et al. (2017).

Subsequently, the diurnal fluctuation of ruminal pH was abstracted as a periodic triangle wave. As visualized in **Figure 2.3**, an equation can be derived in order to compute the TpH<5.8 as a function of $\overline{\mathrm{pH}}$ and ΔpH. Using the theorem of intersecting lines, the marked length of time (x), which corresponds to half of the TpH<5.8, can be described as:

$$x = 12\ \mathrm{h} \cdot \left(1 - \frac{\overline{\mathrm{pH}} + 0.5\Delta\mathrm{pH} - 5.8}{\Delta\mathrm{pH}}\right) \tag{2.5}$$

Assuming a maximum ΔpH of 2 pH units and considering the pH values between 5.0 and 7.0, the TpH<5.8 can be computed as follows:

$$\begin{aligned} &\mathrm{TpH{<}5.8}\left(\overline{\mathrm{pH}},\ \Delta\mathrm{pH}\right) = 24\ \mathrm{h} \cdot \left(1 - \frac{\overline{\mathrm{pH}} + 0.5\Delta\mathrm{pH} - 5.8}{\Delta\mathrm{pH}}\right) \text{ with } \mathrm{TpH{<}5.8} \in [0;\ 24], \\ &\qquad \overline{\mathrm{pH}} \in [5 + 0.5\Delta\mathrm{pH};\ 7 - 0.5\Delta\mathrm{pH}], \text{ and} \\ &\qquad \Delta\mathrm{pH} \in [0.5;\ 2.0] \end{aligned} \tag{2.6}$$

Based on this equation, all TpH<5.8 depending on plausible $\overline{\mathrm{pH}}$ and ΔpH combinations were calculated. For example, if the ΔpH is 0.5 pH units, $\overline{\mathrm{pH}}$ can only lie between 5.25 and 6.75, and if ΔpH is 2 pH units, $\overline{\mathrm{pH}}$ can only be 6.0. Finally, the range of predicted values was compared to actual observations.

With the exception of **Figure 2.1**, **Figure 2.2** and **Figure 2.4**, all figures were programmed using the R (R Core Team, 2018) basis functions.

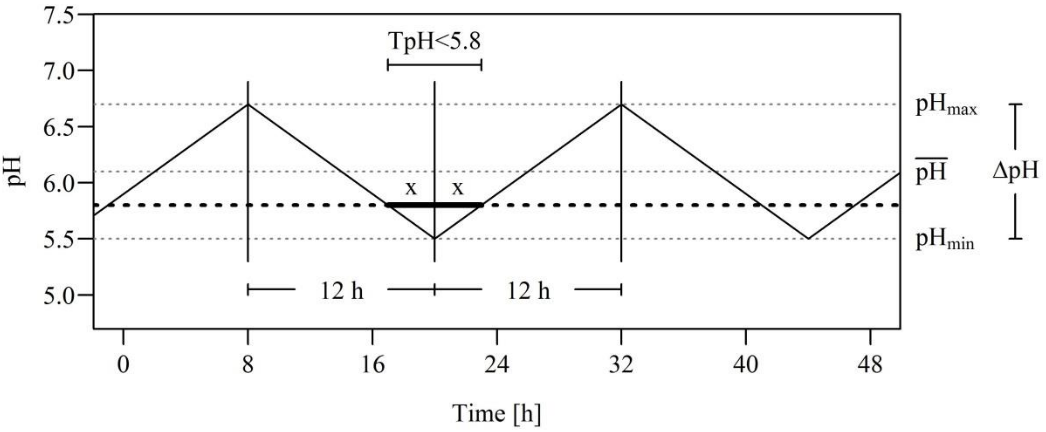

Figure 2.3: Schematic development of the ruminal pH value during the course of the day. $\overline{pH}$ = daily mean ruminal pH; pH_{min} and pH_{max} = minimum and maximum pH; TpH<5.8 = average daily time with pH<5.8; ΔpH = pH range.

RESULTS AND DISCUSSION

Multiple Imputation Based on a Principal Component Analysis Model

Applying the *estim_ncpPCA* (Husson et al., 2019) function on the raw data set, 3 principal components were determined by graphically inspecting the mean squared error of prediction depending on the number of principal components used. A higher number of principal components did not lead to a substantial reduction of the residual mean square error of prediction. Afterwards, the variable factor map shown in **Figure 2.4** was created to assess the quality of the imputation of missing values. This variable factor map is based on the averaged data set, which resulted from 100 imputations created with a bootstrap method. Displayed as arrows, all 20 predictors as well as the 3 pH parameters are shown in the space of the first 2 principal components, which explain together 53.13% of the observed total variance in the data set. The coordinates of the arrowheads show the loadings and correspond to the correlation of the individual variables with the respective principal component. Additionally, the loadings of the variables from the 100 imputed data sets are projected in the same space in order to assess the imputing quality. The point clouds are only slightly scattered around the arrowheads, which indicates that the imputing procedure is appropriate for the available data. Only the variables with the highest proportion of missing values show an increased dispersion and indicate an increased uncertainty of prediction. Especially MUN exhibits by far the highest uncertainty. Moreover, the distributions of the variables before and after imputing were compared graphically and with a 2-sample Kolmogorov-Smirnov test (see supplemental **Figure S2.9**). The null hypothesis assuming that both samples originate from the same continuous distribution could not be rejected for any variable (α = 0.05). This confirmed the applicability and usefulness of the applied imputation procedure.

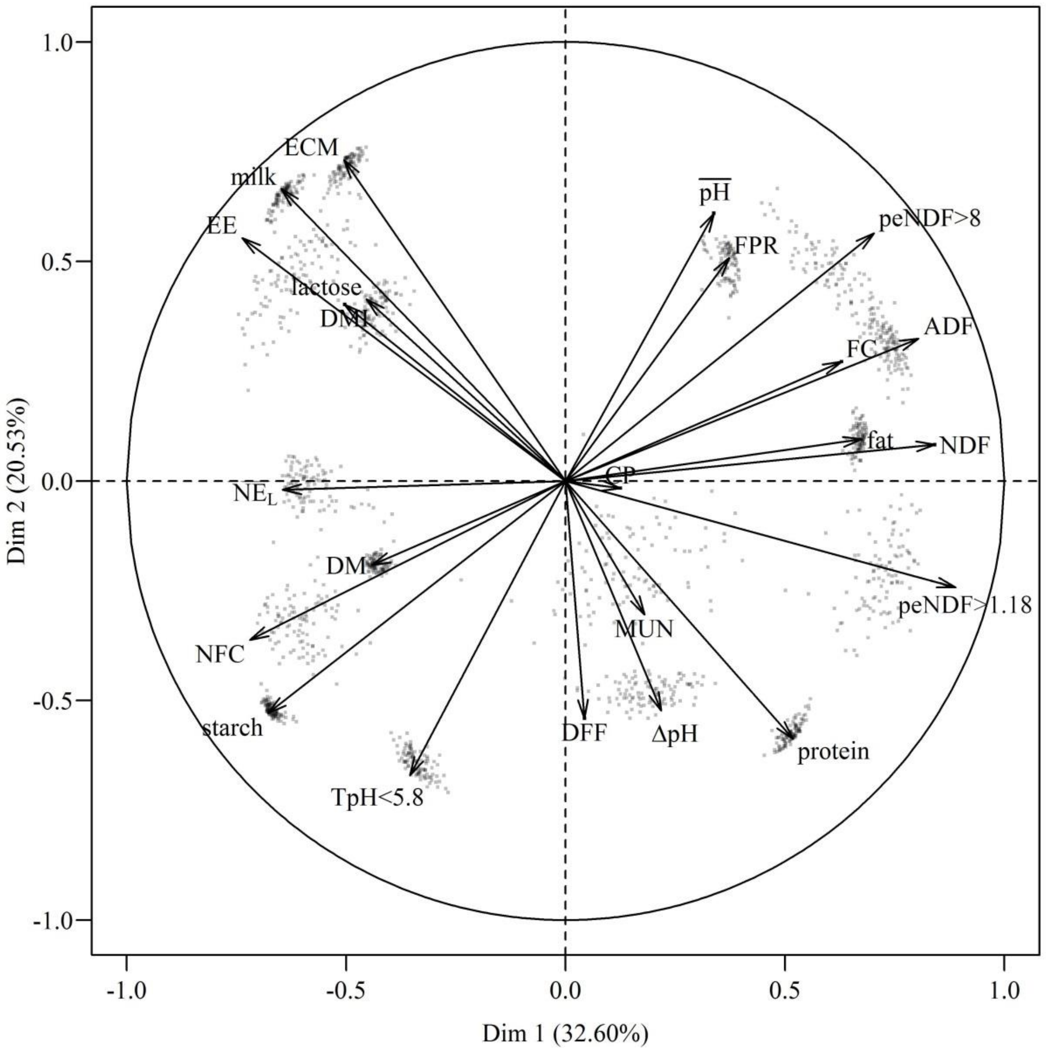

Figure 2.4: Variable factor map (Husson et al., 2019) using the first 2 principal components based on the averaged multiple imputed data set. In addition to the loadings from the variables of the averaged data set (arrows), the loadings from the 100 imputed data sets are projected in the same space (points). DFF = daily feeding frequency; EE = ether extract; F:C = forage to concentrate ratio; FPR = fat to protein ratio; peNDF>1.18 and peNDF>8 = physically effective NDF retained on a 1.18- and 8 mm sieve, respectively; $\overline{\text{pH}}$ = daily mean ruminal pH; TpH<5.8 = average daily time with pH<5.8; ΔpH = pH range.

Within the framework of this multivariate analysis method, it was not only possible to handle the problem of missing values, but also to derive the associations between the variables from the variable factor map. In general, positively correlated variables appear grouped and negatively correlated variables are positioned on the opposite side of the center. As already described by Zebeli et al. (2008), the pH parameters TpH<5.8 and $\overline{\text{pH}}$ are highly negatively correlated. As to be expected, this is also confirmed here by the variable factor map shown in **Figure 2.4**, since the 2 pH parameters are positioned almost exactly opposite to each other. Furthermore, it can be seen that especially the fiber associated variables peNDF>1.18, peNDF>8, ADF, NDF and the forage to roughage ratio are relatively close to each other. On the opposite side are parameters of the diet which are primarily associated with the energy content, including starch, NFC and NE_L. The DM is also positioned opposite to the fiber associated variables, since a higher proportion of concentrate increases the DM content of the diet, *ceteris paribus*.

The interpretation of variables with a shorter distance between the center and the arrowhead has to be carried out with caution. Even though it seems that DFF and pH range cluster together, the variable factor map for the 1st and 3rd principal component (plot not shown) revealed that they are positioned opposite to each other. The same is true for $\overline{\text{pH}}$ and FPR.

Since there are no potential predictors in the immediate neighborhood of the pH parameters, it was concluded that none of them was highly associated with pH parameters. Thus, only several variables together are able to explain the observed variance of $\overline{\text{pH}}$ and TpH<5.8. The multivariate approach via PCA was a pre-analysis in order to give an overview of the relationships between all considered variables. A limitation of this method is that the hierarchical data structure is not considered.

Multi-level Meta-Regression for all Potential Predictors

In the next step, each pH parameter was analyzed in a multi-level meta-regression model (**Equation 2.2**), considering only one predictor at a time in order to identify those which have the strongest association. This analysis was performed on the raw data set with missing values as well as for the imputed data set. To allow for comparability, all predictors were z-transformed so that the estimated regression coefficients ($\hat{b}_1$) of the slope are dimensionless. The results of this analysis are shown in **Figure 2.5**, where all $\hat{b}_1$ are represented separately for all 3 pH parameters by bar charts. The predictors were sorted within the variable groups of milk and diet in descending order of the p-value of the $\hat{b}_1$ based on the raw data set. The error bars correspond to 1.96 times the robust standard error of the estimated value and thus show the robust 95% confidence interval. At first glance, it can be seen that the results tend to be very consistent for both the raw and the imputed data set. The main exceptions are the estimated regression coefficients of MUN for all 3 pH parameters and the estimates of EE for $\overline{\text{pH}}$ and TpH<5.8. With MUN, a greater or even opposite effect is estimated on the basis of the imputed data set. With EE, on the other hand, the effect present in the raw data lost significance by the imputation. Based on the fact that the remaining estimates are very similar for both data sets, further support is gained for the supposition that the imputation of the missing observations to this data set is justifiable.

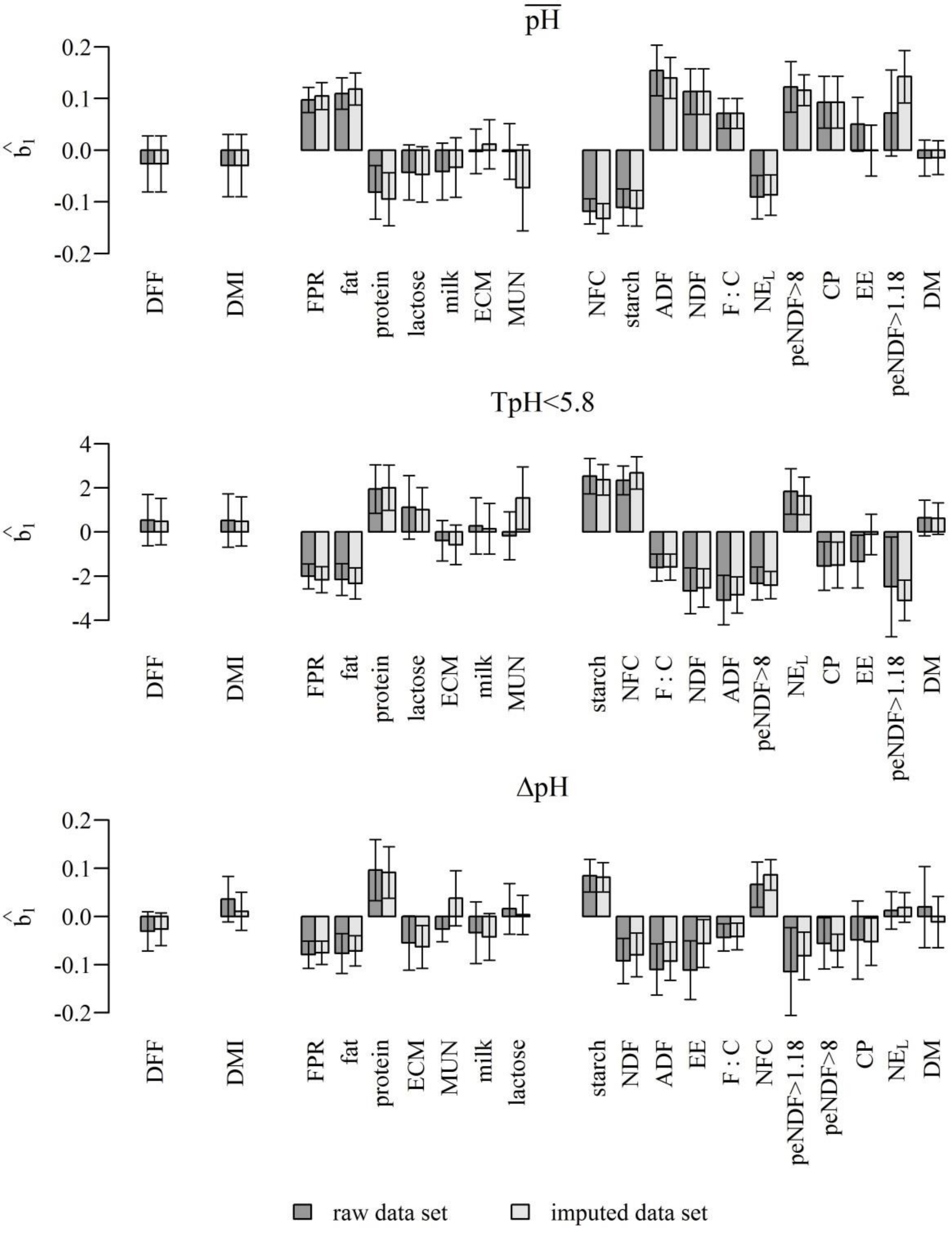

Figure 2.5: Results of regression analysis in which all 3 pH parameters were analyzed individually with each potential predictor in a multi-level meta-regression model. This analysis was performed with both the raw (dark gray) and the imputed (light gray) data set. For better comparability, all predictors were z-transformed previously. The height of the bars represents the estimated regression coefficients ($\hat{b}_1$). The error bars correspond to 1.96 times the robust standard error of $\hat{b}_1$. DFF = daily feeding frequency; EE = ether extract; F:C = forage to concentrate ratio; FPR = fat to protein ratio; peNDF>1.18 and peNDF>8 = physically effective NDF retained on a 1.18- and 8 mm sieve, respectively; $\overline{\text{pH}}$ = daily mean ruminal pH; TpH<5.8 = average daily time with pH<5.8; ΔpH = pH range.

As expected, all $\hat{b}_1$ regression coefficients for $\overline{\text{pH}}$ and TpH<5.8 are negatively correlated, because both pH parameters are strongly negatively correlated as discussed before. It can be seen that the sign of the estimated values for ΔpH corresponds to those for TpH<5.8 and is therefore negatively correlated with those of $\overline{\text{pH}}$. From the group of milk variables, FPR, protein, and fat have a significant association ($P < 0.001$) on all 3 pH parameters with FPR having the lowest p-value. The positive sign of fat for the $\overline{\text{pH}}$ is consistent with the general assumption that low ruminal pH values can also contribute to a milk fat depression as described by Nocek (1997) and Kleen et al. (2003). For example, Danscher et al. (2015) were able to show that significantly ($p = 0.01$) lower pH values tend to be accompanied by lower milk fat values ($p = 0.06$) in SARA-challenged heifers. The authors did not find any significant differences in milk protein between the 2 groups. However, the results of such studies are not consistent in the literature. For example, Krause and Oetzel (2005) could not detect a milk fat depression on the basis of SARA-challenged lactating cows.

The positive association with FPR is consistent with the fat content and supports suggestions that this measure has potential as an indicator of SARA in dairy cows. However, it is interesting to note that although primary milk fat depression is postulated for SARA, there is obviously a negative association between the $\overline{\text{pH}}$ and protein content of milk. In this respect, Rook and Balch (1961) showed that an intraruminal propionate infusion resulted in a decrease of the fat and an increase of the protein content of the milk and thus a decreasing FPR. Assuming that a lower $\overline{\text{pH}}$ can be accompanied with a higher ruminal propionate ratio as described by Russell (1998), the negative sign of the regression coefficient of protein and the positive sign of the FPR is consistent with the aforementioned association (Rook and Balch, 1961). All other milk associated parameters showed no significant effect as a single predictor in a multi-level mixed effect regression model. At this point it has to be noted that the models with FPR as single predictor generally have to be interpreted carefully. As discussed by Kronmal (1993), the use of ratios as an independent variable in a regression model without the 2 independent variables used to calculate the ratio is critical.

In addition to the meta-regression analysis, the pH parameters and potential predictors were examined graphically using scatterplots. **Figure 2.6** shows 3 predictors for each of the 3 pH parameters that are characterized by the smallest p-value (see supplemental **Figure S2.10 - Figure S2.12**). Imputed values, either the predictor, the pH parameter or both, are depicted by gray triangles, while black dots represent observed values. To represent the hierarchical data structure, observations from the same study were linked by lines or, in the case of imputed values, by dashed lines. Moreover, the regression lines, which are based on the raw and on the imputed data set, were added. In all cases, the regression line is based only on the fixed effect part of the model and consists of an intercept and a slope. Obviously, there is an immense heterogeneity among the treatments and across individual studies. It can be seen that single predictors are only able to explain a small part of the observed variance. In each plot, both regression lines are nearly congruent, even though in the case of NFC 49.1% of the observations were imputed.

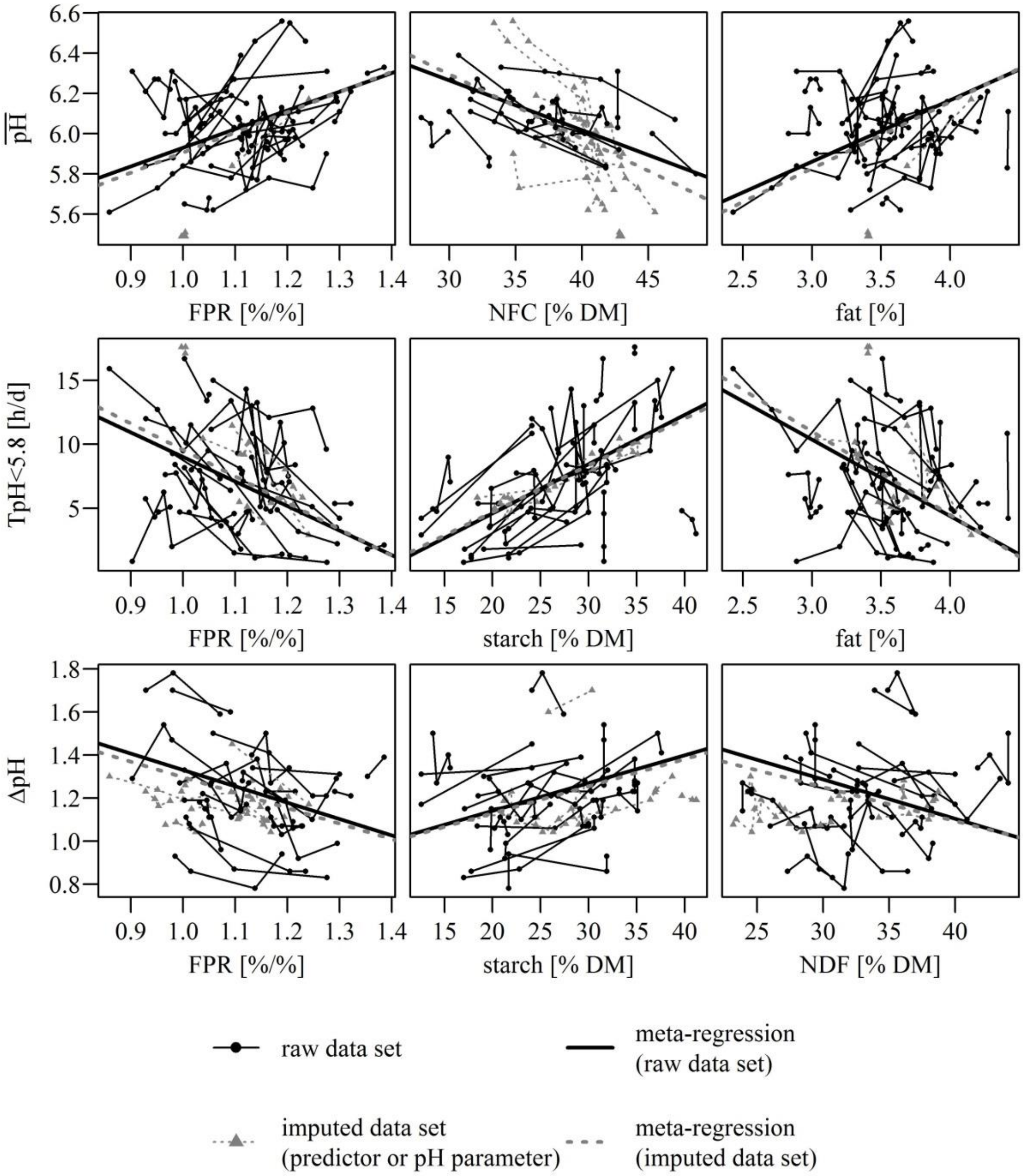

Figure 2.6: Scatterplots for the pH parameters as well as the 3 predictors with highest significance. The actual observed data (black) and the imputed data (gray) are shown. In the latter case the pH parameter, the predictor or both were imputed. Observations from the same study were linked by a line or, in the case of imputed data, by gray dashed lines. Further, the regression lines based on the fixed effect part of a multi-level meta-regression model are drawn. The black bold line shows the regression line estimated using the raw data set, and the gray bold dashed line shows the regression line estimated using the imputed data set. FPR = fat to protein ratio; $\overline{\text{pH}}$ = daily mean ruminal pH; TpH<5.8 = average daily time with pH<5.8; ΔpH = pH range.

Variable Selection and Multi-Model Inference

In total, 6 models (**M1** – **M6**) were generated with the automated variable selection on the imputed data set, which are shown in **Table 2.3**. The uncertainty for the imputed MUN values was by far the lowest and the previous regression analysis led to deviating results. Therefore MUN was not considered in this part of the analysis.

For all 3 pH parameters, the selected models based on the variable selection with diet parameters (M2, M4 and M6) demonstrated a better model accuracy (AICc) compared to the models containing milk parameters (M1, M3 and M5). For the 2 parameters $\overline{\text{pH}}$ and TpH<5.8, similar variables were selected. If only milk variables were considered, DFF, fat, protein, and lactose were selected for both parameters. Taking into account diet variables, DM, EE as well as peNDF>8 were selected for $\overline{\text{pH}}$ and TpH<5.8, whereby NFC was additionally selected for $\overline{\text{pH}}$ and starch for TpH<5.8. The variables chosen for ΔpH corresponded approximately to those selected for $\overline{\text{pH}}$ and TpH<5.8. Instead of lactose, ECM was selected in the variable selection with milk parameters, and DFF instead of DM with respect to diet parameters.

It was surprising that if diet variables were available, EE was selected in all 3 models. The EE content of the diet is generally not linked to a low ruminal pH, although there are indications of relationships reported in the literature. For example, Ikwuegbu and Sutton (1982) investigated sheep and were able to show that the addition of linseed oil led to a change in the mean concentration of acetate, propionate and n-butyrate as well as to reduction of digestibility of energy, OM and ADF. Although the authors could not determine any significant effect of the linseed oil on the pH, the altered VFA profile shows an effect of the dietary fat on the ruminal fermentation process. It is well known that dietary fat and its composition can affect both milk composition and digestibility of the fiber content. Harvatine and Allen (2006a), for example, were able to demonstrate that the addition of PUFA led to a significant reduction in milk fat, energy intake and milk energy yield. In addition, a change in the fatty acid pattern was observed, whereby a higher PUFA content led to a lower proportion of short and medium chain fatty acids. Furthermore, Harvatine and Allen (2006c) demonstrated that the saturation of supplemented fatty acids effected the digestibility of NDF. Thereby, saturated fatty acids caused a decrease and unsaturated fatty acids an increase of ruminal NDF digestibility. The authors also found that the total tract digestibility did not differ due to compensatory post-ruminal digestion. As Harvatine and Allen (2006a) also mentioned, the dietary fat and thus EE can be classified as a fermentation modifier. Interestingly, no effect of fatty acid supple-

Table 2.3: Results of variable selection for daily mean ruminal pH, the average daily time with pH<5.8, and the pH range using the imputed data set (k = 32 studies, n = 112 treatment means)[1]

	M1 (Milk)		M2 (Diet)	
Item	$\hat{b}$	SE	$\hat{b}$	SE
$\overline{\text{pH}}$				
Intercept, pH	8.8849	0.8435 ***	5.9699	0.2354 ***
DFF, 1/d	-0.0571	0.0277 *		
Fat, %	0.2861	0.0372 ***		
Protein, %	-0.5169	0.1048 ***		
Lactose, %	-0.4528	0.1431 **		
DM, % of DM			0.0081	0.0019 ***
EE, % of DM			0.0457	0.0225
peNDF>8, % of DM			0.0173	0.0039 ***
NFC, % of DM			-0.0210	0.0037 ***
AICc	-134.33		-164.11	
I^2, %	88,0		82.6	
I^2_{bs}, %	45.8		43.3	
	M3 (Milk)		M4 (Diet)	
TpH<5.8				
Intercept, h/d	-57.8745	17.2019 **	20.9985	4.4234 ***
DFF, 1/d	0.9958	0.4664 *		
Fat, %	-5.5599	0.9261 ***		
Protein, %	11.4912	2.0459 ***		
Lactose, %	9.8551	2.8286 **		
DM, % of DM			-0.1398	0.0413 **
EE, % of DM			-0.9641	0.4177 *
Starch, % of DM			0.1763	0.0586 **
peNDF>8, % of DM			-0.4957	0.0972 ***
AICc	532.23		514.96	
I^2, %	87.0		82.0	
I^2_{bs}, %	55.0		54.0	
	M5 (Milk)		M6 (Diet)	
ΔpH				
Intercept, pH	0.6874	0.4286	1.8482	0.2709 ***
DFF, 1/d	-0.0592	0.0449	-0.1061	0.0495 *
ECM, kg/d	-0.0075	0.0038		
Fat, %	-0.1663	0.0415 ***		
Protein, %	0.4585	0.1167 ***		
EE, % of DM			-0.1382	0.0346 ***
Starch, % of DM			0.0088	0.0032 *
peNDF>8, % of DM			-0.0133	0.0048 *
AICc	-164.72		-177.78	
I^2, %	77.0		76.4	
I^2_{bs}, %	60.4		47.7	

[1] M1 to M6 = models 1 to 6, respectively; AICc = second order Akaike information criterion for small sample sizes; $\hat{b}$ = estimate, DFF = daily feeding frequency; EE = ether extract; I^2 = test statistic to quantify the overall heterogeneity; I^2_{bs} = test statistic to quantify the between-study heterogeneity; peNDF>8 = physically effective NDF retained on a 8 mm sieve; $\overline{\text{pH}}$ = daily mean ruminal pH; SE = standard error; TpH<5.8 = average daily time with pH<5.8; ΔpH = pH range.

*** *P* < 0.001, ** *P* < 0.01, and * *P* < 0.05.

mentation on $\overline{\mathrm{pH}}$ was observed in the same experiment. Nevertheless, the authors could determine an increased ΔpH and pH variance due to supplementation of saturated fatty acids. This is remarkable because EE also showed the most significant effect on ΔpH compared to the effect on $\overline{\mathrm{pH}}$ and TpH<5.8 in our analysis. When considered as single predictor in the model based on the raw data set (see **Figure 2.5**), EE showed a significant effect on TpH<5.8 ($P < 0.05$) and ΔpH ($P < 0.01$) as well as a trend for $\overline{\mathrm{pH}}$ ($P < 0.1$). The same trend was also observed in the multi-model inference regarding the relative importance of EE (see **Figure 2.7**). This insight supports the decision to exclude studies from this meta-analysis which used diets supplemented with fatty acids in order to reduce the possibility of additional bias.

Fat and protein content were present in all milk production models, although the FPR showed the most significant association when considered alone in a model. This is probably due to the dependency chains, but also means that fat and protein as 2 independent variables are mainly associated with the pH parameters and that the additional consideration of the FPR does not lead to any further model improvement.

If only milk variables were available, lactose was selected for $\overline{\mathrm{pH}}$ and TpH<5.8 in M1 and M3. With regard to lactose, only Enemark et al. (2002) have reported an increased lactose content caused by SARA as a consequence of increased ruminant propionate formation and thus an associated elevated blood sugar level. The lactose content is promising as potential predictor since it is the most constant ingredient of the milk (NRC, 2001) and is therefore only marginally influenced by the lactation stage, whereas the protein and fat content of the milk are affected in the course of lactation (Stanton et al. 1992).

From the estimates from I^2 and $\mathrm{I}^2_{\mathrm{bs}}$ for all models, it can be deduced that the present data set shows a very high overall heterogeneity among the individual treatments, which is mainly caused by between-study heterogeneity. It can be assumed that a certain degree of heterogeneity is due to insufficient modeling of the predictors. Based on this meta-analysis, the concrete sources of heterogeneity cannot be identified and further research is required. In general, a large inter-farm variability for pH measurements in the forestomach system is reported in the literature. For example, Denwood et al. (2018) were able to demonstrate that both the range of reticular measured pH and the pH progressions varied among 13 different farms, which could be traced back to different rations and management influences such as milking frequency.

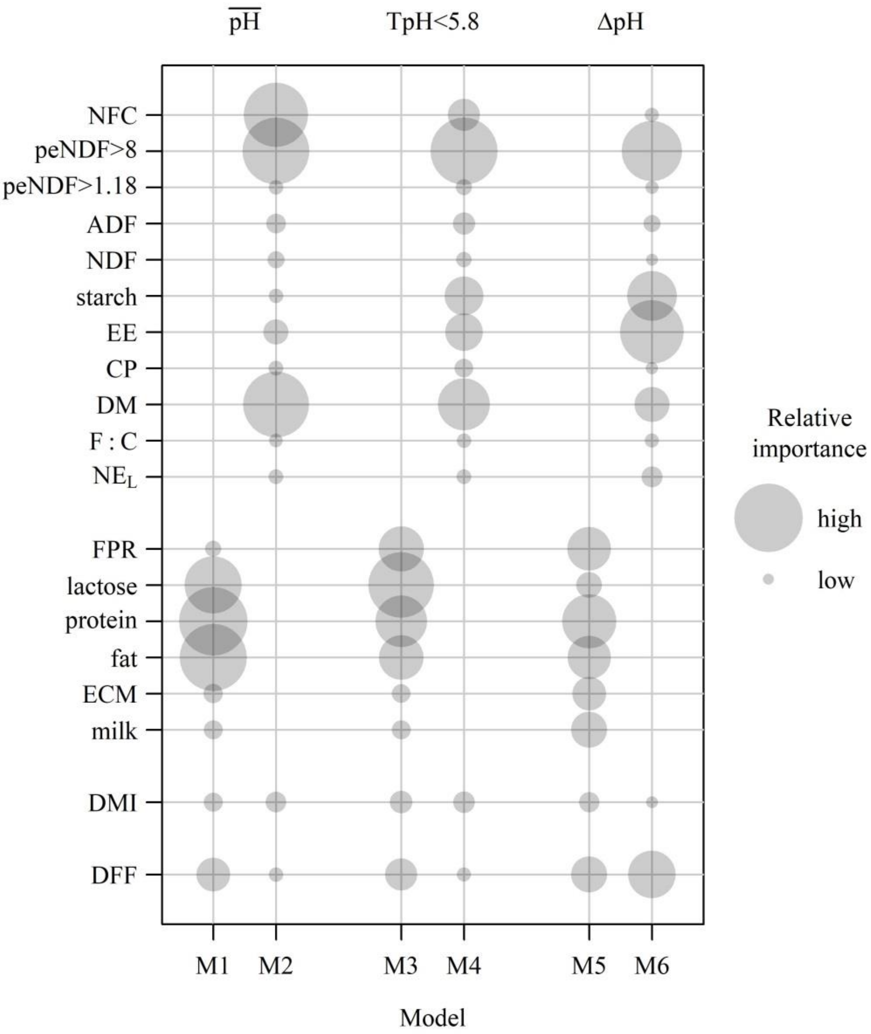

Figure 2.7: Visualization of the estimated relative importance of each variable derived from the multi-model inference for all 6 models. The diameter of the gray circles represents the estimated relative importance of each variable within the model. DFF = daily feeding frequency; EE = ether extract; F:C = forage to concentrate ratio; FPR = fat to protein ratio; peNDF>1.18 and peNDF>8 = physically effective NDF retained on a 1.18- and 8 mm sieve, respectively; $\overline{\text{pH}}$ = daily mean ruminal pH; TpH<5.8 = average daily time with pH<5.8; ΔpH = pH range.

As already derived from the results of the PCA, $\overline{\text{pH}}$, TpH<5.8, and ΔpH are not easily predictable traits, but complex parameters. Based on the results of Golder et al. (2018), it can be assumed that the pH parameters depend on the entire metabolism of the cow as well as the interactions between the cow, ruminal microbiome, and the diet. Since this relationship is not easy to grasp with a single model, a multi-model inference was applied and the relative importance of the individual parameters within each of the 6 model selection procedures was determined. In **Figure 2.7**, the relative importance varying from 0 to 1 of each variable is represented by the diameter of the circle, where a small circle displays a low and a big circle a high relative importance. It can be seen, that the DM, EE, NFC and peNDF>8 content carry the largest relative importance among the diet parameters. With regard to the milk parameters, especially protein, fat and lactose show the highest relative importance. In comparison, Zebeli et al. (2008) identified the squared term of peNDF>1.18, ruminally degradable starch from grain, and the DMI, applying a backward elimination technique to predict the pH in a multiple regression model. In another study, a comparable model using the peNDF>8, the starch content of the diet and DMI were selected to predict pH (Zebeli et al., 2010). Since the starch and NFC content of the diet are highly positively correlated, this is consistent with our results. What differs, however, is that the DMI was not selected in any of the present models in the variable selection and appeared to be only of relatively minor importance.

The modeling of ΔpH showed that DFF also had a significant effect. The signs of the partial regression coefficients of the models M5 and M6 show that a more frequent supply of feed is accompanied by a lower pH range. This is in line with the results of Le Liboux and Peyraud (1999) and indicates that the entire management contributes to the pH development.

Relationship between TpH and $\overline{pH}$

Based on **Equation 2.6**, all possible TpH<5.8 as a function of plausible $\overline{\mathrm{pH}}$ and ΔpH combinations were calculated and are shown in **Figure 2.8**. The predicted range of TpH<5.8 for a certain $\overline{\mathrm{pH}}$ is illustrated by a gray scale gradient. Except for $\overline{\mathrm{pH}} = 5.8$, there is a range of conceivable TpH<5.8 for all other plausible $\overline{\mathrm{pH}}$. The reason for that is that if $\overline{\mathrm{pH}} = 5.8$ the content of the brackets in **Equation 2.6** is cut short by ΔpH and therefore only TpH<5.8 = 12 h is possible. For the remaining value range of $\overline{\mathrm{pH}}$, a variation in TpH<5.8 exists depending on ΔpH. To validate this theory, the observed data from all studies were added to the plot. Although the assumptions are highly simplified in our theoretical relationship between TpH<5.8 and $\overline{\mathrm{pH}}$, approximately 90% of the observed data are in the predicted range. This agreement confirms the association described in **Equation 2.6**. This approach differs from modeling with a broken line model, as conducted by Zebeli et al., (2008).

CONCLUSIONS

In many animal feeding experiments sample size is restricted, particularly if surgical procedures, as in the case of ruminally fistulated cows, are utilized. The application of meta-analytical methods proved to be a powerful tool for obtaining a comprehensive overview of physiological relationships between diet, rumen and milk properties. In the meta-regression analysis, the fat, protein, and the FPR could be identified and confirmed as indicators for rumen pH parameters and thus, in the broader sense, also for SARA. Interestingly, also the lactose content of the milk was selected in the automated variable selection and indicated the ability to improve the predictability for ruminal pH parameters. With regard to diet specific variables, significant effects for peNDF>8, ADF, NDF, NFC, starch as well as the F:C were identified. We can confirm that milk constituents are associated with the ruminant pH value and that these can be used as indicators for monitoring purposes. In the future it should be clarified to what extent further information, e.g. on the fine milk composition, particularly the fatty acid pattern of the milk, can contribute to explaining the association between ruminal pH and the milk in order to find better indicators for SARA.

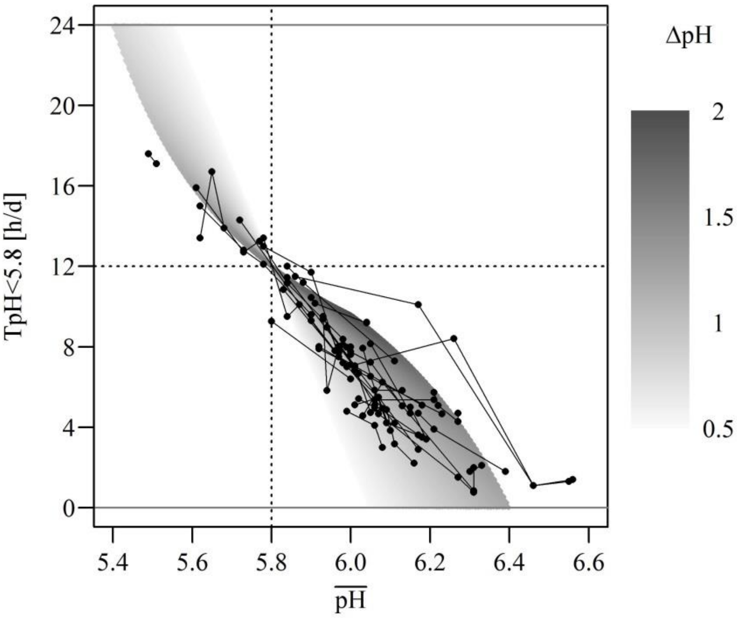

Figure 2.8: Relationship between average daily time with pH < 5.8 (TpH<5.8), daily mean ruminal pH ($\overline{\text{pH}}$) and pH range (ΔpH). All observed treatment means within one study are linked by a line. The gray scale gradient in the background represents the theoretical values for TpH<5.8 depending on a given ΔpH and $\overline{\text{pH}}$ predicted by **Equation 2.6**.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization: AM, JH. Data curation: AM. Formal Analysis: AM. Funding acquisition: JH. Investigation: AM. Methodology: AM, ARS. Project administration: JH. Software: AM, Supervision: JH, ARS. Visualization: AM. Writing – original draft: AM. Writing – review and editing: AM, ARS, JH.

SUPPLEMENTARY

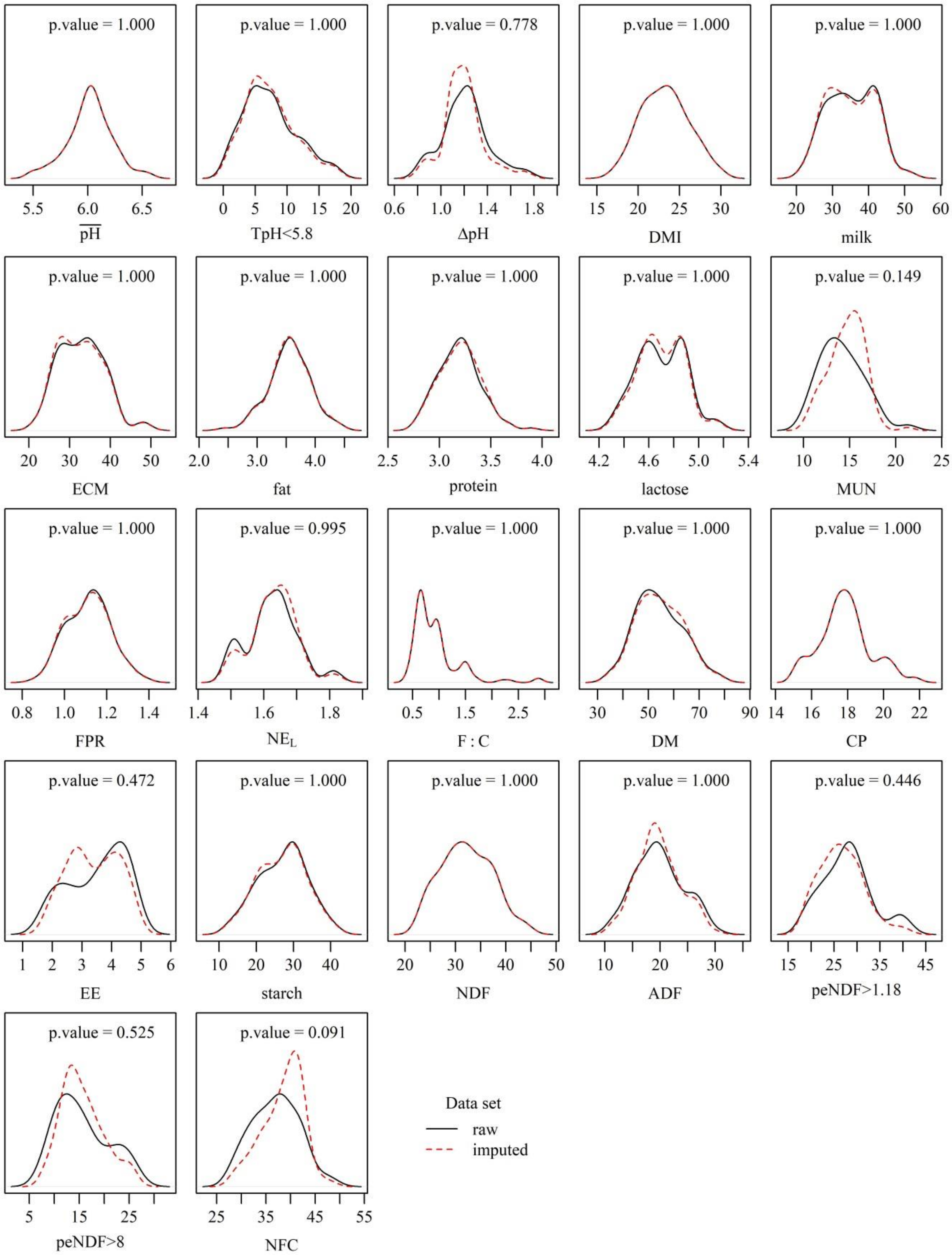

Figure S2.9: Density function of the pH parameters and potential predictors based on the raw and the imputed data set. In each sub plot, the p-value of a 2-sample Kolmogorov-Smirnov test is added. DFF = daily feeding frequency; EE = ether extract; F:C = forage to concentrate ratio; FPR = fat to protein ratio; peNDF>1.18 and peNDF>8 = physically effective NDF retained on a 1.18- and 8 mm sieve, respectively. n = 112 treatment means.

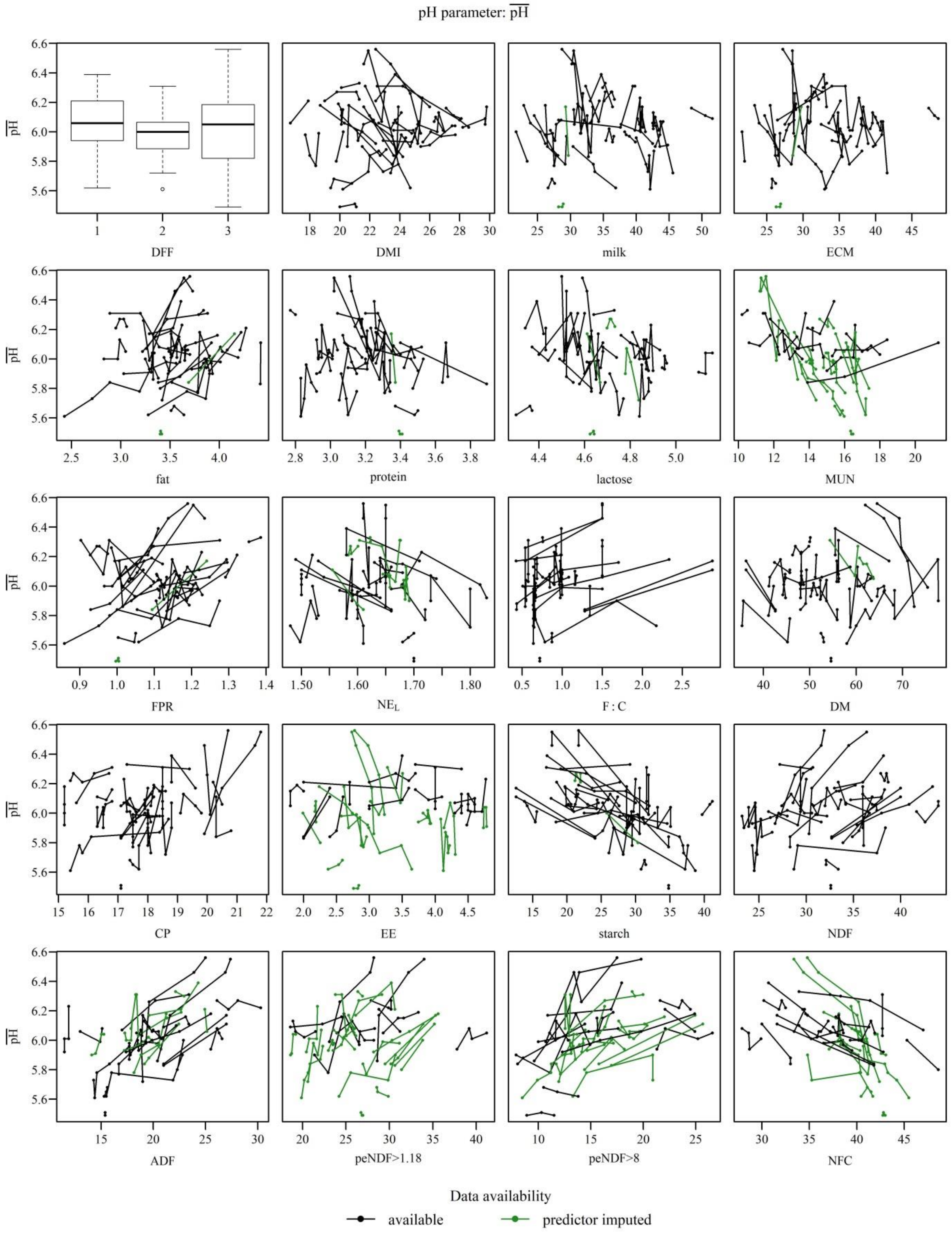

Figure S2.10: Scatterplots for the daily mean ruminal pH ($\overline{\mathrm{pH}}$) and all potential predictors. Observations from one study are linked by a solid line. The color code represents the availability of the data. DFF = daily feeding frequency; EE = ether extract; F:C = forage to concentrate ratio; FPR = fat to protein ratio; peNDF>1.18 and peNDF>8 = physically effective NDF retained on a 1.18- and 8 mm sieve, respectively. k = 32 studies and n = 112 treatment means.

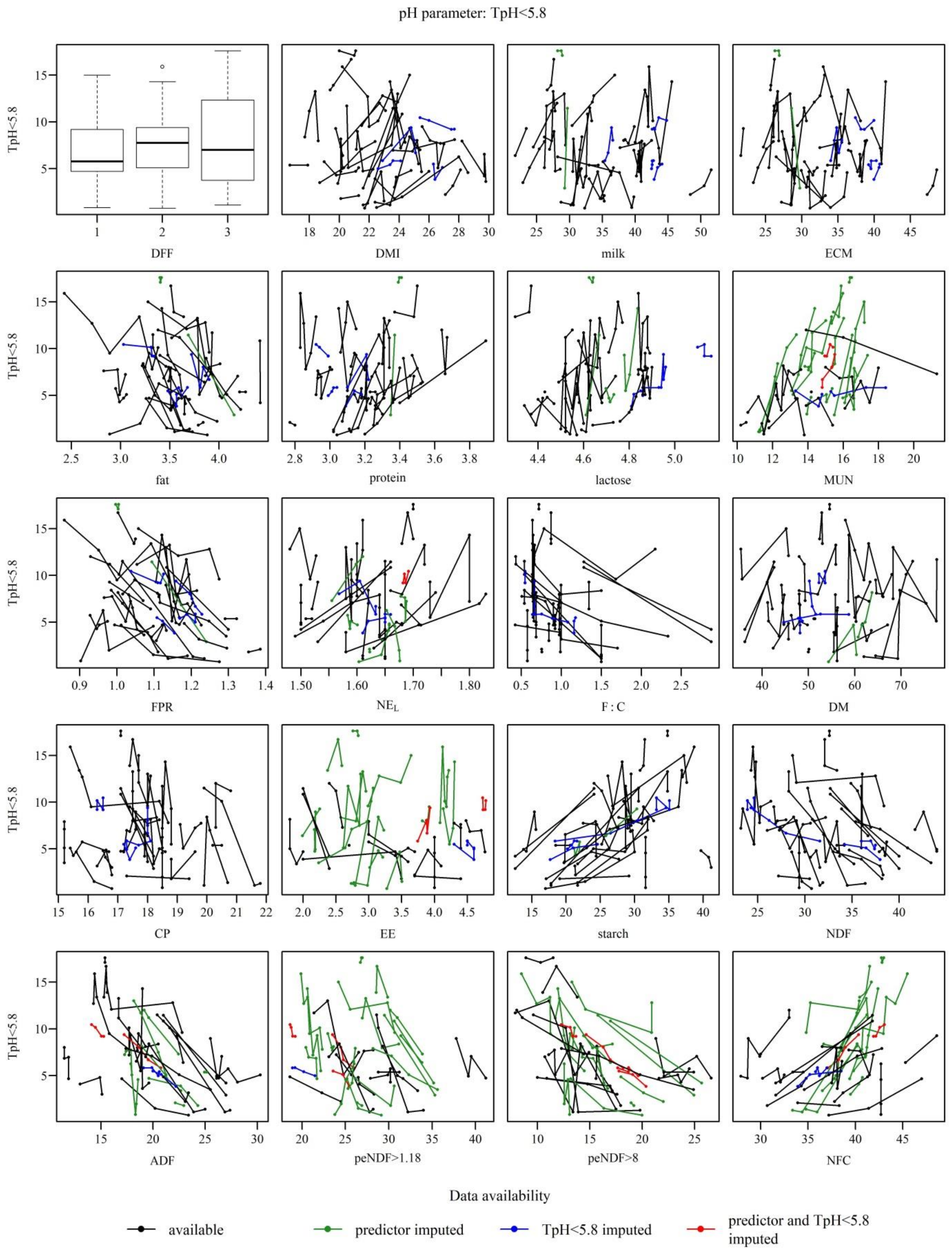

Figure S2.11: Scatterplots for the average daily time with pH<5.8 (TpH<5.8) and all potential predictors. Observations from one study are linked by a solid line. The color code represents the availability of the data. DFF = daily feeding frequency; EE = ether extract; F:C = forage to concentrate ratio; FPR = fat to protein ratio; peNDF>8 = physically effective NDF retained on a 8 mm sieve. k = 32 studies and n = 112 treatment means.

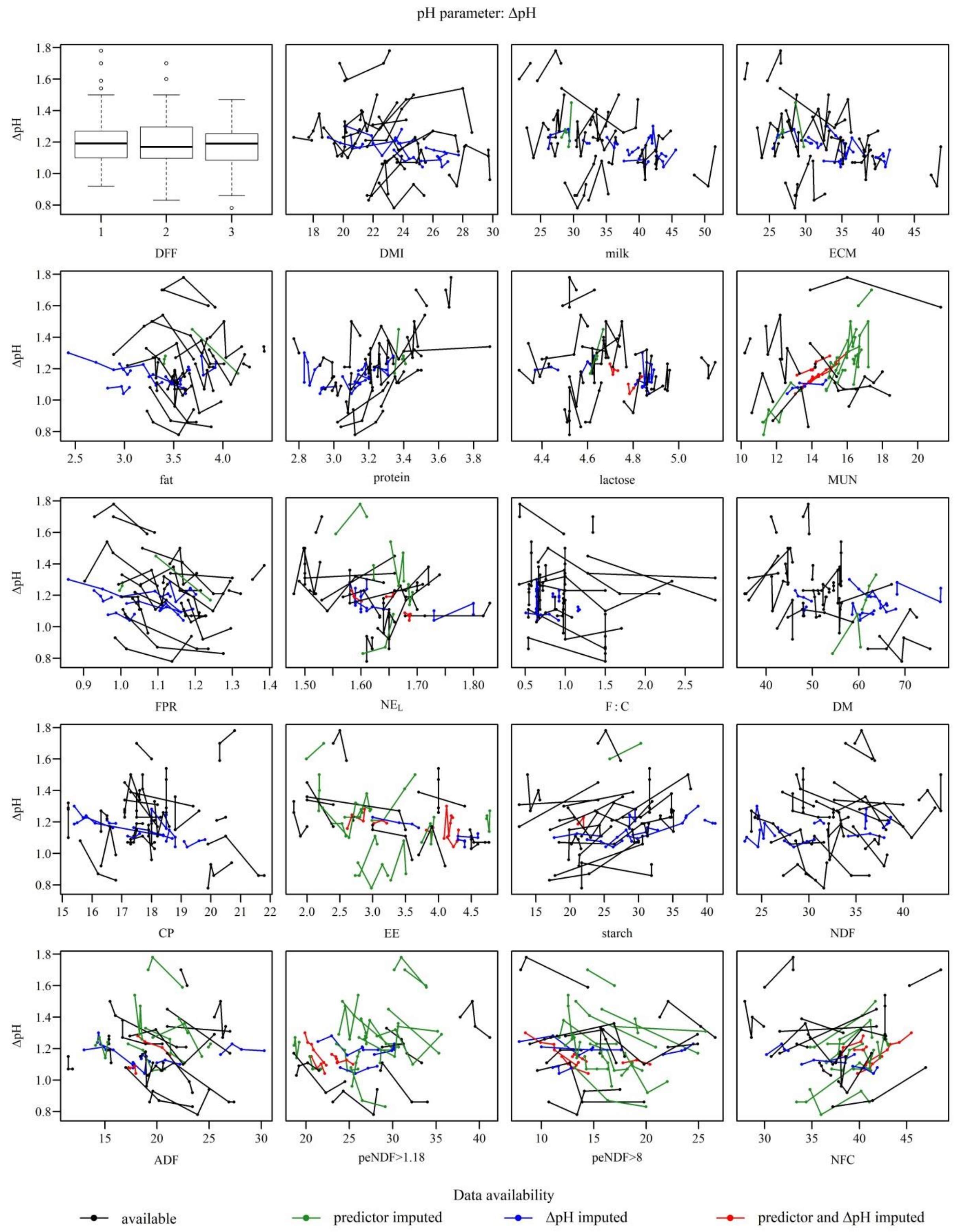

Figure S2.12: Scatterplots for the pH range (ΔpH) and all potential predictors. Observations from one study are linked by a solid line. The color code represents the availability of the data. DFF = daily feeding frequency; EE = ether extract; F:C = forage to concentrate ratio; FPR = fat to protein ratio; peNDF>8 = physically effective NDF retained on a 8 mm sieve. k = 32 studies and n = 112 treatment means.

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Chapter 3

Modeling of reticular and ventral ruminal pH of lactating dairy cows using ingestion and rumination behavior

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ABSTRACT

The prevention and control of metabolic and digestive diseases is an enormous challenge in dairy farming. Subacute ruminal acidosis (**SARA**) is assumed to be the most severe feed-related disorder and impairs both animal health and economic efficiency. Currently, the ruminal pH as well as parameters derived from the daily pH curve are main indicators for SARA. The objective of this study was to explain the daily pH course in the ventral rumen and in the reticulum of dairy cows using ingestion pattern and rumination behavior data gathered by automated data recording systems. The data of 13 ruminally fistulated lactating cows were collected in the experimental station of the Friedrich-Loeffler-Institut (FLI) in Brunswick, Germany. The data included continuous pH measurements, which were recorded simultaneously in the reticulum by pH measuring boluses and in the ventral rumen by a separate data logger. In addition, the rumination behavior was measured with jaw movement sensors and the feed and water intakes were recorded by transponder-assisted systems. Furthermore, milk yield and body weight were determined during or after each milking, respectively. For the statistical evaluation, the data were analyzed using time series modeling with multiple linear mixed regressions. Prior to the application of the developed mathematical statistical modeling, a plausibility assessment was performed to ensure the data quality. The major part of the mathematical statistical modeling consisted of data preparation, where all variables were transformed into a uniform 1-minute resolution. Signal transformations were used to model the individual feed and water intake as well as the rumination behavior events over time. The current study indicates that both diurnal pH curves of the reticulum and the ventral rumen essentially could be predicted with the transformed feed and water intake rates. Rumination events were associated with a marginal temporal increase of the pH. It was observed that the pH of the ventral rumen is delayed by approximately 37 min compared to the pH of the reticulum, which was therefore considered in the modeling. With the models developed in this study, 67.0% of the variance of the reticular and 37.0% of the variance of the ruminal pH curves could be explained by the fixed effects. It can be deduced that the diurnal pH course is to a large extent associated with the animal’s individual feed intake and rumination behavior.

Key words: ruminal pH, time series, statistical modeling, behavior

INTRODUCTION

In dairy farming, the prevention and controlling of metabolic and digestive disorders is an enormous challenge. Particularly subclinical conditions such as SARA are hard to diagnose, but can severely impair both animal health and efficiency in the longer run. Therefore, adequate feeding of lactating cows is of particular importance, especially for high-yielding animals. The 2 most relevant adjustable parameters of a diet are the energy content and the amount of physically effective fiber (Nocek, 1997). However, the energetic upgrading of the diet by concentrates rich in rapidly fermentable carbohydrates can especially predispose to SARA, particularly in the first 3 months of lactation when the need for an increased energy concentration of the diet is high (Gröhn and Bruss, 1990). At a subacute stage, no distinct clinical signs can be detected on an individual animal level in the short term. Nevertheless, associations with various clinical signs at herd level can be observed, including reduced feed intake, lower efficiency of milk production, loose stools (reduced DM content of the feces) and claw diseases, as well as an overall higher culling-rate (Kleen et al., 2003). Even if the same diet is fed, a high inter-animal variability in pH parameters can be observed, suggesting that there are different susceptibilities to SARA among individuals (Humer et al., 2015). In particular, the high inter-animal variance, which is enhanced by factors such as lactation stage or parity, makes the parameters at herd level more meaningful due to the aggregation of data from several animals.

The major challenge with the early diagnosis of SARA is therefore the identification of a suitable and practical diagnostic parameter. In science, pH thresholds are currently regarded to be the most appropriate measure for diagnosing SARA. As already objected by Zebeli et al. (2008), various thresholds for different pH parameters can be found in the literature. In a meta-analysis, these authors derived that the daily mean ruminal pH should be higher than 6.16 and the time with pH < 5.8 should not exceed 5.24 h/d to reduce the risk of SARA. Additionally, orally applied measuring boluses have recently been used for continuous measurement of the intra-reticular pH value (Humer et al., 2017; Denwood et al., 2018). However, these devices are prone to considerable drifts over time (Villot et al., 2017) and it has not yet been sufficiently clarified to what extent their measurements reflect the ventral ruminal pH. The first approaches have already been made to characterize the relationship between reticular and ruminal pH measurements. Falk et al. (2016) studied the reticular pH measured with eCow boluses in association with the ruminal pH measured with LRCpH loggers of fistulated lactating cows. The authors arrived at the conclusion that no clear relationship between the reticular and the ruminal pH can be provided. In contrast, Neubauer et al. (2018a) analyzed the relationship between measured pH by eCow boluses and 3 spot samples collected from the ventral free-rumen liquid with a rumen pump. They concluded that a pH of 5.8 of the free-rumen liquid corresponds to a pH of 6.0 in the reticulum.

Generally, the daily pH course can be associated with the individual feed intake behavior and the daily feeding frequency. Le Liboux and Peyraud (1999) observed a smaller daily pH range if the feeding frequency was increased from 2 to 6 times per day. The milking frequency and time of milking are also reflected in the pH curve and contribute to a farm-specific pH profile (Denwood et al., 2018). Another influencing factor is the composition of the feed. Jiang et al. (2017) showed that the increase of dietary roughage from 40 to 70% led to longer feed intake and higher daily average pH values. Feeding twice a day, the authors observed a decrease of the pH immediately after each feeding, in which a sinusoidal pH progression with 2 periods per day was determined independently of the roughage level.

In a meta-analysis, Mensching et al. (2020) examined the associations between 3 ruminal pH parameters and milk and diet-specific parameters and determined high between-study heterogeneity, even if the 4 most explanatory variables were considered together in a multiple regression model. Consequently, a precise prediction of the pH parameters remains challenging. Newest approaches focus increasingly on indicators, for example the fatty acid composition of the milk fat, which is known to be associated with ruminal fermentation characteristics (Vlaeminck et al., 2006b).

The main objective of the present study was to explain the temporal pH progression in the reticulum and in the ventral rumen with high-resolution sensor data of the animal-specific feed and water intake as well as the rumination behavior using time series analysis methods. We strived to gain a deeper insight into the causality of the daily pH progression in order to identify eventually reliable indicators for SARA.

MATERIALS AND METHODS

The experiment was conducted in accordance with the German legislation on animal protection (Animal Welfare Act). It was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany) in consultation with an independent ethics committee (AZ 33.19-42502-04-15/1858). Further results of this experiment have already been published by Bünemann et al. (2019), who investigated the effect of BCS and concentrated feed on energy metabolism of dairy cows.

To give an overview on the extensive Material and Methods section, **Figure 3.1** provides a summary of all essential steps in a flow chart. This figure was created using the yEd graph editor (yWorks GmbH, 2019).

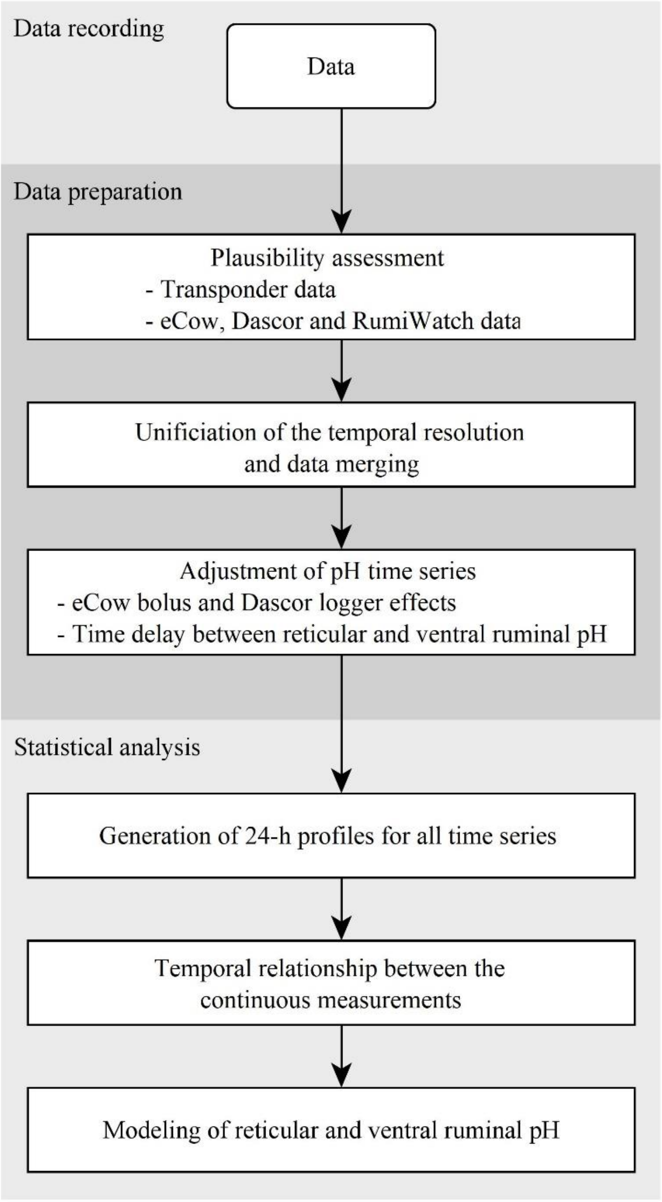

Figure 3.1: A flowchart showing systematically all steps, as described in detail in the Material and Methods section. eCow = eCow pH bolus (eCow Ltd., Exeter, UK); Dascor = LRCpH logger (Dascor Inc., Escondido, CA); RumiWatch = RumiWatch noseband halter (ITIN + HOCH GmbH, Liestal, Switzerland).

Data Recording

Data from 13 ruminally fistulated cows were collected between November 9, 2016 and March 17, 2017 at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut (FLI) Brunswick, Germany. They were kept in a loose-housing barn with resting pens in a group of 60 cows in total. Fresh feed was provided once a day between 11:00

and 12:00 a.m. Information on the diets is given in the supplementary **Table S3.2**. Further experimental details can be found in the publication of Bünemann et al. (2019).

The data examined in this study include pH and temperature data which were recorded simultaneously with pH measurement boluses (eCow Ltd., Exeter, Devon, United Kingdom) in the reticulum and the LRCpH logger (type 4, Lethbridge Research Center ruminal pH measurement system, Dascor Inc., Escondido, California) in the ventral rumen. Seven LRCpH loggers were available for the 13 fistulated cows, such that the devices had to be interchanged between the animals. Before and after each use, the electrode of the LRCpH loggers were calibrated at 39°C in pH 4 and pH 7 buffer solutions. Assuming a linear drift, this 2-point calibration was used to correct the measured values in millivolts which then were converted to pH values as described by Penner et al. (2006). All 13 eCow boluses, which were activated in a water bath at 39°C before insertion, were also calibrated in pH 4 and pH 7 buffer solutions. A feature of the data collection was that, since the animals were ruminally fistulated, the eCow boluses were also exchanged between the animals. As a result, the bolus was not confounded with the cows, so that bolus effects could be determined.

To measure the rumination behavior, RumiWatch (ITIN + HOCH GmbH, Liestal, Switzerland) noseband halters were used. Again, 7 devices were available, such that the devices had to be interchanged between the animals and were used simultaneously with the LRCpH logger. The raw data of the RumiWatch noseband sensor were converted with the help of the RumiWatch Converter V0.7.3.36 into (RumiWatch, ITIN + HOCH GmbH, Liestal, Switzerland) a 1-minute resolution. Thereby, every observation was assigned to one of 6 classifications (0 = other, 1 = ruminate, 2 = eating with head position down, 3 = eating with head position up, 4 = drink, 5 = jaw movement for ripping of grass).

For each animal, the individual feed intake of the partially mixed ration (**PMR**) and the amount of water were recorded using transponder-assisted weighing troughs (RIC, Insentec B.V., Marknesse, The Netherlands). For each single observation of the feed or water intake, namely the visit of the trough, the start and end times as well as the start and end weights of available feed and water were recorded. With this information, the duration of the visit and the amount of PMR or water, respectively, consumed by an animal during its visit were calculated. The weighing troughs are equipped with a pneumatic hatch that opens and closes at the beginning and end of each visit. The PMR was offered *ad libitum* and contained 30% concentrate on DM basis. To create 2 different feeding treatments, additional transponder based concentrate feeders (Insentec, B.V., Marknesse, The Netherlands) were used to offer supplementary concentrate feed so that 6 cows were fed with 35% (**C_{35}**) and the 7 others with 60% concentrate feed (**C_{60}**) on DM basis. The amount of concentrated feed in the C_{60} group was increased from 35 to 60% in the first 3 weeks *postpartum*. In order to attain the targeted concentrate feed proportions while offering the PMR for *ad libitum* consumption at the same time, the amount of concentrate feed required was adjusted weekly depending on the amount of PMR consumed and was provided restrictively. For all concentrated feed observations, the start and end times as well as the quantity of concentrates distributed were recorded. The milk yield was determined twice daily during the milking starting at 5:30 a.m. and 3:00 p.m. using an automatic milk counter (Lemmer Fullwood GmbH, Lohmar, Germany). After leaving the milking parlor, the body weight was also measured using a walkover live weight measurement system (Insentec, B.V., Marknesse, The Netherlands) twice a day.

Data Preparation

Plausibility Assessment of Transponder Data. It is generally known that transponder-based acquisition systems are susceptible to technical errors. This has mainly been investigated in pig farming, e.g. Eissen et al. (1998) and Casey et al. (2005). After visual examination of the data of our study, typical technical errors were found especially in the case of PMR intake. On the one hand this includes observations during which the visit duration at the trough was very long and on the other hand visits during which implausibly large amounts of feed disappeared in a short time. This circumstance is graphically represented in the supplemental **Figure S3.8**, which was created with the *heatscatter* function from the LSD (Schwalb et al., 2018) package. In addition, overlapping visits of the same animal as well as of different animals on one weighing trough were identified. The relatively long visits and time overlaps were found particularly at 4 of the 60 weighing troughs for the PMR intake and were caused by technical errors of the antenna. This malfunction concerned the logout of the transponder from the transponder station. The login, however, has been found to be valid.

In the analysis of high-resolution time series data, where the cumulative effect of ingestion over time is considered, ignoring or excluding these technically caused invalid observations could lead to a bias in the results. Therefore, we decided to identify and correct implausible observations which are caused by systematic technical errors.

The problem of overlapping visits was treated as follows: Since the login is to be regarded as valid, the preceding affected visit was cut off so that its end fell on the beginning of the subsequent visit. The further invalid records were then examined individually for each animal with statistical models. For this purpose, the approximately linear relationship between the visit duration and the amount of feed consumed was estimated. First, both the PMR amounts and the visit durations had to be transformed due to right-skewed distributions. A Box-Cox-transformation was used to determine the square root as an appropriate transformation for both variables. Based on transformed data, a robust standardized major axis (**SMA**) regression (Warton et al., 2006) was applied to describe the relationship between PMR amount and time per visit at the transponder station. In this SMA regression model a linear relationship between the transformed variables is assumed as follows:

$$\sqrt{\mathrm{PMR}_i} = b_0 + b_1\sqrt{\mathrm{VD}_i} \qquad [1]$$

where PMR_i is the amount of PMR intake i and VD_i is the corresponding visit duration, b_0 is the intercept and b_1 is the slope. The analysis with SMA models was carried out with the *sma* function from the smatr (Warton et al., 2018) package in R. Observations with standardized residuals > 3 (i.e. eating rate too high) or < -3 (i.e. visit duration too long) were classified as outliers. In a further step, these implausible observations were corrected using the SMA model; i.e. if the visit duration was too long, the expected visit duration was calculated based upon the plausible eating quantity. If, on the other hand, the eating rate was too high and the visit duration was plausible, the expected amount of feed was calculated using the given visit duration. Within this procedure 0.627% of PMR > 0 records with a visit duration too long and 0.517% of PMR > 0 records with a feeding rate too high were identified and corrected (see supplemental **Figure S3.9** and **Figure S3.10** for examples).

Plausibility Assessment of eCow, Dascor and RumiWatch Data. To assess the plausibility of the records, values on a daily basis, i.e. daily average values for the reticular and ruminal pH, as well as the total daily rumination duration were calculated for all animals. Observations were regarded as outliers if the value was more than 1.5 times the interquartile distance away from the lower or upper quartile. With regard to the eCow data, observations of 3 boluses were identified, which showed an extreme drift after approximately 80 d of measurements and recorded daily average pH values > 7.1. Using this procedure, 109 of 786 d were classified as outliers in the eCow data and only 2 of 339 d in the Dascor data. Regarding the RumiWatch data, 12 of 212 d were classified as outliers. Among them were cases where the cows temporarily dropped the halter which led to measuring gaps. If one day was detected as outlier, the previous and the following day were also excluded.

Unification of the Temporal Resolution of the Data. Since all measured variables have a different temporal resolution depending on the respective technique, the data were transformed into a 1-minute resolution and had to be merged for further statistical analysis. This required different strategies depending on the type of data.

Based on the amount of PMR, concentrate and water consumed and the time required for this intake, the average intake rates per minute were calculated using the plausibility checked transponder data. For example, if 1 kg DM of the PMR were consumed during a 10 min visit, an average intake rate of 0.1 kg/min was assumed for these 10 time periods. Since the start times of the feed and water intakes were recorded in a 1-second resolution, observations of an animal within the same minute were combined. All starting times of the visits as well as the duration of the visits were then rounded to whole minutes. For observations with a visit duration of < 30 seconds, a duration of 1 min per visit was assumed. With this transformation it was ensured that the sum of all intake rates corresponds exactly to the recorded quantities (see supplementary **Figure S3.11** for an example).

The raw pH and temperature data of the eCow measurement boluses were provided as 15 min summaries presented as a mean value by the devices. In order to transform these data into a 1-minute resolution, the time recorded in hours, minutes and seconds was rounded to whole minutes. Afterwards, the eCow pH and temperature mean values of the 15-min summaries were assumed for these time intervals. The resulting step-shaped pH and temperature curves were then smoothed with a moving average of a window length of 15 min (see supplementary **Figure S3.12** for an example).

The pH and temperature data collected with the LRCpH logger already had the required 1-minute data resolution. For the sake of comparability, the data were treated like the eCow data and first aggregated to 15-min summaries and then smoothed again with a moving average with a window length of 15 min.

The RumiWatch data converted to a 1-minute resolution were also prepared. Thereby, a new rumination variable was created, which was set to 1 for ‘rumination’ and else to 0.

The total amount of PMR, concentrate and water consumed per animal and day was calculated by aggregating the individual transponder observations. The resulting observations with a daily resolution were also included in the 1-minute resolution data set together with the daily milk yields and the daily average body weight.

Adjustment of pH Time Series. It is known that the *in vivo* pH measurement of ventral ruminal pH can underlie a random drift after a certain amount of time, which is why a calibration before and after the measurement with a LRCpH logger is common (Penner et al., 2006). However, the calibration of the intra-reticular eCow pH measurement boluses is not possible for the use intended by the manufacturer because they remain in the animal for the rest of its life. Since the cows examined here are rumen fistulated experimental animals, the eCow boluses could be exchanged between animals, so that the data can be adjusted for bolus-specific effects using linear mixed regression models to obtain adjusted pH values. In this procedure, we have taken into account further information on intake and thus the feeding group (C_{35} and C_{60}), DIM, milk performance and body weight of the individual animal. The body weight was used as a covariate because it represents animal-individual information and is highly correlated with the parity. For the adjustment of eCow pH measurements, the following multiple linear mixed regression model was applied:

$$\begin{aligned} pH_{ijkt} = {} & \beta_0 + \beta_1 \Sigma PMR_{it} + \beta_2 \Sigma C_{it} + \beta_3 \Sigma W_{it} + \beta_4 DIM_{it} + \beta_5 BW_{it} + \beta_6 MY_{it} + \\ & A_i + \gamma_{0jk} + \gamma_{1jk} TD_{it} + e_{ijkt} \end{aligned} \qquad [3.2]$$

where pH_{ijkt} is the continuously measured reticular pH of animal i at time point t, β_0 is the intercept, $\beta_1,...,\beta_6$ are the fixed regression coefficients, ΣPMR_{it}, ΣC_{it}, and ΣW_{it} are the daily consumed amounts of PMR, concentrate and water, respectively, DIM_{it} is the days in milk, BW_{it} is the average daily body weight and MY_{it} is the daily milk performance. The individual cow with repeated measurements is considered with a random normally distributed effect A_i. For the interaction of bolus j and month k, a random intercept γ_{0jk} and a random slope γ_{0jk} for time in days (TD) from the beginning of data recording was also considered in the model. Further, e_{ijkt} represents a normally distributed random error. After parameter estimation, the adjusted pH values were determined by removing bolus effects from the raw observations, leaving the variance attributed to the fixed effects, the random animal effects, and the residuals. Since the co-variable TD_{it} was taken into account on a daily basis, the curve remains unchanged within one day so that the adjustment can only result in a slight change of the daily mean pH value.

Since the pH value measurements for each individual animal were carried out using different LRCpH loggers these values could be adjusted by applying the following model:

$$\begin{aligned} pH_{ijt} = {} & \beta_0 + \beta_1 \Sigma PMR_{it} + \beta_2 \Sigma C_{it} + \beta_3 \Sigma W_{it} + \beta_4 DIM_{it} + \beta_5 BW_{it} + \beta_6 MY_{it} + \\ & A_i + \gamma_{0j} + TD_{it} + e_{ijt} \end{aligned} \qquad [3.3]$$

This model basically corresponds to the model from **Equation 3.2**, considering the j-th LRCpH logger as a random intercept γ_{0j}. The determination of the adjusted values was done in the same way as for the eCow data, so that only the variability was left, which was covered by the fixed effects, the random animal effects and residuals.

In this procedure no LRCpH logger effect could be determined, which is attributable to the before and after calibrations. In contrast, the eCow measurements showed considerable bolus effects, with drift also playing a significant role. The supplementary **Figure S3.13** allows a comparison of the raw and adjusted pH values. It can be seen that the adjustment did not cause any visible change in the data of the LRCpH loggers. In the eCow data, however, the drift of some boluses could be fixed by the adjustment.

Cross-correlation analysis was used to analyze the temporal relationship between reticular and ventral ruminal pH. The cross-correlation function ρ_{xy} between 2 time series x_t and y_t as a function of the time lag τ is defined as

$$\rho_{xy}(\tau)=\frac{\gamma_{xy}(\tau)}{\sqrt{\gamma_x(0)\gamma_y(0)}} \text{ with } -1\leq\rho_{xy}(\tau)\leq 1, \qquad [3.4]$$

where

$$\gamma_{xy}(\tau)=\operatorname{cov}(x_{t+\tau},y_t) \qquad [3.5]$$

is the cross-covariance function considering a time lag τ (Shumway and Stoffer, 2017). In addition to analyzing the general relationship between 2 time series, the cross-correlation function is able to analyze lagged relations. Thus, time lags between time series can be determined. Cross-correlation analysis was performed using the *ccf* function from the R basic package. As the pH and temperature respond more quickly to the animal's ingestion behavior in the reticulum than in the ventral rumen, the pH measurements in the ventral rumen were individually adjusted for the identified time lag.

Description of continuous measurements in the course of the day and in relation to each other

To describe the general pH progressions in the reticulum and ventral rumen, the 24-h pH profiles averaged over all days were calculated separately for each feeding regime (C_{35} and C_{60}) as mean and standard deviation for each minute of the day considering the subset of the data in which all measured variables were available. Furthermore, the relative frequency of observed PMR, concentrate and water recordings as well as rumination behavior were also determined per minute of the day to create 24-h profiles. To this end, feed and water intakes were transformed into a 1/0 coding in order to reduce the time series to the information whether an activity has taken place or not. Depending on the feeding group and the time of the day, the mean of each minute of the day was calculated over all test days. For comparability, these profiles were then scaled over both feeding groups with the following max-min transformation to the value range from 0 to 1:

$$T\left[x\right]=\frac{x-x_{\min}}{x_{\max}-x_{\min}} \qquad [3.6]$$

The previously described cross-correlation analysis in **Equations 3.4** and **3.5** was also used to investigate the general temporal relationships between the continuous measurements. To do so, the animal-specific cross-correlation functions for the pH and temperature in the reticulum and ventral rumen, the PMR, concentrate and water intake and the rumination behavior were calculated.

Modeling of Reticular and Ventral Ruminal pH

Since the diurnal pH course corresponds to a continuous and smooth progression, the time series with peak-like observations of feed and water intake rates as well as the rumination behavior in form of spike data had to be transformed. With regard to the amount of PMR, concentrate and water consumed, it is assumed that these not only influence the pH value immediately during or after intake, but also have a sustained and delayed effect over a period of time. Further, it is supposed that the effects of individual intakes accumulate over time and overlap in their effects on the ruminal fermentation and thus the pH course. To take these complex interrelations into account, an animal's individual intake rates were transformed using recursive time series filters as described by Shumway and Stoffer (2017). This recursive filter, which corresponds to a combination of an accumulation and exponential decrease of a time series, can be described as follows:

$$T\left[x\right]_t=x_t+\sqrt[60]{\alpha}\times T\left[x\right]_{t-1} \qquad [3.7]$$

where $T\left[x\right]_t$ is the transformed time series of the original intake rate x_t and $\alpha\in\left(0,1\right)$ corresponds to a rate of change per hour resulting in an exponential decrease. The cumulative part of this filter captures the accumulation of the further intakes in the course of time and the exponential decrease represents the decreasing contribution to the overall fermentation process over time. The latter one will thus include both the decrease in fermentation activity as well as passage rate effects. For better interpretation, the α was considered on an hourly basis, which requires the formation of the 60th root to meet the 1-minute data resolution. For example, $\alpha = 0.96$ corresponds to a rate at which the effect of the transformed PMR intake rate would decrease by 4% per hour. For better understanding, **Figure 3.2 A** illustrates simulated PMR recording rates and the result of 3 filters ($\alpha = 0.90$, $\alpha = 0.85$, and $\alpha = 0.80$) for a period of 8 h. One can see that the larger α is, the higher the curve tends to be. Additionally, the transformed intake rate increases in phases of numerous intake events and decreases exponentially between such events. This kind of filter was used to transform PMR, concentrate and water intake rates. The units of the transformed variables are not changed by the procedures described above. Since the rate of changes will be determined such they best explain the pH, the resulting transformed variables are artificially generated variables. Therefore, the units of these transformed variables are not further specified.

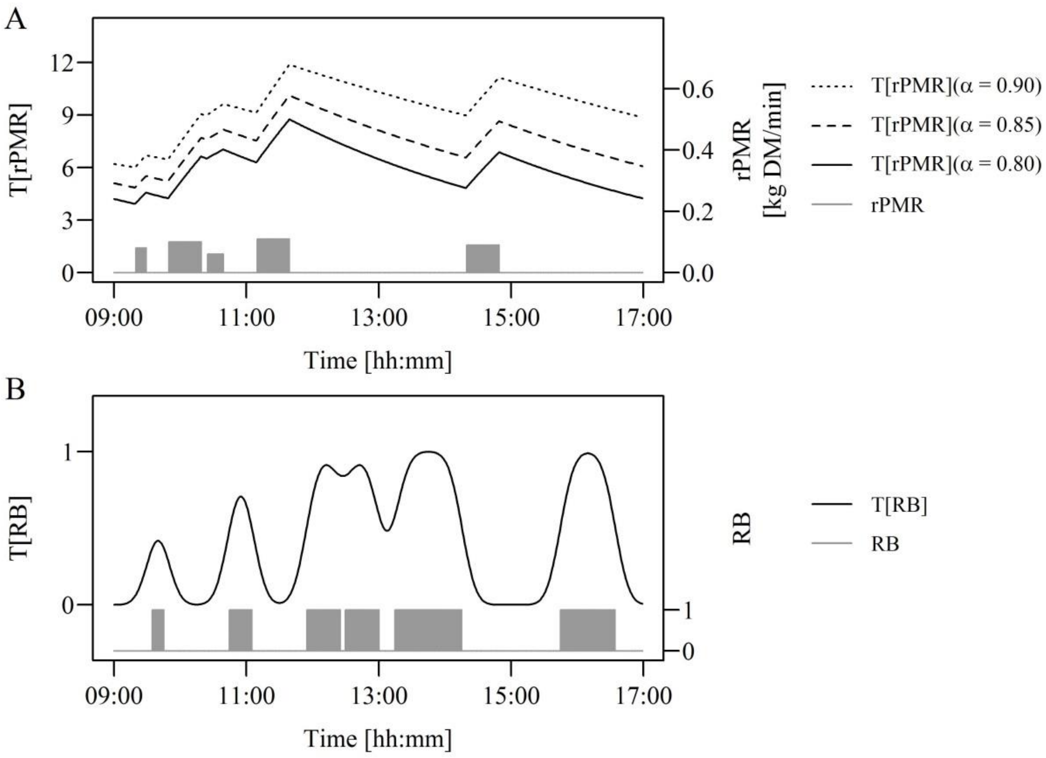

Figure 3.2: Examples for the signal transformation with recursive filters (A) and a symmetric Gaussian filter smoothing (B). Panel A shows the partial mixed ration (PMR) intake rates (rPMR, light gray bars) and the result of 3 recursive filters (T[rPMR]) with α as rate of change for the exponential decrease. Panel B illustrates the rumination observations (RB; light gray bars) and the result of filtering with the symmetric Gaussian filter with a window length of 61 min (T[RB]).

The graphical examination of the intake, pH and rumination time series showed that rumination (**RB**) has an immediate effect on the pH, expressed as a temporal increase. Therefore, RB coded as 1/0 was transformed to $\mathrm{T}\left[\mathrm{RB}\right]$ using a convolutional moving average smoother with a *Gaussian* kernel (Shumway and Stoffer, 2017) and a window length of 61 min, which caused the best fit in the later analysis. This transformation can be described as:

$$T\left[RB\right]_t = \sum_{i=0}^{b} w_i \times RB_{t-\frac{b}{2}+i} \qquad [3.8]$$

where $T\left[RB\right]_t$ is the transformed rumination behavior time series, RB_t is the raw rumination behavior time series, b is the window length of the symmetric kernel and w_i are the weights. The weights correspond to the scaled function values of a Gaussian curve from -3 to +3 standard deviations (**SD**) so that $\sum_{i=0}^{b} w_i = 1$. This transformation is illustrated in **Figure 3.2 B** using a simulated example.

The modeling of the reticular and ventral ruminal pH time series was carried out with the following linear mixed random intercept model:

$$\begin{aligned} pH_{it} = {} & \beta_0 + \beta_1 T\left[rPMR\right]_{it}\left(\alpha_{PMR}\right) + \beta_2 T\left[rC\right]_{it}\left(\alpha_C\right) + \beta_3 T\left[rW\right]_{it}\left(\alpha_W\right) + \beta_4 T\left[RB\right]_{it} + \\ & \beta_5 \Sigma PMR_{it} + \beta_6 \Sigma C_{it} + \beta_7 \Sigma W_{it} + \beta_8 DIM_{it} + \beta_9 BW_{it} + \beta_{10} MY_{it} + \\ & A_i + e_{it} \end{aligned} \qquad [3.9]$$

where pH_{it} is the adjusted continuously measured pH (eCow or Dascor) of the animal i at time point t, β_0 is the intercept and $\beta_1,...,\beta_{10}$ are the fixed regression coefficients, $T\left[rPMR\right]_{it}$, $T\left[rC\right]_{it}$ and $T\left[rW\right]_{it}$ are the transformed intake rates of the PMR, concentrate and water depending on the decreasing rates α_{PMR}, α_C and α_W. $T\left[RB\right]_{it}$ is the transformed rumination behavior. Further, ΣPMR_{it}, ΣC_{it}, and ΣW_{it} correspond to the total consumed amount of PMR, concentrate and water per day, DIM_{it} are the days in milk, BW_{it} is the mean body weight per day and MY_{it} is the daily milk yield. The individual cow is considered with a random effect A_i with $A_i \sim N\left(0,\sigma_A\right)$ and e_{it} is a random error with $e_{it} \sim N\left(0,\sigma_e\right)$. In this model the transformed feed and water intake rates, which are considered as independent variables are dependent on the respective rate of change α. In order to determine α_{PMR}, α_C and α_W so that the transformed time series best explains the pH time series, all combinations between 0 and 1 in 0.01 steps were tested via grid search. As criterion for the selection of the optimal parameters, the root mean square error (**RMSE**) of the model was used. The combination of α_{PMR}, α_C and α_W leading to the smallest RMSE was considered as the best fitting.

For the final models, the marginal R^2 ($\mathbf{R_m^2}$) and the conditional R^2 ($\mathbf{R_c^2}$) according to Nakagawa et al. (2017) were calculated using the estimated variance components. R_m^2 represents the explained variance of the fixed effects and R_c^2 stands for the explained variance of the entire model.

Although observations of continuous measurements during 21.8 ± 5.8 d with RumiWatch data, 34.5 ± 7.9 d with Dascor data and 58.1 ± 27.8 d with eCow data were available after the plausibility check per cow on average, only observations for which all time series were available were used in the final modeling. As shown in the Venn diagram in **Figure 3.3**, a total of 145 d of continuous and parallel records of all considered parameters from the 13 cows were available and included in the final analysis. The Venn diagram was created using the *draw.quad.venn* function from the VennDiagram (Chenn et al., 2018) package in R.

Unless otherwise stated, the entire data preparation, creation of figures and statistical analysis was done within the software environment R (R Core Team, 2019).

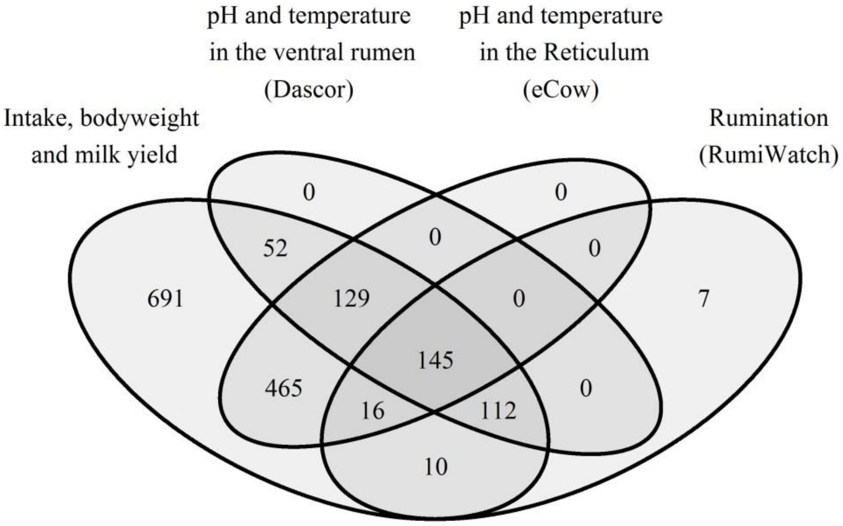

Figure 3.3: Venn diagram (Chenn et al., 2018)of available data quantity from various automated acquisition systems in days. eCow = eCow pH bolus (eCow Ltd., Exeter, UK); Dascor = LRCpH logger (Dascor Inc., Escondido, CA); RumiWatch = RumiWatch noseband halter (ITIN + HOCH GmbH, Liestal, Switzerland).

RESULTS AND DISCUSSION

Description of Continuous Measurements in the Course of the Day

In order to get a general overview of the daily pH progressions in the reticulum and in the ventral rumen as well as of the ingestion and rumination behavior, the time series were first visualized as 24-h profiles (**Figure 3.4**). The graphical representation was done separately for both feeding regimes (C_{35} and C_{60}). In addition, the time windows of the morning (05:30 to 07:30 a.m.) and evening (03:00 to 05:00 p.m.) milking as well as the daily supply of fresh feed between 11:00 and 12:00 a.m. were marked with vertical gray lines. The pH curves represent the adjusted pH values averaged as a function of the time of the day. It can be seen that the pH curves at both measuring sites are essentially comparable for both the C_{35} and the C_{60} group. The averaged curves of the reticular pH values for both groups are about 0.4 pH units higher than those of the ventral rumen. This tends to be in line with the results of Neubauer et al. (2018a), who found a difference of about 0.2 pH units at a similar pH range between the reticular pH and the pH of the free-rumen liquid. In addition, it can be seen that the progression of the ruminal pH has a higher variance compared to the reticular pH, which is also consistent with previous results (Falk et al., 2016).

The profiles of PMR, concentrate and water intake have common characteristics for both groups. The PMR intake is increased from the onset of feeding to shortly after evening milking. This is to be expected as freshly presented feed is preferred and stimulates the feed intake (DeVries et al., 2003; Oberschätzl-Kopp et al., 2016). With concentrated feed, the intake during the day seems to decrease progressively in particular in the C_{60} group. The cows of the C_{35} group already consumed their entitled amount of additional concentrated feed by noon. In comparison, the probability of visits in which concentrate is consumed decreases during the day in the C_{60} group. However, this is partly due to a restriction for the release so that an equal supply of concentrate feed supply is provided during the day. Nevertheless, it appears that the permitted amount of concentrated feed is consumed as early in the day as possible, which corresponds to a preference of the concentrate over the PMR and is associated with the displacement of roughage by additional concentrated feed (Lawrence et al., 2015). When it comes to water intake, it is noticeable that the peaks are similar to those of the PMR intake and, in the case of the C_{60} group, also to concentrate intake. This implies that the feed and water intake events are accompanied. In the average rumination behavior minima can be observed, especially during milking times and fresh feed offering. The phases of highest rumination activity were observed between the evening and early morning hours.

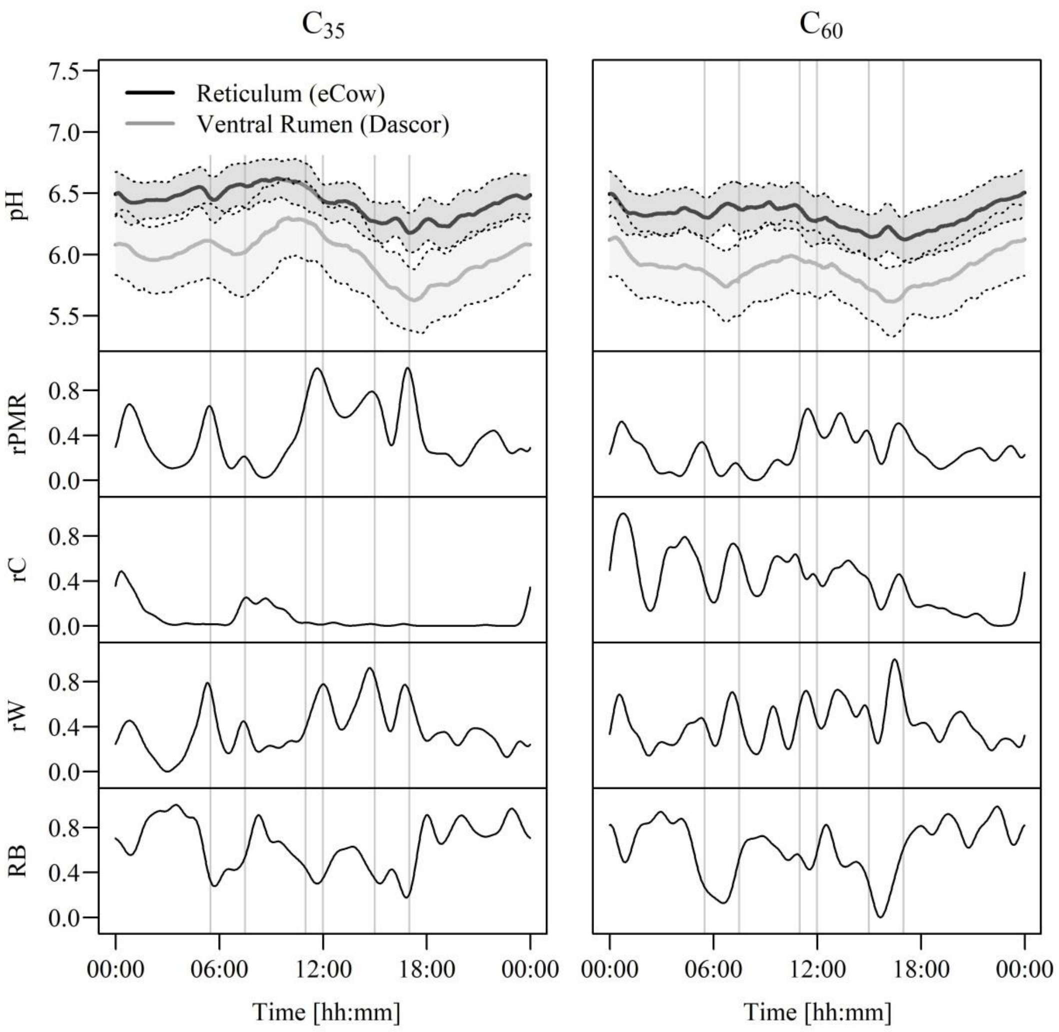

Figure 3.4: Averaged 24-h profiles of reticular and ruminal pH and behavior for 2 feeding groups (C_{35} = 35% concentrate; C_{60} = 60% concentrate) from the periods in which all measured values were available. The light gray areas around the curves correspond to the ±1 SD environment. The other 4 time series [rPMR = partial mixed ration (PMR) intake, rC = concentrate intake, rW = water intake, RB = rumination] correspond to the relative frequencies scaled from 0 to 1 for the respective activity depending on the time of day. The vertical lines indicate the time spans of morning (0530 to 0730 h) and evening milking (1500 to 1700 h) as well as of the fresh feed supply (1100 to 1200 h). eCow = eCow pH bolus (eCow Ltd., Exeter, UK); Dascor = LRCpH logger (Dascor Inc., Escondido, CA).

It is conspicuous that the ingestion behavior shows a local maximum shortly after 00:00 a.m. This can be explained by the calibration of the weighing troughs, which took place between 11:45 p.m. and 00:00 a.m. In the course of this calibration, the pneumatic hatches lead to a development of noise, which presumably triggers the cow’s behavior. In this context, the peak before milking in the morning should also be mentioned. Since the experiment took place in the winter months, the light was switched on every morning at 05:00 a.m. about 30 min before a.m. milking. This also seems to influence the behavior of the animals as well as the pH progressions.

From **Figure 3.4** it can also be derived that the intake of PMR in particular has an effect on the pH value. During the phases with the highest probability (shortly after midnight, before a.m. milking and between fresh feed supply and until shortly after p.m. milking) of PMR intake, the pH value in both the reticulum and the ventral rumen tend to decrease in both feeding groups, whereas the pH value tends to increase in phases with a low probability of intake. This is in line with the effect of concentrate intake and can particularly be observed in the C_{60} group in the first half of the day. Comparing the pH progressions in the reticulum and ventral rumen for the 2 feeding groups, it is noticeable that especially the pH progressions in the reticulum are similar, but the pH of the C_{60} group tends to decrease slightly more in the morning hours than in the C_{35} group, which might be due to the different amounts of concentrate feed intake at this time.

Temporal Relationship between the Continuous Measurements

The calculated cross-correlation functions of the animals between the time series are shown in **Figure 3.5** for $-120\,\mathrm{min} \le \tau \le 120\,\mathrm{min}$. The cross-correlation functions of the 2 pH time series show that they are generally positively correlated in the observed range of τ (pH (eCow) vs. pH (Dascor)). In addition, for all functions a local maximum at -37 ± 24 min can be determined. This means that the reticular pH of 37 min before is maximally correlated with the current ruminal pH or that the ruminal pH value is delayed by this time lag. A comparable time delay can be observed in the results of Falk et al. (2016; Figure 1). Consequently, adjusting the ruminal pH values regarding the time lag determined individually for each animal is justifiable.

Nevertheless, it is not clear to what extent the identified time lag can be attributed to the fact that ruminally fistulated cows were used, in which the fistula could partially affect the motility of the rumen due to its punctual fixation to the rumen wall. Furthermore, a gas exchange between reticulorumen and the atmosphere caused by general leakage and the opening and closing of the fistula is expected. The resulting reduction of the carbon dioxide partial pressure in the forestomach system can lead to an increase of the reticuloruminal pH and therefore affect the bicarbonate-based buffer system (Kohn and Dunlap, 1998). Strictly speaking, the results generated here are therefore only valid for ruminally fistulated cows. However, past studies (e.g., Hayes et al., 1964; MacRae and Wilson, 1977) comparing fistulated and non-fistulated ruminants have shown that there are, if at all, only minor differences in behavior or digestibility. Thus, it can be assumed that the results are transferable to non-fistulated cows.

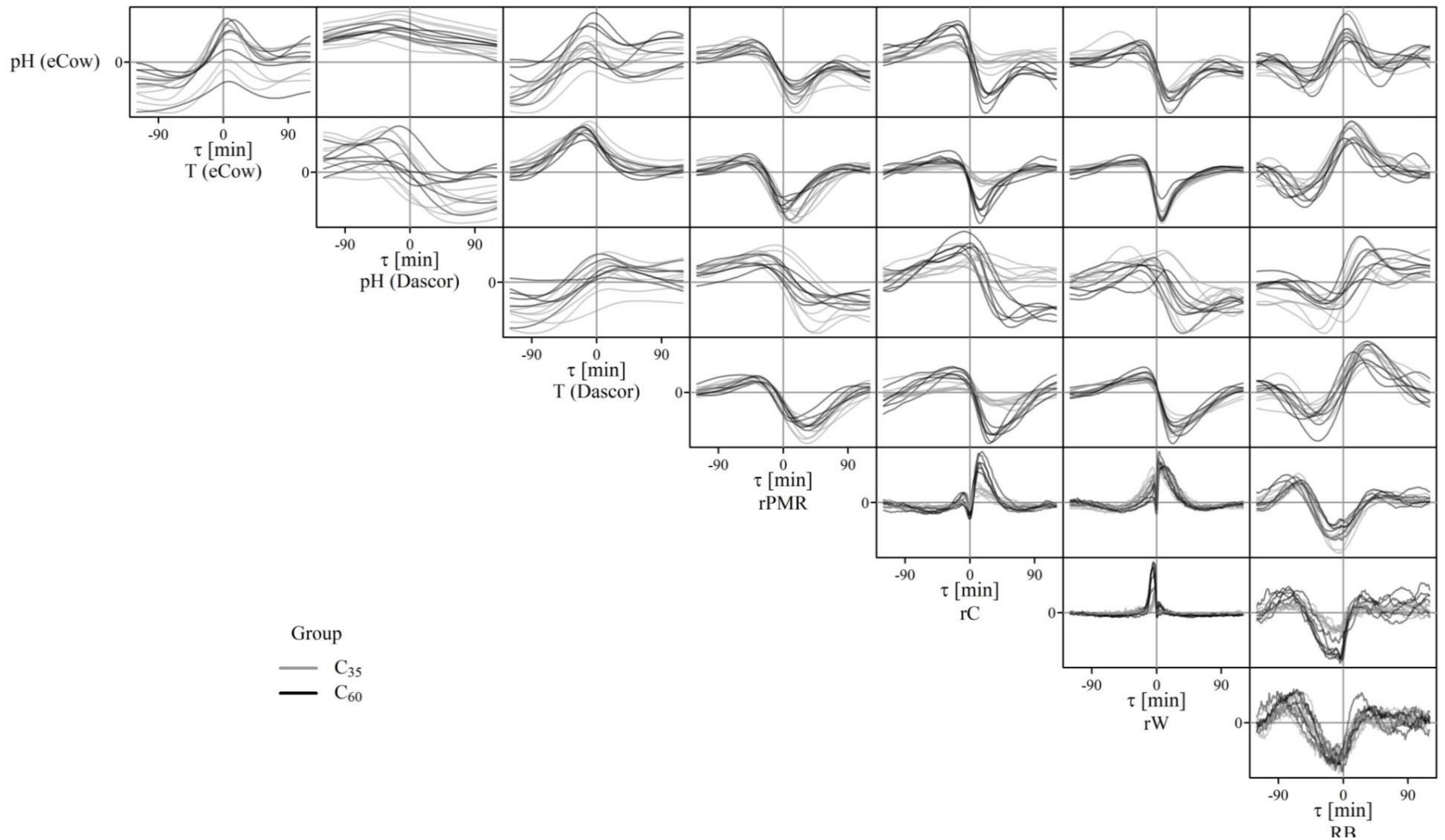

Figure 3.5: Pairwise visualization of cow-specific cross-correlation functions of 8 time series for a time lag of −120 min ≤ τ ≤ 120 min with the feeding group affiliation being illustrated by 2 different gray scales (C_{35} = 35% concentrate; C_{60} = 60% concentrate). The cow-specific curves display the correlation between 2 different time series x (variable name to the left of the row) and y (variable name at the bottom of the column) when the time series x is shifted by the time interval τ (see **Equation 3.4**). Interpretation example for x = T(eCow) and y = T(Dascor): From the uniform local maximum of the 13 curves at approximately τ = −20 min, it can be seen that the temperature in the reticulum recorded with the eCow bolus 20 min ago is maximally correlated with the current temperature recorded with the Dascor in the ventral rumen. TpH (eCow) and pH (Dascor) = reticular and ruminal pH; T(eCow) and T(Dascor) = reticular and ruminal temperature; rPMR = intake rate of partial mixed ration (PMR); rC = intake rate of concentrate; rW = intake rate of water; RB = rumination behavior; eCow = eCow pH bolus (eCow Ltd., Exeter, UK); Dascor = LRCpH logger (Dascor Inc., Escondido, CA).

Further, the cross-correlation functions show that both the reticulum and the ventral rumen experience a drop in temperature after water intake for both feeding groups (T (eCow) vs. rW, T (Dascor) vs. rW) The temperature drop in the reticulum is almost immediate and slightly delayed in the ventral rumen and can be explained by the consumed water, the temperature of which (approximately 8 to 15°C) is far below the body temperature of 39°C. The temporal difference as well as the temperature change can be explained by the anatomy of the cow: the rumen of lactating cows, with a volume of 102-148 l (Budras and Wünsche, 2002), accounts for the largest proportion of the forestomach system, whereas the reticulum is considerably smaller. This difference in size will therefore make the rumen a more inert system for a change of pH and temperature. In addition, the reticulum is closer to the esophagus, which is why the eCow boluses also remain there after the oral insertion. Bewley et al. (2008) also determined that the reticular temperature decreases depending on the amount and temperature of the water, especially in the first 15 min after water intake.

A similar relationship can also be observed between temperature, PMR and concentrate intake (T (eCow) vs. rPMR, T (Dascor) vs. rPMR, T (eCow) vs. rC, T (Dascor) vs. rC). Since the experiment was carried out in the winter, the temperature of the PMR and concentrate was presumably much lower than the body temperature and therefore led to a temperature drop in the reticulum and in the ventral rumen immediately after ingestion.

The analysis with cross-correlations also showed that rumination leads directly to an increase in reticular pH and a delayed increase in ventral ruminal pH (pH (eCow) vs. RB and pH (Dascor) vs. RB). This was to be expected and is due to the increased swallowing of saliva with a pH value of approx. 8.2 during the rumination (Aschenbach et al., 2011).

Furthermore, behavior patterns can be derived: Water intake often follows a previous concentrate intake (rC vs. rW). With regard to PMR and water intake events, it can be seen that water intakes are generally accompanied by PMR intakes, where especially the PMR intakes in the C_{60} group follow more often water intakes (rPMR vs. rW). PMR and concentrate feed intake also tend to coincide, with concentrate feed being consumed more frequently before PMR (rPMR vs. rC).

Modeling of Reticular and Ventral Ruminal pH Progressions

The final analysis of the reticular and ruminal pH course was done using the model presented in **Equation 3.9**. The results of the grid search for the determination of α_{PMR}, α_C and α_W are illustrated in **Figure 3.6**. For the sake of illustration and a better comparability, the ratio between the SD of the pH measurements and the RMSE of the respective model are plotted in 3D wireframe surface plots (Sarkar, 2008) each dependent on 2 rates of change. The higher the ratio, the better the fit of the model. **Figure 3.6 A** and **C** show that there is a unique local maximum at $\alpha_{PMR} = 0.85$, $\alpha_C = 0.91$ and $\alpha_W = 0.86$ for the modeling of the reticular pH. Furthermore, it can be inferred that the change in the α_{PMR} has the highest impact on the fit statistic and therefore the entire modeling. The second largest influence is exerted by α_C. The modeling of the ruminal pH value showed a slightly different relationship (**Figure 3.6 B** and **D**). The local maximum is at $\alpha_{PMR} = 0.87$, $\alpha_C = 0.89$ and $\alpha_W = 0.42$. The change of α_{PMR} shows again the highest impact on the fit statistic. The difference is that the variation of α_W had almost no influence. Furthermore, the SD/RMSE is generally lower than in the grid search for the reticular pH.

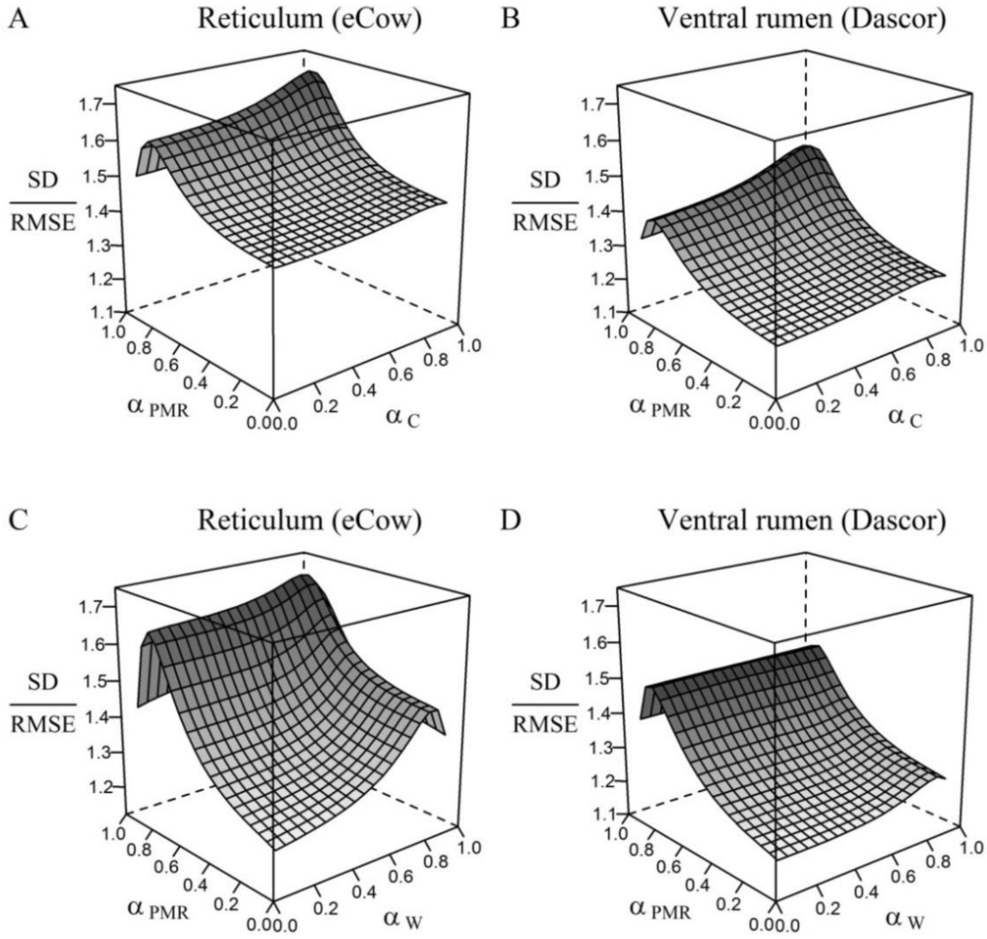

Figure 3.6: Results of the grid search to determine the optimal rates of change (α_{PMR}, α_C, and α_W, respectively) for the recursive filtering of partial mixed ration (PMR), concentrate and water intake for the modeling of the reticular (panels A + C) and ruminal (panels B + D) pH development. Visualized are the ratios between the standard deviation (SD) and root mean square error (RMSE) of the linear mixed models depending on the rate of change for α_{PMR}, α_C, and α_W with 3-dimensional wireframe surface plots (Sarkar, 2008). eCow = eCow pH bolus (eCow Ltd., Exeter, UK); Dascor = LRCpH logger (Dascor Inc., Escondido, CA).

However, the interpretation of the rates of change (α_{PMR}, α_C and α_W) is not trivial. As explained in the Material and Methods section, this is an abstract quantity which is presumably associated with the fermentation properties and the passage rate of the feed through the rumen. Interestingly, the estimated values of α_{PMR} and α_C (0.85 and 0.87, respectively 0.91 and 0.89), which correspond to an hourly rate of change of 0.15 and 0.13, respectively 0.09 and 0.11, are in the same order of magnitude as estimated ruminal degradation rates of the dietary dry matter intake. For example, Hatew et al. (2015) estimated fractional degradation rates between 0.043 and 0.139 per hour for the organic matter part of the ration. In comparison, Maxin et al. (2013) also determined similar degradation rates of DM for different protein-containing dietary supplements with values between 0.05 and 0.09.

The estimates of the regression coefficients for the transformed time series of PMR and concentrate intake have negative signs in both models (**Table 3.1**). The sign of the transformed water intake is also negative for the reticular pH in the modeling, whereas it is positive for the ruminal pH. In both cases the sign for the transformed rumination behavior is positive from which it can be concluded that rumination leads to a temporary increase in pH values which is in line with the results presented in **Figure 3.5**. As examples, these complex relationships are illustrated in **Figure 3.7 A** and **B** by showing the pH curves, the intake rates and rumination behavior as well as their transformations for 2 cows (one from each feeding group; panel A = C_{35}, panel B = C_{60}) over a period of 48 h. Both cows were in the 6th parity. The figures show that the pH curve essentially corresponds to a combination of the reverse transformed PMR and concentrate intake rates, which is in line with the signs of regression coefficients from the models. This means that the pH during feed intake decreases and increases progressively more slowly in the time after intakes (see **Figure 3.7 A** and **B**).

The interpretation of the significances and the F-values has to be done with care. Due to the very large sample size caused by the temporal resolution, the F-values are extremely large, so that even the smallest effects become significant. It is therefore helpful to consider the ratio between the F-values of the covariables. The F-values show that $T[rPMR]$ and $T[rC]$ in particular contribute substantially to the explanation of the variance for both pH measurements. In contrast to the modeling of the ventral ruminal pH, $T[rW]$ and $T[RB]$ also explain a considerable amount of the variance of the reticular pH. Furthermore, the lactation stage has an influence on the reticular pH. The other variables which were measured on a daily basis show

Table 3.1: Estimates of regression coefficients (b), transformation traits, and model statistics of linear mixed models for reticular and ruminal pH (n = 13 animals, in total 145.3 d of continuous measurements)[1]

	Reticulum (eCow)				Ventral rumen (Dascor)			
Variable[2]	b	SE		*F*-value	b	SE		*F*-value
Intercept	6.87437	0.02681	***		6.40964	0.07168	***	
T[rPMR], kg	-0.07508	0.00025	***	86982.6	-0.13304	0.00037	***	127390.3
T[rC], kg	-0.09562	0.00056	***	29343.1	-0.19145	0.00102	***	35374.5
T[rW], kg	-0.00877	0.00006	***	23870.2	0.00340	0.00013	***	642.2
T[RB]	0.09829	0.00082	***	14439.7	0.06733	0.00147	***	2090.2
ΣPMR, kg/d	0.00930	0.00013	***	5082.2	0.02694	0.00022	***	14357.8
ΣC, kg/d	0.01093	0.00054	***	413.6	0.02982	0.00092	***	1049.6
ΣW, kg/d	0.00095	0.00003	***	1042.0	-0.00187	0.00005	***	1555.2
DIM, d	0.00407	0.00002	***	48739.7	0.00123	0.00003	***	1430.1
BW, 100 kg	-0.08243	0.00254	***	1052.6	-0.07939	0.00448	***	313.6
MY, kg/day	0.00749	0.00011	***	4360.5	0.01433	0.00020	***	5156.8
Rate of change								
α_{PMR}	0.85				0.87			
α_{C}	0.91				0.89			
α_{W}	0.86				0.42			
Model statistic								
RMSE	0.124				0.217			
SD	0.212				0.326			
SD/RMSE	1.719				1.501			
σ_A	0.075				0.235			
σ_e	0.124				0.217			
R^2_m, %	67.0				37.8			
R^2_c, %	76.0				71.4			

[1] eCow = eCow pH bolus (eCow Ltd., Exeter, UK); Dascor = LRCpH logger (Dascor Inc., Escondido, CA).

[2] T[rPMR], T[rC], and T[rW] = transformed intake rates of partial mixed ration (PMR), concentrate, and water; T[RB] = transformed rumination behavior; ΣPMR, ΣC, and ΣW = daily amounts of PMR, concentrate, and water; MY = daily milk yield; α_{PMR}, α_C, and α_W = rates of change for the recursive filtering of PMR, concentrate, and water intake rates; RMSE = root mean square error; SD/RMSE = ratio between SD and RMSE; σ_A = standard deviation of random animal effects; σ_e = standard deviation of random errors; R^2_m = marginal R-squared, R^2_c = conditional R-squared.

***$P < 0.001$.

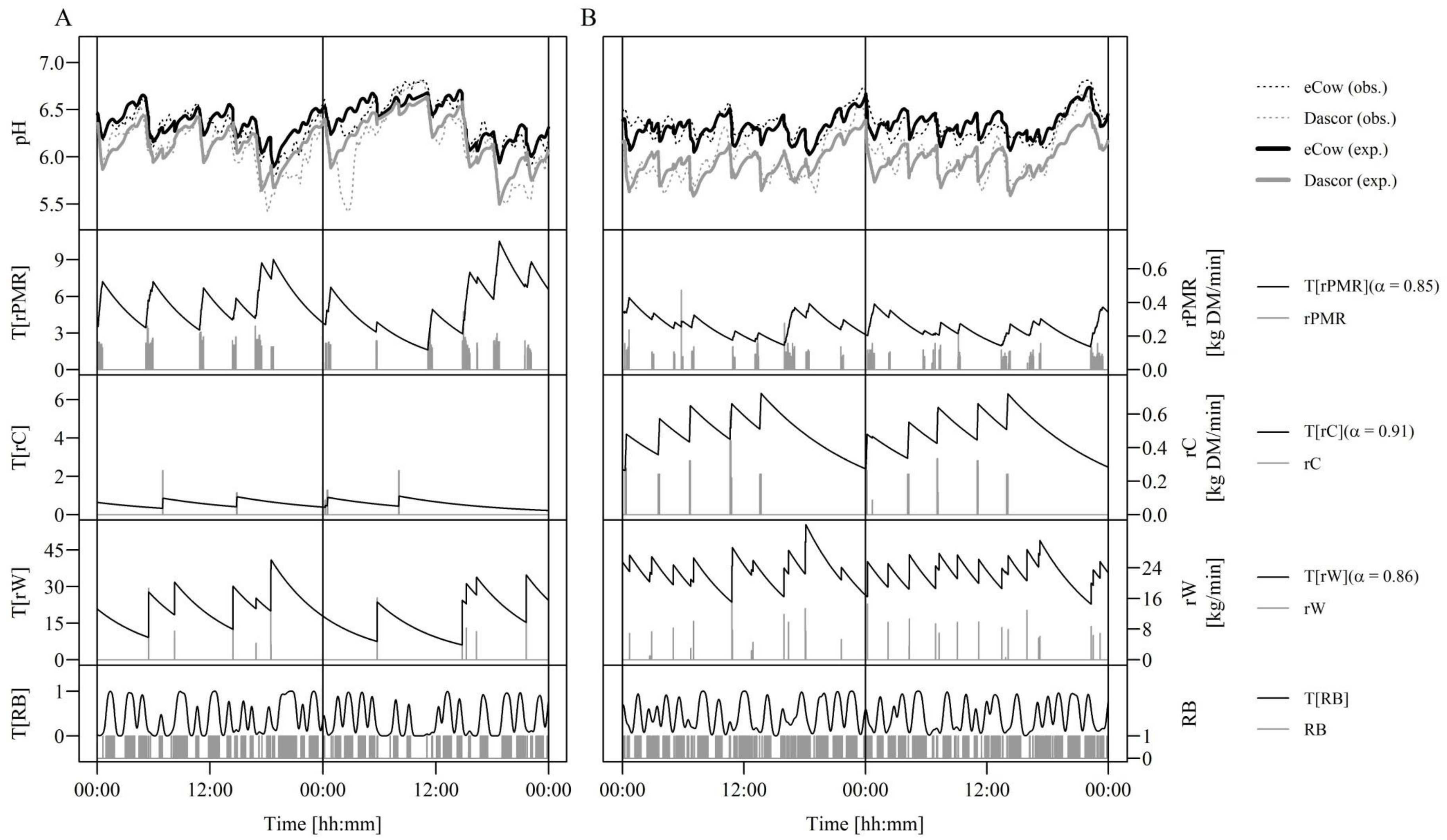

Figure 3.7: Examples for the observed time-series data of a cow from the C_{35} group (A, 35% concentrate) and from the C_{60} group (B, 60% concentrate) for 48 h. Shown are the reticular and ruminal pH progressions, the partial mixed ration (PMR), concentrate, and water intake rates (rPMR, rC, and rW) as well as rumination behavior (RB). In addition, the transformed intake rates T[rPMR], T[rC], T[rW] and the transformed rumination behavior T[RB] were added as black lines. The pH values predicted by the linear mixed models are included as bold lines. α = rate of change for the recursive filtering; eCow = eCow pH bolus (eCow Ltd., Exeter, UK); Dascor = LRCpH logger (Dascor Inc., Escondido, CA).

only small effects, except for the daily consumed amount of PMR in the modeling of the ruminal pH. The 3 regression coefficients for $\Sigma\mathrm{PMR}$, $\Sigma\mathrm{C}$, and $\Sigma\mathrm{W}$ are generally difficult to interpret, since the covariables are also partly represented in the transformed time series $\mathrm{T}[\mathrm{rPMR}]$, $\mathrm{T}[\mathrm{rC}]$ and $\mathrm{T}[\mathrm{rW}]$ and were only included in the model for correction purposes.

The proportion of explained variance of the fixed effects is high for the reticular pH (R_m^2 = 67.0%) and moderate for the ruminal pH (R_m^2 = 37.8%). The variance explained by the entire model has relatively high values with R_c^2 = 76.0% and R_c^2 = 71.4% for both models indicating a good fitting of the models to the data. This also becomes evident when comparing the observed with the predicted values by the model in **Figure 3.7 A** and **B**. The reason that more variance of the reticular pH can be explained by the fixed effects may also be due to the aforementioned fact that the reticulum, due to its smaller size and proximity to the esophagus, reacts faster and is less inert than the ventral rumen. Further, the individual-associated variance is higher at the ruminal pH than at the reticular pH making the absolute pH level and therefore the ruminal daily mean a parameter that is difficult to predict. Nevertheless, the ruminal pH is decisive for the digestive disorder SARA.

The results exhibit that the entire behavior patterns during the day determines the individual reticular and ruminal diurnal pH course of the animal substantially. Thereby, the behavior is influenced by management related factors, such as different milking and feeding times and presumably all other factors that either stimulate or suppress feed intake behavior, e.g. claw disorders, mastitis or other diseases. The fact that the behavior of the animal is directly reflected in the pH is also in line with the results of Macmillan et al. (2017), who found that cows, classified as having a higher risk for SARA, ingested feed less evenly during the day than the low risk cows. It was also found that low and high SARA risk cows differed in sorting behavior (Coon et al., 2019). The low risk cows sorted less feed and thus ate a more balanced diet than cows classified as high risk cows.

A further goal of the modeling was to split the variance in pH caused by feed intake and rumination. However, this is not possible with the modeling applied here. The reason for this is that to a certain extent there is a confounding between feed intakes, respectively the non-feed intakes and rumination, because at a certain time point only one activity is possible. As can be seen in **Figure 3.7 A** and **B**, the probability of rumination is very high when there is no ingestion. For this reason, the transformed feed intake, which is included in the model with a negative coefficient and therefore increases between intake events, will also capture the increase in pH caused by rumination.

The neutralization of the pH by saliva and the release of short chain fatty acids by fermentation were to a certain extent captured by the presented modeling. However, it is not yet clear to what extent the animal-specific mechanisms of absorption and buffering by bicarbonate secreted via the rumen wall contribute to pH development. These 2 mechanisms are difficult to quantify and could vary from animal to animal, which makes the ruminal pH such a complex parameter and hard to estimate.

Although the pH progression in the cow’s forestomach system is the result of a complex and dynamic regulation by endogenous and exogenous factors, this modeling shows an enormous potential to explain the pH development in the course of the day. Since both the pH value in the reticulum and the pH value in the ventral rumen can essentially be modeled using feed intake behavior, a causal relationship between the 2 measurement locations is confirmed.

CONCLUSIONS

The rumen is a complex system in which a multitude of physiological processes take place. The present work indicates that methods which are uncommon in animal sciences, such as high-resolution time series data or the application of signal transformations, can offer new insights into complex physiological processes. In the current study, the collected data were not reduced to parameters on a daily basis, but were considered to full extent in order to elucidate the reticular and ruminal pH development. It was demonstrated that both pH progressions in the course of the day can be modeled using animal transponder based individual ingestion and sensor based rumination behavior. In this approach 67.0% of the variance of reticular and 37.8% of the variance of ventral ruminal pH could be explained by applying linear mixed models on 1-minute resolution time series data. Although all animals were subject to the same management and daily routine, the differing pH profiles could be attributed to the individual temporal distribution of feed and water intake as well as the rumination during the day. Up to our knowledge, the investigated physiological relationships in this study, which are generally well known, were quantified for the first time with such a high degree of precision with the help of these innovative approaches. In a further step, these methods could also be used to investigate the relationship between the 2 pH measurement sites in the reticulum and the ventral rumen in more detail.

The use of time series data is a powerful approach because time delays between different measures can be captured and quantified. Therefore, such approaches should be considered for the investigation of physiological processes in future studies. Also with regard to precision livestock farming, which increasingly relies on automated data acquisition, e.g. with automated milking or heat detection systems, the enhanced consideration of the longitudinal nature of these data has the potential to improve the herd management.

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AUTHOR CONTRIBUTIONS

Conceptualization: AM. Data curation: KB, AM. Formal Analysis: AM. Funding acquisition: JH. Investigation: KB, UM, DvS. Methodology: AM, AOS. Project administration: JH. Resources: SD, UM. Software: AM. Supervision: ARS, JH, SD. Visualization: AM. Writing – original draft: AM. Writing – review and editing: AM, KB, UM, DvS, JH, AOS, ARS, SD.

SUPPLEMENTARY

Table S3.2: Information on concentrates and the chemical composition of the feed[1]. Adapted from Bünemann et al. (2019)

Component [g/kg FM]	Concentrates		Roughage (silage)		Diet	
	C_{35}	C_{60}	Maize	Grass	C_{35}	C_{60}
Rapeseed meal	400	200				
Wheat	150	213				
Barley	144	213				
Maize	200	290				
Dried sugar beet pulp	50	50				
Urea	8	-				
Calcium carbonate	13	12				
Soybean oil	10	10				
Vitamin-mineral premix[2]	25	12				
Chemical composition						
DM, g/kg FM	878	878	361	306		
Crude ash, g/kg DM	76	55	40	94		
Crude protein, g/kg DM	239	170	82	127		
Ether extract, g/kg DM	48	48	32	33		
Crude fiber, g/kg DM	85	66	203	273		
$aNDF_{om}$, g/kg DM	208	178	401	526		
ADF_{OM}, g/kg DM	125	96	230	297		
Starch, g/kg DM	368	490	331	0	285	353
ME[3], MJ/kg of DM	12.3	12.8	10.8	10.4	11.3	11.8
NE_L[3], MJ/kg of DM	7.7	8.1	6.5	6.2	6.9	7.3

[1] $aNDF_{om}$ = neutral detergent fiber after amylase treatment and ashing; ADF_{OM} = acid detergent fiber after ashing; C_{35} = group with 35% concentrate on DM basis; C_{60} = group with 60% on DM basis.

[2] Mineral feed for lactating dairy cows, ingredients per kg according to the manufacturer's specifications: 140 g Ca; 120 g Na; 70 g P; 40 g Mg; 6 g Zn; 5.4 g Mn; 1 g Cu, 100 mg I; 40 mg Se; 25 mg Co; 1,000,000 IU vitamin A; 1,000,000 IU vitamin D3; 1500 mg vitamin E.

[3] Calculation based on equations of the German Society for Nutritional Physiology (GFE, Empfehlungen zur Energie-und Nährstoffversorgung der Milchkühe und Aufzuchtrinder; DLG-Verlags-GmbH: Frankfurt am Main, Germany, 2001).

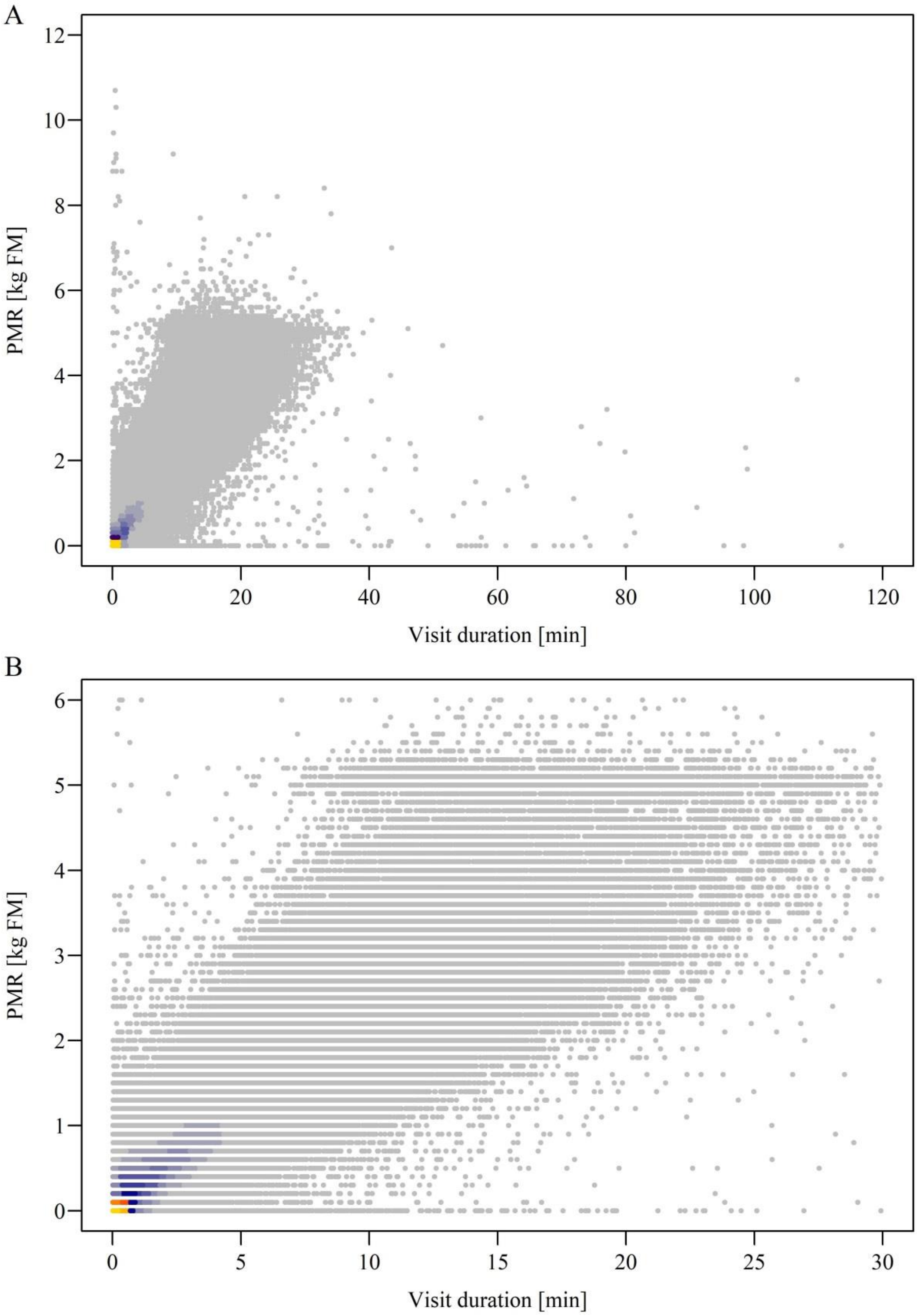

Figure S3.8: Transponder-assisted observations of partially mixed ration (PMR) intake of all 60 animals over the entire experimental period (n = 428,574). The presumably ingested amount of PMR per visit at the weighing trough is shown as a function of the visit duration in a heatscatter plot. Panel B corresponds to a zoomed-in version of Panel A. Gray represents a low density of points, while the color gradient from blue to yellow indicates an increase of density. FM = fresh matter.

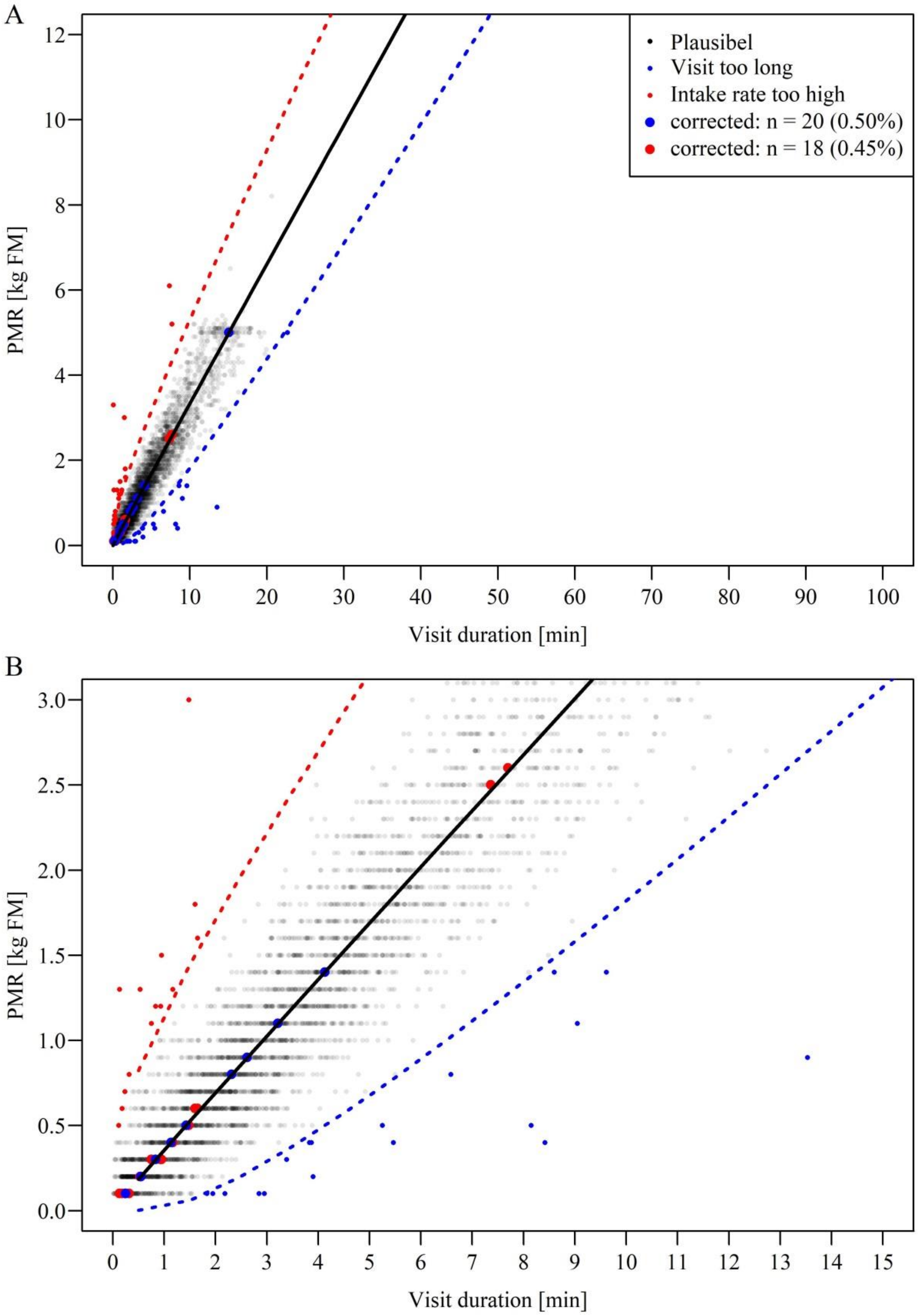

Figure S3.9: Transponder-assisted observations of partially mixed ration (PMR) intakes > 0 of one animal from the C_{35} group over the entire experimental period. The presumably ingested amount of PMR per visit at the weighing trough is shown as a function of the visit duration using a scatter plot (n = 4,372). Panel B shows a zoomed-in version of Panel A. The black bold line corresponds to the back transformed regression curve from the SMA model (**Equation 3.1**). The red and blue dotted lines represent the ± 3 SD border. In addition to the values classified as outliers were marked in color (small and blue dots) and the corrected values (bold red and blue dots) have been added. For more information, see section ‘Plausibility Assessment of Transponder Data’. FM = fresh matter.

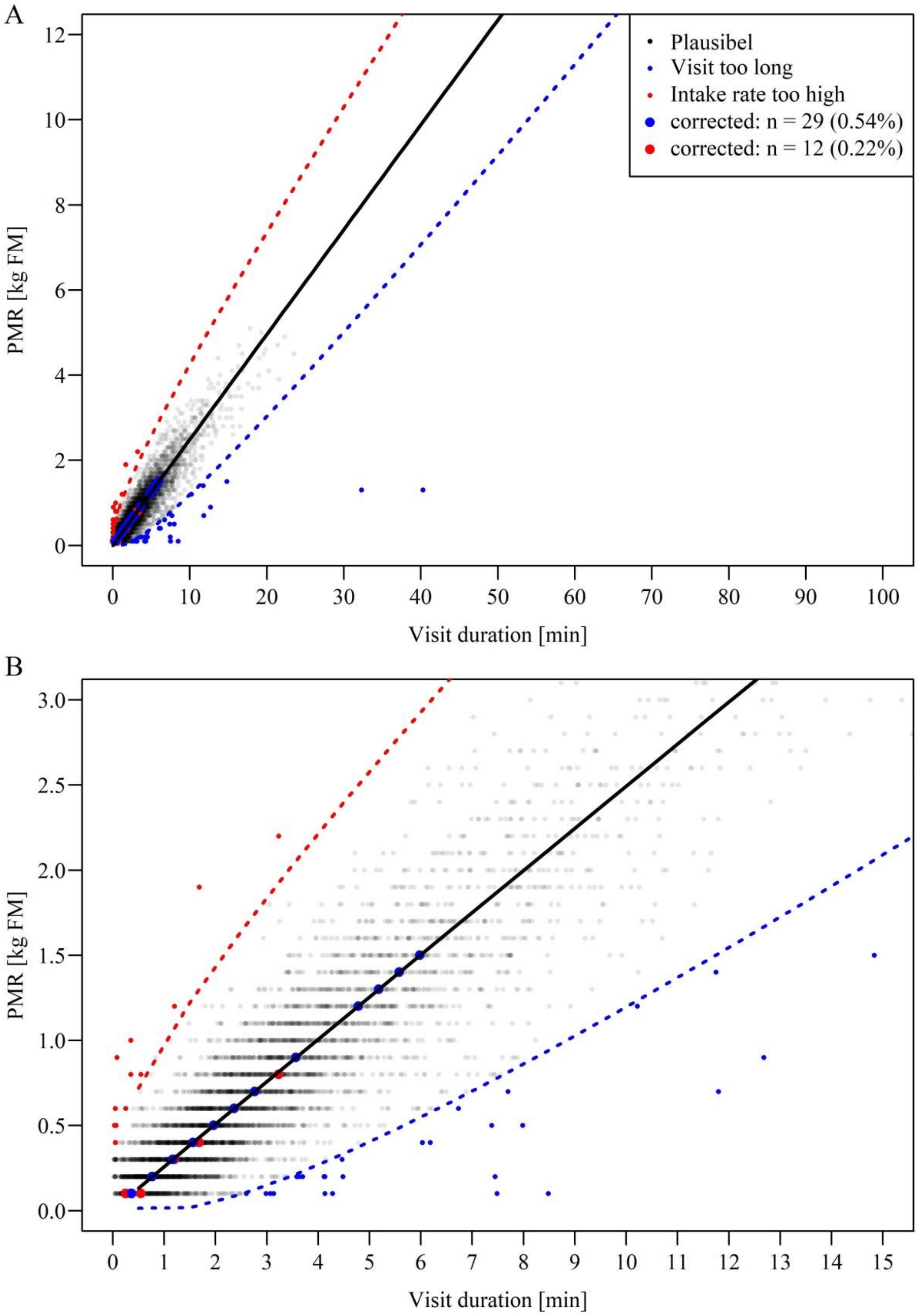

Figure S3.10: Transponder-assisted observations of partially mixed ration (PMR) intakes > 0 of one animal from the C_{60} group over the entire experimental period. The presumably ingested amount of PMR per visit at the weighing trough is shown as a function of the visit duration using a scatter plot (n = 3,705). Panel B shows a zoomed-in version of Panel A. The black bold line corresponds to the back transformed regression curve from the SMA model (**Equation 3.1**). The red and blue dotted lines represent the ± 3 SD border. In addition to the values classified as outliers were marked in color (small and blue dots) and the corrected values (bold red and blue dots) have been added. For more information, see section ‘Plausibility Assessment of Transponder Data’. FM = fresh matter.

Start time	End time	Start Weight [kg DM]	End weight [kg DM]	Visit duration [s]	PMR intake [kg DM]
10:22:13	10:28:58	12.1	11.5	405	0.6
10:31:08	10:34:24	11.5	11.2	196	0.3
10:38:47	10:43:05	11.2	10.8	258	0.5

↓ Time rounding and calculation of PMR intake rates

Start time	End time	Start Weight [kg DM]	End weight [kg DM]	Visit duration [min]	PMR intake [kg DM]	PMR intake rate [kg/min]
10:22:00	10:29:00	12.1	11.5	7	0.6	0.086
10:31:00	10:34:00	11.5	11.2	3	0.3	0.100
10:39:00	10:43:00	11.2	10.8	4	0.5	0.120

↓ Transformation to 1-min resolution

Time	PMR intake rate [kg/min]
…	…
10:22:00	0.086
10:23:00	0.086
10:24:00	0.086
10:25:00	0.086
10:26:00	0.086
10:27:00	0.086
10:28:00	0.086
10:29:00	0
10:30:00	0
10:31:00	0.100
10:32:00	0.100
10:33:00	0.100
10:34:00	0
10:35:00	0
10:36:00	0
10:37:00	0
10:38:00	0
10:39:00	0.120
10:40:00	0.120
10:41:00	0.120
10:42:00	0.120
10:43:00	0
…	…

Figure S3.11: Example of the plausibility checked transponder data for the PMR intake and the transformation to a 1-min resolution.

Time	pH	Temperature [°C]
10:14:20	6.36	38.7
10:29:20	6.27	35.1
10:44:20	6.16	36.2
10:59:20	6.26	37.2
11:14:20	6.29	37.6
11:29:20	6.37	38.0

↓ Time rounding

Time	pH	Temperature [°C]
10:14:00	6.36	38.7
10:29:00	6.27	35.1
10:44:00	6.16	36.2
10:59:00	6.26	37.2
11:14:00	6.29	37.6
11:29:00	6.37	38.0

↓ Transformation of 15-min summary mean values (black dots) to 1-min resolution (black line) and moving average smoothing (green line)

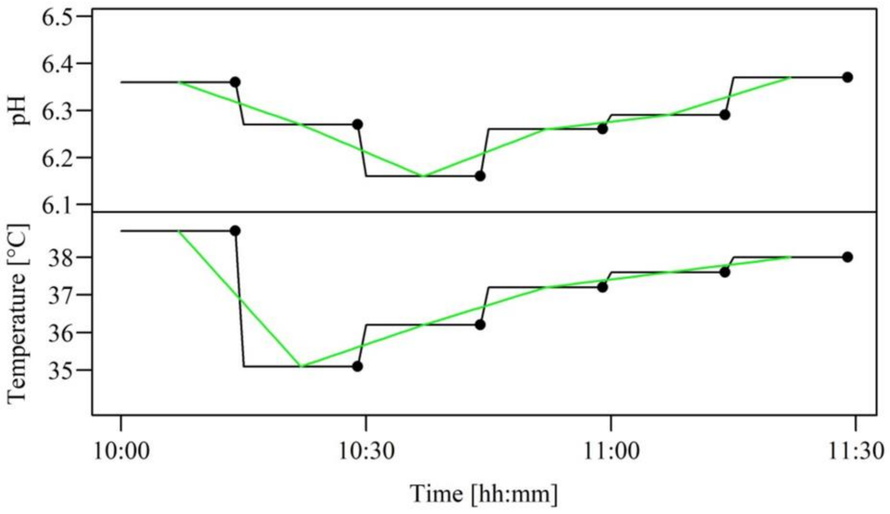

Figure S3.12: Example of raw eCow pH and temperature data and the transformation to a 1-min resolution.

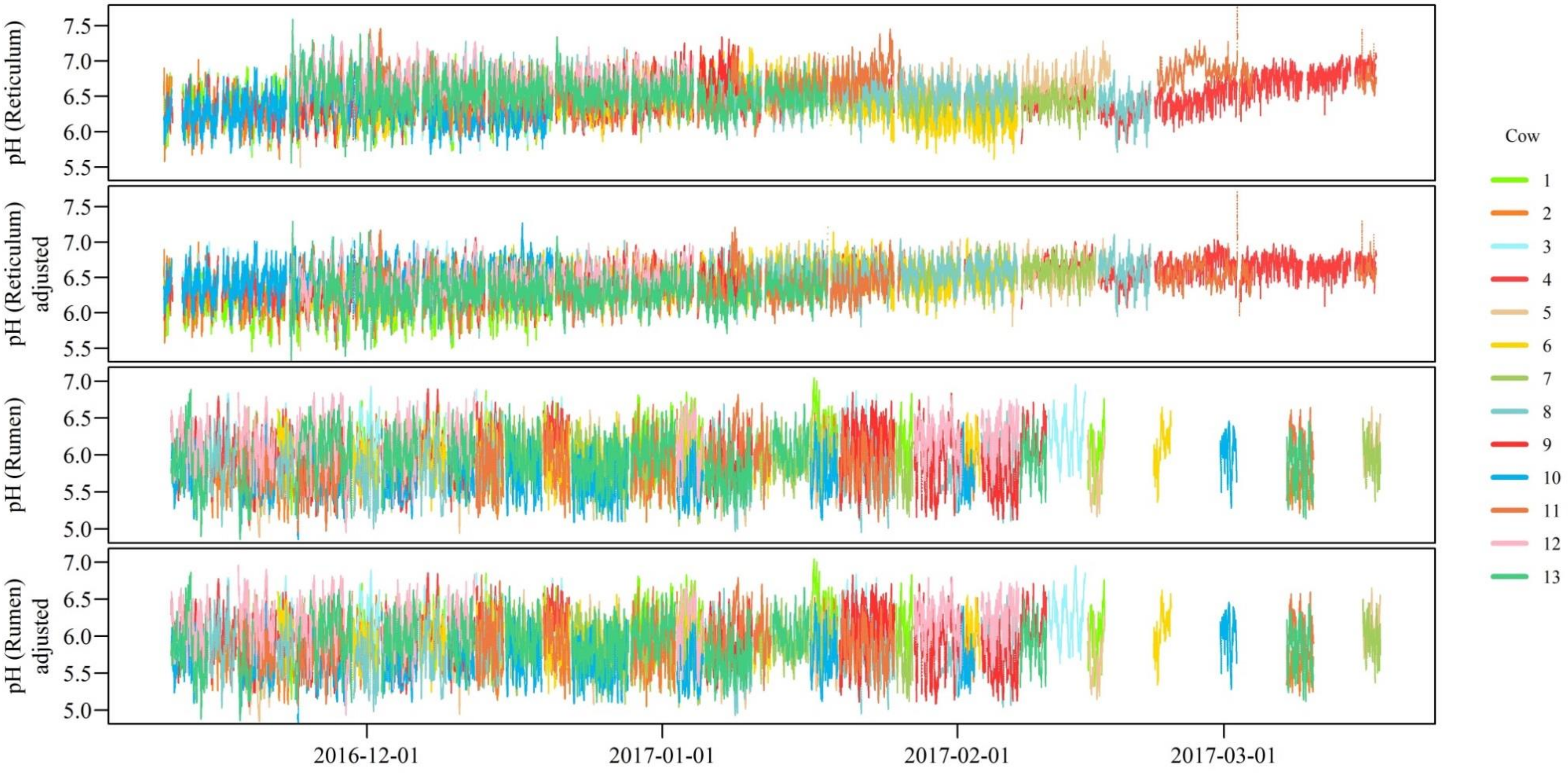

Figure S3.13: Raw and adjusted reticular and ruminal pH time series over the entire experimental period.

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Chapter 4

An innovative concept for a multivariate plausibility assessment of simultaneously recorded data

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SIMPLE SUMMARY

Benefiting from technical progress, it is nowadays easy to collect huge amounts of data using computerized sensor-based acquisition systems for both research and practical applications. However, such data often contain technology-related errors that are difficult to distinguish from physiologically extreme observations and thus can impair the quality of the data and also the statistical analysis. To tackle this, an innovative procedure for a multivariate plausibility assessment was developed to discriminate observations of simultaneously recorded traits between ‘physiologically normal’, ‘physiologically extreme’ or ‘implausible’ cases. In order to evaluate the performance and applicability, it was tested on a comprehensive data set collected from 10 commercial dairy farms. The added value of the developed method can be summarized as the ability to improve the quality of huge data sets with complex structure by distinguishing implausible observations from observations indicating physiological extreme conditions. The underlying concept can be applied to future data collections in science as well as in agricultural practice with regard to precision livestock farming.

ABSTRACT

The aim of this work was to develop an innovative multivariate plausibility assessment (**MPA**) algorithm in order to differentiate between ‘physiologically normal’, ‘physiologically extreme’ and ‘implausible’ observations in simultaneously recorded data. The underlying concept is based on the fact that different measurable parameters are often physiologically linked. If physiologically extreme observations occur due to disease, incident or hormonal cycles, usually more than one measurable trait is affected. In contrast, extreme values of a single trait are most likely implausible if all other traits show values in a normal range. For demonstration purposes, the MPA was applied on a time series data set which was collected on 100 cows in 10 commercial dairy farms. Continuous measurements comprised climate data, intra-reticular pH and temperature, jaw movement and locomotion behavior. Non-continuous measurements included milk yield, milk components, milk mid-infrared spectra and blood parameters. After the application of the MPA, in particular the pH data showed the most implausible observations with approximately 5% of the measured values. The other traits showed implausible values up to 2.5%. The MPA showed the ability to improve the data quality for downstream analyses by detecting implausible observations and to discover physiologically extreme conditions even within complex data structures. At this stage, the MPA is

not a fully developed and validated management tool, but rather corresponds to a basic concept for future works, which can be extended and modified as required.

Key words: multilevel data, plausibility assessment, sensor-based data acquisition systems

INTRODUCTION

In the era of 'Big Data' it is possible to record large amounts of data automatically and cost-effectively. Irrespective of the field of research, data quality plays a major role in statistical analysis. As will be shown by means of several examples, the acquisition of automated mass data in animal sciences carries the risk that technically induced disturbances lead to values that leave the physiological range and are therefore implausible. Nevertheless, it is possible that physiologically extreme values occur, for example evoked by a certain treatment in the course of an experiment, because of diseases or natural extreme situations such as in the peripartum period.

As an example where recorded data exceeds the physiologically normal range and become extreme, clinical mastitis of lactating cows can be mentioned. Fogsgaard et al. (2012) demonstrated that the body temperature increased massively about 18 h after an experimentally induced infection with *Escherichia coli* and rectal temperatures of about 40°C were reached. At the same time, the authors observed an extreme increase in the somatic cell count (**SCC**) of the milk, as well as a decrease in feed intake and milk performance. In the case of clinical ketosis, measurable physiological changes can also occur in early lactation. Known characteristics are a decrease in feed intake and milk performance as well as an increase in ketone bodies in the blood, in particular non-esterified fatty acids (**NEFA**) and β-hydroxybutyrate (**BHB**) (Baird, 1982). Claw disorders can also be mentioned as a further example. Pavlenko et al. (2011) observed significantly lower milk performances and a different standing and lying behavior in cows with digital dermatitis compared to healthy animals. In this context, further diseases could also be mentioned, such as milk fever and a displaced abomasum.

From these examples, it becomes clear that disease-related extreme values can occur simultaneously or with a time delay between observations in various measurable parameters. Such observations can generally be classified as plausible and should not be classified as outliers or erroneous observations from a physiological point of view. For mastitis, ketosis and claw diseases, for example, estimated incidences of 21.6, 1.7 and 19.5% of affected lactations can be found (Fleischer et al., 2001). Therefore, it is likely that physiologically extreme values caused by such diseases will occur in any data collection. Additionally, other factors may induce real extreme values, such as management incidents, stress, unwanted restriction access to water and feed, extreme climatic conditions or heat as they may affect behavior, milk production or intake and consequently other measureable traits.

Real extreme observations or recording errors can be found in both test day milk yields and milk components. In this context, Wiggans et al. (2003) and Gao et al. (2018) have established strategies to handle such extreme observations. Wiggans et al. (2003) developed a procedure in which preceding and subsequent observations of test day records were investigated with a univariate analysis to detect implausible observations. In comparison, Gao et al. (2018) used a multivariate method based on the Mahalanobis distance between fixed effect corrected test day milk performances and protein and fat contents.

In addition to true extreme values, there is the risk of technical random or systematic errors, in every automated sensor-based data acquisition, e.g. with transponder-assisted feed and water intake, sensor-based rumination and locomotion activity measurements or the intra-reticular recording of pH and temperature. In the case of feed intake measurements in pigs, Eissen et al. (1998) presented algorithms based on threshold-based rules to detect implausible observations. The random non-directional drift observed during in continuous intra-reticular pH measurements was discussed by Villot et al. (2017). The authors solved this problem of systematic errors by applying a trend correction with moving averages to calculate relative moving mean centered pH values which served as indicator for subacute ruminal acidosis (**SARA**). However, there are also approaches to further discriminate the source of extreme observations. Alawneh et al. (2011) for example investigated the daily walkover live weight of cows and differentiated identified outliers into 'biologically implausible' or 'potentially erroneous'. The authors made these classifications based on a smoothed live weight curve, where observations outside the ± 4 standard deviation (**SD**) interval were classified as ‘biologically implausible’ and observations outside the ± 1.96 SD interval (95% confidence interval) as ‘potentially erroneous’ cases.

Since the measured values in the statistical evaluations are assumed to be random variables, outlier detections based on the mean ± 3 empiric estimated standard deviations ($\bar{\mathbf{x}} \pm \mathbf{3}\hat{\boldsymbol{\sigma}}$) are often found, as in the study by Liang et al. (2013) where daily reticular temperatures were investigated. Another example is mid-infrared (**MIR**) spectral data, which contains 1060 variables per sample when measured with a Foss spectrometer (Foss Hillerod, Denmark). With such data, it is common to use the standardized Mahalanobis distance (global H-value, **GH**), whereby spectra with a GH > 3 are regarded as outliers (Shenk and Westerhaus, 1991; Vanlierde et al., 2015). Another common method is to detect outliers within the framework of statistical modeling using standardized or studentized residuals between measured and predicted values (Stevens, 1984). As a rule of thumb, the observations with standardized or studentized residuals of ± 3 are regarded as outliers. Regarding the threshold, however, different recommendations can be found in the literature. Rousseeuw et al. (2006), for example, proposed 2.5 as the threshold for outlier classification.

Therefore, without a global visualization of the data it is difficult for specialists to discriminate extreme records and erroneous data. This issue is even more highlighted when data are analyzed by general statisticians having no skills in animal sciences.

The main objective of this study was to develop an innovative approach of data cleaning based on a multivariate plausibility assessment (**MPA**) of simultaneously recorded data, considering both automatically collected sensor data and conventional samples such as milk and blood samples. The aim was to distinguish in an automated way between 'physiologically normal', 'physiologically extreme' and 'implausible' observations. The developed MPA procedure is applied on data collected in agricultural practice in order to obtain an overview of the data quality of the individual measuring systems and to prepare the dataset for downstream analyses. At this stage, the MPA is not a fully developed and validated management tool. It rather corresponds to a basic concept which has been adapted to the available data set, but which can be extended and modified as required in future works.

MATERIALS AND METHODS

Ethical Declaration

The experiment was conducted in accordance with the German legislation on animal protection (Animal Welfare Act) and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany; AZ: 33.9-42502-05-17A106).

Data Recording

The data collection was carried out as part of the project ‘Evaluation of Animal Welfare in Dairy Farming – Indicators for the Metabolism and Feeding’ (IndiKuh). The project focused on metabolic and digestive disorders of early lactating cows and aimed both to validate existing and to develop novel indicators. In this regard, the investigations targeted the disorders sub-clinical ketosis and subacute ruminal acidosis, which is why a wide range of different data was collected.

Farms and Animals. The data collection was carried out in 10 commercial dairy farms in the northwest of Lower Saxony, Germany, between April 2017 and March 2018. The farms are characterized by 305-d milk yields between 9.200 and 11.100 kg and approx. 200 to 600 cows and therefore can be classified above average with regard to performance and size in Germany. Each farm was visited for 3 weeks, whereof the first week was used for preparations and the animals' adaptation to the measuring instruments. In the following 2 weeks, the data recording took place. The data collection scheme used in each farm is illustrated in **Figure 4.1**. On each farm, 10 Holstein cows in early lactation were selected. Attempts were made to ensure that all parities of the cows (1, 2, 3 and ≥ 4) were covered equally in the farms. All farms had a loose-housing outdoor climate barn with resting pens and fed a partially or totally mixed ration with no change in diet during the 3 weeks stay. In each farm the cows were milked twice a day.

Climate Data. On each farm, the temperature and relative humidity in the barn were recorded in 15 min intervals using 3 to 6 Tinytag climate data loggers (Tinytag Plus 2 TGP-4500, Gemini Data Loggers, Chichester, United Kingdom). The data loggers were positioned at a height of about 2 m in the feeding, lying and drinking areas. In addition, climate data were obtained from the German Weather Service (DWD Climate Data Center (CDC), 2018), which was collected at the weather station in Altenoythe, Germany, in the vicinity of the 10 farms.

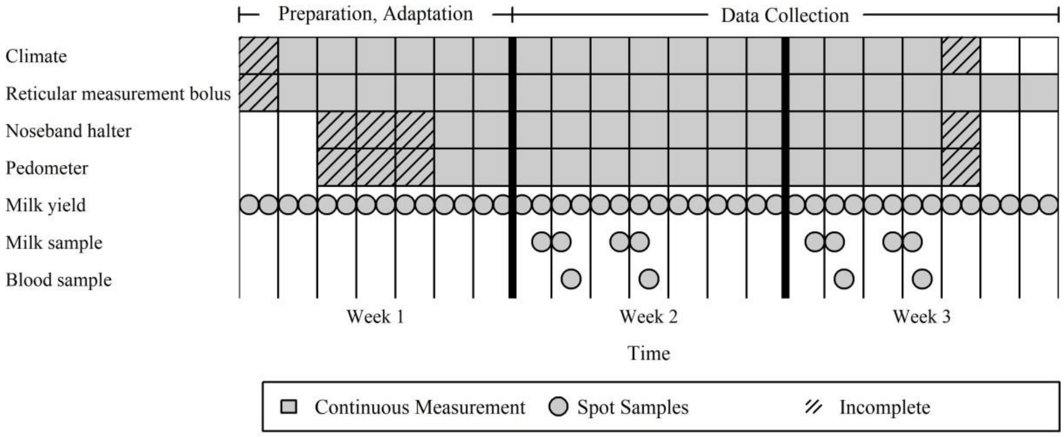

Figure 4.1: Data collection scheme used in each of the 10 farms.

Intra-Reticular pH and Temperature. In total 100 cows were equipped with an eCow measurement bolus (eCow Ltd., Exeter, Devon, UK) for the intra-reticular continuous measurement of the pH and the temperature. Before insertion, all boluses were activated in a water bath at 38.4°C and calibrated in pH 4 and pH 7 buffer solutions. The raw pH and temperature data were provided as mean values of 15 min time periods by the eCow boluses (termed 'boluses' in the remainder of this paper).

Eating, Chewing and Locomotion Behavior. The jaw movement behavior was recorded using noseband-sensor halters (RumiWatch, ITIN + HOCH GmbH, Liestal, Switzerland). Simultaneously, pedometers (RumiWatch, ITIN + HOCH GmbH, Liestal, Switzerland) were equipped to measure locomotion behavior. They were attached to the rear left or right leg. The data recording started at the end of the first week and continued during the following 2 weeks. The raw data of the RumiWatch noseband sensor and the pedometers were converted using the RumiWatch Converter V0.7.4.13 (RumiWatch, ITIN + HOCH GmbH, Liestal, Switzerland) into a 1-hour resolution.

Milk Yield and Milk Samples. The milk yield of all animals was measured with the on-farm milk quantity recording system. In addition, 4 evening milk samples and 4 morning milk samples of the following day were taken during the data collection period. Samples were collected following ICAR guidelines (ICAR, 2017) and were preserved with Bronopol. The samples were analyzed by the Landeskontrollverband Weser-Ems e.V. in Leer, Germany. In addition to the standard components such as fat, protein, lactose, urea and somatic cell count (SCC), the milk MIR spectra were also provided. The somatic cell count was measured using a flow cytometer (Fossomatic FC, FOSS, Hillerød, Denmark). The raw spectral data, which were recorded with a spectrometer (MilkoScan FT+, FOSS, Hillerød, Denmark) were then standardized according to Grelet et al. (2015) using the tools and strategies developed at the Walloon Agricultural Research Center (CRA-W, Gembloux, Belgium).

Blood Samples. Within the data collection period, blood samples from each cow were taken on 4 days from the *vena caudalis mediana* after the a.m. milking. Samples were collected in separate tubes with serum clot activator, one for BHB and NEFA and the other for glucose (**GLU**) analysis, and were centrifuged to harvest serum. The analysis was conducted in the laboratory of the Institute of Veterinary Medicine at the University of Goettingen.

Since the data collection was carried out under field conditions, some individual recordings were disrupted unintentionally for some of the collection systems. These include for example unreadable boluses, noseband halters and pedometers.

Data Preparation

Unification of the Temporal Resolution. Climate data recorded simultaneously with 3 to 6 Tinytag loggers at different locations in the barn displayed very similar values. Nevertheless, some loggers showed outliers particularly for the relative humidity, e.g. single observations with a relative humidity of 0%. Since both the determination of mean- or median-based daily courses led to distorted results, a moving window based method was used taking into account all simultaneous Tinytag measurements in order to get the best estimate of the temperature in the barn. Thereby, all Tinytag time series were summarized in a moving convolutional median with a window length of 9 values, 2 h respectively, to obtain one averaged course for temperature and one for relative humidity. Since the time window is relatively small in relation to the rate of change of the measurements, no significant smoothing behavior was observed with this method, so that the variance of the values was only marginally influenced (See supplemental **Figure S4.7** for an example).

The multivariate plausibility check of the aforementioned measured variables is challenging, as some of them differ in their temporal resolutions. Blood samples with daily values have the coarsest resolution in data collection. For the multivariate plausibility check, all continuous measurements were therefore also reduced to values on a daily basis.

Theoretically, the bolus data should consist of 96 observations per day and the halter and pedometer data of 24 observations per day. In practice this was not always the case. Among the 96 readable boluses, 2 devices recorded on average only 41 and 66 values per day throughout the entire data collection period due technical failure. The same was observed for the halter and pedometers, where, for example, cases were detected with 15 of 24 observations per day. This led to difficulties in the aggregation of these time series to values on a daily basis, where mean-based values should be determined for the bolus measurements (pH or temperature daily mean or median) and the sum of events over the day for the halter and pedometer data (daily duration of rumination, feeding and lying). Since missing observations have less influence on averaging than summation, up to 2 h missing observations were allowed for the bolus data 24 h-averaging and only 1 h for the halter and pedometer data 24 h-summation. If not enough data per day were available, the data of the affected traits from this day were set to missing and thus excluded from further analysis

The milk MIR spectra consisting of transmittance values (**T**) were converted into absorbance values (A) using a logarithm function with A = -log10(T) as common in spectroscopy (Gengler et al., 2016). In the remainder of this paper, only absorbance spectra were considered. To aggregate the evening and the subsequent morning milk samples, the components as well as the MIR spectra were daily averaged with a weighted average proportionally to the milk yield. The aggregated values were then assigned to the day of the evening milking. The blood values were also assigned to the previous day to compensate for the latency of the animal's digestion and metabolism.

After all the preparation steps mentioned above, the data were merged into one data set with a daily resolution. Since all continuous measurement systems were installed from day 5 at the latest, the data from day 6 to day 21 were considered for the plausibility assessment, so that 16 observations per animal were available on a daily basis.

Handling Missing Values. As the daily average temperature in the barn is going to be used in the MPA and some observations are missing, the extremely high correlation between the external climate data from the weather station and the barn climate data were used to predict missing values. For this a linear model ($R^2 = 0.99$, residual standard error (**RSE**) = 0.67°C) was used, in which the temperature in the barn was modeled as a function of the outside temperature and a farm effect.

In around 6% of the cases of days where milk samples were taken, the separation of the total daily milk yield into morning and evening yield was missing. However, this information was needed, for example, to determine the pooled MIR spectra. As this only concerned a few individual cases per animal, all available observations were used to estimate the morning and evening milk quantity for affected days using the available daily total milk yield. This took into account that the morning milk yield corresponds to about 55% of the daily total milk yield when milking twice a day (Quist et al., 2008). Thus, a linear model ($R^2 = 0.94$, RSE = 1.34 kg/d) was used to estimate the morning milk performance considering an animal effect as well as the interaction between animal effect and total milk performance. The evening milk yield was then estimated by subtracting the estimated morning milk yield from the daily milk yield.

Transformations. Since both the BHB and NEFA values in the blood as well as the SCC are extremely right-skewed, the former were Log10 transformed and the latter were transformed by calculation of the somatic cell score (**SCS**) according to Wiggans and Shook (1987) using the following formula:

$$\mathrm{SCS} = \log_2\left(\frac{\mathrm{SCC}}{100}\right) + 3 \qquad [4.1]$$

These transformations resulted in an approximately normal distribution of all 3 variables.

In 3 of the 100 cows, medications affecting rumen fermentation were administered by herd managers due to unspecified clinical signs in or immediately prior to the experimental period. All animal-individual observations of these cows after administration of the medications were discarded to the end of data collection.

Multivariate Plausibility Assessment

Concept and Underlying Assumptions. The developed MPA concept for checking of simultaneously measured data is based on the fact that when real extreme values occur, they are reflected not only in one but most likely in several measurable traits. Therefore, the following assumptions were made:

- Several of the measured traits are (physiologically) associated
- Cases of disease are reflected in more than one of the measured parameters in form of conspicuously high or low values
- The probability that 2 or more measuring systems will display erroneous values at the same time is close to zero

Consequently, it can be concluded that if extreme observations are observed in only one trait, they are most likely not of a physiological nature but rather due to an error in data recording. Furthermore, in the absence of extreme observations, all are considered to be plausible. Therefore, 3 possible classifications are assumed whereby ‘physiologically normal’, ‘physiologically extreme’, and ‘implausible’ cases are distinguished. This classification is illustrated in **Figure 4.2** by 4 simulated case studies taking into account 3 traits in the form of time series data:

- Case A represents a healthy and thus physiologically normal animal,
- Case B is a cow, whose reticular pH sensor had a malfunction with pH as Trait I,
- Case C is a cow with a temporarily dropped halter with daily rumination duration as Trait II and
- Case D shows a cow with a clinical mastitis, where Trait I would be the milk performance, Trait II the SCC or body temperature and Trait III the daily eating or rumination duration.

In summary, simultaneously collected characteristics are used for mutual plausibility checks in order to ensure adequate data quality for downstream analyses.

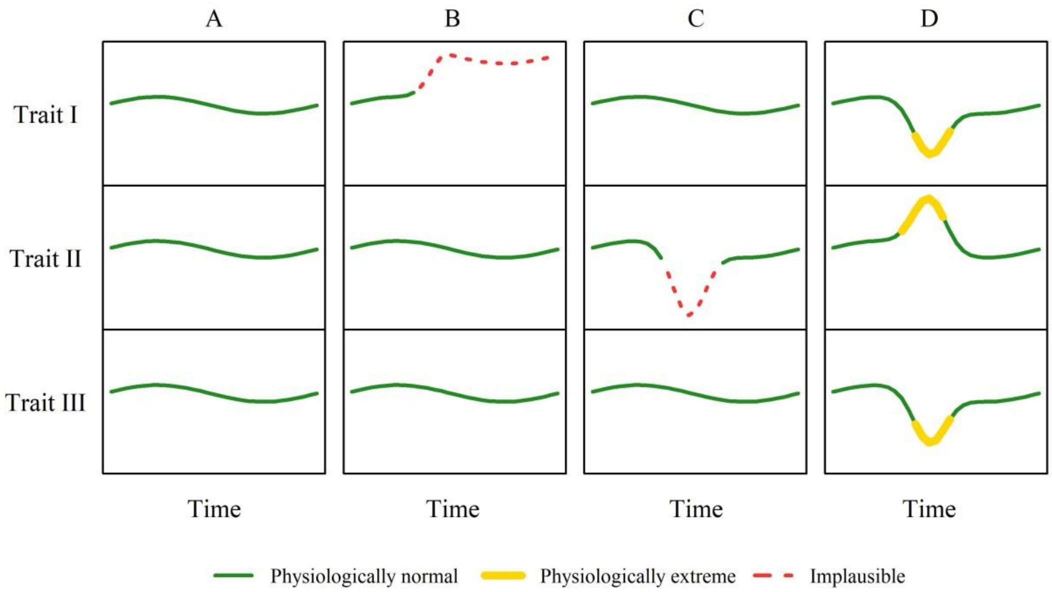

Figure 4.2: Simulated examples illustrating the classifications 'physiologically normal', 'physiologically extreme' and 'implausible' for observations in 3 physiologically associated traits.

Selection of Traits. Since a large number of traits could be derived from the collected data, a target-oriented selection had to be made, because a drift occurring in one sensor is likely to affect all the data collected by it. For all variables measured with a common measuring system, traits were selected being as independent and uncorrelated as possible. For the bolus data, the daily average pH ($\mathbf{r.\overline{pH}}$) and the median of the reticular temperature ($\mathbf{r.T_{med}}$) were selected, since the pH and temperature measurements were recorded with 2 different sensor systems in the bolus. For the temperature, the median was taken as a robust statistic for the daily average instead of the arithmetic mean, since the temperature measurement in the reticulum is temporarily strongly influenced by the water intake (Liang et al., 2013). The use of the arithmetic mean, however, would lead to an underestimation of the daily average temperature. Regarding the halter data the total eating and rumination duration ($\mathbf{\Sigma.Rt}$ and $\mathbf{\Sigma.Et}$) were chosen. For the pedometer data, only the total lying duration ($\mathbf{\Sigma.Lt}$) was selected, since, for example, lying and standing time are highly correlated (r = -0.99). With regard to performance and milk parameters, the daily milk yield (**MY**), the SCS and the entire MIR spectrum was used. As already stated by Gengler et al. (2016), the special feature of the MIR spectrum is that it can be seen as a ‘fingerprint’ of the entire milk composition and thus contains not only information on the standard components (fat, protein, lactose, urea) but also on fine milk composition such as the fatty acid pattern of the milk fat. For this reason, solely the MIR spectrum of milk will be considered in the following. Further, all 3 blood parameters were taken into account because the analyses for BHB and NEFA were performed on the same sample and are only slightly correlated (r = 0.13) and because glucose was determined independently on a separate blood sample.

Technical Implementation of the MPA. Due to the experimental design, the available data are subject to a hierarchical structure with the levels ‘farm’, ‘animal’ within the respective farm and the ‘repeated measurements’ within each animal. In addition, there are other influencing factors, such as the lactation stage and parity, which can also have an effect on the variables under investigation. Since the data collection covered a period of one year, the climate could also have an effect on the respective parameters. Heat stress can lead to a decline in the rumination duration, feed intake and milk performance (Kadzere et al., 2002). For this reason, the temperature in the barn ($\mathbf{s.\overline{T}}$) was also taken into account as a possible abiotic influencing factor. As the MPA approach is based on deviations of observations from expected values both on animal and single measurement level, these deviations have to be quantified first. In order to consider both the hierarchical data structure and the fixed effects, linear mixed models were established as follows for all traits except for the milk MIR spectrum:

$$y_{ijklm} = \beta_0 + \beta_1 s.\overline{T}_{ijklm} + P_i + \sum_{p=1}^{4}\left(\beta_p DIM_{ijklm} \times P_i\right) + F_j + F_j \times TD_k + A_l + e_{ijklm} \qquad [4.2]$$

where y_{ijklm} is the observation m of cow l on the farm j for trait y, $s.\overline{T}_{ijklm}$ is the daily mean temperature in the barn, P_i is the fixed effect of parity class i (1, 2, 3 or ≥ 4), and $DIM_{ijklm} \times P_i$ is the interaction of lactation stage and parity. β_0 is the intercept while β_1 and β_p are regression coefficients. The farm F_j, the interaction of farm and the test day $F_j \times TD_k$ and the animal A_l are considered as normally distributed random effects with $F_j \sim N\left(0, \sigma_F\right)$, $F_j \times TD_k \sim N\left(0, \sigma_{F\times TD}\right)$ and $A_l \sim N\left(0, \sigma_A\right)$. Further, e_{ijklm} is a random error with $e_{ijklm} \sim N\left(0, \sigma_e\right)$.

The cases shown in **Figure 4.2** are limited to the hierarchy level of repeated measurements within an animal. But considering the data structure and the modeling, 4 different scenarios are conceivable, especially for the bolus, noseband halter and pedometer derived traits:

- Neither the animal effect nor the residuals within the animal are extreme
- The animal effects are extreme, but not the residuals within the animal
- The residuals within one animal are extreme, but not the animal effect
- Both the animal effect and the residuals within the animal are extreme

The modeling assumes that both the animal effects and the residuals are normally distributed and with a mean of zero. With regard to the classification of the aforementioned 4 cases, 2 new binary coded auxiliary variables A´ and e´ were added for all traits based on the level of the animal effects as well as on the level of the residuals. Animal effects and residuals were classified as extreme and coded as 1, if the estimated effects were outside the confidence interval of ± 3 $\hat{\sigma}$ with $\hat{\sigma}$ being the empirically estimated standard deviation of the residuals or animal effects; otherwise they were classified as normal and coded as 0. If ≥ 50% of an animal's residuals were extreme, the animal effect was also coded 1 in the auxiliary variable. However, the coding for the animal effect A´ was only done for the bolus, noseband halter and pedometer derived traits, since in these data collections the measuring instruments were confounded with the respective animal. The 3 $\hat{\sigma}$ wide confidence range was chosen, as it is a generally accepted threshold (Stevens, 1984). According to the 3 sigma rule, one would therefore expect 0.3% of the data as outliers for the random animal effects as well as for the residuals with true normal distributions.

A special case is the consideration of the MIR spectral data in this plausibility assessment. Of the 1060 absorbance values of the different wavelengths, the noisy areas were removed first according to Grelet et al. (2015). In order to detect extreme spectra, a principal component analysis (**PCA**) based on the reduced spectra was performed as described by Soyeurt et al. (2019) to determine the number of components that explain 99% of the variability. The GH was then determined from the scores of the required principal components (n = 7). Spectra with GH > 3 (Shenk and Westerhaus, 1991; Vanlierde et al., 2015) were coded as 1 and otherwise 0 in a further auxiliary variable GH'. If more than 50% of the spectra of an animal had a GH > 3, an additional animal-based auxiliary variable for the GH would also have to be coded as 1. However, such case did not occur.

After 1/0 coding for all 11 traits on the individual observation and entire animal level, the procedure described in the section "Concept for Multivariate Plausibility Assessment" had to be applied. The developed R (R Core Team, 2019) program is visualized in **Figure 4.3** in form of pseudo code in a program flow chart. This figure was created using the yEd graph editor (yWorks GmbH, 2019). Time lags ± 2 d were also taken into account in the MPA, since delayed extreme value formation can occur (e.g., Fogsgaard et al., 2012). This means that extreme observations of at least 2 different traits were classified as physiologically extreme if they were not only observed on the same day but also in a time window of ± 2 d.

Unless otherwise stated, the entire data preparation, creation of figures and statistical analysis was done within the software environment R (R Core Team, 2019). The package lme4 (Bates et al., 2015) was used for regression parameter estimation of linear mixed models.

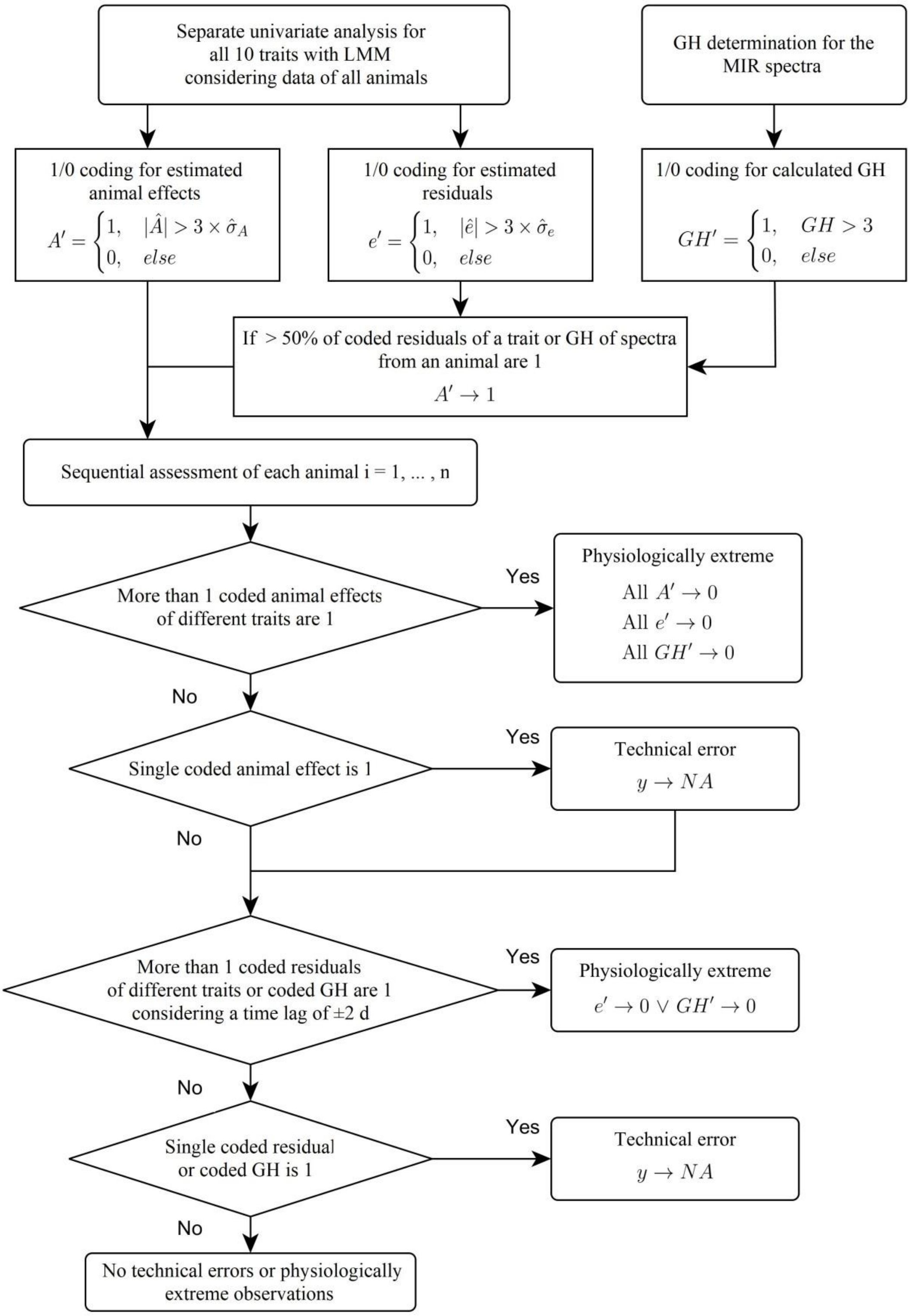

Figure 4.3: Flow chart demonstrating the pseudo-code of the developed multivariate plausibility assessment. GH = global H; standardized Mahalanobis distance; MIR = mid-infrared; NA = not available; y = trait; $\hat{\sigma}$ = empirically estimated standard deviation of the residuals e or animal effects A.

RESULTS AND DISCUSSION

Classification of Observations and Descriptive Statistics

In order to assess the performance of the developed concept of a MPA for automated data acquisition systems, it was applied on data collected under field conditions. Thereby, it was examined which proportions of the data considered in the analysis were classified as ‘physiologically normal’, ‘implausible’ or ‘physiologically extreme’ observations. The result of this classification is illustrated in **Figure 4.4** by showing the relative frequency of 'implausible' and 'physiologically extreme' observations for each trait. In addition, **Table 4.1** provides an overview of the descriptive statistics of the data set before and after the MPA. Observations of traits classified as 'implausible' were set to missing in the data set.

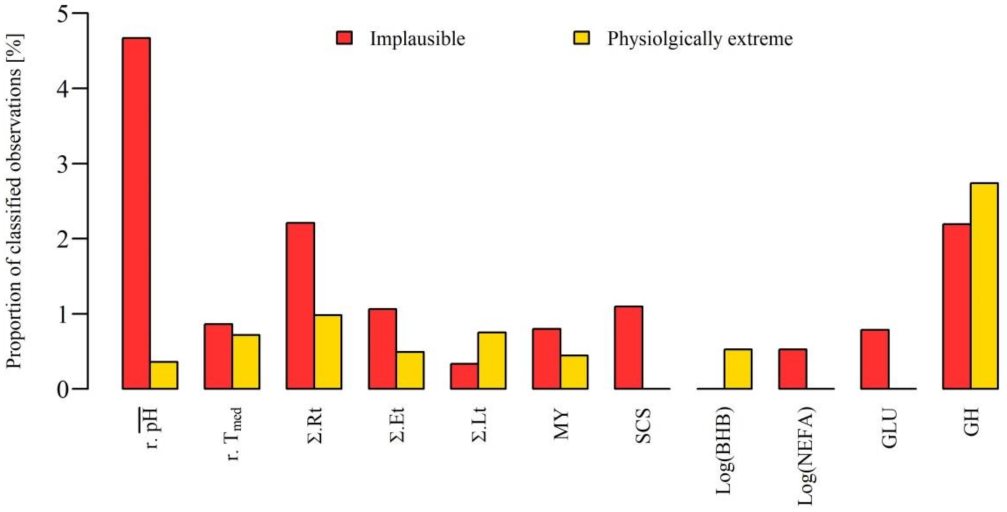

Figure 4.4: Proportion of classified observations assigned by the multivariate plausibility assessment for the classes 'physiologically extreme' and 'implausible' of all available observations for all 11 evaluated traits. BHB = β-hydroxybutyrate in blood; GLU = glucose in blood; GH = global H, standardized Mahalanobis distance of milk mid-infrared spectra; MY = daily milk yield; NEFA = non-esterified fatty acids in blood; $\overline{\text{r.pH}}$ = ruminal daily mean pH; r.T_{med} = median of the reticular temperature; SCS = somatic cell score according to Wiggans and Shook (1987); Σ.Et , Σ.Rt , and Σ.Lt = daily duration of eating, rumination and lying, respectively.

Table 4.1: Descriptive statics of the data set before and after the applied multivariate plausibility Assessment (MPA)[1]

	Before MPA							After MPA						
Variable	n_A	n	Mean	SD	Min	Median	Max	n_A	n	Mean	SD	Min	Median	Max
Climate														
Daily Mean Temperature ($s.\overline{T}$), °C		160	12.1	6.2	-1.3	12.8	25.0		160	12.1	6.2	-1.3	12.8	25.0
Animal														
Lactation stage (DIM), d	100	1,600	20.1	7.5	3	20	41	100	1,600	20.1	7.5	3	20	41
Parity	100	1,600	3.1	1.6	1	3	8	100	1,600	3.1	1.6	1	3	8
Reticular measurement bolus														
Daily mean pH ($r.\overline{pH}$)	93	1,392	6.19	0.25	5.05	6.21	7.76	**89**	**1,327**	**6.18**	**0.16**	**5.35**	6.21	**6.71**
Daily median Temperature ($r.T_{med}$), °C	93	1,392	39.37	0.36	38.30	39.30	41.30	93	**1,380**	**39.36**	**0.34**	38.30	39.30	41.30
Noseband halter														
Rumination duration ($\Sigma.Rt$), h/d	97	1,222	9.32	1.42	1.07	9.58	12.47	**95**	**1,195**	**9.42**	**1.25**	1.07	**9.62**	12.47
Eating duration ($\Sigma.Et$), h/d	97	1,222	5.37	1.39	1.40	5.37	12.45	**96**	**1,209**	**5.31**	**1.27**	1.40	**5.36**	**9.90**
Pedometer														
Lying duration ($\Sigma.Lt$), h/d	98	1,197	10.51	2.80	0.87	10.77	18.38	98	**1,193**	**10.53**	**2.77**	0.87	10.77	18.38
Milk														
Milk yield (MY), kg/d	99	1,126	37.5	8.5	5.2	38.0	63.8	99	1,117	37.5	8.5	5.2	38.1	63.8
Somatic cell count (SCC), 1000/ml	99	365	310.7	974.2	6.5	60.2	13,067.4	99	**361**	**260.1**	**679.8**	6.5	**59.3**	**4,901.6**
Somatic cell score[2] (SCS)	99	365	2.7	2.0	-0.9	2.3	10.0	99	**361**	**2.6**	**1.9**	-0.9	**2.2**	**8.6**
MIR spectrum	99	365						99	**357**					
Blood														
BHB, mmol/l	99	380	0.92	0.60	0.27	0.77	5.13	99	380	0.92	0.60	0.27	0.77	5.13
Log(BHB), log(mmol/l)	99	380	-0.09	0.21	-0.57	-0.12	0.71	99	380	-0.09	0.21	-0.57	-0.12	0.71
NEFA, mmol/l	99	379	0.38	0.24	0.01	0.33	1.89	99	**377**	0.38	0.24	**0.06**	0.33	1.89
Log(NEFA), log(mmol/l)	99	379	-0.50	0.28	-2.00	-0.48	0.28	99	**377**	**-0.49**	**0.26**	**-1.22**	-0.48	0.28
Glucose (GLU), mg/dl	99	381	57.33	9.76	8.90	57.20	147.10	99	**378**	**57.34**	**7.93**	**31.80**	57.20	**79.20**

[1] BHB = β-hydroxybutyrate; DIM = days in milk; MIR = mid-infrared; n_A = number of animals; n = number of observations; NEFA = non-esterified fatty acids. Numbers that have changed in the comparison before and after the MPA are written in bold.

[2] The somatic cell score (SCS) was calculated according to Wiggans and Shook (1987).

For almost each trait both classifications were made for the data set. In particular, the pH values derived from the data recordings with boluses showed the highest relative frequency of implausible observations with almost 5%. For all other traits less than 2.5% observations were classified as implausible. Comparing the relative frequency of classified 'physiologically extreme' and 'implausible' milk performances with 1.2% of all observations to the findings of Wiggans et al. (2003), who identified 1.9% abnormal test day milk records, the same order of magnitude for conspicuous observations can be identified In other automatic measurements, such as walkover live weight recording, Alawneh et al. (2011) found a much higher frequency of conspicuous observations. The authors detected a total of 12% outliers, of which they classified 25% as 'biologically implausible' and 75% as 'potentially erroneous'. In comparison, Eissen et al. (1998) found incorrect observations in about 6% of the automatically recorded feed intakes of pigs. Consequently, the MPA algorithm presented here classified similar proportions of conspicuous observations with regard to the various traits on the basis of the example data set.

Before applying the MPA, 4 of the 100 boluses were not readable at the end of the trial and thus the data could not be downloaded. Two further erroneous boluses were also removed before the MPA as they had on average only 41 or 66 observations per day instead of the 96 theoretical observations. In addition, the observations from another cow were removed as fermentation-influencing medication was administered immediately before data collection. The MPA, however, classified the data of 4 other boluses as implausible, so that only reticular pH data of 89 of the 100 animals were available for further analysis. In the data set examined here, it is remarkable that especially the reticular pH measurements had the biggest losses already during data collection and showed the most implausible values in the MPA. At this point it has to be emphasized that intra-reticular measurement is one of the most complicated and innovative measurement methods used in this study. This is partly due to the milieu in which the bolus is located, the generally sensitive pH measurement electrodes and the fact that each bolus is confounded with the animal and cannot be calibrated after data recording.

In some studies, spectra with a GH > 3 are considered as outliers and excluded from further analyses because in the development of MIR-based prediction equations they could endanger the robustness and accuracy (Shenk and Westerhaus, 1991; Vanlierde et al., 2015). However, in this study 10 of the 18 MIR spectra with a GH > 3 were classified as ‘physiologically extreme’ and thus were accompanied by extreme observations in other traits in the present study. It shows that removing records based only on spectral distances can erroneously deprive the dataset from real extreme values that potentially could be interesting for model development. This also confirms the fact that the fine milk composition reflects the physiological status of the cow, as demonstrated by Grelet et al. (2019) and Colman et al. (2010) for the metabolic status or digestive disorders like SARA.

In total, about 1% of the data could be rescued which would have been classified and removed as outliers in a common univariate plausibility checks, but which could be assigned to physiological extreme conditions by the developed MPA.

Case Studies

To illustrate the applicability of the MPA, **Figure 4.5** visualizes the collected data in the form of time series for a cow of the 3rd parity showing signs of clinical mastitis in several of the traits investigated. Plotted are all 10 variables as well as the GH of the spectra as a function of time. In addition to the individual observations on a daily basis, the estimated regression line of the respective trait was plotted as a function of DIM for all animals of the same parity. The estimated random effect of the farm was taken into account to determine the intercept of the regression line. For illustrative purposes, the temperature in the barn which was also considered as a covariable in the model was mean centered and set to 0 for the prediction of the line. Around this regression line, the 3 $\hat{\sigma}$ confidence range of estimated random animal effects was drawn in dark gray. The regression line of the animal, for which both the estimated random farm effect and the random animal effect were considered for the determination of the intercept, was added as a black dashed line. This individual animal line is also surrounded by the 3 $\hat{\sigma}$ confidence range of the residuals in light gray, which is enclosed by dashed black lines. The figure shows that from the 35th DIM onwards the reticular temperature and the somatic cell counts in the milk temporarily increase and that the milk performance, the rumination and eating duration decrease. Except for the SCS, all measured variables simultaneously show extreme values and are classified as 'biologically extreme' by the MPA. It should also be noted that SCS, which is generally considered to be an essential indicator of mastitis, is not classified as ‘biologically extreme’ for these observations due to the generally high somatic cell counts of this animal and the overall high residual variance of all animals for this trait. If the dataset had been univariately plausibilized using standardized or studentized residuals, the ‘biologically extreme’ observations of the other 4 traits would have been removed from the dataset. However, since the MPA considered all variables in a multivariable procedure, the observed extreme values could be classified as plausible. The observations made here thus correspond to known signs of clinical mastitis and are in line with the results of Fogsgaard et al. (2012), who induced mastitis in cows with Escherichia coli experimentally.

As a further example, **Figure 4.6** visualizes the data of a cow of the third parity in which the intra-reticular pH measurement clearly failed. All other variables show observations classified as 'physiologically normal'. A further example with only observations classified as 'physiologically normal' can be found in supplemental **Figure S4.8**.

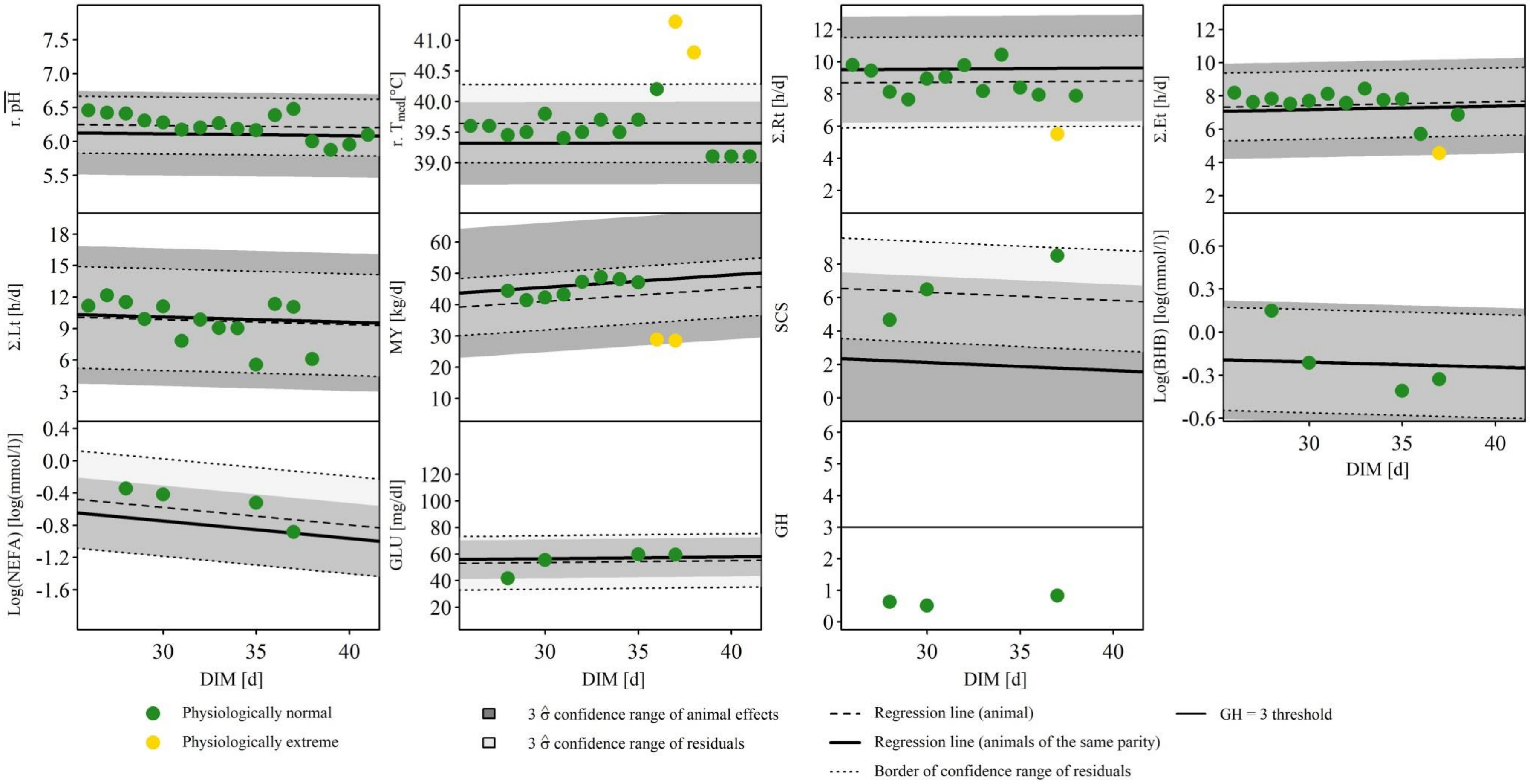

Figure 4.5: Case study of a cow of 3rd parity with signs of a clinical mastitis. The observations of 11 different traits as a function of the lactation stage, investigated in the multivariate plausibility assessment, are illustrated. BHB = β-hydroxybutyrate in blood; DIM = days in milk; GLU = glucose in blood; GH = global H; standardized Mahalanobis distance of milk mid-infrared spectra; MY = daily milk yield; NEFA = non-esterified fatty acids in blood; $\text{r.}\overline{\text{pH}}$ = ruminal daily mean pH; r.T_{med} = median of the reticular temperature; SCS = somatic cell score according to Wiggans and Shook (1987); Σ.Et , Σ.Rt , and Σ.Lt = daily duration of eating, rumination and lying, respectively; $\hat{\sigma}$ = empirically estimated standard deviation of the residuals or animal effects, respectively.

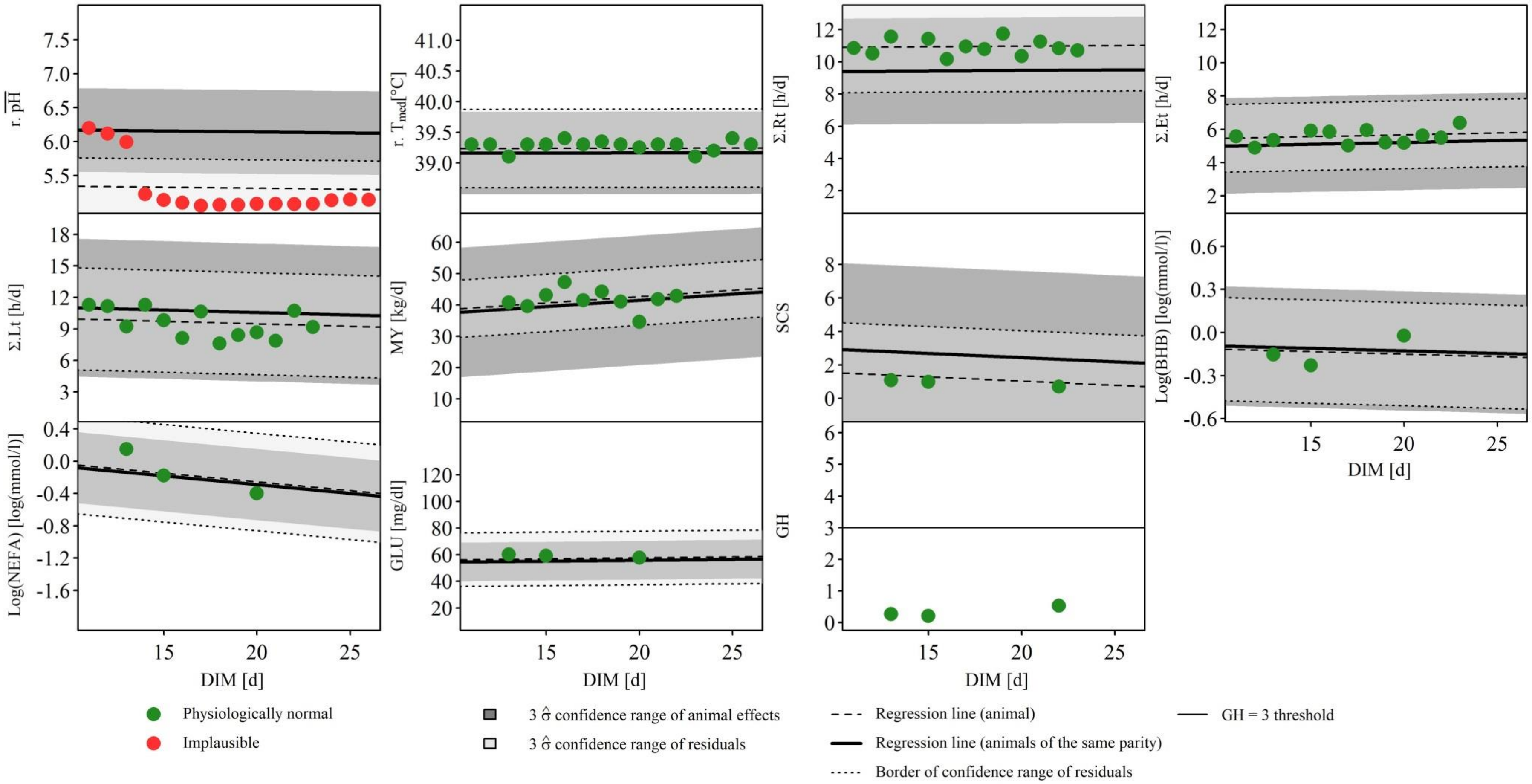

Figure 4.6: Case study of a cow of 3^{rd} parity with an erroneous intra-reticular pH measurement. The observations of 11 different traits as a function of the lactation stage, investigated in the multivariate plausibility assessment, are illustrated. BHB = β-hydroxybutyrate in blood; DIM = days in milk; GLU = glucose in blood; GH = global H, standardized Mahalanobis distance of milk mid-infrared spectra; MY = daily milk yield; NEFA = non-esterified fatty acids in blood; $r.\overline{pH}$ = ruminal daily mean pH; $r.T_{med}$ = median of the reticular temperature; SCS = somatic cell score according to Wiggans and Shook (1987); $\Sigma.Et$, $\Sigma.Rt$, and $\Sigma.Lt$ = daily duration of eating, rumination and lying, respectively, $\hat{\sigma}$ = empirically estimated standard deviation of the residuals or animal effects, respectively.

Further Remarks and Implications for Future Research

On the basis of the data considered here and most of the studies mentioned in the introduction, it can be concluded that automated data acquisition often produces conspicuous observations which are either physiologically justified or caused by technical errors. In the age of ‘Big Data’, the possibility of direct data control is not feasible due to the volume of data, so that the need for systematic and, above all, automatable monitoring strategies to ensure data quality increases. Such data should therefore be examined in detail before being further analyzed in the context of a specific objective.

With an extensive data set from the agricultural practice, there is a risk that extreme values due to different physiological causes may occur. Considering the estimated incidences of diseases such as mastitis, claw diseases or ketosis (see Fleischer et al., 2001), the probability of finding such cases in a data set such as the present one is therefore relatively high. Looking at the case shown in **Figure 4.5**, for example, it can be seen that clinical mastitis is accompanied by a temporary decrease in the duration of rumination and eating. It is highly probable that this will be accompanied by a decrease in dry matter intake. This would have the consequence that the rumen fill level decreases and also the fermentation process and thus the concentration of short-chain fatty acids decreases (Dado and Allen, 1995). This would explain the temporary increase of the reticular pH value, which is an associated trait of the ventral ruminal pH value. Therefore, those values physiologically make sense and may be considered in further analysis, whereas an univariate data cleaning process would have removed these records. Additionally, for further analysis with a specific objective it allows a valuable deep understanding of the data and of such complex and inter-dependent mechanisms occurring, especially if the data were collected in the field and thus not under experimental standardized conditions.

One of the biggest advantages of the MPA presented here is that the classification depends on the distributions of the investigated variables, whereby the entire hierarchical data structure could be considered. Consequently, no static thresholds had to be chosen subjectively, as in the case of Denwood et al. (2018), who, for example, removed reticular measured pH values ≥ 10 before analysis.

Interestingly, the concept presented here is also similar to the Heatime® System (SCR Ltd., Netanja, Israel), in which the simultaneous measurement of neck activity and chewing behavior is used for heat detection. Further resemblances exist to the method of Van Hertem et al. (2013), who developed statistical models for the detection of lameness using multivariate sensor data such as milk performance, rumination behavior, and neck activity. Furthermore, the quality control charts described for the first time by Shewhart (1931) are based on a similar concept. These charts are frequently used in industry for quality management in the monitoring of processes and provide a tool to check whether a statistical measure is under control or not. For this purpose, distribution-dependent thresholds are also used like $\bar{x} \pm 3$ SD.

The remaining question is how to deal with the result of the classification of observations into 'physiologically normal', 'implausible' or 'physiologically extreme'. This depends on the research question. The MPA presented here offers the possibility to automatically locate conspicuous observations. Those classified as biologically extreme should be investigated in particular, as extreme observations are often of scientific interest. However, the observations classified as 'implausible' are likely to occur due to technical and systemic errors in data collection and should be discarded in further analysis. The added value of the method presented here is that on the one hand the data quality can be improved and on the other hand physiologically interesting cases can be detected automatically. One condition for success, however, is that a sufficient number of traits are collected to cover the majority of disease-related changes.

In future studies, other traits are conceivable to be taken into account for such a plausibility assessment of simultaneously collected data. For instance the electrical conductivity of milk as an auxiliary characteristic for udder health (e.g., De Mol and Ouweltjes, 2001) or the development of body weight as it is related to the energy balance (e.g., Alawneh et al., 2011). In this respect, data collection in the dairy sector using automatic milking systems in combination with other monitoring systems, such as for heat detection, is of particular interest. As already summarized by Jacobs and Siegford (2012), the use of such a technology facilitates recordings of numerous traits by means of different sensor systems, which, if properly taken into account, can lead to many benefits for the economy and animal welfare of dairy farming. It is possible that our MPA procedure can be applied to such data collections to ensure data quality and to detect physiologically extreme conditions.

Limitations of the study

The presented MPA should not be seen as a fully developed management tool. At the current stage, the system should be considered more as a warning system to highlight potentially implausible data or observations related to extreme physiological conditions than as a validated cleaning system for routine data. What is decisive is the underlying concept, in which data collected with different sensor systems can be used for mutual plausibility checks. The modeling used in this study was customized to the data set that was used as an example. It is possible to adapt the modeling individually for even more extensive data sets. On the one hand, this could be relevant if not only data from early lactation but from the entire lactation are available. Then, typical lactation curves, such as for milk yield, milk ingredients, energy balance or dry matter intake could be taken into account (Buttchereit et al., 2010; Schmitz et al., 2018). On the other hand, if data are collected from many more animals, more robust models could be set up in which for example interaction effects between herd, parity and DIM could also be considered to adapt the models of each trait individually to the physiological conditions.

The main limitation of this study is that the application of MPA to the sample data set cannot be validated because neither diagnoses of a veterinarian nor other subjective observations of the health status are available. Efforts should therefore be made to validate the MPA on a further data set against real diagnoses, ideally with extensive health data.

CONCLUSIONS

In this study, a concept for multivariate plausibility assessment of simultaneously recorded data was developed, in which individual observations were classified as 'physiologically normal', 'implausible' or 'physiologically extreme'. The probabilistic approach, which is based on statistical models, aims to ensure adequate data quality so that the likelihood of distortions due to technical malfunctions is minimized for further investigations. The application to a hierarchically structured data set with 11 different traits showed the ability to identify most likely disease-related and thus physiologically extreme observations as in the example of clinical mastitis. Furthermore, observations of measurement boluses with non-physiological intra-reticular pH values were detected systematically. Even with such a complex data set as the one used here, where both the hierarchical data structure and different temporal resolutions in the data collection were challenging, the developed concept revealed to be a flexible method to improve the data quality for downstream analyses.

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AUTHOR CONTRIBUTIONS

Conceptualization: AM. Data curation: AM, MZ. Formal Analysis: AM. Funding acquisition: JH. Investigation: MZ. Methodology: AM. Project administration: JH. Software: AM, Supervision: JH, ARS. Visualization: AM. Writing – original draft: AM. Writing – review and editing: AM, MZ, JH, AOS, CG, ARS.

SUPPLEMENTARY

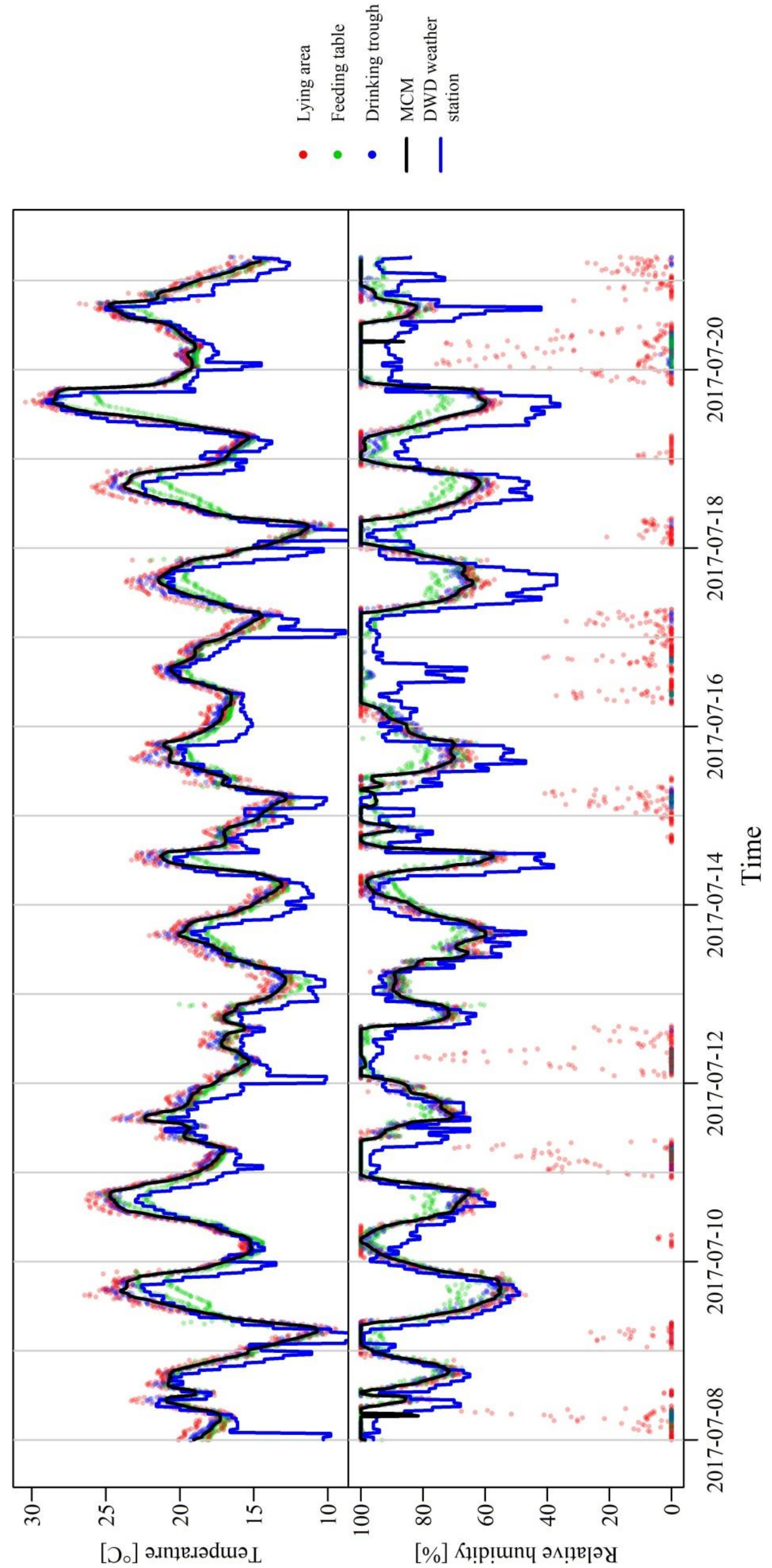

Figure S4.7: Climate data of a farm. Plotted are the individual observations of the Tinytag climate loggers from different positions in the stable as well as the hourly temperature data of a nearby weather station of the German Weather Service (DWD Climate Data Center (CDC), 2018). The black line corresponds to the moving convolutional median (MCM) of the individual Tinytag climate loggers.

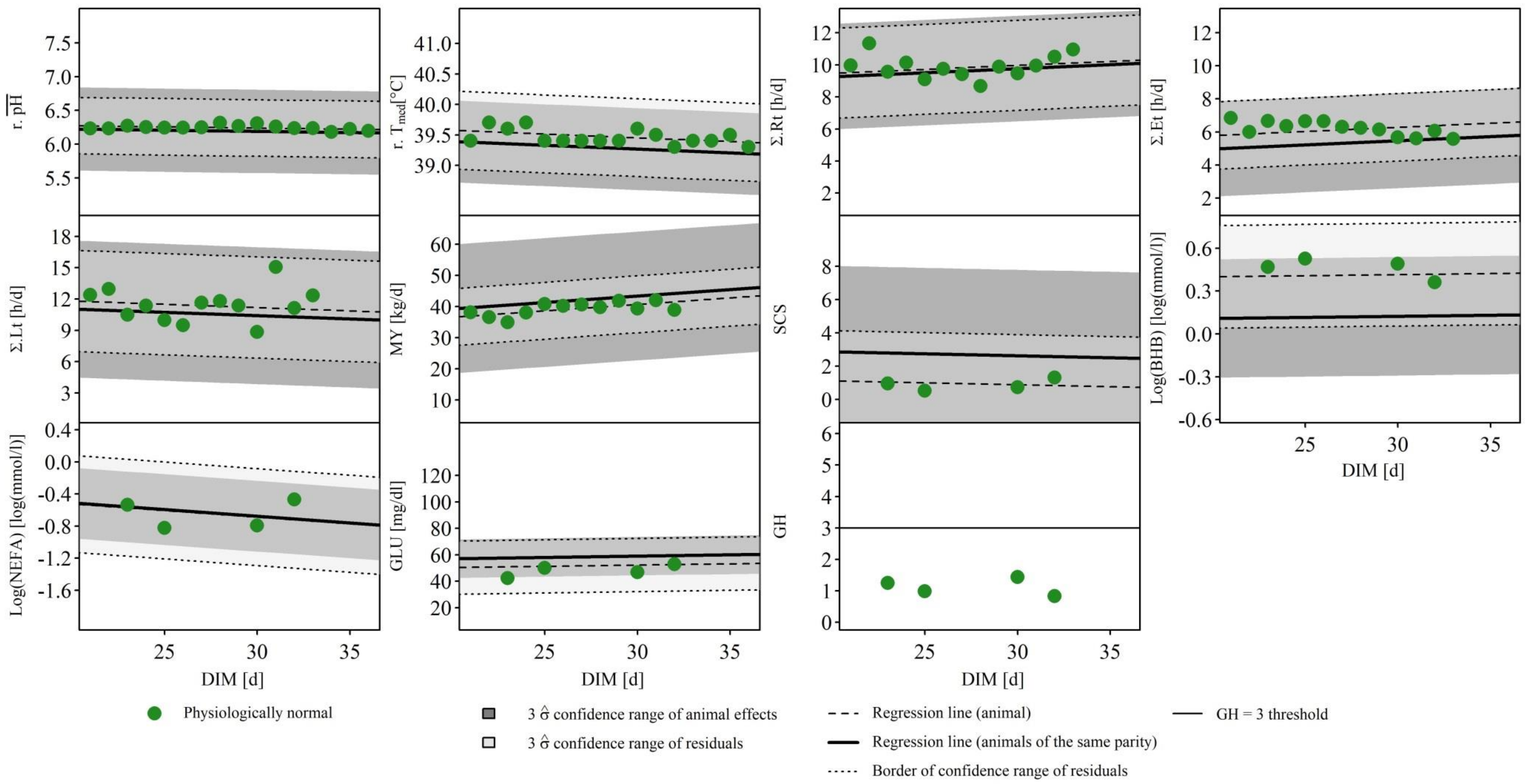

Figure S4.8: Case study of a cow of 5th parity in which all observations were classified as 'physiologically normal'. The observations of 11 different traits as a function of the lactation stage, investigated in the multivariate plausibility assessment, are illustrated. BHB = β-hydroxybutyrate in blood; DIM = days in milk; GLU = glucose in blood; GH = global H, standardized Mahalanobis distance of milk mid-infrared spectra; MY = daily milk yield; NEFA = non-esterified fatty acids in blood; $r.\overline{pH}$ = reticular daily mean pH; $r.T_{med}$ = median of the reticular temperature; SCS = somatic cell score according to Wiggans and Shook (1987); Σ.Et , Σ.Rt , and Σ.Lt = daily duration of eating, rumination and lying, respectively; $\hat{\sigma}$ = empirically estimated standard deviation of the residuals or animal effects, respectively.

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Chapter 5

Development of a SARA risk score and its prediction using milk mid-infrared spectra in early lactation cows

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ABSTRACT

A routine monitoring for SARA on the individual level could support the minimization of economic losses and the ensuring of animal welfare in dairy cows. The objectives of this study were (1) to develop a SARA risk score (**SRS**) by combining information from different data acquisition systems to generate an integrative indicator trait, (2) the investigation of associations of the SRS with feed analysis data, blood characteristics, performance data, and milk composition, including the fatty acid (**FA**) profile, (3) the development of a milk mid-infrared (**MIR**) spectra-based prediction equation for this novel reference trait SRS, and (4) its application to an external data set consisting of MIR data of test day records to investigate the association between the MIR-based predictions of the SRS and the milk FA profile. The primary data set, which was used for the objectives (1) to (3), consisted of data collected from 10 commercial farms with a total of 100 Holstein cows in early lactation. The data comprised barn climate parameters, pH and temperature logging from intrareticular measurement boluses, as well as jaw movement and locomotion behavior recordings of noseband-sensor halters and pedometers. Further sampling and data collection included feed samples, blood samples, milk performance, and milk samples, whereof the latter were used to get the milk MIR spectra and to estimate the main milk components, the milk FA profile, and the lactoferrin content. Because all measurements were characterized by different temporal resolutions, the data preparation consisted of an aggregation into values on a daily basis and merging it into one data set. For the development of the SRS, a total of 7 traits were selected, which were derived from measurements of pH and temperature in the reticulum, chewing behavior, and milk yield. After adjustment for fixed effects and standardization, these 7 traits were combined into the SRS using a linear combination and directional weights based on current knowledge derived from literature studies. The secondary data set was used for objective (4) and consisted of test day records of the entire herds, including performance data, milk MIR spectra and MIR-predicted FA. At farm level, it could be shown that diets with higher proportions of concentrated feed resulted in both lower daily mean pH and higher SRS values. On the individual level, an increased SRS could be associated with a modified FA profile (e.g., lower levels of short- and medium-chain FA, higher levels of C17:0, odd- and branched-chain FA). Furthermore, a milk MIR-based partial least squares regression model with a moderate predictability was established for the SRS. This work provides the basis for the development of routine SARA monitoring and demonstrates the high potential of milk composition-based assessment of the health status of lactating cows.

Key words: SARA, milk mid-infrared spectra, partial least squares regression, indicator trait

INTRODUCTION

Technological progress has facilitated collection, management, and use of “big data,” providing support for the development of innovative and complex functional traits, opening new possibilities in animal research and commercial farming. Particularly an improved monitoring for early detection of subclinical disorders such as SARA could facilitate timely interventions and thus prevent clinical diseases in dairy farming. In this regard, sensor-based data acquisition provides the basis for monitoring systems that can be superior to detection by human sensory perception. Because signs of a disease are generally reflected in several measurable traits, the combination of different data acquisition systems, in particular, can offer the basis for innovative concepts. For sensor-based lameness detection, for example, Van Hertem et al. (2013) used the neck and rumination activity recorded with a heat detection system and combined it with information on the milk performance and achieved a cross-validated sensitivity of 0.89 and specificity of 0.85. However, the situation is different with regard to SARA, as, by definition of the subacute condition, no clear clinical signs on the individual level are present, which makes diagnosis very difficult (Enemark, 2008). In this respect, the integrative combination of different information sources from several sensor systems could therefore be useful and theoretically should provide a better basis for the assessment of the health status.

Dairy herds receiving diets high in concentrate and low in physically effective fiber tend to show various clinical signs of related diseases (Kleen et al., 2003). These include reduced feed intake, lower milk production efficiency, altered fecal consistency, increased frequency of claw diseases, and an overall higher culling rate. Some of these signs are per construction herd-based parameters, and their reliability is based essentially on statistical aggregation of data from the entire herd. However, a routine monitoring system at individual level would be preferable, as this would both improve management and contribute to ensuring animal welfare, and, in addition, the obtained data would be of great value for animal breeding. Current approaches already rely on sensor-based continuous intrareticular pH measurements (e.g., Denwood et al., 2018). Besides SARA, Stangaferro et al. (2016), for example, showed that rumination behavior provides a high potential for individual animal monitoring for ketosis and displaced abomasum.

However, for the monitoring of SARA, one major challenge is that a high individual animal variance concerning susceptibility to SARA has been observed (Humer et al., 2015). Consequently, a need exists to identify innovative indicators such as biomarkers, which provide the highest possible degree of association with SARA on the individual level and which are at best applicable on a large scale in commercial farming.

In the last decade, several studies demonstrated that milk mid-infrared (**MIR**) analysis, which is routinely used to determine standard milk components relevant for milk payment (e.g., fat content), can provide further relevant information for both breeding and management purposes (Gengler et al., 2016). Using MIR spectroscopy, it is possible to differentiate the milk components even further, such as breaking down the fat fraction into its fatty acid (**FA**) composition (Soyeurt et al., 2011). Recent approaches include the MIR-based prediction of more complex traits of animal associated associated characteristics such as the energetic or metabolic status (De Koster et al., 2019) or methane emission (Vanlierde et al., 2018). Because the milk FA profile is associated with ruminal fermentation (Vlaeminck et al., 2006; Jing et al., 2018) and energy metabolism (Gross et al., 2011), it can be suggested that MIR-based models have a high potential for predicting and optimizing the monitoring of functional traits, which is relevant in terms of animal welfare and economy. Once an adequate MIR-based prediction model for healthrelated traits is developed, the main advantage will be that it can be applied to test day data collected during the routine recording and subsequently could be used in commercial farming. A drawback when working with MIR spectroscopy is that intra- and interinstrument variability occurs, and that predictions are unstable over time (Gengler et al., 2016). During routine application, post-prediction corrections are therefore carried out for the standard milk components (fat, protein) using standardized reference samples. However, this is not possible for all other traits that are not covered by the standardized reference samples for post-prediction correction. To solve this problem, Grelet et al. (2015) developed a method to standardize the spectra across different devices and manufacturers, while at the same time eliminating the temporal instability.

Based on data collected under field conditions, the objectives of this study were (1) to develop an integrative indicator trait for detecting SARA by combining information from different data collection systems into one SARA risk score (**SRS**). To evaluate this novel trait, its association with feed composition, blood metabolites, performance data, and fine milk composition was examined (2). Furthermore, a milk MIR spectrabased prediction equation of this novel reference trait SRS was developed (3) and applied to an external data set consisting of test day records to study the association between MIR-predicted SRS and the FA profile of the milk (4).

MATERIALS AND METHODS

The experiment was conducted in accordance with the German legislation on animal protection (Animal Welfare Act) and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany; AZ: 33.9-42502-05-17A106).

Data Recording

The data of this study consisted of a primary and a secondary data set. The primary data set was used for the development of the SRS and the milk MIR-based partial least squares (**PLS**) regression model. The secondary data set served to investigate the associations between predicted SRS and the milk composition under field conditions.

Primary Data Set

Following a standardized sampling scheme (see **Figure 5.1**), data recording was carried out sequentially in 10 commercial farms on a total of 100 Holstein cows in the northwest of Lower Saxony, Germany, between April 2017 and March 2018. Compared with an average 305-d milk yield of 8,500 kg and 63 cows per farm in Germany in 2016 (Lindena et al., 2018), the 10 farms can be classified as above average in terms of performance and size, with 305-d milk yields between 9,200 and 11,100 kg and herd sizes of approx. 200 to 600 cows. All farms had a loose-housing outdoor climate barn with resting pens, fed a partial mixed ration (**PMR**) or TMR, and milked the cows twice a day. Per farm, 10 cows were selected so that the theoretical calving date was as close as possible and at most 2 wk before the actual data collection period. Additionally, we attempted to ensure that all parities of the early-lactation cows (1, 2, 3, and ≥4) were covered equally. On each farm, the 2-wk data collection during early lactation (15 ± 6 DIM on d 8 in each farm; see **Figure 5.1**) was preceded by a 1-wk preparation and adaptation phase, allowing the cows to get used to the measuring equipment.

In each farm, feed samples were collected during the data collection period on d 8, 10, 15, and 17 (see **Figure 5.1**). Samples of equal size were taken immediately after the distribution of PMR or TMR at 3 different positions in the trough (beginning, middle, end) and then were pooled and mixed. At first, measurements with a Penn State Particle Separator (Nasco, Fort Atkinson, WI) were performed on subsamples of the pooled and mixed feed samples to determine the physically effective NDF > 8 mm (**peNDF>8**) of the PMR and TMR, defined as the proportion of material retained on an 8-mm sieve multiplied by the NDF content of the diet. The corresponding procedure was performed by the same person throughout the experiment. Then, additional subsamples of the pooled and mixed feed samples were vacuum-packed and stored at −20°C until further analysis. Concentrated feed samples were also taken at the farms where PMR was fed. The analyses were carried out according to the methods of the Association of German Agricultural Analytic and Research Institute (VDLUFA, 1993), and the fiber analysis was performed using the filter bag system (Ankom Technology, Macedon, NY). The analysis was conducted in the laboratory of the Ruminant Nutrition Group at the University of Goettingen (Goettingen, Germany). Further information on the specific analytical methods of each chemical component can be found in Supplemental **Table S5.2**. An overview of the composition of the diets is given in Supplemental **Table S5.3**. The weight of the distributed feed as well as the leftovers

was also recorded to determine the average DMI of the respective feeding group. Furthermore, the quantities of distributed concentrate via transponder stations were recorded in farms with PMR feeding.

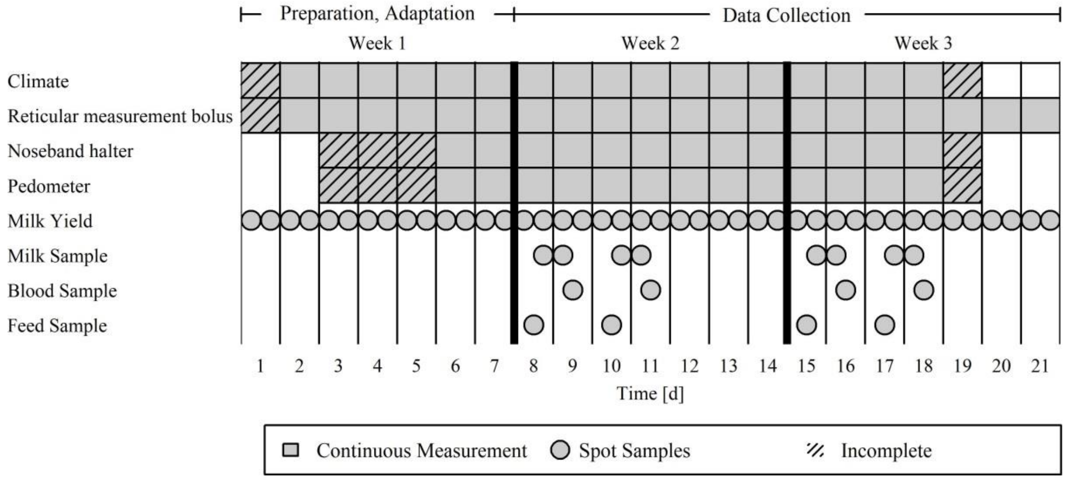

Figure 5.1: Data collection scheme, used sequentially in each of the 10 farms (adapted from Mensching et al., 2020c).

On each farm, 3 to 6 Tinytag climate data loggers (Tinytag Plus 2 TGP-4500, Gemini Data Loggers, Chichester, UK) were used to record climate data in the barn. Thereby, both the temperature and the relative air humidity were recorded at a height of about 2 m in the feeding, lying, and drinking areas, with measurements taken every 15 min. All 100 cows were equipped with a measurement bolus (eCow Ltd., Exeter, Devon, UK) for the continuous intra-reticular measurement of pH and temperature. The boluses were inserted in each of the 10 farms on the first day of the preparation and adaptation phase, so that there were a maximum of 21 d between the insertion and the last day of data collection. Before insertion, all boluses were activated in a water bath at 38.4°C and calibrated in buffer solutions with pH 4 and pH 7. The raw data were provided as mean values of 15-min time intervals for both the reticular temperature and pH.

Jaw movement and locomotion behaviors were recorded using noseband-sensor halters and pedometers (RumiWatch, ITIN + HOCH GmbH, Liestal, Switzerland). The latter were attached to a rear leg. The raw data were converted using the RumiWatch Converter v. 0.7.4.13 (RumiWatch, ITIN + HOCH GmbH) into a 1-h resolution.

Milk yield was measured via on-farm milk quantity recording systems every day at each milking. During the data collection period, milk samples were taken on the evenings of d 8, 10, 15, and 17 and each subsequent morning (see **Figure 5.1**). The samples were taken separately and proportionally to the milk yield according to ICAR guidelines (ICAR, 2017) and were preserved with Bronopol (Georg Hansen e.K., Wrestedt, Germany). Morning and evening samples were analyzed separately by the Landeskontrollverband Weser-Ems e.V. in Leer, Germany, on the same spectrometer (MilkoScan FT+, Foss, Hillerød, Denmark). The standard components (fat, protein, lactose, urea) were estimated using the MIR-based prediction models provided by the manufacturer of the spectrometer. Somatic cell count was measured using a flow cytometer (Fossomatic FC, Foss) combined with the spectrometer. In addition to the standard milk components the MIR spectra were provided (1,060 wavenumbers per sample). The MIR spectra consisting of transmittance values (T) were converted into absorbance values (A) using a logarithm function with $A = -\log10(T)$, as is usually done in spectrometry (Gengler et al., 2016). In the remainder of this paper, only absorbance spectra are considered. The raw spectral data were standardized according to Grelet et al. (2015). Afterward, 27 further traits containing information on the fine milk composition were predicted using models based on the studies of Soyeurt et al. (2007, 2011, 2012) as well as on further unpublished work from the Gembloux Agro-Bio Tech, University of Liège (GxABT, Gembloux, Belgium) and the Walloon Agricultural Research Center (CRA-W, Gembloux, Belgium). Included were predictions of short-chain FA (chain length of 4 to 10 carbons), medium-chain FA (chain length of 12 to 16 carbons), long-chain FA (**LCFA**, chain length of 17 to 18 carbons), and milk lactoferrin content (**LF**). Only the predictions on FA with 15 carbon atoms within the medium-chain FA group are from the unpublished work, whereas all other predictions are based on published results. The applied MIR-based PLS regression models for prediction of FA related traits are characterized by a coefficient of determination of cross-validation of $0.46 \leq \mathbf{R^2_{cv}} \leq 0.99$, a ratio between standard deviation (**SD**) of the trait to the root mean squared error of cross-validation (**RMSE$_{\mathbf{cv}}$**) of $1.4 \leq \mathbf{RPD_{cv}} \leq 10.0$, and a coefficient of determination of validation of $0.39 \leq \mathbf{R^2_{val}} \leq 0.98$. For LF, the model statistics are $R^2_{cv} = 0.72$, $RPD_{cv} = 1.86$, and $R^2_{val} = 0.60$.

During the data collection period, blood samples were collected from the vena caudalis mediana on d 9, 11, 16, and 18, up to a maximum of 3 h after morning milking (see **Figure 5.1**). Separate tubes with serum clot activator were used, one for BHB and nonesterified FA (**NEFA**) in serum and another one for glucose determination. The analysis was conducted in the laboratory of the Institute of Veterinary Medicine at the University of Goettingen (Goettingen, Germany). See Supplemental **Table S5.2** for further details.

Secondary Data Set

The data set for model application consists of the monthly milk test day recordings of the entire herds of the same farms and was gathered for 1 yr from the beginning of the primary data collection described previously. Only data from farms that perform the A4 milk testing scheme according to the ICAR guidelines (ICAR, 2017) with proportional milk test day sampling consisting of evening and morning milk samples were considered. This was the case for 9 out of 10 farms. The pooled samples were analyzed on the same spectrometer as for the primary data set. The resulting secondary data set comprised 21,024 spectra from a total of 3,381 animals up to 305 DIM. In addition to the determination of the standard milk components, the spectra were standardized and the same predictions were made as for the primary data set.

The temperature in the barn was not recorded during collection of the secondary data. However, because it is needed in the further course of this work for correction purposes of fixed environmental effects, it was estimated using a linear model [coefficient of determination (R^2) = 0.99, residual SD = 0.67°C] that was developed by Mensching et al. (2020c) and established on the primary data set. In this model, external climate data from a nearby weather station of the German Weather Service (DWD, 2018), and estimated farm effects were used to predict the temperature in the barn.

Data Preparation

Primary data. To investigate the association between the feed analysis data and the other traits at farm level, the feed analysis data were calculated using the median per trait and farm. For farms with supplementary feeding of concentrates via transponder stations, the composition of the total diet was estimated proportionally to the herd-based average DMI and the average amount of concentrates. In farms with PMR feeding, it was assumed that the peNDF > 8 of the entire diet is traced back only to the PMR and not to the supplemented concentrate feed. Considering the average herd DMI of PMR and concentrate feed, the peNDF>8 of the entire diet was estimated by multiplying the share of PMR of the entire DMI with the calculated peNDF>8 content of PMR. See Supplemental **Table S5.4** for the resulting estimated chemical compositions of the entire diets.

The other data recordings were based on a variety of different data collection systems and methods. They differed particularly in their temporal resolution. Therefore, a data aggregation and a subsequent merging were required. Because the final models are intended to be applicable to proportional sampling test day records, all data were aggregated on a daily basis.

The continuously recorded temperatures of the barn were aggregated by calculating daily arithmetic mean values ($\mathbf{s.\overline{T}}$). The daily reticular mean pH ($\mathbf{r.\overline{pH}}$) and the daily reticular pH range ($\mathbf{r.\Delta pH}$) were derived from the continuously recorded reticular pH data. Because the reticular temperature is significantly influenced by water intake (Liang et al., 2013), the median ($\mathbf{r.T_{med}}$) was used as a robust statistic for the aggregation. An arithmetic mean, however, would systematically underestimate the daily average reticular temperature by about 0.25°C. The jaw movement data in hourly resolution with information on eating and rumination duration per hour were aggregated by calculating the sum of the daily rumination and eating duration ($\mathbf{\Sigma.Rt}$ and $\mathbf{\Sigma.Et}$). In addition, the daily mean rumination chewing frequency ($\mathbf{\overline{RCf}}$) per minute was determined by dividing the sum of daily rumination chews by the daily rumination duration. Based on the hourly pedometer data, the daily lying duration ($\mathbf{\Sigma.Lt}$) was calculated by summation. The milk yield was calculated from the sum of evening and morning milking according to the regular test day sampling procedure. Further, the MIR-predicted milk traits and MIR spectra were pooled proportionally to the morning and evening milk yield. The average milk yields, pooled milk traits, and MIR spectra were assigned to the day of the evening milking sample. The blood values were allocated to the previous day. This approach is also supported by the results of Loor et al. (2007), who observed that NEFA, BHB, and glucose levels responded with a 1-d delay to a feed restriction. Thus, the aim was to compensate for the time delay caused by the ruminal fermentation as well as by the actual digestion, to ensure a realistic time assignment of the individually measured variables.

To normalize the distributions of the BHB and NEFA values of the blood as well as the SCC in the milk, the former were log10 transformed (e.g., Grelet et al., 2019), and the latter were transformed by calculation of SCS according to Wiggans and Shook (1987). The FA traits predicted by standardized MIR spectra were considered as proportion of the total milk fat.

Data acquisitions with automated recording systems and sensitive measuring instruments in difficult environments such as agricultural practice are prone to failure and thus have to be investigated with caution. Therefore, a multivariate plausibility assessment according to Mensching et al. (2020c) was applied to the data set, which was aggregated on a daily basis. In this procedure, the observations of all traits were classified as “physiologically normal,” “hysiologically extreme,” or “implausible,” considering various simultaneously recorded data. Observations classified as implausible were set to missing in the data set. This concerned sensor, performance, blood, and milk MIR spectral data as well as milk MIR-based predictions. However, if it was noticed that extreme spectra were accompanied by extreme observations in other traits, they were not removed from the data set. Observations of 3 animals were removed even before the plausibility of the data was assessed, as they showed unspecific clinical signs and thus were treated by the herd manager with medications, which might affect rumen fermentation. See Mensching et al. (2020c) for more details on this procedure.

The MIR-based prediction equations for the FA traits and lactoferrin were developed in a setting that covered large variability across multiple breeds, production systems, and countries. However, as outliers can always be expected due to variability that is not yet covered but is present in new MIR data, precautions were taken in an additional step using a statistical approach. Not only potential preposterous predictions of the MIRpredicted FA traits and the lactoferrin (values <0) but also extreme values (0.5% of lowest and highest predictions) were filtered out by setting them to missing before associations with the SRS were investigated.

Secondary Data. In the secondary data set, the MIR-predicted FA traits and the lactoferrin were filtered in the same way as in the case of the primary data set. Because the model development based on the primary data set only covers the early-lactation stage ($5 \leq \text{DIM} \leq 37$), the secondary data set was reduced to the same lactation period for PLS model application purposes, with 2,444 remaining observations of 2,254 cows.

SRS: Underlying Concept

For the development of the SRS, different sources of information, or, rather, traits, were combined to generate an integrative SARA-associated phenotype that best reflects the risk of suffering from SARA. It is expected that this will create a trait that is more robust and more accurate for displaying SARA than individual traits alone. A total of 7 different traits were selected, which directly or indirectly can be attributed to SARA. The selection of the traits was based on current knowledge of science derived from the literature as well as on new findings from subsequent data analysis, as follows:

- By definition, a low ruminal pH value and the corresponding acidotic conditions in the rumen are indicators of SARA (e.g., Nocek, 1997). Therefore, a low r.pH reflecting the ruminal pH should increase the SRS.
- As demonstrated by Mensching et al. (2020a), the ingestion behavior in the course of the day is reflected by the pH development. It could be deduced that a uniform ingestion behavior is associated with a limited pH range. However, irregular feed intake is considered a sign of SARA (e.g., Enemark, 2008). This is in line with the fact that the daily average pH and the pH fluctuation range are negatively correlated (Mensching et al., 2020b). For these reasons, high r.ΔpH should increase the SRS.
- The ruminal temperature is negatively correlated with pH (AlZahal et al., 2008) and is also increased by physiological stress (AlZahal et al., 2011). For this reason, elevated r.Tmed is supposed to increase the SRS.
- The daily rumination duration is associated with the amount of secreted saliva and thus the ability to buffer the ruminal pH (Beauchemin, 2018). For this reason, a reduced daily rumination duration (Σ.Rt) should increase the SRS.
- At herd level, a reduced DMI is a sign of SARA (Enemark, 2008). Reduced DMI is basically linked to a reduced eating duration and a reduced amount of secreted saliva during eating (Jiang et al., 2017). Therefore, a reduced daily eating duration (Σ.Et) is supposed to increase the SRS.
- In a preliminary analysis it was determined that from several chewing behavior traits, such as total chews, mean number of ruminate chews per bolus, or mean rumination chewing frequency, the RCf in particular had a significant positive association with the daily mean pH value. Oba and Allen (2000), for example, also observed that higher NDF contents are associated with a higher rumination chewing frequency. Derived from this, low RCf should increase the SRS.
- Khafipour et al. (2009a), for example, observed a decreased milk yield in a SARA induction experiment. Therefore, low milk yields should also be associated with the SRS, where a high SRS should be accompanied by low milk yields.

The directed associations with regard to SARA are visualized in Supplemental **Figure S5.8**.

Statistical Implementation.

The challenge was that the data are subject to a complex hierarchical structure and that factors such as lactation stage, parity, and barn temperature have significant effects on the 7 traits. In a first step, adjusted values were determined for each trait by correcting the raw observations for the influence of fixed effects. For each trait, the following linear mixed model (LMM) was used:

$$y_{ijklm} = \beta_0 + \beta_1 s.\overline{T}_{ijklm} + P_i + \beta_{2,i} DIM_{ijklm} \times P_i + F_j + F_j \times TD_k + A_l + e_{ijklm} \qquad [5.1]$$

where y_{ijklm} is observation m of cow l in farm j on test day k for trait y, $s.\overline{T}_{ijklm}$ is the daily mean temperature in the barn, P_i is the fixed effect of parity (1, 2, 3, or ≥4), and $DIM_{ijklm} \times P_i$ is the interaction of lactation stage DIM_{ijklm} and parity P_i; β_0 is the intercept, and β_1 and $\beta_{2,i}$ are regression coefficients. The farm F_j, the interaction of farm and the test day $F_j \times TD_k$, and the animal A_l are considered as normally distributed random effects $F_j \sim N(0, \sigma_F)$, $F_j \times TD_k \sim N(0, \sigma_{F\times TD})$ and $A_l \sim N(0, \sigma_A)$. Further, e_{ijklm} is a random error with $e_{ijklm} \sim N(0, \sigma_e)$. The lme4 package (Bates et al., 2015) was used for model fitting in R (R Core Team, 2019). After estimating the fixed effects $\beta_0, \beta_1, \beta_{2,i}$, and P_i, the adjusted values y^*_{jklm} were determined as follows:

$$\begin{aligned} y^*_{jklm} &= y_{ijklm} - \left(\beta_0 + \beta_1 s.\overline{T}_{ijklm} + P_i + \beta_{2,i} DIM_{ijklm} \times P_i\right) \\ &= F_j + F_j \times TD_k + A_l + e_{ijklm} \end{aligned} \qquad [5.2]$$

Consequently, the adjusted values contain only variance that is attributed to the random effects of the farm (e.g., feeding and management), test day, and animal, as well as the residuals. To standardize the scaling, a z-transformation was performed as follows:

$$z\left(y^*_{jklm}\right) = \frac{y^*_{jklm} - \mu}{\sigma} \qquad [5.3]$$

with μ being the arithmetic mean and σ the SD of y^*_{jklm} .

Due to loss of data during the data collection and through the plausibility checks, n = 357 of theoretically 400 observations (100 cows with 4 repeated measurements) with MIR spectra remained. Thereof, only 280 showed complete cases for the 7 traits of interest. Because this additional data loss of about 22% of the observations is caused by only 7.6% of missing values, a multiple imputing procedure based on a principal component analysis (**PCA**) model was applied to impute missing data according to Josse and Husson (2016), to permit analysis of at least all 357 cases with available MIR data. This was done using the MIPCA function from the missMDA (Husson et al., 2019) package in R. In this procedure, 100 imputed data sets were created using the bootstrap method from which the final estimates were determined. The optimal number of principal components (**PC**) was determined via crossvalidation using the estim_ncPCA function from the same package. The decision was made based on graphical examination of the mean squared error of prediction depending on the number of used PC.

In a final step, the SRS was calculated as a linear combination of the z-transformed and adjusted values $z\left(y^{*}_{jklm}\right)$ and a directed weight w_{π}, as follows:

$$\mathrm{SRS} = \sum_{\pi=1}^{7} z\left(y^{*}_{jklm}\right)_{\pi} \times w_{\pi} \text{ with } w_{\pi} \in \{-1,1\} \qquad [5.4]$$

Traits for which low values are supposed to cause a high SRS were weighted with $w_{\pi} = -1$ and the others with $w_{\pi} = 1$ (see Supplemental **Figure S5.8**). After calculation of the SRS, an LMM according to **Equation 1** was set up to investigate the variance components of the random effects.

Association with other Traits and Feed Data

To visualize the association between SRS and other reference traits, performance data, blood characteristics, and MIR-predicted traits, the observations were divided into 5 groups of equal size using the SRS, with the quintiles of the SRS as thresholds. Then group affiliation was tested for all traits with an LMM, according to **Equation 1**, with additional consideration of the SRS class as a fixed effect. The estimated group means (**LSM**) were compared with Tukey's post hoc test. For illustration purposes, the LSM of milk main components and MIR-predicted FA were plotted in a spider map, which was created with the radarchart function of the fmsb (Nakazawa, 2019) package in R.

The feed analysis data averaged per farm were examined in a PCA. For the SRS and the other 7 traits, LSM estimates corrected for DIM, parity, and temperature in the barn were determined for the 10 farms using an LMM according to **Equation 1**, considering the farm as fixed instead of random effect. Afterward, these data were projected into the space spanned by the first 2 PC of the feed data.

Development of Mid-Infrared-based Prediction Equations

Milk MIR spectra are characterized by very high correlations between the values of the individual number of wavenumbers. Therefore, PLS regression was used to develop the prediction equations for both the SRS and the available traits derived from the sensor data, most of which were also used for the construction of the SRS. The latter was done to assess how much the individual traits are associated with the milk MIR data. Regarding SARA, prediction equations for the 2 pH parameters $\mathrm{r.\overline{pH}}$ and r.ΔpH are also of interest. An accurate MIR-based prediction of the traits would offer a major advantage over time-consuming and costly measurements, such as those using the eCow bolus or the RumiWatch halter, as it could be implemented cost-effectively and on a large scale in the existing milk recording system.

The supervised learning method PLS regression corresponds to a multiple regression in which the regression coefficients are estimated in an iterative procedure that is analogous to the PCA regression technique. What matters is that the design matrix **X** (MIR spectra) is decomposed in a reduced amount of uncorrelated latent variables (**LV**), consisting of scores and loadings, in such a way that results in maximum covariance to the response variable y (e.g., SRS). In a further step, a regression is performed on those reduced variables to predict y. The number of LV used to derive the calibration model affects model performances in terms of root mean squared error ($\mathbf{RMSE_{cal}}$) and coefficient of determination $\mathbf{R^2_{cal}}$, and is a sensitive parameter to tune, as it may induce under- or overfitting of the model.

The number of LV were determined for each setting with an interleaved stratified 10-fold cross-validation, whereby the folds were divided in such a way that the repeated measurements of an animal were all situated into a common fold, to avoid a single cow having measurements in different folds. Then, for each trait, the model with the smallest RMSEcv was selected, but the number of LV was limited to 15. In this work, the SIMPLS (De Jong, 1993) algorithm was used, applying the PLS (Mevik and Wehrens, 2007) package in R. The resulting model, which can be used for predictions based on new milk MIR data, can be described as

$$y_i = \beta_0 + \sum_{j=1}^{n} \beta_j x_{ij} + e_i, \qquad [5.5]$$

where y_i is the response variable, β_0 is the estimated intercept and $\beta_1,...,\beta_n$ are the regressions coefficients for the 1,...,n values x_{ij} of the respective wavenumber of the optionally pre-treated and reduced spectrum. Further, e_i is a random error with $e_i \sim N(0,\sigma_e)$.

Numerous options for PLS modeling exist, of which the following settings have been systematically evaluated, starting with different spectral pretreatments:

- None
- 0/2/5, 1/2/5, 2/2/5 = Savitkzy-Golay smoothing and 1st and 2nd derivative with a polynomial of 2nd degree and a window length of 5 wavenumbers (e.g., Wallén et al., 2018)
- Gap/1/5, gap/2/5 = 1st and 2nd linear derivative with a gap of 5 wavenumbers (e.g., Soyeurt et al., 2011)
- LP(DIM,1) = Legendre polynomial transformation of the spectrum according to Vanlierde et al. (2015) up to the first degree to account linearly for the DIM

In addition, the part of the spectrum used in the PLS modeling was varied:

- Full spectrum with 1,060 wavenumbers
- Reduced spectrum with 516 wavenumbers according to Grelet et al. (2015)
- Reduced spectrum with 212 wavenumbers according to Grelet et al. (2016)

In each case, the pretreatment was carried out first and then the selection of wavenumbers was performed.

To get an objective view on the model performances, the primary data set was divided into a calibration and a validation data set. This was done individually for each trait, using data from 8 farms for model development and data from 2 farms for external validation. To select the farms, the mean values of the respective traits per farm were calculated and ordered. Observations from the second- and ninth-ordered farm were then selected for validation. This was done to take into account the hierarchical data structure and to ensure that both the calibration and validation data set cover, as far as possible, the range of the respective traits. To evaluate the models, common statistics were calculated. These include the $RMSE_{cal}$, $RMSE_{cv}$, $RMSE_{val}$, R^2_{cal}, R^2_{cv}, R^2_{val} and the RPD_{cv}. For more information on these statistics see Soyeurt et al. (2011) and Williams and Sobering (1993).

Application on the Secondary Data

To further validate the SRS prediction equation, the model was applied to the secondary data set. The milk MIR-predicted SRS values were assigned to an SRS class using the same quintile-based group boundaries as for the primary data set. Then the association between the milk MIR-predicted SRS class and the other MIRpredicted traits was examined, using the same LMM as for the primary data set. Again, a spider map was created to illustrate estimated LSM for the milk main components and the MIR-predicted FA, depending on the milk MIR-predicted SRS class.

All data preparation, creation of figures, and statistical analyses were performed with programs developed in the software environment R.

RESULTS AND DISCUSSION

Descriptive statistics of sensor data-derived traits, blood characteristics, performance, and milk composition-related traits can be found in the Supplemental **Table S5.5** and **Table S5.6**.

SARA Risk Score Development

By combining several traits into one integrative indicator trait, we accounted for the fact that signs of a disease are often reflected not only in one but in several measureable traits. As the health state with regard to SARA can vary in a continuous range between physiologically healthy and clinically diseased (Enemark et al., 2002) the design of the SRS underlies a continuous scale. The traits used for its development were derived from 3 different independent data acquisition systems (intrareticular measurement bolus, noseband-sensor halter, and milk performance). Therefore, the SRS should reflect the health status of the animal more accurately than one trait alone. Alternative approaches are also possible, such as those of Grelet et al. (2019) and De Koster et al. (2019), who divided the metabolic status of cows by k-means clustering of 4 blood metabolites into 3 classes as new trait or phenotype, respectively, which was modeled using MIR data.

The PCA-based imputation procedure for the 7 adjusted and z-transformed traits solved the problem of missing values and maximized the number of observations for the downstream analysis. The optimum number of PC determined for imputing using the *estim_ncPCA* (Husson et al., 2019) function was 3. After imputation, a variable factor map (**Figure 5.2**) was generated, which shows the quality of the imputation as well as visualizing the relationships of the 7 traits. This variable factor map displays the loadings of the variables in the space of the first 2 PC, which together explain 48.81% of the variance. The points correspond to the loadings that were determined during the 100 imputations with the bootstrap method, whereas the thin arrows show the loadings of the final averaged data set. Because the point clouds around the arrowheads are only slightly scattered, the imputing quality can be classified as good enough to use those imputed values. As expected, the signs of the loadings of all traits of the first PC are in line with the directed weights, which were used for the development of the SRS. Additionally, the SRS was projected into the same space as a supplementary variable (bold arrow). The SRS lies almost completely along the first PC, so that it covers a large part of the observed variance of the 7 traits.

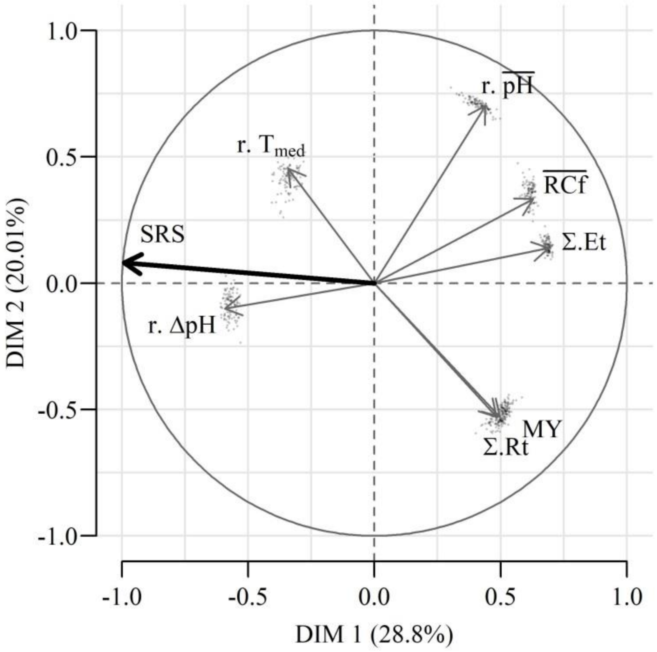

Figure 5.2: Variable factor map using the first 2 principal components based on the averaged multiple imputed data set of the 7 adjusted and z-transformed traits. In addition to the loadings from the variables of the averaged data set (gray arrows), the loadings from the 100 imputed data sets are projected in the same space (points). Furthermore, the SARA risk score was projected into the same space (bold black arrows). $\overline{\mathrm{RCf}}$ = daily mean rumination chewing frequency, r.$\overline{\mathrm{pH}}$ = reticular daily mean pH, r.T_{med} = median of the daily reticular temperature, SRS = SARA risk score, MY = milk yield, r.ΔpH = daily reticular pH range, Σ.Et , Σ.Rt = daily duration of eating and rumination, respectively.

The design of the SRS presented here is only a first approach and can be further optimized and extended. So far, only the direction (−1 or 1) of the selected weights was defined for the SRS calculation. It is conceivable to individually adjust the weights while maximizing the association to a gold standard of the true SARA status−for instance, based on veterinary diagnostics or ventral ruminal pH measurements. However, because such data are not available, the possibilities in the present study are limited.

Analyzing the SRS with an LMM, the following variance components were estimated: $\sigma_F = 1.20$, $\sigma_{F\times TD} = 0.49$, $\sigma_{F\times TD} = 2.81$, and $\sigma_e = 2.02$. Considering the fact that all 10 farms are above average regarding performance and the assumption of high interanimal variance (Humer et al., 2015; Jing et al., 2018), the variance components estimated here seem plausible and meet the expectations with regard to SARA.

Associations of the SARA Risk Score to Other Traits and Feed Data

The relationship between the developed SRS and the other variables used for its development is illustrated in **Figure 5.3**. Furthermore, information on 8 additional variables is reported, including lying duration, 3 performance parameters (fat yield and protein yield), SCS, and milk LF content, as well as 3 blood characteristics (BHB, NEFA, and glucose). Shown are the LSM of each variable depending on the quintile-based SRS class, corrected for the fixed effects of the temperature in the barn, the parity, and the interaction of DIM and parity. The figure shows that the weighting of each variable used to construct the SRS is reflected in the estimated LSM with significant ($P < 0.05$) and directed differences along the means of quintile-based SRS classes, except for r.pH. Nevertheless, a trend can also be seen for r.pH, which would have a significant effect if analyzed with SRS as regression instead of the SRS class in the otherwise identical model. With regard to the lying duration, significant differences can also be observed.

Particularly remarkable is the drop in performance by approximately 20% with increasing SRS classes, which affects not only milk performance but also daily fat and protein yield. The SCS and LF levels are significantly higher in the highest SRS class than in the lowest. Both are well-known indicators of mastitis (Soyeurt et al., 2012). This indicates that the SRS may be linked to other diseases. This was to be expected, particularly as the decrease in performance, eating duration and rumination activity are unspecific signs that can be attributed to clinical conditions of various disorders, such as acidosis, mastitis, or claw disorders (Fleischer et al., 2001; Enemark, 2008). To a certain degree, this is inevitable and generally not a disadvantage, as the superior goal to differentiate between diseased and healthy animals is pursued. In addition, we assume that the value added by taking into account the additional traits compensates for the lower specificity. However, this should be verified with an external data set including veterinary diagnostics or ventral ruminal pH measurements.

The LF content of milk is known to be influenced by the lactation stage and is also negatively correlated with milk yield (Cheng et al., 2008). Consequently, higher LF values are to be expected as a result of lower milk yield. Nevertheless, when considering the daily amount of LF excreted in milk instead of the concentration, a positive trend ($P = 0.058$) was still observed with SRS as regression term instead of the SRS as a class variable in the model. Because LF plays an important role in the immune system of mammals, including ironbinding abilities and bactericidal effects (Baker and Baker, 2004), the increased LF could indicate increased immune responses.

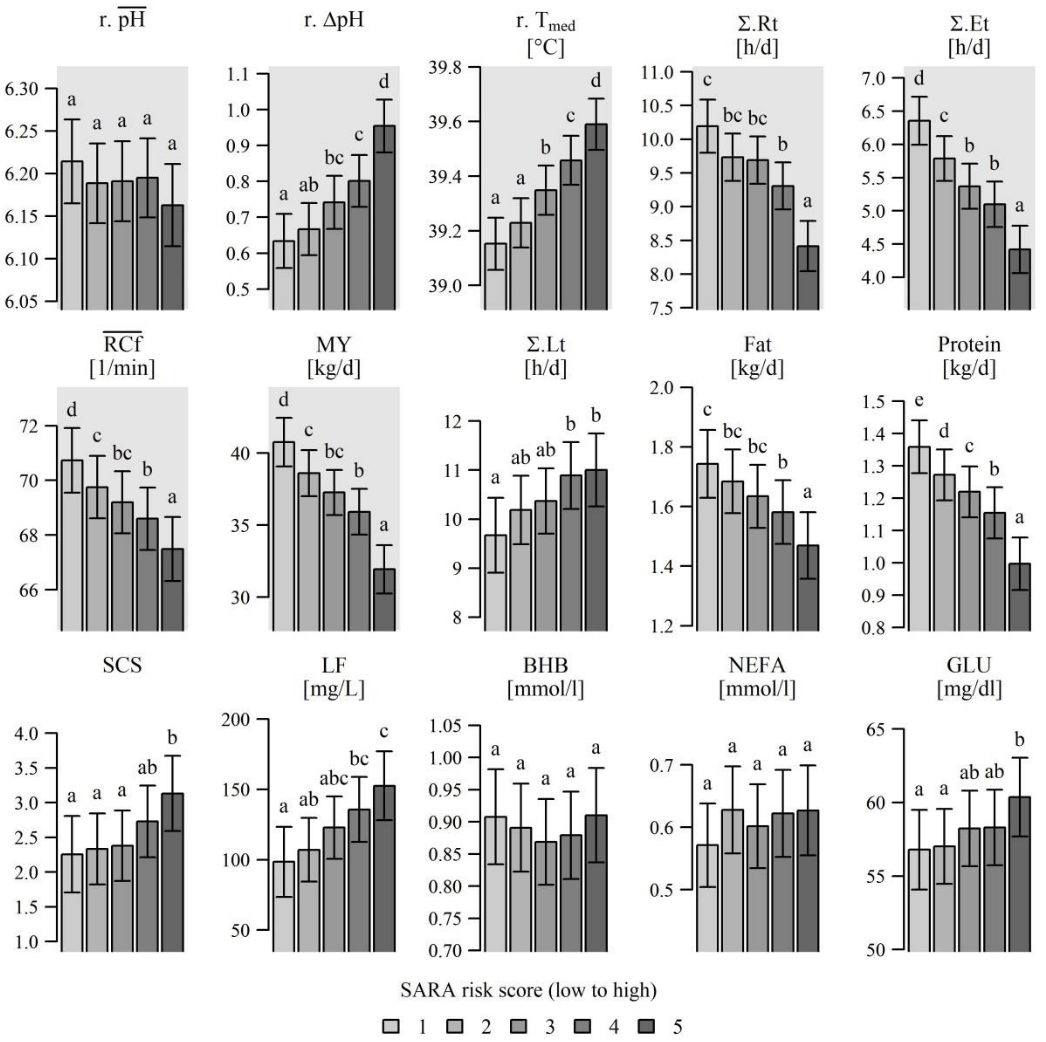

Figure 5.3 LSM (± 1.96 SE) for the 7 reference traits used for the construction of the SARA risk score (SRS; light gray background), further performance traits, and blood characteristics ($307 \leq n \leq 354$) depending on the quintile-based SRS classes. Post hoc comparison of LSM with Tukey's test at $P = 0.05$. Same superscripts indicate nonsignificant differences. The log10 BHB and NEFA estimates were back transformed to the original scale. BHB = blood β-hydroxybutyrate level; GLU = blood glucose; $\overline{\text{RCf}}$ = daily mean rumination chewing frequency; r.$\overline{\text{pH}}$ = reticular daily mean pH; r.T_{med} = median of the daily reticular temperature; SCS = somatic cell score according to Wiggans and Shook (1987); LF = milk lactoferrin; MY = milk yield; NEFA = blood nonesterified fatty acid level; r.ΔpH = daily reticular pH range; Σ.Et , Σ.Rt , and Σ.Lt = daily duration of eating, rumination, and lying, respectively.

Blood contents of BHB and NEFA, which are often used as indicators for metabolic disorders (Oetzel, 2004), do not show a directional relationship to the SRS class. However, an indirect relationship via SARArelated lower feed intake, the resulting reduced energy balance, and the higher BHB and NEFA levels associated with body fat mobilization is conceivable (Baird, 1982; Enemark, 2008). An increase in the blood glucose level is observed if SRS is high. This can be consistent with the results of Khafipour et al. (2009) and Li et al. (2012), who reported increased blood glucose concentration as consequence of a SARA challenge. Considering that propionate is known for its glucogenic role as precursor of gluconeogenesis

(Miettinen and Huhtanen, 1996), a reason for this could be a higher concentration of propionate in ruminal VFA caused by diets rich in concentrate (Lechartier and Peyraud, 2011). This process could also be in line with the fact that SARApromoting diets can lead to an enrichment of bacterial endotoxins in the form of lipopolysaccharides in both the rumen and blood plasma, which in turn can lead to inflammatory reactions (Khafipour et al., 2009b). However, an activated immune system is characterized by increased body temperatures and an increased glucose requirement (Kvidera et al., 2017). Taking into account the fact that glucose is an important precursor of lactose (Kleiber et al., 1955), the significant lower milk yield and thus reduced release of lactose via milk could be linked to the higher SRS.

The PCA results of dietary characteristics as well as the LSM of SRS and the other 7 traits per farm are shown in **Figure 5.4**. The feed data-based variable factor map shows the space spanned by the first 2 PC explaining together 67.23% of the variance. The tips of the gray arrows correspond with the loadings of the feedrelated variables. In addition, the SRS, as well as the other 7 traits were projected into the same space as supplementary variables, as shown using black bold arrows. It can be seen from this figure that the diets of the 10 farms differ mainly with regard to the ratio of properties associated with fiber (NDF after amylase treatment and ashing, crude fiber, peNDF > 8) to energy (starch, nitrogen-free extract, NEL), as they position opposite along the first PC. As expected, the daily average pH also tends to point in the direction of the fiber-associated variables, whereas it is opposite to the variables that are typical for diets rich in concentrate. Thus, a large consensus with the results of Mensching et al. (2020b) can be seen, where feed properties and pH parameters of meta-analytical data were also presented jointly in a variable factor map. Consequently, **Figure 5.4** confirms the basic relationships between diet properties and pH development with regard to SARA (Kleen et al., 2003; Enemark, 2008). Interestingly, the SRS is positioned in the direction of starch and nitrogen-free extract, as well as opposite to the fiber-associated properties and the r.pH. This generally confirms that the SRS has a high potential to indicate SARA.

Presumably, the observed relationships will not only be attributable to the composition of the diet but can also be related to the individual feed intake behavior of the animals. In this respect, Coon et al. (2019) demonstrated that part of the observed interanimal variance regarding susceptibility to SARA is attributable to behavioral differences in sorting against long particles.

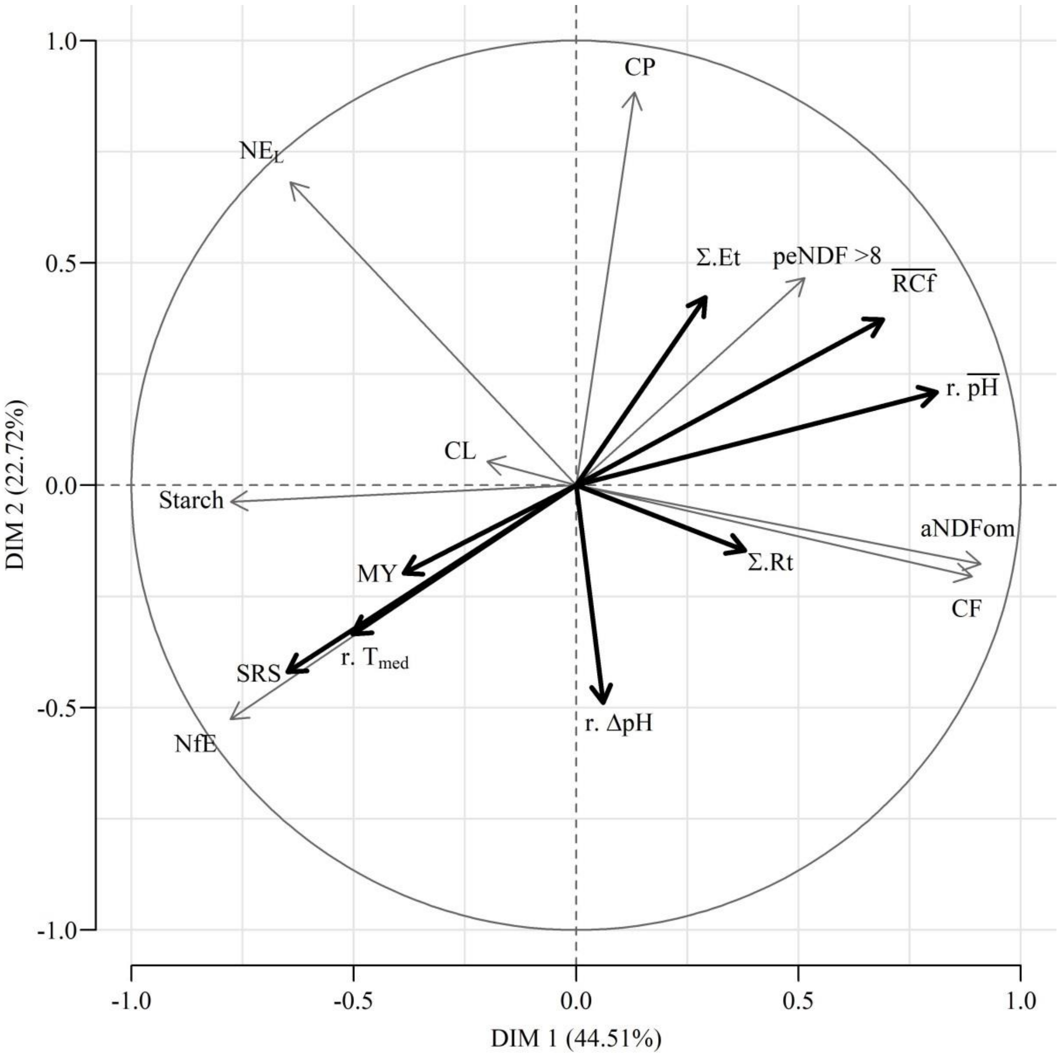

Figure 5.4: Variable factor map using the first 2 principal components of the feed analysis data. In addition to the loadings from the feedrelated variables (gray arrows), the SARA risk score (SRS), and the other 7 traits used for its development were projected into the same space (bold black arrows). aNDFom = NDF after amylase treatment and ashing; CF = crude fiber; CL = crude fat; LF = milk lactoferrin; MY = milk yield; NfE = nitrogen-free extract; peNDF > 8 = physically effective NDF retained on an 8-mm sieve; $\overline{RCf}$ = daily mean rumination chewing frequency; r.$\overline{pH}$ = reticular daily mean pH; r.T_{med} = median of the daily reticular temperature; r.ΔpH = daily reticular pH range; Σ.Et , Σ.Rt , and Σ.Lt = daily duration of eating, rumination, and lying, respectively.

The relationship between the milk main components, FA traits, and SRS are shown in **Figure 5.5** in a spider map. In this figure, one can see that most of the milk traits display a directed relationship with the SRS class. With regard to the milk main components, higher SRS are accompanied by increased fat and decreased protein and lactose. It is known that lower lactose levels are associated with udder diseases, as is the case with the previously discussed increased SCS (Berning and Shook, 1992). In terms of SARA, a milk fat depression would be expected (Enemark, 2008). However, with regard to SRS, the opposite was observed in this study, namely higher fat levels accompanied by significantly lower daily fat yields, the latter being mainly related to the lower milk yield (see **Figure 5.3**). This could be attributed to the fact that all cows were in early lactation with DIM ≤ 37. Enemark et al. (2004) have described a numerically negative but not significant association between pH and milk fat content in early-lactation cows, from which they concluded that the individual fat content is not suitable as an indicator for SARA at this stage of lactation. The increase in milk fat might be caused by a reversed dilution effect as a consequence of reduced milk performance.

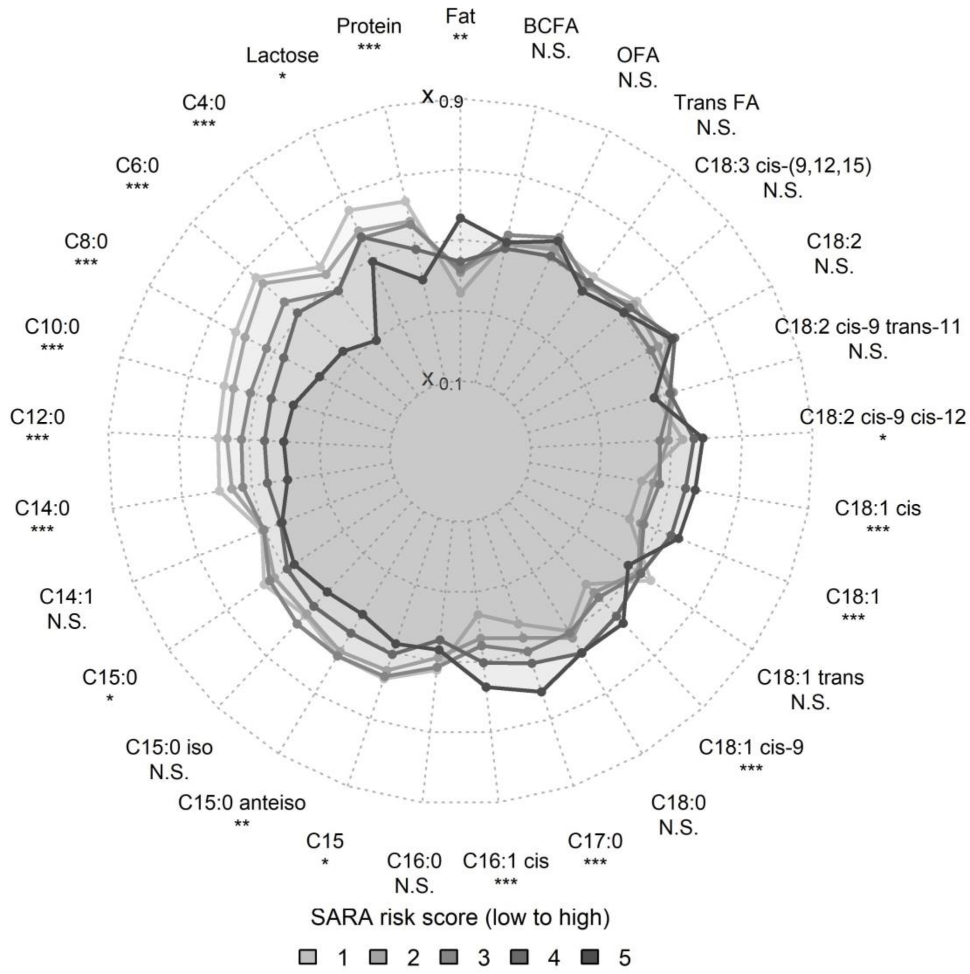

Figure 5.5: Spider plot (Nakazawa, 2019) to visualize standardized LSM of milk main components (%) and mid-infrared‑predicted fatty acid traits (% of fat) depending on a quintile-based classification of the SARA risk score for the primary data set ($351 \leq n \leq 357$). The axes of the individual variables range from the 10 ($x_{0.1}$) to the 90% ($x_{0.9}$) quantile. The Cx:y -coding includes the number of C-atoms (x) as well as the number of double bonds (y) of the respective fatty acid. P-values for the effect of class affiliation on the content of the respective fatty acid from the ANOVA table: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; N.S. = $P \geq 0.05$. FA = fatty acids; BCFA = branched-chain FA; OFA = odd-chain FA

Further, lower levels of short- and medium-chain FA are observed with a higher SRS, whereas both significant and nonsignificant associated FA are found in LCFA. No significant association between SRS class and *trans* LCFA can be observed, whereas significantly higher levels of the *cis* LCFA C18:2 *cis*-9, *cis*-12, C18:1 *cis*, and C18:1 *cis*-9 can be found with an increasing SRS class. Some patterns in the milk FA profile are similar to those already observed by Gross et al. (2011) as a consequence of a negative energy balance. These include elevated C18:1 *cis*-9 as well as lower levels of the FA from the de novo synthesis (≤16 carbon atoms) as a result of a higher proportion of fat resulting from the mobilization of adipose tissue. However, a negative energy balance is plausible, as poor body conditions are also considered to be an indication for SARA (Nocek, 1997).

Additionally, the milk odd- and branched-chain FA, which are essentially derived from bacteria that have been built and left the rumen, are considered to be indicators of ruminal fermentation (Vlaeminck et al., 2006). In this regard, significantly higher levels of C17:0 and lower levels of all C15 FA could be associated with increased SRS in the primary data set. In comparison, Vlaeminck et al. (2006) determined a positive association for C15:0 with the ruminal propionate concentration, whereas they found an opposite association for C15:0 iso. Also, Van Nespen et al. (2005) observed a changed FA profile of the milk with increased iso C17:0, C15:0, C18:2 *cis*-7, *trans*-11, and C18:1 *trans*-10 + C18:1 *trans*-11, and lower contents of iso C14:0 as consequence of increased dietary starch content.

Mid-infrared based Partial Least Squares Regression Models

Descriptive statistics for all traits for which milk MIR-based PLS models were developed are summarized in **Table 5.1** separately for the data sets used for calibration and for external validation. All traits are nearly symmetrical and approximately normally distributed. However, it is noticeable that the r.ΔpH tends to show extreme values in the upper range, and r.pH, daily rumination, and eating duration in the lower range, which can be indicative for SARA or even acute ruminal acidosis-related conditions. Considering the observed SARA prevalence of about 20% in German herds (Kleen et al., 2013), this is plausible, as it can be assumed that the majority of the animals were in a physiologically healthy condition and only a small number were diseased.

Table 5.1 also contains model characteristics, model statistics, and the results of the external validation on individual PLS calibration models. Furthermore, **Figure 5.6** shows for all traits a plot observed against the predicted values from cross-validation and external validation. The use of the entire spectrum led to the best results only for RCF; otherwise, the reduced spectrum with 212 or 516 wavenumbers always led to the best model performances. This was expected, considering that large parts of the spectrum contain noisy regions due to the water content of milk (Grelet et al., 2015). Furthermore, it was observed that the choice of spectral pretreatment is dependent on the respective trait. The fit statistics of the best models only indicate moderate model performances. Looking at R^2_{val} of the 8 traits, only the models for SRS and r.ΔpH show a noteworthy potential, with R^2_{val} = 0.37 and R^2_{val} = 0.49. Consequently, these models explain only a part of the observed variation and are not able to make quantitative accurate predictions. All other models are characterized by R^2_{val} < 0.10 and thus show inferior performance.

Especially for the SRS and for r.T_{med}, models with a relatively high number of LV emerged. As a consequence, a lack of robustness of the model is to be expected, which is also reflected in a large discrepancy between the results of calibration and cross-validation. But this is also true for all other models. Another reason could be the small size of the data set, and associated lack of variability (Davies and Fearn, 2006).

In their current forms, these models cannot be used for individual cow management. However, some models seem to be able to distinguish between extreme values. In this context, Gengler et al. (2016) argued that, under the assumption of random prediction errors, the application of such models to a group of animals could increase the accuracy of the predictions. Considering the fact that these traits are very complex and do not correspond to direct components of the milk, these associations are appreciable and indicate a high potential to obtain additional information that goes beyond the standard components relevant for milk payment. Because the present PLS models are developed with a relatively small amount of data, with few suspected cases of SARA, it is likely that the reduced data volume causes an underestimation of the potential R^2 (Davies and Fearn, 2006). Therefore, it would be of great interest to implement a data set with a higher proportion of SARA-affected cows, to evaluate potential increase of the model performances.

It is interesting that the R^2_{cv} and R^2_{val} for r.pH are very low, whereas they are at least moderate for r.ΔpH. This could have several reasons. In terms of $\mathrm{r.}\overline{\mathrm{pH}}$, it could be that it is generally not well predictable. However, the low accuracy could also be attributable to the reticular pH measurement. Although the reticular and (ventral) ruminal pH are highly associated, it is not possible to infer precisely from reticular pH to ruminal pH, with the latter being the target pH with regard to SARA (Falk et al., 2016). Another reason could be the accuracy of the measurement technique. It is well known that drift is a problem of indwelling boluses (e.g., Villot et al., 2017), although it is more likely to occur several months after insertion of the bolus in the reticulum. However, the drift would primarily affect the accuracy of $\mathrm{r.}\overline{\mathrm{pH}}$ and not of r.ΔpH.

Table 5.1: Descriptive statistics of the calibration and validation data, model characteristics and statistics as well as validation results of the milk mid-infrared spectra based partial least squares calibration models[1]

	Trait							
	SRS	r.$\overline{\text{pH}}$	r.ΔpH	r.$\overline{\text{T}}_{\text{med}}$	Σ.Rt	Σ.Et	$\overline{\text{RCf}}$	Σ.Lt
Unit				°C	h/d	h/d	1/min	h/d
Descriptive statistics of the calibration data								
n	290	253	233	259	277	280	268	268
n$_A$	79	70	70	73	78	78	79	79
Mean	-0.12	6.19	0.76	39.36	9.44	5.32	68.15	10.57
SD	3.48	0.16	0.22	0.33	1.27	1.26	5.13	2.50
Min	-6.51	5.54	0.38	38.30	3.39	1.65	55.48	3.37
Median	-0.56	6.23	0.73	39.30	9.57	5.29	67.94	10.73
Max	16.45	6.52	1.67	41.30	11.94	9.04	79.87	18.38
Descriptive statistics of the validation data								
n	67	54	74	62	59	60	75	66
n$_A$	20	19	19	20	17	18	18	19
Mean	0.32	6.17	0.78	39.41	9.42	5.40	68.74	10.57
SD	4.12	0.17	0.21	0.40	1.33	1.28	5.69	3.61
Min	-7.16	5.77	0.40	38.90	5.00	1.46	59.57	1.38
Median	0.20	6.21	0.74	39.30	9.57	5.45	68.48	10.75
Max	14.07	6.49	1.56	40.70	11.54	7.87	83.23	17.25
Model characteristics								
Pretreat	gap/1/5	gap/1/5	2/2/5	gap/1/5	–	–	0/2/5	2/2/5
LP	–	–	–	–	LP(DIM,1)	–	–	–
WN	516	212	516	212	212	212	1060	212
LV	12	3	3	13	8	8	3	3
Model statistics and validation results								
RMSE$_{\text{cal}}$	2.36	0.15	0.18	0.24	1.02	1.06	4.34	2.30
RMSE$_{\text{cv}}$	2.91	0.16	0.19	0.30	1.10	1.17	4.85	2.40
RMSE$_{\text{val}}$	3.35	0.18	0.15	0.41	1.45	1.29	5.76	3.42
R^2_{cal}	0.56	0.16	0.33	0.52	0.37	0.32	0.29	0.16
R^2_{cv}	0.32	0.06	0.23	0.26	0.26	0.17	0.13	0.09
R^2_{val}	0.37	0.05	0.49	0.09	0.00	0.03	0.04	0.10
RPD$_{\text{cv}}$	1.19	1.02	1.14	1.12	1.16	1.08	1.06	1.04

[1] LP, LP(DIM,1) = Legendre polynomial transformation of the spectrum; see Material and Methods. n = sample size; nA = number of animals; min, max = minimum and maximum. Pretreat = spectral pretreatments; see Material and Methods. R^2_{cal}, R^2_{cv}, R^2_{val} = coefficient of determination of calibration model, of cross-validation, and of external validation. RMSE$_{\text{cal}}$, RMSE$_{\text{cv}}$, and RMSE$_{\text{val}}$ = root mean squared error of the calibration model, cross-validation, and external validation, respectively. RPDcv = ratio of SD of reference to the SE of cross-validation. $\overline{\text{RCf}}$ = daily mean rumination chewing frequency; r.$\overline{\text{pH}}$ = reticular daily mean pH; r.T$_{\text{med}}$ = median of the daily reticular temperature; r.ΔpH = daily reticular pH range; SD = empirically estimated SD; SRS = SARA risk score; WN = number of wavenumbers; LV = latent variables. Σ.Et, Σ.Rt, and Σ.Lt = daily duration of rumination, eating, and lying, respectively.

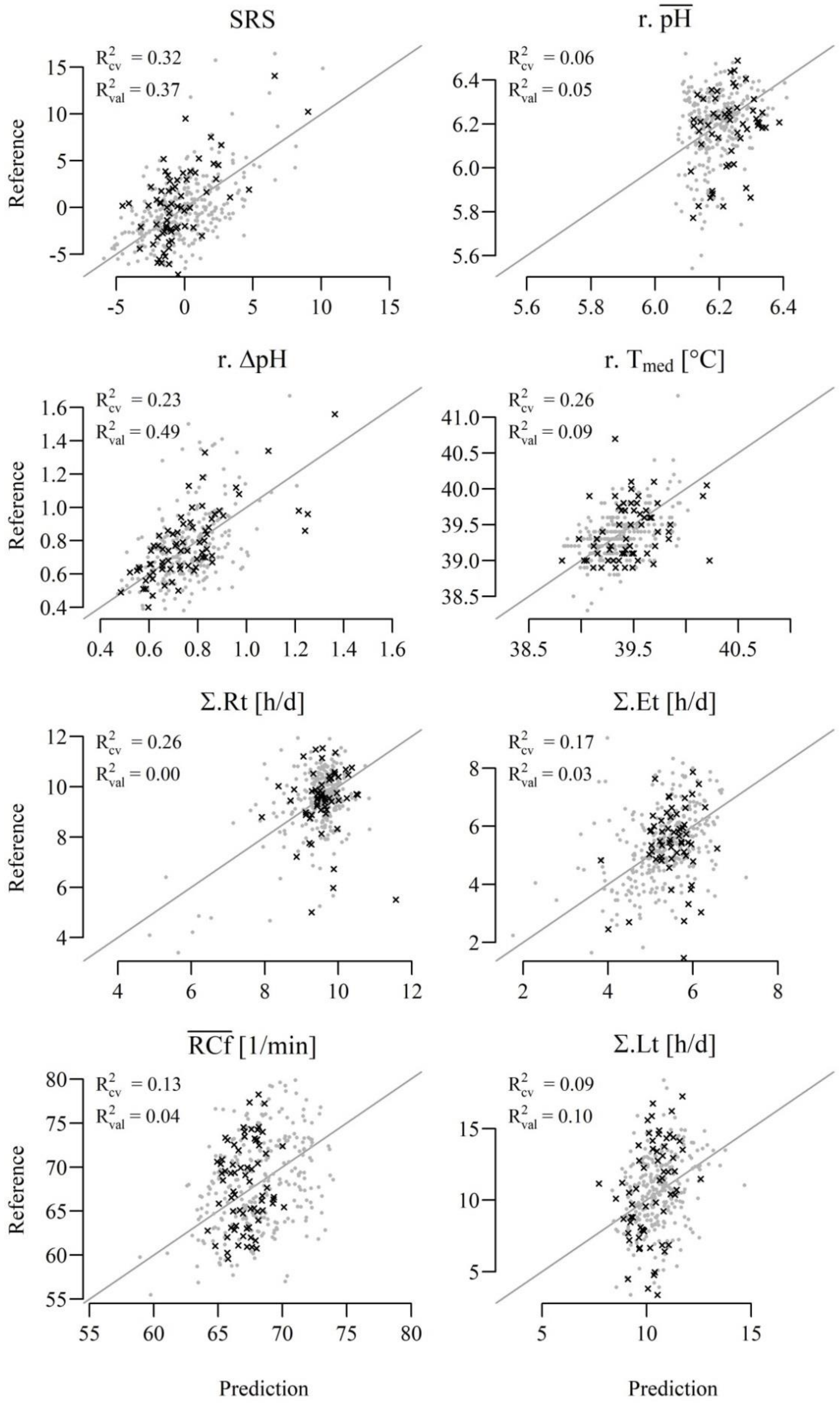

Figure 5.6: Scatterplot for reference values against predicted values from cross-validation (light gray dots) and external validation (crosses) based on mid-infrared–based partial least squares regression models. R^2_{cv}, R^2_{val} = coefficient of determination of cross-validation and of validation, respectively; $\overline{RCf}$ = daily mean rumination chewing frequency; r.$\overline{pH}$ = reticular daily mean pH; r.T_{med} = median of the daily reticular temperature; SRS = SARA risk score; ΔpH = daily reticular pH range; Σ.Et, Σ.Rt, and Σ.Lt = daily duration of eating, rumination and lying, respectively ($306 \leq n \leq 356$).

Comparing the achieved model statistics for the SRS with the results of others who have worked on MIRbased prediction of complex indirect traits, higher performance can be found. McParland et al. (2014), for example, were able to achieve a cross-validated correlation Correlation between observed and predicted values when modeling the energy balance with correlation coefficient r = 0.67 and R^2_{cv} = 0.45, respectively. In the MIR-based prediction of methane emissions measured with a respiration chamber Vanlierde et al. (2018) even achieved an R^2_{cal} of 0.65 and R^2_{cv} of 0.57 using a cross-country data set. However, those studies are based on a large variability, with more than 1,500 records and data from 5 countries. This encourages increase of the current data set with complementary records, which, at best, also cover the entire lactation period.

Because both the FA traits and the SRS can be estimated at least moderately with the milk MIR spectra and they show moderate associations, the predictability of the SRS is likely to be based on altered milk composition. Therefore, the following causality chain can be hypothesized: diet (composition), environment (e.g., temperature), individual animal (e.g., microbiome, genetics, behavior, lactation stage, parity), as well as interactions between them, influence the ruminal fermentation process. This is reflected in the ruminal FA pattern and subsequently also to some extent in the FA pattern of the milk. In turn, this is related to a modified MIR spectrum.

Because milk fat is considered the most variable component of milk, in terms of both concentration and composition (Palmquist, 2006), the major part of spectral variability is attributable to altered milk fat composition. Therefore, it can be assumed that the determined associations of the other 7 traits are also essentially attributable to an altered milk fat composition, whereby the model quality achieved for each trait provides information about the strength of this relationship.

Application on the Secondary Data Set

The developed prediction equation for the SRS was applied to the secondary data for observations in the same lactation stage ($5 \leq DIM \leq 37$). As no reference values of the SRS are available, this is not an external validation in the narrow sense. Nevertheless, the SRS predictions were associated with the main milk components and the FA profile. The results of this investigation are shown in **Figure 5.7** in a spider map (Nakazawa, 2019). Comparing this spider map with the one in **Figure 5.5**, a very high level of agreement is noticeable. It seems that for some of the traits, the directional relationships are even more evident than in the primary data set. Due to the small number of observations in the primary data set, it is likely that more

SARA cases are present in the secondary data set, with an increased discrimination between extreme groups. Complementary, more FA variation is obviously present in the secondary data set.

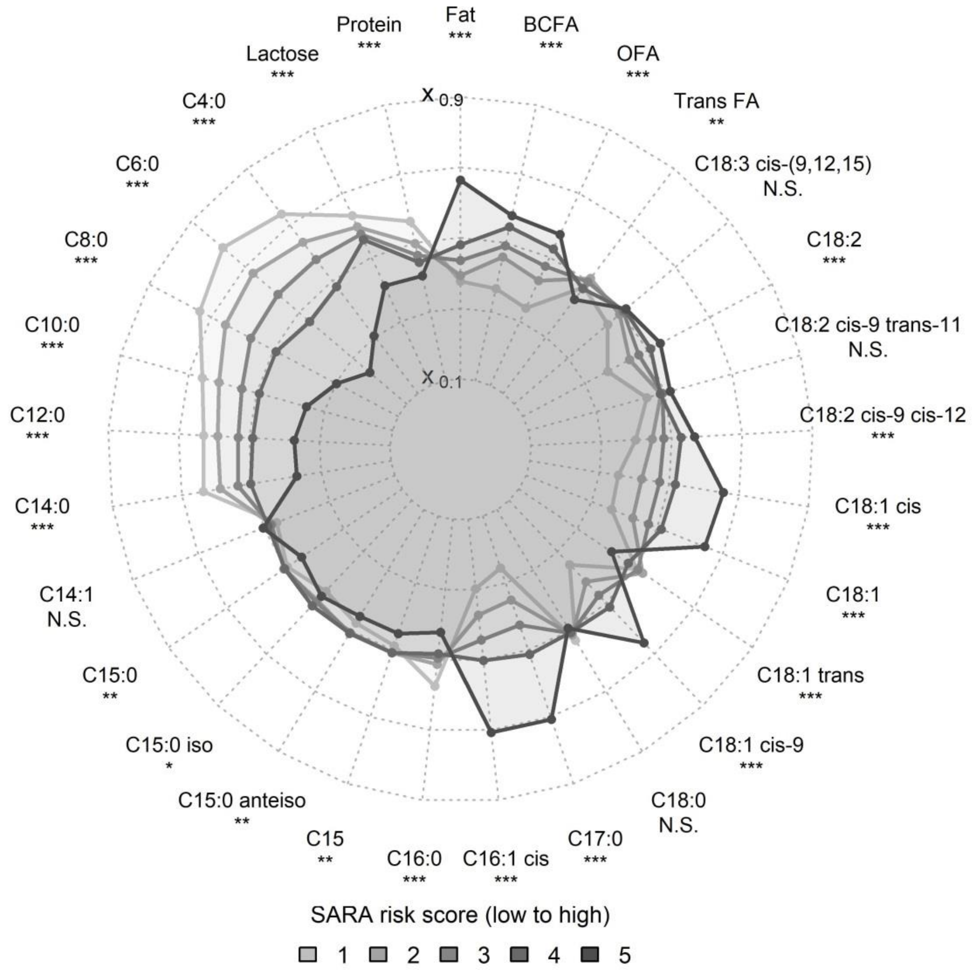

Figure 5.7: Spider plot (Nakazawa, 2019) to visualize standardized LSM of milk main components (%) and mid-infrared (MIR)-predicted fatty acid traits (% of fat) depending on a quintile-based classification of the MIR-predicted SARA risk score for the secondary data set (n = 2,444; $5 \leq \text{DIM} \leq 37$). The axes of the individual variables range from the 10 ($x_{0.1}$) to the 90% ($x_{0.9}$) quantile. The Cx:y -coding includes the number of C-atoms (x) as well as the number of double bonds (y) of the respective fatty acid. *P*-values for the effect of class affiliation on the content of the respective fatty acid from the ANOVA table: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; N.S. = $P \geq 0.05$. FA = fatty acids; BCFA = branched-chain FA; OFA = odd-chain FA.

The fact that higher milk MIR-predicted SRS are accompanied with higher levels of odd- and branchedchain FA, as well as C17:0, validates the SRS approach, as such directed relationships between the milk FA profile and ruminal fermentation properties have already been discussed in terms of SARA as a consequence of diets rich in concentrate (Fievez et al., 2012). However, in view of the results generated here, it is crucial that the observed changes in the FA profile of **Figure 5.7** are attributed to different values of the predicted SRS, as the farm and thus implicitly also the feeding was considered as a random effect in the used LMM.

Nevertheless, farm- and therefore potential feed-specific effects are reflected in the frequency distribution of the predicted SRS classes within individual farms (see Supplemental **Figure S5.9**). But because all SRS classes, and implicitly the varying FA profiles, occur in all farms, a large part of the observed variation is presumably related to high interanimal variance.

CONCLUSIONS

This study developed an innovative approach in which information from different independent data acquisition systems (intrareticular measurement bolus, noseband-sensor halter, and milk performance) were combined to design an integrative indicator trait for SARA called SRS. At the farm level, higher SRS were related to lower daily average pH values, higher levels of easily fermentable carbohydrates, and lower levels of physical structure in the diet. On the individual level, an increased SRS was also found to be linked to a modified FA profile, in which lower levels of saturated and short-chain FA and higher levels of C16:1 cis, C17:0, and odd- and branched-chain FA could be observed. Furthermore, using a PLS regression model, a milk MIR spectra‑based prediction equation with a moderate predictability was established, demonstrating the high potential of milk composition-based characterization of the health status of lactating cows. Based on our research, a first approach for a SARA monitoring could be implemented on a large scale in routine milk performance testing using the MIR-based prediction of the SRS.

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AUTHOR CONTRIBUTIONS

Conceptualization: AM. Data curation: AM, MZ. Formal Analysis: AM. Funding acquisition: JH. Investigation: MZ. Methodology: AM. Project administration: JH. Software: AM, Supervision: JH, ARS, SD. Visualization: AM. Writing – original draft: AM. Writing – review and editing: AM, MZ, JH, CG, NG, SD, ARS.

SUPPLEMENTARY

Table S5.2: Overview of the methods used to determine the chemical composition of the feed and the blood parameters. The feed analyses were performed according to the methods of the Association of German Agricultural Analytic and Research Institute (VDLUFA, 1993). Fiber analysis was carried out using the filter bag system (Ankom Technology, Macedon, New York).

Component	Method
Feed	
Dry Matter content (DM)	VDLUFA 3.1 ‘Determination of moisture content’
Crude Ash (CA)	VDLUFA 8.1 ‘Determination of crude ash’
Crude Protein (CP)	VDLUFA 4.1.2 ‘Determination of crude protein according to Dumas’
Crude fat (CL)	VDLUFA 5.1.1 ‘Determination of crude fat, method B with HCl maceration’
Crude Fiber (CF)	VDLUFA 6.1.1 ‘Determination of crude fiber‘ and Ankom method 7 ‘Crude Fiber in Feeds- Filter Bag Technique’
Neutral detergent fiber after amylase treatment and ashing (aNDFom)	VDLUFA 6.5.1 ‘Determination of neutral detergent fiber after amylase treatment and ashing (aNDFom)‘ and Ankom method 6 ‘NDF in Feeds- Filter Bag Technique’
acid detergent fiber after ashing (ADFom)	VDLUFA 6.5.2 ‘Determination of acid detergent fiber after ashing (ADFom)’ and Ankom method 7 ‘ADF in Feeds- Filter Bag Technique’
Acid detergent lignin (ADL)	VDLUFA 6.5.3 ‘Determination of acid detergent lignin (ADL)’
Enzyme-soluble organic substance (ELOS)	VDLUFA 6.6.1 ‘Determination of Enzyme-soluble organic substance - cellulase method’
24 h gas yield (GY24)	VDLUFA 25.1 ‘Determination of gas formation according to the Hohenheim gas test‘
Starch enzymatic (Starch)	‚Enzymatic starch determination‘ according to Brandt[1] et al. (1987)
Blood	
β-hydroxybutyrate (BHB)	Quantitative *in vitro* determination in serum using a kit from Randox[2] Labroatories Ltd. (London, UK) with an kinetic enzymatic method according to McMurray[3] et al. (1984)
Non-esterified FA (NEFA)	Quantitative *in vitro* determination in serum with a photometric system using a kit from Labor+Technik[4], Eberhard Lehmann GmbH (Berlin, Germany) with an enzymatic method
Glucose	Determination with Hexokinase and Glucose-6-phosphate Dehydrogenase according to Slein[5] (1965) with an optical method

[1] Brandt, M., A. Schuldt, P. Mannerkorpi, and T. Verasilp. 1987. Zur enzymatischen Stärkebestimmung im Darminhalt und Kot von Kühen mit hitzestabiler Amylase. Arch. Anim. Nutr. 37:455 (Abstract).

[2] https://www.randox.com

[3] McMurray, C.H., W.J. Blanchflower, and D.A. Rice. 1984. Automated kinetic method for D-3-hydroxybutyrate in plasma or serum. Clin. Chem. 30:421–425. http://dx.doi.org/10.1093/clinchem/30.3.421.

[4] https://lt-sys.de

[5] Slein, M.W. 1965. D-Glucose: Determination with Hexokinase and Glucose-6-phosphate Dehydrogenase. Pages: 117-130 in Methods of Enzymatic Analysis, 2nd edition, W. Bartley, ed. Verlag Chemie, GmbH, Oxford, UK.

Table S5.3: Composition of the diet on the 10 farms[1]

	Farm									
Component, kg FM/d	1	2	3	4	5	6	7	8	9	10
Maize silage	15.31	13.93	6.97	29.83	21.39	13.44	22.38	27.84	22.15	19.32
Grass silage	24.07	18.87	17.44	24.93	23.93	20.12	11.72	14.97	26.12	14.37
Barley whole crop silage							11.72			
Alfalfa hay	0.51			1.26						
Hay							0.79			0.40
Barley straw	0.49									
Wheat straw			0.34		0.28					
Draff						1.56			4.15	
Soybean extraction meal		1.80	0.69	1.70		1.50			1.51	
Rapeseed extraction meal			1.74	5.10	6.37	1.53			1.51	4.68
Protein premix	3.81[2]						4.65[4]			
Energy premix	2.81[3]						0.64[5]			
Barley (meal)	0.61									
Wheat (meal)						0.81			2.64	1.84
Corn cob mix, ensilaged	2.19		2.79							
AWE meal			2.61[6]							
SM meal		6.71[7]								
Corn meal				5.49	4.43	3.37				4.04
Dried molasses			2.61							
Dried beet pulp				0.84						
Fat	0.22				0.18					0.20
Minerals	0.12	0.33	0.12	0.24	0.29	0.29	0.28	0.37	0.31	0.54
Water	9.37			5.76						8.20
Concentrate in TMR								8.00		
Concentrate (separate)			2.60		1.00	2.29	3.96	4.48	4.78	0.40

[1] FM = fresh matter.

[2] Rapeseed meal, solvent-extracted (50%); soybean meal, solvent-extracted (50%).

[3] Corn (30%); dried molasses (49%); palm-kernel meal (15%); sugar cane molasses (2%); calcium carbonate (2,5%); magnesium oxide (0,5%); sodium chloride (0,7%).

[4] Soybean meal, solvent-extracted (33%); rapeseed meal, solvent-extracted (33%); rapeseed expeller (33%); vinasse (1%).

[5] Rye (37%); dried molasses (30%); corn (30%); vinasse (2.7%); palm-kernel fat (0.3%).

[6] Barley (33%); wheat (33%); corn (33%); molasses (1%).

[7] Corn (33%); dried beet pulp (33%); rapeseed meal, solvent-extracted (33%); molasses (1%).

Table S5.4: Estimated chemical composition of the entire diets in the 10 farms[1]

	Farm									
	1	2	3	4	5	6	7	8	9	10
DM, g/kg FM	373	451	567	374	418	450	456	485	456	409
CA, g/kg DM	71	81	73	78	69	77	76	83	83	67
CP, g/kg DM	163	165	196	180	176	182	158	143	159	134
CL, g/kg DM	45	33	35	36	46	34	38	33	34	43
CF, g/kg DM	190	195	180	177	159	151	184	171	168	170
aNDFom, g/kg DM	381	424	370	365	369	326	363	377	361	345
peNDF>8, g/kg DM	213	199	183	174	162	159	129	148	194	167
Starch, g/kg DM	156	148	181	236	188	234	171	186	146	260
NfE[2], g/kg DM	531	527	517	532	548	562	538	570	548	580
GE[3], MJ/kg DM	18.80	18.42	18.69	18.55	18.83	18.53	18.54	18.11	18.33	18.63
ME[4], MJ/kg DM	11.35	10.74	11.40	11.06	11.40	11.88	10.75	10.84	11.32	11.35
NE_L[5], MJ/kg DM	6.91	6.48	6.95	6.70	6.95	7.33	6.47	6.59	6.92	6.90

[1] DM = dry matter content; CA = crude ash; CL = crude fat; CP = crude protein; CF = crude fiber; aNDFom = neutral detergent fiber after amylase treatment and ashing;peNDF>8 = physically effective NDF retained on an 8-mm sieve; NfE = nitrogen-free extract; GE = gross energy; ME = metabolizable energy.

[2] NfE = 1000 – (CL + CP + CA + CF).

[3] GE (MJ/kg DM) = 0.0239 CP (g/kg DM) + 0.0398 CL (g/kg DM) + 0.0201 CF (g/kg DM) + 0.0175 NfE (g/kg DM) according to: Society of Nutrition Physiologie. 2001. Energie- und Nährstoffbedarf landwirtschaftlicher Nutztiere, Nr. 8: Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder. DLG-Verlag, Frankfurt am Main, Germany.

[4] ME (MJ/kg DM) = 1.5473 + 0.00764 ELOS (g/kg DM) + 0.23292 CL (g/kg DM) – 0.000021 $(\text{CF (g/kg DM)})^2$ – 0.002760 $(\text{EE (g/kg DM)})^2$ according to: Boguhn, J., H. Kluth, O. Steinhöfel, M. Peterhänsel, and M. Rodehutscord. 2003. Nutrient digestibility and prediction of metabolizable energy in total mixed rations for ruminants. Arch. Anim. Nutr. 57:253–266. http://dx.doi.org/10.1080/00039420310001594405.

[5] NE_L (MJ/kg DM) = 0.60 (1 + 0.004 (q - 57)) ME with q = 100 (ME / GE) according to: Van Es, A.J.H. 1978. Feed evaluation for ruminants. I. The systems in use from May 1977-onwards in The Netherlands. Livest. Prod. Sci. 5:331–345. http://dx.doi.org/10.1016/0301-6226(78)90029-5.

Table S5.5: Descriptive statistics of sensor data, blood parameters, SARA risk score, milk yield and milk main components[1]

	n_A	n	Mean	SD	Min	Median	Max
Climate							
Mean temperature (s.$\overline{T}$), °C	100	160	12.1	6.2	-1.3	12.8	25.0
Animal							
Days in milk (DIM), d	100	1,600	20.1	7.5	3	20	41
Parity	100	1,600	3.1	1.6	1	3	8
Reticular measurement bolus							
Daily mean pH (r.$\overline{pH}$)	89	1,327	6.18	0.16	5.35	6.21	6.71
Daily pH range (r.ΔpH)	89	1,327	0.76	0.21	0.35	0.72	1.67
Median of the daily reticular Temperature (r.T_{med}), °C	93	1,380	39.36	0.34	38.30	39.30	41.30
Noseband halter							
Rumination time (Σ.RT), h/d	95	1,195	9.42	1.25	1.07	9.62	12.47
Rating time (Σ.ET), h/d	96	1,209	5.31	1.27	1.40	5.36	9.90
Rumination chewing frequency ($\overline{RCf}$), 1/min	97	1,222	68.28	5.32	54.43	68.06	83.23
Pedometer							
Laying time (Σ.LT), h/d	98	1,193	10.53	2.77	0.87	10.77	18.38
Blood							
β-hydroxybutyrate (BHB), mmol/l	99	380	0.92	0.60	0.27	0.77	5.13
Non-esterified fatty acids (NEFA), mmol/l	99	377	0.38	0.24	0.06	0.33	1.89
Glucose (GLU), mg/dl	99	378	57.34	7.93	31.80	57.20	79.20
SARA risk score							
SRS	99	357	-0.04	3.60	-7.16	-0.38	16.45
Milk: yield and main components							
Milk yield, kg/d	99	1,117	37.48	8.51	5.15	38.10	63.80
Fat, %	99	357	4.45	0.75	2.73	4.32	7.67
Fat yield, kg/d	99	354	1.68	0.41	0.59	1.71	2.83
Protein, %	99	357	3.25	0.29	1.90	3.25	4.31
Protein yield, kg/d	99	354	1.23	0.27	0.28	1.25	2.07
Lactose, %	99	357	4.75	0.23	3.43	4.77	5.36
Lactose yield, kg/d	99	354	1.81	0.41	0.56	1.84	3.00
Somatic cell count (SCC), 1000/ml	99	361	260.08	679.79	6.47	59.27	4,901.62

[1] n_A = number of animals; SD = standard deviation.

Table S5.6: Descriptive statistics of mid-infrared based predictions of lactoferrin and fatty acid composition of the milk (Soyeurt et al., 2007, 2011, 2012)[1]

	n_A	n	Mean	SD	Min	Median	Max
Lactoferrin (LF), mg/L	96	331	128.72	80.00	1.02	111.41	443.86
Fatty acids, % of fat							
C4:0	99	353	2.84	0.29	1.88	2.81	3.96
C6:0	99	353	1.67	0.19	0.84	1.69	2.03
C8:0	98	353	1.07	0.17	0.40	1.08	1.41
C10:0	99	353	2.29	0.58	0.62	2.31	3.90
C12:0	99	353	2.75	0.76	0.72	2.73	5.09
C14:0	99	353	10.11	1.61	5.15	10.25	14.47
C14:1	99	353	0.72	0.13	0.35	0.72	1.12
C15:0	99	353	0.95	0.20	0.51	0.96	1.59
C15:0 iso	99	353	0.20	0.02	0.13	0.20	0.26
C15:0 anteiso	99	353	0.41	0.07	0.22	0.42	0.57
C15	99	353	1.57	0.28	0.86	1.60	2.35
C16:0	99	353	27.98	2.76	21.59	27.80	36.12
C16:1 cis	99	353	1.75	0.26	1.24	1.72	2.78
C17:0	99	353	0.66	0.04	0.56	0.65	0.80
C18:0	99	353	12.16	1.12	8.61	12.28	16.28
C18:1 *cis*-9	99	353	23.88	4.13	14.54	23.56	37.30
C18:1 *trans*	99	353	2.59	0.47	1.37	2.56	3.87
C18:1	99	353	27.93	4.53	17.35	27.66	41.38
C18:1 *cis*	99	353	25.70	4.37	15.74	25.39	39.87
C18:2 *cis*-9 *cis*-12	99	353	1.75	0.15	1.32	1.75	2.34
C18:2 *cis*-9 *trans*-11	99	351	0.40	0.16	0.06	0.39	0.80
C18:2	99	353	2.49	0.18	1.97	2.49	3.02
C18:3 *cis*-9 *cis*-12 c*is*-15	99	353	0.48	0.06	0.30	0.48	0.63
Trans fatty acids (*Trans* FA)	99	353	3.30	0.58	1.78	3.24	4.89
Odd-chain fatty acids (OFA)	99	353	3.42	0.27	2.71	3.42	4.31
Branched-chain fatty acids (BCFA)	99	353	2.00	0.13	1.64	2.00	2.33

[1] n_A = number of animals; SD = standard deviation.

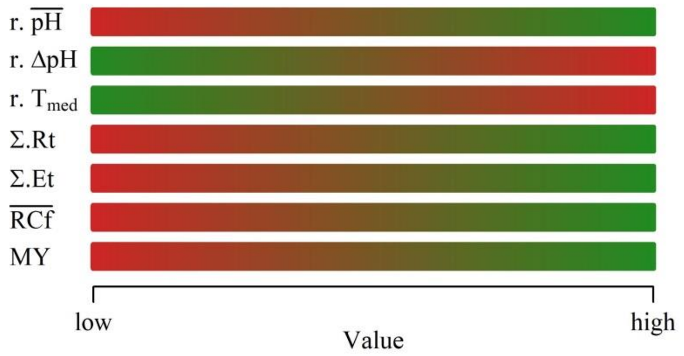

Figure S5.8: Assumed directed associations for the 7 traits with regard to subacute rumen acidosis. Red represents a high risk of SARA, whereas green indicates a low risk. $\overline{\text{RCf}}$ = daily mean rumination chewing frequency; $\text{r}.\overline{\text{pH}}$ = reticular daily mean pH; $\text{r}.T_{med}$ = median of the daily reticular temperature; MY = milk yield; r.ΔpH = daily reticular pH range; $\Sigma.\text{Et}$, $\Sigma.\text{Rt}$, and $\Sigma.\text{Lt}$ = daily duration of eating, rumination and lying, respectively.

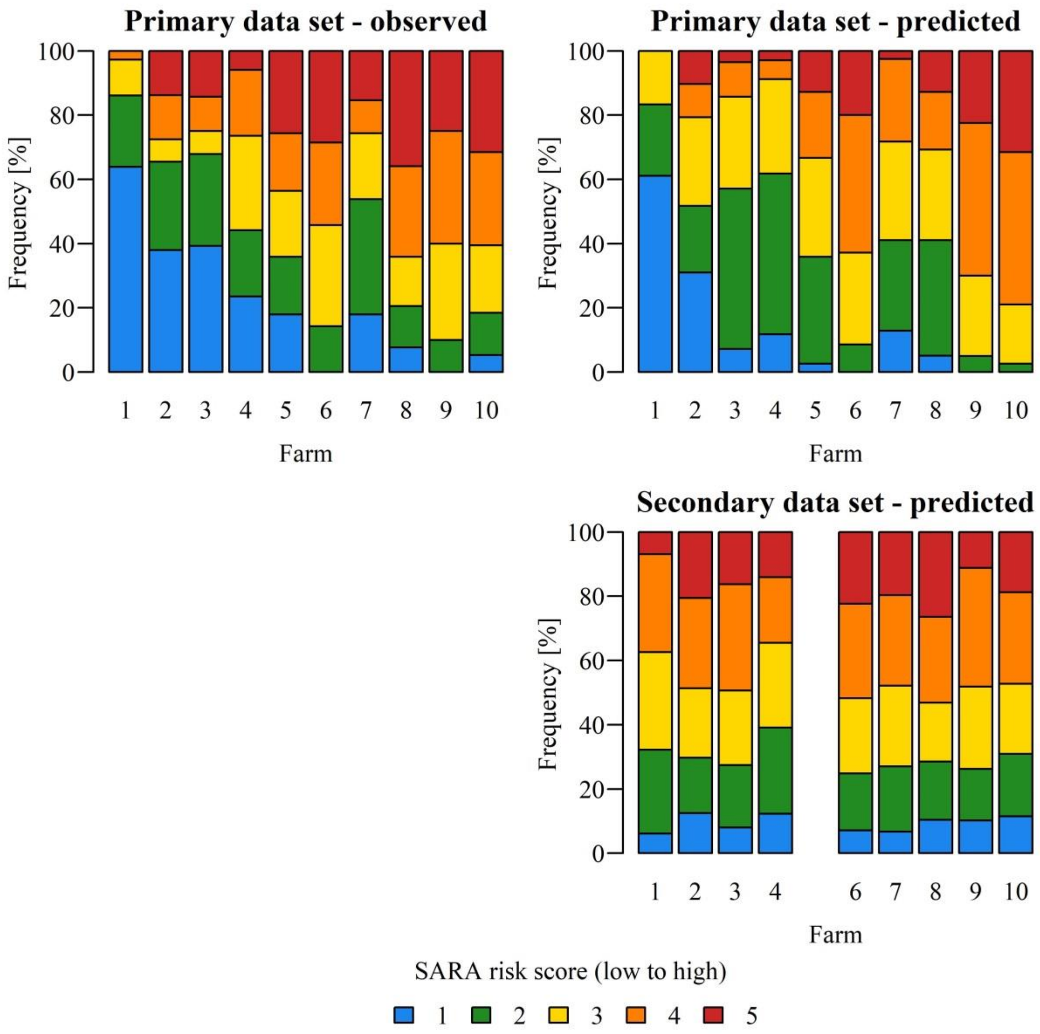

Figure S5.9: Stacked bar charts for the frequency of SARA risk score classes within farms in the primary (observed and predicted) and secondary data set (predicted). The same quintile-based boundaries were used for the class division, which are based on the observed SRS of the primary data set.

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Chapter 6

General discussion

Overview

In this dissertation, different innovative strategies for the statistical modeling of traits associated with subacute ruminal acidosis (**SARA**) were presented. Studies were carried out at the meta-analytical level, on the basis of data collected under experimental conditions and on the basis of data gathered in the field. Since the daily ruminal pH development is crucial for the health status regarding SARA, the different approaches focused in particular on characteristics derived from continuously *in vivo* measured pH data collected with indwelling sensor-based acquisition devices. To create a comprehensive overview of the associations between ruminal pH parameters and properties of the diet as well as pH parameters and the milk composition, a meta-analysis was carried out on the basis of the results of relevant literature studies (**Chapter 2**). In order to explain the daily pH development, high resolution time series data were used to model the daily pH progression in the ventral rumen as well as in the reticulum based on sensor-based data of ingestion and rumination behavior (**Chapter 3**). For the 2 following analyses, the data set collected under field conditions within the 'IndiKuh' project was used. Due to the fact that different sensor-based data acquisition systems were utilized and that these are prone to technical failure and measurement errors, a statistical procedure for a multivariate plausibility assessment was established to prepare the data set for downstream analyses (**Chapter 4**). Afterwards, an innovative 'SARA risk score' (**SRS**) was developed based on several traits derived from data of independent acquisition systems. Then it was examined to what extent the SRS as well as the other available sensor data-based traits were associated with the properties of the milk (**Chapter 5**).

In the following, achieved results and the most important aspects are discussed. To some extent, this will be supported by further results that were obtained during the research but were beyond the scope of the previous chapters. This is followed by an outlook on future research and the main conclusions of this work.

The meta-analytical approach to model ruminal pH parameters

The ruminal pH is the most important biochemical parameter for SARA and its measurement is the basis for current diagnosis approaches (Nocek, 1997; Enemark et al., 2002). The problem, however, is that there is no reliable and economically viable method to date, which allows measurements under field conditions on a large scale. According to the current state of knowledge, it is assumed that SARA often remains undetected due to its subacute state, leading to considerable impairment of animal welfare and also economic losses (Enemark, 2008). Since the ruminal pH is the most important parameter, but can only be determined with great effort, its statistical modeling contributes to the understanding of its physiological background. In general, there are 2 different motivations that can be found in the modeling of the ruminal pH: On the one hand, there is the effort to estimate the effect of the feed on the ruminal pH. In this regard, Nocek (1997) suggested that the prediction of the ruminal pH with dietary properties could help to optimize the design of diets in order to minimize the risk of SARA. On the other hand, indicators are sought that are easy to record on the individual level and reflect the pH as accurately as possible (e.g., Danscher et al., 2015; Jing et al., 2018).

In order to obtain a comprehensive overview of the associations between diet, rumen and milk properties, the meta-analysis presented in **Chapter 2** was carried out as a first step. In comparison to the meta-analyses of Zebeli et al. (2008) and White et al. (2017a; b), only experiments with continuous pH measurements in the ventral rumen were used to model the ruminal pH parameters. In the present meta-analysis, fundamental associations between the properties of the diet and the 3 most frequently reported pH parameters ruminal daily mean pH ($\overline{\mathbf{pH}}$), average daily time with pH < 5.8 (**TpH<5.8**) and daily pH range (**ΔpH**) were confirmed. It was shown that higher contents of fiber associated feed properties such as physically effective NDF retained on a 1.18 and 8 mm sieve (**peNDF>1.18**, **peNDF>8**), acid detergent fiber, neutral detergent fiber, and a high forage to roughage ratio lead to a higher $\overline{\mathrm{pH}}$ and thus minimize the SARA risk. On the contrary, higher values of the energy and carbohydrate related properties of the ration (i.e., starch, non-fiber carbohydrates, and net energy lactation) resulted in lower $\overline{\mathrm{pH}}$ and thus increase the risk of SARA. These results are in accordance with the general knowledge that SARA is promoted by diets with high contents of easily fermentable carbohydrates and low contents of effective fiber (e.g., Nocek, 1997).

With regard to the association analysis between ruminal pH parameters and the properties of milk, particularly the milk fat to milk protein ratio (**FPR**) as well as milk fat and milk protein contents showed significant effects and are thus suitable as indicators for SARA. It was found that lower ruminal $\overline{\mathrm{pH}}$ values are associated with lower milk fat and FPR and higher milk protein contents. Especially the lower fat and FPR values are in line with the milk fat depression, which is often postulated in terms of SARA (Nocek, 1997; Kleen et al., 2003).

Although the investigated relationships between the pH parameters and the properties of the diet, as well as between the pH parameters and the milk properties showed significant associations in most cases, the single predictors explained only a small part of the observed variance. This is mainly attributable to a high degree of heterogeneity in the investigated meta-analytical data within and particularly between studies, as can be seen for example in **Figure 2.6**.

In general, the number of animals in feeding experiments is limited, especially if surgical interventions are required as in the case of ruminally fistulated cows. The studies considered in the meta-analysis are therefore characterized by a very small sample size with only 3 (e.g., Dann et al., 2014) to 13 (e.g., Sun and Oba, 2014) ruminally fistulated cows. Moreover, only few factors of interest can be varied in one study while the rest is kept constant. Since a large number of factors can play a role for ruminal pH development and thus SARA, a single experiment can only cover a small part of all possible factor combinations. The meta-analytical approach is therefore a powerful tool with which the data from a large number of studies with numerous animals and with different formulations of the diet can be analyzed in order to investigate the underlying physiological relationships. Such a meta-analytical approach has already been used in the past to derive nutritional recommendations. In this regard, the work of Zebeli et al. (2008, 2010) can be mentioned, whose results of a conducted meta-analysis is the basis for the current recommendations of the German Society of Nutrition Physiology on the supply of structural fiber in form of peNDF>1.18 and peNDF>8 to lactating cows in mixed rations (GfE, 2014).

However, a critical point is that most of the studies on which this meta-analysis is based used single or replicated Latin square or cross-over designs. This means that different treatments within one experiment are applied sequentially to the same animals. Even though adaptation periods of about 2 weeks to the subsequent treatments are commonly used and data is only collected in the week after adaption (e.g., Yang and Beauchemin, 2007), carry-over effects of one treatment to follow up treatments can occur (Guiard et al., 2003). Considering the fact that the adaptation of the rumen mucosa to a particular diet takes 3-5 weeks (Nordlund et al., 1995) and that the absorption capacity can be impaired for up to 6 months in case of an induced acidosis (Krehbiel et al., 1995), it is likely that especially SARA induction experiments can lead to distorted effect estimates. Thus, it is conceivable that these carry-over effects have at least partly contributed to the observed high heterogeneity between and within the studies.

A further disadvantage is that the lactation stage and also the parity could not be taken into account in the present meta-analysis. One reason for this is that the LSmeans given for the studies are often corrected for time effects and refer to the entire study duration of up to 3 months. Moreover, estimates are based on cows of different parities so that no differentiation is possible. However, a lactation stage and parity dependent analysis would be particularly important with regard to milk characteristics and the association with ruminal pH, as the milk composition varies considerably in the course of lactation and across parities (Stanton et al., 1992; Buttchereit et al., 2010).

Reticular and ventral ruminal pH development in the course of the day

The ruminal pH is a complex parameter, which is influenced by a variety of factors and is subject to a dynamic development in the course of the day. Therefore, complex pH curves can be observed exhibiting farm and animal specific characteristics as well as variation between days (Denwood et al., 2018). The continuous pH measurement with indwelling acquisition systems provides therefore the opportunity to examine the pH development in detail. Although various studies have shown that daily pH development is influenced by management events such as time and frequency of feeding and milking (e.g., Le Liboux and Peyraud, 1999; Denwood et al., 2018), it has not been clarified yet, to what extent the pH development can be attributed to the animal-individual behavior and how exactly the sinusoidal curve is generated.

In order to study the underlying causality of the daily pH development, an innovative time-series based analysis was carried out (**Chapter 3)**. Two particular features of the analysis should be emphasized: Firstly, the pH and its development over time in the reticulum and in the rumen were considered in form of time series and not in form of values aggregated on a daily basis. Secondly, the transponder-based feed and water intake data were transformed in such a way that the cumulative effect of successive feed and water intakes could be considered. The analysis showed that the daily pH developments in the reticulum as well as in the ventral rumen are highly influenced by the animal's individual ingestion and rumination behavior in the course of the day. Feed intake proved to be the most important predictor with regard to the individual feed intake quantities and their distribution during the day. From the results it can be derived, that the more uniform the feed intake and ruminating behavior is during the day, the smaller the pH fluctuations will be. As a consequence, an animal that consumes the feed only in few meals and has an irregular feed intake will show larger pH fluctuations. The ingestion and rumination behavior, albeit difficult to measure in practice, are therefore crucial indicators of the ruminal pH development. Considering the fact that reduced and irregular feed intake behavior is often regarded as an important sign for herds suffering from SARA (Nordlund et al., 1995), it follows that not only low daily mean pH values but also higher pH ranges are an important indicator of SARA affected animals.

Research in pigs has shown that feed intake behavior has a considerable genetic background in traits such as the amount of feed per meal and the number of meals per day with heritabilities of 0.53 and 0.43, respectively (Labroue et al., 1997). If this is also true in cattle and if there are behavioral patterns that create a predisposition for SARA, this could partly explain the observed variation in the susceptibility for this disorder (e.g., Humer et al., 2015; Jing et al., 2018). Further, this could imply that the development of SARA is also due to genetics, thus allowing breeding based approaches to reduce the occurrence of SARA.

Within the framework of the analysis presented in **Chapter 3**, further insights into the associations between the reticular and ventral ruminal pH were obtained. In general, an association of the pH values between the 2 different measuring spots could be confirmed. This can be seen in the comparable average pH curves (**Figure 3.4**) and was particularly confirmed by cross-correlation analysis of animal individual pH data (**Figure 3.5**). These results are in line with the observations described by Sato et al. (2012), Falk et al. (2016) and Neubauer et al. (2018) an can presumably be attributed to the fact that the digesta of the reticulorumen is mixed by regular contractions up to 3 times per minute (Ruckelbusch and Thivend, 1980; Song et al., 2019). Nevertheless, the association is not as strong as expected. This is illustrated in **Figure 6.1 A**, where the reticular and time lag-corrected ventral ruminal pH values adjusted for eCow bolus and LRCpH logger effects are plotted in a 1-minute resolution against each other in a heat scatter plot. It corresponds rather to a point cloud than to a clear relationship. The pH values in the reticulum are mostly higher than in the rumen, but tend to show a higher difference to the ruminal pH at lower values. This observation was also made by Sato et al. (2012) and could be explained by the fact that the cyclic ruminal contractions can be inhibited by lower pH values resulting in a reduced mixing of the digesta (Ash, 1959). Regarding the difference between reticular and ventral ruminal pH, it is assumed that the higher reticular pH is caused by an increased dilution of the reticular digesta with saliva (Sato et al., 2012).

Figure 6.1 B also shows the daily mean pH values of both measurement sites, where the different colors represent different animals. Again, rather a point cloud without a consistent trend between or within animals can be observed. This might also be in accordance with the observations of Falk et al. (2016), who found that the differences between the 2 measuring sites vary over time and suggested that a simple conversion factor is not sufficient to conclude from the reticular to the ventral ruminal pH value. It is interesting that Falk et al. (2016) measured and compared pH values over a period of 10 weeks, with the animals being in lactation during the last 8 weeks. In comparison, Neubauer et al. (2018) carried out measurements on non-lactating cows and covered only a period of about 5 weeks. It is conceivable that the difference of the 2 pH measurement sites is related to time and lactation stage related factors, such as the dry matter intake, which behaves similar to a lactation curve (Schmitz et al., 2018b).

Based on the present data, an unequivocal diagnosis of SARA using reticular measurements is therefore questionable and further research is required. Time series based regression models, as used in the present analysis, could be suitable for further investigations of this complex relationship, as they allow specifically the consideration of the longitudinal nature of such data.

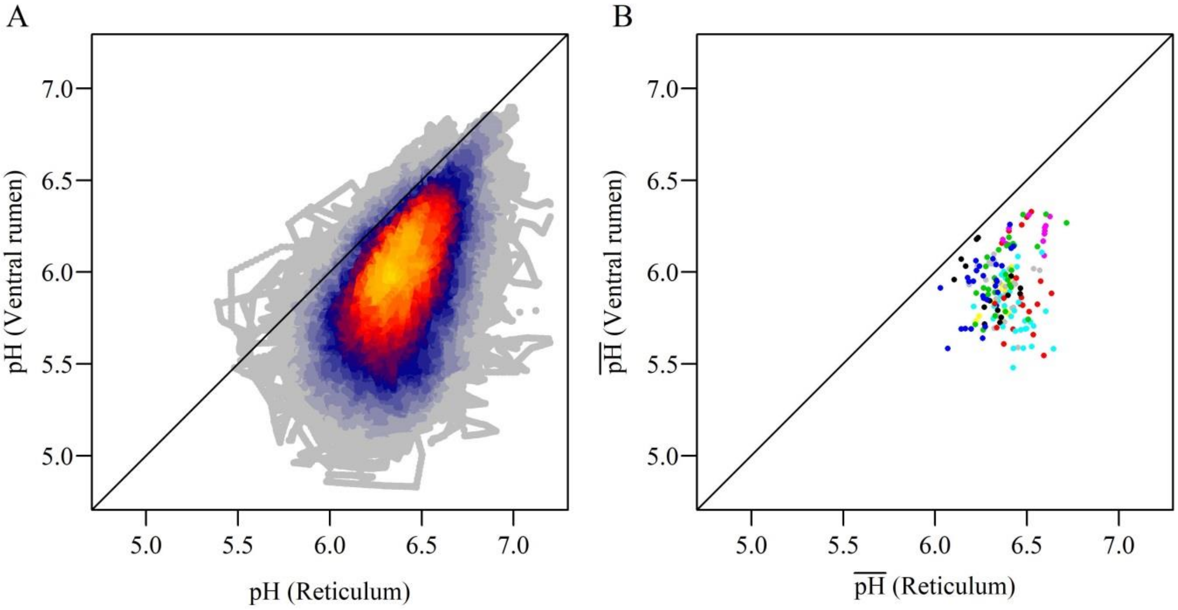

Figure 6.1: Comparison of the reticular and ventral ruminal pH values both in one-minute resolution (A) and on a daily average value basis (B). The diagonal line corresponds in each case to the identity. In A, the density of observation is represented by the annealing colors, yellow representing a high density and gray the lowest density. In B, different colors represent different animals. The data basis is formed by the reticular and lag-corrected ventral ruminal pH values adjusted for eCow bolus and LRCpH logger effects from **Chapter 3**. (A: n = 394.068, B: n = 175).

Plausibility assessment of data from sensor-based acquisition systems

The data collection with automated sensor-based acquisition systems offers numerous applications for both research and commercial farming and has been successfully established in many working areas. Also in the present thesis, data collected with such acquisitions systems formed the basis of the entire work. This type of data acquisition offers 2 major advantages. On the one hand, it enables the possibility to record traits like the continuous *in vivo* pH and temperature measurement in the reticulum and ventral rumen, for example with indwelling sensors (e.g., Penner et al., 2006; Falk et al., 2016). On the other hand, this type of data collection is extremely labor-saving and allows for individual animal data recording. The transponder-assisted recording of feed and water intake with weighing troughs (e.g., DeVries and Von Keyserlingk, 2009), the movement activity with pedometers (e.g., Alsaaod et al., 2015), the ruminating activity with the help of nose-band sensors (e.g., Zehner et al., 2017) or climate data in the barn with data loggers (e.g., Ammer et al., 2016) can be named as examples. However, the collection of data with such systems also has certain disadvantages. Most of these measuring devices are susceptible to technical errors, especially under such difficult conditions as in the barn or even within the forestomach system of the animal. The *in vivo* pH measurement with indwelling sensors is particularly susceptible to systematic errors, with random drift as an often observed problem (Penner et al., 2006). The quality control of such data is therefore of particular importance. However, the challenge is that once a certain amount of data has been collected, it is not feasible to do the quality control ‘manually’ but it should be done using bioinformatics based approaches.

Both data sets, which were collected within the framework of the ‘IndiKuh’ project and formed the basis of the **Chapters 3** to **5** showed conspicuous observations that indicated technical errors and disturbances during data collection. The underlying data set of **Chapter 3** included implausible transponder observations, malfunctions of antennas from transponder stations, defective pH electrodes of LRCpH loggers, non-responding eCow boluses, sensor drift of the eCow boluses, and erroneous measurements of dropped RumiWatch halters. In the second data set, which was collected under field conditions in the 10 farms and was analyzed in **Chapter 4** and **5**, problems with unreadable eCow boluses, sensor drift of the eCow boluses, malfunctions of the RumiWatch halter and pedometer devices as well as implausible climate data observations occurred. Since both technically caused errors and pathophysiological conditions can lead to extreme values, the challenge is to distinguish between these 2 cases. This is particularly important if, as in the present work, the research focus is on a disorder that is likely to actually affect only part of the investigated animals where extreme observations in some traits can be expected. However, in this respect only the extreme and plausible observations are of interest. Implausible observations, which can be attributed to erroneous data, would considerably impair the statistical analysis.

Due to the data volume and the implausible observations already determined during the first visual observation of the data, an innovative concept for a multivariate plausibility assessment was developed for the data set which was collected under field conditions in the 10 farms as described in **Chapter 4**. In this procedure, simultaneously recorded traits were discriminated under mutual consideration between ‘physiologically normal’, ‘physiologically extreme’ or ‘implausible’ observations. The underlying concept is based on the assumption that when a physiological extreme situation occurs, such as in the case of a disease, this is reflected in more than one measureable trait. In contrast, if only one trait of an acquisition system shows implausible values, it is likely that the values are erroneous. The described procedure has 2 major advantages: On the one hand, the procedure is based on a statistical framework, where complex, hierarchical data structures can be handled by the underlying regression models. On the other hand, a distribution-dependent classification of individual observations is applied and does not require subjective decisions in the form of fixed thresholds.

With regard to the sensor-based acquisition systems, it was found that particularly reticular pH measurements showed the most implausible values. In similar work with such measurements, e.g. by Denwood et al. (2018) and Jonsson et al. (2019), the quality control of the data appears to some extent contestable or is simply not explained in detail. Denwood et al. (2018) checked the pH measurements visually and removed observations with pH > 10, on the grounds that they are not compatible with rumen conditions. However, if one looks at the range of values of the raw, non-aggregated observations of the plausible data set in the present work, the 151,041 observations of 89 animals show values between pH 4.68 and 7.73, with the 1% and 99% quantile being pH 5.42 and 6.74, respectively. Assuming that these data are fairly representative, a threshold of pH 10 is questionable. Jonsson et al. (2019) also reported that data of 11 boluses out of a total of 86 were removed, but no further explanation was given. Based on the data collected, it can be concluded that reticular pH measurement with indwelling sensors is a technique prone to failure. A critical point with these devices is that with the current method of use, the boluses remain in the animal and cannot be recalibrated or reused after application.

Potential of the fine milk composition as indicator for subacute ruminal acidosis related traits

A major advantage of using milk-based monitoring is that milk samples are already routinely collected on a large scale in the framework of the breeding value estimation. In Germany, there are approximately 3.6 million (status: 2017/2018) milk performance-tested cows for which milk samples are collected regularly and the main components are determined (Berkemeier, 2018). In this respect, the mid-infrared spectrometric measurement of the milk composition plays an important role, and is an internationally accepted standardized routine method that is used in most milk analyses laboratories (Gengler et al., 2016; ICAR, 2020).

In order to determine, if and to what extent the fine milk composition represented by the MIR spectrum is suitable as an indicator for SARA-related traits, the study presented in **Chapter 5** was conducted. However, the challenge in this work was that the true state of health with regard to SARA was unknown. Therefore, the SRS was developed in order to get a robust indicator trait for SARA by combining several traits based on assumptions derived from literature studies. This approach is thus in line with a proposal of Danscher et al. (2015), who also suggested that due to the complexity of SARA, a combination of different indicators should be used for monitoring purposes.

The special feature of the present work was the MIR spectral data-based partial least squares (**PLS**) modeling of the SRS as well as the sensor data derived traits. Interestingly, of all the reference characteristics considered, the SRS showed the greatest correlation with the milk MIR data, at least for the data set used for calibration. However, it can only be classified as moderate with a coefficient of determination of the calibration model with R^2_{cal} = 0.56 and a coefficient of determination of validation with R^2_{val} = 0.37 (see **Chapter 5** for more details). Regarding the 2 reticular pH parameters, the daily reticular pH range (**r.ΔpH**) also showed a moderate association with R^2_{cal} = 0.33 and R^2_{val} = 0.49, whereas the reticular daily mean pH ($\mathbf{r.\overline{pH}}$) with R^2_{cal} = 0.16 and R^2_{val} = 0.05 showed only a very low association. To date, the literature that has dealt with the MIR-based prediction of pH parameters is rare. So far, only Luke et al. (2018) reported in an abstract moderate associations between MIR spectra and ruminal pH parameters derived from continuous pH measurements with $0.22 < R^2 < 0.59$ in ruminally fistulated cows. However, their results are rather general and do not provide specific details on the model accuracies of the separate traits.

Looking at the model predictions developed in this thesis not at the individual but at the farm level, a stronger relationship between observed and predicted values can be found. For example, the on farm level aggregated observed and predicted values for SRS, $\mathrm{r.\overline{pH}}$ and r.ΔpH show Pearson correlations of 0.99, 0.69 and 0.95. This is also in accordance with the statement of Gengler et al. (2016) that under the assumption of random prediction errors, the accuracy of predictions for the group will increase in comparison to single individual predictions. However, this is a well-known consequence of aggregation in statistical analyses (Clark and Avery, 1976).

If, for example, the association between $\mathrm{r.\overline{pH}}$ and FPR or $\mathrm{r.\overline{pH}}$ and milk fat are investigated with linear mixed regression models considering random farm and random animal effects, a significant ($P < 0.001$) association with positive regression coefficients can be determined in the present data. This is in line with the results of the meta-analysis from **Chapter 2**. However, the predictability of the models, where only the fixed part consisting of an intercept and the regression coefficient for FPR or milk fat can be used for the prediction, is very low. Regarding the $\mathrm{r.\overline{pH}}$, only correlations between observed and predicted values of 0.04 with FPR and 0.03 for milk fat can be found. These correspond to a R^2_{cal} of about 0.002 and 0.001, respectively. Compared to the milk fat or the FPR, the MIR spectrum has obviously much stronger potential regarding the prediction of ruminal pH parameters and thus as indicator for SARA.

Basically, associations between the sensor-based traits and the MIR spectra could be determined, even if they were not very high. Promising in terms of SARA are particularly the MIR-based PLS model performances for the SRS and the r.ΔpH. Consequently, the milk MIR spectrum seems not only to be a ‘fingerprint’ of the milk composition, but also to be indicative for behavior traits and even intra-reticular measured characteristics.

However, it remains to be clarified why especially the reticular daily mean pH is so difficult to model. Either it is in the nature of the trait, or it is attributable to the intra-reticular pH measurement with indwelling boluses, where both the drift of the pH measurement (e.g., Villot et al., 2017) and the insufficient association with the ruminal pH could be relevant (**Figure 6.1**).

A limiting factor in the study presented in **Chapter 5** is that only cows in early lactation with DIM ≤ 37 were examined. Although cows in this period are at risk for SARA (Nordlund et al., 1995), the increased body fat mobilization associated with the negative energy balance and the accompanied modified FA profile (Gross et al., 2011) could have impaired the milk MIR-based predictability. Furthermore, the fact that the differences between R^2_{cal}, R^2_{cv}, and R^2_{val} in the developed models are quite large indicates that the sample size may not be sufficient to consider such complex traits. The models should therefore be regarded as prototypes and are not yet suitable for widespread application in the field. In order to find out to what extent the developed models provide reliable estimates, external validation using a data set in which the same traits were collected is necessary.

Given that the developed models can be updated and improved by additional data, the application to a large external database, which contains MIR spectra as well as other phenotypic and genotypic data, would be of particular interest from a breeder’s point of view. In this regard, the investigation of associations between MIR predicted traits like the SRS and pH parameters with further health traits could provide deeper insights into the understanding of SARA as well as secondary diseases such as laminitis (Nordlund et al., 1995; Nocek, 1997). In addition to associations with claw health traits, the relationship to longevity could also be investigated. Further, quantitative genetic studies could be carried out, in order to estimate the heritability as well as phenotypic and quantitative genetic correlations with other traits of interest. Especially the knowledge of quantitative-genetic correlations is of great importance for the design of breeding schemes, since unfavorable correlations could also lead to unwanted responses (Gengler et al., 2016). Furthermore, a genome wide association study could also be conducted to look for potential genetic markers, which in turn would contribute to the understanding of the genetic background of SARA and would also reveal whether it is possible to select against SARA susceptibility.

Temporal assignment of the sensor data to the milk samples

In the previously discussed approach from **Chapter 5**, the aggregated sensor-based data as well as the SRS were associated with the pooled MIR spectral data of the respective evening and following morning milk sample. Another part of the analysis was the investigation whether this temporal assignment is appropriate. Generally, time delays between feed intake, ruminal fermentation, metabolic processes and the milk synthesis are to be expected. To analyze the temporal assignment, the 24-hour aggregation window for the determination of traits on a daily basis from the continuously recorded sensor data were shifted hourly from -72 to +24 h by a time lag Δt and the influence on the performances of the milk MIR-based PLS models were examined. For the evaluation of the model performance R^2_{cal} and R^2_{cv} were used (see **Chapter 5** for more details). An additional trait based on normally distributed random numbers was considered as a negative control, for which neither an effect of the temporal assignment nor an association with the MIR data should exist. The results of this analysis are shown in **Figure 6.2**.

The model performance of the random number-based negative control trait shows, as expected, that no association exists. However, it also follows that the observed associations of the other traits are most likely not random, although they should generally be considered rather low. The progressions of R^2_{cal} and R^2_{cv} indicate that the lag has only a small effect on the respective model performances, which is most likely attributable to a high auto-correlation of the traits. A further cause for this might be related to general differences of the feeding and milking times in the 10 farms, which thus lead to variations in time between but also within farms. However, since no exact feeding and milking times are available, this approach was the only possibility to estimate this effect at least approximately.

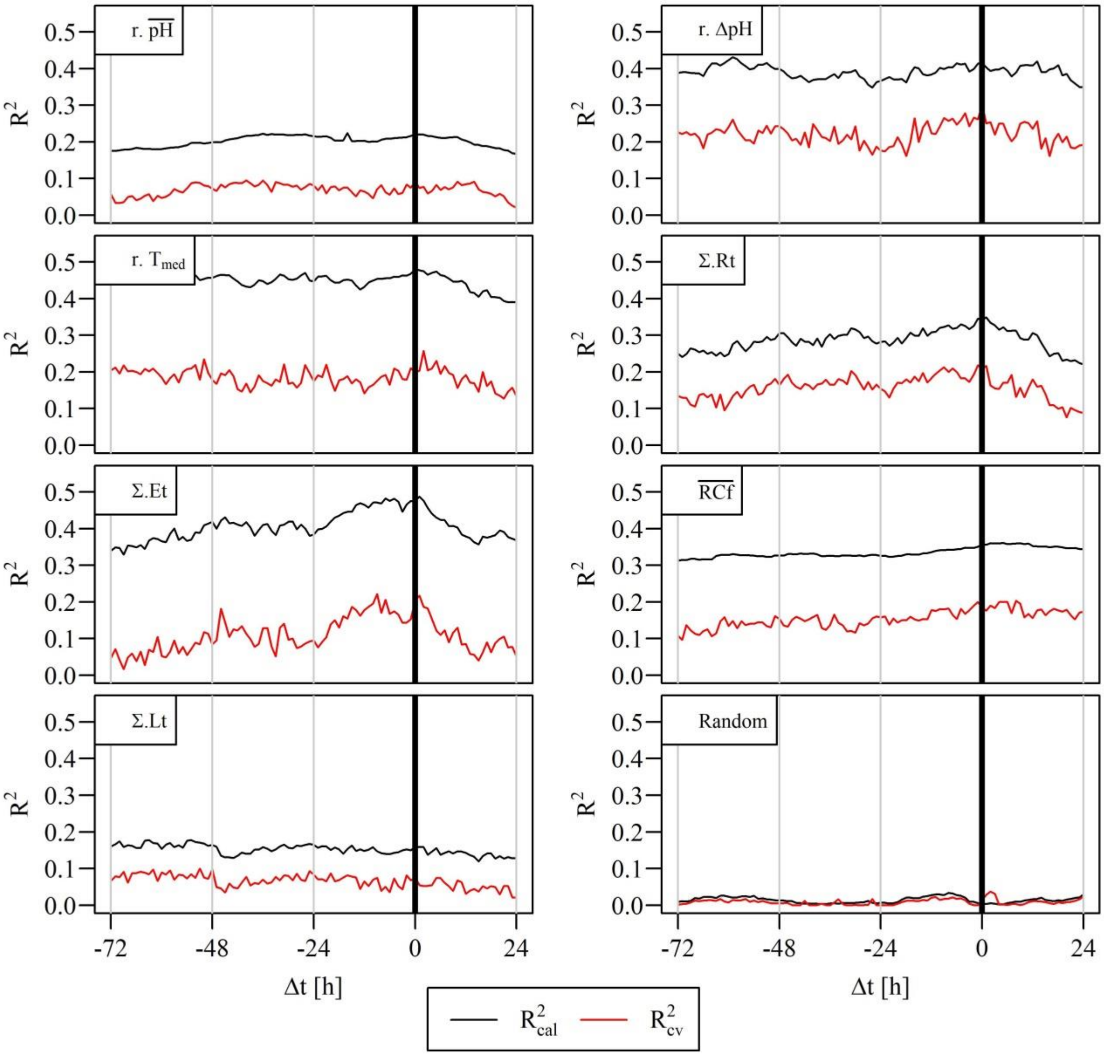

Figure 6.2: Effect of the chosen time assignment between daily aggregated sensor values and the pooled milk sample from evening and morning milk sample of the next day on the model performance of the mid-infrared (MIR) spectra based partial least squares regression models (see **Chapter 5** for details on the underlying models). Different time assignments were obtained by an hourly shifting of the 24-h aggregation window of the sensor-based data by a time lag Δt and taking into account the same pooled MIR spectrum of the milk samples. The time lag Δt = 0 h corresponds to the assignment of the daily aggregated sensor values to the pooled MIR spectrum of the evening milk sample of the same day and the morning milk sample of the next day. Δt = -24 h, for example, means that the aggregated values of the previous day are assigned to the pooled milk MIR spectrum of the evening milk sample and the morning milking sample of the next day. Random = random number-based trait (negative control), R^2_{cal} / R^2_{cv} = coefficient of determination of calibration model and of cross-validation; $\overline{RCf}$ = daily mean rumination chewing frequency; r.$\overline{pH}$ = reticular daily mean pH; r.$\overline{T}_{med}$ = median of the daily reticular temperature; r.ΔpH = daily reticular pH range; Σ.Et, Σ.Rt, and Σ.Lt = daily duration of eating, rumination and lying, respectively ($304 \leq n \leq 344$).

For both the daily rumination and eating duration, a local maximum can be identified approximately at Δt = 0. These results therefore confirm that the temporal assignment, as previously used in **Chapter 5**, is reasonable. In a similar approach, Dehareng et al. (2012) also found in the MIR-based prediction of methane emission that the temporal assignment plays a minor role due to a high auto-correlation of the measured methane emission. They found the highest model performance considering a time lag corresponding to Δt = -36 h.

From the local maxima of R^2_{cal} and R^2_{cv} of the eating and rumination duration it also follows that the behavior is reflected promptly in the properties of the milk. Such a prompt response emphasizes the potential of milk and its fine composition as an indicator for the health status regarding monitoring purposes.

Outlook on future research

SARA is and probably will remain an important disorder in dairy cows that is difficult to detect. The present work confirms many well-known relationships with innovative methods and provides numerous new starting points for follow-up investigations.

Essentially, it would be relevant to find out the cause for observed differences in animal individual susceptibility to SARA, where studies on the interaction of animal behavior, the ruminal microbiome, ruminal fermentation characteristics and the genetic background would be of interest. To date it remains unclear, to what extent certain behavioral patterns, which may be genetically determined, can lead to a predisposition for SARA. It would also be worthwhile to understand in which way the daily ruminal pH fluctuation affects the microbiome as well as the cow’s metabolism and performance.

In this work it was confirmed that the reticular measured pH is basically associated with the ruminal pH, but the association is not sufficiently clarified yet. In this respect, a long-term study with both reticular and ventral ruminal pH measurements should be set up, covering at best both the transition period and the following entire lactation. For such data, a time series based modeling could be considered to further investigate the relationship between the 2 pH measurement locations. Furthermore, it would be interesting to find out whether the pH curve could be used to derive more meaningful pH parameters than, for example, the daily mean pH, the time below a certain pH threshold or the pH range.

Basically, the present milk MIR-based PLS models could be applied to further available milk MIR data of the entire herds as a direct next step. Pedigree based animal models could be used to obtain first estimates of quantitative genetic parameters for the MIR-predicted SARA-related traits. In this regard, the estimation of phenotypic and genotypic correlations with the performance as well as the MIR-predicted FA would be relevant. An external validation of the established models should be carried out, where an extensive data base with further health traits would be beneficial. Preferably, the MIR-based PLS models should be updated with additional data, especially considering more verified SARA cases, which should increase both model robustness and model performance.

In general, the combination of different, independently collected traits to generate new, robust indicator traits seems promising. Analogous to the generation of the SRS, the construction of new additional indicator characteristics, for example for mastitis or ketosis is conceivable. Given that sufficient data are available, it would also be interesting to find out whether new statistical methods, such as deep learning algorithms, are more suitable for the analysis of such data.

Based on the present results, it can be assumed that high-throughput phenotyping methods like MIR-based spectrometry will add to the future development of a routine and comprehensive health monitoring in dairy farming, which will be beneficial for animal welfare and the economic efficiency.

Main conclusions

In this dissertation different strategies of mathematical-statistical modeling of SARA-related traits have been applied in order to contribute to the identification and development of better indicators for SARA. The main conclusions can be summarized as follows:

1) The meta-analysis proved to be an efficient method to obtain a comprehensive overview of the associations between dietary characteristics, milk properties and ruminal pH parameters.

2) Main components of milk are associated with ruminal pH parameters. Thus, information on the milk composition may be indicative for SARA and could be the key for the establishment of a monitoring system in commercial farming.

3) The consideration of the longitudinal nature of continuously recorded data and the analysis with time series methods show a high potential regarding the investigation of complex physiological processes.

4) The overall ingestion and rumination behavior in the course of the day is reflected in the reticular and ruminal pH development and is therefore an important indicator for ruminal fermentation characteristics and SARA.

5) Automated sensor-based data acquisition systems are prone to technical failure, thus the recorded data should always be extensively checked for plausibility to ensure data quality.

6) The investigation of simultaneously used data acquisition systems offers the possibility of mutual plausibility checks to detect both physiologically extreme conditions and implausible observations.

7) The combination of information from various data collection systems is a strategy which may lead to the development of indicators that better reflect the health status of the animal and could therefore be particularly beneficial for subacute disorders such as SARA.

8) Promising associations between the milk MIR data and various SARA-related sensor-based traits were identified, which may form the basis for a monitoring system that could be implemented on a large scale in routine milk performance testing.

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Declaration

1. I hereby declare that this work has not been made available in the same or in a similar form for the purpose of meeting examination requirements.
   I further declare that I have not applied for a doctorate at any other university.
2. I hereby declare that this dissertation was written independently and without unauthorized assistance.

Stadthagen, 11.05.2021

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André Mensching