Ruminant colostrum

Management, Microbes and Maternal immunity

Lisa Robbers

Colofon

Ruminant colostrum: Management, Microbes and Maternal immunity
Lisa Robbers
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Ruminant colostrum

Management, Microbes and Maternal immunity

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(met een samenvatting in het Nederlands)

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CHAPTER 1

An introduction to ruminant colostrum

The importance of colostrum for ruminants

The first weeks of life are a critical period in the development of the newborn ruminant as it is very susceptible to various pathogens. Ruminants are, like other animals, born with a proper, functional but naïve immune system. Because they have an epitheliochorial placenta that does not allow transfer of macro-molecules from mother to fetus during gestation, they are born agammaglobulinemic. Therefore they rely almost entirely on the transfer of immune constituents through the first ingested colostrum for their first immune protection.

Colostrum is best described as the first secretion of the mammary gland after giving birth. There is large variation in colostral composition between species, individual animals, and even within animals. Despite large compositional variability, we know that colostrum contains more nutritive components compared to normal milk. For example, it is well known that bovine colostrum generally contains more proteins and fat as compared to bovine maternal milk (Foley and Otterby 1978; McGrath et al. 2016). In addition, colostrum contains higher concentrations of several growth factors and many vitamins, such as A, D, E, K and several B vitamins (McGrath et al. 2016; Playford and Weiser 2021). The nutritional value of colostrum is essential for growth and regulation of the neonates' body temperature. Next to the great nutritional value, ingestion of colostrum is of the very essence for newborn ruminants because of its immunological features.

Immunological properties of ruminant colostrum

The best known and most studied immune component of ruminant colostrum is immunoglobulin. Immunoglobulins, or antibodies, are proteins that are produced by the B lymphocytes of the adaptive arm of the immune system. Their functions include recognition of antigens of foreign material (pathogens), neutralization of potential threats and targeting them for elimination by other components of the immune system. Immunoglobulins can be bound to the membrane of B lymphocytes, they can circulate in the blood and interstitial fluid, or they are secreted at mucosal sites. The process by which immunoglobulins are transferred from the bloodstream into the mammary secretions is called colostrogenesis. Colostrogenesis and thereby the transfer of immunoglobulins into colostrum is believed to end soon after parturition. Three main immunoglobulin classes are described for colostrum, with immunoglobulin G (IgG) being the most abundant in ruminant colostrum [4]. Immunoglobulin G makes up for approximately 85% to 90% of the total amount of immunoglobulins in ruminant colostrum. Two major subtypes of IgG can be distinguished in colostrum: IgG1 which makes up 80 to 90% of the total IgG, and IgG2. Other types of immunoglobulins in colostrum are IgM and IgA, accounting for approximately 7% and 5% of the total immunoglobulins (Godden et al. 2019; Rudovsky et al. 2008).

Immunoglobulins are not the only immune components present in colostrum. In the past two decades there has been increasing interest in immune cells in colostrum. Colostral leukocytes belonging to the innate immune system such as neutrophils and macrophages have been identified (Stelwagen et al. 2009) as well as T- and B-lymphocytes belonging to the adaptive arm of the immune system (Duhamel et al. 1987). Many studies demonstrate an important role for colostral leukocytes in newborn ruminants. For example Donovan et al. (2007) and Reber et al (2008) describe that colostral leukocytes enhance and protect the neonatal immune development (Donovan et al. 2007; Reber et al. 2008a), and Reber et al. (2005) demonstrate that the antigen-presenting capacity of the neonatal immune system is enhanced by maternal leukocytes (Reber et al. 2005).

Establishing passive immunity in the newborn ruminant

Many studies describe the importance of IgG for the newborn ruminant and thereby the significance of sufficient IgG ingestion through maternal colostrum. Some studies focus on colostrum ingestion in small ruminants such as goats and sheep. Other studies look into colostrum ingestion in beef calves. However, most research focusses on dairy calves. This is because dairy goats and sheep form a minority in livestock, and beef calves usually spend the complete pre-weaning period with their dam and acquire passive immunity through colostrum by suckling. Dairy cattle, however, are often kept on a large scale and often the (replacement) calves are immediately separated from their dam and require colostrum feeding by human intervention. The majority of studies and also this thesis is therefore primarily focused on optimizing colostrum feeding for dairy calves.

For a long time, the aim was to achieve a serum IgG concentration of at least 10 mg/ml in newborn calves, within 24-48 hours of age. This was described as sufficient transfer of passive immunity (TPI). Calves with serum concentrations below this threshold were considered to have failed transfer of passive immunity (FTPI) (Godden et al. 2019), and were consequently at higher risk of early calf morbidity and mortality. However, this dichotomous approach poorly reflects the dose-response association between serum IgG concentration and morbidity risks. Even though mortality risks in pre-weaned calves have decreased since the implementation of the 10 mg/ml threshold, this does not hold for risks of morbidity. Lombard et al. (2020) summarized literature and concluded that there is a difference in disease risks between calves with serum IgG concentrations of just above 10 mg/ml and those with for example >24 mg/ml. The authors aimed to find consensus on the state of passive immunity that should be acquired by the calf that embodies both calf mortality and morbidity risks. In their suggested guideline four categories of serum IgG concentrations are distinguished: excellent, good, fair and poor. This categorization better reflects the dose-response associations between serum IgG concentrations and calf morbidity and mortality risks (Lombard et al. 2020).

Table 1. Adapted from (Lombard et al. 2020). Consensus serum IgG concentrations and equivalent total protein (TP) and Brix measurements for each transfer of passive immunity (TPI) category[1]

TPI category	Serum IgG category (mg/ml)	Equivalent TP (g/dl)	Equivalent %Brix
Excellent	≥ 25.0	≥ 6.2	≥ 9.4
Good	18.0-24.9	5.8-6.1	8.9-9.3
Fair	10.0-17.9	5.1-5.7	8.1-8.8
Poor	< 10	< 5.1	< 8.1

[1]Modified from Godden et al. (2019)

Colostrum management

As described earlier, calves in dairy industry are usually separated from the dam and colostrum needs to be provided by human intervention. It is thus essential that farmers ensure adequate transfer of passive immunity to provide the best start for newborn calves. There are many elements that play a role in establishing the colostral quality, and roughly they can be divided over three main groups. The first group comprises factors that are “cow related” and contains factors that are not or cannot be influenced by human intervention. This group includes, for example, a cow’s parity or the season of calving. The second group is related to all “cow related” factors that can be influenced by human intervention, such as dry period

length and feed intake during dry period. The last section encompasses the colostrum management on farm and covers all human handling between the time of calving and the time of first colostrum feeding, including the milking process, the storage of colostrum and the feeding process.

Objective and research areas covered

Since several years there is increased attention to animal health and welfare in livestock, especially in Dutch dairy industry. High morbidity and mortality rates among dairy calves lead to a search for solutions to improve calf health (Santman-Berends et al. 2014; Santman-Berends et al. 2018).

Since colostrum feeding is one of the key aspects in calf rearing, it is valuable to further investigate its beneficial characteristics. The overall objective of this thesis is to improve knowledge on colostrum management strategies and immunological properties of colostrum, to provide dairy calves with a proper neonatal immune system. Three main research areas will be addressed:

I. Improving colostrum management on dairy farms:

- *How does colostrum management affect colostral quality and calves' immune status?*
- *How is knowledge on colostrum management put into practice?*
- *How does colostrum management influence bacterial contents?*

II. Understanding the role of colostral immune cells in the neonatal immune response

- *Are maternal cells transferred with colostrum functional in the newborn?*

III. Considerations for on-farm colostrum management

Improving colostrum management on dairy farms

How does colostrum management affect colostral quality and calf's immune status?

In **chapter 2** the immunological components of colostrum and how these are affected by potential on-farm colostrum management strategies are studied using the currently available evidence. A systematic literature search strategy is described with which we attempt to identify all research about milking, storing and treating, and feeding of colostrum and their effects on colostrum immune quality and immune status of the newborn calf. The result is a scoping review covering the existing literature of on-farm related colostrum management practices and their effects on the immunological quality of colostrum.

How is knowledge on colostrum management put into practice?

In literature and also in practice the common advice on colostrum feeding is summarized in the three Q's: Quickly, Quality and Quantity. In Dutch this is loosely translated into the

guideline “Veel, vlug, vaak en vers”, the latter one adding the importance of providing fresh colostrum. The essence is clear: to provide a sufficient amount of fresh, high quality colostrum as quickly as possible to ensure adequate transfer of passive immunity. However, it is unknown how these guidelines are exactly interpreted in practice. In **chapter 3** a survey among Dutch dairy farmers is described, in which we inquire about their colostrum practices with their most recently born calf. With this survey we aim to better understand what the general colostrum feeding guidelines mean to Dutch dairy farmers and how they are implemented in practice.

How does colostrum management influence bacterial contents?

Neonatal calves are highly exposed to a variety of pathogens, and therefore immunoglobulins and other immune components transferred with colostrum are essential for first protection. However, freshly milked colostrum can contain high bacterial counts and therefore transfer of passive immunity through colostrum poses a risk of pathogen transmission. The majority of the current research on bacterial content of colostrum is primarily focused on minimizing total bacterial counts to decrease the risk of pathogen transmission (Godden et al. 2019; Lorenz 2021). For example, bacterial contamination can be prevented by hygienic milking, storing and feeding of colostrum (McGuirk and Collins 2004; Stewart et al. 2005). Even though the focus on total bacterial counts of colostrum is important, the study to colostral quality comprises more than just bacterial quantities, and it matters which bacteria are actually present.

From literature we know that duration and temperature of colostrum storage are known to affect total plate counts (TPC), and it is recommended to minimize storage duration and store colostrum preferably at a low temperature (Cummins et al. 2016; Stewart et al. 2005). Less is known about the effects of storage on the composition of TPC. Depending on the condition, colostrum serves as a culture medium in which a certain subset of the present microbial community can thrive. This can result in different microbial compositions as a result of different storage methods. Based on the findings of the survey among Dutch dairy farmers described in **chapter 3**, we selected several storage conditions and investigated the effects of time and temperature on microbial composition of the colostral TPC in an experimental setting. The findings of this research are presented in **chapter 4**.

Understanding the role of colostral immune cells in neonatal immune response

Are maternal cells transferred with colostrum functional in the newborn?

Maternal leukocytes from colostrum can migrate intercellularly across the offspring’s gut wall (Liebler-Tenorio et al. 2002), and a study in sheep lambs shows that these colostral cells are able to reach the neonatal circulation until at least three days postpartum (Schnorr and Pearson 1984). This seems longer than the period until the gut closure for maternal antibodies, which is after 24−36 hours of age (Weaver et al. 2000). While many studies propose an important role for colostral leukocytes in newborn ruminants (Donovan et al. 2007; Langel et al. 2015; Novo et al. 2017; Reber et al. 2008b; Reber et al. 2005; Reber et al. 2006), *in vivo* mechanisms of action and antigen specific functionality in ruminants are not fully understood. In **chapter 5**, two experiments are described in which we study the transfer of antigen specific cellular immunity through maternal colostrum. In addition to previous studies, here we investigate whether these cells are functional in the animal itself. Besides the practical reason that goats are relatively small and therefore easy to keep, a major advantage

of conducting our experiments in goats is that goats are more likely to give birth to twins than for example cows. By conducting our experiment in twin goat kids, we applied a paired experimental design with two treatment groups in which potential maternal influences during gestation are cancelled out.

Considerations for on-farm colostrum management

Finally, in **chapter 6** I will first summarize the results reported in this dissertation and then I will discuss the findings in a broader context with a focus on the potential implications and considerations in on-farm colostrum management.

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PART I

Improving colostrum management on dairy farms


2

CHAPTER 2

A scoping review of on-farm colostrum management practices for optimal transfer of immunity in dairy calves.

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Abstract

Newborn calves are agammaglobulinemic and rely for their first immune protection almost completely on the transfer of immune constituents via colostrum. Inadequate colostrum management practices such as on-farm colostrum storage practices and colostrum feeding methods could affect immune components in colostrum and subsequently immune status of the newborn calf. We conducted a scoping review to identify all literature on the interactions between several colostrum management factors and immunological colostrum quality and passive transfer of immunity. Three major stages were defined: milking methods, colostrum treatment and storage, and administration procedures. Separate CAB Abstracts searches were performed for each of the subjects of interest. The search process was completed on November 9, 2020. Colostrum should be milked as soon as possible, as IgG concentration diminishes over time, probably due to dilution. To minimize bacterial contamination, it is advised to pasteurize colostrum in small batches at maximal 60°C for 30 or 60 minutes. Freeze/thawing of colostrum does not or only slightly affects IgG concentrations, as long as thawing is done au bain-marie and temperature does not exceed 40°C. In on-farm situations, it is difficult to determine the volume that should be fed as the variables contributing to the absorption of IgG by the newborn calf are many and include the quality of the colostrum, the bacterial contamination, the time interval between birth and first moment of feeding and the weight of the calf. Despite all knowledge regarding optimal colostrum management strategies, it remains challenging to predict the effects of certain colostrum management choices in field conditions. Therefore, we recommend measuring the colostral quality, weighing the newborn calf, adjusting the feeding volume accordingly to ensure optimal colostrum intake for each calf.

Introduction

The first weeks of life are a critical period in the development of the newborn calf as it is very susceptible to various pathogens. Cows, just like other ruminants, have an epitheliochorial placenta which prevents the transfer of passive immunity to the neonate during gestation. Hence, newborn calves are agammaglobulinemic and rely for their first immune protection almost entirely on the transfer of immune constituents through the first ingested colostrum. Immunoglobulin (Ig) G is traditionally considered critical in assessing colostrum quality because it is the most abundant Ig (IgG accounts for approximately 85-90% of the total Ig in colostrum (1). Two major subtypes of IgG can be distinguished in colostrum: IgG_1 which makes up 80-90% of the total IgG, and IgG_2. Besides IgG, also IgM (7%) and IgA (5%) are present in colostrum (1). Where IgG appears in monomeric form, IgA and IgM appear in multimeric form. The neonate absorbs all three immunoglobulin classes, but IgA is partly released back into the intestinal lumen to for local, mucosal protection (2). IgM is mainly involved in primary immune response. Because IgA and IgM have a relatively short half-life (3-4 days) compared to IgG (21-28 days) (3, 4), they only perform protective functions in the neonate for a short time. Usually, colostrum quality is expressed in terms of IgG concentration, which is highly variable between cows (5, 6). Colostrum of high quality typically contains a concentration of >50 g/L, while lower concentrations indicate colostrum of low quality (1). It has been widely accepted that a calf should have a serum IgG concentration of at least 10 mg/mL between 24 and 48 hours after birth (1). By definition, there is a failed transfer of passive immunity (FPI) when this criterium is not met, which is correlated with higher incidence of early calf morbidity and mortality as reviewed extensively by many (1, 7, 8). More recently, Lombard et al. suggested to revise this guideline into a new standard, in which the dose-dependent relationship between calf serum IgG concentration and calf morbidity is taken into account (9). In practice, achieving successful transfer of passive immunity through colostrum is one of the main challenges in calf rearing.

Optimal on-farm colostrum management is essential to ensure adequate transfer of passive immunity and provide the best start for newborn calves. Many studies have been conducted to define optimal management strategies for colostrum feeding. Farmers, veterinarians and feed advisors have adopted the “Three Q’s” as a general guideline for providing colostrum: Quantity, Quality and Quickness of feeding. In addition, sometimes two “Q’s” are added: “Quantifying the transfer of immunoglobulins” and “sQueaky clean” (10). In practice, farmers strive to provide a sufficient amount of high quality colostrum as quickly as possible to ensure adequate transfer of passive immunity. Meanwhile, bacterial contamination should be minimized and to ensure proper absorption by the calf, serum IgG concentrations should be monitored between 24-48 hours of age. An overview of all existing knowledge on colostrum management and its effects on colostrum quality and immune transfer to the calf is lacking. We therefore conducted a scoping review on the effect of several on-farm management factors on immunological colostrum quality and transfer of passive immunity by the calf. We grouped the effects into 1) the milking methods, 2) colostrum treatment and storage and 3) administration procedures. The first two are aimed to optimize the quality of colostrum with regards to IgG concentration, while the latter aims to achieve most efficient colostral IgG uptake by the calf. Our research question for this scoping review was defined as: What is the up-to-date evidence on the interactions between on-farm colostrum management, colostrum quality and passive immunity.

Materials and Method

Literature search

Separate CAB Abstracts searches were performed for each of the subjects of interest. All searches included the following terms: (cow OR cattle AND colostrum).mp. The "mp" includes all articles with selected key terms in their abstract, title, original title, broad terms, heading words, identifiers and cabicodes. To identify papers concerning milking methods, these general terms were combined with the term (milking).mp. To identify papers describing storage and treatment methods, the general terms were combined with the terms (storage OR treatment OR heating OR pasteurization OR pasteurization OR temperature OR refrigeration OR thawing OR heat OR frozen).mp. Finally, papers describing feeding methods were identified by extending the general terms to (cow OR cattle OR calves OR calf AND colostrum).mp, and combining these with (bucket feeding OR tube feeding OR esophageal feeding OR feeding method OR feeding technique OR feeding frequency OR bottle feeding OR suckling OR calf feeding).mp.

Inclusion criteria

This review only includes peer-reviewed articles presenting primary research to farm-related management of fresh bovine colostrum for calf feeding and the effects on (colostral and/or serum) immunoglobulins, leukocytes and other potential immune components. Articles were written in English or Dutch, and articles were included only if full text was available. Articles published up until November 9, 2020 were included. Additional selection criteria were included for the separate topics. All selected papers contain studies with common breeds of dairy cattle that were healthy and did not undergo specific treatment before the study period (e.g., no specific vaccination prior to calving or dry feeding strategies). For the selection of papers on milking strategies, only papers addressing milking of dairy cows were included. For selecting papers describing storage and treatments, reports involving bacterial counts were included during the first selection phase. During the second evaluation (full text articles), studies on bacterial counts were only included if they contained information on immune parameters and did not involve in vitro studies on the effects of specific bacterial strains. For selecting the papers evaluating feeding methods, papers were included when colostrum feedings were from fresh bovine colostrum, when feedings occurred within 24h after birth, and when serum immune concentrations were assessed at least within 24-48h after birth. For suckling of the calf, beef cattle studies were selected as well. The first author performed data retrieval. For each search, titles and abstracts were scanned for the selection criteria described above. Of the remaining papers, the main text was evaluated for relevance. In addition, the first author assessed the articles when uncertainty existed on whether or not to include a paper; this was discussed with the second author.

Results

Milking methods

Search methods.

There were 642 records identified using keywords for “milking methods” as described. Two hundred fifty-three records were excluded because they were not in English or Dutch. Title and abstract first screening led to the exclusion of 357 papers that did not contain original research data (such as reviews), were not peer reviewed (such as conference proceedings), and/or did not fit the inclusion criteria. The remaining 32 articles were assessed full text. Three reports were added based on references in full-text read articles. One paper was excluded for it was unavailable full text; six others were excluded because they did not fit the selection criteria. A flowchart summarizing the selection process can be found in Figure 1. In total, 28 articles were included, of which 2 animal studies and 26 population studies. An overview of the study types can be found in Table 1.

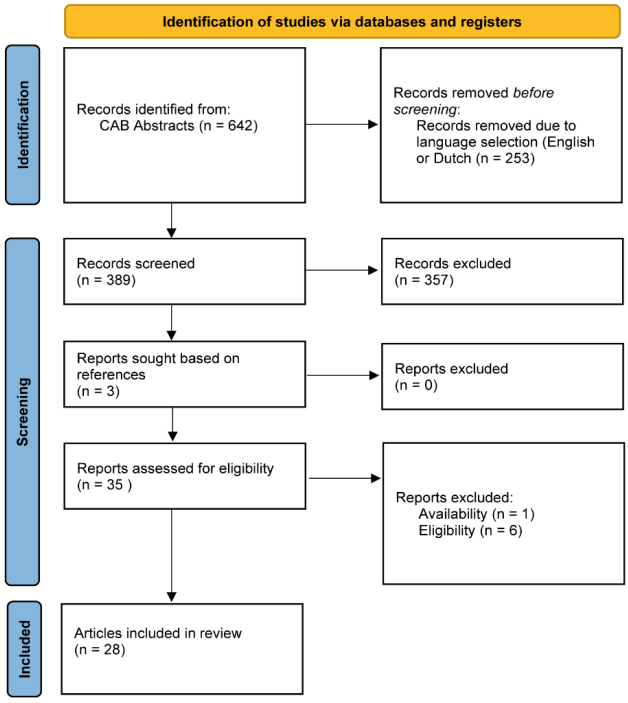


Figure 0-1. Flowchart depicting the article selection process for the subsection “milking methods”.

Table 1. Overview of the study types included in the results, separate columns for each of the sections: colostrum milking, treatments and storage, and administration procedures. Note that the number of total study types for "Colostrum treatments and storage", 56, does not match the number of articles included in our review. This is because some articles present more than one study type, e.g., a combination of laboratory study type and animal study type.

	Milking methods	Colostrum treatments and storage	Administration procedures	Total
Animal studies	2	24	36	62
Laboratory studies		26		26
Population studies	26	6	18	50
Total	28	56	54	138

Time between calving and first colostrum milking

Timing of first colostrum milking is thought to affect immunoglobulin concentrations of colostrum. Many studies agree that by shortening the interval between parturition and milking, the concentration of Ig in colostrum is higher, consequently improving colostrum quality. (6, 11-20). Still, the time after which the immunoglobulin concentration starts to decline is debated. Conneely et al. (2013) found that colostral IgG concentrations in milkings occurring 0-3h, 3-6h or 6-9h post-partum were similar and reports a significant reduction over 9h post-partum (13). These results are confirmed by other studies, that found no negative correlation between colostral IgG and time interval when milked within 3-9 hours post-partum (21-24). Kessler et al. (2020) reported however that milking colostrum within 3 hours post-partum results in significantly higher IgG concentration compared to milking colostrum later. Between 3-12 hours post-partum the concentration remains relatively similar (6). Both Kruse (1970) and Moore (2005) found highest colostral immunoglobulins in colostrum milked within 2 hours (11, 12). Morin (2010) and Conneely (2013) described respectively a 3.7% and 1.1% decrease in colostral IgG concentration with every hour the milking was delayed (13, 14), suggesting milking directly after calving is most optimal. A delay in first colostrum milking may result in dilution of the immunoglobulin content, as colostrogenesis and thereby the transfer of immunoglobulins into colostrum is believed to end at parturition. As the time interval until first milking increases, lactogenesis is initiated and milk yield increases (13). Several studies found a negative correlation between first milking yield and immunoglobulin concentration (13, 18, 20, 22, 25, 26), indicating that delayed milking leads to dilution of the colostrum, thus reducing the antibody concentration. However, Conneely et al. (2013) argue that even after adjusting for the colostral weight, cows that were milked at a later time still produced colostrum with lower IgG concentration. Additionally, some studies did not find an association between increased first colostrum yield and the concentration of immunoglobulins (12, 23, 24, 27, 28). Kessler et al. (2020) propose that the process of colostrogenesis does not abruptly end at parturition and that immunoglobulin transfer continues a few hours after calving. This would explain their findings that colostrum milked within 30 minutes post-partum contains lower IgG concentrations than colostrum milked 3 hours after calving (24).

Fractional milking strategies

Following parturition, colostrum can be milked out completely, but it is also possible to e.g., collect only the quantity needed to cover the needs of the calf and milk the cow partially. Using different fractions of colostrum (other than milking completely) may affect the colostral antibody concentrations. Stott et al. (1981) studied whether colostrum collected

from the cisterns contained higher concentrations of immunoglobulins. From each quarter, 100ml was collected, after which the remaining colostrum was milked completely. For IgG, IgM and IgA no concentration differences were observed between the cisternal and the complete colostrum (29). Other studies found similar results (30-32). However, Ontsouka et al. (2003) obtained samples on day 2 after calving, which usually contains lower levels of immunoglobulins (31). Godden and Hazel (2011) assessed IgG concentrations in different fractions of colostrum. During the entire process of colostrum collection, every 30 seconds a 10mL sample of colostrum was collected into a syringe through a sampling port that was located in the milk line. This enabled them to make a clear distinction of the separate fractions. In contrast to other findings, they describe a clear difference in IgG concentration between cisternal and composite samples. Moreover, they found higher IgG concentrations in cisternal colostrum compared to the first quartile, the first half and the first three quarters of the first milking. Milking 25%, 50% and 75% of colostrum showed no IgG concentration differences (33). Despite contradicting observations regarding fractional milking, the use of cisternal fractions of the first streaks of colostrum to estimate its quality is discouraged, as those samples may not represent total colostrum quality (33). Sroka et al. (1998) suggest milking completely and save the remaining colostrum after first feeding for the next feedings, since consecutive milkings from cows milked out completely contain lower IgG concentrations (34).

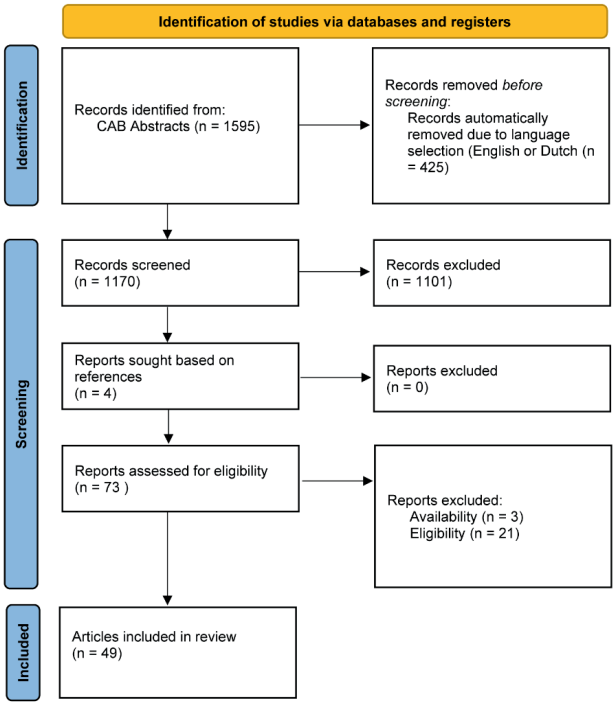
The effect of milking individual quarters at different time points as fractional milking strategy is not extensively studied. Madsen et al. (2004) reported some small differences in IgG concentrations when one quarter was omitted for milking, but omitting one quarter did not affect total IgG yield. This was explained by the neutralization of all quarters in the fourth milking (35). Also Gomes et al. (2011) and Kessler et al. (2020) did not find any significant differences in IgG, IgM or IgA concentration between quarters (24, 36), although large variations in production between quarters were observed (24). Baumrucker et al. (2014) on the other hand found that the front quarters produced colostrum with a higher concentration of IgG compared to the rear quarters (37).

Colostrum treatment and storage

Search methods.

There were 1595 records identified using keywords for "milking methods" as described. Four hundred twenty-five records were excluded because they were not in English or Dutch. Title and abstract first screening led to the exclusion of 1101 papers that did not contain original research data (such as reviews), were not peer reviewed (such as conference proceedings), and/or did not fit the inclusion criteria. The remaining 69 articles were assessed full text. Four reports were added based on references in full-text read articles. Three papers were excluded for they were unavailable full text; twenty-one others were excluded because they did not fit the selection criteria. A flowchart summarizing the selection process can be found in Figure 2. In total, 49 articles were included, of which 24 animal studies, 26 laboratory studies and 6 population studies. An overview of the study types can be found in Table 1. The numbers in table 1 for "Colostrum treatment and storage" add up to 56 instead of 49. This is because 7 articles combined two study types, for example laboratory study and animal study.

To review the effects of storage, we decided to make a distinction between short term and long term storage or treatment effects. We defined short term storage or treatment as on-farm practices applied to freshly milked colostrum used to feed calves within 48 hours after milking. In case dams cannot produce a sufficient volume or high colostrum quality, there is a need for stored colostrum from other cows that calved earlier. Thus, it is important to evaluate the effect of long term storage of colostrum on quality as well. We defined long term storage a method applied to store or treat fresh colostrum for a period longer than 48 hours after milking.



Short term storage

Some studies have investigated the effect of short term storing conditions on the presence of (pathogenic) microorganisms in colostrum. Fewer studies looked into effects on immunological parameters.

Storing at different temperatures

Storing colostrum at room temperature or at 4 °C did not affect IgG concentration (38). However, an association was found between increased bacterial counts (>1.000.000) and decreased calf serum IgG levels after storage at higher temperature (22°C) (39). With respect to storing colostrum in a refrigerator, Langel et al. (2015) note that refrigeration (4°C) up to 8 hours did not affect cell viability (40), whereas effects of refrigeration for a longer period are yet unclear. We did not find other literature investigating effects of short term storage of colostrum such as temperature and/or duration, on colostral Igs or other immune components in colostrum.

Pasteurizing colostrum

Pasteurization of milk is widely used to eliminate the number of microorganisms to a minimum at which milk can safely be used for human consumption. Pasteurization can also be applied to minimize the amount of pathogens in colostrum in order to minimize health risks for the calf. As the effects of pasteurization on pathogen elimination are widely ascertained, we focus on the effects of pasteurization on the immunological content of colostrum by looking at immunoglobulins in particular. In addition, we payed attention to the apparent efficiency of absorption (AEA) of immunoglobulins by the neonate.

Effects on colostrum IgG

Protein structure can be altered by temperature and therefore heating of colostrum can influence availability and functionality of proteins including immunoglobulins. The majority of studies investigating pasteurization effects focus on colostral or calf serum IgG. In all existing literature a variety of combinations regarding temperature and duration of pasteurization is applied and therefore these studies are a challenge to compare. We summarized the results of the existing literature in Supplementary table 1. The majority of studies show that colostral IgG concentration is not (41-53) or only slightly affected (54-57) by heating at less than 60°C for either 30 or 60 minutes. In contrast, heating of colostrum above 60°C frequently resulted in significant loss of colostral IgG (41-48, 50, 54, 58-60). Heating colostrum at a temperature of 60°C for 30 or 60 minutes leads to significant reduction in bacterial counts, while viscosity remains similar and there is only a slight reduction in colostral IgG concentration (54). Several studies noted that the loss of IgG depended on the original quality of colostrum and found that colostrum of high quality suffered from a greater reduction in IgG concentration compared to colostrum of low quality (41, 47, 58). Meylan et al. (1996) argue that high quality colostrum is more likely to aggregate, leading to protein clump formation, which results in denaturation of the protein (58). However, different results were obtained by Balthazar et al. (2015), who observed a greater IgG losses for low-quality colostrum compared to high-quality colostrum (61).

Effects on serum IgG and Apparent Efficiency of Absorption (AEA)

While pasteurization of colostrum can result in slight reduction of colostral IgG concentration, it has been suggested that calf serum IgG levels are potentially increased when

colostrum is pasteurized. The study by Johnson et al. (2007) was one of the first to describe that there was no noteworthy difference in IgG observed between pasteurized and unpasteurized colostrum, but calf serum IgG levels were significantly higher in calves fed pasteurized colostrum (22.34 mg/mL vs 18.07 mg/mL)(42). Subsequent studies revealed similar results: heat treatment of colostrum at 60°C for 30 or 60 minutes preserved both viscosity and IgG levels of colostrum, while serum IgG and AEA was higher in calves fed the heat treated colostrum compared to calves fed unheated colostrum (43, 44, 47-49, 57, 62, 63). Possibly, degradation and denaturation of proteins that would otherwise compete with the intestinal absorption of IgG contributes to increased IgG absorption after pasteurization. Also, immunoglobulins transferred with colostrum could bind to bacteria, thereby inhibiting them from passing the epithelial barrier. When the number of bacteria is reduced by pasteurization, this can lead to increased amounts of free immunoglobulins which can pass the epithelial barrier. This theory is supported by the work of Gelsinger (2015). Colostrum with a high bacterial count – but with similar IgG concentration - resulted in decreased AEA compared to colostrum low in bacterial count (62). However, in an earlier study performed by Elizondo-Salazar no such difference in AEA was noted (44). Perhaps entire bacterial dead cells or their fragments may still attach to colostral immunoglobulins in the gut and thus reduce their absorption.

Although pasteurization of colostrum *above* 60°C is generally associated with decreased colostral immunoglobulins, several studies noted that this does not automatically lead to decreased calf serum concentrations. Tyler et al. (2000) studied the effects of pasteurizing at different temperatures and found that heating colostrum to 63°C for 30 minutes did not affect serum IgG in calves compared to unpasteurized colostrum. Pasteurization at 76°C did lead to lower serum IgG levels (64). Also, Bush et al. (1982) even found calf serum IgG to be increased 12 hours after calves ingested pasteurized colostrum (63°C) compared to calves fed unpasteurized colostrum. In subsequent measurements, this difference diminished (65). Also Lakritz et al. (2000) found no significant effect on serum IgG concentrations between calves fed pasteurized (76°C) or unpasteurized colostrum (66). However this study was performed with a small sample size and results should be interpreted with care. Stabel et al. (2008) reported that over the long term (one month) no differences in calf serum IgG are found between calves fed pasteurized colostrum (65°C) and calves that suckled fresh unpasteurized colostrum (67).

While the majority of the studies focus on loss of colostral IgG concentration, few paid attention to loss of IgG functionality. McMartin et al. performed serum neutralization assay to determine colostral antibody activity in samples pasteurized at either 60°C or 63°C. While no adverse effects of pasteurization on antibody activity were found, the authors emphasize that these results should be interpreted with care, as many samples were lost due to congealing in the process of pasteurization (41).

Effects on other immunological factors

Lactoferrin is an immune component of colostrum, which is known to possess immunomodulatory and antimicrobial properties and is therefore interesting to look into. A few studies have looked into the effects of heating colostrum on lactoferrin levels. Pasteurization at 60°C, 63°C, 72°C and 76°C lead to significant reduction of lactoferrin concentrations in colostrum and in calf serum, as visualized in Supplementary table 2 (66, 68, 69). These results largely agree with the study by Shimo et al. (2015), who recorded

significant aggregation and denaturation of lactoferrin at temperatures above 63°C, especially when pH was increased as well. Below 63°C, no significant denaturation occurred (70). Other immunological compounds in colostrum such as cytokines were studied as well. Gelsinger and Heinrichs (2017) found that neonatal absorption of colostral interferon-γ was not affected by pasteurization at 60°C for 60 minutes. On the other hand, interleukin 1β concentration in calves receiving heat-treated colostrum was decreased. The authors suggest that the neonatal immune response is not inhibited by heat treatment of colostrum (55). In the same study, calves were subcutaneously challenged with ovalbumin to determine B cell activity and thus antibody production. Calves receiving heat treated colostrum showed higher ovalbumin specific IgG levels to this challenge indicating that neonatal B-cell function was not affected by heat treatment of colostrum. However, calves receiving unheated colostrum tended to recover their average daily gain faster after the immune challenge. During the Minnesota Dairy Health Conference in 2012 Godden et al. discussed their study investigating whether viability of colostral immune cells was affected by the process of pasteurization. Indeed, viability was reduced but not completely eliminated after pasteurizing for 60 minutes at 60°C. Also the authors state that although viability of colostral immune cells is reduced, more research is required regarding functionality and biological meaning of colostral cells (45).

Freezing and thawing of colostrum

As freezing will always be inextricably associated with thawing, investigating the effect of freezing on colostrum quality should be combined with thawing, just as thawing should be studied in combination with freezing. Hence, we will review studies investigating freezing and/or thawing and combine results in our analysis.

Effects on colostrum IgG

Several studies examined the effects of freeze/thawing on IgG content of colostrum and serum IgG (Supplementary table 3). To our best knowledge, there are no studies investigating thawing at room temperature.

Wiking and Pedersen (2009) studied several ways to thaw and heat colostrum in a microwave oven. Heating of refrigerated colostrum samples did not directly lead to a loss of IgG. Thawing of frozen colostrum by microwave resulted in unevenly heated colostrum and clotting, however no information of IgG concentration was reported (71). Jones et al. (1987) examined thawing by microwaving at two different microwave settings (325 and 650 Watt) and found no differences in IgG or IgM content as compared to thawing au bain-marie (45°C). However, small losses of IgA were found (72). Balthazar et al. (2015) found that increasing the power of a microwave was associated with a significant greater loss of IgG_1: 20% loss at 200W versus 31% loss at 350W (61). Heating to 50 and 60 °C au bain-marie resulted in a similar IgG loss as heating at 40°C (8%), while heating above 60°C resulted in a significant (26%) reduction in IgG1. This is in line with other studies on pasteurization showing greater loss of IgG when colostrum is heated above 60°C. Losses of IgG_1 were greater for the low quality and thus low IgG_1 fresh samples compared to the high quality fresh samples (61). With regard to repeated freeze/thawing, Haines et al. (1992) did not find significant changes in IgA, IgM or IgG concentrations in a single colostrum sample after multiple freeze-thawing cycles with a water bath at 37°C (73). However, since this study only included one sample these results should be interpreted with care. A larger study by Morrill et al. (2015) showed that freeze/thawing for a single time does not reduce colostral IgG concentration. Compared to fresh colostrum, repeated freeze/thawing leads to a significant decrease of 7.8% and 7.7% for two and three freeze/thaw cycles, respectively (74). To our knowledge, no studies have examined how repeated freeze/thawing affects colostral immunoglobulin stability or function.

Effects on serum IgG

Few studies looked into the effects of feeding colostrum after freezing/thawing on serum immunoglobulin concentration of neonate calves. Olson et al. (1989) found that both serum IgG_1 and IgG_2 levels were lower in calves fed au bain-marie heated (41°C) colostrum compared to calves fed microwave heated colostrum (312W, heated to 41°C), while colostral IgG_1, IgA and IgM concentrations were similar and the IgG_2 was only marginally lower. The authors note that these results should be interpreted with care, since the size of the study was quite small (75). Holloway et al. (2001) and Donovan et al. (2007) found no significant differences in serum IgG concentration between calves fed frozen and thawed colostrum and calves fed fresh colostrum (76, 77).

Effects on other immunological factors

With respect to other immune components of colostrum, Holloway et al. (2003) studied whether freeze/thawing affected lactoferrin concentrations in colostrum and calf serum. No difference in lactoferrin concentrations was observed between fresh and frozen colostrum. Serum lactoferrin concentration on day 2 did not differ between the calves fed fresh or freeze/thawed colostrum, however on days 4 and 7 serum concentrations were higher in the freeze/thawed colostrum fed calves. Unfortunately, the authors were unable to determine whether the measured serum lactoferrin was derived from colostrum or from endogenous origin (78). In addition to immunoglobulins and lactoferrin, colostrum contains maternal leukocytes as well. The general opinion is that leukocytes remain viable and functional under certain specific circumstances: the optimal temperature for mammalian cells is 37°C, increasing the temperature above 42°C leads to denaturation of proteins and destruction of the cell, and freezing leads to intracellular ice crystal formation and thereby to damage and even lysis of cells. The assumption that colostral cells are indeed destroyed during freezing is supported by the studies by Novo et al. (2017). Using Trypan blue to check for cell viability, they report that no viable cells were found in their freeze/thawed colostrum (79, 80). Many papers studying the effects of leukocytes in colostrum make use of freeze/thawing for their control group of cell-free colostrum. Donovan et al. (2007) studied the functionality of maternal colostral cells in neonatal calves. Calves were fed fresh colostrum, freeze/thawed colostrum or cell-free colostrum, all from dams vaccinated against Bovine Viral Diarrhea Virus (BVDV). The group fed fresh colostrum, with colostral cells, showed increased in vitro proliferative responses after stimulation with BVDV. Both the thawed and cell-free colostrum groups did not. The observed difference was attributed to the functionality of transferred maternal cells (77). These results combined with other results of studies using freeze/thawing as a valid method to lyse maternal cells in colostrum, indicate that freezing of colostrum indeed destructs colostral leukocytes (40, 79-81). On the contrary, Stieler et al. (2012) found increased neutrophilic activity in calves fed fresh frozen colostrum compared to calves fed fresh colostrum, although not significant. No differences in activation capacity was observed after follow up with milk replacer for 21 days. The authors attributed the increased neutrophilic activity in the frozen/thawed colostrum fed calves to the release of transfer factors by lymphocytes in response to freezing, leading to stimulation of the cellular immune response (82).

Other treatments

Few studies have experimented with high pressure techniques to eliminate bacterial counts in colostrum. Masuda et al. (2000) reported effective suppression of bacterial growth for 9 days at 4°C after treating colostrum at 300 and 400 MPa for 10 minutes. Up to 300 MPa, IgG remained intact, but application of 400 MPa resulted in altered viscosity of the colostrum and denaturation of IgG (83). Indyk et al. (2008) and Foster et al. (2016) found colostral IgG to be stable up to 400 MPa treatment, as long as duration was limited to 30 minutes. Increasing pressure (500 or 600 MPa) or duration resulted in increased denaturation and aggregation (84). Apparently, IgG in colostrum is more stable compared to IgG isolated from colostrum. Probably the colostral environment enhances the stability of immunoglobulins (85). The use of formaldehyde to preserve colostrum is sometimes applied in warm ambient climates when refrigeration is not an option. Mbuthia et al. (1997) found treatment with formaldehyde to be the best method for preserving immunoglobulin content in colostrum for up to four weeks compared to treatment with formic acid or natural fermentation (86).

Administration procedures

Search methods.

There were 1407 records identified using keywords for "milking methods" as described. Four hundred eighty-four records were excluded because they were not in English or Dutch. Title and abstract first screening led to the exclusion of 923 papers that did not contain original research data (such as reviews), were not peer reviewed (such as conference proceedings), and/or did not fit the inclusion criteria. The remaining 59 articles were assessed full text. Seven reports were added based on references in full-text read articles. Seven papers were excluded for they were unavailable full text; five others were excluded because they did not fit the selection criteria. A flowchart summarizing the selection process can be found in Figure 3. In total, 54 articles were included, of which 36 animal studies and 18 population studies. An overview of the study types can be found in Table 1.

Achieving adequate passive immunity depends on timely ingestion of a certain level of immunoglobulins and the absorption ability of the calf. In this paragraph we discuss studies looking into the effect of time interval between birth and first feeding, volume of first feeding, and equipment used for colostrum feeding on calf serum Ig status.

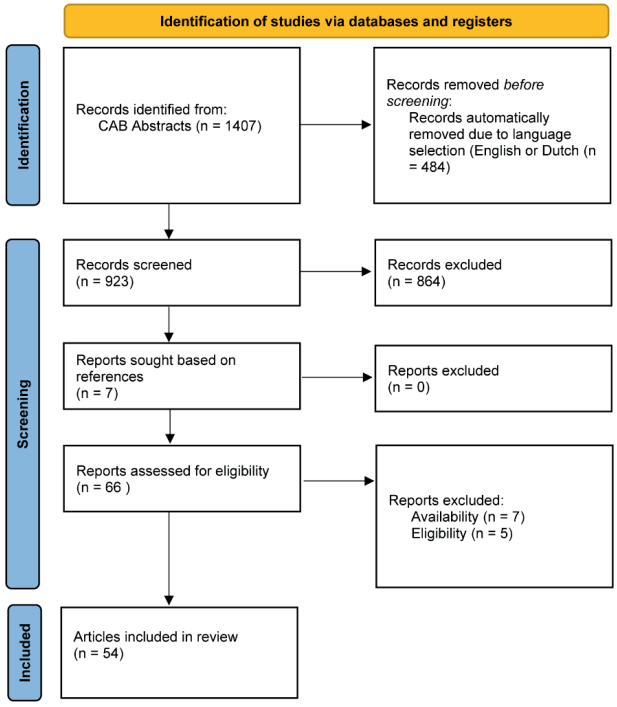


Figure 0-3. Flowchart depicting the article selection process for the subsection "administration procedures".

Timing of first and subsequent feedings

While some studies failed to find an association between timing of first feeding and serum IgG concentration (87, 88), the study by Keulen et al. (1985) showed that besides volume and quality of first colostrum, also the timing of first feeding partially explains variation in calf serum IgG (89). Indeed, most studies agree that the ability to absorb IgG declines with delaying first colostrum feeding. Still, much uncertainty exists about the exact time period to which the first feeding can safely be delayed without affecting the ability to absorb IgG. An early study by Smith et al. (1967) noted that calves receiving their first colostrum at 10-12 hours after birth, showed a larger proportion of low serum immunoglobulin cases compared to calves receiving their first colostrum feeding within 8 hours. The study did not look further into the association between timing of first colostrum feeding and serum IgG or absorption rate (90). While some studies find it safe to feed the first colostrum up until eight hours of age without affecting IgG absorption (91), some claim the absorption rate starts to decline earlier, around six hours of age (92) or even after four hours (93-95). These last studies are in line with the study by Chigerwe et al (2009), where no effect of time (<4 hours) on FPT was observed (96). Another study by Chigerwe et al. (2008) recommended feeding of colostrum within 2 hours of birth (97). The study by Osaka et al. (2014) shows a gradual decline in AEA over time for the first 12 hours. After 12 hours, a more rapid decline is observed (98). Some studies used models to estimate effects of delayed colostrum intake on calf serum IgG. One model showed that calf serum IgG concentration decreased with 2 mg/ml with every 30 minute delay in colostrum intake (99), another found a decrease of with 0,32 mg/ml with each hour the colostrum ingestion is delayed (63). With respect to other immune components, Zanker et al. (2001) showed that delayed feeding of colostrum up to 24 hours post-partum does not affect hematological values such as the total number of leukocytes in the neonate (100).

Absorption of IgG can be affected by the timing of the first colostrum, but also by timing of subsequent feedings. In the study by Fallon et al. (1989) it was shown that an additional feeding within 12h significantly increased the serum immunoglobulin content (101). Timing of first colostrum feeding also affects the effectiveness of subsequent colostrum feedings. Calves fed colostrum within the hour with subsequent feedings at intervals of 8 hours showed higher serum IgG than calves fed their first colostrum at 16h or 24h followed by the same subsequent feedings (91).

Volume of feeding

Many studies have attempted to determine the volume that should be consumed by the newborn calf to obtain first passive immunity. While investigations were carried out carefully, they differ from each other in terms of colostrum quality and timing of the first feeding. Therefore, a quantitative comparison of these studies is hardly possible. Some studies suggest to determine colostrum volume by means of the bodyweight of the newborn calf (102, 103). Conneely et al. (2014) propose that calves should ingest a volume corresponding to 8.5% of their body weight (within 2 hours of age) (102). Some studies however claim that calves need to ingest a fixed amount of IgG to achieve successful transfer of passive immunity (serum IgG concentration >10 mg/ml) within 24-48h of age. Some propose to provide at least 100 grams of IgG in total (26, 104), others deem this insufficient and suggest to feed 150-200 gram of IgG or possibly even more (97, 105). Morin et al. (1997) conducted a series of experiments in which they extensively studied several combinations of colostral quality, volume and timing of feeding on serum IgG in newborn calves. They found

that feeding 4L of high quality colostrum (60.1 mg/ml) within 3 hours after birth, followed by another 2L at 12 hours after birth resulted in highest serum IgG at both 24 (31.1 mg/ml) and 48 hours after birth (30.4 mg/ml). In total, these calves consumed >360 g IgG. Calves receiving 240 grams of IgG, divided over 2 feedings of 2L showed serum IgG concentrations of >20 mg/ml at both 24 and 48h after birth (105), indicating sufficient transfer of passive immunity. For low quality colostrum (32.9mg/ml), they recommend to feed an additional 2L at 6 hours of age next to feeding 2L at birth and 12 hours of age (105). Different conclusions were drawn by Jaster (2005). For low quality colostrum he observed higher serum IgG levels in calves fed 4L at once compared to calves receiving the same amount equally divided over a feeding moment at birth and at 12 hours. For calves fed high quality colostrum, higher serum IgG levels were observed at 24 and 48h when they were fed 2L at birth followed by another 2L at 12h compared to calves fed 4L at once, suggesting a maximal absorption capacity for IgG (106). Kaske et al. (2005) found also that providing 4L containing 213 grams of IgG resulted in highest serum IgG levels (25.2 mg/ml). Feeding 2L containing 97.4 grams of IgG resulted nevertheless in serum concentrations of 14.1 mg/ml, which is still above the threshold of 10 mg/ml (107). Hopkins and Quigley (1997) point out that providing 3.8L in either one or two feedings results in equal serum IgG concentrations, considered colostrum is of good quality (>50g/L IgG) (108). Chigerwe et al. (2009) suggest calves should voluntary ingest as much colostrum as possible within four hours of birth. Depending on the volume that is voluntarily ingested, they recommend a second feeding at 12 hours of age (96). An on-farm study by Halleran et al. (2017) found calves fed 5.6L within 12 hours of age had significant higher serum IgG concentrations than calves fed 4L (109). A large retrospective study identifying risk factors contributing to FPT by Renaud et al. (2020) showed that feeding >6L within the first 24h of life was associated with decreased risk of FPT (110).

Feeding methods

Many studies evaluated the effectiveness of several feeding methods to achieve successful transfer of passive immunity. Many studies looking into suckling with the dam showed that voluntary suckling (111, 112) and assisted suckling (113-115) can result in sufficient serum immunoglobulin concentrations. However, most studies report that suckling with the dam leads to high risks of FPI (26, 101, 116-120). A possible explanation is that there seems to be a large variation in the time it takes calves to voluntarily suckle (121) and this can increase up to more than six hours (122). Studies by McBeath and Logan (1974) and Rajala and Castren (1995) therefore point out the importance of supervision when calves are left with the dam to suckle (123) (99). Additional studies showed that calves assisted to suckle after 6 hours were still able to achieve proper transfer of IgG, while those not assisted showed FPI (95, 122, 124). Altogether, it appears that (assisted suckling) can lead to adequate transfer of passive immunity, but these methods do in general result in lower serum IgG levels when compared to active methods to deliver colostrum, such as esophageal or bottle feeding.

The use of a nipple bottle or (nipple) bucket is a common method for on-farm administration of colostrum. With respect to feeding with a nipple bottle, voluntary intake of colostrum by nipple bottle leads in many cases (31%) to insufficient (<2L) ingestion of colostrum (125). While some studies reported higher immunoglobulin concentrations in calves that were nursed by the dam in comparison with calves fed by nipple bottle (112, 114), others found no difference (115, 126) or higher serum immunoglobulin concentrations for bottle fed calves (113, 127). When comparing feeding colostrum with a nipple bottle or bucket, no significant

difference in serum IgG was observed at 24 or 48h (128) or at 96h (101). The use of an esophageal feeder is effective for achieving adequate passive immune transfer (129, 130). Several studies have compared the use of esophageal feeding of colostrum to other feeding methods (Supplementary table 4). When compared to voluntary suckling, esophageal feeding seems to be the better option for preventing FPI (26). However, some studies in beef calves suggest that when calves are assisted for suckling, serum IgG levels are comparable to that of calves fed with esophageal feeder (131, 132). When bottle and esophageal feeding methods are compared, esophageal feeding results in either higher (107) or comparable serum IgG concentrations (133-138). Kaske et al. (2005) showed increased serum IgG levels in calves fed 4L colostrum by esophageal tube compared to 2L bottle fed calves (107), however these feedings differ both in volume and in method offered and therefore these results are difficult to compare. A more comprehensive study was carried out by Godden et al. (2009) by investigating the differences between esophageal and bottle feeding at two different volumes. When using a fixed volume of 3L colostrum replacer by either bottle or esophageal feeding, no differences in serum IgG were observed, which was later confirmed by Desjardins-Morrissette et al. (2018) as well (104, 136). However, feeding a smaller volume of 1.5L resulted in higher serum IgG levels in bottle fed calves when compared to tube fed calves. This result implies a possible association between the method of feeding, the volume fed and the serum IgG in calves. As pointed out by Lateur-Rowet and Breukink (1983) feeding colostrum with the use of an esophageal feeder led to failure of the esophageal groove reflex, consequently leading to colostrum being led to the rumen and reticulum, instead of the abomasum. Rapid flow from reticulorumen to abomasum was observed though (130). Kaske et al. (2005) estimated that the time of emptying the contents of the reticulum into the abomasum lies between 2-3h (107). This may explain the results found by Godden et al. (2009) (104): when smaller volumes of colostrum are fed by drenching, the reticulum would not easily overflow and colostrum does not enter the abomasum quickly. However, when larger amounts are fed, the reticulum overflows and colostrum is directly transferred to the abomasum where first digestion occurs (138). Chigerwe et al. (2008) calculated the optimal volume fed by esophageal tube for adequate transfer of passive immunity. Calves fed by esophageal tube require at least 150-200 grams colostral IgG, which can be translated to the recommendation of 3L colostrum as soon as possible after birth, given the colostral Ig concentration is at least 50mg/ml (97).

Discussion

We attempted to provide a complete overview of all existing literature about on-farm colostrum management strategies and their effects on colostrum quality and calf immune status. With respect to milking methods, most studies included are observational population studies (26 out of 28). Results obtained from this type of study designs are often a valid reflection of field conditions and therefore have high external validity. The majority of the studies agrees that increasing the interval between calving and first milking decreases the concentration of IgG in colostrum. Probably, this reduction in IgG concentration is largely, but maybe not only, the result of a dilution effect. Dilution also explains decreased IgG concentrations in subsequent milkings. Therefore, milking the cow completely and as soon as possible after parturition is highly encouraged and will likely result in the highest colostral IgG concentrations. We discourage to measure colostral IgG quality by measuring only the first streaks, for it may not represent the quality of the complete milking of colostrum.

Regarding storage and treatments of colostrum after milking but before feeding it to the newborn, study designs in our results include mostly animal studies (n=24) and laboratory studies (n=26). Laboratory studies have high internal validity, and combined with evidence from animal studies and some population studies, we think the associations described here are quite strong. The most studied treatment of colostrum is the use of pasteurization. Effects of both duration and temperature have been studied extensively, of which the latter seems to affect colostral proteins the most. From the cited studies, sixteen studies looked into the effects of pasteurizing at 60°C on IgG levels, of which twelve concluded pasteurization can safely occur at 60°C without drastically affecting colostral IgG. Two out of four studies finding reduced colostral IgG concentrations looked into effects on calf serum IgG as well and concluded that calf serum IgG remained unaffected or even increased. Increasing temperature of pasteurization (>60°C) and prolonged duration of pasteurization lead to decreased levels of colostral and serum IgG. Pasteurizing small batches is associated with a smaller loss of immunoglobulins compared to pasteurizing larger batches, since larger batches require prolonged exposure to heat as opposed to smaller batches. From these results we conclude that heating up until 60°C results in a minimal reduction of colostral and calf serum IgG concentrations. Together with findings that total bacterial counts and coliform counts are diminished by heat treatment (47, 52), pasteurization could provide protection against neonatal disease development (46, 139, 140). Feeding pasteurized colostrum and milk for a prolonged period (21 days) results in long term health effects, including reduced morbidity, increased body weight and increased milk production during the first lactation (141).

Freezing does not, or only slightly affect colostrum IgG concentration or serum IgG levels. Thawing by au bain-marie method up to 40°C is best, using a microwave results in unevenly heated colostrum, leaving some parts to remain frozen and other parts to be heated to a degree that proteins denature. Similar to the pasteurization process, we advise to thaw (and thereby freeze) in small portions to reduce the duration of heat exposure. Repeated freeze/thawing is discouraged, as colostral IgG concentrations diminish after multiple freeze/thaw cycles. We did not find studies in which thawing at room temperature or in a refrigerator was studied, however it is known that total bacterial counts will increase over time when left at room temperature (38, 39). Effects of freeze/thawing on other colostral immune components are not well understood, but seems to reduce immunity cells viability. A minority of studies involved the treatment of colostrum by fermentation, freeze drying or gamma radiation. Fermentation is a method not commonly applied, however freeze drying and gamma radiation are performed by commercial suppliers of fresh colostrum replacements. In this review we aimed

to address colostrum practices with regard to fresh colostrum in on-farm situations, therefore these methods of colostrum treatment were not included within this research.

For administration procedures, we found 36 animal studies and 18 population studies. The animal studies were often randomized controlled studies and thus provide strong internal validation. Together with the population studies with high external validity, we considered this a substantial body of evidence. Most studies agree that the ability to absorb IgG is highest directly after birth and declines with time. The ability to transfer macromolecules such as immunoglobulins decreases due to the process of "gut closure" (8). With respect to suckling with the dam, when calves are not assisted they often lack vigor to start suckling in time and therefore are at risk of FPI. We recommend to use an active form of colostrum feeding either by nipple bottle or esophageal feeding, to ensure a sufficient volume is ingested. Both methods are suitable for obtaining adequate transfer of passive immunity, as out of the nine studies comparing these methods, the majority (6) reports no significant differences in IgG absorption by the calf. For normal healthy calves we advise to use a bottle for colostrum feeding; esophageal tube feeding can be an invasive procedure and should only be used when calves do not voluntarily ingest a sufficient amount of colostrum. Recommendations for feeding volume vary and each of these recommendations is based on a different principle, for example providing a certain mass of IgG or providing a volume adjusted to the size and birthweight of the calf. Following the first recommendation, the volume to be fed is determined according to the *quality* of the colostrum: when quality of colostrum equals or exceeds 50 g/L, a calf should ingest at least 2L or 3-4L according to the recommendations to ingest 100 or 150-200 grams IgG in total, respectively. When colostrum quality is low (<50 g/L), even more should be fed divided over one or two extra feeding times. The second guideline depends on the *birthweight* of the calf. When the recommendation of feeding 8.5% of the BW is applied, 3.4L is required for an average calf weighing 40kg at birth. All of the guidelines described here provide an approximation of the volume to be administered. In fact, the volume that should be ingested to achieve serum IgG >10 mg/mL, depends on a combination of many factors: the quality of the colostrum (105), the bodyweight (103), and the absorption efficiency of the calf, of which the latter is affected by the timing of feeding and possibly by volume (104) and bacterial content of the colostrum (62). A finite answer to the question how much colostrum should be given exactly, remains difficult. Because some of these determinants of calf serum IgG are hardly quantifiable in on-farm situations, such as the absorption capacity and related factors, it is even more important to at least measure those parameters that *can* be quantified, such as colostrum quality and the weight of the newborn calf. By doing so, a farmer can adjust the colostrum volume to satisfy requirements for each individual calf. Which method should be used to determine colostrum volume, whether calculated by birthweight or colostrum quality, is probably depending on the situation on farm, e.g., which parameters *can* be measured and/or adjusted by the farmer. Ideally, all parameters should be taken into account for determining individual colostrum intake.

Searching systematically for milking, storing and feeding strategies of colostrum enabled us to identify current gaps regarding knowledge on colostrum management. One of the limitations in this field of research, is that the emphasis lies on how concentration of immunoglobulin G in colostrum or eventually calf serum is influenced, while the effects on bioavailability and *in vivo* functionality are understudied. We know bacterial contamination of colostrum reduces intestinal uptake of immunoglobulins, however one of the functions of colostral immunoglobulins is to bind to pathogenic bacteria to prevent colonization. Hence, besides looking into systemic effects, local effects of colostrum immune components in the gastro-intestinal tract should be investigated as well. There is a growing body of literature that

recognizes the transfer colostral leukocytes to newborns and its functionality. Liebler-Tenorio et al. (2002) found that colostral leukocytes are transferred through the epithelial barrier via follicle-associated epithelium of Peyer patches in the neonate gut (142). Protective effects of colostral leukocytes on neonatal immune development are described by Donovan and colleagues (143). In addition, Reber et al. (2008) proposed that presence of maternal leukocytes from colostrum was correlated with faster development of neonatal lymphocytes in the first week of life (144). Despite the promising effects of colostral leukocytes, very little is known about how colostrum management affects the functionality of these maternal cells in the newborn. We do know that uptake of colostral cells by the neonate is not limited by gut closure (145), however how storage methods or pasteurization affects viability or functionality of these maternal leukocytes remains uncertain. There are some limitations to our scoping review. Because of the magnitude of the available literature on colostrum management, it was impossible to cover *all* aspects related to colostrum management procedures and we had to delineate our scope. Therefore, we decided not to include, for example, the effects of treatment and storing on colostral microbiome or specific antibodies studied *in vitro*. Furthermore, in many systematic reviews, two independent persons carry out the search and selection process, we only utilized a single person. As we did not aim to quantify the combined search results in a meta-analysis procedure, we feel one person was sufficient for this scoping review.

This review was set out to evaluate current knowledge on-farm colostrum management processes and how these affect immune properties of colostrum. The findings of this study suggest that optimal colostral IgG can be achieved by milking colostrum directly after calving. If possible, we advise pasteurizing fresh colostrum for 30 minutes at 60°C. Colostrum can safely be stored by freezing and thawing by au bain-marie is recommended to minimize IgG loss. In on-farm situations, determining the volume that should be fed is difficult. The variables contributing to absorption of IgG by the newborn calf are many and include the quality of the colostrum, the bacterial contamination, the time interval between birth and first moment of feeding and the weight of the calf. Despite all knowledge regarding optimal colostrum management strategies, it remains difficult to predict effects of certain colostrum management choices in practice. We therefore recommend measuring the colostral quality and/or weighing the newborn calf and adjust the volume of feeding accordingly to ensure optimal colostrum intake for each individual calf. The main emphasis in this field of study is still on the concentrations of IgG, however consequences of colostrum management on colostral leukocytes and other colostral immune compounds are understudied.

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Supplementary Material

Supplementary tables

Supplementary table 1: Comparison of pasteurization methods and their effects on colostral immunoglobulin and serum immunoglobulin concentrations.

Study	Pasteurizaion process					Calf feeding	Outcome	
	Method	Comparison	Temperature (°C)	Time (min)	Volume (L)	Volume (L)	Colostral IgG	Serum IgG
(Godden et al., 2003)	Batch pasteurizing system1	Fresh frozen colostrum	63	30	57 95	2 4	Reduced IgG Larger effect for larger volumes pasteurized	Reduced IgG (2L feeding) No effect (4L feeding)
(Bush et al., 1982)	Pasteurization	Fresh frozen colostrum	63	30	?	?	?	Increased IgG
(Tyler et al., 2000)	Commercial Pasteurizer2	Fresh frozen colostrum	63	?	3	3	NA	No effect
	Commercial Pasteurizer2	Fresh frozen colostrum	76	?	3	3	NA	Reduced IgG
(McMartin et al., 2006)	Rapid Visco Analyser3	Fresh frozen colostrum	59	120	0.05	NA	No effect IgG	NA
		Fresh frozen colostrum	60	120	0.05	NA	No effect IgG	NA
		Fresh frozen colostrum	61	120	0.05	NA	No effect IgG	NA
		Fresh frozen colostrum	62	120	0.05	NA	Loss of IgG	NA

[1] Batch pasteurizing system, DT Silver, DairyTech Inc., Windsor, CO
2 Pres-Vac Home Pasteurizer, Schlueter Company, Janesville, WI 53545, USA
3 Rapid Visco Analyser, Newport Scientific, Warriewood, Australia

		Fresh frozen colostrum	63	120	0.05	NA	Loss of IgG	NA
(Johnson et al., 2007)	Batch pasteurizing system1	Fresh frozen colostrum	60	60	8	3.8	No effect IgG Increased AEA No effect IgM No effect IgA	Increased IgG Increased AEA No effect IgM No effect IgA
(Meylan et al., 1996)	Water bath	Fresh frozen colostrum	63	30	0.05	NA	Loss of IgG	NA
(Stabel, 2008)	Pasteurization	Fresh (suckling)	65	30	?	4	NA	No effect IgG (1 month)
(Elizondo-Salazar and Heinrichs, 2009b)	Commercial batch pasteurizer4	Fresh frozen colostrum	60	30	28	3.8	No effect IgG1, IgG2 and total IgG	Increased IgG1, IgG2 and total IgG
(Elizondo-Salazar and Heinrichs, 2009a)	Commercial batch pasteurizer4	Fresh frozen colostrum	60	30	21	3.8	No effect IgG1, IgG2 and total IgG	Increased IgG1, IgG2 and total IgG
(Elizondo-Salazar et al., 2010)	Water bath	Fresh frozen colostrum	57	30 60 90	0.01	NA	No effect IgG1 and IgG2 No effect IgG1 and IgG2 No effect IgG1 and IgG2	NA

[4] Girton Manufacturing Co., Millville, PA

	Water bath	Fresh frozen colostrum	60	30 60 90	0.01	MA	Reduced IgG1, not IgG2 Reduced IgG1, not IgG2 Reduced IgG1 and IgG2	NA
	Water bath	Fresh frozen colostrum	63	30 60 90	0.01	NA	Reduced IgG1 and IgG2 Reduced IgG1 and IgG2 Reduced IgG1 and IgG2	NA
(Godden et al., 2012a)	Batch pasteurizing system1	Fresh frozen colostrum	60	60	?	NA	No effect	NA
(Godden et al., 2012b)	Batch pasteurizing system1	Fresh frozen colostrum	60	60	?	3.8	No effect	Increased IgG
(El-Zahar et al., 2014)	?	Fresh colostrum	63	30	?	NA	Reduced IgG	NA
	?	Fresh colostrum	72	0.25	?	NA	Reduced IgG	NA
	?	Fresh colostrum	100	10	?	NA	Reduced IgG	NA
(Gelsinger et al., 2014)	Commercial batch pasteurizer4	Fresh colostrum	60	30	?	3.8	No effect	Increased IgG1, IgG2 and total IgG Increased

								AEA
(Gelsinger and Heinrichs, 2017)	Commercial batch pasteurizer4	Fresh frozen colostrum	60	60	?	8% BW	Reduced IgG	No effect IgG No effect AEA
(Sotudeh et al., 2018)	Water bath	Fresh colostrum	60 55	30 60	0.25	NA	Reduced IgG Reduced IgG	NA
(Saldana et al., 2019)	Commercial batch pasteurizer4	Fresh frozen colostrum	60	30 60	?	3.8	Reduced IgG	Increased AEA
(Kryzer et al., 2015)	Perfect Udder system5	Fresh frozen or refrigerated colostrum	60	60	3.8	3.8	No effect	Increased IgG Increased AEA
	Batch pasteurizing system1	Fresh frozen or refrigerated colostrum	60	60	12.6	3.8	No effect	Increased IgG Increased AEA
(Donahue et al., 2012)	Batch pasteurizing system1	Fresh refrigerated colostrum	60	60	?	NA	No effect	NA
(Rafiei et al., 2019)	On-farm colostrum pasteurization system6	Fresh frozen colostrum	60	30	1.5	10% BW	No effect	Increased IgG Increased AEA
(Mann et al., 2020a;Mann et al., 2020b)	Batch pasteurizing system1	Fresh frozen colostrum	60	60	4	8.5% BW	No effect	No effect IgG No effect IgA
(Elsohaby et al., 2018)	Water bath	Fresh frozen colostrum	60	30 60	0.01	NA	No effect No effect	NA

[5] The Perfect Udder colostrum management system, developed by Dairy Tech Inc. (Greeley, CO)
[6] On farm pasteurization system (V4, Shirmark Group)

	63	30	Reduced IgG
		60	Reduced IgG

Supplementary table 2: Effects of pasteurization on lactoferrin (LF) concentrations in colostrum and calf serum.

Study	Temperature in °C	Duration in minutes	LF Control Colostrum (mg/mL)	LF Heat Treated Colostrum (mg/mL)	LF serum Control colostrum (mg/mL)	LF serum Heat Treated colostrum (mg/mL)
(El-Fattah et al., 2014)	60	60	1.0 ± 0.1	0.67 ± 0.0		
(El-Fattah et al., 2014)	63	30	1.0 ± 0.1	0.57 ± 0.0		
(Teixeira et al., 2013)	63	60	0.28	0.21		
(El-Fattah et al., 2014)	72	0.25	1.0 ± 0.1	0.13 ± 0.0		
(Lakritz et al., 2000)	76	15			1.97 ± 1.0	0.42 ± 0.32

Supplementary table 3: Comparison of freeze/thawing methods and their effects on colostral and/or serum IgG concentration.

Study	Treatment	Comparison	Watt	Temp. start (°C)	Temperature reached (°C)	Time (min)	Volume (L)	Volume given to calf (L)	Effect Ig	Effect serum Ig
(Jones et al., 1987)	Microwave	Microwave, Bain-marie	650	-20	?	10	1	NA	No effect IgG, IgM, lower IgA	NA
	Microwave	Microwave, Bain-marie	325	-20	?	17	1	NA	No effect IgG, IgM, lower IgA	NA
	Bain-marie	Microwave		-20	45	25	1	NA	No effect IgG, IgM, higher IgA	NA
(Olson, 1989)	Bain-marie	Microwave	NA	-29	41	20	1.89	3.78	No effect IgG1, IgA and IgM. Lower IgG2	Lower IgG1 and IgG2 (24h) No effect at 48h
	Microwave	Bain-marie	312	-29	41	45	1.89	3.78	No effect IgG1, IgA and IgM. Higher IgG2	Higher IgG1 and IgG2 (24h) No effect at 48h
(Haines et al., 1992)	Bain-marie	Fresh	NA	-20	37	30	0.02	NA	No effect IgG, IgA and IgM	NA
(Holloway et al., 2001)	Bain-marie	Fresh	NA	-20	25	?	1	4	?	No effect IgG (48h)
	Bain-marie	Fresh	NA	-80	50, 37	?	?	1.89	?	No effect IgG
(Wiking and Pedersen, 2009)	Microwave	Unknown	850	4°C	40-42		4	NA	No effect IgG	NA
(Balthazar et al., 2015)	Microwave	Fresh	200	-20	39	14,7	?	NA	20% IgG1 loss	NA
	Microwave	Fresh	350	-20	39	26,6	?	NA	31% IgG1 loss	NA
	Bain-marie	Fresh	NA	-20	40	65	?	NA	8% IgG1 loss	NA
	Bain-marie	Fresh	NA	-20	50	?	?	NA	14% IgG1 loss	NA

Bain-marie	Fresh	NA	-20	60	?	?	NA	15% IgG1 loss	NA
Bain-marie	Fresh	NA	-20	70	37	?	NA	26% IgG1 loss	NA

Supplementary table 4: Comparison between esophageal feeding and other methods of feeding.

Study	Year	N	Treatment	Volume given to calf within 12h (L)	Serum IgG/FPT	Better?
(Logan et al., 1981)	1981	31	Esophageal feeder	0,5-1	Lower FPT in bottle and esophageal fed calves	Esophageal and bottle
		47	Nipple bottle	0,5-1		
		250	Suckling	NA		
(Bradley and Niilo, 1985)	1985	31	Esophageal feeder	1	No sig. difference within 48h	Neither
		31	Suckling	NA		
(Adams et al., 1985)	1985	52	Esophageal feeder	10% BW	No sig. difference	Neither
			Nipple bottle			
(Besser et al., 1991)	1991	334	Esophageal feeder	2,84	10,8% FPT	Esophageal feeder
		83	Nipple bottle	3,8	19,3% FPT	
		165	Suckling	NA	61,4% FPT	
(Kaske et al., 2005)	2005	15	Esophageal feeder	4	Higher IgG esophageal feeder	Esophageal feeder
		21	Nipple bottle	2		
(McGee et al., 2006)	2006	83	Esophageal feeder	50 ml/kg	No sig. difference	Neither
			Suckling	NA		
(Elizondo-Salazar et al., 2011)	2011	40	Esophageal feeder	3,8	No sig. difference	Neither
			Nipple bottle	3,8		
(Chigerwe et al., 2012)	2012	13	Esophageal feeder	2,2	No sig. difference	Neither
		13	Nipple bottle	2,2		
(Bonk et al., 2016)	2016	37	Esophageal feeder	3,5	No sig. difference	Neither
			Nipple bottle	3,5		
(Desjardins-Morrissette et al., 2018)	2018	10	Esophageal feeder	3	No sig. difference	Neither
		10	Nipple bottle	3		

(Shah et al., 2019)	2019	5	Esophageal feeder	10% BW	Higher IgG esophageal feeder compared to suckling. No sig. difference bottle vs esophageal feeder	Suckling< bottle < esophageal feeder
		5	Nipple bottle	10% BW		
		5	Suckling	NA		
(Godden et al., 2009)	2009	97	Esophageal feeder	1,5	1.5L: Higher IgG for bottle feeding. 3L: No sig. difference in feeding methods	Depends on the volume given
			Esophageal feeder	3		
			Nipple bottle	1,5		
			Nipple bottle	3		


3

CHAPTER 3

Survey on colostrum management by dairy farmers in the Netherlands

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Abstract

Colostrum feeding is essential for the transfer of passive immunity and health of newborn calves. Information on current colostrum management practices to reduce calf morbidity and mortality is important but lacking for Dutch dairy herds. We therefore conducted a survey to investigate colostrum management strategies on Dutch dairy farms. The survey was specifically focused on the most recently born calf and was returned by 107 respondents (response rate of 13.4%). The mean amount of colostrum fed at first feeding was 2.9 liters. Overall, 79% of farmers provided the calf with at least 6 liters of colostrum in up to three feedings. The majority of respondents (84%) claimed to provide the calf with colostrum for the first time within two hours post-partum. Using ordinal logistic regression and Wilcoxon rank sum test, we found no differences in time to first colostrum feeding or total amount of colostrum fed between bull calves and heifer calves, respectively. Ordinal logistic regression showed no significant differences in time to first colostrum feeding or time between calving and removing the calf from the dam between AMS and conventional milking herds. Two sample T-test comparing the total volume of colostrum showed no significant difference between AMS and conventional milking herds. Time of day at which a calf was born affected both volume fed at first colostrum feeding and time until first colostrum feeding. Calves born between 00.00 and 06.00 were significantly at risk of receiving the first colostrum later as compared to calves born at other times. Calves born in the evening received on average a lower amount of colostrum at first feeding.

Survey results on colostrum management on most Dutch dairy farms are in agreement with the advice to feed as soon as possible after parturition and to provide at least 6 liters within 24 hours of age. The current study points at time of calving as a potential risk factor for sub-optimal colostrum feeding. Further research is necessary to determine the consequences of this observation.

Introduction

Calf health and mortality are important issues in the dairy industry. Calves are born agammaglobulinemic (1, 2) and rely for their first humoral specific immune protection on antibody transfer via (maternal) colostrum. Maternally derived antibodies from colostrum provide protection both locally in the gut and systemically after intestinal absorption (2, 3). This passive protection is crucial for neonates, as their immune system is fully developed, but lacks immunological memory. Insufficient uptake of maternal antibodies is termed Failed transfer of Passive Immunity (FPI) (2-4), and severely increases the risk of infections, disease and death. FPI used to be defined as a neonatal serum IgG concentration of 10 mg/ml or lower between 24 and 48 hours of age (2-4) and for a long time the aim was to provide as much colostrum as needed to achieve a serum IgG concentration of ≥ 10 mg/mL. However, as new studies indicated that serum IgG concentration has a dose-response effect (5), this dichotomous approach was rather obsolete and a new and extended approach was presented by Lombard et al. (2020) in which four categories of serum IgG concentrations are distinguished (excellent, good, fair and poor) which reflects the dose-response associations between serum IgG concentrations and calf morbidity and mortality risks (6). Multiple causes can lead to FPI and include the quality of freshly produced colostrum, storage and or treatments of colostrum and feeding methods of colostrum to the neonatal calf. Feeding colostrum of insufficient quality or quantity can lead to inadequate uptake of immunoglobulins. Feeding a calf too late hinders the intestinal absorption of maternal antibodies due to the process of gut closure (2, 4). As the antibody concentration decreases with every milking, and with a prolonged time between calving and milking, it is recommended to milk a cow completely and as quickly as possible after parturition (4, 7). In addition the supply of antibodies for the transfer of passive immunity, colostrum fulfills a range of other functions for the newborn calf. One of them is providing newborn calves with an adequate amount of energy to cover their relatively high energy requirements for thermoregulation (8). The nutritional value of colostrum is significantly higher than that of milk. The amount of fat is highly variable, but reported to be approximately 60% higher and the amount of casein is reported to be 90% higher compared to milk (9). Vitamins and minerals are present in a higher concentration as well (4). Moreover, colostrum contains high concentrations of growth factors, antimicrobial factors (10) and hormones (3).

The importance of adequate colostrum intake is undisputed and methods to ensure optimal colostrum feeding in practice are well known. To cover the needs of the newborn calf, many advisors and farmers have adopted the “Three Q’s” strategy: Quickly, Quantity, Quality. Additionally, a fourth Q, “sQueaky clean” is sometimes added (11), further termed “cleanliness”. This strategy highlights the importance of timely feeding of an adequate amount of good quality colostrum with minimal bacterial contamination. Still, calf morbidity and mortality are important issues. Improving on-farm colostrum management may present an opportunity to reduce calf morbidity and mortality. It has been suggested that farmers using an Automatic Milking System (AMS) have a different working routine than farmers using conventional milking systems (12). Also, there are indications that bull calves are treated differently from heifer calves with respect to the timing and volume of feeding colostrum (13). Knowledge on current colostrum management practices on dairy farms in the Netherlands is lacking. Therefore, the aim of this study was to investigate colostrum milking, storage, feeding and other colostrum related management methods currently applied in Dutch dairy farms. Additionally, we were interested whether dairy farms with automatic or conventional milking systems use different colostrum management methods and whether bull calves were treated differently from heifer calves. To this end, a survey was sent out to Dutch dairy farmers to inquire about colostrum management practices.

Materials and methods

Colostrum management survey

The survey was developed by calf health experts at the Faculty of Veterinary Medicine at Utrecht University. Field experts and a communication consultant from the commercial dairy calf sector were involved to ensure content validity of the survey. Psychometric testing of the survey was not performed. Prior to distribution, a prototype survey was field tested with three dairy farmers to identify potential interpretation difficulties, and any unclear questions were adjusted.

We wanted to be able to identify a difference of 40% and 60% between AMS and conventional milking herds with 95% precision and a power of 80%. For this we needed a sample size of at least 100 respondents in each group. We expected a survey response of 25% and thus we sent out 400 invitations to participate in the survey to AMS farms and another 400 to farms with a conventional milking parlor. We could utilize a national database of milk equipment service organizations, which includes contact details of all Dutch dairy herds, following GDPR regulations. In total 800 Dutch dairy farmers were randomly selected and approached for participation.

The selected farmers were invited by an automated email in which they were asked to participate in the online questionnaire. The corresponding cover letter included information on the purpose of the research, a brief outline of the survey and the assurance that participation was completely voluntary and anonymous. Participants did not receive any remuneration. By clicking on a link provided in the email, respondents were directed to a survey software program (EvaSys Survey and Evaluation Software) where the survey started. To comply with national privacy regulations, the contact details of the farmers were not accessible for the researchers. Participants could provide their email address if they were interested in outcome of the study and potential additional in-depth interviews. Informed consent was given by clicking “yes” to the question if they would like to provide their email address, after which participants could provide their email address. It was assured that contact details would not be used for any other purposes. The online survey program was accessible between 11th of February 2019 and 25th of March 2019. A reminder to participate in the survey was send out once at the 4th of March 2019.

The survey included a section on farm management, a section on technical information, a section on colostrum supply to the most recently born calf and a section on farmers’ attitude and aspirations towards colostrum management. A full list of the questions in the survey can be found in supplementary table 1. Questions regarding farm management included whether the farm used an AMS, whether the milk produced was organic or not, and the age of the farm manager. Included technical information were the production results (305 days cumulative milk production (kg) and percentage fat and protein), the yearly percentage of calves that developed either respiratory disease or scours within 14 days of age, as well as the yearly percentage of calves that died withing 14 days of age. With respect to administration of colostrum, farmers were asked to specifically describe the details regarding colostrum management of the calf that was most recently born on their farm. By asking specifically about the most recently born calf we tried to minimize social desirability/response bias (i.e. describing best or generally applied practices rather than the actual on-farm colostrum management practices) and recall bias, given the assumed short period of time between calving and survey participation. In addition to questions related to the colostrum feeding methods performed with the most recently born calf, we inquired farmers’ opinions on standard procedures with respect to colostrum supply. We asked opinions on several

statements to which they could agree or disagree (supplementary table 2) or to which they could indicate a score on a 7 point Likert scale (supplementary table 3).

Data handling and statistical analysis

For practical reasons and to conform to model assumptions of logistical models, levels within some dependent variables from the survey were grouped: Time to first colostrum feeding was grouped to 1) within 1 hour, 2) within 2 hours and 3) after 2 hours. Farmers had the option to either report actual time of birth or, if they did not recall, to report the part of day in which the calf was born. All actual timepoints were converted to the part of day: morning (06:00-12:00), afternoon (12:00-18:00), evening (18:00-00:00) and night (00:00-06:00).

After data cleaning, R version 3.5.3 (Great Truth) and R studio version 1.3.959 were used for all descriptive and statistical analyses. The Shapiro-Wilks test was used to check for normality. When data was normally distributed, continuous data was compared using t-tests, if not, a Kruskal-Wallis combined with a Wilcoxon rank sum test was applied. Binary, categorical and ordered categorical data were assessed with logistic regression, multinomial logistic regression and ordinal logistic regression, respectively. For the ordinal logistic regression, the Brant test was used to assess parallel regression. The p-values of the logistical models were calculated using Analysis of Deviance (ANODE). Variables were dropped if they increased the AIC when included in a model. If dropping a variable from a model increased the AIC by less than 2.0, this variable was dropped in favor of a less complex model.

Results

Survey response

Out of the 800 dairy farmers invited to participate in the survey (400 AMS, 400 conventional milking system) a total of 107 surveys responded, an overall survey response of 13.4%. A total of 62 farmers with an AMS system and 45 famers with a conventional milking system participated in the survey. Nine out of 107 surveys were incomplete (farm characteristics, day of calving and satisfaction with colostrum management). As the number of respondents was quite small and the missing values did not include data on colostrum management with the most recently born calf, we decided to include these incomplete surveys as much as possible to optimize the power of this study. We checked for biologically unrealistic values by histogram plots and checking for outliers. In some surveys some illogical values were reported: two farmers reported to have provided physiologically unlikely high amounts of colostrum with the third feeding (10 and 12 liters) and thus we reported these data as missing values. For a complete overview of the questions and number of corresponding answers, we refer to supplementary table 1.

Population description

Of the 107 respondents, 7 (7%) produced organic milk, 98 (92%) used conventional farming methods, and 2 (2%) reported a different farming strategy. In Figure 1A the distribution of respondents' herd size is given. The 305 days cumulative milk production was grouped in categories of 500 kg and the number of respondents for each category is shown in Figure 1B. The mean 305 days production of the respondents was 9,388 kg (SD 1,282). The mean milk fat and milk protein percentage was 4.43% (SD 0.24) and 3.57% (± 0.12% SD), respectively. From the conventional milking farms, 44 (98%) out of 45 milked 2 times a day, and one milked three times per day. For the AMS farms the mean number of milkings per day was 2.8

(± 0.4 SD). The age distribution of the farm managers is displayed in Figure 1C. Distributions of calf calvings by part of day and by sex are given in Table 1.

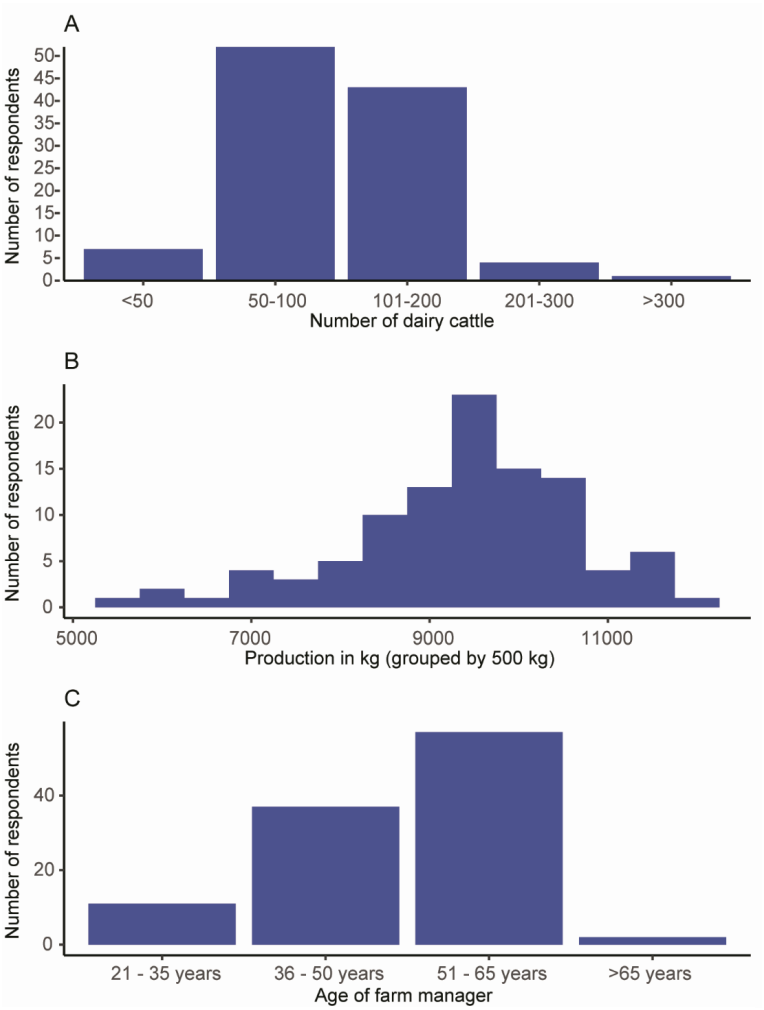

Figure 1. Distribution of respondents. The number of respondents (n=107) grouped according to A) herd size (n=107), B) 305 days milk production (n=102) and C) age of the farm manager (n=107).

Table 1. General calving statistics of the most recently born calf by time of calving and sex

	Morning (6:00 - 12:00)	**Afternoon (12:00 - 18:00)**	**Evening (18:00 - 24:00)**	**Night (24:00 - 6:00)**	total
Heifer	16	17	11	12	56
Bull	20	12	11	7	50
Total	36	29	22	19	106

Amount of colostrum fed

Of the 107 responders, 104 (97%) provided colostrum to the most recently born calf. The majority reported to feed the dams' own colostrum (Table 2). Of these 104, 15 farmers allowed the calf to suckle the dam for at least the first feeding, and another three farmers allowed the calf to suckle after the first feeding(s). Total volume ingested over three feeding moments could be determined for 84 respondents. The mean total amount of colostrum fed by 84 farmers known to feed all colostrum manually was 7.2 liters (SD 2.2). Out of these 84 farmers, 66 (79%) fed at least 6 liters of colostrum. An increase in the number of feedings was significantly ($p<0.01$, Kruskal-Wallis) associated with a higher total colostrum supply. Also, method of feeding significantly ($p<0.01$, Kruskal-Wallis) affected the volume provided at first feeding (Figure 2). Higher amounts were fed with an esophageal tube compared to feeding with a nursing bottle or a nursing bucket ($p<0.01$, Wilcoxon rank sum). The amount of colostrum fed at first feeding was not the same for calves born at different times of the day (Figure 3A). Calves born during the afternoon received significantly more colostrum at first feeding than calves born during the evening ($p<0.001$, Wilcoxon rank sum). However, total volume of feeding was not affected by the part of day in which a calf was born ($p=0.240$, Kruskal-Wallis) (Figure 3B).

Table 2. Sources of colostrum fed to the most recently born calf

	Provided n (% of total)	From dam n (% of total)	From other cow n (% of total)	Mixed colostrum n (% of total)
First feeding	104 (97.2%)	100 (96.2%)	3 (2.9%)	1 (1.0%)
Second feeding	97 (91.0%)	93 (95.9%)	3 (3.1%)	1 (1.0%)
Third feeding	90 (84.1%)	87 (96.7%)	0	3 (3.3%)

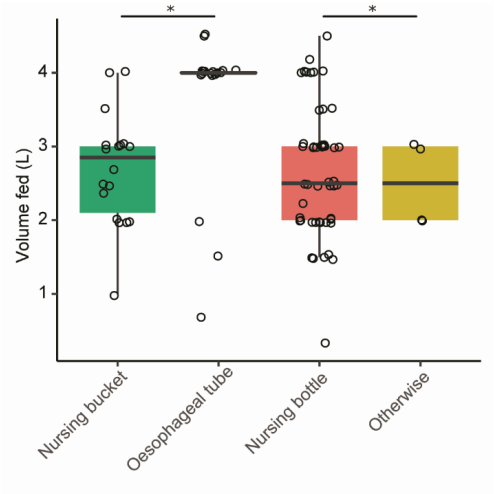

Figure 2. Volume of colostrum fed with the first colostrum feeding with different feeding methods (n=89). * = $p < 0.05$ (Wilcoxon rank sum test). The amount of colostrum fed with an esophageal tube was reported to be 4 liters 13 out of 18 times, resulting in the

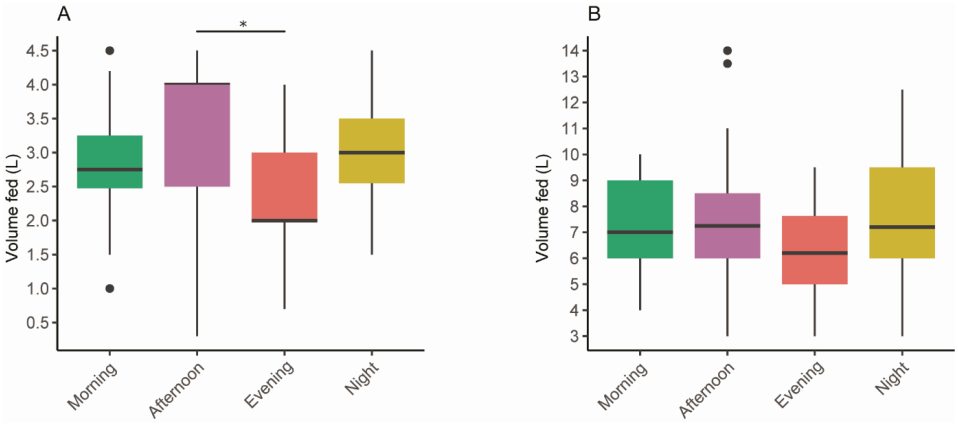

Figure 3. Volume of colostrum fed per calving time. A: The volume fed at first feeding (n=89), B: The total volume of colostrum fed over three feedings (n=84). * = $p < 0.05$ (Wilcoxon rank sum test) .

Time until first feeding

Our results show that out of 104 calves receiving colostrum, 87 (84%) received the first colostrum within 2 hours. Calves born at night had a significant higher risk to receive first colostrum later compared to calves born in the morning (OR = 8.21, CI 2.49-29.09), the afternoon (OR = 5.94, CI 1.76-21.46) and the evening (OR = 20.92, CI 5.35-90.48) (tested with ordinal logistic regression). Calves born in the evening tended to receive their first colostrum feeding sooner than calves born in the afternoon (Figure 4, Table 3).

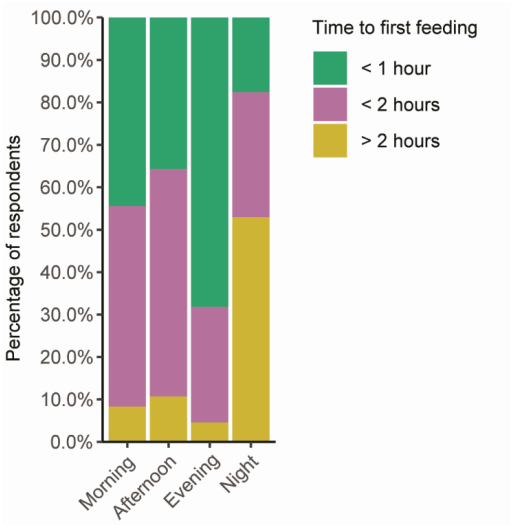

Figure 4. Time to first colostrum feeding with respect to time of calving of the most recently born calf (n=103). Most calves born during morning, afternoon and evening receive first colostrum within 2 hours after birth. At night however, half of the calves receives colostrum later than 2 hours after birth.

Table 3. Estimated odds and 95% confidence interval (in brackets) of the odds to receive colostrum later between different times of birth

	Morning	Afternoon	Evening
Afternoon	1.38 [0.54 – 3.53]		
Evening	0.39 [0.13 – 1.13]	0.28 [0.09 – 0.86]	
Night	8.21 [2.49 – 29.09]	5.94 [1.76 – 21.46]	20.92 [5.35 – 90.48]

As part of day of calving affected the time until and volume at first colostrum feeding, we examined whether this would affect method of feeding. We found that the multinomial logistic models with these dependent variables fitted our data better when part of day was dropped from the models. Thus, we found no influence of the part of day on feeding methods used (data not shown).

Storage, heating and feeding methods used

All respondents together reported a total of 291 feedings. The majority of all colostrum feedings, 163(56%), especially the first feeding, consisted of fresh colostrum given to the calf directly after milking (Table 4). Suckling the dam occurred in 24 (8%) of the total 291 feeding moments. Stored colostrum was almost exclusively used for 2nd and 3rd feeding. Colostrum was most frequently stored in the refrigerator, 46 out of 291 feeding moments(16%), followed by storage at room temperature, 29 out of 291 feeding moments (10%). Colostrum previously frozen and thawed was rarely used for feeding (3 times, 1%). The other 9% of the total amount of feedings was stored differently. Most commonly used storage equipment were both sealed and open buckets (Table 5). A large proportion of the farmers, 29 out of 89 (33%), 37 out of 90 (41%) and 31 out of 88 (35%) reported to store the colostrum in open buckets, for feedings one, two and three, respectively. With respect to feeding equipment, colostrum was most frequently offered in a nursing bottle, particularly at first feeding (Table 6). At subsequent feedings, nursing buckets and normal buckets were increasingly used.

Table 4. Storage methods used (if applicable) prior to first, second and third feeding of colostrum to the most recently born calf

	Given directly after milking n (% of total)	Drank with dam n (% of total)	Stored frozen n (% of total)	Stored at room temperature n (% of total)	Stored refrigerated n (% of total)	Other n (% of total)	Total
First feeding	85 (82%)	15 (14%)	2 (2%)	1 (1%)	0	1 (1%)	104
Second feeding	37 (38%)	7 (7%)	1 (1%)	13 (13%)	28 (29%)	11 (11%)	97
Third feeding	41 (46%)	2 (2%)	0	15 (17%)	18 (20%)	14 (16%)	90

Table 5. Storage containers used prior to first, second and third feeding of colostrum to the most recently born calf

	Sealed box n (% of total)	Sealed bucket n (% of total)	Open bucket n (% of total)	Nursing Bottle n (% of total)	Total
First feeding	7 (8%)	29 (33%)	29 (33%)	24 (27%)	89
Second Feeding	12 (13%)	27 (30%)	37 (41%)	14 (16%)	90
Third Feeding	12 (14%)	33 (38%)	31 (35%)	12 (14%)	88

[1]*The option of using an esophageal feeding tube was not available for the 2nd and 3rd feeding.*

Table 6. Feeding method used at first, second and third colostrum feeding of the most recently born calf

	Nursing Bucket n (% of total)	Normal bucket n (% of total)	Nursing Bottle n (% of total)	Esophageal Tube n (% of total)	Other n (% of total)	Total
First feeding	18 (20%)	0	49 (55%)	18 (20%)	4 (5%)	89
Second Feeding	39 (43%)	7 (8%)	42 (47%)	N/A[1]	2 (2%)	90
Third Feeding	51 (58%)	7 (8%)	29 (33%)	N/A[1]	1 (1%)	88

Four farmers out of the 104 farmer reporting to provide colostrum reported using colostrum from another source than the own dam. Two farmers fed for the first feeding frozen colostrum that was thawed using a hot water bath. One used colostrum from another dam, the other used pooled colostrum. One other farmer fed colostrum from another dam which was heated by a heat element. One farmer fed colostrum from one other dam, that was stored at room temperature. Out of the 90 farmers providing colostrum manually for the second time, 37 (41%) stated to have fed directly after milking the dam. Almost one third (31%) fed colostrum that had been stored in a refrigerator, while 14% fed colostrum that had been stored at room temperature. Only one farmer used frozen/thawed colostrum for feeding the second time. The remaining farmers ticked category "other" when asked about how they stored colostrum used at the second feeding. For the third feeding, 41 farmers (46%) supplied colostrum directly to the calf after milking the dam and two reported the calf drank with the dam. Storage in a refrigerator and at room temperature were reported by 18 (20%) and 15 (17%) of the farmers, respectively. None supplied colostrum that was previously frozen. The remaining respondents, 14, ticked "other" when asked about storage method.

Comparing colostrum management between heifer and bull calves and between farms with conventional and automatic milking systems

Analyzing the survey results on the most recently born calf, sex of the calf indeed did not affect the time to first colostrum feeding (ordinal logistic regression) (Table 7) or the volume of colostrum provided ($p=0.94$, Wilcoxon rank sum test), as demonstrated in Figure 5. Also, no differences in colostrum management between AMS farms and farms with conventional milking systems were found in the current study for any of the dependent variables (time until first feeding of colostrum (ordinal logistic regression), total volume of colostrum fed ($p=0.41$, two sample T-test), time between calving and removing calf from the dam (ordinal logistic regression), and the feeding method used for the first feeding (categorical logistic regression).

Table 7. Time to which heifer and bull calves receive their first colostrum

	Heifer calves	Bull calves	Total
Within 1 hour	25	19	44
Within 2 hours	22	21	43
Later than two hours	8	9	17
Total	55	48	104

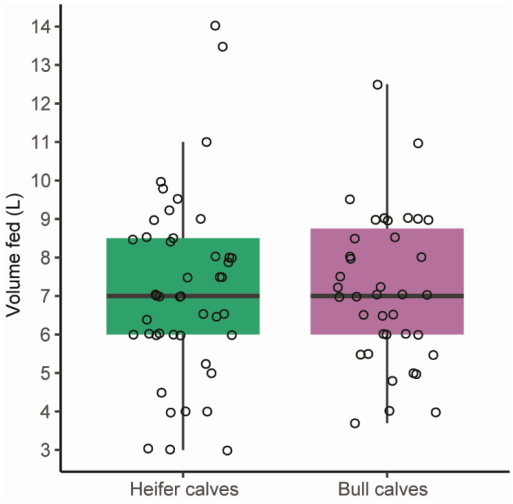

Figure 5. Total volume of colostrum fed to heifer calves and bull calves. Across all farms that provided colostrum manually for all three feedings (n=84), no difference in total volume of colostrum fed was found between heifer and bull calves (p=0.94, Wilcoxon rank sum test).

Farmers opinions and reported standard procedures with respect to colostrum management

Farmers indicated that they did not differentiate colostrum feeding strategies between calves of different sexes, which was in agreement with the analyses of the most recently born calves in the survey. According to the questionnaire, 96 out of 103 (93%) responding farmers were satisfied with the way they provided colostrum to the most recently born calf. Out of all 107 respondents, 50 farmers defined colostrum as the first milking after calving (47%), while 44 (41%) farmers described it as milk from the first three milkings after calving and 11 (10%) described it as the first six milkings. The remaining respondents either defined colostrum as the first two milkings after calving (1%), or declared that it differs per dam (1%). As much as 34 (32%) reported that they routinely measure colostrum quality and 30 (28%) incidentally and the remaining 43 (40%) never measure colostrum quality. With respect to measuring colostrum quality, 45 (70%) farmers reported measuring colostrum with the use of a Brix refractometer, nine (14%) used a densimeter bobber and ten (16%) measured quality with a different method.

Almost half of the farmers, 46 out of 107 (43%), indicated that they never use an esophageal tube to feed calves. Thirty-eight respondents (36%) claimed to sometimes use esophageal tubes (10-40% of the calves), seven (7%) used it routinely (41-60% of the calves) and 4 (4%) frequently (61-90% of the calves). A total of 12 farmers (11%) claimed to always (more than 90% of the calves) use esophageal tubes for feeding colostrum.

Fifty out of the 107 (47%) farmers agreed with the statement that one of the most important reasons why calves sometimes cannot be fed the desired amount of colostrum in time, is that the first feeding of colostrum is delayed for a calf born at night compared to a calf born at other times of the day. However, only 19 (18%) farmers agreed with the statement that colostrum management is different between a calf born in the night compared to a calf born at other times of the day. Only one farmer agreed with the statement that colostrum management differs between bull- and heifer calves. The majority of farmers, 56 out of 107 (52%) stated that they acquired information on colostrum management from their veterinarian. Furthermore, 42 out of 107 farmers (39%) indicated they wanted more information on colostrum management from the veterinarian, 41 (38%) from the feed advisor, and 24 (22%) responded with a written answer: Eleven out of 25 answered that they were not in need of more information and 9 farmers out of 25 indicated that they wanted more information regarding colostrum management from a specialist in calf rearing and one claimed to want more information from scientific research. The other three of the 25 written answers were not specific.

Discussion

Proper management of colostrum feeding practices could potentially positively affect the uptake of immunoglobulins, thereby preventing FPI. In the search for optimal colostrum management, farmers and advisors have adopted the strategy: Quantity, Quickly, Quality and cleanliness. The aim of the survey was to investigate milking, storage, feeding and other colostrum management aspects as currently applied on Dutch dairy farms.

As results from a survey may not always reflect actual methods applied in practice, we specifically asked the farmers about the colostrum management methods they applied to the most recently born calf in order to reduce social desirability bias, and to minimize recall bias. Obviously, recall bias cannot be canceled out completely and therefore results should be interpreted with caution. For this type of survey we expected a response rate of ~25%, however the response rate of our study was relatively low and it is therefore difficult to extrapolate these results to the broader target group of dairy farmers in the Netherlands. We cannot exclude participation bias as it is possible that the subject of the survey may have attracted farmers with a special interest in calf rearing, while the online method may have refrained some farmers from responding.

To further determine the external validity of our study, we assessed the representativity of the respondents by comparing the results on farm characteristics to a national database. The most common reported herd size was 50 to 100 heads of cattle, which is in line with the average herd size of 99 heads of cattle in the Netherlands (14). The mean percentages for fat and protein reported in this survey were 4.47% and 3.57% respectively and are comparable to the means in the Netherlands, 4.38% and 3.59%, respectively (14). The mean 305 days milk production of the respondents was 9,388 kg, compared to 9,155 kg in the Netherlands (14). Considering the similarities, we think that with respect to farm characteristics (except for the high percentage AMS farms), our study population is representative of the situation in the Netherlands.

To identify a difference of 40% and 60% between AMS and conventional milking herds we aimed for a minimum of 100 participants in each group. Due to the low response rate, we only managed to achieve half the sample size we aimed for. Therefore, this sample size allowed us to only identify potential large effects (e.g. a difference of 30% and 60% between types of

milking system). The precision of the study would have increased with a larger sample size, which would have allowed for smaller differences between groups to be detected.

One of our aims was to investigate whether colostrum management methods were different between dairy farms with a conventional and farms with an automatic milking system. Despite suggested differences in working conditions and time division between AMS farms and conventional milking system farms (12), no large differences in colostrum management strategies were found between farms using an AMS or a conventional milking system in survey. Additionally, we were interested whether colostrum management was different across farms between bull calves and heifer calves. Bull calves cannot replace dairy livestock in the herd and as such have less value to a dairy farmer. This might lead to farmers prioritizing colostrum feeding differently for heifers and bulls. Results from the survey indicate that with regard to colostrum management, across all farms both farmers' aims and actual practices applied to the most recently born calf did not differ between sexes. Similar to the results with respect to differences between AMS farms and farms with a conventional milking parlor, potential small effects could have remained undetected due to the limited sample size of the survey. Our results are different from results obtained by Shivley et al. (2019), who reported that bull calves received first colostrum at a later timepoint compared to heifer calves. Also, bull calves were provided with a lower amount of colostrum over 24 hours, compared to heifer calves (13). A possible explanation for these contradicting results could be that bull calves from participating herds left the operation at an average age of 7.6 days, while in the Netherlands bull calves are not allowed to be transported before 14 days of age. In this way, Dutch dairy farmers are responsible for the health status of their bull calves for a longer period.

Timely feeding of colostrum is essential, as the window for absorbing immunoglobulins by the gut is limited to 24 hours post-partum and absorption is most optimal within two hours of age (3, 15). With regards to Quickly feeding of colostrum, the majority of farmers (84%) reported feeding the first feeding of colostrum within 2 hours or earlier, which coincides with commonly given advice to feed as quickly as possible (3). Our results are similar to those of Cummins et al. (2016), who reported that the majority of the farmers in their study (84%) said to feed colostrum within 3 hours after calving (16). The survey by Kehoe et al. (2007), carried out on 55 dairy farms in Pennsylvania, US, revealed that only 44% of farmers fed colostrum within 2 hours, 51% within 2 to 6 hours, and 5% later than 5 hours (17). With respect to timely feeding of colostrum, most progress could be made by paying more attention to calves born at night, as in our study, calves born at night received their colostrum significantly later than calves born at other times of the day. However for practical reasons, this might be difficult to implement. Calves born in the evening receive colostrum the quickest, but also the least amount at first feeding. Possibly, the farmers are more flexible in their time during the evening, as other farm tasks are mostly performed in the morning and afternoon.

With regards to Quantity and frequency of colostrum feeding, we found that most farmers (84%) fed at least three servings of colostrum. In our study, 55% of the farmers that fed colostrum manually, supplied 3 liters or more with the first feeding, which is in line with the advice by Godden et al. (2019). Adequate transfer of passive immunity is not only dependent on the volume, but is also affected by factors such as quality, contamination, and timing of the colostrum intake as well as birthweight. Obviously, farmers are not able to quantify these effects and this probably explains some of differences between the recommendations encountered in the literature. It is possible to maximize colostrum intake up to 3 liters with the use of an esophageal tube when calves do not voluntarily ingest at least 2 liters (15, 18). However, the farmers in our study appear to prefer repeated colostrum feedings to ensure

sufficient uptake of IgG. The majority (79%) fed ≥6 liters in the three subsequent feedings we questioned, indicating small volumes at first feeding are compensated with subsequent feedings. These practices are in line with feeding at least 6 liters within 24 hours as recommended (3, 19).

Quality of colostrum is usually expressed as the concentration of IgG in colostrum (3) and depends on many factors, such as such as breed (20), continuous milking (21), season of calving (22) and the time between calving and first milking (23, 24). Because of the large variation in colostrum quality, it can be useful to measure colostrum quality for example using Brix refractometry. Only about one third of our respondents reported to routinely measure colostrum quality. We recommend to pay more attention to measuring the quality of colostrum, because depending on the quality, it is possible to try to increase the volume to compensate for lower quality colostrum in order to achieve adequate transfer of passive immunity.

Another factor that attributes to quality of colostrum, is bacterial contamination. Storage of colostrum is known to affect total bacterial counts in colostrum (25, 26). While the majority of all feedings collectively was fed directly after milking the dam, many farmers in our survey reported to store colostrum in a refrigerator or at room temperature. Both Stewart et al. (2005) and Cummins et al (2016) showed that storing colostrum at room temperature leads to a rapid increase in total bacterial counts within 24 hours. Storing colostrum in a refrigerator delayed bacterial growth, however the effects were only temporary (25, 26). Not only are increased numbers of total bacteria associated with increased risk of disease, it may also lead to decreased absorption of colostral IgG by the newborn calf (27). We therefore recommend to store colostrum more often in a refrigerator instead of at room temperature to delay bacterial growth up until 24-48 hours. For storing colostrum for a longer time, freezing and thawing of colostrum is recommended, as the immunoglobulin content is preserved while bacterial growth is diminished (28, 29).

Our survey provides insight in colostrum management practices by dairy farmers in the Netherlands. Our results indicate no systematic differences in colostrum management between dairy farms using an automatic or a conventional milking system. Across farms, heifer and bull calves were not treated differently. Furthermore, our results indicate that overall across the farms in our study, the common guidelines with respect to the “Four Q’s” are mostly followed. The majority reported to feed Quickly, within two hours of age). With regard to Quantity, approximately half of the respondents claimed to feed 3-4 liters colostrum at the first feeding and the majority, 79%, provides a total volume of ≥6 liters. More attention should be paid to measuring the Quality of colostrum, as this was not commonly measured in our survey and it helps determining the volume that should be fed. As for cleanliness, storing colostrum in a refrigerator rather than storing at room temperature should be considered to minimize bacterial growth. More attention should be paid to calves born at night, as they are at risk to receive colostrum later than calves born at other times of day, which affects the efficiency with which immunoglobulins are taken up by the calf.

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Supplementary Material

Supplementary Tables

Supplementary table 2: Survey questions, number of respondents, and variable type

Question	Respondents	Variable type
Farm management type	107	Factor
Age of the farm manager	107	Factor
Number of cattle	107	Factor
Milking system	107	Factor
305 days milk production (kg)	104	Continues variable
305 days milk procution: fat %	106	Continues variable
305 days milk production: protein %	106	Continues variable
BSK	105	Continues variable
Number of milkings (conventional milking system)	45	Factor
Number of milkings (automatic milking system)	62	Continue variable
Date of birth of the most recently born calf	107	Date
Is the time of calving known?	106	Factor
What is the time of calving	87	Time
What part of day did calving take place	19	Factor
What was the sex of the calf?	107	Factor
Aim to keep the heifer calf?	56	Factor
Course of parturition	107	Factor
What was the drylenght period of the corresponding dam?	107	Factor
Which lactation stage does the corresponding dam enter?	107	Factor
When was the calf removed from the dam?	107	Factor
Which date was the calf removed from the dam?	31	Date
Is the time known when the calf was removed from the dam?	31	Factor
What time was the calf removed from the dam?	25	Time
Which part of day was the calf removed from the dam?	6	Factor
When was the dam milked first after calving?	107	Factor
In what way was the dam milked?	107	Factor
Was the dam vaccinated against calf scours?	107	Factor
Did you supply the calf with colostrum?	107	Factor
What is the date when the calf received colostrum for the first time?	104	Factor
When did the calf receive colostrum for the first time? (hours after birth)	104	Factor
What was the colostrum source for the first colostrum feeding?	104	Factor
Was the calf nursed by the dam?	100	Factor
Was the dam milked out completely?	85	Factor
What was the volume of colostrum milked?	86	Continues variable
From which milking was the first colostrum given to the calf?	3	Factor
What volume of colostrum was fed to the calf at the first feeding?	89	Continues variable
What was the reason not to provide colostrum from the dam?	4	Factor

How did you store the colostrum that was given to calf?	4	Factor
What method did you use to thaw the colostrum?	2	Factor
Did you warm the colostrum before feeding to the calf?	2	Factor
What method did you use to warm the colostrum?	3	Factor
Did you check the temperature of the colostrum before feeding to the calf?	89	Factor
What was the temperature of the colostrum?	10	Continues variable
How was the colostrum stored before feeding it to the calf?	89	Factor
What method did you use to supply the calf with colostrum?	89	Factor
Did you save leftover colostrum?	89	Factor
After the first colostrum feeding, did you provide a second colostrum feeding?	104	Factor
What was the date of the second colostrum feeding?	97	Date
Do you know the time at which the second colostrum feeding was given?	97	Factor
At what time was the second colostrum feeding given?	88	Time
Which part of day was the second colostrum feeding given?	9	Factor
What was the colostrum source for the second colostrum feeding?	97	Factor
Was the calf nursed by the dam for the second colostrum feeding?	94	Factor
From which milking was the second colostrum given to the calf?	90	Factor
What volume of colostrum was fed to the calf at the second feeding?	89	Continues variable
How did you store the colostrum that was given to calf at second colostrum feeding?	90	Factor
What method did you use to thaw the colostrum for the second feeding?	1	Factor
Did you warm the colostrum before feeding to the calf at the second colostrum feeding?	52	Factor
What method did you use to warm the colostrum for the second feeding?	49	Factor
Did you check the temperature of the colostrum before feeding to the calf at the second colostrum feeding?	90	Factor
What was the temperature of the colostrum of the second feeding?	11	Continues variable
How was the colostrum stored before feeding it to the calf the second colostrum feeding?	90	Factor
What method did you use to supply the calf with colostrum for the second colostrum feeding?	90	Factor
Did you save leftover colostrum from the second feeding?	90	Factor
After the second colostrum feeding, did you provide a third colostrum feeding?	97	Factor
What was the date of the third colostrum feeding?	90	Factor
Do you know the time at which the third colostrum feeding was given?	90	Factor
At what time was the third colostrum feeding given?	81	Time
Which part of day was the third colostrum given to the calf?	9	Factor
What was the colostrum source for the third feeding?	90	Factor
Was the calf nursed by the dam for the third feeding?	87	Factor
From which milking was the third feeding given to the calf?	85	Factor
What volume of colostrum was fed to the calf at third feeding?	88	Continues variable
How did you store the colostrum that was given to the calf at third colostrum feeding?	88	Factor
What method did you use to thaw the colostrum for the third feeding?	0	Factor
Did you warm the colostrum before feeding to the calf at the third feeding?	47	Factor
What method did you use to warm the colostrum for the third feeding?	39	Factor
Did you check the temperature of the colostrum before feeding to the calf at	88	Factor

the third colostrum feeding?		
What was the temperature of the colostrum of the third feeding?	11	Continues variable
How was the colostrum stored before feeding it to the calf the third colostrum feeding?	88	Factor
What method did you use to supply the calf with colostrum for the third colostrum feeding?	88	Factor
Did you save leftover colostrum from the third feeding?	88	Factor
Are you satisfied with the way you have provided colostrum to the most recently born calf?	103	Factor
In your opinion, what is the definition of colostrum?	107	Factor
Additional explanation	2	Text
How do you recognize the quality of colostrum?	105	Text
Do you measure the quality of colostrum?	107	Factor
What method do you use to measure the quality of colostrum?	64	Factor
I measure the IgG concentration with Brix refractometry		
I measure the IgG concentration with a colostrum densitometer		
I measure the quality of colostrum in another way than stated here, namely:	12	Text
What do you aim for? Within how many hours do you aim to provide the first colostrum to heifer calves?	107	Continues variable
In how many times do you succeed in this aim?	107	Factor
What do you aim for? What volume of colostrum do you aim to provide within 12 hours of age to heifer calves?	107	Continues variable
In how many times do you succeed in this aim?	107	Factor
What do you aim for? within how many hours do you aim to provide the first colostrum to bull calves?	107	Continues variable
In how many times do you succeed in this aim?	107	Factor
What do you aim for? what volume of colostrum do you aim to provide within 12 hours of age to bull calves?	107	Continues variable
In how many times do you succeed in this aim?	107	Factor
In general, how often do you make use of esophageal feeding for colostrum supply to the calf?	107	Factor
What are the most important reasons why calves do not receive a desired volume of colostrum in a desired time?	107	Factor
If the calf is born at night, it will receive colostrum later than if it is born during the day.		
If the calf drinks poorly, it will receive less colostrum		
If the dam gives insufficient colostrum, the calf will receive less colostrum		
Other reason, namely:	29	Text
From who did you obtain most information about the way of administering colostrum?	107	Factor
Animal feed advisor		
Veterinarian		
Professional magazines		
Other dairy farmers		
Predecessor (e.g. Parents or other relatives)		
From who would you like to learn more about colostrum management?	107	Factor
Animal feed advisor		
Veterinarian		
Someone else, namely:	24	Factor
Colostrum management has a high priority on my farm	107	Factor
Optimal hygiene while milking and feeding colostrum has a high priority on my farm	107	Factor

In which way do you ensure optimal hygiene during collection and feeding of colostrum?	107	Text
It is important to be present at every parturition, even at night	107	Factor
Colostrum management on my farm is the same for all calves	107	Factor
Agree, every calf needs a sufficient amount of colostrum for a good start		
Disagree, colostrum management is different for calvings during the day or during the night		
Disagree, colostrum management is different and depends on sex of the calf		
Agree, because…		
Disagree, because…		
Explanation agree/disagree colostrum management is the same for all calves	69	Text
I am actively working to optimize colostrum management	107	Factor
Explanation actively working to optimize colostrum management	69	Text
Do you know the KalfOK score of your farm?	104	Factor
What is the KalfOK score on your farm?	56	Continues variable
Over the past 12 months, what percentage of calves until 8 weeks of age has developed diahrea and/or respiratory infections?	107	Factor
Over the past 12 months, what was the percentage of calf mortality on your farm? (percentage stilborn or born within 14 days of age)	106	Factor

Supplementary table 2: Agreement of participating farmers with different statements on colostrum management

Regarding colostrum feeding	**Agreed**	**Disagreed**
If the calf is born at night, it will receive colostrum later than if it is born during the day.	50 (47%)	57 (53%)
If the calf drinks poorly, it will receive less colostrum	42 (39%)	65 (61%)
If the dam gives insufficient colostrum, the calf will receive less colostrum	33 (31%)	74 (69%)

Regarding whom gives information on colostrum management	**Agreed**	**Disagreed**
Animal Feed advisor	**42 (39%)**	**65 (61%)**
Veterinarian	**57 (52%)**	**50 (48%)**
Professional magazines	**36 (34%)**	**71 (66%)**
Other dairy farmers	**17 (16%)**	**90 (84%)**
Predecessor (e.g. Parents or other relatives)	**35 (33%)**	**72 (67%)**

Colostrum management is the same for every calf on my farm	**Agreed**	**Disagreed**
Agree, because every calf needs enough colostrum for a good start	**86 (81%)**	**21 (19%)**
Disagree, because management differs for day and night calvings.	**18 (17%)**	**89 (83%)**
Disagree, because management differs for bull- and heifer calves.	**1 (1%)**	**106 (99%)**
Agree, for different reasons	**4 (4%)**	**103 (96%)**
Disagree, for different reasons	**1 (1%)**	**106 (99%)**

Supplementary table 3: Farmers agreement on a Likert scale (1 – 7) on several topics regarding colostrum and calf management.

	Mean score	Standard deviation
Colostrum Management has a high priority on my farm	6,2	1,1
Optimal hygiene while milking and feeding colostrum has a high priority on my farm	6,0	0,9
It is important to be present at every parturition, even at night	4,5	1,8
I am actively working to optimize colostrum management	5,4	1,5

4

CHAPTER 4

Effects of storage methods on total bacterial count and microbial composition of bovine colostrum

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Abstract

Neonatal calves need to acquire passive immunity from their mother through colostrum. High bacterial counts can hamper antibody absorption from colostrum and pathogens can be transmitted to the calf via colostrum. Duration and temperature of colostrum storage affect bacterial counts, often measured as total plate counts (TPC). Little is known about the effects of storage on the bacterial composition of colostrum.

This study characterizes the effects of different colostrum storage methods on the composition of the viable, aerobic, microbial community. After different storage conditions, bacterial growth was assessed using the aerobic TPC, followed by 16S rRNA gene amplicon sequencing to characterize the bacterial composition at the family level. Differences in bacterial composition of the TPC of the stored colostrum samples were mostly explained by the variation in bacterial composition of colostrum sample directly after milking. This is probably the result of contamination or other environmental influences during the milking process. Furthermore, storage at room temperature for 24 hours or in a refrigerator for a week leads to a significant increase in TPC and affects bacterial community structure. This is most likely due to increased numbers of Enterobacteriaceae.

These results provide a deeper insight in how storage under conditions used in practice affect the microbial composition of colostrum TPC. Prolonged storage of colostrum before feeding leads to bacterial growth, particularly of Enterobacteriaceae, and is therefore not advised. This study serves as a primer for more detailed research into the determinants of bacterial composition of colostrum and the linked health effects.

Introduction

Neonatal calves are highly susceptible to a variety of pathogens. They are born with a fully developed but naïve immune system and they lack maternal antibodies as these are not transferred to the fetus during pregnancy due to the structure of the bovine placenta [1]. Thus, passive immunity in the form of maternal antibodies has to be acquired by early colostrum feeding [2].

Milked colostrum is not sterile and may contain high bacterial counts. Even when extra precautions are taken to minimize contamination, colostrum still contains a viable bacterial community [3]. In on-farm circumstances additional contamination cannot be completely avoided and high total plate counts have been reported [3-6]. Consequently, feeding colostrum poses a certain risk of accumulated pathogen transmission. Additionally, it is hypothesized that a high bacterial load in colostrum hampers the absorption of antibodies [7, 8].

The majority of previous research on the bacterial content of colostrum focused on minimizing total bacterial counts to decrease the risk of pathogen transmission [2, 9]. Bacterial contamination can be prevented by optimizing hygienic conditions during milking, storing and feeding of colostrum [10]. Pasteurization can be applied to reduce total bacterial counts to decrease the risk of pathogen transfer, such as for example *Mycobacterium bovis* or *Escherichia coli* [11].

Even though reducing total bacterial counts in colostrum is essential, bacterial quality of colostrum comprises more than just the number of bacteria. Rapid developments in sequencing and -omics technologies have led to an increased interest in the field of microbiome research, and recently several studies looked into the microbiome of bovine colostrum [12-14]. Rich and diverse microbial communities were found and there is a large

variation in composition between individual animals [14, 15]. In the view of reducing transmission of potential pathogens to the calf it is valuable to identify the composition of the bacterial content of colostrum.

To maximize the transfer of maternal antibodies, it is advised to completely milk the dam after calving to provide the calf with sufficient first milking colostrum as soon as possible [16]. Subsequent colostrum feedings are beneficial [2], and in practice first colostrum milkings are often stored to use for (subsequent) feedings [5, 17-19]. Duration and temperature of colostrum storage are known to affect total plate counts (TPC), and it is recommended to minimize storage duration and store colostrum at a low temperature [3, 4]. Less is known about the effects of storage on the composition of the TPC. Depending on the storage conditions (i.e. duration and temperature), colostrum can be a substrate in which certain bacterial species can thrive. Therefore, storage method can affect the microbial composition of the colostrum fed to the calf. The aim of this study was to characterize the effects of different routinely used colostrum storage methods on the composition of the viable microbial community. Amplicon based 16S rRNA amplicon sequencing characterizes all bacterial DNA, including DNA of non-culturable and dead bacteria. Since we aimed to evaluate the composition of the viable microbial community as measured in the TPC, we assessed bacterial growth using the aerobe plate count culture method, and subsequently performed 16S rRNA gene amplicon sequencing on the bacterial colonies from the plate culture. This allowed us to relate the microbial composition directly with the TPC outcomes.

Materials and Methods

Animal handling and care

Holstein-Friesian cattle were housed at the Farm Animal Health (FAH) clinic or at the organic teaching farm “Tolakker”, both located at the Faculty of Veterinary Medicine, Utrecht University (Table 1). All cows were machine milked immediately post-partum according to standard farm practice to obtain composite colostrum.

Sample collection

Colostrum samples were collected from 5 cows that calved in January and February 2020 within one hour post-partum to standardize the samples and to process them equally. Composite colostrum samples (250-400 ml) of the first milking were obtained from five animals by scooping or pouring from the milk bucket. Samples were immediately transferred to the laboratory and were aliquoted in ten 15 ml tubes. One sample was directly frozen at -20°C (baseline sample), the other nine samples were subjected to one of the nine storage conditions (Table 2).

Table 1. Characteristics of each cow from which the baseline colostrum sample was obtained.

Animal ID	Date of calving and sample collection	Location	Parturition	Parity
#1	12-1-2020	Tolakker farm	Natural	6th
#2	17-1-2020	FAH clinic	Induced, caesarian	1st
#3	21-1-2020	FAH clinic	Induced, caesarian	1st
#4	30-1-2020	Tolakker farm	Natural	1st
#5	2-2-2020	Tolakker farm	Natural	3rd

Table 2. Overview of all storage conditions, their abbreviations and the number of baseline samples subjected to each of the conditions.

Abbreviation	Treatment
B	Baseline sample, directly frozen after collection
RT8	Room Temperature (21 °C). for 8 hours
RT24	Room Temperature (21 °C). for 24 hours
Re8	Refrigerator (5 °C) for 8 hours.
Re24	Refrigerator (5 °C) for 24 hours.
Re168	Refrigerator (5 °C) for 168 hours (1 week) .
Fr24	Freezer (-20 °C) for 24 hours followed by thawing in a 21 °C water bath
Fr168	Freezer (-20 °C) for 168 hours (1 week) followed by thawing in a 21 °C water bath
Re24W	Stored as Re24, then heated in a warm water bath until 38 °C.
Re24M	Stored as Re24, then heated in a microwave (90 Watt) until 38°C

Storage conditions

Colostrum samples subjected to storage at room temperature were kept at 21 °C for 8 (RT8) or 24 hours (RT24). Samples subjected to refrigeration (R) were kept at 5°C for 8, 24 or ±168 hours (1 week), Re8, Re24 and Re168, respectively. Samples subjected to freezing (Fr) were kept at -20°C for either 24 hours (Fr24) or ±168 hours (Fr168), after which they were placed in a water bath of 21°C to thaw to room temperature. Samples undergoing reheating experiments by warm water bath and microwave were first stored at 5 °C for 24 hours (similar to samples Re24), after which the heating experiments started. Samples and accompanying water tubes were heated by placing them in a water bath at 60°C (Re24W). The temperature of the colostrum was inferred by measuring the temperature of an accompanying water tube of initially 5 °C, ensuring no bacterial contamination of the colostrum samples. It took 2.5 minutes to heat samples to a temperature of 38 °C. A similar approach was applied for heating using a microwave: a microwave was set at 90 Watt for reheating after 24 hours of storage at Re (Re24M). As a reference, a water sample of 5 °C was microwaved for 90s and temperature was measured every 10s. Once the water was 38 °C, time was noted and this time was used for reheating the colostrum samples. After emulating the storage conditions both the baseline and all storage samples were frozen at -20 °C until further analyses. Total durations differed as colostrum samples from different animals were obtained on different days, and because storage conditions lasted for different times.

Total Plate Counts

After all samples had been subjected to the storage treatments and had been frozen until further analysis, they were collectively thawed at room temperature. Colony forming units (CFU) of colostrum samples were assessed using a track dilution method. Serial dilutions in DPBS were made of all stored colostrum samples and the baseline samples. We calculated the colony counts per milliliter using in triplicate measurements using the track dilution method Ten µl of the (diluted) samples was pipetted on square plates with Heart Infusion agar (ACU 7269C, Acumedia Manufacturers®) supplemented with 5% defibrinated sheep blood (BT-SG500, bioTRADING®). Plates were tilted to create a track across the plate. After overnight incubation at 37 °C, pictures were taken from all plates and CFUs were counted. Plates were placed back at 37 °C and incubated overnight. Pictures were taken the

next day and CFUs were counted. Afterwards, plates were stored at 4°C prior to DNA extraction.

DNA extraction

Glass beads (2mm) were added to the CFUs grown from the undiluted colostrum samples, together with 5 mL sterile PBS. The plate was swirled until individual colonies were no longer visible. Per culture condition, 2 mL bacterial suspension was collected for DNA extraction. DNA was extracted using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Art.No. 51604) according the manufacturer's instructions, except for the addition of a bead-beating step. Briefly, after resuspending the samples in InhibitEx buffer samples were subjected to repetitive bead-beating (3 times for 30 s with 5 s cooldown in between) using Lysing Matrix B tubes (MP Biomedicals, Art.No. 116911050-CF) and the FastPrep-24 instrument (MP Biomedicals). Microbial DNA extracts were checked on a 2200 Tapestation (Agilent Technologies Santa Clara, CA, United States).

Amplicon library preparation and sequencing

Bacterial community composition was assessed by sequencing the combined V3–V4 hypervariable region of the 16S rRNA gene. Briefly, this region was first amplified by 25 cycles of PCR using the primers CVI_V3-forw CTACGGGAGGCAGCAG and CVI_V4-rev GGACTACHVGGGTWTCT [20]. PCR products were checked on a 2200 Tapestation, and sequencing was performed using v3 paired-end 300 bp sequencing on a MiSeq sequencer (Illumina Inc., San Diego, CA, United States). Negative controls (H2O) were used in each round of amplification to confirm the sterility of reagents, and a mock bacterial community was included in the sequencing run as a control.

Processing of sequence data

All sequence data processing were performed in R 3.6.2 [21]. The 16S rRNA gene sequencing reads were filtered, trimmed, dereplicated, chimera-checked, and merged using the DaDa2 package v.1.12.1 [22] using standard parameters except for TruncLength = (240, 210) and reads were assigned with the GTDB taxonomy database version ssu_r86 [23]. Downstream analyses were performed with the phyloseq version 1.32 [24] and vegan 2.6 [25] packages. Good's coverage was >0.999. For alpha-diversity based analyses the data were rarefied to 29406 reads per sample (rarefy_even_depth, replace=FALSE, seed=2202). For beta-diversity the relative abundance data (at ASV level if not indicated otherwise) were made absolute using total plate counts per sample. The final dataset contained 518 amplicon sequence variants (ASVs) corresponding to 111 genera in 35 bacterial families. Raw fastq sequences are deposited in NCBI's Sequence Read Archive under BioProject accession number PRJNA872909. Obtained phyloseq object can be found at Zenodo.org (https://doi.org/10.5281/zenodo.7033893).

Data analysis

For all descriptive and statistical analyses of the TPC data version 3.6.2 of R was used [21]. As data were not normally distributed (Shapiro-Wilk normality test), differences between the baseline sample and the 9 storage samples and differences between the 9 storage samples were assessed by the non-parametric Kruskal-Wallis test . Despite the paired nature of the sample collection, paired analysis was not possible due to missing values. Post hoc testing (Dunn test) followed, combined with p-value adjustment for multiple testing (Benjamini-Hochberg).

All subsequent data analysis were performed in R version 4.2.0 [26]. Alpha diversity (observed richness, Shannon and Pielou's evenness) indices were calculated at the ASV level on the rarefied data. Multi-variable Principle Coordinate Analysis (PCoA) was performed on non-rarefied relative ASV abundance data that was first transformed into a Bray-Curtis dissimilarity matrix where the treatment parameters of interest were tested with the Animal_ID as a random variable. Differences in microbiota community structure were identified using PERMANOVA (permutational analysis of variance) on the taxonomic data in phyloseq using the adonis2 function from Vegan. Homogeneity of community structural dispersion assumptions were checked using hidsp2 from Vegan to make sure observed significant PERMANOVA results were not a result of variations in dispersion between groups of samples.

Results

Total Plate Counts of colostrum stored under different conditions

Five colostrum samples were collected, with two obtained from the FAH clinic and three from the organic farm "Tolakker". An overview of cow characteristics per colostrum sample is described in table 2. For one sample the condition "frozen for 1 week" (F168) was missing, and for one other sample both the baseline sample and refrigerated storage for a week (R168) were missing. Including the baseline samples, there were a total of 47 colostrum samples post storage conditions (table 3).

Median TPC of baseline colostrum samples was 10^4.78 CFU/ml. Total plate counts for each storage method and the differences in duration are displayed in figure 1. Total plate counts of colostrum stored for 8 hours in a refrigerator or at room temperature did not significantly differ from the baseline sample. However, average TPC was significantly higher in samples stored at room temperature for 24 hours, but this was not the case for samples stored for 24 hours in a refrigerator. Refrigerated samples for a week resulted in a significant increase in TPC. Samples that were refrigerated for 24 hours and reheated using either a water bath or microwave did not differ in TPC compared to refrigeration for 24 hours alone, nor to the baseline samples. Total plate counts in samples that were frozen for 24 hours or one week remained similar to the baseline samples.

Table 3. Overview of stored colostrum samples for each animal from which colostrum was collected

Animal ID	B	RT8	RT24	Re8	Re24	Re168	Fr24	Fr168	Re24W	Re24M
#1	1	1	1	1	1	1	1	1	1	1
#2	1	1	1	1	1	1	1	1	1	1
#3	1	1	1	1	1	1	1	1	1	1
#4	1	1	1	1	1	1	1		1	1
#5		1	1	1	1		1	1	1	1

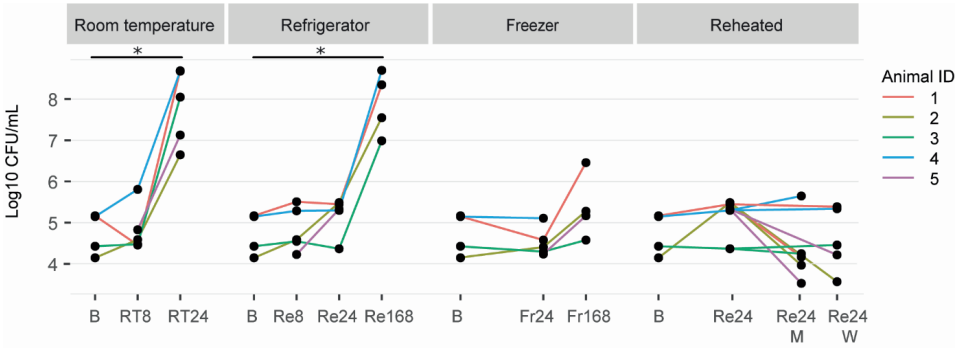

Figure 1. Total plate counts (Log10CFU) for each storage method. A) room temperature including baseline sample (B), room temperature for 8 hours (RT8) and room temperature for 24 hours (RT24); B) refrigerator including baseline sample (B), refrigerated for 8 hours, 24 hours and a week (Re8, Re24 and Re168, respectively); C) freezer, including baseline sample (B), 24 hours frozen storage (Fr24) and a week of frozen storage (FR168); and D) reheated samples with the baseline sample (B), refrigerated storage for 24 hours (Re24), microwaved sample (Re24M) and water bath sample (Re24W). Lines between observational points are merely for illustration purposes and only indicate that they originate from the same baseline sample. They do *not* indicate repeated measures over time. Significant differences ($p<0.05$) are indicated with an asterisk.

Differences in bacterial community structure of stored colostrum

Alpha diversity

First we analyzed bacterial community diversities of the colostrum samples. Observed species richness were not significantly different between the two collection locations (supplementary figure 1A). Refrigerated samples that were heated by microwave showed significantly lower (ANOVA, $p < 0.05$) species richness compared to samples refrigerated for 24 hours only and samples frozen for a week (figure 2A). No differences in species richness were found between other storage conditions. Even though samples obtained from the FAH clinic seemed to show larger variation in evenness (Pielou) than samples obtained from the Tolakker, no significant differences were found between the two locations (supplementary figure 1B). Samples stored in the refrigerator for 168 hours had significantly lower Shannon indices compared to samples stored by freezing (figure 2B). Pielou's evenness of samples frozen for 24 hours was significantly lower compared to samples stored at room temperature and samples stored by refrigeration for 24 hours (figure 2C).

Beta diversity

PERMANOVA showed that difference in community structure was largely explained (48%) by the microbial contents of the baseline samples (R2; $p = 0.001$). This is illustrated by the clustering observed in the principal coordinate analysis in figure 3. The storage conditions of colostrum were of less influence, explaining 16% (R2; $p = 0.012$). Both location of the animals and parity seemed of influence as well, but these factors were strongly correlated with the individual animal ID and therefore with the baseline colostrum sample.

Figure 4 shows the bacterial abundances of the stored colostrum samples over time at family level. For both room temperature and refrigerator conditions, individual temporal dynamics of the top 10 most abundant families are shown in supplementary figures 2A and 2B, respectively. Storing colostrum for 24 hours at room temperature seemed to increase the abundance of *Enterobacteriaceae* compared to the baseline sample and storing it for 8 hours (figure 4A, supplementary figure 2A). Community structure between storing at room

temperature for 24 hours and the baseline sample was significantly different (PERMANOVA, p=0.002). However, community structure was not different between storing 8 and 24 hours at room temperature (p=0.259).

As can be seen in figure 4B and supplementary figure 2B, the relative abundance of Enterobacteriaceae of colostrum stored in a refrigerator remained relatively stable for the first 24 hours and seemed to increase after one week of storage. A similar trend was observed for *Streptococcaceae, Pseudomonadaceae* and *Carnobacteriaceae*. The community structure of samples refrigerated for a week differed significantly from the baseline samples (p=0.010) and samples stored in refrigerator for 8 hours (p=0.001) and 24 hours (p=0.006). As can be seen in figure 4C, community structure seemed to remain stable when heating colostrum samples that were refrigerated for 24 hours using either a microwave or water bath.

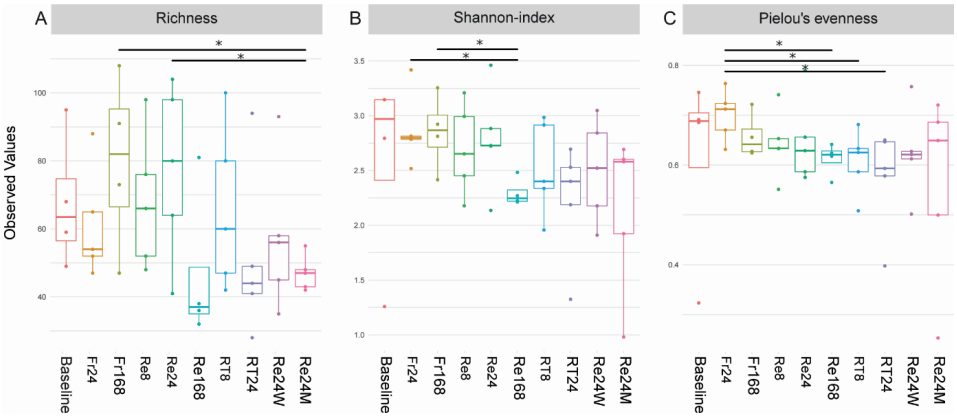

Figure 2. Alpha diversity indices (Richness, Shannon diversity index and Pielou's evenness) between all storage conditions: frozen for 24 hours and for a week (Fr24 and Fr168, respectively), refrigerated for 8 hours, 24 hours and for a week (Re8, Re24 and Re168, respectively), stored at room temperature for 8 and 24 hours (RT8 and RT24, respectively), and refrigerated for 24 hours and reheated by microwave (Re24M) or water bath (Re24W). Significant differences ($p<0.05$) are indicated with an asterisk.

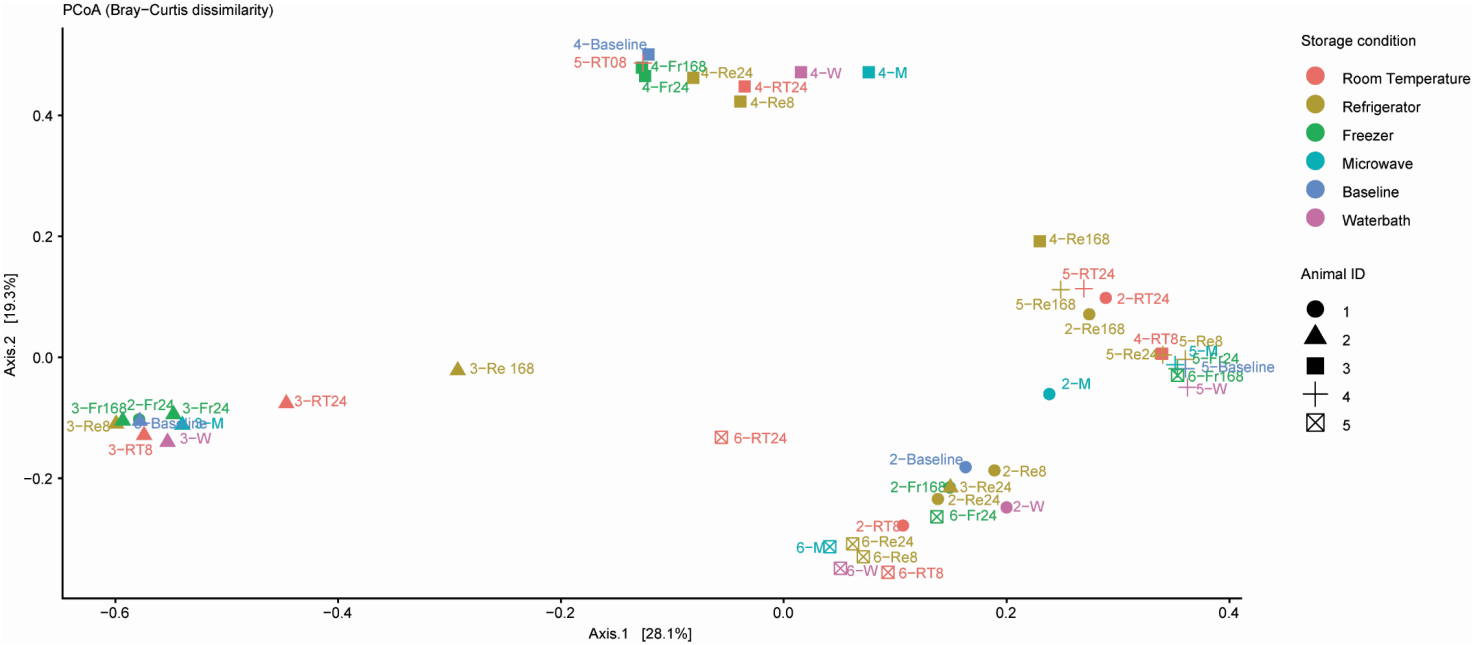

Figure 3. Analysis of beta diversity using principal coordinate analysis (PCoA) of Bray-curtis similarities. Storage conditions include: frozen for 24 hours and for a week (Fr24 and Fr168, respectively), refrigerated for 8 hours, 24 hours and for a week (Re8, Re24 and Re168, respectively), stored at room temperature for 8 and 24 hours (RT8 and RT24, respectively), and refrigerated for 24 hours and reheated by icrowave (Re24M) or water bath (Re24W).

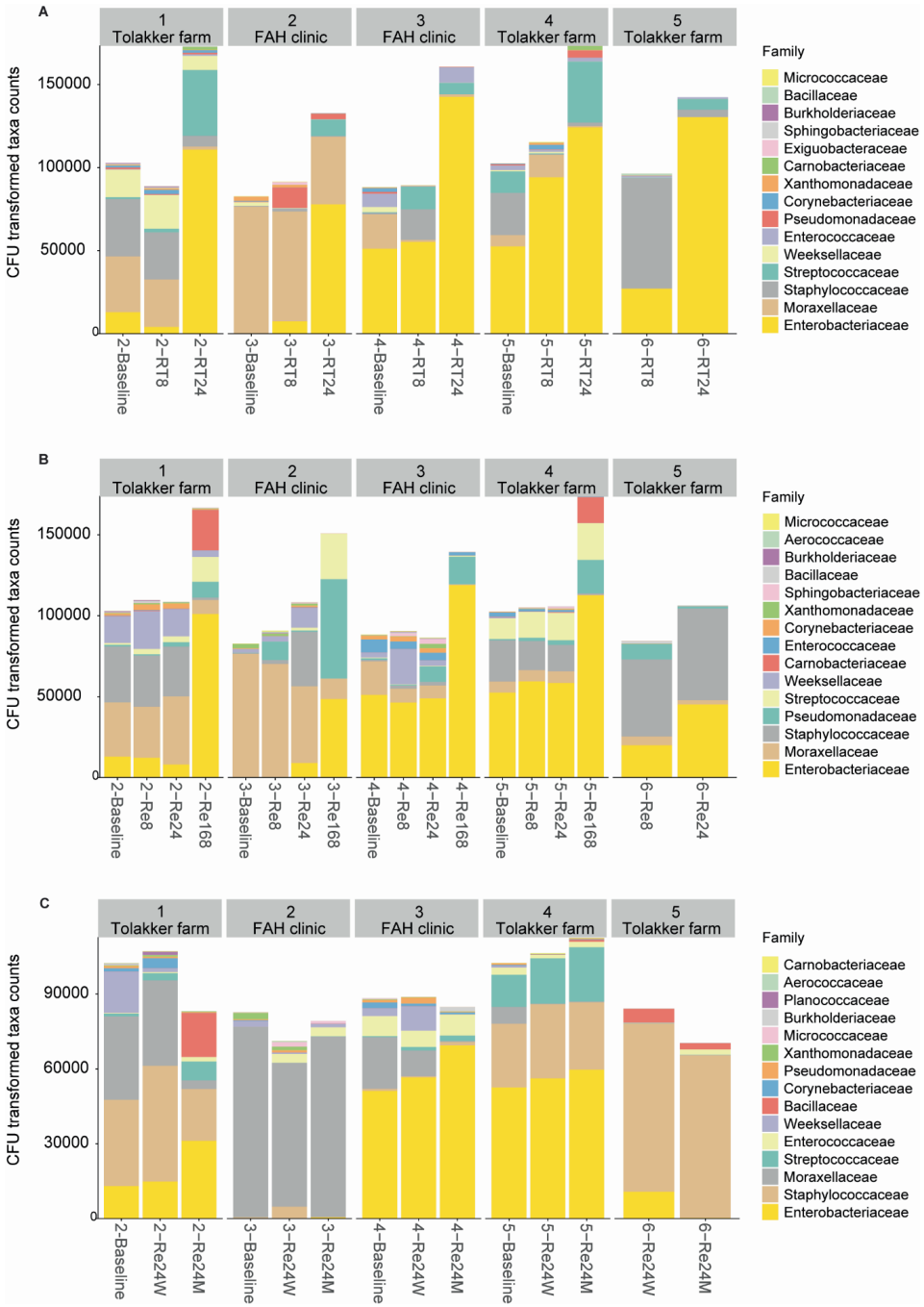

Figure 4. Barplots reflecting temporal dynamics in absolute abundance (relative abundance, corrected for the total CFU counts) on family level per storage condition: A) storage at room temperature including baseline sample (B), room temperature for 8 hours (RT8) and room temperature for 24 hours (RT24); B) storage in a refrigerator (5°C) including baseline sample (B), refrigerated for 8 hours, 24 hours and a week (Re8, Re24 and Re168, respectively); C), reheated samples with the baseline sample (B), microwaved sample (Re24M) and water bath sample (Re24W). Results are clustered according to colostrum sample.

Discussion

The bacterial content of colostrum is an important aspect of colostrum quality, comprising both the total number and the composition of the bacteria [2, 9]. In this study we evaluated the effect of different storage methods on the TPC and composition of the viable microbial community. The main findings were that differences in the TPC compositions of the stored colostrum samples were mostly explained by the variation between baseline samples and that prolonged storage leads to increased counts of predomantly *Enteriobacteriaceae*.

Similar to previous research, our results suggest that contamination during colostrum collection and potentially other environmental factors can have a large influence on the microbial composition of the TPC [6, 15]. This supports the advice to minimize contamination of colostrum during the milking process by improving sanitation procedures, such as hygienic udder preparation (predipping with teat dip, drying the teats with a clean (paper) towel), fore-stripping and sanitation of the milking equipment [3, 10].

Total plate counts in samples obtained directly from the milking bucket were comparable to the results of others [3, 6]. In line with earlier studies [3, 4], the results from our study show increased TPC for colostrum stored for 24 hours at room temperature, but not when refrigerated for the same amount of time. Additionally, the community structure of the TPC was significantly different from the baseline samples. Adjusting the relative abundance data to absolute abundance using the TPC revealed that this change was predominantly due to growth of *Enterobacteriaceae*. Instead, *Enterobacteriaceae* abundance of refrigerated samples seemed to remain stable for the first 24 hours, and had increased exponentially in samples refrigerated for a week. Our results support the hypothesis that colostrum is best stored for 24 hours in a refrigerator [3, 4], but that storing colostrum for shorter periods at RT is not disastrous.

A recent study by Van Hese et all (2022) suggested the composition of the colostrum microbiome may interfere with a calf's ability to absorb IgG. Furthermore, previous research by Godden et al. (2012) suggests that especially coliform bacteria negatively affect intestinal IgG absorption by newborn calves [27]. In our study, storing colostrum for either 24 hours at room temperature or in the refrigerator for a week led to growth of *Enterobacteriaceae*. In this study bacterial composition was determined at the family level. However, the observed increase in *Enterobacteriaceae* under these two storage conditions implies that these conditions also give rise to growth of (fecal) coliform bacteria, probably leading to lower efficiency of IgG absorption by the calf [27]. Furthermore, this also indicates that pathogenic *Enterobacteriaceae* (e.g. enterotoxigenic E. *coli*) could thrive under these circumstances, posing a risk to the neonate. Therefore the use of these storage conditions should be avoided as much as possible.

This study aimed to mimic the on- farm situation and this was achieved by taking colostrum from the milking bucket and emulating colostrum storage conditions in the lab. The TPC and microbial composition of the (stored) colostrum samples, therefore, reflected what would be fed to a calf. Assessment of TPC is commonly used to determine microbial quality of milk and/or colostrum [10]. Since microbial quality comprises more than TPC alone, we aimed to evaluate the composition of the viable microbial community in relation to TPC. Identifying microbial composition directly by using 16S rRNA amplicon based sequencing reveals all present microbial DNA, including DNA of non-culturable and dead bacteria, therefore we assessed bacterial growth using the aerobe plate count culture method. A limitation of this approach is that the microbial composition of the TPC provides a potential biased assessment

of the true microbial variety, since only those bacteria that are able to grow under the specific culture conditions are detected. Furthermore, growth rates may be affected by the culture methods as well, and therefore bacterial abundances obtained by 16S analysis are intrinsically biased. However, careful conclusions can still be drawn from our analysis, since these biases are expected to be similar for TPC of all storage conditions in direct comparisons, justifying this comparative analysis. Even though our chosen methods do not precisely reflect the changes in the actual microbial composition of colostrum, and despite the fact that we use a small sample size, our results still provide a valuable insight in microbial dynamics of storage conditions. Future research with larger sample size comparing results from different culture methods and/or metagenomic sequencing up to species/strain level, should be undertaken to further explore the factors affecting changes in microbial composition of stored colostrum. For management practices it may be relevant to study whether observed changes are primarily caused by the dam (observed differences are a cow factor and represent the microbial community of colostrum independent of milking method) or that most or part of these changes are driven by the environment and milking method hygiene. This was beyond the scope of the current study.

In this study we introduced a novel approach to investigate the bacterial quality of colostrum by characterizing the microbial composition of the observed total viable bacterial plate counts and how this is affected by storage methods commonly used by dairy farmers. The bacterial composition of the baseline samples had a stronger influence on bacterial composition than storage method. Nevertheless, storage at room temperature for 24 hours and refrigerating for a week resulted in significantly altered microbial composition of the colostrum TPC, predominantly resulting in increased numbers of *Enterobacteriaceae*. It is therefore important to prevent bacterial contamination during the milking process and it is advised to store colostrum in a refrigerator for a maximum of 24 hours. How the bacterial composition of colostrum affects calf health is open for investigation.

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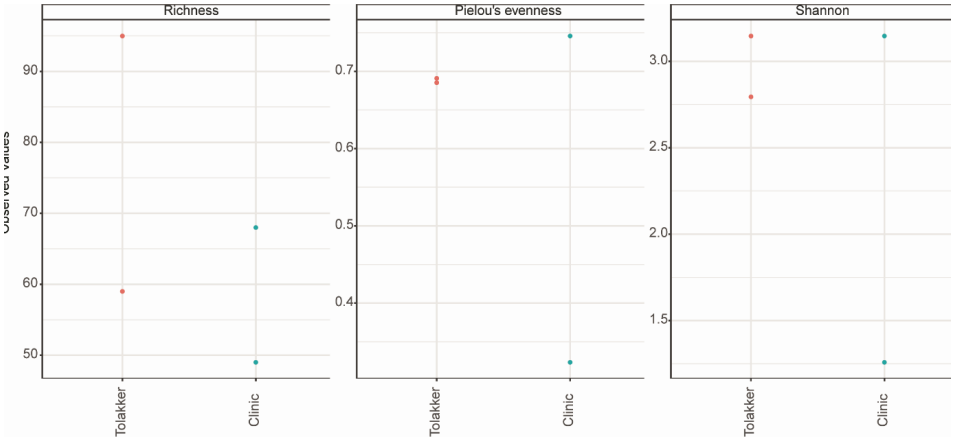
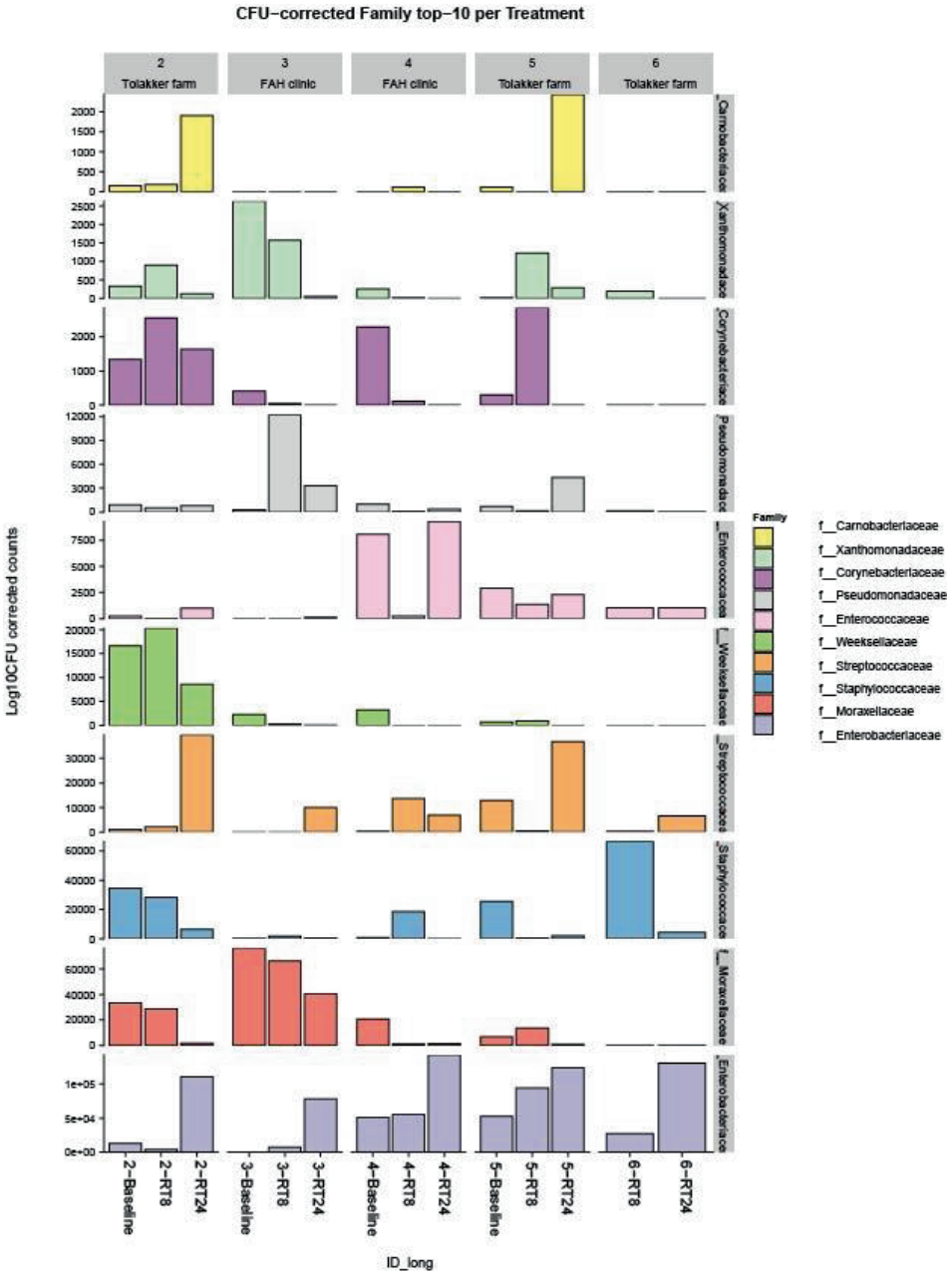
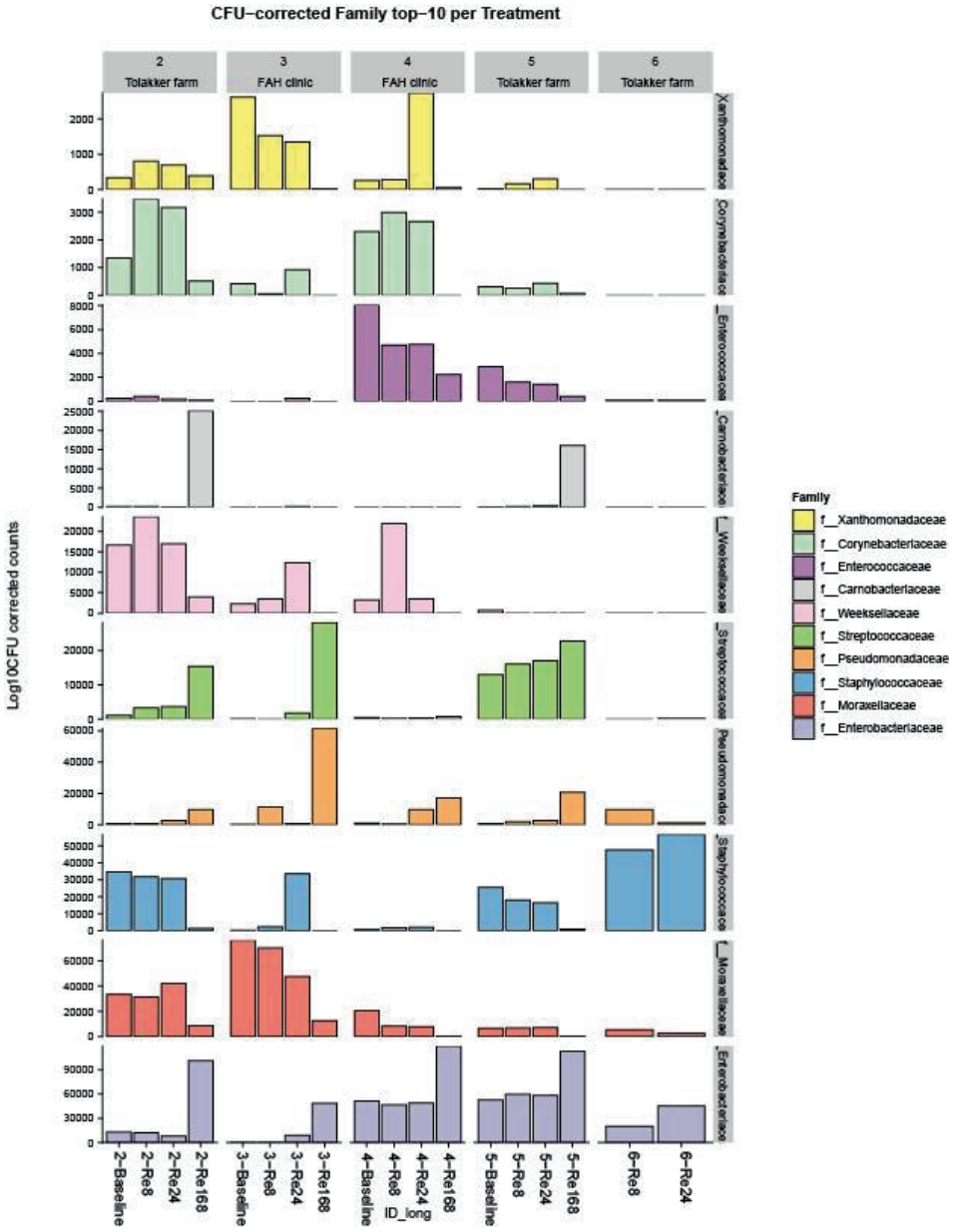
Supplementary figures



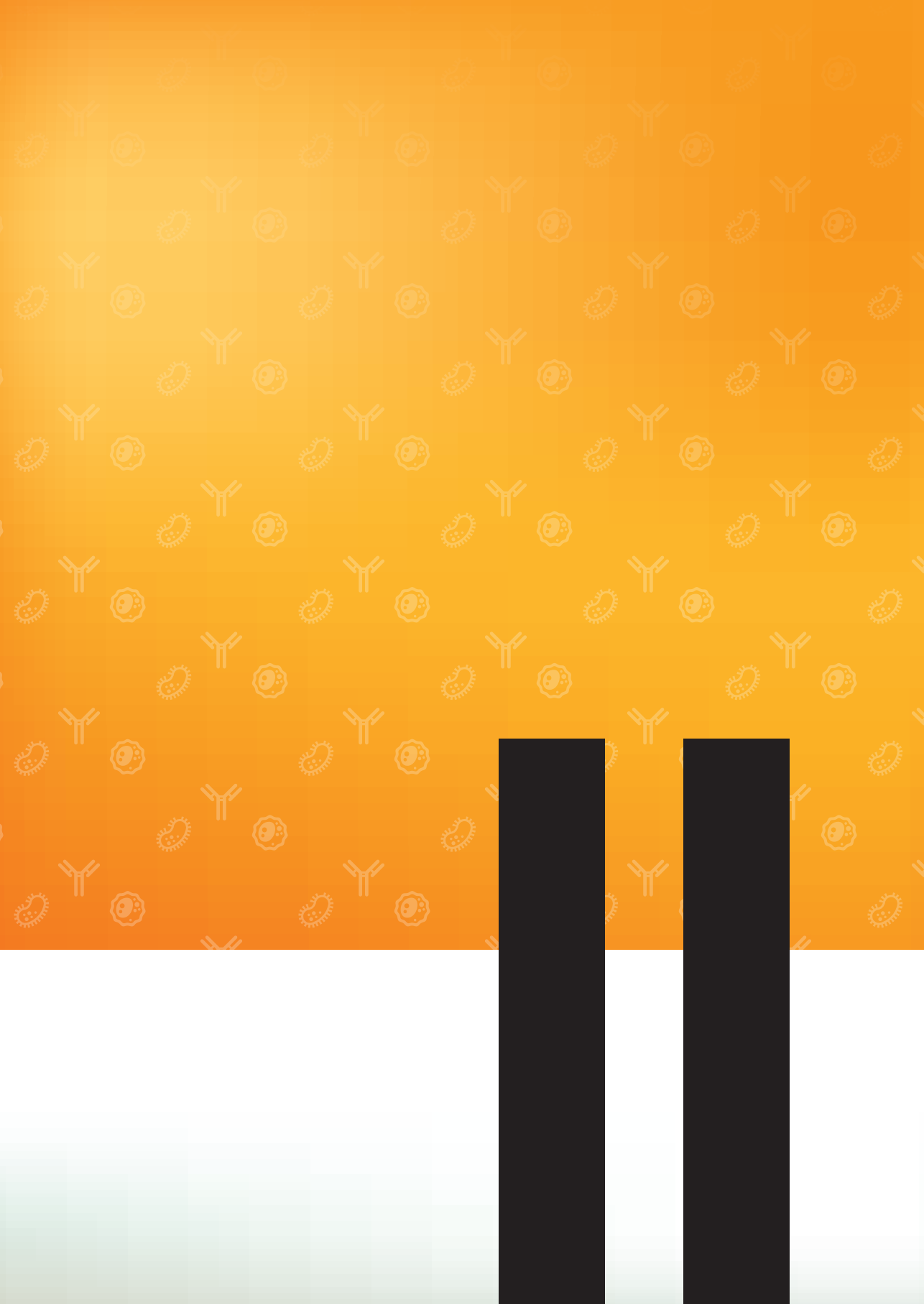
Supplementary figure 1. Alpha diversity indices (Pielou's evenness, richness and Shannon diversity index) between baseline samples from two different collection locations. Red boxplots indicate samples collected at the FAH clinic and blue boxplots indicate samples collected at the Tolakker farm.



Supplementary figure 2A. Individual temporal dynamics in absolute abundance (relative abundance, corrected for the total CFU counts) of the top 10 most abundant families of colostrum samples characterised directly after milking (Baseline) or after storage at room temperature for 8 (RT8) and 24 hours (RT24).



Supplementary figure 2B. Individual temporal dynamics in absolute abundance (relative abundance, corrected for the total CFU counts) of the top 10 most abundant families of colostrum samples characterised directly after milking (Baseline) or after storage at in a refrigerator for 8 hours (Re8), 24 hours (Re24) and 1 week (Re168).



PART II

Understanding the role of colostral immune cells in the neonatal immune response


5

CHAPTER 5

Evidence for transfer of maternal antigen specific cellular immunity against Mycobacterium avium ssp. paratuberculosis via colostrum in a goat twin model

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**equal contribution*

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Abstract

Colostrum intake is one of the most important factors in neonatal health in ruminants, mainly because of its unique immunological properties. Both in practice as well as in research, the attention of lactogenic immunity is focused on the importance of colostral antibodies and less attention is given to the functional role of maternal cells in colostrum. Here we study the transfer of maternal leukocytes via colostrum and the functionality in goat kids. In experiment 1, twenty twin pairs of goat kids from dams previously immunized with an inactivated *Mycobacterium avium* subsp. *paratuberculosis* (MAP) vaccine were fed maternal colostrum from their dam (kid 1) or pasteurized and frozen/thawed bovine colostrum (kid 2). The presence of cell mediated immune response (CMIR) against *Mycobacterium avium* antigens in the kids was assessed using intradermal skin testing with PPD-A tuberculin. Linear mixed effect models showed an increase in skin thickness in response to intradermal PPD-A injection in maternal colostrum fed kids compared to bovine colostrum fed kids. After intradermal PPD-A application, serum concentration of MAP specific antibodies increased in kids fed maternal colostrum, indicating antigen specific activation of the adaptive immune system. We did not detect a similar increase in antibodies in the kids fed bovine colostrum.

In experiment 2, a more reductionistic approach was applied to specifically study the effects of the transfer of maternal colostral leukocytes on CMIR in goat kids. Similar to experiment 1, twin kids from MAP immunized dams were randomly divided over two groups. The experimental group received colostrum replacer supplemented with fluorescently labelled colostral cells of the dam and the control group received colostrum replacer only. No difference in skin response following intradermal PPD-A injection was observed between both groups of kids. Histologic examination of the skin at the intradermal injection site did not show fluorescently labelled cells.

In conclusion, in our initial experiment we observed an antigen specific CMIR in goat kids fed fresh colostrum with colostral leukocytes from vaccinated dams. The lack of a DTH response in kids fed colostrum replacer supplemented with maternal colostrum derived leukocytes indicated that the complete colostral matrix is probably required for colostrum leukocytes to transfer across the intestinal epithelial barrier and modulate the neonatal immune response. In line with earlier studies, our results indicate that caprine maternal leukocytes present in colostrum can functionally contribute to the newborns' early adaptive immune responses adding to the importance of colostrum feeding in ruminant neonates.

Introduction

High neonatal mortality rates in the bovine and caprine dairy industries illustrate that the neonatal period in ruminants is not easily managed. Colostrum intake is one of the most important factors in neonatal health in ruminants, mainly because of its unique immunological properties. At birth, ruminants are immunologically naïve as there is no transfer of maternal immunity such as antibodies across the placenta (Barrington and Parish, 2001). This makes the young ruminants highly susceptible to a variety of pathogens, unless there is sufficient maternal immunity transferred via colostrum. Both in practice as well as in research, the attention of lactogenic immunity is focused on the importance of antibodies absorbed from colostrum by the neonate. The importance of the transfer of maternal immunoglobulins by providing a sufficient amount of high quality colostrum directly post-partum is widely recognized (Robbers et al., 2021). Less attention is paid to the transfer of cellular immune components through colostrum (Gonzalez and Dus Santos, 2017). Research indicates that maternal leukocytes from colostrum can migrate intercellularly across the offspring's gut wall (Liebler-Tenorio et al., 2002). Maternal cells are able to reach the neonatal circulation until at least three days postpartum in sheep lambs (Schnorr and Pearson,

1984), which seems longer than the period until the so-called gut closure for maternal antibodies (24-36 hours) (Weaver et al., 2000). It has been demonstrated that the colostral environment induces phenotypical changes in leukocytes, thereby enabling them to enter the systemic circulation a few hours after ingestion to a point where 1% of circulating cells are maternal leukocytes (Reber et al., 2006). Data from several studies suggests that transferred maternal cells can fulfill protective functions or enhance the neonatal immune response. Donovan and colleagues found that in the first few days after colostrum feeding, leukocytes from newborn calves fed whole colostrum showed enhanced proliferative responses in vitro against antigens to which their dams were previously immunized (Donovan et al., 2007). Others suggested that maternal leukocytes enhance the antigen-presenting capacity of the neonatal immune system (Reber et al., 2005). In addition, (Reber et al., 2008) proposed that presence of maternal leukocytes from colostrum was correlated with faster development of neonatal lymphocytes in the first week of life. Another study showed that calves receiving cell-free colostrum displayed higher incidence of overall disease compared to calves receiving whole colostrum (Novo et al., 2017). Together, these studies all demonstrate an important role for colostral leukocytes in the newborn.

Intradermal skin tests are frequently used to determine *in vivo* cell mediated immune responses (CMIR) in many species (Allen Black, 1999; Bandrick et al., 2008; Hernández et al., 2005; Zhu et al., 1999). Whenever such a cell mediated immune response is provoked in response to an intradermally injected antigen and the response develops over time, it is called a delayed type hypersensitivity (DTH) response, also known as a type IV immune response. This secondary immune response is characterized by activation and proliferation of mainly $CD4^+$ lymphocytes, followed by infiltration of the skin, resulting in a local, inflammatory response (Vohr, 2005). A widely used example of such an immune response is the tuberculin skin test used for clinical diagnosis of (bovine) tuberculosis. Bovine tuberculin (PPD-B) is injected intradermally and in an individual previously exposed to *Mycobacterium bovis* this leads to local inflammation and swelling of the skin caused by infiltrating antigen experienced T lymphocytes and macrophages. *Mycobacterium avium* and closely related subspecies, such as *Mycobacterium avium* ssp *paratuberculosis* (MAP), are common enteric pathogens of ruminants. Infection with *M. avium* and/or related subspecies can lead to false positive skin test reactions to PPD-B, due to the presence of conserved antigens in tuberculins. For this reason a comparative skin test using PPD-B and PPD-A, a *Mycobacterium avium* derived tuberculin, can be performed. The increase in skin thickness 72 hours post injection is used as a diagnostic indicator whether the test subject has been exposed to *Mycobacteria* (OIE, 2018).

While many studies demonstrated an important role for colostral leukocytes in newborn ruminants, *in vivo* mechanisms of action and antigen specific functionality are not clear. We therefore designed two experiments in goat twins in which CMIR was studied *in vivo* with the use of an intradermal skin test. We used a MAP vaccine registered for use in goats to prime MAP specific CMIR in nulliparous goats prior to breeding and evaluated transfer of antigen specific CMIR to the offspring. In the first experiment, twin kids received either maternal colostrum from MAP vaccinated dams or pasteurized frozen/thawed bovine colostrum. In a second experiment, twin kids were fed colostrum replacer supplemented with or without isolated maternal colostral cells (from MAP vaccinated dams). In both experiments intradermal skin testing with PPD-A, a *Mycobacterium avium* derived tuberculin, was used to measure MAP specific CMIR in the goat kids.

Materials and Methods

Ethics

All studies were reviewed and approved by the Utrecht University Animal Ethics Committee and the Dutch Central Committee for Animal Experiments under permit № AVD1080020185064. The animal experiments were conducted in accordance with the Dutch law on Animal Experimentations (Wet op de Dierproeven) and the European regulations on the protection of animals used for scientific purposes (EU directive 2010/63/EU).

Animals and treatments

Experiment 1

Sample size was calculated prior to commencing the study. Because expected means and standard deviations were uncertain and because of the paired design of the experiments, sample size was determined for comparing paired proportions. We aimed to identify a difference of 45% between the pairs of kids with a 95% precision and a power of 80%. For this, a sample size of 20 pairs was required. Considering a potential fallout percentage of 20% (e.g. due to unexpected singletons), we aimed to include 24 twins in this experiment. Dams were selected at a certified CAE/CL free commercial dairy goat farm. There were no indications of endemic paratuberculosis based on farm history and the farmers declaration. In total 40 clinically healthy nulliparous goats were vaccinated against *M. avium* subsp. *paratuberculosis* (Gudair® vaccine) for the purpose of this study approximately two weeks prior to the three-week mating period. Goats were checked twice by transabdominal ultrasound scanning at approximately 9 and 10 weeks of gestation to select goats with twin pregnancies. All twin-carrying goats were subjected to a PPD-A tuberculin skin test at approximately 100 days post vaccination to verify that the Gudair® vaccination induced a DTH response against PPD-A. The 24 dams which showed the highest skin test response were selected. At three weeks before the expected kidding, the animals arrived at the research facilities of the Farm Animal Health division of the Department of Population Health sciences, Faculty of Veterinary Medicine, Utrecht University. The dams were placed in four different compartments of the research facility, each containing a deep-litter pen for six goats. The goats were provided with ad libitum straw and restricted amount of concentrate three times a day according to a commercial feeding scheme and had free access to water. Dams were monitored 24/7 by CCTV with care takers on site to detect kidding and to ensure that kids were immediately separated from the dam at birth. Dams were briefly restrained at parturition and were released back into the group immediately following parturition. Kids were directly moved to individual pens next to the adult goats, which allowed visual, auditory and nose-to-nose contact between kids and dams, however suckling was not possible.

The newborn twins were allotted over two treatment regimens using a predefined pseudo-random block randomization scheme to ensure that 1st/2nd born kids and male/female kids were equally distributed between the two treatments. Group 1 received colostrum and milk derived from their dam (Caprine colostrum, CC). Colostrum was obtained by milking the goats by hand and kids were fed a volume corresponding with 5% of the kid's bodyweight. At 2-4 hours postpartum, goats were machine-milked completely for the first time. Kids in the CC group were again fed 5% of their bodyweight. The kids in group 2 (Bovine colostrum, BC) received pasteurized and frozen/thawed bovine colostrum and milk replacer (Denkamilk Capriplus, 22% crude protein) (28.5 g dissolved in 150 mL water) in similar amount as the kids in the CC group**.** The bovine colostrum was obtained from a certified paratuberculosis-free dairy farm. After feeding the kids in both treatment groups a volume of colostrum equal to 10% of their body weight within 4 hours postpartum, all kids were fed similar quantities of milk following a commercial schedule at 07:00, 12:00 and 19:00, starting at 200ml/feeding at

the first day to 500ml/feeding at the end of week two. Kids in the CC group were fed dam's milk, while kids in the BC group received equal amounts of the commercial milk replacer. Dams were milked twice a day (at 6:00 and 18:00). The required amount was directly fed to the corresponding offspring and surplus milk from the morning milking was stored in a fridge until the afternoon feeding at 12:00. Milk and milk replacer were heated with a water bath and fed at 40°C. Kids were euthanized by intravenous injection of pentobarbital sodium (100 mg/kg BW, Euthanimal 20%) at 27-33 days of age and subjected to necropsy. After completion of the experiment dams were returned to a commercial Dutch goat farm. For a graphical visualization of the experimental design, see Figure 1A.

Experiment 2

Sample size was calculated prior to commencing the study. In 15 out of 20 twins from the first experiment, the highest DTH response was measured in the group that received maternal colostrum. Therefore we aimed to identify a difference of 70% between the pairs of kids with a 95% precision and a power of 80%. For this, a sample size of 10 pairs was required. Considering a potential fallout percentage of 20% (e.g. due to unexpected singletons), we aimed to include 12 twins in this experiment. Similarly to the selection procedure described in experiment 1, 44 clinically healthy nulliparous goats were vaccinated against paratuberculosis (Gudair® vaccine). Approximately 9 and 11 weeks after the start of the mating period goats were checked twice for twin pregnancy by transabdominal ultrasound scanning. Twelve clinically healthy and pregnant dams showing the highest skin test response to intradermal skin testing with PPD-A were selected. Housing and procedures around parturition were similar to experiment 1. Kids were housed in similar conditions as in experiment 1. Since sex and order of birth were not related to the outcome parameters in experiment 1, but birthweight was, the newborn twins were allotted over two treatment regimens using a predefined pseudo-random block randomization scheme in which the kids were equally distributed among the treatment groups according to their birthweight (BW). The first group received colostrum replacer (Capracol®, Arts Food Products BV, 's Hertogenbosch, Netherlands) supplemented with CellTrace Violet (CTV) (CellTrace™ Violet Cell Proliferation Kit, Life Technologies, Paisley, UK,) -labelled maternal cells derived from their dams' first milking colostrum (Cells, C). The kids in the second group (No cells, NC) received colostrum replacer in similar quantity as the C group. A detailed description of the cell isolation and preparations can be found in a subsequent paragraph (***Cell isolation and CTV staining*****).** Both groups received their first colostrum feeding at 2 hours after birth, followed by a second feeding of milk replacer (Denkamilk Capriplus, 22% crude protein) at four hours post-partum. When the first feeding was not voluntarily ingested by bottle feeding, an esophageal tube was used to ensure adequate colostrum intake. After colostrum feeding all kids were fed similar quantities of milk replacer, following the schedule as described in experiment 1. Dams were milked twice a day, at 06:00 and at 18:00. Kids were euthanized by intravenous injection of pentobarbital sodium (100 mg/kg BW, Euthanimal 20%) at 8-10 days of age. After completion of the experiment dams were returned to a commercial Dutch goat farm. For a graphical visualization of the experimental design, see Figure 1B.

Intradermal skin tests in goats and kids

In **experiment 1** the intradermal skin test in goats and kids consisted of a single intradermal injection at the left side of the neck at day 7 postpartum with 0.1 ml PPD-A 2500 IE (obtained from *M. avium* subsp. *avium* strain D4ER) (Thermo Fisher Scientific). Before injection, the injection site was shaved and double fold skin thickness was measured with a Hauptner tuberculin caliper. Injection was performed using a disposable 1 ml plastic syringe

with a 0.45x16 mm needle. In adult goats, the injection site was assessed at 72 hours post injection using a Hauptner caliper. In the kids, the assessment was performed at 24, 48 and 72 hours post injection. The assessment of the reaction in the kids consisted of three separate measurements. First, the diameter of the swelling a the injection site was measured using a Vernier caliper. This measurement included all clinical alterations of the skin, taking induration as well as erythema into account. Second, the double fold skin thickness at the injection site was measured using a Hauptner tuberculin caliper. Third, the reaction site was checked for the presence of edema. All measurements were performed unblinded by the same person.

Intradermal skin tests in the kids in the **experiment 2** consisted of 2 bilateral intradermal injections at day 7-9 post-partum: one with 0.1 ml PPD-A 2500 IE (obtained from *M. avium* subsp. *avium* strain D4ER) (Thermo Fisher Scientific) and one with 0.1 ml sterile PBS. Randomization was applied to correct for the side of injection. Since in experiment 1 the skin reaction of the kids peaked at 24 hours post injection, the assessment in **experiment 2** was only performed at 24 hours post injection. The assessment of the reaction in the kids consisted of two separate measurements. First, the double fold skin thickness at the injection site was measured using a Hauptner tuberculin caliper. Second, the reaction site was checked for the presence of edema. Measuring the double fold skin thickness was performed unblinded by the same person that performed the intradermal injection. Checking for edema was performed blinded by another researcher.

Cell isolation and CTV staining

In experiment 2 goats were milked by hand to obtain a volume corresponding with 10% of the kids' BW, from which colostral cells were isolated. Colostrum was equally divided over 16 50mL tubes (Falcon) and diluted with PBS to 50 mL per tube. Tubes were centrifuged at 400 g at room temperature (RT) for 20 minutes. Supernatant was discarded and pellets were resuspended, pooled in 20mL PBS and centrifuged for 10 minutes at 400 g (RT). After discarding supernatant, the pooled pellet was resuspended in 30 mL PBS. Cell Trace Violet (CTV) was added to a final concentration of 5 μM and the solution was incubated for 20 minutes in the dark at RT. After incubation, 200μl of the cell suspension was added to 1ml of FACS buffer (PBS, 2% FCS, 0.01% Na-azide) and refrigerated until further analysis. Another 1.5 ml of cellular concentrate was used for antigen-specific IFN-γ release assays. The remaining cell suspension was centrifuged and the supernatant with leftover staining without the cells was added to 170mL colostrum replacer, resulting in a solution of 75 grams of colostrum replacer into 200mL of liquids. This colostrum was used for feeding the NC group. The CTV stained cell pellet was resuspended in 30mL of PBS and added to another 170mL of colostrum replacer and fed to the C group kids.

Sample collection

During **experiment 1** jugular blood samples (serum vacutainer tube) were collected from all goat kids prior to colostral feeding. Additionally, blood samples were collected on days 1, 7, 14, 21 and the day of euthanasia (day 27-33). Furthermore, independent of colostrum fed to goat kids, 20 ml of collected colostrum samples were collected in 25ml tubes to determine total and MAP specific IgG levels.

For **experiment 2**, from all goat kids jugular blood samples were collected before first colostrum feeding, at 24 after birth and at the day of euthanasia (d7-10). Two vacutainer blood tubes were collected, a serum sample for antibody measurement and a heparin anticoagulated sample for *in vitro* stimulation with PPD-A. Immediately following parturition, two blood samples were collected from dams for antibody measurement and *in vitro* stimulation with PPD-A as well.

Antibody measurement

In **experiment 1**, fresh colostrum was diluted with PBS (1:2) and centrifuged at 2000G for 20 minutes at 4°C. 2 ml supernatant was collected and divided over 2 micronic tubes and stored at -20°C until further analyses. In **both experiments**, jugular blood samples were centrifuged at 2500g and 4°C for 15 minutes, after which the serum was stored at -20°C. For **both experiments**, caprine and bovine IgG concentrations were analyzed using Bethyl "Goat IgG ELISA Quantitation Set" (Cat. No. E50-104) and Bethyl "Bovine IgG ELISA quantitation Set" (Cat. No. E10-118) according to instructions provided by the manufacturer. For **experiment 1**, the level of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) specific antibodies were analyzed using the Paratuberculosis Screening Ab Test (Cat. No. 06-07130-27, IDEXX) and S/P ratios were calculated using OD450 values subtracted by the negative control samples, with the negative control as the lower limit, according to the manufacturer's instructions.

Antigen-specific interferon (IFN)-gamma release assays

In experiment 2, 1.5 ml whole colostrum, isolated (non-stained) colostral cells incubated in RPMI supplemented with 10% FCS and 1% Antibiotic-Antimycotic (Gibco), and whole heparinized blood samples of dams and kids were stimulated *in vitro* with PPD-A (250 IU/ml), and a mixture of PMA (50 ng/ml)/Ionomycin (2µg/ml) as positive control and PBS as negative control. Samples and their stimulation were incubated for 24h at 37°C. Samples were centrifuged and supernatant was collected and stored at -20°C until further analysis. For IFNγ detection, the BOVIGAM assay (Applied Biosystems, Foster City CA, USA) was used according to manufacturer's protocol.

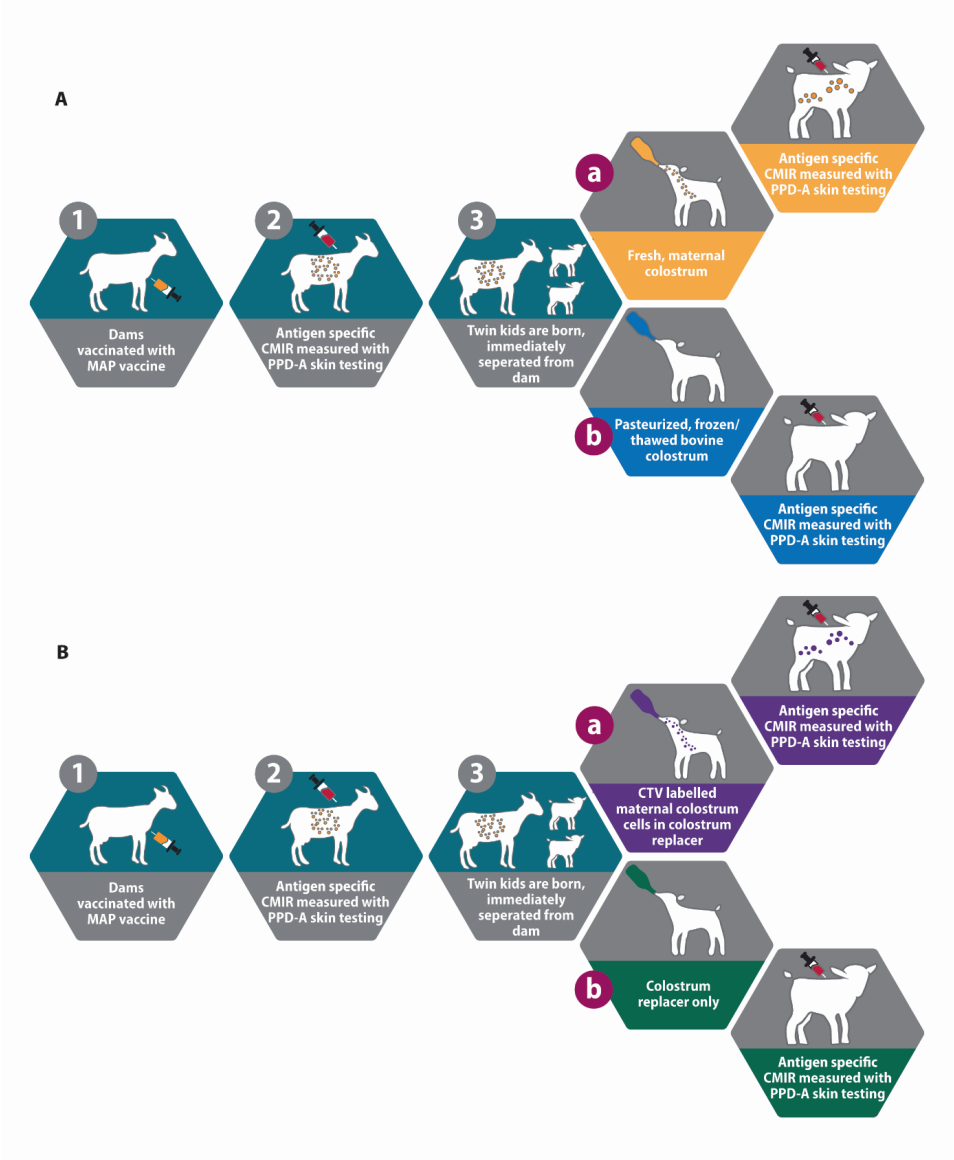

Figure 1. Graphic visualization for experiment 1 (A) and experiment 2 (B). During step 1, dams were vaccinated with MAP vaccine, and this resulted in a CMIR against MAP as confirmed by PPD-A skin testing in step 2. After twin kids were born (step 3), kids were immediately separated from the dam and divided over treatments "a" and "b". Afterwards, in both kids the CMIR against MAP was assessed by PPD-A skin testing.

Flow cytometry

In experiment 1 125ml colostrum of 3 individual goats was obtained for immune cell phenotyping. The colostrum was diluted 1:1 with PBS and centrifuged at 400g for 20' at 4°C. Per goat, the cell pellets were washed twice with 10mL PBS and divided in two. One part was stained with CD4 (Clone 44.38, MCA2213A647, BioRad), CD8 (Clone CC63, MCA837PE, BioRad) and WC1 (Clone CC15, MCA838F, BioRad) in FACSbuffer. The other half of the milk sample was stained with CD68 (Clone ED1, MA5-16654, Invitrogen) after fixation with BD Fix and Perm (BD Cytofix/Cytoperm™ Kit (Cat. No. 554714), according to the manufacturers protocol. Flowcytometry was performed on a BD FACSVerse™ Flow Cytometer.

In **experiment 2**, cell viability of isolated, CTV stained maternal colostral cells was assessed using Fixable Viability Dye ViaKrome 808 (Beckman Coulter, Brea, CA) according to the manufacturer's protocol. In short; cells were harvested, washed with PBS, and stained with 1 μL dye per $5x10^5$ cells for 20 min at RT in the dark. Stained cells were washed with PBS, and fixed in 1% paraformaldehyde (PFA). Cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter) and data were analyzed with FlowJo software (FlowJo LCC, Ashland, OR). Gating strategy can be found in Figure S1.

Post mortem examinations of kids

In a pilot experiment cell culture derived cells were stained using the CTV staining protocol and were subjected to fixation using 4% neutral buffered formaldehyde and checked for presence of CTV derived fluorescent signal. Subsequently fresh CTV stained cells were injected intradermally in fresh goat skin obtained from a slaughterhouse and subjected to fixation in 4% neutral buffered formaldehyde, paraffin embedded and processed for HE staining using routine protocols and checked for the presence of CTV fluorescent signal. In both procedures cells could be visualized using an Olympus BX51 (fluorescence) microscope. In **both experiments** post mortem examination was performed to check for gross pathology and in addition **in experiment 2** both injection sites were examined and a sample was fixed in 4% neutral buffered formaldehyde for 48h for histology. Formaldehyde-fixed samples were processed routinely into paraffin blocks. Paraffin-embedded tissues were cut into 4 μm sections, collected on silane-coated glass sides and dried for at least 48h at 37 °C. After deparaffinization and rehydration in graded alcohols, sections were either stained routinely with haematoxylin and eosin (HE) or examined without further staining for the presence of CTV stained cells using an Olympus BX51 (fluorescence) microscope.

Statistical Analysis

Statistical analyses were performed using R (R Core Team, 2019). When data was normally distributed (Shapiro-Wilks normality test), mean outcome variables of continuous data were compared using paired sample T-tests. For not normally distributed values, the Friedman test was applied using the PMCMRplus package (Pohlert, 2021). For the latter, post-hoc tests were applied (Dunn's many-to-one) with adjustments for multiple testing (Bonferroni).

Univariable regression models were performed to check for crude associations between increase in skin thickness and possible explanatory variables. Based on the outcome of the univariable models, associative linear mixed effects models were developed using the lme4 package (Bates et al., 2015). Residuals of the full models were checked for normality using a normal probability (Q-Q) plot. Backward model reduction based on Akaike Information Criterion (AIC) was performed to determine which variables contributed to a well-fitting model, using the drop1 function (Bates et al., 2015). A variable was dropped from the model when the removal resulted in a reduction in AIC of 2 or more.

Experiment 1. Three linear mixed effect models were fitted: one to evaluate the treatment effect (CC vs BC) and potential confounders, followed by another model to assess which parameters relate to the response observed in the CC group only. A third model was constructed to assess the temporal dynamics of MAP specific antibodies after intradermal injection with PPD-A in the kids.

For the first model, the dependent variable was the increase in skin thickness. The main variables of interest were treatment group (CC vs BC) and time. These variables were included in the model as fixed effects, and the interaction between treatment group and time as well. Two other fixed effects were introduced to check for confounding, being birthweight and the housing compartment of the kids were located. Random intercepts were included for the individual kids, nested in their corresponding mother in order to account for the paired study design. After model reduction, the final model was: Increase ~ factor(group) + factor(time) + birthweight + (1|Dam/KidID).

We used another model to further assess which variables were associated with the increase in skin thickness observed in kids in the CC group. The dependent variable here was the increase in skin thickness in the CC group. The fixed factors time, birthweight, and the compartment of the facility in which the kids were located were included, as well as total and MAP specific antibody levels at the day of kids' intradermal skin testing, and the dams' increase in skin thickness. Since we expected a correlation between total antibody level and MAP specific antibody level, we inserted this as an interaction. A random factor 'Kid ID' was included to adjust for the repeated measures of skin thickness within the kids. After model reduction, the final model was: Increase ~ factor(time) + birthweight + total_antibodies + MAP_specific antibodies + total_antibodies*MAP_specific antibodies + reaction dam + (1|KidID).

The third model looked at the temporal dynamics of MAP specific antibodies within and between treatment groups after intradermal injection with PPD-A. The model with the interaction time*group had the lowest AIC, and the final model was: MAP_specific_antibodies ~ factor(group) + factor(time) + factor(Group):factor(Time) + (1|Kid_ID).

Experiment 2. One linear mixed effects model was fitted to assess the treatment effects. We included group (C vs NC), birthweight and age of the kids as fixed effects. Random intercepts were included for the individual kids, nested in their corresponding mother in order to account for the paired study design. Based on these results, no additional modelling was done.

Results

Experiment 1

MAP specific immune response in dams

Approximately 100 days after MAP vaccination dams were intradermally challenged with PPD-A to assess the MAP specific immune response. At 72 hours post intradermal PPD-A injection, the dams showed a classical DTH response at the site of injection. Average increase in skin thickness following intradermal PPD-A injection for the dams used in our study was 7.52mm (± 2.82 mm) at 72h post injection (Figure 2A). First and second colostrum milkings contained IgG (Figure 2B) and both dams' first and second colostrum milkings and serum contained MAP specific antibodies (Figure 2C).

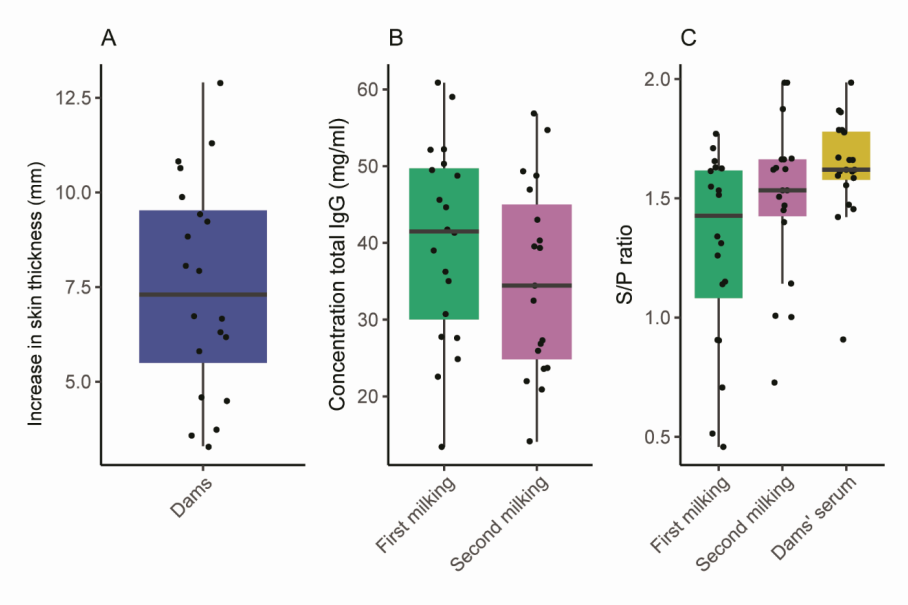

Figure 2. Dams' (n=20) increase in skin thickness (mm) in response to intradermal PPD-A injection (A), concentration of total IgG in colostrum milkings 1 and 2 mg/ml (B) and S/P ratio of MAP specific antibodies in colostrum milkings and serum (C). Box and whisker represent Q1-Q3 and 1.5 x IQR, respectively, with the median indicated by horizontal bar.

In vivo assessment of transfer and functionality of maternal leukocytes in goat kids

The 24 dams gave birth to 18 healthy twins, 2 triplets and the remaining 4 goats gave birth to a stillborn twin, a singleton, and two pairs from which one kid was stillborn. The 18 healthy twins and the two heaviest kids of the triplets were included in the study. Mann-Whitney-Wilcoxon test showed no correlation between sex or order of birth and increase in skin thickness, distributions in Supplementary Table S1. All kids ingested colostrum equal to approximately 10% of their birthweight and this resulted in the rapid increase of blood immunoglobulin concentrations (caprine in CC and bovine in BC groups) 24 hours post-partum (Figure 3). To check for the presence of maternal lymphocytes, colostrum of three dams was collected to assess the phenotype of colostrum cells using flow cytometry. In all three samples, $CD8^+$ lymphocytes appeared most abundant (Supplementary Table S2).

At seven days of age, all kids were subjected to intradermal injection with PPD-A to assess their MAP specific immune response. Mean increase in skin thickness, diameter of the response and proportion displaying edema in both groups are shown in Supplementary Table S3 and Supplementary Figures S2 and S3, respectively. Figure 4 shows the increase in skin thickness in response to challenge with PPD-A for both CC and BC group at several time points post-injection, while data per pair of kids can be found in Figure S4. Backward model reduction was performed to determine variables associated with skin thickness. Our main variable of interest, group, stayed in the linear mixed effect model as an explanatory factor (Table 1). Skin thickness increase in the CC group was 0.20 mm more than in the BC group (95% CI 0.06 mm – 0.35 mm). In addition to the group effect, factors "time" and "birthweight" remained in the final model for illustration purposes as estimates' confidence intervals contained "zero" and are therefore not considered significant. The estimates showed that the mean increase at 24 hours was 0.02 mm higher (CI: -0.07 mm – 0.12 mm) than at 48 hours. Additionally, it was 0.09 mm (CI: -0.01 mm – 0.18 mm) higher when compared to the mean increase at 72 hours (Table 1).

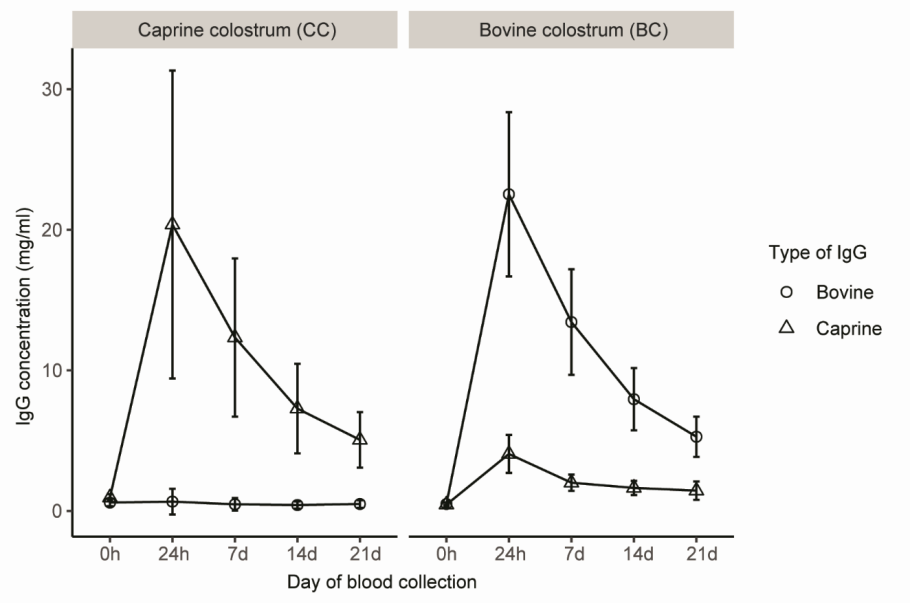

Figure 3. Serum IgG concentration for kids receiving caprine colostrum (CC, n=20) and bovine colostrum (BC, n=20). Circles depict averages of bovine IgG and triangles depict caprine IgG in mg/ml ± 1 x SD per indicated time point.

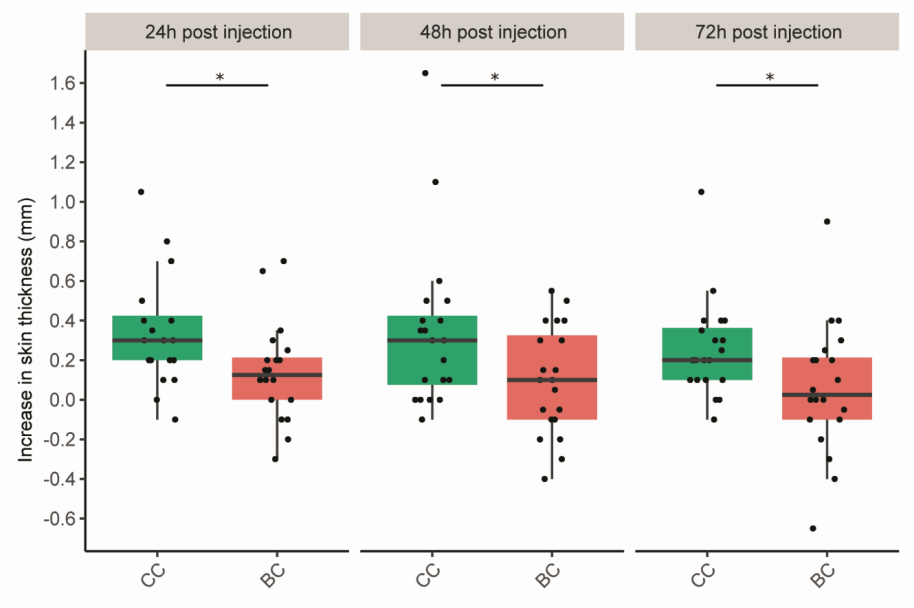

Figure 4. Kids' increase in skin thickness (mm) at 24h, 48h and 72h post intradermal PPD-A injection. Treatment groups are indicated with CC (n=20) as receiving maternal (caprine) colostrum and BC (n=20) as receiving bovine colostrum. Significance ($p<0.05$) between groups within each time point is indicated with an asterisk (Paired sample T-Test). Box and whisker represent Q1-Q3 and 1.5 x IQR, respectively, with the median indicated by horizontal bar.

Table 1. Estimates, standard errors and confidence intervals for the reduced linear mixed effect model for explaining the observed difference in skin thickness between treatment group (CC) and control group (BC).

Variable	Level	β	S.E. (β)	95% CI (β)	Variance (S.D.)
Intercept		0.2190	0.2726	0.2300; 0.4708	
Group	Treatment (CC)	0.2045	0.0741	0.0558; 0.3500	
Time	48 hours post injection	-0.0225	0.0488	-0.1182; 0.0732	
	72 hours post injection	-0.0863	0.0488	-0.1819; 0.0094	
Birthweight		0.0357	0.0720	-0.1081; 0.1766	
Kid/Dam					*0.036 (0.19)*
Dam					*0.010 (0.10)*

Variances of the random effects are in cursive.

Variables affecting increase in skin thickness in the CC group

A second linear mixed effects model was fitted to define which variables were associated with an increase in skin thickness in the CC group. Estimates and corresponding standard errors and confidence intervals are displayed in Table 2. A larger increase in skin thickness and higher concentration of MAP specific antibodies in the mother were associated with a smaller increase in skin thickness observed in the kid. A higher birthweight and a higher

concentration of total antibodies were positively associated with a larger increase in skin thickness in the kids. Interaction between total amount of antibodies and MAP specific antibodies in serum of the kids remained in the model as well, which was associated with less increase in skin thickness in kids. For all of these associations, the confidence intervals of the estimates contained "zero" and are therefore not considered significant.

Adaptive immune response following intradermal PPD-A skin testing

MAP specific IgG concentrations increased rapidly after colostrum feeding and remained similar up until seven days of age. After intradermal injection with PPD-A at day seven an increase in the MAP specific antibody levels was observed in the kids fed maternal colostrum (Figure 5). At experimental day 21, approximately 14 days post intradermal injection there is a peak in the antigen specific antibody response in the kids fed maternal colostrum (CC) only, after which the MAP specific antibody levels decline. According to the linear mixed effects model CC kids showed significantly higher MAP specific antibody levels compared to BC kids at 7, 14 and 21 days after intradermal injection of PPD-A (Supplementary Table S4). Because the residuals were not randomly distributed, we performed non-parametric tests as well, which revealed a significant increase in MAP specific antibodies at 7, 14 and 21 days after intradermal injection in the CC group. In the BC group there was a significant decrease compared to the day of intradermal injection, however almost all values were below the detection limit of the MAP antibody ELISA.

Table 2. Estimates, standard errors and confidence intervals for the reduced linear mixed effect model for associations with skin thickness in treatment group (CC).

Variable	Level	B	S.E. (β)	95% CI (β)	Variance (S.D.)
Intercept		0.0125	0.9702	-1.6581; 1.6830	
Time	48 hours post injection	-0.0025	0.0711	-0.1417; 0.1367	
	72 hours post injection	-0.0900	0.0711	-0.2292; 0.0492	
Birthweight		0.0888	0.1056	-0.0930; 0.2707	
Total antibodies		0.0753	0.1079	-0.1105; 0.2611	
MAP antibodies		-0.0261	-0.5395	-0.9549; 0.9027	
Total antibodies * MAP antibodies		-0.0290	0.0616	-0.1351; 0.0771	
Reaction dam		-0.0360	0.0247	-0.0784; 0.007	
Kid ID					*0.054 (0.23)*

Variances of the random effects are in cursive.

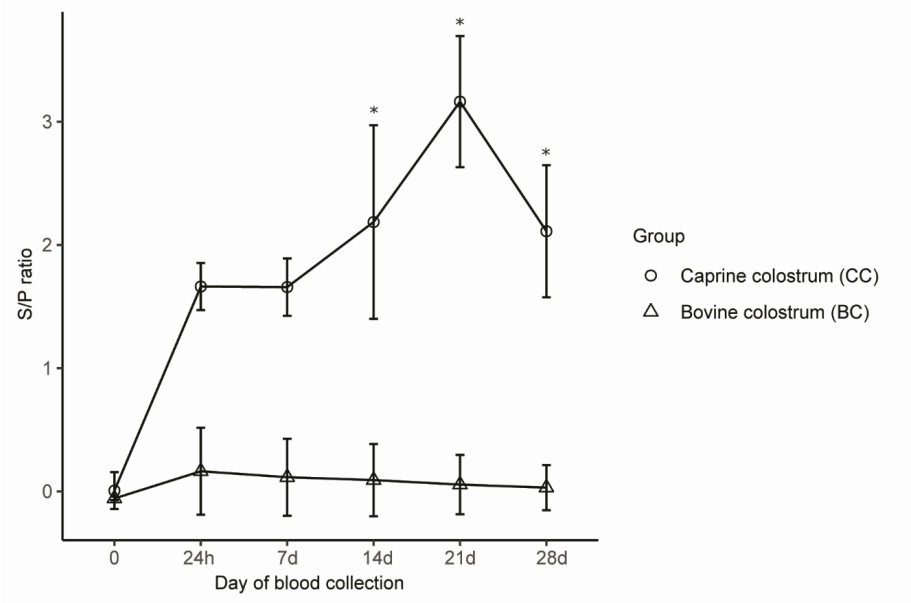

Figure 5. S/P ratio of MAP specific antibodies over time. Asterisks indicate a significant difference (*p<0.05) between time points 14 days, 21 days and 28 days, and the day of intradermal injection at day 7 (Friedman test, Dunn's many to one comparison, Bonferroni adjustment)) in mg/ml ± 1 x SD per indicated time point.

Experiment 2

MAP specific immune response in dams

Approximately 100 days after vaccination with MAP, dams were tuberculinated with PPD-A to assess the MAP specific immune response. At 72 hours post intradermal injection, the dams showed a classical DTH response following intradermal PPD-A injection. Average increase in skin thickness following intradermal injection of PPD-A for the dams used in experiment 2 (n=12) was 8.28 mm (± 4.20 mm) at 72h post injection.

In vivo assessment of transfer and functionality of maternal leukocytes in goat kids

The 12 dams gave birth to 11 healthy twins, the remaining dam gave birth to two stillborn kids and 1 healthy kid. Eleven twin pairs were included in the analyses. Viability of isolated and stained colostral cells was assessed when this was possible within 16 hours after the staining procedure (Figure S1). Mean viability was 91% (± 6.07, n = 5). All kids ingested the intended amount of bovine derived colostrum replacer (200 ml) with or without CTV labeled maternal colostrum cells. Colostrum ingestion by the kids was confirmed by increased bovine IgG concentrations in serum (C and NC groups) 24h post ingestion (Figure 6). At 7-9 days of age, all kids were intradermally injected with PPD-A to assess their MAP specific immune response. Mean increase in skin thickness resulting from PPD-A intradermal injection did not differ significantly between groups, nor did it differ from the increase in skin thickness as a result from injection with PBS (Paired sample T test, Figure 7). A linear mixed effects model was fitted to further explore the effect of treatment group and other factors on the increase in skin thickness. However, treatment was dropped from the model as it did not have an effect on the skin thickness following intradermal PPD-A injection. Furthermore, examination of the

skin at the site of injection using fluorescent microscopy did not show cells that were stained with CTV.

Stimulation assays

Whole blood, whole colostrum and isolated cells were stimulated with PPD-A and IFNγ secretion was assessed by ELISA. Whole blood samples of four of the 11 dams responded to stimulation with PPD-A above the threshold (O.D. > 0.1), while all of the samples responded to the positive control (Figure 8A). Both whole colostrum samples and isolated colostral cells did not produce IFNγ above the threshold (O.D. > 0.1) following stimulation with either PPD-A or the positive control (Figure 8B,C). None of the stimulations of kids' whole blood with PPD-A resulted in production of IFNγ, however these samples did respond to stimulation with the positive control (Figure 8D-F). We found no differences in IFNγ production at any of the time points between the two treatment groups. No associations were found between the four dams with blood samples that showed IFNγ production in response to PPD-A stimulation *in vitro* and other outcome parameters, such as increase in skin thickness in either the dams or their kids.

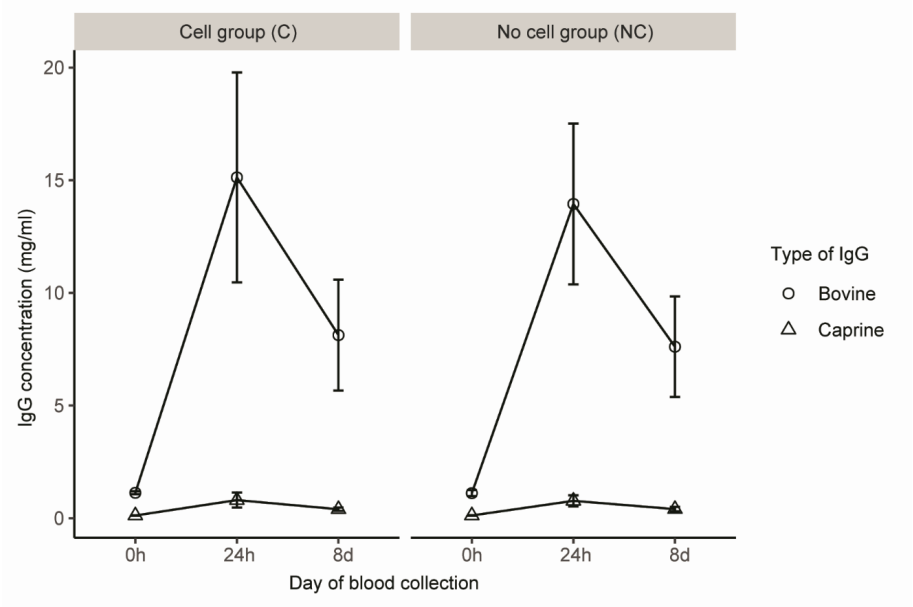

Figure 6. Serum IgG concentration for kids receiving maternal cells (C, n=11) and no maternal cells (NC, n=11). Circles depict presence of bovine IgG and triangles depict caprine IgG in mg/ml ± 1 x SD per indicated time point.

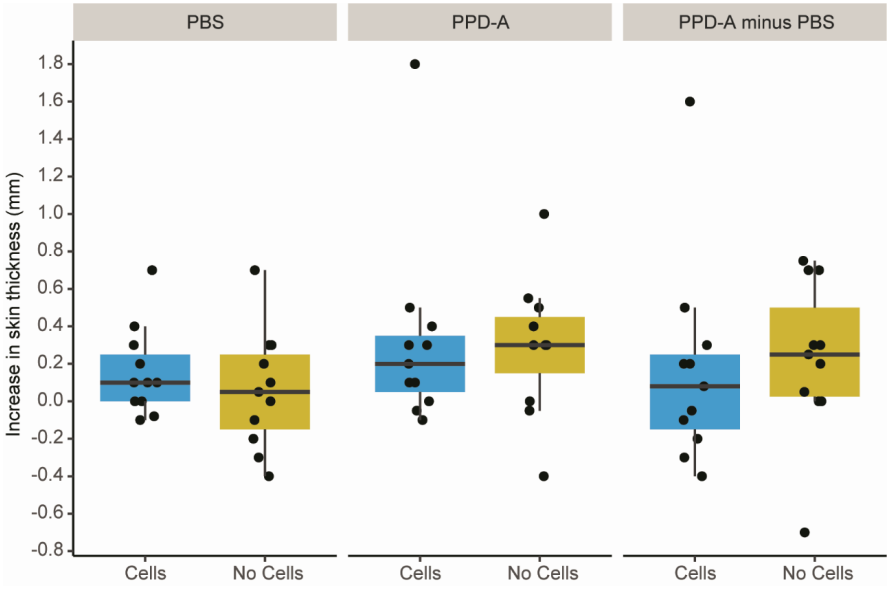

Figure 7. Kids' increase in skin thickness in response to intradermal injection with PBS and PPD-A at 24h post injection. (Cells group n=11; No Cells group n=11). No significant differences were detected (Paired sample T-test). Box and whisker represent Q1-Q3 and 1.5 x IQR, respectively, with the median indicated by horizontal bar.

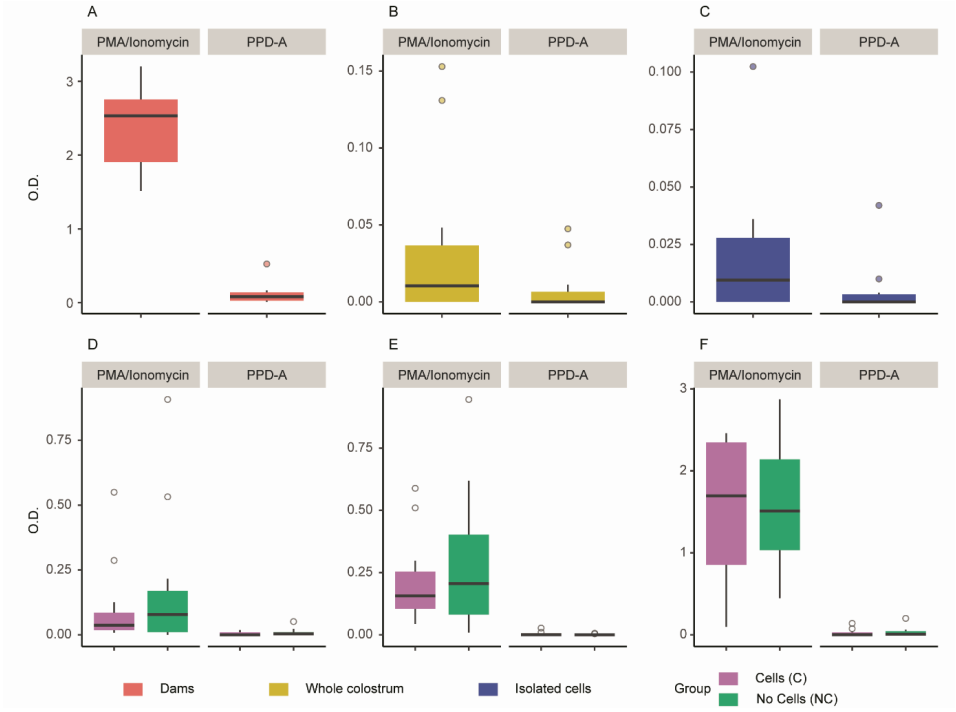

Figure 8. Interferon γ release assays. Top row represents O.D. values for PPD-A stimulation of dams' whole blood samples (A), whole colostrum samples (B) and isolated colostral leukocytes (C). Bottom row shows O.D. values for kids' whole blood stimulations at day 0 (D), 24h (E) and at day 8-10 (F). PMA/Ionomycin was used as positive control. Box and whisker represent Q1-Q3 and 1.5 x IQR, respectively, with the median indicated by horizontal bar.

Discussion

In the first animal experiment we provide evidence for the transfer of a functional and PPD-A specific CMIR from MAP vaccinated dams to the twin receiving the dam's colostrum as opposed to the twin receiving colostrum replacer. We hypothesize that this was due to a direct, antigen specific action of transferred maternal colostral leukocytes contributing to a neonatal CMIR. In the second experiment we isolated the dam's colostral cells and fed kids a bovine colostrum replacer supplemented with labelled maternal colostrum cells. We hypothesize that the lack of a DTH response in kids fed colostrum replacer supplemented with leukocytes derived from maternal colostrum indicates that the complete colostral matrix is required for colostrum leukocytes to transfer across the epithelial barrier and modulate the neonatal immune response.

The aim of the first experiment was to investigate the functionality of colostral leukocytes in goat kids with the use of an intradermal skin test. Kids received maternal colostrum from MAP vaccinated dams (caprine colostrum, CC) or freeze/thawed pasteurized bovine colostrum (BC). The MAP vaccination in the dams led to a strong DTH response following intradermal injection with PPD-A. *In vivo* DTH functionality of maternal cells transferred to kids via colostrum was demonstrated by a statistically significant higher increase in skin thickness in response to intradermal PPD-A injection in the CC group compared to the BC group. Similar to the study by (Fernández et al., 2006) the observed difference between groups was greatest at 24 hours post injection. Our results are similar to those of (Bandrick et al., 2008) who showed that antigen specific maternal leukocytes transferred with colostrum of pigs vaccinated against *Mycoplasma hyopneumoniae* evoke a DTH response in piglets upon intradermal skin injection with *Mycoplasma hyopneumoniae antigens*. With respect to our model to investigate variables contributing to the increase in skin thickness observed in the CC group, we found that antibody concentrations of the dam, antibody concentrations in the kids and birthweight of the kids were not significantly associated with the increase in skin thickness.

Delayed-type hypersensitivity (DTH) is characterized by early (6-12h post injection) activation of antigen-specific memory T lymphocytes upon a second encounter with an antigen, and is therefore also referred to as cell-mediated hypersensitivity. When soluble antigens of *M. tuberculosis* are introduced in the skin, the response typically peaks at 24–72 hours after antigen contact (Vohr, 2005). We used this type of DTH response in order to determine functionality of maternal T lymphocytes in the antigen unexperienced kids. The somewhat early peak in response observed in the kids in our study matches with the observation that there is considerable variation in DTH response between different animal models. In a Guinea Pig Potency Assay (GPPA), the reaction is read 24 hours after intradermal skin testing by measuring the resulting erythema at the site of injection (Steadham et al., 2002). In contrast, when the same tuberculin is applied in cattle for TB diagnosis, the reaction is measured 72 hours post injection by measuring the increase in skin fold thickness (OIE, 2018). Both types of reaction to PPD-A are considered DTH type reactions. In the current study the reactions observed in the adult goat dams followed the reaction type observed in cattle TB diagnostics. On the other hand, in the kids the timing of the reaction resembled those in sensitized guinea pigs with a peak response at 24 hours post injection, and even though significant, increase in skin thickness was rather small in the kids. These differences can be explained by a number of factors such as skin anatomy, maturity of the immune system and number of antigen specific T cells. These characteristics change with age as the dams have a completely developed adaptive immune response including both effector and memory T-cells, while newborns solely depend on their pool of specific but naïve (and

thus not yet clonally expanded) T-cells, which are potentially supplemented with some maternally derived activated mycobacterial antigen-specific T-cells and antibodies.

A linear mixed effects model shows that intradermal injection with PPD-A resulted in an increase of MAP specific antibodies in the goat colostrum fed kids. Despite several attempts to transform the data (squared, square root, log transformations), we did not obtain a normal distribution of the residuals, leading to less reliable predicted values of this model. Based on the data we observed, this is likely caused by the lack of variation in some data points in the model. For the majority of the kids in the BC group the concentration of MAP specific antibodies was zero and the corresponding residuals remained close to zero as well. In contrast, the concentration of MAP specific antibodies in the CC group significantly increased following PPD-A injection, and therefore an increase in variance was to be expected. As a consequence, the combined data results in not normally distributed residuals of the model. To tackle this, we performed some additional testing and found an increase in MAP specific antibodies up to 14 days after intradermal injection with PPD-A in the CC group. This indicates endogenous production of antigen specific antibodies in the kids, suggesting that the adaptive arm of the immune system is activated upon intradermal challenge with PPD-A. Since this is only seen in the kids fed maternal colostrum, we hypothesize that this adaptive immune response is the result of the stimulation of maternal PPD-A specific B and/or T cells (Bandrick et al., 2008; Tuboly et al., 1988).

Our most important finding of the first experiment was that feeding fresh maternal goat colostrum to goat kids increased their skin thickness after intradermal injection with PPD-A compared to feeding pasteurized and frozen/thawed bovine colostrum. The increased skin thickness in maternal colostrum fed kids is indicative of a DTH cell mediated immune response. The increase in MAP specific antibodies in the CC group two weeks after intradermal injection with PPD-A adds to the likelihood that the adaptive arm of the neonatal immune system is activated in the CC kids. We hypothesize that the activation of the adaptive arm of the immune system could be caused or stimulated by maternally derived antigen specific helper T and/or B cells.

In the second experiment we aimed to investigate the role of the cellular fraction of colostrum separate from the other colostrum components including maternal antibodies. Colostral leukocytes were isolated and labelled with a fluorescent dye suitable for tracking viable cells with the aim to track these cells in the kids. The isolated and CTV stained cells of the tested samples were viable at the time of feeding, however this is no indication of functionality. In contrast to the results in the first experiment, we found no difference in skin thickness increase in response to intradermal injection with PPD-A between kids receiving bovine colostrum replacer with maternal colostral cells and kids receiving colostrum replacer only. As a delayed type hypersensitivity response is characterized by lymphocyte infiltration (Hernández et al., 2005), a histopathological evaluation of the injection site was performed to study whether labelled maternal cells were present and involved in the local skin response. None of the skin injection sites at 24 hours post injection showed CTV staining, hence we concluded that no dermal infiltration of labelled maternal leukocytes took place. To determine the transfer and functionality of antigen specific maternal lymphocytes, an antigen stimulation assay measuring IFNγ production was performed on whole colostrum, isolated colostral cells and blood from both dams and kids. However as we were unable to show consistent antigen specific IFNγ production in both dams and kids in all matrices, these tests were not useful for assessing the transfer of antigen specific T cells.

We hypothesize that the lack of a detectable, antigen specific cellular immune response in the second experiment might be explained by the failure of the transfer of maternal immune cells as a result of the absence of the complete colostral matrix. The importance of the colostral

matrix was earlier for the transfer of maternal colostral cells was described by (Reber et al., 2006). In their study, they showed that the colostral environment is essential for colostral cells to pass into the neonatal bloodstream by inducing phenotypical changes on the cellular membrane. We suggest that by isolating colostral cells from their natural environment, trafficking markers on the outer membranes might not have been properly expressed or have been lost and cells were not able to transfer across the epithelial barrier of the neonatal gut.

In both groups of the second experiment, the increase in skin thickness resulting from intradermal PBS (control) injection did not differ significantly from the increase in skin thickness caused by PPD-A intradermal skin testing. Also, the response to intradermal PBS injection did not differ between groups. This implies that the response to PPD-A observed in the second experiment is likely caused by a non-specific and possibly innate immune response, while an antigen specific adaptive response failed to appear. Taken together with the results from experiment 1, in which we did observe a difference in DTH response between the two groups of kids, we infer that the response observed in the CC group in the first experiment was antigen specific and caused by the difference in colostrum feeding regiment.

In conclusion, in our initial experiment we observed a likely direct, antigen specific action of maternally derived colostral leukocytes contributing to a neonatal CMIR. Given the results of the subsequent experiment we hypothesize that the complete colostral matrix is probably essential for maternally derived colostral cells to transfer across the epithelial barrier as viable leukocytes functional in the neonatal environment. In line with earlier studies, our results indicate that caprine maternal leukocytes present in colostrum can functionally contribute to the newborns' early adaptive immune responses.

Acknowledgements

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Supplementary Material

Supplementary Tables

Supplementary table 3: Distribution of sex and order of birth across groups in experiment 1

	Group		
Sex	CC	BC	Total
Female	9	12	21
Male	11	8	19
Total	20	20	40

	Group		
Birth order	CC	BC	Total
1st born	12	8	20
2nd born	8	12	20
Total	20	20	40

	Order of birth		
Sex	1st	2nd	Total
Female	9	12	21
Male	11	8	19
Total	20	20	40

Supplementary table 2: Percentages of CD4+, CD8+ and WC1 type lymphocytes in 125 ml colostrum of three dams in experiment 1, measured by fluorescent-activated cell sorting (FACS).

	Percentages		
Dam	CD4	CD8	WC1
92606	0,02	7,63	0,93
92930	0,024	8,38	0,36
92963	0,051	11,6	1,29

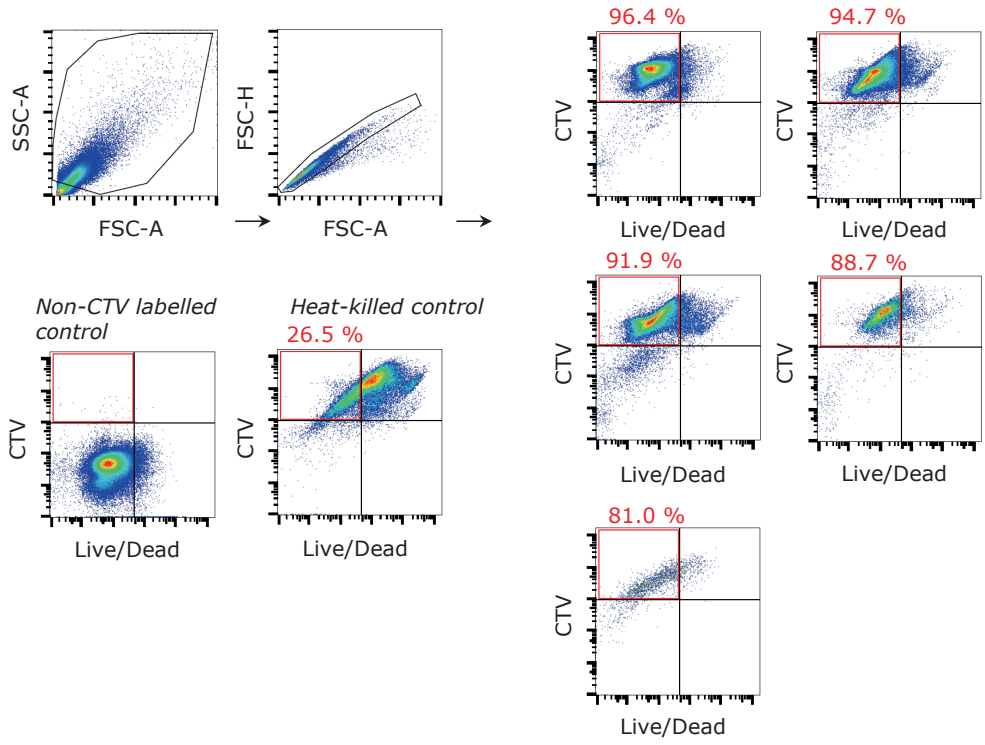
Supplementary table 3: Mean (mm) increase in skin thickness with corresponding standard deviation

Group	n	24 hours post injection	48 hours post injection	72 hours post injection
Caprine colostrum (CC)	20	0.345 (0.28)	0.343 (0.40)	0.255 (0.24)
Bovine colostrum (BC)	20	0.143 (0.24)	0.1 (0.27)	0.06 (0.32)
Mean difference	20	0.203 (0.36)	0.243 (0.41)	0,195 (0.35)

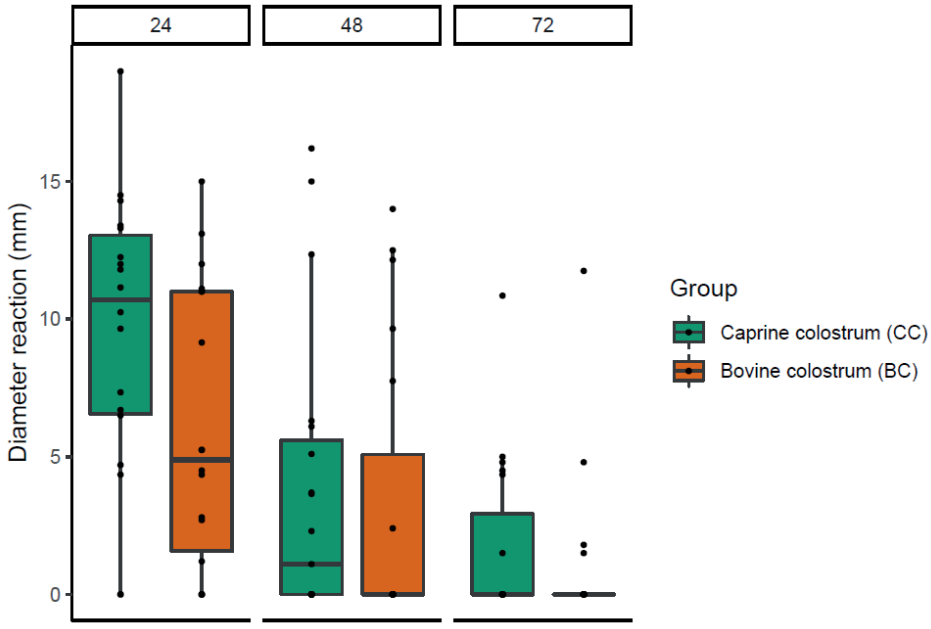
Supplementary table 4. Estimates, standard errors and confidence intervals for the reduced linear mixed effect model for evaluating the temporal dynamics of the MAP specific antibody concentration in the goat kids. Random effects in cursive.

Variable	Level	β	S.E. (β)	95% CI (β)	Variance (S.D.)
Intercept		0.1149	0.0974	-0.0732; 0.3029	
Group	Treatment (CC)	1.5430	0.1377	1.2771; 1.809	
Time	14 days	-0.0233	0.0948	-0.2058; 0.1593	
	21 days	-0.0546	0.0948	-0.2371; 0.1279	
	28 days	-0.0729	0.0948	-0.2554; 0.1097	
Group*Time	14 days (CC)	0.5516	0.1340	0.2935; 0.8097	
	21 days (CC)	1.5602	0.1340	1.3021; 1.8183	
	28 days (CC)	0.5263	0.1340	0.2682; 0.7844	
Kid ID					0.099 (0.32)

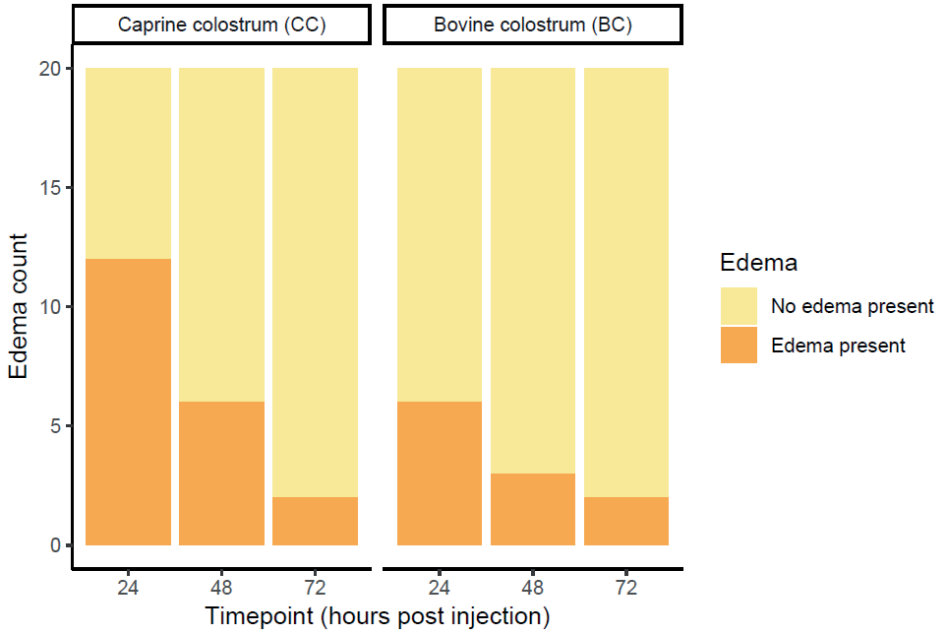
Supplementary figures



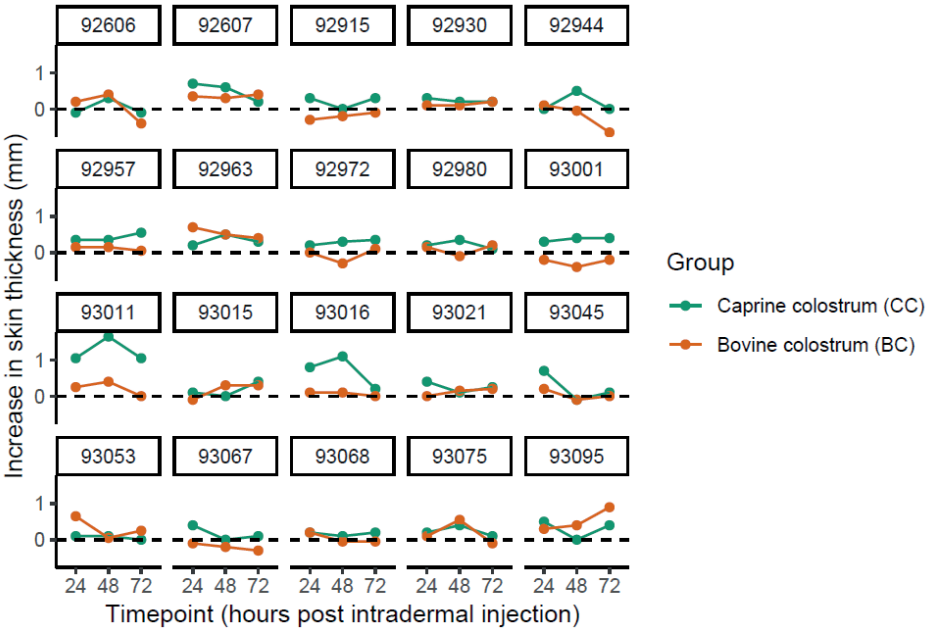
Supplementary figure 1: Gating strategy for maternal cells from colostrum. Gating strategy included exclusion of non-cellular debris (FSC-A vs SSC-A), followed by selection of single cells (FSC-A vs FSC-H). Cells were then analyzed for efficiency of CTV labelling and viability (Live/dead marker negative). Red gates indicate percentage of viable cells of CTV-labelled cells. Samples from five individual goats are presented. Controls show non-CTV labelled live cells, and CTV-labelled heat-killed cells.



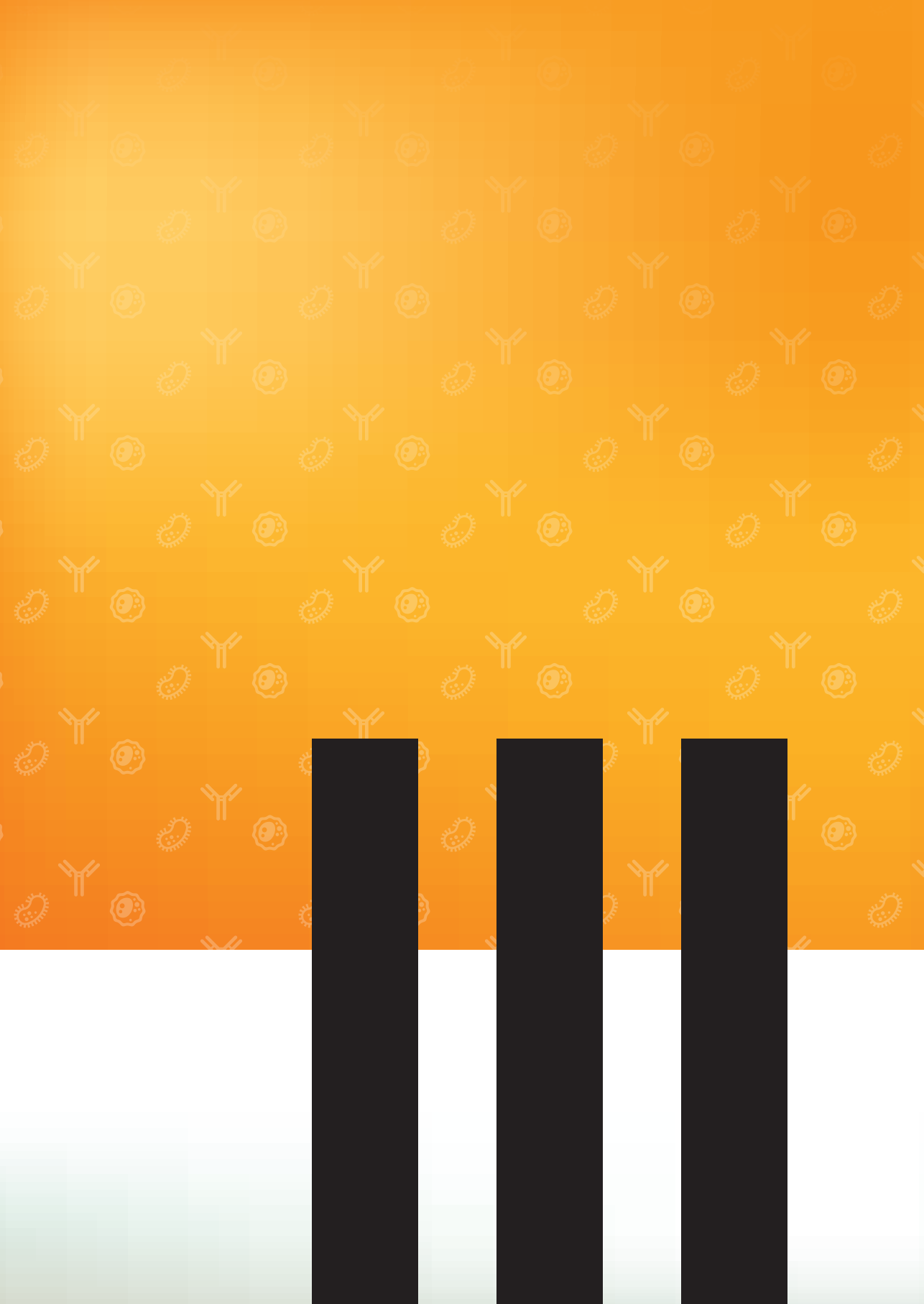
Supplementary figure 2: Diameter of the reaction per group and per measurement time.



Supplementary figure 3: Presence of edema per group and per measurement time.



Supplementary figure 4: Increase in skin thickness following intradermal injection with PPD-A, displayed per pair of kids. Numbers displayed are the individual dam numbers.



PART III

Considerations for on-farm colostrum management

6

CHAPTER 6

General discussion

Main findings of this thesis

Improving colostrum management on dairy farms

How does colostrum management affect colostral quality and calf's immune status?

Chapter 2 provides extensive overview of all on-farm processes between milking and feeding of colostrum, and their effects on colostral immune components and a calf's immune status. Quality of colostrum in terms of IgG is affected by the interval between calving and milking and, depending on temperature and duration, by pasteurization. Besides the quality of colostrum, the timing of feeding and the volume of the provided colostrum affect the transfer of IgG in the newborn. Although it is known that colostrum also contains maternal leukocytes, very little is known about how colostrum management affects the transfer and functionality of these maternal cells in the newborn.

How is knowledge on colostrum management put into practice?

Chapter 3 presents the results of a survey among Dutch dairy farmers regarding the colostrum management practices they applied with respect to their most recently born calf. We found no systematic differences in colostrum management between dairy farms using an automatic or a conventional milking system. Across farms, heifer and bull calves were not treated differently. Time of birth was an important risk factor for sub-optimal colostrum feeding. Calves born at night were at risk to receive colostrum later then calves born at other times during the day, and calves born in the evening received on average less colostrum with the first feeding compared to calves born in the afternoon. Furthermore, the results demonstrate that overall across the farms in our study, the common recommendations with respect to feeding quickly and quantity are mostly followed.

How does colostrum management influence bacterial contents?

Previous literature showed that storage of colostrum can lead to increased total bacterial counts (TBC), which poses a potential risk for the newborn calf. In **chapter 4** we studied whether besides an increase in TBC, different colostrum storing conditions also lead to an altered *composition* of the TBC in colostrum. Here, we described that while storage of colostrum in some cases can lead to altered TBC composition, the composition of the fresh baseline sample is most decisive.

Understanding the role of colostral immune cells in the neonatal immune response

Are maternal cells transferred with colostrum functional in the newborn?

Chapter 5 outlines the study in which the functional transfer of maternal leukocytes via colostrum was investigated. Two experiments were conducted in goat kids of which their dams were immunized with *M. avium* subsp. *paratuberculosis*. To summarize, in the first animal experiment we provided evidence for the transfer of functional and antigen specific maternal leukocytes. An antigen specific cell mediated immune response was observed in the twin receiving the dam's colostrum as opposed to the twin receiving colostrum replacer. We concluded that this was due to a direct, antigen specific action of transferred maternal colostral leukocytes contributing to a neonatal cell mediated immune response. In the second experiment we isolated the dam's colostral cells and fed kids a bovine colostrum replacer supplemented with labelled maternal colostrum cells. We hypothesized that the lack of a cell mediated immune response in kids fed colostrum replacer supplemented with maternal leukocytes indicates that the complete colostral matrix is required for

colostrum leukocytes to transfer across the epithelial barrier and modulate the neonatal immune response.

Considerations for on- farm colostrum management

The studies described in this thesis emphasize the importance of colostrum feeding to newborn ruminants. In literature as well as in practice the common advice on colostrum feeding is based on maximizing calf serum IgG concentrations. This advice is summarized in the three Q's: Quickly, Quality and Quantity. Generally it is assumed that when this advice is followed a sufficient amount of high quality colostrum is provided as quickly as possible to ensure adequate transfer of passive immunity in the form of IgG. However, what to do when for practical reasons farmers have to deviate from this advice?

In practice, the whole process of colostrum feeding to the calf depends on the farm and farm specific characteristics and facilities, and thereby on the farmers' decisions in colostrum management [1]. This can sometimes result in suboptimal colostrum management choices that may differ from what is generally recommended, simply because of feasibility and the circumstances on the farm. For example, as described in **chapter 3**, calves that were born during the night more often received colostrum later than calves born during the day. In cases like this, the advice of the three Q's falls short because there is no clear advice on what the best choice is depending on the actual situation [1]. Furthermore, the three Q's are primarily based on calf serum IgG as an outcome parameter, while perhaps other constituents could be considered as well. In the next paragraphs I will illustrate with some examples that within the generally accepted advice on colostrum management several considerations can be weighed depending on the situation, and that perhaps other outcome parameters could be added to the generally accepted advices as well.

Considerations beyond the current advice

As outlined in **chapter 2**, there are many factors that affect the transfer of passive immunity, and these factors may even affect each other. For instance, the volume that should be ingested to acquire adequate passive transfer of IgG depends on a combination the quality of the colostrum [2], the bodyweight [3], and the absorption efficiency of the calf, among others. Moreover, the latter is affected by the timing of feeding, possibly the volume [4] and bacterial content of the colostrum [5, 6]. The fact that several of these factors interact can make it difficult to make the best colostrum management choices in several common on-farm situations.

First colostrum feeding is recommended within one or two hours after birth at the latest [7] as the efficiency of absorption decreases rapidly afterwards [8-11]. Most calves in the Netherlands seem to receive first colostrum within two hours of birth (**chapter 3**). However, in practice it can occur that colostrum is fed later than two hours, as observed in the Netherlands (**chapter 3**), but also in several dairy herds in Pennsylvania, US [12], Ireland [13], and Australia [14] calves sometimes receive colostrum later. If a calf is fed later than recommended, it is yet unclear if extra colostrum could be supplied to compensate for later feeding, and if so, how much colostrum should be fed to compensate. Should one just provide a larger volume during the first feeding, or is it better to provide several small portions? Probably this is also dependent on the IgG concentration in colostrum and potentially other factors, such as the weight of the calf and perhaps bacterial contamination. Similarly, in the study described in **chapter 3**, calves born in the evening received smaller volumes of first colostrum feeding, however looking at the total volume fed over three feedings, there was no

difference. Probably, some of these farmers have compensated for the small portion in the evening, but does that matter much in terms of IgG absorption?

Thus, the current guidelines do not account for several considerations in actual farm situations, however it is important to study the effects of such considerations. For example, compensation strategies to obtain adequate serum IgG concentrations should be examined for whenever the 3 Q's guidelines cannot be followed in practice and a calf for example receives colostrum later than desired. Therefore, we need to increase our knowledge on the qualitative and quantitative interplay between the different factors affecting IgG absorption efficiency and the subsequent calf serum IgG concentration.

Potential additions to the current advice

Considering lactogenic immunity, colostrum quality is generally determined by its IgG concentration [7]. Also most colostrum management advice is centered around calves achieving the highest IgG concentration possible, sometimes combined with minimizing total bacterial counts as well [15]. Despite the clear association between calf serum IgG concentration and calf health, IgG is probably not the only colostrum constituent important for calf health. For example, as outlined in **chapter 5** and other research papers, maternal colostral immune cells are able to positively influence a newborns immune response [16-21], leading to healthier animals [17, 22]. Any aspect of maternal colostral immune cells is currently only indirectly incorporated in the three Q's. Whenever maternal colostrum is milked directly, contains a high IgG concentration and is fed within two hours after birth, the beneficial effects of maternal cells are likely not negatively affected. However, while colostrum can safely be stored in a refrigerator for 24 hours without affecting IgG concentrations (**chapter 2**) or bacterial counts and composition (**chapter 4**), it is currently unclear what happens to the functionality of colostral immune cells. Furthermore, if a cow produces colostrum with a low IgG concentration, there are several options to consider that are not included in the general advice. Is it better to give the calf frozen and thawed colostrum with a high IgG concentration from another dam? Or is it best to feed the a larger amount of low IgG colostrum of the own dam, because the whole colostrum with all its maternal immunity in the form of immune cells and other factors is still best, despite a low IgG concentration?

Considering the likely added value of the whole colostral matrix including maternal immune cells and potentially other factors as described in **chapter 5**, fresh maternal colostrum is probably favored over frozen stored colostrum from another dam. However this is perhaps also dependent on exactly *what* the IgG concentrations are and whether this can be compensated with additional feedings [23, 24]. A colostral IgG concentration of 45 g/mL is for example still better than a concentration of 10 g/mL, and feeding an extra volume could perhaps compensate a somewhat lower colostral IgG concentration [2]. Still, it is actually unknown exactly *how* important these maternal colostral cells are in the newborn and how large their effect size is, also compared to the already known effects of transferred IgG. Furthermore, up to now we do not exactly know how the immune cells cooperate with other immune components *in vivo*, and to which extent they do. Thus, the effects of maternal cells transferred with colostrum should be further investigated, both the underlying mechanisms of action and interactions with other immunological components of colostrum.

Conclusions

The research presented in this thesis has provided some novel insights into colostrum management and feeding, a well-known vitally important aspect of calf rearing. While the

current guidelines regarding Quickly, Quantity and Quality still stand, the guidelines could be enhanced for colostrum management in practice. In practice, colostrum management choices may differ from what is generally recommended, simply because of feasibility and the circumstances on the farm. In such situation the advice of the three Q's falls short. On the one hand this is because, with respect to achieving the highest serum IgG concentration, there is no clear advice on what the best choice is in a specific field situation. Moreover, the three Q's are primarily based on calf serum IgG as an outcome parameter, while perhaps other constituents could be considered as well. More research is still needed to be able to enhance calf-specific colostrum management guidelines beyond the valid but generic 3 Q's as outlined in this chapter.

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A

APPENDICES

Nederlandse samenvatting
Acknowledgements

Nederlandse samenvatting

Het belang van biest voor herkauwers

De eerste levensweken zijn een kritieke periode in de ontwikkeling van de pasgeboren herkauwer. Pasgeboren herkauwers zijn nog zeer kwetsbaar omdat deze op een bedrijf (en ook in de natuur) worden blootgesteld aan verschillende pathogenen. Omdat ze een placenta type hebben die de overdracht van grote moleculen van moeder naar foetus tijdens de zwangerschap niet toestaat, worden ze zonder afweerstoffen geboren. Voor hun eerste afweer zijn ze bijna volledig afhankelijk van de overdracht van immuun bestanddelen via de eerste biest.

Biest kan het best worden omschreven als de eerste moedermelk die wordt verkregen na de bevalling, ook wel partus genoemd. Tussen verschillende soorten dieren, maar ook binnen soorten en zelfs binnen individuele dieren is er een grote variatie in de samenstelling van de biest. Afgezien van de grote variatie weten we dat biest meer voedingscomponenten bevat in vergelijking met normale moedermelk en dat het veel immunologische bestanddelen bevat, zoals immunoglobulinen en immuun cellen. De opname van immunoglobulinen uit biest wordt ook wel de overdracht van passieve immuniteit genoemd.

Veel studies hebben het belang van immunoglobulinen voor de pasgeboren herkauwer beschreven en de focus ligt dan met name de opname van immunoglobuline G (IgG) via de biest. Het meeste onderzoek is gericht op het optimaliseren van de biestgift van kalveren uit de melkveesector. In de melkveesector worden kalveren direct van hun moeder gescheiden, waardoor de biest vaak door mensen wordt gegeven. Het merendeel van de onderzoeken en ook dit proefschrift is dan ook primair gericht op het optimaliseren van biestgift voor kalveren in de melkveehouderij.

De laatste jaren is er in de agrarische sector meer aandacht gekomen voor diergezondheid en dierenwelzijn, met name in de Nederlandse zuivelsector. Hoge morbiditeit en mortaliteit onder melkkalveren resulteerde in een zoektocht naar oplossingen om de gezondheid van kalveren te verbeteren. Aangezien de eerste biestgift één van de belangrijkste aspecten is in de kalver opfok, is het erg belangrijk dit nader te onderzoeken. Het algemene doel van dit proefschrift was om de kennis over biestgiftstrategieën en immunologische eigenschappen van biest te verbeteren, om zo melkkalveren nóg beter te voorzien van een goed neonataal immuunsysteem.

Biest management op melkveebedrijven

De kwaliteit van de biest wordt door verschillende elementen beïnvloed. Zowel invloeden vanuit managementkeuzes omtrent de koe, zoals voeding, droogstand lengte en vaccinatie, als indirecte factoren zoals seizoen van kalven en pariteit hebben invloed op de kwaliteit van de biest. Vanaf het moment dat een koe gekalfd heeft en de eerste biest gemolken wordt, kunnen we spreken van direct biestmanagement. Biestmanagement behelst het verzamelen van de biest, het al dan niet bewaren van de biest en het voeren van de biest aan het kalf, en alle keuzes op een bedrijf die hiermee samenhangen. In **hoofdstuk 2** van deze dissertatie wordt een uitgebreid overzicht gegeven van alle kritische processen die op het bedrijf plaatsvinden tussen het melken en voeren van biest, en de effecten daarvan op de immunologische biestkwaliteit en immuun status van het kalf. De kwaliteit van biest, in termen van IgG, wordt onder andere nadelig beïnvloed door het interval tussen afkalven en het melken van de biest te vergroten. Het bewaren van biest hoeft de concentratie immunoglobulinen niet te beïnvloeden, maar dat is wel afhankelijk van de behandel- en bewaarmethode. Zo heeft bijvoorbeeld de temperatuur en de duur van de pasteurisatie een groot effect op de IgG

concentratie De timing van de eerste biestgift beïnvloedt de mate van overdracht van passieve immuniteit in de vorm van IgG. Het beste is om de biest zo snel mogelijk, in ieder geval binnen twee uur na de geboorte te geven, dan ligt de absorptie efficiëntie het hoogst. Hoewel er veel bekend is over de effecten van biestmanagement op de IgG in de biest en de opname van IgG door het kalf, is er maar weinig bekend over hoe biestmanagement de functionaliteit van maternale cellen bij de pasgeborene beïnvloedt.

Om inzicht te krijgen in het biestmanagement op Nederlandse melkveebedrijven, wordt in **hoofdstuk 3** een studie beschreven waarin een enquête is uitgezet onder Nederlandse melkveehouders. In de enquête is gevraagd naar de biestgift(en) van het laatstgeboren kalf. De resultaten laten zien dat de meeste Nederlandse melkveehouders biest geven in lijn met het algemene advies om het kalf zo snel mogelijk van biest te voorzien en binnen 24 uur minimaal 6 liter te geven. Een punt van aandacht is de biestverstrekking aan kalveren die 's avonds of 's nachts geboren worden. Kalveren die in de avond werden geboren, kregen gemiddeld minder biest tijdens de eerste biestgift vergeleken met kalveren die in de middag werden geboren. De kalveren die in de nacht worden geboren, lopen het risico om later biest te ontvangen dan kalveren die overdag worden geboren. Omdat de kalveren die 's avonds en 's nachts geboren worden soms wat minder of later biest krijgen, zijn die dieren gevoeliger voor infecties. Wanneer andere omstandigheden voor het kalf optimaal zijn, hoeft dat niet tot ziekte te leiden. Desondanks is het verstandig om op bedrijven waar veel ziekte en/of uitval voorkomt, bedacht te zijn op de biestgift tijdens de nachtelijke uren. Ook is er geen verschil gevonden in het biestmanagement tussen stierkalveren en vaarskalveren of tussen melkveebedrijven met een conventionele melkstal en bedrijven met een automatisch melksysteem.

Het voeren van biest brengt een zeker risico op pathogenen overdracht met zich mee. Door omgevingsfactoren op het melkveebedrijf kan besmetting niet volledig worden vermeden en kunnen zelfs hoge aantallen bacteriën gevonden worden in de. In het algemeen wordt aanbevolen om het totale aantal bacteriën in biest zo laag mogelijk te houden. Het advies is om schoon te werken om te voorkomen dat er veel bacteriën in de biest komen. Desondanks behelst de bacteriële kwaliteit van biest meer dan alleen de hoeveelheid bacteriën, maar ook de samenstelling van het kiemgetal. Hoewel bekend is dat de duur en temperatuur van de biestbewaring het kiemgetal beïnvloeden, is er minder bekend over de effecten van bewaring op de samenstelling van het kiemgetal. We kunnen veronderstellen dat, afhankelijk van de bewaarcondities, biest een substraat is waarin bepaalde bacteriesoorten beter kunnen gedijen dan andere. In **hoofdstuk 4** wordt een studie beschreven waarbij de effecten van verschillende biest bewaarmethoden op de samenstelling van het kiemgetal worden onderzocht. Verschillen in de samenstelling van het kiemgetal tussen de bewaarde biest monsters werden grotendeels verklaard door de variatie in de samenstelling van het kiemgetal van de oorspronkelijke biestmonsters. In overeenstemming met eerdere studies tonen de resultaten van deze studie dat het bewaren van biest op kamertemperatuur gedurende 24 uur leidt tot een hoger kiemgetal dan wanneer het wordt bewaard in de koelkast. De samenstelling van het kiemgetal van biest dat gedurende 24 uur bewaard werd bij kamertemperatuur en dat van biest dat voor een week bewaard was in een koelkast, verschilden significant van de samenstelling van het kiemgetal van de verse biestmonsters. We denken dat dit waarschijnlijk te wijten is aan de toegenomen groei van *Enterobacteriaceae*. De hoeveelheid *Enterobacteriaceae* in gekoelde biest is gedurende de eerste 24 uur stabiel en is duidelijk verhoogd na een week bewaring. De resultaten geven aan dat de microbiële samenstelling van bewaarde biest vooral wordt beïnvloed door de inhoud van het initiële biestmonster, en dus waarschijnlijk door contaminatie tijdens het melkproces.

De rol van maternale immuuncellen uit de biest

In de afgelopen twee decennia is de belangstelling voor immuuncellen in biest toegenomen. Hoewel veel studies het belang van immuuncellen uit de biest beschrijven, zijn *in vivo* werkingsmechanismen en antigeenspecifieke functionaliteit van deze cellen bij herkauwers niet volledig bekend. **Hoofdstuk 5** beschrijft twee studies in geitenlammeren waarbij wordt gekeken naar de overdracht en *in vivo* functionaliteit van antigeenspecifieke cellulaire immuniteit via maternale biest. Tweelingparen geitenlammeren van moederdieren die eerder waren geïmmuniseerd met een geïnactiveerde *Mycobacterium avium* subsp. *paratuberculosis* (MAP) vaccin, kregen verschillende soorten biest: maternale biest van hun moeder versus gepasteuriseerde en ingevroren/ontdooide runderbiest (experiment 1), en kunstbiest met fluorescent gelabelde cellen uit de maternale biest versus enkel kunstbiest (experiment 2). In beide experimenten werd de celgemedieerde immuunrespons van de lammeren tegen *Mycobacterium avium* antigenen getoetst met behulp van intradermale huidtesten met PPD-A tuberculine. In het eerste dierexperiment werd een antigeen specifieke, celgemedieerde immuunrespons waargenomen bij de lammeren die biest van de eigen moeder, en dus met maternale cellen, kregen. Deze response werd niet gevonden in de lammeren die runderbiest kregen. Dit is waarschijnlijk te wijten was aan een directe, antigeen specifieke werking van overgedragen maternale leukocyten uit de biest, die bijdragen aan de neonatale celgemedieerde immuunrespons. Het ontbreken van een celgemedieerde immuunrespons bij de lammeren die kunstbiest met maternale biestcellen kregen in experiment 2 impliceert dat waarschijnlijk de volledige biestmatrix nodig is voor ofwel de overdracht dan wel een functionele response van maternale biestcellen.

Overwegingen voor biestmanagement op het bedrijf

De studies beschreven in dit proefschrift benadrukken het belang van biestvoeding aan pasgeboren herkauwers. Zowel in de literatuur als in de praktijk is het gangbare advies voor biestvoeding gebaseerd op het maximaliseren van de IgG-concentraties in het bloed van de kalveren. Dit advies wordt vaak samengevat in de drie V's: Veel, Vlug en Vaak. Over het algemeen wordt aangenomen dat bij het opvolgen van dit advies zo snel mogelijk een voldoende hoeveelheid biest van hoge kwaliteit wordt verstrekt om een adequate overdracht van passieve immuniteit in de vorm van IgG te verzekeren.

In de praktijk kunnen keuzes omtrent biestmanagement afwijken van wat algemeen wordt aanbevolen. Dit kan simpelweg gebeuren door bedrijfsspecifieke omstandigheden en de daaruit volgende haalbaarheid op een bedrijf. In dergelijke situaties kan het advies van de drie V's tekort schieten. Enerzijds doordat er in specifieke praktijksituaties niet altijd duidelijk is wat de beste management keuze is om de hoogst haalbare IgG concentratie in kalveren te behalen, anderzijds kunnen, op basis van het onderzoek in dit proefschrift, naast de concentratie IgG in de pasgeboren kalveren wellicht ook andere (immuun) parameters uit de biest meegewogen worden als het gaat om kwaliteit en het optimaliseren van de gezondheid van kalveren. Meer onderzoek is nodig om de huidige richtlijnen voor biestmanagement te specificeren en zo verder te kijken dan de geldige, maar vrij generieke 3 V's.

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