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The biological value of bovine colostrum and transition milk: Implications for preweaned calves' intestinal health and long term metabolism

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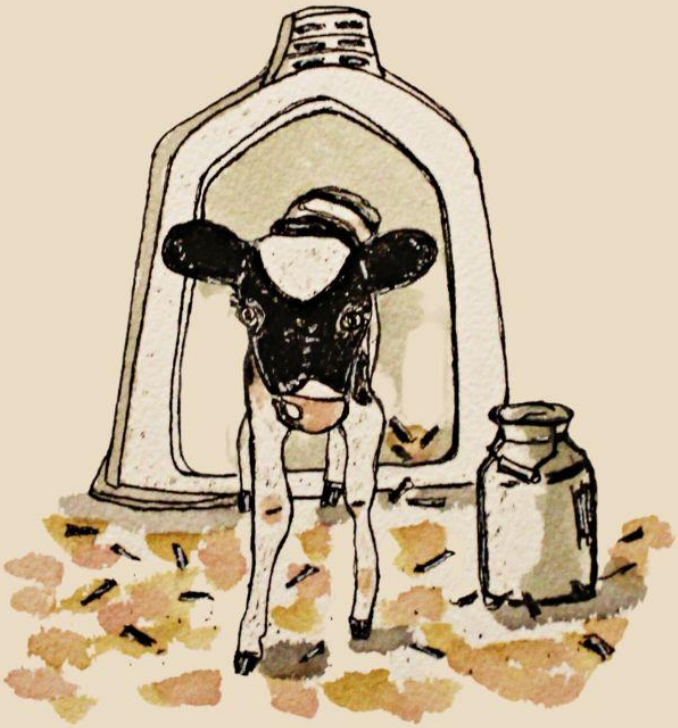

Marina Tortadès i Valero
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The biological value of bovine colostrum and transition milk: Implications for preweaned calves' intestinal health and long-term metabolism.

MEMÒRIA PRESENTADA PER MARINA TORTADÈS I VALERO

DIRIGIDA PER MARTA TERRÉ TRULLÀ

TUTORIZADA PER MARIA DOLORS IZQUIERDO TUGAS

PER ACCEDIR AL GRAU DE DOCTOR DINS EL PROGRAMA DE DOCTORAT EN PRODUCCIÓ ANIMAL DEL DEPARTAMENT DE CIÈNCIA ANIMAL I DELS ALIMENTS

BELLATERRA, SETEMBRE 2023

FACULTAT DE VETERINÀRIA

MARTA TERRÉ TRULLÀ, Investigadora de la Unitat de Remugants de l'Institut de Recerca i Tecnologia Agroalimentària, i

MARIA DOLORS IZQUIERDO TUGAS, Professora Agregada del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona

Certifiquen:

Que la memòria titulada "**The biological value of bovine colostrum and transition milk: Implications for preweaned calves' intestinal health and long-term metabolism"** presentada per **Marina Tortadès i Valero** per optar al grau de Doctor en Veterinària, ha estat realitzada sota la direcció de la Dra. Marta Terré Trullà i, considerant-la acabada, autoritza la seva presentació perquè sigui jutjada per la comissió corresponent.

I per tal que consti els efectes que corresponen, signa la present a Caldes de Montbui, 22 de setembre de 2023.

Marta Terré Trullà	Maria Dolors Izquierdo Tugas
Directora	Tutora

CAT: Les primeres setmanes de vida són clau per al futur rendiment productiu d'un vedell. Per aquesta raó, la gestió eficaç del calostre és fonamental per garantir una immunitat passiva adequada i promoure la salut i el creixement dels vedells. S'han descrit diferents estratègies abans del llevat per aconseguir el màxim potencial genètic del vedell, que varien des de l'extensió de les preses de calostre boví (CB) i llet de transició (LT) fins a l'alimentació amb nivells elevats de lactoremplaçant. En aquesta tesi es van realitzar tres estudis per avaluar el valor biològic dels excedents de llet de transició i calostre, proposant dues estratègies per fomentar-ne l'ús, ja sigui a la granja o a la indústria làctia, per promoure la salut i el rendiment dels vedells.

Així, el primer estudi va comparar les concentracions d'immunoglobulina G, factor de creixement insulínic-I i lactoferrina en vaques lleteres primípares i multípares. En aquest estudi, descrivim que la LT de la segona munyida conté més de la meitat de la concentració de molècules bioactives presents a la CB i com aquestes molècules varien en funció de la paritat de la mare. Aquests troballes poden indicar una possible aplicació futura dels excedents de CB i LT com a nutricèutic en salut animal o humana.

En el segon estudi d'aquesta tesi, es va explorar una de les possibles aplicacions dels excedents de LT i CB. Es van aplicar diferents tractaments terapèutics per fer front als efectes negatius del transport en vedells lactants. Els resultats d'aquest estudi van mostrar que l'alimentació amb CB o LT després d'un episodi de restricció alimentària i dejú (simulant un transport prolongat) va ajudar a recuperar la funcionalitat de la barrera intestinal i a proporcionar protecció immunològica intestinal.

La segona estratègia proposada per utilitzar els excedents de BC va consistir en subministrar diferents quantitats de CB a les vedelles després de nèixer per analitzar si hi havia algun efecte metabòlic després del seu primer part. Les dades de rendiment no van revelar un clar efecte de la impressió metabòlica en ampliar de 2 a 8 les tomes de CB. No obstant això, es van detectar efectes mínims sobre el metabolisme postpart, principalment relacionats amb la via de biosíntesi del colesterol.

En resum, la present tesi proporciona els resultats de l'anàlisi dels compostos bioactius presents a la CB i LT en funció de la paritat i proposa diferents aplicacions dels excedents de CB i LT per millorar la salut dels vedells.

A més, es va realitzar un estudi preliminar de l'impacte de BC, TM i llet en la renovació d'enteròcits.

ESP: Las primeras semanas de vida son claves para el futuro rendimiento productivo de un ternero. Por esta razón, la gestión eficaz del calostro es fundamental para garantizar una inmunidad pasiva adecuada y promover la salud y el crecimiento de los terneros. Se han descrito diferentes estrategias antes del destete para alcanzar el máximo potencial genético del ternero, que varían desde la extensión de las tomas calostro bovino (CB) y leche de transición (LT) hasta la alimentación con niveles elevados de lactoremplazante. En la presente tesis se realizaron tres estudios para evaluar el valor biológico de los excedentes de leche de transición y calostro, proponiendo dos estrategias para fomentar su uso, ya sea en granja o en la industria láctea, para promover la salud y el rendimiento de los terneros.

Así, el primer estudio comparó las concentraciones de inmunoglobulina G, factor de crecimiento insulínico-I y lactoferrina en vacas lecheras primíparas y multíparas. En este estudio, describimos que la LT del segundo ordeño contiene más de la mitad de la concentración de moléculas bioactivas presentes en la CB y cómo estas moléculas varían en función de la paridad de la madre. Estos hallazgos pueden indicar una posible aplicación futura de los excedentes de CB y LT como nutracéuticos en salud animal o humana.

En el segundo estudio de esta tesis, se exploró una de las posibles aplicaciones de los excedentes de LT y CB. Se aplicaron diferentes tratamientos terapéuticos para hacer frente a los efectos negativos del transporte en terneros lactantes. Los resultados de este estudio mostraron que la alimentación con CB o LT tras un episodio de restricción alimentaria y ayuno (simulando un transporte prolongado) ayudó a recuperar la funcionalidad de la barrera intestinal y a proporcionar protección inmunitaria intestinal.

La segunda estrategia propuesta para utilizar los excedentes de BC consistió en suministrar diferentes cantidades de BC a las terneras tras el parto para analizar si había algún efecto metabólico después de su primer parto. Los datos de rendimiento no revelaron un claro efecto de impronta metabólica al ampliar de 2 a 8 las tomas de CB. Sin embargo, se detectaron efectos mínimos sobre el metabolismo postparto, principalmente relacionados con la vía de biosíntesis del colesterol.

En resumen, la presente tesis proporciona los resultados del análisis de los compuestos bioactivos presentes en la CB y LT en función de la paridad y propone diferentes aplicaciones de los excedentes de CB y LT para mejorar la salud de los terneros.

Además, se realizó un estudio preliminar del impacto de BC, TM y leche en la renovación de enterocitos.

ENG: The first weeks of life are the onset for a successful calf's future performance. Effective colostrum management is critical to ensure an adequate passive immunity and promote calf health and growth. Different preweaning strategies have been described to achieve the calf's maximum genetic potential, varying from extending bovine colostrum (BC) and transition milk (TM) feedings to enhanced milk replacer feeding programs. In the present thesis, three studies were conducted to evaluate the biological value of transition milk and colostrum surpluses, proposing two strategies to encourage their use, either on-farm or in the dairy industry, to promote calf health and performance.

Thus, the first study compared the Immunoglobulin G, Insulin Growth Factor-I, and lactoferrin concentrations in primiparous and multiparous dairy cows. In this study, we described that the TM from the second milking contains more than half of the concentration of bioactive molecules present in BC and how these molecules vary depending on the dam's parity. These findings may indicate a potential future application of BC and TM surpluses as nutraceutical in animal or human health.

In the second study of this thesis, we explored one of the possible applications for the TM and BC surpluses. Different therapeutical treatments were applied to cope with the negative effects of transportation in preweaned calves. Results from this study showed that feeding either bovine colostrum or transition milk after an episode of feed restriction and fasting (simulating long transportation) helped to recover intestinal barrier functionality and provide gut immune protection.

The second strategy proposed to use BC surpluses was providing different amounts of BC feedings to female calves after birth to further analyze if there was any metabolic effect after their first calving. The performance data did not reveal a clear imprinting effect of extending BC feedings from 2 to 8. However, minor effects on postpartum metabolism were detected, mainly related to the cholesterol biosynthesis pathway.

In summary, the present thesis provides results of the analysis of bioactive compounds present in BC and TM by parity and proposes different applications for BC and TM surpluses to enhance calf health.

Additionally, a preliminary study of the impact of BC, TM and milk on enterocyte turnover was accomplished.

ABBREVIATIONS USED

ADG: average daily gain

ADF: acid detergent fiber

AHAW: Animal Health and Welfare

BC: bovine colostrum

BHB: β-Hydroxybutyrate

BW: body weight

CR: colostrum replacer

CP: crude protein

Ct: threshold cycle

DIM: days in milk

DM: dry matter

DNA: deoxyribonucleic acid

ECF: extended colostrum feeding

ECM: energy-corrected milk

EDTA: Ethylenediaminetetraacetic acid

EE: ether extract

EFSA: European Food Safety Authority

EU: European Union

F/B: firmicutes to bacteroidetes

FcR: Fc receptor

FDR: false discovery rate

FE: feed efficiency

FRF: feed restriction and fasting

GIT: gastrointestinal tract

HPG: hypothalamic-pituitary-gonadal

HTST: high temperature short time

IGF: insulin growth factor

Ig or Igs: immunoglobulin/s

IRTA: Institut de Recerca i Tecnologia Agroalimentària

LPS: lipopolysaccharide

LTF: lactoferrin

LTLT: low temperature long time

M1/2/3/10: first/ second/ third/ tenth milking

MR: milk replacer

NDF: neutral detergent fiber

NEFA non-esterified fatty acids

OS: oligosaccharides

PBS: phosphate-buffered saline

PCA: principal component analysis

PCR: polymerase chain reaction

PLT: platelet

RBC: red blood cells

SCC: somatic cell count

TM: transition milk

VFA: volatile fatty acids

WBC: white blood cells

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Chapter 1

LITERATURE REVIEW

1. LITERATURE REVIEW

Introduction

The first weeks of calf's life are the onset of a successful future performance. Effective colostrum management is critical to ensure adequate immunity and promote calf health and growth (Godden et al., 2019). Different preweaning strategies have been described to achieve the calf's maximum genetic potential, varying from extending bovine colostrum (**BC**) and transition milk (**TM**) feedings to enhanced milk replacer (**MR**) feeding programs (Soberon and Van Amburgh, 2013; Hare et al., 2020; Pyo et al., 2020). For instance, Faber et al. (2005) observed that an increase in colostrum amounts (from 2 to 4 L) during the first newborn intake resulted in an increase of 322 kg in milk yield during the first lactation. Alternatively, BC and TM have been used as a nutraceutical to decrease mortality and incidence of disease in dairy calves (Conneely et al., 2014; Kargar et al., 2020; Van Soest et al., 2020).

This review will cover different aspects of the current use of BC and TM to promote calves' health and performance, including gut dysfunctionality during transportation and the long-term effects of their extended feeding.

1.1. Calf liquid feeding and its benefits

1.1.1. Definition and uses of colostrum, transition milk, waste milk, and milk replacer.

Bovine colostrum is the first mammary gland secretion after calving. In ruminants, the intake of colostrum during the first hours after birth is crucial due to two main factors. First, females have a syndesmochorial placenta that does not allow the passage of immunoglobulins (**Igs**) from the mother to offspring in the uterus (Peter, 2013). Second, the calf's intestine only absorbs Igs from colostrum during the first 24 h after birth, when the "gut closure" occurs, and this absorption capacity is gradually lost (Quigley, 2002). These maternal Igs will provide passive immunity to calves to protect them against external pathogens until the maturation of their own immune system (Elizondo-Salazar and Heinrichs, 2008). For this reason, early administration of good-quality colostrum to boost effective passive immunity is the main goal to ensure calf survival (Godden et al., 2006). The mammary secretion from

the second to the sixth milking after calving is considered TM, in which the solid constituents (including Igs and other bioactive substances) gradually decrease (Foley and Otterby, 1978; Godden, 2008). Then, the mammary secretion becomes the saleable whole milk (see **Figure 1.1**) (Godden, 2008). Feeding BC plus further TM feedings improved growth rates and enhanced health scores in neonatal calves (Conneely et al., 2014; Van Soest et al., 2020). However, TM could be treated as a by-product of the dairy industry instead of being treated as waste milk. In this context, further studies are required to provide recommendations on how to manage TM feedings in commercial farms to enhance calves' health (USDA, 2010; National Academies of Sciences, Engineering and Medicine, 2021).

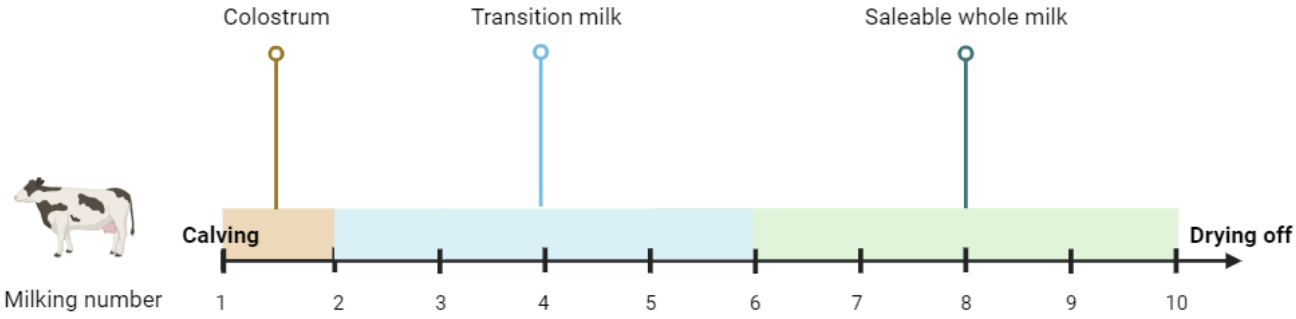

Figure 1.1. Mammary secretions denomination from calving to drying off. Image created using biorender.com

Another strategy to acquire an adequate passive immunity is feeding colostrum replacer (**CR**). According to USDA regulations, CR should be able to raise above 10 mg/mL of calves' serum IgG concentrations, which is achieved when CR contains more than 100 g of IgG per dose (Quigley et al., 2001). Immunoglobulins of CR can come from different biological origins, such as lacteal secretions (milk and colostrum) or bovine serum (Cabral et al., 2013). However, the cost of each dose (30-50 € per dose) and the need to reach at least 200 g of IgG (equivalent to two doses of CR) to ensure an adequate passive transfer, make save this resource to research or particular situations when no good quality BC is available, or when disease preventive protocols do not allow to use BC from infected cows (Pithua et al., 2009; Chigerwe et al., 2012; Leach, 2021). In research, the use of CR is extended because it is used to equalize and homogenize treatments using the same source of IgG (Hiltz

and Laarman, 2019; Carter et al., 2022), or even to simulate TM feeding using a 1:2 dilution rate of CR in whole milk or milk replacer (**MR**) (Pyo et al., 2020).

Alternatively, some dairies feed pasteurized waste milk in an attempt to reduce costs and recycle this discarded unsaleable milk (Godden et al., 2005). Waste milk is mainly comprised of colostrum surpluses, milk from cows with clinical mastitis, or milk obtained during the withholding period after cows' treatment with veterinary drugs (Kesler, 1981). However, the main concern of feeding waste milk is that pasteurization does not completely eliminate the bacterial toxins or spores from the mastitis bacteria. Also, the antibiotic residues from milk-treated cows are spread, creating the adverse effect of antimicrobial resistances in the fed calves (Aust et al., 2013).

After the first colostrum feeding, the calf's liquid feeding program is followed by whole milk or MR until weaning (defined as the transition from a liquid-based diet to a solid-based diet). Although whole milk is intended for the market and it comes with storage and disease transmission challenges, it can be an alternative to MR when fed at the same energy requirements (Van Amburgh and Drackley, 2005; Hawkins et al., 2019). Commonly, farmers apply different MR feeding programs depending on the future use of the animal (e.g. replacement heifers vs. calves surpluses), the economics of the farm, or the market price of the whole milk and MR (Kertz and Loften, 2013). Conventional MR feeding programs consist of feeding a restricted amount of liquid to stimulate an early intake of starter feed and promote early weaning using MR nutrients quality between 20-22 % CP and 18-20 % fat (Davis and Drackley, 1998; Erickson and Kalscheur, 2020). Alternatively, accelerated or enhanced-growth feeding programs are based on feeding a high protein MR (28 % CP and 20 % fat) and are usually accompanied using automatic milk feeders, in an attempt to simulate ad-libitum feeding, and to promote a gradual weaning to encourage further concentrate feed intake (Erickson and Kalscheur, 2020). Accelerated feeding programs are costly, but the improved growth gains during preweaning have been described to have long-term benefits on heifers' performance (Raeth-Knight et al., 2009; Soberon and Van Amburgh, 2013). Although some publications (Raeth-Knight et al., 2009; Terré et al., 2009; Davis Rincker et al., 2011) conducted by different research groups did not find differences between

conventional and accelerated feeding programs on first lactation milk yield, a meta-analysis conducted by Soberon and Van Amburgh (2013) indicated that heifers fed greater amount of nutrients from MR or milk were twice more likely to produce more milk (400 to 500 kg) than a calf fed with restricted nutrient intake. Similarly, Gelsinger et al. (2016) also included the effect of the starter intake on a preweaned calf nutrition metanalysis, concluding that either liquid or starter dry matter intake in conjunction improved the projected first lactation performance. Overall, the main goal of the preweaning phase is to encourage rumen development, enhance growth, and provide the fundament for a lifetime performance of calves (Soberon et al., 2012; Rosadiuk et al., 2021).

1.1.2. Bioactive compounds in bovine colostrum and transition milk

Bovine colostrum is well known to contain high concentrations of nutrients as well as a wide range of bioactive substances such as immune components (Igs, leukocytes, cytokines) (Oyeniyi and Hunter, 1978; Hagiwara et al., 2000; Reber et al., 2008), growth factors (Bagwe et al., 2015), antimicrobials factors (lactoferrin (**LTF**), lysozyme, lactoperoxidase) (Pakkanen and Aalto, 1997) and hormones (leptin, insulin) (Hammon et al., 2000) that play an important role in the development of the intestinal epithelium, maturation of the immune and metabolic systems of the calf (Godden, 2008). These components are accumulated in the mammary gland during late gestation, and during the first milkings, their concentrations are greater than in the mature milk (Barrington et al., 2001). Particularly, colostrogenesis (the process in which the mentioned bioactive substances are transferred from the maternal circulation into mammary secretions) mostly occurs during 2 to 4 weeks prepartum and is driven by hormones including estrogens, progesterone, and prolactin (Barrington et al., 2001; Baumrucker and Bruckmaier, 2014; Godden et al., 2019; Baumrucker et al., 2021). During colostrogenesis, certain components may have synergic effects such as the cellular transcytosis mechanism in which LTF facilitates the recycling of IgG1 into colostrum by interacting with its receptor (Baumrucker et al., 2021). After the first mammary secretion, the bioactive components present in TM diminish gradually as they dilute in the mammary gland (**Table 1.1**) (Foley and Otterby, 1978; Stott et al., 1981; Blum and Hammon, 2000). Osmotic molecules like lactose create a positive osmotic pressure, which causes the incorporation of more

water and a reduction of the concentration of the biomolecules accumulated in the mammary gland before parturition (Baumrucker et al., 2010). Although BC has been widely studied in the literature, further research on TM's bioactive substances is needed to obtain the entire scope of components such as cytokines, enzymes, nucleotides, and nucleosides (**Table 1.1**) (McGrath et al., 2016). Despite the benefits of BC and TM on calves, they are unsaleable for human consumption as early lactation mammary secretion contains elevated epithelial cells from the mammary gland. Both epithelial cells and leukocytes are considered somatic cells, which can be pathologically increased during mastitis or injury (Schultz, 1977). In fact, the European Regulation (EC) Nº 853/2004 (European Community, 2004) established a maximum somatic cell count (**SCC**) in the saleable milk of <400.000 cells/mL.

1.1.2.1. Benefits of bovine colostrum and transition milk bioactive substances in gut health

Several authors report how the nutritional, immune, antimicrobial, and growth factors detected in BC and TM could have a nutraceutical potential as a therapeutic and preventive treatment for calves' gastrointestinal disorders as well as the gastrointestinal tract (**GIT**) and immunological maturation (see **Table 1.1**) (Pakkanen and Aalto, 1997; Bagwe et al., 2015; Carter et al., 2021).

Particularly in newborn calves, passive immunity is immediately provided by ingested Igs from BC, which are absorbed into systemic circulation across the small intestine lumen. The transfer of colostral IgG is a non-selective macromolecular transport system that occurs within the 24-36 h of birth (Besser and Gay, 1994; Pakkanen and Aalto, 1997). Immunoglobulin G prevents the adhesion to the intestinal barrier of pathogens, by opsonizing them and activating the Fc receptors (**FcR**) on phagocytes that initiate immune responses (Ulfman et al., 2018). In addition to the passive immunity conferred by Igs, growth factors including insulin growth factor (**IGF**)-I, IGF-II, and TFG-β, have been demonstrated to stimulate the growth and differentiation of mammalian cells, including the GIT ones (Pakkanen and Aalto, 1997).

Moreover, other bioactive compounds related to the innate and acquired immune response have been also identified in BC. These include neutrophils, macrophages, oligosaccharides (**OS**), acute-phase proteins, immunomodulatory factors (such as

cytokines), and peptides and proteins with antimicrobial activity (LTF, lactoperoxidase, and lysozyme) (Stelwagen et al., 2009). Concretely, when neutrophils and macrophages are activated by their surface FcR, they are accountable for eliminating pathogens through phagocytosis or the production of cytokines, reactive oxygen species, or antimicrobial peptides (Ulfman et al., 2018). Similarly, cytokines, including IL-1β, IL-6, TNF-α, INF-γ, and IL-1ra, modulate immune function and the mediation of inflammatory processes by participating in the pathogen recognition, cell recruitment, and immune system maturation (Hagiwara et al., 2000; Rathe et al., 2014).

Finally, since OS are not the predominant carbohydrate in BC, TM, and bovine milk compared to lactose in eutherian animals, they may have a principal biological function rather than a nutritive one (Fischer-Tlustos et al., 2020; Urashima et al., 2022). Bovine milk OS may protect against pathogens, probably acting as competitive inhibitors in the intestinal surface, and promoting the growth of colonic beneficial bacteria such as in *Bifidobacterium* that uses OS as a fermentative substance (Gopal and Gill, 2000), playing an important role in the maintenance of gut homeostasis, and developing the host immune system (Urashima et al., 2022).

Table 1.1. Composition of colostrum, transition milk, and whole milk of Holstein cows

Item	Concentration[1]				References
	Colostrum	TM	TM	Milk	
Milking	1	2	3	>6	
Composition					
Total solids, %	23.9-24.5	17.9-19.9	14.1-16.0	12.2-12.9	(Foley and Otterby, 1978; Blum and Hammon, 2000; Hammon et al., 2000)
Fat, %	6.4-6.7	5.4-5.6	3.9-4.6	3.9-4.0	
Protein, %	13.3-14.0	8.4-8.5	5.1-6.2	3.1-3.2	
Lactose, %	2.7	3.9	4.4	5.0	(Foley and Otterby, 1978)
Bioactive substances					
Immunoglobulins					
IgG, mg/ml	32.0-81.0	25.0-58.0	15.0-17.0	0.4-0.6-	(Foley and Otterby, 1978; Stott et al., 1981; Newby et al., 1982; Blum and Hammon, 2000)
IgA, mg/ml	4.4-17.8	10.7	4.1	0.05-	(Stott et al., 1981; Newby et al., 1982; Borad and Singh, 2018)
IgM, mg/ml	4.2-12.0	7.0	3.1	0.04	(Stott et al., 1981; Pakkanen and Aalto, 1997)
IGF-I, µg/l	341-310	242-195	144-105	15-<2	(Blum and Hammon, 2000; Hammon et al., 2000)
Insulin, µg/l	66	35	16	1	(Blum and Hammon, 2000; Hammon et al., 2000)

Item	Concentration[1]				References
	Colostrum	TM	TM	Milk	
Milking	1	2	3	>6	
Lactoferrin, g/l	1.53-1.84	0.51-0.86	0.26-0.46	0.09	(Korhonen, 1977; Blum and Hammon, 2000)
Lactoperoxidase, µg/mL	11-45	14-54	15-156	13-30	(Korhonen, 1977; Pakkanen and Aalto, 1997)
Lysozyme, µg/mL	0.14-0.70	0.20-0.70	0.57-0.78	0.07-0.60	(Korhonen, 1977; Pakkanen and Aalto, 1997)
Oligosaccharides, µg/ml					(Nakamura et al.; Fischer-Tlustos et al., 2020)
3′-sialyllactose (3′SL)	590-850	310-560	180-440	40	
6′-sialyllactose (6′SL)	105-120	80-85	60-80	20	
disialyllactose (DSL)	225	95	45	10	
6′-sialyllactosamine (6′SLN)	140	75-115	35-100	2-15	

[1]Ranges are shown when available.

1.1.3. Current knowledge of colostrum management

To achieve adequate passive transfer is fundamental to provide a correct quality and quantity of colostrum as well as perform adequate colostrum management. Indeed, is during the first 4 h that the passive transfer of IgG is optimal and then, it is promptly reduced 12 h after birth (Weaver et al., 2000). A failure of passive transfer results in an increase in calf mortality during the preweaned period (Nocek et al., 1984). In fact, the neonatal calves' mortality rate that is associated with a failure of the passive transfer ranges from 8 to 25 % (Raboisson et al., 2016; Urie et al., 2018).

Firstly, BC is of acceptable quality when it has more than 50 g/L of IgG. On the farm, the BRIX refractometer (>22°) is used to do an indirect estimation (Godden et al., 2019). As mentioned in section 1.1.1., when there is no availability of high-quality colostrum, providing CR or supplementing the poor-quality colostrum can be strategies to reach the quality standards. Secondly, the recommended volume of BC administration is 7-10 % of the calf's body weight (**BW**), reaching an ingestion of 150-200 g of total IgG (Robbers et al., 2021b). Thus, in an average Holstein calf weighing 40-43 kg of BW, it corresponds at least to 3-4 L of high-quality colostrum in the first feeding, which ensures a successful passive transfer of immunity ensuring the threshold of 10 mg of IgG/mL in serum (McGuirk and Collins, 2004). Calves administered with 2 feedings of BC had 4 times lower odds of having a failure of passive transfer than the ones that were fed once with BC (Abuelo et al., 2021). Thirdly, some factors related to the producer's management of BC affects significantly colostrum quality, including time to colostrum collection, bacterial contamination during harvest, pasteurization, storage, and feeding of colostrum (**Table 1.2**) (Godden, 2008). Bacterial count levels, used as a measure of BC hygiene, should not exceed 100,000 CFU/ml since they interfere with the absorption of Igs (Godden et al., 2006). A common strategy to avoid bacterial contamination during on-farm management practices, and transmissible diseases such as paratuberculosis or bovine leukosis is BC pasteurization (Godden, 2008). Godden et al. (2006) and Elizondo-Salazar and Heinrichs (2008) established a colostrum heat treatment for pasteurization of 60ºC for 30 to 60 min. This treatment allowed to maintain colostrum quality (avoid protein denaturalization) and reduce the pathogens such as *Mycoplasma bovis*, *Listeria monocytogenes, E. coli O157:H7, Salmonella*

enteritidis or *Mycobacterium avium subsp. paratuberculosis.* Other methods to preserve colostrum quality by limiting microbial proliferation and metabolism include refrigeration, freezing, and chemical additives (Foley and Otterby, 1978; Borad and Singh, 2018). Within 1 hour after collection, colostrum may be frozen for 1 year, avoiding freeze-thaw cycles, or refrigerated for up to 2 d (to avoid unacceptable bacterial counts, if no preservatives are added) (Godden, 2008). When thawing, overheating (temperatures > 60ºC) should be avoided to not denaturalize the proteins (McMartin et al., 2006). Finally, the method of delivery could enhance the acquisition of immunity (esophageal tuber feeder vs. nipple bottle) (McGuirk and Collins, 2004). On one hand, esophageal feeding of colostrum may affect the efficiency of Igs absorption negatively, but on the other hand, delivering a known volume quickly in time using an esophageal tube boosts immunity acquisition (McGuirk and Collins, 2004; Chigerwe et al., 2012). In addition, the management of the dam is also involved in the final delivery quality (**Table 1.2**). In fact, some factors of risk to obtaining low-quality colostrum are first parity/lactation, high production breed (Holstein), short dry period (<21 d), the production of a large volume of colostrum during first milking (>8.5 kg), delay on the first milking (3.7 % reduction for each hour of delay) and health disorders (e.g. mastitis or heat stress) (Godden, 2008; Mendoza et al., 2017).

Indeed, an adequate colostrum management (or passive immunity transfer) in the short term reduces mortality before weaning (McGuirk and Collins, 2004), improves the metabolic state of the calf (enhances the gastrointestinal system maturation as well as nutrient absorption) (Hammon et al., 2013) and promotes the establishment of beneficial bacteria in the neonate's gut and might reduce the odds of pathogenic bacterial colonization and subsequent disease (Malmuthuge et al., 2015).

Table 1.2. Management risk factors for a failure of the passive transfer. Data extracted from Godden et al. (2019).

DAM

- Breed (↓IgG Content Holstein < Brown Swiss < Jersey)
- Age of the dam (↓IgG Content in primiparous cows)
- Season of calving (↓IgG Content ↑ T[a])
- Preparturient vaccination (↑Antigen-specific Antibodies)
- Dry period length (↓IgG Content in short period (<21d))
- Volume of colostrum produced at first milking (↓IgG Content, >8.5 kg)
- Health disorders (e.g. mastitis or heat stress)

CALF

- Time to first colostrum feeding (↑ Time ↓ AEA[1] of Ig)
- Method of BC delivery (↓ AEA[1] of Ig in nipple bottle < esophageal feeder)

FARM

- Bacterial contamination of colostrum (↑ bacteria ↓ AEA[1] of Ig)
- Volume delivered at first feeding (↓IgG Content, ↓ colostrum volume)
- Delayed colostrum collection (↓ 3.7 % of IgG content per each hour)

[1]Apparent efficiency of absorption

1.1.4. Extended bovine colostrum and transition milk feedings after the first bovine colostrum administration

New approaches to BC and the subsequent TM feedings have been suggested in the literature, tending to smooth the transition between colostrum and MR (Conneely et al., 2014; Fischer et al., 2019; Kargar et al., 2020). Contrary to the dairy industry calves, nature-born calves experience a gradual transition from colostrum to mature milk instead of going directly from colostrum to MR. To simulate this progressive transition, some authors propose an extended colostrum feeding strategy to expand the benefits of colostrum on health and survival as occurs in nature (McGuirk and Collins, 2004; Pyo et al., 2020).

Several studies have described how feeding BC and TM from farm surpluses enhanced preweaned calf health (Blum and Hammon, 2000; Berge et al., 2009; Conneely et al., 2014). The supplementation of MR with BC from d 2 to 14 of life

decreased the detection of diseases as well as the associated use of antibiotics (Berge et al., 2009; Chamorro et al., 2017). Similar effects had TM on calf health status when colostrum feeding was followed by 4 subsequent feedings of TM (Conneely et al., 2014). Besides, when TM feeding was extended until 21 d of life, calves improved growth performance and reduced the occurrence of diarrhea (Kargar et al., 2021). Recently, Carter et al. (2022) compared the effects of feeding MR or a mixture of CR: MR 50:50 % (simulating TM) for 2 or 8 feedings. They showed that calves fed 8 meals of colostrum had a faster resolution of diarrhea as well as a greater preweaning growth rate (+98 g/d) than MR-fed ones. Furthermore, Blättler et al. (2001) demonstrated in a histopathologic study that feeding BC or TM for 3 d to calves furthered GIT maturation, enhancing intestinal epithelial cell proliferation, compared to MR-fed calves, being more accentuated in the ones fed BC. Similar effects reported Pyo et al. (2020) and Hare et al. (2020) on small intestine development, including a greater (+15.9 %) serum apparent IgG persistency during 72 h of life while feeding a short period of 2-3 d at 50:50 % CR:MR after first colostrum compared to whole milk.

Thus, the research available offers dairy producers a possible strategy of feeding neonatal calves TM or diluting colostrum with milk or MR (after an initial colostrum meal) to promote intestinal development and function. However, the length of the treatments added to the on-farm management such as preservation and storage needs further studies. Despite BC has been used as a therapeutic and prophylactic nutraceutical in newborn calves, BC potential in older calves (>21 d of life) remains uncertain (Ulfman et al., 2018).

1.1.5. The effect of bioactive molecules of bovine colostrum after the gut closure: further applications in health and performance

Although the main bioactive molecules present in BC are not absorbed after gut closure, the local functionality of their immune, antimicrobial, and anti-inflammatory bioactive molecules is still being investigated (Blum and Hammon, 2000; McGrath et al., 2016).

Due to its high abundance in BC, the main studied bioactive molecule in BC has been IgG (Foley and Otterby, 1978). It plays a role in innate and adaptative immunity in

the GIT by binding to FcR, leading to phagocytosis and killing bacteria (innate) and promoting the uptake of bovine IgG-pathogen immune complexes via FcR (Ulfman et al., 2018). This process results in increased T-cell, and ultimately B-cell responses to the pathogens and the induction of secretory IgA (adaptative) (Ulfman et al., 2018). In fact, hyperimmune BC (with high concentrations of IgG) supplements have been used as a nutraceutical against human gastrointestinal and respiratory infectious diseases, showing an alternative or combined therapy to antimicrobials (Kelly G. S., 2003; Ulfman et al., 2018). In preweaning calves, Carter et al. (2022) have reported the addition of bovine CR (65 g/L) to the MR ration during 8 feedings (4 d) as an effective therapy for diarrhea from 5 d to 3 wks of life.

Some growth factors of BC, including IGF-I, IGF-II, and insulin, are not absorbed in significant amounts in neonatal calves and, therefore, it would not be expected that they had a systemic action (Hammon et al., 2013). Locally on the GIT mucosa, the IGF-I receptor is most highly expressed in the ileum and colon, and the IGF-II receptor in the duodenum, while the insulin receptor expression is similar throughout all the GIT (Georgiev et al., 2003). These mentioned growth factors, especially IGF-I and insulin, enhanced intestinal growth and maturation through mitogenic signaling and other undefined mechanisms in calves up to 8 days old (Roffler et al., 2003; Hare et al., 2023).

Moreover, milk LTF has been demonstrated to have several roles (Joslin et al., 2002) beyond antimicrobial properties (Joslin et al., 2002). Lactoferrin is also a regulator of the immune system (stimulating the phagocytic activity of human neutrophils), an inhibitor of solid tumor growth and, it enhances *in vitro* bovine epithelial cell proliferation (Rejman et al., 1992; Bezault et al., 1994; Miyauchi et al., 1998). Lactoferrin supplementation in calves' MR has been proven to increase the average daily gain (**ADG**) and reduce the occurrence of diarrhea (Robblee et al., 2003) and it has been administered as a nutraceutical in humans for antioxidant, anti-tumor, anti-inflammatory, and antimicrobial purposes (Superti, 2020).

Finally, OS promote the establishment of a balanced neonatal microbiome, as explained by the correlation between *Bifidobacterium* and colostrum OS in the small intestine of calves within 12 h of life (Fisher, 2018). Besides an in vitro research demonstrated that BC and milk OS are highly efficient (more than 80 %) at inhibiting

K99-fimbriated enterotoxigenic *E. Coli* strains isolated from diarrheic calves (Martín et al., 2002).

Overall, BC contains various bioactive molecules that have potential health benefits for both calves and humans, including the regulation of the immune system, growth enhancement, and antimicrobial effects.

1.1.6. Further implications

As mentioned in the previous sections 1.1.2.1. and 1.1.4, TM has a great potential for its use in dairy calves' benefit. On one hand, TM is not commonly supplied to neonatal calves and is either discarded or conducted to the milk tank (increasing riskily the tank's SCC) (Fischer et al., 2019; Kargar et al., 2020). In fact, TM is often considered waste milk by the dairy industry and some government associations (e.g. USDA), achieving the same level as mastitis milk or milk with antibiotic residues (USDA, 2010). On the other hand, despite its richness in bioactive molecules compared to whole milk, their use is not extended to dairy farms and therefore, its management could be challenging (Blum and Hammon, 2000) for different reasons. First, the homogeneity of TM milk is highly variable since its quality decreases with each milking and management considerations such as biological factors of the dam (parity, genetic) and on-farm management practices (number of milkings per day, nutrition) (Oyeniyi and Hunter, 1978; Godden, 2008). Second, there is a lack of standardized protocols for the usage of TM on-farm: how to harvest, store, and preserve the quality and the availability of storage facilities to accomplish them. Hopefully, some of the protocols could be easily adaptable from BC (e.g. harvest and storage), but others require (e.g. preservation, dam's miking, and parity inclusion criteria) further research. Third, even though TM's positive impact on health has been described (Conneely et al., 2014; Van Soest et al., 2020), there is a lack of a standard protocol (e.g. amount, feedings, or TM milking number) for feeding it. Finally, there is limited knowledge available concerning the mid and long-term effects of feeding TM to calves on growth performance, health, reproduction, and future milk yield performance that requires further research to fully describe its benefits. Alternatively, some authors propose the use of CR mixed with MR to simulate TM, which could solve some preservation, hygiene, and storage problems, but the presence of bioactive compounds is diminished and comes with an extra cost

for the farm (Foley and Otterby, 1978; Kargar et al., 2020). Although it has been studied in IgG, it remains unknown if the absorption and bioavailability of other bioactive compounds are preserved (Arthington et al., 2000; Quigley et al., 2002; Lopez et al., 2020).

1.2. The impact of long transportation on unweaned calves' health

1.2.1. Calves transport from farm surpluses

In the European Union (**EU**), around 22.6 million calves are born each year (Eurostats, 2019). After achieving the future female replacement needs of the dairy farm, the remaining female and male unweaned calves are often considered a by-product of low financial value (Devant and Marti, 2020). As a result, their postnatal care is not a priority for the dairy sector and these dairy beef calves are rapidly sold at auction markets for then be reared in intensive systems. Concretely in the EU, around 1.5 million unweaned calves are transported annually, being the Netherlands, Spain, and Italy the main importing countries (Eurostats, 2019). Usually, preweaned calves are transported from multiple origins to assembly centers. After that, calves are sold to rearing and fattening farms until they reach the optimal weight to be slaughtered (Pardon et al., 2014; Wilson et al., 2020).

Multiple stressors involved during transportation (transport, feed restriction, regrouping batches, and environmental factors) have been shown to negatively affect calves' health, energetic balance, and immune status (Knowles et al., 1999; Renaud et al., 2018). This makes these animals more likely to suffer infectious diseases such as bovine respiratory disease and enteric pathogens (*Escherichia coli*, rotavirus, coronavirus, *Salmonella* spp., and *Cryptosporidium parvum)* (García et al., 2000; Step et al., 2008; Earley et al., 2017). Additionally, unweaned calves between 2 and 4 weeks of age experience an immunological gap period due to the transition between passive (acquired from colostrum) and active immunity (developed from vaccination or in response to an infection), which unfortunately matches with the minimum age to transport lactating calves in the EU (Chase et al., 2008; Velarde et al., 2021; EFSA Panel on Animal Health and Welfare (AHAW) et al., 2022). Overall, the mentioned conjunction of factors could explain why preweaned calves are more susceptible to long journeys than weaned calves (Velarde et al., 2021; EFSA Panel on Animal Health and Welfare (AHAW) et al., 2022).

1.2.2. European Union's transport legislation

Nowadays, the current legislation for the protection of animals during transport is the Regulation (EC) No 1/2005 (European Community, 2005). This law established for long transport (>100km) of unweaned calves a minimum age of 14 d and a maximum journey duration of 19 h, consisting of two travels of 8 h with 1 h rest in between. After the 19 h journey, animals must be unloaded, fed, and watered into an EU-approved control post and rest for a 24 h period until further transport. Also, the legislation implements minimum welfare requirements regarding the transport of unweaned calves such as fitness for transport, watering and feeding intervals, transport temperature range, or transport conditions including space allowance, bedding, water supply, and ventilation. In addition, the assembly centers are described in the Regulation (EC) No 1/2005 (European Community, 2005) as holdings, collection, and markets, at which domestic animals of bovine, ovine, caprine, or porcine species originating from different holdings are grouped together to form consignments. However, transiting through assembly centers confers a higher risk of impaired welfare, primarily as a result of hurried handling (associated with slips or falls during loading and unloading), and feed and water deprivation (as feeding electrolytes instead of MR does not fulfill calves' nutritional requirements) (Marcato et al., 2020; Velarde et al., 2021).

Nonetheless, the European Food Safety Authority (EFSA)'s Panel on Animal Health and Welfare (AHAW) has recently exposed its concerns about the current legislation related to the transport of cattle and developed recommendations to enhance their welfare. Regarding preweaned calves' transportation, the EFSA Panel on AHAW (2022) suggested delaying the present minimum age of 2 weeks to 5 weeks of life to avoid the immune gap period and deal with more mature calves.

1.2.3. Assessing calf gut dysfunctionality during transportation

During stressful episodes including transportation and feed restriction, cortisol is released in response to stress, compromising the intestinal barrier functionality (Lambert, 2009). This condition, also known as "leaky gut", undergoes with a loosening of the tight junction in between enterocytes and increased permeability to harmful molecules and microorganisms to the systemic circulation (Fischer et al., 2019). Several research conducted on adult cattle (Zhang et al., 2013; Kvidera et al.,

2017) and calves (Pisoni et al., 2022) described how either progressive or short-term feed restriction affected intestinal barrier integrity and functionality, which generated energy metabolism disbalances and altered gastrointestinal permeability. Nowadays, the most common practice for feeding calves in assembly centers is providing a rehydration solution to recover them from transportation (Devant and Marti, 2020). However, the present rehydration protocols are highly variable and mostly addressed to diarrheal diseases to reverse dehydration and metabolic acidosis, but lack to cope with the negative energy balance caused by transportation (Smith, 2009; Miqueo et al., 2018). Marcato et al. (2020) studied the effects of the pre-transport diet (electrolytes vs. MR) on the physiological status at the rearing farm's arrival. This study revealed that feeding MR (1.5 L at 12.5 % DM) before 6 h transportation softened the negative energy balance associated with fat and protein mobilization as well as BW losses compared to electrolyte-fed calves (1.5 L at 2 % DM).

In the literature, the most common serological biomarkers (see **Figure 1.2**) to assess intestinal permeability and barrier functionality in bovine include Chromium-EDTA (Udén et al., 1980; Hunt et al., 2002), lactulose: D-mannitol test (Hall, 1999) and citrulline (Gultekin et al., 2019). The ratio of lactulose to mannitol is the most described biomarker in calves. They are oligosaccharides that have the propriety of being non-digestible and non-metabolizable by the host, being lactulose a much larger molecule than mannitol (Celi et al., 2019). In healthy animals, on one hand, lactulose passes through the GIT without being digested, whereas in a damaged intestinal barrier, it can leak through the tight junctions and enter the bloodstream. On the other hand, mannitol acts as a "control molecule" and passes freely through the GIT barrier (Galipeau and Verdu, 2016). However, lactulose and mannitol are metabolized by the colonic microbiota, and therefore, this ratio is not useful for assessing colonic permeability (Galipeau and Verdu, 2016). Then, Cr-EDTA is a non-nutritive compound that was first validated as a paracellular permeability biomarker for calves by Zhang et al. (2013). Elevated Cr-EDTA serum concentrations indicate an increase in paracellular permeability, which makes blood more accessible to large molecules (Amado et al., 2019).

Finally, citrulline is a non-essential amino-acid synthesized by small intestine enterocytes (Gultekin et al., 2019). Indeed, levels of circulating citrulline are

indicative of the functionality of the enterocyte mass, and therefore, a decrease of enterocyte cell mass in the small intestine results in declined circulating levels of citrulline, suggesting an impaired GIT functionality (Crenn et al., 2008).

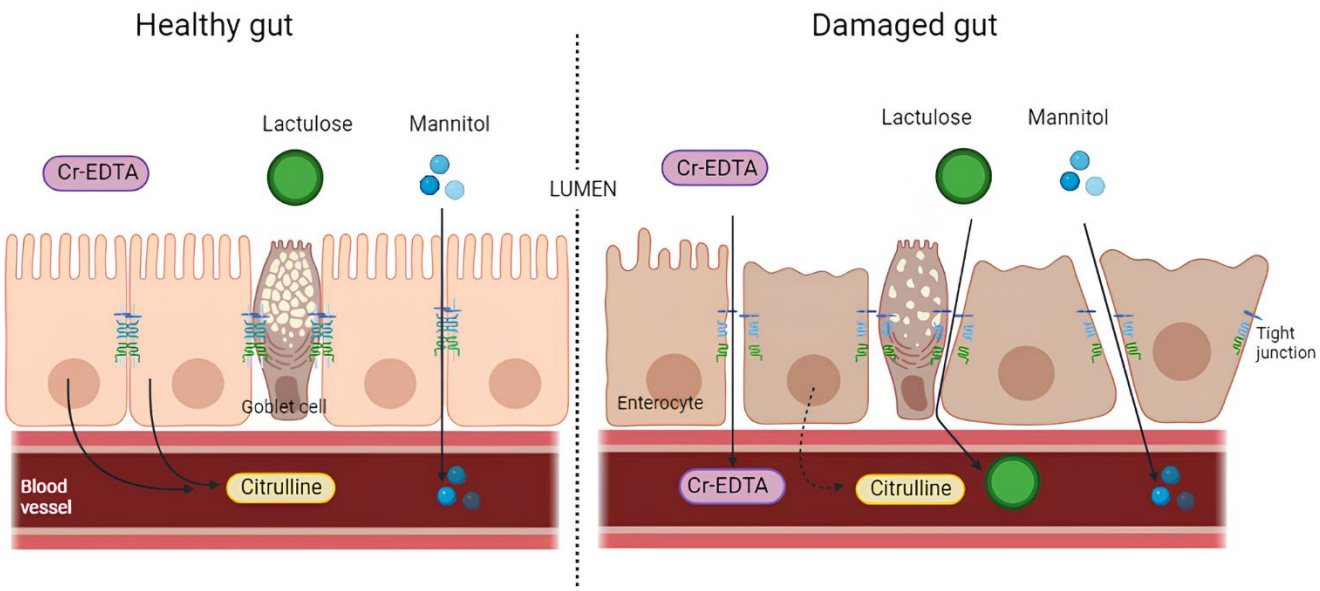

Figure 1.2. Serological biomarkers of intestinal permeability. Image created using biorender.com.

1.2.4. The importance of maintaining gut health

The digestive system of neonatal calves undergoes an enzymatic and immune system maturation. During the pre-ruminant phase (up to 30 d of life) calves acquire the adaptative immunity (mainly through BC) and develop the digestive enzymes (though the suckling behavior when ingesting milk) for the breakdown and assimilation of nutrients (Longenbach and Heinrichs, 1998; Chase et al., 2008). For that reason, since the first ingestion of colostrum, the GIT mucosa epithelium plays a double role, absorbing nutrients and, at the same time, protecting against pathogen colonization (Maynard et al., 2012).

Inside the larger immune barrier that represents the mucosa epithelium, the first line of defense are the epithelial cells and mucus layer (mucin glycoproteins secreted by Goblet cells) and their interaction with calf's microbiome, which maintains the gut's homeostasis (Belkaid and Hand, 2014; Chase, 2022). This diverse microbiome produces beneficial metabolites like volatile fatty acids that enhance barrier integrity and improve mucosal immunity (Belkaid and Hand, 2014). The

microbiome also initiates the maturation of the epithelial mucosa and the gut-associated lymphoid tissue formed by Peyer's patches of the distal ileum, isolated lymphoid follicles, and mesenteric lymph nodes and prepares the host for the adaptative immune response (Maynard et al., 2012). Once the mucosal epithelium is breached, the innate systems rapidly respond activating a battery of cellular and humoral components such as macrophages, neutrophils, interferon, and cytokines (proinflammatory and T stimulatory) (Chase, 2022). Then, adaptative response (or memory response) is developed in naïve animals (previously unexposed animals) 10 to 14 d later mediated by secretory IgA and IgG, and T and B lymphocytes (Chase, 2022). Finally, the antigen presentation executed by intestinal dendritic cells prevents the over-response of the pro-inflammatory cytokines, by supporting the development of regulatory CD4+ T cells, and therefore, maintains intestinal barrier homeostasis (Maynard et al., 2012). To sustain the barrier homeostasis to respond actively and eventually, develop an effective and persistent immune response, the energy uptake is essential for its success (Chase, 2022).

Moreover, the microbiota also plays an important role in preserving the GIT's health. Symbiotic bacteria enhance neonatal GIT immune homeostasis and promote protection against pathogen colonization (Pickard et al., 2017). They described two direct mechanisms of pathogen colonization resistance involving symbiotic bacteria (**Figure 1.3**). These GIT's microbiota either compete for nutrients and space that would be otherwise available to pathogens or kill/inhibit growth by producing bacteriocins, short-chain fatty acids (e.g. butyrate, acetate, and propionate), or enzymes that convert conjugated, primary bile acids to secondary bile acids. There have been described other indirect mechanisms, such as mucus production containing glycans, IgA and IgG production from B cells, activation of cytokines (IL-22) that promote the secretion of antimicrobial peptides, oxidation of sugars via reactive nitrogen species, production of microbe-associate molecular patterns produced by bacteria and viruses that stimulate host innate immunity on epithelial cells or dendritic cells, and the production of butyrate which decreases oxygen concentration by stimulating host epithelial cell metabolism, and it also contributes to the stimulation of the innate and adaptative defenses in the gut barrier.

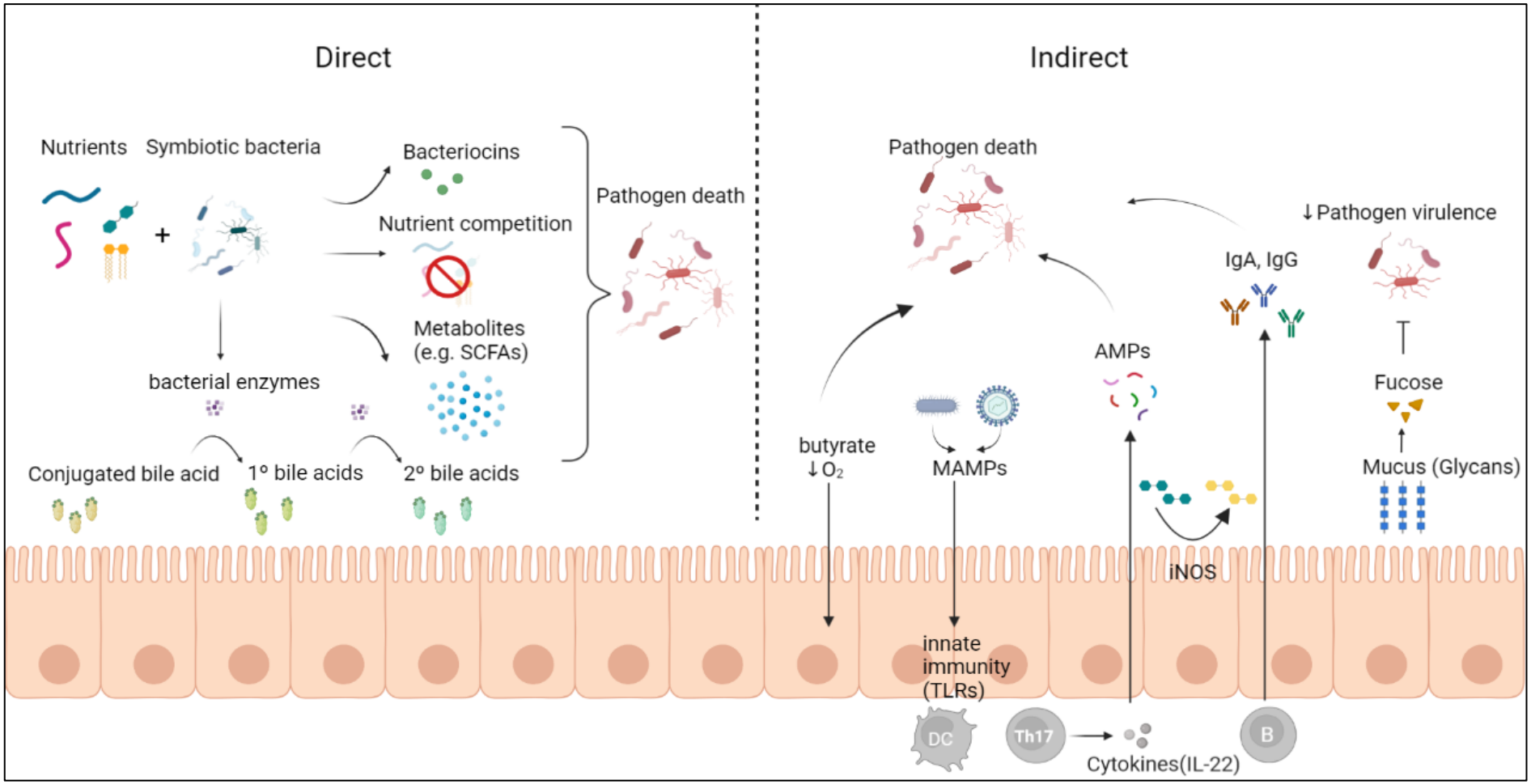

Figure 1.3. Direct and indirect mechanisms of colonization resistance. Image adapted from Pickard et al. (2017) using biorender.com. In this graft, some molecules are abbreviated as follows: AMPs = antimicrobial peptides, DC= dentritic cell, iNOS= inducible nitric oxide synthase, MAMPs = microbe-associate molecular patterns), SCFA = short-chain fatty acids, TLRs= toll-like receptors

1.3. Early nutritional strategies on future performance: the long-term effects of feeding colostrum.

1.3.1. Neonatal Imprinting and literature precedents of early nutritional strategies on future performances

The impact of maternal-fetal imprinting and early nutrition in humans and animals has been described for years (Dyer and Rosenfeld, 2011; Bartol et al., 2013; Cassidy and Charalambous, 2018). During the postgestational period of all mammals, neonates receive maternal support coming uniquely from milk and colostrum (Langer, 2008). During this period a unique maternal epigenetic programming, also known as the "Lactocrine" effect, occurs in which neonates adapt to environmental changes (Yan et al., 2006; Hochberg et al., 2011). The epigenetic control of genome function is developed by the genomic imprinting phenomenon (Ferguson-Smith, 2011). Concretely, genomic imprinting consists of expressing genes based on their parental origin, in which one of the two identical copies of the gene is either expressed or silenced by deoxyribonucleic acid (**DNA**) methylation, leaving only one monoallelic copy of the gene (Ferguson-Smith, 2011; Cassidy and Charalambous, 2018).

Until now, studies on maternal imprinting on offspring development have been focused mainly on perinatal stress (based on adverse conditions such as heat stress or the circulating levels of corticoids) and diet (over and under nutrients requirements) (Edwards et al., 2021). Therefore, the concept of *The Developmental Origins of Health and Disease (DOHaD)* has been described to refer to how early life environments can modulate non-infectious disease risks and lifetime health (McKerracher et al., 2019).

Focused on dairy cattle, early nutritional strategies can influence their future metabolism and performance, affecting the economic benefits of the farms (Hammon et al., 2012; Yunta et al., 2015; Kenéz et al., 2018). An increase in the growth rate during the first weeks of life has been associated with increased milk yield at first lactation, which generates profitable economic returns (Bach, 2012; Soberon et al., 2012; Bach et al., 2021a). Particularly, Kenéz et al. (2018) showed how increasing MR or whole milk allowance during calves' early age exerted long-

term metabolic profile alterations and greater milk yields. Principally, the restricted availability of MR during the preweaning period negatively affected acylcarnitine heifers' plasma concentrations, which are involved in the metabolic pathway of mitochondrial fatty acid oxidation, suggesting indicating adaptive mechanisms of energy expenditure (Kenéz et al., 2018). Furthermore, providing a high plane of nutrition to young calves may program metabolic changes in the hypothalamic-pituitary-gonadal (HPG) axis resulting in an early onset of puberty (Keogh et al., 2021; Sánchez et al., 2021). These changes in the HPG axis are mediated by metabolic signaling molecules (including leptin, insulin, and IGF-I) which regulate the gonadotropin-releasing hormone release (Sánchez et al., 2021).

Although recent literature avouches the imprinting effects of calves' milk intake on future milk yield performance (Bach, 2012; Soberon et al., 2012; Rosadiuk et al., 2021), there are scarce studies relating the imprinting of colostrum feeding on long-term performance and metabolism.

1.3.2. Neonatal imprinting and colostrum

Literature regarding the effects of maternal imprinting during colostrum feeding in any species is very limited. Probably this fact could be due to that colostrum imprinting is a growing area of research, with costly studies due to the complex nature of the investigations (e.g. the use of -omics science), and the specie-specific relationship between colostrum composition and placentation (Bigler et al., 2022). Moreover, in literature found in humans and mice, colostrum is included as breast milk, which makes it unable to differentiate the maternal effects of colostrum from the TM and milk ones (Verduci et al., 2014; Cacho and Lawrence, 2017)

Focusing on the mentioned colostrum-placenta relationship, the different degrees of involvement of fetal and maternal placenta tissues among mammalian species and the ability of each type of placenta to transfer passive immunity could determine the concentration of the main Ig isotypes (Furukawa et al., 2014; Bigler et al., 2022). In ruminants and pigs, the multiple tissue layer of the epitheliochorial placenta restricts the diffusion of large molecules (including Igs), the reason why colostrum's biological components (mainly IgG) are essential to ensure neonate survival

(Furukawa et al., 2014; Burton and Fowden, 2015; Godden et al., 2019). Alternatively, female primates, rabbits, and rodents develop a hemochorial placenta, which is the most histologically invasive placenta and less restrictive (Furukawa et al., 2014). Therefore, these species have prepartal transfer in utero and the most concentrated component in colostrum is IgA, which coats the intestinal mucosa surfaces (Bigler et al., 2022).

Indeed, looking for studies using "maternal imprinting" without including "colostrum", the main repeated topics were the effects of maternal stress (induced by glucocorticoids or extreme temperatures) and diet as reported by Edwards et al. (2021) in mammals. Similarly, changes in milk composition as a result of maternal diet (overnutrition or undernutrition) lead to dysregulated metabolic imprinting of offspring in rodents (Bautista et al., 2016). A maternal obesogenic diet, five times richer in fat than the control one, reduced the milk yield, carbohydrate and omega-3 fatty acid content in milk, which are associated with lower brain weight and impaired neurodevelopment in offspring (Bautista et al., 2016). Interestingly, other collateral effects of maternal diets are the regulation of metabolic hormones (including leptin, adiponectin, and insulin) in offspring (Edwards et al., 2021). Feeding formulas with a lack of leptin to infants leads to a high tendency to accumulate visceral fat deposition and consequently, gain BW in adulthood (Picó et al., 2007). Finally, in calves, the mid-term effect of colostrum imprinting on performance and metabolism calves has been only evaluated in a small-scale study about delaying the first colostrum feeding (Zanker et al., 2001). In this study, calves were able to compensate for the 24 h of fasting after birth before 7 d of life, which might indicate that there was no permanent imprinting on the hematological, metabolic, or endocrine parameters studied.

1.3.3. Impact of colostrum intake on calves' lifetime performance

In ruminants, the low placental transfer of maternal antibodies produces that most of the maternal immunity programming occurs through colostrum ingestion during the first 24 h of life. Colostrum provides lactocrine nutritive, protective, and adaptative advantages to newborn calves due to its high content of nutrients and

bioactive compounds, as mentioned in section 1.1.2. (Godden, 2008; Van Hese et al., 2020; Playford and Weiser, 2021).

A systematic search in PubMed, Web of Science, and Scopus about the effect of colostrum feedings on long-term performance and metabolism was performed using the keywords "colostrum", "long-term", "calf", "heifer", "performance" and "metabolism". After refining the outcomes, the literature available about the effect of feeding different amounts of colostrum in female calves is scarce and authors did not report the same parameters. In 2004, Kertz and Chester-Jones disclosed the lack of complete data, materials, and methods in calves and heifer studies. These authors elaborated a guideline for calves and heifer experimental reporting to facilitate better experimental design and planning, collection of data, and summation. Similar to them, after reviewing long-term effects of colostrum studies (**Table 1.3**), it was detected a lack of a uniform period to calculate preweaning and postweaning ADG or different reproductive parameters and terminology for describing heifers' performance. Thus, the incompleteness of data encouraged us to perform a guideline on how to report experimental data to assess the long-term effect of colostrum on calves (manuscript unpublished).

According to the scarce literature available, long-term implications of feeding BC on milk performance were first reported by Faber et al. (2005), who observed an increase of 305-d mature equivalent milk at first (+322 kg) and second lactation (+1,349 kg) when Brown Swiss female calves were fed with 4 instead of 2 L in the first colostrum feeding. They also reported a decrease in veterinary cost and an increase in growth rate up to 500 d of age for the 4 L treatment, but no differences were observed in age at conception. Similarly, Abuelo et al. (2021) fed either 1 or 2 meals of BC feedings, consisting of a first 3 L feeding with a second one optional of 2 L. They observed that offering an extra colostrum meal increased 120 g/d of preweaning ADG, tended to increase first lactation milk yield and reduce the number of inseminations needed for conception. Additionally, other authors also have described preweaning growth benefits (until 1 month of life) of feeding maternal BC instead of CR at birth while reporting similar IgG absorption (Jones et al., 2004). Contrary to that, Furman-Fratczak et al. (2011), using γ-globulin concentrations as a criteria to assess the failure of the passive transfer, reported that animals with

higher serum γ-globulin concentrations (> 15 g/L) at 30-60 h of life did not have any differences in growth rates compared to calves with failure (< 5 g/L) or partial failure (5 to 10 g/L) of the passive transfer during the first 6 months of life. However, heifers with higher γ-globulin (> 15 g/L) reduced by an average of 30 d the age at first insemination compared with heifers with passive transfer failure, being established a minimum age of 15 mo and BW of 390 kg to be inseminated.

Moreover, in the shorter term, extended colostrum supplementation compared to feeding MR (Chamorro et al., 2017) or whole milk (Kargar et al., 2020) for 2 weeks had different results. After a first common colostrum feeding, calves fed 3 L of MR supplemented with CR (2:1 ratio) had similar preweaning ADG than MR-fed calves (Chamorro et al., 2017); whereas calves fed daily 0.7 kg of pasteurized BC and 4.3 kg of whole milk had greater ADG (+100 g/d) and final BW (+7.8 kg) on d 81 of life than calves fed 5 kg of whole milk (Kargar et al., 2020). This fact suggests that possibly the source of colostrum and the amount delivered could interfere with growth performance. Furthermore, some authors have recently described how colostrum intake shapes the neonatal calf metabolome at 8- 36 h after birth, having systemic effects on energy metabolism pathways, including amino acid, carbohydrate, and fat oxidation (Qi et al., 2018; Zhao et al., 2018; Liermann et al., 2020).

To conclude, the current knowledge of the biological value of BC has been widely studied during the last 40 years, offering different applications as a nutraceutical in human and animal health. However, in TM's research including biological compounds, management, and further implementation, there is still much progress to be made.

Table 1.3. Compilation of the literature available about the effect of feeding different amounts of colostrum in female calves.

Study	Treatment	Effect
Jones et al. 2004	Maternal colostrum vs. CR	No differences in ADG and structural measures (length, hip, withers height, and heart girth) at d 29 of age.
Faber et al., 2005	First feeding of 2 L vs 4 L of BC	Greater preweaning ADG and greater first lactation yield in 4 L animals. No differences at age of conception.
Furman-Fratczak et al., 2011	Different colostral immunoglobulin concentration on serum at 30-60 h after birth	No differences in growth rates but lower age at insemination for higher γ-globulin (> 15 g/L).
Chamorro et al., 2017	Extended CR and MR (2:1) for 2 weeks vs. MR	Similar preweaning ADG.
Kargar et al., 2020	Extended colostrum feeding for 2 weeks. Daily 5 kg whole milk (WM) vs 4.65 kg WM+ 0.350 kg BC vs 4.3 kg WM + 0.7 kg BC	Calves fed daily 0.7 kg of BC had greater ADG and final BW (d 81) than calves fed 5 kg of WM.
Abuelo et al., 2021	1 feeding (3 L) vs 2 feedings (3+2 L) of BC	Greater ADG at weaning, lower num. of inseminations and greater first lactation yield with 2 feedings.

Chapter 2

OBJECTIVES

2. OBJECTIVES

The main objective of this thesis was to expand the current knowledge about the biological value of TM and BC surpluses to encourage their use to promote calf health and performance. The specific objectives were:

1. Comparing the biological value of TM and BC considering the parity of the dam.
2. Evaluating the digestive tract recovery and metabolism of feeding either BC, TM, or MR after an episode of feed restriction and fasting (**FRF**) in dairy calves.
3. Determining the impact of the amount of colostrum fed to calves on their metabolomic profile after the first parturition.

To achieve these objectives, three studies were conducted:

- In study 1, IgG, IGF-I, and LTF concentrations in primiparous and multiparous dairy cows were evaluated.
- In Study 2, performance parameters and serum and fecal biomarkers of immune, energy status, intestinal barrier functionality, and gut microbiota were evaluated to determine whether BC or TM enhances calves' recovery from FRF.
- In Study 3, possible long-term benefits of feeding 2 vs. 8 feedings of BC to dairy female calves on their future performance and metabolism after first calving were evaluated.

Chapter 3

THE BIOLOGICAL VALUE OF TRANSITION MILK: ANALYSES OF IMMUNOGLOBULIN G, IGF-I AND LACTOFERRIN IN PRIMIPAROUS AND MULTIPAROUS DAIRY COWS

A fraction of this research has been published in:

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3.1. Introduction

After the first milking of BC, the mammary secretion from the second milking to the sixth is considered TM (Godden et al., 2019). Although it is sometimes treated as waste milk, it contains at least half of the IgG, IGF-I, and LTF found in BC (Blum and Hammon, 2000). An appreciation of the biological value of TM is needed to exploit its potential for future applications on animal and human health. To maximize the industrial process of obtaining the molecules, the parity and milking number of the cow should be considered to evaluate the economic viability of the process.

The objective of the present study was to determine the biological value of TM compared to BC for its use in veterinary and human medicine. Herein we present interesting results from evaluating IGF-I, IgG and LTF concentrations in primiparous and multiparous cows which encourages further research in this topic.

3.2. Materials and methods

3.2.1. Collection and sample analysis

One hundred mL of BC (first milking-**M1**), TM from second (**M2**) and third (**M3**) milkings and milk from the tenth milking (**M10**) were collected by farm personnel from a total of 45 primiparous and 45 multiparous Holstein-Friesian cows randomly selected from three different commercial farms (2 x 15 cows from each farm) in the North-East of Spain and frozen at -20ºC until its use. In all farms, cows were dried at 60 d before parturition, but Farms B and C vaccinated dry cows against Rotavirus, Coronavirus, and *Escherichia coli* K99, and Farm A did not. All cows were fed a unique dry cow diet, but it slightly differed between farms (**Table 3.1**). Furthermore, cows in Farms B and C were milked three time per day, and twice in Farm A. Bulk milk tank from each farm was sampled at each milk delivery to the buyer and analyzed for SCC and total bacteria count at the Central Laboratory for Milk Recording (ALLIC, Catalonia, Cabrils, Spain) to assess a general farm overview of milk quality. The samples were obtained after cleaning and disinfecting cows' teats and collected in sterilized 50 mL Falcon tubes.

After thawing at room temperature, IgG, IGF-I and LTF concentrations were quantified.

Table 3.1. Nutrient composition of the dry cows' rations and the average milk tank quality of farms enrolled in the study.

Item	Farm A	Farm B	Farm C
Nutrient, g/kg DM			
DM	440	522	633
CP	125	125	125
EE[1]	21	26	27
NDF	450	562	522
ADF	293	354	288
Ash	77	80	55
NE_l[2], MJ/kg DM	5.52	5.76	5.44
Milk tank quality[3],			
Milk SCC[4], cells/L	215	207	229
Total bacteria count, cfu/L	11	26	14

[1]EE=Ether extract
[2]NE_l=Net energy of lactation
[3]Mean of SCC and cfu/L values measured in tank milk samples every two days during the study
[4]SCC=Somatic Cell Count

Immunoglobulin G quantification.

Samples were analyzed for IgG concentrations using a single radial immunodiffusion (SRID) kit (Radial Immunodiffusion Test, Triple J Farms, Bellingham, WA, USA) according to the manufacturer's recommendations. Bovine colostrum and TM were centrifuged for 15 min at 2,000 x *g* at 4ºC to remove the upper fat layer of the sample. Whey fraction was collected and diluted with Phosphate-buffered saline (**PBS**) 1:10 (M1), 1:5 (M2) and 1:2 (M3), respectively. Five µL of sample was added per well and incubated at room temperature for 24 h until plate reading.

Insulin Growth Factor- I quantification.

Samples were analyzed for IGF-I concentrations using an IGF-I ELISA kit (Mediagnost, Reutlingen, Germany). According to the kit manual, samples were initially diluted with PBS 1:20 but afterwards, some of them were repeated at 1:40 (M1 and M2), 1:21 (M3) or 1:10 (M10) as they were out of the detection range. Plates were read at 450 nm using EMS Reader MF V.2.9-0. from Labsystems (Vantaa, Finland).

Lactoferrin quantification.

Samples were analyzed for LTF concentration using Bovine Lactoferrin ELISA kit from Cloud-Clone Corp. (Katy, TX, USA). Following the manufacturer's instructions, samples were centrifuged for 15 min at 10,000 x *g* at 4ºC. The aqueous fraction was collected and centrifuged twice more for a total of 3 cycles. After that, samples were diluted with PBS 1:100,000 (M1 and M2), 1:20,000 (M3) and 10,000 (M10). Plates were read at 450 nm using Model 680 Microplate Reader from Bio-Rad (Hercules, CA, US).

3.2.2. Statistical analyses

Data were examined using descriptive statistics and Tukey outlier boxplots were performed to detect outliers using JMP®, version 16.0.0 (SAS Institute Inc., Cary, NC, USA). Outlier exclusion criteria was 1.5-fold interquartile range considering each biomolecule, milking number and lactation, which was indicated by the length of boxplot's whiskers. Finally, 16, 8 and 24 observations of IgG, IGF-I and LTF data, respectively, were excluded, leaving 315, 267 and 311 observations for IgG, IGF-I

and LTF analysis, respectively. The remaining missing values were mainly from M10 samples because they were below the technique detection range. Furthermore, data from one cow was removed due to clinical mastitis.

The final dataset obtained was statistically analyzed using SAS software (version 9.4, Institute, Cary, NC, USA). Data were analysis with a mixed-effect model accounting for the random effects of cow and farm, as well as the fixed effects of parity (primiparous or multiparous), milking number and their interaction. To explore the effect of farm on the parameters studied, a mixed-effect model accounting cow as a random effect, and milking number, farm and their interaction as fixed effects was done. In all cases, milking entered to the model as a repeated measure using an autoregressive covariance matrix (the structure with the lowest Bayesian criterion), except for the IGF-I, in which was used an unstructured covariate matrix. Additionally, on the LTF analysis, plate was included as a block in the fixed effects. Data were previously transformed to logarithm to achieve a normal distribution.

3.3. Results and discussion

Although this study was not designed to elucidate the effects of farm practices on milk bioactive compounds, it was observed that Farm A had lower ($P < 0.05$) IgG concentrations than the two other farms. Dry cow vaccination against calf diarrhea in Farm B and C, but not in Farm A (Denholm et al., 2018) might have influenced in this parameter. Although Baumrucker et al. (2021) found a positive correlation of IgG1 with LTF, in this study IgG and LTF were not correlated. Contrarily, LTF was lower ($P < 0.05$) in farm C compared with Farm A and B. Cheng et al. (2008) suggested stage of lactation, daily milk production, and SCC as the main factors associated with milk LTF concentration. Individual SCC and milk volume of the different milkings may explain differences among the three farms in LTF concentrations. Since different on-farm management practices like vaccination protocols or milk volume can affect the concentration of these bioactive compounds, farm was included as a random effect in the statistical model (Godden et al., 2019). Despite IGF-I concentrations being similar in all farms, it was observed a positive correlation with IgG concentrations (R-square = 0.388, $P < 0.001$), which might suggest a common mechanism of secretion of both components.

The results of the current study showed that the concentration of IgG, IGF-I and LTF decreased (P < 0.001) from the first milking to the tenth. Multiparous cows produced colostrum/ TM significantly richer in IGF-I and LTF than the primiparous cows (P < 0.01, P < 0.05, respectively, **Table 3.2.**), and parity affected the change in IGF-I between the M1 and M2 milkings (P < 0.001, **Table 3.2.**). As the milking progressed, multiparous cows, despite having initially greater concentrations on M1 and M2, had similar IGF-1 dilution rate than primiparous cows until M3 (**Fig. 1**).

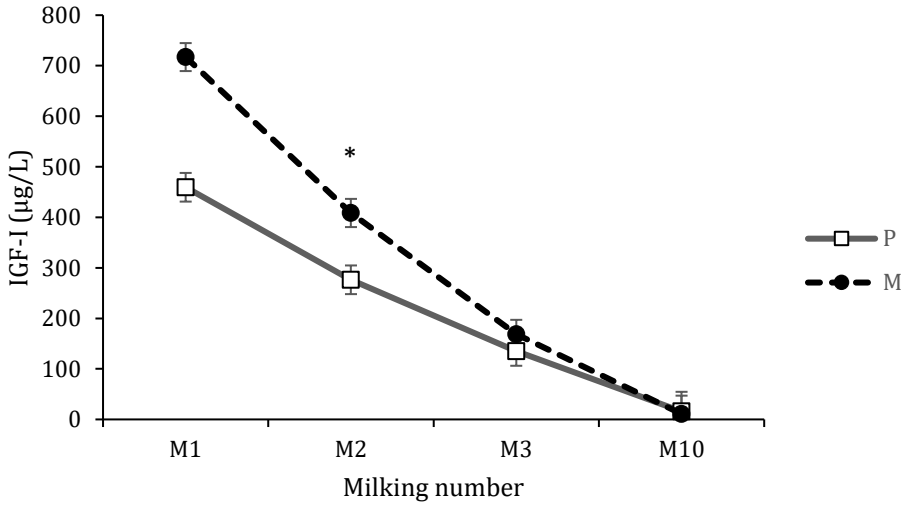

Figure 3.1. Milk IGF-I concentration evolution by milking number and parity primiparous (P) and multiparous (M). Statistical differences in milking number are indicated as follows: * denotes P< 0.001

Focusing on the IgG concentration, M1 and M2 met the quality standard of 50 g IgG/L to ensure calf survival (Godden et al., 2019). After parturition more than a half of the IgG concentration was maintained between the first two milkings. Particularly, a total of 54 %, 36 % and 5 % of the IgG BC content remained in the M2, M3 and M10 milking, respectively. This decrease could be explained not only because a reduction in production but also because of the osmotic pressure in the mammary gland caused by molecules like lactose, which incorporates more water and reduces the concentration of the biomolecules accumulated in the mammary gland entirely before parturition (Baumrucker et al., 2010). Although Oyeniyi and Hunter (1978) observed IgG differences from the 4th parity with a greater IgG disappearance rate

in younger animals (first to third lactation vs fourth to seventh lactations), in the present study primiparous and multiparous cows evolved following a similar disappearance rate.

Regarding IGF-I concentrations, M2, M3 and M10 conserved 58 %, 25 % and 3 % of M1 IGF-I amounts, and our values were within the wide range of IGF-I described in Meyer et al. (2017)'s review. Blum and Hammon (2000) also found similar IGF-I concentration conservation from first to second (63 %) and from first to third milking (34 %), with very low concentrations in mature milk. There is limited literature reporting LFT concentration in TM. This study differed slightly from that of Blum and Hammon (2000), obtaining greater LFT concentration conservation from M1 to M2, 72 % vs 47 %, respectively, and from M1 to M3, 38 % vs 25 %, respectively. Most of literature concerning LFT concentration is focused on mature milk with stage of lactation and SCC being positively correlated, and milk yield negatively correlated with LFT concentration in milk (Cheng et al., 2008).

This study revealed that TM from M2 contains at least 54 % of the concentration of BC bioactive molecules. When the concentration of BC components in M2 vs M1 samples were compared, LTF was conserved the most with 72.3 %, followed by IGF-I with 58 % and finally, IgG with 54 %. Parity also affected the concentration of biomolecules on M2 samples. Overall, multiparous cows produced more than primiparous cows (26 %, 31 % and 34 % for IgG, IGF-I and LTF, respectively), being significant for IGF-I and LTF.

At the present time, the surplus of colostrum is being processed and transformed into different by-products for multiple purposes (Kelly G. S., 2003), such as IgG or LTF supplements for humans (BioNatIn BV, Son en Breugel, The Netherlands) or calves (Saskatoon Colostrum Company Ltd., Saskatoon, SK, Canada) to enhance immune and digestive health or to promote sports nutrition by improving performance and recovery. Even a TM replacer is currently available on the market (Transformula, Bonanza Calf Nutrition, Dundalk, Ireland). These commercial examples demonstrate the industry's potential to exploit these nutraceutical surplus by-products from dairy farms.

Table 3.2. Least square means of milk immunoglobulin G (IgG), IGF-I and lactoferrin (LTF) concentrations measured in the first, second, third and tenth milkings by parity.

Item	Primiparous				Multiparous				SEM[1]	*P*-value[2]		
	1	2	3	10	1	2	3	10		LN	M	LNxM
IgG, g/L	98	50	20	1	122	68	22	1	6	0.139	<0.001	0.263
IGF-I, µg/L	465^{b}	280^{c}	133^{d}	21^{e}	717^{a}	407^{b}	166^{d}	13^{e}	32	0.006	<0.001	<0.001
LTF, g/L	0.72	0.57	0.34	0.18	1.28	0.87	0.42	0.19	0.14	0.022	<0.001	0.264

[1] Standard error of the mean

[2]LN = effect of lactation number (Primiparous vs Multiparous); M= effect of milking number; LNxM = effect of the interaction between lactation and milking number. Means in a row with different superscripts differ (P<0.05) in the effect of the interaction between lactation and milking number (represented in lowercase letters).

3.4. Conclusion

Our study demonstrates that TM from the second milking contains more than a half of the concentration of bioactive molecules present in BC. This may indicate a potential future application of this by-product whether on animal and human health. However, further studies are required to investigate the effect of parity in order to ensure that the biological value of TM is maintained. Therefore, additional analysis is required to apply this knowledge of TM to farm management to avoid being treated as waste milk and obtain a greater profit return in calf's health.

Chapter 4

FEEDING COLOSTRUM AND TRANSITION MILK FACILITATES DIGESTIVE TRACT FUNCTIONALITY RECOVERY FROM FEED RESTRICTION AND FASTING OF DAIRY CALVES

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4.1. Introduction

The bioactive compounds contained in BC and TM modulate the GIT's microbial population, development, and immune system functionality (Blum, 2006). Until 24 h after birth the small intestine non-selectively absorbs macromolecules which let a direct access to the circulatory system (Staley et al., 1972; Stott et al., 1979). However, after the GIT closure, prolonged BC and TM feedings may promote more local benefits (Blum, 2006), enhancing small intestine cell development (Blättler et al., 2001; Van Soest et al., 2022). Also, intestinal microbiota changed with age and feeding program of preweaned calves mainly due to the variable fermentable subtract (carbohydrate and amino acid) available for bacterial communities (Song, 2018), which produce different microbial fermentation products, such as short chain fatty acids, that may interfere with host metabolism and gut health (Lührs et al., 2002; Tolhurst et al., 2012; Wang et al., 2012).

The effects of BC and TM feeding on immune and nutritional status have been studied after birth, however BC and TM supplementation later in critical stages during the rearing phase have not been evaluated. Considering the negative impact of FRF period in young calves (3 weeks of age) after transportation (Pisoni et al., 2022), the potential benefits of BC and TM and the need to reduce the systematic use of antibiotics, we hypothesized that BC and TM may help to overcome the negative effects of FRF on preweaned calf intestinal integrity, energy metabolism, and health status. Therefore, the objective of the present study was to evaluate the digestive tract recovery and metabolism of feeding either BC, TM, or MR after an episode of FRF on performance, physiological parameters, gastrointestinal permeability, gastrointestinal immune status, microbiota changes and general health indicators.

4.2. Materials and methods

The current study was conducted at IRTA Torre Marimon facilities (Caldes de Montbui, Spain) from October to December 2021 and was approved by the Animal Care Committee of the Government of Catalonia (authorization code 11477).

4.2.1. Animals, treatments and feeding

Thirty-five Holstein male calves were born at a commercial farm located in Lleida, Spain (Selergan S.A.) and fed 4 L of colostrum within the first 6 hours and 2 L within

the first 12h after birth via esophageal tube. Thereafter, calves were fed 3 L of whole milk twice daily until transportation for 2 h to IRTA facilities (Caldes de Montbui, Spain). Calves entered to the study in two different batches of 15 and 20 calves, respectively, with a delay of 2 weeks. At arrival, calves were weighed (22 ± 4.8 days old and 48 ± 6.0 kg of BW), distributed in individual pens (1.20 m x 1.97 m) and vaccinated against Bovine Respiratory Syncytial Virus (BRSV) and bovine Parainfluenza virus type 3 (PI3) (Bovilis ® Intranasal RSP™ Live; MSD Animal Health, UK).

To simulate calves staying at an assembly center and further transport, calves were fed restrictively 2.5 L of a rehydration solution (Coriosal, Dilus Laboratories, Santa Eugènia de Berga, Spain) at a concentration of 50 g/L twice daily with ad libitum access to water during 3 days, and a subsequent 19 h of fasting, which represents the maximum journey duration allowed by the Regulation (EC) nº 1/2005 (European Community, 2005) for unweaned calves. This simulation was based on the severe treatment reported by Pisoni et al. (2022). After that, calves were blocked by BW and age, and randomly assigned to one of the 5 feeding treatments (n=7; established as d 1 of study): calves were fed either pooled BC (**C4**) or TM (**TM4**) twice daily during 4 days and thereafter MR (22.6 % CP and 18.5 % fat, Trouw Nutrition España S.A., Madrid, Spain) for 6 days (short duration feeding treatments); or BC (**C10**) or TM (**TM10**) twice daily for 10 days (long duration feeding treatments); or MR twice daily for 10 days (**CTRL**). To feed the same BC and TM quality to all the calves and feedings, 265 L of BC and 373 L of TM were collected from several farms and stored at -20ºC. Then, they were thawed, pooled in a 200 L milk tank, and individually bottled to achieve 720 g of DM content per bottle (one serving) assuming an average of 25.5 % DM content in BC and 18.1 % DM content in TM as previously measured in BC and TM samples from our laboratory. Finally, bottles were frozen at -20ºC until their use. One hour before feeding, bottles were thawed in a water bath at 55ºC. Then, the contained liquid was transferred to feeding bottles and brought up to 3 L at 39ºC with warm water to assess the same volume as MR.

All treatments were initially prepared to be fed at the rate of 720 g/d DM content. Afterward, all calves were fed the same feeding program, restricted amounts of MR at 12.5 % DM: 3 L twice daily from d 11 to 15, 2.5 L twice daily from d 16 to 22, 2 L

twice daily from d 23 to 29, and finally 2L once daily from d 30 to 43 when calves were weaned. Concentrate feed (14.8 % CP, 13.6 % NDF; **Table 4.1**), water, and straw (3.9 % CP, 72.6 % NDF, 46.2 % ADF) were offered ad libitum until d 50 after fasting.

4.2.2. Measurements and sample collection

Feed, milk, and straw intake were daily recorded throughout the experiment. Before the morning feeding on d -3, 1, 2, 5, and 11 calves were weighed, and blood samples were collected from the jugular vein, using a non-additive serum and K2 EDTA tubes (BD Vacutainer®, Eysins, Switzerland). Serum tubes were centrifuged at 1,500 × g at 4°C for 15 min, and serum was obtained and stored at −20°C until further analysis of non-esterified fatty acids (**NEFA**), β-Hydroxybutyrate (**BHB**), and citrulline as an intestinal enterocyte mass marker. Whole blood was used for a subsequent hematological analysis. After that a permeability test was performed by giving orally to calves, 0.1 g of Cr-EDTA/kg of BW (Sigma-Aldrich.®, Merck KGaA, Darmstadt, Germany) dissolved in 100 mL of warm water (Amado et al., 2019). Two hours later a blood sample was collected using non-additive serum tubes and following the same procedure described above. All blood samples were analyzed on d -3, 1, 2, 5 and 11, except from BHB and NEFA where d -3 was not determined. Fecal samples were collected by rectal stimulation, on d -3, 2, 5 and 11 in 50 mL sterile containers and frozen at -20 ºC for further LTF, IgA, volatile fatty acids (**VFA**), microbiota (Firmicutes to Bacteroidetes (**F/B**) ratio and *Faecalis prausnitzii*) and dry matter analysis. Finally, on d 13, 2 h after the morning feeding, a blood sample was collected using sodium heparin tubes (BD Vacutainer®, Eysins, Switzerland) and stored at 4 ºC to perform an in vitro lipopolysaccharide (**LPS**) challenge. After d 11 of the study, calves were weekly weighed before the morning feeding until the end of the study.
Health scores were recorded daily from d -3 to d 14 as well as d 23 and 30. Calf health scoring criteria were rectal temperature, cough, nasal discharge, eye discharge, ear disposition and fecal consistency using the Calf Health Scoring Chart (University of Madison–Wisconsin School of Veterinary Medicine; https://www.vetmed.wisc.edu/fapm/svm-dairy-apps/calf-health-scorer-chs/). Clinical parameters were scored on a scale of 0 through 3, representing 0 for a healthy calf and 3 for a severely affected one. Rectal temperature was also analyzed as a continuous variable.

4.2.3. Chemical analyses

Concentrate feed, straw, MR, BC and TM samples were collected and analyzed for DM (24 h at 103 °C; Regulation (EC) nº 152/2009 (European Community, 2009)), ash (4 h at 550 °C; Regulation (EC) nº 152/2009), CP (Kjeldahl method; Regulation (EC) nº 152/2009) and fat (gravimetry with acid hydrolysis preparation; Regulation (EC) nº 152/2009). Furthermore, the concentrate feed sample was analyzed for ADF and NDF (method 973.18; AOAC International, 1996), and BC and TM samples for lactose content using HPLC—Refractive Index (method 984.22; AOAC International, 1995). Gross energy was determined using an adiabatic calorimeter IKA C 2000 basic (IKA, Staufen, Germany) using the method DIN 51900 for MR and an isoperibol calorimeter (Parr 6400, Moline, IL, USA) for TM and BC. Osmolarity was analyzed using a thermistor cryoscope (European Phamacopoeia, 2012).

Bovine colostrum, TM and MR samples were analyzed for IgG concentrations using a single radial immunodiffusion kit (Radial Immunodiffusion Test, Triple J Farms, Bellingham, WA, USA) according to the manufacturer's recommendations. Bovine colostrum and TM were centrifuged for 15 min at 2,000 x *g* at 4 ºC to remove the fat from the sample. Whey fraction was collected and diluted with PBS 1:10 (BC) and 1:5 (TM). Five µL of sample was added per well and incubated at room temperature for 24 h until plate reading.

Table 4.1. Nutrient and bioactive compounds composition of the feed, milk, colostrum, and milk replacer used in this study.

Item	Colostrum	Transition Milk	Milk Replacer	Concentrate	Straw
Gross Energy, kcal/kg DM	5,776	5,567	4,831	-	-
Nutrient, % DM					
DM	21.7	16.2	96.0	87.6	90.6
CP	58.5	56.2	22.6	14.8	4.3
NDF	-	-	-	13.6	80.1
ADF	-	-	-	5.2	51.0
EE[1]	23.5	24.7	18.5	5.2	-
Ash	4.6	6.8	7.5	4.1	6.3
Lactose[2]	12.9	20.9	51.4	-	-
Osmolarity, mOsm/kg	377	404	362	-	-
Biomolecules					
IgG, mg/dL	6864	3834	<280	-	-
IgA, mg/dL	657	397	2	-	-

[1]EE=Ether extract

[2]Lactose in milk replacer was determined by difference (100%-CP%-fat%-ash%) based on Quigley et al. (2006)

Serum NEFA concentrations were determined by the enzymatic colorimetric method ACS-ACOD-MEHA (NEFA-HR(2) Assay; Fujifilm Wako Chemicals GmbH, Neuss, Germany). Serum BHB concentrations were determined using a kinetic enzymatic method (D-3-Hydroxybutyrate (Ranbut), Randox Laboratories Ltd., Crumlin, UK). Serum NEFA and BHB values were measured using a Beckman Coulter AU400 platform (Beckman Coulter, Brea, CA, USA). Serum citrulline concentrations were measured using a spectrophotometric kit (L-Citrulline Kit, Immundiagnostik AG, Bensheim, Germany). Plates were read at 540 nm using Model 680 Microplate Reader from Bio-Rad (Hercules, CA, USA). Serum Cr-EDTA concentrations were determined by inductively coupled plasma-optical emission spectrometry, using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7900, Santa Clara, CA, USA). Blood samples were analyzed for hematology using Element HT5® Veterinary Hematology Analyzer (Heska, Loveland, CO, USA). Hematological variables consisted of hematocrit, hemoglobin, number of white blood cells (**WBC**) (including neutrophils, lymphocytes and monocytes), number of red blood cells (**RBC**) and platelet (**PLT**) counts.

The LPS challenge was performed within 6 h after sampling. Before the challenge, lyophilized LPS (*Escherichia coli* O111:B4, ref: L3012-5MG, Sigma Aldrich, Merck KGaA, Darmstadt, Germany) was diluted in PBS to obtain a 1mg/mL LPS solution. In a laminar airflow cabinet, two aliquots with 1 mL of whole blood per calf were stimulated either with 50 µL of PBS (nonchallenged control samples) or with 50 µL of 1 mg/mL LPS solution (challenged samples). Aliquots were incubated for 2 h at 39°C with a rotated shaking. Whole blood samples were centrifuged at 2,000 x *g* for 10 min at 4 ºC. Serum was collected and stored at −80°C until the determination of TNFα. Serum TNFα concentrations were analyzed using a Bovine TNFα ELISA kit (orb437514, Biorbyt LLC, San Francisco, USA). Samples were diluted 1:10 with the kit's dilution buffer to overcome sample matrix interference. Plates were read at 450 nm using Model 680 Microplate Reader from Bio-Rad (Hercules, CA, USA) and the increment of absorbance between the challenged and non-challenged samples for each calf was calculated.

All fecal samples were analyzed for DM (103ºC during 24 h) and results concerning IgA, LTF and VFA were expressed on DM basis. Fecal extracts for IgA analysis were prepared as Schaut et al. (2019) described with minor modifications. Briefly, 1 mg

fecal samples were washed with 10 mL PBS containing 1 % Triton-X. Samples were centrifuged at 3,200 × *g* for 30 min at 4ºC. The supernatant was transferred to 15-mL tubes centrifuged for a second time at 12,000 × *g* for 20 min at 4ºC to further clarify the samples. Supernatants were filter sterilized using 0.2 µm syringe filters and samples were stored at −20°C until analysis. Fecal extracts as well as BC, TM and MR samples were analyzed for IgA concentration using Bovine IgA ELISA kit from Bethyl Laboratories, Inc. (Cat. No. E11-121, Montgomery, TX, USA). Plates were read at 450 nm using EMS Reader MF V.2.9-0. from Labsystems (Vantaa, Finland).

Fecal LTF was extracted adapting Cooke et al. (2020)'s protocol and LTF concentrations were determined using Bovine Lactoferrin ELISA kit from Bethyl Laboratories, Inc. (Cat. No. E11-126, Montgomery, TX, USA). Plates were read at 450 nm using EMS Reader MF V.2.9-0. from Labsystems (Vantaa, Finland).

Semiquantitative analysis of VFA including acetic acid, propionic acid, isobutyric acid, acid butyric, isovaleric acid, and valeric acid in fecal samples was performed using Gas Chromatography with Flame Ionization Detection (GC-FID; HP 6890 Series II GC System, Agilent, Santa Clara, CA, USA) with an HP-5 capillary column (P/N:19091J-413, 30 m x 0.32 mm x 0.25 µm, Agilent, Santa Clara, CA, USA).

To quantify the F/B ratio and *Faecalis prausnitzii*, the DNA of each fecal sample was extracted using Stool DNA Isolation Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's instructions with minor modifications. The changes consisted of adding an extra centrifugation step (16,100 x *g* for 2 min) before binding the DNA to the column and discarding the pellet as well as eluting the DNA with 50 µl of elution buffer. Real-time PCR was performed using specific primers assembled from Yang et al. (2015) for Firmicutes and Bacteroidetes, Schwiertz et al., (2010) for *Faecalis prausnitzii* and Takai and Horikoshi (2000) for prokaryotic universal rDNA (referred in this article as universal) (**Table 4.2.**). For these quantifications, a total reaction volume of 20 µL was used, containing 10 ng of DNA, 10 µL of SYBR Green (TaKaRa Bio Inc, Kusatsu, Japan), and the optimized primer concentration for each target (**Table 4.2.**). The PCR reactions consisted of an initial denaturing cycle of 10 min at 95°C, followed by 40 cycles of 10 s at 95°C (denaturation), 30s at 60°C for Firmicutes, Bacteroidetes and universal, and 30s at 53°C for *Faecalis prausnitzii* (annealing; temperature defined at **Table 4.2.**) and a final extension of 10 min at 72°C. Bacteria quantification was evaluated using the

threshold cycle (**Ct**) values, defined as the number of PCR cycles over which product amplification occurs. Therefore, the greater the Ct value, the lower the concentrations of fecal bacteria.

To obtain the F/B ratio, the average Ct value obtained from each primer pair was transformed into a percentage using the following formula (Yang et al., 2015):

$$X = \frac{(Eff.Spec)^{CtSpec}}{(Eff.Univ)^{CtUniv}} \times 100$$

where Eff.Univ is the calculated efficiency of the universal primers (2 = 100 % and 1 = 0 %) and Eff.Spec refers to the efficiency of the taxon-specific primers. CtUniv and CtSpec are the Ct values registered by the thermocycler. All fecal samples were analyzed with the qPCR assay, and the Ct values were used to calculate the proportion of bacterial taxa in the feces. Then, the Ratio between the Firmicutes and Bacteroidetes proportion was calculated.

Table 4.2. Primers, annealing temperature (Tm), and optimized DNA primer concentrations of 16S ribosomal RNA for Firmicutes, Bacteroidetes, universal and *Faecalis prausnitzii.*

Target group	Primer	Sequence (5'→ 3')	Tm, ºC	Concentration, µM
Bacteroidetes	Bac960F	GTTTAATTCGATGATACGCGAG	60	0.50
	Bac1100R	TTAASCCGACACCTCACGG	60	0.50
Firmicutes	Firm934F	GGAGYATGTGGTTTAATTCGAAGCA	60	0.50
	Firm1060R	AGCTGACGACAACCATGCAC	60	0.50
Universal	926F	AAACTCAAAKGAATTGACGG	60	0.50
	1062R	CTCACRRCACGAGCTGAC	60	0.50
Faecalis	PrausF480	CAGCAGCCGCGGTAAA	53	0.50
prausnitzii	PrausR631	CTACCTCTGCACTACTCAAGAAA	53	0.50

4.2.4. Statistical analyses

Performance, Blood and Fecal parameters

The study design was a randomized complete balanced design with a covariance adjustment. A power analysis was conducted to determine the experimental units (calf) needed. The type I error rate (α) was 0.05, and the power (1 − β) was set at 80

%. The concentration of citrulline was considered our primary outcome based on the differences in citrulline between the severe and control treatment in Pisoni et al. (2022). Data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) with repeated measures. The statistical model for performance, blood and fecal parameters and rectal temperature accounted for the random effect of calf as well as the fixed effects of treatment, time and their interaction. Initial BW and batch were included as covariate and batch, respectively, in all the parameters as well as day -3 values in the performance ones. Covariates were removed when they were not significative ($P < 0.05$). Performance parameters and rectal temperature were summarized in 3 periods consisting of from d -3 to -1 (pretreatment period), from d 1 to d 10 (treatment period) and from d 11 to the end of the study (posttreatment period). The first-order autoregressive covariance matrix, the spatial power and the unstructured covariance matrix were tested according to the time points, and the Kenward–Roger degrees of freedom were used based on the lower Bayesian information criterion value. Data were tested for normality using the Shapiro-Wilk test. WBC, PTL, Neutrocytes, BHB, NEFA, Cr-EDTA, IgA and LTF data were transformed to logarithm to achieve normal distribution. One observation was removed for hemogram analysis because it was an outlier (Mohri et al., 2007) and we suspected of a detection error. Initial BW and age were analyzed using the MIXED procedure of SAS with treatment as a fixed effect and batch was included as a block. Differences were declared significant at $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.10$ for all models. Reported means and SEM in the figures were obtained from the output of SAS as well as the *P*-values except for the transformed data where *P*-values came from the normalized data.

Calf Health Scores

Individual health criteria scores were categorized binarily considering 0=healthy calf (score 0) and 1= unhealthy calf (score 1 to 3) for the statistical analysis. Calf general health score (the overall sum of health criteria score for each calf and day) were categorized binarily as follows: if the resulting sum was <5 it was codified as 0, and if it was ≥ 5 it was considered as 1. Data were organized in 3 periods to fit the model: from d -3 to -1 (pretreatment period), from d 1 to 10 (treatment period) and from d 11 to the end (posttreatment period). Either the binary general or the individual health criteria scores were analyzed using the PROC GLIMMIX procedure

of SAS using a cumulative logit link function and assuming a binary distribution. The statistical model accounted for the fixed effects of treatment, period, and their interaction as well as calf as a random effect. Batch was included as a block.

4.3. Results

4.3.1. Intake and performance parameters

Initial age and BW values on the pretreatment period (d-3 to -1) before applying the FRF were similar among the different treatment groups (**Table 4.3.**). During the pretreatment period, animals lost an average of 2.5 kg BW because of the FRF. After, during the treatment and posttreatment periods, calves increased their BW and maintained their ADG similarly among the different feeding treatments. During the application of the feeding treatments (from d1 to 10), no differences in concentrate feed intake were observed among treatments, but all calves increased concentrate feed intake on d1 followed by a drastic decline the following days to overcome the FRF. Milk DM intake in TM4, C4, and TM10 calves was lower ($P < 0.001$) than in CTRL calves, and the greater amount of milk refusals was observed in C10 calves compared with the other treatments. All liquid feeds, BC, TM, and MR had similar osmolarity (**Table 4.1**). Despite the differences in DM intake, protein and energy intakes were greater ($P < 0.001$, **Table 4.3**) in BC and TM treatments than in CTRL calves, being C10 and TM10 diets richer in calories and protein content than the others. Finally, DM intake increased during the posttreatment period (from d11 to the end) without differences among treatments (**Table 4.3**). No differences were observed in feed efficiency (**FE**) in the pretreatment and treatment periods, but FE differed among treatments during the posttreatment period. Long duration treatments (C10 and TM10) had greater ($P < 0.05$) FE than CTRL, TM4 and C4 the week after the end of the feeding treatments (d 11 to 16), and they were similar afterward.

Table 4.3. Least Square means of intake and performance parameters by period of preweaned calves.

	Treatment[1]						***P*-value[2]**		
Item	**CTRL**	**TM4**	**TM10**	**C4**	**C10**	SEM	*T*	*time*	*T x time*
Pretreatment period (d -3 to 1)									
Initial age, d (d-3)	21.2	22.4	22.2	22.1	21.9	0.81	0.87	-	-
Initial BW, kg (d-3)	48.0	47.3	47.8	48.2	48.1	2.22	1.00	-	-
Rehydrating solution, g of DM/d	250	246	246	248	247	2.9	0.87	0.16	0.62
ADG, kg/d	-0.82	-0.85	-0.73	-0.89	-0.87	0.124	0.90	-	-
Treatment period (d 1 to 10)									
Pre-treatment BW, kg (d 1)	45.5	44.8	45.6	45.5	45.5	2.15	1.00	-	-
Milk/Colostrum[1], g of DM/d	720^{a}	696^{b}	691^{b}	693^{b}	647^{c}	5.9	<0.0001	<0.0001	<0.0001
Concentrate, g of DM/d	225	194	232	212	181	23.4	0.51	<0.0001	0.73
Total DMI, g of DM/d	946^{a}	891^{ab}	923^{a}	906^{a}	828^{b}	25.7	0.02	<0.0001	0.82
Milk/Colostrum[3] GE Intake, kcal/d	3478^{d}	3560^{cd}	3847^{a}	3595^{c}	3735^{b}	32.2	<0.0001	<0.0001	<0.0001
Milk/Colostrum[3] CP Intake, g/d	163^{d}	247^{c}	388^{a}	250^{c}	378^{b}	2.9	<0.0001	<0.0001	<0.0001
Milk/Colostrum[3] lactose Intake, g/d	370^{a}	276^{b}	145^{d}	255^{c}	83^{e}	1.7	<0.0001	<0.0001	<0.0001
ADG, kg/d	0.73	0.68	0.66	0.72	0.47	0.067	0.07	0.84	0.50
FE[2], kg/kg DM	0.77	0.76	0.74	0.79	0.57	0.068	0.16	0.17	0.51

Table 4.3 (continued).

Item	Treatment[1]					SEM	*P*-value[2]		
	CTRL	**TM4**	**TM10**	**C4**	**C10**		*T*	*time*	*T x time*
Posttreatment period (d 11 to 50)									
Post-treatment BW, kg (d11)	52.7	51.7	52.1	52.8	50.2	2.32	0.93	-	-
Final BW, kg (d51)	90.4	88.7	91.1	86.1	88.9	4.44	0.94	-	-
Milk replacer, g of DM/d	440	438	440	440	440	1.0	0.46	<0.0001	0.62
Concentrate, g of DM/d	1472	1417	1510	1243	1431	75.0	0.11	<0.0001	0.89
Straw, g of DM/d	65	72	73	61	72	9.2	0.86	<0.0001	0.33
Total DMI, g of DM/d	1903	1851	1947	1669	1867	80.5	0.14	<0.0001	0.88
ADG, kg/d	0.94	0.93	0.98	0.83	0.97	0.045	0.13	<0.0001	0.53
FE[4] kg/kg DM	0.52	0.53	0.55	0.52	0.56	0.019	0.43	<0.0001	0.048

[1] CTRL= Milk replacer (MR) twice daily for 10 days; TM4 = Transition milk (TM) twice daily during 4 days and thereafter MR for 6 days; TM10 = TM twice daily for 10 days; C4 = Bovine colostrum (BC) twice daily during 4 days and thereafter MR for 6 days; C10 = BC twice daily for 10 days

[2]T = effect of feeding treatment; time= day of study; T × Time = effect of the time by treatment interaction

[3]According to the established treatment: CTRL, TM4, TM10, C4, C10

[4]FE= Feed efficiency

[a–e] Within a row, least squares means without a common superscript differ (P < 0.05) among treatments.

4.3.2. Blood parameters

Energy Balance

Serum NEFA concentrations were lower ($P < 0.05$, **Table 4.4**) in CTRL compared with TM4, TM10 and C10 calves from d 1 to 11. Although no differences were observed in all treatments, on day 1, after the FRF, serum concentration of BHB was greater ($P < 0.001$, **Figure 4.1.**) than that on day 2. On d 5, serum concentration of BHB was lower ($P < 0.001$) in CTRL calves compared with the other treatments, and on d 11, serum BHB concentration in the long treatments (C10 and TM10) remained greater than that in the shorter ones (C4 and TM4) and CTRL.

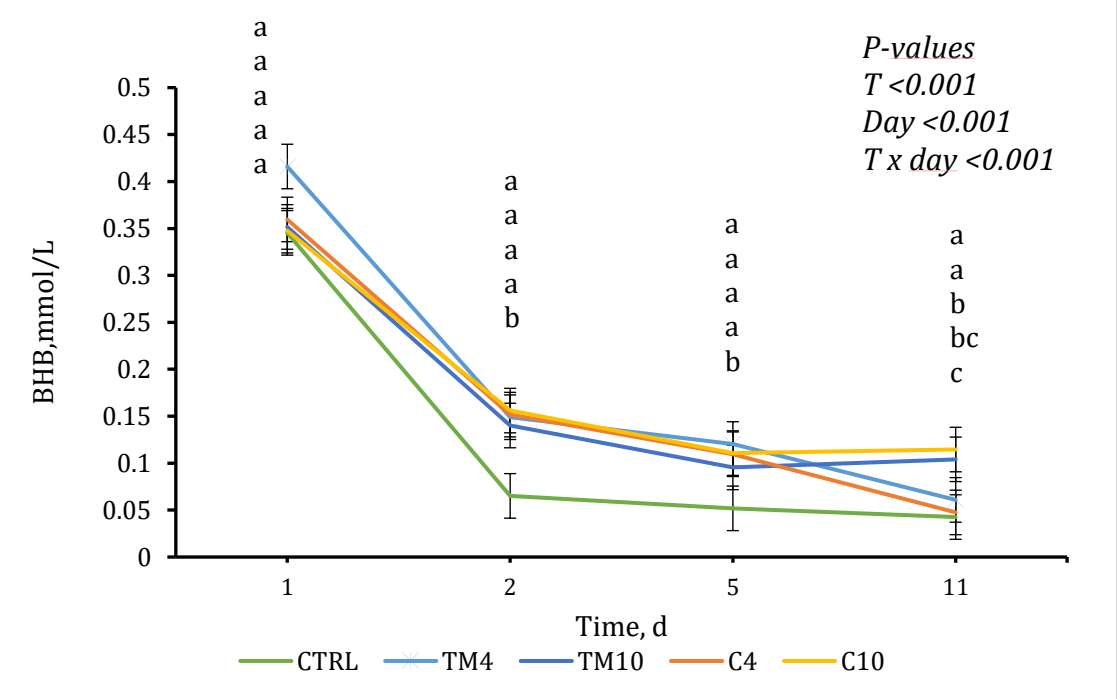

Figure 4.1. Serum BHB evolution by experimental day after a feed restriction and fasting period in preweaned calves fed either milk replacer (MR) twice daily for 10 days (CTRL), transition milk (TM) twice daily for 4 days and thereafter MR for 6 days (TM4), TM twice daily for 10 days (TM10), bovine colostrum (BC) twice daily during 4 days and thereafter MR for 6 days (C4) or BC twice daily for 10 days (C10) from day 1-10 of study.

Biomarkers of intestinal barrier functionality

Although no differences among treatments were detected on serum Cr-EDTA concentrations, serum Cr-EDTA concentrations increased (P < 0.001, **Table 4.4**) during the FRF in all treatments, having the greatest serum Cr-EDTA concentrations peak on d 1 followed by a gradual decrease until d 11. Serum citrulline concentrations on d -3 and d 1 were similar in all treatments. Once treatments were applied (d 2), serum citrulline concentration rose (P < 0.01; **Figure 4.2**) in all treatments except for the CTRL. At the end of the short treatment application (d 5), C4 and C10 calves had greater serum citrulline concentration than the CTRL treatment. Finally, when the application of the long treatments finished (d 11), serum citrulline concentrations tended (P = 0.06) to be greater in C10 and TM10 calves than in the CTRL, TM4 and C4 ones; and the short treatments (TM4 and C4) had similar serum citrulline concentrations than CTRL.

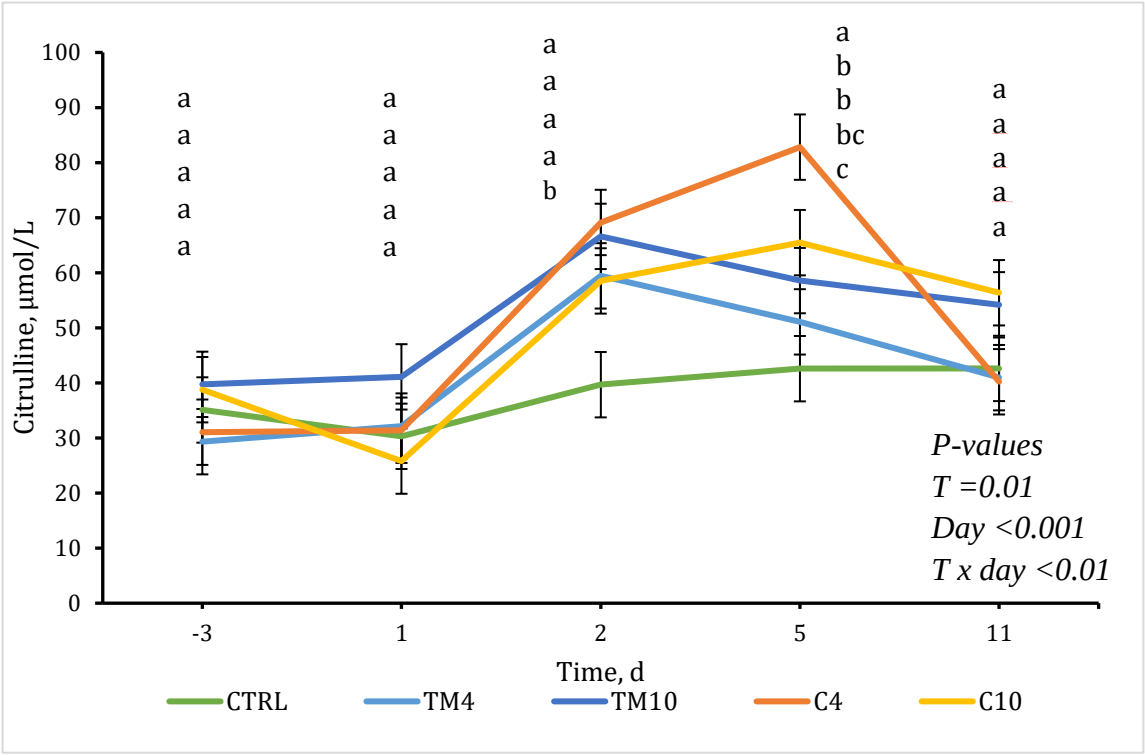

Figure 4.2. Serum citrulline evolution by experimental day before and after a feed restriction and fasting period (from d -3 to -1) in preweaned calves fed either milk replacer (MR) twice daily for 10 days (CTRL), transition milk (TM) twice daily during 4 days and thereafter MR for 6 days (TM4), TM twice daily for 10 days (TM10), bovine colostrum (BC) twice daily during 4 days and thereafter MR for 6 days (C4) or BC twice daily for 10 days (C10) from day 1-10 of study.

Biomarkers of immune status

None of the hematological parameters showed differences among treatments. Hemogram values changed ($P < 0.05$, **Table 4.4)** during the sampling days except for neutrophils that maintained their count. During the FRF period, all hematological parameters, except WBC and neutrophils, increased from d -3 to 1 (data not shown). Then, from d 2 to 11 (treatment period) all hematological parameters gradually decreased. In addition, the production of TNFα did not differ ($P = 0.46$, **Table 4.4)** among blood cells of the different treatments after an LPS challenge.

4.3.3. Fecal parameters

Gastrointestinal biomarkers of immune status

No differences among treatments were detected in fecal LTF concentrations, but they decreased ($P < 0.05$; **Table 4.5**) from day 5 to day 11 in all treatments. However, fecal IgA differed among feeding treatment and time (**Figure 4.3**). Before the application of the feeding treatments (d -3), all calves had similar fecal IgA concentrations. On d 5, all calves increased fecal IgA concentrations except for CTRL calves. Finally, at the end of the feeding treatments application (d 11), TM10 and C10 (long duration treatments) had greater ($P < 0.05$; **Figure 4.3)** fecal IgA concentrations than CTRL and TM4 treatments, being C10 feeding treatment above all. In addition, analyses of IgA indicated that BC was 40 % richer in IgA content than TM, whereas MR contained less than 0.6 % of IgA determined in BC and TM.

Gut microbiota and microbiota metabolites

To study the gastrointestinal microbiome and its interaction with the feeding treatment, fecal VFA, the F/B ratio and the quantification of *Faecalis prausnitzii* were determined. There were no differences in total fecal VFA concentrations among treatments. However, the molar percentage of fecal propionate was lower ($P < 0.05$, **Table 4.5**) in C10 than in CTRL, TM4 and TM10 calves, and like C4 calves. The proportion of fecal butyrate was greater ($P < 0.05$, **Table 4.5**) in C4 and C10 calves than in CTRL and TM4 ones, as well as it decreased ($P < 0.01$) from day 5 to 11 in all treatments. The proportion of fecal acetate tended to be greater ($P = 0.07$, **Table 4.5)** in TM4 and CTRL compared with TM10 calves, but it increased ($P < 0.05$) from day 5 to 11 in all treatments.

The F/B ratio, the *Faecalis prausnitzii*, and the bacterial taxons did not differ among treatments, but *Faecalis prausnitzii* specific Ct values increased from d 5 to d11 ($P < 0.01$, **Table 4.5**). The overall mean relative abundance of Firmicutes and Bacteroidetes were 34.15 % and 65.5 %, respectively.

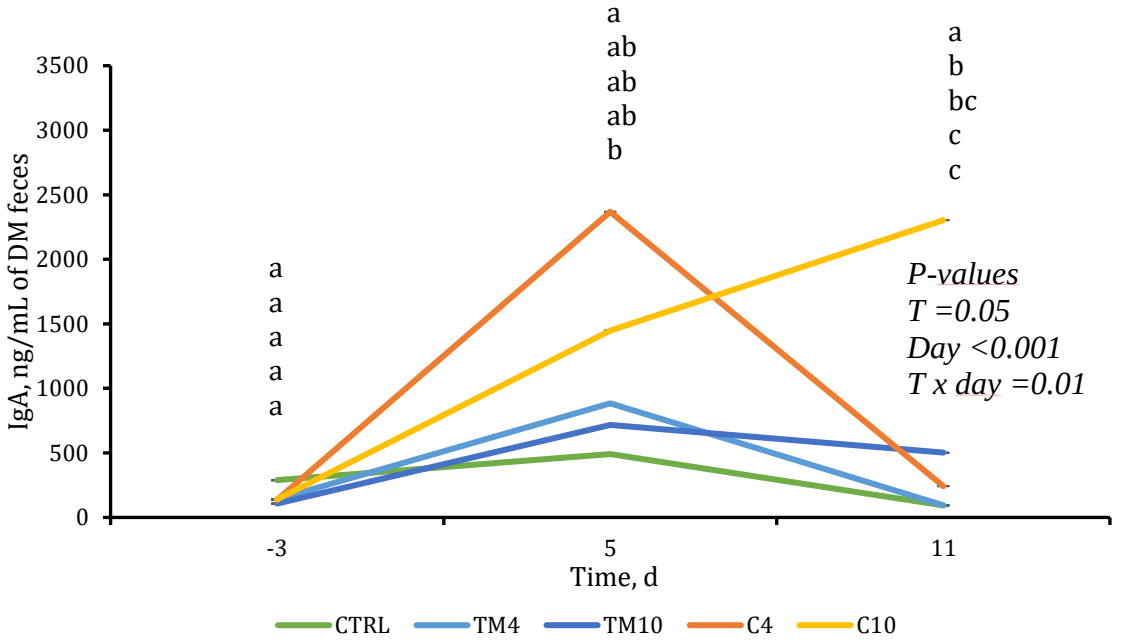

Figure 4.3. Fecal IgA evolution by experimental day before and after a feed restriction and fasting period (from d -3 to -1) in preweaned calves fed either milk replacer (MR) twice daily for 10 days (CTRL), transition milk (TM) twice daily during 4 days and thereafter MR for 6 days (TM4), TM twice daily for 10 days (TM10), bovine colostrum (BC) twice daily during 4 days and thereafter MR for 6 days (C4) or BC twice daily for 10 days (C10) from day 1-10 of study. Data was back transformed from fecal IgA logarithm least square means while error bars represented fecal IgA logarithm standard errors.

4.3.4. Calf health scores

The general proportion of animals considered ill using the binary scale (being 0=healthy calf and 1= unhealthy calf) did not differ among treatments. The binary general score indicated that during the treatment period (d 1 to 10) calves were less healthy ($P < 0.001$, **Figure 4.4** and **Table 4.5**) than during the pretreatment (d-3 to d 1) and posttreatment period (d 11 to the end). Moreover, no differences in individual health criteria scores were found among treatments (data not shown) except for the fecal scores, which varied throughout the experiment. During the

pretreatment period, the fecal proportion of non-normal feces was similar among treatments, but once the feeding treatments were applied (treatment period, from d 1 to 10) fecal scores increased in all the treatments except from TM4. The proportion of non-normal fecal scores of C10 fed calves was greater ($P < 0.05$; **Table 4.5**) than the ones fed with TM (TM4 and TM10). During period 3 (from d 11 to 50), C10 calves had a greater fecal proportion of non-normal feces than TM4 ones, while C4, TM10 and CTRL did not differ between C10 and TM4. No differences in mean rectal temperature were found among treatments.

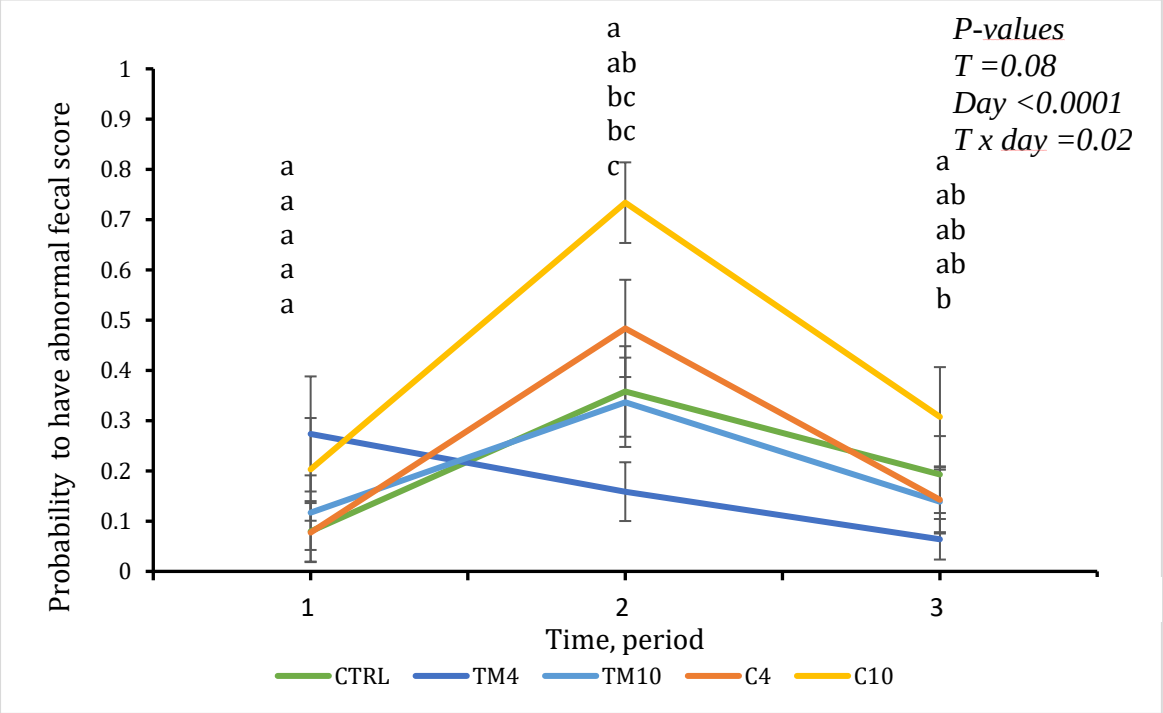

Figure 4.4. Probability to have abnormal feces (1) versus normal ones (0) by period of time (pretreatment period (with feed restriction and fasting) = from d -3 to -1, treatment period = from d 1 to 10 and posttreatment period = from d 11 to the end of the study) in preweaned calves fed either milk replacer (MR) twice daily for 10 days (CTRL), transition milk (TM) twice daily during 4 days and thereafter MR for 6 days (TM4), TM twice daily for 10 days (TM10), bovine colostrum (BC) twice daily during 4 days and thereafter MR for 6 days (C4) or BC twice daily for 10 days (C10) from day 1-10 of study.

Table 4.4. Least square means of energy balance, intestinal permeability, hemogram and immune parameters in blood on days -3 to 10 relative to feed restriction and fasting (FRF) of preweaned calves.

	Treatment[1]						***P*-value[2]**		
Item	CTRL	TM4	TM10	C4	C10	SEM	*T*	*time*	*T x time*
Energy balance parameters									
BHB[3,7,8], mM/L	0.12^{b}	0.18^{a}	0.17^{a}	0.17^{a}	0.18^{a}	0.012	<0.0001	<0.0001	<0.0001
NEFA[4,7,8], mM/L	0.31^{c}	0.37^{a}	0.33^{ab}	0.31^{bc}	0.31^{ab}	0.023	0.02	<0.0001	0.41
Intestinal barrier functionality parameters									
Citrulline, µmol/L	37.7^{c}	42.2^{bc}	51.7^{a}	50.6^{ab}	48.7^{ab}	3.26	0.01	<0.0001	<0.01
Cr-EDTA[7], mg/L	1.00	0.98	1.21	0.99	1.08	0.116	0.81	<0.0001	0.74
Hemogram parameters									
Hematocrit, %	33.37	30.65	29.38	33.76	31.38	2.032	0.51	<0.0001	0.71
Hemoglobin, g/dL	10.82	10.05	9.46	10.97	10.23	0.728	0.59	<0.0001	0.54
RBC[5], x10^{12}/L	9.32	8.85	8.71	9.27	9.12	0.475	0.87	<0.0001	0.47
WBC[6,7], x10^{9}/L	9.58	10.88	10.36	9.77	10.13	0.878	0.83	<0.0001	0.54
Neutrophiles[7], x10^{9}/L	4.49	5.14	4.78	4.80	5.08	0.602	0.87	0.008	0.79
Lymphocytes, x10^{9}/L	4.78	5.40	5.26	4.66	4.75	0.415	0.63	<0.0001	0.27
Monocytes, x10^{9}/L	0.25	0.28	0.26	0.26	0.23	0.023	0.71	<0.0001	0.04

Table 4.4 (continued)**.**

Item	Treatment[1]					SEM	*P*-value[2]		
	CTRL	TM4	TM10	C4	C10		*T*	*time*	*T x time*
Hemogram parameters									
Platelets[7], x10^7/L	8.82	8.68	10.23	9.52	9.07	0.911	0.88	<0.0001	0.78
Immune parameters									
TNFα, increase of absorbance	0.011	0.020	0.020	0.006	0.009	0.0067	0.46	-	-

[1] CTRL= Milk replacer (MR) twice daily for 10 days; TM4 = Transition milk (TM) twice daily during 4 days and thereafter MR for 6 days; TM10 = TM twice daily for 10 days; C4 = Bovine colostrum (BC) twice daily during 4 days and thereafter MR for 6 days; C10 = BC twice daily for 10 days

[2]T = effect of feeding treatment; time= day of study; T × time = effect of the time by treatment interaction

[3] BHB= β-Hydroxybutyrate

[4]NEFA= non-esterified fatty acids

[5]RBC= Red blood cell

[6]WBC=White blood cell

[7]*P*-values were obtained from the logarithm transformed data

[8]Least square means were obtained from d 1 to d 10 relative to FRF

[a–c] Within a row, least squares means without a common superscript differ (P < 0.05) among treatments.

Table 4.5. Least square means of fecal immune and microbiota biomarkers of preweaned calves on d 5 and 11, and health scores from d -3 to d 14, d 23, and d 30 of study.

	Treatment[1]						***P*-value[2]**		
Item	CTRL	TM4	TM10	C4	C10	SEM	*T*	*time*	*T x time*
Immune biomarkers									
IgA[5], µg/dL of DM feces	79.0^{b}	101.7^{b}	98.5^{ab}	164.5^{ab}	219.9^{a}	48.63	0.05	<0.001	0.01
Lactoferrin[5], ng/mg of DM feces	226.5	207.4	227.8	213.9	263.3	26.75	0.51	0.02	0.22
Microbiota biomarkers									
Total VFA, µmol/g of DM feces	426	377	467	350	412	49.5	0.50	0.31	0.33
Molar % of VFAs									
Acetate	56.7	57.2	49.0	53.4	52.7	2.15	0.07	<0.01	0.88
Propionate	25.1^{a}	24.1^{a}	24.3^{a}	22.9^{ab}	20.1^{b}	1.11	0.03	0.19	0.27
Butyrate	11.5^{bc}	11.3^{bc}	13.1^{ab}	13.5^{a}	13.8^{a}	0.69	<0.05	<0.01	0.77
F/B Ratio, Ct	0.56	0.59	0.62	0.47	0.57	0.060	0.49	0.59	0.47
Faecalis prausnitzii[5], Ct	24.7	24.8	25.3	25.6	26.5	1.20	0.83	<0.01	0.96
Bacterial taxa relative abundance, %									
Firmicutes	36.8	35.7	34.6	30.1	34.7	2.11	0.24	0.41	0.48
Bacteroidetes	70.8	62.7	61.7	65.2	65.2	3.37	0.38	0.60	0.20

Table 4.5 (continued).

Item	Treatment[1]					SEM	*P*-value[2]		
	CTRL	TM4	TM10	C4	C10		*T*	*time*	*T x time*
Health scores									
General score[3]	0.13	0.17	0.07	0.13	0.19	0.058	0.58	<0.001	0.78
Fecal score[4]	0.19[b]	0.15[b]	0.19[b]	0.20[ab]	0.42[a]	0.065	0.08	<0.001	0.02
Rectal temperature, ºC	38.7	38.5	38.5	38.6	38.5	0.08	0.33	<0.001	0.99

[1] CTRL= Milk replacer (MR) twice daily for 10 days; TM4 = Transition milk (TM) twice daily for 4 days and thereafter MR for 6 days; TM10 = TM twice daily for 10 days; C4 = Bovine colostrum (BC) twice daily during 4 days and thereafter MR for 6 days; C10 = BC twice daily for 10 days.
[2]T = effect of feeding treatment; time= day of study for all the parameters except for the health score where time units were period; T × time = effect of the time by treatment interaction
[3] General health score (the overall sum of health criteria for each calf and day) was categorized binarily: if the resulting sum was <5 it was codified as 0, and if it was ≥5 it was considered as 1. Finally, the proportion contemplates 0=healthy calf (score 0) and 1= unhealthy calf.
[4] Fecal score was binary transformed considering 0=normal feces (score 0) and 1= diarrheic feces (score 1 to 3).
[5]*P*-values were obtained from the logarithm transformed data.
[a–c] Within a row, least squares means without a common superscript differ (P < 0.05) among treatments

4.4. Discussion

In the present study different performance traits, blood parameters, fecal biomarkers, and health scores were used to evaluate recovery of feeding calves either BC, TM, or MR after an episode of FRF. Until now, several authors described how FRF associated with transport negatively affected calf energy balance (Knowles et al., 1999), immunity (Marcato et al., 2020), health status (Renaud et al., 2018), and their intestinal barrier integrity (Zhang et al., 2013).

One of the main findings in this study was that FRF and feeding treatments affected serum BHB and NEFA concentrations. During the prolonged FRF period, calves underwent to a negative energy balance status and NEFAs were released from adipose tissue to the blood due to the insufficient intake of glucose-precursors. Consequently, calves were unable to metabolize the increasing NEFA concentration to triacylglycerol through the glucose-depending Krebs's cycle, and the cycle precursor acetyl-CoA was accumulated (Adewuyi et al., 2005). Then, acetyl-CoA was completely oxidated into ketonic bodies such as BHB. In our study, serum BHB and NEFA concentrations increased twice after the FRF period (day 1) compared to expected values for this age reported in Knowles et al., (2000), which was also described in Pisoni et al. (2022) for the severe FRF treatment. In fact, during this period our calves lost an average of 2.5 kg of BW, which may indicate the need of calves to mobilize body fat reserves into NEFA (**Table 4.3**). After that, all calves recovered their age-expected normal serum NEFA concentrations 24 hours after the FRF period, suggesting a similar decrease of the need of fat mobilization in all treatments. However, BC and TM fed calves had still greater serum BHB concentrations than CTRL calves on d 2 and d 5, indicating a possible effect of the energy source of feeds on serum BHB levels recovery. Calves in CTRL groups received MR, which was richer in lactose and poorer in fat content in contrast to BC and TM (**Table 4.1**), and it is consistent in the literature on the ketogenic effect of rich-fat diets (Helge, 2002; Vidali et al., 2015). This hypothesis was reinforced by the values of BHB serum concentration on d11, because when short treatments (C4 and TM4) fed calves changed their diet to MR, serum BHB concentration decreased.

In the literature, Welboren et al. (2021) showed that calves fed at the same DM level with a high-fat MR (39.9 % lactose and 24.6 % crude fat) had greater ADG than those

fed a high-lactose MR (46.1 % lactose and 18 % crude fat) due to a greater energy intake in high-fat fed calves. However, calves fed ad libitum a high-lactose/low-fat MR (44 % lactose and 17 % fat) had greater ADG than those fed a high-fat MR (37% lactose and 23 % fat) during the preweaning period because they were able to regulate their intake according to the caloric density of the diet (Echeverry-Munera et al., 2021). In our study, differences in milk DM intake were due to the lower DM content of TM and BC obtained from the pooled samples (**Table 4.1**) compared with the assumed DM content when bottled the individual feedings (25.5 % DM content in BC and 18.1 % DM content in TM). In addition, BC and TM fed calves still consumed more energy and protein than the MR fed ones, without differences in performance among treatments, this may be explained partially, due to the short duration of the feeding treatments, and/or the low number of animals per treatment used. The FRF period also affected concentrate feed intake. On d 1 after a 3 days of feed restriction when calves had access to a concentrate feed, they increased their concentrate feed intake. However, the day after, calves decreased concentrate feed intake. In a similar study, Pisoni et al. (2022) also observed this concentrate consumption pattern after a FRF period. This may be due to a transient subclinical ruminal acidosis for the sudden concentrate feed consumption (Pederzolli et al., 2018) and or to gut transient functionality loss (Pisoni et al., 2022).

In our study, hemogram parameters, health scores and cytokine TNFα were used to assess the general health status of animals. Hematology mean values obtained were within the corresponding age range (Mohri et al., 2007), when references were found, or the adult reference range for cattle (Radostits et al., 1994) when no references values were found for these young age, except for a mild neutrophilia and thrombocytosis found in all treatments. During FRF calf hemogram values suggested worse calf health, because of the increase in hematocrit, lymphocytes and platelets, probably indicating a mild dehydration (Roland et al., 2014), or/and reactive thrombocytosis to stress (Roland et al., 2014). Besides, the increase in lymphocytes could be also related to an innate immune response to vaccination applied at calf arrival to our facilities (Ellis, 2017). Furthermore, the proportion of animals with a bad general health score increased from the FRF period to the treatment period, whereas it decreased afterward. The general health status and hemogram recovery within the first 10 days of study may be related to the lack of differences in the LPS

challenge among treatments. Apart from a decrease in fecal consistency of BC fed calves during the treatment period (which will be discussed later), neither respiratory nor rectal temperature was affected by the feeding treatments, which agrees with Berge et al. (2009) and Chamorro et al. (2017) health studies related with feeding MR supplemented with CR to dairy calves after an initial colostrum feeding.

Focusing on calf GIT functionality, we used different biomarkers of intestinal barrier functionality (Cr-EDTA and citrulline), immune status (LTF and IgA) and microbiota (VFA, F/B ratio, and *Faecalis prausnitzii*) together with fecal scores to assess the effects of our feeding treatments. Foley and Otterby (1978) reported a laxative effect of colostrum when it was undiluted compared with diluted BC with water or whole milk. They concluded that when total solids were equalized to whole milk levels, an increase in the incidence of scours should not be expected. In addition, some authors described improvements in gut health whether they prolonged BC or TM administration after the first day of life (Berge et al., 2005; Kargar et al., 2021; Van Soest et al., 2022). In fact, when MR was supplemented with CR, newborn calves reduced the incidence of diarrhea as well as the need for antibiotic therapy (Berge et al., 2009; Chamorro et al., 2017). Kargar et al. (2021) described how feeding TM for 3 weeks period improved the newborn growth performance and reduced the occurrence of diarrhea. Also, feeding TM for 4 days after one colostrum feeding stimulated intestinal development as well as improved health scores compared with MR fed calves (Van Soest et al. 2022). Contrary to the previous authors' reports and despite diluting the BC and TM with warm water, getting similar osmolarity values in BC, TM, and MR, we observed a decrease in fecal consistency in C10 treatment between d 1 and 10. Although our investigation was not designed to study the incidence of diarrhea, the poor fecal scores in C10 treatment cannot be attributed to differences in the feed osmolarity. To assess the intestinal barrier functionality, it was tested with Cr-EDTA as a marker of gut permeability and citrulline, a non-protein amino acid, as an enterocyte mass biomarker (Amado et al., 2019; Gultekin et al., 2019). Elevated Cr-EDTA serum concentrations indicate an increase in paracellular permeability, which makes blood more accessible to large molecules (Zhang et al., 2013). In our study, the elevated serum Cr-EDTA concentrations during the FRF period could be explained as a dysfunctionality of the gut barrier by the feed

restriction and stress for the long hours of fasting in all treatments, possibly producing an increase in gastrointestinal permeability. Moreover, a high citrulline concentration would be indicative of greater enterocyte mass and a healthier intestinal barrier (Gultekin et al., 2019). Concretely in mammals, citrulline is a product of the glutamine conversion in enterocytes and it is metabolized in the kidney to provide Arg to the urea cycle (Curis et al., 2005). In our investigation, all calves maintained the same citrulline levels at arrival and 24 hours after the FRF period. However, CTRL calves sustained their lower citrulline levels throughout the study, while calves fed either BC or TM increased their serum citrulline, suggesting a benefit of BC and TM on intestinal cells. Based on literature (Krácmar et al., 2007, Terré et al., 2021), BC is richer in Glu than conventional MR, which it may explain the greater serum citrulline concentration observed in BC or TM fed calves. Biomarkers used to assess immune status were IgA, that plays an important role in the defense against the entry of enterotoxins and pathogenic organisms, and LTF as a marker of neutrophil activity in the GIT(Celi et al., 2019). IgA is not only secreted by the intestinal mucosa, it could be also provided with the diet. Although human colostral IgA has antitrypsin resistance, it is sensible to pepsin in acid pH (2.5), and the digestion of IgA depends on the development of the proteolytic activity of digestive enzymes in human neonates, which is very low up to 3 mo of life (Parkin et al., 1973). However, no literature about colostral IgA digestion in calves was found, but the intestinal secretion of IgA was described in preruminant calves (Porter et al., 1972). In our study, fecal IgA was highly detected on BC fed calves' feces compared to the CTRL ones on d 5, and continued in greater concentrations until d11 in C10 calves, which could be due to the differences in IgA concentrations present in BC, TM, and MR. The detection of fecal IgA could explain a possible role of IgA in the preservation of the intestinal epithelial barrier and the development of immune tolerance to commensal gut microbiota depending on the colostrum treatment provided (Celi et al., 2019). Unfortunately, we cannot distinguish if these differences could come from either feeding treatments or intestinal secretion. On the other side, LTF is excreted from neutrophils and is found in most exocrine secretions, including milk and intestinal mucus (Celi et al., 2019), but it is also present in BC (1.84 g/L) and TM (0.86 g/L) (Blum and Hammon, 2000). In our study, fecal LTF decreased during the feeding treatments, but it was similar among them, which might suggest a reduction

of gastrointestinal inflammation (Celi et al., 2019) after the stress period of feed restriction. Similar to IgA, we could not assure if fecal LTF detected came from the feeding treatments or intestinal mucosal secretion.

Finally, some authors described the role of the gut microbiome in metabolic health in preweaned calves, since they contribute in the development of the intestinal mucosal epithelium, mucosal immune system, and hindgut digestion and fermentation (Guan, 2022). Particularly, Dietert and Silbergeld (2015) suggest multiple interactions between and within the microbiome, host immune system and the exposure to exogenous actions, which may modulate the composition and metabolism of gut microbiota by altering fatty acid production and regulating epigenetic modifications. Undigested carbohydrates and proteins are the main substrates for bacterial fermentation, which result in a wide range of metabolites such as VFA (Macfarlane and Macfarlane, 2012). In the present study we used fecal VFA to assess intestinal microbial fermentation (Macfarlane and Macfarlane, 2012) and the F/B Ratio as a marker of gut dysbiosis (Yang et al., 2020). This ratio represents the proportion of "potential beneficial microbes" (including major species from the genera *Bifidobacterium, Lactobacillus, Faecalibacterium, Eubacterium* and *Ruminococcus*) to "potential detrimental microbes" (that include some species from the genera *Clostridium, Enterobacter, Enterococcus, Bacteroidetes*, and *Ruminococcus*) (Yang et al., 2020). Some human and animal studies report that an increase in the F/B ratio is associated with several pathologies such as obesity and inflammatory bowel disease (Magne et al., 2020; Stojanov et al., 2020; Yang et al., 2020). In our study, despite the decrease of fecal consistency during the feeding treatments (treatment period), especially in C10 calves, we did not detect differences the in the F/B ratio among treatments. However, we observed a different VFA profile, especially in BC calves, in which they increased butyrate proportion in their feces in comparison to CTRL calves. Bovine colostrum is richer in oligosaccharides compared with mature milk (Fischer-Tlustos et al., 2020), and milk enrichment of oligosaccharides increased the abundance of *Bifidobacterium*, but also a small increase of the butyrate-producing bacterium *Faecalis prausnitzii* (Fu et al., 2019). However, in our study, we observed a decrease in *Faecalis prausnitzii* concentrations from d 5 to 11, indicating that the increase in butyrate could not be attributed to this species. Butyrate plays an important role in the regulation of cell

growth and differentiation, the regulation of energy metabolism and the promotion of intestinal barrier function due to its anti-inflammatory activity or decreasing epithelial permeability in Caco-cell lines (Macfarlane and Macfarlane, 2012; Magne et al., 2020). Although we did not observe differences in the F/B ratio and *Faecalis prausnitzii*, we are aware that to study the effects of BC and TM on microbiota, we would need more statistical power to draw accurate conclusions about microbiota populations. However, our preliminary results suggest that neither of the feeding treatment caused a severe dysbiosis to calves to alter the F/B ratio, and BC feeding might promote butyrate-producing bacteria. Although propionate only represented a 20 % of the molar proportion of VFA, C10 fed calves had lower propionate concentrations than the CTRL and TM fed. Propionate is the primary precursor for ruminant gluconeogenesis and improves the development of ruminal epithelium (Zhang et al., 2018). This decrease in propionate could be linked to the prolonged mobilization of glucose precursors as BHB in C10 fed calves.

In summary, the results of the present investigation showed that BC and TM supplementation after a FRF period influenced calf recovery. On one hand, differences in nutrient composition (fat and lactose) changed the recovery of energy status (higher BHB concentrations) of BC and TM fed calves. On the other hand, although no performance differences among treatments were observed, results from serum citrulline, and the molar proportion of butyrate in feces, especially in calves fed BC, support the hypothesis that bioactive molecules present in BC and TM may help to restore the intestinal absorptive function and provide gut immune protection after the FRF period. Although extended BC and TM feeding maintained enterocyte mass and increased the IgA detected at d11, BC supplementation had an undesirable effect on calves scouring. Overall, we would recommend the use of BC for 4 days or TM for 10 days to cope with the negative effects of the FRF period on calf health, but fecal scours should be monitored. However, research increasing the statistical power, number of calves per treatment, is needed to further evaluate the effects of BC and TM after a FRF period on health status and microbiota.

4.5. Conclusions

Feeding either BC or TM after an episode of FRF helps to recover intestinal barrier functionality and provide gut immune protection. The feeding treatments with the most desirable health outputs were feeding BC for 4 days or TM for 10 days. However, further research is needed to confirm the undesirable effects on fecal consistency when colostrum was fed for 10 days.

Chapter 5

LONG-TERM EFFECTS OF EXTENDED COLOSTRUM FEEDING IN METABOLIC IMPRINTING OF HOLSTEIN COWS

5.1. Introduction

In dairy cattle, early nutritional strategies can influence their future metabolism and performance, affecting the economic benefits of the farms (Hammon et al., 2012; Yunta et al., 2015; Kenéz et al., 2018). Colostrum is well known to provide lactocrine, nutritive, protective, and adaptative advantages to newborn calves due to its high content of nutrients as well as bioactive compounds (Godden, 2008; Playford and Weiser, 2021). Also, BC intake shapes the neonatal metabolome, having systemic effects on energy metabolism pathways, including amino acid, carbohydrate, and fat oxidation (Qi et al., 2018; Zhao et al., 2018; Liermann et al., 2020).

Although recent literature avouches the imprinting effects of calves' milk intake on future milk yield performance (Bach, 2012; Soberon et al., 2012; Rosadiuk et al., 2021), there are scarce studies relating the imprinting of colostrum feeding on long-term performance and metabolism. The hypothesis of our study is that extended feeding of colostrum may provide different long-term advantages in performance and metabolism. The objective of our study was to evaluate the impact of the amount of colostrum feedings on performance and metabolism of dairy heifers.

5.2. Materials and methods

5.2.1. Animals and treatments

Thirty-four female Holstein calves born between November 2019 and June 2020 were enrolled in the study at the Blanca from the Pyrenees dairy farm (Hostalets de Tost, Lleida, Spain) under the approval and supervision of the Animal Care Committee of the Government of Catalonia (authorization code 10745). Calves were removed from their dams immediately after birth and placed in individual pens. Animals (n=17) were fed frozen colostrum from primiparous and multiparous cows either for 2 feedings (**SHORT**) or 8 feedings (**LARGE**) of 2.5 L each at the same feeding times. During the 6 feedings in which LARGE calves were fed colostrum, SHORT calves were fed a MR (24.0 % CP and 18.5 % fat, Nukamel Blue, Nukamel, Weert, Holland) at 12.5 % DM. From d 5 to 13 of life calves were fed 2.5 L twice daily of the same MR at 12.5 % DM. At d 14 of life, calves were transported to a contract heifer operation (Rancho Las Nieves, Mallén, Zaragoza, Spain), where they were submitted to the same management regardless of the treatment. Upon arrival, they were weighed and housed in individual hutches (1.07 x 1.60 m) bedded with straw.

Then, all calves were fed the same feeding program, decreasing MR gradually from 3 L twice daily to 2 L once daily at 15 % DM dilution rate until weaning (d 56 of age), offering water and straw ad libitum. All calves received the same MR (Nukamel Blue, Nukamel, Weert, Holland) in feeding bottles twice daily at 7.00 and 17.00 and the same calf starter concentrate feed until 1 wk after weaning (**Table 5.1**).

Table 5.1. Nutrient and bioactive compounds composition of colostrum, milk replacer and starter feed used in this study

Item	**Feed**[1]	**Colostrum**	**Milk Replacer**[2]
Nutrient, % DM			
CP	24.3	62.0	24.5
NDF	17.8	-	-
ADF	10.4	-	-
EE[3]	4.5	21.3	18.9
Ash	6.9	4.4	7.7
Lactose[4]	-	7.4	48.9

[1]Estimated nutrient analysis from the feed composition
[2] Data provided by Nukamel Blue, Nukamel, Weert, Holland.
[3] EE=Ether extract
[4] Lactose in milk replacer was determined by difference (100%-CP%-fat%-ash%) based on Quigley et al. (2006).

Housing and breeding practices were the same as the ones described in Terré et al. (2009). Briefly, calves were housed individually until 1 wk after weaning. Then, animals were moved to groups of 6 calves until 72 d of age and they reached the target BW of 116 kg. Then, pens were combined, and heifers were progressively transferred to bigger pens five times while they were growing (combining animals that did not belong to the study). Until they reached 160 d of age and a target BW of 164 kg, they stayed in groups of 24 heifers. Then, pens were merged to form again groups of approximately 70 heifers until they were 215 d old and reached a target BW of 213 kg. Later, these pens were moved and assembled into single groups of 130 heifers until a target BW of 261 kg at an age of 270 d. Then, at the age of 330 d

all heifers weighing >310 kg were moved into other pens of the same dimension. Lastly, at the age of 400 d heifers weighing above 380 kg were moved to a breeding pen. Estrus was checked 3 times per day and heifers were inseminated 12 h after estrus was detected. Two months before calving they returned to the Blanca from the Pyrenees farm, where all animals were fed the same prepartum diet (57.4 % NDF, 36.3 % ADF, 10.4 % CP, 1.4 % EE and 6.7 % ash, on a DM basis). After calving, all animals were managed according to the farm protocol until 60 days in milk (**DIM**), cows were milked twice daily.

5.2.2. Measurements, health events, and blood extraction

Heifers were weighed at weaning and bimonthly thereafter until first calving. All health and reproductive events were recorded along each calf's life to evaluate the potential long-term impact of colostrum feedings. Milk yield was recorded until 60 DIM using electronic milk meters (AfiMilk, Afikim Ltd., Israel) as described by Bach et al. (2020). Milk fat and protein contents were determined electronically in every milking using the AfiLab system (Afikim Ltd., Israel), which was calibrated fortnightly. Energy-corrected milk (**ECM**), following the International Farm Comparison Network's equation, was calculated as:

ECM (kg/d) = 0.327 × milk yield (kg/d) + 12.95 × fat yield (kg/d) + 7.65 × protein yield (kg/d) (Tyrrell and Reid, 1965).

At 21 d after parturition blood samples were collected from the coccygeal vein, using evacuated K2E (EDTA) tubes (BD Vacutainer®, Eysins, Switzerland). After blood sample collection, they were centrifuged at 1,500 × *g* for 15 min, and they were kept frozen at -20 ºC for subsequent metabolomic analysis.

5.2.3. Colostrum management and chemical analyses

First-milking colostrum was collected by farm personnel from primiparous and multiparous cows in plastic bags (ColoQuick, Skive, Denmark) and stored at −20 °C. Colostrum 3 L-plastic bag was fitted with agitators into a thawing unit to ensure even heating and thawed at 42 °C for 30 min. Only colostrum with ≥25º BRIX was included in the study. Colostrum samples from each dam were collected, pooled and analyzed for DM (24 h at 103 °C; Regulation (EC) nº 152/2009 (European Community, 2009)), ash (4 h at 550 °C; Regulation (EC) nº 152/2009), CP (Kjeldahl

method; Regulation (EC) nº 152/2009), fat (gravimetry with acid hydrolysis preparation; Regulation (EC) nº 152/2009), and lactose (HPLC—Refractive Index; method 984.22; AOAC International, 1995).

5.2.4. Metabolomics analysis

A non-targeted metabolic analysis was performed on metabolic extracts from plasma samples using ultra-high-performance liquid chromatography (UHPLC) coupled to electrospray ionization quadrupole time of flight (ESI-Q-TOF) tandem mass spectrometry (MS/MS, model 6545, Argilent Technologies, Spain) following a previously published method (Sol et al., 2023). Data filtering and peak picking of the chemical constituents was performed using the Mass Profiler Professional Software (Argilent Technologies, Spain) following the manufacturer's recommendations. Briefly, the variation among samples was evaluated using Principal Component Analysis (**PCA**) and the metabolic profile was visualized using heatmaps. Retention time and mass charge were used to identify the metabolites present in the analyzed samples by associating them with libraries of the Mass Profiler Professional Software. Lastly, relevant features were searched against the Human Metabolome Database (Wishart et al., 2008) to identify differential metabolites.

5.2.5. Statistical analyses

One animal from the SHORT treatment was excluded from the reproductive parameters due to premature calving (253 d). Two animals from each treatment were excluded from milk yield analysis due to death or sale before 30 DIM. Milk yield and components from day 1 after calving were removed as they were considered colostrum. Performance growth (from weaning to breeding) and daily milk yield and milk components data were analyzed using the PROC MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) accounting for the random effect of animal, and the fixed effects of treatment, time, and their interaction. Calving month was included in milk and reproduction performance analysis as a block, whereas the month of birth in the growth performance. For milk performance and ADG, an unstructured covariate matrix was used because it was the one with the lowest Bayesian criterion. Reproduction parameters, accumulated ECM at 60 DIM, peak

yield, and day of peaking were analyzed using the PROC GLM procedure of SAS for a generalized linear model (one-way ANOVA) to assess the effect of treatment. The PROC FREQ procedure of SAS was used to perform a Chi-square test to analyze differences between treatments in health reports. Comparisons of the observed metabolite abundances among treatments were executed by t-tests using MetaboAnalyst 5.0 (Xia et al., 2009). The obtained raw-*P* values were false discovery rate (**FDR**) adjusted. Significance threshold was set at 0.01 for raw-*P* values while at 0.05 for FDR.

5.3. Results and discussion

The present study sought to evaluate if maternal programming was partially conferred through colostrum feedings and thus exerting long-term effects on growth, postpartum metabolism, and milk performance. Herein no differences on ADG or reproductive parameters, including age at first calving, interval between calving and conception, and the number of services per conception were observed between treatment groups (**Table 5.2**). However, BW was greater (P = 0.03) in LONG than in SHORT fed heifers at 14 mo. The main afflictions detected were diarrhea, respiratory disorders, and hyperkeratosis representing 21 %, 54 %, and 13 % of the incidence, respectively, but these health reports did not differ (P = 0.66) between treatments. Although accumulated and daily ECM milk yield at 60 DIM were similar between treatments, ECM milk yield peak at 60 DIM tended (P = 0.10) to be greater in SHORT than LONG cows, occurring in both treatments at 49 and 46 d, respectively. Milk fat and protein contents did not differ between treatments, but they decreased (P < 0.001) at the onset of lactation during 2 and 5 d, respectively. Although these performance results remain tentative, it is important to note that the sample size (n = 17/ group) is insufficient to draw definitive conclusions.

Table 5.2. Least square means of reproductive and milk performance, of the cows fed 2 (SHORT) or 8 (LONG) colostrum feedings after birth.

Item	**Treatment[1]**			**P-value[2]**		
	SHORT	LONG	SEM	*T*	*time*	*T x time*
From weaning to first calving						
ADG, kg/d	0.90	0.90	0.020	0.88	<0.001	0.14
Reproductive parameters						
Age at calving, d	713	720	9.89	0.71	-	-
Interval conception-calving, d	275	275	1.0	0.98	-	-
AI per conception , n	1.0	1.1	0.08	0.19		
Milk performance at 60 DIM						
Cumulative ECM[3], kg/d	1824	1705	60.24	0.16	-	-
Daily ECM[3], kg/d	31.2	30.4	0.98	0.59	<0.001	0.44
ECM peak[3], kg/d	38.5	35.8	1.20	0.10	-	-
Milk peak day[4], d	49.9	45.2	2.33	0.15	-	-
Daily Fat yield[4], g/d	1,068	1044	441	0.71	<0.001	0.55
Fat, [4], %	3.6	3.6	0.10	0.83	<0.001	0.97
Daily Protein yield[4], g/d	1,000	971	317	0.51	<0.001	0.54
Protein[4], %	3.4	3.4	0.05	0.95	<0.001	0.32

[1] SHORT= Colostrum for 2 feedings; LONG= Colostrum for 8 feedings
[2]T = effect of feeding treatment; time= effect of day of study; T × Time = effect of the interaction between T and time.
[3] Energy-corrected milk (**ECM**), was calculated as: ECM (kg/d) = 0.327 × milk yield (kg/d) + 12.95 × fat yield (kg/d) + 7.65 × protein yield (kg/d)
[4] Measured using the AfiLab system (Afikim Ltd., Israel)

Contrary to our findings, the literature available reported differences in ADG or milk yield when the colostrum intake is increased (Faber et al., 2005; Abuelo et al., 2021).

However, age at calving was similar between treatments (713 (SHORT) vs 720 d (LARGE)) as also observed by other authors. Faber et al. (2005) observed an increase in 305-d mature equivalent milk at first (+322 kg) and second lactation (+1,349 kg) when Brown Swiss female calves were fed 4 L instead of 2 L in their first colostrum feeding. They also reported a decrease in veterinary costs (-9.74 $ per calf) as well as an increase in growth rate up to 500 d of age (+ 0.23 kg/day), but they did not find differences in age at conception. Similarly, Abuelo et al., (2021) fed to all calves 3 L of colostrum at birth, and a group of calves receive 2 more L later. They observed that offering an extra colostrum meal increased preweaning ADG (+120 g/day) and tended to increase first lactation milk yield (+984 kg) and decrease the number of inseminations needed to conceive (-0.29 inseminations) (Abuelo et al., 2021). In both studies in which short-term effects of colostrum intake are observed, the high colostrum treatments are similar to our SHORT treatment, suggesting that the most common practice of 2 feedings of BC are enough to achieve main health benefits of colostrum. Nevertheless, there is scarce literature evaluating long-term effects (milk yield performance, reproductive, health, and growth parameters in bovine species) of colostrum treatments, with inconsistent parameters reported that render a difficult comparison across studies (Faber et al., 2005; Abuelo et al., 2021).

Regarding other mammalian species, the impact of early nutritional programming on development and survival has been studied mostly in piglets and infants, highlighting the importance of a low birth weight and postnatal growth rate on impaired health status and neonate survival (Guilloteau et al., 2009; Picone et al., 2018). Moreover, it has been described in rats how the growth pattern and the amount of visceral fat constrains the metabolic capacity later, predisposing to lifelong alterations as glucose intolerance or insulin resistance (Guilloteau et al., 2009). One example of that is the development of obesity risk in infants with early overweight or low BW with rapid catch-up growth (Ong, 2006). Considering the previously mentioned authors, they may also suggest that greater effects of early nutrition on performance may come from providing diets in a sustained way, rather than punctually.

Table 5.3. Identified compounds by non-targeted metabolic analysis in LONG versus SHORT cows.

			Regulated LONG		
Compound	***t* -statistic**	***P*- value**	**vs SHORT[1]**	**Compound**	**HMDB ID**
M_N_466.3643.13.22301	2,7712	0,0092273	Down	5β-Cholestane-3α,7α,27-triol	HMDB0060138
M_P_466.3503.10.868	2,9071	0,0065751	Down	LysoSM(d18:0)	HMDB0012082
M_N_429.108.11.11601	-3,6448	0,00093911	Up	Succinyladenosine	HMDB0000912

[1] SHORT= Colostrum for 2 feedings; LONG= Colostrum for 8 feedings

In this study, samples were obtained within the postpartum phase to investigate whether supplying supplemental colostrum feedings could render metabolic advantages during this physiologically demanding phase. After calving, the dam mobilizes lipid reserves towards the mammary gland to sustain milk demands (Contreras and Sordillo, 2011). This fact increases plasma NEFA concentrations, which are known to alter immune and inflammatory responses, including leukocytes and mediators of COX2 expression (Contreras and Sordillo, 2011). During the metabolomic analysis, only the molecular features that were at least in 70 % of the samples per treatment group were selected from raw data. After peak filtering, 4,625 and 2,446 molecular features for the positive and negative ionizing modes were obtained, respectively. Although, the non-supervised PCA did not reveal distinct metabolic profiles between treatments, the variability explained by PC1 and 2 was 19.3 % and 9.3 %, respectively (**Figure 5.1**). In this study, t-tests found differences (raw $P < 0.01$), but none when applying a 5 % FDR, in twenty-two plasma metabolites, from which it was possible to identify three of them (**Table 5.3**). Moreover, the hierarchical clustering heatmap showed a good sample clusterization of individual samples according to its treatment for the top 22 identified components (**Figure 5.2**).

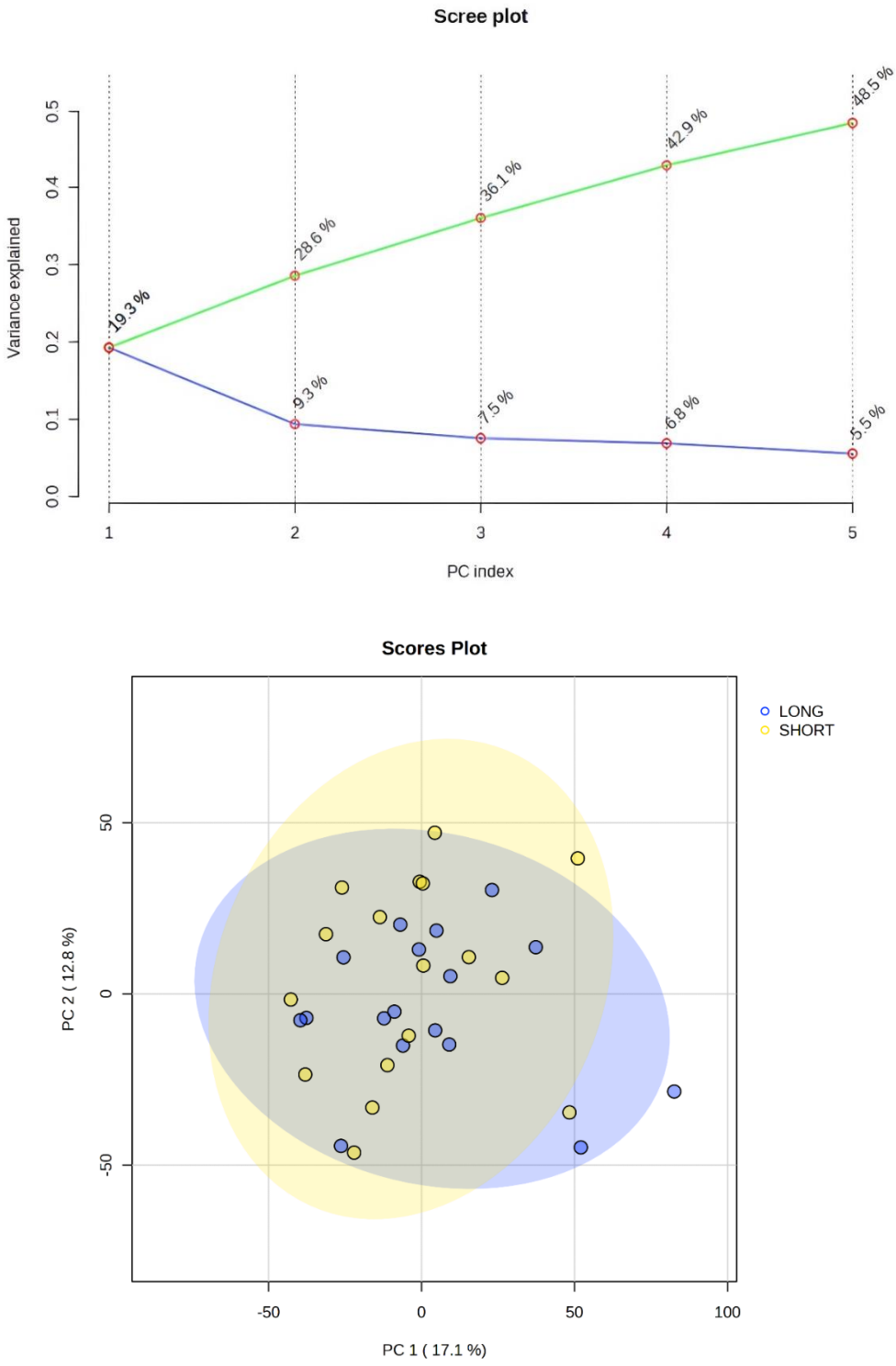

Figure 5.1. Representation of the principal component analysis (PCA). a) Variance explained per accumulated principal component. The green line represents the cumulative variances while the blue one the fraction of variance accounted by each PCA. b) PCA scores plot. Plasma samples from SHORT animals (colostrum for 2 feedings) were compared against LONG (colostrum for 8 feedings) ones.

Concretely, the analyzed data revealed down-regulation of 5β-cholestane-3α,7α,27-triol, and lysoSM (d18:0) along with an up-regulation of succinyladenosine in LONG compared to SHORT heifers, suggesting that increasing the colostrum feedings had a minor effect on heifers' future metabolism at the onset of first lactation. The 5β-cholestane-3α,7α,27-triol is involved in bile acid and cholesterol synthesis pathways, while LysoSM (d18:0) participates in the biosynthesis of sphingolipids (Jewison et al., 2014; Wishart et al., 2022). Particularly, 5β-cholestane-3α,7α,27-triol is an intermediate metabolite of bile acids biosynthesis, in which its steroid side-chain is oxidated in the hepatocyte mitochondria to form the cholestanoic acid THCA (Chiang, 2013; Wishart et al., 2020). To a lesser extent, 5β-cholestane-3α,7α,27-triol participates in cholesterol homeostasis, given that it can inhibit by negative feedback the activity of HMG-CoA reductase (the rate-limiting enzyme of the cholesterol biosynthetic pathway) when intracellular cholesterol levels are high and increase the expression of the LDL receptor, which leads remove LDL cholesterol from the blood and inhibit lipogenesis (Chiang, 2013). At the onset of lactation, cows are reported to have upregulated biosynthesis of cholesterol to transport liver triglycerides to the mammary gland and consequently, low cholesterol and triglycerides in plasma (Kessler et al., 2014). Particularly, after parturition the mammary secretion is high in cholesterol and phospholipids, but rapidly decreases within 48 h (Contarini et al., 2014). In this study, SHORT cows had greater plasma 5β- cholestane-3α,7α,27-triol, but no differences between treatments in milk fat content during the first 60 DIM were observed.

LysoSM (d18:0) is found in animal cell membranes, especially in axons' membranous myelin sheath surrounding the nerve cells, which is why they play a role in signal transduction (Wishart et al., 2022). Sphingomyelins are catalyzed to ceramide and phosphorylcholine in the sphingolipid biosynthesis and the opposite when the lipid metabolism requires it (Wishart et al., 2020). Particularly, the degradation of sphingomyelin from the plasma membrane leads to cholesterol moving from the membrane to the endoplasmic reticulum (Gupta and Rudney, 1991; McCluskey et al., 2022). This fact generates the accumulation of cholesterol, which also downregulates the previous mentioned enzyme HMG-CoA reductase (Gupta and Rudney, 1991). Additionally, other authors described that during the onset of lactation, sphingomyelins were greater than in mid-lactation (Contarini et al., 2014).

Alternatively, the upregulated metabolite succinyladenosine is involved in the de novo purine synthesis pathway (Wishart et al., 2022). In humans, it accumulates in body fluids when the adenylosuccinate lyase enzyme is deficient (Zikánová et al., 2005), whereas in bovine species it has been described an downregulation of succinyladenosine when cows were fed with high-concentrate diet (to cause subacute ruminal acidosis) (Mu et al., 2023). Finally, the preliminary identification of these metabolites remains tentative, however, it should be confirmed using the corresponding MS/MS standards to fully confirm the chemical structures of the metabolites. Moreover, further research in the neonatal programming of cholesterol metabolism by colostrum vs MR is needed, including data from the preweaning phase until first lactation, since literature suggests a different effect on fetal imprinting if high fat diets are fed only during gestation or during the lactation period (Bringhenti et al., 2015). The offspring of maternal high-fat fed diets alters long-term behavioral and physiological changes increasing the preference for fat and hyperlipidemia (Chang et al., 2008). In contrast, when high-cholesterol milk formula was fed to neonatal male rats, it prevented them to suffer dietary-induced hypercholesterolemia (that could predispose to cardiovascular diseases) later in life (Reiser and Sidelman, 1972). This suggests that early exposure to maternal high fat diets could potentially unchain metabolic imprinting mechanisms in the adulthood, differing from the ones induced by maternal high-fat diets during the pregnancy period.

5.4. Conclusions

Overall, performance data did not reveal the potential maternal lactocrine effect of extending colostrum feedings from 2 to 8. However, minor effects on postpartum metabolism were detected, mainly related to the cholesterol biosynthesis pathway.

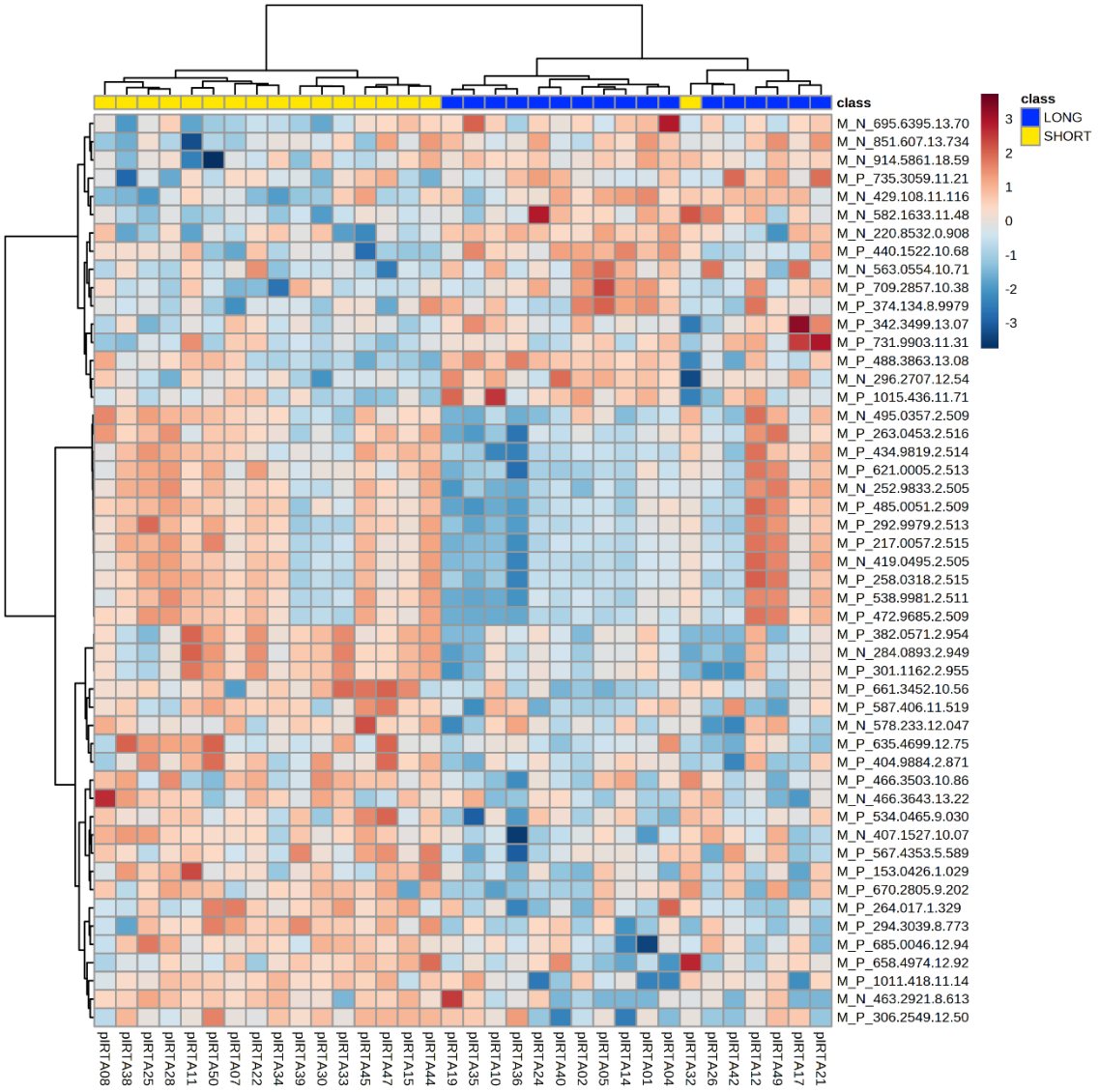

Figure 5.2. Hierarchical clustering heatmap of the top 50 identified compounds. Individual samples (vertical axis) are separated using hierarchical clustering (horizontal axis) with the dendrogram being scaled to represent the distance between each brand. The clusters containing SHORT (colostrum for 2 feedings) and LONG (colostrum for 8 feedings) samples are highlighted in yellow and blue, respectively. Relative abundance of targeted metabolites is represented by color; red indicates high relative abundance, while blue indicates low abundance.

Chapter 6

GENERAL DISCUSSION

6. GENERAL DISCUSSION

All the studies in this thesis aimed to investigate the effect of the bioactive compounds of TM and BC on calves' health and performance. The first study was performed with the objective of exploring the biological value of TM and BC depending on the dam's parity to promote calves' health and performance. Therefore, the other studies were conducted to evaluate two possible applications of the surpluses of TM and BC such as reducing the negative health impact of the transportation in preweaned calves in Europe or extending the feeding of colostrum to further analyze the long-term effect (after calving) in heifer's metabolome.

When analyzing the biological value of BC and TM, we observed that dairy farms produce BC and TM that, after the nearly mandatory first feeding of BC, their surpluses are either discarded, conducted to the milk tank (increasing riskily the tank's SCC) or to a lesser extent, feed to dairy calves as waste milk (Urie et al., 2018; Fischer-Tlustos et al., 2020). In the Study 1, we described how the TM from the second milking contains more than half of the concentration of bioactive molecules present in BC and how these molecules vary depending on the dam's parity. These findings make us want to inquire into potential second milking applications as a nutraceutical in animal or human health and further investigate which on-farm and dam factors may modulate the concentrations of the bioactive molecules on TM, as it is already described in BC (Shivley et al., 2018; Godden et al., 2019).

During the first study, as we obtained the milking samples from several commercial farms, we had limited control over other aspects, a part of lactation number and milking number, that influenced the concentrations of bioactive molecules, being aware that biomolecules concentration studied could have been affected by other uncontrolled factors. In BC, as described in section 1.1.3., Godden et al. (2019) explained which management factors could interfere with the production of colostrum, especially with the concentrations of IgG. In the first study, several management practices such as the number of daily milkings, the protocol of prepartum vaccination, dry cows' rations, or the milking routine, were already established by the farmers. Fortunately, we inquired about their management to attribute some differences in IgG to the effects of farm practices including vaccination, but 3 farms were not enough to infer the cause-effect of the different

managements within the farms. Other aspects that could be involved in BC's IgG concentrations that were not mentioned in the study are the time interval between calving and first milking or the volume of BC produced (Morin et al., 2010; Kessler et al., 2020). Colostral IgG seems to decrease when BC yield increases and the first milking relative to calving is delayed (Kruse, 1970; Pritchett et al., 1991). In relation to the other bioactive molecules studied (LTF and IGF-I), scarce literature is available regarding the factors that might influence their presence in BC. Colostral LTF is reported to vary among breeds (Tsuji et al., 1990) while the use of exogenous bovine growth hormone increased IGF-I in mammary secretions around parturition (Hadsell et al., 1993). Concerning the TM, the inherent factors that modulate BC quality may also interfere in TM to a lesser extent, since in the first study, as the milkings progressed the bioactive compounds experience a diluting effect in the mammary gland due to the osmotic pressure (Baumrucker et al., 2010). Regarding the management practices that may enhance IGF-I and LTF concentrations in milk., it has been described that increasing milking frequency could boost IGF-I bioavailability in mammary tissues, while LTF concentrations in milk were correlated with the stage of lactation, daily milk yield, and, at a lower rate, the SCC (Cheng et al., 2008; Murney et al., 2015; Meyer et al., 2017). However, further research is needed to clarify the importance of the mentioned BC management factors that could also have an impact on TM.

In the first study of this thesis, it was found that BC samples from M1 and TM from M2 met industry quality standards of 50 g IgG/L to ensure calf survival (McGuirk and Collins, 2004). Considering that a healthy cow may produce on average 6.5 L of BC (with at least 30 % of variability within farms) and that this amount exceeds the requirements of the newborn calves (7-10 % of the calf's BW; 3-4 L), the remaining surplus could be commercially exploited (Kehoe et al., 2011; Conneely et al., 2013). Thus, the main goal for the industry is to transform the BC into a saleable final product, without compromising its bioactive substances that have nutraceutical properties and at the same time, avoiding microbial growth (Godden et al., 2006). However, apart from a limited amount of BC surpluses, its processability and storage come with different technological and preservation challenges that difficult its exploitation in large-scale situations (Marnila and Korhonen, 2002; Kaplan et al., 2022). On one hand, to facilitate BC collection and transport to the industry, farms

require nearby freezing and cooling equipment to preserve its bioactive substances (mainly IgG), which could be a limiting factor in transforming the BC (Stewart et al., 2005; Borad and Singh, 2018; Godden et al., 2019). Once the farm has collected enough volume of BC to be processed, the BC is transported to the processing plant, tested for IgG quality (to reach a minimum quality standard of IgG), and pooled to be transformed into the final product. Depending on its intended final application, the raw BC will undergo different thermal or drying treatments to prevent bacterial proliferation of different origins (as explained in section 1.1.3), each of them with its benefits and disadvantages (Chelack et al., 1993; Jay, 1998; Sotudeh et al., 2018). On the other hand, BC's viscosity increases with temperature, which becomes a challenge for heat treating (McMartin et al., 2006). This could be mainly due to BC's high protein content and the low coagulation temperature of the whey protein (Marnila and Korhonen, 2002; Pelegrine and Gasparetto, 2005). Other issue that could interfere in colostrum manipulation is that colostrum's antimicrobial components may inhibit fermentation processes, which could alter the antibiotic residue tests based on microbial growth, implying false positives (Marnila and Korhonen, 2002; Romero et al., 2014). Specifically, the majority of the microbial inhibitor tests used commercially are based on a colorimetric assay that measures the growth of the microorganism *Geobacillus stearothermophilus var. calidolactis*, therefore, the results could be interfered by a variety of substances that could inhibit microbial growth as IgG1, milk protein, SCC and BC preservatives (Andrew, 2001; Romero et al., 2014).

Once the colostrum reaches the industry plant, the first step would be the thermal treatment to reduce bacterial load. Pasteurization was one of the first developed methods that reduced the microbial load while heat treating BC (Elizondo-Salazar and Heinrichs, 2008). Although the standard protocol of the dairy industry for pasteurizing milk is at high-temperature short-time (HTST; 72ºC for 15 s), the HTST procedure denaturalizes the thermolabile protein like LTF or IgG, reducing their bioactivity (Tacoma et al., 2017). Nowadays, the recommended colostrum on-farm pasteurization protocol to reduce bacterial contamination, while minimally affecting IgG concentration and colostrum viscosity, is 60ºC for 60 min (Godden et al., 2006; Elizondo-Salazar and Heinrichs, 2008; NAHMS, 2016). However, under the last conditions mentioned, other bioactive compounds seem to be affected, including a

decrease in insulin and IGF-I concentrations and the loss of protein abundance (Mann et al., 2020). From an industrial point of view, several authors proposed a gentle pasteurization of BC (low-temperature long-time (LTLT), 63ºC for 30 min) to maintain product sterility and to potentiate the bioactive compounds (Nguyen et al., 2019; Salar et al., 2021). These authors also suggest adding an extra process of gamma-irradiation for delicate patients as infants (Nguyen et al., 2019) or combining it (57ºC for 30 min) with freeze drying or spray and freeze drying methods to improve microbial quality (Salar et al., 2021). Additionally, in some cases, prior to pasteurization, an extra step of filtration is added to concentrate Igs from defatted BC, causing a total loss of IgM and a 30 % reduction of the IgG2 concentrations but not affecting IgG1 ones (Elfstrand et al., 2002).

After the pasteurization, in case of willing a final powder product that could be stored at room temperature, a drying method is required. Several authors described freeze-drying or spray-drying as the most suitable preservation methods for large-scale commercializing BC while enhancing its quality, extending its shelf life, and reducing its volume and weight (Sotudeh et al., 2018; Gomes et al., 2021). On one hand, freeze-drying, also known as lyophilization, consists of dehydrating the BC in vacuum conditions while lowering the temperature (Chelack et al., 1993), and on the other hand, spray-drying involves the evaporation of the moisture present in BC while spraying it (Chelack et al., 1993). Regarding Igs retention after drying treatments, Chelack et al. (1993) reported that after freeze-drying BC maintained the 99 % of total Igs, whereas Elfstrand et al. (2002) observed a retention of 34 % in total Igs of defatted colostrum whey. Although freezing-drying prevents the heat denaturalization of proteins, this technique is 20-fold more time-consuming and 2.5 times less efficient for the same volume of BC than spray drying (Chelack et al., 1993; Elfstrand et al., 2002), suggesting freezing-drying as an appropriate process for small BC batches of high biological value. Concerning the preservation of Igs content, Borad et al. (2021) noted that spray drying retained 88 % of Igs in spray-dried zebu colostrum protein preparations while Chelack et al. (1993) reported 94 % of total Igs retention during spray drying of BC. Spray-drying of BC costs 2.5 times less than freeze-drying, is less time-consuming and it is easily scalable (Chelack et al., 1993). Nonetheless in both techniques, bacterial microbiological proliferation has to be controlled since extreme temperatures that may sterilize the BC are avoided

(Chelack et al., 1993). In the case of sensitive patient groups (preterm infants, chemotherapy-treated cancer patients, or antibiotic-associated diarrhea), Chatterton et al. (2020) recommended a LTLT pasteurization (63 °C, 30 min) process followed by spray-drying as the most suitable treatment to preserve the bioactive components at the same time that removing the microbial contamination.

Concerning the outcomes of BC, the majority of products that are commercially available for human use, are either complete BC powder or defatted BC powder and, to a lesser extent, BC with subfractions removed (casein or lactose) or enriched with Igs and growth factors (Playford and Weiser, 2021). The applications of BC supplementation for humans vary from exercise performance enhancers to treating GI diseases in humans (Kelly G. S., 2003; Linehan et al., 2023). Currently, there are more than 130 clinical trials involving BC supplementation in adults and infants (see https://trialbulletin.com/lib/trials/term=Colostrum). However, Playford et al., (2020) revealed concerns about the variability among BC trials regarding the bioactivity of the IgG and other bioactive substances given that it is only mandatory to report protein and IgG content. In the mentioned author's study, the bioactivity of different commercial BC supplements was evaluated, showing a high range of variability (6-fold) at similar IgG and protein content, which could cause also inconsistencies among clinical trials and difficult the interpretation of the results (Playford et al., 2020; Playford, 2021).

Regarding the veterinary applications, as explained in the Introduction, in bovine species CR (>100 g IgG) could be an alternative option to maternal BC when it is unavailable (Godden et al., 2019). Additionally, BC supplements are used to boost IgG content in poor-quality BC to ensure a successful transfer of passive immunity (Godden et al., 2019). Even though CR represents an advantage in storage and preparation, it supposes an extra cost to the farmers, and their antibodies might not be farm-specific (Cabral et al., 2013). In both cases, human and veterinary applications, the fewer processing steps to obtain the desired product, the more economically viable and efficient the transformation will be (Fasse et al., 2021).

As mentioned in Chapter 3, either M1 or M2 reached the target quality of > 50 g of IgG/L (McGuirk and Collins, 2004). Literature reported greater IgG concentrations when the parity increases (Kehoe et al., 2011; Conneely et al., 2013; Gross et al.,

2017), but the first study of this thesis only showed that LTF and IGF-I were greater in multiparous than primiparous cows. In our study, IgG was numerically greater in M1 and M2 as lactation number increased, which suggests that possibly analyzing each lactation separately (instead of primiparous vs multiparous) the effect of the lactation number would have been appreciated. Moreover, several authors further analyzed the Igs concentrations of the second milking and the obtained TM yield in Holstein cows (Chelack et al., 1993; Gross et al., 2017). Chelack et al. (1993) reported an average milk yield in the second milking of 7.2 L vs 4.3 L and 50.3 g/L vs 34.7 g of Igs /L concentrations in multiparous vs primiparous cows, respectively, whereas Kessler et al. (2020) observed 5.6 kg at 29.9 g of IgG/L in the second milking of multiparous cows. Additionally, Kessler et al. (2020) reported that the milk yields from the second milking depended strongly on the milking interval between the first and the second milking. If we consider previously reported BC and TM from the second milking's yield and IgG concentrations, and the statistics of the dairy cow population reported in the dairy industry reports from the Spanish Ministry of Agriculture, Fisheries and Food, there would be annually produced 2.4 million of BC liters (after a 3L first feeding to the offspring) and 3.3 millions of liters of TM surpluses available for their use on-farm or to be commercialized (**Table 6.1**). If we extrapolate the BC and TM surpluses in Spain to IgG mass, it would be obtained a total amount of over 450 tons of IgG per year. Additionally, Spain's dairy cows represent the 4 % of the European census (MAPA, 2022), which suggests a great potential market to scale up the use of farm surpluses in the future.

Regarding the other TM bioactive compounds studied in the present thesis, the industry mainly isolates LTF from BC and milk, whereas IGF-I could be synthesized from bacteria specifically for human use (Ballard et al., 1987; Iranpoor et al., 2015; Cui et al., 2022). Thus, the potential marketable components of TM from the second milking would be IgG and LTF, without the need to discard primiparous cows according to our data.

Table 6.1: Estimated bovine colostrum and transition milk yield and IgG available in Spain every year**.**

	Number of female breeding animals (>24h)[1]	**Number of calves per cow and year[1]**	**Average volume per cow[2], L**	**Total of liters**	**Average IgG concentrations, g/L[3]**	**Total IgG mass, kg**
Bovine colostrum	797,878	0.68	7.5 (4.5)[4]	4,069,178 (2,441,507) [4]	110.0	447,610 (268,566) [4]
Transition milk	797,878	0.68	7.0	3,351,088	59.4	199,055
Total annual				7,420,266 (5,792,595) [4]		646,665 (467,621) [4]

[1] Data extracted from Subdirección General de Producciones Ganaderas y Cinegéticas y Dirección General de Producciones y Mercados Agrarios. (2020) and Subdirección General de Producciones Ganaderas y Cinegéticas Dirección General de Producciones y Mercados Agrarios (2022).

[2] Data obtained from the annual BC and TM yield records of Holstein-Friesian cows from EVAM (IRTA Monells, Girona, Spain).

[3] Data obtained from the study 1 of this thesis.

[4] Between parenthesis, subtracting the first feeding of BC to the offspring. Data extracted from Shivley et al. (2018) and Robbers et al. (2021).

In the second study of this thesis, we explored one of the possible applications for the TM and BC surpluses. Different therapeutical treatments were applied to cope with the negative effects of transportation in preweaned calves. Considering that this study was performed on a small scale, some challenges in storage and transportation were observed, mainly maintaining the cold chain, and dealing with a large volume of liquid. In our case, 294 L of BC and 392 L of TM surpluses were collected from farms in 25 L frozen bottles and transported to IRTA facilities once they were filled. Once at the processing plant, bottles were thawed and homogenized separately for each milking. Finally, BC and TM were bottled for each calf meal, frozen until use, and transported to a large freezer next to the experimental farm. Moreover, in the current production system, at least in Southern Europe, the fattening farms are separate from the dairies, which makes difficult the reuse of surpluses in the same business. For that reason, counting with the equipment that allows to reduce the volume liquid into powder along with preserving it at room temperature, would be a huge advance in the management of surpluses. However, further economic analysis is needed to analyze if compiling and drying the surpluses is a viable solution instead of preserving them in liquid form. Also, the possibility of joining forces among local farms or commercializing the surpluses on a larger scale could be considered.

Furthermore, this study was performed on calves with more than three weeks of age in accordance with the current regulation on lactating calves' transportation. However, several therapeutic BC and TM studies have been accomplished with newborn calves, which have a more immature gastrointestinal system and they are acquiring passive immunity (Conneely et al., 2014; Hare et al., 2020; Kargar et al., 2020). In our study, we focused on the local effects that bioactive substances remaining in the lumen could have on the GIT's health, from enhancing their local immune system to protecting against the colonization of pathogens after the period of FRF (Blum and Hammon, 2000; Carter et al., 2021). Also, in this study, TM and BC provided nutritional induced metabolic change, since calves fed with BC and TM maintain their ketogenic glucose metabolism from FRF compared to the MR-fed ones, suggesting a possible effect of energy source (rich-fat diets instead of lactose-based ones) (Helge, 2002; Vidali et al., 2015). Furthermore, in this study there was no evidence of sustained effects of feeding BC or TM at 3 weeks of age, since after

changing the diets from BC or TM to MR, the monitored parameters were similar among treatments. Hence, this study concluded that the feeding treatments with the most desirable health outputs were feeding BC for 4 d or TM for 10 d.

Thus, considering the available surpluses of TM in Spain and the reported benefits of TM as a nutraceutical, there is an arising a need to transform TM into a biologically safe and durable product. However, until now only Chelack et al. (1993) reported a methodology for dry preservation of TM, but the techniques available for BC could be also useful for TM, with the processing differences that TM contains less fat than BC and it is less concentrated in components (Foley and Otterby, 1978). Therefore, based on the available literature on BC, the process to obtain TM powder could follow these steps (Elfstrand et al., 2002; Fasse et al., 2021; Linehan et al., 2023):

1. On-farm collection and preservation: The TM from the second milking will be collected and visually inspected (to avoid blood or raw materials in it). Then, it will be transferred to a sterile bottle, properly marked to ensure traceability and kept in the freezer.
2. Transportation to the freezing facility: Once the farm freezer is full, it will be emptied and frozen transported from different farms to a freezing facility (external, already in the processing plant). Before transportation, another visual inspection will be performed to ensure that the TM is viable.
3. Thawing and main quality check: Bottles will be thawed in a cold chamber at 4ºC and each of them will be tested for IgG concentration, bacteriological contamination, and antimicrobial residues. Regulation (EC) no 853/2004 (European Community, 2004), describes the hygiene of food of animal origin for food business operators producing raw milk and dairy products (including colostrum) intended for human consumption. Contrary to raw milk that follows common EU, for BC applies a national criterion regarding plate count, SCC or antibiotic residues. Hence, the Spanish Real Decree 1086/2020 establishes that the colostrum should be produced by animals free of brucellosis and tuberculosis.
4. TM homogenization and defatting: TM will be merged into one batch and will be defatted using a centrifugal cream separator until 3 % to ensure adequate powdering.

5. Mild pasteurization: TM will be pasteurized in batches without exceeding the 60ºC (LTLT) to preserve the bioactive molecules and avoid excessive viscosity, while reducing bacterial load (McMartin et al., 2006). Other alternatives or added steps could be filter-sterilization for skimmed samples, for instance, microfiltration and ultrafiltration (Borad et al., 2019)
6. Low-temperature spray drying: TM will be powdered at low temperatures to prevent bioactive substances' denaturalization. For instance, literature available in BC reported the following conditions (feed temperature of 32 ºC, feed rate of 160 mL/h, inlet air temperature of 125 ºC, outlet air temperature of 49 ºC and drying air flow rate of 0.78 m^3/ min) to obtain the colostrum powder (Borad and Singh, 2018; Salar et al., 2021).
7. Storing: Before storing at room temperature, a quality and food safety check is required. Then, the TM powder will be packed into the desired format in a protected atmosphere to secure the durability of the product. Nonetheless, Playford et al., (2020) currently reported that there is a wide variability in the bioactivity of commercially available products derived from BC, using cell proliferation and migration assays in addition to immunoneutralization and heat stability studies. This marked variability created the need to establish *in vitro* assays for standardizing BC and TM by-products' biological quality measurement as well as a minimum quality threshold.

Finally, in the third study, we provided different amounts of BC feedings to female calves after birth to analyze if there was any metabolic effect after their first calving. In this study, we delivered either 2 or 8 feedings with at least ≥ 25º BRIX. The SHORT treatment was the commercial farm's protocol to raise female calves while the LONG treatment was selected according to a feasible number of feedings manageable on-farm considering the size of the farm, the due dates of cows, and the expected colostrum yield to be obtained. Also, as the experimental trial was continuously conducted as the calves were born, it wasn't necessary to increase the storage and processing capacity as it started at once. Furthermore, this is one of few studies available from a long-term perspective, considering long-term after first calving (Faber et al., 2005; Furman-Fratczak et al., 2011; Abuelo et al., 2021). However, as

mentioned in the Introduction, the incompleteness of the data reported complicated comparisons among studies about the long-term effects of BC. Concretely, the provision of extended colostrum feeding (**ECF**, understanding colostrum in this section as either maternal colostrum, CR or TM) feeding beyond day one of life has been a research topic of increasing popularity throughout the last 20 years. For instance, from 2005 to 2013, there were 48 papers published related to the ECF topic in Pubmed[1] versus 136 papers published from 2017 to 2023. Similarly, an increasing number of farms have begun to implement this feeding strategy in their calf rearing system, with > 50 % of surveyed Canadian dairy farms reporting that they feed TM for 2 – 3 days after the initial colostrum meals (Uyama et al., 2022).

Therefore, the incompleteness of data encouraged us to perform a guideline for reporting and measuring the experimental data related to ECF trials, and the effects on performance, health, and reproduction to assess the long-term effect of colostrum practices on calves (manuscript in preparation). Briefly, recommendations for each growth stage and production phase of heifers are described in **Tables 6.2, 6.3 and 6.4** based on the most relevant parameters reported in the reviewed articles in section 1.3.3. of this thesis.

[1] The available literature search was performed using the keywords colostrum (or transition milk), calf (or heifer) and performance (or metabolism or reproduction) following the systematic review guide by Leenaars et al. (2012).

Table 6.2. Reporting recommendations for newborn calves.

Parameter	Key points
Performance	• Birth Weight
Health	• Treatment protocol (i.e. navel dipping, vitamin and mineral supplements, etc.) • Vaccination protocol
Nutrition	• Colostrum protocol including source, processing, quality, quantity, mode of delivery, and time of delivery relative to birth.
Blood Parameters	• Time of sample relative to birth • Parameters measured and laboratory methods used

Table 6.3. Reporting recommendations for the preweaning period.

Parameter	Key points
Performance	• Passive transfer level, age at sampling, and laboratory methods used
Health	• Morbidity rates for all diseases (diarrhea, respiratory disease, navel infections, etc.) • Mortality rates along with cause • Vaccination protocol
Nutrition	• Colostrum protocol • Feeding level, source, composition, physical nature, and method of delivery for all liquid and solid feeds delivered. • Experimental treatments/diets
Housing	• Stocking density, pen sizes, housing type, bedding type, pen movements, grouping, etc.
Transport	• Age at transport, length of transport, calf source, and health upon arrival

Table 6.4. Parameters to be reported throughout heifer breeding and calving.

Parameter	Key points
Performance	• ADG (indicating the age and period of time) • Body weight: critical timepoints (weaning, breeding, or calving) and monthly • Minimum age, weight or wither height required to inseminate. • Age at calving
Health	• Health events and therapies (classified by cause) • The use of growth promoters (if allowed in the country) • Morbidity and mortality of all diseases during the postweaning (respiratory and gastrointestinal)
Nutrition	• Culling rate, age and reason • Intake, feedings/day vs ad libitum, composition and ingredients • Pasture access • Medications included (if applicable) • Report the plane of nutrition for each group or lactating phase
Reproduction	• Basic reproduction parameters (age at first breeding, age at pregnancy, AI per conception, parity number and calving interval) • Breeding protocol (natural, synchronization or heat detection) • Reproductive problems by pathology • The use of reproductive technologies (embryo transfer, sexed semen)
Housing	• Housing system (animal density, type of bedding and type of enclosure) • Interventions to ensure comfort (ventilation, heater, cooling systems)
Lactation	• Milk production parameters (305d ME, fat and protein %, lactation length (DIM) and somatic cell count • Colostrum harvesting: amount, quality of colostrum and parity of the dam.

Chapter 7

CONCLUSIONS

7. CONCLUSIONS

The results obtained in this thesis allow to conclude that in our experimental conditions:

1. Transition milk from the second milking contains more than half of the concentration of bioactive molecules analyzed (IgG, IGF-I and LTF) present in BC. However, only LTF and IGF-I concentrations are greater in multiparous than primiparous cows.
2. Feeding either BC or TM compared to MR after an episode of feed restriction and fasting helps to recover the GIT functionality and provide gut immune protection without altering dairy calves' performance.
3. Extending BC feedings to calves beyond the standard of 2 meals have minor effects on their metabolomic profile after the first parturition without affecting their long-term performance.

Chapter 8

ANNEX I

8. Annex I: The impact of bovine colostrum and transition milk on enterocyte turnover.

This research was performed during my stay at the Department of Agricultural, Food & Nutrition Science of the University of Alberta (AB, Canada) under the supervision of Dr. Anne Laarman and the technical support of Alison Mark.

8.1. Introduction

8.1.1. Gut closure and IgG absorption

With the ingestion of the first colostrum, the newborn GIT allows the passive absorption of macromolecules (including Igs and lactoferrin) into the bloodstream via immature enterocytes until the first 24h (Stott et al., 1979; Sangild, 2003). However, Jochims et al. (1994) observed that immature enterocyte populations, which actively absorb Igs, are rapidly replaced by mature enterocytes, that have lost the absorptive activity, during calves' gut closure. Similarly, Castro-Alonso et al. (2008) reported from a histological point of view that the disappearing rate of absorption of IgG was associated with the rate of duodenal immature enterocyte apoptosis in goat kids, given that the upcoming mature enterocytes contain tight junctions that disable the pinocytosis (defined as the uptake of extracellular fluids and solutes) and passive diffusion of Igs.

Moreover, this first intake of colostrum results in an ordered establishment of microbiota as well as the naïve immune system maturation (mucosa and gut-associated lymphoid tissue), which will become the first layer of protection against external pathogens (Maynard et al., 2012). Also, the established microbiome will produce beneficial metabolites like volatile fatty acids that enhance barrier integrity and improve mucosal immunity (Belkaid and Hand, 2014).

8.1.2. Apoptosis in the gastrointestinal tract

Apoptosis is a programmed type of cellular death that is mediated by caspases that cleave specific regulatory proteins to induce cell death (Reed, 2000). The activity of caspases produces the characteristic morphological features of apoptosis, such as DNA fragmentation, phosphatidylserine externalization, and an increase in membrane permeability, that causes cell contraction and plasma membrane blebbing (Ramachandran et al., 2000). Particularly in the GIT, enterocyte apoptosis

is part of the continuous turnover of the intestinal barrier without losing functionality in humans (Bullen et al., 2006). The shedding of epithelial cells at the villus tip occurs as a result of the migration from the crypt to the top of the epithelium (Bullen et al., 2006). Research studies from primary or secondary cultures (immortal cells) have been used to perform an apoptosis model using LPS, an enterotoxin produced in the outer membrane of Gram-negative bacteria (Yu et al., 2005; Hirotani et al., 2008).

Moreover, studies treating Caco-2 (human colon carcinoma) cells with processed BC powder have been shown to enhance small intestine integrity in BC milk protein concentrate and alter colon motility in skimmed BC powder (Anderson et al., 2019). Prolonged BC feedings (5d) to neonatal calves decreased apoptosis rates in Peyer's Patches of the ileum compared to single colostrum-fed calves, suggesting that colostrum intake reduces lymphoid tissue apoptosis in the ileum (David et al., 2003).

8.1.3. Research gap, hypothesis, and objectives

The existing literature focuses on either the performance and metabolism effects of feeding colostrum or on the histological changes in the enterocytes (see Chapter 1 revision). However, the specific apoptosis mechanisms involved in GIT cell turnover remain unknown in calves. Identifying *in vitro* these mechanisms involved within the interaction of colostrum feedings and gut closure could provide the tools to delay apoptosis and increase the success of the passive transfer of IgG.

The hypothesis of this study was providing colostrum may reduce the rate of apoptosis of calves' enterocytes *in vitro*. Thus, the main objective was to determine in vitro the effect of feeding colostrum on enterocyte turnover, especially on the apoptosis rate. The specific objectives were (1) to set up of primary and secondary cell cultures and characterization by flow cytometry and immunofluorescence microscopy, (2) to establish primary cell cultures of neonatal bovine intestine enterocytes, (3) to establish an LPS challenge model to induce apoptosis on the secondary cell cultures of human colon enterocytes, and (4) to study the impact of colostrum, transition milk, and milk in mitigating LPS-induced cellular necrosis and apoptosis in secondary cell cultures.

8.2. Material and Methods

8.2.1. Animals and collection of intestinal tissues

All animal experiments were conducted in accordance with the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. Three newborns were euthanized by stunning with a captive bolt followed by exsanguination.

8.2.2. Cell culture establishment

To obtain primary cell culture of bovine enterocytes, 10 long cm segments of jejunum were collected from newborn calves and placed in ice-cold washing solution containing PBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1X Penicilin-Streptomycin-Amphotericin (Antibiotic-Antimycotic, Gibco).
Then, the isolation of the epithelial cell was performed following previously described protocols Domènech et al. (2014). The isolated epithelial cells were maintained in Dulbecco's modified Eagle's medium, Nutrient Mixture F12 (DMEM F12, Gibco) supplemented with 2mM L-Glutamine (Glutamax™, Gibco) + 8ug/mL Insulin-Transferrin-Selenium (ITS-G, Gibco) + 50ug/mL Hydrocortisone (Sigma-Aldrich, Sant Louis, MO, USA) + 1X Penicillin-Streptomycin-Amphotericin + 10ug/mL Gentamicin (Gibco) + 10 % fetal bovine serum (Sigma-Aldrich)

To check the epithelial cell phenotype, a tissue immunofluorescence technique was applied following Tsang et al. (2017)'s protocol. Briefly, the cell culture was grown on a coverslip at the bottom of 12-well plate during 24 h at 5 % of CO_2, then the cells were fixed and immunostained using Anti-Fibroblast-specific Protein 1 Primary Antibody (1:500, ABF32, Sigma-Aldrich) and a secondary Antibody tagged with Alexa Fluor 488 (1:250, A11008, Invitrogen, Waltham, MA, USA). The slide was mounted with ProLong AntiFade reagent, including DAPI nuclear stain (Life Sciences, Burlington, ON, Canada). A negative control of cells without a primary antibody was included in the study. Immunofluorescence was detected using a Fluoview FV3000 Confocal Laser Scanning Microscope and analyzed using Fluoview FV31S-SW software for image acquisition (Olympus, Shinjuku, Japan).

In parallel, the secondary culture of Caco-2 cell line (ATCC, Manassas, VA, USA) was thawed according to the manufacturer's protocol and maintained at 37ºC in a culture medium of Dulbecco's modified Eagle's medium supplemented with 4.5 mg/mL glucose + 50 U/mL penicillin + 50 U/mL streptomycin + 4 mmol/L glutamine + 25 mmol/L HEPES + 10 % fetal bovine serum and 5 % CO2 as described by Nighot et al. (2017). The experiment was performed on cells between passages 5 and 10.

8.2.3. Dose-response challenge to Lipopolysaccharide in Caco-2 cells

The experimental design consisted of two main factors, time and LPS (O111:B4, Sigma-Aldrich) in a 5 x 5 factorial arrangement of treatments. The studied time points were 0, 6, 12, 18, and 24 h whereas the LPS treatments were 0, 10, 10^2, 10^3, and 10^4 ng/mL. The LPS was reconstituted in PBS at 1mg/mL and serially diluted in the culture medium to obtain the desired concentrations. Afterward, the dose-response challenge was repeated with the higher dose to observe a dichotomic separation between the CTRL (0 ng/mL) and LPS MAX (10^4 ng/mL) in the time points 0, 6, 18 and 24 h.

Cell culture was performed in biological triplicate for each treatment. Cells were seeded in 12-well plates at $1x10^6$ cells in each well and grown at 37ºC until achieving a confluence of approximately 70 % to 80 % which took approximately 24 h. Then the medium was carefully replaced by the respective LPS treatment which lasted until the set time point for measurement.

Prior to measuring the cell viability, treatment media was removed, and cells were washed with PBS and detached using 1 mL trypsin-EDTA (0.25 %, Thermo Fisher Sci.). The trypsin enzymatic process was neutralized with the same amount of growth media and cells were pipetted up and down 10 times to mix the well. Then 10µL of sample were mixed with an equal volume of Trypan Blue (dilution factor = 2) (0.4 %, Thermo Fisher Sci.) and cells were counted in a Neubauer chamber.

The percentage of dead cells and the concentration of viable cells (live cells/mL) were calculated using the following equations:

$$\text{Viable Cell Count} = \left(\frac{Number\ of\ total\ \ cells}{Number\ of\ \ large\ squares\ counted}\right)\ x\ Dilution\ factor\ x\ 10^4$$

$$\% \text{ of cell mortality} = \left(\frac{Number\ of\ blue\ cells\ (death)}{Number\ of\ total\ cells}\right) x\ 100$$

8.2.4. Bovine colostrum, transition milk, and milk treatments

Bovine colostrum from the first milking, TM from the second day after calving, and milk from 40 d in milk were collected from four multiparous cows from the Palouse Dairy at the University of Idaho (US) and frozen at -80ºC until use.

Before applying the BC, TM, and milk treatments, samples were analyzed for bacterial and fungal contamination. Mammary secretions diluted 1:5 in growth medium were applied for 16 h to 12-well CaCo-2 seeded plates previously treated with LPS 10^4 ng/mL for 6 h. Then, attached Caco-2 cells and incubation treatment (supernatant) were collected separately in 0.75 mL of TRIzol reagent (Invitrogen). DNA was extracted using TRIZol reagent's experimental protocol for cells grown in suspension. Extracted DNA was quantified, and quality checked using the Nanodrop (Thermo Fisher Sci.). PCR was performed using the Amplification of bacterial full-length 16S gene with barcoded primers (Pacific Biosciences, CA, USA) and following the manufacturer's protocol whereas for the fungal ITS amplification, we used the primer pair of ITS4ngs/ITS1 as reported by Huseyin et al. (2017) . The thermocycling conditions consisted of an initial denaturing cycle of 3 min at 95°C, followed by 27 cycles of 30 s at 95°C (denaturation), 30 s at 57°C (annealing), and 60 s at 72ºC (DNA Synthesis) and a final extension of 10 min at 72°C. DNA visualization on stained agarose gels (1 % agarose gel stained with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA)) and visualized under UV light.

The experimental design consisted of two factors, LPS, and mammary secretion, in a 2 x 4 factorial arrangement of treatments. The LPS were CTRL (0 ng LPS/ mL) and LPS (10^4 ng/mL) while the mammary secretion was 1:5 dilutions of BC, TM, milk, and PBS in Caco-2's growth medium. Each mammary secretion treatment was performed in quadruplicated (one per each cow) while the cell culture was performed in biological triplicate for each treatment combination.

8.2.5. Flow cytometry

An Attune NxT flow cytometer (Life Technologies, Thermo Fisher Sci.) was used to analyze cell properties and fluorescent staining. Prior to LPS treatment, Caco-2 cells were stained using CellTrace™ Violet (Invitrogen, Thermo Fisher Sci.), following the alternate method to label adherent cells from the manufacturer's protocol with minor modifications. Concretely, the constituted Cell Tracer™ vial was diluted 1:1000 in PBS and 100µL of the loading solution was inserted into each well. After the mammary secretion treatment, cells were also stained with a Dead Cell Apoptosis Kit with Annexin V-FITC and Propidium iodide (**PI**, V13242, Invitrogen, Thermo Fisher Sci.) to differentiate between apoptotic and necrotic dead cells, respectively. Negative and compensation controls for each dye were included in the experiment. The analysis was performed on the VL1 channel of the flow cytometer for the CellTrace™ Violet, the BL1 channel for the FITC-labeled, and the YL1 channel for the PI. The gate strategy was the following: Total cells were first gated based on size (Forward scatter-area (FSC-A)) and granularity (Side Scatter Area (SSC-A)) to exclude sub-cellular debris, then FSC-A single cells were gated as FSC-Height(H) vs. FSC-A to eliminate doublets. The single cells FSC threshold was set at 10,000 events to eliminate debris and gates were considered to indicate positive and negative staining cells were set based on fluorescence minus one (FMO) tests on the control samples.

8.2.6. Statistical analysis

Cell culture experiments were analyzed by ANOVA and Tukey's multiple comparisons test (GraphPad). All experiments were performed in triplicate and represented as the mean of non-transformed data ± non-transformed standard error of the mean (SEM).

8.3. Results and discussion

In this study, we aimed to determine *in vitro* the effect of feeding colostrum on intestinal cells turnover, especially on the apoptosis rate.

First, neonatal enterocytes were isolated from the bovine jejunum. However, when the epithelial cell phenotype of the obtained cell culture was analyzed,

contamination with fibroblast was observed (**Figure 8.1**). This fact could be due to the fact that the mucosa and muscular layers of the neonatal calf's intestine are less developed than those older calves that received postnatal feeding, complicating the separation of layers during primary cell isolation (Bittrich et al., 2004). One possible solution for future experiments could be, before establishing the primary cell culture, the application of a Trypsin-Versene solution for 1-2 min to selectively remove fibroblast from the primary cell culture, since they are more sensible than enterocyte to the enzymatic digestion (Al-Yaman and Willenborg, 1984; Kaushik et al., 2008).

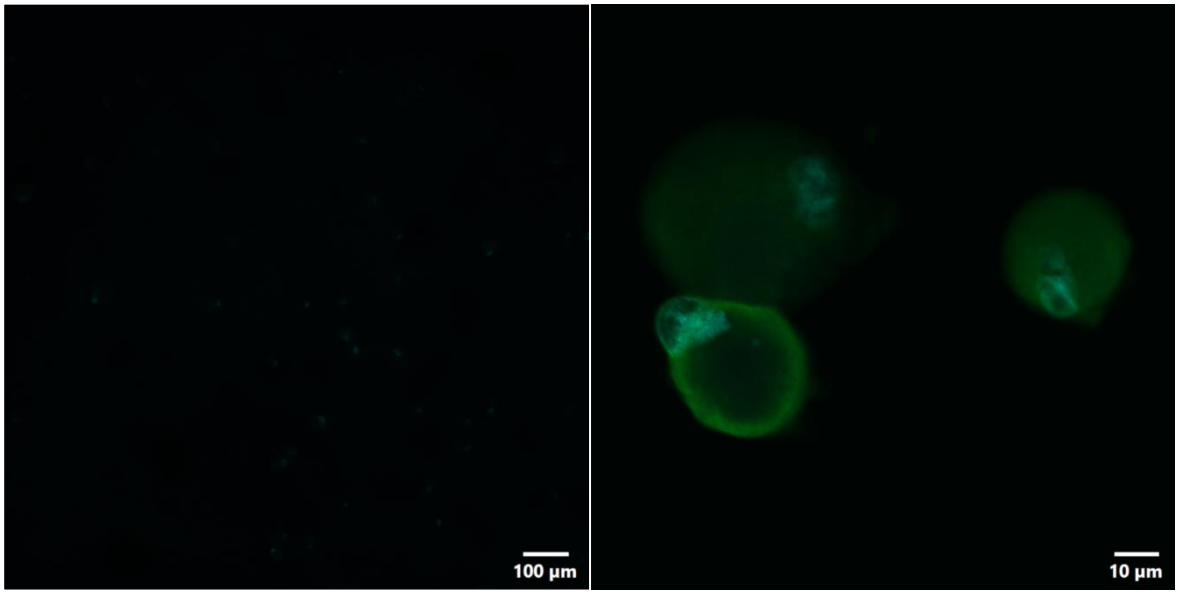

Figure 8.1. Evaluation of the primary cell culture of neonatal calves' enterocytes obtained from jejunum. The images from the fluorescence microscopy show that the cells isolated (cell nucleus, in blue) were fibroblasts (positive in Anti-Fibroblast-specific Protein 1, in green).

After the impossibility of using the isolated primary cell culture due to fibroblast contamination, the study was performed with a commercial Caco-2 cell line. First, a dose-response curve with 5 timepoints and 5 LPS doses was executed to determine the desired timepoint and dose to induce cellular necrosis and apoptosis with LPS. However, unlike the other experiments, dose 0 ng/mL at timepoint 0 h was used as a negative control. In this experiment, the number of live cells decreased ($P<0.05$;

Figure 8.2) with the increase of LPS dose, without interacting with the timepoint factor. The experiment was repeated with the maximum dose of LPS (LPS MAX) looking for the dichotomic separation at a set time point to proceed with the colostrum treatments. In this case, the dichotomic separation in cell mortality between the LPS MAX ($1x10^4$ ng/mL; 25.5 %) and the CTRL (0 ng/mL;10.0 %) was appreciated at 6 h (*P* = 0.08; **Figure 8.3**). These results agree with the literature available since LPS have been shown to induce apoptosis at doses ranging between 1-50 μg/mL, reducing the number of viable cells with increasing LPS concentrations (Yu et al., 2005; Hirotani et al., 2008; Calvello et al., 2016). Additionally, from 1 μg/mL LPS also provokes inflammatory responses that result in the release of reactive oxygen species to protect against infections (Calvello et al., 2016).

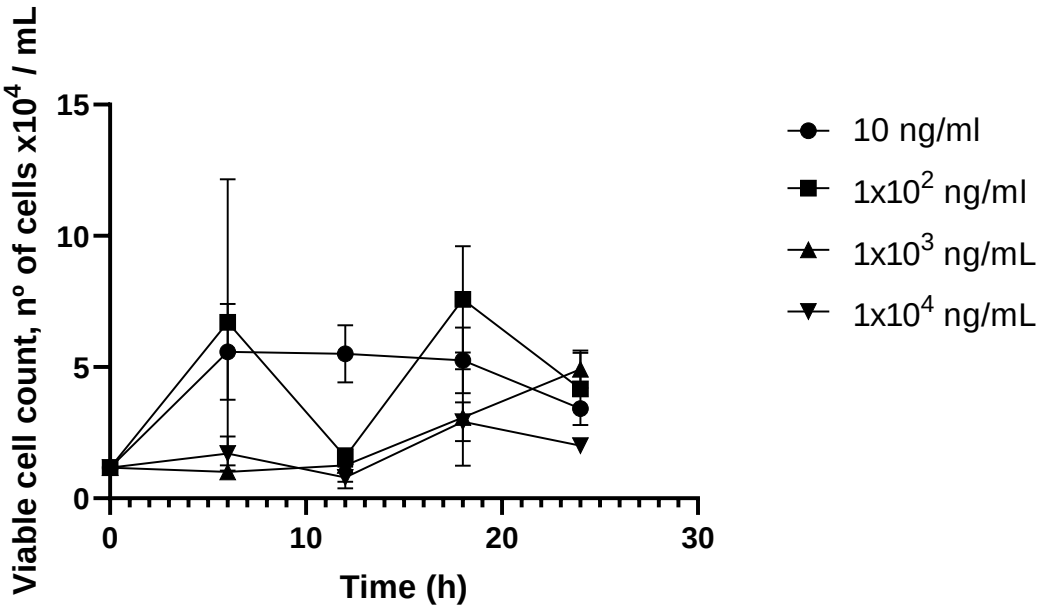

Figure 8.2. Effects of increasing LPS doses to Caco-2 cells at 0, 6, 12, 18 and 24 h. Values are the mean ± standard error of the mean (SEM) (n = 3). Results showed a decrease in the number of live cells (P<0.05) with the increase of LPS dose, without interacting with the timepoint.

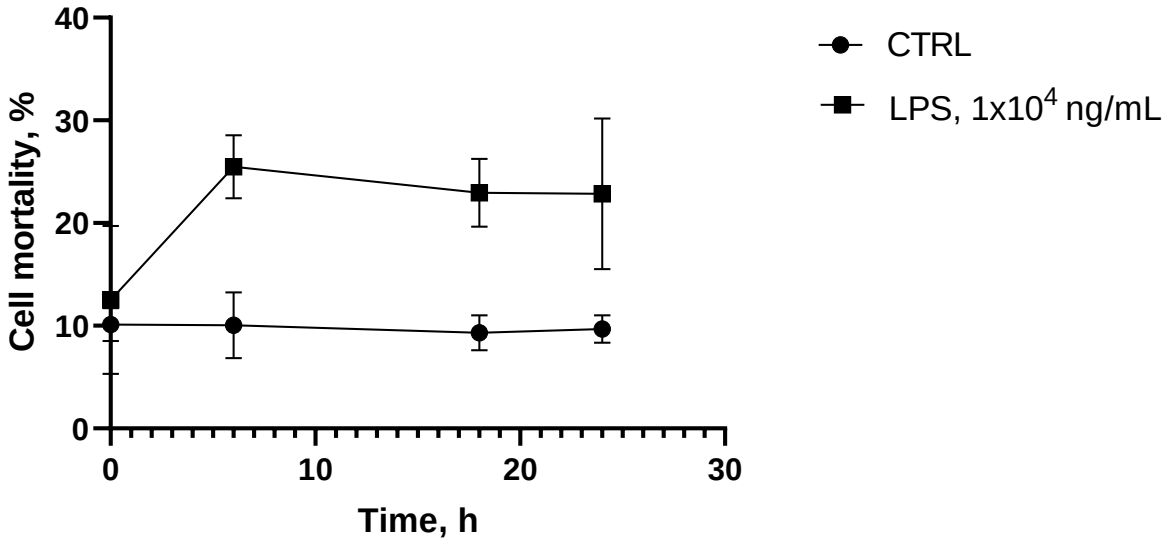

Figure 8.3. Effects of LPS MAX in Caco-2 cells at 0, 6, 18 and 24 h. Values are the mean ± standard error of the mean (SEM) (n = 3). Results showed a dichotomic separation between the LPS MAX and CTRL treatments from 6 to 24 h.

After setting a desired timepoint and dose, BC, TM, and milk samples were checked for microbial contamination. The resulting agarose gel showed no contamination either in Caco-2 cells or incubation treatment, not posing a biological risk to introduce the mammary secretion treatments to the Caco-2 cells.

Finally, preliminary results of applying BC, TM, and milk on apoptotic LPS-damaged cells showed no differences neither in the number of dead cells (marked with PI) or apoptotic cells (marked with FITC-Annexin). However, the positive and negative events of PI-Height were gated against the FITC-Annexin V-Height ones, showing that the samples treated with LPS and TM or milk had greater (P = 0.003; **Figure 8.4**) cellular death (indicated by positive PI events) caused by apoptosis (indicated by positive FITC events) than the LPS-PBS ones. These results suggest that there might be different inherent factors of the mammary secretions involved in cellular apoptosis (as the protein concentration) since the BC treatment to LPS-damaged cells had any protective effect (Anderson et al., 2019). Moreover, our findings are contrary to those reported by David et al. (2003), where the extended BC feedings to calves reduced the apoptotic rates in Peyer's Patches of the ileum. Also, our protocol

for flow cytometry could be optimized, for instance, combining the biological replicates before PI and FITC-Annexin staining to be more time and cost-efficient.

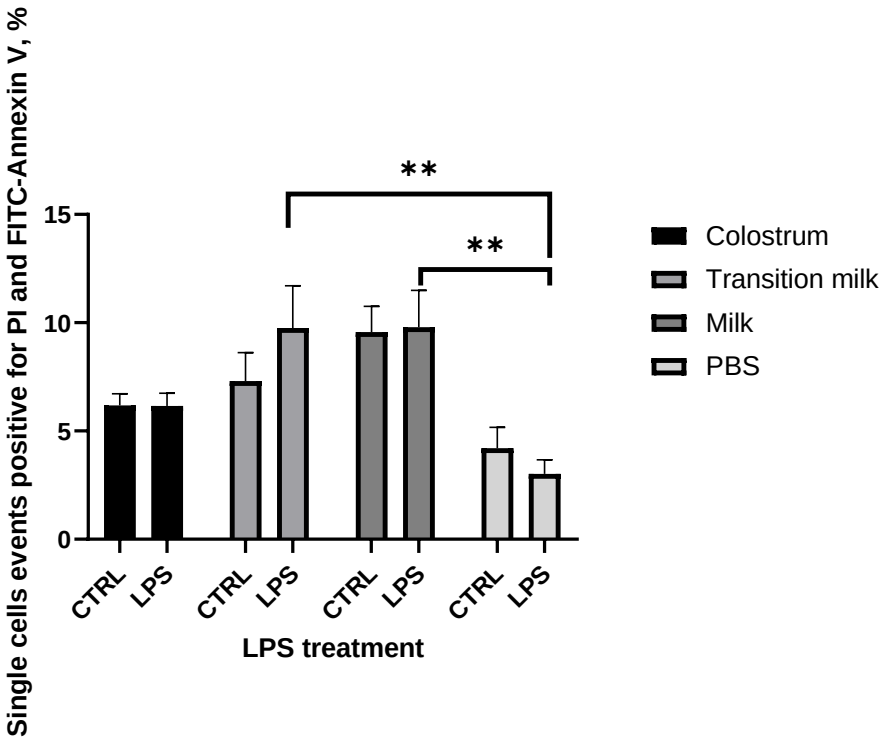

Figure 8.4. Flow Cytometry preliminary results after applying Colostrum, Transition milk, and milk on LPS-damaged Caco-2 cells. The positive and negative events of PI-Height (dead cells biomarker) were gated against the FITC-Annexin V-Height (apoptotic cells biomarker) ones. Values are the mean ± standard error of the mean (SEM) (n = 3). The samples treated with LPS and TM or milk had greater ($P < 0.01$; **) cellular death (indicated by PI) caused by apoptosis (indicated by FITC) than the LPS-PBS ones.

8.4. Conclusions

LPS induced cellular death to Caco-2 cells at doses of $1x10^4$ ng/mL and time of 6 h. In addition, preliminary results of applying TM and milk to Caco-2 cells seem to have a protective effect against cellular death caused by apoptosis. Therefore, further investigations on the mammary secretions' inherent factors that could affect cellular apoptosis are needed.

Chapter 9

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Chapter 10

ACKNOWLEDGEMENTS

Aquesta tesi és una història resiliència i capacitat d'adaptació. Des del camp a la taula, passant pel laboratori. Aquesta és una història de com sortir de la zona de confort, de com sobreposar-te a les inseguretats i de com gràcies a la gent del voltant el camí es fa més planer. Aquesta tesi és una història multidisciplinària, transversal, com la ciència o el coneixement, que no tenen límits. I perquè per entendre el tot, s'ha de començar des de res.

Doncs bé, en aquesta tesi es parteix des de l'esglaó més baix, el calostre que rep el nounat fins arribar al efectes a llarg termini en l'edat adulta. De com composició molecular del calostres pot afectar el creixement, la salut i, fins i tot, a la lactació. Els grans reptes venen amb grans responsabilitats.

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Vaig entrar a l'IRTA com una estudiant de Veterinària i en surto com una jove científica amb ganes de menjar-se el món amb nous reptes.

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