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Phenotypic and genomic analyses of udder health in compost bedded pack barns based on microscopic differential cell count and specific mastitis pathogens

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List of Abbreviations

°C	Degree Celsius
BSA	Bovine serum albumin
BTA	*Bos taurus* (autosome)
C.bovis	*Corynebacterium bovis*
CHL1	Cell adhesion molecule L1 like
CNS	*Coagulase negative staphylococcus*
COR	*Corynebacterium*
ct	Cent
CTNNA3	Catenin alpha 3
DSCC	Differential somatic cell count
E.coli	Escherichia coli
EVA1A	Eva-1 homolog A
GEN	Genetic (latent variable)
GWAS	Genome-wide association study
GxE	Genotype by environment interaction
h^2	Heritability
HEMK1	HemK methyltransferase family member 1
IL	Interleucin
IMI	Intramammary infection (latent variable)
JAKSTAT	Janus kinase and signal transducer and activator of transcription proteins
MAJOR	Major pathogens
MAPK	Mitogen-activated protein kinase
MINOR	Minor pathogens
mL	Milliliter
PMN	Polymorphonuclear neutrophils
PROD	Production (latent variable)
S.agalactiae	*Streptococcus agalactiae*
S.aureus	*Staphylococcus aureus*
S.uberis	*Streptococcus uberis*
SCC	Somatic cell count
SCS	Somatic cell score
SEM	Structural equation model
SNP	Single-nucleotide polymorphism
TIME	Time (latent variable)

ZUSAMMENFASSUNG

Neue alternative Haltungssysteme, wie Kompostierungsställe finden aufgrund des höheren Tierwohls, einer höheren Langlebigkeit und einer verbesserten Gesundheit zunehmend mehr Beachtung in der Milchrinderhaltung. Durch die Mischung von Einstreu und Exkrementen sowie einer höheren Feuchtigkeit in diesem Lauf- und Liegebereich, kann es zu einem erhöhten Vorkommen von Bakterien kommen, die in das Euter eindringen und zu einer Mastitis führen können. Die Eutergesundheit ist einer der wichtigsten Faktoren bei Milchkühen. Mastitiden verursachen erhebliche Kosten für den Betrieb und führen zudem zu erheblichen Einbußen durch einen geringeren Milchertrag und Abzüge in der Molkerei durch erhöhte Zellzahlen in der Milch. Daher führt die Mastitis auch zu den größten wirtschaftlichen Verlusten in der Milchproduktion weltweit. Das Ziel dieser Arbeit war es daher, die Eutergesundheit bei Kühen zwischen dem neuen Haltungssystem, dem Kompostierungsstall, und dem klassischen Liegeboxen-Laufstall auf phänotypischer und genomischer Ebene zu untersuchen und gegenüber zu stellen. Zudem wurde für neue, detailliertere Merkmalsdefinitionen der Eutergesundheit geprüft, ob diese für eine verbesserte Zucht auf Eutergesundheit geeigneter sind, als die bisherigen Merkmale.

Um in das Thema der Dissertation allgemein einzuleiten, wird in **Kapitel 1** zunächst die Eutergesundheit bei Milchrindern thematisiert. Im weiteren Verlauf wird auf neue Haltungssysteme und die Genotyp-Umwelt-Interaktion in Bezug zur Eutergesundheit eingegangen. Im weiteren Verlauf wird in Strukturgleichungsmodelle zur Analyse der Eutergesundheit eingeführt.

Kapitel 2 befasst sich mit der Evaluation der spezifischen somatischen Zellfraktionen und Mastitispathogene in der Kuhmilch in Kompostierungs- und konventionellen Liegeboxen-Laufställen. Die Zellfraktionen sowie die Mastitispathogene wurden insbesondere unter dem Fokus auf Interaktionen von System x Laktationsstadium, System x Milchertrag und System x somatischer Zellzahl analysiert.

Hoch signifikant ($P < 0,001$) war der Interaktionseffekt von System und Laktationsstadium für die Zellfraktionen Lymphozyten, Makrophagen und PMN. Auch die Interaktion zwischen somatischer Zellzahl und System war hoch signifikant ($P < 0,001$) für die Zellfraktionen Makrophagen, Lymphozyten, PMN sowie deren Subfraktionen segmentkernige und stabkernige Neutrophile. Mit steigender Zellzahl sinkt der Gehalt an Lymphozyten in beiden Haltungssystemen. Hingegen steigt der Gehalt an Makrophagen und PMN mit ansteigender Zellzahl. Der Anstieg an PMN ist besonders deutlich, wenn die somatische Zellzahl 200.000 Zellen/mL übersteigt.

Insgesamt wurden signifikant weniger bakteriell positive Viertel in Kompostierungsställen im Vergleich zu konventionellen Liegeboxen-Laufställen gemessen. Die höchsten Prävalenzen wurden für CNS (*Coagulase negative Staphylococcus*) (27%) und COR (*Corynebacterium*) (18%) identifiziert, wobei die Prävalenzen im konventionellen Liegeboxen-Laufstall jeweils höher waren als im Kompostierungsstall. Auch für Minor-Pathogene lag die Prävalenz in Liegeboxen-Laufställen (54%) signifikant höher als in Kompostierungsställen (35%).

In Bezug zu den Pathogenen gab es bei der Interaktion von Milchertrag und dem jeweiligen Haltungssystem nur kleine, aber nicht signifikante Untertschiede. Während die Anteile bakteriologisch negativer Viertel über verschiedene Milchertragslevel in Kompostierungsställen weitgehend konstant blieben, war in Liegeboxen-Laufställen ein leichter Anstieg mit steigendem Milchertrag zu verzeichnen.

Die verschiedenen Indikatoren für die Eutergesundheit sowie deren Reaktion sind abhängig von Umweltmerkmalen, wie dem Haltungssystem in Kombination mit unterschiedlichen kuhbezogenen Faktoren wie Laktationsstadium, somatische Zellzahl oder Milchleistungsniveau. Daher ist es unerlässlich, spezifische Strategien zur Mastitisprävention in Abhängigkeit vom Haltungssystem und betriebsindividuellen Spezifikationen zu entwickeln und anzuwenden.

Auf Grundlage der in Kapitel 2 erlangten Erkenntnisse über den Einfluss des Haltungssystems auf die verschiedenen Indikatoren der Eutergesundheit wurden in **Kapitel 3** genomische Assoziationen zwischen den Haltungssystemen und den Eutergesundheitsparametern näher untersucht. Die Eutergesundheit ist abhängig von vielen verschiedenen Faktoren, wie beispielsweise der Umwelt, Fütterung, Pathogenspezies und von genetischen Mechanismen, die das Immunsystem beeinflussen und aktivieren. Für eine spezifischere Evaluation dieser Mechanismen im Bezug zur Eutergesundheit wurden detailliertere Indikatoren, wie die einzelne Zusammensetzung der somatischen Zellfraktionen (Makrophagen, Lymphozyten und PMN) und die ausdifferenzierten Mastitispathogene in einer genomweiten Assoziationsstudie integriert. Zudem wurde das genetisch-statistische Modell durch die Interaktion von SNP x Haltungssystem weiterentwickelt. Für den Haupteffekt wurden 35 signifikante SNPs auf 14 verschiedenen Chromosomen für Zellfraktionen und Pathogene detektiert. Für den Interaktionseffekt von SNP mit dem Haltungssystem waren insgesamt sechs SNPs signifikant.

Für den Haupt SNP-Effekt wurden die beiden potentiellen Kandidatengene *EVA1A* (eva-1 homolog A) und *CTNNA3* (catenin alpha 3) identifiziert. *EVA1A* wirkt in verschiedenen Signalwegen der Autophagie und des programmierten Zelltods. *CTNNA3* greift ein in den MAPK (mitogen-activated protein kinase) Signalweg und in den adhärens-junktionalen Komplex in Epithelzellen für die zelluläre Adhäsion. Zudem konnten zwei potentielle Kandidatengene für den Interaktionseffekt SNP x Haltungssystem identifiziert werden. Das

Gen *HEMK1* (HemK methyltransferase family member 1) ist an der Entwicklung des Immunsystems in Abhängigkeit von umweltbedingten Stressoren beteiligt. *CHL1* (cell adhesion molecule L1 like) zeigt einen immunosuppressiven Effekt unter Stressbedingungen.

Zukünftige Zuchtstrategien zur Verbesserung der Eutergesundheit sollten zum einen auf eine präzisere Definition der Eutergesundheitsmerkmale bedacht sein, um die unterschiedlichen genetischen Immunabwehrmechanismen zu berücksichtigen. Zum anderen sollte das jeweilige Haltungssystem mit in die Analysen einfließen, um gezielt gesundere Tiere für das entsprechende System auszuwählen.

Das in **Kapitel 4** aufgeführte Strukturgleichungsmodell diente der Identifikation von Kausalitäten von umweltbedingten und genetischen Faktoren in Bezug zur Eutergesundheit. Strukturgleichungsmodelle können besonders komplexe Zusammenhänge für die Eutergesundheit genauer und vereinfacht darstellen mittels latenter (nicht messbarer) Variablen, die durch manifeste (messbare) Variablen erläutert werden. Zum anderen lassen sich durch diese Modelle nicht nur direkte, sondern auch indirekte Einflüsse auf Merkmale analysieren. Dieses Modell beinhaltet vier latente Variablen (intramammäre Infektion, Produktion, Alter und Genetik), die durch unterschiedliche manifeste Variablen, wie die spezifischen Mastitispathogene, die einzelnen somatischen Zellfraktionen, Milchzusammensetzung und signifikante SNPs aus der genomweiten Assoziationsstudie in Kapitel 3 beschrieben werden. Insbesondere konnte hier gezeigt werden, dass der direkte Effekt des Haltungssystems auf die latente Variable „Intramammäre Infektion“ mit einem Ladungskoeffizienten von -0,05 gering ist. Jedoch ist der direkte Effekt (0,37) auf die latente Variable „Produktion“ hoch, wie auch der Effekt der „Produktion“ (0,17) auf die „Intramammäre Infektion“. Der indirekte Effekt des Haltungssystems auf die Eutergesundheit ist somit deutlich höher, als der direkte Effekt. Zudem konnte durch den Effekt der „Intramammären Infektion“ auf die latente Variable „Genetik“ sowie die Konstellation zu den anderen latenten Variablen eine Genotyp-Umwelt-Interaktion für die Eutergesundheit nachgewiesen werden. Die Weiterentwicklung solcher Strukturgleichungsmodelle kann zur Verbesserung der Zucht auf Eutergesundheit in unterschiedlichen Haltungssystemen beitragen, da die Beziehungen von Einflussfaktoren auf komplexe Merkmale untereinander abgebildet werden können.

Abschließend werden die wichtigsten Ergebnisse dieser Arbeit in **Kapitel 5** noch einmal zusammenhängend diskutiert. Hier wird insbesondere darauf eingegangen, warum für eine bessere Beurteilung der Eutergesundheit vor dem Hintergrund verschiedener Haltungssysteme neue Merkmale zur Definition von Mastitiden notwendig sind und somit auch zu einer Verbesserung in der Zucht führen können.

SUMMARY

New alternative housing systems, such as compost-bedded pack barns, attract increasing attention in dairy cow housing due to higher animal welfare, longevity and improved animal health. The mixture of bedding and cow excreta as well as an increased moisture in the compost can result in an increased presence of pathogens, which can invade the udder and cause mastitis. Udder health is one of the most important factors for dairy cows. Mastitis implies considerable costs for the farm and additionally causes severe losses due to a lower milk yield and deductions in the dairy due to increased somatic cell counts in the milk. Hence, mastitis is leading to one of the most important economic losses in milk production worldwide. The aim of this study was therefore to analyse and compare udder health in cows between the two housing systems, i.e., the compost-bedded pack barn and the conventional cubicle barn on a phenotypic and genomic level. Furthermore, new and even more detailed trait definitions for udder health were analyzed to evaluate their potential in the context of improving breeding strategies for udder health compared to previous traits.

To introduce to the subject of the dissertation in general, **Chapter 1** first focusses on a discussion on udder health in dairy cattle. Afterwards, the background for the new housing systems and possible genotype by environment interactions in relation to udder health is presented. In the following, the framework and possibilities for structural equation model applications, is addressed.

Chapter 2 focuses on the evaluation of specific somatic cell fractions and mastitis pathogens in cow milk in compost-bedded pack barns and conventional cubicle barns. The cell fractions and mastitis pathogens were analyzed with a special focus on system x lactation, system x milk yield and system x somatic cell count interaction.

The interaction effect of system and lactation stage was highly significant ($P < 0.001$) for the cell fractions lymphocytes, macrophages and PMN (Polymorphonuclear neutrophils). The interaction of somatic cell count and system was also highly significant ($P < 0.001$) for the cell fractions macrophages, lymphocytes, PMN and their sub-fractions segmented and banded neutrophils. The amount of lymphocytes decreased with increasing cell count in both housing systems. In contrast, the content of macrophages and PMN increased with increasing cell count. The increase of PMN was especially high when the somatic cell count exceeds 200,000 cells/mL.

In total, significantly fewer bacterial positive quarters were measured in compost-bedded pack barns compared to cubicle barns. The highest prevalences were identified for CNS (*Coagulase negative staphylococcus*) (27%) and COR (*Corynebacterium*) (18%), whereby the prevalences were higher in conventional barns than in compost-bedded pack barns. The prevalence of

minor pathogens was also significantly higher in cubicle barns (54.1%) than in compost-bedded pack barns (35%).

In relation to the pathogens, there were only small but not significant differences in the interaction between milk yield and the respective housing system. While the proportions of pathogen-negative quarters remained constant across different milk yield levels in compost-bedded pack barns, there was a slight increase with increasing milk yield in cubicle barns.

The different indicators of udder health and their response depend on environmental factors such as the housing system in combination with different cow-related factors such as lactation stage, somatic cell count or milk yield level. Therefore, it is essential to develop and apply specific strategies for mastitis prevention depending on the housing system and individual farm characteristics.

Based on the knowledge from Chapter 2, i.e., the effects of the housing system on the different indicators of udder health, genomic associations between the housing systems and the udder health parameters were examined in more detail in **Chapter 3**. Udder health is dependent on many different factors, such as the environment, feeding, pathogen species and genetic mechanisms that influence and activate the immune system. For a more specific evaluation of these mechanisms in relation to udder health, more detailed indicators such as the individual composition of somatic cell fractions (macrophages, lymphocytes and PMN) and the differentiated mastitis pathogens were integrated in a genome-wide association study. In addition, the model was newly developed and enhanced by considering the interaction of SNP and housing system. For the main effect on cell fractions and pathogens, 35 significant SNPs were detected on 14 different chromosomes. For the interaction effect of SNP with the housing system, six significant SNPs were found.

The two potential candidate genes *EVA1A* (eva-1 homolog A) and *CTNNA3* (catenin alpha 3) were identified for the main SNP effect. *EVA1A* acts in different signaling pathways of autophagy and programmed cell death. *CTNNA3* is involved in the MAPK (mitogen-activated protein kinase) signaling pathway and in the adherens-junctional complex in epithelial cells for cellular adhesion. In addition, two potential candidate genes for the SNP x housing system interaction effect, were identified. The gene *HEMK1* (HemK methyltransferase family member 1) is involved in the development of the immune system in response to environmental stressors. *CHL1* (cell adhesion molecule L1 like) shows an immunosuppressive effect under stress conditions.

To improve udder health, future breeding strategies should focus on a more precise definition of udder health traits in order to take into account the different genetic immune defense

mechanisms. On the other hand, the respective housing system should be included in the analyses in order to select animals that are healthier for the respective system.

The structural equation model described in **Chapter 4** was used to identify causalities of environmental and genetic factors for udder health. Structural equation models better represent and simplify relationships for complex udder health traits, by introducing latent (non-measurable) variables that are explained by manifest (measurable) variables. On the other hand, these models can be used to infer not only direct but also indirect effect-trait mechanisms and causalities. The applied structural equation model included four latent variables (intramammary infection, production, age and genetics), which are described by different manifest variables, such as the specific mastitis pathogens, somatic cell fractions, milk composition and significant SNPs from the GWAS described in Chapter 3. Especially the direct effect of the housing system on the latent variable intramammary infection with a loading coefficient of -0.05 was quite low. However, the direct effect (0.37) on the latent variable production is high as it is the effect of production (0.17) on intramammary infection. The indirect effect of the housing system on udder health is therefore significantly higher than the direct effect. In addition, the effect of intramammary infection on the latent variable genetics as well as the constellation with the other latent variables indicated a genotype by environment interaction for udder health. The further enhancement of such structural equation models can significantly improve the breeding of udder health in different housing systems through a better and more detailed understanding of the trait complexity.

Finally, the most important results of this work are discussed in **Chapter 5**. In particular, the discussion focusses on a) the new trait definitions for mastitis in the context of a detailed understanding of udder health mechanisms, b) housing system effects on udder health and c) potentials to improve breeding strategies.

CHAPTER 1

General Introduction

1 Introduction

Producing 31.9 million tons of cow's milk in 2022, Germany is the biggest cow's milk producer in the European Union, followed by France (24.1 million tons), the Netherlands (13.9 million tons) and Italy (13.0 million tons) (Eurostat, 2024). In Germany, the breeding and housing of dairy cows is of high importance. In November 2023, 3.71 million dairy cows were counted on 50,581 farms (Statistisches Bundesamt, 2024).

1.1 Importance of udder health

Mastitis is the most common disease in dairy cattle herds and the reason for one of the main economic losses in milk production worldwide (Le Maréchal et al., 2011). In 2009, the annual costs of mastitis caused by *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Escherichia coli* were estimated at 4896 euro in a herd of 100 dairy cows (Halasa et al., 2009). Today, the costs in Europe are estimated between 215 and 250 euro per intramammary infection (Bonestroo et al., 2023).

In addition to the high costs for the farm, the changing composition of the milk also causes difficulties in the further processing of milk as a nutritional product, e.g. in the production of yoghurt, cheese, etc. (Le Maréchal et al., 2011).

1.1.1 Anatomy of the udder and impact on udder health

The udder of the dairy cow and the udder health play an important role in the assessment of breeding value and conformation (Vereinigte Informationssysteme Tierhaltung w.V., 2023). The anatomical features do not only have an influence on milkability, they also affect udder health.

The udder has two mammary gland complexes on each side, dividing it into front and back quarters. All udder quarters are separated from each other. The quarters on both sides are separated by a strong connective tissue septum. In the equilateral quarters, the glandular lobes are interconnected (Loeffler and Gäbel, 2009). This means that each quarter can be affected by udder inflammation by itself. In addition, cell counts and pathogens can differ in the individual quarters (Leitner et al., 2006; Le Maréchal et al., 2011).

The parenchyma of the quarters is divided into glandular lobes, which are connected by an interstitium of fibrous tissue and are composed of alveoli and milk ducts. Several glandular lobes unite to form a glandular lobe. The alveoli open into sinusoidal ducts, which are highly branched and continue into highly branching mammary ducts. The sinusoidal extensions of

the duct systems serve as storage reservoirs. The milk ducts flow into the milk cistern. This in turn is divided into a glandular part and a teat part. The teat part finally ends in the teat canal. The mouth of the teat canal has a decisive influence on udder health. It should not be flat, pointed or funnel-shaped, as this can lead to soiling. The increased soiling can be a reservoir for pathogens. Such malpositions can predispose to increased infections in the udder (Loeffler and Gäbel, 2009).

1.1.2 Milk production and composition

The composition of bovine milk depends on various influencing factors, such as genetic, physiological and environmental factors (Loeffler and Gäbel, 2009; Töpel, 2016). Between the different breeds exist considerable variations in the composition of the milk (Loeffler and Gäbel, 2009; Le Maréchal et al., 2011). The physiological influencing factors include the stage of lactation, age and health of the cow (Le Maréchal et al., 2011; Töpel, 2016). In the course of lactation, the milk yield decreases while the milk fat content increases. During oestrus, the quantity of milk often decreases. The fat content can both increase and decrease during this time. The milk yield also decreases from the third month of pregnancy (Loeffler and Gäbel, 2009). External factors include feeding, skincare and strain on the udder as well as milking technique (Loeffler and Gäbel, 2009), but also the environmental temperature (Kipp et al., 2021).

Milk consists of anorganic as well as organic components. The main components are water, lactose, milk fat and milk proteins (Töpel, 2016). The milk proteins consist of caseins (84%), lactalbumin (15%) and immunoglobulins (1%). Casein and alpha-lactalbumin are formed in the mammary gland cells, whereas milk serum albumin and immunoglobulins are transferred in unchanged form from the blood (Loeffler and Gäbel, 2009).

1.1.3 Clinical and subclinical mastitis

Bovine mastitis is an intramammary infection caused by an inflammatory reaction in the udder triggered by physical trauma or microorganism infection (Cheng and Han, 2020). It can be divided into three different types of mastitis: clinical, subclinical and chronic mastitis. Clinical mastitis is characterized by very watery milk with flakes, possibly fever or even a swollen and painful udder (Khan and Khan, 2006). Clinical mastitis can be divided into the stages of per-acute, acute and sub-acute mastitis (Kibebew, 2017). Subclinical mastitis, on the other hand, is much more difficult to recognize and is characterized by a reduction in milk yield and a higher somatic cell count (SCC), whereby these components depend highly on the pathogen (Le Maréchal et al., 2011; Abebe et al., 2016). Due to the less obvious clinical appearance of

subclinical mastitis, losses are difficult to quantify. However, the losses caused by subclinical mastitis are estimated to be greater than those caused by clinical mastitis (Zhao and Lacasse, 2008; Romero et al., 2018). Chronic mastitis is an inflammatory process that lasts for months, with repeated clinical flare-ups at different intervals (Cheng and Han, 2020).

Several risk factors can lead to bovine mastitis. These include pathogenic, host and environmental factors (Klaas and Zadoks, 2018). The main cause of mastitis is usually a pathogenic bacterial intramammary infection. These pathogenic infections can be divided into two different types, contagious and environmental (Lakew et al., 2019). The contagious pathogens are transmitted from cow to cow and colonize and grow in the teat canal. These include, for example, *Staphylococcus aureus* (*S.aureus*), *Streptococcus agalactiae* (*S.agalactiae*), *Mycoplasma bovis* and *Corynebacteria* (Kibebew, 2017). Contagious pathogens can be well controlled by reducing contact between healthy and affected cows (Smith and Hogan, 1993). Environmental pathogens, on the other hand, do not live in the udder or on the udder skin, but in the bedding and housing of the herd. They can mainly invade during milking and lead to mastitis, especially in animals with an already compromised immune system. Examples include *Escherichia coli* (*E.coli*), *Streptococcus uberis* (*S.uberis*), *Klebsiella spp.*, *Enterobacter spp.* and coliforms (Bradley, 2002).

Host risk factors include breeding and genetics, the anatomy and structure of the udder and the age of the cow (Cheng and Han, 2020). High yielding breeds such as Holstein Friesian appear to be more susceptible than medium yielding breeds such as Jersey (Washburn et al., 2002; Shaheen et al., 2016).

As already mentioned in Chapter 1.1.1, the structure of the udder has a decisive influence on the susceptibility of the udder to an intramammary infection. Funnel-shaped teats, swing-shaped udders and blind udder quarters offer a higher risk of mastitis (Leitner et al., 2006; Waller et al., 2014). Teat size and the distance from the teats to the ground also have an influence on udder health (Sharma et al., 2017). With increasing age, the teat canal expands due to regular milking and is sometimes permanently open, making the cow more susceptible to infections (Kibebew, 2017). Additionally, the risk of infections increases with age due to irreversible damage to the tissue and thus an increase in epithelial permeability as a result of previous inflammation in the udder (Król et al., 2013). Within a lactation, cows are particularly susceptible to infection during the transition period. The transition period is defined as the time period between three weeks before and after birth (Drackley, 1999). Due to an increasing oxidative stress level and a low antioxidant defense, animals tend to immunosuppression and are significantly more susceptible to infections (Sharma et al., 2011; Abebe et al., 2016). During lactation, cows have a higher energy and nutrient demand for synthesis of milk and colostrum at the beginning of lactation. If this demand is not supplied, a negative energy balance results

(Kibebew, 2017). In turn, this is associated with deficits in the nutrient balance, amino acids and trace elements, which leads to immunosuppression at cellular and humoral level and increases susceptibility to infection (Matsui, 2012; Shaheen et al., 2016).

Furthermore, environmental factors and management of the housing system play a major role in animal health and welfare. A good herd management can significantly reduce the incidence and severity of mastitis (Weigel and Shook, 2018). This will be discussed in more detail in chapter 1.2.

1.1.4 Cellular immune response in the udder

The immune response in the cow's udder consists of a humoral and a cellular immune defense. The humoral immune defense will not be discussed in detail here. Instead, the focus will be on the predominant cellular immune defense. This is mainly induced by macrophages, polymorphonuclear neutrophils (PMN) and lymphocytes (Paape et al., 1979; Concha, 1986). PMN and macrophages are primarily responsible for the non-specific immune response and phagocytosis (Lee et al., 1980), while the lymphocytes are responsible for a specific immune response (Riollet et al., 2001).

Macrophages have a central role as "signal cells". Following invasion by pathogens, macrophages induce an increased expression of cytokines and inflammatory mediators. The synthesis of chemoattractants during an initial infection also ensures a rapid influx of neutrophils into the mammary gland (Cassatella, 1995; Wittmann et al., 2002). The specific immune response to the corresponding pathogen is mediated by lymphocytes, which recognize antigens of membrane receptors from past infections and produce appropriate antibodies (Riollet et al., 2001).

This knowledge about the different functions of somatic cells plays a central role in better understanding and analyzing intramammary infections and thus better assessing the udder health of the animals and the herd. Until now, the somatic cell count (SCC) has been used as a general health parameter to assess udder health (Le Maréchal et al., 2011). This includes the somatic cells, consisting of lymphocytes, macrophages, PMN and epithelial cells (Sarikaya et al., 2005). An inflammatory reaction is said to begin from 100,000 cells per mL of milk in quarter milk (Deutsche Veterinärmedizinische Gesellschaft, 2000). However, the SCC does not consider the exact distribution of the cell populations (Sarikaya et al., 2005). Furthermore, the SCC is susceptible to fluctuations regardless of an infection, such as the stage of lactation and lactation number. The number of somatic cells and their composition are highly dependent on the physiological status of the mammary gland (Sarikaya et al., 2005). This physiological condition in the udder as well as the effects depend strongly on the conflict substance and thus

not only influence the SCC, but also the milk yield and milk composition (Le Maréchal et al., 2011).

Not only SCC, but also the course of SCC during an intramammary infection depends mainly on the pathogen and the type of mastitis (clinical, subclinical, chronic) (Sarikaya et al., 2005; Le Maréchal et al., 2011). In addition to the different SCC course, there is also a variation in the cytokine profile depending on the response of the pathogen (Le Maréchal et al., 2011). Each cytokine modifies the permeability of the milk barrier, the gene expression profile or the recruitment of PMN and hence the composition of somatic cells (Le Maréchal et al., 2011). Some of them, such as IFN-Y, Interleucin (IL)-6, IL-10, IL-12 and C5a are generally induced, regardless of the pathogen (Shuster et al., 1997; Riollet et al., 2000a; Bannerman et al., 2004b). For other cytokines, such as IL-8, Winter et al. (2003) observed differences in the time of induction depending on the pathogen. IL-8 and TNF-α are released by the macrophages upon invasion of a pathogen, which in turn triggers the recruitment of PMN from the blood into the milk (Shuster et al., 1996; Lehtolainen et al., 2004; Watanabe et al., 2008). In order to ensure this transport path for PMN from the blood into the milk, endothelial permeability is necessary. By increasing endothelial permeability, other substances can also be transported from the blood into the milk, such as bovine serum albumin (BSA). However, how much BSA enters the milk depends highly on the permeability, which is in turn influenced by the respective pathogen. Coulona et al. (2002) showed that the highest BSA content in milk was measured in *E. coli*, followed by *S. aureus* and *S. uberis*. Thus, the overall host immune response is dependent on the pathogen, making it very difficult to generalize the processes in the cellular immune response (Bannerman et al., 2004a; Leitner et al., 2006).

1.1.5 Mastitis pathogens

There is a large number of pathogens that cause intramammary infection and can be classified in many different ways. As already mentioned in Chapter 1.1.3, pathogens can be fundamentally divided into contagious and environmental pathogens. For example, *S.aureus*, *S.agalactiae*, *Corynebacterium* and coagulase-negative *Staphylococci* (CNS) (*Staphylococci* other than *S.aureus*) are predominant in the group of contagious pathogens. Predominant environmental pathogens include *E.coli*, *S.uberis*, *S.dysgalactiae* and various gram-positive and catalase-negative *Cocci* (Dufour et al., 2019).

S.aureus colonizes the teat skin and mainly causes subclinical mastitis, which subsequently turns chronic (Riollet et al., 2000b; Bannerman et al., 2004b). *S.agalactiae*, on the other hand, infects the ducts and cisterns of the mammary gland and mostly leads to subclinical mastitis. Infected cows usually show clinical symptoms, but without abnormalities in the milk, for

example in form of flakes. Infection with *S.agalactiae* results in a loss of milk yield and in high SCC (Hillerton and Berry, 2003). The prevalence of this pathogen has decreased significantly over the last 30 years and ranges from 4.4% (Canada) to 0.3% (Argentina) depending on the country (Calvinhol and Tirante, 2005; Olde Riekerink et al., 2010). *Mycoplasma spp.* are highly contagious microorganisms that can cause severe damage to secretory udder tissue, abscesses and lymph node fibrosis. The incidence of this pathogen is much lower compared to *S.agalactiae* or *S.aureus* (National Mastitis Council, 1999; Fox et al., 2005). It is typical for this pathogen that many animals in the herd suddenly show a significant decrease in milk yield without any general deterioration in condition. The infection transfers from udder quarter to udder quarter or atrophies individual quarters, why treatment is not successful (Deutsche Veterinärmedizinische Gesellschaft, 2000). CNS colonize the teat skin and are currently the most frequently isolated pathogen in heifers with mastitis (Pyörälä, 2008). It is also the most isolated pathogen in milk samples with high SCC (Piepers et al., 2008; Sampimon et al., 2009). Over the years, the prevalence of these pathogens has increased in North America, Europe and Latin America (Sampimon et al., 2009). In most cases, CNS lead to subclinical mastitis (Fox, 2009). However, as this group includes over 50 species and subspecies, the virulence factor and host response to infection vary widely between species (Pyörälä, 2008; Taponen and Pyörälä, 2009). Quarters infected with *Staphylococcus chromogenes*, *Staphylococcus capitis* or *Staphylococcus xylosus* have a significantly higher SCC than bacteria-free udder quarters. In contrast, the SCC of affected quarters with *Staphylococcus epidermides* and *Staphylococcus hyicus* only tends to be higher than in bacterial-free quarters, but does not differ significantly (Borm et al., 2006).

Streptococcus spp. are environmental pathogens. They usually cause subclinical or clinical mastitis. They increased in the course of control strategies for contagious pathogens (especially *S.aureus*) on farms, while the number of mastitis caused by contagious pathogens decreased (Carrillo-Casas and Miranda-Morales, 2012). The most common representatives here are *S.uberis* and *S.dysgalactiae* (National Mastitis Council, 1999; Olde Riekerink et al., 2008). Others are *Enterococcus* such as *Enterococcus faecium* (Osterås et al., 2006), but *Aerococcus*, in particularly *Aerococcus viridans*, are also counted as environmental pathogens (Zadoks et al., 2004).

1.1.6 Mastitis and the impact on milk

Not only the course of SCC, but also the course of mastitis and the change in milk composition depend on each individual pathogen (Haas et al., 2002b; Leitner et al., 2006). For example, in the case of an intramammary infection caused by coliforms, the SCC is lower before and after clinical mastitis than during the actual infection. In case of infections with *S.aureus*, the SCC is especially high at the beginning and end of mastitis, whereas infections caused by Streptococci pathogens result in a continuous increase in SCC during clinical mastitis. Only when the inflammation abates, the SCC level decreases again (Le Maréchal et al., 2011). In addition, different peak values of SCC can be measured depending on the pathogen. For *Corynebacterium bovis* (*C.bovis*) the level was 105,000 cells·ml^{-1}, for *S.aureus* 357,000 cells·ml^{-1}, for *S.uberis* 1,024,000 cells·ml^{-1} and for *E. coli* 1,151,000 cells·ml^{-1} (Djabri et al., 2002).

The various pathogens affect milk composition differently. Some pathogens, such as *C. bovis*, have a small influence on milk composition, while others, such as *E. coli*, have a very large influence (Coulona et al., 2002). These differences are due to the fact that each pathogen induces specific changes in the milk during mastitis (Hettinga et al., 2009). For example, *S.uberis* is typically associated with increasing protein, casein, calcium and lactoferrin content (Coulona et al., 2002; Chaneton et al., 2008). *S.dysgalactiae*, on the other hand, is associated with increasing protease peptones and plasmin, but it does not change fat, casein and protein content (Leitner et al., 2006). It is therefore very difficult to make a general statement about the change in milk composition during mastitis (Le Maréchal et al., 2011).

Nevertheless, some fundamental reasons for the changes in milk composition caused by intramammary infection can be identified. During mastitis, physiological damage to the udder tissue occurs as the synthesis and secretory function of the mammary gland is reduced, which changes the milk composition (Kitchen, 1981). Another reason is the modification of the gene expression profile of the udder cells during mastitis, as expression of genes coding for antimicrobial proteins is increased (Le Maréchal et al., 2011). In addition, the improved endothelial permeability increases the passage of components from the blood. In particular, enzymes which are necessary for the defense against invading pathogens can destroy some milk components resulting in a change of milk composition (Chaneton et al., 2008).

However, some basic changes in the composition of the milk components can be summarized: During mastitis, an increase in protein content can occur due to inflammatory processes. In addition, the composition within the caseins changes. While β- and α-casein decrease, the proportion of γ-casein increases (O'Brien et al., 2001; Le Maréchal et al., 2011). Reduced fat synthesis and secretory capacity of the mammary gland can lead to a decrease in fat content,

but this is controversially discussed (Raynal-Ljutovac et al., 2007). On the other hand, an increase in free fatty acids was observed by Erwin and Randolph (1975), due to the change in the milk fat globule membrane caused by leukocyte lipase, which can increase lipolysis. Lactose content (Wickström et al., 2009) and milk yield (Singh et al., 2005; Le Maréchal et al., 2011) decrease during mastitis. The decrease in milk yield is due to the loss of epithelial cells through necrosis and apoptosis during mastitis, as well as to the down-regulation of genes for milk production (Singh et al., 2005; Le Maréchal et al., 2011). The down-regulation of genes for milk production can be explained by the invasion of pathogens into the udder and by the increase in cytokines during infection (Watanabe et al., 2008).

1.2 Housing systems

The housing system has a strong impact on animal health and welfare (Fregonesi et al., 2009; Kester et al., 2014). Conventional cubicle barns, which are the standard housing system used for decades in the dairy industry, enable the best possible hygiene by separating manure and urine from the lying area however, at the expense of animal welfare (Bewley et al., 2017; Petersen, 2018). In addition, the high proportion of liquid manure contributes to greenhouse gas emissions (Petersen, 2018). New freewalk housing systems, such as the compost-bedded pack barns, grow in attraction due to better animal welfare, longevity and health (Leso et al., 2020).

1.2.1 Structure and differences between compost-bedded pack barns and cubicle barns

Conventional cubicle barns typically have a feed alley with a solid floor alleys with slatted concrete surfaces and individual resting areas (Leso et al., 2020). The lying area can vary between deep bedded and synthetic mattresses. A wide variety of bedding materials can be used in the deep bedded areas. Sand, straw, sawdust and solids from recycled manure are used most often. They usually provide good comfort, but are very labor-intensive and require a lot of bedding material (Bewley et al., 2017). Synthetic mattresses, on the other hand, reduce labor intensity and material consumption, but reduce lying comfort and increase hock lesions and lameness (Cook et al., 2004). Manure and urine are excreted onto the concrete slatted floor, where it is trodden through the slats or moved away using a scraper. If this concrete floor is not roughened, it can become very slippery for the cows (Albright J.C., 1995).

The system of compost-bedded pack barns was developed in Virginia in the 1980s (Wagner, 2002). This housing system attracted increasing interest worldwide in the last 15 years. In Europe, the first compost-bedded pack barns were established in 2009/2010 (Leso et al., 2020).

The compost-bedded pack barn also contains a feeding corridor with a solid floor (Bewley et al., 2017) and access to water (Janni et al., 2007). The rest of the area consists of an open bedded pack where the animals can move and rest. The open bedded pack area consists of a mixture of organic bedding and cattle excrement. This area is cultivated one to three times per day to get fresh manure and air into the pack, allowing for an aerobic composting process (Leso et al., 2020). This mixture also has the advantage that it is solid and already has undergone a composting process enabling it to be used directly for soil application (Bewley et al., 2017). However, the area per cow in the compost-bedded pack barn is greater than in the cubicle barn or straw yard (Bewley et al., 2017; Leso et al., 2020). Depending on various factors, such as climate, bedding, pack management and cow characteristics, between 7.4 - 15m^2/cow are required (Leso et al., 2020). The pack depth varies between 20cm and 1m, depending on pack management and bedding use (Leso et al., 2020). Galama et al. (2014b) was able to show that around 50cm are required to maintain sufficient heat in the pack to support the composting process.

As evaporation occurs on the surface, more surface area per cow results in dryer bedding, reduced use of bedding material and therefore lower running costs (Leso et al., 2020). Another factor influencing the drying rate of the pack is the heat development within the pack (Smits and Aarnink, 2009). Ideally, the temperature at a depth of 15 to 30cm should be between 43.3°C and 65°C (Janni et al., 2007; Bewley et al., 2013). For good cultivation of the pack, the manure level should ideally be between 40 and 65% (Janni et al., 2007; Black et al., 2013).

Climate, especially air conditions, also have a high impact on the drying rate (Eckelkamp et al., 2016a). In warm, dry and windy regions, the drying rate is higher and therefore the consumption of bedding is lower. In cold and humid regions, on the other hand, the drying rate is limited. Therefore, more space per cow is needed to achieve a higher drying rate (Smits and Aarnink, 2009). In addition, the outside temperature plays a central role in the processing of the pack. In warm temperatures, more frequent processing of the pack leads to an increase in temperature of up to 10°C and thus a better composting process (Black et al., 2013). At cold temperatures, more frequent processing leads to a significant loss of temperature in the pack. Therefore, in this case processing should be reduced (Galama, 2014a; Leso et al., 2020). With regard to the weather, the building design should also consider the structural orientation of the barn in order to optimize utilization of natural ventilation (Gooch, 2008).

Manure removal is usually done every six months to once a year, then the composting process starts all over again (Barberg et al., 2007a). The time to add fresh bedding is essential for a well functioning system, clean animals and healthy udders. It should be added before the pack sticks to the animals (Barberg et al., 2007a; Janni et al., 2007). There is a wide variety of bedding materials worldwide. In Kentucky, for example, green, kiln-dried shavings or soy hulls mixed with sawdust are used (Black et al., 2013). In Italy, a mixture of sawdust and wood shavings is used (Leso et al., 2013). As well as in Brazil, where rice straw or coffee husks are also used (Mota et al., 2017). Whereas in the Netherlands wood chips where used (Galama et al., 2011).

1.2.2 Effects of the housing system on animal wellfare

Animal health and welfare depend significantly on the housing system and management (Fregonesi et al., 2009; Kester et al., 2014). Particularly the lying time affects rumination time and consequently milk production (Schirmann et al., 2012). Uncomfortable barns have a major impact, particularly in cubicle barns. Poorly designed lying areas result in less lying time and more standing time on slatted floors, influencing rumination, milk production and claw health (Fregonesi et al., 2009; Kester et al., 2014). Additionally, the soil has an effect on walking performance and thus on total behavior. Especially in the case of non-roughened concrete floors, the heat behavior changes and thus affects the fertility rate (Frankena et al., 2009; López-Gatius, 2012). In Fregonesi et al. (2009), it was observed that the animals spent significantly more time in the open pack than in the cubicle barn when given a free choice between cubicle barn and straw yard. The animals showed synchronized lying behavior, as well as longer lying times and thus an increased rumination time in straw yard (Fregonesi and Leaver, 2001). However, the disadvantages of the straw yard were shown by a higher degree of soiling of the animals and lower udder health. The somatic cell count and the incidence of clinical mastitis was significantly higher in straw yards than in cubicle barns (Fregonesi and Leaver, 2001).

Compost-bedded pack barns were developed to improve cow comfort and longevity (Janni et al., 2007) and to provide more natural living conditions for the animals (Endres and Barberg, 2007). The pack management should be hygienic and offer the cows a comfortable lying area (Leso et al., 2020).

Due to the shorter standing times on concrete surfaces, cows suffer significantly less diseases, when compared to cubicle barns (Bewley et al., 2017). Various studies have shown that animals in compost-bedded pack barns have significantly reduced lameness and hock lesions compared to cubicle barns (Fulwider et al., 2007; Lobeck et al., 2011; Ofner-Schröck et al.,

2015). Due to the softer bedding, the animals rest longer, stand more frequently on soft ground and lie down more often than in cubicle barns (Haley et al., 2001).

The animals' cleanliness and udder health depend primarily on the management (Black et al., 2013; Eckelkamp et al., 2016b). Wet bedding can stick to the cows and lead to dirty animals with an increased risk of mastitis and a longer preparation time of the udders before milking (Black et al., 2013). Especially, manure levels (Eckelkamp et al., 2016a) and the frequency of pack preparation have a major impact (Janni et al., 2007; Damasceno, 2012).

Udder health in compost bedded pack barns is discussed controversially. Lobeck et al. (2011) found a higher SCC in compost-bedded pack barns than in cubicle barns. An increased proportion of *Klebsiella spp.* in the pack, as well as an increased prevalence in the udder, can occur with intensive use of wood shavings and sawdust (Janni et al., 2007). In contrast, Eckelkamp et al. (2016b), found no significant differences between the two systems in SCC and mastitis prevalence. Other studies even showed a lower herd mastitis infection prevalence (Barberg et al., 2007b), a lower SCC (Fulwider et al., 2007) and higher milk production as a result of the lower stress level of the animals in compost-bedded pack barns (Black et al., 2013; Borchers, 2018). However, optimized bedded pack conditions and management are essential for udder and cow hygiene (Fávero et al., 2015; Albino et al., 2018). Nevertheless, no relationship was found between the SCC and the count of bacteria in the pack (Shane et al., 2010).

1.3 Genetic and environmental effects on udder health

When the same genotype changes its phenotype differently in various environments, it is referred a genotype by environment interaction (GxE) (Hammami et al., 2009). As the environment changes, the genotype responds differently (Lynch and Walsh, 1998) due to differences in gene regulation (Via et al., 1995).

Udder health and milk composition depend on many different environmental factors, such as feeding, hygiene or temperature (Hayes et al., 2003; Streit et al., 2013; Tiezzi et al., 2015). Therefore, there are also different approaches to detect GxE. Tiezzi et al. (2017) used a cross-classified research design for e.g. climate or feeding groups. He was able to prove a GxE for milk yield. However, he could not detect a GxE for milk composition and SCC. Others calculated correlations between the same traits in different environments (Cromie et al., 1998; Veerkamp and Goddard, 1998; Hayes et al., 2003). Hayes et al. (2003) focused on continuous environmental descriptors such as temperature, herd production level or herd size applying random regression methodology.

The choice of environmental descriptor is important for analyzing the GxE. Often an average value is taken for the total herd in order to include a maximum of unmeasured environmental factors (Hayes et al., 2003). For the udder health traits, the SCC or the somatic cell score (SCS) are usually used as indicators, even in Germany, but indicators of hygiene or other environmental factors are completely neglected (Streit et al., 2013). However, the SCC or the average herd SCS, have their limits in the analysis, when considering the complexity of the udder health, and the different effects of pathogens on SCC (Barkema et al., 1998; Streit et al., 2013). Nevertheless, in most countries, selection for mastitis resistance has been implemented based on the linear change in SCC (Rupp and Boichard, 2003).

Susceptibility to intramammary infection or mastitis resistance are highly complex and depend on genetic components as well as physiological and environmental factors (including infection burden) (Rupp and Boichard, 2003). Mastitis resistance is a trait with a high polygenic mode of inheritance with a large number of genes being expressed differently. Many of these genes show a small but additive effect (Detilleux, 2009; Pighetti and Elliott, 2011; Tiezzi et al., 2015).

Due to its complexity, the udder health trait is difficult to define and record. The focus is usually on SCC and clinical mastitis, as these data are often recorded by the farm. However, subclinical mastitis often remains undetected (Rupp and Boichard, 2003; Tiezzi et al., 2015). In addition, the trait is usually recorded on individual base but it is not recorded for each udder quarter separately. Generally, only one quarter is infected and not all four quarters. Due to the dilution effect of all quarters on the cow's individual SCC, recording the individual SCC is not a useful way for describing udder health. (Rupp and Boichard, 2003). Welderufael et al. (2018) also shows that the definition of mastitis as a binary trait (healthy, affected) does not provide any useful results for breeding. Due to the very low heritabilities (h^2) of $h^2 \approx 0.03$ for mastitis, selection is very difficult (Heringstad et al., 2000; Carlén et al., 2005; Koeck et al., 2012; Tiezzi et al., 2015). Whereas Nash et al. (2000) and Haas et al. (2002a) found varying but low heritabilities for the different species of pathogens infecting the udder. For *S.aureus*, CNS, *E.coli*, *S.uberis* and *S.dysgalactiae* the heritabilities are 0.05, 0.06, 0.06, 0.04 and 0.05 (Haas et al., 2002a). Similar heritabilities are estimated by Schafberg et al. (2006) for *S.aureus* (0.068) and CNS (0.093) and Sørensen et al. (2009) for *S.aureus* (0.035), CNS (0.051), *E.coli* (0.049), *S.uberis* (0.076) and *S.dysgalactiae* (0.044). Correlations between the different mastitis pathogens are between 0.45 and 0.77. *E.coli* and *S.aureus* have the lowest correlation (0.45), indicating that the individual pathogens can be treated as separate traits (Sørensen et al., 2009). In addition, Bannerman et al. (2004a) showed that an infection with *S. aureus* and *E. coli* trigger different cascades in the immune system.

Due to this large variety of influencing factors and different immune responses, genome-wide association studies (GWAS) seem more suitable to find traits through direct markers and

increase the frequency of favored alleles (Tiezzi et al., 2015; Welderufael et al., 2018). GWAS use information on genetic markers such as SNPs to determine associations with a trait of interest. This assumes that a marker is in linkage equilibrium with or close to a causative mutation (Hirschhorn and Daly, 2005; Goddard and Hayes, 2009). Various studies have shown that significant SNPs for udder health occur primarily in regions that play a role in the regulation of the immune system. Genes that are part of the major histocompatibility complex and are involved in the regulation of neutrophils have a major influence on the susceptibility and disease of the gland (Detilleux, 2009). Significant SNPs for susceptibility to clinical mastitis are located on chromosomes BTA (*Bos taurus*) 3, 7 and 13, and for the ability to recover on BTA 7, 12, 13 and 15 (Welderufael et al., 2018). Welderufael et al. (2018) also detected candidate genes for autophagy, macrophage recruitment, inflammatory processes and immune responses. Tiezzi et al. (2015) found 10 neighboring SNP windows at regions on chromosomes BTA 2, 14 and 20 that have an influence on genetic variations for clinical mastitis. In particular, genes that influence the JAK-STAT (Janus kinase and signal transducer and activator of transcription proteins) signaling pathway, as well as the cytokine-cytokine receptor interaction, responsible for cell proliferation and apoptosis, play a major role. The expression of cytokines is strongly dependent on the pathogen (Lee et al., 2006; Lahouassa et al., 2007; Gutiérrez-Barroso et al., 2008).

1.4 Structural equation models

Structural equation models (SEM) are used to empirically test theoretical statements about complex cause-effect relationships (Geiser, 2010; Fuchs, 2011). The SEM is intended to test whether a theoretical model is consistent with the observed data and shows if and how a theoretical model is appropriate. However, it does not serve the classical null hypothesis, i.e. it is not about disproving a model (Urban and Mayerl, 2014).

The special feature of the SEM is the differentiation between manifest, measurable variables and latent, non-measurable variables (Fuchs, 2011; Urban and Mayerl, 2014). The advantage of SEM with manifest and latent variables is that the use of latent variables allows measurement errors to be explicitly taken into account in the analysis. In addition, correlations can be estimated more correctly than in correlation, regression or path analyses (Geiser, 2010). In addition, non-linear relationships can also be modeled in these models and non-normally distributed variables can be taken into account in the SEM (Urban and Mayerl, 2014). The SEM allows the multivariate analysis of causal models in which a differentiation is also made between independent (exogenous) variables and dependent (endogenous) variables. The estimation of direct, indirect and total effects is also possible (Urban and Mayerl, 2014).

SEM or causal models are composed of different sub-models. These usually consist of an inner structural model and outer measurement models (Figure 1.1). In the structural model, latent variables that explain other variables in this model are referred to as exogenous (ξ). Those that are explained by exogenous latent variables are referred to as endogenous (η) (Fuchs, 2011).

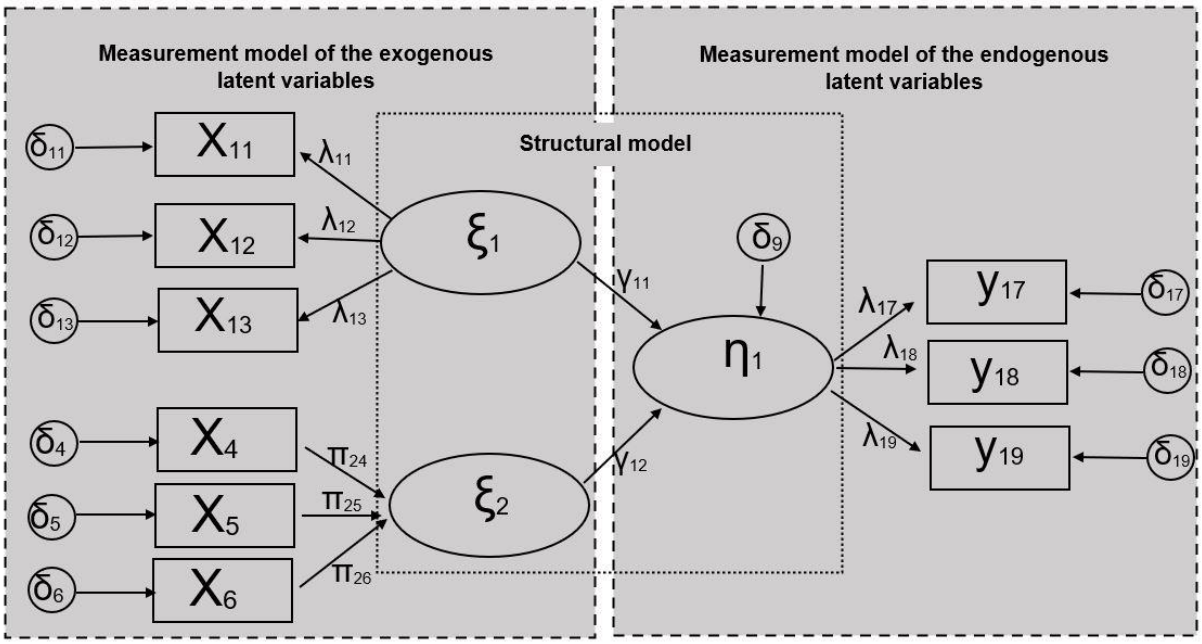

Figure 1.1. Construction of a complete causal structural equation model (Fuchs, 2011).

The measurement models are used to estimate the structural relationships of the latent variables. Since latent variables cannot be measured directly, they must be represented by suitable empirical indicators (manifest indicators) that describe the latent construct as accurately as possible. Residual terms (δ or ε) are also essential in the measurement model, as the measured variables are generally associated with errors (Tenenhaus et al., 2005; Ringle et al., 2006; Backhaus et al., 2006).

The shown model (Figure 1.1) represents an SEM with two exogenous (ξ) and one endogenous (η) construct. The hypothesized relationships and the power of the causal relationships between the latent constructs are represented by loading or path coefficients (γ) (Fuchs, 2011). The direction of the arrow also describes the direction of the effect of the causal relationship (Wright, 1934).

Additionally, this model shows both a reflective (ξ1) measurement model and a formative (ξ2) measurement model. The basis for reflective measurement models is a factor-analytical approach. The manifest variables are selected in that way that they reflect the latent construct in its entirety as accurately as possible (Weiber and Mühlhaus, 2010).

Formative measurement models, on the other hand, are based on a regression analysis approach. In this model, the manifest variables are the source of expression of the latent construct. As there are no measured values for the dependent variable (the latent construct) of the regression equation in this approach, the latent variable must first be estimated in relation to other latent constructs. The choice of measurement models should be carefully selected based on content-related considerations about the relationships between latent and manifest variables (Homburg et al., 2008; Weiber and Mühlhaus, 2010).

So far, SEM has not been used very often in the agricultural sector. Nevertheless, the advantages of this methodology are demonstrated and SEM is gaining in importance. In the livestock production sector, Peñagaricano et al. (2015) used an SEM to analyze growth and meat quality traits in pigs. Rauber et al. (2021) used the methodology to evaluate health parameters in broilers.

The first models for dairy cows were already developed in 2006. Los Campos et al. (2006) developed a recursive SEM to investigate the relationships between SCS (somatic cell score) and milk yield in more detail, especially the interaction between them. For example, not only the SCS affects the milk yield during mastitis, but also the milk yield affects the SCS by dilution effects, even though this effect is much smaller (Los Campos et al., 2006). In further analyses about efficiency in milk production, Drews et al. (2018) showed that biological efficiency (0.644) is three times higher than economic efficiency (0.266) in relation to the efficiency of milk production.

A great advantage of SEM is the analysis of direct and indirect factors, simultaneously. Using path models, Sharma et al. (2020) showed that the direct effect of lactation order (-0.002) and lactation length (-0.18) on milk yield is significantly lower than the indirect effects of these influencing factors (-0.16 for lactation order, 0.21 for lactation length). However, the direct effect of age of animal (0.68) is higher than the indirect effect (-0.002).

Direct and indirect factors are also evaluated in relation to udder health and mastitis. For example, Detilleux et al. (2012) integrated risk factors for subclinical and clinical mastitis, herd demography, housing conditions, feeding aspects, milking routine, mastitis prevention, in their SEM. Especially pre- and post-milking teat disinfection, cleanliness in the housing system and milking routine were the most notable direct effects (Detilleux et al., 2012). When compared to conventional models, the risk and tolerance of mastitis infections is better analyzed applying SEM. The different mastitis pathogens are a strong direct factor. On the other hand, the indirect effect for the manifest variable post dipping and the latent variable production was shown to be greater than the direct effect (Detilleux et al., 2013).

1.5 Aims of the study

The present thesis analyzes udder health in compost-bedded pack barns compared to conventional cubicle barns on a phenotypic and a genomic level using alternative traits such as differential somatic cells and specific mastitis pathogens. In addition, new methodological approaches are chosen, such as the development of a structural equation model and the integration of SNP x housing system interaction and SNP main effects in a GWAS. The chapters of this thesis had the following aims:

I. In **chapter 2**, udder health is analyzed by differential somatic cell composition and specific mastitis pathogens at the phenotypic level between compost and loose housing. In addition, the relationships to lactation stage, milk yield and somatic cell count are examined.

II. **Chapter 3** focuses on genome-wide association studies in relation to udder health in different housing systems. Possible candidate genes are identified in this chapter, which are expressed differently by the environment.

III. **Chapter 4** includes the development of a structural equation model to explain the direct and indirect influence of different traits and their relationship to one another.

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CHAPTER 2

Microscopic differential cell count and specific mastitis pathogens in cow milk from compost-bedded pack barns and cubicle barns

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Abstract

Compost-bedded pack barns (compost) as a new free walk housing system favorably influence udder health due to improved animal welfare and lying comfort. On the other hand, unfavorable effects on udder health are possible, due to the open bedded pack and the associated larger bacterial content in moisture. For in-depth farming system comparisons, the present study aimed to evaluate the specific cell fractions and mastitis pathogens in milk from cows kept in compost and in conventional cubical barns (cubicle). For milk sample collection we used a repeated measurement data structure of 2,198 udder quarters from 537 Holstein cows kept in six herds (3 in compost and 3 in cubicle). Differential cell counting was conducted including lymphocytes, macrophages and polymorphonuclear leukocytes (PMN). Specific mastitis pathogens comprised major and minor pathogens. Mixed models were applied to infer environmental and cow associated effects on cell fractions and on prevalences for pathogen infections, with specific focus on system × lactation stage, system × milk yield and system × somatic cell count effects. The interaction between system and lactation stage showed significant differences ($P < 0.01$) between the systems. A significantly smaller number of bacteriologically positive quarters and lower prevalences for minor pathogens were detected in compost compared to cubicle. Least squares means for pathogen prevalences indicated a quite constant proportion of bacteriologically negative udder quarters across milk yield levels in compost, but a slight increase with increasing milk yield in cubicle. Cell fraction responses in both systems differed in relation to the overall bacteriological infection status and farming system particularities. In conclusion, different cell fractions and specific mastitis pathogens should be considered as an indicator for udder health in different production systems, taking into account cow associated factors (lactation stage, milk yield).

Introduction

New free walk housing systems including compost-bedded pack barns attract increasing attention due to improved animal welfare, longevity and health (Leso et al., 2020). Astiz et al. (2014) associated higher milk production and lower somatic cell count in compost with a lower environmental stress level than in conventional cubicle barns. Improved rumination due to a longer lying time in compost was favorably associated with milk production (Schirmann et al., 2012; Leso et al., 2020). However, regarding udder health impact, opinions and practical impressions are quite controversial. On the one hand, compost implies less stress and wellbeing through the implemented free walk area, but on the other hand, the open bedded pack area as a mixture of organic bedding and cattle excreta might increase the risk for bacterial infections (Barberg et al., 2007). The combination and amount of pathogens in the compost depends on different factors, such as temperature (Black et al., 2013). According to

Janni et al. (2007), the optimal bedding temperature in a depth of 15 to 30 cm ranges between 43.3 to 65.0°C. However, Barberg et al. (2007) and Black et al. (2013) identified large numbers of temperatures beyond the suggested range. Black et al. (2013) investigated the bacterial flora in compost in relation to the bedding temperature. Coliforms as well as staphylococci and streptococci grew in a wide range of temperatures. Hence, with regard to udder health, Black et al. (2013) identified milking hygiene and the dry lying surface as the most important parameters.

A detailed evaluation of udder health in various farming systems requires proper udder health indicator traits, reflecting the pathogen-specific infection status. Somatic cell count is only moderately associated with impaired udder health or prevalences for clinical mastitis, and genetic correlations depend on the infection status (Riggio et al., 2010). A more accurate evaluation of udder health may be possible on the basis of microscopic cell fraction differentiations of leukocytes in milk or on prevalences for specific mastitis pathogens (Rivas et al., 2001). Microscopic cell fraction differentiations of leukocytes in milk are proper early indicators for health abnormalities, because specific fractions indicate intramammary infections in early stages (Kehrli and Shuster, 1994; Rivas et al., 2001). In this regard, lymphocytes, macrophages and polymorphonuclear leukocytes (PMN) represent different immunological functions. High proportions of macrophages indicate earliest defense mechanisms against infections (Paape et al., 2003), whereas lymphocytes become dominant in the later course of infection (Sordillo and Streicher, 2002). Regarding infection levels, a very large proportion of PMN (>90%) is an obvious hint for severe clinical mastitis cases (Sordillo and Streicher, 2002). PMN can further be divided into segmented neutrophils (mature neutrophils) and banded neutrophils (immature neutrophils). Banded neutrophils are detectable at the beginning of acute infections (Sellon, 2004).

Consideration of specific mastitis pathogens allows a clear differentiation of infections with detailed suggestions for cow treatments, and recommendations for breeding and selection strategies (Schafberg et al., 2006). The mastitis pathogens are subdivided into major (e.g. *Staphylococcus aureus*) and minor pathogens (e.g. *Corynebacterium sp.*) according to the severity of the inflammatory process triggered in the udder (Ariznabarreta et al., 2002). Pathogen species that infect the udder quarter can vary widely among housing systems (Lobeck et al., 2012). Comparisons in this regard considering compost systems focused on bulk tank milk (Leso et al., 2020). Astiz et al. (2014) analyzed the dry period and reported fewer cases of bacteriologically positive udder quarters for cows kept in compost. Consequently, also in lactating cows, we assume udder health differences in compost compared to cubicle systems, due to the positive impact of cow associated well-being parameters. On the other

hand, the pathogen burden in the bedding material might be a challenge for cows kept in compost barns.

The aim of the present study was to compare microscopic differential cell counts for lymphocytes, macrophages, PMN, banded and segmented neutrophils, and prevalences for specific mastitis pathogens in compost and cubicle on cow and udder quarter levels during lactation. In this regard, we implemented a specific sampling design in selected herds to inferring the impact of cow associated effects such as production level and lactation stage and of farm characteristics via mixed model applications.

Material and methods

Data for this study comprised milk samples from routine milkings. No additional experiments were conducted. Thus, in concordance with the German animal welfare legislation, no ethical approval was required for this study.

Herds, animals and traits

For milk sample collection, we used six Holstein dairy cow herds. Three herds were kept in compost and the three others in cubicle systems. Interestingly, two farms had both systems compost and cubicle, and they randomly allocated their milking cows to either the compost or the cubicle group. The remaining compost and remaining cubicle herds were selected considering various criteria to make farming system comparisons as objective as possible. Each pair of herds was located in the same region implying identical climatic impact and the same herd size with a similar cow age structure. Cows from the same herd pair were fed the same feed ratio through cooperations in harvesting food. The guidelines for selecting case (i.e. compost) and control (i.e. cubicle) herds were defined by an expert group from the European Freewalk Consortium as stated by Blanco-Penedo et al. (2020). The six herds were located in the German federal states North Rhine-Westphalia and Hesse.

Milk sampling comprised 2,198 udder quarters from 537 different Holstein dairy cows. The farms were visited on eleven dates between October 2018 and April 2019, implying a repeated measurement data structure within cow. Some 44% of all samples represented the compost and 56% the cubicle system. Some 61% of all samples were taken from cows in first lactation and 39% from cows in second lactation. Diseased cows treated with antibiotics were excluded from all analyses. Before milking, the teats were disinfected with 70% ethanol and the first five squirts of milk were discarded. A 10ml milk sample per udder quarter was taken following the DVG guidelines (Deutsche Veterinärmedizinische Gesellschaft, 2000) and subsequently examined for specific mastitis pathogens and somatic cell count in the laboratory of Landesbetrieb Hessen. Isolates of specific mastitis pathogens were classified into major

pathogens including *Aerococcus sp.*, *Aesculin hydrolyzing streptococci*, *Candida krusei*, *Enterococcus sp.*, *Escherichia coli*, *Lactococcus sp.*, *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *mold fungus* and *Proteus sp.* and minor pathogens including *Coagulase-negative staphylococci* and *Corynebacterium sp*. In addition, the following pathogens were analyzed individually: *Coagulase-negative staphylococci* (CNS), *Corynebacterium sp.* (COR), *Aesculin hydrolyzing streptococci* and *Aerobic bacilli* (AER). An udder quarter diagnosis of at least one pathogen within the defined groups major and minor implied the score = 1 for the respective overall group, otherwise the score = 0 was assigned. Also, for the individual pathogen analyses for CNS, COR, AER and *Aesculin hydrolyzing streptococci* scores 1 (pathogen detected) or 0 (no pathogen detected), were used. For the ongoing analyses, bacteriological determinations were available from 2,145 udder quarters.

Differential cell counting was conducted in the laboratory of the Institute of Animal Breeding and Genetics, University of Giessen. In this regard, we followed the protocol of Sarikaya et al. (2005). Cell counting comprised determination of lymphocytes, macrophages, PMN, segmented and banded neutrophils. The total count comprising all cell fractions was set as 100%. Fractions for differentiated cells within udder quarters were considered as percentages in the ongoing analyses, comprising 2,053 udder quarters with at least 30 counted cells. Descriptive statistics for all udder health traits are given in online Supplementary Table S2.1.

Statistical analyses

Linear mixed models were applied to infer the effect of the farming system (compost vs. cubicle) on the different cell fractions lymphocytes, macrophages, PMN, segmented and banded neutrophils. From a theoretical perspective for the statistical analyses of count data, generalized linear mixed models with a Poisson link might be more appropriate. However, based on previous experiences in this regard, we found very similar results compared to linear modeling approaches, and partly failed convergence when fitting a large number of effects. In the context of farming system comparisons, special focus addressed interactions with the lactation stage, with cow productivity in milk yield and with the overall infection status as indicated by somatic cell count levels. In analogy, generalized linear mixed models with a logit-link function were used for the analyses of the binary distributed mastitis pathogens (major, minor, CNS, COR, AER, *Aesculin hydrolyzing streptococci*). For all statistical analyses, the lme4 package R, version 3.6.2, was applied (Bates et al., 2015). This package was also used to compute least squares means for the different levels of effects. Details for statistical model definitions and corresponding fixed and random effects are specified in the online Supplementary File.

Results

Cell differentiation

The mean values of the cell fractions in udder quarters were 29% (±21%) for macrophages, 60% (±25%) for lymphocytes and 10% (±14%) for PMN, with a maximum of 97% PMN in infected quarters as shown in the online Supplementary Tables S2.1 and S2.2. Least squares means for the differential somatic cell fractions from the two production systems depending on lactation stage, cow productivity in milk yield per day and the total somatic cell counts in 1000 ml/l are shown in online Supplementary Table S2.3.

Impact of lactation stages within housing system on differential cell counts

The interaction effect of system and lactation stage was highly significant ($P < 0.001$) on cell fractions for lymphocytes, macrophages, PMN and the segmented subfraction. The significant interaction indicates that specific cell counts strongly depend on the cow lactation period within the both systems compost and cubicle. Figure 2.1 shows the least squares means for lymphocytes, macrophages and PMN in response to lactation stage variations in compost and cubicle. The proportion of macrophages increased with increasing days in milk, but the slope differed in compost and cubicle. Regarding the lactation period after 100 days, fractions for macrophages were very similar in cubicle, but steadily increased by lactation stage in compost. Also, for PMN, cell fractions increased with increasing lactation stage. In the late lactation beyond 300 days in milk, PMN and the segmented subfraction rapidly increased in both systems. For PMN, the system × lactation stage effect was highly significant ($P < 0.001$). The segmented subfraction strongly fluctuated by days in milk in both systems. Opposite reactions were observed for lymphocytes, i.e. a decline of cell fractions with increasing lactation stage. The decrease of lymphocytes with increasing days in milk was stronger in compost than in cubicle.

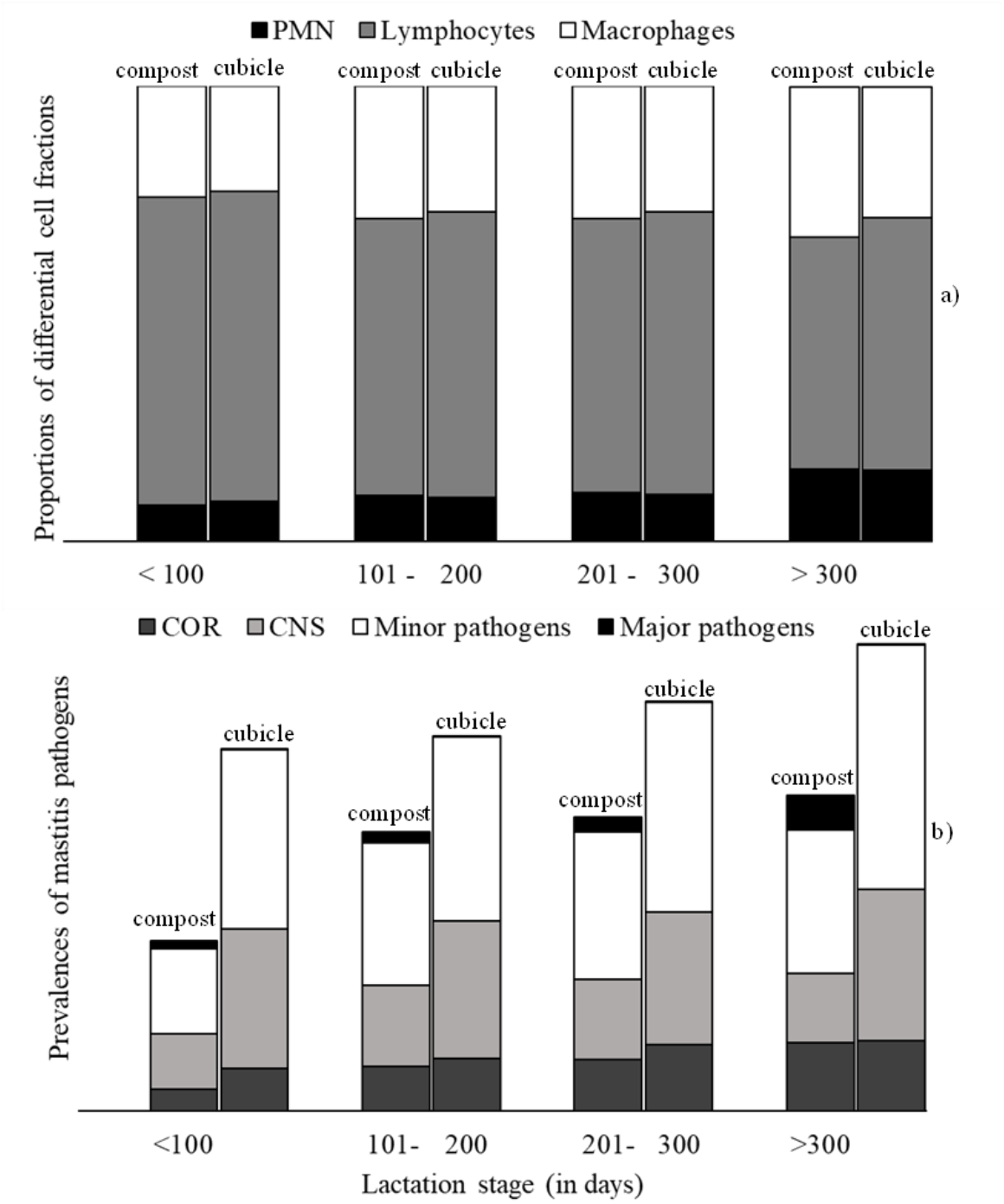

Figure 2.1. (a) Least squares means for the differential somatic cell fractions lymphocytes, macrophages and polymorphonuclear leukocytes (PMN) from the two production systems compost-bedded pack barns (compost) and conventional cubicle barns (cubicle) depending on lactation stage (Standard errors for least squares means were in a narrow range from 0.009 to 0.025). (b) Least squares means for prevalences of major pathogens, minor pathogens, *Coagulase-negative staphylococci* (CNS) and *Corynebacterium sp.* (COR) from the two production systems compost-bedded pack barns (compost) and conventional cubicle barns (cubicle) depending on lactation stage. (Standard errors for least squares means were in a narrow range from 0.006 to 0.051).

Impact of milk yield level within housing system on differential cell counts

The interaction system × milk yield only was significant for lymphocytes ($P < 0.05$). The macrophages remained relatively constant in relation to daily milk yield of the cow, in compost between 25.3% (±1.92%) and 32.2% (±1.78%), and in cubicle between 25.5% (±1.55%) and 29.8% (±1.8%) (Figure 2.2). The lymphocytes differed slightly between the systems in relation to milk yield. In cubicle, lymphocytes steadily increased up to a milk yield level of 40 kg per day with a maximal value of 68%. In compost, lymphocyte fractions were quite constant with 58% in low yielding cows (<25 kg milk per day) up to 65% in the neighboring milk yield class (25–30 kg milk per day). Percentages for PMN and for the segmented neutrophils varied across milk yield classes and housing systems. Fractions for segmented decreased with increasing milk yield, but increased in the high yielding cows with more than 40 kg milk per day in both systems. The proportions of banded neutrophils varied across all milk yield classes, with different responses in compost and cubicle.

Impact of somatic cell count levels within housing system on differential cell counts

The interaction term, somatic cell count class × system, was highly significant for all cell fractions macrophages, lymphocytes, PMN, segmented and banded neutrophils ($P < 0.001$). The proportion of lymphocytes decreased with increasing somatic cell count in both systems, in compost from 71.5% (± 1.34%) to 33.7% (±1.97%), and in cubicle from 72% (±1.55%) to 40.2% (±1.83%) (Figure 2.3). The proportions of macrophages increased with increasing somatic cell count, with larger percentages (~ plus 2%) of macrophages in compost than in cubicle for all somatic cell count classes. The sub-fractions of PMN (banded and segmented neutrophils) showed a strong increase with increasing somatic cell count, especially when the somatic cell count exceeded 200,000 cells. In most of the somatic cell count classes, proportions for PMN and segmented neutrophils were larger in compost than in cubicle. Fractions for banded neutrophils fluctuated across somatic cell count classes and housing systems.

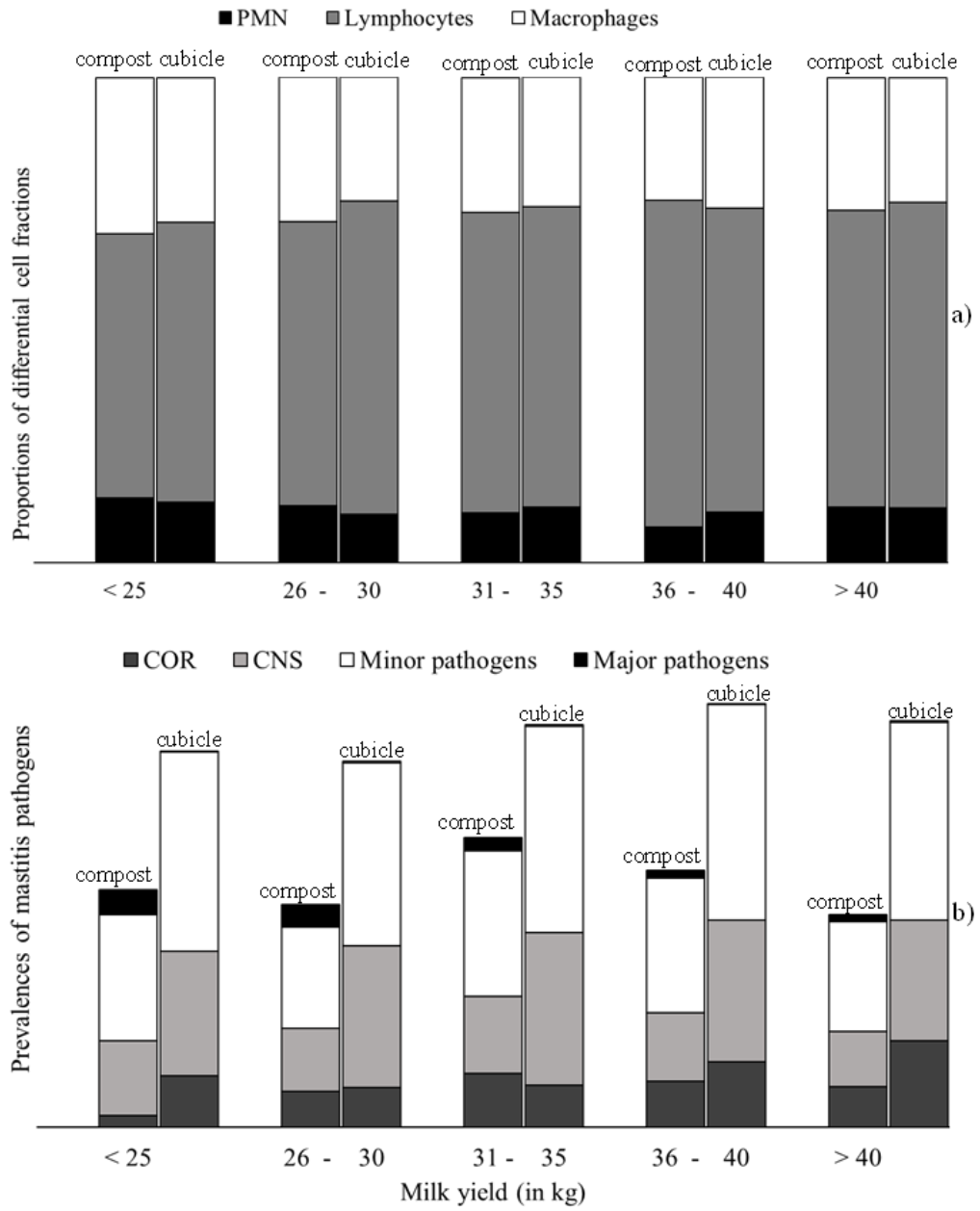

Figure 2.2. (a) Least squares means for the differential somatic cell fractions lymphocytes, macrophages and polymorphonuclear leukocytes (PMN) from the two production systems compost-bedded pack barns (compost) and conventional cubicle barns (cubicle) depending on cow productivity in milk yield per day (Standard errors for least squares means were in a narrow range from 0.012 to 0.026). (b) Least squares means for prevalences of major pathogens, minor pathogens, *Coagulase-negative staphylococci* (CNS) and *Corynebacterium sp.* (COR) from the two production systems compost-bedded pack barns (compost) and conventional cubicle barns (cubicle) depending on cow productivity in milk yield per day. (Standard errors for least squares means were in a narrow range from 0.01 to 0.045).

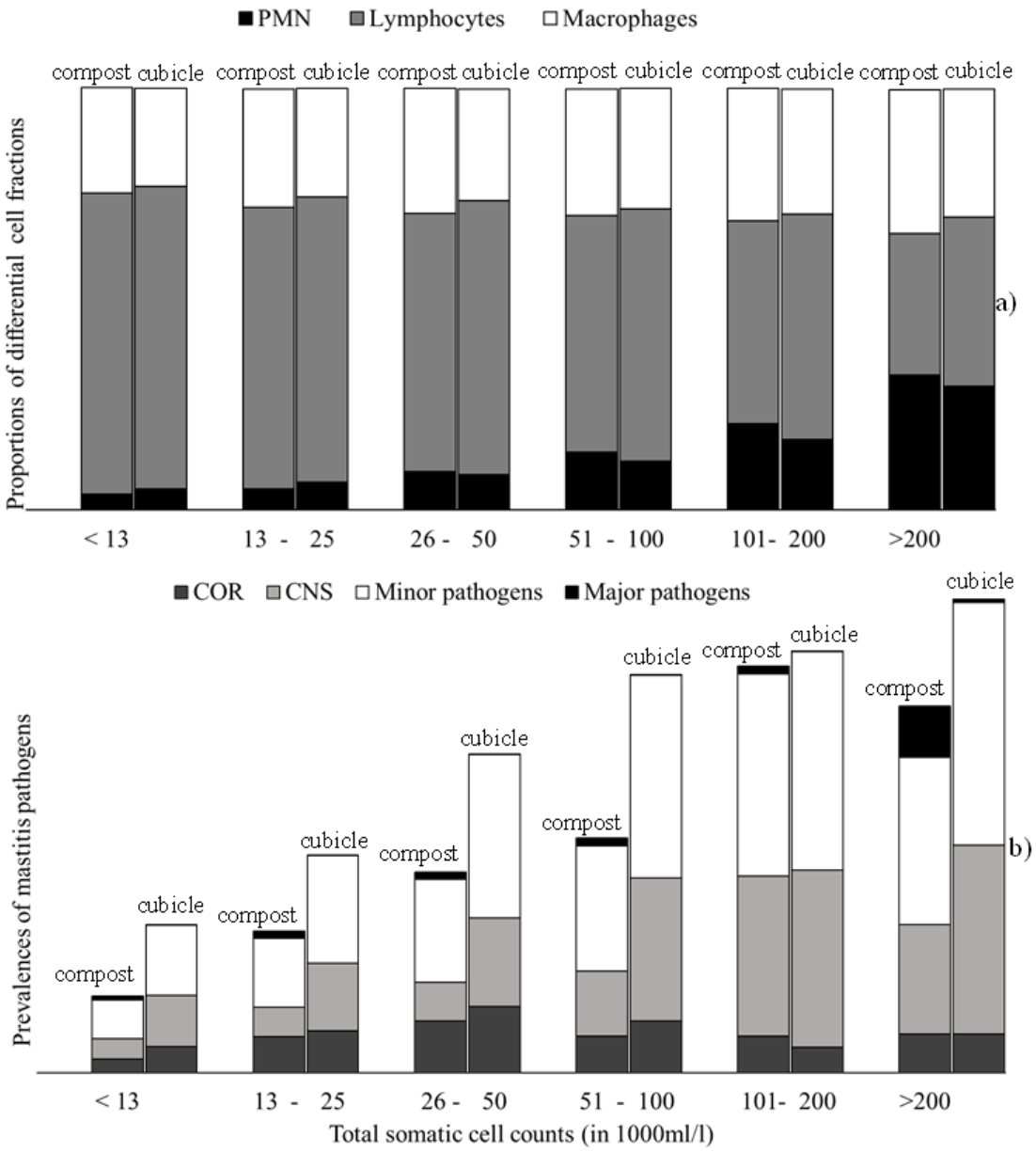

Figure 2.3. (a) Least squares means for the differential somatic cell fractions lymphocytes, macrophages and polymorphonuclear leukocytes (PMN) from the two production systems compost-bedded pack barns (compost) and conventional cubicle barns (cubicle) depending on the total somatic cell counts in 1000 ml/l (Standard errors for least squares means were in a narrow range from 0.008 to 0.021). (b) Least squares means for prevalences of major pathogens, minor pathogens, *Coagulase-negative staphylococci* (CNS) and *Corynebacterium sp.* (COR) from the two production systems compost-bedded pack barns (compost) and conventional cubicle barns (cubicle) depending on the total somatic cell counts in 1000 ml/l. (Standard errors for least squares means were in a narrow range from 0.005 to 0.049).

Mastitis pathogens

Least squares means for prevalences of mastitis pathogens in both systems compost and cubicle are given in online Supplementary Table S2.2 and in Supplementary Table S2.4 related to lactation stage, cow productivity in milk yield per day and total somatic cell counts in 1000ml/l. Overall, fewer bacteriologically positive quarters were detected in compost than in

cubicle. Among all bacterial species, largest prevalences were identified for CNS (27%) and COR (18%). The system comparison indicated higher prevalences for minor pathogens in cubicle (54.1%) than in compost (35%). Prevalences for CNS were substantially larger in cubicle (36.7%) compared to compost (18.6%). Also, prevalences for COR were larger in cubicle than in compost. Regarding major pathogens, prevalences were generally low with 2.9% in compost and 0.1% in cubicle. We found no significant difference ($P > 0.05$) between compost and cubicle for AER prevalences, which were on a low level in compost (2.9%) and in cubicle (0.2%). Least squares means for the prevalences of *Aesculin hydrolyzing streptococci* were 1.13% in compost and 0% in cubicle. The difference was not significant ($P > 0.05$).

Impact of lactation stages within housing system on mastitis pathogens

The number of bacteriologically negative samples decreased with increasing lactation stage in both systems. The lactation stage had a significant effect on prevalences for COR ($P < 0.05$) and for minor pathogens ($P < 0.01$). Least squares means for COR prevalences increased with increasing lactation stage in compost from 5.1% (±1.34%) to 16.2% (±5.1%), and from 10.2% (±2.69%) to 16.5 (±3.87%) in cubicle (Fig. 2.1). Hence, we observed substantial differences in COR prevalences between both systems during early lactation, but very similar prevalences beyond 300 days in milk, explaining the significant system × lactation stage interaction effect ($P < 0.05$). Least squares means for CNS only slightly increased by days in milk, from 13.3% (±2.11%) to 16.2% (±3.87%) in compost, and on a much higher prevalence level in cubicle from 32.8% (±4.22%) to 35% (±4.53%). AER and *Aesculin hydrolyzing streptococci* increased with increasing lactation stage, but their prevalences were quite low. Least squares means for major pathogens increased with increasing lactation stage, in compost from 1.84% (±0.69%) to 8.33% (±0.32%), and in cubicle from 0.14% (±0.23%) to 0.22% (±0.37%).

Impact of milk yield levels within housing system on mastitis pathogens

The interaction term, system × milk yield, did not differ in regard to mastitis pathogens ($P > 0.05$). The proportion of bacteriologically negative udder quarters was quite constant in compost across milk yield levels, but slightly decreased with increasing milk yield in cubicle. Least squares means for prevalences of minor pathogens and CNS were almost independent from milk yield alterations (Fig. 2.2), with higher estimates across all milk yield levels in cubicle than in compost. Least squares means for CNS prevalences varied in a narrow range in compost between 13.4% (±3.28%) and 18.5% (±3.38%). In cubicle, CNS prevalences altered between 29.2% (±4.35%) and 36.8% (±4.25%). In both systems compost and cubicle, cows with an average production level in milk yield had the highest CNS prevalences. Least squares means for COR prevalences increased with increasing milk yield in cubicle up to 20.7% (±4.79%), but opposite reactions were observed in compost. Least squares means for AER

prevalences substantially increased beyond production levels larger than 40 kg per day in both systems, but displaying higher prevalences in compost. Least squares means for *Aesculin hydrolyzing streptococci* prevalences were independent from the cow production level, with larger prevalences in compost than in cubicle. Least squares means for prevalences of major pathogens decreased with increasing milk yield in compost from 6.02% (±2.32%) to 1.67% (±1.02%), but were quite constant and close to zero in cubicle.

Impact of somatic cell count levels within housing system on mastitis pathogens

The interaction system × somatic cell count had a significant effect on prevalences for CNS ($P < 0.001$), COR ($P < 0.001$), major pathogens ($P < 0.001$) and minor pathogens ($P < 0.001$). Least squares means for prevalences of minor pathogens, CNS and major pathogens increased with increasing somatic cell count in both systems compost and cubicle (Fig. 2.3). Regarding udder quarters with less than 50,000 cells/ml, COR prevalences were higher in cubicle than in compost. Afterward, with increasing somatic cell count, COR prevalences decreased in cubicle, but remained relatively constant (12%) in compost.

Discussion

Cell fractions in housing systems

In our study, the main cell type in both compost and cubicle systems comprised lymphocytes, as previously observed by Dosogne et al. (2003) and Schwarz et al. (2011) in conventional farming systems. In contrast, macrophages were the predominant cell fraction in conventional farming systems in the study by Riollet et al. (2001). The proportions of leukocytes in healthy quarters were highly variable in different studies and in different environments (Paudyal et al., 2018). As reported by Yang et al. (1997) and Paudyal et al. (2018), fractions for macrophages and lymphocytes strongly depended on the lactation stage, but as shown in our present study, response pattern can differ between compost and cubicle. Also, the proportion of lymphocytes changed during lactation. In agreement with results by Dosogne et al. (2003) and Paudyal et al. (2018) in conventional systems, our study indicated a lymphocyte decrease as lactation advanced. In contrast to the previous studies, proportions of macrophages and PMN increased, especially in compost. Fractions for lymphocytes were highest in cows with highest test-day milk yield, confirming results by Paudyal et al. (2018), and especially increased with increasing milk yield in compost. The higher bacterial burden in the compost bedding material might trigger immune responses in compost, initiating the circulation of lymphocytes (Derakhshani et al., 2018). In contrast, fractions for macrophages increased with decreasing productivity. The proportions of PMN were quite constant across milk yield levels in previous studies (see for example Paudyal et al. 2018), but small PMN fluctuations were observed in

the present study, especially in compost. In compost, the bacterial flora showed obvious variations due to the different properties of added materials and cultivation processes (Ferraz et al., 2020). The bedding material can be a primary source of exposure to environmental mastitis pathogens (Ferraz et al., 2020). Ferraz et al. (2020) reported the highest levels of *Klebsiella spp.* in flax straw and conifer forest litter, as well as in Miscanthus and spelt husk. However, *Klebsiella spp.* was not detected in any of our quarter milk samples. Coliforms were detected by Ferraz et al. (2020) in barley husk, bark mulch, flax straw, wheat husk and barley straw. Black et al. (2013) identified coliforms, *Bacillus spp.*, Staphylococci and Streptococci in the bedding material. However, the occurrence of specific bacteria in the bedding material was unrelated with compost characteristics such as bedding temperature, which complicates the formulation of specific compost management guidelines toward udder health improvements.

Varying bedding materials and the cultivation of the pack may have an impact on PMN fluctuations. The cell fraction PMN has shorter half-life periods than lymphocytes or macrophages, and reacted more sensitive on environmental alterations (Anwer et al., 2017), possibly explaining the observed differences in PMN fractions between both farming systems compost and cubicle. Independent from the farming system, the count for segmented neutrophils substantially increased with increasing milk production. Such dependencies, i.e. segmented fractions in relation to milk yield, were previously elaborated by Le Maréchal et al. (2011).

Specific mastitis pathogens in housing systems

Overall, cows kept in compost had fewer infected udder quarters (48.9%) compared to cows from cubicle (66.1%). Favorable effects of free access farming systems on bacteriological udder infections were stated by Astiz et al. (2014) and Eckelkamp et al. (2016), and especially in compost, lower incidences for clinical mastitis were observed. Eckelkamp et al. (2016) detected different bacteria species in specific housing systems. In the present study, we excluded cows with severe clinical mastitis and antibiotic treatments, causing potential biases in pathogen prevalences. Nevertheless, due to the implementation of mastitis prevention programs in the past two decades, minor pathogens are of greater importance in well-managed farms than major pathogens, as shown in the present study and by Tenhagen et al. (2006) and Piessens et al. (2011). Furthermore, field tests have shown that quarters infected with COR as a minor pathogen were partly resistant against major pathogens due to an intensified stimulation of the immune system (Sordillo et al., 1989). Prevalences for CNS differed between compost and cubicle, confirming that the occurrence of CNS positive udder quarters are highly dependent on housing system characteristics and the type of bedding (Supré et al., 2011; Condas et al., 2017). As previously shown by Schukken et al. (2009), the number of COR increased with increasing cow lactation stage in both systems compost and cubicle.

Cows producing more than 40 kg milk per day displayed the highest prevalence for AER, which invade through the teat canal. Galama et al. (2015) indicated that heat-resistant compost bacteria may also enter into the udder, complicating ongoing milk processing. Nevertheless, among the different species from *Bacillus spp.*, only *Bacillus cereus* causes mastitis (Bergmann and Seffner, 1994). Recently, Giambra et al. (2021) investigated compost and milk samples, but they could not detect *Bacillus cereus* in bulk tank milk. Interestingly, the number of bacteriologically negative quarters decreased with increasing milk yield in cubicle, but increased in compost. The combination of a good hygiene management and animal welfare in compost has positive impact on the udder health of physiologically (productionrelated) stressed cows.

Cell fractions and specific mastitis pathogens: possible associations

An exclusion of negative bacteriological results from the dataset implied a small increase of proportions for banded neutrophils, with 1.6% in cubicle and 1.5% in compost. In contrast, proportions of segmented neutrophils were larger in compost than in cubicle, especially for increasing SCC levels. Hence, cell fraction responses in both systems differed in relation to the overall infection status and farming system particularities. Larger levels for banded neutrophils in cubicle than in compost for a somatic cell count exceeding 200,000 cells indicate strong inflammatory reactions due to the pathogens in cubicle systems (Paape et al., 2003). Physiologically, an increase of banded neutrophils implies that the number of immature band cells released from the bone marrow is larger than the demand in body and udder tissue (Sellon, 2004). The type of pathogen species influences the increase of specific cell fractions and of somatic cell count. In this regard, different CNS species induce different mechanisms within the immune system, with specific impact on somatic cells. Some of the CNS species (e.g. *Staphylococcus chromogenes*, *Staphylococcus simulans*) induce an increase in somatic cell count similarly to *Staphylococcus aureus* and are more pathogenic than other species of the CNS group (Supré et al., 2011; Condas et al., 2017).

In conclusion, different udder health indicator traits depended on specific environmental (farm) characteristics, with different responses in combination with cow associated factors such as lactation stage and milk yield levels. In cubicle, a stronger acute immune response to mastitis pathogens was shown by higher proportions of segmented neutrophils in milk. The number of bacterial positive quarters was lower in compost than in cubicle, as suggested in our hypothesis. Compost was associated with a significantly lower prevalence of minor pathogens. Especially environmentally associated mastitis pathogens depend on milking hygiene and barn management including a dry lying surface and ventilation of the compost material. Based on the results from the present study and according to Lobeck et al. (2012) and Leso et al. (2020), it is imperative to apply specific mastitis prevention strategies adapted to the housing system

characteristics and individually to each farm. Ongoing studies associating compost characteristics with cow milk criteria and udder health are imperative in this regard.

Supplemental material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000844.

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Supplementary File

Supplementary Materials and Methods

Statistical models

For statistical analyses of the quasi Gaussian distributed percentages of cell fractions for macrophages, lymphocytes, PMN, segmented and banded neutrophils, the linear mixed model included the fixed effects for the lactation number (1 or 2), for the lactation stage classes (0-100 days, 101-200 days, 201-300 or >300 days after calving), for the farming system (compost or cubicle), for classes for the daily production level in milk yield at the recording date (< 25 kg, 25.1-30 kg, 30.1-35 kg, 35.1-40 kg, > 40 kg), for the herd-test-date at milk sampling and for the person counting the cells in the laboratory, for the interaction between the system with milk yield classes, for the interaction between the system with lactation stage classes, and for the interactions between the system with SCC classes. For the separation of the system effect from remaining farm management effects, a hierarchical design was considered, implying to nesting the system effect nested within in herd-test-day effect. A further fixed effect was the individual udder quarter. Due to the repeated measurements within cow (512 cows), the cow effect was treated as random. For the analyses of binary mastitis pathogens (major pathogens, minor pathogens, CNS, COR, AER, *Aesculin hydrolyzing streptococci*), we considered the same fixed and random effects as defined for the linear mixed model. The only difference is the consideration of a logit-link function in the generalized linear mixed model, implying the exclusion of a random error term.

Supplementary Table S2.1

Descriptive statistics for the differential somatic cell counts and mastitis pathogens per udder quarter

Traits[1]	Mean	Min	Max	SD
Cell fractions (in relation to the				
Total sum of all cell counts)				
Macrophages	0.292	0.000	0.980	0.208
Lymphocytes	0.608	0.000	1.000	0.246
PMN	0.100	0.000	0.971	0.143
Banded neutrophils	0.005	0.000	0.130	0.013
Segmented neutrophils	0.095	0.000	0.951	0.140
Mastitis pathogens (in prevalences)				
Negative samples	0.514	0.000	1.000	0.500
CNS	0.274	0.000	1.000	0.446
COR	0.183	0.000	1.000	0.387

AER	0.035	0.000	1.000	0.184
Aesculin hydrolyzing streptococci	0.015	0.000	1.000	0.122
Minor pathogens	0.407	0.000	1.000	0.491
Major pathogens	0.030	0.000	1.000	0.171

[1]Polymorphonuclear neutrophils (PMN), *coagulase-negative staphylococci* (CNS), *Corynebacterium sp.* (COR), *Aerobic bacilli* (AER), minor pathogens (including CNS and COR), major pathogens (including *Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia Coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, mold fungus* and *Proteus sp.*)

Supplementary Table S2.2

Least squares means (LSM) and corresponding standard errors (SE) for the differential somatic cell counts and prevalences of specific mastitis pathogens in the housing systems compost-bedded pack barn (compost) and conventional cubicle barn (cubicle) and P-Values for the LSM differences in the two farming systems

	compost		cubicle		
Traits	LSM [%]	SE [%]	LSM [%]	SE [%]	p-value
Cell fractions (in relation to the Total sum of all cell counts)					
Macrophages	29.700	0.800	27.500	0.869	0.0533
Lymphocytes	56.300	0.988	59.400	1.075	0.0307
PMN	13.900	0.582	13.100	0.630	0.3291
Segmented neutrophils	13.100	0.570	12.300	0.616	0.3153
Banded neutrophils	0.795	0.054	0.801	0.058	0.9409
Mastitis pathogens (in prevalences)					
Negative samples	51.100	2.850	33.900	2.920	<0.0001
CNS	18.600	1.900	36.700	2.720	<0.0001
COR	10.600	1.630	12.800	2.020	0.3939
AER	0.004	2.349	0.001	0.704	0.9989
Aesculin hydrolyzing streptococci	1.130	0.460	0.000	0.043	0.9945
Minor	35.000	2.500	54.100	2.920	<0.0001
Major	2.925	0.748	0.153	23.323	0.9845

[1]Polymorphonuclear neutrophils (PMN), *coagulase-negative staphylococci* (CNS), *Corynebacterium sp.* (COR), *Aerobic bacilli* (AER), minor pathogens (including CNS and COR), major pathogens (including *Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia Coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, mold fungus* and *Proteus sp.*)

Supplementary Table S2.3

Least squares means (standard errors) for the differential somatic cell fractions lymphocytes, macrophages and polymorphonuclear leucocytes (PMN) from the two production systems compost-bedded pack barns (compost) and conventional cubicle barns (cubicle) depending on lactation stage, cow productivity in milk yield per day and the total somatic cell counts in 1000ml/l.

Trait	Lactation stage (in days)	compost	cubicle
Macrophages	< 100	0.243 (0.011)	0.230 (0.015)
	101-200	0.291 (0.017)	0.276 (0.014)
	201-300	0.290 (0.018)	0.275 (0.013)
	>300	0.330 (0.019)	0.289 (0.016)
Lymphocytes	< 100	0.679 (0.016)	0.682 (0.020)
	101-200	0.608 (0.023)	0.628 (0.019)
	201-300	0.603 (0.024)	0.622 (0.017)
	>300	0.510 (0.025)	0.554 (0.021)
PMN	< 100	0.079 (0.010)	0.088 (0.013)
	101-200	0.101 (0.015)	0.097 (0.012)
	201-300	0.107 (0.015)	0.103 (0.011)
	>300	0.159 (0.016)	0.157 (0.013)
	Milk yield (in kg per day)		
Macrophages	< 25	0.322 (0.018)	0.298 (0.018)
	26-30	0.297 (0.016)	0.255 (0.016)
	31-35	0.278 (0.016)	0.267 (0.014)
	36-40	0.253 (0.019)	0.270 (0.017)
	> 40	0.273 (0.017)	0.257 (0.016)
Lymphocytes	< 25	0.544 (0.024)	0.578 (0.024)
	26-30	0.586 (0.022)	0.646 (0.021)
	31-35	0.620 (0.021)	0.620 (0.019)
	36-40	0.675 (0.026)	0.626 (0.023)
	> 40	0.612 (0.023)	0.631 (0.021)
PMN	< 25	0.134 (0.015)	0.124 (0.016)
	26-30	0.117 (0.014)	0.099 (0.013)
	31-35	0.102 (0.013)	0.113 (0.012)
	36-40	0.072 (0.016)	0.104 (0.015)
	> 40	0.115 (0.015)	0.112 (0014)
	Total somatic cell count (in 1000ml/l)		
Macrophages	<13	0.251 (0.011)	0.232 (0.013)
	13-25	0.282 (0.012)	0.258 (0.012)
	26-50	0.297 (0.014)	0.265 (0.012)
	51-100	0.301 (0.016)	0.286 (0.013)
	101-200	0.314 (0.018)	0.297 (0.015)
	>200	0.341 (0.016)	0.303 (0.015)

Lymphocytes	<13	0.715 (0.013)	0.720 (0.016)
	13-25	0.668 (0.015)	0.679 (0.014)
	26-50	0.613 (0.017)	0.652 (0.015)
	51-100	0.563 (0.019)	0.600 (0.016)
	101-200	0.483 (0.021)	0.536 (0.018)
	>200	0.337 (0.020)	0.402 (0.018)
PMN	<13	0.036 (0.008)	0.048 (0.010)
	13-25	0.050 (0.009)	0.064 (0.009)
	26-50	0.090 (0.011)	0.083 (0.009)
	51-100	0.136 (0.012)	0.115 (0.010)
	101-200	0.204 (0.014)	0.167 (0.012)
	>200	0.319 (0.012)	0.293 (0.012)

[1]Polymorphonuclear neutrophils (PMN)

Supplementary Table S2.4

Least squares means (standard errors) for prevalences of cultural negative samples, major pathogens, minor pathogens, *Coagulase-negative staphylococci* (CNS) and *Corynebacterium sp.* (COR) from the two production systems compost-bedded pack barns (compost) and conventional cubicle barns (cubicle) depending on lactation stage, cow productivity in milk yield per day and the total somatic cell counts in 1000ml/l.

Trait	Lactation stage (in days)	compost	cubicle
cultural negativ samples	< 100	0.716 (0.033)	0.506 (0.050)
	101-200	0.595 (0.055)	0.488 (0.046)
	201-300	0.533 (0.060)	0.381 (0.041)
	>300	0.493 (0.063)	0.342 (0.048)
Major	< 100	0.018 (0.007)	0.001 (0.229)
	101-200	0.025 (0.013)	0.003 (0.498)
	201-300	0.035 (0.016)	0.003 (0.512)
	>300	0.083 (0.033)	0.002 (0.370)
Minor	< 100	0.201 (0.027)	0.425 (0.047)
	101-200	0.337 (0.050)	0.435 (0.044)
	201-300	0.348 (0.053)	0.497 (0.044)
	>300	0.340 (0.058)	0.580 (0.049)
CNS	< 100	0.133 (0.021)	0.328 (0.042)
	101-200	0.192 (0.038)	0.324 (0.039)
	201-300	0.189 (0.038)	0.313 (0.035)
	>300	0.162 (0.039)	0.358 (0.045)
COR	< 100	0.051 (0.013)	0.102 (0.027)
	101-200	0.104 (0.031)	0.125 (0.029)
	201-300	0.122 (0.037)	0.157 (0.031)
	>300	0.162 (0.051)	0.165 (0.039)

	Milk yield (in kg per day)		
cultural negativ samples	< 25	0.551 (0.059)	0.423 (0.062)
	26-30	0.623 (0.053)	0.490 (0.053)
	31-35	0.537 (0.052)	0.412 (0.047)
	36-40	0.603 (0.063)	0.384 (0.054)
	> 40	0.632 (0.055)	0.412 (0.052)
Major	< 25	0.060 (0.023)	0.001 (0.086)
	26-30	0.053 (0.021)	0.002 (0.245)
	31-35	0.031 (0.014)	0.004 (0.422)
	36-40	0.019 (0.013)	0.002 (0.187)
	> 40	0.017 (0.010)	0.004 (0.451)
Minor	< 25	0.305 (0.051)	0.482 (0.061)
	26-30	0.246 (0.043)	0.442 (0.051)
	31-35	0.352 (0.047)	0.498 (0.046)
	36-40	0.326 (0.058)	0.519 (0.055)
	> 40	0.265 (0.047)	0.477 (0.052)
CNS	< 25	0.180 (0.038)	0.301 (0.051)
	26-30	0.151 (0.032)	0.343 (0.046)
	31-35	0.185 (0.034)	0.368 (0.043)
	36-40	0.165 (0.041)	0.343 (0.049)
	> 40	0.134 (0.033)	0.292 (0.044)
COR	< 25	0.083 (0.029)	0.123 (0.036)
	26-30	0.086 (0.027)	0.095 (0.026)
	31-35	0.129 (0.033)	0.102 (0.026)
	36-40	0.110 (0.038)	0.157 (0.042)
	> 40	0.097 (0.030)	0.207 (0.048)
	Total somatic cell count (in 1000ml/l)		
cultural negativ samples	<13	0.808 (0.028)	0.716 (0.041)
	13-25	0.658 (0.043)	0.589 (0.043)
	26-50	0.595 (0.055)	0.413 (0.047)
	51-100	0.527 (0.067)	0.276 (0.044)
	101-200	0.262 (0.062)	0.194 (0.043)
	>200	0.219 (0.050)	0.093 (0.026)
Major	<13	0.011 (0.006)	0.001 (0.132)
	13-25	0.023 (0.011)	0.001 (0.090)
	26-50	0.022 (0.013)	0.000 (0.064)
	51-100	0.024 (0.016)	0.001 (0.220)
	101-200	0.026 (0.017)	0.002 (0.343)
	>200	0.163 (0.050)	0.017 (2.487)
Minor	<13	0.123 (0.021)	0.222 (0.035)
	13-25	0.219 (0.035)	0.340 (0.039)
	26-50	0.325 (0.049)	0.517 (0.046)

	51-100	0.397 (0.064)	0.642 (0.048)
	101-200	0.639 (0.066)	0.691 (0.053)
	>200	0.528 (0.063)	0.769 (0.045)
CNS	<13	0.065 (0.015)	0.161 (0.028)
	13-25	0.094 (0.022)	0.213 (0.030)
	26-50	0.123 (0.030)	0.280 (0.038)
	51-100	0.206 (0.050)	0.453 (0.050)
	101-200	0.507 (0.070)	0.561 (0.058)
	>200	0.347 (0.059)	0.596 (0.056)
COR	<13	0.044 (0.013)	0.084 (0.021)
	13-25	0.114 (0.028)	0.134 (0.028)
	26-50	0.164 (0.041)	0.210 (0.041)
	51-100	0.116 (0.038)	0.163 (0.038)
	101-200	0.115 (0.040)	0.081 (0.027)
	>200	0.122 (0.038)	0.124 (0.036)

[1]*coagulase-negative staphylococci* (CNS), *Corynebacterium sp.* (COR), minor pathogens (including CNS and COR), major pathogens (including *Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia Coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, mold fungus* and *Proteus sp.*)

CHAPTER 3

Genome-wide associations for microscopic differential somatic cell count and specific mastitis pathogens in Holstein cows in compost-bedded pack and cubicle farming systems

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Simple Summary

New free walk housing systems such as compost-bedded pack barns might positively influence animal welfare. However, udder health can be negatively affected due to the microbial environment in the pack. Udder health depends on many factors, such as the environment, the feed, the pathogen species, and the genetic mechanisms of the cow's immune system. For a more precise evaluation of udder health, we examined novel traits including specific mastitis pathogens and differential somatic cell fractions in milk. In order to identify possible candidate genes for udder health, a genome-wide association study, including single-nucleotide polymorphisms (SNP) by housing system interactions (compost-bedded pack barn and conventional cubicle barn), was performed. We identified two potential candidate genes for the interaction effect in relation to udder health. The identified potential candidate gene *HEMK1* (HemK methyltransferase family member 1) is involved in immune system development, and *CHL1* (cell adhesion molecule L1 like) has an immunosuppressive effect during stress conditions. The results suggest housing system-specific breeding strategies in order to improve udder health in compost-bedded pack and conventional cubicle barns.

Abstract

The aim of the present study was to detect significant SNP (single-nucleotide polymorphism) effects and to annotate potential candidate genes for novel udder health traits in two different farming systems. We focused on specific mastitis pathogens and differential somatic cell fractions from 2198 udder quarters of 537 genotyped Holstein Friesian cows. The farming systems comprised compost-bedded pack and conventional cubicle barns. We developed a computer algorithm for genome-wide association studies allowing the estimation of main SNP effects plus consideration of SNPs by farming system interactions. With regard to the main effect, 35 significant SNPs were detected on 14 different chromosomes for the cell fractions and the pathogens. Six SNPs were significant for the interaction effect with the farming system for most of the udder health traits. We inferred two possible candidate genes based on significant SNP interactions. *HEMK1* plays a role in the development of the immune system, depending on environmental stressors. *CHL1* is regulated in relation to stress level and influences immune system mechanisms. The significant interactions indicate that gene activity can fluctuate depending on environmental stressors. Phenotypically, the prevalence of mastitis indicators differed between systems, with a notably lower prevalence of minor bacterial indicators in compost systems.

Introduction

Genotype by environment interactions (GxE) in dairy cows have been reported widely [1–4]. There are many different approaches to prove GxE, such as creating a cross-classified research design for, e.g., climate or feeding groups [2], or calculating correlations between the same trait recorded in different environments [3–5]. Another approach focusses on continuous environmental descriptors such as temperature, herd production level, or herd size and the application of random regression or reaction norm methodology [3].

In genomic studies, Lillehammer et al. [3] and Hayes et al. [6] enhanced their genomewide association studies (GWAS) by considering GxE effects. In a two-step approach, they estimated intercept and slope effects for each single-nucleotide polymorphism (SNP) and found environment-sensitive and environment-robust SNPs. Similarly, Streit et al. [1] conducted a GWAS for milk protein yield using average herd somatic cell score (SCS) as an environmental descriptor. Dependent variables in their genome-wide association models were intercept and slope from reaction norm models.

New animal-friendly “free walk” housing systems, such as compost farming, reduce environmental stress, implying improved animal welfare [7]. However, the open-bedded pack area, as a mixture of composting material, microbes, and feces, increases the risk for specific bacterial infections and mastitis [7–9]. In this regard, several studies pointed out the associations between bedding management and physiological cow responses and addressed overall immunity [7,10,11]. A possible breeding approach is the selection of mastitis-resistant animals in a challenging environment [11,12]. Selection of animals in harsh environments implied a pronounced genetic differentiation in udder health indicator traits [13]. Nevertheless, such a selection strategy, in specific environments, raises concerns regarding site suitability for conventional farming systems, and vice versa.

Furthermore, selection on mastitis resistance is hampered due to the trait definitions and the complex polygenic mode of inheritance [12,14]. Heritabilities for SCS and overall clinical mastitis were very small and lower than 0.05 [15–17]. Regarding accurate trait definitions, difficulties in distinguishing between cases of clinical and subclinical mastitis were reported [11,16,18]. SCS is considered a mastitis indicator in overall dairy cattle breeding goals or selection indices, due to a genetic correlation of approximately 0.75 [19]. However, the genetic correlation being substantially smaller than one suggests alternative and more precise mastitis indicators, such as differential somatic cell count, should be considered.

Previous studies indicate that mastitis is caused by different pathogens to which the cow may respond differently [11]. Sørensen et al. reported that genetic correlations between pathogen-specific mastitis traits ranged widely from 0.45 to 0.77 [14]. In their study, the lowest correlation

of 0.45 was estimated between *Staphylococcus aureus* and *Escherichia coli*, possibly due to the different immune responses of these two pathogens. The highest correlation of 0.77 was detected between *Streptococcus dysgalactiae* and *Streptococcus uberis*. Both pathogens belong to the same genus and trigger a similar cascade of immune reactions in the udder [14]. The moderate genetic correlations suggest consideration that different mastitis pathogens can impact different traits in genetic evaluations for udder health. Addressing the farming effect, different bedding materials and different compost temperatures were associated with specific types of mastitis [7]. In this regard, *Coagulasenegative staphylococci* (CNS) and *Corynebacterium sp.* (COR), as well as minor pathogens, are of increasing importance [20,21]. Accordingly, due to pathogen-specific reactions of the immune system, Kurz et al. [22] suggested pathogen-specific mastitis analyses instead of the only moderately correlated indictor trait SCS. Hence, we decided to focus on novel mastitis traits in the present genomic study, including specific mastitis pathogens and differential somatic cell fractions comprising lymphocytes (LYM), macrophages (MAC), polymorphonuclear leucocytes (PMN), and their subgroups of PMN segmented neutrophils (sN) and banded neutrophils (bN), and considered these for detailed phenotypic analyses in free walk housing systems [23].

In a previous GWAS without farming interaction effect, dense SNP marker panels were used to infer possible candidate genes playing a role in defense mechanisms against pathogens and mastitis resistance [11]. Possible candidate genes or quantitative trait loci (QTL) and SNPs for clinical mastitis were detected on nearly all chromosomes [11,12], again raising the question of proper mastitis trait definitions. Nevertheless, in gene annotations, detected genes such as *LY6K, LY6D, LYNX1, LYPD2, SLURP1*, and *PSCA* are part of the lymphocyte antigen 6 complex and influence the regulation of PMN [12]. Further detected genes, e.g., *LY75*, *NR4A2*, and *ITGB6*, are involved in regulating immune response or were regulated during the presence of specific pathogens [12]. Kurz et al. [22] identified *RASGRP1* as a potential candidate gene based on differential gene expression studies, considering different levels of pathogen burden.

The aim of the present study was to identify significant main and interaction effects for specific mastitis pathogens and differential somatic cell counts in conventional cubicle and compost-bedded farming systems. Significant SNP markers were annotated with potential candidate genes with the aim of improving mastitis resistance in specific farming systems, taking GxE into account. In this regard, our own software package, allowing GWAS with SNP by housing system effects, was applied.

Materials and Methods

Animal Ethics Statement

Data for this study considered milk samples from routine milking. No additional experiments were conducted. Thus, in concordance with German animal welfare legislation, no ethical approval was required for this study.

Farms and Animals

Cow milk samples were collected from six selected Holstein dairy cattle herds. The herds were located in the German federal states of North Rhine-Westphalia and Hesse. Herd selection for cubicle and compost-bedded farming systems considered the herd criteria as defined in the collaborative EU FreeWalk project [24]. The case (compost) and control (cubicle) farms were identical regarding herd size, production level, climate conditions, location, and feeding and milking systems, as well as management aspects [24]. The only major difference was the bedding component. In two cases, in the same location with the same climatic conditions and geographical coordinates, and using the same milking technique and feed ration, both compost and cubicle systems were available. Three herds or sub-herds for each farming system compost and cubicle were selected. Trait recording spanned the period from October 2018 to April 2019. The final dataset comprised milk samples from 2198 udder quarters from 537 first and second parity Holstein Friesian dairy cows. A total of 44% of the samples represented the compost system, while 56% of the samples represented the cubicle system.

Milk Sample Preparation and Udder Health Trait Determination

Teats were disinfected with 70% ethanol before milking and the first five squirts of milk were discarded. A quantity of 10 mL milk per udder quarter was examined for specific mastitis pathogens and somatic cell count in the laboratory of Landesbetrieb Hessen, following the DVG guidelines [25]. The specific mastitis pathogens were classified into major pathogens (MAJOR) including *Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia Coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, mold fungus,* and *Proteus sp.*; minor pathogens (MINOR) included *Coagulase-negative staphylococci* and *Corynebacterium sp.* The following pathogens were additionally analyzed separately: *Coagulase-negative staphylococci* (CNS), *Corynebacterium sp*. (COR), *Aesculin hydrolysing streptococci* (AESC), and *Aerobic bacilli* (AER). The pathogens were defined as binary traits. A cow received a score of 1 for the overall group definition (MAJOR or MINOR) or for CNS, COR, AESC, or AER when the respective pathogen was detected in at least one udder quarter, otherwise a score of 0 was assigned. At the individual cow level, 32.9% and 12.6% of all samples were culturally negative in the compost and in the cubicle system, respectively. The prevalence of minor pathogens was

57.2% in compost and 80.6% in cubicle. A fraction of 16.7% of cows kept in compost and 6.1% of cows kept in cubicle had infections with AER. The prevalence of AESC was 5.0% in compost and 4.8% in cubicle.

Microscopic differential cell counting from a 50 mL sample was conducted in the laboratory of the Institute of Animal Breeding and Genetics, University of Giessen, according to the protocols by Sarikaya et al. and Pappenheim [26,27]. We considered LYM, MAC, PMN, and the PMN subgroups sN and bN. The sum of these cell fractions was 100%. The individual cell fractions were defined as percentages in the subsequent analyses. Quarters with less than 30 counted cells were excluded from the study. Mean values for LYM were 82.0% in compost and 79.4% in cubicle. The mean value for MAC in compost was 47.6% and 49.4% in cubicle. Mean values for PMN were 21.6% in compost and 20.4% in cubicle.

Cow Genotyping

The 273 Holstein cows from both systems were genotyped based on extracted DNA from hair samples in the laboratory of the veterinary institute (The Center for Molecular Diagnostics), Georg-August-Universität Göttingen, using the Illumina BovineSNP50 Bead Chip V3. The SNP genotypes from the Illumina BovineSNP50 Bead Chip V2 for the 277 cows (compost and cubicle) were provided by the Vereinigte Informationssysteme Tierhaltung w.V. (VIT). A total of 53,217 SNPs from Bead Chip V3 and 45,613 SNPs from Bead Chip V2 were available for quality control. In this regard, only the SNP available in both genotype datasets and located on *Bos taurus* autosomes were considered. The SNP positions were remapped according to the ARS1.2 assembly [28]. Quality control was performed using PLINK software, version 1.9 [29]. The SNP markers with a minor allele frequency <0.01, a minimum call rate <90%, and which were in Hardy–Weinberg equilibrium ($p \geq 10^{-3}$) were discarded. After quality control, we considered 43,095 SNPs from 550 genotyped cows (235 genotyped cows from compost and 315 genotyped cows from cubicle). For the ongoing GWAS, 537 cows with genotypes and phenotypic information were included.

Genome-Wide Associations

Single-trait GWAS with interaction effects between farming systems and SNP effects were conducted using the self-written R program “GWAInter.R”. All relevant data and the program source code are available at JLUdata at the University of Giessen (https: //jlupub.ub.uni-giessen.de/, accessed on 7 January 2021). The application of GWAInter.R focuses on the detection of possible GxE for SNP markers in a one-step approach. The approach simultaneously enables the estimation of main SNP effects, as well as of SNP by environmental interaction effects, for the two different farming systems – compost and cubicle. Compared with other approaches, it is possible to estimate main SNP effects and interaction effects in one model without splitting the data set. The corresponding statistical model was:

$$y = Xb + x_{snpi}\, b_{snpi} + x_{interi}\, b_{interi} + Zg + e \quad (1)$$

where y was a vector of observations for the traits LYM, MAC, PMN, sN, bN, MAJOR, MINOR, CNS, COR, AER, and AESC; b was a vector of fixed effects including parity, the general health status of the cow based on somatic cell count levels (with >200,000 cells indicating inferior cow health), farming system (compost or cubicle), farm, herd-test day for trait recording, and the person analyzing the milk samples in the laboratory; x_{snpi} was a vector of genotypes for marker i; b_{snpi} was a regression coefficient for the i*th* SNP (the main SNP effect); x_{interi} was a vector of genotypes for cows; b_{interi} was a regression coefficient of i*th* SNP (the interaction effect); and g was a vector of genetic effects following $N(0, G\sigma^2_g)$ and contained the polygenic effects, where G was a relationship matrix [30] and σ^2_g was the genetic variance. The G matrix was constructed considering all SNPs, but we excluded the SNP markers from the same chromosome where the candidate SNP was located, i.e., the "leave one chromosome out strategy"; e was the vector of random residual effects following $N(0, I\sigma^2_e)$, where I was an identity matrix and σ^2_e was the residual variance. X and Z were incidence matrices for b and g, respectively. The "leave one chromosome out approach", as also implemented in most of the commercial GWAS software packages, was realized by connecting GWAInter.R with the R package "gaston" [31], implying the permanent estimation of variance components per trait and chromosome. That is to say, we gave 29 G matrices (G matrix without chromosome 1, chromosome 2 . . . chromosome 29) to "gaston", separately for each trait. For the estimation of main SNP and SNP interaction effects, we implemented a generalized least squares approach in GWAInter.R. The p-value for each SNP was calculated by applying a test statistic which follows a chi-square distribution (Wald-test). The test statistic for the i*th* main SNP effect was:

$$\chi^2_{snpi} = \frac{\widehat{\mathbf{b}_{snpi}}^2}{\mathrm{var}(\widehat{\mathbf{b}_{snpi}})}$$, with 1

degree of freedom (df). The test statistic for the i*th* interaction effect was:

$$\chi^2_{interi} = \frac{\widehat{\mathbf{b}_{interi}}^2}{\mathrm{var}(\widehat{\mathbf{b}_{interi}})}$$, with 1 df.

Significantly associated SNPs were detected according to the Bonferroni corrected significance threshold p_{Bonf} = 0.05/43,095 SNP markers. Additionally, a less conservative threshold was defined as p_{sugg} = 0.05/(number of independent SNPs). The number of independent SNPs was 4479 when setting the linkage disequilibrium as ≤ 0.15 (squared correlation between two SNPs) for consecutive genomic windows including 500 SNPs along the bovine genome. Potential candidate genes were queried and assigned to associated SNP markers using the current gene annotations from the ENSEMBL and NCBI databases, considering a window 50 kb upstream or downstream from the significantly associated

candidate SNP [32,33]. Physiological functions and positions of potential candidate genes were further manually reviewed in the String (version 11) [34], KEGG, and NCBI databases [33,35].

Results

Genome-Wide Associations

A total of 35 significant (according to the Bonferroni corrected genome-wide significance and less conservative threshold) SNPs for the main effect were detected on 14 different chromosomes for the traits PMN, sN, bN, MAJOR, MINOR, cultural negative, AER, and CNS (Tables 3.1 and 3.2). A subset including eight SNPs surpassed the Bonferroni significance threshold, and 27 SNPs were significant according to the less conservative threshold.

The chromosome including the largest number of significantly (Bonferroni corrected genome-wide significance and less conservative threshold) associated SNPs was chromosome 14. For the cell fractions PMN and sN, the same significant SNPs when considering the less conservative threshold were detected on chromosome 6. One significant (less conservative threshold) SNP on chromosome 28 was significantly associated with bN. For MAJOR, 18 significant (Bonferroni corrected genome-wide significance and less conservative threshold) SNPs were located on chromosomes 1, 2, 4, 5, 11, 15, 17, 18, 22, and 26. Five SNPs exceeded the Bonferroni significance threshold, and 13 exceeded the less conservative threshold. For MINOR, four significantly associated SNPs (according to the less conservative threshold) were detected, and three of them were located on chromosome 14. In addition, for cultural negative, all three significant SNPs (two passed the less conservative threshold and one passed the Bonferroni genome-wide significance) were located on chromosome 14. Four SNPs were identified on chromosomes 4, 5, and 15 for AER. For CNS, three SNPs were detected on chromosome 8.

Table 3.1. Genome-wide significant SNPs for the main effect and potential candidate genes associated with the differential somatic cell fractions.

Trait	SNP	Chromosome	Position	*p*-Value SNP	Gene Name
PMN	BTA-110591-no-rs	6	16024226	0.00001080976	*COL25A1*
sN	BTA-110591-no-rs	6	16024226	0.000005703675	*COL25A1*
bN	BTB-00944057	28	22970021	0.000002966474	*CTNNA3*

PMN, polymorphonuclear neutrophils; sN, segmented neutrophils; bN, banded neutrophils; p value: All SNP passed the less conservative threshold (p_{sugg} = 0.05/4479 = 1.12 x 10^{-5}).

Table 3.2. Genome-wide significant SNPs for the main effect and potential candidate genes associated with the specific mastitis pathogens.

Trait	SNP	Chromosome	Position	*p*-Value SNP	Gene Name
MAJOR	ARS-BFGL-NGS-60721	1	35809354	0.000002509339	-
	ARS-BFGL-NGS-26782	1	152561941	0.0000007190281*	-
	Hapmap23088-BTA-151194	1	152612216	0.000001737926	*HACL1*
	ARS-BFGL-NGS-45691	2	127889562	0.000003947047	-
	ARS-BFGL-NGS-110081	4	41230144	0.000003844456	-
	ARS-BFGL-NGS-29150	5	108921269	0.000004774763	-
	ARS-BFGL-BAC-14274	11	44153677	0.0000008244985*	*EVA1A*
	Hapmap57340-rs29010501	11	44928962	0.000001551728	-
	ARS-BFGL-NGS−116393	11	104186003	0.00000764746	*ABO*
	ARS-BFGL-NGS−24368	15	44660806	0.000006775498	-
	ARS-BFGL-NGS-67343	15	56153143	0.00000157791	-
	ARS-BFGL-NGS-39731	15	56501007	0.000004006894	*CAPN5*
	ARS-BFGL-NGS-113915	17	32550404	0.000008442227	-
	Hapmap47619-BTA-43853	18	4489809	0.000002801936	-
	Hapmap40333-BTA-10479	18	10989533	0.0000002207883*	*CHRISPLD2*
	BTA-86068-no-rs	22	26048787	0.000000002563736*	*CHL1*
	BTA-77184-no-rs	22	26490348	0.000001678371	-
	ARS-BFGL-NGS-39928	26	38508625	0.0000008220506*	-
MINOR	ARS-BFGL-NGS-27512	8	25393606	0.0000079641	*ADAMTSL1*
	UA-IFASA-8766	14	42871327	0.000001380271	-
	Hapmap47921-BTA-34862	14	45867755	0.00001113587	*SAMD12*

	ARS-BFGL-NGS-112964	14	68578807	0.000002708568	-
cultural negative	BTB-01709715	14	44933472	0.000002761847	-
	Hapmap47921-BTA-34862	14	45867755	0.000003396697	*SAMD12*
	ARS-BFGL-BAC-23102	14	68228943	0.0000009770646*	-
AER	ARS-BFGL-NGS-93391	4	8794043	0.000003244757	-
	BTB-00909994	5	3684232	0.00001095756	-
	ARS-BFGL-NGS-40368	15	2048607	0.000009708123	*GRIA4*
	ARS-BFGL-NGS-9407	15	2578791	0.000000220117*	-
CNS	Hapmap51393-BTA-113111	8	23814719	0.000004550739	*MLLT3*
	BTA-103194-no-rs	8	24224606	0.000009541055	-
	ARS-BFGL-NGS-27512	8	25393606	0.0000005630255*	*ADAMTSL1*

MAJOR, *Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia Coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, mold fungus,* and *Proteus sp.*; MINOR, *Coagulase-negative staphylococci* and *Corynebacterium sp.*; AER, *Aerobic bacilli*; CNS, *Coagulase-negative staphylococci*; * Bonferroni-corrected genome-wide significance, otherwise less conservative threshold (p_{sugg} = 0.05/4479 = 1.12 x 10^{-5}). "-" no gene was found inside the 50kb window.

Manhattan plots from the GWAS indicate the most significant SNP (Bonferroni corrected genome-wide significance and less conservative threshold) for PMN as representative of the cell fractions and for MAJOR as representative of the specific mastitis pathogens. Hence, Manhattan plots for main effects and for interaction effects are given in Figure 3.1a,b, respectively for PMN, and in Figure 3.2a,b, respectively for MAJOR. Manhattan plots for sN, bN, MINOR, cultural negative, CNS, AER, and AESC are shown in Figure S3.1.

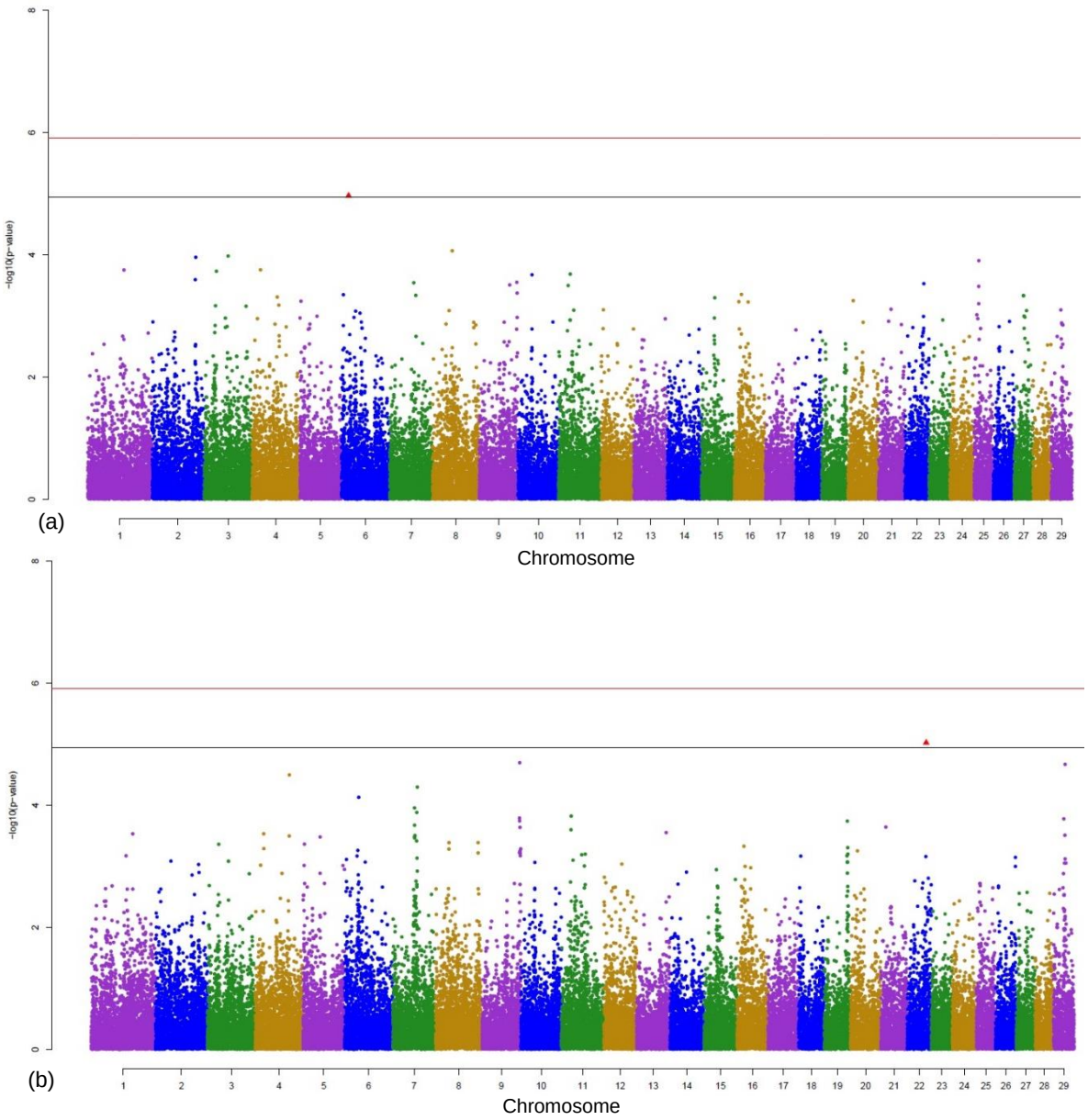

Figure 3.1. Manhattan plots displaying the GWAS results (p-values) for polymorphonuclear neutrophils (PMN): (a) indicates the main SNP effect, and (b) the interaction effect between SNPs and housing system. The Bonferroni-corrected genome-wide significance (red line) and the less conservative threshold (grey line) (p_{sugg} = 0.05/4479 = 1.12 × 10^{-5}) are shown. Red triangles highlight the significant SNP

Regarding SNP interaction effects, significances (Bonferroni corrected genome-wide significance and less conservative threshold) were detected for PMN, MAJOR, MINOR, cultural negative, AER, and AESC. In total, six SNPs were identified as displaying significant interaction effects with the farming system (Table 3.3), whereas five passed the less conservative threshold. The SNPs were located on chromosomes 3 (three SNPs), 15 (one SNP), and 22 (two SNPs). Only the significant SNP for MAJOR on chromosome 22 surpassed the strict Bonferroni significance threshold.

Gene Annotations

Among the 35 significant SNPs for main effects, 14 were located in different genes (Tables 3.1–3.3). With regard to the remaining significantly associated SNPs, no genes in close proximity were detected. A significantly associated SNP was identified for PMN and sN on chromosome 6. The annotated *COL25A1* (collagen type XXV alpha 1 chain) gene is a proteincoding gene which is involved in protein digestion and absorption (KEGG entry: bta04974). Functional annotation of the bN candidate gene reveals the involvement of adherent junction (KEGG entry: bta04520), leucocyte transendothelial migration (KEGG entry: bta04670), and bacterial invasion of epithelial cells (KEGG entry: bta05100) for *CTNNA3* (catenin alpha 3). For some potential candidate genes for MAJOR, functional relationships and their roles in biological or functional pathways are not yet known, e.g., for *CRISPLD2* (cysteine rich secretory protein LCCL domain containing 2), *HACL1*(2-hydroxyacyl-CoA lyase 1), and *CAPN5* (caplain 5). Nevertheless, we inferred four other potential candidate genes for MAJOR. *CHL1* (cell adhesion molecule L1 like) on chromosome 22 is a cell adhesion gene playing a role in cell migration and in the suppression of neuronal cell death (KEGG entry: bta04515). *EVA1A* (eva-1 homolog A) on chromosome 11 also mediates autophagy and apoptosis and acts in the regulation of programmed cell death [30]. Further functional annotations of potential candidate genes for MAJOR reveal the involvement of the metabolic pathway (KEGG entry: bta01100) and glycosphingolipid biosynthesis (KEGG entry: bta00601) for *ABO* (alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase).

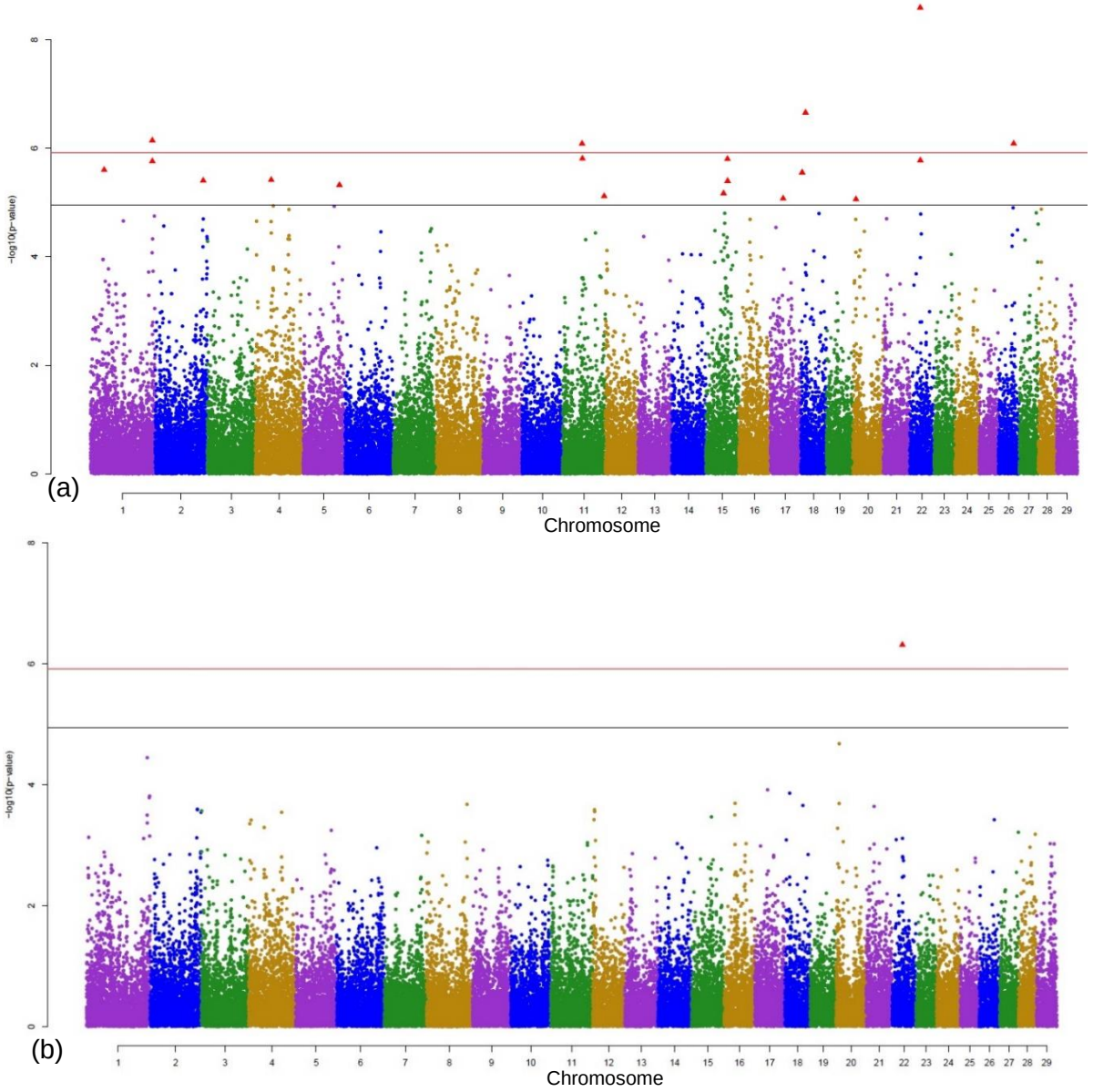

Figure 3.2. Manhattan plots displaying the GWAS results (p-values) for MAJOR pathogens (*Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia Coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, mold fungus*, and *Proteus sp.*): (a) indicates the main SNP effect, and (b) the interaction effect between SNPs and housing system. The Bonferroni-corrected genome-wide significance (red line) and the less conservative threshold (grey line) (p_{sugg} = 0.05/4479 = 1.12 × 10^{-5}) are shown. Red triangles highlight the significant SNP.

Table 3.3. Genome-wide significances for interactions between SNPs and housing systems and annotated potential candidate genes for differential somatic cell fractions and for mastitis pathogens.

Trait	SNP	Chromosome	Position	*p*-Value Interaction	Gene Name
PMN	ARS-BFGL-NGS-16330	22	49768282	0.000009423205	*HEMK1*
MAJOR	BTA-86068-no-rs	22	26048787	0.0000004836461*	*CLH1*
MINOR	ARS-BFGL-NGS-111815	3	118644571	0.000004458816	-
cultural negative	ARS-BFGL-NGS-111815	3	118644571	0.000002214425	-
AER	INRA-611	3	51240435	0.00000619058	*BTB 8*
AESC	BTB-00591978	15	31666125	0.000009842745	*TBCEL*

PMN, polymorphonuclear neutrophils; MAJOR, *Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia Coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, mold fungus*, and *Proteus sp.*; MINOR, *Coagulase-negative staphylococci* and *Corynebacterium sp.*; AER, *Aerobic bacilli*; AESC, *Aesculin hydrolysing streptococci*; * Bonferroni-corrected genome-wide significance, otherwise less conservative threshold (p_{sugg} = 0.05/4479 = 1.12 x 10^{-5})."-" no gene was found inside the 50 kb window.

For MINOR, two SNPs were located in the genes *ADAMTSL* (ADAMTS like 1) and *SAMD12* (sterile alpha motif domain containing 12). The same SNP localized in *SAMD12* was detected for the cultural negative group. Likewise, the same SNP in gene *ADAMTSL* was significantly associated with CNS. With regard to CNS, the candidate gene *MLLT3* (MLLT3 super elongation complex subunit) was also identified for CNS, which is involved in the transcriptional misregulation mechanism of cancer (String). Functional annotation of AESC candidate genes indicates the involvement of the cAMP (adenylyl cyclase) signaling pathway for *GRIA4* (glutamate ionotropic receptor AMPA type subunit 4) (KEGG entry: bta04024).

Based on the significant SNP by housing system interaction effects, four different potential candidate genes carrying one significant SNP were detected (Table 3.3). An associated SNP was identified for PMN on chromosome 22. The annotated *HEMK1* (HemK methyltransferase family member 1) gene is a protein-coding gene which is involved in the methylation of the mitochondrial translation release factor [30]. The SNP located in the *CHL1* gene was significant for the main SNP effect, as well as for the interaction effect between SNPs and farming system. The gene *BTB/POZ 8* (BTB (POZ) domain containing 8) was identified as a possible candidate gene for AER. For AESC, the *TBCEL* (tubulin folding cofactor E like) gene was detected on chromosome 15.

Discussion

Genome-Wide Association Analyses

The new free walk housing system (compost) implies an enriched bacterial concentration in the bedded pack, depending on management [7]. Thus, the cows are exposed to a higher pathogen burden, which triggers their immune system [7,36]. Due to the higher load on the immune system, the first and memory immune responses change over time, accompanied by alterations to genetic mechanisms [36,37]. Housing-specific mastitis resistances and differing cellular immunological mechanisms suggest specific udder health breeding strategies for alternative farming systems [11,14,37]. Genome-wide associations revealed several potential candidate genes, reflecting the complex nature of udder diseases [12,14]. In our study, among the 41 significant SNPs (Bonferroni corrected genome-wide significance and less conservative threshold), we identified 18 (14 significant SNPs for the main effect and four significant SNPs for the interaction effect) in different genes. However, in the context of udder health, the majority of the detected SNPs and annotated potential candidate genes are reported here for the first time. This is not surprising to us, as our study design, combined with novel udder health indicators, differed widely compared with most previous approaches [11,22]. In addition, compost bedding reflects a challenging and new environmental system, and this study was the first time that GWAS with interaction effects were applied.

Most of the previous genomic udder health studies focused on easier trait definitions such as producer-recorded clinical mastitis or overall somatic cell score. In a study based on specific pathogens [22], significant SNPs on 14 different chromosomes (2, 3, 7, 8, 10, 11, 13, 15, 16, 17, 18, 26, 27, and 28) were reported [22]. Tiezzi et al. [12] used producer records to determine udder health status, and focused only on first parity cows. Significant SNPs were identified on chromosomes 2, 8, 11, 14, 16, 19, 20, 24, and 29. Sørensen et al. [14] estimated genetic correlations among specific mastitis pathogens in the range from 0.45 to 0.77, suggesting that each pathogen should be considered as a specific udder health trait. The lowest correlation of 0.45 was found between *Staphylococcus aureus* and *Escherichia coli*, also explained by the different immune responses of these two pathogens. The highest correlation (0.77) was detected between *Streptococcus dysgalactiae* and *Streptococcus uberis* [14]. Both pathogens belong to the same genus and trigger a similar cascade of immune reactions in the udder [14]. Accordingly, in our GWAS, we identified different significant SNPs (Bonferroni corrected genome-wide significance and less conservative threshold) for the different pathogens, as well as for the differential somatic cell fractions, supporting the quantitative genetic associations [14]. Only four SNPs were significantly associated with two different traits, but these traits were closely related. For example, sN represents a subgroup of PMN, and in such a case overlapping significant SNPs were expected. Similarly, CNS is a fraction of MINOR. Hence,

the same SNP located on chromosome 8 was significantly associated with both pathogens. The second significant SNP for MINOR on chromosome 14 was also significant for the cultural negative group. Interestingly, MAJOR displayed a SNP which was significant for both housing systems, as well as for the interaction component.

The different pathogens, which can vary widely between housing systems (compost and cubicle), initiate different immune responses and thus modulate many different genes [38]. These differences are evident especially in the case of *Escherichia coli* and *Staphylococcus aureus* [38]. However, in the last ten years, the diversity of pathogens has shifted from MAJOR to MINOR due to improved stable hygiene management [20,21]. Accordingly, we identified quite high incidences for MINOR, which, in particular, can cause subclinical mastitis. The corresponding immune reactions are less pronounced with weak clinical signs, which complicates the distinction between affected and unaffected animals [39,40]. However, we detected six significant SNPs for the main effect, and one significant SNP by housing system interaction. Consequently, we annotated three potential candidate genes (*ADAMTSL1*, *SAMD12*, and *MLLT3*) for minor pathogens.

Gene Annotations and Functional Pathways

We identified *CTNNA3* as a possible candidate gene. *CTNNA3* is part of the *CTNNA* family with three catenin subtypes (*CTNNA1, CTNNA2*, and *CTNNA3*) displaying close functional relationships [41,42]. The catenin subtypes effect a wide range of diseases, such as Alzheimer's disease or cancer development in humans [42]. CTNNA3 is a key protein of the adherens-junctional complex in epithelial cells and plays a central role in cellular adherence [41]. It also acts as an activator in the MAPK (mitogen-activated protein kinase) pathway [42]. MAPK signaling further regulates the production of target inflammatory genes during clinical mastitis [43].

EVA1A is involved in autophagy and programmed cell death [44,45]. Since the invasion of a pathogen into the udder activates phagocytic cells, which afterwards have to be eliminated by apoptosis [46], it is possible that *EVA1A* is also involved in this process.

HEMK1 was identified as a possible candidate gene. The SNP located in *HEMK1* was significant for the interaction effect, indicating alterations of gene activities with farming system particularities. *HEMK1* acts as a regulator in the JAK-STAT (Janus kinase/signal transducer and activator of transcription) pathway. The JAK-STAT pathway is pivotal for the development and function of the immune system, displaying different mechanisms in different studies [47]. Hence, it is not surprising that *HEMK1* is differently regulated in different housing systems, such as compost and cubicle, due to different environmental stressors. Previous studies demonstrated that immune-related genes such as *IL17*, *IL17F*, and *LIF* indicate GxE [48].

Furthermore, these genes also influence fertility traits, depending on environmental conditions [48].

The *CHL1* gene on chromosome 22, belonging to the L1 family cell adhesionmolecule [49], was also detected on the basis of SNP by housing system interactions. In humans, *CHL1* plays a role in diseases triggered by chronic stress [49]. Yang et al. showed that *CHL1* expression in monocytes was significantly downregulated in depressed patients with chronic stress [50]. In addition, these patients showed a reduced number of positive CD19+ and CD20+ B cells. The downregulation of these two immune cells has a negative effect on the immune system and implies an increased disease susceptibility [50]. Due to improved animal welfare and lower stress levels in compost barns [7], we assume that a possible upregulation of *CHL1* is associated with a positive effect on the immune system and therefore on udder health. A gene expression analysis was conducted in relation to different floor types in Holstein dairy cows [51]. Various types of floor implied different comfort behaviors of cows. Improper lying and walking areas increased stress levels and triggered several cellular processes. In this way, the expression of various genes may be influenced [51,52]. Consequently, a rapid increase in the expression of the gene *HSPA1A* during stress was identified, with ongoing effects on immune system functions [51].

Conclusions

Based on a novel modelling approach allowing GWAS with SNP by farming system interaction effects, we identified significant main and interaction effects for specific pathogens and cell fractions in milk. The detailed udder health phenotyping strategy, combined with the novel GWAS, contributed to the detection of significant SNPs for PMN, sN, bN, MAJOR, MINOR, cultural negative, CNS, AER, and AESC. For the traits PMN and sN, MINOR and CNS, and MINOR and cultural negative, we identified overlapping significant SNP effects, indicating similar genetic and physiological mechanisms. In this regard, we inferred the potential candidate genes *EVA1A* and *CTNNA3* which are involved in the pathways for autophagy and programmed cell death (*EVA1A*), in the MAPK pathway, and in the adherens-junctional complex in epithelial cells for cellular adherence (*CTNNA3*). Interestingly, for the traits PMN, MAJOR, MINOR, cultural negative, AER, and AESC, we identified significant SNP by farming system interactions, indicating opposite effects of the same SNP markers in cubicle and compost. Annotated potential candidate genes were *CHL1* and *HEMK1*, which are involved in pathways regulating the immune system dependent on stress levels and in the development of the immune system. From a practical perspective, our results suggest farming system-specific breeding strategies in order to improve udder health in both systems. In addition, our new modelling approach considering SNP main and interactions effects simultaneously

simplifies the estimations and removes the need to split the data. For future breeding strategies to improve udder health, a very precise definition of mastitis traits is imperative as we identified different genetic mechanisms in response to specific pathogens.

Supplementary Materials:

The following are available online at https://www.mdpi.com/article/10.3390/ani11061839/s1, Figure S3.1: Manhattan plots displaying the GWAS results (p-values) for the main SNP effects for: (a) segmented neutrophils, (c) banded neutrophils, (e) MINOR pathogens (*Coagulase negative staphylococci* and *Corynebacterium sp.*), (g) cultural negative, (i) *Aerobic bacilli*, (k) *Aesculin hydrolyzing streptococci*, and (m) *Coagulase-negative staphylococci*. Manhattan plot displaying the GWAS results (p-values) for the interaction of the SNP and housing system effects for: (b) segmented neutrophils, (d) banded neutrophils, (f) MINOR pathogens (*Coagulase-negative staphylococci* and *Corynebacterium sp.*), (h) cultural negative, (j) *Aerobic bacilli*, (l) *Aesculin hydrolyzing streptococci*, and (n) *Coagulase-negative staphylococci*. Bonferroni-corrected genome-wide significance (red line) and less conservative threshold (grey line) (p_{sugg} = 0.05/4479 = 1.12 x 10^{-5}) are shown. Red triangles highlight the significant SNP.

Author Contributions:

Conceptualization, S.K.; data curation, P.W., K.B., P.E. and C.W.; formal analysis, P.W., T.Y. and K.B.; investigation, P.W., K.B., P.E. and C.W.; methodology, P.W. and T.Y.; laboratory, P.W., K.B., P.E., C.W. and K.S.; project administration, K.B., P.E. and S.K.; supervision, S.K.; validation, P.W. and K.B.; visualization, P.W.; writing—original draft, P.W.; writing—review and editing, T.Y., K.B. and S.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement:

Ethical review and approval were waived for this study, due to the data for this study considered milk samples from routine milking. No additional experiments were conducted. Thus, in concordance with German animal welfare legislation, no ethical approval was required for this study.

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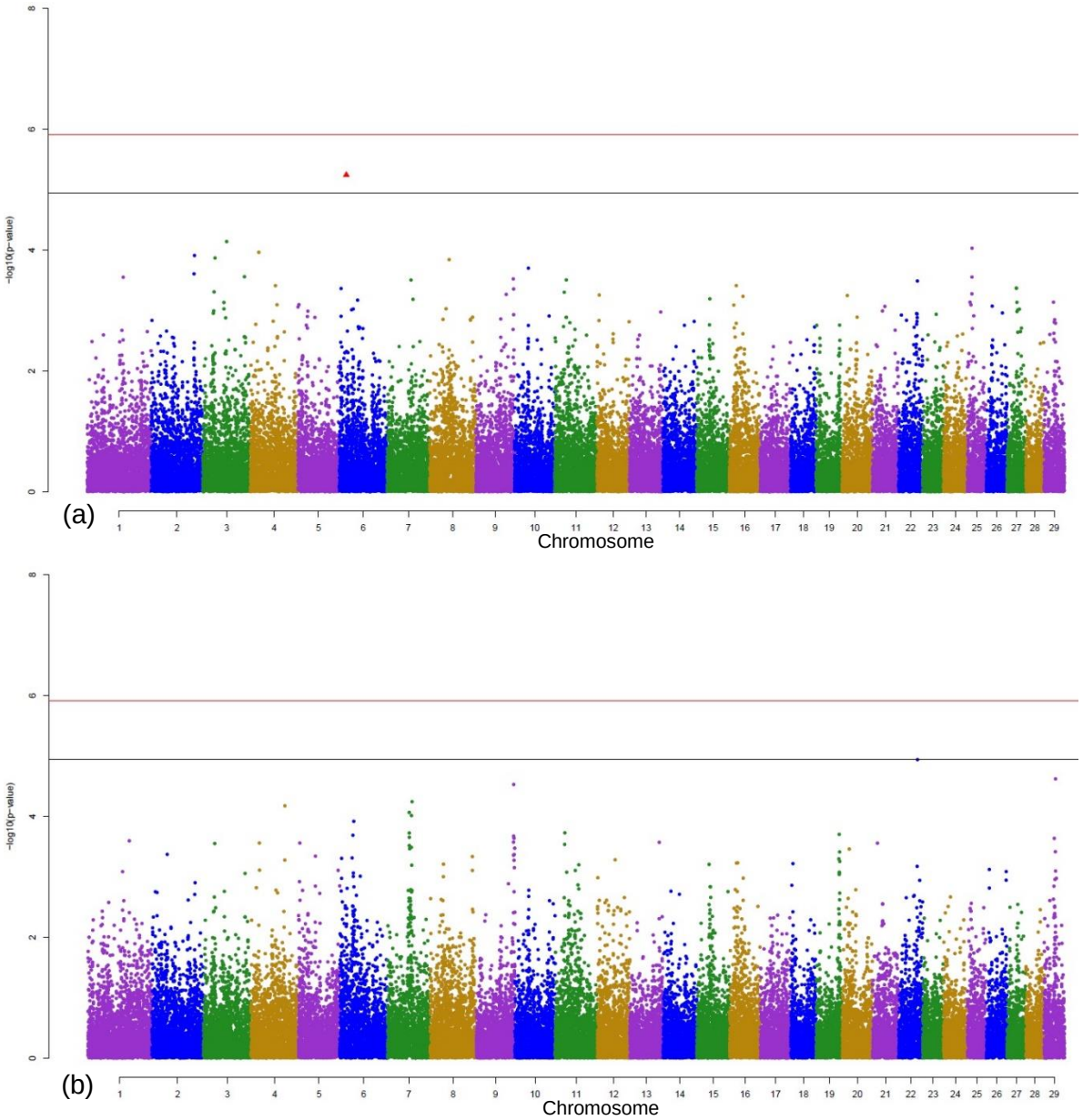
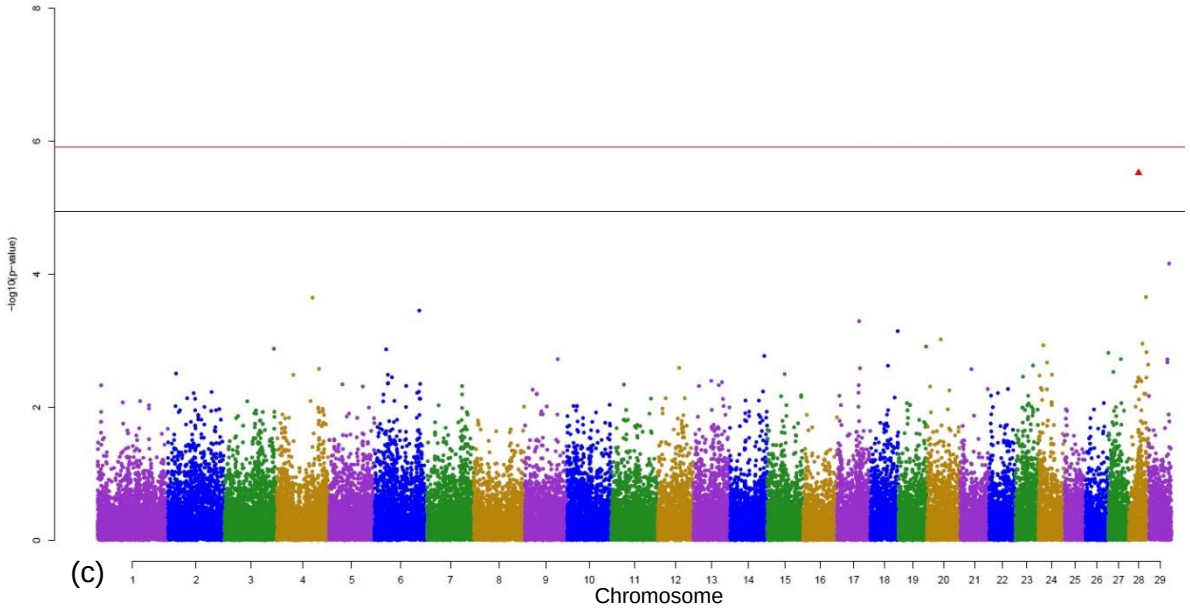
Supplementary File

Supplementary Figure 3.1:




−log10(p−value)
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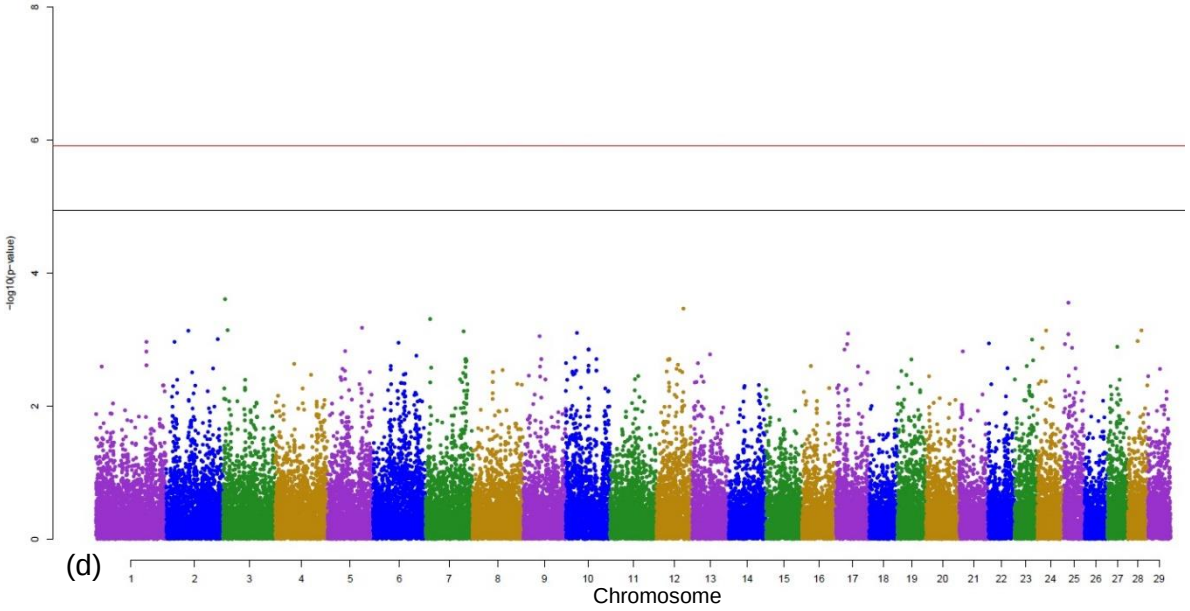
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Chromosome

(c)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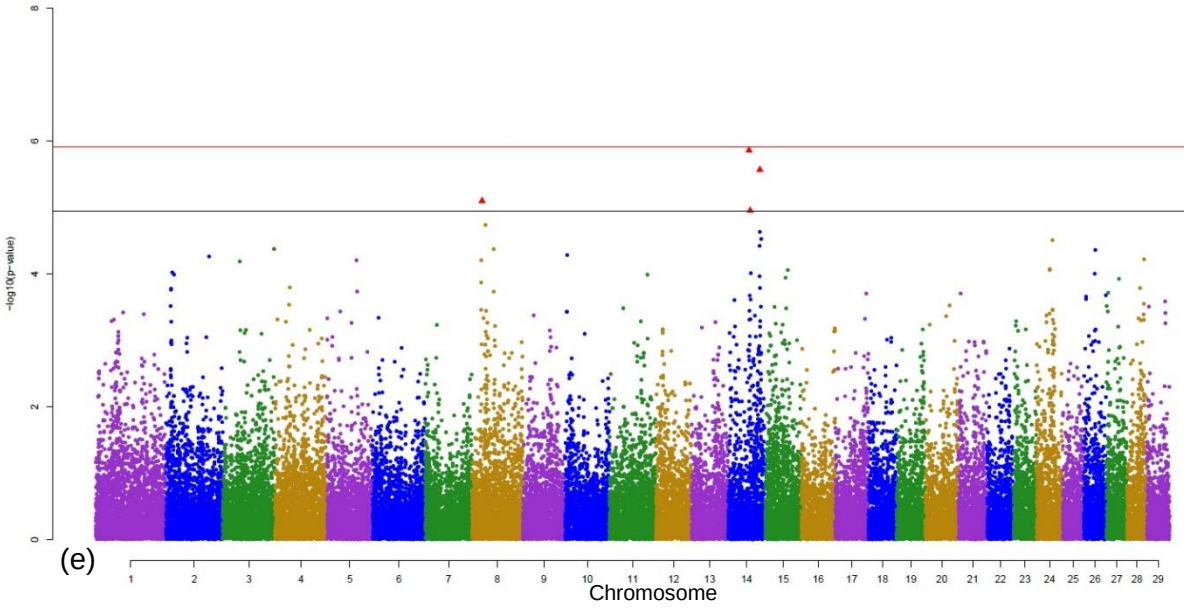
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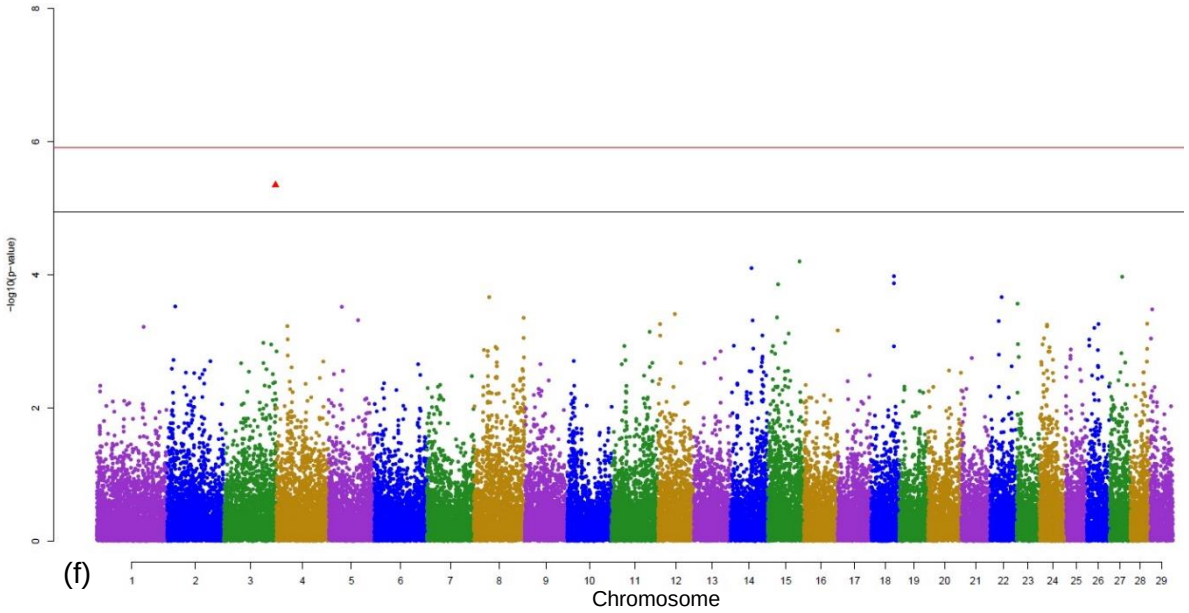
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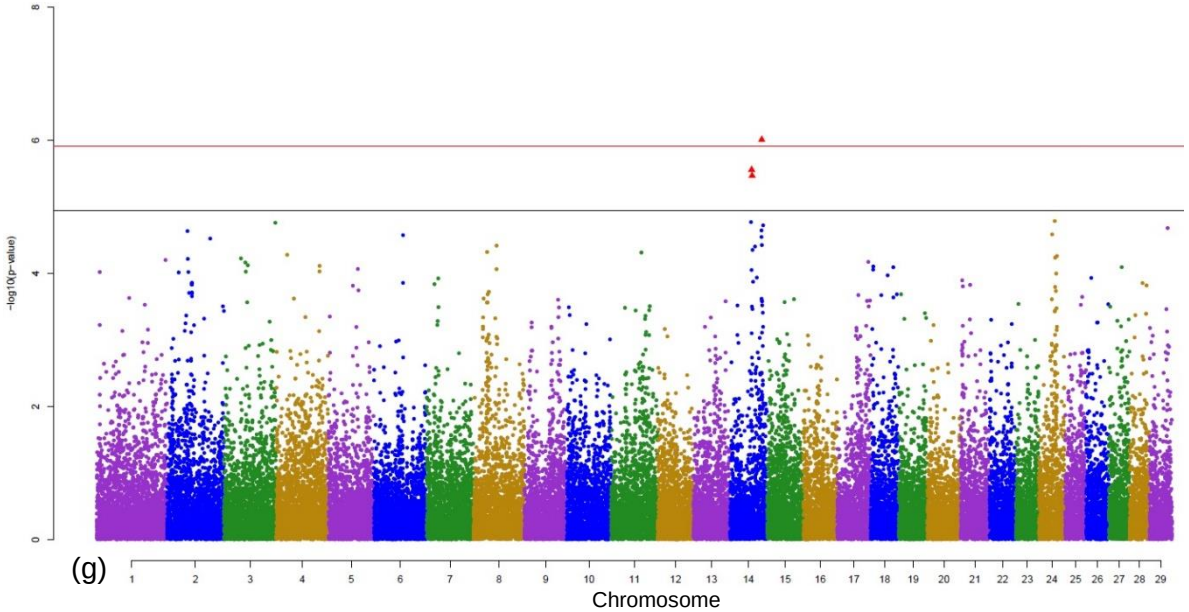
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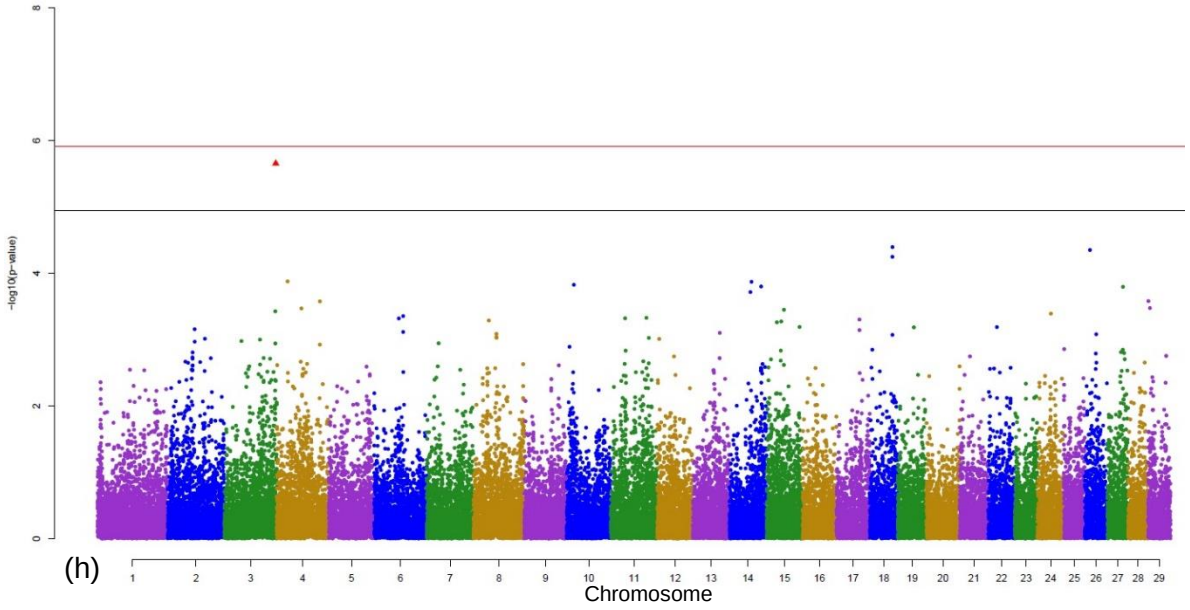
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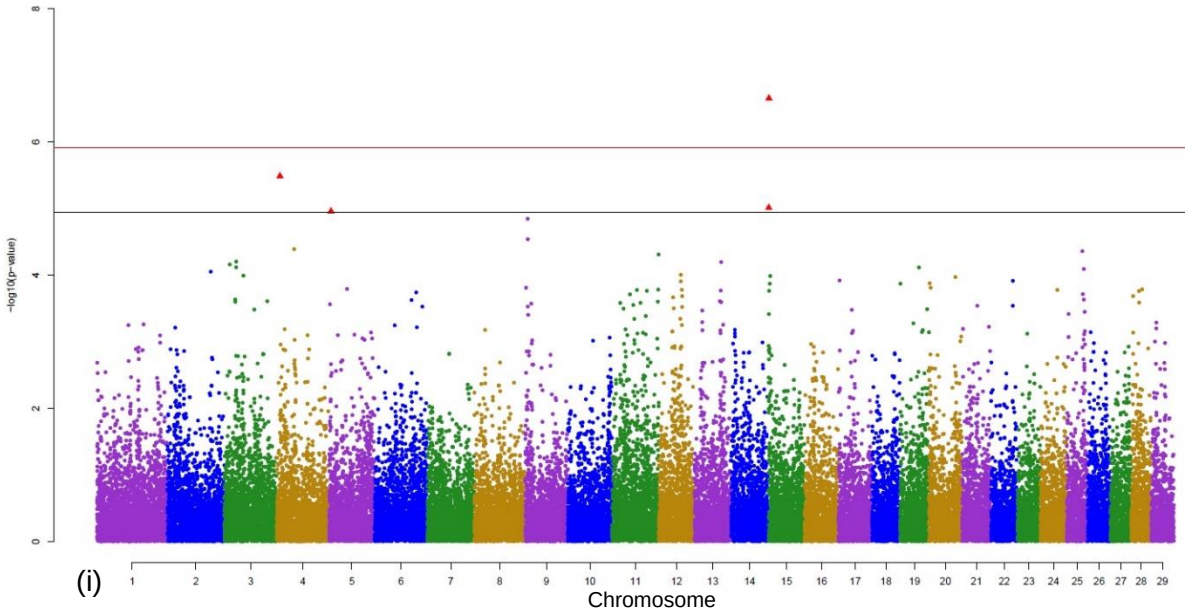
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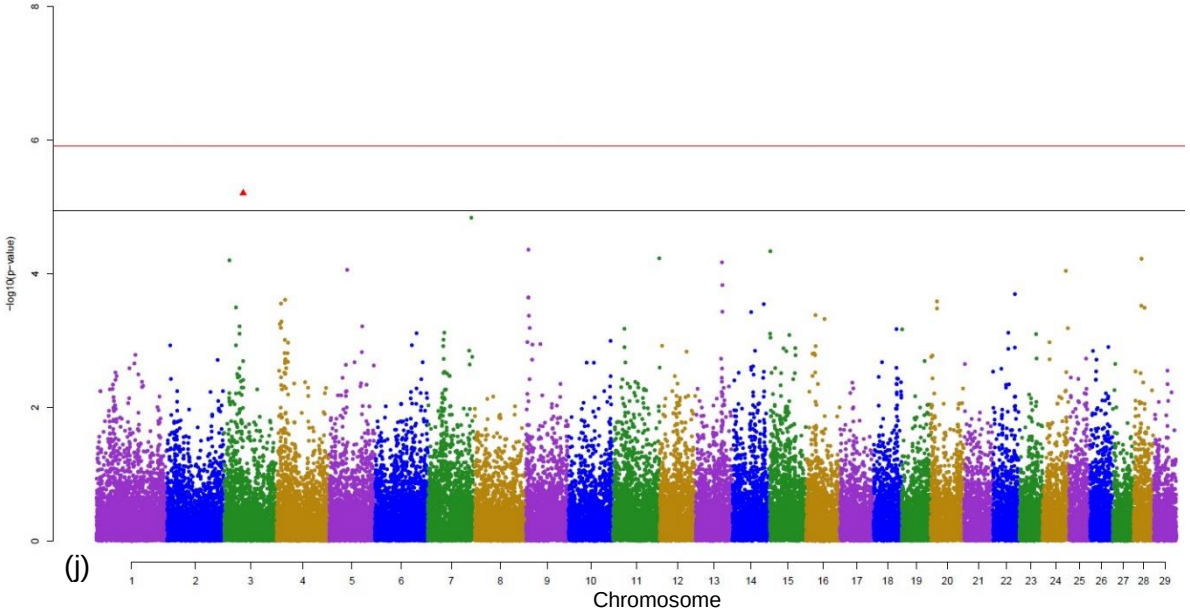
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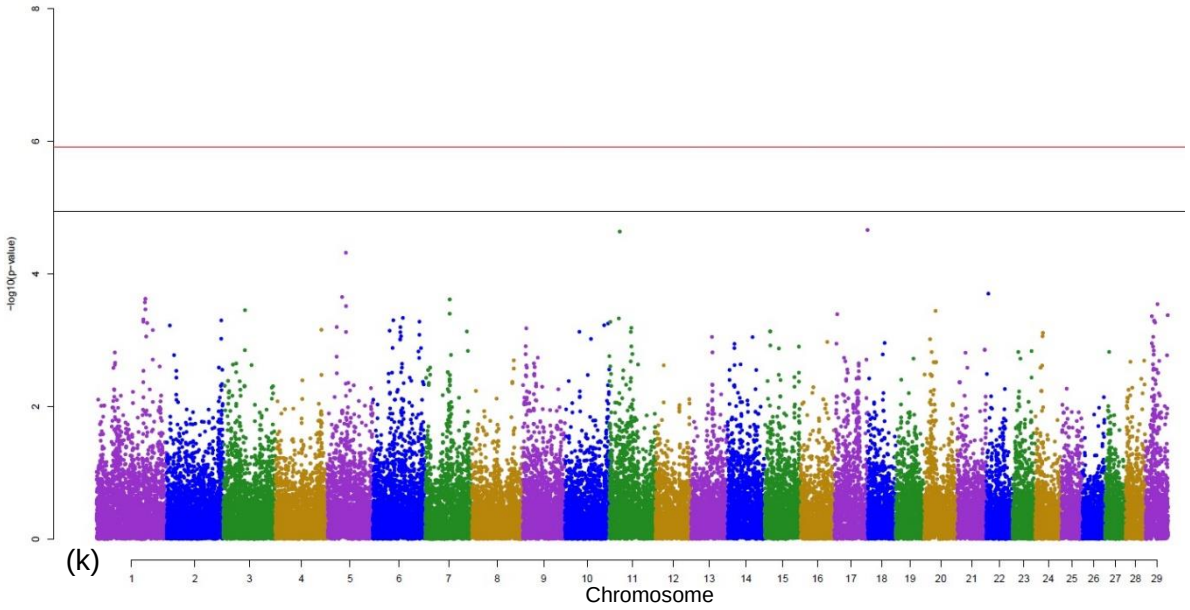
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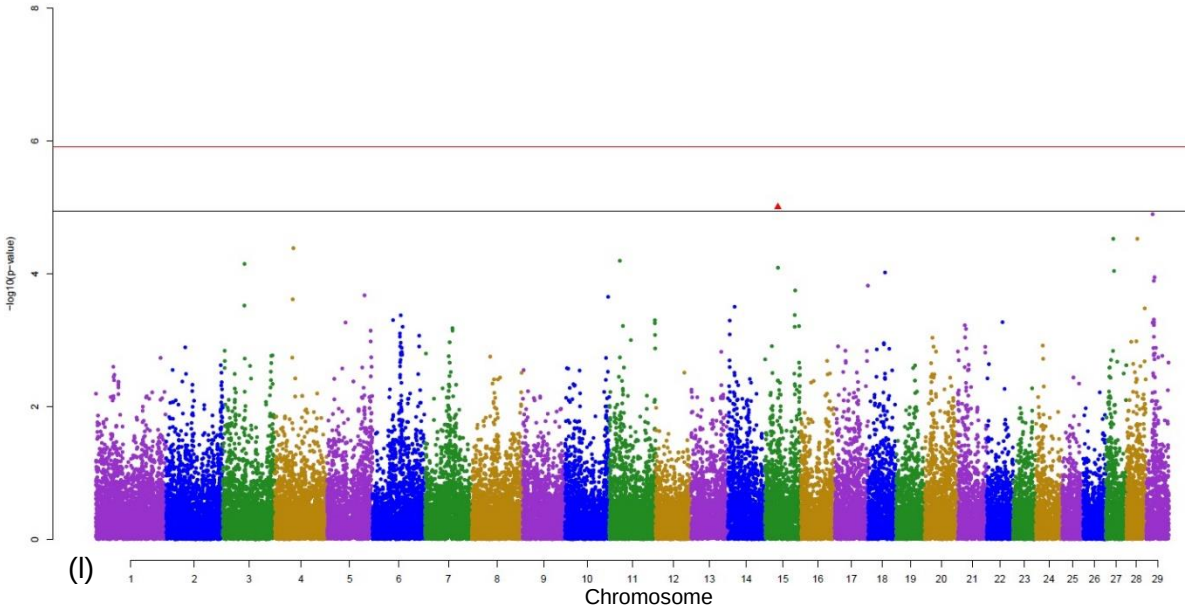
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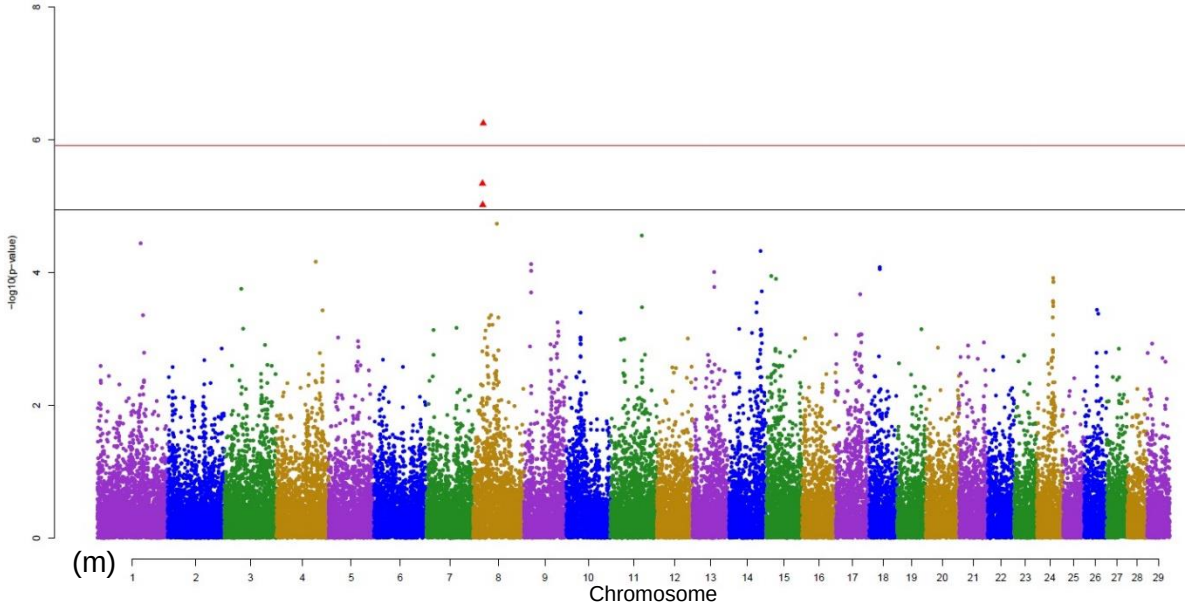
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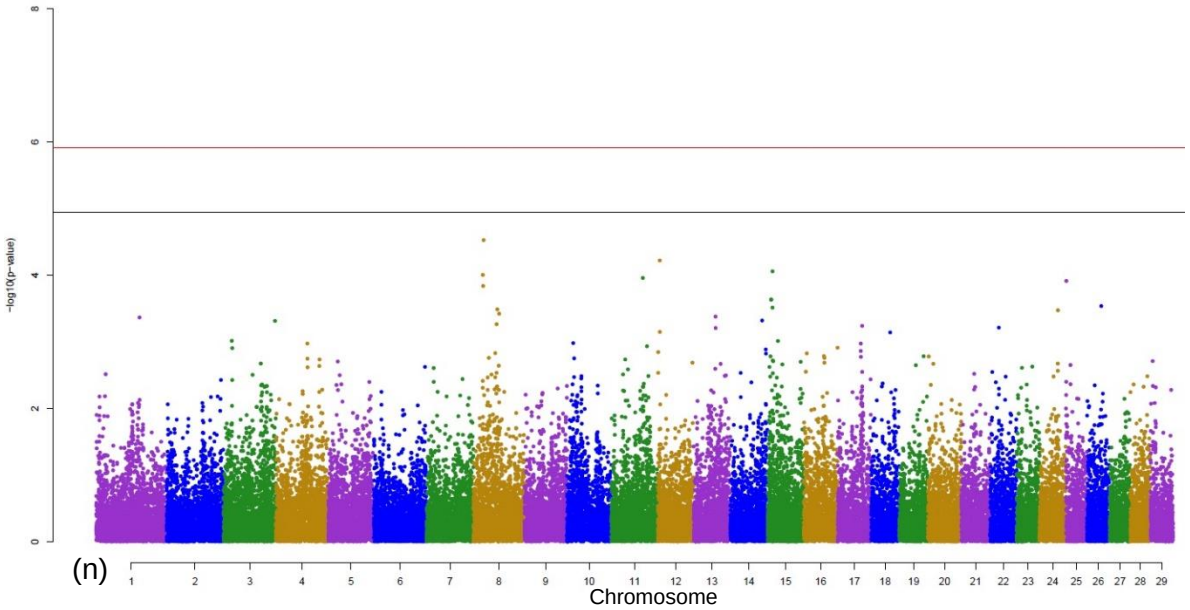

Supplementary Figure S3.1. Manhattan plot displaying the GWAS results (p-values) of the main SNP effects for a) segmented neutrophils, c) banded neutrophils, e) MINOR pathogens (*Coagulase-negative staphylococci* and *Corynebacterium sp*.), g) cultural negative, i) *Aerobic bacilli, k) Aesculin hydrolyzing streptococci* and m) *Coagulase-negative staphylococci.* Manhattan plot displaying the GWAS results (p-values) of interaction of the SNP effects and the effects of cows in the housing systems for b) segmented neutrophils, d) banded neutrophils, f) MINOR pathogens (*Coagulase-negative staphylococci* and *Corynebacterium sp*.), h) cultural negative, j) *Aerobic bacilli, l) Aesculin hydrolyzing streptococci* and n) *Coagulase-negative staphylococci*. Bonferroni-corrected genome-wide significance (red line) and less conservative threshold (grey line) (P_{sugg} = 0.05/4479 = 1.12 × 10^{-5}) are shown. Red triangles highlight the significant SNP.

CHAPTER 4

Inferring causalities of environmental and genetic factors for differential somatic cell count and mastitis pathogens in dairy cows using structural equation modelling

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Abstract

The aim of this study was to establish and evaluate a structural equation model to infer causal relationships among environmental and genetic factors on udder health. For this purpose, 537 Holstein Friesian cows were genotyped, and milk samples were analyzed for novel traits including differential somatic cell counts and specific mastitis pathogens. In the structural model, four latent variables (intramammary infection (IMI), production, time and genetics) were defined, which were explained using manifest measurable variables. The measurable variables included udder pathogens and somatic differential cell counts, milk composition, as well as significant SNP markers from previous genome-wide associations for major and minor pathogens. The housing system effect (i.e., compost-bedded pack barns versus cubicle barns) indicated a small influence on IMI with a path coefficient of -0.05. However, housing system significantly affected production (0.37), with ongoing causal effects on IMI (0.17). Thus, indirect associations between housing and udder health could be inferred via structural equation modeling. Furthermore, genotype by environment interactions on IMI can be represented, i.e., the detection of specific latent variables such as significant SNP markers only for specific housing systems. For the latent variable genetics, especially one SNP is of primary interest. This SNP is located in the *EVA1A* gene, which plays a fundamental role in the MAPK1 signaling pathway. Other identified genes (e.g., *CTNNA3* and *CHL1*) support results from previous studies, and this gene also contributes to mechanisms of the MAPK1 signaling pathway.

Introduction

Diseases of the udder are considered as one of the most important clinical infections in dairy cows with strong detrimental effects on farm economy [1]. However, in terms of underlying genetic and physiological mechanisms and with regard to pathogenesis, udder infections are very complex and depend on a variety of factors, including milk yield, lactation stage, genetics, type of pathogens, and also on farm-specific characteristics [2,3]. Farm characteristics address alternative animal friendly housing systems appreciated by the society, such as compost-bedded pack barns. From an animal perspective, compost-bedded pack barns improve animal welfare, animal health and longevity [4]. On the other hand, due to the mixture of the substrate of bedding materials and manure, there may be an increased risk of bacterial infections in the udder [5].

As pointed out in some publications [6], the cow milk quality requirements as defined by the European Union are based on herd averages for somatic cell count (SCC) with a maximum of 400,000 cells/mL and a bacterial standard plate count. However, the type of pathogens can greatly vary. The type of pathogens not only determine whether an intramammary infection induces an acute or a subclinical mastitis but also has specific effects on the overall immune

system. Specific defense mechanisms might be activated, thus affecting the somatic cell count in milk, which is the standard parameter for indicating udder health status [7]. However, the composition of the somatic cells might also vary depending on the type of infection, suggesting differential somatic cell count analyses. Furthermore, genetic cascades are triggered by the immune system, which can vary individually. Intramammary infection impairs milk yield and milk composition. Additionally, in recursive biological systems, the level of milk yield and composition might influence the susceptibility of an udder infection [8,9]. In addition, from an "environmental" perspective, the age of the animal, the status of nutrition and many other factors such as climate play a role in determining an animal's susceptibility for an infection [2,3].

In order to fully understand and resolve such complex and various influencing factors and their interrelationships, alternative modeling approaches are needed. Regression analyses are used as a common tool to explore one-way relationships by neglecting possible recursive or mutual associations [10]. Structural equation models (SEM), on the other hand, allow for flexible and comprehensive approaches to examine the relationships between variables in a hypothetical model [11]. Extended SEMs depict associations among measurable parameters (manifest variables), and additionally enable the estimation of parameters that cannot be measured or recorded directly among themselves, the so-called underlying latent variables [12]. De los Campos et al. [13] and Wu et al. [14] have already used SEM to infer relationships between udder health (via somatic cell count) and milk yield both phenotypically and genetically, but in their modeling approach, they ignored mutual associations among environmental effects. Another advantage of an SEM is that both direct and indirect effects can be modeled simultaneously, also in a recursive framework [15]. Detilleux et al. used SEM to obtain a basic understanding of the many different factors involved in clinical mastitis in the risk for infections and tolerance mechanisms [3,16]. The relationships among the latent variables, among the manifest variables as well as among the latent and manifest, are denoted as loading coefficients or path coefficients.

The objective of the present study was to apply SEM to infer causal relationships on IMI based on a detailed recording for differential somatic cell counts and specific mastitis pathogens as well as specifically selected SNP markers. In this regard, a two-step strategy was applied; first, a genome-wide association study (GWAS) was used to detect significant SNPs, and afterwards, in step 2, these SNPs were analyzed using enhanced SEM approaches.

Materials and Methods

Animal Ethics Statement

Data considered in the present study are based on milk samples from routine milk recording and genotypes used for the official national genetic evaluations. No extra animal experiments were conducted. Thus, following the guidelines of the German animal welfare legislation, a specific ethical approval was not required.

Farms, Animals and Sampling

Cow milk samples were collected from the individual udders of six Holstein dairy cattle herds located in the German federal states of Hesse and North Rhine-Westphalia for all ongoing studies. The herds for this project were selected based on the criteria for selecting case (i.e., herds with the compost farming system) and control herds (i.e., conventional cubicle barns) as defined in the collaborative EU FreeWalk project [17]. The two housing systems compost-bedded pack barns and cubicle barns were identical with regard to herd size, production level, location, climatic conditions, milking system, feeding and breeding aspects in order to ensure an objective comparison. The only, and a large, difference was the housing system; i.e., compost-bedded pack barns in contrast to conventional cubicle barns. Overall, three farms represented the compost system and three other farms had the cubicle system, while two farms had both the systems. Allocation of cows to the different sub-herds in the farms with both systems is described in our previous study by Wagner et al. [18]. The current study considered 587 first and second parity Holstein Friesian dairy cows. A large fraction of all cows, 79%, was in first lactation. A total of 44% of all cows were kept in compost farms, and 56% of all cows were in the conventional cubicle farms, indicating a very similar data distribution across both the housing systems.

For ongoing laboratory milk analyses, we considered milk samples from 2198 udder quarters of these 587 cows. The milk analyses for specific pathogens in the Landesbetrieb Hessen followed the DVG guidelines [19]. The specific mastitis pathogens were classified into major pathogens (MAJOR) or minor pathogens (MINOR). The classification was conducted according to the severity of an infection and immune responses, and does not indicate any prevalence. The same classification of mastitis pathogens according to the infection status was considered in several previous udder health studies, especially when inferring genetic and physiological mechanisms [20–23]. Accordingly, the category MAJOR included the pathogens *Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, mold fungus* and *Proteus sp*. The category MINOR included *Coagulase-negative staphylococci* and *Corynebacterium sp*. One udder quarter of at least one pathogen

within the defined groups, MAJOR and MINOR, resulted in score = 1 for the respective total group; otherwise, score = 0 was assigned. At cow level, the score = 1 was assigned for the total group (MAJOR, MINOR) if the respective pathogen was detected in at least one udder quarter; otherwise, score = 0 was applied.

Differential cells were counted under the microscope in our own milk analysis laboratory at Justus-Liebig-University, Giessen. Differential cell counting included the cell fractions for lymphocytes, macrophages and polymorphonuclear leucocytes (PMN) in a 50 mL milk sample per udder quarter following official guidelines and protocols [24,25]. Milk samples with a small number of counted cells (<30 counted cells per sample) were excluded from the ongoing modeling approaches. The sum of all determined lymphocytes, macrophages and PMN was defined as 100%, and the respective percentages of the specific cell types were used as variables in the SEM. The descriptive statistics of the udder health traits are shown in Table 4.1.

Table 4.1. Descriptive statistics for the microscopic differential somatic cell counts and mastitis pathogens per udder quarter.

Traits[1]	Mean	Min	Max	SD
Cell fractions (in relation to the				
Total sum of all cell counts)				
Macrophages	0.292	0.000	0.980	0.208
Lymphocytes	0.608	0.000	1.000	0.246
PMN	0.100	0.000	0.971	0.143
Mastitis pathogens (in prevalences)				
Negative samples	0.514	0.000	1.000	0.500
Minor pathogens	0.407	0.000	1.000	0.491
Major pathogens	0.030	0.000	1.000	0.171

[1]Polymorphonuclear neutrophils (PMN), minor pathogens (including *Coagulase-negative staphylococci* and *Corynebacterium sp.*), major pathogens (including *Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, mold fungus* and *Proteus sp.*).

Genome-Wide Associations

Only the significant SNPs from GWAS were included in the ongoing SEM. Genomewide associations were performed for SNP main effects and SNP x housing system interaction effects. A significant interaction means that the SNP significantly affects udder health in compost-bedded pack barns, but not in cubicle barns. In this regard, we followed SNP data preparation and the statistical methods as outlined in our previous study by Wagner et al. [6]. For this, 277 Holstein Friesian (HF) cows were genotyped with the Illumina BovineSNP50 Bead

Chip V2. An additional dataset including 273 first parity HF cows was genotyped with the Illumina BovineSNP50 Bead Chip V3. After quality control of the SNP data via the software package PLINK, version 1.9 [26], 43,095 SNPs from 550 genotyped cows were available for the ongoing genomic studies. Criteria for SNP quality control implied consideration of *Bos Taurus* autosomes, the exclusion of SNPs with a minor allele frequency lower than 0.01, the exclusion of SNPs with a call rate lower than 0.90 and the exclusion of SNPs significantly deviating ($p < 0.001$) from the Hardy–Weinberg equilibrium. ARS1.2 assembly [27] was used for remapping the positions of the SNP markers.

The algorithm for the estimation of SNP effects and significances is incorporated into our own R package, named GWAInter.R, which can be downloaded at https://jlupub.ub.uni-giessen.de/ (accessed on 20 April 2023). The respective statistical model 1 in matrix notation for the GWAS was defined as follows:

$$y = Xb + x_{snpi}\, b_{snpi} + x_{interi}\, b_{interi} + Zg + e \qquad (1)$$

where y = a vector of observations for the pathogens MAJOR or MINOR (cow level) in consecutive runs; b = a vector of fixed effects including the herd test day, the housing system cubicle barn or compost-bedded pack barn, parity, and the person from the milk laboratory analyzing the milk samples; X = incidence matrix for fixed effects; x_{snpi} = a vector of SNP genotypes; b_{snpi} = a regression coefficient for the main effect of the i*th* SNP marker; x_{interi} = a vector of genotypes for cows kept in compost-bedded pack barns; b_{interi} = a regression coefficient for the SNP x housing system interaction effect of the i*th*-SNP marker; g = a vector of random additive-genetic effects following $N(0, G\sigma^2_g)$; G = the genomic relationship matrix which was constructed as defined by Yang et al. [28] by excluding the respective candidate SNP; σ^2_g = additive-genetic variance; Z = incidence matrix for the random additive-genetic effects; e = vector for the random residual effects following $N(0, I\sigma^2_e)$, I = identity matrix; σ^2_e = residual variance.

The estimation for the required additive-genetic and residual variances for MAJOR and MINOR was performed using the model y = Xb + Zg + e, with the effects as stated above for model 1 and applying the software package “gaston” [29]. Our software package GWAInter.R version 1.0 utilizes generalized least squares approach as outlined by Halli et al. [30] for the estimation of the SNP main and interaction effects. In this regard, we used a Wald-test statistic [31]. The respective chi^2 value for the main and interaction effects is the ratio of the respective squared regression coefficient divided by the variance of the regression coefficients at 1 degree of freedom. Significance thresholds were defined based on the strict Bonferroni correction with P_{Bonf} = 0.05/number of SNPs and the more relaxed suggestive threshold with P_{sugg} = 0.05/number of independent SNPs. The number of SNPs was 43,095, and the number of

independent SNPs was 4479. The number of independent SNPs was calculated considering linkage disequilibrium ≤ 0.15 in chromosomal segments with 500 markers.

The last step was the annotation of potential candidate gens. The definition of a candidate gene implied at least one significantly associated SNP, which is directly located in the gene or located in a surrounding segment 200kb up- and downstream. For gene annotations, we used the databases ENSEMBL and NCBI [32,33]. For the interpretation of gene functions and related physiological pathways with focus on the modelings in the ongoing SEM, we referred to the Kyoto Encyclopedia of Genes and Genomes and the NCBI database [33,34].

A total of 41 SNPs were significant for both SNP main and SNP interaction effects. Based on SEM evaluations for goodness of fit criteria (see Section: Results – Overall Structural Equation Model Evaluation), 13 SNPs from both categories, MAJOR and MINOR, were integrated into the final SEM. These significant SNPs are listed in Table 4.2 along with respective candidate gene information and location.

Table 4.2. Genome-wide significances for SNP main effects (superscript M) and interaction effects (superscript I) with housing systems and annotated potential candidate genes for mastitis pathogens which were integrated into the SEM considering estimates and findings by Wagner et al. [6].

Trait	SNP	CHR	Position	P-value SNP	SNP located in a gene	Gene name
MAJOR	BTA-86068-no-rs (y = 11)^{M,}	22	26048787	0.000000002563736[a]	yes	*CHL1*
	BTA-86068-no-rs (y = 11)^{I}	22	26048787	0.0000004836461[a,]	yes	*CHL1*
	ARS-BFGL-NGS-39928 (y = 22)^{M}	26	38508625	0.000002509339[a]	-	-
	ARS-BFGL-BAC-14274 (y = 17)^{M}	11	44153677	0.000001737926[a]	yes	*EVA1A*
	Hapmap57340-rs29010501 (y = 18)^{M}	11	44928962	0.000003947047[b]	-	-
	Hapmap23088-BTA-151194 (y = 13)^{M}	1	152612216	0.000003844456[b]	yes	*HACL1*
	ARS-BFGL-NGS-60721 (y = 12)^{M}	1	35809354	0.000004774763[b]	-	-
	Hapmap47619-BTA-43853 (y = 21)^{M}	18	4489809	0.0000008244985[b]	-	-
	ARS-BFGL-NGS-110081 (y = 15)^{M}	4	41230144	0.000001551728[b]	-	-
	ARS-BFGL-NGS-45691 (y = 14)^{M}	2	127889562	0.00000764746[b]	-	-
	ARS-BFGL-NGS-29150 (y = 16)^{M}	5	108921269	0.000008442227[b]	-	-
	ARS-BFGL-NGS-116393 (y = 19)^{M}	11	104186003	0.000002801936[b]	yes	*ABO*
	ARS-BFGL-NGS-113915 (y = 20)^{M}	17	32550404	0.0000008220506[b]	-	-
MINOR	ARS-BFGL-NGS-112964 (y = 23)^{M}	14	68578807	0.000002708568[b]	-	-

MAJOR *Aerococcus sp., Aesculin hydrolyzing streptococci, Candida krusei, Enterococcus sp., Escherichia Coli, Lactococcus sp., Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus*

uberis, mold fungus and *Proteus sp.*, MINOR *Coagulase-negative staphylococci* and *Corynebacterium sp.*, a Bonferroni-corrected genome-wide significance and b less conservative threshold, y represent the vectors of the indicator variables of the structural equation model, described in Table 4.3.

Structural Equation Model

The package lavaan (version 0.6–9) in R (version 3.6.2) [35] was used for the development and application of the SEM [36]. The SEM is composed of a structural model, several reflective measurement models and a formative measurement model. The general SEM (Model (2)) was

$$\eta = B \cdot \eta + \Gamma \cdot \xi + \zeta \text{ (structural equation model) (2)}$$

The formative measurement Models (3) and (4) were

$$x = \Lambda x \cdot \xi + \delta \text{ (Measurement model for latent exogenous variables } \xi) \text{ (3)}$$

$$y = \Lambda y \cdot \eta + \varepsilon \text{ (Measurement model for latent endogenous variables } \eta) \text{ (4)}$$

The reflective measurement Models (5) and (6) were

$$\xi = x \cdot \Pi\xi + \delta\xi \text{ (Measurement model for latent exogenous variables } \xi) \text{ (5)}$$

$$\eta = y \cdot \Pi\eta + \delta\eta \text{ (Measurement model for latent endogenous variables } \eta) \text{ (6)}$$

where η = a vector representing endogenous latent variables (η_1 = production, η_2 = intramammary infection und η_3 = genetics); ξ = a vector of exogenous latent variables (ξ_1 = time); ζ = indicating the residual variable, since endogenous latent variables are not completely explained by the exogenous latent variables, and the complete impact for factors that were not considered in the model. The coefficient matrices B and Γ show the interdependence relationships B between latent endogenous variables and Γ between latent endogenous and exogenous variables); x and y = vectors representing the indicator variables, as described in Table 4.3; Λx and Λy = vectors of the path or loading coefficients (Λn); Π_ξ and Π_η = vectors of multiple regression or weighting coefficients between an (endogenous or exogenous) latent variable and the assigned indicator variables; δ or ε = vectors of the exogenous or endogenous residuals.

Table 4.3. Overview of the latent variables and their associated measurement variables and (if possible) their minimum and maximum values, mean values and standard deviation or their groups. Each of the latent variables are estimated by three or more measurement variables; η = the vector of endogenous latent variables; ξ = the vector of exogenous latent variables; ζ = the residual variable; x and y = the vectors of the indicator variables.

Latent variables	Indicator variables (manifest variables)	Range (min-max) or groups	Mean	SD
η1=Poduction (PROD)	y1 = fat content [in %]	2.43–7.6	4.88	0.73
	y2 = protein content [in %]	2.7–4.93	3.56	0.37
	y3 = lactose content [in %]	3.4–5.28	4.88	0.19
	y4 = milk yield [in kg]	<25, 25.1-30, 30. 1–35, 35.1–40, >40	-	-
η2 = Intramammary infection (IMI)	y5 = average somatic cell count of the herd	5.05–5.58	5.30	0.14
	y6 = lymphocyte content [in %]	0–100	61.00	0.25
	y7 = PMN content [in %]	0–97	9.80	0.14
	y8 = somatic cell count of test day (the exact test day of our sampling)	2.64–11.16	2.35	2.09
	y9 = MAJOR	0, 1	-	-
	y10 = MINOR	0, 1	-	-
η3 = Genetic (GEN)	y11—23 = significant SNP from GWAS			
ξ1 = Time (TIME)	x1 = barn age [in years]	1, 2, 3	-	-
	x2 = average first calving age [in days]	750–760, 760.1–775, 775.1–780, 780.1–800, >800	-	-
	x3 = average calving interval [in days]	382–432	407	0.17
	x4 = lactation stage [in days]	0–100, 100.1–200, 200.1–300, >300	-	-
formative model	y24 = housing system	compost, cubicle	-	-

Results

Overall Structural Equation Model Evaluation

The overall goodness fit of the model was assessed by applying a 2 goodness of fit test and alternative fit indices. Such applications induce the standardized root-mean-square residual (0.101) (SRMR), the root-mean-square error of approximation (0.135) (RMSEA), the Tucker – Lewis index (0.244) (TLI) and the comparative fit index (0.312) (CFI). Based on these evaluation criteria, failed convergence status and unrealistic parameter estimates when including parity as indicator variable for the latent variable time, we decided to exclude parity as a cow-specific parameter from the SEM. This might be due to the extremely high proportion of first parity cows in our dataset and the strong auto-correlations between parity with other parameters including age at first calving and production traits.

Completely standardized estimates of the parameters for the final SEM are shown in Figure 4.1. Four relationships were inferred among the latent variables in the SEM. In total, there are 28 measures (indicator or manifest variables) associated with latent variables enabling

estimations of the respective latent variables. Information on the impact of latent constructs were obtained by assessing the path coefficients (λn). The possible range is from -1 to +1. A value of ≥ 0.2 or ≤ -0.2 is generally considered as a relevant correlation [37].

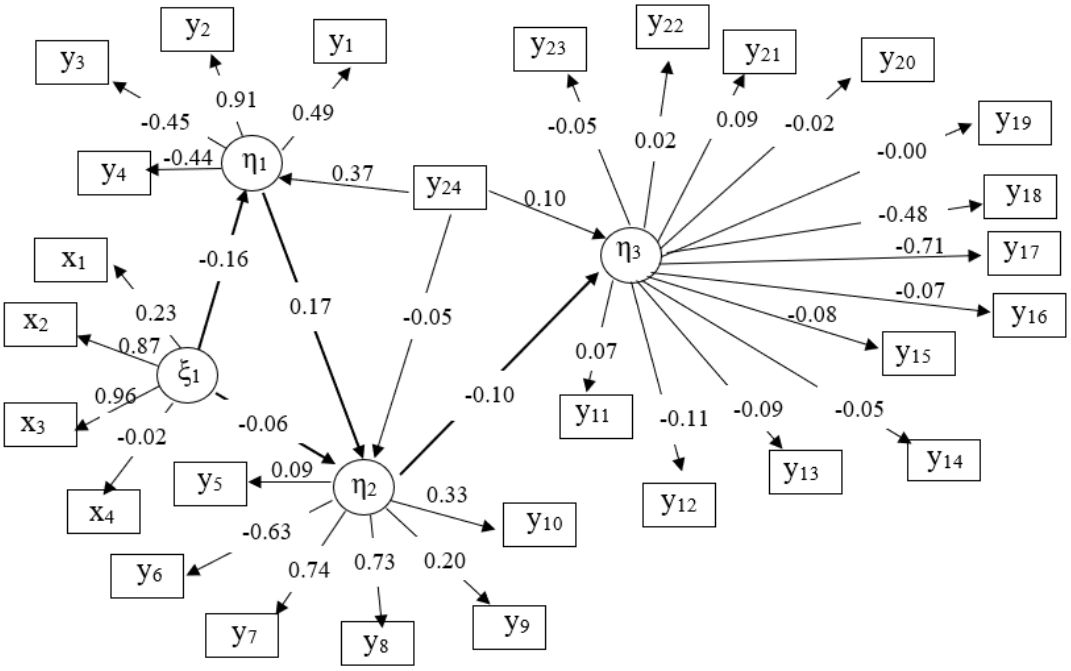

Figure 4.1. Path coefficients for causal relationships from the structural equation model with four latent variables (η_1 = production, η_2 = intramammary infection, η_3 = genetics, ξ_1 = time); x_1 = barn age, x_2 = average first calving age, x_3 = average calving interval, x_4 = lactation stage, y_1 = fat content, y_2 = protein content, y_3 = lactose content, y_4 = milk yield, y_5 = average somatic cell count of the herd, y_6 = lymphocytes, y_7 = PMN, y_8 = somatic cell count of test day, y_9 = MAJOR, y_{10} = MINOR, $y_{11\text{-}23}$ = significant SNPs from the previous GWAS as indicated in Table 2 , y_{24} = housing system.

Latent Variable Intramammary Infection

According to the measurement models, the overall average somatic cell count of the herd is a weak indicator (0.09) to explain an IMI (Figures 4.1 and 4.2). In contrast, the influence of the individual somatic cow cell count from the test day explains a very accurate IMI (0.73). The path coefficients for lymphocytes and PMN (0.74) are also quite large (i.e., values close to -1 or close to 1), with a negative value for lymphocytes (-0.63). The path coefficients for the major and minor pathogens with 0.20 and 0.33, respectively, are slightly smaller than the estimates for the specific cell fractions.

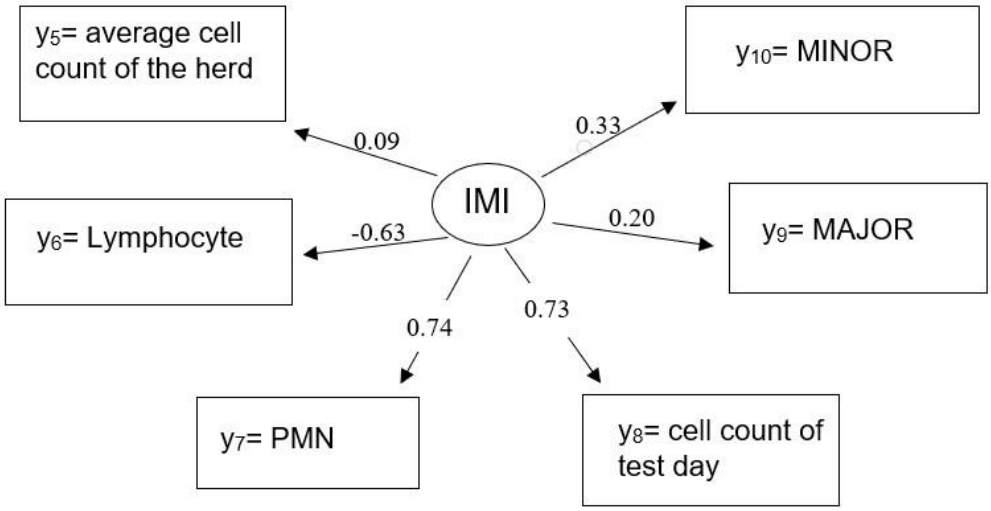

Figure 4.2. Path coefficients for causal relationships from the measurement model for the latent endogenous variable η_2 = intramammary infection (IMI) and the manifest variables y_5 = average somatic cell count of the herd, y_6 = lymphocytes, y_7 = PMN, y_8 = individual somatic cell count of test day, y_9 = MAJOR, y_{10} = MINOR.

Latent Variable Production

For the latent variable production (PROD), all four measurement variables indicate quite a large effect on production (Figures 4.1 and 4.3). The highest path coefficient was found for protein content with 0.91, followed by fat content with 0.49. Lactose and milk yield are also important determinants with −0.45 and −0.44, respectively.

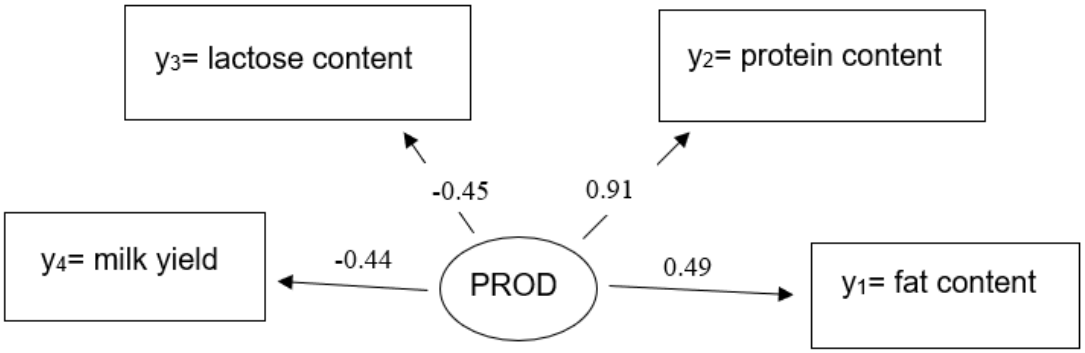

Figure 4.3. Path coefficients for causal relationships from the measurement model for the latent endogenous variable η_1 = production (PROD) with the manifest variables y_1 = fat content, y_2 = protein content, y_3 = lactose content, y_4 = milk yield.

Latent Variable Genetic

The latent variable genetic (GEN) is determined by 13 measured variables, i.e., SNP effects. Two of these SNPs (y17 = ARS-BFGL-BAC-14274, y18 = Hapmap57340-rs29010501, both located on chromosome 11) indicate quite a strong effect, but the path coefficients of the remaining SNPs are close to zero (Figures 4.1 and 4.4).

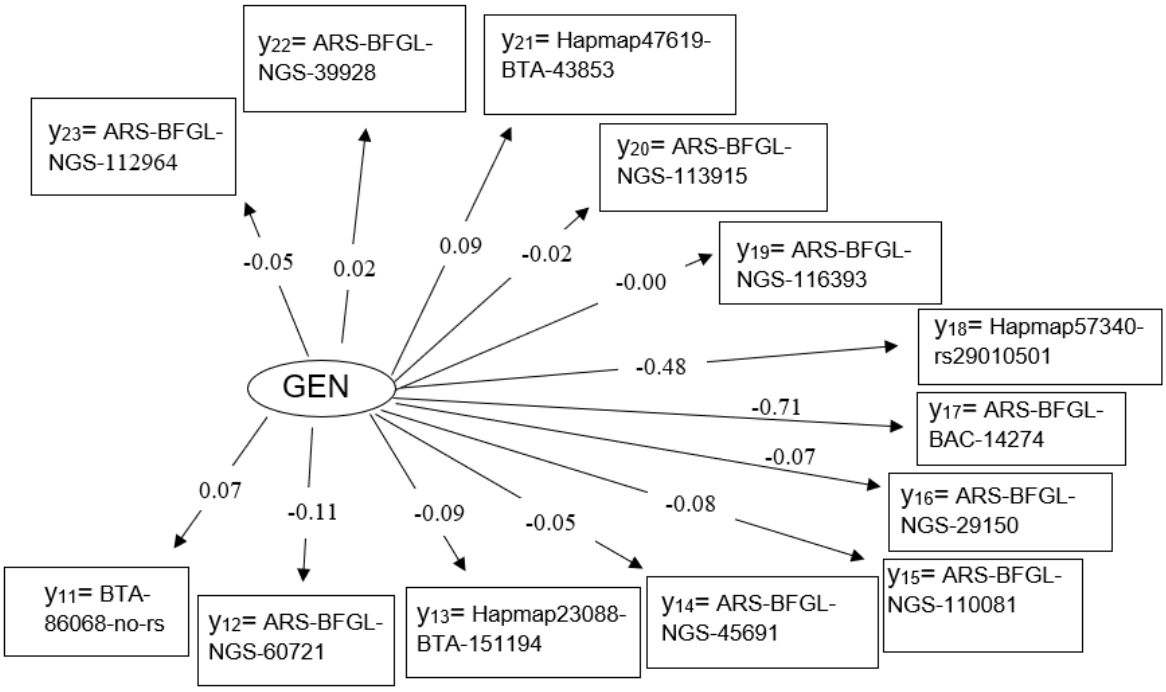

Figure 4.4. Path coefficients for causal relationships from the measurement model for the latent endogenous variable η_3 = genetic (GEN) with the manifest variables $y_{11\text{-}23}$ = significant SNP from the previous GWAS (Table 4.1).

Latent Variable Time

For the exogenous latent variable time (TIME), the path coefficients for average calving interval with 0.96 and the average calving age with 0.87 are quite large (Figure 4.5). A moderate effect was identified for barn age (0.23) and a negligible effect was identified for lactation stage (-0.02).

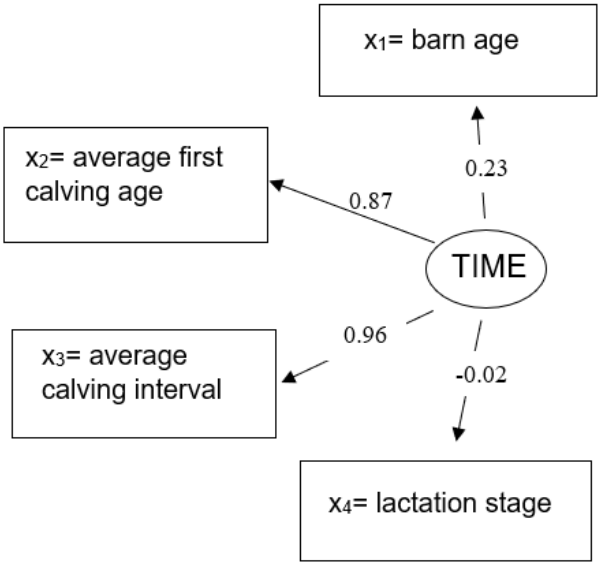

Figure 4.5. Path coefficients for causal relationships from the measurement model for the latent exogenous variable ξ_1 = time (TIME) with the manifest variables x_1 = barn age, x_2 = average first calving age, x_3 = average calving interval, x_4 = lactation stage.

Relationships among Latent Variables

In the SEM framework, it is evident that the effect of the latent variable TIME on the latent variable IMI is quite small, with a path coefficient of -0.06 (Figure 4.6). The effect of TIME on the latent variable PROD was moderate (-0.16). Of similar magnitude was the effect of path coefficient of PROD on the latent variable IMI with 0.17, and the effect of the loading coefficient of the latent variable IMI on the latent variable GEN with -0.10.

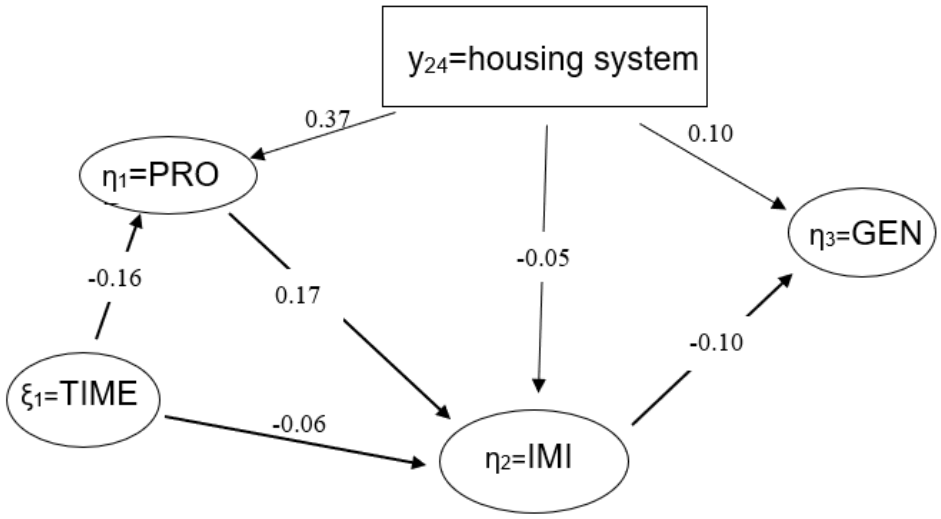

Figure 4.6. Path coefficients for causal relationships from the structural model with four latent variables (η_1 = production, η_2 = intramammary infection, η_3 = genetics, ξ_1 = time); y_{24} = housing system.

In this model, the manifest variable housing system shows an effect on three latent variables. However, the respective path coefficient was quite small (0.05) for the latent variable IMI and for the latent variable GEN (0.10). The highest path coefficient from this model was 0.37, i.e., the effect of the measurement variable “housing system” on the latent variable PROD.

Discussion

Manifest Variables on Intramammary Infection and Production

In the present study, we used a holistic approach, which contributed to a deeper understanding of the mechanisms of udder health in dairy cattle in different housing systems simultaneously considering time, environmental effects and cow effects, combined with a variety of udder health indicators.

With regard to the overall measurement models, it is shown that the effect of the average herd somatic cell count is very small (0.09) to explain an intramammary infection. Accordingly, Beaudeau et al. [38] reported that herd cell count is a weak predictor for intramammary infections, implying the detailed recording of individual cell counts, preferably the generation of a longitudinal data structure by time. Especially, the effect of the individual somatic cell count at the nearest official test day on IMI was quite large (0.73), again supporting the results from the comprehensive udder health study by Beaudeau et al. [38]. The individual somatic cell count considerably changes in the course of an intramammary infection [39], and the large variation also explains the substantial SCC effects from the modeling approach. However, SCC as a single indicator is not sufficient to understand the mechanisms of udder health in detail. Riggio et al. [40] already showed strong associations between the increase in SCC and the status of an infection. Since the cell count and the cell composition depend on the type of pathogen [20], both lymphocytes and PMN, as well as major and minor were integrated into the SEM for the latent variable IMI. All four manifest variables had an effect on udder health and should be simultaneously considered to infer the physiological pathways of an intramammary infection [40].

The path coefficient for the specific cell fraction of the lymphocytes was negative (-0.63), since this cell fraction predominates, especially in the healthy udder quarter [41,42]. In contrast, the path coefficient of the PMN was positive (0.74). In the process of acute infections, the content of lymphocytes decreases, while the content of PMNs in the udder increases [18,43]. Hence, the results from the structural equation modeling approach support well-known physiological mechanisms. Both major and minor pathogens displayed moderate effects on the latent

variable IMI. Interestingly, the influence of major pathogens (0.20) was smaller than the influence of minor pathogens (0.33). This could be due to the shift generally observed in the importance of mastitis pathogens, i.e., with a greater importance of minor than of major pathogens nowadays in the context of severe udder infections [18,44,45]. Accordingly, in the present study, more cows were significantly affected due to minor pathogens. Consequently, the results from the present SEM suggest the evaluation of alternative classifications of pathogens.

The four manifest variables to explain the latent variable PROD indicated a moderate to large effect in a range from -0.44 to 0.91. In this regard, the strongest effect with 0.91 was identified for protein content. Craig et al. [46] already showed that protein content is closely related to productivity. With increasing milk yield, the amount of protein decreases, explaining the opposite signs of the path coefficients of milk yield and protein content in our model [46]. Regarding udder health, protein content reacted very sensitively, especially to somatic cell count alterations, while fat content was more stable [9]. In our SEM analogy, path coefficients were larger for protein than for fat content. For milk quality traits, França et al. [9] indicated sensitivity of lactose contents to the udder health status, with pronounced contrast for infections with *Streptococcus spp.* or *Staphylococcus aureus*. Since our SEM is influenced by both types of pathogens and additional pathogens within the MAJOR and MINOR groups, this may depress individual path coefficients due to different inter-trait relationships of each pathogen, thereby explaining the smaller influence of lactose in our study.

Genetic Influence in the Structural Equation Model

For the latent variable GEN, 13 SNPs were included, but most of them only show a small influence in the SEM when additionally considering a large number of environmental characteristics. Nevertheless, these SNPs were significant for the major and minor pathogens in previous GWAS [6], but mostly located in chromosomal segments outside of functional genes. From a physiological perspective, the two groups, MAJOR and MINOR, contain many different species of pathogens that initiate very different immune responses, and thereby are modulated by many different genes [7]. For example, with regard to MAJOR, the immune response mechanisms of *Staphylococcus aureus* and *Escherichia coli* are different, triggering a cascade of specific genes for specific immune responses [47].

However, the SNP (ARS-BFGL-BAC-14274) of pathway y17 has a quite strong effect with -0.71. This SNP is located directly in the gene *EVA1A*. *EVA1A* is involved in autophagy and the programming of cell death [48,49]. In addition, this quite under-researched gene plays an important role by up- or down-regulating in the MAPK (mitogen-activated protein kinase) signaling pathway [28]. The MAPK pathway plays a fundamental role in udder health, with interactions of the *CHL1* gene [50,51]. However, direct consideration of *CHL1* in the SEM

implied an only small path coefficient of y11 = 0.07. With regard to functional mechanisms and pathways of udder health, *CHL1* plays a role in the activation of the MAPK signaling pathway [50,51]. In a GWAS for the cell fraction PMN, ignoring housing system interaction effects, the potential candidate gene *CTNNA3* was identified, which also intervenes in the MAPK pathway [6]. In the case of clinical mastitis, MAPK signaling regulates inflammatory gene expression [52].

Overall Structural Equation Model Evaluations, Limitations and Prospects

In the SEM, the exogenous latent variable TIME contains four manifest variables. In this regard, the effect of lactation stage was quite small with a path coefficient of -0.02. In contrast, a stronger effect with 0.23 was identified for the age of the cow barn. This variable was integrated in the SEM to consider the experiences of management practices, especially in the context of the quite new compost farming system. Ivemeyer et al. [53] highlighted the significance of herd management on udder health and productivity, especially in alternative or novel housing systems. The effect of the average age was very strong at first calving (0.87) and the average calving interval (0.96). Accordingly, in standard mixed models, age at first calving significantly influenced milk yield and milk composition [54,55], as well as udder health [56]. A late age at first calving was associated with increased milk yield and an increased risk for udder infections [57], supported by the signs for the latent variables PROD and IMI in the present study. Drews et al. [58] assigned a separate latent variable to the two manifest variables age at first calving and calving interval in his SEM. The high path coefficient for the age at first calving with 0.98 reflect the estimate from the present study. In contrast, Detilleux et al. [3] considered average parity in the herd and the percentage of heifers in their modeling approach. However, when developing a SEM and assembling the manifest and latent variables, it is imperative to exclude similar variables with similar explanatory power or high collinearity. Otherwise, estimates from the SEM might be biased [59]. This was also a reason to exclude the variable parity from our finally applied SEM.

The SEM inferred causal relationships among a variety of udder health indicators and respective environmental and cow-associated factors. The direct housing system effect on udder health was quite small (-0.05). However, the housing system moderately affected the latent variable PROD (0.37), implying an indirect housing system effect on IMI through this pathway. Both the housing system and the latent variable IMI were moderately associated with the latent variable GEN. Hence, the SEM also indicates possible genotype by environment interactions, because of the specific reactions of udder health and immune response traits depending on the housing conditions. Overall, the present SEM revealed its potential to depict complex structures of udder health in dairy cows via the modeling of direct and indirect pathways. For the structural equation modeling, we considered the four latent variables IMI,

PROD, TIME and GEN and integrated the variable housing system as a formative measurement model in this SEM. Furthermore, we modeled direct and indirect pathways for trait and effect relationships. However, such comprehensive analyses require a broad data structure based on different types of data, i.e., novel cow traits, cow-associated factors, housing characteristics, as well as “classical” environmental effects. Generation of such data structure implies tremendous efforts regarding labor, time and logistics. Consequently, in the present study, we neglected some additional possible environmental effects on an intramammary infection such as the climatic conditions in the barn. Gernand et al. [60] identified temperature and humidity close to the official test-day as major effects on clinical mastitis. However, overloading a SEM with more detailed environmental effects might lead to failed convergence, or to biased parameter estimates [61]. An alternative in this regard is to enlarge the cow trait dataset, but this is also a challenge for novel health traits as considered in the present study. Attempts to establish the so-called “cow training sets” for genomic selection, comprising innovative health traits for a large number of genotyped cows [62], might be the perfect database for ongoing and even more detailed SEM applications.

Conclusions

The applied SEM clearly inferred effects among response variables indicating udder health and environmental and cow-associated factors. Trait and modeling complexity was reduced by considering the latent variables. The direct effect of the housing system on the latent variable IMI was quite small, but the indirect pathway via PROD indicated housing system–IMI associations. For the latent variable GEN, especially one SNP is of primary interest. This SNP is located in the *EVA1A* gene, which plays a fundamental role in the MAPK1 signaling pathway. Other identified genes (e.g., *CTNNA3* and *CHL1*) support results from previous studies, and this gene also contributes to mechanisms of the MAPK1 signaling pathway. Overall, our SEM emphasizes the importance of this pathway for udder health in a very complex modeling context including a larger number of further environmental effects.

Author Contributions:

Conceptualization, S.K.; data curation, P.W., K.B. and P.E.; formal analysis, P.W., T.Y. and K.B.; investigation, P.W., K.B., P.E. and T.Y.; methodology, P.W.; laboratory, P.W., K.B. and P.E.; project administration, K.B., P.E. and S.K.; supervision, S.K.; validation, P.W. and K.B.; visualization, P.W.; writing—original draft, P.W.; writing—review and editing, K.B. and S.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement:
Ethical review and approval were waived for this study, because all data were recorded in the process of routine milk recording as used for official genetic evaluations. Hence, no extra impairment of animals was associated with this study.

Informed Consent Statement: Not applicable.

Data Availability Statement:
The data presented in this study are available on request from the corresponding author. The data are not publicly available due to their use in official genetic evaluations for dairy cattle.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 5

GENERAL DISCUSSION

5 General Discussion

The analyses in this study show the differences in udder health between compost and cubicle housing systems at phenotypic and genomic level, as well as the relationships between various influencing factors on udder health. Chapter 2 indicates that compost bedded pack barns are not negatively affecting udder health. It also shows that the different indicators of udder health depend on specific environmental characteristics, with different responses in combination with cow-related factors such as lactation stage and milk yield level. Based on the phenotypic trait definitions, Chapter 3 shows in genome-wide association analyses that the specific and very precise definitions of mastitis traits are necessary to improve future breeding strategies. As the different pathogens trigger different responses in the immune system, a precise definition of traits is essential to improve the understanding of the genetic mechanisms involved in udder health. In order to obtain a general overview of the phenotypic, environmental and genetic influences on udder health, Chapter 4 focuses on a structural equation model. Here the relationships between the traits and their strength of influence on udder health, are shown.

In the following part of this chapter, the new trait definitions are discussed in more detail, how they are related to each other, how the relationships might change in different housing systems, and how they could be used to improve breeding strategies. Finally, concluding remarks and recommendations for practical applications are given.

5.1 Udder health in different housing systems

Udder health in compost-bedded pack barns is discussed controversially. Lobeck et al. (2011) found significantly higher somatic cell count (SCC) and mastitis prevalence in compost-bedded pack barns than in conventional cubicle barns. However, Janni et al. (2007) and Black et al. (2013) found that udder health is highly dependent on the type of bedding material and the management of bedding, especially the temperature inside the pack and moisture content. Materials such as wood shavings and sawdust are predestined for an increased prevalence of *Klebsiella spp*. Eckelkamp et al. (2016a), on the other hand, found no difference in udder health between compost-bedded pack and conventional cubicle barns. In agreement with our results presented in Chapter 2, also Barberg et al. (2007b) showed better udder health for cows kept in compost-bedded pack barns due to higher animal welfare. Due to lower stress, improved animal welfare and a longer lying time, milk production is positively influenced and the SCC level decreased (Borchers, 2018). However, various authors have shown that udder health depends primarily on the management of the lying area and udder hygiene (Janni et al., 2007; Lobeck et al., 2012; Black et al., 2013; Fávero et al., 2015; Albino et al., 2018). The following

chapter outlines the perspectives of novel udder health traits in the context of compost and cubicle housing systems.

5.1.1 Differential cell fractions as alternative traits for the assessment of udder health

The somatic cell count serves as a health parameter and indicator for mastitis. The individual cell count per cow is also the data basis for the quality assessment of bulk tank milk (Kruif et al., 2007).

With respect to the tank milk cell count, the official limit in Germany is 400,000 cells/ml (geometric mean of three months). Exceeding this limit leads to economic losses due to a deduction of 1 ct/kg milk price, up to a blocking of milk collection if there is no improvement (Bundesministerium für Ernährung und Landwirtschaft, 2021). The aim is to achieve values of less than 250,000 cells/ml per month and less than 150,000 cells/ml as an annual average (Kruif et al., 2007). However, the cell count of the tank milk is not a good parameter for a prognosis of udder health in the herd, due to the strong dilution effects caused by cows with very low cell counts. This effect increases with increasing herd size. In addition, milk from already infected animals with a very high cell count does not even reach the tank (Kruif et al., 2007). The somatic cell count per cow, all four quarters represented in one value, on the other hand, is very often used as an indicator for udder health (Sarikaya et al., 2005; Le Maréchal et al., 2011). However, even here the informative value is clearly limited, as dilution effects also occur. As usually only single udder quarters are affected by an infection, the other quarters with low SCC improve the mean value of the cow (Rupp and Boichard, 2003). In healthy tissue, the somatic cell count is between 10,000 and 30,000 cells per ml milk at quarter level (Kruif et al., 2007). In the course of an infection, the strong increase in PMN is usually attributed to the increase in the somatic cell content in milk, without an explicit determination of the cell composition (Sarikaya et al., 2005; Kruif et al., 2007). In addition, the SCC fluctuates strongly even without infection, for example due to the stage of lactation and the lactation number. However, the type of conflict agent or the species of pathogen has a great influence on the SCC. The various pathogens are detected differently by the immune system and trigger different cascades in the immune system. In turn, this has a major impact on the increase of somatic cells, the cell count and their composition and consequently, also on the type of mastitis (clinical, subclinical or chronic) (Sarikaya et al., 2005; Le Maréchal et al., 2011).

5.1.2 Specific mastitis pathogens in housing systems

Due to the mixture of organic material and manure in the bedding of the compost-bedded pack barn, it is expected that udder health is impaired and animals are more frequently affected by mastitis. Astiz et al. (2014) and Eckelkamp et al. (2016a) already disproved this statement and observed even lower incidences of clinical mastitis were measured in compost bedded pack barns. Also in our analyses, less cows were bacteriologically positive in compost-bedded pack barns than in cubicle barns (Chapter 2). Furthermore, our evaluations showed that the trend to lower prevalences of major pathogens in the course of mastitis prevention programs over the last two decades is initially not dependent on the housing system. Especially in well-managed farms, minor pathogens are more important than major pathogens, as already shown by Tenhagen et al. (2006) and Piessens et al. (2011). Thus, our values for minor pathogens also showed prevalences of 35% in compost-bedded pack and 54% in cubicle barns.

Regardless of the housing system, the management of the bedded lying area has a significant influence (Leso et al., 2020; Freu et al., 2023), which covers much more space in the compost-bedded pack barn than in the cubicle barn (Leso et al., 2020). Temperatures less than 55°C (Black et al., 2013) and an excessive moisture content (> 60%) (Albino et al., 2018) can have a negative impact on the pack. In addition, meteorological conditions, such as the ambient temperature and humidity, play a significant role (Leso et al., 2020). With a lower ambient temperature and higher humidity, more bedding area is required to enable sufficient evaporation (Smits and Aarnink, 2009). In addition, the timing of the bedding is crucial for the moisture content in the pack (Barberg et al., 2007a; Janni et al., 2007). Wet bedding leads to dirtier udders (Eckelkamp et al., 2016b) and consequently to a higher risk of mastitis (Fávero et al., 2015).

Several studies discuss the concentration of pathogens and the pathogen species living in the compost as well as their influence on udder health and the presence of pathogens in milk controversially. Freu et al. (2023) found that coliforms in the pack are positively associated with the prevalence of subclinical mastitis. However, it should be noted that the definition of subclinical mastitis in this study is limited to the definition of subclinical mastitis if the SCC is higher than 200,000 cells/ml milk. Kull et al. (2017), on the other hand, did not define clinical or subclinical mastitis, but analyzed the levels and species of pathogens at the teat ends in relation to sand as bedding material. With recycled sand, the teats of the animals were more frequently colonized with gram-negative bacteria and *Staphylococcus spp*. However, it is not inevitable that these pathogens will cause mastitis. It shows, as indicated by Ray et al. (2022), that the bedding material serves as a reservoir for some pathogens and can act as an additional stress factor for an intramammary infection. In addition, the type of pathogens

strongly depends on the different bedding materials and therefore may but does not necessarily have to contain mastitis pathogens (Ray et al., 2022; Wu et al., 2022). With intensive use of wood shavings and sawdust, Janni et al. (2007) found an increase in *Klebsiella spp*. in the pack and an increasing prevalence in the udder. In addition, these types of bedding can be a source for mycobacteria, which can be a health risk for the udder (Ghielmetti et al., 2017). In contrast, Eckelkamp et al. (2016a) found no difference between compost and sand-bedding with regard to cell counts, likewise Lobeck et al. (2012) when comparing milk bacteria counts in different systems with milk bacteria counts in compost-bedded pack barns. Many of these studies highlight the importance of good management of the bedded pack for the respective housing system and the influence on udder hygiene and health (Fávero et al., 2015; Albino et al., 2018; Ray et al., 2022; Freu et al., 2023).

5.1.3 Associations between cell fractions and pathogens

As already mentioned in the previous chapters, the somatic cell count in tank milk, the animal-specific SCC and the farmer's subjective evaluation of clinical mastitis are not the best parameters for assessing the complex trait udder health in detail. Especially if new housing systems should be adequately assessed in relation to udder health, more detailed indicators have to be chosen to evaluate udder health (Pillai et al., 2001; Rivas et al., 2001).

A novel analysis method, i.e., the determination of the differential somatic cell count (DSCC), is now being used by some laboratories in addition to the SCC. Schwarz et al. (2010) performed a large number of DSCC analyses. The DSCC reflects the combined proportion of PMN (polymorphonuclear neutrophils) and lymphocytes in the milk. The macrophage content results from 100 - DSCC. In case of acute udder infections, the PMN usually increase rapidly at first, which is then reflected in the DSCC (Schwarz et al., 2010; Schwarz et al., 2011; Damm et al., 2017; Kirkeby et al., 2020). In our study, we deliberately decided to differentiate microscopically between the subcategories segmented neutrophils and banded neutrophils in addition to lymphocytes, macrophages and PMN. The immature banded neutrophils (bN) especially are only released from the bone marrow in the early stages of very acute infections (Paape et al., 2003). These results could already provide information on the type of infection or possible pathogens without analyzing them, as the different pathogens trigger the immune system in different ways (Paape et al., 2003; Le Maréchal et al., 2011). In order to be able to assess in more detail whether this measurement could be used profitably, further analyses would be necessary in order to sample the individual animals not only once. Instead, it is recommended to sample them repeatedly over a certain period, for example on a weekly basis.

The influence of the pathogen on the cell count and the course of an intramammary infection is also not considered in the case of SCC and only to a limited extent in the case of DSCC. This is demonstrated especially in the case of infections caused *by Escherichia Coli (E. coli)* or *Staphylococcus aureus* (*S. aureus)*, which are detected completely differently by the immune system and therefore trigger the immune system to initiate different cascades (Bannerman et al., 2004). In addition, the two pathogens influence the composition of somatic cells, which is reflected in the course of the increase in SCC (Bannerman et al., 2004) and also the permeability, which is essential for the transport of substances and cells from the blood into the udder. These two factors can then significantly influence the composition of banded neutrophils and segmented neutrophils. Finally, all these cascades determine whether clinical or subclinical mastitis develops (Bannerman et al., 2004; Taponen and Pyörälä, 2009).

It would be very interesting in further analyses to differentiate the *Coagulase negative staphylococcus* (CNS) and *Corynebacterium* (COR), the minor pathogens, as these are the most common pathogens since mastitis prevention programs began, while the major pathogens are decreasing. Especially within the CNS group, the individual species have very different effects in the udder. For example, *Staphylococcus chromogenes, Staphylococcus capitis* or *Staphylococcus xylosus* lead to a significantly higher SCC, while *Staphylococcus epidermides* or *Staphylococcus hyicus* increase the SCC by a smaller degree (Borm et al., 2006). An improved understanding of the cascades that are triggered in the immune system and how the cell fractions respond in these different species could also significantly improve the quality of genomic analyses of udder health in the future.

5.2 The effects of genotype by environmental interactions

Genotype by environment interactions reflect how a genotype leads to different phenotype expressions in different environments (Hammami et al., 2009). However, the assessment of genotype by environment interactions on a specific trait highly depends on the quality of the definition of the trait and on the amount of factors influencing this trait (Barkema et al., 1998; Streit et al., 2013). This is especially difficult for a complex trait highly dependent on a wide range of interacting factors, ie., udder health (Streit et al., 2013). Hayes et al. (2003) already used herd descriptors, e.g. average herd protein yield or herd test-day coefficient of variation for protein yield or temperature humidity index, to reduce unmeasured environmental factors. To reduce environmental “noise parameters”, the herds in our study were selected in advance according to predefined criteria, i.e., to make the study as objective as possible. The herds were selected by the European FreeWalk consortium considering criteria as defined by Blanco-Penedo et al. (2020). Hence, the region of the farm, herd size and feeding

characteristics for both farm types were selected as similar as possible to isolate disturbing environmental effects apart from the bedding system. Hence, the main objective of this study was to determine genotype-housing interactions for udder health, in relation to the two different housing systems (compost-bedded pack barns and cubicle barns).

The majority of previous studies also used the SCC, SCS (somatic cell score) or different definitions of clinical and subclinical mastitis in genetic and genomic analyses to define udder health (Barkema et al., 1998; Rupp and Boichard, 2003; Streit et al., 2013). As already explained in the previous chapters, these definitions are partly weak to moderate indicators of an udder infection, implying difficulties when inferring detailed genomic associations. Following several studies, heritabilities (h^2) were determined for different mastitis pathogens. The heritabilities vary, for example, between 0.035 and 0.068 for *S. aureus*, between 0.049 and 0.06 for *E. coli* and between 0.051 and 0.093 for CNS (Haas et al., 2002; Schafberg et al., 2006; Sørensen et al., 2009). Thus, heritabilities for mastitis pathogens are only slightly larger compared to clinical mastitis with $h^2 \approx 0.03$ (Heringstad et al., 2000; Carlén et al., 2005; Koeck et al., 2012; Tiezzi et al., 2015). Since the occurrence of the respective pathogen mainly depends on the environmental conditions, especially in the case of environmental mastitis pathogens, small heritabilities can be expected. Hence, Tiezzi et al. (2015) and Welderufael et al. (2018) already concluded that GWAS for the indicator trait clinical mastitis is more suitable to detect potential candidate genes that influence udder health. Welderufael et al. (2018) found significant SNPs for clinical mastitis on chromosomes BTA 3, 7, 12 and 13, and Tiezzi et al. (2015) on chromosomes 2, 14 and 20. Yet, no more detailed definition has been used in any GWAS except SCC, SCS, clinical and subclinical mastitis. Consequently, a new approach was chosen in our GWAS to focus on the detailed individual indicators rather than using the traditional definitions on their own to describe udder health. Thus, the individual cell fractions (macrophages, lymphocytes, PMN, segmented neutrophils and banded neutrophils) and the individual mastitis pathogens were included in the models. In addition, the SNP x housing interaction was considered in this modelling approach, implying that there was no need to split the data set between the animals in the compost-bedded pack barn and cubicle barn. In our GWAS, we identified 35 significant SNPs for the main effect and 6 significant SNPs for the interaction effect on different chromosomes. As observed by Tiezzi et al. (2015) and Welderufael et al. (2018), some significant SNPs were located on chromosomes 2, 3 and 14. It is not surprising that we found some significant SNPs on other chromosomes or identified other candidate genes in our analyses due to the completely new definitions of traits (differentiated cells and individual pathogens). The identified genes where the SNPs are located and where a function is already known are all involved in immune system mechanisms. Strikingly, in other studies, these genes were also found to be regulated by the stress factor with ongoing effects on gene expression. *HEMK1*, for example, is involved in the development

of the immune system in response to environmental stressors (Liongue et al., 2012). *CHL1* has an immunosuppressive effect during stress conditions (Huang et al., 2013). The *EVA1A* gene is also involved in autophagy and programmed cell death (Li et al., 2016; Shen et al., 2017), as well as in the MAPK signaling pathway, which plays an important role in udder health and also interacts with the *CHL1* and *CTNNA3* genes (Shen et al., 2017). The SNP located in this gene was significant in GWAS and additionally showed a strong impact within the structural equation model. Gene expression analyses and further GWAS analyses with better indicator markers could lead to better knowledge about the exact role of these genes in udder health and how they are regulated.

In order to gain even better knowledge of genetic mechanisms in relation to the housing systems compost-bedded pack barns and cubicle barns, it would be useful to consider more detailed information of the bedding material in statistical models. Since the compost farms in our analyses used very different bedding materials and also vary in their management, pathogens also varied. If these detailed bedding parameters could be taken into account, the quality of the analyses could be significantly improved and a better assessment of the genotype-environment interaction in the housing systems could be achieved.

5.3 Structural equation models as a new method for determination of udder health factors

Udder health is a very complex trait. It is influenced by many factors, via direct or via indirect effects. However, as already described, this complexity is not usually taken into account in the definition of the trait or in the analysis of breeding values. It is no longer expedient to use only SCC, SCS or producer recorded clinical mastitis as representative traits for udder health, because these traits do not reflect the disease progression sufficiently. Detilleux et al. (2013) already showed in their structural equation model (SEM) approach that indirect resistance to mastitis pathogens is dependent on the existing pathogen and pathogen counts. In our SEM, the pathogen groups MAJOR (major pathogens) and MINOR (minor pathogens) also show a significant effect on the latent variable intramammary infection (IMI). Our analyses, as well as the SEM defined by Los Campos et al. (2006), also show that the latent variable production influences the latent variable intramammary infection. Again, the latent variable production is influenced by the latent variable time (TIME) in our model. In contrast to the other studies using structural equation models, we also include the manifest variable housing system (compost-bedded pack and cubicle barns). In our case, it directly affects IMI, GEN (genetics) and PROD (production). This shows that the indirect influence on the latent variable PROD (0.37) is significantly higher than the direct influence on IMI (-0.05). Our SEM thus shows that for the

assessment of udder health and improved breeding for udder health, the influencing factors must be determined. These influencing factors, which can represent the complexity of udder health, must first be assessed by SEM. This is in agreement with Detilleux et al. (2012) and Detilleux et al. (2013). Subsequently, using the structural equation models, it is also possible to reduce modelling complexity again by using the latent variables. Such approach allows the identification of new traits as health indicators, which can be considered in other models and breeding value estimations.

In order to further expand and enhance our SEM and to be able to better assess udder health between compost-bedded pack and cubicle barns, it would be useful to integrate additional parameters. In the case of the latent variable IMI, it would be possible to use the presence of individual bacteria instead of the pathogen groups major and minor, since bacteria like *E.coli* and *S.aureus*, which are in the major group, trigger completely different immune reactions in the udder. Furthermore, with additional integration of the bacteria in the pack and the composition of bedding materials, relationships with existing pathogens in the udder could be better examined. In order to be able to assess udder health in new housing systems such as compost-bedded pack barns, the course of udder health could be much more conclusive. For this purpose, the composition of the cell fractions and the presence of mastitis pathogens in the udder have to be integrated into the model during the period and the animals have to be sampled several times over a longer period. Cortisol levels in the animals' blood would also reflect the stress factor of the animal in the different housing systems and could significantly improve the model. In addition, explicit meteorological data, as well as temperature and humidity in the pack over time, would provide a significant benefit for this model and thus contribute to a better assessment of the housing system for practice and breeding.

5.4 General conclusion and recommendations

The results of this thesis show that cows in compost-bedded pack barns have a better udder health, especially for minor pathogens, than cows in conventional cubicle housing systems. However, it also shows that udder health in both housing systems is dependent on the barn management.

It was also shown that alternative, more detailed traits such as the differential somatic cells in combination with the individual mastitis pathogens enable a significantly better assessment of udder health in different housing systems and provide a better understanding of the response cascades of the immune system. These more detailed traits can be used in genome-wide association studies to identify new gene loci that influence udder health and can then be used for further analyses and further development of breeding strategies. Thus, breeding in the context of udder health can be better and more precisely than in the case of traits such as SCC, SCS or the subjective evaluation of clinical mastitis.

In addition, structural equation models can be used as a new approach to better understand the complex structures of udder health and to re-evaluate influencing factors, especially indirect factors, and incorporate them into models and breeding value estimates accordingly.

For the practice, the conclusion should be that animals in the composting barn not only have better welfare and generally better health. They can also have improved udder health if udder hygiene and good management of the pack are ensured.

Based on these findings, we recommend the following measures:

- A detailed phenotyping strategy of differential somatic cell count and specific mastitis pathogens that contributed to the detection of immune pathways.
- GWAS with interactions inferred genes such as *HEMK1* or *CHL1* with specific effects only in the compost system.
- SEM applications detected direct and indirect influencing factors for traits and reduced their complexity.
- In practice, good bedding management and proper udder hygiene should be ensured.

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Curriculum Vitae

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Formal Declaration

Erklärung gemäß der Promotionsordnung des Fachbereichs 9 vom 7. Juli 2004 § 17 (2)

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht.

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Gießen, den 13. Februar 2024

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Patricia Wagner