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Recovery of unweaned male dairy beef calves after transportation: impact of colostrum consumption at birth and marketing feeding strategies

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PhD Thesis

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Autonomous University of Barcelona

November 2023

PhD in Animal Production

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PhD Thesis, 2023

Thesis submitted by Lucía Pisoni as partial fulfilment of the requirements for the PhD Degree in Animal Production by the Autonomous University of Barcelona.

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This work has been mainly conducted in the Institute of Agriculture and Food Research and Technology, under the supervision of the doctors Sonia Marti Rodriguez and Maria Devant Guille.

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SUMMARY

Male calves and non-replacement heifers from the dairy industry are fattened for meat production in a system known as dairy beef. Various characteristics inherent to this system involve an initial collection of calves from their dairy farms of origin, stays of different durations at assembly centers or auction markets, and final transportation to the rearing facilities. During this process, transportation and fasting subject calves to physical, nutritional, and physiological stressors that compromise their health, recovery, and welfare. This thesis aimed to characterize the impact of feeding strategies before transport on the nutritional status and gastrointestinal functionality of unweaned calves subjected to transportation.

To respond to our objective, three studies have been conducted. In the first and second studies, we evaluated the effects of colostrum consumption at birth and feed restriction during an assembly center simulation on the nutritional status, the gastrointestinal permeability, and welfare in calves after a long-distance transportation. Results from the first study demonstrated that feeding rehydrating solutions had a detrimental effect on pre-transport energy balance and body weight; in addition gastrointestinal permeability, body weight, and concentrate intake recovery after arrival were also impaired. Feeding low volumes of colostrum at birth numerically decreased concentrate intake recovery during the first week after arrival. Finally, feeding milk replacer instead of rehydrating solutions was not enough to prevent body weight losses, drops in concentrate intake, and alterations of energy balance and gut permeability markers.

The second study was a companion to the first but in this case, we evaluated the effects of colostrum consumption at birth and feed restriction previous to transport on indicators of gut functionality in transported calves. In this study, different segments of the small intestine were used for the assessment of ex vivo gut permeability, histomorphology, tight junction proteins gene expression, and microbiota analysis. Additionally, in vivo gut permeability tests and fecal markers of intestinal inflammation were evaluated. Results from this study demonstrated that colostrum shortage at birth and feed restriction and fasting during transportation negatively impacted gut functionality.

Results showed an increase in the ex vivo gut permeability of the ileum, a downregulation of gene expression of the tight junction protein *CLDN2* in the jejunum, and an increase in the fecal concentration of lactoferrin.

After confirming our hypothesis on the detrimental effects of colostrum shortage at birth and feed restriction during the assembly center and transportation, we conducted a third study to evaluate the nutritional supplementation in the assembly center. The nutritional treatments studied were based on concentrate and/or acidified milk in addition to milk replacer. Results from this experiment showed that increasing the nutrients and energy provided by pre-transport supplementation with concentrate or acidified milk improved body weight and concentrate intake recovery at arrival. Additionally, the combination of concentrate with acidified milk offered ad libitum showed improvements in the gastrointestinal permeability of calves.

RESUMEN

Los terneros machos y las terneras no destinadas a la reposición en la industria láctea son engordados para la producción de carne en un sistema conocido como "*dairy beef*". Varias características inherentes a este sistema implican una recolección inicial de los terneros de sus granjas lecheras de origen, estancias de diferentes duraciones en centros de concentración o mercados de subasta, y su posterior transporte a las instalaciones de engorde. Durante este proceso, el transporte y el ayuno someten a los terneros a factores estresantes físicos, nutricionales y fisiológicos que comprometen su recuperación y bienestar. Esta tesis tuvo como objetivo caracterizar el impacto de las estrategias de alimentación previas al transporte en el estado nutricional y la funcionalidad gastrointestinal de terneros no destetados sometidos a transporte.

Para responder a nuestro objetivo, se han llevado a cabo tres estudios. En el primer y segundo estudio, evaluamos los efectos del consumo de calostro al nacimiento y la restricción alimentaria simulando una estancia en un centro de concentración en el estado nutricional, la permeabilidad gastrointestinal y el bienestar después de un transporte de larga duración. Los resultados del primer estudio demostraron que la alimentación con soluciones rehidratantes tuvo un efecto perjudicial en el balance energético y el peso corporal previo al transporte. Así como en la permeabilidad gastrointestinal y la recuperación del peso corporal y la ingesta de concentrado después de la llegada. Además, alimentar con bajos volúmenes de calostro al nacer disminuyó numéricamente la recuperación de la ingesta de concentrado durante la primera semana después de la llegada. Finalmente, la sustitución de la solución rehidratante por lactoreemplazante no fue suficiente para prevenir las pérdidas de peso corporal, las disminuciones en la ingesta de concentrado y las alteraciones del balance energético y los marcadores de permeabilidad intestinal.

El segundo estudio fue complementario al primero; en este caso se profundizó en la evaluación de los efectos del consumo de calostro y la alimentación previa al transporte sobre la funcionalidad digestiva. En este estudio, se utilizaron diferentes segmentos del intestino delgado para la evaluación de la permeabilidad intestinal ex vivo, la histomorfología, la expresión génica de

proteínas de unión estrecha, y el análisis de la microbiota intestinal. Además, se realizaron pruebas de permeabilidad intestinal in vivo y se midieron marcadores fecales de inflamación intestinal. Los resultados de este estudio demostraron que la escasez de calostro al nacer y la restricción alimentaria y el ayuno durante el transporte impactaron negativamente la funcionalidad gastrointestinal. Los resultados mostraron un aumento en la permeabilidad intestinal ex vivo del íleo, una menor expresión génica de la proteína de unión estrecha *CLDN2* en el yeyuno, y un aumento en la concentración fecal de lactoferrina.

Después de confirmar nuestra hipótesis sobre los efectos perjudiciales de la escasez de calostro y la restricción alimentaria previa al transporte, llevamos a cabo un tercer estudio para evaluar la suplementación nutricional en el centro de concentración. Los tratamientos nutricionales estudiados fueron a base de concentrado y/o leche acidificada ad libitum. Los resultados de este experimento mostraron que aumentar los nutrientes y la energía proporcionados por la suplementación previa al transporte con concentrado o leche acidificada tuvo un efecto positivo en la recuperación del peso corporal y la ingesta de concentrado a la llegada. Además, la combinación de concentrado con leche acidificada ofrecida ad libitum mostró mejoras en la permeabilidad intestinal de los terneros.

RESUM

Els vedells mascles i les vedelles no destinades a reposició en les granges lleteres són engreixats per la producció de carn en el sistema conegut com el vedell mamó (*"dairy beef"*).Les característiques diferencials d'aquest sistema de producció són que els vedells són recollits de diferents granges lleteres, traslladats a mercats o centres de concentració on hi poden passar varius dies fins que són traslladats finalment a les granges d'engreix. Durant aquest procés els vedells pateixen estrès del propi transport i restricció alimentaria que comprometen la seva salut, recuperació i benestar animal. Aquesta tesi va tenir com objectiu caracteritzar l'impacte de les estratègies alimentaries prèvies al transport en l'estat nutricional, la funcionalitat gastrointestinal dels vedells mamons que han sigut transportats.

Per respondre al nostre objectiu, s'han dut a terme tres estudis. El primer i segon estudis, vam avaluar els efectes del consum de calostre al néixer i la restricció alimentaria simulant la seva estada en un centre de concentració sobre l'estat nutricional, la permeabilitat gastrointestinal i el benestar després d'un transport de llarga durada. Els resultats del primer estudi van demostrar que l'alimentació amb rehidratant va tenir un efecte perjudicial en el balanç energètic i el pes corporal abans del transport; així com en la permeabilitat gastrointestinal i la recuperació del pes corporal i la ingesta de concentrat després de l'arribada. A més, alimentar amb volums baixos de calostre al néixer també va disminuir el consum de pinso numèricament durant la primera setmana després de l'arribada. Finalment, la substitució de la solució rehidratant per lactoreemplaçant no va ser efectiva per prevenir les pèrdues de pes corporal, la reducció de consum de pinso i els increments de biomarcadors de balança energètic i permeabilitat gastrointestinal.

El segon estudi va ser complementari al primer; en aquest cas es va aprofundir en avaluar els efectes del consum de calostre i l'alimentació prèvia al transport sobre la funcionalitat digestiva. En aquest estudi es van avaluar diferents segment dels intestí a través de l'estudi de permeabilitat intestinal ex vivo, la histomorfologia, l'expressió gènica de proteïnes d'unió estreta, i l'anàlisi de la microbiota. A més es va realitzar proves de permeabilitat in vivo i es van mesurar marcadors fecals

d'inflamació intestinal. Els resultats van mostrar un augment de la permeabilitat intestinal ex vivo del ili, una menor expressió gènica *CLDN2* dal jejú, i un augment de la concentració de lactoferina en femtes.

Després de confirmar la nostra hipòtesis sobre els efectes negatius de l'escassetat de calostre i la restricció alimentaria prèvia al transport, vam dur a terme un tercer estudi per avaluar la suplementació nutricional en el centre de concentració. Els tractament nutricionals estudiat va ser a base de concentrat i/o llet acidificada a lliure elecció. Els resultats van mostrar que augmentar els nutrients i l'energia proporcionada per la suplementació prèvia de concentrat o llet acidificada va tenir un efecte positiu en la recuperació del pes corporal i el consum de concentrat a l'arribada. A més, la combinació de concentrat i llet acidificada van mostrar millores en la permeabilitat gastrointestinal dels vedells.

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CHAPTER VI: General Discussion

Annex 1: Simulation of feed restriction and fasting: effects on animal recovery and gastrointestinal permeability in unweaned Angus-Holstein calves

Annex 2: Effects of different management strategies at arrival to improve dairy beef calf recovery after marketing and transportation: preliminary data

CHAPTER I

INTRODUCTION

1. The dairy beef production system

1.1. Surplus calves: "by-products" of the dairy industry

The dairy beef production system consists of fattening male dairy calves and dairy heifers that will not be used as replacements with the ultimate goal of meat production. Because the primary goal of the dairy industry is milk production, these calves have traditionally been considered a "surplus" or "by-product" and intended to be removed from the farm relatively quickly to avoid feeding and management costs (Haskell, 2020). The most common destinations for these calves include on-farm euthanasia after birth, "bobby" calf production, veal and rosé beef production, and the dairy beef production. On-farm euthanasia is conducted right after birth (Creutzinger et al., 2021a). This practice is allowed in some countries, such as the UK, where 15% of male calves born at dairy farms were euthanized in 2018 (Rutherford et al., 2021a). However, public concern is putting strong pressure on legislation to abolish this practice (AHDB Annual Report & Accounts, 2020-2021). On the other hand, countries like Australia and New Zealand produce "bobby" calves which are reared at dairy farms and, between 1 week and 14 d of age, transported to slaughter for leather, pet food, and by-products of the pharmaceutical industry (Cave et al., 2005; Haskell, 2020). Only in Victoria, Australia, approximately 600,000 bobby calves are slaughtered each year (Cave et al., 2005), and 5% of male calves born in Canada are used for this productive system (Renaud et al., 2017a). In Europe, veal production is a strong and well-developed industry (Pardon et al. 2014). Veal calves are slaughtered unweaned between 0 to 8 months of age while are still being fed milk. White veal calves are fed specific milk diets, therefore on a low-iron, low-fiber diet and represent the largest proportion of veal in Europe, whereas rosé veal calves are grain-fed and slaughtered before 8 months of age (Pardon et al., 2014a). On the other hand, surplus calves raised for red meat in the dairy beef system, may be fattened up to 12 months of age and are classified as young beef or up to 16 months of age and classified as beef. In other countries like United States or Canada the dairy beef calves can be fattened up to 24 months of age (European Commission Publication Office, 2014; Sanchez et al., 2021; Renaud and Pardon, 2022). In Spain, calves arrive at rearing facilities unweaned and are milk fed with ad libitum access to concentrate until 6-8 weeks of age and after are moved to the fattening unit where they will be fed concentrate and straw separately (Sánchez et al., 2022). In other countries with greater forage production capacity, dairy calves are fed a total mixed ration (TMR) during the fattening phase. The use of surplus calves as a dairy beef production system has been growing steadily in Europe and the rest of the world in recent decades because it is linked to dairy production. The dairy beef production system is the one of interest in this review and its inherent characteristics are described in more detail below.

1.2. Importance of the dairy industry in meat production

Lactation in cows starts with calving (Tucker and Lansing, 1981). In a beef production system, calves suckle from their mother for 6-8 months until weaning (Reinhardt and Reinhardt, 1981). In the dairy industry, on the other hand, calves are separated from their dams immediately after birth and fed milk replacers for economic (increased sellable milk), presumed ethical (to prevent the formation of a maternal bond that is increasingly difficult to break), and health reasons (reduced risk of pathogen transmission from the dam to the newborn calf) (Beaver et al., 2019a). Considering that the probability of a dairy cow delivering a male calf is 50% and that it has been shown that 60% of the lactating cows are enough to deliver the number of replacement heifers required for the herd (De Vries et al., 2008a), the outcome is a significant increase in the number of calves from the dairy industry (Haskell, 2020) that are later fattened for meat production.

The dairy beef industry's contribution to total beef production can be significant, exceeding the national beef herd in some countries (Berry, 2021). Approximately 11 million non-replacement dairy calves are born annually in the European Union (Mekonnen et al., 2019). Available records for the estimation of female surplus calves are limited, however, it is expected to increase with the implementation of sexed semen in the dairy industry (De Vries et al., 2008a). The number of dairy calves slaughtered for beef is unclear, however, it is estimated that 2/3 (approximately 30%) of the beef supply in Europe is produced by dairy cows (Vinci, 2022). Only the veal industry slaughters around 4 million calves a year producing 600000 Tn of meat in 2022 (Agridata, European Commission and FEFAC, 2021) in countries like France, The Netherlands, and Italy with a veal production of 27%, 25%, and 16%, respectively, in 2010 (Pardon et al., 2014b). In England, the proportion is about half of the total beef being produced by the dairy beef industry according to the AHDB Dairy Mobility Scoring System (AHDB Annual Report & Accounts, 2020-2021). Meanwhile, the number of male calves in the USA and Canada coming from the dairy industry in 2020 was greater than 5 million (Renaud and Pardon, 2022) and it is estimated that U.S. dairy beef accounts for approximately 18% of all beef and veal marketed (Lowe et al., 2009). In New Zealand, 65 % of beef production comes directly or indirectly from dairy cattle (Morris, 2008).

1.3. Dairy beef production and sustainability

Sustainability has been previously defined as the overlap between what the current generation wants, reflecting social and economic concerns, and what is ecologically possible in the long term (Vavra, 1996). Whereas the USDA defines agricultural sustainability as "the efficient production of food that meets the current generations' needs for food and quality of life, enhances the environment and natural resources, and does not compromise the productive capability of future generations" (USDA, 2007). The three main pillars of sustainability are economic, social, and environmental (Purvis et al., 2019). Predictions of the world population increasing over the next 30 years will lead to a surge in animal product consumption, therefore, addressing the sustainability of livestock systems is essential (Chemineau, 2016a).

The social pillar of sustainability in livestock production is mainly concerned with how animal production is perceived by society. Within dairy-beef production, certain characteristics inherent to the industry create consumer discomfort. One characteristic is the management of calves based on their gender. Male calf management is typically more neglected when compared to heifer care (Fecteau et al., 2002; Renaud et al., 2017b; Shivley et al., 2019a). The main differences are related to the lower quantity and quality of colostrum and feed offered to male calves, and negligence in navel disinfection and vaccination (Renaud et al., 2017b, 2020). The result is an increased prevalence of failure in the transfer of passive immunity (**FTPI**) and increased susceptibility to suffering from disease. In practice on dairy farms, the amount of milk offered, the timing, the temperature at the time of feeding, access to starter feed, bedding conditions, ad libitum access to fresh and clean water, etc., are usually neglected when it comes to unweaned male calves.

Furthermore, these calves are transported at a young age (approximately 14 d of age in Europe and a few days of age in North America and Australia) and subjected to feed and water restrictions. At this time, calves are still dependent on strict milk consumption and these nutrient restrictions further reduce their body reserves and ability to cope with the negative energy balance and stress associated with transport and market periods. These challenges have not gone unnoticed by consumers, who have expressed discontent with the handling of these calves, negatively impacting their consumer preferences (Haskell, 2020). Some welfare concerns have to do with the methods of castration and the age at which this is performed (Rutherford et al., 2021a). Others have to do with subjecting these surplus calves used in the veal and dairy beef production systems to long hours of transport and fasting (Roadknight et al., 2021). Additionally, because the time inside the truck is equivalent to time without being fed, transportation directly impacts welfare (Bolton and von Keyserlingk, 2021). Maternal separation and access to pasture versus indoor housing are also controversial welfare issues of concern to consumers (Mandel et al., 2022). Maternal separation raises concern because calves are not allowed to suckle milk or exhibit normal behavior between the dam and the calf (Beaver et al., 2019b; Webb et al., 2023a). Indoor housing and limited space are also detrimental to calf welfare. The use of individual hutches in the dairy industry isolates calves and it has been shown to impair their social skills and affect their emotions and moods (Costa et al., 2016; Bučková et al., 2019). Also, confinement in concrete flooring has negative effects on lameness (Rutherford et al., 2021a). In a study analyzing the impact on animal welfare depending on the type of production (dairy vs. beef), animals from dairy herds were more likely to suffer negative welfare due to a greater level of intervention compared with those used solely for meat production, something that was particularly true for dairy calves raised for veal production (Mandel et al., 2022). Disease control at the rearing facilities is another difficult task when considering that surplus calves come from different farms of origin and may have been exposed to different infectious agents. The poor condition of calves at arrival gets worsened by navel infections, scours, respiratory disease, and FTPI (Wilson et al., 2000; Pempek et al., 2017). Previous research has shown that 20% of male calves arriving at auction markets had health abnormalities, being navel disease the most prevalent (Wilson et al., 2020a). In addition, transportation and time spent at the assembly center may act as amplifiers in the spread of disease (Creutzinger et al., 2021b). Because of the high vulnerability to disease and exposure to pathogens, the first weeks after arrival at the rearing facilities are a high-risk period for calves. The increase in morbidity and mortality rates (USDA, 2017) on the one hand, and the subsequent increase in the use of antibiotics, especially their use as a metaphylactic measure, (Renaud and Pardon, 2022) on the other hand, are two serious drawbacks of this production system rising social concern. In previous studies conducted in Canada, researchers found that a total of 68% of calves were treated with antibiotics and 42% of calves died during the first 3 weeks after arrival (Renaud et al., 2018a; Scott et al., 2019a). However, Renaud and Pardon (2022) argued that the intensive use of antibiotics is not only a problem in terms of generating antimicrobial resistance, especially in the veal sector (Catry et al., 2005), but that previous studies have shown inconclusive results on their positive effects in preventing disease in calf herds, indicating that their indiscriminate use may not even have a positive effect.

The economic pillar of livestock sustainability is associated to the degree of specialization of the farms (Chemineau, 2016b). Fattening calves with high concentrate diets at conventional feedlots lowers the slaughter age and increases slaughter weight when compared with grass-fed systems (Capper, 2012). Conventional beef production can be differentiated based on the origin of calves (calves sourced from sucklers and calves from the dairy industry), and the type of feed used during fattening (roughage or concentrate-based) (de Vries et al., 2015). The conventional beef production system can provide a more uniform product in contrast to pasture-based meat production, where carcass quality differs greatly depending on factors such as region, forage quality, and climate (Klopatek et al., 2022). Another distinction between the two systems concerning their ultimate products is that in the dairy beef system, a cow provides milk, meat, and a surplus calf that will be fattened for meat, whereas in the grazing system meat is produced by the cow and its calf (de Vries et al., 2015). Considering these differences between systems, the analysis of their environmental and economic impacts may vary. Despite being conventional or grass-based, beef production has a major environmental impact accounting for 41% of global emissions in greenhouse gases (**GHG**) (Opio et al., 2013), nitrogen losses contributing to acidification and eutrophication, land use, and deforestation, transportation, among others (Rutherford et al., 2021b; Faverdin et al., 2022).

Due to the projected rise in worldwide demand for beef in the coming years, a need exists for further study of strategies to reduce the environmental impact of the beef production system (De Vries et al., 2008b). The third pillar of livestock sustainability explores this environmental impact via life cycle assessments (**LCA**) analysis. Previous studies have shown that the carbon footprint of dairy beef production using surplus calves is low because these calves have a high conversion rate and are slaughtered at a young age compared to calves from beef suckler herds, demonstrating the efficiency of the dairy beef production system (de Vries et al., 2015; Hietala et al., 2021). Additionally, dairy x beef crossbreds have higher efficiencies than pure Holstein calves (Hessle et al., 2017a) and some studies in European Nordic countries and France have demonstrated that the environmental impact on global warming of producing 1 kg of beef meat was lower when the origin was from dairy calves than from suckling cattle (Nguyen et al., 2013; Mogensen et al., 2015). Hessle et al. (2017) argued that to diminish the environmental impact of dairy and beef production systems, production efficiency must improve. However, the dairy and beef production systems are closely connected and the potential for environmental improvements of both has to be analyzed simultaneously (Hessle et al., 2017b). For example, reductions in the environmental impact are possible through the use of dual-purpose dairy-bred beef calves or from dairy cows crossbred with beef, which improves the growth performance and feed efficiency of dairy beef (Hietala et al., 2014). The use of sexed semen, crossbreeding, or dual-purpose cattle breeds increases the value of surplus calves (Webb et al., 2023b). The sum of highly specialized dairy and beef breeds and the interconnection between both systems results in a higher emission efficiency with a lower environmental impact on global warming, eutrophication, and land use (Webb et al., 2023).

One of the biggest weaknesses of the dairy beef industry nowadays is the disinformation gap that exists between the dairy farms producing surplus calves and the rearing facilities receiving them (Creutzinger et al., 2021c). This disconnection is linked to the lack of knowledge in the management of calves in terms of colostrum consumption at birth, animal care, nutritional and health status, vaccinations, and performance, among others. This thesis will address some of the main concerns related to welfare and health issues during the first weeks of life of dairy beef calves.

1.4. The importance of colostrum administration

During prenatal development, the transfer of immunoglobulins (Ig) between the fetus and the dam is limited due to the low permeability of the syndesmochorial cotyledonary placenta (Furukawa et al., 2014). This characteristic makes the newborn reliant on consuming colostrum to obtain immune factors and transfer them into the bloodstream in a process known as “transfer of passive immunity” (Lombard et al., 2020). The fact that calves are born agammaglobulinemic does not mean they lack an immune system, but the ability to defend against potentially harmful pathogens, in other words, they lack immunocompetence (Hulbert and Moisá, 2016). From birth until the first 24 hours after birth, the linear absorption of Ig in the gut is almost completely restricted by a phenomenon called gut closure (Bush and Staley, 1980; Godden et al., 2019). In general terms, to avoid FTPI, calves need to consume a minimum of 150 to 200 g of IgG shortly after birth in approximately 3-4 L of colostrum (10-12% of their BW) (Godden et al., 2019). Unfortunately, in practice, it has been shown that between 12 % to 43% of the calves entering the veal or dairy beef industry suffer FTPI (Renaud and Pardon, 2022). This problem is recurrent in many countries and is entirely related to the fact that male calves are considered a by-product in the dairy industry and therefore their care and management are inferior to that of replacement heifers (Lovell and Hill, 1940; Rutherford et al., 2021c). Usually at dairy farms, it has been shown that heifers receive higher volumes of less contaminated colostrum compared to male calves (Fecteau et al., 2002) who in some cases receive no colostrum at all (Renaud et al., 2017c). Male calves are also more likely to consume colostrum directly from the dam and at delayed times compared to heifers (Shivley et al., 2019b). The end result of this poor management is a lower concentration of serum total protein in male calves which translates into FTPI and increases disease susceptibility and mortality rates (Godden et al., 2019).

However, the importance of colostrum consumption goes beyond Ig absorption, it also determines the passage of nutrient and non nutrient components that are fundamental to the health, growth, and development of newborn calves. For example, the energy provided by lactose and fat is key for thermoregulation in newborn calves (Godden et al., 2019). In addition to micronutrients such as minerals and vitamins, and macronutrients such as protein, fat, lactose, etc., colostrum is composed of non nutrient or bioactive compounds (Table 1) that have been shown to positively influence morphological and functional changes in calves (Blum and Baumrucker, 2008).

Table 1. Composition (especially of nitrogen-containing and bioactive compounds) of bovine colostrum

		Colostrum milkings				
		1	2	3	4	5/6
Essential amino acids	mmol/l	390	230	190	140	115
Nonessential amino acids	mmol/l	490	290	240	170	140
Immunoglobulin G	g/l	81	58	17	12	ND [b]
Lactoferrin	g/l	1.84	0.86	0.46	0.36	ND
Transferrin	g/l	0.55	0.44	0.39	0.21	ND
γ-glutamyltransferase	µkat / l	509	284	145	102	83
Alkaline phosphatase	µkat / l	19	8	3	2	1
Aspartate aminotransferase	µkat / l	1.5	0.9	0.5	0.3	0.20
Tumor necrosis factor-α	µg/l	5	ND	ND	ND	3
Insulin	µg/l	65	35	16	8	7
Glucagon	µg/l	0.16	0.08	0.08	0.05	0.03
Prolactin	µg/l	280	180	150	120	ND
Growth hormone	µg/l	1.4	0.5	<1	<1	<1
IGF-I	µg/l	310	195	105	62	49
IGF-II	µg/l	150	ND	ND	ND	ND

[a] Modified from Blum, J. W., and H. Hammon. 2000. Colostrum effects on the gastrointestinal tract, and on nutritional, endocrine, and metabolic parameters in neonatal calves. Livest. Prod. Sci. 66:151–159. doi:10 .1016/S0301-6226(00)00222-0.
[b] Not determined.

Most of these bioactive compounds are present in higher concentrations in the first colostrum and decrease in subsequent milkings (Blum and Hammon, 2000). This concept is particularly important when feeding colostrum to newborn calves, as calves fed subsequent colostrum milkings (transition milk) will have less availability of these substances and therefore less chance of these bioactive compounds exerting beneficial effects. Some bioactive compounds such as lactoferrin, lactoperoxidase, and lysozyme have antimicrobial activity whereas it has been suggested that oligosaccharides may contribute to the development of the gut microbiome by acting as a substrate for beneficial microorganisms such as *Bifidobacterium* (Elfstrand et al., 2002; Fischer et al., 2018). Evidence in the literature suggests that the gastrointestinal tract (**GIT**) may be the most affected organ by the bioactive compounds in colostrum and several studies have demonstrated their positive influence on its development and function (Blum and Hammon, 2000; Nissen et al., 2017; Hammon et al., 2020). Colostrum consumption has been shown to increase crypt cell proliferation, decrease apoptosis, and stimulate villus growth in calves (Blum, 2006). Increased crypt cell proliferation in calves fed high amounts of colostrum compared to calves fed milk replacer or formula has been demonstrated previously (Blättler et al., 2001). This concept is quite important when considering that an increase in villus growth in the small intestine due to colostrum consumption translates as an increase in the absorptive capacity (Blättler et al., 2001; Blum and Baumrucker, 2008). Emphasizing the relevance of the bioactive compounds, a previous study showed that supplementation of a milk replacer formula with human insulin-like growth factor I (**IGF-I**) increased intestinal villus size in neonatal calves (Roffler et al., 2003). Bioactive factors such as IGF-I and insulin are poorly absorbed and therefore exert their growth-stimulating effects locally on the intestinal mucosa of calves (Baumrucker et al., 1994; Donovan and Odle, 1994; Blum, 2006; Hammon et al., 2013). However, research has shown that it is not the individual action of the bioactive compounds, but rather the interaction of a large number of factors that are responsible for the beneficial effects on intestinal cell proliferation and growth (Hammon et al., 2012).

1.5. Marketing and transportation

As previously commented, one of the weaknesses of the dairy beef industry in terms of management is the marketing and transportation phase of unweaned calves due to their structure. Although the number of dairy farms in the EU has decreased by 62% since 2005, the number of cows has not decreased proportionately resulting in the disappearance of farms and a higher cow concentration (Requena-i-Mora and Barbeta-Viñas, 2023). The average herd size is still low with an average of 55 cows/farm (Augère-Granier, 2018). For example, Germany has a range between 7 and 2000 cows/herd with an average of 122 cows/farm (Lindena and Hess, 2022), the Netherlands has an average of 101 cows/herd, Ireland has an average of 93 cows/herd, France makes 70 cows/herd, and the UK has an average herd size of 160 cows in 2021 (AHDB Annual Report & Accounts, 2020-2021). This means that when the number of cows/herd is low, the number of calves born each week is also low, so more transportation is needed to multiple farms to collect the number of calves needed by the veal producers (Schnyder et al., 2019). For example, a previous study conducted in Belgium showed that the median number of dairy farms per 10 transported calves was 10 (Schnyder et al., 2019). This limitation of the system increases the risk of contagious diseases due to the multiple origins of the calves. In addition, moving dairy calves from dairy farms to beef farms requires a structure based on auction markets and concentration centers. Therefore, the structure of the dairy industry increases the risk of morbidity and mortality of calves due to the massive commingling of the system.

1.5.1. Legislation

The transportation of young calves is regulated by the legislation of each country. In the USA, for instance, the transport of unweaned calves is governed by the "Twenty-eight Hour Law" which states that farm animals cannot be transported for more than 28 consecutive hours. After that time, the animals must be unloaded for at least 5 hours to get feed, water, and rest (National Archives and Records Administration, USDA,1974). In Canada, the Health of Animals Regulations (**HAR**) limit the transport time of livestock 8 d of age or less to 12 hours, after which the animals must be unloaded, fed, and allowed to rest. Especial attention is put on the transport of male dairy calves during the first week of life in section 141 of this regulation. This section considers age-specific risk factors that make calves more susceptible to long-distance transportation like the dependence on milk as their only food source, their inherent risk of suffering dehydration, and their immature immune system, among others. Additionally, it also defines guidelines for the loading and unloading of calves and identification of unfit animals for transport [Health of Animals Regulations (HAR), 2019]. Other countries like Australia, New Zealand, and the UK, also count on their legislation on transport policies for calves (Australian Animal Welfare Standards and Guidelines, 2009, Australia; The Welfare of Animals During Transport, 2006, England; Code of Welfare: Transport within New Zealand, 2018, New Zealand). In Australia, unweaned calves younger than 5 days of age can only be transported for 6 hours, while the maximum time off water for calves between 5 to 30 days of age is 18 hours, according to the Australian Animal Welfare Standards-Land Transport of Livestock

(Animal Health Australia, 2012). In the European Union (**EU**), Regulation (EC) Nº 1/2005 on the protection of animals during transport regulates transport characteristics for live animals. For unweaned calves, this legislation limits transport to animals that are at least 14 days old. Additionally, after traveling for 9 hours, unweaned calves must be given a break of at least 1 hour and be offered water and "if necessary" fed. After this rest period, they can be transported for another 9 hour (European Council, 2005). Even though Regulation (EC) Nº 1/2005 applies to all member states, some countries have adopted additional rules in their national legislation. For example, Germany has recently added new amendments regarding the age of calves allowed to be transported, increasing the transport age to 28 days. Additionally, Germany set a maximum national transport duration of 8 hours for unweaned calves. Other countries like Sweden do not permit long distance transport of unweaned animals (Reenen et al., 2022).

1.5.2. Transport within the EU

According to recent reports, nearly 1.4 million calves were transported across the EU borders between 2015 and 2020, with 42% (580,000) of these calves subjected to long journeys of more than 8 h (Reenen et al., 2022) (Figure 1). The typical dynamic of the dairy beef industry relies on a network of short and/or long-distance transports connecting those countries specialized in milk production, and therefore producing dairy calves, with rearing countries dependent on imports. According to Velarde et al. (2021), the main countries exporting calves in Europe in 2019 were Germany (44% of all calves exported in the EU), France (18%), and Ireland (8%). Whereas the main importers were The Netherlands (57% of all imported calves), Spain (30%), and Italy

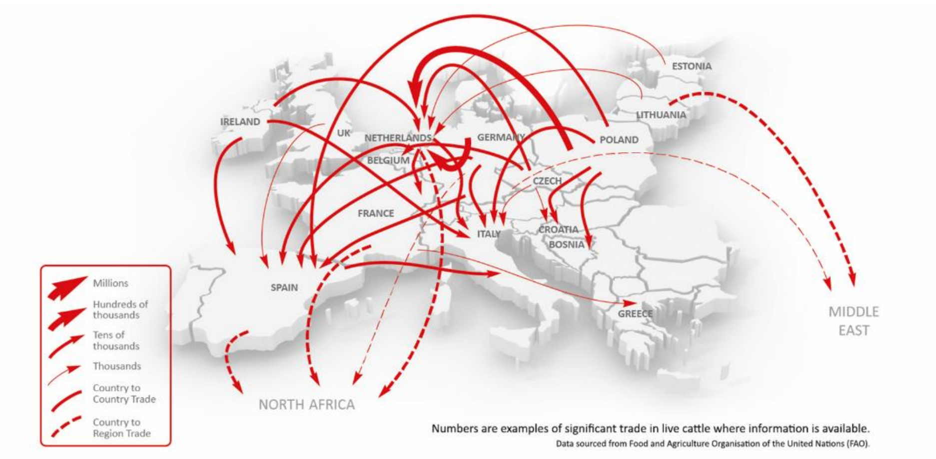

Figure 2. *Transportation of unweaned calves among member states of the European Union. © 2019 Compassion in World Farming*

(6%). Of interest, most of the European countries seemed to import calves from neighboring member states (**MS**). For example, The Netherlands imports calves mainly from Germany (69%) and Belgium (7%) and Spain does it mainly from France (64 %) (Velarde et al., 2021; SITRAN, 2021). However, imports from MS located further away are also a fact, for example Spain imports calves form Ireland (10%) and the Czech Republic (9%) (SITRAN, 2021). In addition to the international market, there is a national marketing network within different regions of the same country. For example, in Spain, the internal movement of calves involves transport distances between 4 to 12 hours. However, when importing calves from other MS, distances can go up to more than 20 hours if coming from Germany, 4 to 19 hours if coming from France (depending on the region), and up to 4 to 5 days when calves are being imported from Scotland. Catalonia is one of the most important beef production regions of Spain. It receives 51% of the imports from which 70% are male calves and 30% are females (SITRAN, 2021). Of the total of the imported calves, 54% were younger than 2 months of age, weighing approximately 56-60 kg, with the Holstein Friesian breed or crossbred accounting for the majority of males and the crossbreed for the majority of females (SITRAN, 2021). Once calves arrive at the rearing facilities they are fatten before being sent to slaughter.

1.5.3. Transport dynamics

Depending on their market destination, calves may be transported from their origin farms directly to rearing facilities or slaughterhouses, or they can be held at assembly centers, or auction markets before reaching rearing facilities (Figure 2). In an international calf-buying scenario, calves from a nearby dairy farm arrive at the assembly centers or auction markets, where they are

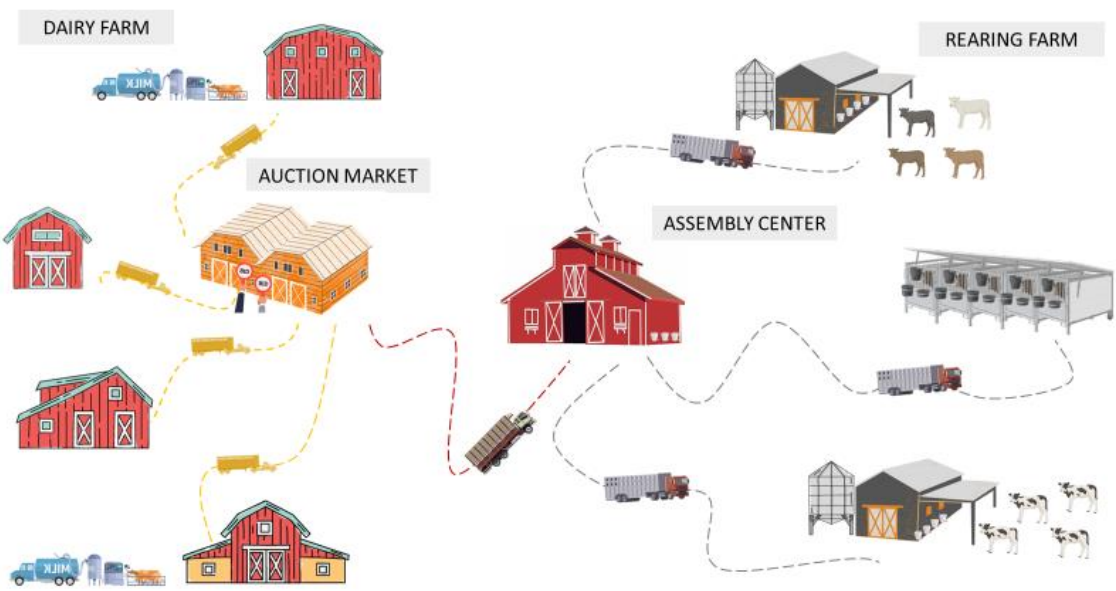

Figure 2. An overview of the transport dynamics in the marketing of dairy beef calves.

grouped into lots based on buyers` requests and later transported, usually over long distances, to be raised for veal or beef (Velarde et al., 2021). Calves can remain at these collection centers for several hours up to a few days. Electrolyte-based diets or milk replacers are the two diets typically provided at collection centers and auction markets (Velarde et al., 2021). After sorting and regrouping, calves undergo a second transportation phase before reaching their final destination at either a veal or beef-rearing facility. This second transportation typically involves longer distances. However, as mentioned above, and as required by law, after 9 hours of transportation calves must be given access to water, and in the case of trips exceeding 19 hours, it is mandatory to take a 24 hour-break to allow calves to rest and to have access to feed (European Council, 2005). The designated livestock facilities used for these breaks are referred to as control posts (CPs). At these CPs, calves stay for a minimum of 24 hours before continuing their journey and are provided with milk replacer, water, and the opportunity to rest. The Council Regulation (EC) No. 1255/1997 requires official veterinarians for the inspection of the trucks, documentation, and the fitness for transport of the calves (Velarde et al., 2021). These CPs must be located in areas free from animal health restrictions, undergo twice-a-year inspections, comply with the EU legislation on animal health, and respect hygiene and health measures (Council Regulation (EC) No. 1255/1997). Although there has been a lot of pressure on the importance of these CPs in the recovery of animals before subsequent transport, research has not shown consistent agreement on the positive effects of CPs on animal recovery and welfare (Padalino et al., 2018), and few studies on this topic are available to date.

1.5.3.1. Hours of fasting and nutrient restriction

As mentioned earlier, the marketing of surplus calves involves periods of fasting and nutrient restriction due to the hours spent in transport and at the assembly centers or auction markets. At auction markets, feed or water are usually not offered as calves get sorted and marketed within 24 hours (Wilson et al., 2020b). However, at assembly centers, staying times are longer, ranging from a few hours up to 6 days (Reenen et al., 2022). During this time, calves are typically fed only rehydrating solutions or, in some centers, milk replacer. However, electrolyte supplementation does not meet the calves' nutritional needs, so the feed and water restrictions imposed during the first transport persist, resulting in longer fasting periods for the calves. Accordingly, a previous study investigating the use of electrolytes Vs. milk replacer as pre-transport diets in young veal calves showed that during a 6-hour-length transport, feeding milk replacer increased glucose and decreased non-esterified fatty acids (**NEFA**) serum concentrations and BW losses compared with calves fed only electrolytes (Marcato et al., 2020). Feeding electrolytes at the assembly centers therefore prolongs nutrient restriction and ends up creating a negative energy balance in calves whose body reserves and ability to cope with fasting are very limited (Marcato et al., 2020). Implementing feeding strategies at assembly centers to reverse this negative energy balance before transport becomes urgent. The length of time calves can be on feed restriction during marketing varies depending on when was their last feeding, their final destination, the distance between the countries involved in the market, and the applicable legislation. Feeding only rehydrating solutions during long-distance transportation does not fulfill energy requirements in unweaned calves, which at this time, are dependent on milk consumption (Marcato et al., 2020). An alternative would be feeding milk to calves on board the truck. In some regions of Germany, the long-distance transportation of calves is restricted if calves cannot be fed during the journey. For this reason, trucking companies have designed a system to feed milk to the calves on the truck. These systems exist and are used in some countries like Germany. However, some would argue that the milk powder is difficult to mix completely, the amount of milk consumed is unknown, and it is difficult to assist and monitor the calves to ensure consumption. Furthermore, no studies have been published on the feeding of calves with milk replacer on board and its positive or negative effects after arrival. The few studies that have evaluated the possibility of feeding calves during transport breaks and its potential beneficial effects on energy balance have been performed providing electrolyte solution to the calves. These studies have demonstrated minimal benefit from mid-transport electrolyte supplementation in unweaned calves to increase serum glucose concentration and prevent dehydration during 24 hour and 19 hour trips (Knowles et al., 1997, 1999). However, as suggested by Knowles et al. (1997; 1999), feeding during a journey of those lengths may not be advantageous due to the negative impact on the calves caused by the additional stress resulting from handling and feeding. In commercial practice, these drawbacks are likely to outweigh any benefits, and further research exploring these practices under various transport lengths and feeding strategies are urgent as, according to EFSA recommendations (Nielsen et al. 2022), the new regulation on the protection of animals during transport is likely to incorporate the milk feeding on board in long distance transportation.

In addition to the nutrient restriction, according to EU legislation, trucks used for long-distance transportation of unweaned calves must be equipped with water troughs (European Council, 2005). However, these are normally not adapted for young calves which are also not familiar to them. Additionally, the number of these water troughs is usually insufficient for the number of calves being transported and the amount of water a calf can drink from them is very limited (Nielsen et al., 2022).

Since, as commented, calves have only 1 h of rest to drink water within 2 consecutives 9 h trips (European Council, 2005), these difficulties exacerbate their water restriction and stress during transport. Water consumption during transport is another area to explore specially during summer.

1.5.3.2. Transport factors that extend recovery

Before even being traded, calves are exposed to nutritional and management challenges from birth at their origin dairy farms. As previously commented, there is a huge gap in information related to the background of male calves being sold from dairy farms to rearing facilities. On many occasions, this information is missing but in others it is not recorded at all. This data is essential to evaluate the level of risk of these calves before being transported.

One of the critical points of discussion within the EU is the age at transport. According to EU legislation, calves are allowed to be transported at a minimum of 14 d of age. However, this time coincides with a period of low immunocompetence where maternal antibodies from colostrum decline and the calf is just beginning to develop its own antibodies through exposure to environmental antigens (Figure 3) (Hulbert and Moisá, 2016). Overall, the additive effect of poor colostrum consumption and a lower plane of nutrition at the origin dairy farm, results in calves being exposed to future transport with a low energy balance, an immature immune system, and light weights (Wilson et al., 2000) increasing their disease susceptibility.

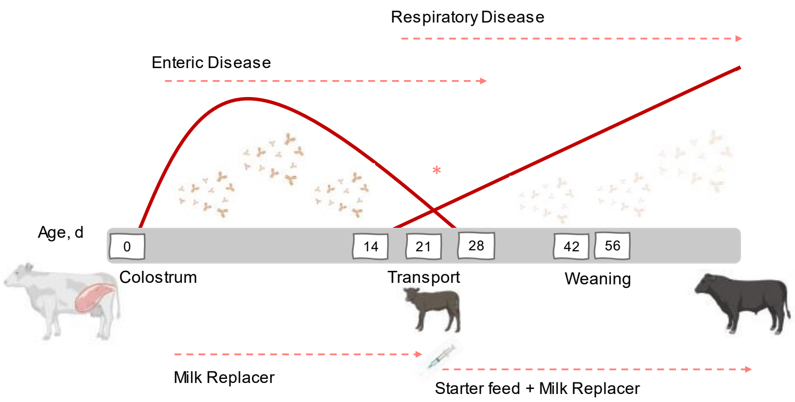

Figure 3. Immunological development during the first months of age in calves (Adapted from Hulbert and Moisa, 2016).

In the trade process, fasting and nutrient restriction during transport are probably two of the most important stressors that calves face. However, they are not the only ones, as calves are also exposed to the stress of commingling, mixing with calves from different origins, exposure to new pathogens, environmental conditions, fatigue, loading and unloading from trucks, and physical trauma, among others. (Warriss, 1990; Cooke, 2017). These stressors, in addition to those experienced on the dairy farm of origin, can result in immune system inhibition due to prolonged exposure to stressful stimuli, directly affecting the health and performance of these calves (Van Engen and Coetzee, 2018a). It has been shown that calves arriving at rearing facilities have lower BW and that a lower body mass index is associated with greater morbidity and mortality in calves (Scott et al., 2019b). Others have shown that heavier calves are at lower risk for respiratory disease and diarrhea, especially in the first 21 d after arrival, which have been described as the most challenges and the ones with the greater morbidity and mortality rates (Pardon et al., 2012a; Winder et al., 2016a; Scott et al., 2019b; Wilson et al., 2020a). Renaud and Pardon (2022) have proposed a BW >50 kg as a tentative threshold for BW at arrival in order to lower the risk for digestive and respiratory diseases. Transport itself is another predisposing factor to the development of bovine respiratory disease (**BRD**) due to the inflammatory process stimulated by cytokines and acute phase proteins (Van Engen and Coetzee, 2018a). The dehydration status of calves at arrival, the presence of fever, and umbilical infection have also been associated with increased mortality risk (Renaud et al., 2018a; Wilson et al., 2020a). Regarding transportation, previous studies on farm animals have demonstrated that it is the length of transportation (including stationary waiting time) rather than the distance traveled that increases mortality risk (Warriss et al., 2007; Nielsen et al., 2011) because factors such as space allowance or climate conditions, among others can extent the stress during transport. When transported at low space allowances, calves reduced their opportunity to move and lying time (Todd, et al.2000; Uetake et al. 2011; Grigor et al., 2001), which increases their lying behavior upon arrival because calves are fatigated (Grigor et al. 2001, Fisher et al. 2014). Additionally, climatic conditions can be critical for young calves as their thermoneutral zone ranges from 15 ºC to 25 ºC (Bianca et al. 1970; Spain et al. 1996; Davis et al. 1998), and some metabolic changes can be observed at temperatures above 26 ºC (McDowell et al. 1954; McLean, 1963). Taken together, these are all factors inherent in calf management that increase the susceptibility of calves to nutrient restriction and fasting during transport.

1.6. Recovery at the rearing farm

The nutritional and sanitary management of calves at arrival varies considerably depending on the type of production, legislation, and the producer's preferences for calf management. In the case of veal production in Europe, calves are frequently allocated in individual hutches for 6 weeks before being housed in collective pens (2 to 8 calves) with slatted floors (Pardon et al., 2012b). Regarding the dairy beef industry, housing conditions can vary. In the U.S., for instance, calves may be individually housed until 9-10 weeks of age before being moved to collective pens, or they may be group housed immediately upon arrival to the rearing facility. The usual number of calves per pen can range from 5 to 6 up to 25 calves. In any case, it is important to consider that larger groups often have a less consistent growth rate, and it is more difficult for producers to monitor diseases (Felix et al., 2017). Running “all-in, all-out” systems is a common practice. In this system, barns are emptied at once and disinfected completely before the arrival of a new lot. By doing this, the probability of disease transmission between new and old groups of animals is minimized. Correct bedding is crucial and should be regularly changed to avoid lameness and respiratory problems.

Some producers feed calves with rehydrating solutions instead of milk replacer upon arrival at the rearing facilities. This practice aims to restore the mineral homeostasis and hydration status of the calves while providing sugars that are used as a quick source of energy. However, others argue that feeding only electrolytes alone, without milk replacer, may not be sufficient to meet nutritional requirements and may contribute to increasing the negative energy balance of calves at arrival. Stressors like diarrhea, irregular feeding, cold temperature of the milk, and stress due to transport, among others, cause esophageal groove dysfunction in calves (Lateur-Rowet and Breukink, 1983; Gentile, 2004; Khan et al., 2007). For this reason, some producers prefer to feed calves with rehydrating solutions instead of MR the first hours after arrival. Additionally, increased osmolality of milk replacers can lead to digestive disturbances such as diarrhea (Kertz and Loften, 2013; Glosson et al., 2015), which can worsen the condition of fasted calves under the stress of transport. In any case, rehydration therapies could be a nutritional complement but not a substitute for the nutritional value of milk. In cases of post-transport rehydrating therapies, it is recommended to use less concentrated solutions than those used for treating diarrhea and to feed the electrolytes several hours after the milk replacer feeding because electrolytes may inhibit the formation of casein clot in the abomasum reducing digestibility and intensifying scours (Constable et al., 2009).

Although EU legislation states that "calves older than 2 weeks must have access to sufficient water at all times" (European Council, 2008), in some cases, water is not always offered at arrival. The rationale for this practice is that calves arrive at the rearing farms dehydrated and thirsty after many hours of transport without access to water. Offering water ad libitum at arrival therefore means that many calves will drink water to excess and then refuse milk intake. To avoid this problem, some producers do not offer water to calves for the first 2 to 3 d after their arrival. Additionally, water availability is sometimes neglected because of the idea that calves consuming milk replacer do not need to drink water, which has been shown to be incorrect (Jensen and Vestergaard, 2021). Calves still need a fresh source of clean water to stay hydrated, especially during hot weather seasons due to water loss through evaporation (West, 2003; Quigley et al., 2006). Additionally, the lack of water consumption decreases starter feed intake (Davis and Drackley, 1998; Broucek, 2019). Because calves are typically fed restricted amounts of milk (Drackley, 2005), calves need to fulfill their nutritional requirements through the ingestion of solid feed which is, at the same time, associated with the amount of water consumed (Jensen and Vestergaard, 2021). Additionally, calves that are malnourished or dehydrated may lack the nutrients and energy required to mount a proper immune response to a vaccine, which can negatively affect the effectiveness of vaccination (Richeson et al., 2019).

The supply of long fiber on rearing farms is also not a common practice. In Spain, and probably in other countries as well, it is generally believed that calves get their source of fiber from bedding if needed. However, the importance of fiber in the preweaned calf in terms of rumen development and performance demands further attention. Consumption of fiber contained in straw and forages is important in young ruminants to avoid the drops in rumen pH caused by the ingestion of starter feed, especially pellets and grounded types (Türkmen and Muruz, 2023). The starter feed given to calves at rearing facilities will be the initial introduction to solid feed for most male dairy calves. This is mainly because, on dairy farms, concentrate is mostly reserved for replacement heifers to reduce feed costs, and male calves are not exposed or trained to it until later. This practice contradicts the idea that concentrate should be introduced early to promote rapid weight gain and early slaughter weight, as dairy calves have a higher feed-to-weight gain ratio than conventional beef steers (Duff and McMurphy, 2007). Although this would be the ideal situation, it is not always the case in the dairy beef industry. Once at the rearing farm, calves usually have ad libitum access to concentrate to promote rumen development and to transition from a liquid to a solid-based diet as soon as possible (6-8 weeks) to reduce labor and fed cost. To encourage consumption, the starter offered should be of high quality and palatable.

Finally, in terms of milk replacer, conventional calf rearing systems use restrictive milk feeding programs that aim to increase concentrate intake by reducing the amount of milk replacer offered. The CP and fat content of milk replacer in this system can vary but are around 20% CP and 18-22% fat, whereas the CP and fat content of whole milk are 25 and 30%, respectively, on a dry matter basis [(National Research Council (NRC), 2001)]. According to the NRC (2001), milk replacers contain 10 to 20 % less energy than whole milk because of the lower fat content. This means that milk replacer is not only offered at low volumes but also their nutrient composition is lower. Consequently, these programs differ much from what a calf would consume in nature and often, due to their composition, they provide levels of protein and fat below the calves' requirements, limiting early growth. Although reducing the quantity of milk replacer provided is frequently practiced for encouraging concentrate consumption, solid feed intake is often limited during the initial 3 weeks due to the immaturity of the forestomaches, resulting in poor solid feed digestion (Khan et al., 2016) that often is exacerbated by the negative consequences due to marketing and transport. Some of the demonstrated benefits of intensive milk-feeding programmes are: increased body weight and growth, GIT development, less signs of hunger, activated immune response and health, enhanced muscle and body fat growth, among others (Hammon et al., 2020). In addition, previous studies have shown that providing high-milk-feeding programs (>20% of BW in milk) decreases the incidence of diarrhea and disease treatments and improves the immune system in the preweaning phase (Khan et al., 2007; Ollivett et al., 2012; Ballou et al., 2015; Renaud and Pardon, 2022). Also, higher planes of nutrition have been shown to increase calf body weight and welfare while reducing disease risk, ultimately improving growth and feed efficiency (Khan et al., 2011). Thus, ensuring proper milk replacer feeding during these first weeks is critical for the growth and development of these calves. However, although the benefits are clear, feeding higher levels of milk replacer to male calves is not typical in practice. Some of the reasons may be due to the low investment in these surplus calves, the high cost and the labor of providing milk replacer.

1.7. Short and long-term consequences on performance and health

Low body weight at arrival has been associated with increased mortality risk in veal calves (Winder et al., 2016b; Renaud et al., 2018a). During transport, calves may lose between 3 to 11 % of their BW (Warriss, 1990) being dehydration and shrinkage two of the principal triggers in body weight losses. Lighter body weight has also been associated with a higher incidence of bovine respiratory disease (**BRD**), immune activation, and diarrhea (Brscic et al., 2012; Chamorro et al., 2017; Masmeijer et al., 2019; Wilson et al., 2020a). In addition to body weight losses, calves can take up from 2 to 4 weeks to recover their normal intake upon arrival at the rearing facility (Hutcheson and Cole, 1986; Loerch and Fluharty, 1999), exacerbating body weight losses in young calves. Reduced feed intake due to short or progressive feed restriction has been previously studied as a trigger for intestinal permeability in young calves and pigs (Pearce et al., 2013; Zhang et al., 2013; Kvidera et al., 2017b). The mechanism behind intestinal barrier disruption appears to be associated with the release of glucocorticoids in response to stress (Lambert, 2009a), such as the physical and psychological stress caused by marketing and transportation. Any disruption in the normal functioning of this gut barrier would allow the entrance of antigenic macromolecules from the lumen leading to or perpetuating an increase in its permeability and leading to local or systemic inflammatory reactions (Lambert, 2009b; Bischoff et al., 2014). During an inflammatory response, glucose and amino acids are used as fuel by the activated immune system (Kvidera et al., 2017a).

The energy cost of immune activation caused by an increase in gastrointestinal permeability has been shown to reach values of ~1.0 g glucose/ kg BW0.75/ hour in lactating cows (Kvidera et al., 2017a). A process that, considering glucose requirements on a BW basis, is expected to be similar in calves (Sanz-Fernandez et al., 2020). The negative impact of an activated immune system resides in the fact that the glucose and amino acids intended to be used for productive phenotypes (i.e., growth) are not available affecting the metabolism and performance of these calves (Kvidera et al., 2017a).

Furthermore, the first 21 days after arrival is the time of greatest mortality risk for calves (Pardon et al., 2012b), especially for those that did not have adequate nutritional and sanitary management at the dairy farm of origin. The occurrence of diseases like BRD or scours during this stage has long-term implications in performance through decreases in average daily gain (**ADG**), dry matter intake (**DMI**), and feed efficiency (Van Engen and Coetzee, 2018b), growth and morbidity and mortality rates (Renaud and Pardon, 2022).

2. Indicators of calves' status at arrival to the rearing facilities

The immunological, metabolic, and health status of the calves arriving at the rearing facilities is usually impaired. In this scenario, the need for indicators of health, metabolic, immunological, and physiological status upon arrival becomes critical to develop preventive strategies to differentiate calves that are more susceptible to disease.

2.1. Indicators of maternal immunity

Measurements of immunoglobulins (Ig), particularly IgG, have long been considered the gold standard method to assess FTPI in calves. Immunoglobulin G has 2 subclasses: IgG1 and IgG2. Measurements of IgG1 are considered to be more accurate for assessing FTPI because failed transfer of passive immunity has been described as < 1.0 g/dL serum IgG concentration in calves that are between 1-7 d of age (Tyler et al., 1996a). From the different methods used to determine serum IgG, radial immunodiffusion is considered the reference test (Beam et al., 2009), however, refractometers are commonly used in practice. They measure total protein (**TP**), which has a very high correlation with IgG (Tyler et al., 1996; Wilm et al., 2018). However, one of the disadvantages of using refractometers is that the readings may be altered in cases of dehydration which is particularly important when using them in transported calves which are normally dehydrated at arrival. Previous studies have-shown that age can also influence serum IgG concentration, mainly due to a process of protein degradation (Cuttance et al., 2019; Souza et al., 2021). Cuttance et al. (2019) suggested that calves should not be older than 1 week and de Souza et al. (2021) suggested 24-48 hours. This is another difficulty when trying to assess FTPI in dairy calves, as they arrive at the farm at around 14 days of age, which makes using this technique impractical. Cholesterol has also been studied as a potential biomarker of colostrum consumption (Marcato et al., 2018a). Cholesterol is found in greater concentrations in colostrum compared with milk and, consequently, its concentration in serum might be indicative of the amount of colostrum consumed (Renaud et al., 2018b). Another proposed biomarker of colostrum consumption is gamma-glutamyl transferase (GGT). Gamma-glutamyl transferase is a bioactive component of colostrum found in high concentrations in young calves (> 500 to 1,000 IU/L) (Buczinski et al., 2020). Some studies have shown positive correlations between serum GGT and serum immunoglobulin concentrations (Thompson and Pauli, 1981; Perino et al., 1993; Parish et al., 1997; Weaver et al., 2000). Serum concentrations of GGT are not indicative of IgG concentrations as there is no biological relationship between them (Weaver et al., 2000; Souza et al., 2021), but they can estimate whether or not a calf had access or not to colostrum at birth. An advantage of using GGT for this assessment is that serum concentrations decline to adult activity by approximately 5 weeks of age (Thompson and Pauli, 1981; Yu et al., 2019), providing a larger window for detecting differences in serum concentration, which is particularly important for dairy beef calves that arrive at the rearing facility at approximately 2 weeks of age. Finally, greater serum concentrations of alkaline phosphatase (ALP) have been positively correlated to increments in serum GGT and IgG concentrations (Thompson and Pauli, 1981; Zanker et al., 2001; Britti et al., 2005), suggesting that ALP may be a good indicator of colostrum intake. However, inconsistent results in the literature on calves and lambs and the lack of current studies looking at its use in farm animals make it difficult to validate ALP as a biomarker of colostrum intake (Thompson and Pauli, 1981; Pauli, 1983).

2.2. Indicators of gut functionality and inflammation

Another important health parameter that can be affected by feed restriction is gastrointestinal functionality. Gut integrity can be affected by short or long-term feed restriction, and stressful events like transport, fasting, and weaning, among others (Zhang et al., 2013; Kvidera et al., 2017b; Chase, 2018; Wilson et al., 2020a). Measurements of gut permeability (an indicator of gastrointestinal disruption) in calves have been widely conducted via in vivo tests. During these tests, markers are given orally to the calves and collected afterward via a total collection of feces urine, or blood samples. Some of the most commonly used markers of gut permeability in calves are lactulose, D-mannitol (and their ratio), Cr-EDTA, and ovalbumin (Zhang et al., 2013; Araujo et al., 2015; Amado et al., 2019; Deluco and Wilson, 2021; Cangiano et al., 2022). Lactulose and D-mannitol are saccharides with the ability to cross the intestinal layer through paracellular (lactulose) and transcellular (D-mannitol) routes. Lactulose is a large disaccharide that, under normal physiological conditions, is not absorbed in the small intestine but is later fermented by bacteria in the large intestine (Bischoff et al., 2014). When the integrity of the small intestine is compromised, lactulose crosses the intestinal layer via the paracellular route (Bischoff et al., 2014). D-mannitol is a small-size monosaccharide that is normally absorbed in the small intestine via a transcellular pathway without significant metabolism (Wang et al., 2015). The final assessment of gut integrity is made by calculating the ratio between the concentration of the two sugars in plasma. This ratio will reflect the functionality of the paracellular pathway, therefore, a low serum lactulose: D-mannitol ratio would reflect a normal intestinal layer, whereas a high serum lactulose: D-mannitol ratio would indicate a failure of intestinal integrity (Hall, 1999). Following a similar principle, Cr-EDTA and ovalbumin have been used as markers of gastrointestinal permeability in several species (Nejdfors et al., 2000; Hunt et al., 2002; Wood et al., 2015; Wilms et al., 2019). Under normal physiological conditions, tight junction proteins, adherent junctions, and desmosomes regulate the passive movement of molecules < 600 Da through the gap between adjacent enterocytes (Ménard et al., 2010). Large molecular weight (**MW**) markers such as Cr-EDTA (340kD) and ovalbumin (42.7kD) are expected to pass by the paracellular route in the event of an increase in intestinal permeability due to disruption of the intestinal layer (Wood et al., 2015). These indigestible probes pass through the paracellular space of adjacent enterocytes indicating an increase in the gastrointestinal permeability to large molecules (Bjarnason et al., 1995). These markers can be later measured in plasma, urine, and feces (Bischoff et al., 2014). However, measurements in feces and urine are tedious and require specific facilities for total collection for 24 hours. In addition to assessing losses in gut integrity, serum concentration of citrulline has been previously used as a marker of enterocyte mass function in calves (Gultekin et al., 2019). Because citrulline is produced strictly by healthy enterocytes in the small intestine, variation in its serum concentration can be indicative of health and function of the intestine in calves (Windmueller and Spaeth, 1981; Gultekin et al., 2019). When evaluating the functionality and integrity of the gastrointestinal tract other approaches such as histology, gene expression of tight junction proteins, in vitro test of gut permeability, among others, provide valid information on the health status of the gut. However, these techniques would not be feasible for dairy beef calves because they require animal sacrifice, are laborious, and are expensive to perform. Easily measurable and inexpensive markers are needed for rapid assessment of gut function in calves at arrival. In this scenario, fecal concentration of lactoferrin could be proposed as a feasible marker of intestinal inflammation. Plasma lactoferrin is a bioactive compound contained in colostrum in great concentration (Donovan, 2016) and in secondary granules of neutrophils (Kane, 2003; Langhorst et al., 2008). In the event of intestinal inflammation, these neutrophils invade the mucosa and release lactoferrin in response to inflammation, increasing its concentration in the feces (Guerrant et al., 1992; Legrand et al., 2005). To date, lactoferrin has been mostly studied as a marker of intestinal bowel disease in humans (Walker et al., 2007; Langhorst et al., 2008; Borkowska et al., 2015), however, to our knowledge, this has not been tested in calves and its use appears promising.

2.3. Indicators of energy status

During events of stress and nutrient restriction, measurements of serum concentration of glucose, NEFA, and BHB have been extensively used for the assessment of energy balance in calves. Marketing and transportation in young dairy beef calves induce periods of fasting and feed restriction that negatively impact their energy metabolism. Feed restriction induces a decrease in serum glucose concentration and a switch to lipid oxidation, which increases fat mobilization as an energy source, increasing blood NEFA and beta-hydroxybutyrate (**BHB**) concentrations (Pénicaud et al., 2000; Grigor et al., 2001; Marcato et al., 2018b). However, fat mobilization is more limited in unweaned compared to weaned calves because of the minor energy stores (Knowles et al., 1997; Zhang et al., 2013), further compromising the metabolic status of these calves. Energy metabolism is altered synergistically by dietary restriction and the stress derived from hunger. It has been shown that the activation of the hypothalamic-pituitary-adrenal axis (**HPA**) increases glucocorticoids and catecholamine concentrations that alter glucose concentration and mobilization of fat reserves (Kent and Ewbank, 1986; Frohli and Blum, 1988; Herman et al., 2016). Previous studies have shown a decrease in plasma glucose concentration during transportation that increased immediately following feeding in calves (Mormede et al., 1982; Knowles, 1999; Tadich et al., 2005) and others have found lower serum glucose concentrations to be positively correlated with a greater incidence from BRD at later stages (Cusack et al., 2003). Hypoglycemia after transport has also been shown to affect the performance of young calves beyond the first week after arrival (Mormede et al., 1982). On the other hand, higher levels of serum NEFA have been shown to induce disease and suppress the immune system in dairy cattle (Collard et al., 2000).

2.4. Indicators of health

Cortisol is a glucocorticoid hormone involved in the stress response and regulated by the HPA axis that has been proposed as a potential biomarker of disease in young calves (Marcato et al., 2018a). Increases in serum cortisol during transport have been previously noted in young calves (Fazio et al., 2005; Bernardini et al., 2012). In addition to being a marker of stress, cortisol has also been shown to negatively affect the immune system by inducing changes in cytokine levels (Vegas et al., 2011) and to cause increases in intestinal permeability affecting normal digestive functions and decreasing feed intake (Lambert, 2009b). Chronic rises in plasma cortisol (> 4 to 5 days) have been shown to affect glucose metabolism making animals less resilient to diseases (Marcato et al., 2018a).

Other proposed biomarkers of disease and physical challenge are creatine kinase (**CK**), haptoglobin (**Hp**), lactate, body temperature, and behavior. Increased levels of CK have been previously described in calves during transportation and handling procedures (Minka and Ayo, 2010). Direct trauma like knocks and bruises during transportation and the consequent tissue damage, hypoxia, fatigue, poor muscle tissue reperfusion, and increased muscle membrane permeability are some of the consequences associated with transport and handling increasing its serum concentration in transported livestock (Warriss et al., 1995; Averós et al., 2008; Guàrdia et al., 2009). The reasons behind an increment in CK due to transportation may not be directly associated with transport itself but with the fact that greater times on transportation increase the risk of bruises that induce CK elevation (Knowles et al., 1997; Fisher et al., 2014). Transport also induces the release of acute-phase proteins **(APPs)** such as Hp in response to cytokine release (Tothova et al., 2014). Because cytokines are released in response to disease, an increase in Hp might be used as a marker for identifying sick calves (Murray et al., 2014). Previous studies have found greater Hp concentrations in young calves following transportation (Murata and Miyamoto, 1993; Arthington et al., 2003). Additionally, the increase in APPs concentration, especially Hp, has been proposed as a predictor of acute and chronic respiratory disease in calves (Tóthová et al., 2010; Moisá et al., 2019). The threshold for distinguishing between healthy and sick calves based on Hp concentration in serum has been proposed as 0.13 g/L of Hp (Gånheim et al., 2003). Its highly reported sensitivity in detecting disease suggests Hp is the most useful APP for discriminating between sick and healthy calves (Marcato et al., 2018b). The degradation of muscle glycogen due to stress and exhaustion (frequently suffered during long-distance transport and handling) raises lactate levels in the blood (Todd et al., 2000; Chacon et al., 2005). Additionally, lactate concentration has been shown to increase prior to infection, which makes it a good predictor of future disease (Aich et al., 2009). Bovine respiratory disease and neonatal calf diarrhea, two of the most important diseases in young calves, have been positively correlated with rises in lactate serum concentration (Coghe et al., 2000; Lorenz, 2004; Buczinski et al., 2015). Marcato et al. (2018) proposed L-lactate as a predictor of death in clinically ill calves within 24 hours, although contradictory results were found in the bibliography. Another useful biomarker of disease in calves is body temperature. Young calves have a limited capacity to maintain their body temperature as they have limited body reserves and are dependent on their hair coat and body fat as an insulation mechanism (Borderas et al., 2009). This ability to thermoregulate may be compromised during transport, especially if the transport includes a period of water and feed restriction that worsens the condition. During transport, calves may experience body weight losses due to shrinkage and dehydration, which forces the mobilization of nutrients and ultimately increases rectal temperature (Marcato et al., 2018b). An increment in body temperature can also be indicative of glucocorticoids and catecholamine secretion due to stress (Burdick et al., 2010). Monitoring behavior during transport can also indicate stress and, ultimately, the risk of disease. A useful tool for monitoring behavior during transport is the use of accelerometers. A previous study using pedometers (accelerometers) in transported heifers showed longer lying times 2 days after transport compared to non transported control heifers, indicating fatigue after transport (Theurer et al., 2013). Finally, another useful marker of health and recovery of calves at arrival to the rearing facility is the concentrate intake recovery. It has been previously shown that newly arrived calves might not recover their intake of concentrate until the second or fourth week after arrival (Hutcheson and Cole, 1986; Loerch and Fluharty, 1999) aggravating their poor general condition post-transport and marketing. The reasons for this decrease in concentrate intake may be varied and include a novel source of feed and water, adaptation to new facilities, grouping, hierarchy, and other social interactions. Among the nutritional factors that cause this decrease in concentrate intake, changes in the rumen environment due to feeding and water deprivation during transport have been studied (Loerch and Fluharty, 1999). After a 48-hour period of feed and water deprivation, between 10 to 25% of the number of bacteria in the rumen has been reported to be reduced (Baldwin, 1967). Disease incidence is also negatively associated with concentrate intake in calves. Two typical disease behaviors in calves with BRD are reduced feed intake and, related to the above, reduced activity levels recorded by accelerometers (Costa et al., 2021; Morrison et al., 2021). In a previous study evaluating the use of precision technology in young calves that suffered BRD, results showed a positive association between health recovery and improvements in starter intake and lying bouts (activity) in BRD-recovered calves (Cantor et al., 2022).

All of the arguments outlined above highlight the importance of knowledge and the use of biomarkers to determine the status of calves upon arrival at the rearing facilities. Their proper use would allow understanding the health status of the calves and developing management strategies appropriate to their needs, with the ultimate goal of reducing the incidence of disease and mortality rates while improving calf performance and welfare.

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CHAPTER II

OBJECTIVES

1. General objective

A previous study conducted in the framework of this thesis (Annex 1) indicatedd that exposing unweaned calves to periods of feed restriction and fasting when simulating a marketing and transportation, negatively affected calf energy balance, gastrointestinal functionality, and recovery of body weight and concentrate intake during the first days upon arrival at a rearing facility. Additionally, previous studies conducted on young calves have indicated the importance of colostrum intake in the development of the gastrointestinal tract and functionality of the immune system (Blum and Hammon, 2000; Blättler et al., 2001).

Based on our previous studies and literature results, the general objective of this thesis was to evaluate potential strategies to improve unweaned dairy beef calf recovery at the arrival farm and to characterize their impact on nutritional status and gut functionality.

2. Specific objectives

1. To evaluate the effects of colostrum consumption and feed restriction in the recovery of BW and concentrate intake after long-distance transportation in male dairy beef calves.

2. To examine the effects of colostrum consumption and feeding milk replacer or a rehydrating solution before a long-distance transport on the nutritional status of calves.

3. To characterize gastrointestinal tract functionality in calves fed different volumes of colostrum at birth and subjected to feed restriction and fasting during transport.

4. To evaluate how feeding higher planes of nutrition before transportation influences BW, concentrate intake, and gastrointestinal tract functionality recovery at arrival to the rearing facility.

To accomplish these specific objectives, three studies have been conducted:

STUDY 1: "The effects of colostrum consumption and feed restriction during marketing and transportation of male dairy beef calves: Impact on pre-transport nutritional status and on farm recovery"

STUDY 2: "The effects of colostrum consumption and feed restriction during marketing and transportation of male dairy beef calves: Impact on gastrointestinal tract functionality"

STUDY 3: "Evaluation of feeding strategies applied at assembly centers on pre-transport status and recovery at the rearing farm in unweaned dairy beef calves"

CHAPTER III

The effects of colostrum consumption and feed restriction during marketing and transportation of male dairy beef calves: Impact on pre-transport nutritional status and on farm recovery.

Published at the Journal of Dairy Science
https://doi.org/10.3168/jds.2023-23374

“The effects of colostrum consumption and feed restriction during marketing and transportation of male dairy beef calves: Impact on pre-transport nutritional status and on farm recovery“

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ABSTRACT

The aim of this study was to evaluate the effects of colostrum consumption and feed restriction on biomarkers of stress, nutritional and health status, gut functionality, and behavior in male dairy beef calves being marketed and transported. A total of 82 male Holstein calves [42 ± 1.2 kg of body weight and 14 ± 0.9 d of age] were used to study the amount of colostrum given at birth at the dairy farm of origin, the degree of feed restriction suffered at an assembly center simulation (d -4 to d -1), and the effects of a 19 h transportation (d -1). Treatments were as follows: control calves (**CTRL**; n=16) were fed 10 L of colostrum at the dairy farm of origin, milk replacer (**MR**) and concentrate at the assembly center, and were not transported; high colostrum-milk replacer fed calves (**HCMR**; n = 17) were fed 10 L of colostrum at the dairy farm of origin, MR at the assembly center, and were transported; high colostrum-rehydrating solution fed calves (**HCRS**; n = 16) were fed 10 L of colostrum at the dairy farm of origin, a rehydrating solution (**RS**) at the assembly center, and were transported; low colostrum-milk replacer fed calves (**LCMR**; n = 17), were fed 2 L of colostrum at the dairy farm of origin, MR at the assembly center, and were transported; and low colostrum-rehydrating solution fed calves (**LCRS**; n = 16) were fed 2 L of colostrum at the dairy farm of origin, RS at the assembly center, and were transported. Transported calves mimic a 19 h long transportation.

After transport, all calves were fed 2.5 L of MR twice daily and had ad libitum access to concentrate, straw, and water. Calves' recovery was followed during 7 d. Concentrate intake and health records were collected daily from d -4 until d 7 and BW and blood samples were collected on d - 4, - 1, 0, 1, 2, and 7 of the study. Results showed that the feeding regime provided at the assembly center reduced BW for the HCRS and LCRS calves compared with the CTRL, HCMR, and LCMR calves. Concentrate intake peaked on d 0 in the transported calves followed by a drop in intake on d 1 after transportation. Concentrate intake recovery was lower for the LCRS and LCMR calves. On d -1, non-esterified fatty acids and beta-hydroxybutyrate concentrations were greater for the HCRS and LCRS calves compared with the CTRL, HCMR, and HCRS calves. After transportation, serum Cr-EDTA concentration was greater for the HCRS and LCRS calves than the HCMR, LCMR, and CTRL calves. The LCRS calves had the lowest serum concentration of citrulline. Finally, health scores were greater for the LCRS calves from d 0 to d 7. In summary, both the greatest degree of feed restriction during the assembly center and the low colostrum consumption at birth negatively affected the recovery of concentrate consumption and BW, gut functionality, health status, and behavior in calves after arrival at the rearing farm.

Key words: dairy beef calf, colostrum, feed restriction, gut functionality.

INTRODUCTION

In recent years, the dairy beef industry has developed significantly as an important supply of meat in many countries. However, even though these surplus calves represent an income for dairy producers, efforts put into their postnatal care and nutrition are usually not a priority (Devant and Marti, 2020). Previous studies have revealed differences in management practices with minor attention put on male calves' nutrition, colostrum quality, navel disinfection, and vaccination when compared with heifer calves (Fecteau et al., 2002; Shivley et al., 2016; Renaud et al., 2017a; Renaud et al., 2018b). Additionally, it has been demonstrated that male calves receive lower volumes of colostrum when compared to females (Renaud et al., 2020a) or, in a minority of cases, no colostrum at all (Renaud et al., 2017). Because of poor colostrum management practices, the risk of mortality and morbidity increases due to failure on the transfer of passive immunity (Renaud and Pardon, 2022). Colostrum consumption in newborn calves is not only important to gain passive immunity from maternal antibodies (Hulbert and Moisá, 2016), but also because it is composed of nutrients (carbohydrates, lipids, proteins, etc.) and bioactive substances (growth factors, cytokines, enzymes, etc.) that have been shown to modulate the development and function of the gastrointestinal tract **(GIT)** (Blum and Hammon, 2000). Some of the effects that colostrum exerts on the GIT are related to enhanced cell proliferation and protein synthesis, modulation of microbial population, absorption and motility, vascular tone, among many others (Blum, 2006). Therefore, colostrum provision may exert long-term effects on calves' life.

In addition to the poor colostrum management, male calves destinated to the dairy beef production are also exposed to physical and psychological challenges during marketing and transportation from their dairy farm of origin to the rearing facilities. In the marketing process, calves can be transported directly from the dairy farm to the rearing facilities or be mixed and regrouped at auction markets or assembly centers (Pardon et al., 2014a; Marcato et al., 2020b; Rot et al., 2022a) before being transported to the rearing farms. In Europe, when subjected to assembly centers, calves can stay in these establishments for a period of up to 6 days (Reenen et al., 2022). During this period, calves are normally fed only rehydrating solutions **(RS)** or in some centers, milk replacer **(MR)** and, in addition to the feed and water restriction, they are exposed to commingling, mixing, exposure to new pathogens, loading and unloading from the truck, physical trauma, etc. (Warriss, 1990).

The final transport to the rearing farms involves short or long-distance transportations. According to the European legislation, trucks used for long-distance transportation of unweaned calves must be equipped with water troughs (European Council, 2005). However, these are normally not adapted for young calves, the number of them are not sufficient, and calves are not familiar to them. Because calves have only 1 h of rest to drink water within two consecutives 9 h trips (European Council, 2005), these difficulties aggravate their water restriction and stress during transport.

Altogether, the stress and fasting due to transportation lead to losses on BW that are visible at arrival to the rearing facilities. Research has shown that dairy beef calves arrive at veal farms with low BW and compromised health status (Renaud et al., 2018). The weight loss during marketing and transportation and the decrease in intake during the first days after arrival to the rearing facilities (Hutcheson and Cole, 1986; Loerch and Fluharty, 1999) aggravate their general condition. Hutcheson and Cole (1980) estimated that it can take between 1 to 3 weeks for calves to recover their expected feed intakes after arrival. In accordance, our research group has demonstrated that feed-restricted and fasted unweaned calves take approximately 21 d to recover their concentrate intake after a period of fasting and feed restriction (Pisoni et al., 2022a). Back to the importance of colostrum consumption, it has been recently demonstrated that a low colostrum consumption at birth (2 L) can generate losses of approximately 2 kg of BW at arrival to the rearing facilities when compared with calves that received higher amounts of colostrum at birth (10 L) (Pisoni et al., 2022b). Altogether, these data suggest that there may be many factors associated and acting together in incrementing BW losses and decreasing feed intake in calves at arrival at the rearing phase.

Finally, another variable associated with drops in concentrate intake and stress is the alteration of gut permeability. Feed restriction can cause modifications in the normal functioning of the intestinal barrier (Zhang et al., 2013; Kvidera et al., 2017d; Pisoni et al., 2022a). Under normal conditions, the GIT barrier prevents the absorption of harmful antigens and pathogens while allowing the absorption of nutrients (Groschwitz and Hogan, 2009). Consequently, an alteration in this normal functioning allows pathogens' translocation from the intestinal lumen to the bloodstream, leading to immunological activation and increased susceptibility to infection (Berg, 1999). Altogether, it has been shown that the loss of integrity in the gut can directly affect production, metabolic, and inflammatory parameters (Kvidera et al., 2017a).

Poor colostrum provision, marketing, and long-distance transportation are common practices that are detrimental to the recovery of calves upon arrival at the rearing facilities. To potential advice new strategies for improving calves' welfare and performance during this period, it is important to elucidate if these factors exert an individual impact and/or if they synergistically deteriorate calves` recovery; a concept that, to our knowledge, has not yet been elucidated. Therefore, our initial hypothesis is that male dairy calves with a low colostrum consumption at birth, which suffer feed restriction during marketing and transportation, arrive at the rearing facilities with a compromised nutritional status and gut functionality which will negatively affect their performance, health, and behavior. Therefore, the objectives of this study were to evaluate the effects of colostrum consumption and feed restriction on biomarkers of stress, nutritional and health status, gut functionality, and behavior in male dairy beef calves being marketed and transported.

MATERIALS AND METHODS

Handling of calves at their dairy farm of origin

All calves used in this study were managed following the principles and guidelines of the Animal Care Committee of Institut de Recerca i Tecnologia Agroalimentàries (Barcelona, Spain; RD 53/2013; project no. 11211). For this study, a total of 82 male Holstein calves (42 ± 1.2 kg of BW and 14 ± 0.9 d of age; mean ± standard error) born at a commercial dairy farm (Granja Selergan S.A., Lleida, Spain) were used. At the dairy farm of origin, calves were divided into two groups depending on the amount of colostrum consumed at birth: high-colostrum (**HC**; n= 49) calves received 4 L of colostrum within the first 2 h after birth, and 2 L of colostrum in the next 3 feedings within the first

24 h after birth; and low-colostrum (**LC**; n= 33) calves received only 2 L of colostrum (Besser et al., 1991) within the first 2 h after birth. Calves were balanced by birth BW, cow parity (primiparous or multiparous), and birth time (day or night). For all calves, only high-quality colostrum (McGuirk and Collins, 2004) was used and administered via esophageal tube [56.28% CP, 29.48% fat, and 10.07% lactose on a DM basis, and 36,910 UI/L gamma-glutamyl transferase (**GGT**), 1.83 mg/L lactoferrin, 145.44 mg/mL IgG, and 33.11mg/mL IgG1; analyzed from a single pool of colostrum collected and mixed from the 43 pools fed to the calves]. After colostrum consumption, calves were allocated in individual hutches and received 2 L of MR at a concentration of 125 g /L as fed twice daily (21.86% CP, 16.59% fat, 45.50% lactose; Schils, The Netherlands) and ad libitum access to concentrate, following the standard operating procedures of the commercial dairy farm. Calves remained at the dairy farm of origin until approximately 14 d of age (minimum age required for transportation; European Council, 2005) and then were transported for 2.5 h to an experimental research unit located at IRTA (Torre Marimon, Spain). This short trip was intended to mimic the typical transportation of calves from their dairy farm of origin to the assembly centers or auction markets. In order to obtain 82 pure Holstein male calves from the same commercial dairy farm we entered them in 4 groups of 20 with a 2-week interval between each group.

Handling of calves during an assembly center simulation (d -4 to d -1)

Upon arrival at the experimental research unit, calves were assigned to an assembly center diet during 3 d (from d -4 to d -1 of the study) by BW and age to simulate the feed restriction suffered at assembly centers. During this period, calves were only fed either MR or RS in order to simulate two of the typical diets normally offered in these establishments. A control group that did not suffer any nutritional challenge and was fed high-colostrum at birth was also incorporated as follows: control (**CTRL**) calves were fed 2.5 L of MR at a concentration of 125 g /L as fed twice daily, had ad libitum access to a pellet concentrate (17.1% CP, 16.9% NDF, 5.7% ADF, 29.2% starch, 5.5% ether extract, 5.1% ashes on a DM basis, with the main ingredients being 39% cornflake, 17% barley, 14% wheat middlings, 13.9% soybean meal, 9% wheat bran, 3% sunflower meal, 1.9% palm oil, and 1.3% calcium carbonate, 0.5% premix, and 0.4% salt), and water during 3 days at the assembly center simulation. The **MR** calves were only fed 2.5 L of MR at a concentration of 125 g /L as fed twice daily with no access to concentrate during 3 days at the assembly center simulation; and **RS** calves were only fed 2.5 L of a RS at a concentration of 60 g /L as fed twice daily (0.39% CP, 0.05% fat, 84.75% dextrose; Corion®, Spain) with no access to concentrate during 3 days at the assembly center simulation. The CTRL and MR calves were fed the same MR offered at their dairy farm of origin (21.86% CP, 16.59% fat, 45.50% lactose; Schils, The Netherlands).

Final treatments

Based on the amount of colostrum fed at birth at the dairy farm of origin (HC or LC) and the type of diet offered during the assembly center simulation (MR, RS, or MR and concentrate), calves were assigned to the final treatments by BW and age as follows: high colostrum-milk replacer fed calves with ad libitum access to concentrate **(CTRL**; n= 16); high colostrum-milk replacer fed calves **(HCMR**; n= 17); high colostrum-rehydrating solution fed calves (**HCRS;** n = 16); low colostrum-milk replacer fed calves (**LCMR;** n= 17); and low colostrum-rehydrating solution fed calves (**LCRS**; n= 16). The technical staff working on this experiment was not blinded to treatments.

Handling of calves during the long distance transportation (d -1)

In the early afternoon of d -1 of the study (at 1300; after the 3-day assembly center simulation), all calves fitted in the MR and RS treatments were transported during 19 h in order to mimic a long distance transportation arriving at the rearing facility in the early morning of d 0 (0800) of the study. The CTRL calves were not transported and stayed at the experimental research unit where they were fed MR twice daily and ad libitum concentrate fed and water during the 19 h period. The long-distance transportation was intended to simulate an international purchase of male dairy beef calves. During the 19 h trip, calves had access to water inside the truck during the 1 h rest stop after 9 h of transport following the regulations from the European Commission for the transport of unweaned calves (European Council, 2005). However, instead of using the water troughs installed in the truck, calves drank from buckets to assure that all calves had access to water during the rest stop. The vehicle was a commercial semi trailer with 3 decks with side vents with sliding panels. Straw was used for bedding. In total, 4 trips (replicates) with approximately 20 calves each were done following the same route to ensure similar road conditions during the month of October and November 2020 [average ambient temperature of 11°C (range = 4.4–17°C) and 87.5% humidity (range = 82–90%)]. Calves were not transported at commercial densities as only the 20 calves of the study for each replicate were transported in one deck at a time. The journey was completed with two drivers to comply with the legislation.

Handling of calves at arrival to the rearing facility (d 0 to d 7)

After transportation, calves returned to the experimental research unit. From d 0 until d 7 of the study, all calves were fed 2.5 L of MR (21.86% CP, 16.59% fat, 45.50% lactose; Schils, The Netherlands) at a concentration of 125 g /L as fed twice daily and had ad libitum access to the same pelleted concentrate used for the CTRL calves during the assembly center simulation in addition to straw and water. This period was equivalent to the arrival at a rearing facility where all calves are fed the same diet.

In order to facilitate the understanding of the experimental design, the reader can refer to Table 1 and Figure 1 for a summarized description of the treatments and the timeline of this study. As previously explained, in order to obtain the total number of calves for this trial we entered them in 4 groups of 20 calves. Therefore, the timeline was repeated 4 times in total, 1 time for each group entering the trial.

Table 1. Treatments description of the study on the effects of colostrum consumption and feed restriction during marketing and transportation in the recovery of dairy beef male Holstein calves.

Treatments[a]	Colostrum given at birth[b]		Diet offered during the assembly center simulation (d -4 to -1)			19 h transportation (d -1)	
	2 L (LC)	10 L (HC)	Concentrate	Milk replacer (MR)	Rehydrating solution (RS)	Yes	No
CTRL		X	X	X			X
HCMR		X		X		X	
HCRS		X			X	X	
LCMR	X			X		X	
LCRS	X				X	X	

[a]CTRL = control; HCMR = high-colostrum /milk replacer fed; HCRS = high-colostrum / rehydrating solution fed; LCMR = low-colostrum / milk replacer fed; LCRS = low-colostrum / rehydrating solution fed.
[b]LC= low-colostrum; HC= high-colostrum.

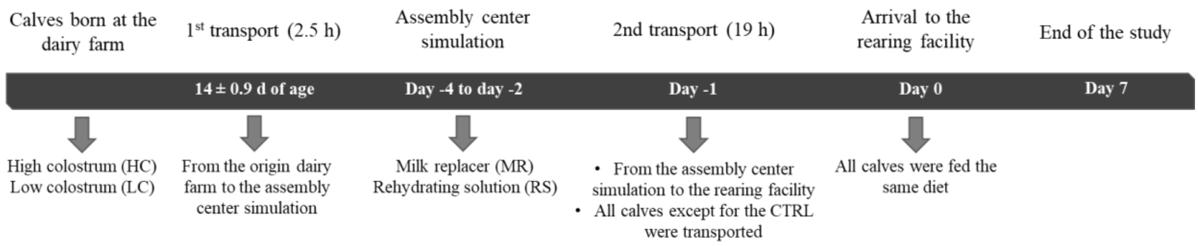


Figure 1. Temporal overview of the study setup of the effects of colostrum consumption and feed restriction during marketing and transportation in the recovery of dairy beef male Holstein calves.

Measurements and Sample Collection

Rectal temperature and health and fecal scores were recorded daily from d -4 until d 7 of the study to assess the general health status of the calves and to identify those that might need medical treatment. For the assessment of respiratory disease, the calf health scoring from the University of Wisconsin-Madison was used by scoring rectal temperature, nasal discharge, eye discharge, ear disposition, and cough using a scale of 0 to 3 as previously described (McGuirk, 2008). If the sum of each health criteria for each calf and day was ≥ 5 a calf was considered sick and treated accordingly. In the event of respiratory disease, calves were treated with an intramuscular injection of Florfenicol 300 mg/mL (Florvex, SP Veterinaria S.A., Spain) at a dose of 20 mg/ kg of BW twice with an interval of 48 h. Fecal scores were also recorded daily and scored following Larson et al. (1997). A score of 0 was considered normal (firm but not hard, original form is distorted slightly after dropping to the floor and settling); a score of 1 was for soft feces (does not hold the form, piles but spreads slightly); a score 2 was for runny feces (spreads readily); and a score 3 for watery feces (liquid consistency, splatters). Calves were considered diarrheic when they presented a score ≥ 2 (Renaud et al., 2020a). Diarrheic calves were treated with a unique intramuscular injection of Marbofloxacin 100 mg/mL (Marbox, CEVA Salud Animal S.A., Spain) at a dose of 8 mg/kg of BW. Body weight was recorded at birth and on d - 4, - 1, 0, 1, 2, and 7 of the study. From d - 4 until d 7, concentrate offered and refused was recorded and used to calculate daily dry matter intake (**DMI**).

In vivo gut permeability tests were conducted on d -4, -1, 0, 1, 2, and 7 of the study using Cr-EDTA as a marker of total tract permeability. Cr-EDTA was orally administered to the calves 2 h after the morning feeding at a concentration of 0.1 g/kg of BW (Sigma-Aldrich Corp.) and a blood sample was taken 2 h after the administration of the marker. The preparation of Cr-EDTA and the concentration given to the calves were based on a previous study (Amado et al., 2019). Blood samples were collected before the morning feeding on d - 4, - 1, 0, 1, 2, and 7 from the jugular vein by using evacuated tubes (BD Vacutainer). Plasma was obtained using 4 mL vacuum tubes with a glycolytic inhibitor (BD Vacutainer® Fluoride Tubes) while serum was obtained using 10 mL vacuum tubes with clot activator and silicon coated (BD Vacutainer® Serum Tubes). Samples were centrifuged at 1,500 × g at 4°C for 15 min and the obtained serum was aliquoted in individual polypropylene tubes and stored at -20 ºC until analysis. Serum was collected to analyze energy balance markers [nonesterified fatty acids (**NEFA**), beta-hydroxybutyrate (**BHB**), and glucose)], markers of stress (cortisol), inflammation [haptoglobin (**Hp**)], muscular damage [creatine kinase (**CK**)], dehydration [total protein (**TP)**], and gut functionality (citrulline, Cr-EDTA, and D-lactate). On d -4, blood samples were collected for measurements of IgG1 at arrival at the rearing facility. Accelerometer data loggers (Hobo Pendant G Data Logger, Onset Computer Corporation) were used for measurements of standing time, standing duration, and standing bouts records from d - 4 to d 7 of the study. Standing behavior was recorded at 1-min intervals and accelerometers were placed on the right hind limb of each calf. Each accelerometer was covered with a foamed rubber to protect the calves from abrasions and attached with a self-adhesive bandage wrap. HOBOware software version 3.7.23 (Onset Computer Corporation) was used for data processing.

Chemical Analysis

Concentrate and MR samples were analyzed for DM, ash, CP, ADF, and NDF, and DM, ash, CP, and sugars, respectively, as previously described (Pisoni et al., 2022a). Colostrum samples were analyzed for fat, CP, lactose, and ashes as previously described (Pisoni et al., 2022b) and for the concentration of lactoferrin, GGT, IgG, and IgG1. Colostrum lactoferrin concentration was measured using a bovine lactoferrin ELISA kit (Cat. No. E11-126, Bethyl Laboratories, Inc., Montgomery, TX, USA). Concentrations of GGT in colostrum were analyzed using a Beckman Coulter AU480 analyzer, and IgG and IgG1 were measured by an ELISA kit (species-specific Bovine IgG and Bovine IgG1, Bethyl Laboratories Inc.). Serum concentration of NEFA was measured by the enzymatic colorimetric method with NEFA-C reagent (Wako Chemicals GmbH). Serum concentration of glucose was determined following the hexokinase method (OSR 6121, Beckman Coulter Inc.) A kinetic enzymatic method (Randox Laboratories Ltd.) was used to determine serum BHB concentration. Serum cortisol concentrations were determined by an enzyme immunoassay (DRG International, Inc.). Serum Hp concentrations were determined by a colorimetric assay (Tridelta Development Ltd.). Serum CK catalytic concentration was determined by the IFCC kinetic method (OSR 6179, Beckman Coulter Inc.). D-lactate was determined by a fluorescence-based assay (Cayman Chemical, USA). Serum IgG1 concentrations were determined by an enzyme-linked immunosorbent assay (specie specific Bovine IgG1, Bethyl Laboratories, Inc.). Serum concentration of TP was analyzed by the method of Biuret (OSR 6132). NEFA, glucose, BHB, Hp, CK, and TP were determined in a Beckman Coulter AU analyzer. Serum citrulline concentration was measured using a spectrophotometric kit (L-Citrulline Kit, Immundiagnostik AG). Serum Cr-EDTA was determined by inductively coupled plasma-optical emission spectrometry, using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500ce). The intra- and interassay coefficients of variation for NEFA, BHB, glucose, cortisol, Hp, CK, D-lactate, IgG1, TP, citrulline, and Cr-EDTA were 1.62 % and 4.46 %; 0.44 % and 2.69 %; 0.58 % and 1.11 %; 2.6 % and 6.6 %; 4.9 % and 5.8 %; 1.34 % and 3.68 %; 3.1 % and 3.5 %; 3.37 and 9.26 %, 0.38 % and 0.74 %, 9.75%, and 5.12%; and <10% and <15%, respectively.

Statistical Analysis

Calf was the experimental unit. A power analysis was conducted to determine the experimental units needed. The type I error rate (α) was 0.05, and the power (1 − β) was set at 80%. Concentrate intake on d 2 was considered our primary outcome and was based on Pisoni et al. (2022a). We expected that concentrate intake of control calves would be around 240 g at d 2 and to find differences of 50% less in concentrate intake in the treatments applied in this study. The study design was a randomized balanced design with a covariate adjustment (BW and age at d - 4). The model included the random effect of pen and the fixed effect of treatment, time, and their interaction. Paired-based randomization based on BW at birth, cow parity (primiparous/multiparous), and calving time (AM / PM) was used to allocate calves in either the HC or the LC treatment at birth. The same criterion was used for the application of the second treatment based on MR or RS feeding and calves were paired-based randomized based on their age and BW at arrival to the assembly center simulation. Data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc.) with repeated measurements for those continuous variables with multiple sampling over time. Non-normal data were log-transformed to achieve normal distributions. The compound symmetry covariance structure and the first-order autoregressive covariance structure were tested according to the time points. Kenward–Roger degrees of freedom were used based on the lower Bayesian information criterion value. Body weight and age at d - 4 were analyzed using the MIXED procedure with treatment as a fixed effect. Health scores were binarily categorized considering 0 as indicative of a healthy calf (score 0) and 1 as indicative of a sick calf (scores 1 to 3). A score ≥ 5 considering the sum of health criteria for each calf and day and was also binary categorized. In this case, if the sum of the health criteria was < 5 it was codified as 0 (healthy calf) and if the sum was ≥ 5 it was considered 1 (sick calf). Health scores were divided into two periods: period 1 (from d -4 to d -1) and period 2 (from d 0 to d 7). Fecal score was analyzed as described for health score with 0 as indicative of normal feces (scores 0 and 1) and 1 as indicative of diarrhea (scores 2 and 3). All health parameters were analyzed using the GLIMMIX procedure of SAS with a binomial distribution. The model included calf as a random effect and treatment, period, and their interaction as fixed effects. The model with lower Bayesian information criterion value was selected. Differences were declared significant at $P \leq 0.05$, and trends were discussed at $P \geq 0.05$ and $P \leq 0.10$ for all models.

RESULTS AND DISCUSSION

Verifying differences between treatments based on the amount of colostrum consumed

To verify that treatments based on the amount of colostrum consumed at birth (HC or LC) were correctly applied, serum concentration of IgG1 was measured on d -4 of the study (right before starting the assembly center simulation). Results showed treatment differences being greater for the CTRL, HCMR, and HCRS calves when compared with the LCMR and LCRS calves ($P < 0.01$; Figure 2). Serum concentration of IgG1 is used as a biomarker for the transfer of passive immunity. Immunoglobulin G1 is the most predominant subclass in colostrum (Korhonen et al., 2000) and, because of this, a better estimate of colostrum consumption. As expected, calves in the HC treatment had greater concentrations of IgG1. There is a great amount of research investigating Ig concentration in young calves and it has been estimated that maternal Ig has a half-life of approximately 10 d after colostrum consumption (Hassig et al., 2007). However, results from this investigation showed that differences in IgG1 between HC and LC calves can remain beyond 14 d of age (14.2 ± 0.81 d of age; mean ± standard error).

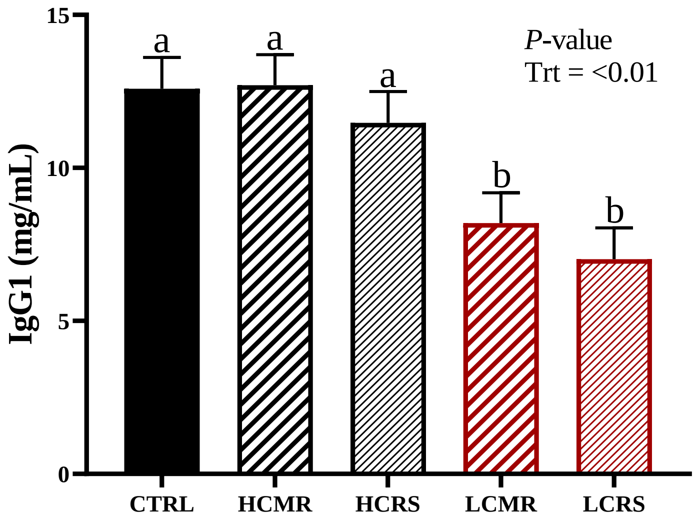


Figure 2. Serum concentration (mean ± SE) of immunoglobulin G1 (IgG1) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS) on d -4 of the study. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

Body weight and concentrate intake recovery

On d -1, after the assembly center simulation period, BW was lesser ($P < 0.01$; Figure 3) for the HCRS and LCRS calves compared with the CTRL, HCMR, and LCMR calves. Correspondingly, a time by treatment interaction ($P < 0.01$; Figure 3) was observed for concentrate intake during the assembly center simulation (from d - 4 to d - 1). This significant interaction indicated that only CTRL calves were able to consume concentrate (average concentrate intake was 78.52 ± 10.98 g/d; data not shown). As expected, there was a clear negative impact on BW losses depending on the type of diet offered during the assembly center simulation period. During this period, in addition to the access to concentrate, the CTRL calves had access to MR and their average daily DMI was 669 g DM/ d compared with HCMR and LCMR calves which consumed only MR (DMI= 604 g DM/ d), and

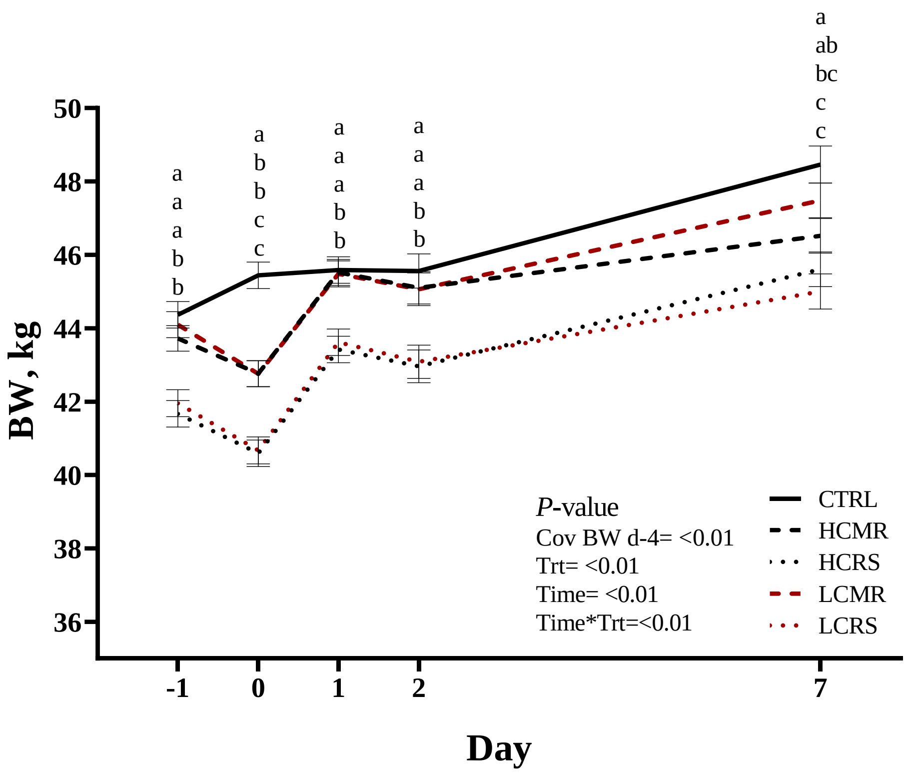


Figure 3. Body weight recovery (mean ± SE) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and

transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), from d −1, 0, 1, 2, and 7. Body weight from d −4 was used as a covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

HCRS and LCRS calves which only consume a RS (DMI= 243 g DM/ d; Figure 5). Crude protein and ME intake were also higher for the CTRL calves during this period. On average the CTRL calves consumed 14.5 g/d of CP and 3.2 Mcal/d of ME, whereas the HCMR, HCRS, LCMR, and LCRS calves consumed an average of 11.4 g/d of CP and 2.6 Mcal/d; 4.5 g/d of CP and 1.0 Mcal/d of ME; 11.5 g/d of CP and 2.6 Mcal/d of ME; and 4.7 g/d of CP and 1.1 Mcal/d of ME, respectively (Figure 6 A and B). Differences in DM, CP, and ME intake during the assembly center period are responsible for the BW losses for the RS calves by d -1 of the study. These results agree with a previous experiment conducted by our research group where the effects of fasting and feed restriction were evaluated in unweaned male calves (Pisoni et al., 2022a). Results from Pisoni et al. (2022a) showed lower BW for calves being fed RS compared to calves fed MR. Additionally, these differences remained almost 21 d after arrival at the rearing farm. Marcato et al. (2020) found similar results in transported calves fed MR vs. RS. In their study, calves that were fed a RS lost BW to a greater extent than calves fed MR after 6 h of transportation (Marcato et al., 2020a). The amount of nutrients and energy contained in the MR might have helped to reduce fat mobilization and the consequent use of body reserves as a source of energy (Marcato et al., 2020a), something that is unlikely to occur by feeding RS which main composition is quickly metabolizable sugars. In the present study, nutritional differences in the diet fed to each treatment caused similar effects. These results confirm that an inadequate feeding regime (low energy and protein intake) at the assembly centers in young calves causes BW losses before calves are transported.

After 19 h of transport (d 0), all transported calves suffered a shrinkage of approximately 1 kg (Figure 3). However, at d 1 and 2 after transportation, HCMR and LCMR calves had similar BW than CTRL while LCRS and HCRS continued exhibiting lesser ($P < 0.01$; Figure 3) BW compared with the rest of the treatments. These results suggest that feeding a RS might delay the recovery of BW when compared with MR-fed calves. Finally, 7 d after arrival all calves increased their BW, however, the HCMR, HCRS, and LCRS calves continued to show lower BW compared with CTRL calves. After the 19 h transportation period (d 0), concentrate was offered to all calves, and HCMR, LCMR, HCRS, and LCRS calves showed a pronounced increment in concentrate intake ($P < 0.01$; time effect; Figure 4) achieving concentrate intake values close to the CTRL calves; this behavior is most likely explained by the hunger experienced during transportation. However, on d 1 of the study, a significant drop in concentrate intake was observed for all transported treatments ($P < 0.01$; time effect; Figure 4). Only HCRS calves showed a lesser drop in concentrate intake close to CTRL calves. The potential reasons for this drop might be in relation to a digestive disorder caused by sudden access to a source of concentrate after a period of feed restriction and transportation. The sudden

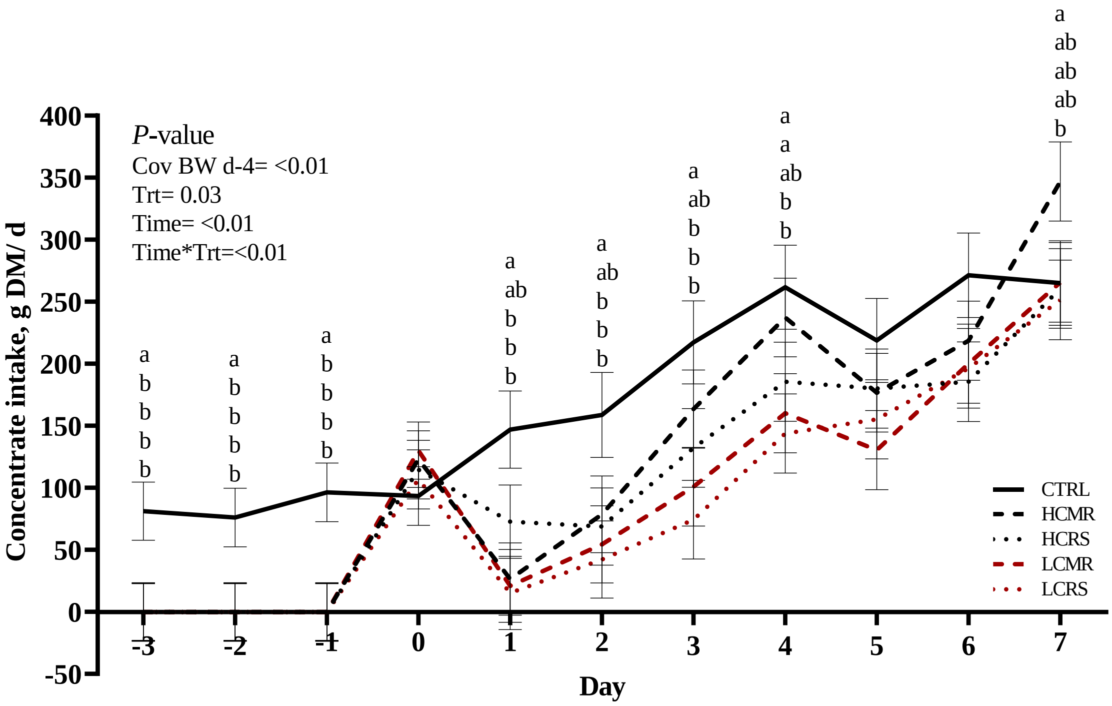


Figure 4. Concentrate intake recovery (mean ± SE) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), from d −3 (assembly center simulation) to d 7. Body weight from d −4 was used as a

covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

increment in intake, in addition to an undeveloped or damaged digestive tract, could have potentially caused ruminal acidosis after rapid overconsumption (Owens et al., 1998). Another possible explanation could be that under a condition of stress due to long transport and feed restriction followed by the sudden access to a rich source of rapidly fermented carbohydrates like concentrate, the gut integrity might have suffered a certain level of disruption allowing the passage of toxins or feed compounds to the bloodstream. Under this circumstance, calves might have reduced their feed intake as part of a defensive physiological mechanism. Also, from d 1 until d 7 all transported calves had a numerically lower concentrate intake recovery (Figure 4) when compared to the CTRL calves. This could be indicative of a process of gut restoration taking place after the distress caused by the abrupt concentrate intake on d 0. The drop in concentrate intake 24 h after a period of fasting when concentrate was re-offered to the calves was previously observed (Pisoni et al., 2022a). In Pisoni et al. (2022a), however, the drop in concentrate intake was on average 35.9 g DM on d 1 compared with the 84.8 g DM from the present study for the same day (Figure 4). Differences found between both trials might be related to the fact that in Pisoni et al. (2022a) calves were not transported but fasted for 9 or 19 h. It is possible that, in addition to the feed and water restriction, the stress due to transportation (mixing, loading, unloading, environmental conditions, etc.) in the present study could have caused greater BW losses and greater compensatory concentrate intake after transport with a consequent greater drop on d 1. Also, the type of calves and the type of concentrate in Pisoni et al. (2022a) were different; calves were Angus-Holstein instead of pure Holstein, and the concentrate was a texturized concentrate with a different ingredient and nutrient composition. From d 2 until d 7 of the study, concentrate intake differences began to decrease for the high-colostrum calves (HCMR and HCRS) but continued to be visible for the low-colostrum calves (LCMR and LCRS) until d 4 showing lower concentrate intake when compared with CTRL, HCMR, and HCRS. These results support the idea that a lower amount of colostrum received at birth could negatively impact the digestive capacity to assimilate concentrate making calves take longer times to recover their consumption to levels like those for the high-colostrum calves. In agreement with our hypothesis, previous studies have demonstrated that colostrum consumption in newborn calves is not only important because of the Ig content but also because it is constituted with bioactive compounds and nutrients that are relevant for the development of the GIT (Blum and Hammon, 2000; Blum, 2006). In the present study, the effects of feed restriction in addition to a lower colostrum consumption at birth might produce changes at a GIT level in these calves that could increase the time needed to recover intake after a feed restriction and transportation period. Both factors (colostrum consumption and feed restriction) probably interact synergistically and seem to be relevant for feed intake recovery in ruminants.

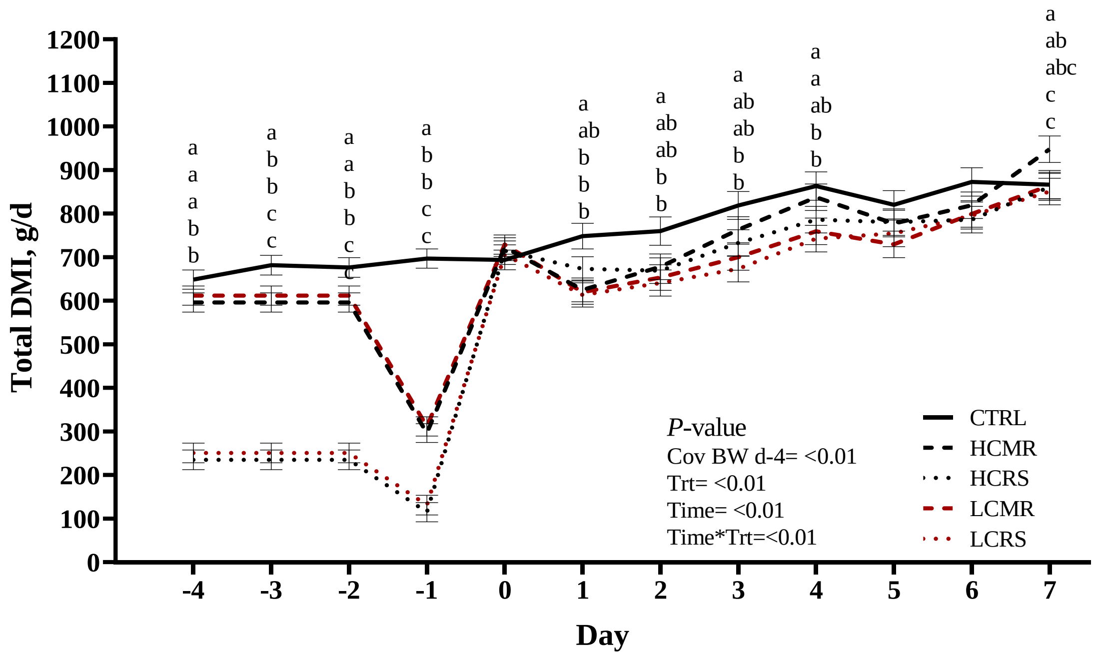


Figure 5. Total dry matter intake (DMI) (mean ± SE) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), from d −4 (assembly center simulation) to d 7. Body weight from d −4 was used as a

covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

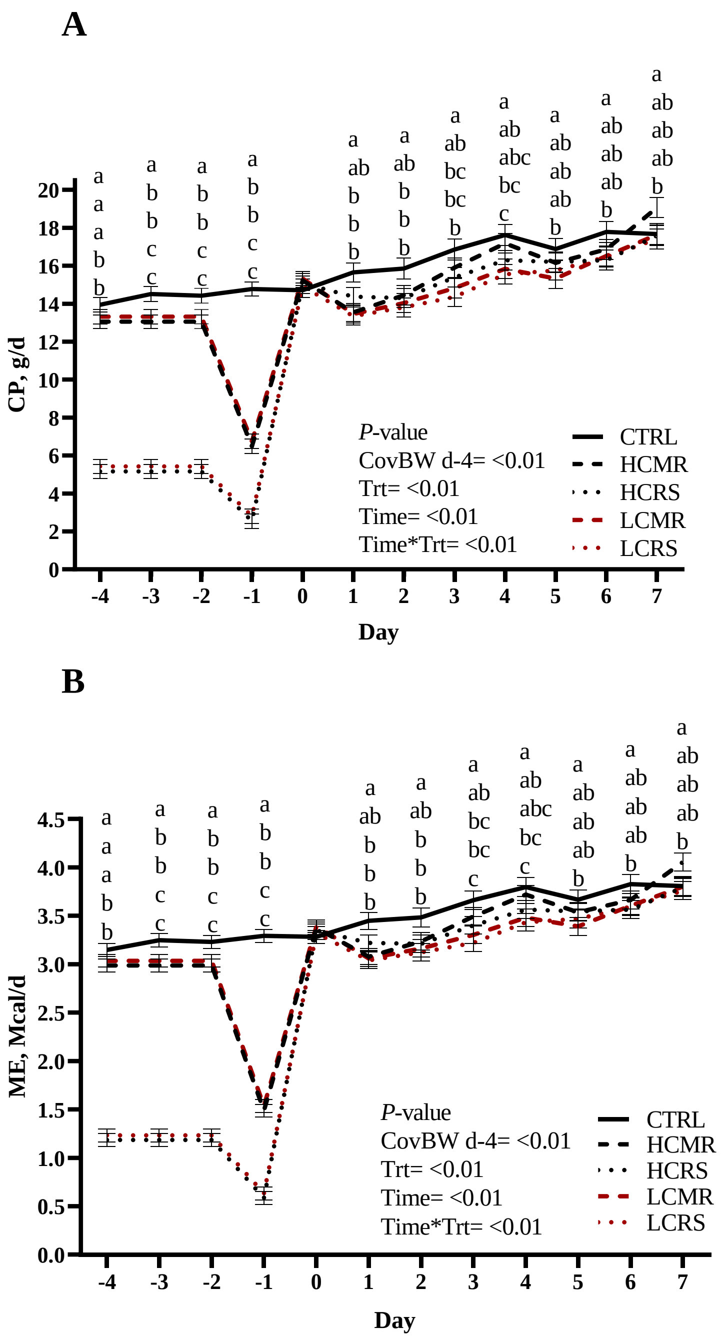


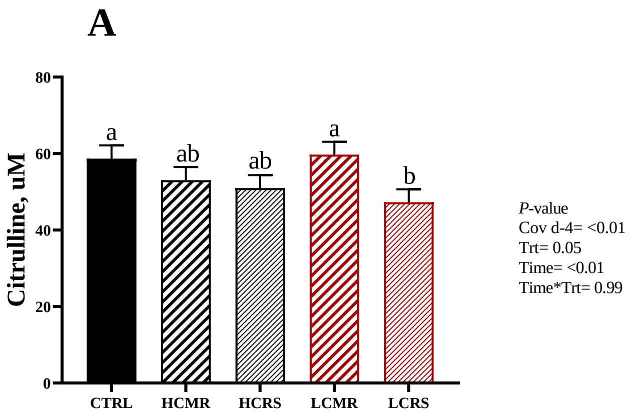
Figure 6. Crude protein (CP, A) and metabolizable energy (ME, B) intake (mean ± SE) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the

assembly center simulation period and transported during 19 h (LCRS), from d −4 (assembly center simulation) to d 7. Body weight from d −4 was used as a covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

Gut functionality

Serum concentrations of Cr-EDTA, citrulline, and D-lactate were used to evaluate the effects of feed restriction and transportation on the gut functionality of calves. Results from serum citrulline concentration showed differences between treatments being lower ($P = 0.05$; Figure 7 A) for LCRS when compared to CTRL and LCMR treatments, while calves in the HCMR and HCRS treatments showed intermediate values. Citrulline is a nonessential amino acid key intermediate in the urea cycle formed exclusively in enterocytes (Kaore and Kaore, 2014). Thus, a decrease in plasma citrulline concentration is associated with a decrease in the functional mass of enterocytes (Oliverius et al., 2010). Results from serum citrulline concentration for the LCRS treatment were expected due to the potential negative effects of low-colostrum consumption (LC) and the greater severity of the feed restriction (SV) on the normal physiology and functioning of the GIT. However, HCRS calves did not show a decrease in serum citrulline. In agreement with our hypothesis, there might be an additive effect of the small amount of colostrum consumed and the severity of the feed restriction on gut functionality. Similar results were observed in a previous study conducted by our research group where serum citrulline concentration was lower in calves fed a RS and fasted during 9 or 19 h when compared with a control group fed MR and not fasted (Pisoni et al., 2022a).

Results from serum Cr-EDTA concentration showed a time by treatment interaction ($P < 0.01$; Figure 7 B). Serum Cr-EDTA is a biomarker of intestinal permeability to large molecules through the paracellular space of adjacent enterocytes (Bjarnason et al., 1995) that has been extensively used in calves (Hunt et al., 2002; Wood et al., 2015b; Wilms et al., 2019). Because it is an indigestible probe it can be used for assessments of total tract permeability and it can be measured in plasma, serum, and urine (Bischoff et al., 2014). In the present study, no differences between treatments were observed by d -1 after the assembly center simulation. However, on d 0 after 19 h of fasting due to transportation, HCRS calves showed greater ($P < 0.01$) serum Cr-EDTA concentrations when compared with CTRL, HCMR, and LCMR calves, whereas CTRL calves showed the lowest concentrations of serum Cr-EDTA. The lack of differences on d -1 suggests that neither feed restriction nor colostrum consumption at birth or their combination might have been challenging



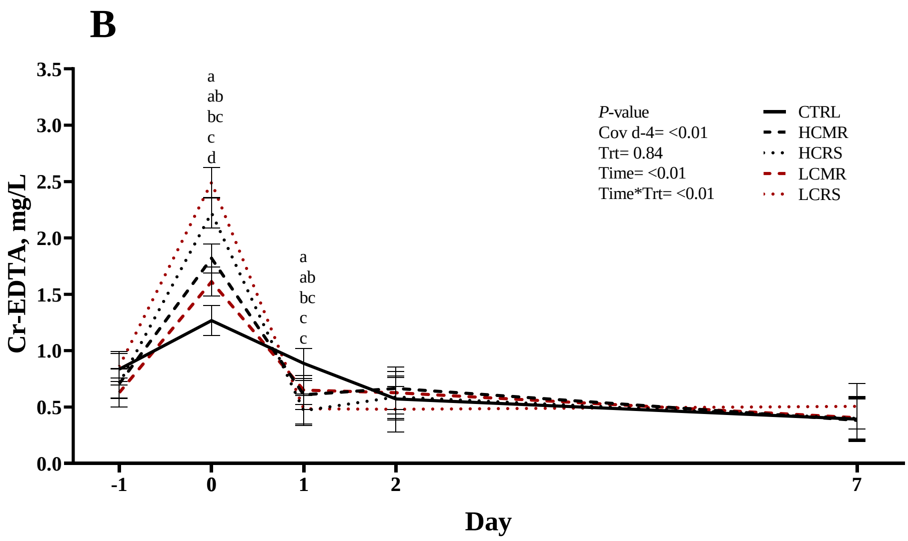


Figure 7. Serum concentration (mean ± SE) of citrulline (A) and Cr-EDTA (B) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period, and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), at d −1, 0, 1, 2 and 7. Serum concentrations of

citrulline and Cr-EDTA from d −4 were used as a covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

enough to exert differences in gut permeability between treatments. However, when calves were exposed to the stress and fasting of a 19 h transportation, those RS-fed calves (HCRS and LCRS) showed greater increments of serum Cr-EDTA. Previous studies have demonstrated that short-term or progressive fasting and feed restriction suffered during marketing and transportation can generate changes in the intestinal function of ruminants (Zhang et al., 2013; Kvidera et al., 2017d; Pisoni et al., 2022a). Reduction in the number of intestinal cells and villus height, increments in cell apoptosis (Ferraris and Carey, 2000), and a decrease in the absorptive capacity of the gut (Moeser et al., 2012) due to fasting and malnutrition have been demonstrated. Other effects on gastrointestinal functionality can also be explained based on colostrum consumption at birth. As previously stated, colostrum is composed of bioactive substances that play an important role in GIT development and modulation (Blum et al., 2002; Blum, 2006). These bioactive substances are components of colostrum that are more predominant in high-quality colostrum which coincides with the first milkings (Blum and Hammon, 2000). Some of the described effects are related to small intestine epithelial growth (Bühler et al., 1998), enhanced survival of epithelial cells, crypt cell proliferation, and enzymatic activity (Blättler et al., 2001). Based on our hypothesis, it was expected that a higher colostrum consumption with a greater amount of bioactive substances would have incremented gut growth and development and made it more resilient to the stress caused by feed restriction and transportation. However, under the conditions of the present study, no clear effect of colostrum consumption itself explained differences in serum Cr-EDTA or serum citrulline concentration. However, as mentioned previously, those calves with LC consumption showed numerically lower concentrate intake recovery when compared with HC-fed calves. Taking this into consideration, it seems to be a potential relationship between the affection of the gut barrier integrity due to stress and feed restriction and its capacity for restoration afterward depending on the previous access to bioactive substances contained in colostrum. Based on these results, it can be reasoned that calves with the lowest colostrum consumption and the highest degree of feed restriction had greater intestinal dysfunctionality and lower intake recovery rates.

Finally, no differences were observed among treatments for serum concentration of D-lactate ($P = 0.61$; data not shown). D-lactate is a normal product from bacterial fermentation (Terpstra et al., 2016). In cases of intestinal ischemia, the gut loses integrity increasing its permeability and the subsequent translocation of bacteria and D-lactate to the bloodstream (Sun et al., 2001; Nielsen et al., 2011). In this study, and considering that feed restriction has been shown to alter the normal functioning of the intestinal barrier (Zhang et al., 2013; Kvidera et al., 2017b), a rise in serum D-lactate was expected. However, results from the present study showed no differences among treatments suggesting that D-Lactate might not be an indicator of gut permeability as sensitive as serum citrulline and Cr-EDTA.

Energy balance

There was a time by treatment interaction ($P < 0.01$) for serum concentrations of NEFA and BHB (Figure 8 A and B, respectively); on d -1 after the assembly center simulation period, these concentrations were greater for the HCRS and LCRS calves when compared with calves in the rest of the treatments. Additionally, a time by treatment interaction ($P< 0.01$; Figure 8 C) was found for serum concentration of glucose being lower for HCRS and LCRS calves in the same period. Measurements of NEFA, BHB, and glucose are normally used as indicators of energy balance. Rises in serum NEFA and BHB concentrations right after the assembly center simulation reflect a greater body fat mobilization to use free fatty acids as a source of energy (Pénicaud et al., 2000; Grigor et al., 2001). This energy deficiency was caused by the negative energy balance triggered by the administration of a RS instead of MR during the assembly center simulation in HCRS and LCRS calves which could not satisfy their energy demands based on the energy obtained by the absorption of nutrients in the diet. In addition to the lack of energy from the diet, the stress derived from hunger raises plasma catecholamine concentrations which also mobilize fat reserves (Frohli and Blum, 1988). Comparable results in these parameters were observed in previous studies where serum NEFA concentrations increased after a period of transportation and feed restriction (Bernardini et al., 2012;

Pisoni et al., 2022a). Concerning serum BHB, its concentration was influenced to the same extent by the negative energy balance suffered. These results on serum NEFA and BHB were in accordance with the losses on BW found for HCRS and LCRS calves after the assembly center simulation period previously described. Moreover, on d -1, there were differences in NEFA and BHB between HCRS and LCRS calves being greater for the HCRS calves. It could be hypothesized that because HCRS calves received greater amounts of colostrum at birth than LCRS calves, these animals might have had greater body fat reserves and were able to mobilize more lipids in that negative energy balance condition. Another hypothesis could be a possible positive effect triggered by the bioactive compounds contained in colostrum that could impact gut physiology and, consequently, its metabolite absorption capacity. In any case, the influence of the feed restriction was, as expected, more pronounced than the influence of colostrum consumption. As expected, on day 0 after transportation, serum concentrations of NEFA and BHB were greater ($P < 0.01$) for all transported calves compared with the CTRL. Calves in the CTRL treatment showed the lowest concentrations of these metabolites during the assembly center simulation and transportation periods as those calves were not transported and were fed MR and concentrate during this time. At d 1, the LCRS calves showed lower concentrations of serum NEFA compared with the rest of the treatments and the HCRS calves had higher concentrations of serum BHB when compared to the CTRL calves. From d 2 until d 7 no differences among treatments were observed neither for serum NEFA or BHB concentrations. Following a similar approach regarding energy balance, calves that were fed only a RS were expected to show a lower concentration of glucose when compared to calves being fed MR. On d 0, after the 19 h transportation, serum glucose concentrations were greater for the CTRL calves when compared with the rest of the treatments due to the access to MR and concentrate during this time. At d 1 and stimulated by the increment in concentrate intake after transportation, serum glucose concentration increased for all transported calves, showing greater concentrations for the HCRS and LCRS calves when compared with CTRL calves. Differences in serum glucose concentration between the HCRS and LCRS and the CTRL calves cannot be explained based on the concentrate intake because no differences were seen between these treatments. Previous research has shown a negative correlation between serum NEFA and BHB concentration and reduced insulin responsiveness (Oikawa and

Oetzel, 2006). Based on Oikawa and Oetzel (2006), we hypothesized that the higher increment in NEFA and BHB concentrations for the HCRS and LCRS calves might have led to an insulin resistance condition or lower insulin secretion that incremented glucose concentration compared to the CTRL calves on d 1. From d 2 until d 7, no differences among treatments in serum glucose concentrations were observed. Rapid increments in serum glucose concentration after transportation when calves start eating concentrate have been previously described (Mormede et al., 1982; Knowles, 1999b; Marcato et al., 2020a). However, because low serum concentrations of glucose have been associated with increments in mortality in newly arrived calves (Mormede et al., 1982; Renaud et al., 2018), avoiding large drops in its concentration should be taken into consideration. This is especially important when these drops last a couple of days like in the case of LCRS and HCRS calves which had serum low glucose concentrations during d -1 to 0.

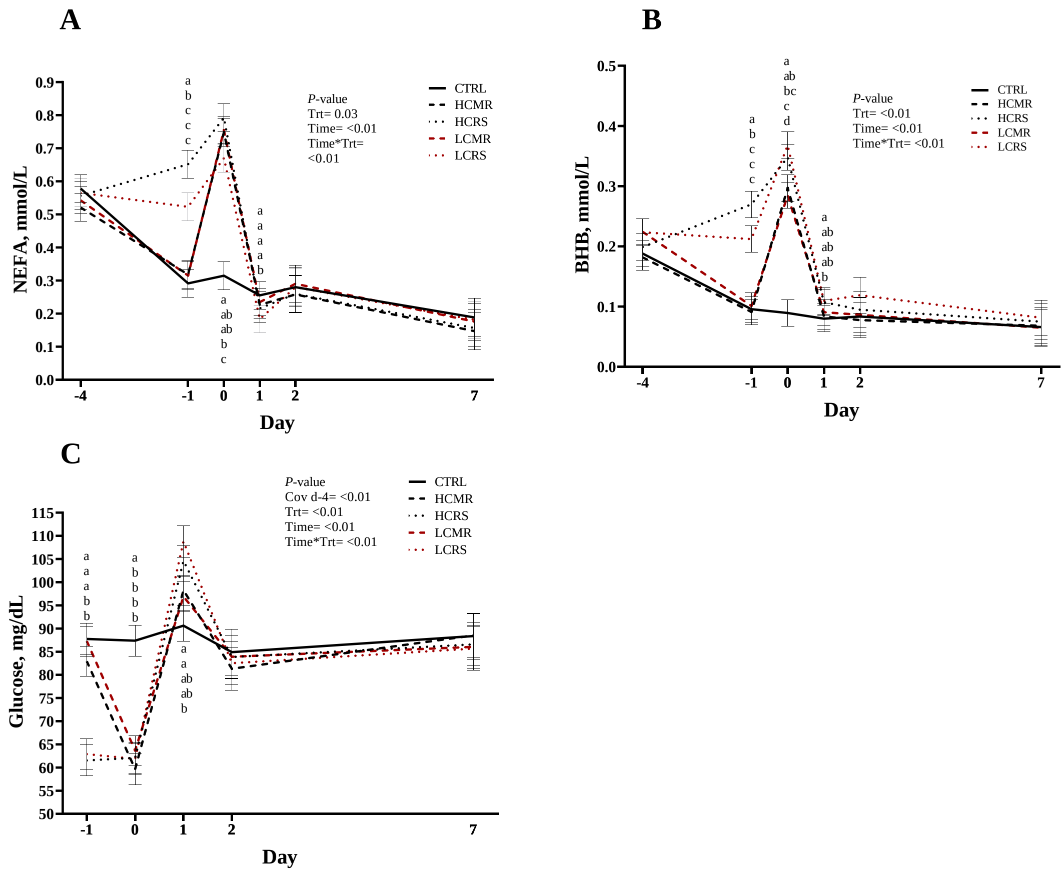


Figure 8. Serum concentration (mean ± SE) of nonesterified fatty acids (NEFA, A), beta-hydroxybutyrate (BHB, B), and glucose (C) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period, and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during

19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), at d −4, -1, 0, 1, 2 and d 7 for NEFA and BHB, and d -1, 0, 1, 2 and d 7 for glucose. Serum concentration of glucose from d −4 was used as a covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

Biomarkers of stress and health status

No differences were observed in serum cortisol concentration during the assembly center simulation (d-4 to d-1). However, a time by treatment tendency ($P = 0.10$; Table 2) was observed at d 0 of the study for serum cortisol concentrations being greater for the LCRS compared with the CTRL, HCMR, and LCMR calves, while LCRS and HCRS calves had similar concentrations. This increment in cortisol was most certainly associated with the stress due to transportation with a higher impact in the RS-fed calves. Cortisol is a glucocorticoid hormone involved in the stress response and regulated by the hypothalamic-pituitary-adrenocortical axis. It is the primary hormone used as a biomarker in studies of stress response. Rises in plasma cortisol levels in unweaned transported calves have been previously described (Fazio et al., 2005; Bernardini et al., 2012). Additionally, high levels of circulating cortisol are responsible for changes in cytokine levels and, therefore, in alterations of the immune function (Vegas et al., 2011). For this reason, cortisol has been proposed as a potential biomarker of disease in young calves (Marcato et al., 2018). In addition, and of interest in this investigation, cortisol produced under stressful situations was shown to disrupt intestinal permeability affecting normal digestive functions and decreasing feed intake (Lambert, 2009a). However, based on the present results, it could be inferred that chronic exposure to stressful conditions with the consequent rises in cortisol might be necessary to cause visible changes in the immune system or to a gut permeability level. Bernardini et al. (2012) found cortisol levels just slightly higher than normal values (18.4 nM; Marcato et al., 2018) in transported calves (37 ± 6 d of age) which returned to normal values after 2 d. In the present study, the average cortisol concentration for all treatments was 14.1 ng/mL (equivalent to 38.8 nM) doubling the reference value for calves.

Additionally, just for the LCRS calves the average concentration of serum cortisol was 15.05 ng/mL (equivalent to 41.4 nM), meaning that when feed restriction was severe and colostrum provision was low, serum cortisol concentrations increased well above reference values.

Surprisingly, serum concentrations of Hp showed no differences between treatments during the assembly center simulation neither for post-transport days (*P* = 0.77; Table 2). Levels of Hp in serum are incremented by cytokines in response to several stressors (Tóthová et al., 2008). On the other side, Hp is considered one of the most reliable markers in detecting diseases in calves since it is mainly triggered by pathological damage and has high sensitivity (Marcato et al., 2018b; Saco and Bassols, 2022). In the present study, the average concentration of Hp for all treatments was 0.13 mg/mL, which has been proposed as a threshold to differentiate healthy from sick calves of approximately 2 (Yu et al., 2019) and 5 weeks of age (Gånheim et al., 2003b). However, in the present study, Hp was not able to differentiate feed-restricted and transported calves from the control. However, the increase from 0.10 to 0.13 mg/mL observed in LCRS from the assembly center simulation period to the post-transport period may be in relation to the increased number of calves with a score > 5 for the same treatment shown in Figure 9 (period by treatment tendency; *P*= 0.07). The sum of health criteria (score > 5) for each calf day was also calculated and showed a period by treatment tendency (*P* = 0.06; Table 3 and Figure 8). During period 1, no differences were seen between treatments for the sum of health criteria. During period 2, after the assembly center simulation and transportation (d 0 to d 7), LCRS calves increased their health scores from period 1 to period 2. From these results, it can be hypothesized that after the feed restriction suffered during the assembly center simulation and transportation, the additive effect of low colostrum consumption and feed restriction based on feeding a rehydrating solution, aggravated the calves` condition making them more prone to get sick. Results from the fecal scores showed period by treatment differences (*P* < 0.01; Figure 10) with an increment in the diarrhea score for the LCRS calves on period 2 compared to CTRL, HCMR, and HCRS calves. Additionally, the HCMR calves had higher fecal scores during period 1 that decreased by period 2. This reduction in the fecal score in the HCMR calves vs. the increase in the LCRS calves could be associated with the greater impact of feed restriction and lower colostrum consumption on the health status of the calves.

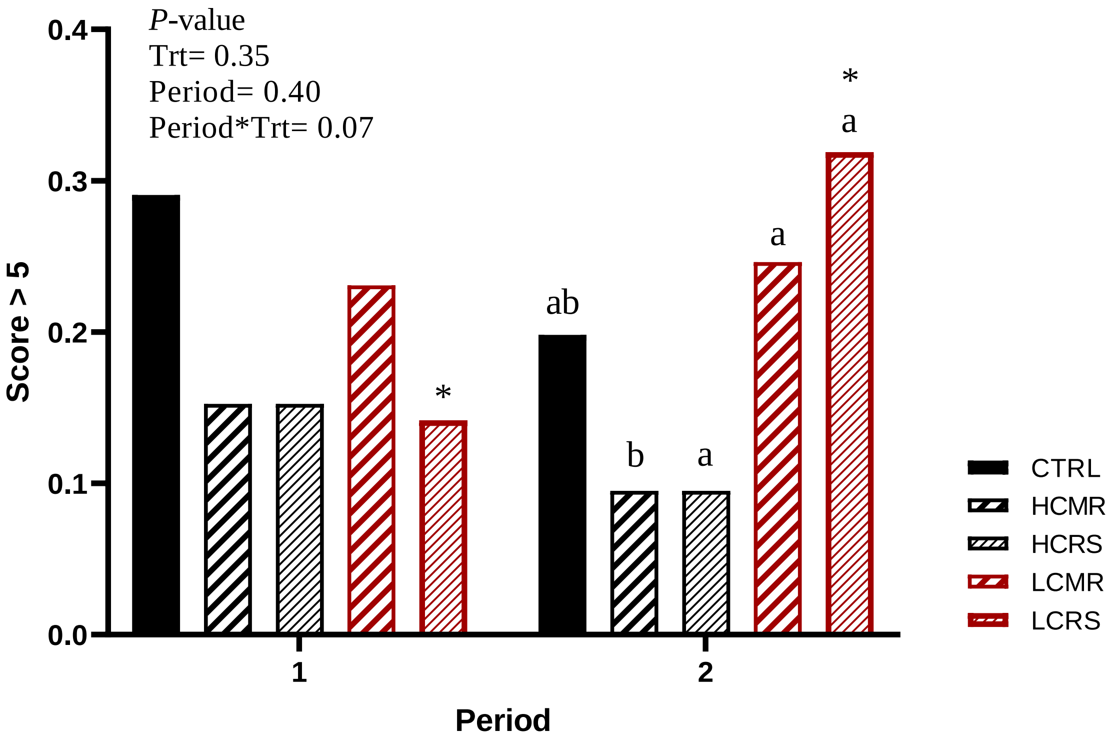


Figure 9. Sum of health criteria for each calf and day (score > 5) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), from d -4 to d -1 (period 1) and from d 0 to d 7 (period 2) of the study. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

Measurements of serum CK have been described as a potential biomarker of physical challenges associated with tissue damage, fatigue, and muscle degradation (Marcato et al., 2018). Previous studies have found increments in serum CK activity in young cattle after a period of transportation (Warriss et al., 1995b; Knowles, 1999b; Averós et al., 2008b). These increments in CK are associated with muscle damage and physical exhaustion and, because of this, CK has been considered an indicator of welfare in calves during transportation (Averós et al., 2008). No differences between treatments were observed for serum catalytic concentration of CK in the present study (P = 0.25; Table 2) indicating that there was no substantial muscular damage during transport. In addition, CK serum catalytic concentrations from the present study were within the reference range for calves in the first weeks of age (Knowles et al., 2000). Some differences between previous research and the

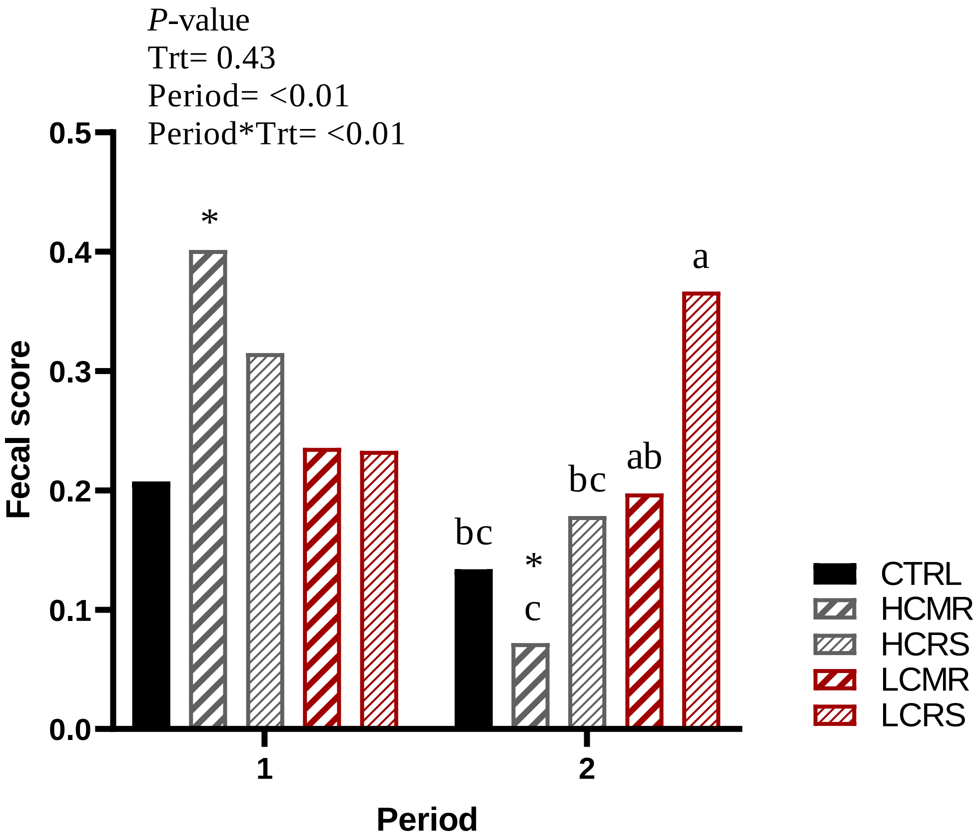


Figure 10. Fecal scores in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), from d -4 to d -1 (period 1) and from d 0 to d 7 (period 2) of the study. Different letters within a time point denote differences among treatments (P < 0.05); the order of the letters denotes the treatment with the highest value.

Table 2. Serum concentration (mean ± SE) of cortisol, haptoglobin (Hp), and total protein (TP) in male Holstein calves measured at d – 4 and -1 (pre-transport), and at d 0, 1, 2, and 7 (post-transport), and on d 0 and 1 for CK.

	Treatments[1]						*P*-value[2]		
Item[3]	CTRL	HCMR	HCRS	LCMR	LCRS	SEM	Trt	Time	Time x Trt
D -4 and -1 (pre-transport)									
Cortisol, ng/mL	11.50	12.55	15.21	13.18	14.98	0.129	0.32	-	-
Hp, mg/mL	0.17	0.12	0.14	0.12	0.10	0.025	0.14	-	-
TP, g/dL	5.70[a]	5.56[a]	5.57[a]	4.98[b]	4.96[b]	0.103	<0.001	-	-
D 0, 1, 2, and 7 (post-transport)									
Cortisol, ng/mL	9.62	10.72	10.83	10.38	15.05	1.736	0.31	<0.001	0.10
Hp, mg/mL	0.15	0.13	0.15	0.12	0.13	0.018	0.93	<0.001	0.16
TP, g/dL	5.26[a]	5.23[a]	5.04[ab]	4.78[bc]	4.74[c]	0.094	<0.001	<0.001	0.84
CK, UI/L	115.69	118.75	115.60	116.79	120.55	2.736	0.26	<0.001	0.31

[a,b] Values with different superscripts within a row differ with a *P* value ≤ 0.05.
Treatments= CTRL (fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported); HCMR (fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h); HCRS (fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h); LCMR (fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h); and LCRS (fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h).
[2]Trt = effect of colostrum consumption and feed restriction, Time x Trt = effect of the time by treatment interaction.
[3] D -4 was used as a covariate in the pre-transport analysis.

Table 3. Proportion of male Holstein calves with calf health score parameters greater than 0 measured from d - 4 (beginning of the assembly center simulation) to d 7 (end of the study).

	Treatments[1]						*P*-value[2]		
Item	CTRL	HCMR	HCRS	LCMR	LCRS	SEM	Trt	Period	Period x Trt
Health score[3],									
Nose	0.09	0.07	0.12	0.06	0.17	0.04	0.46	0.64	0.15
Eye	0.14	0.12	0.16	0.14	0.10	0.04	0.91	<0.01	0.56
Cough	0.04	0.04	<0.01	0.03	0.07	0.02	0.78	0.94	0.98
Fecal[4]	0.17	0.19	0.24	0.22	0.30	0.29	0.43	<0.01	<0.01
Rectal temperature	0.41	0.39	0.34	0.37	0.25	0.07	0.57	0.29	0.11
Score > 5[5]	0.24	0.12	0.19	0.24	0.22	0.05	0.35	0.40	0.07

[1] Treatments= CTRL (fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported); HCMR (fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h); HCRS (fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h); LCMR (fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h); and LCRS (fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h).

[2] Trt= effect of colostrum consumption and feed restriction; Period = period 1 was considered from d -4 to d -1, and period 2 was considered from d 0 to d 7. Period x Trt = effect of the period by treatment interaction.

[3] Health scores greater than 0. The health score "ear" was excluded because of its low incidence.

[4] Fecal score was binary transformed considering 0= normal feces (scores 0 and 1) and 1= diarrheic feces (scores 2 and 3).

[5] Score > 5 (the sum of health criteria for each calf and day) was categorized binary: if the resulting sum was < 5 it was codified as 0, and if it was ≥ 5 it was considered as 1. The proportion contemplates 0= healthy calf (score 0) and 1= sick calf. Because a score > 5 evaluates respiratory disease, the "fecal" score was not considered in the calculation.

present study are in relation to the origin of the calves and the transport conditions. In the present study, all calves belonged to the same dairy farm and did not go through a process of commingling and mixing with calves from different origins. The stocking density in the truck was lower during the 19 h transportation than in commercial situations as only 20 calves were transported in a commercial trailer without space limitation in one deck, therefore, there was plenty space for all calves to lie down during the journey. Finally, the straw bedding provided during transportation was sufficient to cushion calves and avoid bruises. These differences in management might have ameliorated the negative impacts of transportation and therefore diluted differences in CK catalytic concentration between treatments.

Greater concentrations in serum TP pre- and post-transport reflect increments in colostral Ig (Hogan et al., 2015) and this is because, in addition to albumins, Ig are the greatest constituents of TP in newborn calves' blood (Elsohaby et al., 2015) allowing them to be used as an estimator of colostrum consumption in calves. Serum TP levels are also indicative of the hydration status of calves (Heller and Chigerwe, 2018), and their concentration increases during a dehydration condition. In the present study, treatment differences were observed for serum TP concentration being greater for CTRL, HCMR, and HCRS calves when compared with LCMR and LCRS calves ($P < 0.01$; Table 2). Because calves did not exhibit signs of dehydration at the time of sampling, the differences observed in TP concentration may be indicative of the amount and quality of colostrum consumed at birth and not an indication of the hydration status of the calves.

Behavior

Finally, behavior results showed time by treatment differences for standing time ($P = 0.05$; Figure 10). At d -3, LCRS and HCRS calves showed longer and shorter standing times, respectively. These differences disappeared by d -2. During transportation on d -1, LCRS continued showing longer standing times compared with the other treatments. These increments in standing times could reflect the stress originated from hunger in RS-fed calves compared with the MR-fed ones. However, this was only true for LCRS but not for HCRS calves. On d 0, standing times continued to increase coinciding with the previously mentioned raise in concentrate intake at arrival at the rearing facility

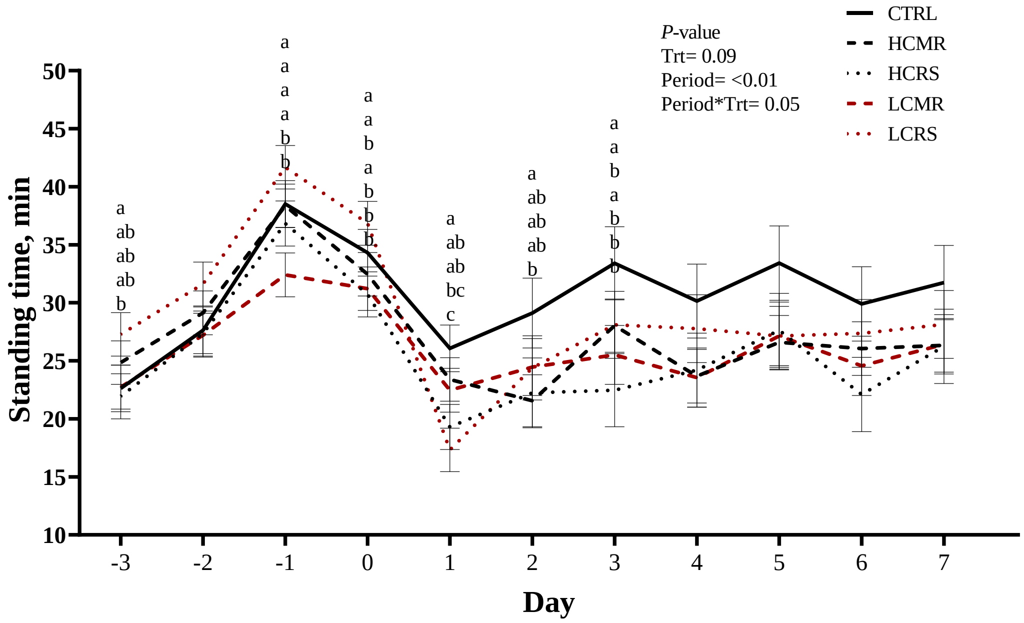


Figure 11. Pendant data loggers' records (mean ± SE) on standing time (min) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), from d - 3 to d 7. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

for all transported calves. In addition, by d 1 standing times decreased for all treatments also coinciding with the drop in concentrate intake observed for the same day. The RS-fed calves (LCRS and HCRS) showed lower standing times when compared with the other treatments for d 1 (Figure 11). From d 1 to d 7, CTRL calves showed longer standing times compared with the transported calves. However, a decrease in the standing time for the transported calves (HCMR, HCRS, LCMR, and LCRS) compared with CTRL was observed as a consequence of exhaustion during the periods under feed restriction and transportation. No differences between treatments were observed for the standing duration and standing bouts. The stress derived from hunger during the assembly center simulation and the 19 h transportation not only affected serum concentrations of NEFA, BHB, glucose, and the corresponding losses on BW but also increased standing times, something that was particularly evident for the calves in the LCRS treatments. Again, an additive effect of these two factors (colostrum consumption and feed restriction) was observed. Results from the present study are in agreement with results observed in a previous study conducted by our research group where increments in standing times during a period of fasting and feed restriction in Angus-Holstein male calves that were being fed RS and fasted during 19h were observed compared with calves fed MR and fasted during 9h (Pisoni et al., 2022a). Finally, the present study results encourage to further investigate other effects of normal commercial situations (commingling, stocking densities, mixing, physical trauma during loading or unloading, among others) and the role these might have as aggravators of the general conditions of calves at arrival to the rearing facilities.

CONCLUSIONS

The degree of feed restriction negatively affected parameters related to BW and intake recovery, gut permeability, and behavior. Moreover, a synergistic effect between the amount of colostrum consumed at birth and the degree of feed restriction suffered during marketing and transportation was also observed in concentrate intake recovery and biomarkers of energy balance (serum NEFA and BHB) and enterocyte mass (serum citrulline). However, the effect of colostrum consumption and feed restriction on health status and stress and gut permeability biomarkers like Hp, CK, cortisol, and D-lactate was not so evident. Results from this study showed that it is clear that feeding RS has a severe negative impact on BW losses but feeding 2 L of MR twice daily also does not cover the requirements of those animals. This practice of feeding low amounts of MR in surplus calves that is common at dairy farms of origin, at assembly centers, or at rearing farms needs further evaluation as improper nutrition has a direct impact on animal health, behavior, and welfare.

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CHAPTER IV

The effects of colostrum consumption and feed restriction during marketing and transportation of dairy beef calves: Impact on gastrointestinal tract functionality

"The effects of colostrum consumption and feed restriction during marketing and transportation of dairy beef calves: pacto n gastrointestinal functionality"

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ABSTRACT

The aim of this study was to investigate how colostrum consumption, feed restriction, and fasting affect the gastrointestinal tract functionality of unweaned dairy beef male Holstein calves being transported during long distances. For this study, 82 male Holstein calves (42 ± 1.2 kg of BW and 14 ± 0.9 d of age; mean ± standard error) born at a commercial dairy farm were used. Calves were assigned to treatments depending on the amount of colostrum fed at birth, the level of feed restriction suffered during an assembly center simulation (d -4 to d -1), and whether or not they were transported during 19 h (d -1). Treatments were as follows: control calves (**CTRL**; n = 16) were fed 10 L of colostrum at birth, milk replacer (**MR**) and concentrate at the assembly center simulation, and were not transported; high colostrum-milk replacer fed calves (**HCMR**; n = 17) were fed 10 L of colostrum at birth, MR at the assembly center simulation, and were transported; high colostrum-rehydrating solution fed calves (**HCRS**; n = 16) were fed 10 L of colostrum at birth, a rehydrating solution (**RS**) at the assembly center simulation, and were transported; low colostrum-milk replacer fed calves (**LCMR**; n = 17), were fed 2 L of colostrum at birth, MR at the assembly center simulation, and were transported; and low colostrum-rehydrating solution fed calves (**LCRS**; n = 16) were fed 2 L of colostrum at birth, RS at the assembly center simulation, and were transported. At arrival at the rearing facility (d 0) all calves were fed the same diet consisting on 2.5 L of MR twice daily and ad libitum access to concentrate, straw, and water. Twenty-four hours after arrival (d 1), blood and fecal samples were collected and a gut permeability test was performed using Cr-EDTA as a marker. After sampling, 8 calves/ treatment were slaughter for intestinal tissue collection. Results showed an increase in the ex vivo gut permeability in the duodenum of LCRS calves when compared with the other treatments whereas the ex vivo permeability from the ileum was increased in all transported calves compared with the CTRL treatment. Intestinal inflammation assessed by the concentration of lactoferrin in feces showed greater concentrations for the HCRS and LCRS calves compared with the CTRL calves. A tendency for downregulation of the tight junction protein *CLDN2* showed lower gene expression in the jejunum of LCRS calves. From the histomorphology analysis, Goblet cells showed a greater counting in the LCMR calves compared with the HCMR, HCRS, and LCRS treatments. Finally, microbiota analysis showed a greater abundance of the family *Bifidobacteriaceae* for the LCMR and LCRS calves. In conclusion, colostrum consumption, feed restriction, and fasting during transportation affected the gastrointestinal functionality of unweaned calves. Calves that consumed a low volume of colostrum at birth and were fed RS (LCRS) showed a greater deterioration in their gastrointestinal tract function. Contradictory results for serum Cr-EDTA and citrulline concentrations and microbiota analysis may be associated to variations in the sampling time, rate of intestinal epithelial recovery, and region and section of the small intestine evaluated, respectively, rather than merely attributable to treatments.

Key words: calves, dairy beef, gastrointestinal functionality, transport

INTRODUCTION

Despite clear evidence of the importance of gut health for immune system development, microbiome establishment, and overall growth, there are still fundamental deficiencies regarding gut functionality and care in young calves. This is especially true for those unweaned calves born on dairy farms and destined to meat production. The poor physiological and nutritional status that these calves usually endure, combined with the stress and feed restriction derived from marketing and transport, worsens their overall health and ability to survive (Mormede et al., 1982; Hulbert and Moisá, 2016; Marcato et al., 2020a). Feed restriction has been shown to increase gut permeability in unweaned calves (Pisoni et al., 2022a), therefore, especial care must be taken when transporting calves during this period .

The gastrointestinal tract (**GIT**) serves a dual purpose: it facilitates nutrient digestion and absorption while preventing the passage of dietary antigens and microorganisms to the bloodstream (Mani et al., 2012). The reticulo-rumen and omasum are composed of a stratified squamous epithelium whose surface area is increased by papillae responsible for the absorption of short-chain fatty acids and minerals, and the secretion of bicarbonate into the lumen (Aschenbach et al., 2011; Steele et al., 2016) . On the other hand, the intestinal epithelium of the lower gut is composed of a single layer of intestinal epithelial cells (columnar epithelium) which in conjunction with gut-associated lymphoid tissue (Spahn and Kucharzik, 2004; Celi et al., 2017), and the lamina propria maintain the gut immune balance and homeostasis (Ahluwalia et al., 2017). The lower gut is constituted by absorptive, mucus-secreting, immune and enteroendocrine cells, which determine its main functions (Meale et al., 2017). These epithelial cells form a physical barrier that selectively favors the transport of molecules across the gut wall in a process commonly known as intestinal permeability (Turner, 2009). Any disruption in the normal functioning of this gut barrier would allow the entry of antigenic macromolecules from the lumen, leading to or perpetuating an increase in its permeability and, consequently, local or systemic inflammatory responses (Lambert, 2009b). Some of the main triggers of gut permeability dysfunction in farm animals are stressful events like weaning (Moeser et al., 2012; Wood et al., 2015a; Mayorga et al., 2020), grouping, transportation (Chase, 2018b; Pisoni et al., 2022a), heat stress (Pearce et al., 2013b), rumen acidosis and ketosis (Abuajamieh et al., 2016; Pederzolli et al., 2018), and feed restriction (Zhang et al., 2013; Kvidera et al., 2017b; Horst et al., 2020). Unfortunately, unweaned male calves destined for dairy beef production are normally exposed to the majority of these stressors, specifically to feed and water restrictions during marketing and transportation (Wilson et al., 2020a; Pisoni et al., 2022a). The development and function of the GIT depend on the early consumption of nutrients and bioactive substances (growth factors, cytokines, hormones, enzymes, among others) contained in the colostrum (Blum and Hammon, 2000; Blättler et al., 2001; Blum, 2006a). Unfortunately, colostrum shortage is another ongoing problem with 12% to 43% of newborn calves entering the dairy beef industry presenting failure in the transfer of passive immunity (**FTPI**) (Renaud and Pardon, 2022). Previous research has shown that feeding calves colostrum during 7 d after birth enhances duodenal villus size and thus the potential for intestinal absorption (Blättler et al., 2001). Similarly, feeding colostrum within 1 h after birth increased the number of beneficial bacteria like *Bifidobacterium* and decreased the prevalence of *E. coli* in calves (Malmuthuge et al., 2015a) which might translate into a lower prevalence of enteropathogens and enteric diseases (Malmuthuge et al., 2015b).

It seems quite evident that maintaining a healthy and functional gut would ensure normal development and growth in young calves. However, in unweaned male calves, the high incidence of FTPI and the limited body reserves available for use as an energy source may reduce their ability to cope with the inherent challenges of marketing and transport. Therefore, we hypothesized that male dairy calves with a low colostrum consumption at birth which suffer feed restriction during marketing and transportation, arrive at the rearing facilities with a compromised gastrointestinal tract functionality. Hence, this study aimed to investigate the effects of colostrum consumption and feed restriction on markers of in vivo and ex vivo gut permeability, enterocyte mass functionality, inflammation and histomorphology, tight junction proteins gene expression, and microbiota in the small intestine of male dairy beef Holstein calves.

MATERIALS AND METHODS

This study was conducted at a commercial dairy farm located in Lleida, Spain (Granja Selergan S.A.) and the experimental research unit of IRTA - Institute of Agrifood Research and Technology (Barcelona, Spain) during October and December 2020. All procedures described herein were conducted as per the guidelines of the Generalitat de Catalunya (Spain; RD 53/2013; project Nº 11211).

Animals and Experimental Design

This project is described in greater detail in the companion to this paper (Pisoni et al., 2023). Briefly, eighty-two male Holstein calves (42 ± 1.2 kg of BW and 14 ± 0.9 d of age; mean ± standard error) born at a commercial dairy farm (Granja Selergan S.A.) were used to evaluate the effects of colostrum consumption at birth and feed restriction during marketing and transportation on gastrointestinal tract functionality. At birth, calves were immediately separated from the dam for colostrum administration. At this moment, newborn calves were divided into 2 groups depending on the amount of colostrum fed. Calves in the high-colostrum group (**HC**; n= 49) were offered a total of 10 L of colostrum (4 L within the first 2 h after birth followed by 3 feedings of 2 L fed within the first 24 h after birth), and calves in the low-colostrum group (**LC**; n= 33) were offered only 2 L of colostrum administered in 1 feeding within the first 2 h after birth. High-quality colostrum (McGuirk and Collins, 2004) was fed via esophageal tube to all calves ([56.28% CP, 29.48% fat, and 10.07% lactose on a DM basis, and 36,910 UI/L gamma-glutamyl transferase (GGT), 1.83 mg/L lactoferrin, 145.44 mg/mL IgG, and 33.11mg/ mL IgG1). All calves were balanced by birth BW, cow parity (primiparous or multiparous), and birth time (day or night) for treatment allocation. After colostrum feeding, calves were reallocated to outdoor individual hutches and were fed 2 L of milk replacer (**MR**) at a concentration of 125 g /L as fed (21.86% CP, 16.59% fat, 45.50% lactose, with the main ingredients being skimmed milk powder, whey powder, vegetable oil, starch, and dextrose; Schils, The Netherlands) twice daily with ad libitum access to concentrate (17.1% CP, 16.9% NDF, 5.7% ADF, 29.2% starch, 5.5% ether extract, 5.1% ashes on a DM basis, with the main ingredients being 39% cornflake, 17% barley, 14% wheat middlings, 13.9% soybean meal, 9% wheat bran, 3% sunflower meal, 1.9% palm oil, and 1.3% calcium carbonate, 0.5% premix, and 0.4% salt) and water. Calves remained at the dairy farm of origin until approximately 14 d of age when they were transported for 2 h to the experimental research unit of IRTA (Barcelona, Spain). The experimental unit was a closed barn with 78 individual pens (1.20 m x 1.97 m) bedded with sawdust. At arrival, an assembly center was simulated during 3 d, from d -4 to d -1 of the study. During the assembly center simulation, calves were fed either MR or a rehydrating solution (**RS**; at a concentration of 60 g/ L; 0.39% CP, 0.05% fat, 84.75% dextrose; Corion®, Spain), two typical feeding strategies used during the commercialization of dairy beef calves in Europe. In practice, calves can stay at assembly centers from a couple of hours until up to 6 d (Reenen et al., 2022), so for this study 3 d was considered the average time used to mimic this period. At the end of the assembly center simulation on d -1 of the study, part of the calves was transported for 19 h, which is the maximum amount of time according to regulations from the European Commission for the transport of unweaned calves (Regulation (EC) 1/2005) without being unloaded for feed and rest. This transport was intended to simulate an international purchase of calves between countries of the European Union. Combining the amount of colostrum fed at birth, the feeding strategy applied during the assembly center simulation, and if calves were transported or not, final treatments were as follow: control calves (**CTRL**, n= 16) were fed 10 L of colostrum at birth and 2.5 L of MR twice daily during the assembly center simulation with ad libitum access to concentrate and water; these calves were not transported during 19 h as this treatment was intended to be representative of an ideal scenario where calves do not suffer colostrum or nutritional shortages and are not exposed to long-distance transportation; high colostrum-milk replacer fed calves **(HCMR**; n= 17) were fed 10 L of colostrum at birth, 2.5 L of MR twice daily during the assembly center simulation, and were transported 19 h on d -1 of the study; high colostrum-rehydrating solution fed calves (**HCRS;** n = 16) were fed 10 L of colostrum at birth, 2.5 L of RS twice daily during the assembly center simulation, and were transported 19 h on d -1 of the study; low colostrum-milk replacer fed calves (**LCMR;** n= 17) were fed 2 L of colostrum at birth, 2.5 L of MR twice daily during the assembly center simulation, and were transported 19 h on d -1 of the study; and low colostrum-rehydrating solution fed calves (**LCRS**; n= 16) were fed 2 L of colostrum at birth, 2.5 L of RS twice daily during the assembly center simulation, and were transported 19 h on d -1 of the study. After transport on d 0 of the study, calves returned to the experimental research unit and were fed the same diet consisting of 2.5 L of MR twice daily with ad libitum access to concentrate, straw, and water. Variations in the MR formula and concentrate may affect the results of the study due to indigestion and/or diarrheas, so for this reason the same MR and concentrate offered at the dairy farm was used throughout the experiment. Finally, on d 1 of the study (24 h after arrival), 8 calves/ treatment were chosen based on BW and age and were euthanized for gut tissue collection. The reader can refer to Table 1 and Figure 1 for a summarized explanation of the treatments and the temporal overview of the study setup.

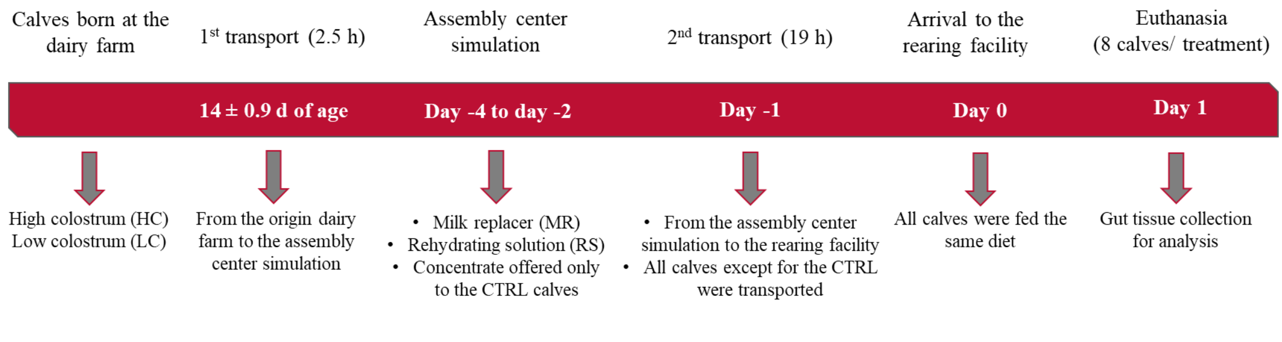


Figure 1. Temporal overview of the study setup on the effects of colostrum consumption and feed restriction during marketing and transportation on gastrointestinal tract functionality of male dairy beef calves.

Table 1. Treatments description on the study of the effects of colostrum consumption and feed restriction during marketing and transportation on gastrointestinal tract functionality of male dairy beef calves.

Treatments[a]	Colostrum given at birth[b]		Diet offered during the assembly center simulation (d -4 to -1)			19 h transportation (d -1)	
	2 L (LC)	10 L (HC)	Concentrate	Milk replacer (MR)	Rehydrating solution (RS)	Yes	No
CTRL		X	X	X			X
HCMR		X		X		X	
HCRS		X			X	X	
LCMR	X			X		X	
LCRS	X				X	X	

[a]CTRL = control; HCMR = high-colostrum /milk replacer fed; HCRS = high-colostrum / rehydrating solution fed; LCMR = low-colostrum / milk replacer fed; LCRS = low-colostrum / rehydrating solution fed.
[b]LC= low-colostrum; HC= high-colostrum.

Sample collection

On d 1 (24 h after transport), a blood sample was collected before the morning feeding for measurements of serum citrulline concentration. Afterward, calves were fed 2.5 L of MR and, 2 h after the morning feeding, in vivo gut permeability tests were conducted using Cr-EDTA and ovalbumin. These markers were given orally to the calves using 100 mL syringes and their serum concentration was determined by a second blood sample collected 2 h after their administration. The concentration of Cr-EDTA was 0.1 g/kg of BW (Sigma-Aldrich Corp, Saint Louis, MO) and it was based on a previous study (Amado et al., 2019). Whereas ovalbumin (Sigma-Aldrich Corp, Madrid, Spain) concentration was calculated based on dose translation (Reagan-Shaw et al., 2008) of the concentration used for rats (González-Quilen et al., 2019).

All blood samples were collected from the jugular vein of calves using 10-mL evacuated serum tubes (BD Vacutainer®, San Agustín del Guadalix, Spain). Tubes were left at room temperature for half an hour and later centrifuged at 1,500 *x g* at 4ºC for 15 min. The obtained serum was aliquoted in 1.5 mL microcentrifuge tubes and frozen at -20ºC until analysis.

Fecal samples for fecal lactoferrin determination were also collected on d 1. Fresh feces were obtained by rectum stimulation and collected using 60 mL polypropylene containers. Afterward, samples were immediately stored at -20ºC until further analysis.

After blood and fecal sampling, 8 calves/ treatment were selected based on BW and age for intestinal tissue and digesta sample collection. Calves were euthanized following the European Guidelines for Animal Welfare (Directive 86/609 EEC). Before euthanasia, calves were rendered fully unconscious via an intramuscular injection of xylazine (Xilasyn® 2, 20 mg/mL, Virbac, Carros, France) at a concentration of 0.3 mg/kg. Final slaughter was done via jugular intravenous injection of sodic pentobarbital (DOLETHAL 200 mg/mL, Vetoquinol, Lure, France) at a concentration of 100 mg/kg. Within 5 min after euthanasia, the portion of the intestinal tract contained within the pylorus and the rectum was ligated, excised, and removed from the abdominal cavity. At the end of each segment, zip-ties were placed to avoid digesta contamination between segments before sampling. Segments from the small intestine were collected for tissue and content samples for later assessment of histomorphology, ex-vivo gut permeability tests, tight junction gene expression, and microbiota analysis.

For histomorphology, 5-cm samples from the jejunum and ileum were collected and washed with sterile PBS solution before being immediately stored in 20 mL biopsy containers filled with a 10 % formalin solution and methanol (BiopSafe®, Hellerup, Denmark). For the jejunum, the portion was located ~5.5 m proximal to the ileocecal junction, and for the ileum, the portion selected was collected 10 cm from the ileocecal junction. Segment length and location were chosen based on previous studies (Malmuthuge et al., 2015; Kvidera et al., 2017; Welboren et al., 2021).

For the ex-vivo gut permeability analysis, segments of 5 cm length from the duodenum, jejunum, and ileum were used. Tissue samples from the jejunum and ileum were taken 10 cm caudal to those for histomorphology, and from the duodenum 10 cm caudal to the pyloric orifice of the abomasum. The technique used to assess ex-vivo gut permeability was developed by a research group at the Department of Biochemistry and Biotechnology from the Universitat Rovirai Virgili (Tarragona, Spain). This set up called Ap-to-Bas (Apical-to-Basolateral) has been initially tested in poultry and pigs to assess vectorial transepithelial processes simulating a Ussing chambers approach (Ginés et al., 2018). For this technique, the obtained tissue samples were immediately stored in ice-cold oxygenated KRB buffer (Hepes 11.5 mM, $CaCl_2$ 2.6 mM, $MgCl_2$ 1.2 mM, KCl 5.5 mM, NaCl

138 mM, NaHCO3 4.2 mM, NaH2PO4 1.2 mM) (Sigma-Aldrich, Madrid, Spain) with d-mannitol (10 mM; Sigma-Aldrich, Madrid, Spain) and transferred to the laboratory at 4ºC. The time between tissue excision and ex-vivo analysis was inferior to half an hour for each calf sampled.

Gene expression analysis of the tight junction proteins claudin 2 and 4 (***CLDN2*** and ***4***), occludin, and zonula occludens-1 (***ZO-1***) was performed on jejunum samples. For this analysis, a 0.5 cm^2 section of jejunum was taken and immediately incubated in a 1.5 mL microcentrifuge tube containing RNAlater (Invitrogen, Madrid, Spain) at 4ºC for 24 h to preserve the integrity of the RNA. After incubation, the liquid was removed and the tissue was stored at -80ºC until further RNA extraction and gene expression analysis.

Finally, mucosa and content samples from the jejunum were collected for microbiota analysis. A 20 cm long segment of the jejunum was sealed in both extremes to isolate the liquid content which was later collected in 50 mL centrifuge tubes. Afterward, the same segment was cut longitudinally to expose the mucosa which was cleaned with a sterile saline solution and subsequently scraped with ice-cold glass slides for sample collection. The mucosa was then stored in 1.5 mL microcentrifuge tubes. After collection, all samples were frozen in liquid nitrogen and stored at -80ºC until further analysis.

Sample analysis

Serum citrulline concentration was determined via a spectrophotometric kit (L-Citrulline Kit, Immundiagnostik AG, Bensheim, Germany). The intra and interassay coefficients of variation were 9.75% and 5.12%, respectively. Serum concentration of Cr-EDTA was measured using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500ce, Agilent, Santa Clara, CA) as previously described (Pisoni et al., 2022a). The intra- and inter-assay coefficients of variation were <10% and <15%, respectively.

Fecal lactoferrin was extracted following (Cooke et al., 2020) extraction, and the determination was done using a commercial kit (Bovine Lactoferrin ELISA kit, E11-126, Bethyl Laboratories, Montgomery, Texas, USA). The intra and inter-assay coefficients of variations were

5.8% and 7.6%, respectively. After determination, fecal samples were dried at 103ºC for 24h, and the final fecal lactoferrin concentration was expressed on a DM basis.

Intestinal tissues for histomorphology analysis were conducted according to a previous study (Nofrarías et al., 2007). Briefly, intestinal section samples were fixed in 10% neutral-buffered formalin. Each tissue sample was processed routinely for histopathology. Tissue capsules were dehydrated in serial alcohol baths, cleared in xylene, and embedded in paraffin wax, using an automatic tissue processor system. Tissue blocks were mounted, and ~4 µm sections were performed with a microtome. Sections were automatically stained with Haematoxylin and Eosin. Morphometric measurements were performed with a light microscope (BHS, Olympus, Barcelona, Spain) as previously described (Nofrarías et al., 2006) using a linear ocular micrometer (Olympus, Ref. 209-35040, Microplanet, Barcelona, Spain). Villus height, crypt depth, Goblet cell, and intraepithelial lymphocyte (**IEL**) numbers were measured in 10 well-oriented villi and crypts from each animal. Measurements of villus height and crypt depth were done at 10x magnification and 20x magnification for Goblet cell and IEL number by the same operator. Villus height was obtained by measuring each villus from the tip to the base and crypt depth was measured by drawing a line from the top of the crypt to the junction of the muscular layer to the bottom of the crypt (Wongdee et al., 2015). The same villus and crypts were used to count the total number of Goblet cells and IEL; these variables were expressed as total per villi and per 100 µm.

The Ap-to-Bas technique evaluated the ex-vivo assessment of gut permeability based on Ginés et al. (2018) and adapted to calves intestinal tissue. Figure 2 shows the steps followed during the sample preparation. Briefly, after arrival at the laboratory, the intestinal segments were rinsed with KRB buffer and placed in a plastic tube to remove the outer muscular layer. Afterward, the intestinal segment was cut longitudinally and the mucosa was exposed. A biopsy punch was used to produce circular subsamples with a diameter of 14 mm that were later randomly placed in a beaker glass. A total of 4 replicates from each segment/calf were used. Subsequently, a silicon tube (internal diameter= 8 mm; external diameter= 12 mm, and 1.5 cm long) was cut perfectly flat and was embedded with tissue adhesive to be later gently pressed onto the apical side of the intestinal segment. The insert containing the tissue sample and the tube was placed in a 12-well plate prefilled with 1 mL of KRB buffer. The apical side of the tube was filled with 400 µL of KRB buffer and incubated at 37ºC for 15 min. Later, a 70-kDa fluorescein isothiocyanate (**FITC**)-dextran (Sigma-Aldrich, St. Louis, MO, USA) was added apically and incubated for 60 min. The apical and basolateral media were collected, centrifuged, and stored at -20ºC until analysis. The concentration of (FITC)-dextran that crossed from the apical to the basolateral side was measured using a PerkinElmer LS- 30 fluorimeter (Beaconsfield, U.K.).

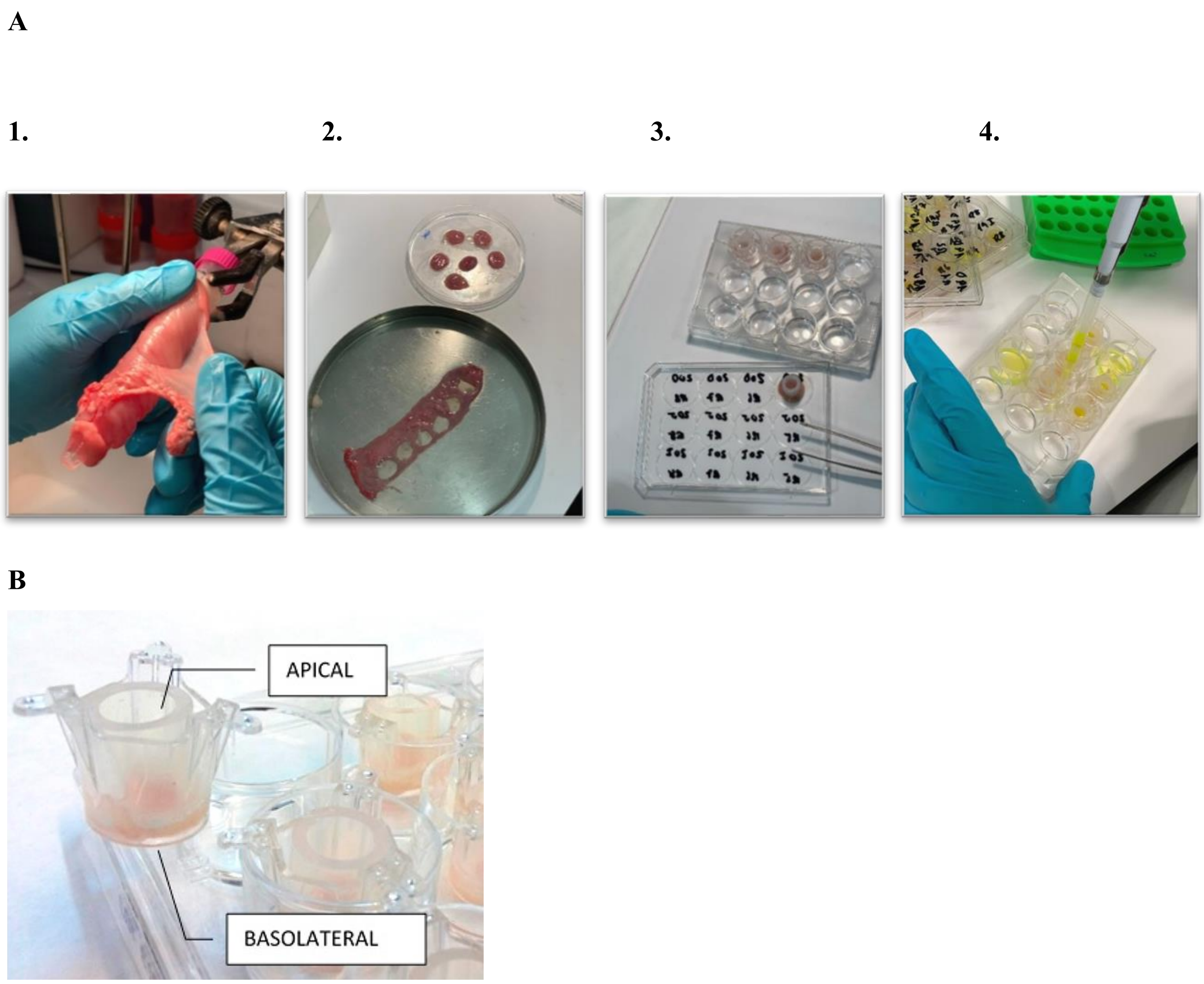
Total RNA for gene expression determination of *CLDN2*, *CLDN4*, occludin, and *ZO-1* was extracted from jejunum samples using TRIzol reagent (Invitrogen) as previously described (Devant et al., 2016). The RNA purity was assessed with a NanoDrop instrument (ThermoFisher, Madrid, Spain) obtaining 260:280 nm ratios between 1.9 and 2.0. The quantification of gene expression was performed by quantitative PCR using β-actin (ACTB) as a housekeeping gene (Table 2).

Table 2. Primer pair sequences of genes analyzed from the jejunum of 14 ± 0.9 d of age male Holstein calves.

Gene[1]	Forward primer (5′-3′) Reverse primer (5′-3′)	At, ºC	Efficiency (%)	Concentration (µM)	Amplicon size bp
ACTB	CTGGACTTCGAGCAGGAGAT CCCGTCAGGAAGCTCGTAG	57	82	0.125	75
CLDN 2	CATGCTAGGCCTGCCCGCTG AAGACTCCGCCCACAACCGC	60	90	0.25	165
CLDN 4	CATGATCGTGGCCGGCGTG AGGGCTTGTCGTTGCGGG	62	82	0.125	226
OCLN	ATCAACCCCGGTGCCGGAAG GTGGTCTTGCTCTGCCCGCC	57	82	0.5	162
ZO-1	TTGCCTGCTGCGGCGTACC GCCCTTCTCCCCAAACACGACA	60	94	0.5	157

[1] *CLDN2*= claudin-2, *CLDN4*= claudin-4, *OCLN*= occludin, *ZO-1*= zonula occludens-1.

For the analysis of the microbiota, microbial DNA was extracted with the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). After DNA extraction, agarose gel electrophoresis was performed in the mucosa and content samples of the jejunum for DNA identification. After quality control, the amount of genetic material from the jejunum content of 18 samples was insufficient for subsequent amplification analysis and the samples were discarded. Thus, the results described herein were focused on treatment effects on mucosa-associated microbiota. Extracted DNA was sent to the University of Illinois Keck Center and amplified with full-length 16S primers and sequenced on a Pacbio Sequel IIe. Amplicons were converted to a library with the SMRTBell Express Template Prep kit 2.0. The library was sequenced on 1 SMRTcell 8M on a PacBio Sequel IIe. The pb-16S-nf Nextflow pipeline was employed to analyze the PacBio HiFi full-length 16S data. Raw read count was cumulative sum scaling normalized and differential abundance among treatments was performed using SAS.



From Ginés et al. (2018)

Figure 2. Description of the Ap-to-Bas setup used for ex vivo assessment of gut permeability. **A.1.** The outer muscular layer is first removed and then the intestinal tube is opened longitudinally to expose the mucosa. **A.2.** Once the mucosa is exposed, a biopsy punch is used to cut circles of tissue (approx. 1.54 cm^2). **A.3.** A silicon tube was glued to the apical side of the intestinal segment and placed in a 12-well plate. **A.4.** Later, 70-kDa fluorescein isothiocyanate (FITC)-dextran was added into the apical side of the tube and incubated for 60 min after which the apical and basolateral media were collected. **B.** A representation of the final insert containing the tissue sample and the tube with the apical side facing the lumen of the tube and the basolateral side facing the 12-well plate bottom.

Statistical Analysis

Calf was the experimental unit. The total sample size of this study was described in the companion to this paper (Pisoni et al., 2023). In contrast, the sample size used to calculate the number of calves euthanized in the present study was calculated based on intestinal villus height from a previous investigation (Kvidera et al., 2017).The study was designed as a randomized balanced design with a covariate adjustment. The model included the random effect of pen and the fixed effect of treatment, and initial BW and initial age as covariates. Data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc.). Non-normal data were log-transformed to achieve normal distribution. The ANOVA test was used for the analysis of the microbiota data in SAS. Differences were declared significant at $P \leq 0.05$, and trends were discussed at $0.05 \leq P \leq 0.10$ for all models.

RESULTS

Results from the in vivo gut permeability test showed treatment differences for serum Cr-EDTA concentrations ($P<0.01$; Figure 3 A). Calves in the CTRL and LCMR treatments showed greater serum concentrations when compared with HCRS and LCRS calves, whereas HCMR calves showed intermediate values (Figure 3 A). Regarding gut permeability to ovalbumin, no differences in its serum concentration were detected among treatments ($P= 0.11$; Figure 3 B).

Evaluation of enterocyte mass function by serum citrulline concentration showed treatment differences ($P= 0.05$; Figure 4 A). Calves in the CTRL and LCMR treatments showed greater concentrations compared with HCMR and LCRS calves, whereas HCRS calves had intermediate values. Regarding the assessment of intestinal inflammation, HCRS and LCRS calves showed greater ($P= 0.05$; Figure 4 B) fecal lactoferrin concentrations when compared with the CTRL treatment. Calves in the HCMR and LCMR treatment showed intermediate values within CTRL and HCRS but were significantly lower than the LCRS calves.

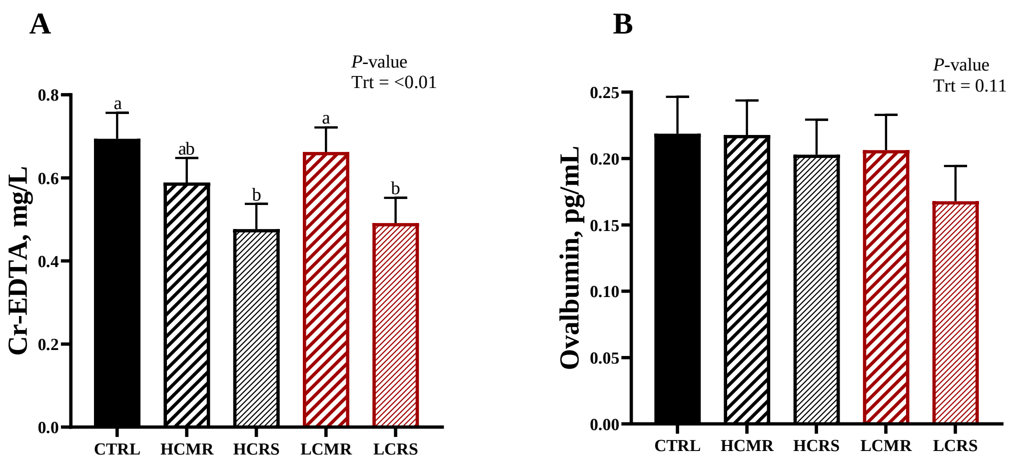


Figure 3. Serum Cr-EDTA concentration measured at d 1 of the study in male Holstein calves fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period, and not transported (**CTRL**), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (**HCMR**), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (**HCRS**), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (**LCMR**), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (**LCRS**). Different letters denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

For the ex vivo determination of gut permeability, the setup Ap-to-Bas was used, and the final results were expressed on % of fluorescence units (**FU**) based on the amount of FITC-dextran on the basolateral side of each segment. Results showed a greater % of FU in the duodenum of the LCRS calves when compared with the rest of the treatments ($P= 0.03$; Table 3), whereas no differences between treatments were observed in the jejunum ($P= 0.46$; Table 3). Results from the ileum section showed treatment differences ($P= 0.03$; Table 3) with a lower % of FU for the CTRL calves compared with the rest of the treatments.

Table 3. Serum concentration of fluorescence units (FU) of fluorescein isothiocyanate (FITC)-dextran recovered on the basolateral side of the duodenum, jejunum, and ileum of male Holstein calves.

	Treatments[1]						*P*-value[2]
Item	CTRL	HCMR	HCRS	LCMR	LCRS	SEM	Trt
Duodenum	45.6[b]	94.0[b]	116.5[b]	159.6[b]	1110.5[a]	209.10	0.03
Jejunum	1188.2	109.3	444.1	235.8	135.4	362.23	0.46
Ileum	5022.4[b]	6160.4[a]	6315.3[a]	6602.9[a]	7842.6[a]	1380.76	0.03

a,b Values with different superscripts within a row differ with a *P* value ≤ 0.05.
[1] Treatments= CTRL (fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported); HCMR (fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h); HCRS (fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h); LCMR (fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h); and LCRS (fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h).
[2] Trt= effect of colostrum consumption and feed restriction.

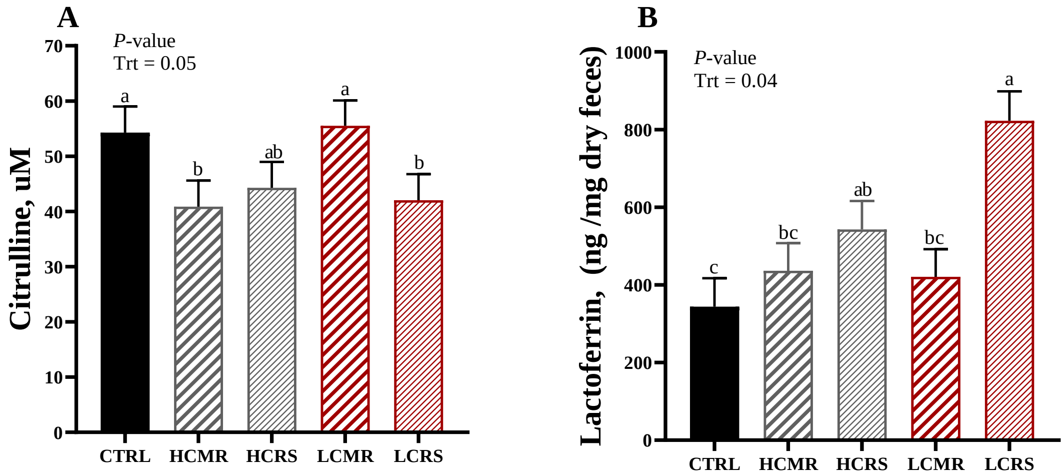


Figure 4. Serum citrulline (A) and fecal lactoferrin (B) concentration measured at d 1 of the study in male Holstein calves fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period, and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS). Different letters denote differences among treatments (*P* < 0.05); the order of the letters denotes the treatment with the highest value.

There was a tendency (*P*= 0.10; Table 4) for the gene expression of the tight junction protein *CLDN2* in the LCRS calves, which showed a reduced jejunal expression of this protein when compared with the other treatments. No differences were observed for the gene expression of *CLDN4*, occludin, and *ZO-1*, however, gene expression of *ZO-1* was numerically lower for LCMR and LCRS calves.

Histomorphological analysis of the jejunum samples could not be performed due to extensive edema and intestinal epithelial shedding (Figure 5; A). The reasons behind these findings are unknown, but research is being carried out to classify the samples according to the degree of edema and epithelial shedding to evaluate if there are any treatment effects that could explain the results. In the histomorphology analysis of the ileum, no differences were found between treatments for VH, CD, and IEL number (*P*= 0.21; *P*=0.79; and *P*= 0.88, respectively; Table 5). However, the number of Goblet cells was greater (*P*= 0.03; Table 5) in the LCMR calves compared with the HCMR, HCRS, and LCRS treatments. Calves in the CTRL treatment showed intermediate values within the HCMR, LCMR, and LCRS but were significantly greater compared with the HCRS calves.

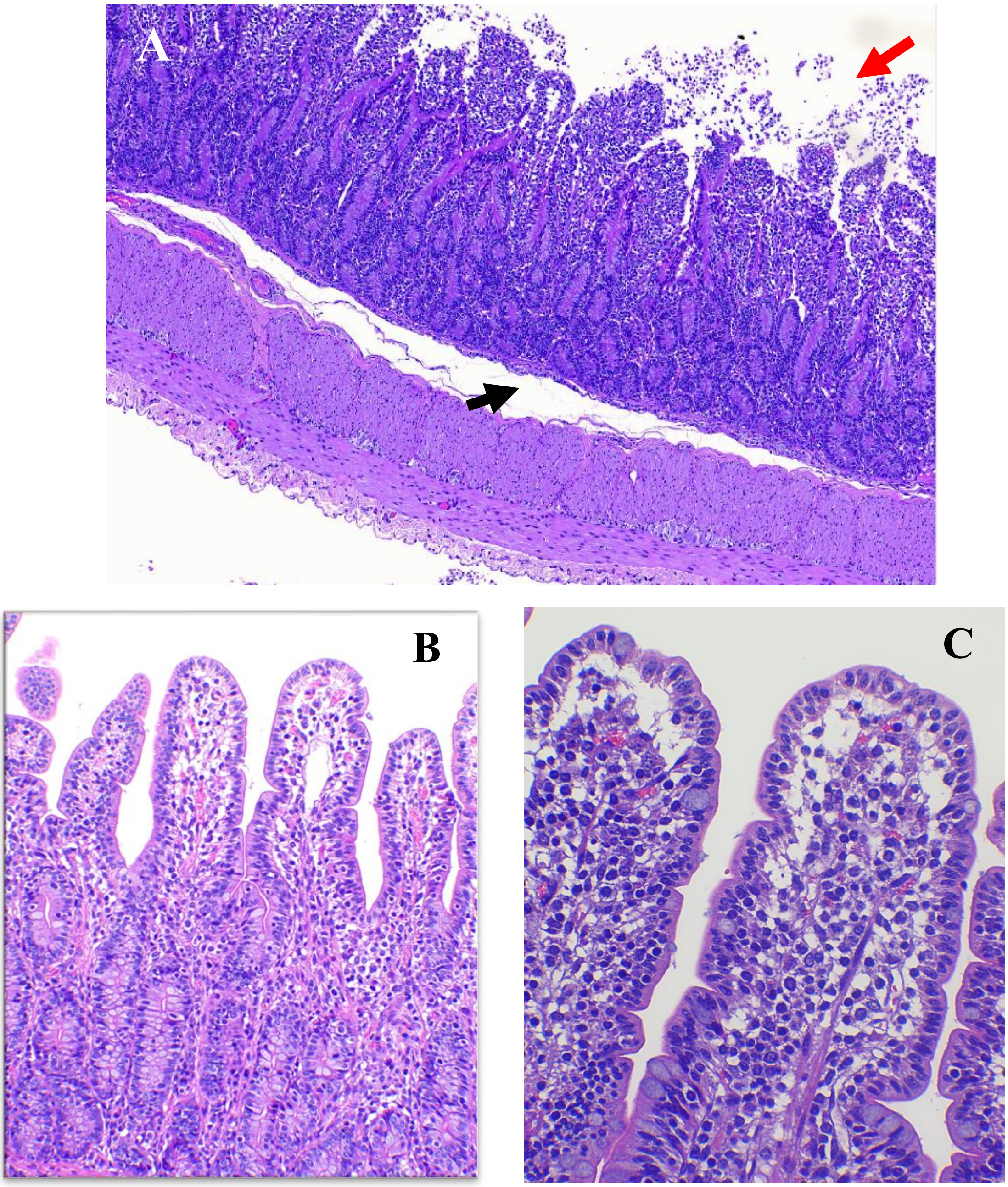


Figure 5. Histological images from the jejunum and ileum collected for histomorphology analysis. Figure **A** show the process of edema (black arrow) and intestinal shedding (red arrow) in the jejunum mucosa that made it impossible to perform histomorphology analysis. Figure **B** shows normal papillae from the ileum. Figure **C** shows intraepithelial lymphocytes (black arrow) and a Goblet cell (red arrow).

Table 4. The effects of colostrum consumption and feed restriction on the mRNA expression of tight junction proteins of interest in the jejunum of male Holstein calves.

	Treatments[1]						*P*-value[2]
Item[3]	CTRL	HCMR	HCRS	LCMR	LCRS	SEM	Trt
CLDN2	0.94	0.93	0.85	1.07	0.69	0.098	0.10
CLDN 4	0.01	0.01	0.01	0.02	0.01	0.003	0.76
OCLN	0.08	0.07	0.05	0.06	0.05	0.008	0.15
ZO-1	1.10	1.80	1.77	0.62	0.65	0.634	0.89

[1] Treatments= CTRL (fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported); HCMR (fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h); HCRS (fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h); LCMR (fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h); and LCRS (fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h).
[2] Trt= effect of colostrum consumption and feed restriction.
[3] *CLDN2*= claudin-2; *CLDN4*= claudin-4; *OCLN*= occludin; and *ZO-1*= zonula occludens-1.

Finally, regarding microbiota analysis, the Shannon index results showed a tendency for a greater (*P*= 0.08; Figure 6) alpha diversity in the mucosa-associated microbiota of LCRS calves compared with CTRL, HCMR, and HCRS calves while LCMR showed intermediate values. Additionally, family *Bifidobacteriaceae* (*P*= 0.10; Figure 7), genus *Bifidobacterium* (*P*= 0.02; Figure 7), and species *Bifidobacterium infantis* (*P*= 0.02; Figure 7), *Bifidobacterium longum* (*P*= 0.03; Figure 7), and *Bifidobacterium pseudocatenulatum* (*P*= 0.05; Figure 7) abundance were greater for the LCMR and LCRS calves.

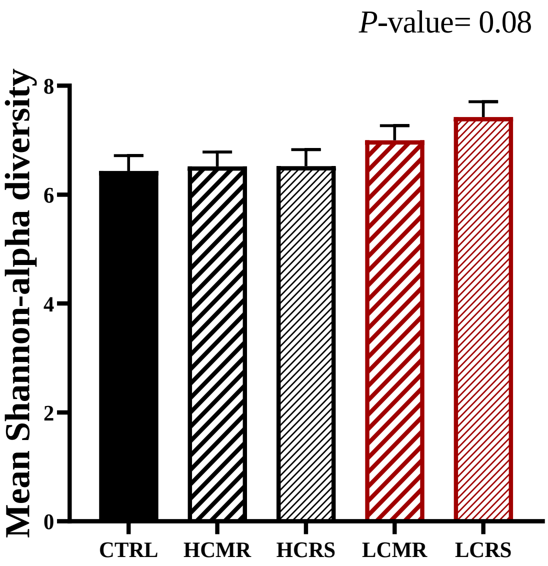


Figure 6. Mean Shannon-alpha diversity used for microbiota evaluation from the jejunal mucosa of male Holstein calves fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period, and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), at d 1 of the study.

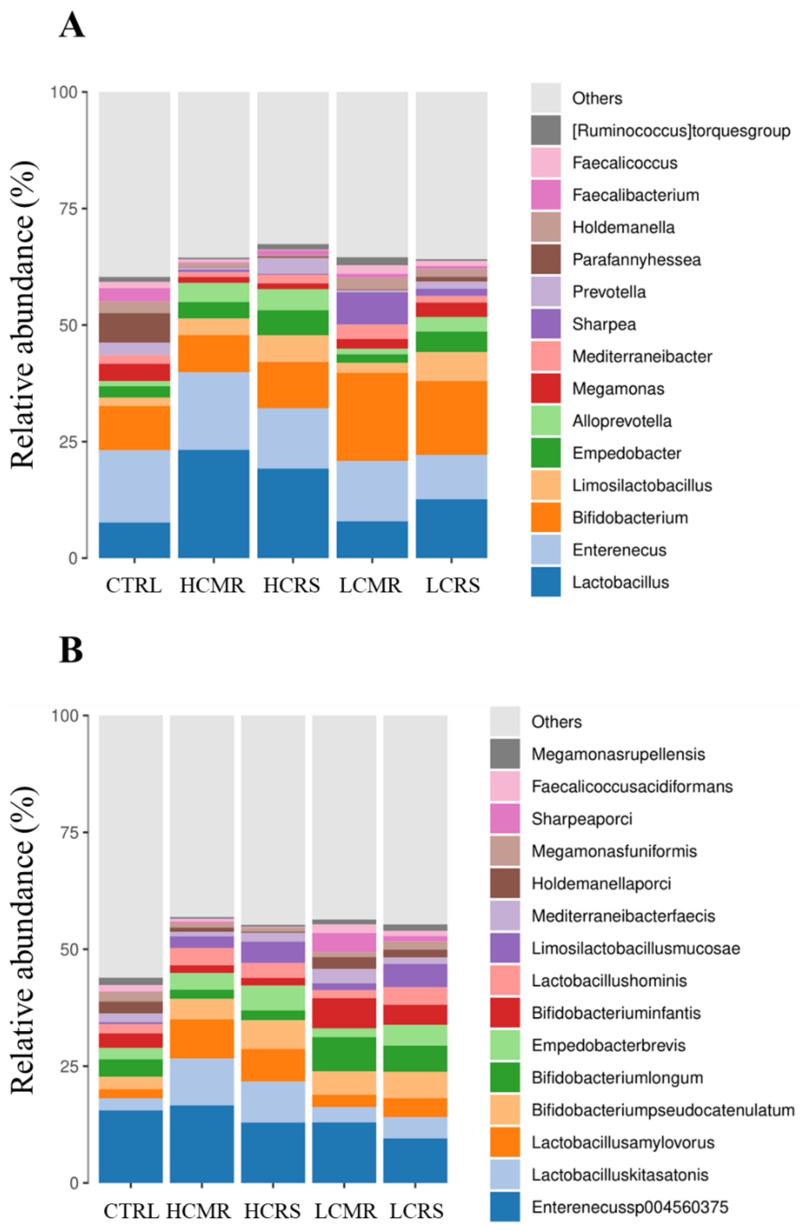


Figure 7. Relative abundance of bacterial composition in jejunum mucosa of male Holstein calves at genus (**A**) and species (**B**) level. Bars represent the relative abundance of the identical bacteria phyla.

DISCUSSION

This study aimed to investigate how colostrum consumption and feed restriction before arrival at the rearing farm, two important nutritional restrictions faced by surplus calves, affect their gut functionality after a long-distance transport. The evaluation of gut functionality was approached by ex vivo (Ap-to-Bas) permeability tests from the three segments of the small intestine, in vivo (Cr-EDTA and ovalbumin) total tract permeability tests, TJ protein gene expression from the jejunum, histomorphology analysis of the duodenum, gut microbiota from the jejunum, enterocytes mass (citrulline), and fecal lactoferrin analyses.

Tight junction proteins, adherent junctions, and desmosomes located in the gap between two adjacent enterocytes regulate the passive movement of molecules < 600 Da through the paracellular pathway (Ménard et al., 2010). The use of large molecular weight markers like Cr-EDTA (340 kDa) and ovalbumin (42.7 kDa) and its posterior recovery in urine (Wood et al., 2015a) or plasma (Uhnoo et al., 1990; Pisoni et al., 2022) indicate an increase in the paracellular permeability. Because of their MW and intestinal indigestibility, Cr-EDTA and ovalbumin have been previously used as markers of total tract permeability in calves (Rothman et al., 1985; Nejdfors et al., 2000; Wood et al., 2015; Wilms et al., 2019; Cangiano et al., 2022). Based on the negative effects that feed restriction (Zhang et al., 2013; Horst et al., 2020), fasting (Pisoni et al., 2022), and stress (Lambert, 2009) exert on intestinal barrier integrity, we hypothesized that calves in the LCRS and HCRS treatments, which suffered more severe feed restriction and were transported afterward, would have the greatest serum concentrations of Cr-EDTA and ovalbumin compared with the other treatments. However, and in disagreement with our expectations, Cr-EDTA concentrations 24 h after transport were greater for the CTRL and LCMR calves, whereas no differences were observed for serum ovalbumin concentration among treatments. In previous studies investigating feed restriction and gut permeability, our research group observed that the increase in gut permeability to Cr-EDTA occurred rapidly after a feed restriction insult but recovered to levels similar to control as soon as calves resumed concentrate intake approximately 24 h after transport (Pisoni et al., 2022). In the present study, focused on day 1, despite the differences in Cr-EDTA between treatments, the concentrations of Cr-EDTA are within the range of a healthy calf of this age, which means that 24 h after the insult we may be able to observe a potential epithelial recovery. In a previous study in acclimated vs. non-acclimated transported pigs, an intestinal ischemia model was used to evaluate differences in intestinal permeability and epithelial repair responses (Ziegler et al., 2020). This study found that acute stress due to transport in non-acclimated ischemic injured pigs caused the greatest intestinal epithelial loss but a more robust repair response afterward (Ziegler et al., 2020). This increase in the recovery of the intestinal tissue was thought to occur due to an endogenous rise in prostaglandin production promoting tissue restitution and tight junction protein recovery (Ziegler et al., 2020). Based on the results from Ziegler et al. (2020) and the present study, we could hypothesize that the observed differences in gut permeability may reflect a protective mechanism with subsequent epithelial restoration, rather than disruption of the intestinal epithelium due to feed restriction and transport. On the other hand, the lack of differences among treatments in ovalbumin concentration may be due to its lower MW (compared to Cr-EDTA), which would allow the passage of this marker between adjacent enterocytes that are in process to recover.

On the other hand, the additive effect of feed restriction and colostrum shortage appears to be related to a downregulation of the TJ protein *CLDN2*, with calves in the LCRS group tending to have lesser gene expression of this protein compared to the other treatments. Also, although not significant, the two treatments fed the lowest amount of colostrum (LCMR and LCRS) showed numerically less expression of the TJ protein *ZO-1*, inferring that there is some degree of responsibility for colostrum shortage in the normal expression of these proteins. As observed in piglets (Grześkowiak et al., 2020; Vodolazska et al., 2023) or mice (He et al., 2021), the colostrum or the exosomes of the colostrum upregulated TJ protein ZO-1 making those animals more robust to insults affecting the gastrointestinal tract. The *CLDN2* gene expression in the jejunum of the LCRS calves were in agreement with the results of the ex vivo gut permeability test (Ap-to-Bas), where there was a significant increase in duodenal permeability in the LCRS calves when compared with the remaining treatments. The increase in the basolateral concentration of % of FU in the LCRS calves followed our assumption that the most challenged calves (LC consumption at birth and RS fed during the assembly center simulation) would express an increase in their gut permeability to this marker due to a loss of intestinal integrity. Tight junction proteins are one of the major components of the apical junctional complex responsible for regulating the paracellular pathway between intestinal epithelial cells (Turner, 2009b). Claudin proteins are the backbone of the TJ (Ulluwishewa et al., 2011), and, in particular, *CLDN2* mediates gut permeability by sealing the space between 2 adjacent enterocytes (Bücker et al., 2010). Downregulation of TJ proteins may lead to increases in gut permeability with the consequent development of local or systemic inflammation. It has been seen in animal models, that feed restriction (Zhang et al., 2013; Kvidera et al., 2017a; Horst et al., 2020) induces damage to the intestinal mucosa, leading to the opening of tight junctions and dysfunction of the intestinal barrier, something that has been hypothesized to occur in the present investigation.

The ex vivo models are intricate systems with the main limitations being related to the difficulty of controlling the experimental conditions, the short viability of tissues, and the absence of a blood supply (Xu et al., 2021). One of the most widely used setups in both humans and animals is the Ussing Chambers. This technique has been previously used in ruminants and young calves (Hansen et al., 2010; Wood et al., 2015c; Dengler et al., 2023). Some limitations of this technique based on price and the difficulty of using replicates to minimize variability were intended to be eliminated with the Ap-to-Bas setup (Ginés et al., 2018). This device allows the use of numerous replicates from different parts of the intestinal segment conferring less variability between samples while using the vectoriality principle through a system that mimics a Ussing Chamber approach (Ginés et al., 2018). An increase in the ex vivo ileal permeability in all transported calves when compared with the CTRL was also observed. The lowest % of FU concentration in the ileum of CTRL calves would be indicative of a conserved intestinal barrier that remains impermeable to large solutes in those calves that were not deficient in colostrum or feed and were not transported. It seems that those calves exposed to colostrum shortage and transportation responded worse to these challenges and that this could hypothetically be linked to an immature gut due to the shortage of colostrum suffered at birth. In general terms, the methodology used for measurements of intestinal permeability varies depending on the animal model, the setting (ex vivo, in vivo, or in vitro), the type of markers used, and the final measurement of the marker (Bischoff et al., 2014; Schoultz and Keita, 2020). In the present study, we expected to find a robust response between the ex vivo and in vivo techniques in terms of intestinal permeability results post transport. We assumed that in the event of loss of intestinal integrity, both methods would yield similar results. However, there appears to be variability in response between different segments of the small intestine and between both methods. We hypothesize that this may be due to the degree of epithelial damage and recovery processes that vary in speed between segments and/or due to the integrative physiological processes (gastric emptying, intestinal motility and digestibility, clearance, and blood flow, among others) that occur internally in calves but are absent in an ex vivo setup.

The results from *CLDN2* gene expression in the LCRS calves are also in line with those for fecal lactoferrin concentration for the same treatment. In a condition of loss in the gut integrity due to TJ protein downregulation, food antigens and microbes contained in the lumen of the intestine could access the bloodstream causing local and systemic inflammation. An increase in fecal lactoferrin is indicative of an active inflammatory condition in the GIT of calves. Lactoferrin is an iron-binding glycoprotein present at low levels in plasma and at much higher levels in milk (Legrand et al., 2005; Donovan, 2016). Plasma lactoferrin is primarily contained in secondary granules of neutrophils (Kane, 2003b; Langhorst et al., 2008). During intestinal inflammation these neutrophils invade the mucosa and release lactoferrin in response to inflammation, increasing its concentration in the feces (Guerrant et al., 1992; Legrand et al., 2005). Once released, lactoferrin exerts bactericidal, immunomodulatory, and inflammatory responses (Donovan, 2016). Lactoferrin has been extensively studied as a marker of intestinal inflammation in inflammatory bowel disease in humans (Walker et al., 2007; Langhorst et al., 2008; Borkowska et al., 2015; Wang et al., 2015). Results from the present study were consistent with our hypothesis that calves subjected to nutrient restriction (colostrum shortage and electrolyte as a pre-transport diet) and fasting during transport (LCRS) would express some degree of GIT inflammation due to the negative effects of feed restriction on gut integrity (Zhang et al., 2013; Kvidera et al., 2017b). Additionally, these results are related to those of *CLDN2* and ex vivo gut permeability. In the scenario of the current study, TJ protein disruption due to feed restriction and stress may have caused the increase in duodenal permeability observed ex vivo, which may have triggered a local inflammation with the consequent lactoferrin secretion into the intestinal lumen.

Regarding histomorphology analysis, and as previously mentioned, the extensive edema and intestinal epithelial shedding from the jejunum samples made histomorphological analysis impossible to perform and further studies to evaluate a scoring system for edema and epithelial shedding are under evaluation.

Feeding high planes of MR has been shown to positively influence both intestinal development and TJ gene expression in calves (Weikard et al., 2018; Koch et al., 2019; Hammon et al., 2020). In line with the benefits of feeding high planes of MR, feed restriction to 40% of ad libitum intake has been shown to reduce villus height and crypt depth in the ileum of lactating cows (Kvidera et al., 2017b). Consistent with this previous evidence, the numerical decrease in VH in the HCRS and LCRS calves could be hypothesized as a consequence of nutrient restriction due to RS feeding during the assembly center simulation, which was later exacerbated by the 19 h of fasting during transport. As previously stated, the consumption of bioactive compounds contained in colostrum was expected to positively influence the development and maturation of the gut in these calves. Numerous studies have confirmed the positive impact that colostrum consumption has on stimulating crypt cell proliferation (Blum, 2006), villus length, crypt depth, and mucosal thickness (Yang et al., 2015), duodenal villus size (Blättler et al., 2001), and enterocyte cell proliferation due to the interaction of growth-stimulating substances contained in it (Hammon et al., 2020). On the other hand, the absence of differences observed in the number of IELs could be hypothesized to reflect a lack of immune activation against enteric pathogens (Ray Waters et al., 1996; Rothkötter et al., 1999). However, the number of Goblet cells was greater in the LCMR calves compared with the HCMR, HCRS, and LCRS treatments. Goblet cells are also associated with immunological responses as they are responsible for synthesizing mucin, a glycoprotein that constitutes the protective mucus layer of the gut (Kim and Ho, 2010). In the presence of acute inflammation due to intestinal infection, mucin secretion is induced to remove enteric pathogens and preserve the mucus layer, thus ensuring the integrity of the intestinal epithelium (Kim and Ho, 2010). It could be assumed that a process of immune activation occurred in the LCMR calves due to the negative effects of poor colostrum consumption and the stress of transport on the integrity of the GIT. However, this would have been the case in the HCMR, HCRS, and LCRS treatments, and only the CTRL calves, which were not expected to show an increase in the number of Goblet cells infiltrating the gut, had similar cell counts. Another theory could be that the numerical decrease in VH in the HCRS and LCRS calves could translate into a loss of epithelial surface area and this loss in the surface area led to a decrease in the Goblet cell counts seen for these treatments. In other words, instead of accounting for the increase in Goblet cells for the LCMR, this effect could be due to a reduction in the number of Goblet cells due to the shorter VH in the other treatments.

Results from the mucosa-associated microbiota analysis in the jejunum showed a greater abundance of *Bifidobacteria* for the LC-fed calves (LCMR and LCRS calves). The early establishment of a functional intestinal microbiota during the first week after birth has been linked to long-term health, performance, development, and maturation of the immune system, and lower susceptibility to enteric disease in calves (Stappenbeck et al., 2002; Hooper et al., 2012; Oikonomou et al., 2013; Malmuthuge and Guan, 2017). *Bifidobacterium* species are present at higher levels during the first 4 weeks of life in calves and then progressively decrease with growth (Oikonomou et al., 2013). Carbohydrates contained in milk, principally lactose, are an important source of energy enriching for *Bifidobacteria* in the GIT environment (Kelly et al., 2016). The presence of *Bifidobacterium* exert anti-inflammatory, antibacterial, and antiviral activities making their presence desirable for the protection of the intestinal barrier from pathologic microorganisms stimulating the immune system (Picard et al., 2005). This has been previously observed in the increase in the susceptibility of calves to suffer colonization of pathogens when *Bifidobacteria* counts were low (Vlková et al., 2008). Colostrum consumption has also been shown to positively enrich *Bifidobacteria* and influence the growth of nonbeneficial bacteria in the gut. A previous study showed that feeding colostrum within 1 h after birth increased the number of *Bifidobacterium* and decreased the prevalence of *E. coli* in calves (Malmuthuge et al., 2015a) which might translate into a lower prevalence of enteropathogens and enteric diseases (Malmuthuge et al., 2015b). *Bifidobacteria* also exerts effects to a TJ protein expression level. It has been previously demonstrated that feeding prebiotics with *Bifidobacterium infantis* increase the expression of *ZO-1* and occludin while decreasing *CLDN2* expression enhancing the effect on transepithelial resistance and ion secretion (Corridoni et al., 2012). In the present study, the increase in the *Bifidobacteriaceae* family in the calves receiving the lower amounts of colostrum (LCMR and LCRS calves) was not consistent with our speculations. It could be hypothesized that the unfavorable environment in the gut of LC calves may stimulate the growth of these bacteria to cope with the inflammatory state in the gut when the epithelia restoration mentioned above is also happening 24 h after feed restriction and fasting. These differences could not be associated with the plane of MR feeding because LCMR and LCRS calves were fed different diets with different nutrient compositions and still showed similar results. However, anti-inflammatory, antibacterial, and antiviral activities have been described for *Bifidobacterium pseudocatenulatum, Bifidobacterium longum,* and *Bifidobacterium infantis*, and beneficial effects on gut permeability have also been shown for *Bifidobacterium infantis* (Underwood et al., 2014; Lim and Shin, 2020). Therefore, these protective functions could be hypothesized to be an immunological response to an inflammatory process occurring in those calves fed only 2 L of colostrum at birth, possibly due to an immature and more susceptible to enteric diseases microbiota. The amount of colostrum consumed at birth and feed restriction showed changes in the microbiota of calves. However, the mechanisms behind the increase of *Bifidobacterium* in the gut of calves fed on 2 L of colostrum at birth compared to calves fed 10 L of colostrum remain unclear and require further elucidation. Finally, it is important to note that the intestinal bacterial population varies considerably depending on the region and the type of sample used for its analysis (Malmuthuge et al., 2014), and these results reflect a unique sector of the GIT (jejunum), so their interpretation should be made carefully. The lack of more conclusive results may be since a larger sample size would be required to see differences between treatments in a tissue with a rapid recovery process and considerable variability in response between individuals. Another limitation of this study is the lack of measurements at the level of the immune system, such as Toll-like receptor concentrations, which could help interpret inflammatory responses in the gut, gut microbiota, and serum cytokines. In addition, and of importance for the interpretation of the results, the calves used in this study are animals that have not suffered FTPI, regrouping, and belong to the same farm of origin, so their care and feeding since birth have been the same. If market calves from different origins and with different nutritional and sanitary management since birth had been used, the results would probably have been influenced by these factors and could have shown greater variability and differences between them.

CONCLUSIONS

Colostrum consumption at birth, feed restriction at the assembly center, and fasting during transportation impacted the gastrointestinal tract functionality of unweaned calves. Some contradictory results for serum Cr-EDTA and citrulline concentrations and microbiota analysis may be associated to variations in the sampling time, rate of intestinal epithelial recovery, and region and section of the small intestine evaluated, respectively, rather than merely attributable to treatments. Gastrointestinal functionality was mainly negatively affected in calves fed low colostrum consumption at birth and that suffered feed restriction due to rehydration solution feeding during the assembly center simulation and transported. In unweaned calves to reduce the risk of gastrointestinal disturbances on arrival at the rearing farms, nutritional strategies for marketing and transportation should exclude those that only provide rehydration solutions.

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CHAPTER V

Evaluation of feeding strategies applied at assembly centers on pre-transport status and recovery at the rearing farm in unweaned dairy beef calves: animal recovery at rearing farm.

"Evaluation of feeding strategies applied at assembly centers on pre-transport status and recovery at the rearing farm in unweaned dairy beef calves: animal recovery at rearing farm"

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ABSTRACT

This study aimed to investigate the implementation of feeding strategies at the assembly centers before long distance transportation on the pre-transport status and the recovery of unweaned male calves upon arrival at the rearing facilities. For this study, 65 male Holstein calves (45.1 ± 1.5 kg of BW and 16.3 ± 0.8 d of age; mean ± standard error) were used and allocated to treatments according to the pre-transport feeding strategy assigned and whether or not they were subjected to transport as follows: control calves (CTR; n = 13) were fed 10 L of colostrum at birth, 2.5 L of MR twice daily, had ad libitum access to concentrate and water during an assembly center simulation, and were not transported 19 h; milk replacer calves (MR; n = 13) were fed 2 L of colostrum at birth, 2.5 L of MR twice daily, had ad libitum access to water during the assembly center simulation, and were transported during 19 h; milk replacer and concentrate calves (MRC; n = 13) were fed 2 L of colostrum at birth, 2.5 L of MR twice daily, had ad libitum access to concentrate and water during the assembly center simulation, and were transported during 19 h; milk replacer and acidified milk calves (MRA; n = 13) were fed 2 L of colostrum at birth, 2.5 L of MR twice daily, had ad libitum access to acidified milk and water during the assembly center simulation, and were transported during

19 h; finally, milk replacer, acidified milk, and concentrate calves (MRCA; n = 13) were fed 2 L of colostrum at birth, 2.5 L of MR twice daily, had ad libitum access to concentrate, acidified milk, and water during the assembly center simulation, and were transported during 19 h. After transport, calves were fed 2.5 L of MR twice daily and ad libitum access to concentrate. Concentrate intake was recorded daily whereas BW was recorded on d -4, -1, 0, 1, 2, 7, and weekly thereafter until weaning (d 42). Gut permeability tests were performed on d -4, -1, 0, 1, 2, 7, and 14. Additionally, blood samples were collected on d -4, -1, 0, 1, 2, 7, and 14 to analyze energy balance parameters, while health records were recorded from d -4 to d 14. Finally, standing time was measured from d -4 to d 7 of the study. Results showed that by the end of the assembly center, MR calves had lower BW compared with MRCA calves whereas no significant differences among the remaining treatments that had intermediate BW values were seen after transport. Compared with CTR concentrate intake peaked at d 0 and dropped on d 1 with the MR calves showing the lowest drop; in addition, MRA and MRC showed a numerically greater concentrate intake recovery from d 8 to d 14. On d -1 serum glucose concentration was greater compared with the other treatments, and in all transported calves on d 0 it dropped compared to non-transported calves (CTR) recovering on d 1 and d 2 after arrivals in a different manner depending on assembly center simulation treatment. On d -1 serum NEFA concentration was also greater in MR calves compared with the other treatments, and on d 0 in all transported calves it drastically increased compared with CTR calves while on d 1 decreased; no more differences among treatments were observed. Finally, serum BHB concentration followed a similar pattern to NEFA with the exception that in all treatments on d 2 concentrations increased and thereafter concentrations slowly decreased. Gut permeability assessed by serum Cr-EDTA concentration varied throughout the study and main differences were observed after the 19 h transport on d 0; the lowest serum Cr-EDAT values were observed in CTR and MRCA calves and the highest in the remaining treatments. In summary, implementing pre-transport feeding strategies like supplementing concentrate or acidified milk to conventional MR feeding improved calves' BW and concentrate intake recovery after transport and decreased gut permeability after transportation indicating that it's a good strategy to improve calf's recovery at the rearing farm.

Key words: dairy beef calves, feeding strategies, acidified milk, intake recovery.

INTRODUCTION

Among the European Union and The United Kingdom, 11 million surplus male dairy calves were produced in 2022 (Renaud and Pardon, 2022). The future of these surplus dairy calves will depend on the country of origin and the country of destination where they will be raised. These calves can be raised for veal (up to 8 months of age and still being fed milk replacer), young dairy beef (up to 12 months of age) and dairy beef (up to 16 month of age) both to produce red meat (Romieu et al. 2014). However, this production system continues to raise ethical and animal welfare concerns (Klein-Jöbstl et al., 2014; Wilson et al. 2020; Winayamohan et al. 2022; Webb et al. 2023).

Although the EU has legislation in place to protect live animals during transport (Regulation (EC) No 1/2005; European Council, 2005), marketing and transportation remain among the most challenging production stages for young and unweaned surplus dairy calves. The commingling of calves from different origins and the periods of feed and water restriction and fasting during marketing and transportation make surplus calves more susceptible to respiratory and digestive diseases. Additionally, unweaned calves' immune response capacity is limited (Hulbert and Moisá, 2016) and their gastrointestinal tract is not completely developed (Davis and Drackley, 1998; Diao et al., 2019) worsening their ability to cope with these challenges. There is therefore a need to improve the management of surplus dairy calves at this stage, as it is estimated that almost 1.4 million surplus calves were transported across EU borders between 2015 and 2020, with 40.8% of these journeys lasting more than 8 h (Reenen et al., 2022).

The application of pre-transport feeding strategies previous to long-distance transports has been shown to have physiological and metabolic effects on veal and other dairy beef calves during transport and at arrival to the rearing farm (Marcato et al., 2018, 2020; Pisoni et al., 2023). However, the literature published to date on pre-transport diets at assembly centers has only evaluated the use of either milk replacer (**MR**) or rehydrating solution (**RS**) (Marcato et al. 2020; Pisoni et al. 2023). Marcato et al. (2020) observed that feeding 1.5 L of MR at 125 g/L 2 h before short-distance transport

(6 h) reduced glucose utilization and fat mobilization compared with calves fed 1.5 L of RS at 20 g/L. However, when the transport duration increased to 18 h, feeding MR did not prevent glucose utilization and fat mobilization. In another study, Pisoni et al. (2023) observed that calves staying 3 d at an assembly center being fed 2 L of RS at 50 g/L twice daily and later transported for 19 h had greater fat mobilization and greater gastrointestinal permeability compared with calves fed 2 L of MR at 125 g/L twice daily during the same period. However, as observed by Marcato et al. (2020), feeding MR to calves in Pisoni et al. (2023) did not prevent BW shrinkage after transport, fat mobilization, and increases in gastrointestinal permeability. These results imply that feeding either MR or RS did not cover calves` nutritional requirements and provide them enough energy to face transportation. Therefore, we hypothesized that implementing pre-transport feeding strategies with a higher plane of nutrition during the assembly center would ameliorate the negative effects of fasting during transportation in unweaned dairy beef calves improving their pre-transport status and recovery upon arrival at the rearing facilities. Therefore, our objective was to evaluate the potential benefits of supplementing MR with a starter concentrate and/or acidified milk ad libitum during an assembly center simulation before a long-distance transportation on pre-transport nutritional status, gut functionality and the recovery of BW and concentrate intake, in unweaned male dairy beef calves at arrival at the rearing facilities.

MATERIALS AND METHODS

Animals, treatments, and feeding

This study was approved by the Animal Care Committee of Generalitat de Catalunya (Barcelona, Spain; RD 53/2013; project number 11566). A total of 65 male Holstein calves (45.1 ± 1.5 kg of BW and 16.3 ± 0.8 d of age; mean ± standard error) were used to evaluate pre-transport feeding strategies on pre-transport nutritional status, gut functionality, recovery of BW and concentrate intake, and behavior. Calves were born at a commercial dairy farm in Lleida, Spain (Granja Selergan, S.A.) At birth, 13 calves were fed 10 L of colostrum (4 L within the first 2 h after birth and 6 L in three consecutive feedings within 24 h after birth (22.9 ± 0.30% of birth BW) and 52 calves were fed 2 L of colostrum within the first 2 h after birth (4.8 ± 0.36% of birth BW). With the aim of improving immunization and gastrointestinal development, 10 L of colostrum were fed to the first group, which was considered our "golden standard" (control) group. The feeding of 2 L of colostrum was intended to be representative of the normal practice of colostrum administration to male dairy beef calves on Spanish dairy farms (Pisoni et al., 2023). All treatments used high-quality colostrum (average of 24.5 % Brix) fed via an esophageal tube. After colostrum consumption, calves were allocated in individual hutches and were fed 2 L of MR twice daily at a concentration of 125 g /L as fed twice daily (21.86% CP, 16.59% fat, 45.50% lactose, with the main ingredients being skimmed milk powder, whey powder, vegetable oil, starch, and dextrose; Schils, The Netherlands). Calves stayed at the dairy farm a minimum of 14 d since they were born (16.3 ± 0.8 d of age; mean ± standard error) which is the minimum age required for transportation > 8 h for unweaned calves (Regulation (EC) No 1/2005; European Council, 2005).

The study was conducted between June and August of 2021. To avoid large age differences between calves and considering the calving prediction of the commercial dairy farm, three batches of 18, 19, and 18 calves were enrolled within 3 weeks. At that time, calves were transported for 2.5 h from the dairy farm to the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) experimental research unit located in Caldes de Montbui (Barcelona, Spain) to simulate a typical first short-distance transportation from the origin farm to an assembly center. The experimental unit was a closed barn with 78 individual pens (1.20 m x 1.97 m) bedded with sawdust. Once at the experimental research unit, an assembly center was simulated during 3 d (d -4 to d -1 of the study). The effects of different feeding strategies before a 19 h transport (on d -1) were studied during this period. Based on the amount of colostrum offered at birth and the feeding strategy applied during the assembly center simulation, calves were distributed by BW and age into 1 of 5 treatments as follows: control calves (CTR; n = 13) were fed 10 L of colostrum at birth, 2.5 L of twice daily (125 g /L as fed) and had ad libitum access to concentrate and water during the assembly center simulation (d -4 to d -1); these calves were not transported during 19 h on d -1 as this treatment was used to represent an ideal situation where calves remained in the dairy farm and did not experience colostrum shortage nor feed restriction and transportation; milk replacer calves (MR; n = 13) were fed 2 L of colostrum at birth, 2.5 L of MR twice daily (125 g /L as fed) and had ad libitum access to water during the assembly center simulation, and on d -1 were transported during 19 h; milk replacer and concentrate calves (MRC; n = 13) were fed 2 L of colostrum at birth, 2.5 L of MR twice daily (125 g /L as fed) and had ad libitum access to concentrate and water during the assembly center simulation, and on d – 1 were transported during 19 h; milk replacer and acidified milk calves (MRA; n = 13) were fed 2 L of colostrum at birth, 2.5 L of MR twice daily (125 g /L as fed) and had ad libitum access to acidified milk and water during the assembly center simulation, and on d -1 were transported during 19 h; finally, milk replacer, acidified milk, and concentrate calves (MRCA; n = 13) were fed 2 L of colostrum at birth, 2.5 L of MR twice daily (125 g /L as fed) and had ad libitum access to concentrate, acidified milk, and water during the assembly center simulation, and on d -1 were transported during 19 h. To avoid gastrointestinal problems related with changing the MR formula that may affect the results of the study, the same MR offered at the dairy farm was used during the experiment. Acidified milk was prepared using the same MR but acidified with a combination of formic acid and ammonium formate (SalmoSTAT®, Madrid, Spain) at a concentration of 4 mL SalmoSTAT®/ L of MR to achieve a final pH of 4.3. The nutrient composition of the concentrate used was 17.1% CP, 16.9% NDF, 5.7% ADF, 29.2% starch, 5.5% ether extract, 5.1% ashes on a DM basis, with the main ingredients being 39% cornflake, 17% barley, 14% wheat middlings, 13.9% soybean meal, 9% wheat bran, 3% sunflower meal, 1.9% palm oil, and 1.3% calcium carbonate, 0.5% premix, and 0.4% salt.

After the first 9 h of transport, and according to Regulation (EC) 1/2005, calves had a rest-stop of 1 h where they were offered water without being unloaded from the truck (Regulation (EC) Nº 1/2005; European Council, 2005). After the resting time, calves were transported for the remaining 9 h until returning to the experimental research unit (Table 1 and Figure 1 summarize treatment description and the timeline of the study, respectively). Calves were transported in the lower deck of a 3-deck semi-trailer with straw bedding and side vents with sliding panels. The calf density inside the truck did not follow normal commercial densities as the number of calves transported for each batch was low (18, 29, and 18 calves/batch). For each batch, the same two drivers and routes were used to ensure equal replicates. After 19 h of transport (d 0 of the study), calves arrived at the rearing facility where all calves were fed 2.5 L of MR at 125 g /L as fed twice daily until d 7. At d 7 of the study, following an 8-week weaning program, MR was reduced to 2 L at 125 g /L as fed twice daily. On d 26 only 2 L at 125 g /L as fed were offered in the morning feeding, and on d 42 calves were completely weaned. Concentrate was offered ad libitum since d 0. The MR and concentrate used from d 0 to d 42 were the same as the ones described previously during the assembly center simulation.

Table 1. Treatment description on the study of the effects of pre-transport feeding strategies on pre-transport nutritional status and the recovery of male dairy beef calves after transport.

Treatments[a]	Colostrum provision at birth		Diet during the assembly center simulation (d -4 to -1)			19 h transportation (d -1)	
	2 L	10 L	Milk replacer	Concentrate	Acidified milk	Yes	No
CTR		X	X	X			X
MR	X		X			X	
MRC	X		X	X		X	
MRA	X		X		X	X	
MRCA	X		X	X	X	X	

[a]**CTR** = control; **MR** = milk replacer; **MRC** = milk replacer and concentrate; **MRA** = milk replacer and acidified milk; **MRCA** = milk replacer, concentrate and acidified milk.

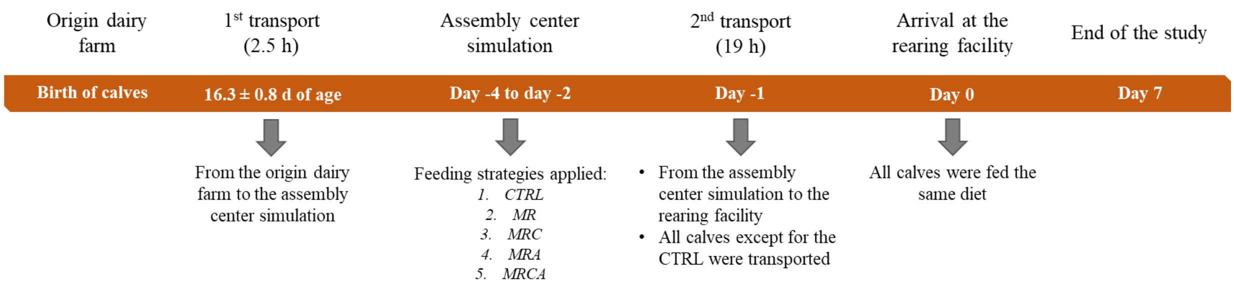

Figure 1. Temporal overview of the study setup on the effects of pre-transport feeding strategies on pre-transport nutritional status and the recovery of male dairy beef calves after transport.

Sample Collection and Chemical Analysis

Individual BW was recorded on d -4, -1, 0, 1, 2, 7, and weekly thereafter until weaning on d 42 of the study.

Concentrate intake was recorded daily and refusals were weighed for further dry matter intake (**DMI**) calculations. Milk replacer refusals were weighed and recorded daily. Samples from concentrate and MR were sent to a referral laboratory to determine DM, ash, CP, ADF, NDF, and DM, ash, CP, and sugars, respectively, as previously described (Pisoni et al., 2022a).

Blood samples were collected before feeding on d -4, -1, 0, 1, 2, 7, and 14 for determination of markers of energy balance (NEFA, BHB, and glucose), and enterocyte functional mass (citrulline). Blood samples were also collected on d -4 to determine colostrum consumption by measuring gamma-glutamyl transferase **(GGT)** and immunoglobulin G and G1 **(IgG** and **IgG1)**. All blood samples were obtained by jugular venipuncture and serum was obtained using 10 mL vacuum tubes (BD Vacutainer® Plus Plastic Serum Tubes, San Agustín del Guadalix, Spain). For glucose determinations, 4 mL vacuum tubes (BD Vacutainer® Fluoride Tubes, San Agustín del Guadalix, Spain) with a glycolytic inhibitor were used. Tubes were placed in a refrigerated polystyrene box and transferred to the laboratory. Blood samples were centrifuged at 1,500 × *g* at 4°C for 15 min and the remaining serum was aliquoted and stored at -20ºC until analysis. An additional 4 mL vacuum tube sprayed with anticoagulant (BD Vacutainer spray coated K2EDTA Tubes, San Agustín del Guadalix, Spain) was used for the collection of whole blood for hematology determinations. Whole blood was immediately processed by using an automated veterinary hematology analyzer (Element HT5, Mindray, Heska, Shenzhen, China). Chemical analysis of all serum and plasma samples was previously described by Pisoni et al. (2022; 2023). Plasma glucose, and serum NEFA, BHB, and GGT were analyzed using a Beckman Coulter AU480 analyzer (AU480, Beckman Coulter International S.A., Nyon, Switzerland), serum citrulline concentration was analyzed using a spectrophotometric kit (l-Citrulline Kit, Immundiagnostik AG), and serum IgG and IgG1 concentrations by an ELISA kit (species-specific Bovine IgG and Bovine IgG1, Bethyl Laboratories Inc., Montgomery, TX).

A gut permeability test using Cr-EDTA as a marker of gut permeability was conducted before feeding on d -4, -1, 0, 1, 2, 7, and 14. Based on a previous study, Cr-EDTA (Sigma-Aldrich Corp, Saint Louis, MO) was used at a concentration of 0.1 g/kg of BW (Amado et al., 2019). The marker was given orally to the calves and a blood sample was collected 2 h after administration for serum determination of the marker. Serum Cr-EDTA concentration was determined using an inductively coupled plasma mass spectrometer (Agilent 7500ce, Agilent, Santa Clara, CA). The intra- and inter-assay coefficients of variation for NEFA, BHB, glucose, GGT, IgG, IgG1, citrulline, and Cr-EDTA were 1.62% and 4.46%, 0.44% and 2.69%, 0.58% and 1.11%, 0.8 and 1%, 3.2 and 3.3%, 3.37 and 9.26%, 9.75% and 5.12%, and <10% and <15%, respectively.

Accelerometers (Hobo Pendant G Data Logger, Onset Computer Corporation, Bourne, MA) were used to record standing behavior (standing time, standing duration, and standing bouts) from d -4 until d 7 of the study. The devices were attached to the right hind limb of the calves with a self-adhesive bandage and covered with foam to avoid abrasions. HOBOware software version 3.7.23 (Onset Computer Corporation, Bourne, MA) was used for the data analysis.

Statistical Analysis

The study was designed as a randomized balanced design with a covariate adjustment. Calf was the experimental unit. For the sample size calculation, the type 1 error rate (α) was 0.05, the power ($1 - \beta$) was set at 80%, and concentrate intake was considered the primary outcome (Pisoni et al., 2022a). The MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) including treatment as a main effect, and pen as a random effect was used for the analysis of GGT, IgG, IgG1, and BW and age at d -4. Serum concentration of NEFA, BHB, glucose, citrulline, Cr-EDTA, hematologic parameters, and standing behavior were analyzed using the MIXED procedure of SAS with repeated measures for those continuous variables with multiple sampling over time. The model included treatment, time, and its interaction as the fixed effect, pen as a random effect and BW and age on d -4 as covariates. The first-order autoregressive and the compound symmetry covariance structure were tested and the Kenward–Roger degrees of freedom were used based on the lower Bayesian information criterion value. When needed, data were log-transformed to achieve normal distribution. Differences were declared significant at $P \leq 0.05$, and trends were discussed at $P \geq 0.05$ and $P \leq 0.10$ for all models.

RESULTS

Immunological status assessment before treatment application

Serum concentrations of IgG, IgG1, and GGT were used as biomarkers of colostrum consumption (Weaver et al., 2000; Godden, 2008; Pisoni et al., 2022b). Results showed no significant differences (*P* > 0.10; data not shown) among treatments for GGT and IgG1. Even though results from IgG concentration showed a tendency to be greater (*P* = 0.09; data not shown) for those calves fed 10 L of colostrum, none of the calves (included those fed 2 L of colostrum) exhibited failure in the transfer of the passive immunity (IgG threshold for FTPI = <1.0 g/dL; Tyler et al., 1996).

Impact of the feeding strategy applied during the assembly center simulation before the long distance transport (d -4 to d -1)

An interaction between time and treatment was observed in BW (*P* < 0.01; Figure 2). By the end of the assembly center simulation (d -1), BW was greater for MRCA calves compared with MR calves, while CTR, MRC, and MRA calves showed intermediate values (Figure 2). A time by treatment interaction (*P* < 0.001; Table 2) was observed in total DMI, total metabolizable energy intake, and CP intake among treatments. On day -4 calves fed acidified milk (MRCA and MRA) had greater total DMI than MRC, CTR, and MR calves, while MR calves had the lowest total DMI. On d -3, the MRCA treatment had the greatest total DMI but did not differ from the MRC treatment; and the MRC total DMI was similar to MRA, CTR, and MR. On d -2, MRCA calves had the greatest total DMI, while MRC, MRA, and CTR calves had an intermediate total DMI and MR calves had the lowest DMI. Similar results were observed for total ME and CP intake from d -4 to d -2.

When considering the type of feed consumed during this period, the results for concentrate intake showed a time by treatment effect (*P* < 0.01; Figure 3). No differences in concentrate intake were observed among the three treatments where concentrate was offered on d -4, -3, and -2 (average concentrate intake = 65.5 g, 80.2 g, and 38.9 g for CTR, MRC, and MRCA calves, respectively). On d -1, concentrate intake was greater for the CTR calves compared with the other treatments.

Regarding acidified milk intake, MRA and MRCA had an average AM intake of 143 g and 192 g from d -4 to d -2, respectively.

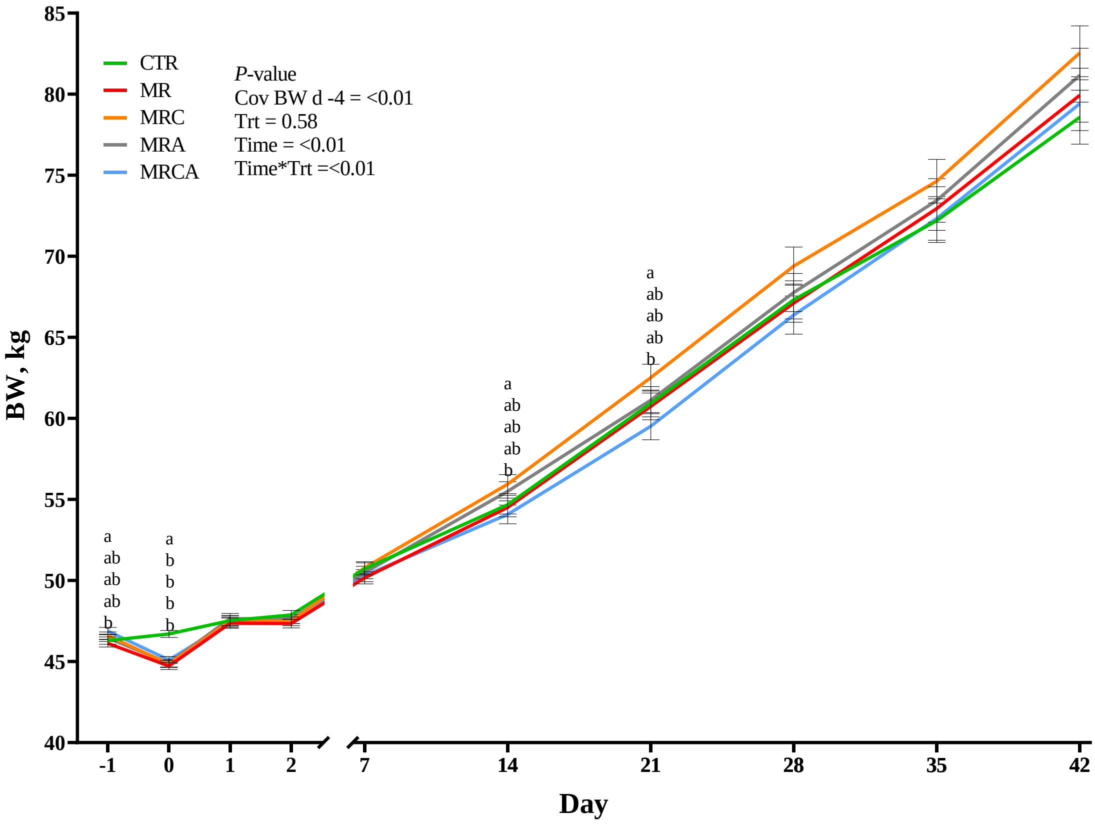

Figure 2. Body weight (mean ± SE) measured daily from d -4 to d 2 and weekly from d 7 to d 42 of the study in male Holstein calves fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported during 19 h (CTR); fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (MR); fed 2 L of colostrum at birth, MR and concentrate at the assembly center simulation period and transported during 19 h (MRC); fed 2 L of colostrum at birth, MR and acidified milk at the assembly center simulation period and transported during 19 h (MRA); and fed 2 L of colostrum at birth, MR, concentrate, and acidified milk at the assembly center simulation period and transported during 19 h (MRCA). Body weight from d -4 was used as a covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

Table 2. The effect of pre-transport feeding strategies on body weight (**BW**), shrinkage, average daily gain (**ADG**), total dry matter intake (**DMI**), and total intake of concentrate, milk replacer, acidified milk, metabolizable energy (**ME**), and crude protein (**CP**) in male Holstein calves.

Item[1]	Treatments[2]					SEM	*P*-value[3]		
	CTR	MR	MRC	MRA	MRCA		Treatment	Time	Time*Trt
BW, kg									
Initial (d -4)	45.0	45.0	45.4	45.2	45.0	1.46	0.99	-	-
Final (d 42)	78.7	80.1	82.4	81.2	79.6	1.34	0.34	-	-
Shrinkage (d -1 to d 0), kg	-0.41[a]	1.39[b]	1.75[b]	1.58[b]	1.78[b]	0.142	<0.001	-	-
ADG (d -4 to d -2), kg/d	0.43	0.37	0.52	0.53	0.62	0.074	0.15	-	-
Total intake (d -4 to d -2), gr DMI/d									
Concentrate	64.45	-	82.63	-	37.68	-	-	-	-
Milk replacer	587.06	572.77	589.36	584.8	584.61	11.304	0.86	0.23	0.74
Acidified milk	-	-	-	143.05	192.71	-	-	-	-
Total DMI (d -4 to d -2), g DMI/d	651.68	573.38	671.55	727.45	812.66	23.463	<0.001	<0.001	<0.001
ME intake (d -4 to d -2), Mcal/d									
From concentrate	0.20	-	0.25	-	0.11	-	-	-	-
From milk replacer	2.94	2.86	2.95	2.92	2.91	0.057	0.86	0.23	0.74
From acidified milk	-	-	-	0.72	0.96	-	-	-	-
Total ME intake (d -4 to d -2), Mcal/d	3.14[cd]	2.86[d]	3.20[c]	3.64[b]	3.98[a]	0.105	<0.001	<0.001	<0.001
CP intake (d -4 to d -2), g/d									
From concentrate	1.10	-	1.41	-	0.64	-	-	-	-
From milk replacer	12.83	12.52	12.88	12.78	12.74	0.247	0.86	0.23	0.74
From acidified milk	-	-	-	3.12	4.20	-	-	-	-
Total CP intake (d -4 to d -2), g/d	13.93[c]	12.52[d]	14.29[c]	15.90[b]	17.58[a]	0.481	<0.001	<0.001	<0.001

[a,b] Values with different superscripts within a row differ ($P \leq 0.05$).

[1] The assembly center simulation period took place between d -4 to d -1 of the study, however, because calves in the MR, MR+C, MR+AM, and MR+C+AM were transported at noon on d -1, their concentrate intake consumption and MR intake were not recorded for the entire d and so the comparisons on ADG, total DMI, and CP, ME, and total intake were made considering intake from d -1 to d -2.

[2] Treatments = CTR (fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported during 19 h); MR (fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h MR); MRC (fed 2 L of colostrum at birth, MR and concentrate at the assembly center simulation period and transported during 19 h); MRA (fed 2 L of colostrum at birth, MR and acidified milk at the assembly center simulation period and transported during 19 h); and MRCA (fed 2 L of colostrum at birth, MR, concentrate, and acidified milk at the assembly center simulation period and transported during 19 h).

[3] Trt = effect of pre-transport feeding strategy; Time × Trt = effect of the time by treatment interaction.

For energy balance metabolites, time by treatment differences were found for serum NEFA, BHB, and plasma glucose concentrations (*P* <0.01; Figure 4). On d -1 serum NEFA concentration was greater (*P* < 0.01; Figure 4 A) for MR calves compared with the MRCA calves whereas the remaining treatments showed intermediate values. In addition, on d -1, MRA calves had greater serum BHB concentration when compared with CTR and MRCA calves, while MR and MRC calves showed intermediate values (*P* < 0.01; Figure 4 B). Plasma glucose concentration was greater for MRCA calves compared with the other treatments by d -1. Finally, total blood cell count analysis showed a time by treatment effect for monocyte concentration (*P* =0.03; Table 3), however, no differences were seen in this period (-4 to -1; data not shown). An interaction between time and treatment was observed in serum Cr-EDTA concentration (*P* = 0.02; Figure 5 A). At the end of the assembly center simulation (d -1), serum Cr-EDTA concentration was greater for MRA and MRCA calves when compared with MRC calves, while CTR and MR calves showed intermediate Cr-EDTA concentrations. There was a time effect for serum citrulline concentration (*P*<0.01; Figure 5), however, no differences between treatments were seen during this period (-4 to -1). No differences among treatments during the pre-transport period were observed in the proportion of calves with respiratory disease or diarrhea (Table 4). Finally, results for pendant data loggers showed time by treatment differences (*P* <0.01; Figure 6) on d -1 where CTR calves showed the lowest standing time compared with the transported treatments.

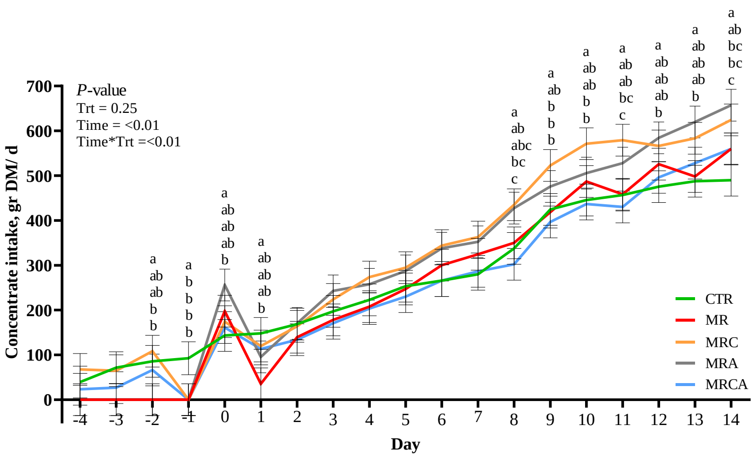

Figure 3. Concentrate intake (mean ± SE) measured daily from d -4 to d 42 of the study in male Holstein calves fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported during 19 h (CTR);

fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (MR); fed 2 L of colostrum at birth, MR and concentrate at the assembly center simulation period and transported during 19 h (MRC); fed 2 L of colostrum at birth, MR and acidified milk at the assembly center simulation period and transported during 19 h (MRA); and fed 2 L of colostrum at birth, MR, concentrate, and acidified milk at the assembly center simulation period and transported during 19 h (MRCA). Body weight from d -4 was used as a covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

Effect of the feeding strategy applied during the assembly center simulation after the 19 h transport at arrival to the rearing facility (d 0)

Time by treatment differences were observed in BW ($P < 0.01$; Figure 2) and concentrate intake ($P < 0.01$; Figure 3) on d 0 after transport. After 19 h of transport, all transported calves had a 1 kg BW shrinkage compared to CTR calves ($P < 0.001$; Table 2). Regarding intake, all transported calves had a peak of concentrate intake the first day after arrival (d 0). The amount of concentrate consumed was greater for MRA calves compared with CTR calves whereas MR, MRC, and MRCA calves showed intermediate values. Results from biomarkers of energy balance showed that at arrival to the rearing facility, all transported calves had significantly greater serum NEFA and BHB concentrations and lesser plasma glucose concentrations compared with CTR calves ($P < 0.01$; Figure 4 A, B, and C, respectively). On d 0, monocyte concentrations were greater ($P = 0.03$; Table 3) in the CTR calves compared with MRC, MRA, and MRCA calves, whereas MR calves had intermediate concentrations. On d 0, the evaluation of gastrointestinal permeability based on serum Cr-EDTA concentration showed the lowest concentration in the CTR calves, the highest concentrations in MR, MRC, and MRA calves, and intermediate concentrations in the MRCA calves. There was a time effect for serum citrulline concentration ($P < 0.01$; Figure 5), with a lower citrulline concentration on d 0 compared with d -1 for all treatments. Although results from pendant data loggers showed time by treatment differences ($P < 0.01$; Figure 6), no differences between treatments were seen for d 0 of the study.

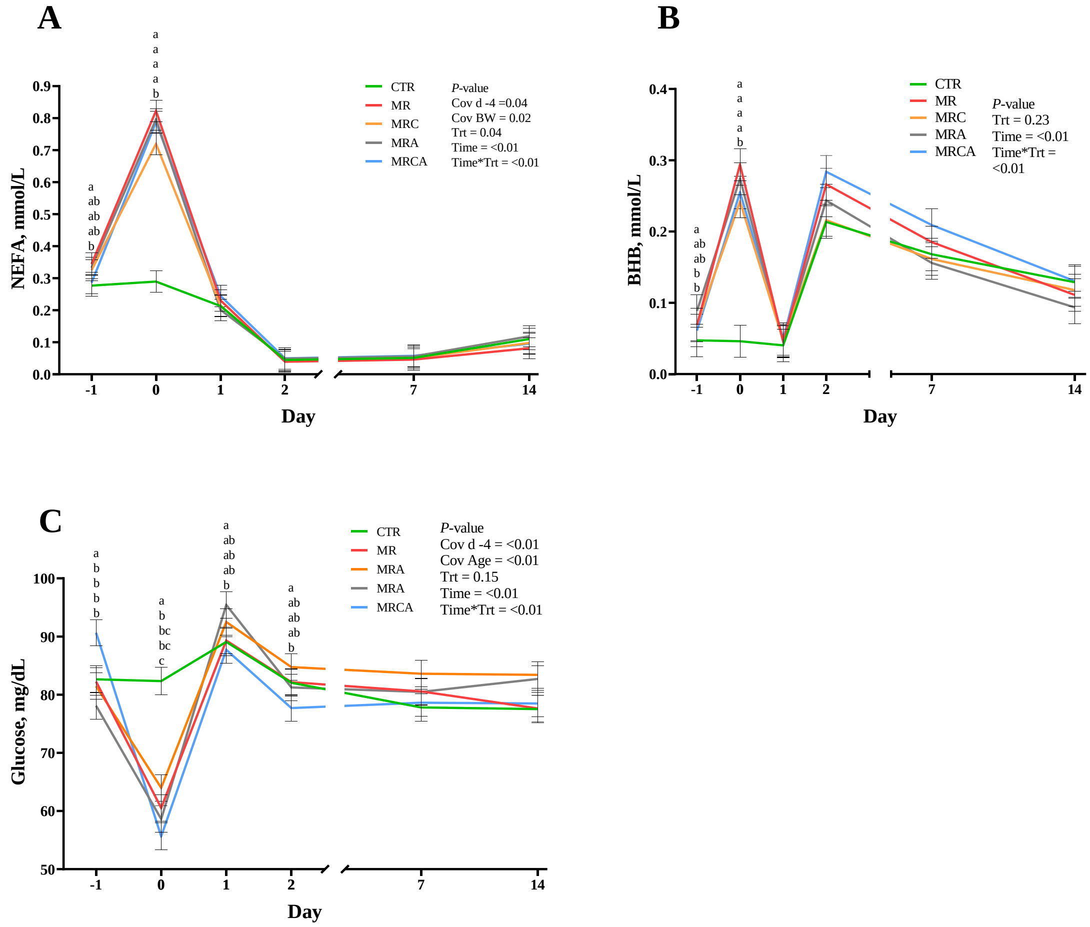

Figure 4. Serum concentration of nonesterified fatty acids (NEFA; A), β-hydroxybutyrate (BHB; B), and glucose (C) measured daily from d -4 to d 14 of the study in male Holstein calves fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported during 19 h (CTR); fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (MR); fed 2 L of colostrum at birth, MR and concentrate at the assembly center simulation period and transported during 19 h (MRC); fed 2 L of colostrum at birth, MR and acidified milk at the assembly center simulation period and transported during 19 h (MRA); and fed 2 L of colostrum at birth, MR, concentrate, and acidified milk at the assembly center simulation period and transported during 19 h (MRCA). Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

Table 3. Hematology parameters in male Holstein calves between 16 ± 3.1 and 33 ± 3.1 d of age.

	Treatment[2]						*P*-value[3]		
Item[1]	CTR	MR	MRC	MRA	MRCA	SEM	Trt	Time	Time x Trt
RBC, 10^12/L	8.35	8.50	8.10	8.13	8.41	0.13	0.17	<0.01	0.38
HCT, %	29.51	30.60	28.87	29.21	30.26	0.48	0.06	<0.01	0.18
HGB, g/dL	9.87	10.14	9.61	9.77	10.05	0.14	0.06	<0.01	0.43
MCV, fL	35.17[b]	36.00[a]	35.76[a]	35.84[a]	36.11[a]	0.19	<0.01	<0.01	0.98
MCH, pg	11.80	11.91	11.89	11.94	12.00	0.06	0.29	<0.01	0.96
MCHC, g/dL	33.36	33.18	33.33	33.35	33.21	0.13	0.87	<0.01	0.90
RDW, %	25.46	25.37	25.06	25.04	25.40	0.26	0.71	<0.01	0.98
WBC, 10^9/L	8.19	7.67	7.18	7.62	7.36	0.38	0.38	<0.01	0.71
Neutrophils, 10^9/L	3.30	3.07	2.70	2.99	3.09	0.27	0.58	<0.01	0.64
Lymphocytes, 10^9/L	4.19	4.27	4.13	4.25	4.02	0.13	0.71	<0.01	0.39
Monocytes, 10^9/L	0.47	0.38	0.36	0.37	0.38	0.04	0.32	<0.01	0.03
Eosinophils, 10^9/L	0.05	0.05	0.05	0.05	0.04	0.003	0.74	<0.01	0.94
PLT, 10^9/L	967.58	1010.48	980.59	994.33	960.52	46.3	0.94	<0.01	0.64
MPV, fL	4.62	4.68	4.73	4.68	4.74	0.03	0.50	<0.01	0.51

[1]RBC: Red blood cell count; HCT: Hematocrit; HGB: Hemoglobin concentration; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red cell distribution width; WBC: White blood cell count; PLT: Platelets; MPV: Mean platelet volume.

[a,b] Values with different superscripts within a row differ ($P \leq 0.05$).

[2] Treatments = CTR (fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported during 19 h); MR (fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h MR); MRC (fed 2 L of colostrum at birth, MR and concentrate at the assembly center simulation period and transported during 19 h); MRA (fed 2 L of colostrum at birth, MR and acidified milk at the assembly center simulation period and transported during 19 h); and MRCA (fed 2 L of colostrum at birth, MR, concentrate, and acidified milk at the assembly center simulation period and transported during 19 h).

[3] Trt = effect of pre-transport feeding strategy; Time × Trt = effect of the time by treatment interaction.

Table 4. The proportion of male Holstein calves with calf health score parameters greater than 0 measured from d - 4 (beginning of the assembly center simulation) to d 14.

Item[1]	CTR	MR	MRC	MRA	MRCA	SEM	Trt	Period	Period x Trt
Health score[3],									
Nose	0.03	0.02	0.01	0.02	0.06	0.014	0.14	0.49	0.79
Eye	0.77	0.02	0.05	0.03	0.05	0.036	0.10	0.13	0.40
Cough	0	0.01	0.01	0.01	0.01	0.003	0.96	0.99	0.94
Fecal[4]	0.01	0.02	0.01	0.01	0.02	0.009	0.84	0.002	0.54
Rectal temperature	0.22	0.14	0.20	0.20	0.18	0.015	0.61	0.01	0.06
Score > 5[5]	0.07	0.07	0.11	0.07	0.14	0.029	0.27	0.06	0.46

[1] Treatments= CTR (fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported during 19 h); MR (fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h); MRC (fed 2 L of colostrum at birth, MR and concentrate at the assembly center simulation period and transported during 19 h); MRA (fed 2 L of colostrum at birth, MR and acidified milk at the assembly center simulation period and transported during 19 h); and MRCA (fed 2 L of colostrum at birth, MR, concentrate, and acidified milk at the assembly center simulation period and transported during 19 h).

[3] Health scores greater than 0. The health score "ear" was excluded because of its low incidence.

[4] Fecal score was binary transformed considering 0= normal feces (scores 0 and 1) and 1= diarrheic feces (scores 2 and 3).

[5] Score > 5 (the sum of health criteria for each calf and day) was categorized binary: if the resulting sum was < 5 it was codified as 0, and if it was ≥ 5 it was considered as 1. The proportion contemplates 0= healthy calf (score 0) and 1= sick calf. Because a score > 5 evaluates respiratory disease, the "fecal" score was not considered in the calculation.

Effect of the feeding strategy applied during the assembly center on calves` recovery during the rearing period (d 1 to d 42)

Twenty-four hours after arrival at the rearing facility (d 1), differences in BW between treatments disappeared and equaled the CTR calves (Figure 2). There were no differences in BW until d 14 and d 21 of the study where MRC calves showed greater ($P < 0.05$) BW compared with MRCA calves. However, these differences disappeared by d 28, and the BW of the calves among treatments remained similar until weaning (d 42 of the study). Regarding concentrate intake, time by treatment differences were seen during this period ($P < 0.01$; Figure 3). After the increment in concentrate intake on d 0 in all transported calves, a sudden drop in their concentrate intake was observed on d 1 (24 h after arrival). The MR calves showed the greatest drop ($P <0.01$) compared with all transported, and their concentrate intake was significantly lower than the CTR calves. Calves supplemented with concentrate and/or acidified milk also showed a peak in concentrate intake by d 0 but did not differ between CTR and MR calves on d 1. No differences among treatments were observed from d 2 to d 8 of the study and all calves showed a linear increase in their concentrate intake (Figure 3). From d 8 to d 14, time by treatment differences ($P < 0.01$; Figure 3) were observed. From d 8 to d 11 in MRC and from d 12 to d 14 in MRA calves, concentrate intake was greater when compared with CTR and MRCA calves.

Differences among treatments on NEFA and BHB serum concentrations disappeared from d 1 until the last sampling on d 14 of the study ($P > 0.01$; Figure 4 A and B, respectively). On the other hand, serum glucose concentration showed time by treatment differences ($P < 0.01$; Figure 4 C). Calves in the MRA treatment showed greater serum glucose concentration on d 1 when compared with the MRCA calves, and MRC calves showed greater concentrations compared with the MRCA calves on d 2 of the study. No differences in glucose concentrations were seen for d 7 and 14 (Figure 4 C). Time by treatment differences in monocyte concentration showed greater ($P = 0.03$; Table 3) concentrations for the CTR calves when compared with MRC and MRA calves on d 2, and greater concentrations for CTR calves compared with MRCA calves on d 7 of the study. Additionally, treatment differences ($P = 0.03$; Table 3) were observed for MCV values being lower for the CTR

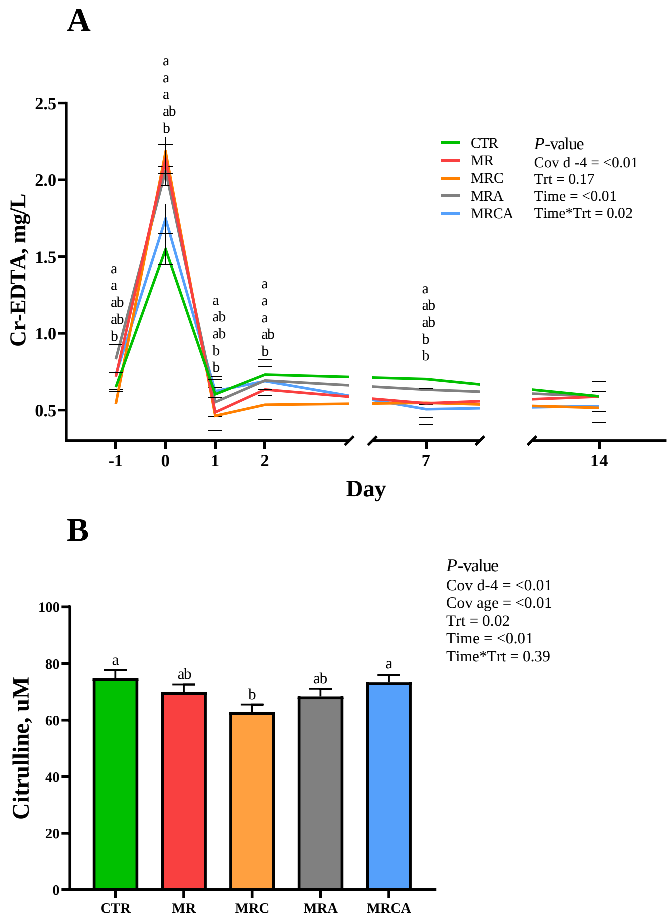

Figure 5. Serum concentration of Cr-EDTA (A) and citrulline (B) measured daily from d -4 to d 14 of the study in male Holstein calves fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported during 19 h (CTR); fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (MR); fed 2 L of colostrum at birth, MR and concentrate at the assembly center simulation period and transported during 19 h (MRC); fed 2 L of colostrum at birth, MR and acidified milk at the assembly center simulation period and transported during 19 h (MRA); and fed 2 L of colostrum at birth, MR, concentrate, and acidified milk at the assembly center simulation period and transported during 19 h (MRCA). Concentrations at d -4 were used as a covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

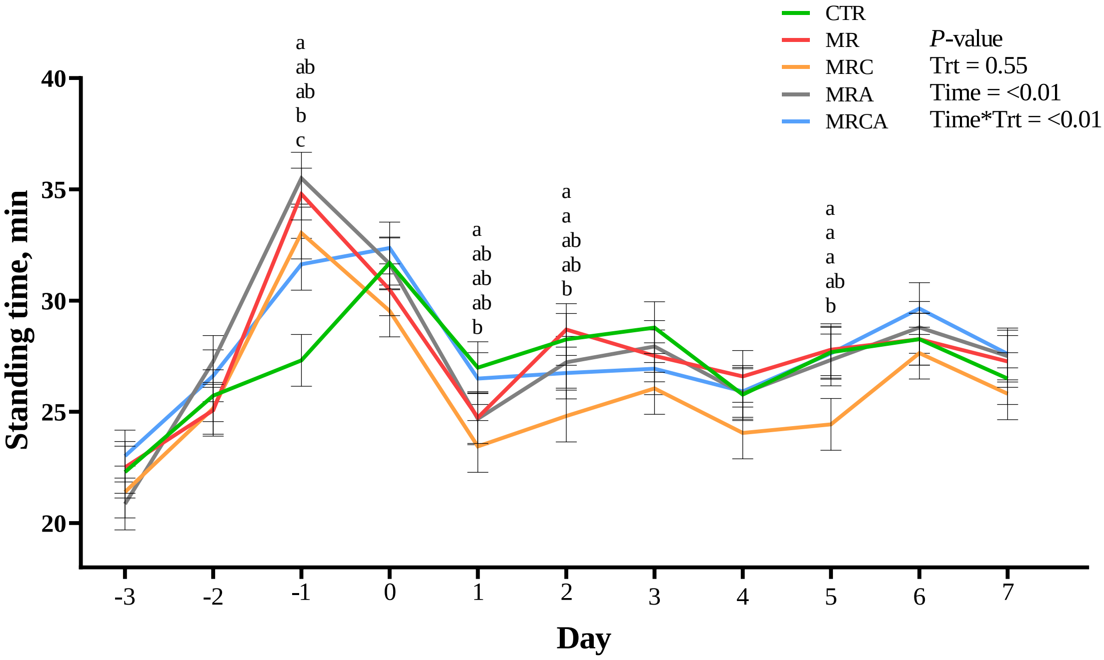

Figure 6. Pendant data loggers' records (mean ± SE) on standing time (min) measured daily from d -4 to d 7 of the study in male Holstein calves fed 10 L of colostrum at birth, MR and concentrate at the assembly center simulation period and not transported during 19 h (CTR); fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (MR); fed 2 L of colostrum at birth, MR and concentrate at the assembly center simulation period and transported during 19 h (MRC); fed 2 L of colostrum at birth, MR and acidified milk at the assembly center simulation period and transported during 19 h (MRA); and fed 2 L of colostrum at birth, MR, concentrate, and acidified milk at the assembly center simulation period and transported during 19 h (MRCA). Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

calves when compared with the transported treatments (*P* < 0.01; Table 3). A tendency (*P*= 0.06; Table 3) was observed for HCT and HGB among treatments. Calves in the MR and MRCA treatments tended to have greater values of HCT and HGB compared with MRC and MRA calves, respectively. When gastrointestinal integrity was evaluated at arrival at the rearing farm after transport (d 1), serum Cr-EDTA concentration was reduced in all treatments (*P* < 0.001; Figure 5 A) compared with serum Cr-EDTA concentrations on d 0. In addition, an interaction (*P* = 0.02) between time and treatment was observed on d 1, 2, and 7 among treatments. On d 1, CTR calves had greater serum Cr-EDTA concentrations than MR and MRC calves, while on d 2 these differences were observed among CTR, MRA, and MRCA versus MRC. On d 7 serum Cr-EDTA concentration was greater for CTR calves compared with MRC and MRCA. Additionally, a time effect was observed on the serum concentration of citrulline (*P* >0.01) with an increase in its serum concentration from d 2 until d 14 of the study (data not shown). Differences among treatments showed that MRC calves exhibited lower (*P* = 0.02; Figure 5 B) concentrations of citrulline when compared with CTR and MRCA calves whereas MR and MRA showed intermediate serum citrulline concentrations. No differences among treatments were observed after transport in the proportion of calves with scores > 5 and > 2 for respiratory disease and diarrhea, respectively. Finally, a time by treatment interaction (*P*< 0.01; Figure 6) on standing behavior showed the lowest standing time on MRC calves in d 1, 2, and 5 of the study.

DISCUSSION

Feeding diets that fulfill energy requirements might allow young calves to better cope with the challenges of transport (Rot et al., 2022b). Therefore, this study aimed to evaluate the beneficial effects of implementing feeding strategies during the assembly center period before long-distance transportation on the pre-transport status and on farm recovery in unweaned male dairy beef calves. To our knowledge, the only feeding strategies investigated to date in unweaned calves assessed the use of either MR or RS (restricted liquid feeding strategies) before transport (Bernardini et al., 2012; Marcato et al., 2020a; b; Pisoni et al., 2023). These authors observed that even though feeding MR seemed beneficial for the calves before being loaded into the truck for a short-distance transport, for long-distance transportation, neither MR nor RS were able to reduce the negative effects of fasting during transport. In the present study, the effects of continuous (ad libitum) provision of solid feed (concentrate) or liquid feed (acidified milk) supplementing conventional MR feeding were evaluated. The strategies studied herein showed that at the assembly center simulation, from d -4 to d -1 of the study, calves did not lose BW as observed when calves were fed RS previous to transport in Pisoni et al. (2023). On the other hand, when calves were supplemented with concentrate and/or acidified milk ad libitum in addition to MR, the BW before loading was greater for those calves compared with the ones that were only fed MR. The differences in BW between MR and MRCA calves may be linked to the nutrient intake differences between these treatments in this period. The amount of ME and CP for both treatments was 3.98 Mcal/d of ME and 17.51 g/d of CP for the MRCA calves and 2.86 Mcal/d of ME and 12.52 g/d of CP for the MR calves. In addition, the small amount of concentrate consumed and progressive increase of concentrate intake during the assembly center simulation, calves with ad libitum access to AM consumed more metabolized energy and CP than the ones with only supplemented with concentrate. However, differences in total DMI were not reflected in BW as expected. Calf BW before transport has been described as an important criterion for reducing the risk of respiratory disease, diarrhea (Brscic et al. 2012; Marcato et al. 2018; Wilson et al. 2020), and mortality (Windeyer et al. 2016; Marcato et al. 2018; Renaud et al. 2018; Wilson et al. 2020) during the 3 weeks after arrival to the rearing farm. The threshold for BW established to reduce the risk of morbidity and mortality before transport is between 46 kg and 51 kg (Brscic et al. 2012; Renaud et al. 2018; Masmeijer et al. 2019; Renaud and Pardon, 2022). In the present study, none of the treatments lost weight during the assembly center simulation, and there were no differences in the health scores previous and after transportation. All these positive outcomes were observed even when BW before loading was lower than the established threshold. These results may indicate that avoiding weight loss in the days prior to transport may have a greater impact on the risk of morbidity and mortality than the weight of the calf at the time of loading for transport. Loss of BW may be a better predictor of health problems in calves that are already mobilizing energy stores before the long hours of fasting during transport (Pisoni et al. 2023). As expected, transported calves lost weight due to transport shrinkage (excretory losses and the lack of feeding and/or drinking) and dehydration (Coffey et al., 2001). The average shrinkage in the present study was 3.5% of the total BW. Other studies have described a shrinkage of 1.5% and 2.7% of the total BW in calves with similar weight (42-45 kg) and age (18 d) fed MR or RS as a pre-transport diet (Marcato et al. 2019; Pisoni et al. 2023), and losses of 5.2% of BW in calves of 78 kg of BW and 37 d of age fed RS previous to transport (Bernardini et al., 2012). Shrinkage during transportation is important to be considered because it has been linked to increased morbidity and reduced performance (Camp, 1983).

The peak in concentrate intake on d 0 of the study stimulated by hunger due to the fasting suffered during transportation was expected as it has been previously described by our research group in other studies evaluating MR and RS as feeding strategies previous to transportation in unweaned calves (Pisoni et al., 2022; Pisoni et al., 2023). The objective of supplementing MR twice daily with ad libitum access to AM was to provide a rich source of energy and fat so that calves would not exacerbate their impaired nutritional status by mobilizing energy as the energy and protein consumption is already below their requirements (Nocek & Braund, 1986; Chen et al., 2020), and also to try to reduce the peak of concentrate intake caused by hunger as it may cause a subclinical acidosis and/or gut functionality disorders (Owens et al., 1998; Pisoni et al., 2023) . However, it has been described that large intake of lactose, as in the MR used in the present study (45.5%), can cause hyperglycemia as lactose is transformed into glucose and galactose during digestion (Hostettler-Allen et al. 1994). Also, decreasing feeding frequencies while maintaining the same milk allowance has negative effects on glucose metabolism (Vicari et al., 2008) leading to a reduction of energy efficiency and protein utilization (Van den Borne et al., 2006). In the present study, it was observed that the greater amount of milk replacer consumed in MRCA (in the form of MR and AM) together with the ad libitum allowance to it, might have caused the greater serum glucose concentration on d -1, and the lower serum glucose concentration (d 2) and concentrate intake the days following the long-distance transport for MRCA calves. Other authors studying enhanced feeding programs in calves have also described that solid feed intake was compromised when large amounts of milk or MR were fed (Jasper and Weary, 2002; Kristensen et al. 2007; Davis Rincher et al., 2011). However, in these studies, MR amounts were larger compared with the present study. Other hypothesis could be that the impact of the fasting hours could cause more stress to calves with greater DM intake negatively affecting the concentrate recovery for those in the MRCA treatment. Besides the impact of the combination of AM and concentrate as a pre-transport diet on concentrate intake recovery in the MRCA treatment, supplementing a certain amount of AM at assembly centers should be taken into consideration as calves may be able to consume more energy before transport. Other benefits of using AM are the reduction in total bacteria and *E. coli* counts in feces and the improvements in fecal consistency (Jaster et al. 1990). Fecal consistency is one of the major concerns as, under commercial practices, MR quality varies between the farm of origin, the assembly center, and the rearing farm. In addition, the use of AM minimizes bacterial growth and contamination (Hill et al. 2013), which is important when calves are rotated in the same environment, which is the case in assembly centers. However, the challenge in providing AM is to get the adequate final pH of the solution, as very low pH can compromise voluntary intake (Thickett et al., 1983; Bush and Nicholson, 1987; Hepola et al., 2008).

As observed by Pisoni et al. (2023) when the pre-transport diet was MR or RS, the increases in gastrointestinal permeability after the long hours of fasting during transport could not be avoided. Increases in Cr-EDTA serum concentration were expected in the transported calves as fasting and stress have been shown to exert negative effects on gut integrity (Zhang et al., 2013; Kvidera et al., 2017; Pisoni et al., 2022a). However, the greater serum NEFA concentration previous to transport in addition to the increase of gastrointestinal permeability observed after transport for the MR calves might explain the greater drop in concentrate intake on d 1 after arrival. A similar slope of the drop in concentrate intake for MR calves was observed for MRA on d 1. It was hypothesized that the bigger the drop, the slower the recovery of concentrate intake afterward (Pisoni et al. 2022; 2023) however, this pattern was not observed for MRA in the present study. In addition, it would be expected that MRC and MRCA calves had greater concentrate intake the following 14 days after transport due to the smaller slope of concentrate intake on d 1. However, as mentioned previously, MRCA calves had lower concentrate intake during the first 14 d together with CTR calves and it may be linked to the greater amounts of lactose consumed previous to transport, the change of allowance to liquid feeding or the greater stress caused by the hours of fasting in calves with greater intake. In previous studies, we were able to see clearer associations between concentrate intake recovery and enterocyte functional mass (citrulline) than in this study (Pisoni et al. 2022a; Pisoni et al. 2023). Contrary, in the present study, calves with lower serum citrulline concentration as observed in MRC calves were one of the treatments with greater concentrate intake after arrival. Supplementing ad libitum access to concentrate in addition to MR twice daily at assembly centers should also be considered as a way of providing calves with more energy before transport. In addition, feeding concentrate before transport could stimulate rumen function providing nutrients in the rumen and helping to mitigate the peak and drop of concentrate intake on d 0 and d 1 after arrival.

CONCLUSIONS

Results from the present investigation showed that feeding ad libitum acidified milk or concentrate in addition to two feedings of milk replacer at assembly centers increased the nutrient and energy provided previous to transport and improved calves'BW and concentrate intake recovery at arrival. However, the feeding strategies proposed herein did not prevent the increase of permeability due to the fasting hours during transport.

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CHAPTER VI

General Discussion

For this discussion, a descriptive analysis of the main findings from Chapters III, IV and V where the effects of the amount of colostrum consumption and different pre-transport feeding strategies were evaluated, and Annexes 1 and 2 was conducted. The Annex 1 had the objective to evaluate the effects of feed restriction during marketing and fasting simulating transportation on calves performance, physiological parameters, behavior, and gastrointestinal permeability, while the Annex 2 had the objective to evaluate the delay of the access to concentrate intake at arrival and substitute the access to concentrate intake by acidified milk between the first two milk replacer feedings, and the use of a COX-2 selective NSAID on concentrate intake and gastrointestinal permeability of commercially unweaned dairy beef calves subjected to marketing and transportation.

The most relevant results from the different studies were analyzed together to have a general overview of all factors studied and to analyze them in more detail, to discuss potential associations or interferences in the elaboration of conclusions and, to reinforce some outcomes or conclusions and, finally, to look for knowledge gaps and future research needs.

The results described herein are focused over a 7-day period (the week after arrival) and in how, during this short period of time, different volumes of colostrum consumed at birth, feed restriction and pre-transport feeding strategies during marketing, and fasting during transport affected the recovery of concentrate intake, body weight, and indicators of energy balance and intestinal functionality in unweaned male dairy beef calves. The reader can refer to Table 1 for a summary of the treatments and experimental designs used in each discussed study.

1. Concentrate intake recovery

Figure 1 represents the recovery in concentrate intake from all the treatments in the 4 different studies included in this thesis. Main interpretations made below include those findings within d 0 after fasting or transportation, depending on the study, until d 7.

Control groups calves did not suffer feed restriction or fasting due to transport; these animals are the "Golden Standard". In case we need to transport animals, any strategy to avoid the negative side effects of marketing and transportation should at least mimic control outcomes (intake, intestinal permeabilty, BW losses, etc.). When evaluating the results of the control groups from each study, clear differences can be seen in the recovery of concentrate intake among treatments (Figure 1, marked in dark blue). The first interesting result is the variability among the control treatments in the amount and evolution of concentrate intake among them. These differences can be explained based on the experimental design used in each of the studies. For example, control calves of the Annex 1 study, which showed the greatest recovery of concentrate intake, were crossbred Angus-Holstein bull calves that belonged to the same dairy farm with high quality standards on the care of the animals. In addition, the low number of calves used allowed us to select them based on their health status, specifically without any lung lesion after ultrasound evaluation. The type of concentrate used in these calves was more palatable concentrate than the one used in other studies. Finally, unlike Chapters III and V, and Annex 2 where calves were transported, in this study calves were subjected only to 9 h or 19 h of fasting intended to simulate transport.

Table 1. Description of the colostrum consumption protocol, the marketing feeding strategies used, and the time spent in transport and fasting in Chapters III, IV, and V and Annexes 1 and 2.

Chapter/ Annex Nº	Trt	Colostrum given at birth			Pre-transport diet				Hours in fasting				Hours in transport			Post-transport diet		
		2 L	6 L	10 L	MR	RS	Concentrate	AM	0	8	9	19	0	8	19	MR	Concentrate	AM
CHAPTER III	**CTRL**			**X**	**X**		**X**		**X**				**X**			**X**	**X**	
	HCMR			**X**	**X**							**X**			**X**	**X**	**X**	
	HCRS			**X**		**X**						**X**			**X**	**X**	**X**	
	LCMR	**X**			**X**							**X**			**X**	**X**	**X**	
	LCRS	**X**				**X**						**X**			**X**	**X**	**X**	
CHAPTERS IV AND V	**CTR**			**X**	**X**		**X**		**X**				**X**			**X**	**X**	
	MR	**X**			**X**							**X**			**X**	**X**	**X**	
	MRC	**X**			**X**		**X**					**X**			**X**	**X**	**X**	
	MRA	**X**			**X**			**X**				**X**			**X**	**X**	**X**	
	MRCA	**X**			**X**		**X**	**X**				**X**			**X**	**X**	**X**	
ANNEX 1	**CT**		**X**		**X**		**X**		**X**							**X**	**X**	
	MD		**X**		**X**						**X**					**X**	**X**	
	MO		**X**		**X**							**X**				**X**	**X**	
	SV		**X**			**X**						**X**				**X**	**X**	
ANNEX 2	**F-CT**	**?**	**?**	**?**	**?**	**?**	**?**	**?**		**X**				**X**		**X**	**X**	
	F-MR	**?**	**?**	**?**	**?**	**?**	**?**	**?**		**X**				**X**		**X**	**X ***	
	F-AC	**?**	**?**	**?**	**?**	**?**	**?**	**?**		**X**				**X**		**X**	**X**	**X**
	O-CT	**?**	**?**	**?**	**?**	**?**	**?**	**?**		**X**				**X**		**X**	**X**	
	O-MR	**?**	**?**	**?**	**?**	**?**	**?**	**?**		**X**				**X**		**X**	**X ***	
	O-AC	**?**	**?**	**?**	**?**	**?**	**?**	**?**		**X**				**X**		**X**	**X**	**X**

The main differences observed in concentrate recovery in these calves may be attributed to these differences regarding breed, health, and type of feed and the fact that these calves were not exposed to the stress of transport (mixing, loading, unloading, environment, fatigue, etc.) which could have influenced their intake recovery afterward. The breed of these calves may have been an influencing factor as breed may influence the resilience to stressful periods (Husseini et al., 2022) besides its effect on carcass quality (Huuskonen et al., 2013; Bown et al., 2016; Hessle et al., 2017; Vestergaard et al., 2019). However, looking at the results of the calves used on Annex 2 study, this hypothesis of crossbreeding was discarded. Other factor that could be accounted for the greater concentrate intakes might have also been linked to the greater BW for the crossbreed calves in Annex 1 compared to the pure Holsteins used in Chapters III, IV and V (shown below). However, calves used in Annex 2 had greater BW than calves in Annex 1 study and the lowest concentrate intake recovery. Even though these calves were also crossbred and had greater BW at the moment of transport, the main differences observed are related to their previous management. These calves were born at different dairy farms with unknown postnatal care, were marketed at auction in France, with also unknown staying times at assembly centers and if they were fed or not during that time, and were then subjected to a commercial 8-h transport to Spain. These differences are important to consider especially when we compare them with the calves from Annex 1 which were only fasted or calves from Chapters III and V that were marketed and transported under experimental conditions. As mentioned above, it seems quite evident that the negative effects of the stress of mixing, comingling, fasting, and transport during marketing in these calves may have worsened their status at arrival prolonging their recovery of concentrate intake even when those calves were enrolled in the study with greater BW. Another difference was that calves in the Annex 1 study were offered a texturized concentrate with a high-quality nutrient and ingredient composition which could have been more palatable and stimulated intake to a greater extent than the pellet concentrate used in Chapters III, IV and V. However, the use of high-quality concentrates is not common in the practice of rearing male calves. In Chapters III, IV and V and Annex 1 studies, the same concentrate used at the dairy farm of origin was used during the study to avoid drops in concentrate intake due to changes in the formulation of the solid feed. In addition, another important difference between Annex 2 and the rest of the studies is that, in the first case, the feeding strategies aimed to stimulate concentrate intake recovery were applied after transport, whereas for the rest of the studies, the strategies were applied before transport during the assembly center simulation. Overall, the difference in concentrate intake recovery between crossbred calves born on the same dairy farm and not transported, and calves born at different origins and exposed to the adverse effects of marketing and transport, is approximately 350 g of concentrate DM/day one week after arrival at the rearing facility. These results highlight the impact of marketing and fasting and call for improvements in the nutritional management of these unweaned calves, as it will be discussed in more detail later in section 1.2 (Figure 3). Moreover, looking at Figure 1, calves in the HCMR (Chapter III), and MRC and MRA (Chapter V) treatments also showed greater recovery of concentrate intake indicating that calves fed high amounts of colostrum at birth and MR during the assembly center, and calves fed low amounts of colostrum at birth but supplemented with concentrate or acidified milk during the assembly center could face better the effects of transportation and recover concentrate intake to a great extent than control calves of the same origin.

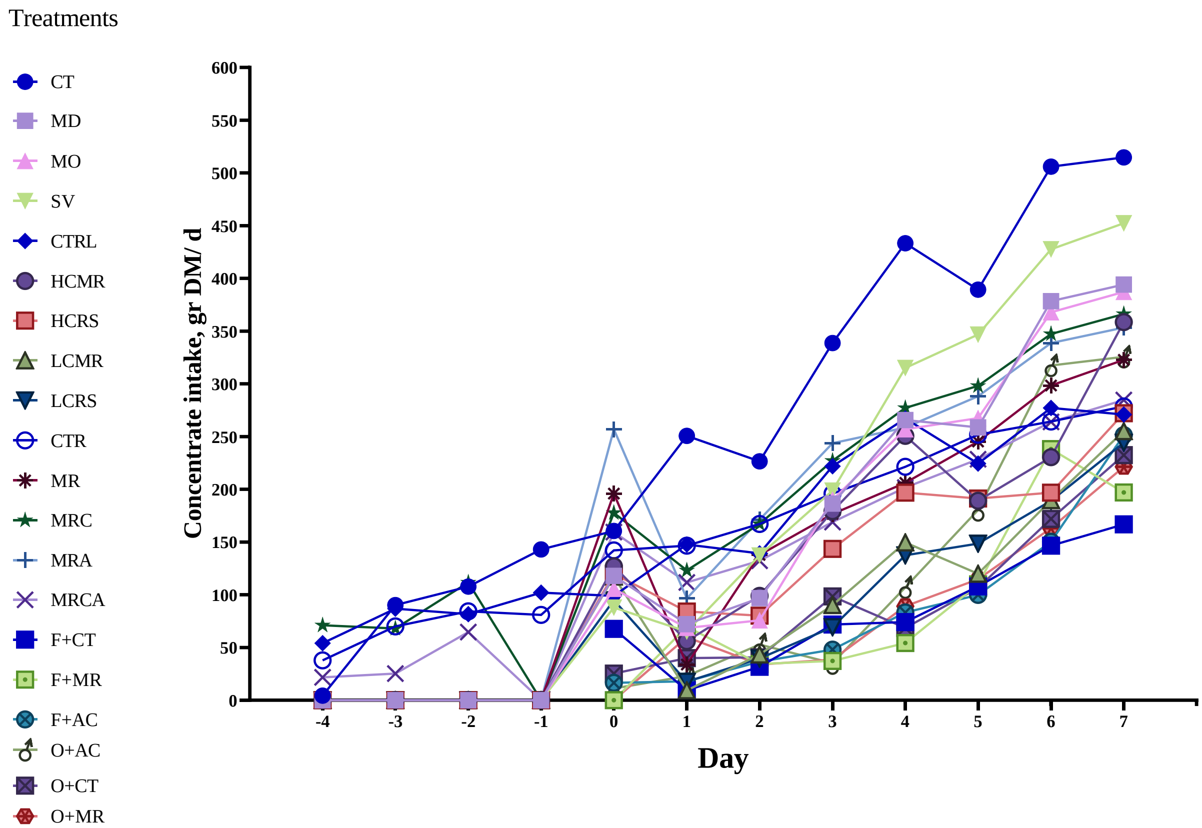

Figure 1. Recovery of concentrate intake during the first week after application of treatments (feed restriction/ fasting and transportation) considering different treatments within studies.

Treatments= CTRL (control); HCMR (high-colostrum milk replacer-fed calves); HCRS (high-colostrum rehydrating solutions-fed calves); LCMR (low-colostrum milk replacer-fed calves); LCRS (low-colostrum rehydrating solution-fed calves). These treatments belong to the Chapters III and IV studies.
CTR (Control); MR (milk replacer calves); MRC (milk replacer and concentrate calves); MRA (milk replacer and acidified milk calves); MRCA (milk replacer, acidified milk, and concentrate calves). These treatments belong to Chapter V study.
CT (control); MD (mild); MO (moderate); SV (severe). These treatments belong to Annex 1study.
F+CT (anti-inflammatory-control calves); F+MR (anti-inflammatory-milk replacer calves); F+AC (anti-inflammatory-acidified milk calves); O+CT (no anti-inflammatory-control calves); O+MR (no anti-inflammatory-milk replacer calves); and O+AC (no anti-inflammatory-acidified milk calves). These treatments belong to the Annex 2 study.

Finally, showing the lesser concentrate intake recovery there are those treatments belonging to the Annex 2 study and the LCMR and LCRS calves, the 2 treatments fed low amounts of colostrum at birth from Chapter III. When comparing these calves with the other treatments from the same study, although numerical, concentrate intake recovery was lower in the low-colostrum-fed calves. We have previously hypothesized that these results may be due to lower ingestion of nutrients (carbohydrates, proteins, lipids) and bioactive substances contained in the colostrum for the LCMR and LCRS calves.

Bioactive compounds in the colostrum such as growth factors, glucagon, insulin, lactoferrin, TNF-α, and amino acids, among others, have been shown to modulate the development and function of the gastrointestinal tract of newborn calves (Blum, 2006). The mechanisms behind these beneficial effects appear to be related to increased growth of intestinal epithelial cells promoting intestinal absorption (Blättler et al., 2001), increased crypt cell proliferation, decreased apoptosis, stimulated villus growth (Blum, 2006), and promotion of intestinal growth and cell proliferation (Hammon et al., 2020). Therefore, feeding lower amounts of colostrum at birth may negatively impact the digestive capacity of these calves, worsening concentrate intake digestion and prolonging the time needed for the calves to achieve concentrate intake levels like those for the high-colostrum-fed calves. In addition, calves that are fed less colostrum at birth may be less robust at the time of facing the challenges of this system.

After analyzing all studies together and observing that some factors may be critical for the concentrate intake recovery, and in order to assess which might be the most important factors influencing the recovery of concentrate intake, the effects of colostrum consumption at birth, transportation, and pre-transport and post-transport feeding strategies have been evaluated separately.

1.1. Effects of colostrum consumption on concentrate intake recovery on arrival

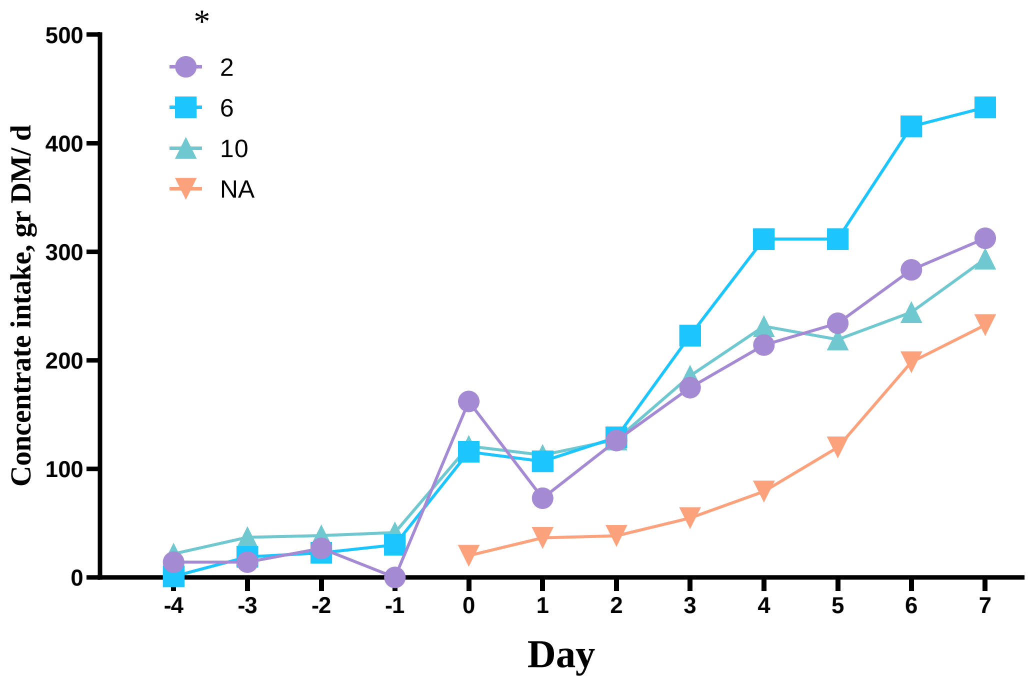

Figure 2. Recovery of concentrate intake during the first week after application of treatments (feed restriction/ fasting and transportation) considering variation in colostrum consumption at birth. * = 2 (2 L of colostrum fed at birth); 6 (6 L of colostrum fed at birth); 10 (10 L of colostrum fed at birth); and NA (not applicable, refers to the Annex 2 study where the amount of colostrum fed at birth is unknown).

Results from Figure 2 compare the recovery in concentrate intake between calves from the Chapters III, V, and Annex 1 and 2 considering the amount of colostrum consumed at birth. In practice, most dairy farms in Spain feed their male calves with at least 2 L of colostrum at birth. In a previous study investigating biomarkers of colostrum consumption at arrival to the rearing facilities, our research group demonstrated that when feeding 10 or 2 L of colostrum at birth, calves would not exhibit failures in their transfer of passive immunity if administration was done within the first 2 h after birth (Pisoni et al., 2022b). However, not only Ig and its protective immune function are essential for these newborn calves but, as previously commented, the bioactive compounds contained in it will exert positive effects on gut development and maturation. Results from Figure 2 show that calves fed 6 L of colostrum had greater recovery of concentrate. However, these calves belong to the Annex 1 study and, as commented above, were crossbreeds, not transported, healthy calves fed a high-quality concentrate, all factors that might obscure the interpretation of these results. When comparing calves fed 2 vs. 10 L of colostrum at birth, there were almost no differences among them. In this case, these calves belong to Chapters III, IV and V, and in these studies in addition to the colostrum consumption, different feeding strategies containing rehydrating solutions, MR, concentrate, and acidified milk and their combinations were studied. These dietary variations could also interfere in the interpretation of concentrate recovery based merely on colostrum consumption. Finally, results under “NA” refer to those calves arriving from a commercial market and whose consumption of colostrum at birth was unknown (Annex 2). In these calves, the effect of colostrum is diluted by other variables affecting concentrate intake like the pre-transport feeding strategy applied and all the previously mentioned challenges that calves had going through market and transportation (mixing, physical and psychological stress, diseases, etc.). It is important to note that in all the studies conducted in this thesis, the quality of the colostrum was previously assessed and only high-quality colostrum was fed to the calves. Except for those calves from the Annex 2 study where colostrum consumption at birth was unknown. The quality and quantity of colostrum fed in practice are usually neglected in male compared to female calves (Renaud et al., 2017), and this difference may condition the relationship between the present results and the actual reality on dairy farms.

1.2.Effects of transportation on concentrate intake recovery on arrival

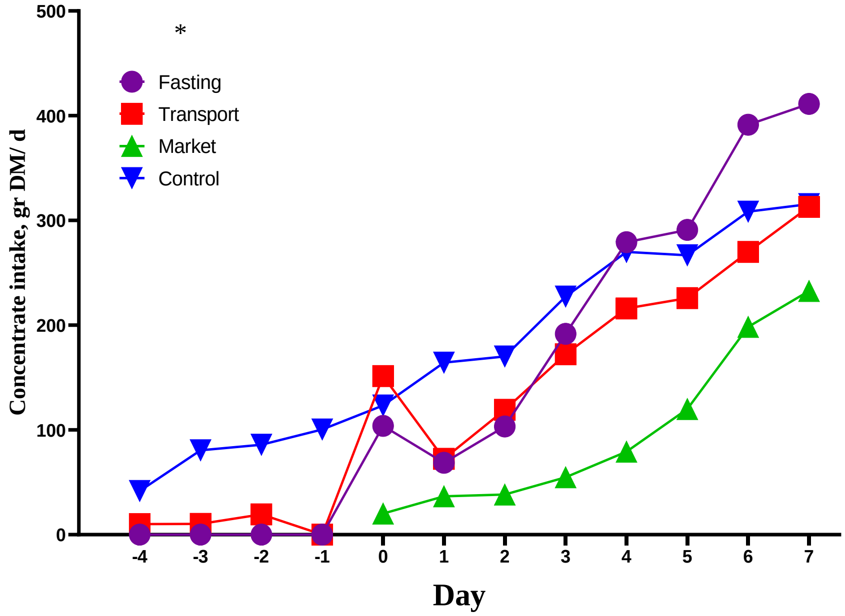

Figure 3. Recovery of concentrate intake during the first week after application of treatments (feed restriction/ fasting and transportation) considering transport.
*= Fasting (calves fasted during either 9 or 19 h; Annex 1 study); Transport (calves transported during 19 h; Studies 1, 2, and 3); Market (calves coming from the auction and transported 8 h; Annex 2 study); Control (not transported or fasted calves).

The results in Figure 3 summarize the recovery on concentrate intake during the first week after arrival taking into account whether the calves were fasted, transported, marketed, or none of the above (controls). Those calves that were not transported and instead fasted for 9 or 19 h showed greater concentrate intake recovery by d 7 after arrival than transported calves. These results may suggest that when calves are not exposed to the challenges of transport, their overall performance status is almost unaffected and they are better able to cope with feed restriction and fasting recovering intake more quickly. On the contrary, but in direct relation to these results, those commercially marketed calves from the Annex 2 study showing lesser concentrate intake recovery had to deal with the stressful process and challenges of marketing and were most likely not fed MR or concentrate until arrival at the rearing facility. Offering male calves ad libitum access to concentrate since birth is usually not a practice in most dairy farms. This solid feed restriction usually gets prolonged as calves are not fed concentrate at assembly centers or during transportation. Therefore, for some calves, concentrate will be first offered at the rearing facilities. As calves are not familiarized with this feed it might take a time until calves start consuming adequate amounts of it. Additionally, the physical and psychological stress induced by transport increases GIT permeability (Meddings and Swain, 2000; Lambert, 2009) which directly affects concentrate intake and absorption initially, and production, metabolic, and inflammatory parameters in the long-term (Kvidera et al., 2017). In addition to concentrate, male calves are usually fed insufficient amounts of milk or MR at the dairy farm of origin, a situation that gets worse when arriving at the assembly centers. At the assembly centers, calves are normally not fed MR and in some cases, they are fed rehydrating solutions instead. In consequence, the nutrients and energy contained in these solutions are not sufficient and calves end up losing weight. All these factors make calves more prone to disease and mortality and directly affect their recovery at arrival. Finally, results from control and transported calves over concentrate intake recovery were similar. It was expected that those non-transported calves would have greater intake recovery compared with the transported ones. However, the feeding strategies applied before transport in Chapter V may have diluted differences and confounded these results. The results observed in concentrate intake when comparing non-transported calves, fasted calves, transported calves under experimental conditions, and transported calves in commercial conditions showed a clear impairment of the recovery as additional challenges were added to the calves during the marketing and transportation periods, as calves coming from commercial conditions had the lowest concentrate recovery.

1.3. Effects of pre-transport diet on concentrate intake recovery on arrival

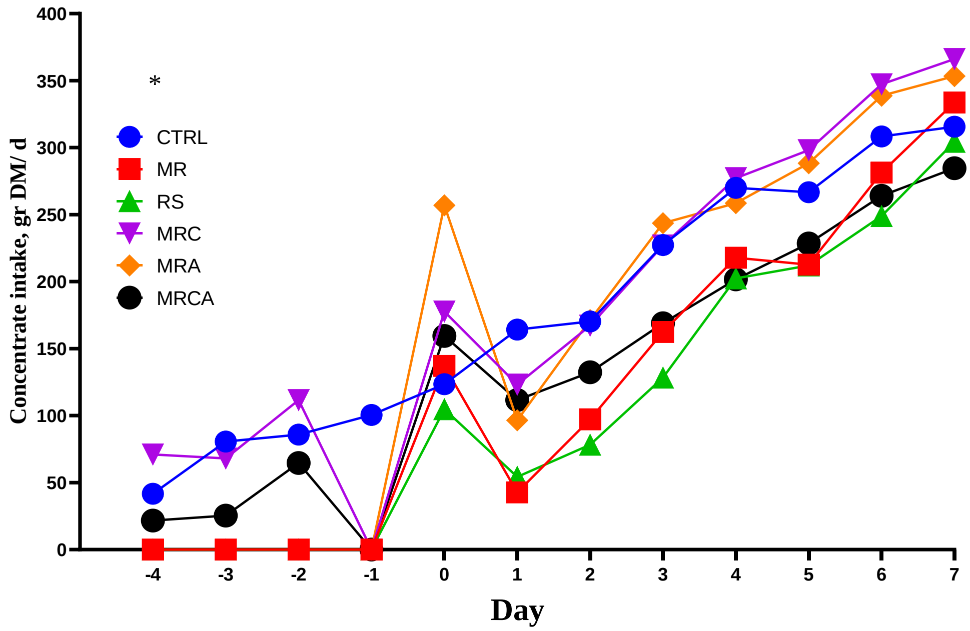

Figure 4. Recovery of concentrate intake during the first week after application of treatments (feed restriction/ fasting and transportation) considering the pre-transport diet administered.
*= CTRL (control calves fed MR and concentrate ad libitum); MR (calves fed only MR during the assembly center simulation); RS (calves fed only RS during the assembly center simulation); MRC (calves fed MR and ad libitum concentrate during the assembly center simulation); MRA (calves fed MR and ad libitum access to acidified milk during the assembly center simulation); and MRCA (calves fed MR and ad libitum access to concentrate and acidified milk during the assembly center simulation). See Table 1 for further details on treatments and feeding strategies for each study.

Figure 4 reflects the effect that pre-transport feeding strategies have on the recovery of concentrate intake at arrival. When calves arrive at the rearing facility after a period of fasting due to transportation their concentrate intake peak as a response to hunger. Additionally, a consequent drop in concentrate intake can be observed for all transported treatments 24 h after the peak. This intake pattern was consistent between all the studies of this thesis, even for Chapter V where we implemented pre-transport feeding strategies aiming to prevent these acute changes in intake.

Interestingly, the lowest concentrate intake at d 1 was in the treatments where calves were not supplemented with either concentrate and/or acidified milk (RS and MR calves). Previous intake of concentrate (MRC and MRCA calves) may have improved rumen function and provided a continuous supply of nutrients in the lower gut. In addition, feeding higher volumes of milk by providing an ad libitum source of AM (MRA and MRCA) may also have increased nutrient availability and improved recovery of concentrate intake on arrival. In addition, this would confirm, as discussed in this thesis, that feeding only RS or MR was not enough to prepare calves for fasting and fat mobilization to such stressful events as transport and fasting. Results also showed that feeding calves MR and concentrate or MR and acidified milk (MRC and MRA calves from Chapter V) before transport increased the concentrate intake recovery at arrival to levels similar to those of the controls which are not even being transported or fasted. These treatments have a greater intake recovery until d 7 when compared with calves fed only RS, MR, or the most complete feeding strategy (MRCA). The main outcome from these results is that there is a clear impact on intake recovery depending on the diet offered before transport. Plus, increasing nutrient and energy availability by feeding concentrate or acidified milk may positively affect this recovery. The reasons for these differences are not fully understood, but it may be that these calves, being better prepared from a nutritional standpoint, might be more resilient to the stress of fasting and transportation and therefore able to recover more quickly at arrival.

In the evaluation of pre-transport feeding strategies, commercially marketed calves from Annex 2 were not included in the analysis as their pre-transport feeding was unknown. However, further consideration should be given to the fact that these older calves may have had access to solid feed on the farm of origin and that, marketing these calves under only RS or MR feeding for several days before fasting them during transport could have caused severe digestive distress. This fact may explain the low concentrate intake seem during the first 7 d after arrival (Figure 3) and the prolonged recovery of the intestinal permeability, which will be described below.

2. Recovery of BW and energy balance and gut functionality markers

2.1. Effect of colostrum consumption at birth

Recovery of BW after transport seems to be affected by the amount of colostrum consumed at birth. In Figure 5 A, it can be observed that calves fed only 2 L of colostrum at birth had a lower BW after transport compared to 10 L calves and controls. These losses of BW are probably associated with lower fat reservoirs due to colostrum shortage. When considering calves arriving from the market (Annex 2) even though their BW was higher, the slope in the losses of BW after transport is similar to those calves fed 2 L of colostrum. Market calves may have a higher BW because they were older animals, but the similarity of the slope with 2 L-fed calves suggests that they may have been fed lower amounts of colostrum at their dairy farm of origin. Feeding colostrum at birth is fundamental to providing immunity and nutrients like carbohydrates, proteins, and lipids (Blum, 2006). Therefore, feeding high levels of colostrum to calves may increase nutrient availability and stimulate fat reserves as a reservoir. In calves fed lower amounts of colostrum, mobilization of energy from these reservoirs may be insufficient in a state of negative energy balance caused by feed restriction and transportation, and this can be observed as greater shrinkage compared to calves fed higher amounts of colostrum. Calves fed 6 L of colostrum had greater BW by d 7 compared with calves fed 10 and 2 L. These are Annex 1 calves and, as mentioned previously, most of the differences observed with these animals are in relation to their crossbreed and the fact that they did not undergo transportation but were fasted. So, differences in BW by d 7 may therefore be mainly related to these factors and not to a significant influence on the amount of colostrum consumed.

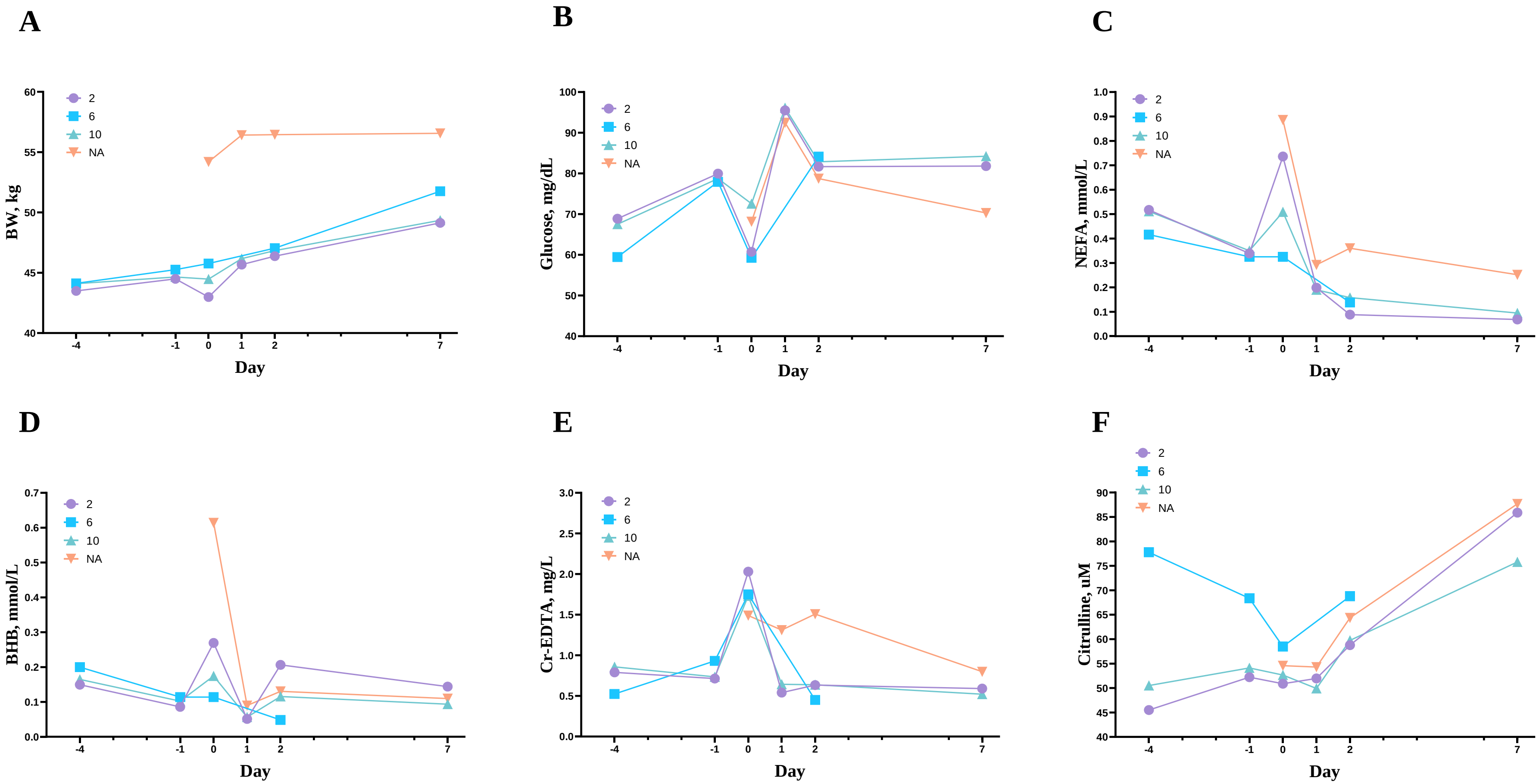

Figure 5. Recovery of BW (A) and markers of energy balance (B, C, and D) and gut functionality (E and F) during the first week after application of treatments (feed restriction/ fasting and transportation) considering variation in colostrum consumption at birth.
* = 2 (2 L of colostrum fed at birth); 6 (6 L of colostrum fed at birth); 10 (10 L of colostrum fed at birth); and NA (not applicable, refers to the Annex 2 study where the amount of colostrum fed at birth is unknown).

Regarding markers of energy balance, greater serum NEFA and BHB concentrations (Figure 5; B and C, respectively) are observed for the 2 L-fed calves and those arriving from the market after transportation. These results are in close relation to the greater BW losses for these calves for the same period. As discussed, calves fed lower amounts of colostrum with a more limited capacity to generate reservoirs would have to mobilize more fat as a source of energy to cope with the energy demands of transportation and feed restriction. Although fat reserves in unweaned calves are scarce, calves can switch to lipid oxidation (represented by the increment in serum NEFA and BHB) to use lipid as a source of energy during negative energy balance conditions (Grigor et al., 2001; Bernardini et al., 2012). The decrease in glucose concentration for the NA (market) calves from d 1 to d 7 is most likely related to their low intake. Firstly, these calves were being fed 2 L instead of 2.5 L of MR as, per experience, commercially marketed calves do not tolerate 2.5 L of MR twice daily, and secondly, their concentrate intake was much lower compared to Annex 1 and Chapters III and V calves. These characteristics may be the cause of the observed differences in serum glucose concentration rather than an effect of colostrum consumption.

Gut permeability assessed by Cr-EDTA serum concentrations (Figure 5; E) showed an increment in all calves after transport/fasting (depending on the study). As mentioned, bioactive compounds contained in the colostrum have been shown to stimulate gut epithelium development and function (Blum and Hammon, 2000). However, results showed no protective effect on gut permeability for the high plane of colostrum-fed calves compared with those fed only 2 L or the commercially marketed calves on arrival. The subsequent differences in gut permeability in commercially marketed calves may be due to the combination of challenges that these calves were exposed to, rather than as a result of colostrum intake alone. Finally, citrulline results (Figure 3; F) showed no clear differences, which cannot be attributed to colostrum consumption, but may be related to dietary factors which will be discussed later.

2.2. Effect of transportation

Body weight was greater in the calves arriving from the market even though they went through commingling and mixing conditions during marketing (Figure 6; A). However, marketed calves were older than the other treatments upon arrival at the rearing facility. From d 0 to d 1 after transport, the slope of the commercially marketed and transported calves was similar, showing that loading and transporting calves in a truck causes greater losses due to shrinkage than fasting. In addition, it seems that fasted calves may have compensatory growth from d 2 to d 7 due to the greater concentrate intake described above (Figure 3). On the other hand, the slope of the BW recovery during the first week in the commercially marketed calves remained on a plateau, not gaining much weight from d -1 to d 7. As mentioned in the concentrate intake results (Figure 3), marketed calves had surprisingly lower intakes, resulting in lower BW recovery. It could be hypothesized that the negative effects of commingling, mixing of calves from different origins, exposure to disease and environmental conditions, etc., associated with marketing from a different country could negatively affect BW recovery in these marketed calves.

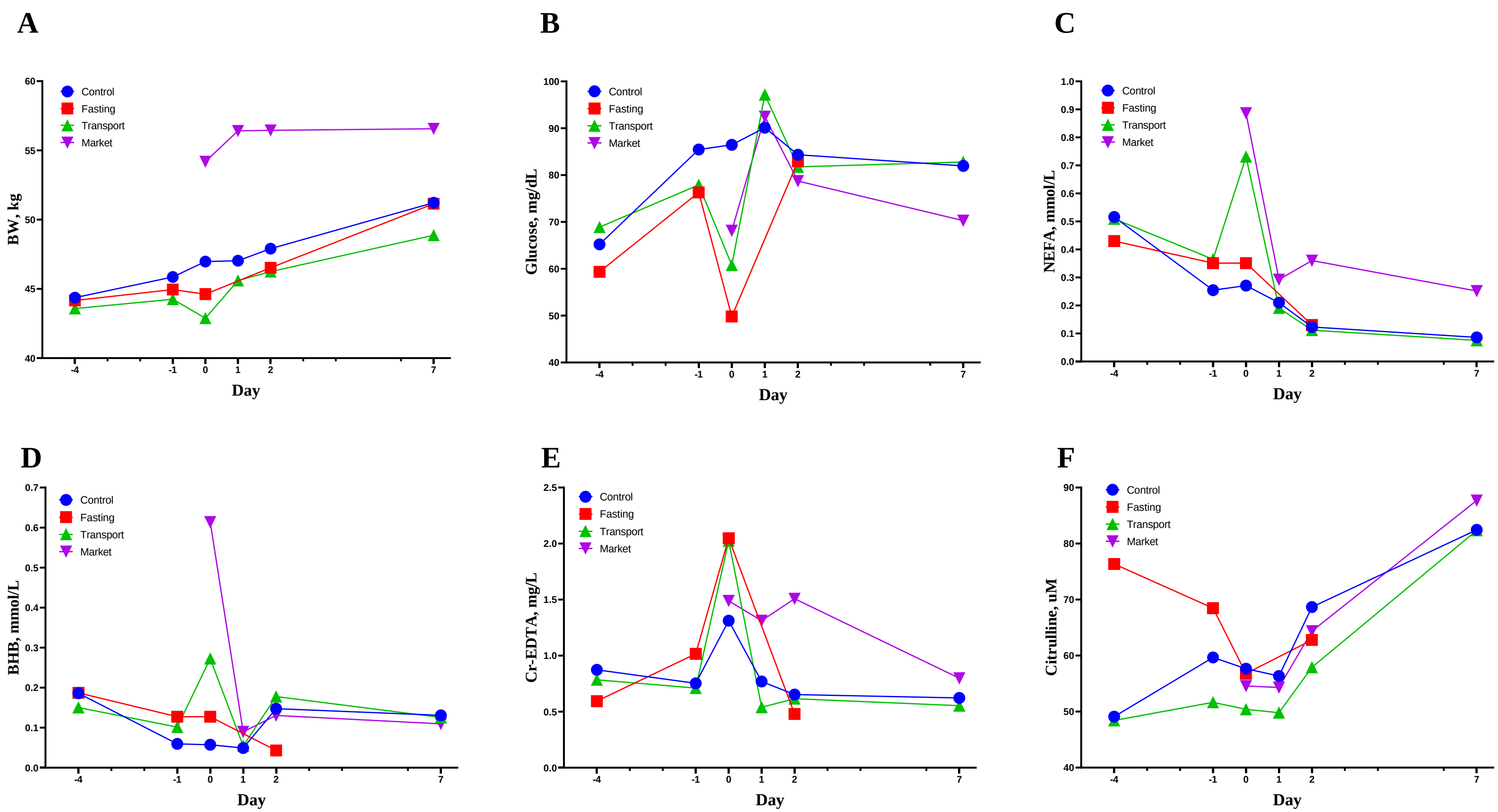

Figure 6. Recovery of BW (A) and markers of energy balance (B, C, and D) and gut functionality (E and F) during the first week after application of treatments (feed restriction/ fasting and transportation) considering transport.

*= Fasting (calves fasted during either 9 or 19 h; Annex 1 study); Transport (calves transported during 19 h; Studies 1, 2, and 3); Market (calves coming from the auction and transported 8 h; Annex 2 study); Control (not transported or fasted calves).

Drops in glucose as a response to fasting and transportation are conserved within treatments (Figure 6; B). However, in relation to markers of lipid mobilization, there was an increase in serum NEFA and BHB concentrations due to transport in the transported and commercially marketed calves (Figure 6; C and D, respectively). This response has been previously commented and is mainly associated with fasting and nutrient restriction in addition to the stress of transport. The greater the stress and nutrient restriction, the greater the use of fat as a source of energy with the consequent increase in NEFA and BHB concentration in blood. For the same reason, calves undergoing transportation mobilize more fat than fasted calves. These results are directly associated with those from BW losses and diet previously mentioned. The lower glucose concentration in commercially marketed calves from d 2 to d 7 is probably related to the diet, as these calves were fed 2 L instead of 2.5 L of MR, and also to their lower concentrate intake during this period, which in turn depends on the mobilization of fat for energy.

Serum concentration of Cr-EDTA was higher for the transported and fasted calves compared to the control (Figure 6; E). Interestingly, serum Cr-EDTA concentration in the commercially marketed calves perdured in time (from d 0 to d 7). This could be associated with a greater degree of intestinal injury or disruption during marketing which might take longer to recover and not just a response to acute stress due to transport or feed restriction. Finally, both transportation and fasting decrease citrulline concentrations, likely due to the lack of nutrients and the negative effect of stress on the GIT (Figure 6; F). These levels recover as calves regain concentrate and MR intake, reaching their highest point one week after the insult.

2.3. Effect of pre-transportation diet

Body weight during the assembly center simulation (d -4 to d -1) was lower for the RS-fed calves (Figure 7; A). This is as expected based on the fact that these calves were being fed electrolytes whose nutrient composition was below their requirements. On the other hand, even though feeding only MR prevented BW losses when compared with RS calves, the BW of these calves was still lower compared with controls and animals being fed pre-transport feeding strategies with higher planes of nutrition. By d 0 after transport/fasting, all calves, without exception with respect to previous feeding, suffered a decrease in BW due to shrinkage. The recovery of BW after transportation/fasting showed similar incremental slopes for all calves over the following 7 d, meaning that BW recovery was similar for all treatments. However, those RS and MR calves, which suffered greater BW losses during the assembly center, did not recover BW to the same extent as the other treatments by d 7. Overall, the implementation of pre-transportation feeding strategies during the stay at assembly centers increases the BW of calves before transportation, better preparing them to cope with shrinkage and stimulating faster recovery on arrival at the rearing facilities.

As expected, serum concentrations of glucose were lower whereas serum NEFA and BHB were greater at the end of the assembly center simulation in calves fed RS because of the poor nutrient composition of electrolytes (Figure 7; B, C, and D, respectively). Low glucose levels before transport are detrimental and feeding strategies based solely on electrolytes should be discouraged in unweaned calves. As previously discussed, variations in markers of energy balance associated with fasting and transportation (↓ glucose and ↑ NEFA and BHB serum concentrations) behaved similarly across treatments, regardless of the pre-transport feeding strategy applied. On the other hand, the peak in serum glucose concentration observed for the RS calves after transport on d 1 was most likely stimulated by the mentioned increase in concentrate intake for these calves for the same period. By d 7, serum BHB was greater in those calves that had previous access to concentrate or acidified milk, especially when comparing them with the RS-fed calves (Figure 5; D).

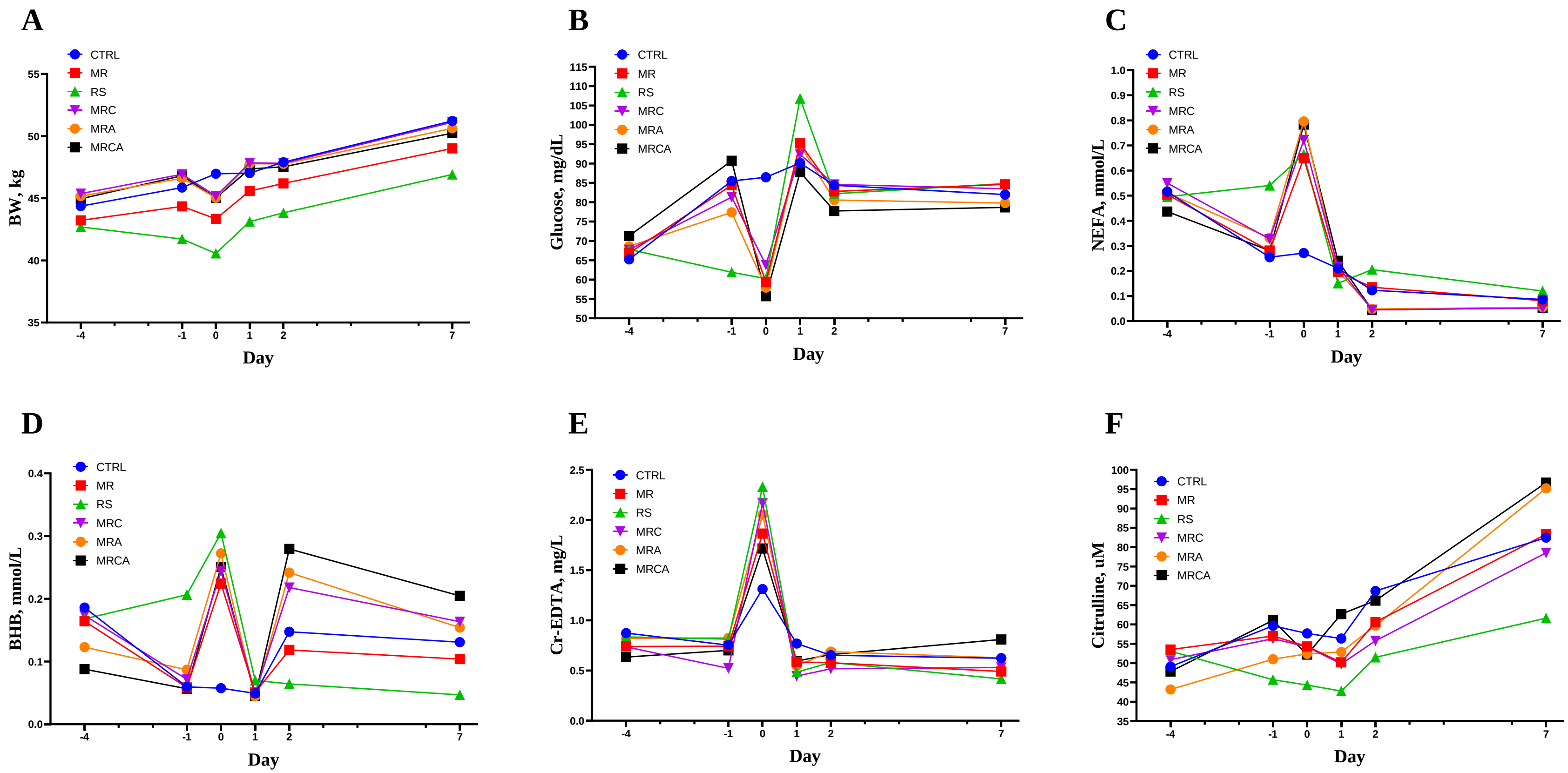

Figure 7. Recovery of BW (A) and markers of energy balance (B, C, and D) and gut functionality (E and F) during the first week after application of treatments (feed restriction/ fasting and transportation) considering the pre-transport diet administered.

*= CTRL (control calves fed MR and concentrate ad libitum); MR (calves fed only MR during the assembly center simulation); RS (calves fed only RS during the assembly center simulation); MRC (calves fed MR and ad libitum concentrate during the assembly center simulation); MRA (calves fed MR and ad libitum access to acidified milk during the assembly center simulation); and MRCA (calves fed MR and ad libitum access to concentrate and acidified milk during the assembly center simulation). See Table 1 for further details on treatments and feeding strategies for each study.

This could be associated with an increase in rumen development in calves being earlier exposed to solid feed (MRC, MRCA) or with an additional energy source provided by MR or MR and acidified milk feeding (MRA and MRCA). This may have prevented greater GIT impairment compared with severely restricted calves (RS), where an undeveloped rumen structure and function may be affected. Finally, serum Cr-EDTA concentration after transportation increased in all transported calves, even in those offered a higher plane of nutrition feeding strategy previous to transport (Figure 7; E). Results from serum citrulline concentration showed lower values for the RS-fed calves overall (Figure 7; E). This could be associated with the negative impact of a poor nutritional strategy that affects gut growth and development in RS calves, resulting in a loss of enterocyte mass functionality.

3. Impact of the present thesis and potential future research steps

The dairy beef industry represents an important productive sector in many countries to date, however, there are several limitations inherent to its activity that deserve further consideration. Some of the main concerns that have been evaluated during this thesis are those related to transportation, fasting, and nutritional strategies to ameliorate the negative consequence of this management on the welfare of unweaned calves. After a comprehensive analysis carried out through the studies presented, a series of final comments and future directions are proposed below.

3.1. Avoid the use of electrolyte feeding as a unique feed source at assembly centers

One of the key messages of the present study is to avoid the use of electrolytes during the assembly center as a unique feed. Results showed that calves under this feeding strategy resulted in lower BW before and after transportation or fasting. Additionally, the recovery of that BW in these calves took longer when compared with calves being fed milk replacer or other feeding strategies. When feeding electrolytes, nutrient restriction is increased and relies on a greater mobilization of reserves to try to fulfill the energy and nutrient requirements of calves. In addition, a detrimental effect of feeding only electrolytes was also observed in the functionality of the enterocyte mass, as observed in the decreased serum citrulline concentrations that not only decreased after transport but remained low until the first week after arrival.

3.2. Origin of animal: a single dairy farm origin vs. animals from commercial assembly centers - Its impact needs to be explored more deeply

One of the first results drawing attention from the discussion section is the difference in overall recovery (intake, gut functionality, and energy balance parameters) in calves coming from the same dairy farm origin and those coming from a commercial assembly center. The recovery of animals coming from a commercial assembly center is worse and slower. For example, considering concentrate intake by d 7 after arrival, crossbred calves were consuming approximately 350 g DM/day of concentrate more than those coming from a commercial assembly center (Annex 3) which were collected from many different dairy farms, marketed, and transported during 8 h. Apart from the low intake, these calves coming from commercial assembly center did not have the same consumption pattern as those coming from a controlled dairy farm, as they did not have the peak in concentrate intake that we attribute to hunger at arrival. This low concentrate intake and the lack of the peak of concentrate intake at arrival could also be associated with a greater degree of stress due to mixing, transportation, and feed restriction. In addition, throughout the experiments carried out in Chapters III, IV, and V we were able to observe how the acute stress of transport negatively impacts gut functionality and energy balance markers. These parameters were normally increased the day after transportation but recovered to control levels 24 hours afterward. However, in the marketed calves, serum NEFA and Cr-EDTA concentrations remained elevated for longer times. The unknown origin of the market calves leaves gaps in important management procedures such as colostrum consumption at birth, amount of milk replacer fed, access to solid feed, disease, and vaccination protocols, among many other aspects that provide important information of the background in these calves. The management of calves during the first weeks after birth is fundamental in the future development of their immune system and digestive tract. These, in addition to the stress of marketing, worsen the survival chances of unweaned calves on arrival at the rearing farms.

The differences among the present study results and the once pointed out in the study of Annex 2 need to be studied in depth. The impact of the strategies studied in the present thesis should be contrasted under commercial conditions, as they probably would have greater benefits on animal recovery than the once observed in this thesis.

CHAPTER VII

Conclusions

This thesis was focused on the study of the effects of colostrum consumption at birth and marketing feeding strategies in the recovery of unweaned dairy beef calves subjected to marketing and transportation. As our main goal was to find strategies to ameliorate the consequences of management previous to arrival, all conclusions will be elaborated in relation the control treatment calves, which are the golden standard as these animals were fed high quantities of colostrum at birth and were not restricted and fastened and were not transported. Any strategy that achieves results close to control animals should be considered a good strategy to be implemented to improve animal welfare and recovery after marketing and transportation.

- *Regarding the performance parameters:*

  1. Feeding rehydrating solutions during the assembly center period caused a body weight loss before transport worsening transport shrinkage and delaying its recovery during the first week after arrival.
  2. Alternative pre-transport feeding strategies studied, providing concentrate and/or acidified milk, did not prevent the loss of body weight due to shrinkage after transport; however, seven days after arrival, body weight was even greater compared with control calves.
  3. A consistent response was observed across studies, showing a pronounced peak in concentrate intake after transport for all treatments, followed by a marked drop in concentrate intake 24 h after.
  4. Feeding 10 L of colostrum at birth numerically increased the recovery in concentrate intake after marketing and transportation.
  5. At arrival concentrate intake fluctuation were ameliorated when previously at the assembly center concentrate and/or acidified milk was supplemented to calves. Moreover, after arrival, those calves previously fed at the assembly center with concentrate and acidified milk showed numerically greater recovery in concentrate intake the first 2 weeks after arrival.

6. However, feeding only milk replacer before transportation did not avoid the concentrate intake drop at arrival.

- *Regarding markers of energy balance:*

7. Calves fed rehydrating solutions during the assembly center period had significantly greater serum non-esterified fatty acids and β-hydroxybutyrate and lower glucose concentrations indicating that animals suffered a negative energy balance.
8. None of the studied feeding strategies in the assembly center were effective reducing this negative energy balance that take place during transportation.
9. However, all these energy balance parameters were quickly recovered after arrival when animals were offered milk replacer, concentrate and straw.

- *Regarding the gut functionality:*

10. The rise in the intestinal permeability to Cr-EDTA is a short-term response to the stress and fasting suffered during transport that typically returns to previous levels shortly after the insult.
11. Moreover, when calves are fed low amount of colostrum birth and rehydrating solutions before transport an additive detrimental effect on the following intestinal functionality parameters was observed:
    a. A decreased serum citrulline concentration, indicating a low enterocyte mass functionality.
    b. An increased fecal concentration of biomarkers of gastrointestinal inflammation.
    c. An increased in vitro gut permeability in the duodenum.
    d. A tendency downregulating the gene expression of the tight junction protein *CLDN2*.

12. Feeding rehydrating solutions, concentrate ad libitum, or acidified milk during the assembly center period did not prevent the increase of the serum concentration of Cr-EDTA after marketing and transportation indicating an impaired gastrointestinal integrity.
13. However, when the pre-transport feeding strategies were the combination of milk replacer and ad libitum access to concentrate only a small impact decreased serum Cr-EDTA concentrations and had serum citrulline concentrations greater compared with the other pre-transport feeding strategies studied.

Annex 1

Simulation of feed restriction and fasting: effects on animal recovery and gastrointestinal permeability in unweaned Angus-Holstein calves

Published in the Journal of Dairy Science

https://doi.org/10.3168/jds.2021-20878

"Simulation of feed restriction and fasting: effects on animal recovery and gastrointestinal permeability in unweaned Angus-Holstein calves"

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ABSTRACT

Feed restriction and fasting experienced during commercial production negatively affects unweaned calves' behavior and health status. Transportation and stays at assembly centers are the main factors generating these disorders. For this study, twenty unweaned Angus-Holstein bull calves (44.1 ± 2.04 kg and 14.7 ± 0.63 d age; mean ± standard error) were used to evaluate the effects of feed restriction and fasting on performance, energy status (serum concentration of glucose, BHB, and non-esterified fatty acids), and on markers of gastrointestinal permeability (serum concentration of citrulline, Cr-EDTA, lactulose, and D-mannitol). Calves were randomly assigned to 1 of 4 treatments that simulated the feed restrictions of an assembly center situation on one hand, and the fasting hours during transportation on the other. Treatments were as follows: Control (CT): from d - 4 to -1 calves were fed 2.5 L of milk replacer (MR) twice daily, concentrate and straw were offered *ad libitum*; Mild: calves (MD) were fed only MR (d - 4 to -1) as described for CT, and on d -1 calves were subjected to a 9-h feed withdrawal; Moderate (MO): calves were fed only MR (d - 4 to -1) as described for CT and on d -1 subjected to a 19-h feed withdrawal; and Severe (SV): calves were fed only 2.5 L of a rehydrating solution twice daily (d - 4 to -1) and on d -1 subjected to a 19-h feed withdrawal. From d 0 to d 42 (weaning) all calves were fed the same feeding program (MR, concentrate, and straw *ad libitum*). Results showed that body weight (BW) was greater for the CT treatment compared with the others from d 0 to d 7, while BW of SV was lesser compared with the others from d -1 to d 7. No differences between treatments were observed at weaning. At d 2 concentrate intake of MD, MO and SV was lesser compared with CT. By d 4, concentrate intake of SV was similar to that for CT, and it was greater than MD and MO. Similarly to BW, no differences in concentrate intake between treatments were observed at weaning on d 42 of the study. At d -1 for SV and d 0 in all restricted calves, serum glucose concentration was lesser when compared with CT. At d -1 and 0 non-esterified fatty acids and BHB serum concentrations were greater in the SV calves compared with the other treatments. By d 2, serum concentrations of non-esterified fatty acids, BHB and glucose were restored to CT levels. At d -1 serum citrulline concentration was lesser in SV and greater in MD calves. The CT calves had lower serum concentrations of Cr-EDTA (d -1 and d 0), lactulose (d 0), and D-mannitol (d 0) compared with the other restricted calves. Results showed that the degree of dietary restriction, the type of liquid diet (milk replacer or rehydrating solution) and fasting hours (9 vs 19 h) affected calves' BW, concentrate intake, and serum concentration of markers indicative of energy status and gastrointestinal permeability.

Key words: calves, feed restriction, fasting, gastrointestinal permeability.

INTRODUCTION

Unweaned male dairy calves are considered a byproduct of the dairy industry and their marketing for meat production has become a common practice. These calves can be marketed and transported from their origin farms directly to their final destinations or be marketed through assembly centers before being transported to calf-rearing facilities (Pardon et al., 2014; Wilson et al., 2020).

These calves encounter many challenges during transportation due to the stress caused by commingling, mixing of animals from different origins, physical trauma, environmental conditions, handling, and deprivation of feed and water (Gonzales et al., 2012; Cooke, 2017). In addition to the transportation phase in the truck, calves spend from several hours up to 3-4 days at assembly centers where they are usually fed only rehydrating solutions, aggravating their feed restriction. Overall, these conditions have been shown to negatively affect calves' health, immunological status, and energetic balance (Cernicchiaro et al., 2012; Renaud et al., 2018; Marcato et al., 2020).

Another important health parameter that can be affected by feed restriction is the gastrointestinal permeability. Under normal physiological conditions, gut permeability can regulate the passage of solutes through and between adjacent intestinal cells (Hall, 1999). However, under stressful situations like transportation or feed restriction, cortisol produced in response to stress has been shown to disrupt intestinal permeability affecting normal digestive functions and decreasing feed intake (Lambert, 2009). If the integrity of the epithelial barrier is affected so is its capacity to adequately regulate gut permeability. Consequently, the free passage of molecules, microorganisms, and other pathogens from the intestinal lumen to the bloodstream increases the chances of developing diseases. From the many triggers in gastrointestinal permeability dysfunction, feed restriction has been noted as a risk factor for passage of endotoxins from the intestinal lumen to the bloodstream (Deitch et al., 1990). Several studies conducted in bovines have demonstrated that short term (Zhang et al., 2013) or progressive (Kvidera et al., 2017) feed restriction can affect tract barrier function and generate changes in intestinal architecture.

Altogether, these situations condition calves' health status and might influence their future performance. Efforts should be made to the look for strategies to alleviate the negative effects of transportation and feed restriction. Marcato et al. (2020) have demonstrated that pre-transport feeding strategies can influence calves' health in the short term after arrival at the rearing farm. However, there is still a lack of knowledge on the impact of previous feeding regimens on gut integrity in young calves. We hypothesized that the degree of feed restriction (period where calves were fed nutrients below their nutritional requirements) and duration of fasting (period of complete absence of feed and water) during transportation and marketing negatively affects young calves´ gut integrity and recovery.

Therefore, the objectives of the present study were to simulate the effects of feed restriction during marketing and fasting during transportation on calves to evaluate their impacts on performance, physiological parameters, behavior, and gastrointestinal permeability.

MATERIALS AND METHODS

Animals, treatments, and feeding

All calves used in this study were managed following the principles and guidelines of the Animal Care Committee of Institut de Recerca i Tecnologia Agroalimentàries (RD 53/2013; project number: 10885). Calves were born at a commercial dairy farm located in Lleida, Spain (Granja San José, S.A.). At birth, they were fed 4 L of colostrum within the first 6 hours after birth and 2 L of colostrum 12 hours after the first colostrum feeding. All feedings were administered via esophageal tube. After colostrum consumption, calves were allocated in individual hutches and fed milk replacer (**MR**) twice daily (1.75 L/feeding from d 1 to d 10 of life, and 2.6 L/feeding from d 11 to d 15 of life at a concentration of 140g/ L). One week before enrolling the calves to the study, a pulmonary ultrasound (Easi Scan linear, BCF Technology Ltd, Scotland, UK) was performed to exclude calves with pulmonary lesions. Finally, twenty crossbred Angus-Holstein bull calves (44.1± 2.04 kg and 14.7 ± 0.63 d) were transported 1 h from their origin dairy farm to a commercial beef farm located in Lleida, Spain (Agropecuaria Montgai S.L). At arrival, calves were weighed, received an intranasal vaccine against Respiratory syncytial virus (RSV) and Parainfluenza virus 3 (PI3) (Bovilis® INtranasal RSP™ Live, MSD Animal Health), and were distributed in individual hutches. Commonly, unweaned calves are collected from their origin farms and transported to an assembly center prior to a short or long transport to the rearing facilities. At the assembly centers, calves can be fed milk replacer or rehydrating solutions for a period of 1 to 3 d. Regulations from the European Commission require a maximum of 9 h of transportation for unweaned calves (short transport) or two trips of 9 h with a 1-hour rest stop in between when longer distances are needed (long transport) (European Council Regulation (EC) No 1/2005). For this experiment, in order to simulate calves staying at an assembly center, different feed restriction levels were applied, and to simulate calf transport, different fasting duration times were combined to the feed restriction levels resulting in the following 4, randomly assigned, treatment groups: Control treatment (**CT**; n=5), calves were fed 2.5 L of MR at a concentration of 140g/L as fed twice daily, water, concentrate (Table 1) and straw were offered *ad libitum*. These animals were not subjected to feed restriction and to any type of fasting. Mild treatment (**MD**; n= 5), calves were fed 2.5 L of MR at a concentration of 140g/L as fed twice daily from d -4 to d -1 and had *ad libitum* access to water, at the end of d -1 these animals were fasted for 9 h. Moderate treatment (**MO**; n= 5), calves were fed 2.5 L of MR at a concentration of 140g/L as fed twice daily from d -4 to d -1 with *ad libitum* access to water, at the end of d -1 these animals were fasted for 19 h. Severe treatment (**SV**; n= 5), calves were fed 2.5 L of a rehydration solution (**RS**) at a concentration of 50g/L as fed twice daily from d -4 to d -1 with *ad libitum* access to water, at the end of d -1 these animals were fasted for 19 h. The feeding schedule for the MR was 8 AM for the morning feeding and 5 PM for the afternoon feeding. Milk refusals were recorded if present. The fasting period in the MO and SV group took place from 4 PM on d -1 to 11 AM on d 0, simulating a calf transport of 19 h (calves missed two MR feedings and spent a total of 26 h on fasting considering the last MR feeding).

While for the MD treatment the fasting period went from 2 AM until 11 AM, simulating a 9 h transport (calves missed one MR feeding and spent a total of 17 h on fasting considering the last MR feeding). During the fasting period, calves were not offered MR, concentrate, or water. After application of treatments, from d 0 until weaning on d 42 all calves were fed equal amounts of MR and had *ad libitum* access to concentrate, straw, and water. This phase was intended to mimic the arrival of calves to a calf-rearing facility. During this period, calves were fed 2.5 L of MR at a concentration of 140 g /L as fed twice daily (following the feeding protocol explained earlier) from d 0 to d 7 of the study. From d 8 until d 24 calves were fed 2 L of MR at a concentration of 125 g /L as fed twice daily. Finally, from d 25 until weaning on d 42 calves were fed 2 L of MR at a concentration of 125g / L as fed once a day (AM). From d -4 to d 24 calves were fed the same MR offered at the origin dairy farm (24.3 % CP, 20.7 % fat; Serval S.A.S., France) to be sure that the potential dietary effect of MR could not be confounded with the treatment effect. Then, from d 25 until d 42, a 22.8 % CP and 18.7 % EE (Karizoo S.A., Spain) MR was used. Milk replacer was prepared on milk taxis (H&L Milk Taxi, Holm&Laue, Germany). At each milk feeding time, milk concentration (g/ L) and temperature (60°C at manufacture and between 40-45 °C at feeding) were recorded by using a clinical refractometer and a thermometer, respectively. The RS used in the SV group contained 84.5 % glucose monohydrate, 11.0 % salt, 4.5 % monopotassium phosphate, 76.0 % glucose, 14.5 % ashes, 1.0 % phosphor, 4.1 % sodium, 10.4 % chloride, 1.3 % potassium, and 2.8 Mcal of ME/kg; dry matter [DM] basis (Corion, DLB laboratories, Spain). From d -4 for CT, and d 0 for MD, MO, and SV until d 11 of the study calves were fed a commercial texturized concentrate and from d 12 until weaning concentrate pellets were used (Table 1). A transition between the two concentrates was done by mixing them at a concentration of 50-50 % for 6 d (from d 12 to d 17) to avoid a drop on concentrate intake. From d -4 for CT, and d 0 for MD, MO, and SV until weaning on d 42, chopped barley straw was offered. Both, concentrate and straw, were offered *ad libitum*. Water was offered *ad libitum* since d -4 for all treatments except during the fasting period for MD, MO and SV calves simulating transportation.

Table 1. Ingredient composition and nutrients of the concentrates used in the study.

Item		Concentrate	
		Texturized (From d -4 to 11)	Pellet (From d 12 to 42)
Ingredient composition, %			
	Soybean meal	28.3	17
	Cornflake	21.2	-
	Wheat bran	20.7	-
	Beet pulp	12.2	-
	Beet molasses	5.8	-
	Whey powder	5.2	-
	Extruded soybean	4	-
	Calcium carbonate	0.54	1.74
	Dicalcium Phosphate	0.21	0.4
	Sodium chloride	0.11	0.4
	Corn	-	33
	Barley	-	30.5
	Wheat middling	-	8
	Premix	1.74	9
Nutrients, % DM			
	CP	19.1	15.63
	EE	5.4	4.72
	NDF	19.5	13.45
	ADF	9.6	7.05
	Ashes	5.3	4.44
	Starch	26	44.26

Measurements and Sample Collection

Body weight was recorded at arrival on d -4, on d -1, 0, 2, 7, 14, 21, 28, 35, and at weaning on d 42 of the study. Concentrate feed offers and refusals were daily recorded for further concentrate feed intake calculations. Daily water intake was also recorded. Straw was provided to stimulate rumen growth; however, measurements were difficult to perform because hutches were exposed to environmental conditions that interfere with its record.

On d -4, -1, 0, 2, 14, 21 and 42, blood samples were collected. Samples were collected before the morning feeding from the jugular vein using serum vacutainers (BD Vacutainer® Plus Plastic Serum Tubes, Belliver Industrial Estate, Plymouth, Devon, UK). Serum was used for measurements of energy balance markers (non-esterified fatty acids **(NEFA)** and BHB), and gut integrity (citrulline). An additional blood sample was collected with BD Vacutainer® Fluoride Tubes (Belliver Industrial Estate, Plymouth, Devon, UK) for plasma glucose concentration analyses. Blood was centrifuged at 1,500 × *g* at 4°C for 15 min and serum and plasma were obtained and stored at -20°C until further analysis.

A permeability test was conducted on d -4, -1, 0, and 2 of the study. For this test, lactulose, D-mannitol, and Cr-EDTA were used as markers of intestinal permeability. The concentration of these markers was based on a previous study (Amado et al., 2019). For this test, lactulose (0.4 g/kg of BW; Duphalac, Madrid, Spain), D-mannitol (0.12 g/kg of BW; Sigma-Aldrich Corp., St. Louis, MO), and Cr-EDTA (0.1 g/kg of BW; Sigma-Aldrich Corp., St. Louis, MO) were dissolved separately in 100 mL of warm water (Amado et al., 2019). Markers were given to the calves 2 hours after the morning feeding on d -1 and 2 and before the morning feeding on d -4 (at arrival to the rearing facility) and 0 (after the fasting period) using 100 mL syringes. Each marker was charged in separated syringes and given directly into the oral cavity of each calf. Blood samples were collected 120 min after markers' administration. To decide the optimum sampling time for markers of intestinal permeability, blood samples were collected at 60, 120, 180, and 240 min after administration of the markers. Major differences in AUC of markers between CT and the restricted treatments were observed at 120 min (data not shown). Measurements of these markers are normally expensive and time consuming, for that reason the optimization of the technique and the number of samples to be processed is required. Samples were obtained from the jugular vein and collected in serum vacutainers (BD Vacutainer® Plus Plastic Serum Tubes, Belliver Industrial Estate, Plymouth, Devon, UK). After collection, blood was centrifuged at 1,500 × *g* at 4°C for 15 min. The obtained serum was aliquoted in 1 mL microcentrifuge tubes and stored at -20°C until further analysis.

From d -4 to 7, accelerometer data loggers (Hobo Pendant G Data Logger, Onset Computer Corporation, Bourne, MA) were placed between the hock and the fetlock in the right hind limb of each calf for measurements of standing time, standing duration, and standing bouts. The accelerometers were covered with a foam pad to protect the calves from abrasions and attached with self-adhesive elastic bandage. Standing behavior was recorded at 1-minute intervals. Data from the data loggers were recovered using HOBOware software version 3.7.23 (Onset Computer Corporation, Bourne, MA).

Chemical Analysis

Feed samples from both concentrates used in the study were collected and analyzed for DM (24h at 103°C), ash (4h at 550°C), CP by the Kjeldahl method (method 981.10; AOAC, 1995), and ADF and NDF (AOAC Official Method 973.18 (1996) 16th. Edition). Milk replacer samples were collected and analyzed for DM (24 h at 103°C), ash (4h at 550°C), CP by the Kjeldahl method (method 981.10; AOAC, 1995), and sugars (HPLC- Refractive Index; (method 984.22; AOAC, 2002).

Serum glucose concentration was determined following the hexokinase method (Beckman Coulter, OSR 6121, Ireland Inc.) The intra-assay coefficient of variation was < 0.70%. Serum NEFA concentration was determined by the enzymatic colorimetric method NEFA-C reagent (Wako Chemicals GmbH, Neuss, Germany). The intra-assay and interassay coefficients of variation were 4.46% and 1.62%, respectively. Serum BHB was determined using a kinetic enzymatic method

(Randox Laboratories Ltd, Crumlin, UK) The intra-assay and interassay coefficients of variation were 2.69% and 0.44%, respectively. Serum citrulline concentration was measured by using a spectrophotometric kit (L-Citrulline Kit, Immundiagnostik AG, Bensheim, Germany) The intra-assay and interassay coefficients of variation were 9.75% and 5.12%, respectively. Analysis of lactulose and D-mannitol in serum extracts was performed in an Acquity UPLC (Waters Corp., Mildford, MA, USA) connected to a Xevo-G2 Qtof mass spectrometer (Waters Corp., Mildford, MA, USA) operating in a negative mode (100 to 1200 m/z). Serum extracts (2 μL) were injected into an ethylene bridged hybrid (**BEH**) amide column (2.1 mm × 100 mm, 1.7 μm, Waters Corp., Mildford, MA, USA) and analytes eluted using mobile phases comprising A = 10 mM ammonium acetate in ACN:H2O (acetonitrile:water) (9:1) and B = 10 mM ammonium acetate in ACN:H2O (4:6). Flow rate was set to 0.5 ml/min and column and auto-sampler chamber temperatures were maintained at 40 and 10°C, respectively. Leucine-enkephalin (200 ng/ml) was used as lock mass for mass accuracy and infused at a flow of 10 μL/min. Serum samples were subjected to protein precipitation with 20 μL of internal standard solution + 130 μL of ACN:H2O (8:2) and analyzed in four independent replicates per sample. Chromatograms were processed using QuanLynx software (v 4.1, Waters Corp., Mildford, MA, USA). Ion areas were used for quantification based on standard curves prepared using authentic standards and raffinose as internal standards (IS). Serum Cr-EDTA was determined by inductively coupled plasma-optical emission spectrometry, using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500ce). The intra-assay and interassay coefficients of variation were <10% and <15%, respectively.

Statistical Analysis

A power analysis was conducted to determine the experimental units needed. The type I error rate (α) was 0.05 and the power ($1 - \beta$) was set at 80%. Concentration of Cr-EDTA, was considered our primary outcome. Based on the differences on Cr-EDTA in Amado et al. (2019) and the severity of the fasting treatments, assuming differences among treatments of 30% the minimum sample size was 5. Calf was the experimental unit. The study design was a randomized complete balanced design with a covariance adjustment. The model included the random effect of pen and the fixed effect of treatment, time, and their interaction and initial BW as a covariate. Data was analyzed using the MIXED procedure of SAS (9.4, Institute, Cary, NC) with repeated measurements for those continuous variables with multiple sampling over time. The compound symmetry covariance structure, the first-order autoregressive covariance structure, and the unstructured covariance structure were tested according to the time points, and the Kenward–Roger degrees of freedom were used based on the lower Bayesian information criterion value. Initial BW and age were analyzed using the MIXED procedure with treatment as fixed effect. Differences were declared significant at $P \leq 0.05$, and trends were discussed at $0.05 \leq P \leq 0.10$ for all models.

RESULTS AND DISCUSSION

Recovery: Body weight, feed consumption and behavior

During the feed restriction period (period where calves were fed nutrients below their nutritional requirements) simulating an assembly center (from d - 4 to d-1), feeding an oral rehydration solution impaired calf´ BW in contrast to MR fed calves ($P < 0.01$; Figure 1). After the fasting period (period of complete absence of feed and water) simulating the transport, from d 0 to d 7 all restricted treatments (MD, MO and SV) recovered their BW although they did not achieve CT calves` BW ($P < 0.01$; Figure 1). Within the restricted groups, MD calves had greater BW recovery compared with MO and SV. Differences in BW at d 7 (Figure 1) among MD, MO and SV treatments could be related to the severity of the feed restriction (MR or RS) and the duration of fasting (9 vs 19 h). A time by treatment interaction was observed on energy intake ($P < 0.01$; Table 2). Calves under the SV treatment received 1.34 ± 0.228 Mcal/d (± SE) during their feed restrictive phase while MD and MO consumed 3.36 ± 0.228 Mcal/d and CT calves consumed 3.61 ± 0.228 Mcal/d. No differences were observed on protein intake (Table 2). In addition, minor BW gains were observed for MO and SV calves after the transport simulation (from d 2 to d 7) due to the longer hours of fasting (19 h)

Table 2. Initial age, body weight, average daily gain, and intake of crossbred Angus-Holstein bull calves fed MR and not subjected to feed restriction or fasting (CT), fed MR and fasted for 9 h (MD), fed MR and fasted for 19 h (MO), and fed a rehydration solution and fasted for 19 h (SV) from d-4 to -1 (feed restriction and fasting period) and from d0 to d42 (weaning).

	Treatment[1]					P-value[2]		
Item	CT	MD	MO	SV	SEM	Trt	Time	Time x Trt
Initial age, d	15.8[a]	14.2[ab]	13.2[b]	15.6[a]	0.63	0.03	-	-
Body weight, kg								
Initial (d -4)	44.0	44.2	44.2	44.2	2.05	0.99	-	-
Post treatment (d 0)	49.2	46.2	42.7	44.9	1.90	0.15	-	-
Final BW (d42)	91.5	89.7	85.9	90.5	2.05	0.31	-	-
Intake (d -4 to d -1),								
Milk replacer, g DM/d	672	588	588	-	-	-	-	-
Rehydrating solution, g DM/d	-	-	-	210	-	-	-	-
Concentrate, g DM/d	86.3	-	-	-	-	-	-	-
Total DMI, g DM/d	759[a]	588[b]	588[b]	210[c]	18.5	<0.01	<0.01	<0.01
Water, mL/d	28	210	89	51	0.1	0.36	<0.01	0.36
Intake (d 0 to d 42),								
Milk replacer, g DM/d	405	405	405	405	-	-	-	-
Concentrate, g DM/d	1044	980	957	1076	72.6	0.63	<0.01	0.74
Total DMI, g DM/d	1429	1364	1344	1463	72.0	0.63	<0.01	0.57
Water, L/d	2.5	2.2	2.2	2.1	0.23	0.69	<0.01	0.62
ADG (from d - 4 to d 42), kg /d	1.03[a]	0.95[ab]	0.86[b]	0.92[ab]	0.043	0.05	<0.01	<0.01
Protein intake (from d -4 to d 42), g/d	19.4	18.0	17.6	19.8	1.47	0.65	<0.01	0.15
Energy intake (from d -4 to d 42), Mcal/d	4.61	4.42	4.37	4.35	0.183	0.78	<0.01	<0.01
Serum lactulose:D-mannitol[3]	2.68	2.16	1.82	2.17	0.14	0.74	<0.01	0.15

[1]Values with different superscript within a row differ with a P value ≤ 0.05.
[2] Trt = effect of feed restriction and fasting, Time x trt = effect of the time by treatment interaction.
[3]The values presented herein correspond to non-transformed means; however, SEM and P-values correspond to the ANOVA analyses using log-transformed data.

which probably caused a shrinkage due to content loss (manure, urination;(González et al., 2015) and dehydration (Von Borell, 2001). These differences among treatments, although minor, remained until d 21 of the study (time by treatment interaction (P = 0.04; Figure 2). Unexpectedly, at weaning on d 42 MO calves had the lowest BW when compared with the other treatments (Figure 2).

Monitoring BW changes in the first weeks of age provides relevant information as the first week after arrival to the feedlot has been described as a critical period where calves' intake normally decreases (Hutcheson and Cole, 1986; Loerch and Fluharty, 1999). Marcato et al. (2018) observed that pre-transport diet and transport duration have a direct effect on these animals´ performance in the first weeks after arrival to the rearing facility. In the present study, even though calves were subjected to different degrees of feed restriction and duration of fasting, the recovery on concentrate intake attenuated differences on BW by d 7 after application of treatments. Similarly, BW recovery after a few days post transportation and feed restriction has been previously reported in calves (Hutcheson and Cole, 1986; Marques et al., 2012). Bernardini et al. (2012) showed that losses in BW due to long-haul transportation (19 h) of male Holstein calves recovered by d 3 after transportation and access to feed and water. Similarly, previous research has shown that pre-transport BW was regained in transported calves between 8-16 h (Knowles et al., 1999) and 24 h (Knowles et al., 1997) after transportation. Altogether, results from these studies reinforce the idea that a quick intake recovery can have an almost immediate impact on calves BW. Likewise, but considering the age difference with the calves from our study, previous research evaluating continuous transportation vs. transportation with 2 h- rest stops in recently weaned Angus × Hereford steers showed that even though ADG was lesser for transported animals compared with the control (not transported ones), those differences were not sufficient to impact BW by d 28 after arrival to the feedlot (Cooke et al., 2013).

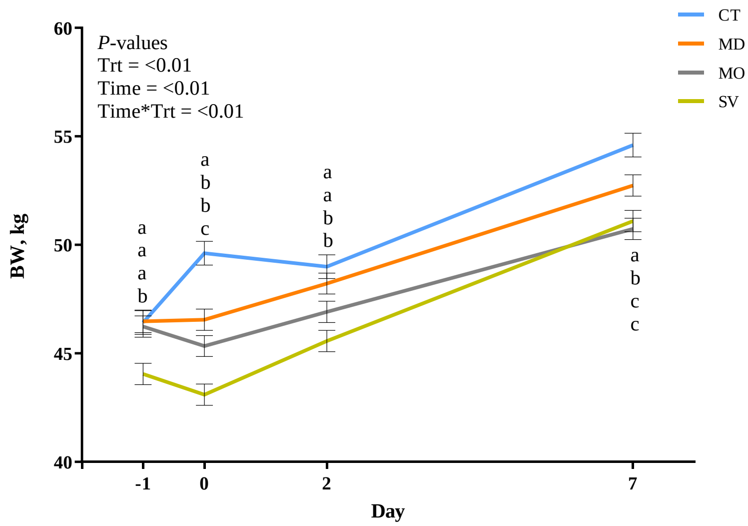

Figure 1. Evolution of BW recovery (Mean ± SE) in crossbred Angus-Holstein bull calves fed MR and not subjected to feed restriction or fasting (CT), fed MR and fasted for 9 h (MD), fed MR and fasted for 19 h (MO), and fed a rehydration solution and fasted for 19 h (SV) from d -1 (end of the feed restriction and fasting period) to d 7. The BW from d -4 was used as a covariate. Different letters within a timepoint denotes differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

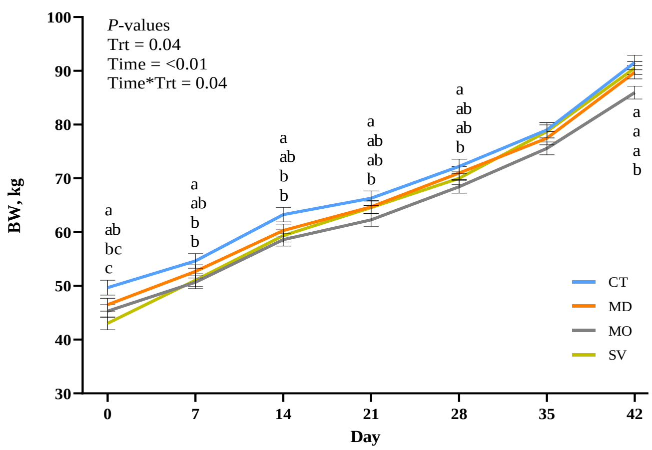

Figure 2. Evolution of body weight recovery (Mean ± SE) in crossbred Angus-Holstein bull calves fed MR and not subjected to feed restriction or fasting (CT), fed MR and fasted for 9 h (MD), fed MR and fasted for 19 h (MO), and fed a rehydration solution and fasted for 19 h (SV) from d-0 to d 42. The BW from d -4 was used as a covariate. Different letters within a timepoint denotes differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

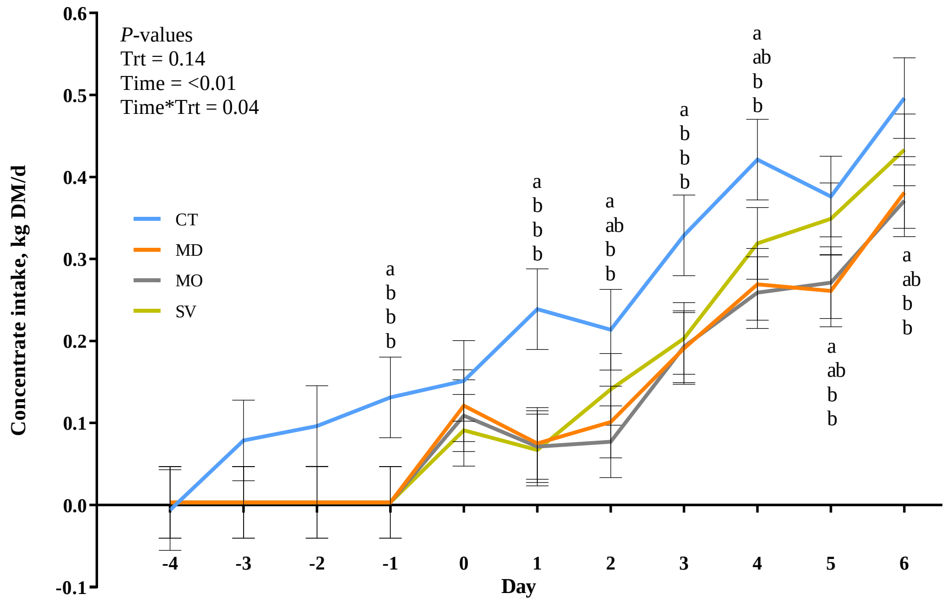

Figure 3. Evolution of concentrate intake (Mean ± SE) in crossbred Angus-Holstein bull calves fed MR and not subjected to feed restriction or fasting (CT), fed MR and fasted for 9 h (MD), fed MR and fasted for 19 h (MO), and fed a rehydration solution and fasted for 19 h (SV) from d-4 (start of the feed restriction period) to d 42 (weaning). Different letters within a timepoint denotes differences among treatments (*P* < 0.05); the order of the letters denotes the treatment with the highest value.

An interaction between time and treatment (*P* = 0.04; Figure 3) in concentrate feed intake was observed. From d -4 to d -1, only CT calves were offered concentrate and their average concentrate intake was 86 ± 0.1 g/d. Restricted calves (MD, MO, and SV) were not offered concentrate until d 0 of the study, however at d 0 they consumed similar amounts of concentrate to CT calves. At d 1 in the restricted calves (MD, MO, and SV) an important drop in concentrate was observed. Gradually, SV calves recovered their intake close to CT values, although this recovery was not observed in MO and MD calves. The sudden access to concentrate after a hunger period with a digestive tract (rumen and small intestine) unprepared for such great increases in consumption could have provoked subclinical ruminal acidosis (Kim et al., 2016), rumen acidosis (Pederzolli et al., 2018) or other digestive disturbances causing the drop in consumption observed on d 1. According to the behavior data on d 1 (Figure 4 A), when calves recovered their intake and showed the drop on

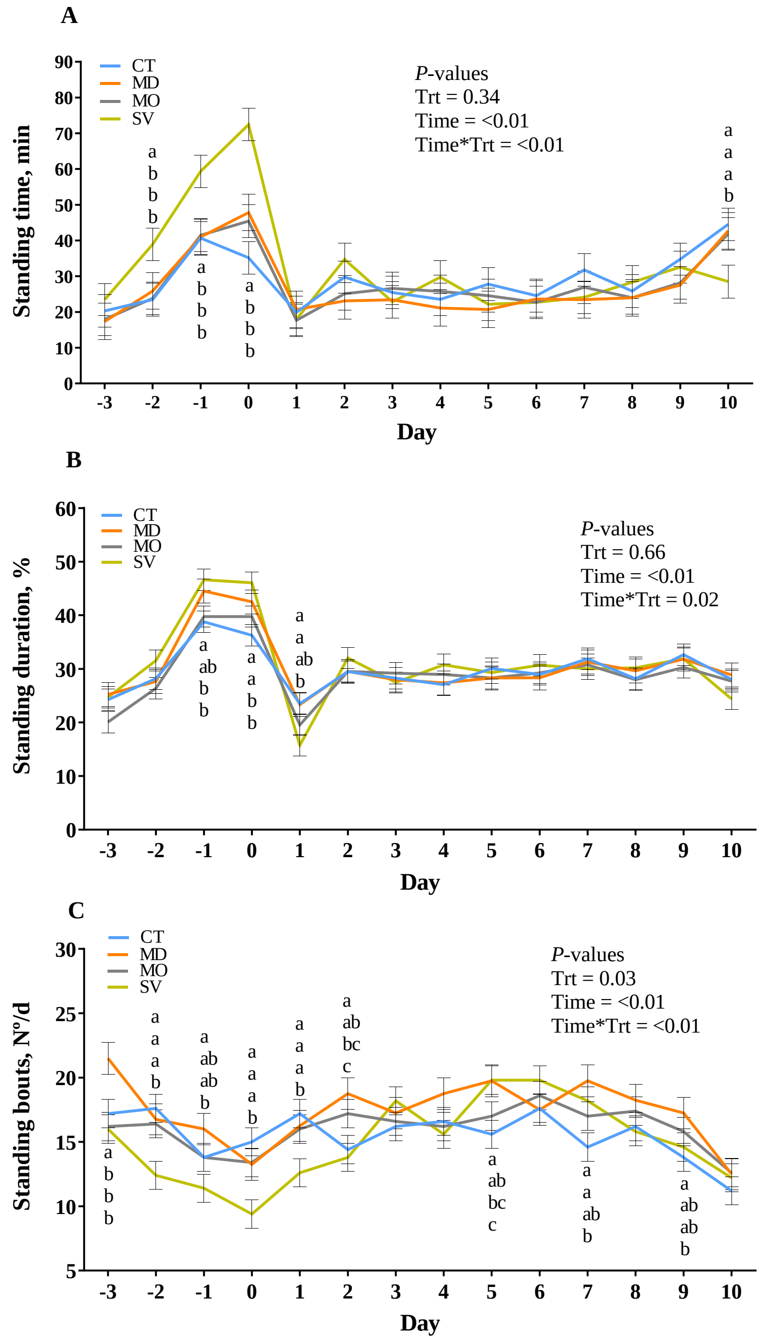

Figure 4. Pendant data loggers' records (Mean ± SE) on standing time (A), standing duration (B), and standing bouts (C) in crossbred Angus-Holstein bull calves fed MR and not subjected to feed restriction or fasting (CT), fed MR and fasted for 9 h (MD), fed MR and fasted for 19 h (MO), and fed a rehydration solution and fasted for 19 h (SV) from d −3 to d 10 of the study. Different letters within a time point denote differences among treatments (P < 0.05); order of the letters denotes the treatment with the highest value. Trt = treatment.

concentrate intake, all treatments decreased their standing duration with no differences between groups after recovery of milk and concentrate intake from d 2 until d 10. Regarding calf behavior, SV calves spent more time standing (time by treatment interaction, *P* <0.01; Figure 4 A), had greater standing duration (time by treatment interaction, *P* = 0.02; Figure 4 B) and lesser standing bouts (time by treatment interaction, *P* <0.01; Figure 4 C) from d -3 until d 0 (end of the restriction and fasting period) when compared with the others during the restriction and fasting period. This behavior denoted the stress derived from hunger. Stress could have potentially generated a higher fat mobilization as expressed by the increment on serum NEFA concentration on d -1 and d 0 (Figure 5 B). Mobilized fat used as a source of energy could explained the major BW losses in SV calves. Back to concentrate intake, by d 6 SV calves showed the greatest concentrate increment reaching values close to those for the CT. Although the fasting hours between MO and SV calves were the same, concentrate intake recovery was lesser for MO calves. This could be explained as a process of compensatory growth after a period of feed restriction (Alves et al., 2019). Compensatory growth has been widely studied in cattle as a physiologically process where animals increase their muscle protein synthesis after a period of restricted development, usually due to a reduction in feed intake (Therkildsen, 2005). Additionally, it has been demonstrated that the level of feed restriction is proportional to the magnitude of the compensation (Coleman and Evans, 1986). The SV calves were exposed to a higher degree of feed restriction because of their diet based on a rehydrating solution and because of that they might have experienced a higher degree of compensatory growth compared with the MO calves. Accordingly, Hutcheson and Cole (1986) demonstrated that calves arriving to the feedlot after a period of marketing and transportation only consume 0.5 to 1.5 % of their BW during the first week, recovering normal intake among the second and fourth week after arrival to the feedlot. In agreement, in the present study calves consumed 0.7, 0.4, 0.4, and 0.5 % of their BW for CT, MD, MO and SV, respectively from d 1 to d 7 of the study. At weaning on d 42, concentrate feed intake did not show any significant differences among treatments (*P* = 0.39).

Finally, water consumption was similar among treatments from d 0 until d 42 ($P = 0.62$; Table 2). Even though results from intake and BW were equal by the time of weaning for all treatments, the potential long-term effects that feed restriction might have on the overall performance and health status of these animals remains unclear and further research should be conducted on this topic.

Energy balance

Serum concentration of glucose, NEFA, and BHB can be used as indicators of energy metabolism in calves. The hypothalamic-pituitary-adrenal axis and the consequent release of glucocorticoids and catecholamine during stressful conditions like marketing and transportation generates changes in plasma glucose concentration (Herman et al., 2016). In the present study, serum concentration of glucose was lower at d -1 right after the feed restriction period (time by treatment interaction, $P < 0.01$; Figure 5 A) for the SV calves when compared with the rest, while MD, MO, and CT showed similar concentrations. These results were expected due to the energy deficit in the SV calves´ diet based on a rehydrating solution with sugar as a source of energy when compared to the MR offered to the other restricted groups. By d 0, CT calves showed the highest ($P < 0.01$) concentration of glucose, whereas the restricted groups showed lower values. From the restricted groups, MD calves which were fasted during 9 h, had higher glucose concentration ($P= 0.01$) when compared to MO and SV calves which were fasted during 19 h. These differences between the restricted groups show that the drop in glucose concentration during fasting is lower in those calves fasted for less amount of hours (9 vs. 19 h). After d 2, when calves recovered their intake, serum concentrations of glucose in feed restricted calves reached similar concentrations to the CT and no differences among treatments were observed until weaning. Similarly to our results, previous studies showed a decrease on plasma glucose concentration during transportation that increased immediately following feeding in young calves (Mormede et al., 1982; Fröhli and Blum, 1988), Friesian-cross steers transported for 16 h with 12 h of lairage (Tadich et al., 2005) and 2-weeks old calves transported during 19 h with a 1 h- rest stop (Knowles et al., 1999). Marcato et al. (2020) studied the effects of pre transport diet (MR vs. RS), transport duration (6 vs. 18 h) and transport condition (opened vs.

conditioned trucks) in physiological parameters of 368 unweaned male calves. Their study showed that calves fed either MR or RS and transported during 18 had similar glucose concentrations, however, when transported during 6 h calves that were fed MR had higher glucose concentration compared with those fed RS (Marcato et al., 2020). In addition, calves fed MR and transported during 6 h had higher glucose and lower NEFA concentrations compared to calves fed MR but transported during 18 h (Marcato et al., 2020). This evidence could suggest that transportation length might influence calves´ health status at arrival to the rearing facilities in a higher degree than pre transport diet itself. Low plasma glucose concentrations have been associated with a higher mortality risk in calves after the first days following transportation (Mormede et al., 1982; Trunkfield and Broom, 1990; Renaud et al., 2018) although none of the calves in the present study died after the feed restriction and fasting suffered during the simulation of an assembly center and transportation.

Serum concentrations of NEFA and BHB showed differences between treatments throughout the study (time by treatment interaction, $P < 0.01$; Figure 5 B, and Figure 5 C, respectively). Both serum NEFA and BHB concentrations were greater in SV calves at d -1 and 0 compared with the other treatments while MD, MO, and CT showed similar concentrations. The greater concentrations of NEFA and BHB for the SV group might indicate that those calves mobilized body fat reserves, as observed by the lower BW described previously, causing a negative energy balance since these animals were offered only a rehydration solution instead of MR during the restriction period. Even though adipose reserves in unweaned calves are limited, calves use lipids as an energetic source to cope with negative energetic balance conditions (Grigor et al., 2001; Bernardini et al., 2012). This switch to a lipid oxidation is represented by the increment on plasma NEFA and BHB concentration during feed withdraw, especially noticeable for the most restricted group (SV). Serum concentration of NEFA and BHB did not differ between d -1 and d 0 (fasting period) for CT, MD and MO treatments. The lack of differences between MD and MO, could indicate that 9 h or 19 h of fasting in MR-fed calves might not cause major effects on the energy balance. However, these differences could be masked due to the larger differences respective to the SV treatment. From d 2 until weaning, serum

NEFA concentrations were similar for all treatments with the exception of the CT calves, which showed an unexpected increase at d 14 but equaled the others by d 42. In accordance, Marti et al. (2017) found no differences on NEFA concentrations 2 d after a 20 h transportation in newly weaned beef calves. In all treatments, serum BHB concentration continued increasing after d 2 reaching the greatest concentration at weaning. Increments on BHB´s concentrations near weaning are related to ruminal function development in calves triggered by an increment on dry feed intake (Quigley et al., 1991).

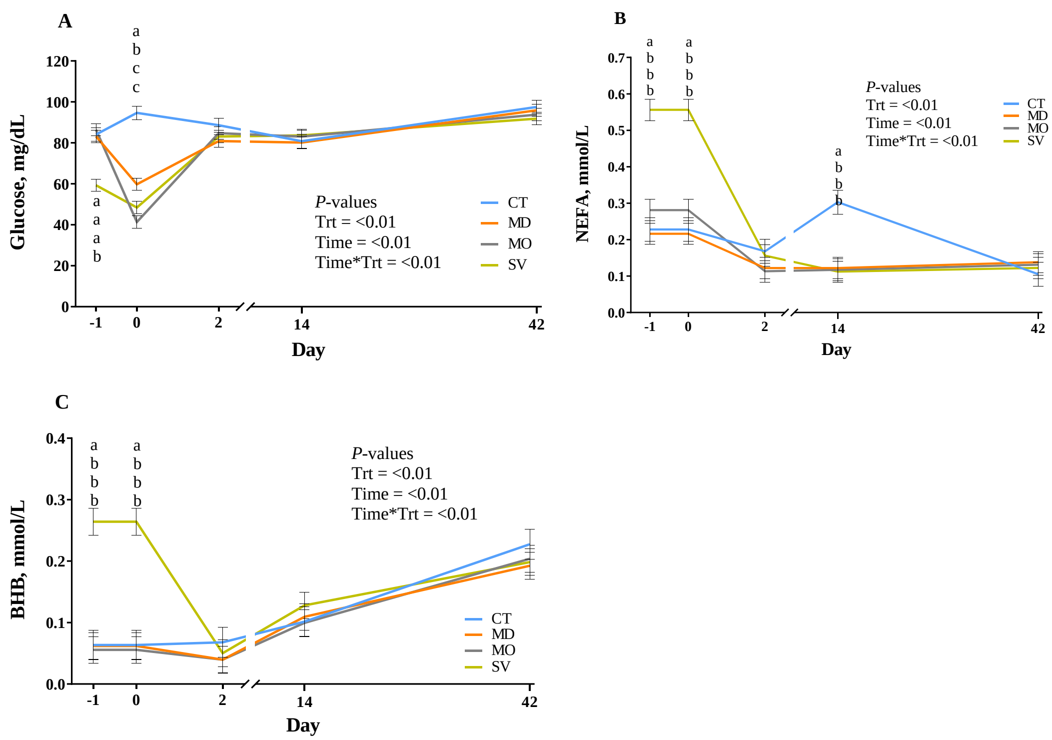

Figure 5. Serum concentrations (Mean ± SE) of glucose (A), non-esterified fatty acids (NEFA, B), and β-hydroxybutyrate (BHB, C) in crossbred Angus-Holstein bull calves fed MR and not subjected to feed restriction or fasting (CT), fed MR and fasted for 9 h (MD), fed MR and fasted for 19 h (MO), and fed a rehydration solution and fasted for 19 h (SV) from d-1 (end of the feed restriction and fasting period) to d 42 (weaning). Values from d -4 was used as a covariate. Different letters within a timepoint denotes differences among treatments (*P* < 0.05); the order of the letters denotes the treatment with the highest value.

Intestinal permeability

Fasting and malnutrition have been related to a reduction in the number of intestinal cells, villus height, and increments in cell apoptosis (Ferraris and Carey, 2000; Moeser et al., 2012). In the present study different markers (serum citrulline, Cr-EDTA, lactulose, and D-mannitol) were used to evaluate the gastrointestinal permeability after a feed restriction and fasting period. Serum citrulline concentration after the feed restriction period (d - 4 to d -1) was lesser in SV calves, followed by MO and MD and greater in CT (time by treatment interaction, $P < 0.01$; Figure 6 A). Surprisingly, at d 0 even MD, MO, and SV treatments went through a fasting period (9 or 19 h), no differences among treatments in serum citrulline concentration were observed. However, and as a consequence of an increment on concentrate intake at d 1, a serum citrulline concentration increment was observed by d 2 for the CT calves. At d 14, an unexpected reduction on citrulline concentration was seen for the CT calves, probably associated with the unexpected increment on serum NEFA concentration in the same day for this group. However, by weaning on d 42 no differences were found among treatments. Citrulline is a nonessential amino acid produced in the enterocytes of the small bowel (Windmueller and Spaeth, 1981) that has recently been studied as a potential biomarker of enterocyte mass and intestinal absorptive function (Gultekin et al., 2019) and as a negative inflammatory marker during acute or chronic intestinal insufficiency (Fragkos and Forbes., 2018). From the present results, it can be assumed that feed restriction might have affected the intestinal epithelium of the restricted calves to a certain extent, reflected on the lower levels of citrulline on those animals when compared with the CT. Levels of citrulline incremented for all groups after the restriction period, probably due to a reestablishment of the normal function of the intestinal epithelium after recovering their intake. In agreement with our results, Gultekin et al. (2019) showed that citrulline levels in plasma were lower in neonatal calves with acute diarrhea when compared with healthy calves.

In relation to Cr-EDTA serum concentration, no time by treatment interaction was observed (P =0.33; Figure 6 B). The CT calves showed the lowest serum concentration of Cr-EDTA followed by MD and SV with intermediate concentrations, and MO calves presenting the highest concentration

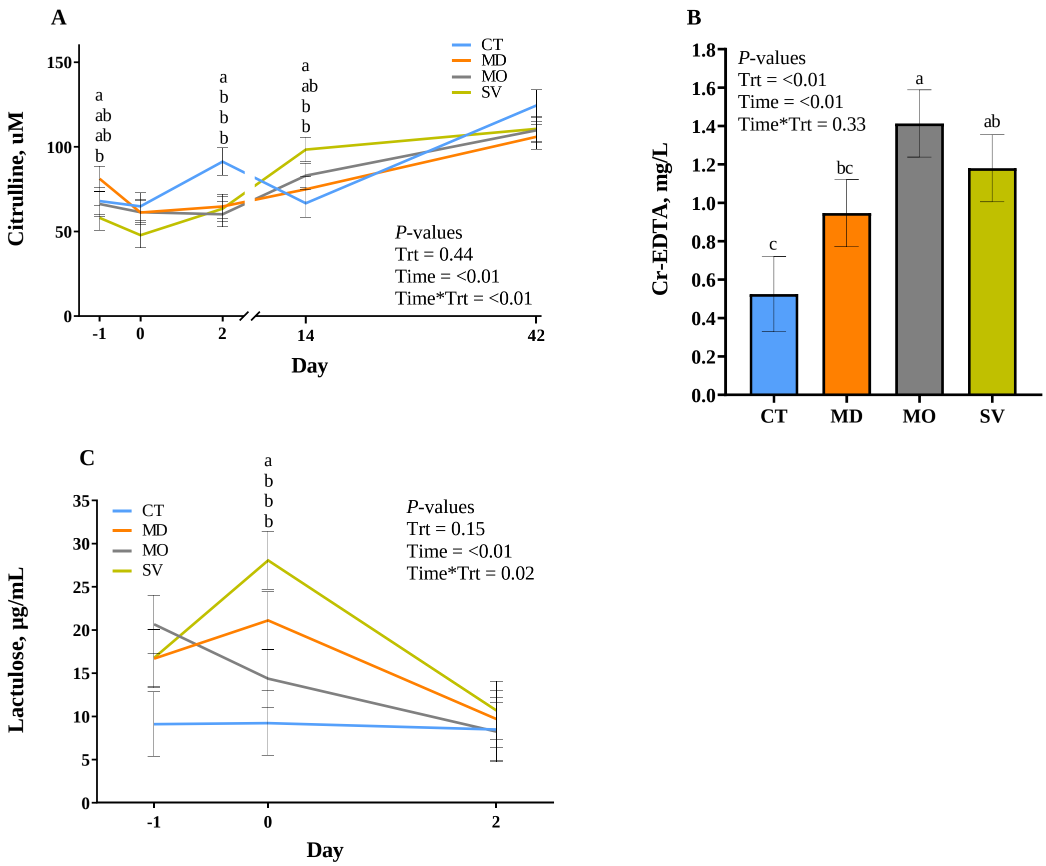

Figure 6. Serum concentrations (Mean ± SE) of citrulline (A), Cr-EDTA (B), and lactulose (C) in crossbred Angus-Holstein bull calves fed MR and not subjected to feed restriction or fasting (CT), fed MR and fasted for 9 h (MD), fed MR and fasted for 19 h (MO), and fed a rehydration solution and fasted for 19 h (SV) from d-1 (end of the feed restriction and fasting period) to d 42 (weaning) for citrulline and d 2 for Cr-EDTA, lactulose and D-mannitol. Values from d -4 was used as a covariate. Different letters within a timepoint denotes differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

($P < 0.01$). Cr-EDTA has been widely used as a gastrointestinal permeability marker in calves (Hunt et al., 2002; Wood et al., 2015; Wilms et al., 2019). It is an indigestible probe that can be measured in plasma, serum, and urine (Bischoff et al., 2014) after its passage through the paracellular space of adjacent enterocytes (Bjarnason, 1995) indicating an increment in the intestinal permeability to large molecules. Cr-EDTA it is not fermented by bacteria in the gut (Aabakken, 1989) and this characteristic makes it a trustable maker of total tract barrier function (Zhang et al., 2013). Previous studies have found increments on intestinal permeability in calves exposed to high-fat MR (Amado et al., 2019) and hypertonic MR (Wilms et al., 2019) by using Cr-EDTA as a total tract permeability marker. Results from the present study showed that CT calves had the lowest Cr-EDTA concentrations, whereas restricted calves showed the greatest concentration of this marker, demonstrating that the level of feed restriction suffered can be positively associated with the recovery of Cr-EDTA in serum. It was expected that SV calves would have greater serum Cr-EDTA concentration than MO calves as they suffered a greater feed restriction. Despite this, our results are in accordance with a previous study using Cr-EDTA as a marker of total tract permeability and conducted on heifers subjected to a severe short-term feed restriction (25 % of voluntary intake for 5 d) which showed a reduction in the intestinal barrier function of the total GIT (Zhang et al., 2013).

Finally, serum concentration of lactulose and D- mannitol were also used as gastrointestinal permeability markers. Lactulose and D-mannitol have been extensively used for assessment of intestinal permeability in various species (Vilela et al., 2008; Araujo et al., 2015; Gilani et al., 2017). Lactulose is a disaccharide that under physiological conditions does not cross the intestinal layer and is normally fermented by bacteria in the colon (Schumann, 2002). When the integrity of the intestinal barrier is compromised, lactulose reaches the bloodstream via paracellular transport (Bischoff et al., 2014). On the other hand, D-mannitol, a monosaccharide, is normally absorbed in the small intestine via transcellular pathway (Arrieta, 2006). Recovery of lactulose and D-mannitol in blood or urine are commonly used for assessments of intestinal integrity. In the present study at d 0, serum lactulose concentration for SV and MD calves was greater compared with CT and MO ($P = 0.02$; Figure 6 C). A tendency ($P = 0.07$) was observed for serum D-mannitol concentration. As observed from Cr-EDTA, serum concentration of D-mannitol tended to be lower for CT (5.2 ± 0.38 µg/mL) compared with MO (11.56 ± 0.34 µg/mL) and SV (10.08 ± 0.34 µg/mL) while no differences were seen between CT and MD (9.33 µg/mL ± 0.34) and between MD and SV treatments. An increment in serum lactulose and D-mannitol concentration was expected for the restricted groups assuming that feed restriction and fasting negatively affects the physiological functionality of the GIT. Our assumptions were partially confirmed when CT calves consistently showed the lowest concentrations of all biomarkers used in this study. However, the lack of differences on serum lactulose concentration between CT and MO calves on d 0 cannot be explained based on our assumptions. One possible explanation could be an effect based on the sample size. Additionally, lactulose and D-mannitol could have suffered some level of fermentation in the reticulorumen due to the oral administration of these markers. At d 2 after the restriction period there were no significant differences among treatments for serum concentration of lactulose. Kvidera et al. (2017) showed that progressive feed restriction in lactating cows increased intestinal permeability due to reductions in the mucosal surface area, ileum villus height, and goblet cell area. In addition, the stress suffered during feed restriction and the consequent increment in cortisol have been proposed as a potential mechanism of increased intestinal permeability (Kvidera et al., 2017; Horst et al., 2020). Results from the present study showed that there is a clear effect of feed restriction and fasting on the gastrointestinal permeability of these calves during a short-time restriction period. Results from Cr-EDTA and D-mannitol are close to those for serum citrulline. A lower serum citrulline concentration (low enterocyte mass) and a high serum concentration of Cr-EDTA (increment of the gastrointestinal permeability to large molecules) in the restricted groups in comparison with the CT supports the hypothesis that feed restriction has a negative impact on the normal physiological conditions of the intestinal barrier. However, further research needs to be done on the effects that feed restriction and fasting exerts on morphological parameters of the gut wall as well as the development of new techniques for assessment of intestinal and total tract permeability in order to make them less laborious and expensive to analyze. In this scenario, citrulline could be good indicator of gut permeability that does not require the oral administration of biomarkers and whose determination can be easily done via serologic assays.

CONCLUSIONS

The greater the degree of feed restriction and fasting before arrival at the rearing farm the greater the negative effects on physiological parameters and markers of intestinal permeability. Additionally, a great degree of restriction together with a long fasting period alters animal's behavior probably due to the stress caused by hunger. Most of the physiological and performance results equaled after the recovery of feed intake in the restricted calves. By understanding how feed restriction affects performance, new feeding strategies could be implemented at assembly centers to enhance calves' health, gut function, and behavior.

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Annex 2

Effects of different management strategies at arrival to improve dairy beef calf recovery after marketing and transportation: preliminary data

"Effects of different management strategies at arrival to improve dairy beef calf recovery after marketing and transportation: preliminary data"

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INTRODUCTION

Spain accounted with 17.400 fattening farms that fattened a total of 2.8 million calves in 2021. Of these, almost 900.000 were dairy beef calves less than 2 months old, originating from farms in provinces other than the rearing facilities (SITRAN, 2021). In addition, the fattening farms dedicated to dairy beef production import calves from other countries to fill their capacity, and it is estimated that more than 500.000 dairy beef calves less than 2 months old are imported into Spain (SITRAN, 2019), mainly from France (69%), Ireland (12%) and the Czech Republic (10%) (SITRAN 2021). In regions like Catalonia and Aragon most of these calves are raised and this dairy beef production system needs to face how to ameliorate the negative consequences of the stressful period caused by feed restriction and fasting among others due to the marketing and transportation at very young ages.

Calves that have been marketed and transported are hungry on arrival and, therefore, a peak in concentrate intake during the first 24 hours after arrival at the rearing facility and after several days of feed restriction and fasting has been observed. This abrupt concentrate intake upsets an undeveloped gastrointestinal tract and paradoxically reduces the concentrate intake thereafter (Pisoni et al., 2022a, 2023). In addition, feed restriction and fasting caused by marketing and transportation increase gastrointestinal permeability (Figure 1), reduce enterocyte mass and induce inflammation in the small intestine (Chapter IV). These conditions, together with the peak in concentrate intake mentioned above, prolong the recovery of concentrate intake by a week or more compared with a calf not subjected to marketing and transportation (Figure 2).

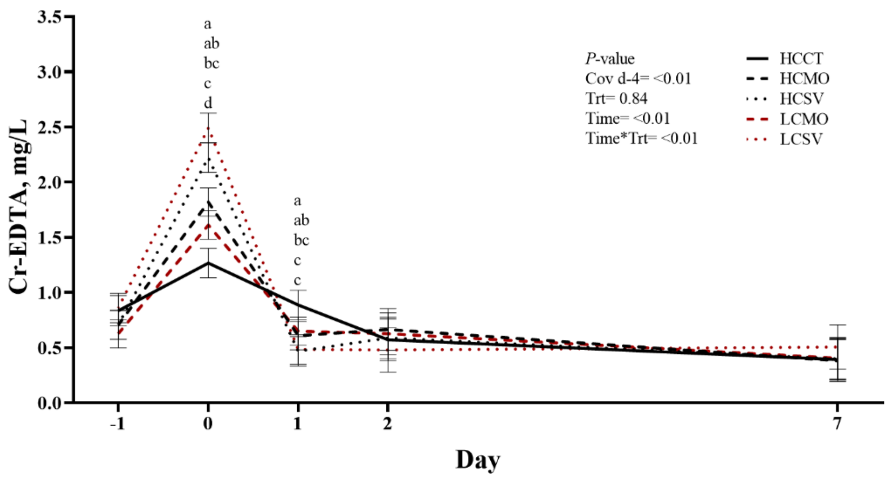

Figure 1. Serum concentration (mean ± SE) of Cr-EDTA in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period, and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), at d −1, 0, 1, 2 and 7. Serum concentrations of citrulline and Cr-EDTA from d −4 were used as a covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

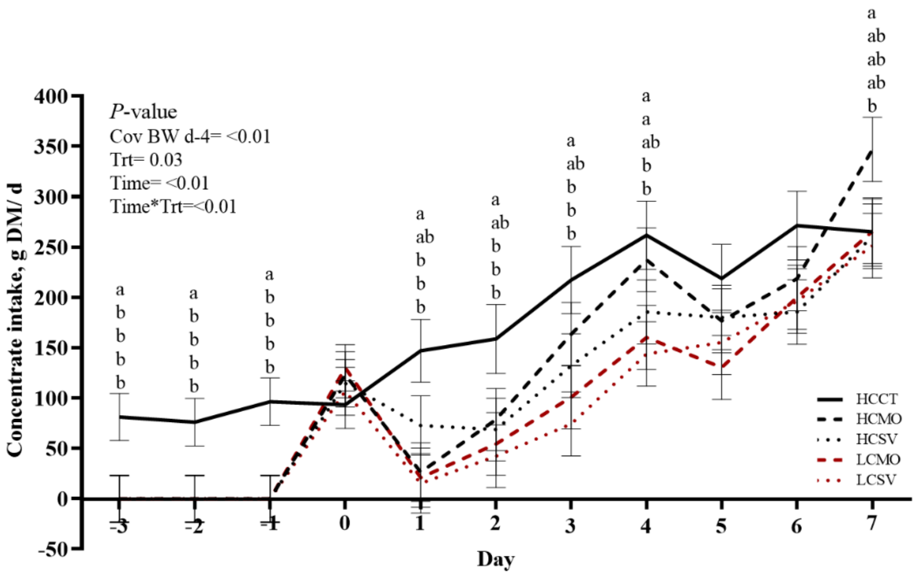

Figure 2. Concentrate intake recovery (mean ± SE) in male Holstein calves fed 10 L of colostrum at birth, MR at the assembly center simulation period and not transported (CTRL), fed 10 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (HCMR), fed 10 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (HCRS), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h (LCMR), and fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h (LCRS), from d −3 (assembly center simulation) to d 7. Body weight from d −4 was used as a covariate. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value.

Previous research conducted by our research group has suggested supplementing the two feedings of milk replacer (**MR**) normally offered to calves at assembly centers with acidified milk (**AM**) and/or starter concentrate, based on the positive results observed in energy status before transport and faster recovery of intake at arrival (Chapter V). However, farmers often lack control over calf nutrition at the assembly centers, meaning they cannot intervene in the calves' feeding management. Additionally, calves may not spend enough time in the assembly center to receive more than one feeding of MR. Therefore, it is necessary to investigate feeding management practices upon calves' arrival at rearing facilities, to prevent the surge of concentrate intake that could aggravate small intestine damage.

Different strategies are based on the hypothesis that the concentrate intake peak at arrival, when gut functionality (permeability) is impaired, has negative effects on concentrate intake recovery during the first week after arrival. The first strategy of this study is based on the potential effects of non-steroidal anti-inflammatory (NSAID) drugs. NSAID drugs used to reduce pain and inflammation inhibit cyclooxygenases (COX-1 and COX-2) and block the production of prostaglandins exerting anti-inflammatory effects (Gunaydin and Bilge, 2018). Because COX-1 plays an important role in maintaining the homeostasis of the intestinal barrier function, selective inhibition of the COX-2 isoenzyme is expected to prevent increases in intestinal permeability in the event of intestinal inflammation (Jackson and Hawkey, 2000). In horses, it has been observed that the use of a modestly COX-2 selective NSAID (meloxicam) or the highly COX-2 selective NSAID (firocoxib) significantly lowered intestinal permeability compared with horses treated with nonselective COX inhibitors (Ziegler et al., 2019). Calves that are marketed and transported may benefit from the administration of selective COX-2 NSAIDs as these drugs can reduce gastrointestinal inflammation without impairing intestinal permeability. The second strategy is the avoidance of concentrate access during the first 24 h after arrival while the gut recovers its functionality. And finally, providing acidified milk during the first hours at arrival could decrease the calf's hunger and consequently the concentrate intake peak could be smaller without decreasing nutrient intake. Providing acidified milk ad libitum could allow the calf to self-regulate nutrient intake and also decrease digestive tract pH improving nutrient digestibility and potentially decreasing gut pathogens. Moreover, calves used in the present study came from a commercial action market~~, more a lot of stressors take place (mixing, feed restriction, fasting).~~ We expected that feed recovery of those calves could be more compromised than in previous studies (Chapters III and V), as in previous studies animals were collected from only one dairy farm with good management procedures. In consequence, with these calves, the impact of the suggested strategies should be more evident.

Thus, this study aimed to evaluate whether delaying access to concentrate intake upon arrival and substituting it with AM between the first two MR feedings, as well as administering a COX-2 selective NSAID, affects the concentrate intake and gastrointestinal permeability of unweaned dairy beef calves exposed to marketing and transportation.

MATERIALS AND METHODS

This study was conducted at the experimental research unit of IRTA - Institute of Agrifood Research and Technology (Barcelona, Spain) during April and July 2023. All procedures described herein were conducted as per the guidelines of the Generalitat de Catalunya (Spain; RD 53/2013; project Nº 12075).

Animals and Experimental Design

For this study, a total of 78 crossbred calves (54.2 ± 6.6 kg of BW and 27 ± 8.8 d of age; mean ± standard error) born at commercial dairy farms located in France were used to evaluate the effects of post-transport feeding strategies and anti-inflammatory treatment on the recovery of concentrate intake and BW, gut functionality, nutritional status, and behavior at arrival to the rearing facility. Calves were collected from their dairy farms of origin and transported to an auction market. The management of the calves during this period was unknown, which is common in commercial farms. Afterward, calves were transported to the experimental research unit of IRTA (Caldes de Montbui, Spain). Transport duration between France and Spain was approximately 8 h, and calves were transported in two batches of 39 animals each with a two-week interval to achieve the total number of experimental units. Upon arrival, on d 0 of the study, calves were assigned to an enclosed barn with a capacity of 78 individual pens (1.20m x 1.97m) bedded with sawdust and received an intranasal vaccine against respiratory syncytial virus and parainfluenza 3 (Bovilis INtranasal RSP Live, MSD Animal Health). After allocation, calves were balanced by BW and randomly assigned treatments based on the post-transport feeding strategy and whether or not they received the anti-inflammatory drug in a 3 X 2 factorial design. Calves receiving the anti-inflammatory drug (**NSAID**) were orally dosed with 0.3 mg/kg of a nonsteroidal anti-inflammatory COX-2 selective (Equioxx®, Boehringer Ingelheim Animal Health USA Inc., Duluth, GA). This anti-inflammatory is labelled for use in horses but concentrations were adjusted to calves based on a previous study (Ziegler et al., 2019). The second factor under study was the post-transport feeding strategy (**FS**). In this case, 3 strategies were implemented as follows: control (**CT**) strategy, where calves were fed 2 L of MR at a concentration of 125 g /L as fed twice daily with ad libitum access to concentrate, straw, and water at arrival to the experimental research unit; milk replacer (**MR**) strategy, where calves were fed 2 L of MR at a concentration of 125 g /L as fed twice daily with ad libitum access to straw and water, but concentrate was offered 10 h after arrival at the research unit; and acidified milk (**AM**) strategy, where calves were fed 2 L of MR at a concentration of 125 g /L as fed twice daily with ad libitum access to straw, water, concentrate, and acidified milk during the two milk feedings (approximately 8 h) at arrival to the experimental research unit. Based on the combination of these factors, there were 6 final treatments with a total of 13 calves each as follows: 1) no anti-inflammatory-control calves (**0-CT**); 2) no anti-inflammatory-milk replacer calves (**0-MR**); 3) no anti-inflammatory-acidified milk calves (**0-AM**); 4) anti-inflammatory-control calves (**A-CT**); 5) anti-inflammatory-milk replacer calves (**A-MR**); and 6) and anti-inflammatory-acidified milk calves (**A-AM**). On d 1, 24 h after the application of treatments, all treatments were offered the same diet consisting of 2 L of MR at a concentration of 125 g /L as fed twice daily with ad libitum access to concentrate, straw, and water, and were followed until weaning on d 49 of the study. The MR composition was 22.56% CP, 17.92% fat, and 50.36% lactose, with the main ingredients being skimmed milk powder, whey powder, vegetable oil, starch, and dextrose (Schils, The Netherlands). Acidified milk was prepared using the same MR acidified with 60% formic acid and 40% lignosulfonic acid (PROTACID, Higienizo, Madrid, Spain) at a concentration of 1.5 mL PROTACID/ L of MR to achieve a final pH of 5.2. Concentrate composition was 14.9% CP, 13.7% NDF, 6.4% ADF, 43.9% starch, and 5.3% ether extract on a DM basis, with the main ingredients being 13.9% soybean meal, 38.9% corn, 9% wheat, 17% barley, 3% sunflower expeller, 1.9% palm oil, and 1.3% calcium carbonate). The experimental design of this study is illustrated in Figure 3.

Sample collection and analysis

Individual BW was recorded on d 0, 1, 2, 7, and weekly thereafter until d 49 of the study. Concentrate intake and MR refusals were recorded daily for further dry matter intake (**DMI**) calculations. Samples from concentrate and MR were sent to a referral laboratory for analysis of nutrient composition (DM, ash, CP, ADF, ether extract, and NDF for concentrate, and DM, ash, CP, and sugars for MR). Chemical analyses were performed as previously described (Pisoni et al., 2022). Blood samples were collected before the morning feeding of d 0, 1, 2, and 7 of the study for serum determination of energy balance markers [nonesterified fatty acids (NEFA), β-hydroxybutyrate (BHB), and glucose)] and enterocyte mass functionality (citrulline). On d 0, markers of colostrum consumption [(IgG and gamma-glutamyl transferase (GGT)] were also measured in blood. All blood samples were obtained via jugular venipuncture using 10 mL serum (BD Vacutainer® Plus Plastic Serum Tubes, San Agustín del Guadalix, Spain) and 4 mL plasma (BD Vacutainer® Fluoride Tubes, San Agustín del Guadalix, Spain) vacuum tubes. During the sampling period on the farm, blood samples were stored in a cooler for preservation until arrival at the laboratory. Upon arrival at the laboratory, blood was centrifuged at 1,500 × *g* at 4°C for 15 min and the obtained plasma and serum were aliquoted in individual polypropylene tubes and stored at −20°C until analysis. An extra 4 mL vacuum tube sprayed with anticoagulant (BD Vacutainer spray coated K2EDTA Tubes, San Agustín del Guadalix, Spain) was collected for hematology determinations of whole blood. These vacuum tubes were not centrifuged and were immediately analyzed using an automated veterinary hematology analyzer (Element HT5, Mindray, Heska, Shenzhen, China). On d 0, 1, 2, and 7 in vivo total tract permeability tests were conducted using Cr-EDTA as a marker. Chromium-EDTA was administered to the calves before the morning feeding at a dose of 0.1 g/kg of BW (Sigma-Aldrich Corp, Saint Louis, MO) based on a previous study (Amado et al., 2019) using 100 mL syringes. Two hours after administration of the marker a blood sample was collected from the jugular vein for serum concentration determination. Chemical analysis of all serum and plasma markers evaluated have been previously described in greater detail in Pisoni et al. (2023). Shortly, serum NEFA, BHB, glucose, and GGT were analyzed using a Beckman Coulter AU480 analyzer (AU480, Beckman Coulter International S.A., Nyon, Switzerland). Serum IgG was measured by an ELISA kit (species-specific Bovine IgG and Bovine IgG1, Bethyl Laboratories Inc., Montgomery, TX), whereas serum citrulline was measured using a spectrophotometric kit (l-Citrulline Kit, Immundiagnostik AG, Bensheim, Germany). Finally, serum Cr-EDTA was determined using an inductively coupled plasma MS (Agilent 7500ce, Agilent, Santa Clara, CA). The intra- and inter-assay coefficients of variation for NEFA, BHB, glucose, GGT, IgG, citrulline, and Cr-EDTA were 1.62% and 4.46%; 0.44% and 2.69%; 0.58% and 1.11%; 9.75% and 5.12%; 0.8 and 1%; 3.2 and 3.3%; and <10% and <15%, respectively.

RESULTS

No differences were observed among treatments on initial BW, final BW, or ADG at the end of the study (d 49) (Table 1). Additionally, no interactions of FS, NSAID, or FS×NSAID with time were observed on concentrate intake (Figure 3), total DM intake, MR intake, and straw intake (data not show).

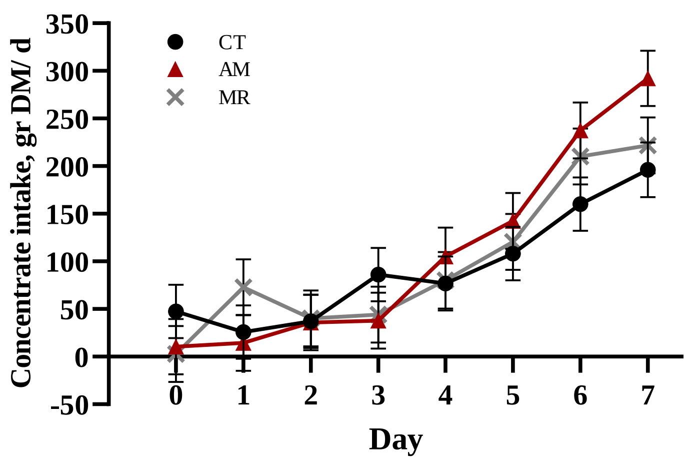

Figure 3. Concentrate intake (*P* >0.10) from d 0 to d 7 after arrival to the rearing facility of dairy beef calves fed 2 MR feedings and had ad libitum access to concentrate and straw (CT), fed 2 MR feedings, ad libitum access to concentrate and straw, and ad libitum access to AM between the two milk feedings (AM), and fed 2 MR feedings, had ad libitum access to straw, and ad libitum access to concentrate after the second milk feeding.

However, a FS effect and NSAID effect were observed in MR intake during the first week of the study. As observed in Figure 4, calves administered with COX-2 selective NSAID consumed more ($P = 0.01$) MR than O calves during the first week of the study. Moreover, a tendency ($P = 0.08$) was observed among treatments on the FS treatment, calves fed AM during the first two milk feedings consumed less MR during the first week compared to CT and MR calves (figure 5).

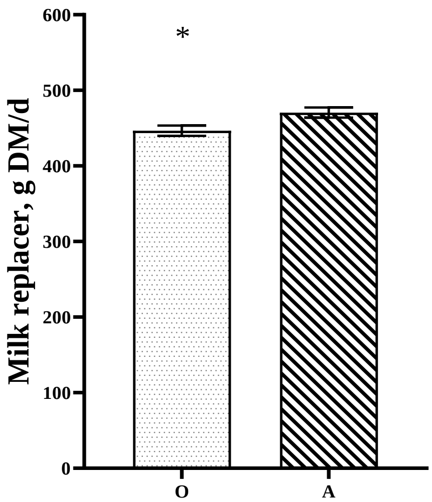

Figure 4. Milk replacer intake from d 0 to d 7 after arrival to the rearing facility of dairy beef calves orally administered with COX-2 selective NSAID (**A**) or not (**O**).

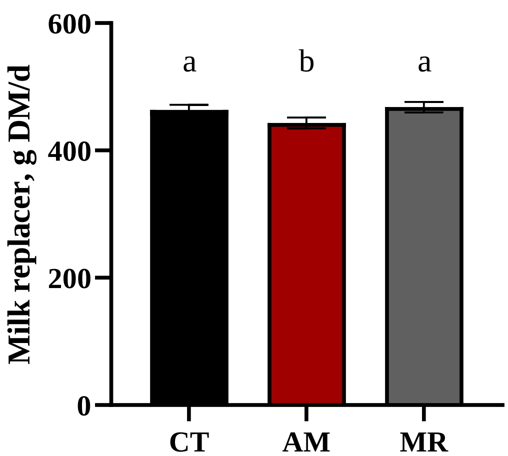

Figure 5. Milk replacer intake from d 0 to d 7 after arrival to the rearing facility of dairy beef calves fed 2 MR feedings and ad libitum access to concentrate and straw (CT), fed 2 MR feedings, ad libitum access to concentrate and straw, and ad libitum AM between the two milk feedings (AM), and fed 2 MR feedings, ad libitum access to straw, and ad libitum access to concentrate after the second milk feeding.

Table 1. The effect of post-transport feeding strategies and use of COX-2 selective NSAID on body weight (**BW**), average daily gain (**ADG**), total dry matter intake (**DMI**), and total intake of concentrate, milk replacer (**MR**), acidified milk (**AM**), and straw in male crossbred dairy beef calves.

	Treatments[1]							*P*-value[2]		
	O			A			SEM	FS	NSAID	FS×NSAID
Item	CT	AM	MR	CT	AM	MR				
BW, kg										
Initial, d 0	54.1	54.2	54.2	54.1	54.3	54.3	1.93	0.99	0.97	0.99
Final, d 49	95.3	94.2	96.4	93.2	97.1	97.8	2.52	0.53	0.72	0.60
ADG, kg/d	0.83	0.81	0.86	0.80	0.87	0.89	0.051	0.52	0.70	0.60
Total intake (d0 to d7), gr DMI/d										
Concentrate	100.0	130.0	105.0	84.5	88.9	92.2	22.85	0.74	0.21	0.79
Milk replacer	452	421	465	474	465	472	11.7	0.08	0.01	0.31
Acidified milk	-	155.5	-	-	144.6	-	-	-	-	-
Straw	21.0	29.6	28.7	25.0	28.8	26.5	5.63	0.50	0.94	0.83
Total DMI (d0 to d7), g DMI/d	571	588	598	583	600	590	28.9	0.78	0.81	0.92

[1]Treatments= calves fed 2 MR feedings and ad libitum access to concentrate and straw (CT); calves fed 2 MR feedings, ad libitum access to concentrate and straw, and ad libitum access to AM between the two milk feedings (AM); calves fed 2 MR feedings, had ad libitum access to straw, and ad libitum access to concentrate after the second milk feeding; calves orally administered with COX-2 selective NSAID (A); and not administered antiinflammatory drugs (O).

[2]FS = effect of feeding strategy; NSAID = effect of COX-2 selective NSAID; FS x NSAID = effect of the feeding strategy by NSAID interaction.

Table 2. The effect of post-transport feeding strategies and use of COX-2 selective NSAID on serum concentration of non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), glucose, Cr-EDTA, and citrulline in male crossbred dairy beef calves.

	Treatments[1]							*P*-value[2]					
	O			A			SEM	FS	NSAID	FS×NSAID	FS×T	NSAID×T	FS×NSAID×T
Item	CT	AM	MR	CT	AM	MR							
NEFA, mmol/L	0.44	0.45	0.44	0.44	0.44	0.46	0.026	0.91	0.89	0.77	0.52	0.37	0.98
BHB, mmol/L	0.24	0.25	0.25	0.22	0.22	0.23	0.020	0.19	0.89	0.86	0.43	0.87	0.95
Glucose, mg/dL	78.5	79.6	78.7	74.8	75.9	79.1	1.96	0.15	0.51	0.49	0.73	0.39	0.84
Cr-EDTA, mg/L	1.39	1.22	1.10	1.35	1.26	1.27	0.085	0.08	0.45	0.43	0.07	0.07	0.57
Citrulline, uM	65.1	63.2	62.0	69.1	70.3	61.1	4.13	0.32	0.31	0.61	0.99	0.33	0.84

[1]Treatments= calves fed 2 MR feedings and ad libitum access to concentrate and straw (CT); calves fed 2 MR feedings, ad libitum access to concentrate and straw, and ad libitum access to AM between the two milk feedings (AM); calves fed 2 MR feedings, had ad libitum access to straw, and ad libitum access to concentrate after the second milk feeding; calves orally administered with COX-2 selective NSAID (A); and not administered antiinflammatory drugs (O).

[2]FS = effect of feeding strategy; NSAID = effect of COX-2 selective NSAID; FS x NSAID = effect of the feeding strategy by NSAID interaction; FS x T = effect of the feeding strategy by time interaction; NSAID x T = effect of NSAID by time interaction; and FS x NSAID x T = effect of the feeding strategy by NSAID by time interaction.

A tendency between the administration of a COX-2 selective NSAID and time ($P = 0.07$) was observed for serum Cr-EDTA concentration (Figure 6). On day 2 after arrival, "A" calves had a greater concentration of Cr-EDTA compared with O calves.

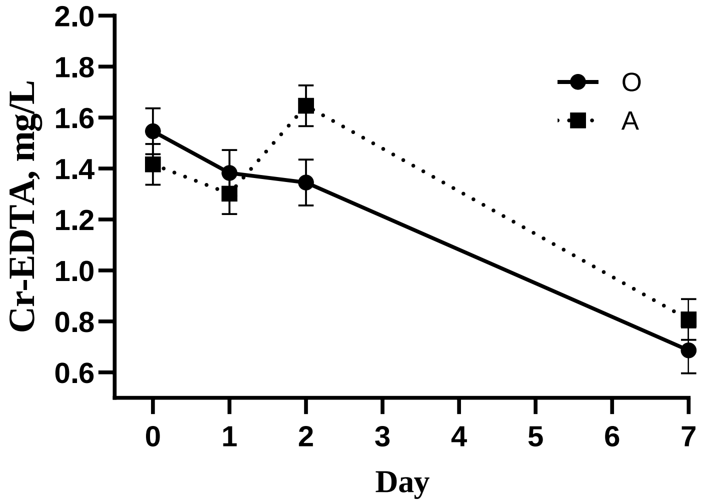

Figure 6. Serum concentration of Cr-EDTA on the day of arrival (d 0), d 1, d 2, and d 7 after arrival to the rearing facility of dairy beef calves orally administered with COX-2 selective NSAID (A) or not (O).

Another tendency ($P = 0.07$) was observed among the FS on serum Cr-EDTA concentration (Figure 7). On d 1, AM calves had lesser serum Cr-EDTA compared with CT and MR, however on d 2, MR calves had lesser serum Cr-EDTA compared with CT and AM, and on d 7 compared with CT calves.

Finally, no differences among treatments were observed on serum citrulline, NEFA, BHB, and glucose concentration on arrival day (d 0), or d 1, 2, and 7 after arrival to the rearing facility (Table 2).

Results regarding total concentrate intake until d 49 of the study, fecal lactoferrin concentration, and standing and lying behavior are still under analysis.

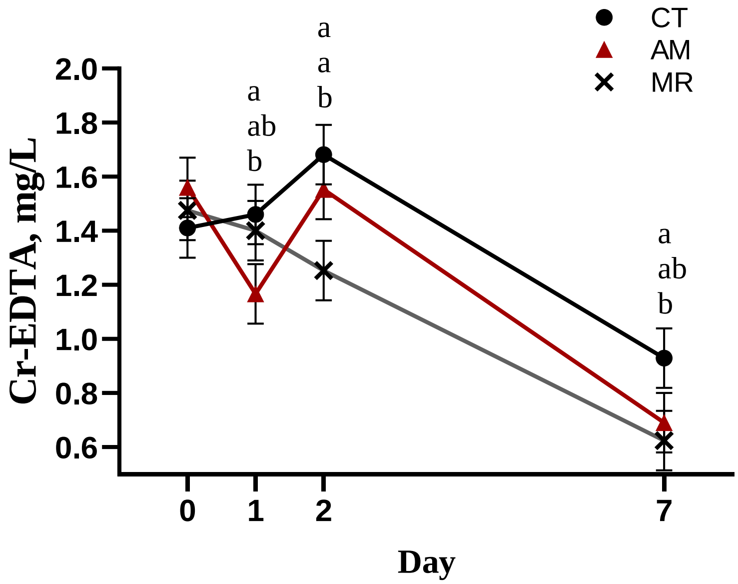

Figure 7. Serum concentration of Cr-EDTA on the day of arrival (d 0), d 1, d 2 and d 7 after arrival to the rearing facility of dairy beef calves fed 2 MR feedings and ad libitum access to concentrate and straw (CT), fed 2 MR feedings, ad libitum access to concentrate and straw, and ad libitum AM between the two milk feedings (AM), and fed 2 MR feedings, ad libitum access to straw, and ad libitum access to concentrate after the second milk feeding.

PRELIMINARY CONCLUSIONS

The increase in MR intake by calves administered with COX-2 selective NSAID during the first week could be linked to the NSAID's anti-inflammatory properties in relieving fatigue and pain that may have been present after marketing and transportation. This, in turn, could have made the calves feel healthier and more motivated to consume more MR. The increase in MR for the calves administered with COX-2 selective NSAID may also explain the numerically lower concentrate intake seen for these calves when compared to the ones not receiving the antiinflammatory treatment. However, the observed increase in intestinal permeability, as indicated by the rise in Cr-EDTA levels in COX-2 selective NSAID calves, did not match our expectations. We predicted that this NSAID would protect the gut against the harmful effects of prostaglandins, however, it remains to be investigated whether the calves may have consumed more milk due to their well-being, despite having a permeable intestine and its potential effect on final BW.

Regarding the feeding strategies studied, calves offered acidified milk ad libitum at arrival consumed 150 g of acidified milk replacer and decreased the consumption of milk replacer offered twice daily by approximately 25 g; so total milk replacer was greater. In addition, gut permeability initially decreased increasing thereafter at the second day. Moreover, when acidified milk was offered arrival together with COX-2 selective NSAID a numerical overall growth was observed. Data that need to be analyzed can help us to interpret these preliminary findings.

Regarding the strategy of delaying concentrate consumption after the second milk feeding, we observed a decrease in gut permeability after arrival, and numerically a good growth overall of the animals, mainly when they were administered a COX-2 selective NSAID. As the initial total dry matter intake was not drastically modified, we expect an overall better efficiency.

As mentioned previously, these are preliminary results that need to be complemented with the remaining data that are being currently analyzed.

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