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Feeding strategies to improve performance and health of Holstein calves

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El creixement i el desenvolupament dels vedells durant l'alletament és crucial per la seva futura productivitat. En aquesta tesi es van realitzar quatre estudis per avaluar diferents estratègies de millora dels paràmetres productius i de salut dels vedells alletats Holstein. Al primer estudi es va avaluar la integritat intestinal dels vedells recent nascuts i es va associar aquesta amb la incidència de diarrees durant els primers dies de vida. Es va veure que els vedells que desenvolupaven diarrees durant els primers 7-14 dies de vida havien nascut amb una permeabilitat intestinal elevada, la qual cosa suggereix que els vedells que més endavant desenvolupen diarrees possiblement hagin nascut més predisposats a patir-les. Al segon estudi es van avaluar els efectes de l'addició del probiòtic *L. rhamnosus* en dues dosis diferents (10^7 or 10^9 CFU/50kg de pes viu) durant 14 dies en el període de deslletament sobre la microbiota fecal, la immunitat i el creixement. Va resultar que els vedells complementats amb el probiòtic no van tenir millor immunitat ni creixement possiblement perquè la microbiota fecal no es va veure afectada per la ingestió de *L. rhamnosus.* Al tercer estudi es van avaluar els efectes de l'addició de butirat en forma de butirat sòdic o tributirina al lactoreemplaçant, amb dosis de 3 g/kg de matèria seca, durant 42 dies en el creixement, el metabolisme de la glucosa i la incidència de diarrees. Els vedells complementats amb butirat van tendir a tenir una major incidència de diarrees i els vedells complementats amb tributirina van tenir un creixement menor que els vedells control. A més, l'addició de butirat al lactoreemplaçant no va influenciar el metabolisme de la glucosa. Al quart estudi es van avaluar els efectes en el creixement d'incrementar el nivell d'alimentació dels vedells durant el període d'alletament i fins a dues setmanes després. Els vedells prenien 4 o 6 L/d de lactoreemplaçant en combinació amb un pinso baix (4.1%) o alt (11.2%) en greix. Es va veure que els vedells que prenien més lactoreemplaçant creixien més durant el període d'alletament i quan aquests menjaven un pinso alt en greix seguien creixent més també després del deslletament, a diferència dels que menjaven un pinso baix en greix que van disminuir el seu creixement un cop es va deixar d'oferir lactoreemplaçant.

En conclusió, els vedells que pateixen diarrees durant el seu desenvolupament van néixer amb una permeabilitat intestinal elevada. A més, suplementar els vedells alletats amb *L. rhamnosus* i amb butirat al lactoreemplaçant després del deslletament (amb dosis de 10^7 o 10^9 CFU/50kg de pes viu durant 14 dies) i abans del deslletament (amb dosis de 3 g/kg de matèria seca durant 42 dies), respectivament, no va resultar en avantatges sobre la salut i el creixement. En canvi, incrementar el nivell de greix del

pinso (fins al 11.2%) quan els vedells prenien 6 L/d de lactoreemplaçant va resultar en un major pes viu abans i després del deslletament.

Growth and development of young calves is crucial for the later productive parameters. In this thesis, four studies were conducted to evaluate different strategies to improve performance and health of young Holstein calves. In the first study, intestinal integrity of newborn calves was evaluated and associated with the development of diarrhea during the first days of life. It was found that calves that incurred diarrhea during the first 7-14 days of life had been born with an increased intestinal permeability suggesting that diarrheic calves may have been more predisposed to suffer scours. In the second study, the effects of supplementing calves with the probiotic *L. rhamnosus* around weaning in two different doses (10^7 or 10^9 CFU/50kg of body weight) for 14 days on the modulation of the fecal microbiota, performance and immunity around weaning were evaluated. Calves that had been supplemented with the probiotic showed no improved performance or immunity possibly due to the lack of modulation of the fecal microbiota by the ingestion of *L. rhamnosus*. On the third study, the effects of supplementing preweaned calves with butyrate in the form of sodium butyrate or tributyrin in the milk replacer, at doses of 3 g/kg of dry matter, for 42 days on performance, glucose metabolism and incidence of diarrhea were evaluated. Calves that had been supplemented with butyrate tended to have increased incidence of diarrhea and calves supplemented with tributyrin showed retarded growth compared to control calves. Also, butyrate addition in the milk replacer resulted in no improved glucose metabolism. The fourth study evaluated the effects on performance parameters of an enhanced feeding program before and after weaning. Calves were fed either 4 or 6 L/d of milk replacer in combination with a low fat (4.1%) or a high fat (11.2%) starter feed. Results showed that calves fed increased daily amount of milk replacer had improved growth before weaning and calves that were also fed a high-fat starter feed had the greatest body weight after weaning in contrast with calves fed 6 L/d of MR and a low-fat starter feed that showed decreased growth once milk replacer was no longer provided.

In conclusion, calves that experienced diarrhea early in life were born with increased intestinal permeability. Moreover, supplementing calves with *L. rhamnosus* or with butyrate in the milk replacer at weaning (at doses of 10^7 or 10^9 CFU/50kg of body weight for 14 days) and during the preweaning period (at doses of 3 g/kg of dry matter for 42 days), respectively, had no beneficial effects on performance and health.

However, increasing the amount of fat in the starter feed to 11.2% when calves were fed 6 L/d of milk replacer increased overall body weight before and after weaning.

ABBREVIATIONS USED

ACTB: β-actin
ADF: acid detergent fiber
ADG: average daily gain
ANOVA: analysis of variance
AOAC: Association of Official Analytical Chemists
BHBA: β-hydroxybutyrate
BW: body weight
cDNA: complementary deoxyribonucleic acid
CFU: colony forming units
CV: coefficient of variation
CP: crude protein
DM: dry matter
DMI: dry matter intake
DNA: deoxyribonucleic acid
EE: ether extract
EDTA: Ethylenediaminetetraacetic acid
FDR: false discovery rate
GCR: glucose clearance rate
GIT: gastrointestinal tract
GLP-1: glucose-like peptide 1
GTT: glucose tolerance test
ICR: insulin clearance rate
Ig: immunoglobulin
IL-1β: Interleukin-1 beta
IL-6: Interleukin-6
IL-8: Interleukin-8
IL-10: Interleukin-10
INF-γ : Interferon gamma
IRTA: Institut de Recerca i Tecnologia Agroalimentària
ME: metabolizable energy
MR: milk replacer
mRNA: messenger ribonucleic acid
NDF: neutral detergent fiber
NFC: non-fiber carbohydrates content
NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NRC: National Research Council
OTU: operational taxonomic unit
PCR: polymerase chain reaction
qPCR: quantitative polymerase chain reaction
RNA: ribonucleic acid
SE: standard error
SEM: standard error of the mean
TGF-β: transforming growth factor beta
TNF-α: tumor necrosis factor alpha
VFA: volatile fatty acid

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LITERATURE REVIEW

1. LITERATURE REVIEW

Introduction

The association between nutrition and health has been of increasing interest in farm animals reared in intensive systems. For instance, several direct effects of the ingested feed on the immune system, specifically in the intestines, have been described. Dairy calves are born with a naïve immune system. Moreover, they are typically fed restricted amounts of MR or whole milk for a limited period of time (6-8 weeks) and then abruptly weaned.

This review will cover different aspects influencing health and performance of dairy calves during the preweaning and weaning periods along with several strategies to improve them.

1.1. Feeding of dairy calves

Dairy calves are usually fed restricted daily amounts of milk (about 10% of their BW) along with *ad libitum* starter feed to promote early solid intake and accelerate rumen development. A common practice is to provide dairy calves with MR instead of cow milk. Even though the quality of the MR has been notably improved in the last decades, the lack of bioactive compounds, such as hormones and growth factors, and the type of fat and protein sources used in MRs may influence performance and immune function.

1.1.1. Liquid feeding

1.1.1.1. Colostrum

When dairy calves are born, they depend on milk until they have a GIT mature enough to digest solids. In fact, at birth the abomasum is well developed and highly functional allowing milk digestion (Short, 1964). The first meal offered to calves is colostrum: the first secretion of the mammary gland produced after calving. Colostrum contains greater amounts of nutrients and vitamins than milk and also high amounts of Ig, leukocytes and cytokines that provide calves with passive immunity. Absorption of the colostrum Ig occurs by passive transfer due to an increased permeability of the GIT during the first 24h of life. Apparently, the permeability of the intestines depends on the amount of colostrum fed and the time elapsed between birth and first colostrum intake (Stott et al., 1979; Bush and Staley, 1980). Failure of passive transfer results in increased mortality

during the preweaning period (Nocek et al., 1984a) and high concentrations of Ig in serum of calves at 24 to 48h after birth is associated with greater survival for the first weeks of life (Robison et al., 1988).

1.1.1.2. Milk

After colostrum intake, calves are fed either whole milk, waste milk (mastitic or with antibiotics), transition milk or high quality MR. Even though feeding MR is the most common practice when rearing dairy calves, some producers use cow milk. Whole milk has higher energy content and better nutrient balance than MR and it contains non-nutritional components such as a variety of hormones and growth factors (Campbell and Baumrucker, 1989; McGrath et al., 2008; D'Alessandro et al., 2011). For instance, some have reported increased growths when providing calves with whole milk instead of MR (Dvorak et al., 1986) even though gross composition of the MR was equalized to that of whole milk (Lee et al., 2009) possibly due to the bioactive components in whole milk. Nevertheless, Jaster et al. (1990) reported similar growth rates when comparing calves fed MR and whole milk. Waste and transition milk are economic options that should be used cautiously. In fact, calves fed mastitic milk have greater risk of mortality than calves fed whole milk (Losinger and Heinrichs, 1997) due to bacterial contamination and calves fed waste milk containing antibiotics may develop antibiotic resistance (Langford et al., 2003). Pasteurization of waste milk has been proved to be a promising solution to reduce bacterial contamination and calves mortality (Jamaluddin et al., 1996).

The quality of MR depends on the quality and quantity of protein and fat sources used for their formulation. Nutrient composition of MR often ranges from 18 to 28% of CP and from 10 to 22% of fat which is lower than the protein and fat contents in whole milk. The two main protein sources are milk or hydrolyzed vegetable proteins (soy, wheat, potato). Milk proteins used in MR formulations are either whey resulting from cheese making after curd formation or casein from skim milk. The main difference between these two protein sources is the ability of casein (present in skim milk) to clot in the abomasum of calves 10 min after ingestion which results in a slower digestion in the abomasum and a controlled release of caseinate proteins and milk fat into the duodenum. However, similar performance parameters have been reported in calves fed both protein sources (Terosky et al., 1997). For economical reasons, most formulations

include a proportion of vegetable protein sources to reduce production costs, with soy protein being the most commonly used. Despite the generally favorable amino acid profile, soy protein is less digestible than milk protein (Akinyele and Harshbarger, 1983; Dawson et al., 1988; Khorasani et al., 1989; Montagne et al., 2001) and it may contain antinutritional factors such as trypsin inhibitors and antigenic proteins that can be detrimental for the performance of calves if not properly processed (Gorrill et al., 1967; Dawson et al., 1988). Disadvantages of feeding calves MR including large proportions of soy protein are low digestibility and increased diarrhea due to villus atrophy (Seegraber and Morrill, 1986; Silva et al., 1986). Fat sources are of animal origin (tallow, lard) or vegetable oils (such as coconut and palm oils). Even though the preruminant calf is able to utilize a wide variety of fats in the MR, its fatty acid profile may determine palatability, intake, and performance (Jenkins et al., 1986). Also, fatty acid composition of the MR fat has an influence on growth and immunity of the preruminant calf (Hill et al., 2007a,b and 2011). For instance, Hill et al. (2007a) reported improved ADG and reduced days with abnormal fecal scores after increasing the amount of butyrate, medium-chain, and polyunsaturated fatty acids in the MR compared with a MR containing all animal fat from lard. Similarly, Hill et al. (2007b) reported improved ADG and reduced days with abnormal fecal scores after increasing the concentration of short-chain (C4:0), medium-chain (C8:0, C10:0, C12:0 and C14:0), and essential fatty acids (C18:3) of a MR compared with a MR containing all animal fat from lard. Also, Hill et al. (2011) reported improved growth rates and feed efficiency of calves, reduced scours and medical treatments for *Clostridium* sickness, reduced the inflammatory response and increased antibody titers against bovine viral diarrhea and respiratory parainfluenza-3 vaccinations after supplementing a MR with NeoTec4 (a commercial blend including butyrate, coconut oil and flax oil) so that the fatty acid profile of the supplemented MR differed notably from the controls, especially for C4:0, C12:0, C14:0, and C18:2 fatty acids. From this concern, different MR formulations including fatty acid supplements such as butyrate have been considered for calves (Hill et al., 2007b; Guilloteau et al., 2009; Górka et al., 2011a).

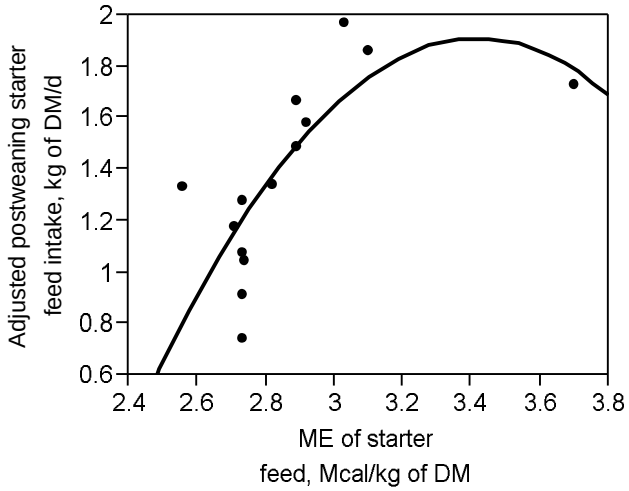
1.1.2. Solid feeding

The GIT of calves at birth is immature to digest solids efficiently. Early consumption of dry feed is needed to stimulate rumen maturation. Then, calves are usually offered starter feed *ad libitum* from the first days of life. High quality ingredients are used to

formulate starter feeds as its composition and palatability influences intake and, thus, rumen development. The main ingredients are cereal grains; being corn, sorghum, barley, wheat, oats and rice the most commonly used. Soybean meal is the most common protein source due to its favorable amino acid profile and meal or ground alfalfa is the most commonly used fiber source. The NRC (2001) recommendations for starter feed protein content is about 18%. However, in a cafeteria study in which calves were allowed to perform their own solid diet from different ingredients, the final diet composition that calves constructed by eating different proportions of all offered ingredients contained 29% of CP and 6.3% of fat (Montoro and Bach, 2012), which differs from the commonly used nutrient composition of commercial starter feeds in Europe that typically contain 20% CP and 4% fat (Montoro and Bach, 2012) but did not result in improved growths. Similarly, increasing CP amounts of the starter feeds over 19.6% failed to increase preweaning starter intake and daily gain (Stamey et al., 2012) or postweaning starter intake (Akayezu et al., 1994) of calves. Moreover, increasing fat content of starter feeds may be a recommended practice when rearing calves in cold environments. However, reduced DMI and weight gain have been reported after increasing the fat content of the starter feeds up to 7.3% DM using whole soybeans (Kuehn et al., 1994) or to greater amounts (up to 20%) using brown grease and hydrogenated cottonseed oil (Miller et al., 1959). Contrary, Johnson et al. (1956) reported that feeding a high-fat starter (up to 10% fat) to calves limit-fed whole milk did not affect solid feed intake and increased feed efficiency.

The effects of increasing the fat content of the starter feeds on intake and growth found in the mentioned studies may have been related to the increase in the ME of the resulting feeds. Satiety signals controlled by the energy balance of calves may have reduced starter intake when the ME content was high (Montgomery and Baumgardt, 1965; Litherland et al., 2014). This observation has been evaluated in Figure 1.1. Data from 5 studies providing calves starter feeds differing in ME content were adjusted for the random effect of each study and a mixed-effect linear regression was performed between the study-adjusted starter feed intake 2 weeks after weaning and the ME content of the starter feed consumed. It can be noticed in the figure that ME of the starter feed reduces postweaning starter feed intake after a certain level (around 3.4 Mcal/kg of DM).

Figure 1.1. Linear relationship between ME of the starter feed and study-adjusted starter feed intake 2 weeks after weaning of calves from different studies: adjusted-postweaning starter intake = -3.166323 + 1.6133244 * ME of starter feed - 1.493993 * (ME of starter feed - 2.87714)^2; $R^2 = 0.56$; $P < 0.01$; Luchini et al., 1993; Kuehn et al., 1994; Lesmeister and Heinrichs, 2004, 2005; Brown et al., 2005.



Starter feed can be offered to calves in different physical forms such as finely ground, pelleted, extruded (mash form) or texturized. The particle size of the feed has an impact on the health of the rumen, especially the fiber fraction. Digestibility, typically is highest in steam-flaked grains, followed by finely-ground, then dry-rolled grains, and is lowest in whole grains (Theurer, 1986; Huntington, 1997). Moreover, generally, feeds with a small particle size decrease rumen pH and cellulolytic bacteria populations (Beharka et al., 1998) and are associated with rumen parakeratosis, an excess of keratinized squamous epithelial cells in the papillae (Greenwood et al., 1997). However, even though it is commonly believed that the texturized and extruded starters increase calf performance compared with finely ground or pelleted starter feeds, limited and controversial results exist in the literature about the effects on performance of providing calves with starter feed in different physical forms. Nevertheless, it has been recently reported that pelleted and texturized starter feeds seem to improve performance compared with a mashed form (Nejad et al., 2012).

Providing preweaning calves with a forage source is not a common practice in dairy farms. However, it has been demonstrated that feeding chopped grass hay to young dairy calves increased total DMI and growth rates and improved rumen health (Castells et al., 2013), even if compared at same levels of fiber when provided in the starter feed

(Terré et al., 2013). Moreover, Terré et al. (2015) recently demonstrated that providing chopped hay to calves fed a pelleted starter feed improved growth and rumen pH compared with calves fed a texturized starter feed.

1.2. Weaning

Weaning is the transition from a liquid-based diet (milk) to a solid-based diet (grains and forage). Even though calves are ruminants, they are born with a physiologically nonfunctional forestomach. However, the GIT of calves at birth is prepared to digest milk as the abomasum is well developed and highly functional (Short, 1964). During the preweaning period, once calves start eating solids a transformation on the digestive capacity of the GIT of calves begins, facilitating digestion of solid feeds efficiently, which has been suggested to happen 2-3 weeks after the first solid feed intake (Lallès and Poncet 1990). Therefore, calves should be weaned with a mature ruminant function able to digests solids efficiently to guarantee the postweaning growth capacity.

In this section, main physiological changes of the GIT of calves during the weaning transition will be reviewed along with the most common milk feeding and weaning methods and its consequent effects on intake, health and growth before and after weaning.

1.2.1. Changes at weaning

The main change in the GIT of calves during the preweaning period is the development of the rumen capacity to digest solid feeds. At birth, calves main energy source is glucose from the digestion of milk lactose. With the transition from a liquid (milk) to a solid (grains and forage) diet during the preweaning period, the immature rumen is progressively colonized by anaerobic bacteria that are responsible for the fermentation of solids and the production of VFA, the main energy source in adult ruminants.

Establishment of the rumen microbiota appears to follow a pattern dependable on the substrates available. In fact, Rey et al. (2013) recently demonstrated that ruminal microbiota, detected by 454 DNA sequencing, may follow a three-stage implantation process that is largely dependent upon the age and type of diet of the calf and that solid feed intake progressively shapes the rumen bacterial community. At day 2, the bacterial community of newborn calves was mainly composed of Proteobacteria (70%) and Bacteroidetes (14%), and *Pasteurellaceae* was the dominant family (58%). After that

and until day 12 of life, the bacterial community was mainly composed of the genera *Bacteroides* (21%), *Prevotella* (11%), *Fusobacterium* (5%) and *Streptococcus* (4%). However, when solid feed intake increased, *Prevotella* was the dominant genera (42%) and many genera detected during the first days of life were strongly reduced or no longer detected (Rey et al., 2013). Even though it has been suggested that calves have a mature ruminal function 2-3 weeks after the first solid feed intake (Lallès and Poncet 1990), it has been recently demonstrated that functional microbiota may be established before the arrival of solid substrate. For instance, the presence of fibrolytic and cellulolytic bacteria have been detected even before first solid feed intake takes place (Jami et al., 2013; Rey et al., 2012, 2013). Moreover, main enzymatic activities (fibrolytic, amylolytic, proteolytic, and ureolytic) have been found in the rumen of calves during the first week of life (Sahoo et al., 2005; Rey et al., 2012). Therefore, enzymatic activities, fermentative products and bacterial community detected in the rumen of calves during the first days of life suggest a functional microbiota before the arrival of solid substrates in the rumen.

The main end-products from microbial fermentation of solid feeds in the rumen are VFA, which are responsible for the rumen epithelial development, and also ammonia and lactate. The rumen epithelium is constituted by papillae, a structure that enables absorption and utilization of the fermentation end-products, and its development is stimulated by the most abundant VFA produced in the rumen: acetate, propionate and butyrate. In fact, butyrate and, to a lesser extent, propionate have the greatest influence on rumen epithelial development as they are the main energy sources used by the rumen papillae (Sander et al., 1959; Harrison et al., 1960; Tamate et al., 1962).

In addition to the mucosal development, muscular tissue of the rumen and its motility needs to be promoted to achieve a functional rumen. Whereas papillae growth depends on the fermentation products, muscular tissue appears to be influenced by the rumen fill weight, as it develops by constantly moving and mixing the rumen contents (Harrison et al., 1960). The presence of non-fermentable substrate in the rumen such as wood shavings or bulky material has been associated with increased muscularization with little effect on papillae development (Harrison et al., 1960; Tamate et al., 1962). Moreover, it has been proposed (Nocek et al., 1984b) that effective fiber was able to stimulate ruminal contractions as shown by the thicker rumen muscle layer of calves fed hay compared with calves fed concentrate.

In summary, the main challenge at weaning is to obtain a healthy calf with a functional rumen that guarantees growth when fed exclusively on solids (grains and forage) which may be achieved by different weaning methods.

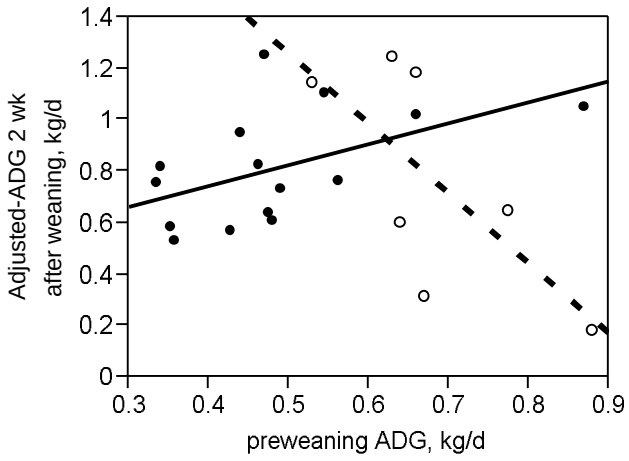
1.2.2. Weaning methods

1.2.2.1. Milk feeding

Calves fed milk *ad libitum* have consumption rates of about 10 kg/d (Appleby et al., 2001). However, common feeding programs for dairy calves are based on restricted amounts of liquid diets (8-10% of BW) to reduce costs, and to encourage early solid feed consumption which promotes rumen development and thus lower weaning age. Moreover, calves are usually fed twice a day which contrasts with the 7 to 10 times a day calves would suckle their dam. This leads to a small supply of nutrients above maintenance to be used for growth and the immune function, which results in reduced growth rates (Jasper and Weary, 2002) and to a depressed immune response in neonatal calves because of a protein and energy malnutrition (Griebel et al., 1987). For instance, restricted milk feeding has been associated with reduced performance because of low nutrient availability (Appleby et al., 2001, Jasper and Weary, 2002) and plane of nutrition has been shown to affect the immune system of young calves, as detailed in section 1.3.3.1.

In the recent years, though, enhanced feeding programs, such as increasing daily milk allowance, have been associated to increased growths, reduced age at first calving, and increased milk production during first lactation (Davis Rincker et al., 2011; Bach, 2012; Soberon et al., 2012). However, feeding calves with intensified milk programs have also been associated to decreased solid feed intake (Cowles et al., 2006; Hill et al., 2010; Davis Rincker et al., 2011), delayed rumen development (Terré et al., 2007; Suárez-Mena et al., 2011), and postweaning ADG (Cowles et al., 2006; Hill et al., 2007d). Therefore, feeding calves increased amounts of MR seems to be a promising strategy to improve preweaning growths but may reduce ADG after calves are weaned because of reduced solid feed intake and rumen development. This observation has been evaluated in Figure 1.2. Data from 15 studies providing calves different MR feeding programs were adjusted for the random effect of each study and a mixed-effect linear regression was performed between the study-adjusted ADG during 2 weeks after weaning and the ADG of the preweaning period.

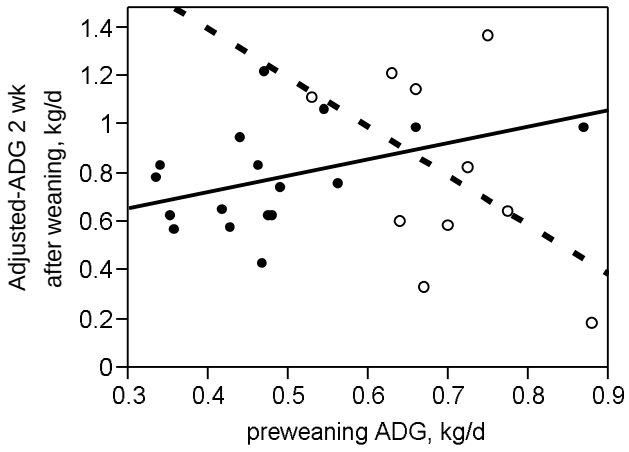
Figure 1.2. Linear relationship between preweaning ADG and study-adjusted ADG 2 weeks after weaning of calves from different studies: adjusted-postweaning ADG = 0.417 + 0.817 * preweaning ADG; R^2 = 0.27; $P < 0.05$ for calves in conventional milk feeding programs (solid line and solid dot) and adjusted-postweaning ADG = 2.616 - 2.717 * preweaning ADG; R^2 = 0.49; P = 0.07 for calves in enhanced milk feeding programs (dashed line and open dot); Abdelgadir et al., 1996; Lesmeister and Heinrichs, 2004, 2005; Cowles et al., 2006; Jensen, 2006; Terré et al., 2006; Hill et al., 2007d; Raeth-Knight et al., 2009; Davis Rincker et al., 2011; Stamey et al., 2012; Jarrah et al., 2013: Margerison et al., 2013; Beiranvand et al., 2014; Pezhveh et al., 2014; Terré et al., 2015.



Usually, preweaning duration in dairy calves is around 6-8 weeks in which calves are typically fed MR at 10% of BW at 12.5% dilution twice a day. However, the milk feeding period is more extensive in some countries, such as in Switzerland, where calves are fed 6 L/d of MR from 3 to 8 weeks of age and milk is progressively reduced from weeks 8 to 12 of age and weaned at 12 weeks of age (Roth et al., 2009). Main weaning criteria are calf BW, calf age, or a minimum amount of dry feed daily consumption (e.g. 1,000 g/d). Calves depend on milk until they are able to consume enough energy from solid feed to cover at least the maintenance requirements. Then, weaning according to calves age does not consider individual variation on their adaptation to solid feed consumption. Adjusting weaning to the ability of calves at eating solids has been shown to be a promising practice as it can reduce age at weaning and improve postweaning intake, growth and health (Roth et al., 2009; de Passillé and Rushen, 2012).

The main weaning practice, however, is according to the age of calves by either progressively reducing the MR dilution rate for a few days before weaning, reducing milk allowance to half the amount for one or two weeks before weaning or abruptly abolishing milk feeding. Similar postweaning intakes and growths have been reported when calves were fed reduced MR allowances (10% of BW) and were either abruptly weaned at 35 d of age or the daily milk allowance was reduced to half the amount the week before weaning (Quigley, 1996). However, when calves are fed on enhanced milk feeding programs and following the common weaning practice to reduce the MR allowance for a few days before weaning, reduced postweaning ADG have been reported (Cowles et al., 2006; Terré et al., 2006; Hill et al., 2007d; Stamey et al., 2012). Nevertheless, Khan et al. (2007b) has proposed a milk feeding strategy for calves fed increased amounts of MR that notably reduced the postweaning ADG slump. It consisted on gradually reducing to half the amount of MR provided from days 26 to 30 of age and feed this rate for the following 15 d, and then progressively dilute the MR with water for the last 5 d before weaning. This method resulted in improved postweaning ADG (Khan et al., 2007a,b, and 2011a). For instance, when including these 3 studies to the evaluation performed in Figure 1.2, linear regressions between the study-adjusted ADG 2 weeks after weaning and the ADG of the preweaning period for the conventional milk feeding it goes from significant to a tendency and for the enhanced milk feeding it goes from a tendency to not significant, as shown in Figure 1.3.

Figure 1.3. Linear relationship between preweaning ADG and study-adjusted ADG 2 weeks after weaning of calves from different studies: adjusted-postweaning ADG = 0.459 + 0.664 preweaning ADG; R^2 = 0.18; *P* = 0.09 for calves in conventional milk feeding programs (solid line) and adjusted-postweaning ADG = 2.202 - 2.018 preweaning ADG; R^2 = 0.23; *P* > 0.1 for calves in enhanced milk feeding programs (dashed line); Abdelgadir et al., 1996; Lesmeister and Heinrichs, 2004, 2005; Cowles et al., 2006; Jensen, 2006; Terré et al., 2006; Hill et al., 2007d; Khan et al., 2007a,b, and 2011a; Raeth-Knight et al., 2009; Davis Rincker et al., 2011; Stamey et al., 2012; Jarrah et al., 2013: Margerison et al., 2013; Beiranvand et al., 2014; Pezhveh et al., 2014; Terré et al., 2015.



Therefore, calves may benefit from enhanced milk feeding programs during the preweaning period to improve growth and health by following a step-down method for the weaning period to improve postweaning ADG.

1.2.2.2. Solid feed intake

Starter feed consumption should be well established at weaning as it will be the main source of nutrients for calves after weaning. Therefore, it is important to stimulate starter feed intake the weeks before weaning. Several factors affect starter feed intake before weaning such as MR allowance and its composition, solid feed characteristics, and forage provision.

Starter feed intake is inversely dependent on the amount of MR offered to calves. In fact, it has been reported that feeding calves MR *ad libitum* reduced starter feed intake to almost half the amount when compared with calves fed at 10% BW (Jasper and

Weary, 2002; Cowles et al., 2006; Raeth-Knight et al., 2009). Moreover, nutrient composition of MR can influence starter intake as shown by the decrease in starter feed consumption when calves were offered a high-fat MR (Kuehn et al., 1994; Hill et al., 2009c; Stamey et al., 2012). Kuehn et al. (1994) fed calves two MR differing in fat content (15.6 and 21.6%) and reported reduced starter intake before and after weaning when calves were fed the high-fat MR, probably because of its high ME content that may have induced satiety signals. Hill et al. (2009c) fed calves with MR containing different amounts of fat (14, 17, 20 and 23%) and found that starter intake of calves before weaning responded quadratically with the amount of fat in the MR, being lowest at 14 and 23% of fat. Also, satiety signals may have been induced in calves fed the MR with 23% fat and thus reduce starter intake. However, the reduction in starter feed intake of calves fed the MR with the lowest fat content (14%) remains inexplicable. Stamey et al. (2012) found reduced starter intakes when calves were fed an enhanced (28% of CP and 15% of fat MR fed at 15% dilution) compared to a conventional (20% of CP and 20% of fat MR fed at 12.5% dilution) milk feeding program possibly due to the increased ME intake.

As stated above, the physical form of the starter feed and its CP and fat content may influence performance parameters of calves as it modulates intake. Another factor affecting DMI is the fiber content of the starter feed (Kang and Leibholz, 1973). Even though it has been shown that increasing the fiber content of the starter feed increased DMI and growth of preweaned and weaned calves (Thomas and Hinks, 1982; Hill et al., 2009a), some found similar solid feed intakes when comparing two starters with different NDF contents (Porter et al., 2007; Terré et al., 2013). Moreover, they reported increased performance (Terré et al., 2013) and digestibility (Porter et al., 2007) parameters in calves fed low compared with high-fiber starter feeds.

Considering the challenge that calves go through during the transition from a liquid to a solid nutrition, high quality and palatable starter feeds should be offered to calves before weaning to foster dry feed consumption. High-energy and high-protein ingredients should be considered of good palatability for young calves as they are the most preferred ingredients when calves are allowed to choose (Montoro and Bach, 2012; Miller-Cushon et al., 2014a,b). In particular, soybean meal, followed by corn and whole soybean were the most preferred ingredients for calves in a cafeteria study (Montoro and Bach, 2012). Moreover, in a similar preference test study in recently-weaned calves,

Miller-Cushon et al. (2014a) found that wheat meal and sorghum were the most preferred high-energy feed types and that soybean meal and dried distillers grains were the most preferred high-protein feed types. As expected, inclusion of the preferred ingredients in mixed starter feeds were found to be the most consumed by recently-weaned calves, suggesting that palatability of starter feeds may be improved by the inclusion of the most preferred ingredients identified (Miller-Cushon et al., 2014a).

Other strategies to improve DMI and performance of dairy calves is the addition of different supplements in the starter feed formulation such as sugars, fatty acids, and probiotics, among others. Sweet taste has been reported to be palatable for young calves (Hellekant et al., 1994). Moreover, increasing sugar content of the starter feed has been associated with increased butyrate production (Waldo and Schultz, 1960). However, some have reported no advantages when adding sugar or molasses in the starter feed on performance parameters (Lesmeister and Heinrichs, 2005; Hill et al., 2008; Beirnvand et al., 2014), but some found a slight effect on rumen fermentation, such as decreased rumen acetate concentration (Beiranvand et al., 2014) or increased blood VFAs (Lesmeister and Heinrichs, 2005). Inclusion of long-chain fatty acid salts, such as calcium salts of flax oil, in the starter feeds resulted in improved intake and performance (Hill et al., 2009b). Moreover, the addition of short chain fatty acids salts such as sodium butyrate and calcium propionate in a starter diet had no effect on intake and growth of calves around weaning (Ferreira and Bittar, 2011). Others, however, reported increased DMI and rumen development when calves where fed a starter feed supplemented with sodium butyrate (Górka et al., 2011a). Microbial feed additives (known as probiotics) are living organisms that can modulate the gastrointestinal microbiota of the host and have been recently tested as growth promoters substituting antibiotics with promising results in piglets. However, there are few studies regarding the effects on performance of the addition of microbes in the starter feed of calves. Indeed, some reported no clear effects on starter intake and growth when supplementing a starter feed of calves with a *Lactobacillus* fermented product (Schwab et al., 1980) whereas other found increased intake and performance after supplementing a starter feed with *Bacillus subtilis* (Sun et al., 2010). Finally, the use of other feed additives such as certain trace minerals and essential oils in the starter feed of calves has also been investigated with variable results on intake and performance parameters (Koong et al., 1970; Spears et al., 1986 and 1991; Kincaid et al., 1997; Vakili et al., 2013).

1.3. Immune function and intestinal integrity

The GIT is the organ responsible for the digestion and absorption of nutrients. Moreover, it is a prominent part of the immune system. The intestinal mucosa is composed, from the lumen, of: the epithelium, the lamina propria, and muscle, all of them with different protective mechanisms. Resistance to disease is a balance between the microorganisms the calf is exposed to and the ability of its immune system to detect and eliminate the undesired ones. Then, the intestinal mucosa is the first physical barrier of the immune system separating the numerous microorganisms of the intestinal lumen from the rest of the body and allowing only the selected substances to be absorbed into the bloodstream. For this reason, nutrition can influence the immune system homeostasis of calves both directly by supplying specific nutrients to modulate the immune response and indirectly by influencing the exposure to microorganisms. The intestinal integrity is, therefore, crucial as a first step of the immune response.

1.3.1. Immune system of young calves

Calves are born with an immature immune system. However, during gestation, they develop an immune function able to deal with the microbial environment to which they will be exposed since birth, as indicated by the presence of functional phagocytes and its antimicrobial capacity (Menge et al., 1998). In fact, from the sterile environment of the placenta, the GIT of the newborn calf is progressively colonized by the surrounding bacterial community. Establishment of the microbiota in the GIT of calves is influenced by diet, genetics and factors associated to dam. The first contact with external bacteria is with the vagina during delivery where Enterococci and Staphylococci are the predominant genera (Otero et al., 2000). Some microflora is also transferred to newborn calves through colostrum intake in which Lactobacillus and Bifidobacteria are predominant (Taschuk and Griebel, 2012). It is, therefore, hypothesized that commensal bacteria, present in various maternal tissues and fluids, contributes to the GIT development of the newborn calves (Taschuk and Griebel, 2012). Moreover, host genetics may contribute to commensal bacterial discrimination which may have an influence on the establishment of the gastrointestinal microbiota (Benson et al., 2010, Mayer et al., 2012). In addition, environmental factors, such as diet, may also modulate the gastrointestinal microbiota of young calves, such as the initiation of the solid feed intake (Malmuthuge et al., 2013).

The gastrointestinal microbiota homeostasis is crucial for the maintenance of the mucosal barrier function and the maturation of the immune system. Gut flora prevents colonization of pathogens by competitive exclusion. Also, it influences the expression of tight junction proteins affecting intestinal permeability of calves (Malmuthuge et al., 2013), creates a protection biofilm from pathogens (Mills et al., 2013), and modulates the secretion of protection peptides such as bacteriocins (Klaenhammer, 1988). Moreover, commensal microbiota appears to regulate IgA secretion, an Ig that, in turn, regulates host interaction with commensal bacteria (Tsuji et al., 2008). Also, commensal bacteria induce the development of the intestinal regulatory T-cells, as reviewed recently (Veenbergen and Samsom, 2012). Furthermore, the intestinal microbiota, either pathogen or commensal flora, express pathogen associated molecules which are detected by toll-like receptors that act as sensors in the intestinal mucosa and thus modulate the mucosal immune system (Malmuthuge et al., 2012). Kelly and Conway (2005) suggested that toll-like receptors are able to discriminate between pathogenic and non-pathogenic commensal microbiota. For instance, toll-like receptor 2 can detect peptidoglycan from Gram-positive bacteria, lipopeptide from Mycobacteria and other fungal elements, as well as lipopolysaccharides of leptospirosis and other spirochetes, as reviewed by Chang (2010). Therefore, this communication between host and commensal bacteria through toll-like receptors is crucial for the regulation of mucosal inflammation, because the correct identification of the antigen is necessary for a proper immune response.

1.3.2. Intestinal integrity

The digestive tract is open to the external environment through feed ingestion so that it is potentially exposed to organisms and toxic agents. The first protective physical barrier from pathogen invasion is the gastrointestinal mucosa which is composed by the epithelia and the lamina propria. Therefore, an adequate barrier function of the intestinal mucosa ensures an appropriate nutrient absorption while preventing pathogens and antigens from entering the bloodstream.

The epithelial cells can be classified as absorptive enterocytes, goblet cells that produce mucus into the lumen as a protective barrier from bacterial invasion (Specian and Oliver, 1991), hormone-producing enteroendocrine cells (Lee and Kaestner, 2004), and Paneth cells that secrete antimicrobial peptides into the lumen (Jones and Bevins, 1992).

They are all interconnected by small proteins called tight junctions forming a semi permeable surface that actively and passively allows passage of fluids, nutrients, electrolytes, and large molecules such as Ig after birth (Staley et al., 1972). Intestinal permeability can be transcellular (through epithelial cells by active or passive transport) for small molecules absorption or paracellular (through tight junctions) for macromolecules absorption. Therefore, tight junctions are key modulators of the intestinal barrier function. In fact, for the following 24h after birth, paracellular intestinal permeability is high in order to allow the passive absorption of the Ig of the colostrum (Stott et al., 1979).

The lamina propria contains the lymphatic and vascular channels, as well as the gut-associated lymphoid tissue containing the macrophages and the dendritic cells, B and T-cells, and also secretes IgA onto the mucosal surface (Liebler et al., 1988; Macpherson et al., 2001; Fries et al., 2011). Finally, the mucosal muscle is responsible for the intestinal motility that prevents stasis of nutrients and bacteria.

Microorganisms present in the GIT, such as viruses and bacteria, can damage the intestinal mucosa by degrading the mucosa layer, destroying the epithelial cells, and interrupting the vascular supply (Salim et al., 1990; Sansonetti et al., 1999). This may compromise intestinal integrity allowing the entrance of pathogens causing disease. Then, maintaining a proper intestinal integrity is of great interest for preventing diseases and the use of living bacteria for this purpose has been lately considered. For instance, there is evidence that exposure to commensal bacteria, such as lactic acid bacteria, may modulate intestinal permeability by affecting the expression of tight junction throughout the epithelia (Malmuthuge et al., 2013). Moreover, germ-free and gnotobiotic animal models are a useful tool to understand the relationship between commensal bacteria and the immune system, as recently reviewed (Umesaki, 2014).

1.3.3. Factors affecting the immune system

The immune system of young calves is mostly affected by diet, stress and genetics. For instance, nutrition can influence disease resistance of calves both directly by supplying specific nutrients and indirectly by influencing the exposure to microorganisms. Moreover, stress, as caused by weaning and transportation, has been associated to a depression of the immune function (Kelley, 1980). Then, management of dairy calves

has great influence on the immune status and thus, survivability during the first weeks of life (when calves are most vulnerable).

1.3.3.1. Diet

Colostrum is the first feed provided to the newborn calves and is the main source of antibodies in addition to many bioactive and growth-promoting compounds, such as growth factors, peptide hormones, nucleotides, enzymes, and cytokines that modulates their intestinal development and immune function. However, the failure of passive transfer of the Ig due to low quality of some colostrums has prompted the development of colostrum replacers (products containing high amounts of IgG, i. e. >100 g of IgG/dose and nutrients required by newborn calves) and colostrum supplements (containing exogenous IgG from bovine lacteal secretions, eggs or bovine serum) that are provided in conjunction with colostrum because colostrum supplements (containing tipically 25 to 45 g of IgG/dose) alone do not provide sufficient IgG to replace maternal colostrum. However, apparent efficiency of IgG absorption of colostrum supplements seems to differ depending on the formula and the method of processing. For instance, poor apparent efficiency of IgG absorption has been reported from colostrum supplements derived from lacteal secretions (Abel Francisco and Quigley, 1993, Morin et al., 1997), whereas acceptable IgG transfer using colostrum supplements derived from serum have been reported (Arthington et al., 2000, Quigley et al., 2002). Also, colostrum replacers are used with inconsistent results on immune function. Even though it has been reported that when calves are fed similar IgG amounts from a commercial colostrum replacer the IgG absorption was 60% the IgG absorption from maternal colostrum (Quigley et al., 1998), some commercial colostrum replacers have been found to result in successful IgG transfer (Quigley et al., 2002; Foster et al., 2006; Poulsen et al., 2010). In addition, it has been recently reported that colostrum replacer resulted in more uniform levels and duration of antibodies to common bovine respiratory viruses than maternal colostrum (Chamorro et al., 2014).

Colostrum bacterial load may modulate the immune system both by providing commensal bacteria that influence the immune system development and by exposing the intestinal barrier to pathogens. For instance, several possible pathogens can be transmitted from colostrum and milk (Selim and Cullor, 1997; Fecteau et al., 2002) to newborn calves. Preventing contamination during harvesting and storage of colostrum

by freezing, refrigerating and using preservatives are recommendable strategies (Stewart et al., 2005). Also, pasteurizing or heat-treating colostrum has successfully reduced its microorganism load and the spread of several diseases in cattle such as bovine paratuberculosis (Streeter et al., 1995). However, the main disadvantage of heat treating colostrum is the loss of Ig, lactoferrin and neutrophil antimicrobial activity that also affects the immune response (Lakritz et al., 2000). In fact, several studies have been conducted studying the effects of lactoferrin, an immunoregulatory protein with multiple functions, in the MR on the immune response of preweaned calves with promising results (Joslin et al., 2002; Robblee et al., 2003; Prenner et al., 2007; Prgomet et al., 2007). For instance, Joslin et al. (2002) and Prenner et al. (2007) reported beneficial effects on performance and health (reduced disease and diarrhea incidences) after supplementing lactoferrin in the colostrum and the MR and Prgomet et al. (2007) and Robblee et al. (2007) reported improved performance and immune system (reduced diarrhea and morbidity) from supplementing calves with lactoferrin in the MR, all of them throughout the preweaning period.

After colostrum intake, dairy calves are usually fed MR instead of whole milk to reduce costs. However, fatty acid composition of MR and whole milk differ notably. Whereas cow milk is mostly composed of short-chain, mid-chain and some polyunsaturated fatty acids (Caboni et al., 1984), fat in MR, which is usually lard or tallow, contain much lower concentrations of these fatty acids (NRC, 2001). This has implications in health and performance of calves as fatty acid profile of the diet has been shown to affect immunity and growth parameters. In fact, some (Hill et al., 2007a,b and 2011; Esselburn et al., 2013) found increased growth rates and improved humoral immunity, inflammatory response and reduced scours and medical treatments after increasing the concentration of short-chain, mid-chain and essential fatty acids of a MR based on all animal fat. Also, Hill et al. (2007c) reported increased growth rates and feed efficiencies when calves were fed a starter feed containing a blend of sodium butyrate, canola oil and coconut oil (thus increasing the content of C4:0, C8:0, C10:0, C12:0, C18:2, and C18:3 fatty acids) compared with a control starter feed and Hill et al. (2009b) reported improved performance in calves fed a corn and soybean meal-based diet supplemented with linoleic acid (C18:3) as a calcium salt of flax oil. Moreover, bovine milk C10:0 and C12:0 fatty acids have been shown to have antimicrobial properties against

Escherichia coli O157:H7, *Salmonella enteritidis*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Clostridium perfringens* in vitro (Sprong et al., 2001).

Also, plane of nutrition has an influence on the immune system of the young calves. It has been shown that the somatotropic axis of the neonatal calves is affected by plane of nutrition (Hammon and Blum, 1997), and the somatotropic axis has an influence on the development of the immune system in cattle, as reviewed by Elsasser et al. (1997). Moreover, a protein to energy malnutrition has been associated to a depressed inmmune response in neonatal calves (Griebel et al., 1987). Then, it has been hypothesized that an enhanced feeding program may benefit the immune system. Many have, in fact, reported an effect of the plane of nutrition on the immune status of calves. For instance, Pollock et al. (1993 and 1994) reported a modulation of the immune response on the cellular and the humoral immunity of calves fed on different planes of nutrition. In particular, Pollock et al. (1993) reported an increased skin sensitivity responses to keyhole limpet haemocyanin and decrease lymphocyte blastogenesis test responses to concanavalin A and pokewood mitogen in calves fed 400 g/d DM of MR compared to 1000 g/d and Pollock et al. (1994) reported that calves fed 1000 g/d DM of MR decreased anti-horse erythrocyte titers and serum IgG2 and IgA responses to keyhole limpet haemocyanin. Also, Nonnecke et al. (2003) found that blood mononuclear leukocytes from calves fed increased dietary fat and protein produced less INF-γ and more inducible nitric oxide, thus affecting leukocyte functions associated with cellular immunity in calves. Recently, Ballou (2012) showed that Jersey calves in an intensive milk feeding program improved the innate immune response after weaning compared to conventionally fed calves. In addition, Foote et al. (2005a,b) reported that higher milk feeding plane of nutrition affected the composition and functional capacities of peripheral blood mononuclear cell populations (i. g. by producing less IFN-γ and more nitric oxide), although the influence of these effects on susceptibility to infection of calves needs further investigation. However, no improvement on incidence of diarrhea or bovine respiratory diseases have been reported in calves fed high MR allowances (Uys et al., 2011; Bach et al., 2013b).

Finally, other aspects of the diet of young calves and its relationship with the immune function have been investigated. For instance, it has been demonstrated that introduction of solid feeds may modulate the gastrointestinal microbiota and thus influence the immune system of young calves (Malmuthuge et al., 2013). Therefore, the presence or

the addition of certain bioactive compounds in the young calf diet has been of increased interest in animal production research, being prebiotics and probiotics widely investigated in the recent years. Prebiotics are defined as food ingredients that survive the enzymatic digestion of the upper GIT and are fermented by the colonic flora stimulating growth and activity of one or more bacterial species already residing in the colon (Gibson and Roberfroid, 1995). Also, prebiotics have been shown to increase the concentration of short chain fatty acids of the lower intestinal tract as a result of the fermentation (Campbell et al., 1997) and fatty acids have been shown to affect immunity. The prebiotics mostly commercialized are oligosaccharides and dietary fibers (mainly inulin) and their main mode of action is to modulate the colonic microbiota by increasing the beneficial bacteria such as bifidobacteria (Gibson et al., 1995) which have inhibitory effects on pathogenic bacteria such as *E. coli*, *Salmonella*, and *Listeria* among others (Gibson and Wang, 1994) and inulin-type fructans which have been shown to reduce *Salmonella* counts in the caecum of supplemented chickens (Bailey et al., 1991). In the recent years, the use of prebiotics in calves has been tested with promising results on growth and health (Heinrichs et al., 2003, 2009; Masanetz et al., 2010; Gosh and Mehla, 2012; Grand et al., 2013; Samanta et al., 2013).

On the other hand, probiotics are living non-pathogenic organisms (mainly bacteria) that, after ingested, are considered to induce beneficial effects on the health of the host by contributing to the homeostasis of the gastrointestinal microbiota (Blum and Schiffrin, 2003). As stated above, intestinal microbiota plays a role in the immune system of the host as it influences the development of the gut-associated immune system, prevents the pathogen infection and modulates the development of the systemic immunity and its imbalance has been associated to several diseases in humans and animals (Frick and Autenrieth, 2013). The main action of probiotic bacteria in the immune system are, as recently reviewed (Howarth and Wang, 2013), competition with pathogenic bacteria by adhesion to mucosal surfaces and epithelial cells, maintenance of intestinal integrity, influence on cell kinetics, production of IgA, modulation of the inflammation, and stimulation of dendritic cells maturation. The main probiotics found in the literature are *Lactobacillus* and *Bifidobacterium*, but also *Lactococcus*, *Streptococcus* and *Enterococcus* as well as some yeast strains (Borchers et al., 2009). Beneficial effects of lactic acid bacteria as probiotics have been tested in calves with

promising results on growth and immune function (Timmerman et al., 2005; Frizzo et al., 2012; Signorini et al., 2012; Qadis et al., 2014a,b).

1.3.3.2. Stress

Stress has been widely demonstrated to negatively affect the immune system, increasing the susceptibility to diseases (Kelley, 1980). In dairy calves, different environmental and management stressors may affect the immune status such as transportation, extreme weather, weaning, and allocating changes.

Effects of transportation on stress are associated to handling, deprivation of food and water, animal density, ventilation, temperature, and humidity. For instance, long distance transportation has been shown to induce dehydration and increase susceptibility to disease (Mormede et al., 1982), increase plasma fibrinogen concentration (Phillips, 1984), increase oxidative stress (Chirase et al., 2004), and exert detrimental effects on the liver function of young calves (Uetake et al., 2009), and modify clinical and hematological variables at the end of the journey (Bernardini et al., 2012). In addition, it has been recently shown that transported prepubertal male calves experiment a decrease in the pulse frequency of growth hormone which may suppress metabolism and growth (Kadokawa et al., 2013). González et al. (2012) reported that young calves are the most susceptible cattle to transportation stress and that the factors affecting mortality and weakness are mostly duration of the journey, insufficient or excessive space allowances, extreme ambient temperatures, and the truck drivers experience. Then, measures to guarantee a proper management of dairy calves during long distance transportation are necessary. In fact, in the European Union, the legislation regarding animal transportation specifies several measures to guarantee animal welfare and reduce related stress and mortality.

Young calves are susceptible to extreme weather conditions as their heat regulation mechanisms are not fully developed. In fact, it has been shown that calves up to 6 weeks old are unable to tolerate extreme temperatures (5 ºC cold and 35 ºC hot) for longer than 4 h (Elmer and Reinhold, 2002). Cold stress usually happens when calves are born in environments below the lower critical temperature of 8ºC (Young, 1981) and energy required for maintenance is greater than the energy provided by the diet, limiting the amount of energy available for the immune function. For instance, cold stress has been associated with increased severity of disease after *Moraxella bovis* infection

(Kopecky et al., 1981) and some have reported a detrimental effect of cold stress on the immune response in young calves (Kelley et al., 1982a,b). One strategy to improve the capacity of newborn calves to cope with cold stress has been to modulate the CP and energy contents of the diet of dams with inconsistent results (Woodard et al., 1980a,b; Olson et al., 1981; Bull et al., 1991; Dietz et al., 2003). Another proposed strategy is to increase the energy content of the diet of the newborn calves, which has been shown to have short-term beneficial effects on growth of calves under cold stress conditions (Jaster et al., 1990; Litherland et al., 2014). On the other hand, heat stress has been shown to negatively affect the passive immunity acquisition in newborn calves (Stott et al., 1976; Monteiro et al., 2014), increase lymphocites and decrease neutrophiles (Broucek and Kovalcik, 1989), and reduce intake and growth (Broucek et al., 2009). Then, several measures have been tested to reduce heat stress of young dairy calves exposed to high temperatures such as supplemental shade in calves reared outdoors (Spain and Spiers, 1996), improving ventilation (Moore et al., 2012), and covering hutches with reflective insulation (Carter et al., 2014).

Weaning of dairy calves has been widely associated with stress and the consequent negative effects on immunity. For instance, the suppression of one of the daily milk feedings has been shown to produce a transient neutrophilia (Hulbert et al., 2011a). Also, abrupt weaning has been shown to negatively affect cellular (Pollock et al., 1993) and humoral (Pollock et al., 1994) immunity and, independently of age, weaning has been shown to suppress many innate immune responses (Hulbert et al., 2011b). Moreover, weaning is also associated with a change in the diet of young calves from liquid to solid feed, which has been shown to modulate the gut microbiota and thus influence the host mucosal immune function (Malmuthuge et al., 2013). Moreover, weaning is often followed by moving calves from individual hutches to group pens which may increase the exposure to new pathogens. Indeed, reduced disease incidences have been reported after keeping animals in the individual hutches for an additional week after weaning (Quigley, 2001), even though some have reported increased health after moving calves to transition small group pens (Bach et al., 2010). Keeping calves in individual hutches has the main purpose to minimize spread of disease, but calves are therefore deprived from social contact. For this reason, the European Union has mandated that calves over 8 weeks old should be reared in group (Directive 97/2/EC). For instance, rearing calves in transition small group pens after weaning (before moving

to larger group pens) has been shown to be a mild stressor without major effects on the innate immune system (Hulbert and Ballou, 2012) and even improve performance and health possibly due to the well-being of animals resulting from the social contact (Bach et al., 2010). Also, calves raised in group have been shown to reduce respiratory incidences when reared in small groups under 10 calves (Svensson and Liberg, 2006). Moreover, no detrimental effects on immunity have been reported in calves raised in groups of five animals before and after weaning (Terré et al., 2006).

Chapter 2

OBJECTIVES

OBJECTIVES

The main objective of this thesis was to evaluate different strategies to improve performance of young dairy calves by enhancing intestinal health and their metabolic function. The specific objectives were:

1. Evaluating the possible influence of the colostrum on intestinal permeability and incidence of diarrhea in newborn calves.

2. Evaluating the effects of supplementing a milk replacer with a probiotic on the modulation of the microbiota and health of calves at weaning.

3. Evaluating the effects of supplementing a milk replacer with butyrate on the performance, metabolism, and diarrhea incidences of calves before weaning.

4. Evaluating the effects of an enhanced feeding program on performance and glucose metabolism of calves before and after weaning.

To achieve these objectives, four studies were conducted:

- Study 1: Intestinal permeability and colostrum quality were compared between healthy and diarrheic calves.

- Study 2: This study assessed the hypothesis that supplementing calves with a probiotic at weaning would modulate the fecal microbiota and improve health at weaning.

- Study 3: Performance, metabolism and diarrhea incidences of calves were evaluated after supplementing a milk replacer with different forms of butyrate during the preweaning period.

- Study 4: Performance and glucose metabolism of calves fed either an enhanced or a conventional feeding program was evaluated before and after weaning.

Chapter 3

INTESTINAL PERMEABILITY AND INCIDENCE OF DIARRHEA IN NEWBORN CALVES

A fraction of this research has been sent to the Journal of Dairy Science

3.1. Introduction

The intestinal epithelium is the first protective barrier from exogenous pathogens (Deitch and Berg, 1987). Thus, the integrity of the intestinal mucosa ensures proper nutrient absorption while avoiding translocation of pathogens into the lamina propria. Intestinal permeability can be transcellular (through epithelial cells by active or passive transport), which is mainly used for absorption of small molecules or paracellular (through tight junctions that connect epithelial cells), which is mainly used for absorption of macromolecules (Bjarnason et al., 1995; Hall, 1999). Intestinal permeability is high in newborn calves during the first 24-36 h of life (Bush and Staley, 1980; Besser and Gay, 1994), which is crucial to facilitate transfer of Ig from the colostrum into the calf bloodstream via a non-selective macromolecular transport system across the small intestinal epithelium (Staley and Bush, 1985). However, on the other hand, this increased permeability during the first few hours after birth renders the intestinal wall of newborn calves highly susceptible to bacterial translocation and increases the susceptibility to infections (Berg, 1995; Uil et al., 1997).

Our hypothesis was that calves that suffer diarrhea would have an altered intestinal permeability Thus, the objective of this study was to evaluate potential changes in intestinal permeability before and after an incidence of diarrhea in newborn calves.

3.2. Materials and methods

3.2.1. Animals and treatments

Seventy-six singleton newborn Holstein calves (44.4 ± 6.15 kg BW) born to calves that needed no assistance for calving were involved in this study from birth until 21 d of age. Within 2 h after birth, calves were fed 4 L of colostrum either frozen, refrigerated, or fresh (either from their dam or from another cow) using an esophageal tube, followed by 3 meals of 2 L of late colostrum or transition milk in a bucket. The following 3 wk, calves were fed 1.5 L of a commercial (Naturmilk Super 60, Ouest Elevage, France) MR containing, on a DM basis, 22.9% CP and 20.1% fat twice daily (0830 and 1700 h) at a 15% DM concentration. Animals were raised in individual hutches (3.1 × 1.2 m) and had *ad libitum* access to water and starter feed (containing 19.7% CP and 3.9% fat on a DM basis) throughout the study.

3.2.2. Measurements and sample collection

Individual BW was recorded at 0, 7, 14 and 21 d of age. Fecal consistency of calves was evaluated on a daily basis following a 5-scale fecal score (Lesmeister and Heinrichs, 2004). Also, a permeability test using lactulose and D-mannitol as markers (Hall, 1999) was performed in all 76 calves at birth (day 0; coinciding with the first colostrum feeding), 7, 14 and 21 d of age (while offering the morning MR allowance). The permeability tests consisted of administering (via colostrum or MR) at birth 20 g of lactulose (Duphalac, Madrid, Spain) and 4 g of D-Mannitol (Sigma-Aldrich, MO 63103, USA), at 7 and 14 d of age 21 g of lactulose and 4.2 g of D-mannitol, and at 21 d of age 22 g of lactulose and 4.4 g of D-mannitol dissolved either in the colostrum or MR. In all permeability tests, 60 min after dosing the makers via colostrum or MR, blood samples were collected (BD Vacutainer® clot activator, Belliver Industrial Estate, UK) for subsequent determination of lactulose and D-mannitol serum concentrations and estimate intestinal permeability based on these concentrations.

Two replicates of first- and late-colostrum (or transition milk) samples were taken in sterile 50 mL collection tubes and immediately frozen at -20ºC for later IgG and bacterial load determinations. At birth, a blood sample was collected from the jugular vein (BD Vacutainer® spray-coated K2EDTA 4 mL Tubes, Belliver Industrial Estate, UK) from all calves to later determine basal plasma IgG concentrations. Additional blood samples for IgG determination were taken 6 h, 24 h, and 12 d after first colostrum consumption. All blood samples were kept on ice for a minimum of 20 min and then centrifuged at 3,500xg for 10 min. Serum and plasma samples were stored at -20ºC until subsequent analyses.

3.2.3. Chemical analyses

Samples of MR were analyzed for DM (24 h at 103°C), ash (4 h at 550°C), and N content according to the method of the AOAC (method 988.05; AOAC, 1990) adapted for an automatic distiller Kjeldahl (Kjeltec Auto 1030 Analyzer, Tecator) with copper sulfate/selenium as a catalyst instead of copper sulfate/titanium dioxide and for EE following method 920.39 of AOAC (1990) with petroleum ether used for distillation instead of diethyl ether (AOAC, 1990).

Determination of serum lactulose and D-mannitol concentrations were performed by ultra high performance liquid chromatography-mass spectrometry (Xevo G2 TOF, Waters) with an electrospray ionization source operating in negative mode. Serum extract was injected (5 µL) onto a BEH amide column (2.1 mm x 100 mm, 1.7 µm, Waters). The mobile phases were: water + 0.1% NH4OH, and methanol + 0.1% NH4OH. Elution conditions, at a flow rate of 0.3 mL/min, were as follows: 90% methanol + 0.1% NH4OH maintained for 2 min, linear gradient from 90 to 60% in 4 min, and equilibration to initial conditions over 4 min. Column and auto sampler chamber temperatures were maintained at 45 and 4°C, respectively. The operating conditions were as follows: source temperature = 120 °C; desolvation temperature = 350 °C; desolvation gas = 900 L/h; cone gas = 10 L/h; capillary voltage = 0.5 kV; cone voltage = 30 V; and extraction cone = 4 V. Leucine enkephalin at a concentration of 2 µg/mL was used as a lock mass for mass accuracy and infused at a flow of 5 µL/min. Chromatograms were processed using Quanlynx software (v 4.1, Waters).

Concentrations of IgG in plasma and colostrum were measured by radial immunodiffusion (Triple J Farms, Washington, USA). Determination of colostrum bacterial loads were performed by colony counting using 3 dilutions (1:10, 1:100 and 1:1000) of each specimen. For total bacterial counts, 100 µL of the dilutions 1:10 and 1:100 were plated onto trypticase soy agar (TSA; Difco, Detroit, Michigan, USA) with 5% bovine blood and the plates incubated at 37°C in an atmosphere of 5% CO2 for 24 h. For total Enterobacterial counts, 100 µL of dilutions 1:100 and 1:1000 were plated onto MacConkey agar plates and incubated at 37°C for 24 h.

3.2.4. Calculations and statistical analysis

Whenever an animal presented a fecal score ≥ 3 for three consecutive days they were considered diarrheic. That allowed classifying calves as ‘healthy’ or ‘diarrheic’. After all samples and observations from all 76 calves were collected, a random subset of 15 diarrheic (fecal score ≥3 for at least three consecutive days) and 15 healthy (no history of scours) calves was taken and used for all subsequent analyses. Therefore, results presented herein refer to data of the 30 selected calves.

Absorption of IgG at 6 h was calculated as: [plasma IgG (mg/L) at 6 h x estimated plasma volume (L)] / [first-colostrum IgG (mg/L) x first-colostrum intake (L)]. Absorption of IgG at 24 h was calculated as: [plasma IgG (mg/L) at 24 h x estimated

plasma volume (L)] / [first-colostrum IgG (mg/L) x first-colostrum intake (L) + late-colostrum IgG (mg/L) x late-colostrum intake (L)], considering that plasma volume was 6.5% of calf BW (Mollerberg et al., 1975). Serum concentration values of D-mannitol were log-transformed and data from serum lactulose concentrations and serum lactulose to D-mannitol ratio were root squared to obtain normal distributions. Bacterial counts were obtained by multiplying the number of colonies counted by the dilution factor and the result divided by the volume plated. Means presented herein correspond to non-transformed data whereas SEM correspond to transformed data.

Prior to conducting the statistical analyses, correlations were made to identify factors that may have influenced permeability, such as colostrum IgG concentration and bacterial load. Factors significantly correlated with permeability were included as covariates in the statistical model. Then, data were analyzed with a mixed-effects model for repeated measures. The statistical model included initial BW, colostrum bacterial load, and colostrum IgG concentration as covariates, type of animal (healthy or diarrheic), day of study, and the 2-way interaction between type of animal and day of study as fixed effects, plus animal as a random effect.

3.3. Results and discussion

3.3.1. Performance

Even though diarrhea is usually associated with weight loss in calves (Lewis and Phillips, 1978), no differences were found on BW, ADG, and total BW gain between calves that experienced diarrhea for at least three consecutive days and those that did not (data not shown). As most of the diarrhea episodes appeared between 7 to 14 d of age, monitoring BW until 21 d of life may have been too short of a period to detect any potential impact of diarrhea on growth. Furthermore, diarrhea incidences in the current study were relatively mild (with a mean fecal score between 7 to 14 d of study of 1.1 ± 0.07 for healthy and 2.2 ± 0.07 for diarrheic calves) and other authors have reported no significant impact of mild diarrhea on growth of young calves (Woode, 1978).

3.3.2. Colostrum quality and IgG transfer

Colostrum quality (bacterial load and IgG concentration) and IgG absorption are depicted in Table 3.1. Concentrations of IgG in colostrum consumed by diarrheic and healthy calves did not differ and in all cases they were much greater than the threshold

of 50 mg/mL, which is commonly used to classify colostrum as good quality (McGuirk and Collins, 2004; Chigerwe et al., 2008). Concentration of IgG in late colostrum was also similar for all calves. Moreover, there were no differences in total bacteria and enterobacteria counts in first- or late-colostrums (or transition milk) offered to healthy and diarrheic calves. McGuirk and Collins (2004) concluded that colostrum with a mean total bacterial load over 100,000 CFU/mL should be considered of low quality. All first colostrums used herein had bacterial loads slightly above this concentration, but transition milk was well below this figure (Table 3.1).

Table 3.1. Colostrum quality parameters and transfer of IgG in calves classified either as healthy or diarrheic.

	Calf classification			*P*-value[1]		
	Healthy	Diarrheic	SEM	C	t	Cxt
First colostrum						
IgG, mg/mL	101.5	100.5	8.52	0.94	-	-
Total bacterial load, CFUx10^6/mL	1.47	2.73	0.343	0.58	-	-
Enterobacterial load, CFUx10^4/mL	6.12	1.43	0.317	0.12	-	-
Late colostrum or transition milk						
IgG, mg/mL	17.5	18.1	2.84	0.89	-	-
Total bacterial load, CFUx10^4/mL	4.67	1.71	0.199	0.13	-	-
Enterobacterial load, CFU x10^4/mL	7.42	2.97	0.349	0.42	-	-
Plasma IgG, mg/mL[2]	15.2	15.1	1.15	0.98	<0.001	0.86
IgG absorption, %[3]	14.3	17.8	2.51	0.29	<0.01	0.12

[1]C: effect of calf classification (healthy or diarrheic); t = effect of sampling time; Cxt = interaction between calf classification and sampling time.

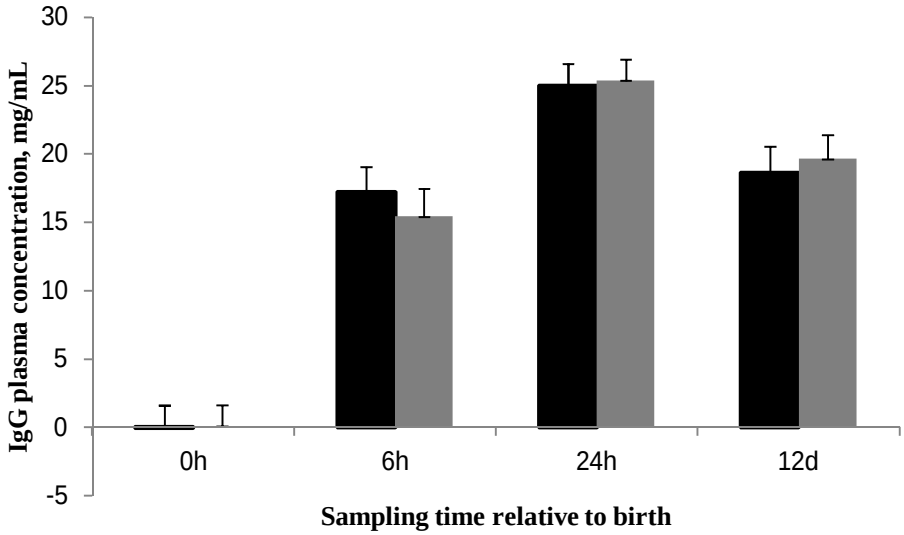
[2]Measured at birth, at 6 h, 24 h, and 12 d of life.

[3]Calculated at 6 h and 24 h of life. See materials and methods for calculation details.

It has been suggested that the presence of bacteria in the small intestine may impair IgG absorption (James and Polan, 1978 and 1981; Staley and Bush, 1985). However, plasma IgG concentrations at birth, 6 h, 24 h, and 12 d relative to first colostrum intake were

similar for all calves (Figure 3.1), suggesting that diarrhea incidences found herein may not have been related to differences in plasma IgG concentration. This is consistent with Rajala and Castrén (1995) who also found no differences in plasma Ig concentrations when comparing diarrheic and non-diarrheic calves. The lack of differences in plasma IgG found in the present study was probably due to the lack of differences found in first- and late-colostrum in bacterial loads and IgG concentrations between healthy and diarrheic calves. Moreover, an adequate IgG transfer is considered to be achieved when serum IgG concentrations are ≥ 15 mg/mL (Furman-Fratczak et al., 2011), and the mean plasma IgG concentration found herein at 24 h in both diarrheic and healthy calves was 24.9 and 25.4 mg/mL, respectively. Therefore, it could be concluded that all calves involved in the study had a correct passive transfer. It has been previously reported that calves with low plasma gamma globulin concentrations have a greater risk of incurring diarrhea (Gay et al., 1965; Thornton et al., 1972). However, even though all calves in the present study had a correct passive transfer, some presented diarrhea.

Figure 3.1. Evolution of plasma IgG concentrations in calves that were classified as healthy (black) and diarrheic (grey). Bars denote SE of the mean at each time point.



3.3.3. Intestinal permeability

Mucosal integrity in the present study was measured by the lactulose/mannitol test (Uil et al., 1997; Hall, 1999). The principle of the test is based on the assumption that the monosaccharide D-mannitol passes the intestinal epithelia through small pores on the apical surface of enterocytes (transcellularly), whereas the disaccharide lactulose passes

the epithelia through the tight junctions located between the epithelial cells (paracellularly). However, differently from previous studies that measured intestinal permeability of calves by quantifying D-mannitol and lactulose in urine (Branco Pardal et al., 1995; Klein et al., 2007 and 2008), in the present study both saccharides were quantified in serum. Even though extracting blood is an invasive technique, in farm animals it is more practical than collecting urine.

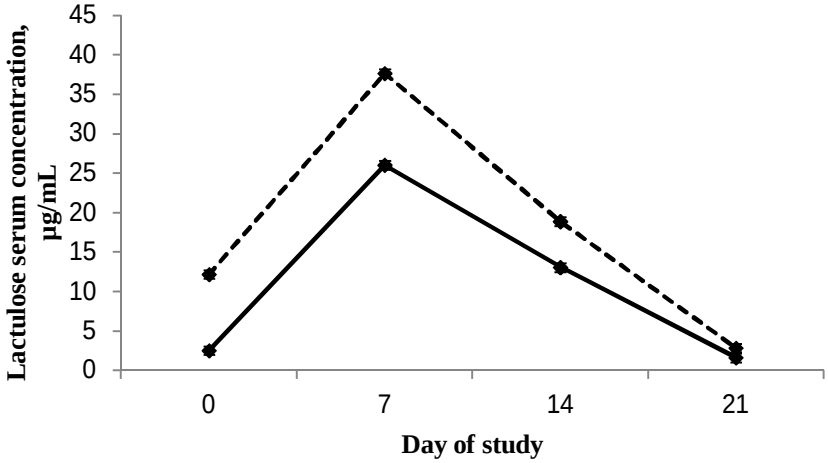
Mean D-mannitol serum concentrations were similar between the two types of calves. However, serum lactulose concentrations were greater ($P < 0.01$) for diarrheic than for healthy calves throughout the 21 d of study (Table 3.2). This observation suggests that paracellular permeability was high in diarrheic calves from the first day until day 21 of life. In fact, even though the interaction with time was not significant (Table 3.2), the evolution throughout the study of the serum lactulose of healthy and diarrheic calves (Figure 3.2) suggests that the largest differences may be due to days 0 and 7. Therefore, it seems that calves that presented diarrhea were more predisposed to scouring right after birth, as they were apparently born with an increased intestinal permeability compared with calves that did not incur diarrhea throughout the study.

Table 3.2. Permeability parameters of calves classified either as healthy or diarrheic, measured at 0, 7, 14 and 21 d of age.

	Calf classification			*P*-value[1]		
	Healthy	Diarrheic	SEM	C	t	Cxt
Serum D-mannitol, µg/mL	9.6	9.9	0.02	0.61	<0.001	0.80
Serum lactulose, µg/mL	8.4	15.3	0.37	0.01	<0.001	0.45
Serum lactulose to D-mannitol ratio	0.81	1.26	0.161	0.06	<0.001	0.51

[1]C: effect of calf classification (healthy or diarrheic); t = effect of sampling time; Cxt = interaction between calf classification and sampling time.

Figure 3.2. Evolution of serum lactulose of calves that were classified as healthy (solid line) or diarrheic (dashed line). Bars denote SE of the mean (as obtained from the statistical model using square root transformed data to achieve normality) at each time point.



Calves that were born with increased intestinal paracellular permeability presented diarrhea between 7 and 14 d of life. This is consistent with the study by Klein et al. (2008) who reported an increased intestinal permeability after a Cryptosporidium parvum challenge. Surprisingly, the novel finding in the current study is that calves that incurred diarrhea had an increased intestinal permeability well before the scours occurred. Reasons that could influence gut permeability at birth are unknown. Effects of nutrition of the calf may be considered; however, since permeability tests were performed 1 h after first colostrum intake, it is unlikely that factors in colostrum may have affected intestinal permeability. Moreover, colostrum storage differences (fresh, refrigerated or frozen) had no influence on the incidence of diarrhea, as indicated by a Chi-square test (69 vs 31% for fresh, 66 vs 34% for refrigerated and 48 vs 52% for frozen colostrum, for healthy and diarrheic calves, respectively). However, we speculate that one factor affecting intestinal permeability at birth may be differences in the establishment of commensal bacteria, which are present in various maternal tissues and fluids in the GIT of calves during delivery (Taschuk and Griebel, 2012). Moreover, the genome of the host may also influence the commensal bacterial population, which may have an influence on the establishment of the gastrointestinal microbiota (Benson et al., 2010; Mayer et al., 2012). Other potential reasons could be nutritional and/or immune status of the dam. For instance, González-Recio et al. (2012) described that the

physiological status of the dam (primiparous vs multiparous) during pregnancy had an impact on milk production and longevity of the offspring. Furthermore, Bach (2012) reviewed the potential long-term effects of the parity of the dam on milk production, and metabolic and immune functions, and described several differences between offspring born to primiparous and offspring born to multiparous, and highlighted the potential consequences of parity and stress and metabolic load of the dam on IgG passive transfer. For example, Meyer et al. (2010) illustrated that a moderate (i.e., ~80% of recommendations) nutrient restriction during early- to mid-pregnancy of beef cattle altered the jejunal proliferation and total intestinal vascularity of the fetus, which could alter the capacity for IgG absorption. In the current study, calves born to multiparous dams (n = 17) had a lower ($P < 0.05$) efficiency of IgG absorption at 6 h after first colostrum consumption (9.0 ± 3.2%) than calves born to primiparous (n = 13) dams (19.4 ± 2.99%), and calves born to multiparous dams tended ($P = 0.09$) to have a lower efficiency of IgG absorption at 24 h after first colostrum consumption (17.6 ± 2.27%) than calves born to primiparous dams (23.5 ± 2.59%). However, there were no differences ($P = 0.71$) in the incidence of diarrhea between calves born to primiparous (46.2 ± 0.14%) or multiparous dams (52.9 ± 0.13%).

Serum lactulose:D-mannitol ratios found herein are within the range of those reported by Branco Pardal et al. (1995), but greater than those reported by Klein et al. (2007 and 2008). Intestinal integrity can be estimated by the serum lactulose:D-mannitol ratio, which is based on the assumption that a normal villus may take up many small but few large molecules. Therefore, a low serum lactulose:D-mannitol ratio would be indicative of adequate intestinal integrity; whereas a high serum lactulose:D-mannitol ratio would be indicative of damaged villi, which may be more permeable to large molecules (Hall, 1999). An increase in serum oligosaccharide to monosaccharide ratio (assessed by the lactulose:D-mannitol ratio) has been frequently ascribed to intestinal villus flattening in both humans and animals (Strobel et al., 1984; Hall and Batt, 1991; Quigg et al., 1993). In the present study, serum lactulose:D-mannitol ratio tended ($P = 0.06$) to be greater in diarrheic than in healthy calves, indicating a tendency towards a poorer intestinal integrity as indicated by a greater absorption of large particles (Travis and Menzies, 1992). Typically, the absorption of small molecules by the intestinal epithelium is mostly affected by a reduction of the absorption surface (Hall, 1999). However, no differences were found in transcellular permeability between diarrheic and healthy

calves as serum D-mannitol concentrations were not different between both types of calves. Therefore, results suggest that, compared with healthy calves, the absorption surface of diarrheic calves might have not been affected, whereas the barrier function of the intestinal mucosa might have been damaged (i.e., increased paracellular permeability), because intestinal integrity tended to be poorer and paracelluar permeability was greater than in healthy calves.

Diarrhea may be due to several factors including enteric pathogens, which may affect different segments of the gastrointestinal tract. In the present study, intestinal permeability was assessed by the lactulose/mannitol test which has been validated to assess small intestine permeability in many species including calves (Hall, 1999; Klein et al., 2007). For instance, lactulose may reflect small intestine permeability since it is rapidly metabolized by colonic bacteria (Hall, 1999) and flux rates of mannitol have been shown to be greatest in the jejunum of calves (Penner et al., 2014). Therefore, it could be concluded that permeability results in the present study correspond to small intestine, and may not be representative of the permeability of the whole GIT.

3.3.4. Intestinal permeability and colostrum quality

Stott et al. (1979) found that intestinal closure in calves was shortened after colostrum intake as a potential mechanism to avoid pathogen entrance. Even though in the present study IgG absorption was similar for diarrheic and healthy calves (Table 3.1), intestinal permeability was greater for diarrheic than for healthy calves throughout the study (Table 3.2, Figure 3.2). This observation suggests that colostrum consumption was unable to promote intestinal closure in diarrheic calves, which had already greater paracellular permeability within the first 3 h after birth (when the first permeability test was conducted) than calves that never incurred diarrhea later on in life. Although the interaction of IgG absorption with time was not significant (P = 0.12), it can be observed in Figure 3.3 that the largest differences may be found at 6 h after birth, in which diarrheic calves had increased IgG absorption, coinciding with the increased paracellular permeability of day 0.

Interestingly, in diarrheic calves, there were no correlations between either serum lactulose or D-mannitol concentrations and IgG content in first- and late-colostrums (Figures 3.4A, 3.5A, and 3.6A). In contrast, in healthy calves, serum lactulose concentrations tended (P = 0.07) to be negatively correlated with IgG concentrations in

late colostrum (Figure 3.4B) on day 21 of study, and D-mannitol (Figure 3.5B) concentrations, as well as serum lactulose to D-mannitol ratio (Figure 3.6B) on day 21 of study were negatively ($P < 0.05$) correlated with late colostrum IgG concentrations, suggesting a regular response to colostrum intake on intestinal closure in healthy calves, but a deficient or unresponsive closure in diarrheic calves. Reasons for this outcome are unknown and deserve further study.

Figure 3.3. Efficiency of intestinal absorption of IgG from colostrum at 6 and 24 h after birth in calves that were classified as healthy (solid line) or diarrheic (dashed line). Bars denote SE of the mean at each time point.

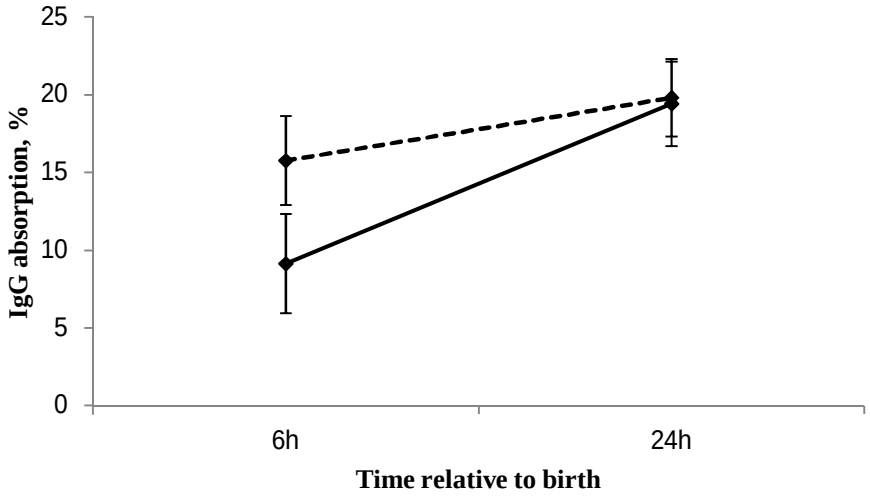
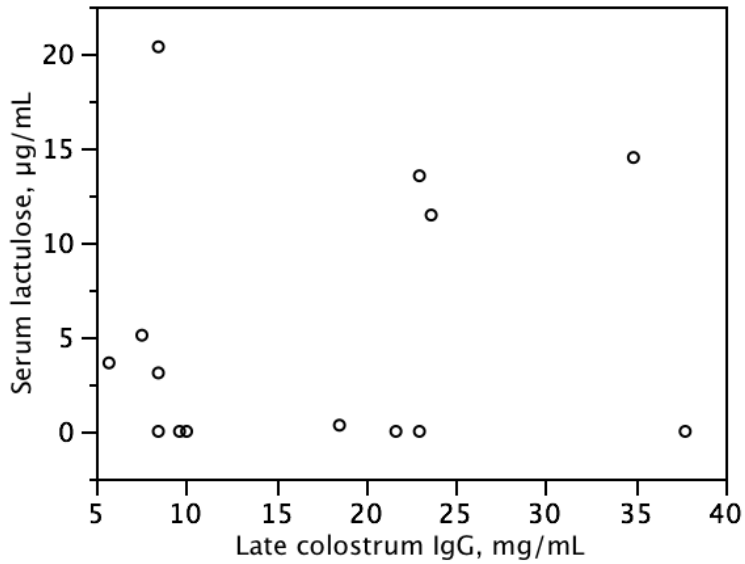


Figure 3.4. Correlation between concentration of IgG in late colostrum or transition milk and serum lactulose concentration at day 21 of study for diarrheic (A) and healthy (B; $R^2 = 0.44$; $P = 0.07$) calves.

A)



B)

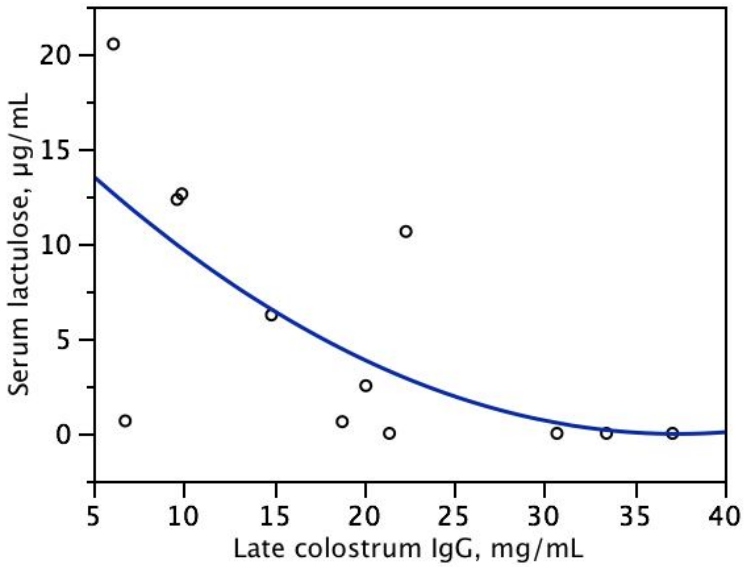
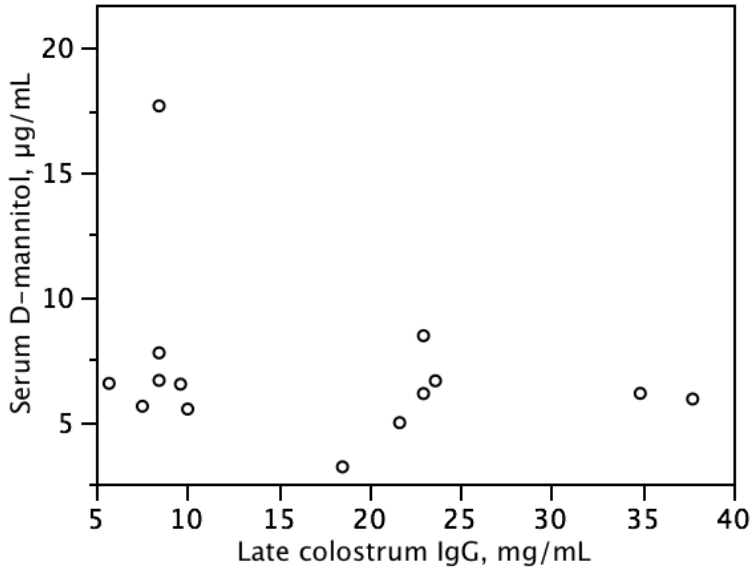


Figure 3.5. Correlation between concentration of IgG in late colostrum or transition milk and serum D-mannitol concentration at day 21 of study for diarrheic (A) and healthy (B; $R^2 = 0.49$; $P < 0.05$) calves.

A)



B)

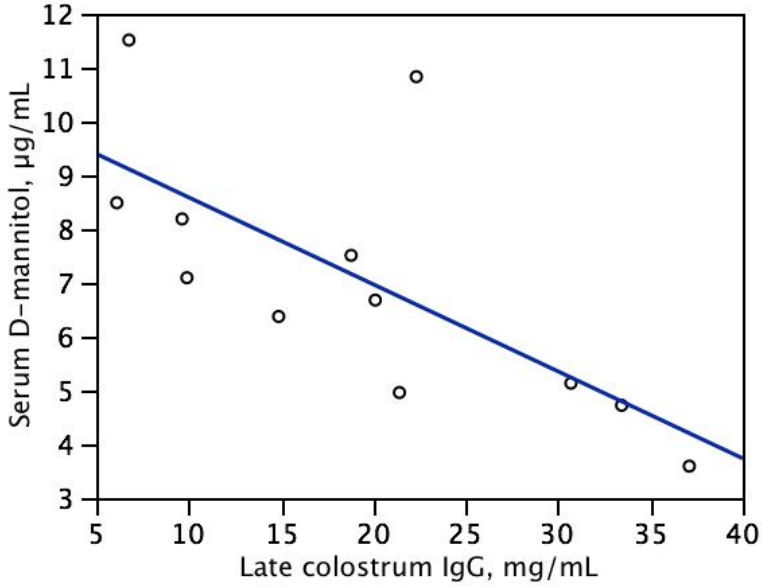
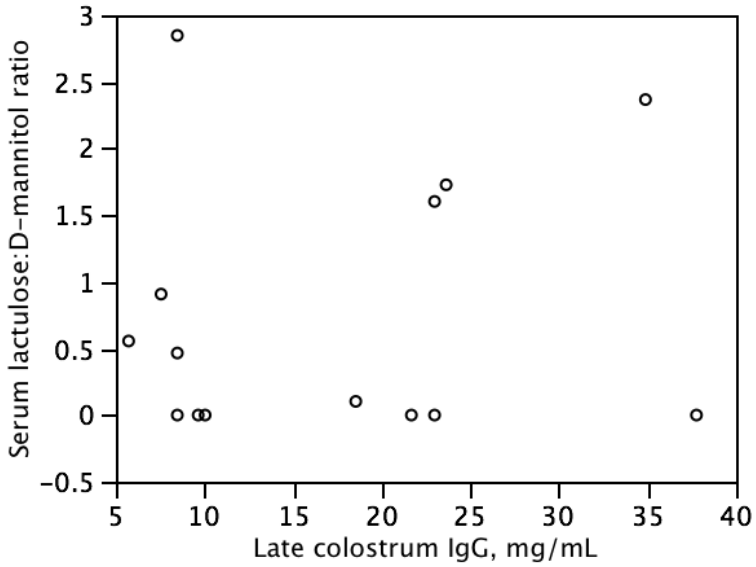
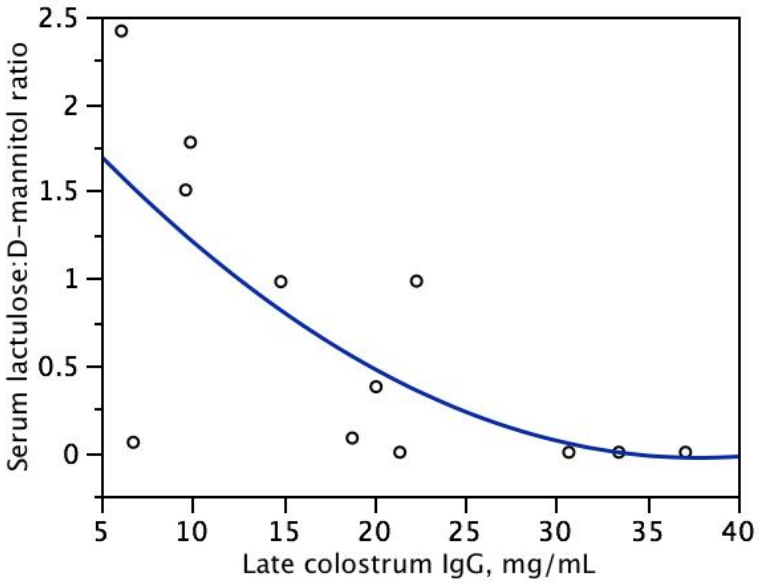


Figure 3.6. Correlation between concentration of IgG in late colostrum or transition milk and serum lactulose:D-mannitol ratio at day 21 of study for diarrheic (A) and healthy (B; $R^2 = 0.48$; $P < 0.05$) calves.

A)



B)



No differences were found in total bacterial and enterobacterial load in first- and late-colostrum samples fed to healthy and diarrheic calves. However, diarrheic calves had greater paracellular permeability compared with healthy calves. Thus, the increased intercellular space (responsible for the increased paracellular permeability) may have enabled environmental bacterial present in colostrum or MR to translocate into the lamina propria and impaired intestinal barrier function leading to diarrhea (Swank and

Deitch, 1996). Furthermore, some strains of commensal bacteria may have also affected the intestinal permeability by modulating the expression and distribution of the tight junction proteins, which seal the paracellular space between the epithelial cells (Ulluwishewa et al., 2011). For instance, it has been found that *E. Coli* given to calves before first colostrum intake could penetrate the intestinal epithelia, whereas given along with first colostrum or 1 h after were unable to colonize the intestinal epithelia (Corley et al., 1977). Therefore, even though all calves received their first colostrum within 2 h after birth, another possible explanation for the increased intestinal permeability in newborn calves may be an early invasion of enterobacteria previous to first colostrum intake.

3.4. Conclusions

In calves with a correct IgG passive transfer, intestinal paracellular permeability is greater in those that incur diarrhea than in those that do not suffer scours from birth until at least 21 d of life. Thus, calves that incur diarrhea may be predisposed to suffer scours due to altered intestinal permeability within the first 3 h of life. The factors modulating intestinal permeability in newborn calves need further investigation.

3.5. Acknowledgements

Authors would like to thank Lucta SA for partial funding to conduct this research. Also, a special thanks for Josep Lluís and Míriam Allué (Allué Dairy, Lleida, Spain) for allowing the performance of this study in their facilities. Last, a special thanks to José J. Pastor from Lucta for conducting the laboratory determinations of serum lactulose and D-mannitol.

Chapter 4

EVALUATION OF THE EFFECTS OF SUPPLEMENTING *L. RHAMNOSUS* ON PERFORMANCE, FECAL MICROBIOTA AND HEALTH PARAMETERS OF HOLSTEIN CALVES AT WEANING

4.1. Introduction

Weaning is a widely recognized stressor for the young calf and a critical period affecting their future health and growth. Stress during weaning has been associated with a depression of the humoral (Gwazdauskas et al., 1978; Pollock et al., 1992 and 1994) and cellular (Pollock et al., 1993) immune response. For this reason, research has focused on management practices and feed additives to reduce the stress and its immunosuppressant effects during weaning.

The microflora of the GIT plays a key role on the immune system and the nutritional status of the host (Frick and Autenrieth, 2013). A probiotic is a living microorganism that after ingested contributes to the homeostasis of the gastrointestinal bacterial flora, thus improving animal health in different ways, such as inhibiting pathogenic bacteria, modulating the immune response and improving intestinal integrity (Howarth and Wang, 2013) Moreover, gut microbial population can modulate performance parameters. In fact, high *Firmicutes/Bacteroidetes* ratios in gut microflora have been associated with increased weight gain in mouse (Ley et al., 2005) and calves (Oikonomou et al., 2013).

Gastrointestinal beneficial effects of lactic acid bacteria as probiotics have been tested in calves with promising results in growth and health (Timmerman et al., 2005; Frizzo et al., 2012; Signorini et al., 2012; Qadis et al., 2014a,b). *Lactobacillus rhamnosus* is a lactic acid bacteria belonging to the *Firmicutes* phyla that has been extensively tested as a probiotic in farm animals. Even though it is isolated from the human GIT, it has been shown, to survive the GIT of dogs (Weese and Anderson, 2002) and calves (Ewaschuk et al., 2004) and to have beneficial effects on the health of piglets (Zhang et al., 2010; Zhu et al., 2014). Moreover, *L. rhamnosus* has positive effects on ex vivo and in vitro bovine intestinal cultures. It regulates the inflammatory response after an infection with *E. coli* EPEC and also modulates the basal inflammatory response and enhances cell viability (Fàbregas et al., 2013). However, to our knowledge, the effects of the direct-fed *L. rhamnosus* on the gastrointestinal microbiota and the modulation of the immune response have not been investigated in cattle.

It is hypothesized that *L. rhamnosus* may be a promising probiotic for calves to reduce the detrimental effects of stress on health that may occur at weaning. Then, the objective

of the present study is to describe the effects of the supplementation with *L. rhamnosus* on immune function of dairy calves at weaning.

4.2. Materials and methods

4.2.1. Animals and treatments

Sixty-six Holstein male calves (75 ± 12.0 kg of BW and 64 ± 5.3 d of age) that were raised in the facilities of IRTA at Torre Marimón (Caldes de Montbui, Spain) were involved in the study. Calves were kept in individual hutches (1.6 × 1.0 m) bedded with sawdust, and managed according to the guidelines of the Animal Care Committee of IRTA. Previous to the beginning of the trial, calves had been fed 4 L/d of MR (22.4% CP and 20.5% fat of DM) for 5 weeks and 2 L/d of MR for an additional week. The day after, on day 0 of the present study, calves were immunized against ovalbumin containing 0.5 mg of crystallized ovalbumin from chicken egg white (Grade VII, Sigma-Aldrich, Saint Louis, MO, USA) and 0.5 mg of coadjuvant Quil-A (Brenntag, Fredderikssund, Denmark) in 1 ml of saline solution for later analyses of plasma antibody titers. Then, animals were randomly assigned to three treatments consisting of: no supplementation (CT), $1x10^7$ cfu/50kg of BW (LD), or $1x10^9$ cfu/50kg of BW (HD) of *Lactobacillus rhamnosus* suspended in saline solution (< 1.5 ml final volume) mixed with 0.5 L of MR (to ensure calves had the complete probiotic dose) once a day in the morning for 14 consecutive days. Animals were offered water, starter feed (Table 4.1) and straw at libitum throughout the 21 d of the study.

Table 4.1. Starter feed ingredients and chemical composition.

Item	Composition, % of DM
Ingredient	
Corn	34.60
Corn gluten feed	15.00
Wheat middlings	14.50
Soybean 47	10.90
Barley	9.50
Caromic	3.50
Wheat	3.00
Palm fat	2.09
Beet pulp	2.00
Whole soybean	2.00
Calcium carbonate	1.86
Premix[1]	0.60
Sodium chloride	0.32
Nutrient	
CP	15.6
NDF	18.8
ADF	7.5
EE	5.8
Ash	5.8

[1]Premix composition: vitamin A 14,999 IU/kg; vitamin D3 2,820 IU/kg; vitamin E 15 ppm; Mn 50 ppm; Fe 7,093 mg/kg; Cu 8 ppm; Co 0.8 ppm; Zn 49 ppm; I 0.7 ppm; Se 0.3 ppm.

4.2.2. Measurements and sample collection

Individual starter feed and straw intake were recorded daily and BW was measured weekly to adjust the probiotic dose. Blood and fecal samples were collected on days 0, 14 and 21.

Blood samples were taken from the jugular vein using 10-ml tubes containing Na-heparin (BD Vacutainer® spray-coated with Na-heparin tubes) for plasma separation

and immediately placed on ice for at least 20 min. One ml subsample of whole blood was placed in a 1.5-ml eppendorfs, centrifuged at 3,500 x g at 4°C for 15 min, then plasma was decanted and the blood pellet was stored at −20°C until later RNA extraction. The rest of the whole blood of all samples was also centrifuged at 3,500 x g at 4°C for 15 min, and plasma was decanted, divided in two different 1.5-ml eppendorfs and stored separately for later analyses of total antioxidant capacity (−80°C) and antibody titers against ovalbumin (−20°C) until subsequent analysis.

Fecal samples were collected with sterile swaps and immediately stored at −20°C for later DNA extraction.

4.2.3. Sample processing and analyses

4.2.3.1. RNA

Total RNA was extracted from blood cells using Trizol® (Invitrogen) following manufacturer's instruction and quantified using Nanodrop. The RNA was retrotranscribed to DNA using IScript cDNA synthesis kit (Bio-Rad, California, USA). Quantitative PCR was performed for genes encoding for ACTB, IL-6, IL-8, IL-10, TGF-β, TNF-α, INF-γ, IL-1β and NF-kβ using iQ5 Termocycler (Bio-Rad) and qPCR conditions for each set of primers were individually optimized (Table 4.2). The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in DNA agarose gel and a single peak in the qPCR melting curves. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve of Ct versus log concentration was plotted to obtain the efficiency, which is calculated using the formula 101/slope, with an acceptable range of 1.8-2.2 (Chow et al., 2010). A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µ of SYBER Green Fluorescent (Bio-Rad), and the optimized primer concentration for each gene (Table 4.2). The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 15 s at optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C. Relative gene expression was calculated using the 2ΔCt method with ACTB as reference gene.

Table 4.2. Primers optimization.

Gene	Primer Fw (5'-3')	Primer Rv (5'-3')	C[1], µM	A[2], bp	HT[3], ºC	E[4], %
ACTB	CTGGACTTCGAGCAGGAGAT	CCCGTCAGGAAGCTCGTAG	0.125	75	57	82
IL-1b	TGGGAGATGGAAACATCCAG	TTTATTGACTGCACGGGTGC	0.313	232	50	82
IL-6	GGGCTCCCATGATTGTGGTA	GTGTGCCCAGTGGACAGGTT	0.5	69	51.5	86
IL-8	TTGAGAGTGGGCCACACTGTG	TGCACCCACTTTTCCTTGG	0.5	93	55	94
IL-10	ACTTTAAGGGTTACCTGGGTTG	GAAAGCGATGACAGCGCCGC	0.5	170	57	90
INF-g	ATAACCAGGTCATTCAAAGG	ATTCTGACTTCTCTTCCGCT	0.5	217	50	92
NFkb	TAACTCTGTTTTGCACCTCGCT	TGGCTACATGGATGGCATTC	0.5	131	56	92
TGFb	TGAGCCAGAGGCGGACTACT	TGCCGTATTCCACCATTAGCA	0.5	61	60	91
TNF-a	AACAGCCCTCTGGTTCAAAC	TCTTGATGGCAGACAGGATG	0.5	296	60	89

[1]C: concentration

[2]A: amplicon size

[3]HT: hybridization temperature

[4]E: efficiency

4.2.3.2. DNA

Each fecal swab was placed in 1 ml of sterile phosphate-buffered saline (PBS) and vortexed for at least two minutes. The swab was then removed and the sample centrifuged for 10 min at 13,200 g. The supernatant was discarded and the remaining pellet was used for immediate DNA extraction. Total fecal microbial DNA were extracted using the RBB+C method which employs bead beating in the presence of high concentrations of sodium dodecyl sulphate (SDS), salt and EDTA and subsequent DNA purification with QIAamp columns (QIAGEN). Concentration of DNA of all samples was determined by nanodrop (Nanodrop® ND-1000, Thermo Scientific, Wilmington, DE, USA) and 20µl aliquots of pooled samples balanced by DNA concentration (from 4-5 animals of the same treatment) were obtained and sent to Molecular Research ADN (Shallowater, TX, USA) for diversity assays. There, the 16S rRNA gene V4 variable region PCR primers 515/806 with a barcode (or tag) on the forward primer were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple

samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at Molecular Research DNA (Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using a proprietary analysis pipeline (Molecular Research DNA, Shallowater, TX, USA). In summary, sequences were joined, depleted of barcodes then sequences <150bp removed, and sequences with ambiguous base calls removed. Sequences were denoised, and OTUs were defined by clustering at 3% divergence (97% similarity) and chimeras removed. Final OTUs were taxonomically classified at Molecular Research DNA (Shallowater, TX, USA) using BLASTn against a curated database derived from GreenGenes. A selection of the most abundant OTUs were taxonomically reclassified at IRTA (Caldes de Montbui, Spain) using the RDP Classifier 2.2 with a bootstrap cutoff of 80% (Wang et al., 2007).

4.2.3.3. Decanted plasma

Plasma was analyzed for antibodies specific for ovalbumin by indirect ELISA using Maxisorp 96-microtiter plates (Nunc, Roskilde, Denmark) coated with 0.015 mg of ovalbumin per well. The plate was incubated overnight at 4ºC to allow ovalbumin to adhere to the wells. Following the 18 h incubation the plate was emptied and washed three times with 200 µl PBS-0.05%Tween 20 (PBS-T) and further blocked with PBS-T for 2 h at 37 ºC. One hundred µl of serum samples from d 0, 14 and 21 were added to the plate at dilution 1/160 with PBS-T. This dilution was previously determined with a minimum of 6 different animals as the dilution giving the maximal signal. The plate with diluted serum was incubated for 1 h at 37 ºC and then washed three times with PBS-T. Horseradish Peroxidase anti-bovine IgG (A5295 Sigma Aldrich, St Louis, Missouri) was diluted 1:20,000 with PBS-T and 100 µl were added to the wells and incubated for 1 h at 37 ºC. After 3 times PBS-T washes the HRP reaction was developed with 100 µl of TMB substrate (Sigma Aldrich, St Louis, Missouri) and stopped with Stop Reagent for TMB substrate from Sigma Aldrich, St Louis, Missouri. Finally, the ELISA plate was read at 450 nm. All samples were analyzed by duplicate and the non specific binding that occurred at day 0 was subtracted to the signals obtained at days 14 and 21. Variations in readings among different ELISA plates were

corrected by normalizing the readings from each sample within a plate to a reference control sample included in each plate.

Total antioxidant capacity of plasma samples from days 14 and 21 was determined using a commercial kit (Antioxidant Assay Kit, Cayman Chemical Company, Ann Arbor, MI, USA).

4.2.4. Calculations and statistics

Performance data were analyzed with a mixed-effects model for repeated measures of days 0, 14 and 21. The statistical model included initial BW and age as covariates and accounted for the fixed effects of probiotic dose, day of measurement, and their interaction, plus the random effect of calf. Data from the total antioxidant capacity test, antibody titers against ovalbumin and mRNA cytokines expression were analyzed with a mixed-effects model for repeated measures of days 14 and 21. Cytokine expression data were previously log-transformed to achieve normality so that the means presented herein correspond to the non-transformed data. The statistical model accounted for the fixed effects of probiotic dose, day of measurement, and their interaction, plus the random effect of calf.

A selection of the 82 most abundant OTUs was performed out of the 1,268 OTUs found in the feces. OTUs were selected when their relative abundance was over 1% and were present in more than one replica for treatment.

Incremental values of the relative abundance from day 0 to days 14 and 21 were calculated for taxonomical phylum, class, order, family, and OTU data from the fecal microbiota. The resulting data were analyzed with a mixed-effects model for repeated measures of the two increments (0-14 and 0-21) except for one taxonomical family that was transformed to absence or presence and analyzed using a Chi-square. The mixed-effects statistical model accounted for the fixed effect of probiotic dose, increment, and their interaction, plus the random effect of calf. Then, all *P*-values were reanalyzed for a FDR of 5% significance level because an abundant number of variables were analyzed at the same time. Moreover, family and OTU data were also analyzed with a principal component multivariate test and variables were grouped in 12 and 16 rotated factors, which explained 86.2% and 89.8% of the variability, respectively. Families and OTUs associated to factors were determined when correlation values were over 0.5. Only

when all families and OTUs were associated to just 1 factor, were the factors considered. Then, 6 families (out of 43) and 14 OTUs (out of 82) that were not associated to the 12 and 16 factors, respectively, were excluded from the model and recalculated. Resulting factors were later analyzed with the same mixed-effects model for repeated measures used for the family and OTU data. All *P*-values were corrected for a FDR of 5% significance level.

4.3. Results

4.3.1. Intake and performance

Results from intake and growth parameters are depicted in Table 4.3.

Table 4.3. Feed and nutrient consumption (DM basis) and performance of calves as affected by the level of supplementation with *L. rhamnosus* in the MR once a day for fourteen consecutive days.

	Treament[1]				*P*-value[2]		
	CT	LD	HD	SEM	T	D	TxD
Starter feed intake, g/d	2,740	2,749	2,585	95.4	0.40	<001	0.71
Straw intake, g/d	65	84	92	13.2	0.16	<001	0.09
Total intake, g/d	2,805	2,833	2,677	100.8	0.54	<001	0.56
BW, kg	90.4	90.5	89.4	0.55	0.29	<001	0.87
ADG, kg/d	1.05	1.06	0.96	0.048	0.29	<001	0.92
Gain to feed ratio	0.40	0.35	0.39	0.024	0.28	0.34	0.95

[1]CT: non supplemented calves; LD: calves supplemented with *L. rhamnosus* in the MR at dose of $1x10^7$ cfu/50kg of BW; HD: calves supplemented with *L. rhamnosus* in the MR at dose of $1x10^9$ cfu/50kg BW.

[2]T: effect of treatment; D: effect of day of study; TxD: interaction between treatment and day of study.

4.3.2. Fecal microbiota

No significant differences were found on the relative abundance of fecal eubacteria of calves among treatments. After the ANOVA analyses, once the *P*-values were corrected for the FDR, no differences were found among treatments in the relative abundance of the incremental values relative to day 0 of eubacteria phylum, class, order, family and OTU. Also, similar results were obtained after grouping family and OTU data in rotated factors among treatments (Tables 4.4 and 4.5).

Table 4.4. Results of the ANOVA of the rotated factors from the Principal Components multivariate analyses associating the incremental values relative to day 0 of the relative abundance of the Eubacteria taxonomical families from DNA sequencing of the fecal microbiota of calves as affected by the level of supplementation with *L. rhamnosus* in the MR once a day for fourteen consecutive days.

	Treament[1]				*P*-value[2]			FDR *P*-value[3]		
Rotated Factor	CT	LD	HD	SEM	T	D	TxD	T	D	TxD
F1	0.06	0.11	-0.17	0.462	0.89	0.23	0.62	0.89	0.68	0.82
F2	-0.24	0.30	-0.15	0.270	0.76	0.67	0.99	0.89	0.94	0.99
F3	-0.33	0.05	0.14	0.552	0.71	0.54	0.33	0.89	0.94	0.60
F4	0.37	0.00	-0.57	0.537	0.30	0.02	0.28	0.89	0.26	0.60
F5	-0.07	-0.06	0.00	0.401	0.40	0.71	0.35	0.89	0.94	0.60
F6	0.12	0.21	-0.32	0.446	0.49	0.66	0.30	0.89	0.94	0.60
F7	0.51	-0.09	-0.33	0.488	0.25	0.78	0.45	0.89	0.94	0.68
F8	0.32	0.09	-0.46	0.450	0.56	0.95	0.17	0.89	0.95	0.60
F9	0.16	0.13	-0.28	0.446	0.85	0.21	0.27	0.89	0.68	0.60
F10	-0.33	-0.08	0.41	0.444	0.85	0.90	0.81	0.89	0.95	0.88
F11	-0.45	0.88	-0.43	0.316	0.02	0.16	0.77	0.23	0.68	0.88
F12	-0.09	0.74	-0.48	0.471	0.11	0.39	0.01	0.65	0.93	0.16

[1]CT: non supplemented calves; LD: calves supplemented with *L. rhamnosus* in the MR at dose of $1x10^7$ cfu/50kg of BW; HD: calves supplemented with *L. rhamnosus* in the MR at dose of $1x10^9$ cfu/50kg BW.

[2]T: effect of supplementation treatment; D: effect of day of study; TxD: interaction between supplementation treatment and day of study.

[3]FDR: *P*-values corrected for the FDR of 5% significance level.

Table 4.5. Results of the ANOVA of the rotated factors from the principal components multivariate analyses associating the incremental values relative to day 0 of the relative abundance of the OTU from DNA sequencing of the fecal microbiota of calves as affected by the level of supplementation with *L. rhamnosus* in the MR once a day for fourteen consecutive days.

	Treament[1]				*P*-values[2]			FDR *P*-values[3]		
Rotated Factor	CT	LD	HD	SEM	T	D	TxD	T	D	TxD
F1	-0.46	0.00	0.40	0.447	0.38	0.28	0.21	0.80	0.61	0.66
F2	-0.29	0.20	0.21	0.535	0.68	0.13	0.91	0.80	0.60	0.91
F3	-0.09	-0.56	0.65	0.408	0.15	0.03	0.32	0.77	0.54	0.66
F4	-0.55	0.19	0.36	0.419	0.30	0.44	0.10	0.80	0.78	0.66
F5	0.39	-0.47	0.09	0.444	0.41	0.87	0.52	0.80	0.87	0.66
F6	-0.02	-0.40	0.31	0.505	0.53	0.80	0.29	0.80	0.87	0.66
F7	0.09	0.30	-0.47	0.493	0.47	0.82	0.53	0.80	0.87	0.66
F8	0.18	0.16	-0.30	0.477	0.71	0.67	0.36	0.80	0.87	0.66
F9	0.17	-0.59	0.42	0.415	0.24	0.76	0.47	0.77	0.87	0.66
F10	-0.63	0.27	0.36	0.422	0.23	0.30	0.47	0.77	0.61	0.66
F11	0.02	0.10	-0.11	0.196	0.75	0.71	0.22	0.80	0.87	0.66
F12	-0.29	0.28	-0.03	0.439	0.64	0.76	0.34	0.80	0.87	0.66
F13	0.30	0.29	-0.49	0.407	0.23	0.19	0.58	0.77	0.60	0.67
F14	-0.59	0.47	0.11	0.356	0.15	0.23	0.44	0.77	0.61	0.66
F15	0.00	0.02	-0.02	0.385	1.00	0.08	0.16	1.00	0.60	0.66
F16	0.27	-0.22	-0.06	0.406	0.70	0.18	0.69	0.80	0.60	0.74

[1]CT: non supplemented calves; LD: calves supplemented with *L. rhamnosus* in the MR at dose of $1x10^7$ cfu/50kg of BW; HD: calves supplemented with *L. rhamnosus* in the MR at dose of $1x10^9$ cfu/50kg BW.

[2]T: effect of treatment; D: effect of day of study; TxD: interaction between treatment and day of study.

[3]FDR: *P*-values corrected for the FDR of 5% significance level.

Some descriptive information can be obtained from the fecal microbiota results before correcting the *P*-values for the FDR. Resulting family associations in factors from the principal components analyses is indicative of similar behavior of the associated families through time and treatments. A rotated factor in which the relative abundance

of *Anaeroplasmataceae* and *Rikenellaceae* families were associated, was most abundant ($P < 0.05$) in the microbiota of calves in the LD treatment, indicating that these 2 families were more relatively abundant in the fecal microbiota of LD compared with CT and HD calves. Indeed, this coincides with the tendencies in the results of the individual ANOVA performed for these 2 families. Another rotated factor including the relative abundances of *Enterobacteriaceae* and *Nitrosomonadaceae* families was increased ($P < 0.05$) from days 0 to 21 in the fecal microbiota of calves in the LD treatment, indicating that the relative abundance of these 2 families increased throughout the study. This coincides with the individual results of the ANOVA performed for the *Nitrosomonadaceae* family. Results from the individual ANOVA of the *Rhodospirillaceae* and *Dermatophilaceae* families indicated greatest ($P < 0.05$) relative abundances for LD and CT calves, respectively, throughout the 0 to 21 d period of study. However, such effect was not seen in the factors associated to any of these 2 families. The purpose of associating families to the principal components rotated factors was to reduce the number of ANOVA analyses. Significant results of factors coinciding with individual ANOVA results of the associated families suggest that, although not significant after the FDR correction, possible effects of treatments may be considered to such families. Descriptive results, then, suggest that supplementing calves with *L. rhamnosus* at a dose of 10^7 cfu/50kg of BW, in the conditions of the present study, had a slight effect on the modulation of the fecal microflora by increasing the relative abundance of *Anaeroplasmataceae*, *Rikenellaceae* and *Nitrosomonadaceae* taxonomical families. However, results are not conclusive due to the lack of statistical significance after the FDR correction.

4.3.3. Immunity indicators

Results from the different immunity indicators measured are depicted in Table 4.6. General immunity status as indicated by the total antioxidant capacity measured in plasma was not affected by the probiotic supplementation. However, responses to vaccine with egg white ovalbumin, as indicated by the measured antibody titers, showed a different pattern through time among treatments. For instance, compared with CT, calves in LD treatment tended ($P < 0.09$) to have lower antibody titers on day 21 compared with day 14 of study.

Inflammation response of calves was evaluated by different inflammatory cytokine expression in mRNA from peripheral blood cells. Cytokines TNF-α, INF-γ, IL-1β, and IL-6 were not expressed in most of the samples and thus they were not analyzed statistically. Expression of anti-inflammatory cytokines TGF-β and IL-10 and pro-inflammatory cytokine IL-8 were similar among treatments.

Table 4.6. Immunity parameters of calves as affected by the level of supplementation with *L. rhamnosus* in the MR once a day for 14 consecutive days.

	Treament[1]				*P*-value[2]		
	CT	LD	HD	SEM[3]	T	D	TxD
Total antioxidant capacity, mM	3.16	3.04	3.10	0.097	0.69	<0.001	0.26
Ovoalbumin antibody titers, Δ Abs 650nm	2.67	2.63	2.75	0.099	0.68	<0.001	0.09
Anti-inflammatory cytokines, RQ[4]:							
TGF-β	0.49	0.82	0.89	2.520	0.42	0.02	0.35
IL-10	0.080	0.062	0.062	0.0033	0.70	0.06	0.49
Pro-inflammatory cytokines, RQ[4]:							
IL-8	0.0024	0.0032	0.0041	0.29497	0.26	0.13	0.17

[1]CT: non supplemented calves; LD: calves supplemented with *L. rhamnosus* in the MR at dose of $1x10^7$ cfu/50kg of BW; HD: calves supplemented with *L. rhamnosus* in the MR at dose of $1x10^9$ cfu/50kg BW.

[2]T: effect of treatment; D: effect of day of study; TxD: interaction between supplementation treatment and day of study.

[3]SEM: SE of the log-transformed mean.

[4]RQ: relative quantification non-transformed mean.

4.4. Discussion

Calves in the present study showed acceptable intake and growth rates values (Table 4.3). Then, supplementing calves with *L. rhamnosus* in the MR at doses of $1x10^7$ and $1x10^9$ cfu/50kg of BW for 14 consecutive days had no detrimental effects on performance of Holstein calves.

4.4.1. Fecal microbiota

L. rhamnosus has been suggested to be a potential probiotic for calves (Ewaschuk et al., 2004) and has been shown to modulate the microflora of supplemented piglets (Zhang et al., 2010; Li et al., 2012). However, to our knowledge, there are no previous results of fecal microbiota modulation in calves supplemented with *L. rhamnosus*. In the present study, no effect was found on the fecal microbiota of calves after *L. rhamnosus* supplementation. This is consistent with previous works with piglets (Trevisi et al., 2011) and veal calves (Timmerman et al., 2005). However, Li et al. (2012) reported an increase in the fecal counts of *Lactobacillus* and *Bifidobacterium* after supplementing piglets with *L. rhamnosus*. Nevertheless, the techniques used in the cited works to detect bacteria in feces may not be comparable to the results from DNA sequencing of the present study. In fact, analyzing the fecal microbiota by DNA sequencing has the limitation that it detects all microorganisms present whether they were active or not, whereas culturing bacteria in selective media, as performed in the previous works cited, can detect only living organisms. Extracting RNA from the fecal samples and sequencing the cDNA would have provided information on the active microorganisms of the fecal microbiota. Even more interesting, simultaneously extracting both DNA and RNA and comparing both diversity results would have given a more complete vision of the potential changes induced by *L. rhamnosus* supplementation. In fact, some researches in our institute are obtaining interesting results from this double analysis and found significant effects from the RNA analyses that were not detected on the DNA sequencing (Viñas, personal communication). Unfortunately, the results reported herein are based on DNA sequencing of all bacteria present in feces, regardless of their biological activity.

Results from phylum, order, class, and family were used for statistical analysis in addition to OTUs. Operational taxonomic units are operational definitions of a species or group of species often used when DNA sequence data are available, corresponding to a cluster of reads with 97% similarity. In the present study, OTUs were taxonomically classified against two different databases (GreenGens and RDP Classifier) and used to describe the fecal microbiota in a more concrete level than that of taxonomical families. Even though some OTU corresponding to *Lactobacillus spp.* were found, no OTU was found corresponding to *L. rhamnosus*. Fecal microflora is assumed to represent only the luminal flora and not the flora associated to the surfaces of the mucosal epithelia which

have been reported to differ consistently, as reviewed by Van den Abbeele et al. (2011). Then, the lack of presence of the *L. rhamnosus* in feces may be indicative of inexistent epithelia colonization. Nevertheless, recovery of *L. rhamnosus* in feces after supplementation has been shown to be temporary in both humans (Alander et al., 1999) and calves (Ewaschuk et al., 2004), even though it actually colonized the mucosa. In fact, Alander et al. (1997) recovered *L. rhamnosus* in colonic biopsies but not in feces, indicating that fecal microbiota is no truly reflective of the mucosal microbiota. Furthermore, it has been demonstrated that microbiota characterisation from fecal samples may be representative of only a portion of the gut microbiome, as microorganisms in the mucosa of the different GIT regions differ substantially (Malmuthuge et al., 2012a and 2014). These findings suggests that the lack of effects of the probiotic supplementation found in the fecal microbiota in the present study may not be indicative of a lack of effect of the *L. rhamnosus* supplementation on the mucosa-associated microbiota of certain gastrointestinal regions.

Moreover, it has been shown that relative abundance of *Lactobacillus*, involved in milk digestion, of the intestinal microbiota of calves is increased during the first 4 weeks of life, after which, it gradually decreases (Oikonomou et al., 2013). Calves in the present study were in average 9 weeks old and had been recently weaned, and relative abundance of the *Lactobacillaceae* family was low (from 2-4%). Then, doses provided in the present study may have been insufficient to notably increase the *Lactobacillaceae* population and thus modulate the intestinal microbiota.

Furthermore, transition from liquid to solid feed diet during weaning has been associated to changes in the microbial community of calves (Malmuthuge et al., 2013). Then, changes in the fecal microbiota induced by diet may have masked the possible microbiota modulation effects of *L. rhamnosus* supplementation in the present study.

4.4.2. Immunity indicators

Temporary stress caused by weaning may have detrimental effects on the immune status of young calves. Despite the proven antioxidant ability of lactic acid bacteria (Lin and Yen, 1999), in the present study, total antioxidant capacity, as a measure of the general immunity status, was not affected by the probiotic supplementation.

However, responses to vaccine with egg white ovalbumin followed a different pattern through time among treatments suggesting a negative effect of supplementing calves with *L. rhamnosus* at dose of $1x10^7$ cfu/50kg of BW on the antibody response to egg white ovalbumin. Trevisi et al. (2011) also found a decreased humoral immunity response as they reported a reduction in blood serum IgA in weaned piglets supplemented with *L. rhamnosus* at the dose of 10^{10} cfu/day for 14 d. In the present study, the discrepancy between the effects of the low and high doses suggests that probiotic supplementation doses may have different effects on the humoral immunity of calves. In fact, contradictory effects of *L. rhamnosus* on humoral immunity after challenging with *E. coli* have been reported among studies and different supplementation doses. Sharma et al. (2014) reported increased antibodies against *E. coli* in aging mice supplemented with *L. rhamnosus* and Zang et al. (2010) described an increase in jejunum and ileum secretory IgA concentration in piglets supplemented with *L. rhamnosus*. Nevertheless, Suda et al. (2014) found no effects on antibody levels after supplementing piglets with *L. jensenii*. Moreover, some (Li et al., 2012; Zhu et al., 2014) have found contradictory effects on health after supplementing piglets with low and high doses of *L. rhamnosus*, although no results were reported of humoral immunity.

The pro-inflammatory cytokine IL-8 was expressed similarly for all animals suggesting a possible health impairment due to the stress of weaning. However, inflammation response of calves in the present study was not affected by *L. rhamnosus* supplementation. Supplementing with probiotics as *Lactobacillus* for a few days has been shown to modulate the inflammatory response after *E. coli* challenge in piglets and mice (Zhang et al., 2010; Li et al., 2012; Suda et al., 2014; Zhu et al., 2014) or in scouring calves (Qadis et al., 2014a). Nevertheless, some reported no advantages (Trevisi et al., 2011). Although *L. rhamnosus* is isolated from the human GIT, it has been shown to survive the GIT of calves and thus it has been proposed as a potential probiotic for calves (Ewaschuk et al., 2004). However, *L. rhamnosus* supplementation, in the conditions of the present study, was insufficient to reduce the inflammation response of calves at weaning (as indicated by the similar IL-8 expression found among treatments), which may have been due to the lack of modulation of the microbiota. In fact, *Faecalibacterium spp.* has been shown to have anti-inflammatory effects by blocking NF-kB activation and IL-8 production (Sokol et al., 2008). In the present

study, however, mRNA expression of the protein involved in cytokine expression, NF-kB, was similar for all calves (data not shown) and no differences were found in the OTU corresponding to *Faecalibacterium spp.* between treatments.

4.5. Conclusions

Supplementing Holstein calves at weaning with *L. rhamnosus* in the MR for fourteen consecutive days at doses of 10^7 and 10^9 cfu/50kg of BW had no effects on health parameters. Doses and duration of the probiotic supplementation provided in the present study were insufficient to elicit an effect on the immune response of Holstein calves at weaning.

4.6. Acknowledgements

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Chapter 5

EFFECTS OF SUPPLEMENTING A MILK REPLACER WITH SODIUM BUTYRATE OR TRIBUTYRIN ON PERFORMANCE AND METABOLISM OF HOLSTEIN CALVES

A fraction of this research has been considered for publication in:

Animal Production Science

5.1. Introduction

Feed additives have been widely investigated as a strategy to promote growth and health of pre-weaned calves. Butyrate, a short chain fatty acid produced during anaerobic fermentation in the GIT of ruminants, has been of interest in the recent years as a potential feed additive for calves and piglets.

Before weaning, calves are fed mostly MR and the abomasum and small intestine are the main sites of feed digestion; thus, the development of those GIT compartments is crucial for nutrient absorption, performance and health of calves. Butyric acid is naturally present in cow's milk (Parodi, 1997) and thus, supplementing with butyrate the MR of calves may be a good strategy to improve intestinal development (Górka et al. 2011b).

Several advantages in performance, health and glucose metabolism have been attributed to butyrate addition both in starter (Górka et al. 2011a) and MR (Hill et al. 2007b; Guilloteau et al. 2009; Górka et al. 2011a) of newborn calves. Some authors have found enhanced growth due to an improvement of the GIT development (Guilloteau et al. 2009, 2010a), which has possibly resulted from increased energy availability for epithelial cells (Baldwin VI et al. 2004) and indirectly from increased secretion of some gut hormones and growth factors (Górka et al. 2011b and 2014; Kato et al. 2011). Other studies have reported reduced incidence of diarrhea and improved health status when supplementing MR with butyrate (Hill et al. 2007b; Guilloteau et al. 2009; Górka et al. 2011a). Lastly, several authors have reported an improvement in glucose metabolism after butyrate supplementation in calves and mice (Guilloteau et al. 2010b; Kato et al. 2011; Vinolo et al. 2012). However, contradictory effects from butyrate supplementation in calves have been found among similar studies, mostly regarding performance parameters (Hill et al. 2007a; Ferreira and Bittar, 2011; Kato et al. 2011) and further investigations are needed to provide a possible explanation for the discrepancies.

Butyrate is usually added to diets in the form of sodium butyrate. However, salts have the disadvantage of having a very short half-life in plasma (Miller et al. 1987, Daniel et al. 1989). Tributyrin is a triglyceride composed of butyric acid and glycerol containing equivalent content of butyric acid than sodium butyrate, on a molar basis. It has a more potent direct effect on cells and maintains greater and longer serum levels than butyrate

salts and thus having more favorable pharmacokinetics than sodium butyrate (Chen and Breitman 1994; Egorin et al. 1999). In fact, tributyrin has been reported to improve performance and intestinal morphology in weaned piglets (Hou et al. 2006). However, to our knowledge, it has not been tested in calves.

The hypothesis of the current study was that butyrate supplemented in the MR of pre-weaned calves would result in improved growth and glucose metabolism with a more pronounced effect when supplied as TRB rather than SB. Then, the aim of the present study was to evaluate the effects of SB and TRB supplementation in the MR on performance, glucose metabolism and blood metabolites of pre-weaned calves.

5.2. Materials and methods

5.2.1. Animals and treatments

Fifty-one Holstein calves (being mean ± SD: 45.7 ± 5.8 kg of BW and 11.8 ± 3.1 d of age) were used for the study. Calves were purchased from commercial farms, raised in the facilities of IRTA (Institut de Recerca i Tecnologia Agroalimentaries) -Torre Marimon (Caldes de Montbui, Spain) and managed according to the recommendations of the Animal Care Committee of IRTA. Before arriving to IRTA, calves were fed colostrum (between 2 and 3 L) and then 2 L of MR twice daily. After arrival, calves were given a broad-spectrum antibiotic (Draxxin, tulathromycin, Zoetis, Madrid, Spain) to prevent respiratory disease and 3 d later, calves were vaccinated against respiratory syncytial virus (Rispoval RS, Pfizer Animal Health). A commercial MR (97.7% DM content, of which 25% CP and 19.2% EE; Sprayfo Excellent 60, Sloten BV, Deventer, Holland) was offered in 2-L bottles twice daily at 0700 and 1700 h. All calves were fed 4 L/d of MR reconstituted to 15% DM during the 6 weeks of study. Calves were allowed a 6-d adaptation period to the new facilities and management. During this period, the DM content of the MR was gradually increased from 10 to 15% DM.

After the adaptation period, animals were distributed according to BW and age into 3 groups: control animals (CTR), tributyrin animals (TRB) that were supplemented with TRB at 0.3% DM (3 g per kg of DM) in the MR, and sodium butyrate animals (SB) that were supplemented with SB at 0.3% DM (3 g per kg DM) in the MR. For all MR offerings, both sodium butyrate and tributyrin were added to the reconstituted MR manually, and tributyrin was previously emulsified in 5 ml of distilled water by mixing

vigorously. After the addition of supplements to the reconstituted MR, bottles were filled and immediately provided to calves. Calves were housed in individual hutches (1.6 × 1.0 m) and bedded with sawdust for the 6-wk study.

All animals were offered *ad libitum* water and starter feed (Table 5.1) in buckets except for 5 calves per treatment which were offered a daily limited amount of the same starter feed (100 g for 2 wk, 150 g for the next 2 wk and 200 g for the last 2 wk of study).

Table 5.1. Ingredient and chemical composition (% of DM) of the starter feed.

Item	Starter
Ingredient	
Barley	11.2
Wheat	24
Corn	22
Soybean meal	24
Soybean hulls	5
Wheat middlings	12
Calcium carbonate	0.5
Dicalcium phosphate	0.3
Sodium chloride	0.8
Mineral-vitamin premix[A]	0.2
Nutrient composition	
CP	20
NDF	19.8
ADF	8.4
EE	3.8
Ash	5.6
NFC	50.8

[A]Premix composition: vitamin A 2,007,000 IU/kg; vitamin D3 433,000 IU/kg; vitamin E3 685 mg/kg; vitamin B1 52 mg/kg; vitamin B2 197 mg/kg; vitamin B6 98 mg/kg; vitamin B12 0.76 mg/kg; vitamin K3 52 mg/kg; nicotinic acid 656 mg/kg; pantothenic acid 394 mg/kg; Mn 5,877 mg/kg; Fe 7,093 mg/kg; Cu 2,026 mg/kg; Co 46 mg/kg; Zn 8,112 mg/kg; I 304 mg/kg; Se 46 mg/kg. NFC = 100 – CP – NDF – EE – ash.

5.2.2. Measurements and sample collection

Starter feed and MR intakes were recorded daily on an individual basis and calves were weighed fortnightly. Diarrhea, electrolyte therapies and other veterinary treatments were recorded. A sample of starter feed and MR were taken at the beginning of the experiment from different bags and immediately sent to an external laboratory (Laboratorio de Mouriscade, Pontevedra, Spain) for chemical analysis.

5.2.3. Blood collection

Blood samples were taken from the jugular vein of all calves involved in the study on days 0, 14, 28 and 42 after overnight fasting and 60 min after MR feeding. Four-milliliter tubes containing EDTA and aprotinin (BD Vacutainer® spray-coated K3EDTA Aprotinin Tubes) for plasma separation were used.

Glucose tolerance tests consisting of an i.v. infusion of 180 mg/kg of BW of glucose were performed in the 5 calves per treatment that were fed a limited amount of starter feed throughout the study on days 0 and 35 of study starting 4 h after the morning offer of MR and the fixed amount of starter feed when plasma glucose and insulin concentrations were expected to be close to basal levels (Kamalu and Trenkle, 1978). Feed offer was limited in these 5 calves per treatment to avoid any interference of potential differences in level of intake on glucose metabolism. An indwelling catheter (Abbocath®-T 18G x 51 mm; Hospira Inc., Lake Forest, IL) was placed in the left jugular vein and used to infuse glucose (Glucose 50% Braun) and to collect blood samples. Blood was harvested into 4 mL evacuated tubes containing a glycolysis inhibitor (BD Vacutainer® Fluoride Tubes) to determine blood glucose concentrations and 4 mL evacuated tubes containing EDTA (BD Vacutainer® spray-coated K2EDTA Tubes) for plasma insulin determinations. Blood samples were taken at -15, -5, 0, 4, 8, 12, 18, 25, 35, 45, and 60 min relative to glucose infusions. Catheter patency was maintained by flushing 5 mL of heparinized saline solution (1,000 USP units of heparin/ml).

5.2.4. Chemical analyses

Samples of MR were analyzed for DM (24 h at 103°C), ash (4 h at 550°C), and N content according to the method of the AOAC (method 988.05; AOAC, 1990) adapted for an automatic distiller Kjeldahl (Kjeltec Auto 1030 Analyzer, Tecator) with copper

sulfate/selenium as a catalyst instead of copper sulfate/titanium dioxide, and for EE following method 920.39 of AOAC (1990) with petroleum ether used for distillation instead of diethyl ether (AOAC, 1990). Samples of starter feed were analyzed for DM (24h at 103°C), ash (4 h at 550°C), CP with Kjeldahl analyses (method 988.05; AOAC, 1990), NDF using sodium sulfite and heat-stable α-amylase (van Soest et al. 1991), ADF following AOAC (1990) method 973.18, and for EE following method 920.39 of AOAC (1990) with petroleum ether used for distillation instead of diethyl ether (AOAC, 1990).

Blood samples were centrifuged at 1,500 x g for 15 min at 4°C and plasma was stored at −20°C until further analysis. Plasma glucose, insulin, GLP-1 and BHBA were determined at an external laboratory (Department of Cell Biology, Physiology and Immunology, Faculty of Bioscience, Universitat Autònoma de Barcelona, Bellaterra, Spain). Concentrations of glucose were measured by hexoquinase method (OSR, Beckman Coulter, Ireland; with an intra- and inter-assay CV 0.6%, CV and 1.6%, respectively), insulin was measured by ELISA (Millipore porcine insulin, St. Charles, Missouri, USA; with an intra- and inter-assay CV of 5.7 and 10.5%, respectively), GLP-1 was measured by RIA (Phoenix Pharmaceuticals, Burlingame, California, USA; with an intra- and inter-assay CV of 2.3% and 7.9%, respectively), and BHBA was analyzed using a colorimetric technique and commercial kit (Randbut, Randox Laboratories Limited, Crumlin, United Kingdom).

5.2.5. Calculations and statistical analyses

Intake data was summarized by week and animal prior to conducting statistical analyses. All statistical analyses were performed using SAS for Windows version 9.3 (SAS Institute, 2012). Data from the 15 limit-fed calves (5 calves per treatment) were not included in statistical analyses of growth performance. Data of 2 *ad libitum* fed calves in the TRB and 1 calf of the SB group were eliminated from the study because the doses of TRB and SB were not fully supplied due to frequent MR refusals during the first 2 weeks of study.

Blood samples collected at -10 and 0 min relative to glucose infusion during the GTT were used to determine baseline concentrations of glucose and insulin. The AUC (concentration/min) for these 2 metabolites was calculated as the increase with respect to the baseline using the trapezoidal method. Then, ICR (%/min) and GCR (%/min)

were computed as described elsewhere (Bach et al. 2013a). Insulin sensitivity was estimated using a simplification of the minimal model (Bergman, 1989) following Christoffersen et al. (2009).

Plasma glucose and insulin concentrations at 0 min before feeding were subtracted from postprandial plasma concentrations (60 min) to later analyze relative changes in these metabolites as affected by dietary treatments. To obtain a normal distribution, data from insulin plasma concentrations and insulin to glucose ratio on days 0, 14, 28 and 42 were log-transformed. Least squares means presented herein for these 2 parameters correspond to non-transformed data, and SE and *P*-values correspond to the results from the mixed-effects model using log-transformed data.

Growth, intake, and blood data were analyzed with a mixed-effects model for repeated measures. For intake data, the repeated measure was the week of study; for growth data, the repeated measure was the 2-week period; for GTT data and blood metabolites the repeated measure was the sampling day. The models included initial BW and age as a covariate and accounted for the fixed effects of supplementation, week of study, and their interaction, plus the random effect of calf within treatment. Following a significant *F* test ($P < 0.1$), least squares means were separated using the PDIFF (probability value for testing difference between two means) option of SAS (SAS Institute, 2012). Lastly, incidence of diarrhea was calculated as the number of calves that had to be treated for diarrhea and data were evaluated using a Chi-square.

5.3. Results

5.3.1. Intake and growth

Performance data of the 15 limit-fed calves are depicted in 5.2. All limit-fed calves had similar intake and growths. Performance data of the *ad libitum* fed calves are depicted in Table 5.3. No differences in starter and total DM intake were found among treatments. Control calves had the greatest BW and had greater ADG than calves in TRB. However, some significant interactions with time were found but results from different weeks are not described in the table. In particular, during the first week of study, calves in the SB treatment had lesser ($P < 0.001$) MR intake (refused more MR) than calves in the TRB treatment, which had lesser ($P < 0.001$) MR intake than CTR

calves. Moreover, calves in TRB treatment tended (P = 0.09) to have lesser BW than CTR at the end of study.

Table 5.2. Feed intake (DM basis) and performance of 15 limit-fed calves as affected by treatments, week of study, and their interaction.

	Treatment[1]				*P*-values[2]		
	CTR	TRB	SB	SEM	Trt	W	TrtxW
MR intake, g/d	478	481	475	8.1	0.87	<001	1.00
Starter feed intake, g/d	112	108	107	7.6	0.89	<001	0.89
Total DMI, g/d	591	585	581	19.6	0.94	<001	1.00
Initial BW, kg	43.1	45.4	43.7	2.63	0.83	-	-
Average BW, kg	51.4	49.9	50.0	1.47	0.73	<001	0.13
Final BW, kg	58.2	56.4	56.1	1.14	0.38	-	-
ADG, kg/d	0.35	0.29	0.29	0.043	0.56	<001	0.57
Gain to feed ratio	0.58	0.51	0.51	0.103	0.87	0.32	0.91

[1]CTR: control diet; TRB: control diet + tributyrin in the MR at rate of 0.3% DM; SB: control diet + sodium butyrate in the MR at rate of 0.3% DM.

[2]Trt: effect of treatment; W: effect of week of study; TrtxW: effect of interaction between treatment and week of study.

Table 5.3. Feed intake (DM basis) and performance of calves as affected by treatments, week of study, and their interaction.

	Treatment[1]				*P*-values[2]		
	CTR	TRB	SB	SEM	Trt	W	TrtxW
MR intake, g/d	487	482	480	2.3	0.07	<001	<001
Starter feed intake, g/d	448	323	416	44.7	0.14	<001	0.20
Total DMI, g/d	935	805	895	45.3	0.13	<001	0.15
Initial BW, kg	45.5	46.4	45.3	1.67	0.88	-	-
Average BW, kg	57.7[a]	54.6[b]	55.2[b]	0.87	0.04	<001	0.09
Final BW, kg	68.0[a]	63.6[b]	65.3[b]	0.95	0.01	-	-
ADG, kg/d	0.53[a]	0.42[b]	0.48[ab]	0.029	0.04	<001	0.16
Gain to feed ratio	0.80	0.68	0.77	0.055	0.29	<001	0.77

[1]CTR: control diet; TRB: control diet + tributyrin in the MR at rate of 0.3% DM; SB: control diet + sodium butyrate in the MR at rate of 0.3% DM.

[2]Trt: effect of treatment; W: effect of week of study; TrtxW: effect of interaction between treatment and week of study.

[a,b]Within rows, means followed by different letters differ significantly ($P < 0.05$).

Calves in the CTR group tended ($P = 0.09$) to have lesser incidence of diarrhea (defined as proportion of animals needing to be treated for scours) than the calves supplemented with TRB or SB (being mean ± SD: 5.6 vs 15.7 ± 4.3%).

5.3.2. Blood parameters

5.3.2.1. Hormones and metabolites

Postprandial blood metabolite concentrations relative to preprandial concentrations are depicted in Table 5.4. Overall, supplementation of TRB or SB in MR had no effect on postprandial glucose, insulin, BHBA and GLP-1 plasma concentrations relative to those found preprandial in calves throughout the study.

Table 5.4. Postprandial (60 min) blood metabolite concentrations of calves relative to preprandial concentrations as affected by treatments, day of study, and their interaction.

	Treatment				*P*-values		
	CTR	TRB	SB	SEM	Trt	D	TrtxD
BHBA, mmol/L	-0.05	-0.05	-0.05	0.010	0.94	0.01	0.22
GLP-1, pg/mL	5.8	4.7	6.4	0.96	0.47	<0.01	0.17
Glucose, mmol/dL	43.3	42.5	41.1	3.39	0.90	<0.001	0.24
Insulin, μU/mL	30.5	22.2	20.3	0.18	0.25	<0.001	0.96
Insulin to glucose ratio, μU/mmol	0.83	0.65	0.72	0.191	0.65	0.04	0.32

[1]CTR: control diet; TRB: control diet + tributyrin in the MR at rate of 0.3% DM; SB: control diet + sodium butyrate in the MR at rate of 0.3% DM.

[2]Trt: effect of treatment; D: effect of day of study; TrtxD: effect of interaction between treatment and day of study.

5.3.2.2. GTT

Data from the two GTTs performed on the 15 limit-fed calves (5 calves per treatment) on days 0 and 35 (when half the amount of MR was offered) of study are depicted in Table 5.5. On day 35 of study, all calves had greater ($P < 0.05$) mean plasma insulin concentration, insulin peak concentration, and a longer time to insulin peak compared with day 0, but the time spent to reach plasma insulin baseline was shortened. No effects of TRB or SB supplementation were found on insulin sensitivity.

Table 5.5. Plasma glucose and insulin responses to an i.v. GTT performed on days 0 and 35 of study on 15 limit-fed calves as affected by treatments, day of study, and their interaction.

	Treatment[1]				*P*-values[2]		
	CTR	TRB	SB	SEM	Trt	D	TrtxD
Glucose, mmol/L	6.24	6.33	5.98	0.16	0.36	0.01	0.10
Insulin, µU/mL	23.72	31.56	20.68	3.72	0.15	0.01	0.86
Glucose AUC, mmol/L x 60 min	66.2	53.7	65.2	7.85	0.47	0.86	0.31
Insulin AUC, µU/mL x 60 min	670.9	1,060	503.4	189.4	0.14	0.23	0.89
Maximum glucose increase, mmol/L	3.81	5.11	4.24	0.532	0.24	0.51	0.19
Maximum insulin increase, µU/mL	34.4	60.3	33.0	9.92	0.13	0.05	0.66
Time glucose baseline, min	43.3	29.8	43.6	5.94	0.21	0.70	0.13
Time to insulin baseline, min	34.2	42.5	41.3	3.98	0.32	0.02	0.42
GCR, %/min	9.28	14.37	8.58	2.47	0.22	0.43	0.25
ICR, %/min	12.27	10.16	10.74	1.39	0.56	0.44	0.28
Insulin to glucose ratio, µU/mmol	3,631	4,935	3,658	662	0.41	0.20	0.96
Insulin sensitivity[3], mL/min x pM^{-1} per kg of BW	2.50	0.97	1.55	0.67	0.29	0.91	0.97

[1]CTR: control diet; TRB: control diet + tributyrin in the MR at rate of 0.3% DM; SB: control diet + sodium butyrate in the MR at rate of 0.3% DM.

[2]Trt: effect of treatment; D: effect of day of study; TrtxD: effect of interaction between treatment and day of study.

[3]Calculated following Christoffersen et al. (2009).

5.4. Discussion

5.4.1. Intake and growth

During the first week of study, SB and TRB supplemented calves refused greater amounts of MR than CTR calves (Table 5.3). As the butyrate additives used in the study were added in the MR, they may have modified the taste and that might have resulted in greater refusals for the supplemented calves during the first week of the study. However, other studies (Hill et al. 2007b; Guilloteau et al. 2009; Kato et al. 2011) have not reported increased MR refusals when sodium butyrate was supplemented in the MR of calves. After the first week of study, all treatments had similar MR intakes, which

indicate a potential adaptation to the potentially altered taste of the MR by the addition of TRB or SB. Nevertheless, calves supplemented with SB and TRB tended to have more incidence of diarrhea than CTR and scouring calves usually have a reduced MR consumption.

The lack of differences in starter and total DM intake found among treatments is in accordance with previous findings (Hill et al. 2007a,b,c; Guilloteau et al. 2009; Ferreria and Bittar, 2011; Górka et al. 2011a; Kato et al. 2011) from studies in which calves were supplemented with sodium butyrate in the MR or the starter feed. Other studies (Hou et al. 2006; Vinolo et al. 2012) also reported no differences in starter feed intake when weaned piglets or mice were supplemented with tributyrin.

Supplementing calves with TRB or SB showed no advantages in performance in the conditions of the current study (Table 5.2). The findings herein are in contrast with previous studies (Hill et al. 2007b; Guilloteau et al. 2009) that reported greater BW and improved feed conversion efficiencies in calves supplemented with sodium butyrate in the MR. However, Guilloteau et al. (2009) supplemented calves from 12 d of age until slaughter. In contrast, other studies have also found no differences in BW (Hill et al. 2007a; Ferreira and Bittar, 2011), feed conversion efficiency (Kato et al. 2011), and Górka et al. (2011b) reported just a tendency for an improved BW when supplementing calves with sodium butyrate. In addition, Vinolo et al. (2012) described a decreased efficiency when tributyrin was supplemented (2 g/kg BW every 48h) to mice fed a high-fat diet.

Moreover, the increased ADG found herein for CTR compared to TRB calves (Table 5.3) is in agreement with previous findings supplementing 10 g/kg for 6 wk in piglets and with 2 g/kg of BW in the commercial diet or every 48h for 10 wk in mice by oral gavage (Piva et al. 2002; Vinolo et al. 2012). However, it is contrast with other reports involving piglets (Hou et al. 2006). Nevertheless, doses of tributyrin used in the mentioned reports were greater than the ones in the current study (2 g/kg BW in mice every 48h, 5-10 g/kg in piglets) and animals were supplemented in the commercial diet or by oral gavage. Differences in doses and way of supplementation among studies may explain the controversial results after adding butyrate to diets. Also, rates of gastrointestinal tissue development might differ across species and thus the duration of

the supplementation may have different effects on the performance parameters in every species.

Despite the described attenuating effects of butyrate on inflammation and mucosal lesion (Saëmann et al. 2000; Vinolo et al. 2011; Vieira et al. 2012), an increase on scouring was found for calves supplemented with TRB and SB suggesting a negative effect of butyrate on the incidence of diarrhea, which in turn may have negatively affected growth. This is in contrast with previous studies where abnormal fecal days decreased in calves that were supplemented with sodium butyrate in the MR, which then resulted in improvements in growth performance (Hill et al. 2007b). Nevertheless, another study (Górka et al. 2011b) found no health improvement when calves were supplemented with sodium butyrate in the MR.

Several studies with piglets and calves supplemented with sodium butyrate have reported advantages in performance, gut peptides and pancreatic secretions and GIT development. Differently from what has been reported in piglets and calves, several studies with mice, have found a decrease in obesity (Gao et al. 2009) and reduced BW (Vinolo et al. 2011) after supplementing butyrate or tributyrin. Gao et al. (2009) attributed this effect of butyrate to the promotion of energy expenditure through enhanced mitochondrial expression of thermogenic markers. Vinolo et al. (2012) also reported increases in energy expenditure and oxygen consumption in mice supplemented with tributyrin possibly by different mechanisms from that of sodium butyrate. To our knowledge, no previous studies have been conducted supplementing TRB to calves, but in this study, performance results are more in accordance to previous work with mice than with calves supplemented with sodium butyrate or with piglets supplemented with tributyrin. Overall, no substantial advantages of SB or TRB supplementation in the MR were found on performance parameters of preweaning calves.

Controversial results among studies may be related to differences in species studied and also doses and duration of the supplementation. In fact, butyrate is a fermentation product naturally present in the forestomach of ruminants and in the colon of monogastrics. Therefore, differences in site of fermentation and diets of ruminants and monogastrics may influence differences in minimal and maximal amounts of butyrate to be used in the GIT beneficially among species. Regarding ruminants, to our knowledge,

only four trials have been conducted supplementing butyrate to calves in similar conditions than the current study (Hill et al., 2007b; Guilloteau et al., 2009; Górka et al., 2011a,b). Differences in experimental designs among studies may explain the controversial results between this study and the rest. For instance, Hill et al. (2007b) supplemented the calves with 3-fold the amount of sodium butyrate of the present study. Even though Guilloteau et al. (2009) supplemented the same dose of sodium butyrate, the MR daily amounts provided and duration of the supplementation was 3-fold those of the present study. However, Górka et al. (2011a,b) supplemented the MR with similar daily amounts of sodium butyrate than the present study. A possible factor affecting GIT development and consequent growth in the present study may have also been starter feed characteristics and resulting butyrate fermentation in the rumen of calves. In fact, rumen development (and consequent implications on growth) has been suggested to be more dependent on starter feed intake than butyrate supplementation (Ferreira and Bittar, 2011). For instance, in the present study, starter feed intake was 3 to 4-fold those in Górka et al. (2011a,b) studies. Also, starter feed NFC in the butyrate study of the present work was 50.8%, whereas in Górka et al. (2011a,b) studies starch content of the starter diet was 39% and butyrate in the rumen is mainly produced by carbohydrates fermentation. In addition, 50% of the starter diet in Górka et al. (2011a,b) studies was composed of corn (in contrast to the 22% of the butyrate study), and corn starch has been shown to be less rapidly fermented than barley or wheat (Philippeau et al., 1999).

5.4.2. Blood hormones and metabolites

The supplementation of TRB or SB in the MR had no effect on blood BHBA concentration in calves (Table 5.4), which is in accordance to previous findings (Ślusarczyk et al. 2010; Ferreira and Bittar, 2011). However, Górka et al. (2011a) reported reduced plasma BHBA during the first week after supplementing SB in the MR. Some authors (Manzanilla et al. 2006; Guilloteau et al. 2009) have speculated that BHBA is not found for sodium-butyrate supplemented pigs or calves in peripheral blood because it is metabolized in the GIT wall and/or in the liver. If true, then butyrate may be used as an energy source directly by the GIT cells which in turn might improve calf performance. However, in the present study no advantages in performance were found for butyrate-supplemented calves. Other authors (Ferreira and Bittar, 2011) have also suggested that rumen development is more dependent on starter intake than on butyrate supplementation.

Glucose-like peptide-1 is an incretin hormone secreted by the L-cells of the intestine in two pulses, with the second pulse occurring about one hour after nutrient load (the time in which blood samples were taken in the current study). It has been demonstrated that GLP-1 regulates intake by delaying gastric emptying and gut motility (see review by Shah and Vella, 2014). Then, similar GLP-1 plasma concentrations reported herein may be related to the lack of differences in starter feed intake among treatments.

Even though some authors (Ferreira and Bittar, 2011; Kato et al. 2011) have reported effects of butyrate on glucose metabolism of calves, in the present study no effects of butyrate in the form of sodium butyrate or tributyrin were found on plasma insulin and glucose concentrations. Butyrate has been shown to increase gluconeogenesis in lactating cows in response to intravenous sodium butyrate supplementation (Anand and Black, 1970). However, in the present study, butyrate was supplemented in the MR, and perhaps the amount of butyrate that actually reached the bloodstream after undergoing metabolism in the gut and liver was insufficient to affect the postprandial insulin to glucose ratio. However, Ferreira and Bittar (2011) reported a progressive reduction of postprandial plasma glucose concentration as calves supplemented with sodium butyrate in the starter feed aged. In addition, Kato et al. (2011) found reduced postprandial plasma insulin concentration after supplementing MR of calves with increasing amounts of sodium butyrate for 42 days. They attributed the lack of a postprandial plasma insulin rise on a possible improvement of insulin sensitivity when supplementing sodium butyrate. In agreement with this hypothesis, Vinolo et al. (2012) also reported improved insulin sensitivity when mice were supplemented with tributyrin. However, in the current study such effects were not found (Table 5.5).

Butyrate and other short chain fatty acids have been suggested to improve glucose metabolism in humans and rodents by increasing pancreatic secretion (Guilloteau et al. 2004 and 2010b) and stimulating GLP-1 secretion (Freeland et al. 2010; Lin et al. 2012; Tolhurst et al. 2012). However, no effect of TRB or SB supplementation on GLP-1 plasma concentration of calves was found in the current study, which may explain the lack of response on glucose metabolism. Also, the effects of butyrate on glucose metabolism may be more detectable when there is a risk of impairment of the metabolism as in high-fat diets or large MR allowances (Vinolo et al., 2012; Bach et al. 2013a).

5.4.3. GTT

The two GTT performed on days 0 and 35 of study to the 5 limit-fed calves per treatment were designed to evaluate the effects of butyrate supplementation on glucose metabolism in a more controlled context by eliminating the effects of individual intake variations. However, no clear effect of butyrate on glucose metabolism was observed in the present study. On day 35 of study all calves, independently of treatment, showed increased plasma concentration of insulin mean and peak concentrations compared with those found on day 0 (Table 5.5).

No effects of TRB or SB supplementation were found on insulin sensitivity. However, butyrate and TRB have been suggested to prevent the development of insulin resistance in milk-fed calves (Kato et al. 2011) and in obese mice (Gao et al. 2009; Vinolo et al. 2012). The protective effect of butyrate has been associated with a reduction in BW and a regulation of the production of gut hormones (Vinolo et al. 2011; Lin et al. 2012) such as GLP-1. In the present study, though, effects on GLP-1 concentration and insulin resistance were not detected probably because experimental diets did not challenge sufficiently the glucose metabolism of calves. Certainly, Vinolo et al. (2012) found an effect of tributyrin on insulin sensitivity in mice fed a high-fat diet. Then, the lack of effects of butyrate supplementation on glucose metabolism found in the present study may be due to the fact that glucose metabolism was not impaired.

5.5. Conclusions

Supplementing calves with butyrate in the form of sodium butyrate in MR (at doses of 3 g/kg of DM) for 42 days showed no advantages on growth performance. On the other hand, supplementing calves with butyrate in the form of tributyrin in the same manner as sodium butyrate had a detrimental impact on calf growth. Moreover, no effects of butyrate supplementation in the form of sodium butyrate or tributyrin on glucose metabolism were found in the conditions of the present study.

Chapter 6

INTERACTION BETWEEN MILK ALLOWANCE AND FAT CONTENT OF THE STARTER FEED ON PERFORMANCE OF HOLSTEIN CALVES

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6.1. Introduction

In the last years, the dairy industry has progressively changed from a restricted allowance of about 4 L/d (0.5 kg/d of DM) of milk or MR to more generous allowances (6 L/d or more), partly because it has been shown that fostering rapid growth early in life might have positive consequences on future milk performance (Davis Rincker et al., 2011; Bach, 2012) and survivability to second lactation (Bach, 2011). However, increasing milk or MR allowances to more than 0.8 kg/d reduces starter feed intake (Cowles et al., 2006; Hill et al., 2010; Davis Rincker et al., 2011), rumen development (Terré et al., 2007; Suárez-Mena et al., 2011) and post-weaning ADG (Jasper and Weary, 2002; Cowles et al., 2006; Hill et al., 2007d) mainly due to an insufficient starter feed intake and depressed feed efficiency and digestibility after weaning. Thus, when offering increased amounts of milk or MR, it is especially important to stimulate solid feed consumption to avoid declines in performance and impairments of health after weaning.

A plausible method to stimulate solid feed intake consists of offering palatable ingredients. In a cafeteria study evaluating energy and protein feeds, Montoro and Bach (2012) showed that the most preferred ingredient for young calves was soybean meal (rich in protein), followed by corn and whole soybean (rich in fat and protein). The increased consumption of soybean products by young calves could have obeyed to oro-sensory preferences, but it could also have been to due to an attempt of calves to meet specific nutrient requirements. In the study by Montoro and Bach (2012), it was surprising that calves consumed the different ingredients offered free-choice in such a way that the final nutrient composition of the diet was 29% CP, 6.3% fat, and about 3.73 Mcal of ME/kg (DM basis). These figures differ from the commonly used nutrient composition of commercial starter feeds in Europe containing 20% CP, 4% fat, and 3.20 Mcal of ME/kg (DM basis). Thus, it could be hypothesized that solid feed intake of calves may be fostered by feeding starter feeds with greater amounts of energy or fat than those currently provided. However, increasing fat content of starter feeds up to 7.3% DM using whole soybeans (Kuehn et al., 1994) or to greater amounts (up to 20%) using brown grease and hydrogenated cottonseed oil (Miller et al., 1959) has been reported to reduce DM intake and weight gain. Contrary, Johnson (1956) reported that feeding a high-fat starter (up to 10% fat) to calves limit-fed whole milk did not affect solid feed intake and increased feed efficiency. Nevertheless, all former studies

evaluating the effects of fat addition to starter feeds were conducted in conjunction with low milk allowances (≤ 0.5 kg of DM/d). Moreover, providing large volumes of MR in two daily meals (Bach et al., 2013a) or feeding diets rich in fat (Kubota et al., 1999) may lead to insulin resistance.

Thus, the aim of the present study was to evaluate the consequences on growth performance and insulin sensitivity of calves fed starter feeds with two different amounts of fat (and energy density) when offering either a restricted or a high milk allowance.

6.2. Materials and methods

6.2.1. Animals and treatments

Sixty-six Holstein male calves (42 ± 6.0 kg of BW and 12 ± 3.1 d of age) were purchased from commercial farms, and brought and raised in the facilities of IRTA at Torre Marimon (Caldes de Montbui, Spain). Prior to the arrival to our facilities calves received two daily allowances of 2 L of MR (at 12.5% dilution) and had free access to a starter feed. The composition of MR and starter feeds offered to calves before the beginning of the study differed among farm sources. Calves were kept in individual hutches (1.6 × 1.0 m) bedded with sawdust, and managed according to the guidelines of the Animal Care Committee of IRTA. A MR (Sprayfo Excellent 60, Sloten BV, Deventer, Holland) containing 25% CP and 19.2% fat (on a DM basis) was offered twice daily (at 0800 and 1630h) at 10% DM concentration for the first 2 d, and then raised to 12.5% DM thereafter. Animals were randomly assigned to a 2x2 factorial experiment in which half of the calves were fed 4 L of MR per day and the other half received 6 L of MR per day, both at 12.5% DM concentration. From day 0 of study, calves had *ad libitum* access to a pelleted starter feed (Table 6.1) containing either 4.1 or 11.2% fat (DM basis). Thus, 4 treatments were evaluated: a low-fat (4.1%; LF) starter feed offered with 4 L/d of MR (4LF), a high-fat (11.2% fat; HF) starter feed in conjunction with 4 L/d of MR (4HF), a LF starter feed offered with 6 L/d of MR (6LF), and a HF starter feed offered with 6 L/d of MR (6HF). Animals received MR for five consecutive weeks and half the amount (either 3 or 2 L/d depending on treatment) of MR for one additional week at 0800 h until weaning at day 42 of study. The study was completed 2 wk after weaning.

Table 6.1. Ingredient and nutrient composition (DM basis) of the starter feeds.

	Low fat	High fat
Ingredient, %		
Barley	25	5
Wheat	15	5
Corn	26	42.8
Soybean meal	24.8	.
Soybean full fat	.	37.1
Wheat middlings	9.1	10.0
Sodium chloride	0.03	0.03
Premix[1]	0.04	0.04
Nutrient composition		
CP, %	21.4	22.3
NDF, %	16.0	14.9
ADF, %	7.6	6.2
EE, %	4.1	11.2
Ash, %	4.4	4.6
ME[2], Mcal/kg	3.35	3.74
NFC[3], %	54.1	47.0

[1]Premix composition: 10,000,000 IU/kg of vitamin A; 2,000,000 IU/kg of vitamin D_3; 6,000 UI/kg of vitamin E; 0.5 g/kg of vitamin B_1; 0.5 g/kg of vitamin B_2; 48 g/kg of Mg; 35 g/kg of Zn; 30 g/kg of Mn; 23 g/kg of Fe; 10 g/kg of Cu; 0.6 g/kg of I; 0.4 g/kg of Co; 0.1 g/kg of Se.

[2]Calculated following NRC (2001).

[3]NFC: non-fiber carbohydrate (calculated as 100 – CP – NDF – EE – ash)

6.2.2. Measurements and sample collection

Individual MR and starter feed intakes were recorded daily and calves were weighed weekly. A GTT was performed to 24 calves (6 calves per treatment) on day 30 of study as described elsewhere (Bach et al., 2013a). The sample size was decided by performing a power analysis using variability figures for the outcome variables from the literature. All blood samples were immediately placed on ice for at least 20 min, centrifuged at 3,500 x g at 4°C for 15 min, and plasma or serum was decanted and stored at −20°C until subsequent analysis. Concentrations of serum glucose were measured by spectrophotometry and plasma insulin concentration was determined by ELISA (Millipore porcine insulin, St. Charles, Missouri, USA, with an intra- and inter-assay CV of 5.7 and 10.5%, respectively).

One week after weaning (days 49 to 55 of study), plastic bags were glued to 6 animals per treatment (randomly selected) to determine apparent DM, CP, and fat apparent digestibility of the diet as described by Terré et al. (2007). During 5 consecutive days, all feces were collected and weighed. Bags were changed 3 times a day and fecal samples stored at 4°C meanwhile. At the end of each day, a subsample equivalent to 30% of total daily feces weight was obtained and dried at 60°C for 72 h. Subsamples of the 5 d were composited by animal, ground using a 1-mm screen (Cyclotech 1093 mill, Tecator, Hoganas, Sweden) and analyzed for DM, OM, CP, and NDF following the methods described below.

6.2.3. Chemical analyses

Samples of MR were analyzed for DM (24 h at 103°C), ash (4 h at 550°C), and N content according to the method of the AOAC (method 988.05; AOAC, 1990) adapted for an automatic distiller Kjeldahl (Kjeltec Auto 1030 Analyzer, Tecator) with copper sulfate/selenium as a catalyst instead of copper sulfate/titanium dioxide. Samples of starter feed were analyzed for DM (4 h at 103°C), ash (550°C calcination), CP with Kjeldahl analyses, NDF was analyzed with sodium sulfite and heat-stable α-amylase (van Soest et al., 1991), ADF following AOAC (1990) method 973.18, and EE following method 920.39 of AOAC (1990) with petroleum ether used for distillation instead of diethyl ether (AOAC, 1990).

6.2.4. Calculations and statistical analyses

Nutrient apparent digestibility was calculated dividing the difference between the quantity of nutrient consumed and the quantity of nutrient defecated by the quantity of nutrient consumed. Metabolizable energy of MR and starter feeds as well as requirements of ME and CP of calves were calculated according to NRC (2001). Intake and growth data were summarized by week and animal prior to conducting statistical analyses.

Blood samples collected at -10 and 0 min relative to glucose infusion during the GTT were used to determine baseline concentrations of glucose and insulin. The AUC (concentration/min) for these 2 metabolites was calculated as the increase with respect to the baseline using the trapezoideal method. Then, ICR and GCR were computed as described elsewhere (Bach et al., 2013a). Insulin sensitivity was estimated using a simplification of the minimal model (Bergman, 1989) following Christoffersen et al. (2009).

Data pertaining to the GTT and apparent nutrient digestibility were analyzed using an analysis of variance accounting for the effects of MR allowance (4 or 6 L/d), fat content of the starter feed (4.1% or 11.2%), and their interaction. Performance data were analyzed with a mixed-effects model for repeated measures. The statistical model included initial BW and age as covariates and accounted for the fixed effects of MR allowance, fat content of the starter feed, week of measurement, and their interactions, plus the random effect of calf.

6.3. Results and discussion

6.3.1. Intake, growth, and digestibility

Intake and performance data are depicted in Table 6.2. The BW data for wk 8 of study for most calves were lost due to an electronic problem with the scale. Thus, all data related to BW (BW, ADG, and gain to feed ratio) end at wk 7 of study. Also, due to the study design, consumption of MR was greater by calves offered 6 L/d than by those offered 4 L/d. Overall, fat content of the starter feed had no effect on MR and solid feed intake. Interestingly, starter feed intake overall was not affected by MR allowance. The lack of differences in starter feed intake between the 2 MR allowances, was mainly due to the low starter feed intake of 4HF calves. Calves in the 4LF treatment tended (P =

0.1) to have the greatest and 4HF the lowest starter feed intake. Probably, the greater fat content of the starter feed may have induced satiety signals preventing 4HF calves to reach similar starter feed intake levels than 4LF. Moreover, starter feed intake was greater ($P < 0.05$) in LF compared with HF calves 2 wk after calves had been weaned (Figure 6.1).

Table 6.2. Feed and nutrient consumption (DM basis) and performance of calves as affected by MR allowance (4 vs 6 L/d) and level of dietary fat in the starter feed (4.1 vs 11.2% of the DM).

	Treatment[1]					*P*-values[2]						
	4LF	4HF	6LF	6HF	SEM	SF	MR	SFxMR	W	SFxW	MRxW	SFxMRxW
MR intake[3], g/d	463	479	687	687	7.4	0.33	<0.001	0.29	<0.001	0.81	<0.001	0.84
Starter feed intake, g/d	1,150	957	964	1,003	84.4	0.30	0.34	0.10	<0.001	0.02	0.15	0.32
Total DMI, g/d	1,497	1,317	1,479	1,519	84.2	0.34	0.21	0.14	<0.001	0.02	0.12	0.32
CP intake, g/d	300	267	307	315	15.6	0.36	0.05	0.15	<0.001	0.02	0.01	0.32
Fat intake, g/d	107	161	133	194	4.5	<0.001	<0.001	0.45	<0.001	<.001	<0.001	0.63
ME intake, Mcal/d	5.56	5.30	5.74	6.23	0.248	0.64	0.03	0.13	<0.001	0.02	0.01	0.30
BW[4], kg	55.4	54.6	57.8	60.6	1.31	0.43	<0.001	0.13	<0.001	0.23	<0.001	0.06
ADG[4], kg/d	0.56	0.53	0.56	0.67	0.043	0.25	0.04	0.06	<0.001	0.40	<0.001	0.51
Gain to feed ratio[4]	0.43	0.45	0.46	0.52	0.016	0.03	0.001	0.26	<0.001	0.42	<0.001	0.13

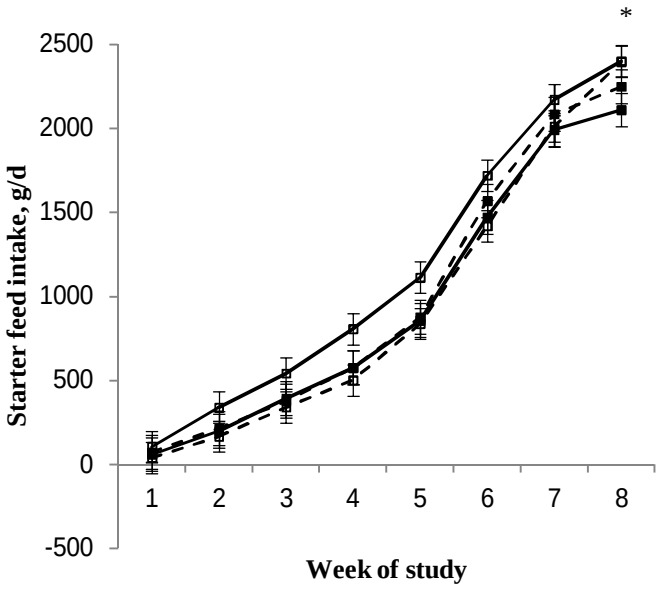
[1]4LF = 4 L/d of MR and low fat starter feed; 6LF = 6 L/d of MR and low fat starter feed; 4HF = 4 L/d of MR and high fat starter feed; 6HF = 6 L/d of MR and high fat starter feed.

[2]SF = Effect of fat content of the starter feed; W = effect of week of study; SFxMR: interaction between MR and fat content of the starter feed; SFxW = interaction between fat content in the starter feed and week of study; MRxW = interaction between MR and week of study; SFxMRxW = interaction among MR, fat content of the starter feed, and week of study.

[3]Provided until day 42 of study.

[4]Values represent data until week 7 of study (data for BW of most calves at week 8 of study were lost due to an electronic problem with the scale).

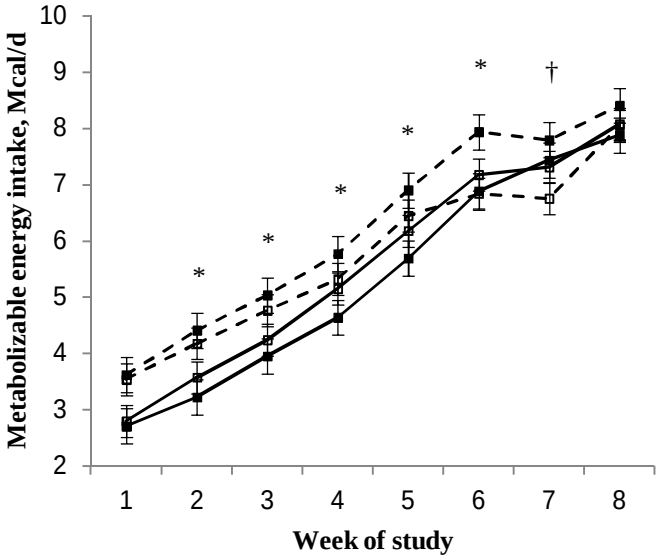
Figure 6.1. Starter feed intake of calves consuming 4 L/d of MR (solid line) and a low fat (□; 4LF) or a high fat (■; 4HF) starter feed, and calves consuming 6 L/d of MR (dashed line) and a low fat (□; 6LF) or a high fat (■; 6HF) starter feed. * denotes differences ($P < 0.05$) between high- and low-fat starter feeds.



Despite the lesser DM consumed by HF calves after weaning, ME intakes of HF and LF calves at wk 8 of the study (2 wk after weaning) were similar due to the greater fat content (and energy density) of the starter feed (Figure 6.2). In fact, fat intake was greater ($P < 0.05$) in HF compared with LF treatments the second week of study and thereafter. Again, satiety signals controlled by the energy balance of the animal may have been activated in calves consuming the HF starter feed, resulting in a milder increase in starter feed consumption (Montgomery and Baumgardt, 1965) after calves were weaned. Energy balance, calculated according to NRC (2001), was unaffected by the MR allowance or fat content of the starter feed (data not shown). However, LF calves had a lesser ($P < 0.05$) energy balance (0.26 ± 0.058 Mcal of ME/d) during the week following weaning than HF calves (0.51 ± 0.058 Mcal of ME/d), suggesting that perhaps energy balance was limiting DM intake in HF calves after weaning. Litherland et al. (2014) have recently reported a lesser starter feed intake when calves were supplemented with fat via MR although consumed similar ME intakes, and suggested that satiety mechanisms may have been responsible for the decrease in solid feed intake when supplementing fat in the MR. Metabolizable energy and fat consumption were greater for calves consuming 6 than for those consuming 4 L/d of MR from wk 1 to 5 of

study (when the full amount of MR was offered; Figure 6.2). After that, all calves consumed similar daily ME except for the first week after weaning (wk 7 of study), when HF calves had a greater ($P < 0.05$) ME intake than LF calves (due to the greater fat content of the HF starter feed). As expected, after weaning, starter feed intake increased notably for all calves. However, solid feed consumption of HF calves stalled during the second week after weaning (wk 8 of study), and as a consequence the differences in ME intake observed on wk 7 of study (greater in HF than in LF calves) disappeared. It could be speculated that feed intake of weaned calves was controlled by energy balance, which curtailed consumption of the starter feed with a high fat content.

Figure 6.2. Metabolizable energy intake of calves consuming 4 L/d of MR (solid line) and a low fat (□; 4LF) or a high fat (■; 4HF) starter feed, and calves consuming 6 L/d of MR (dashed line) and a low fat (□; 6LF) or a high fat (■; 6HF) starter feed.* denotes differences ($P < 0.05$) between 4 and 6 L/d of MR. † denotes differences ($P < 0.05$) between high- and low-fat starter feeds.



As expected, protein intake was greater ($P < 0.01$) for calves consuming 6 than for those on 4 L/d of MR from wk 1 to 5 of study when the full amount of MR was offered. However, protein intake was lower ($P < 0.05$) in HF than in LF calves at 8 wk of study, due to the lesser starter feed intake observed for HF calves.

Apparent DM digestibility was unaffected by dietary treatments (Table 6.3). Interestingly, compared with LF calves, HF calves had greater ($P < 0.05$) CP (74.3 vs

77.9 ± 1.72%, respectively) and greater ($P < 0.001$) fat apparent digestibilities (72.0 vs 83.3 ± 2.45%; respectively). The lesser DM intake observed in HF calves during the week that digestibility was assessed may have slowed passage rate of the digesta and this may be the reason for the increased protein and fat apparent digestibilities of HF compared with LF calves.

Table 6.3. Apparent digestibility (%) as affected by MR allowance (4 vs 6 L/d) and level of dietary fat in the starter feed (4.1 vs 11.2% of the DM) of calves during the second week after weaning.

	Treament[1]					*P*-values[2]		
	4LF	4HF	6LF	6HF	SEM	SF	MR	SFxMR
DM	80.8	83.1	80.2	81.4	1.55	0.20	0.36	0.74
CP	74.5	79.6	74.1	76.2	1.72	0.04	0.15	0.47
Fat	72.8	84.6	71.0	81.9	2.45	<0.001	0.40	0.85

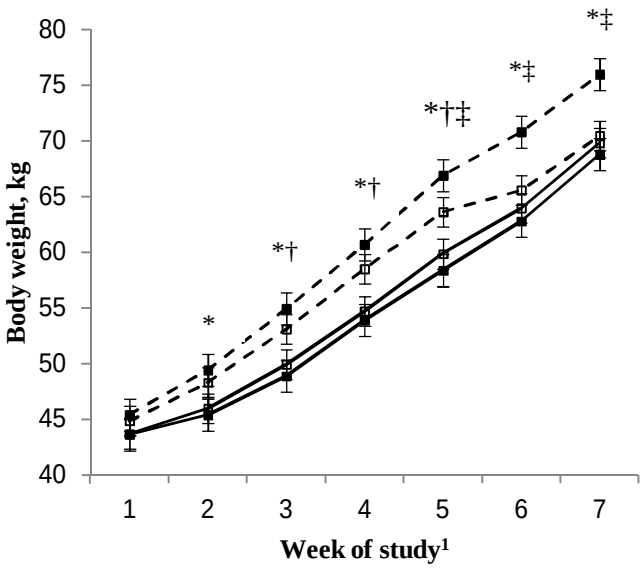
[1]4LF = 4 L/d of MR and low fat starter feed; 6LF = 6 L/d of MR and low fat starter feed; 4HF = 4 L/d of MR and high fat starter feed; 6HF = 6 L/d of MR and high fat starter feed.

[2]SF = effect of fat content of the starter feed; SFxMR = interaction between MR and fat content of the starter feed.

Despite the lack of differences in DM and ME intakes until weaning, calves in the 6HF treatment were heavier than calves in 4LF and 4HF treatments between the second week and the end of the study, and were heavier than 6LF calves from week 5 to the end of the study (Figure 6.3). Furthermore, 6LF calves presented greater BW than 4HF calves between the third week of study and the week before weaning, and greater BW than 4LF calves between the fourth week of study and the week before weaning (Figure 6.3). Average daily gain was greater in calves on 6 L/d of MR than in those on 4 L/d of MR from wk 1 to 5 of study (right before starting to decrease the daily MR offer), but it was lower on the 6 and 7 wk of study (around weaning). However, overall, 6HF tended ($P = 0.06$) to grow more than calves in the other three treatments (Table 6.2). Gain to feed ratio during the first 3 wk of the study was greater ($P < 0.001$) for animals on 6 L/d of MR compared with calves receiving 4 L/d of MR (Table 6.2) as reported in other studies (Raeth-Knight et al., 2009; Terré et al., 2009; Stamey et al., 2012). This difference could be attributed to the greater MR intake in the calves on 6 L/d of MR

than in those on 4 L/d of MR, which resulted in an increased CP and ME intakes (Table 6.2) during the preweaning period (up to wk 6 of study). Moreover, gain to feed ratio was greater ($P < 0.05$) for calves consuming the HF compared with calves consuming the LF starter feed (Table 6.2). This was probably due to the greater CP and fat apparent digestibilities found for calves in the HF treatment. Similar results have previously reported when supplementing fat in the MR (Tikofsky et al., 2001; Litherland et al., 2014) of pre-weaned calves.

Figure 6.3. Body weight of calves consuming 4 L/d of MR (solid line) and a low fat (□; 4LF) or a high fat (■; 4HF) starter feed, and calves consuming 6 L/d of MR (dashed line) and a low fat (□; 6LF) or a high fat (■; 6HF) starter feed. * denotes differences ($P < 0.05$) between 6HF, and 4LH and 4HF. † denotes differences ($P < 0.05$) between 4HF and 6LF. ‡ denotes differences ($P < 0.05$) between 6HF and 6LF. [1]Data pertaining to BW of calves at week 8 were lost due to an electronic problem with the scale.



In general, when feeding calves on intensive feeding programs (~ 6 L/d of MR), the two main problems observed are an insufficient starter feed intake the weeks before weaning and a consequent stall or slump in ADG during the weaning process (Cowles et al., 2006; Terré et al., 2009; Stamey et al., 2012). Some studies have reported a decrease in DM intake and growth when the fat content of the starter feed was above 4% of DM (Miller et al., 1959, Kuehn et al., 1994). However, in the present study, differences in starter feed intake around weaning were not significant, but a decrease in ADG the

weeks before and after weaning (weeks 6 and 7 of the study) was observed for calves fed 6 L/d of MR compared with those fed 4 L/d. The advantage in BW reported in calves offered 6 L/d of MR compared with those on 4 L/d of MR during the weeks before weaning disappeared for 6LF calves when MR was reduced to one allotment per day, but interestingly, not for 6HF calves that continued to be at least 5 kg heavier than the calves in the other treatments at wk 7 of study. Others have also reported an increase in BW before weaning when calves were fed large MR allowances (8 vs 6 L/d; Bach et al., 2013b) or a fat supplement in MR (Litherland et al., 2014). Although Litherland et al. (2014) speculated that due to a potential decrease in solid feed intake when supplementing MR with fat, the dietary CP:ME ratio may decrease and limit growth. However, in the current study, dietary CP:ME ratio ranged between 50.4 and 54.0 g of CP/Mcal of ME, and thus, as suggested by Donnelly (1983) who reported that dietary CP:ME ratios around 54 g of CP/Mcal of ME and Hill et al. (2010) who reported maximized growths with dietary CP:ME ratios between 51.5 and 55.0 g of CP/Mcal of ME, it is unlikely that CP intake in the current study may have limited growth. Nevertheless, some studies have reported that increasing fat in diets of calves resulted in an increased body fat deposition (Tikofsky et al., 2001; Bascom et al., 2007) when calves were fed a high fat diet a dietary CP:ME ratio between 45.8 and 50.1 g of CP/Mcal of ME. In the current study, calves in the 6HF treatment consumed 50.6 g of CP/Mcal of ME, and thus they might have increased body fat accretion. However, efficiency of fat deposition is lesser than that of muscle and no differences were found in gain to feed ratio between 6LF and 6HF treatments in the present study, which would indicate that fattening in 6HF may have been minimal. In fact, both, Tikofsky et al. (2001) and Bascom et al. (2007) reported differences in gain to feed ratio between high- and low-fat diets; whereas calves herein consuming HF treatments did not have lesser feed efficiencies than calves on LF treatments. Moreover, calves in the studies by Tikofsky et al. (2001) and Bascom et al. (2007) were fed exclusively MR whereas in the present study fat was supplemented in the starter feed, which may lead to different metabolic responses. Then, it is likely that the extra energy consumed by 6HF calves may have resulted in more growth without incurring in increased fat deposition. Therefore, it can be concluded that a starter feed high in fat may help to attenuate the stall or slump in growth around weaning in calves raised on an intensive feeding program (~6 L/d of MR).

6.3.2. GTT

We hypothesized that an increased fat or energy supply to young calves might alter glucose metabolism. However, the parameters evaluated with the GTT were not affected by treatments (Table 6.4) conducted on day 30 of the study except for basal serum glucose concentration that was greater ($P < 0.05$) for calves fed 6 L/d of MR compared with those receiving 4 L/d of MR. Mean serum glucose concentration during the GTT tended ($P = 0.10$) to be greater for calves consuming 6 L/d of MR than those consuming 4 L/d of MR. Metabolic disorders such as impaired insulin function may be present when large amounts of MR are fed to calves at a single allotment (Bach et al., 2013a). However, in the current study, no differences on plasma insulin levels were found for calves fed 6 L/d of MR compared with those on 4 L/d of MR and insulin sensitivity was similar among treatments. This is contrary to the insulin resistance previously reported by Bach et al. (2013a) when offering 8 L/d of MR (two daily allotments of 4 L) and to the increase in insulin to glucose ratio reported by Terré et al. (2009) when calves received 2 allotments of 4 L per day. Probably, offering 6 L/d of MR (3 L per allotment) to suckling calves is not sufficient to elicit the negative changes in insulin function observed when offering larger volumes.

Table 6.4. Glucose and insulin responses to an i.v. GTT (performed on day 30 of study) of calves as affected by MR allowance (4 vs 6 L/d) and level of dietary fat in the starter feed (4.1 vs 11.2% of the DM).

	Treament[1]					*P*-values[2]		
	4LF	4HF	6LF	6HF	SEM	SF	MR	SFxMR
Basal glucose, mmol/L	4.9	4.7	5.8	5.1	0.28	0.15	0.02	0.29
Glucose, mmol/L	5.9	5.9	6.6	6.3	0.32	0.62	0.10	0.68
Insulin, µU/ml	42.3	39.5	45.6	48.9	8.03	0.97	0.44	0.71
Glucose AUC, mmol/L	73.2	77.4	60.7	80.5	12.06	0.33	0.70	0.53
Insulin AUC, µU/mL	1,313	1,323	1,241	1,403	335.2	0.80	0.99	0.82
Relative glucose peak, mmol/L	3.7	3.9	3.3	3.7	0.28	0.32	0.24	0.73
Relative glucose nadir, mmol/L	0.4	0.4	0.4	0.6	0.14	0.25	0.58	0.98
Insulin peak, µU/ml	67.0	70.4	59.2	66.1	5.00	0.32	0.24	0.73
Insulin nadir, µU/ml	7.8	7.7	7.4	11.0	2.44	0.48	0.56	0.46
Time to glucose peak, min	4.0	4.0	4.0	4.0	0.00	-	-	-
Time glucose nadir, min	41.7	46.7	35.8	43.3	5.61	0.28	0.42	0.83
Time to insulin peak, min	15.5	10.0	11.3	12.2	2.60	0.38	0.70	0.24
Time to insulin nadir, min	50.8	57.5	46.7	50.8	4.04	0.20	0.20	0.76
GCR, mmol/Lxmin	7.7	5.7	8.1	5.7	1.20	0.09	0.90	0.88
ICR, µU/mLxmin	8.6	6.8	7.6	8.3	1.53	0.74	0.87	0.41
Time to basal glucose, min	37.7	42.7	31.8	39.3	5.61	0.13	0.41	0.41
Time to basal insulin, min	35.3	46.3	35.3	38.7	4.52	0.13	0.41	0.41
Insulin to glucose ratio	118.1	104.2	119.8	126.7	35.22	0.92	0.73	0.77
Insulin sensitivity[3], mL/min x µU/mL	0.79	0.56	0.64	0.50	0.134	0.18	0.46	0.74

[1]4LF = 4 L/d of MR and low fat starter feed; 6LF = 6 L/d of MR and low fat starter feed; 4HF = 4 L/d of MR and high fat starter feed; 6HF = 6 L/d of MR and high fat starter feed.

[2]SF = effect of fat content of the starter feed; SFxMR = interaction between MR and fat content of the starter feed.

[3]Calculated following Christoffersen et al. (2009).

Interestingly, GCR tended (P = 0.09) to be greater for calves consuming LF than for calves consuming HF. This is in contrast with previous studies (Palmquist et al., 1992) that found that calves fed low-fat diets tended to decrease glucose utilization rates with

age and had lower glucose and insulin areas under the curve. Due to differences in nutrient composition of the starter feed, calves fed LF diets consumed proportionally more non-fiber carbohydrates than calves fed HF after weaning. Then, the transition from a diet rich in fat from MR to a diet rich in non-fiber carbohydrates from the starter feed was more abrupt in calves fed LF than in those fed HF. This may have influenced glucose metabolism of calves fed the LF starter feed.

6.4. Conclusions

Growth performance of calves consuming 6 L/d of MR was greater than that of calves consuming 4 L/d before weaning, and calves on 6 L/d of MR and a high-fat starter feed tended to grow the most. However, feeding a high-fat starter feed depressed intake after weaning, but when offering 6 L/d of MR, increasing fat content of the starter feed up to 11.2% increased overall BW and tended to increase growth. Thus, feeding a starter feed high in fat may help to attenuate the stall or slump in growth at weaning in calves raised on intensive feeding programs.

Chapter 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

The first study of the present thesis aimed at determining the effects of intestinal integrity at birth on the later development of diarrhea and it was demonstrated that calves that would later develop diarrhea were born with increased intestinal permeability possibly due to the endogenous microbiota establishment. Then, the second study evaluated the effects on immunity of a probiotic supplementation via MR by modulation of the fecal microbiota but no major effects were found. After that, modulation of performance and diarrhea incidences by supplementation of butyrate in the MR was evaluated and detrimental effects on growth were detected for tributyrin. Lastly, an enhanced feeding strategy to improve performance was evaluated which resulted in beneficial effects on growth.

In this chapter, the results of the four studies conducted throughout the present thesis will be discussed together in order to describe the overall effects of the different feeding strategies evaluated on health and performance of young Holstein calves. In addition, effects of health on performance results of calves will be discussed considering results of all four studies.

7.1. Effects of diet on health of young Holstein calves

In all four studies of the present thesis at least one health parameter was measured. In the study evaluating the intestinal permeability of newborn calves, it was demonstrated that calves with a correct passive transfer that develop scours may have been more predisposed to suffer diarrhea due to an increased intestinal permeability at birth when fed colostrum with high bacterial load. Also, the effect of colostrum intake on intestinal closure was not sufficient to improve the altered intestinal integrity of newborn calves that would later incur diarrhea (Stott et al., 1979). In the study supplementing *L. rhamnosus,* no effects on immunity were observed by the probiotic addition in the diet of weaned calves. Therefore, in the two mentioned studies, the intestinal microbiota may have influenced the immune response.

Indeed, calves that presented diarrhea in the colostrum study were born with an altered intestinal permeability which may have been due to the establishment of the microbiota in the GIT of calves whilst in the placenta (Fichorova et al., 2011) or during delivery (Mackie et al., 1999). In fact, it is hypothesized that commensal bacteria, present in

various maternal tissues and fluids, contribute to the GIT development of the newborn calves (Taschuk and Griebel, 2012). Alternatively, host genetic factors may have influenced the composition of the intestinal microbiota rather than environmental factors (Mayer et al., 2012) suggesting that some calves are more susceptible than others to suffer from diarrhea. Moreover, in the probiotic study, factors such as limitations in the microbiota detection technique (e.g. no detection of active microorganisms or actual epithelial colonization) may have masked the possible effects on the intestinal microbiota of *L. rhamnosus* supplementation at weaning. For instance, Malmuthuge et al. (2012a) showed that fecal microbiota is not representative of the mucosa-associated populations. On the other hand, effects of dietary changes during weaning may have influenced the intestinal microbiota (Malmuthuge et al., 2013), and thus the immune response, rather than the effects of the *L. rhamnosus* supplementation. In addition, host genetic factors may have also influenced the lack of immunity modulation observed (Mayer et al., 2012), as it seemed to happen in the permeability study.

In the study supplementing the MR with butyrate, a negative effect on health of the inclusion of butyrate in the diet of preweaned calves was observed as control calves tended to have a lower diarrhea incidence than the supplemented calves. This is in contrast with previous studies reporting reduced abnormal fecal score days and medical treatments (Hill et al., 2007a,b; Górka et al., 2011a; Hill et al., 2011; Esselburn et al., 2013). In the enhanced feeding study, it was observed that consuming 6 L/d of MR tended to increase the probability of suffering diarrhea compared with 4 L/d. This is consistent with previous studies reporting increased fecal scores in enhanced milk fed-calves (Díaz et al., 2001; Nonnecke et al., 2003; Brown et al., 2005; Quigley et al., 2006; Raeth-Knight et al., 2009; Davis Rincker et al., 2011).

In both studies, diet was modified either by increasing the short-chain fatty acid content of the MR or by increasing its fat and protein contents by feeding more MR. Increasing the amount of MR fed to calves resulted in increased intake of certain fatty acids present in the MR, with major impact for those most abundant. Thus, one common change in the diet of both studies is related to its fatty acid profile. Modifying the fatty acid profile of the MR has been reported to influence health in calves (Hill et al., 2007a,b; Ballou and DePeters, 2008; Hill et al., 2011; Bowen Yoho et al., 2013; Esselburn et al., 2013). However, differences in doses, MR fatty acid profiles and amounts fed among the

different studies resulted in differences in the daily intake of several fatty acids, such as butyrate. In fact, in the butyrate study of the present thesis, the increased daily amounts of butyrate fed on both treatment groups (in the form of sodium butyrate or tributyrin) relative to the control were 0.96 and 0.98 g/d, respectively, whereas in other studies such increases ranged from 1.5 to 4.4 g/d and they reported reduced abnormal fecal score days and medical treatments (Hill et al., 2007a,b; Górka et al., 2011a; Hill et al., 2011; Esselburn et al., 2013) or increased abomasal and colonic heat-shock proteins that act in cell protection (Guilloteau et al., 2009). Then, the daily increase of butyrate (in the form of sodium butyrate or tributyrin) of the supplemented MR relative to the control MR in the present thesis may not have been adequate to elicit a beneficial effect on health.

It should also be considered that some of the studies that reported beneficial effects of butyrate on health supplemented the MR with only sodium butyrate (Hill et al., 2007b; Guilloteau et al., 2009; Górka et al., 2011a), whereas other studies supplemented the MR with NeoTec4 which is a commercial blend including coconut oil and flax oil (Hill et al., 2011; Esselburn et al., 2013) or a mix of fat sources (Hill et al., 2007a) in addition to butyrate, thus the fatty acid profile of the supplemented MR differed notably from the controls, especially for C4:0, C12:0, C14:0, and C18:2 fatty acids. Such differences in the fatty acid composition of the MR may have enhanced the beneficial effects of the butyrate supplementation on health parameters, since fatty acid antimicrobial properties have been shown to be additive (Oh and Marshall, 1994; Tangwatcharin and Khopaibool, 2012). Also, butyrate (C4:0) and linoleic (C18:2) acids are known to reduce the inflammatory response (Hill et al., 2011). Moreover, lauric (C12:0), palmitic (C16:0), linolenic (C18:3), linoleic, oleic (C18:1), stearic (C18:0) and myristic (C14:0) fatty acids are known to have potential antibacterial and antifungal agents (McGaw et al., 2002; Seidel and Taylor, 2004). Indeed, myristic acid has been shown to be the saturated fatty acid with the highest inhibitory effects against gram-positive bacteria among 30 fatty acids and derivatives evaluated (Kabara et al., 1972) and lauric acid has been found to be the fatty acid in milk fat with the highest antimicrobial effect on *Enterococcae*. Also, *Escherichia Coli* and *Helicobacter pilori* have been found to be susceptible to lauric acid (Petschow et al., 1996; Sprong et al., 2001). However, long chain fatty acids, such as oleic (C18:1), palmitic (C16:0) and stearic (C18:0), have been shown to be indigestible for suckling calves and thus cause diarrhea when added at rate

of 2% in the MR (Okada et al., 2009). For instance, studies that supplemented a MR with butyrate along with changes in middle and long-chain fatty acids reported decreased inflammatory responses and increased antibody response in addition to the reduced abnormal fecal score days and medical treatments (Hill et al., 2007a,b; Hill et al., 2011; Esselburn et al., 2013), which may have been due to the increase in the diet of certain fatty acids susceptible to improve immunity along with a decrease in long-chain fatty acids that have been reported to be indigestible for suckling calves (Okada et al., 2009). Table 7.1 summarizes the effects on health, reported in 6 studies, of modifying the fatty acid profile of the MR of calves with either increasing butyrate or in conjunction with modifications in other fatty acids. It should be mentioned that the study of Guilloteau et al. (2009) was not included in Table 7.1 because the only health parameters measured was the relative levels of heat-shock proteins mRNA throughout the GIT. It can be observed in the table that the effects on health were more evident when the fatty acid profile of the MR was modified in middle and long-chain fatty acids in addition to an increase in butyrate content.

Table 7.1. Effects on different health parameters, summarized from 6 studies, of modifying the fatty acid profile of the MR of calves with either increasing butyrate content or in conjunction with modifications in other fatty acids.

			Effects on health[1]			
Study	Fatty acids modified[2]	Fatty acid increase or decrease[3]	Abnormal fecal scores	Inflammatory response	Antibody response	Medical treatments
Hill et al., 2007b						
Trial 1	C4:0	>	+**	NR	NR	+*
Hill et al., 2007a						
Trial 3	C4:0	>				
	C12:0	>				
	C16:0	<	+**	NR	NR	=
	C18:0	<				
	C18:1	<				
Górka et al., 2011a[3]	C4:0	>	+*	NR	NR	NR
Górka et al., 2011b[3]	C4:0	>	=	NR	NR	NR
Hill et al., 2011						
Trial 1a	C4:0	>				
	C10:0	>				
	C12:0	>	+**	+**	+**	+*
	C14:0	>				
	C18:2	<				
	C18:3	>				
Esselburn et al., 2013						
Trial 1	C4:0	>				
	C12:0	>	+**	NR	+**	+**
	C14:0	>				
	C18:2	<				
Trial 2	C4:0	>				
	C12:0	>	+**	NR	NR	+**
	C14:0	>				
	C18:2	>				

[1]Effects are either: not reported (NR), positive (+) or null (=).
[2]Relative to control diet.
[3]> denotes an increase and < denotes a decrease relative to control diet.
*indicates *P*-values < 0.1.
**indicates *P*-values < 0.05.

Nevertheless, some studies (Hill et al., 2007b; Guilloteau et al., 2009; Górka et al., 2011a) have been conducted supplementing the MR of calves with only sodium butyrate and found some positive effects on health. In two of these studies (Hill et al., 2007b; Guilloteau et al., 2009) the daily doses of sodium butyrate were increased in 4.1 and 4.4 g/d relative to the control MR, respectively, which differ from the increased daily amount of sodium butyrate or tributyrin used in the butyrate study of the present thesis of 0.96 and 0.98 g/d relative to the control group, respectively. However, Górka et al. (2011a) supplemented calves with sodium butyrate in more similar daily amounts than the butyrate study of the current thesis (1.5 g/d) and found that supplemented calves tended to have decreased number of days with electrolyte therapies from days 0 and 7 of study and tended to have increased fecal consistency from days 8 to 14 of study. This is in contrast with results from the butyrate study of the present thesis in which control calves tended to have reduced incidences of diarrhea compared with supplemented calves throughout the study. Even though reasons for such a discrepancy are unknown, differences in study design among trials should be considered. In the study by Górka et al. (2011a) calves started the trial with a mean age of 5 d whereas in the butyrate study of the present thesis, calves started the trial with a mean age of 12 d. Since the GIT and immune system of newborn calves develops rapidly during the first weeks of life (Flaga et al., 2011; Tamao et al., 2011), the effects of butyrate supplementation on incidence of diarrhea may be influenced by the development status of the young calf. Moreover, calves in the Górka et al. (2011a) study were born in the same facilities where the trial was conducted, whereas calves used in the butyrate study of the present thesis were purchased from commercial farms and transported to the facilities of the trial. Therefore, differences in origin of calves and immunization protocols of the respective farms may have also influenced the immune status of calves and thus the results found herein.

In the enhanced feeding study of the present thesis, it was found that calves consuming 6 L/d of MR tended to increase the probability of suffering diarrhea compared with 4 L/d. This finding is consistent with previous studies reporting increased fecal scores

when increased amounts of MR were provided to calves (Díaz et al., 2001; Nonnecke et al., 2003; Brown et al., 2005; Quigley et al., 2006; Raeth-Knight et al., 2009; Davis Rincker et al., 2011). However, (Jasper and Weary, 2002) found no differences on fecal scores and incidence of diarrhea when calves were fed whole milk *ad libitum* compared with restricted amounts. The main difference between whole milk and MR is usually the fat amount and its fatty acids profile. Thus, a possible explanation for the increases in incidences of diarrhea when feeding large amounts of MR may have been an inappropriate fatty acid intake. For instance, in the mentioned studies that reported increased fecal scores when feeding increased amounts of MR, all calves had been fed increased amounts of fat and protein (Nonnecke et al., 2003; Brown et al., 2005; Quigley et al., 2006; Raeth-Knight et al., 2009; Davis Rincker et al., 2011) or increased amounts of fat and decreased amounts of protein (Díaz et al., 2001), suggesting that the amount of fat may have been notably influent on the increase of fecal scores.

In order to find a possible explanation on the effects of different fatty acids intake on the increase in fecal scores, the theoretical fatty acid profile of the commercial MR (Sprayfo Excellent 60, Sloten BV, Deventer, Holland) used in the butyrate and enhanced feeding studies for this thesis was calculated according to its fat composition (1/3 of coconut hydrogenated oil and 2/3 of palm hydrogenated oil) and the respective fatty acid profiles (http://www.chempro.in/fattyacid.htm). The resulting fatty acid profile of the experimental MR was used to compare treatments in the butyrate and enhanced feeding studies and also with other studies, when the fatty acid profile of the MR used was available. Moreover, daily intake of the specific fatty acids of calves consuming 4 L/d and 6 L/d of MR (as well as different treatments in other studies) was calculated according to the mean daily MR intake of each treatment group and the fatty acid profile of the MR (Table 7.2). It was, therefore, observed that calves fed 6 L/d of MR were consuming 6.6, 12.0 and 13.3 g/d more lauric (C12:0), palmitic (C16:0) and oleic (C18:1) acids, respectively, than calves fed 4 L/d. Such increase in the daily intake of the mentioned fatty acids may have negatively influenced health. Indeed, long chain fatty acids, such as oleic, palmitic and stearic acids, have been shown to be indigestible for suckling calves and thus cause diarrhea when added at a rate of 2% in the MR resulting in daily intakes increases of about 5.6 g relative to control (Okada et al., 2009). In addition, middle-chain fatty acids have been found to benefit health and performance. For instance, replacing 20% of the fat content of a MR containing all fat from edible

lard with coconut oil (rich in lauric acid (C12:0)) resulted in increased performance and reduced fecal scores (Bowen Yoho et al., 2013) and bactericidal properties have been reported in vitro for C10:0 and C12:0 fatty acids from bovine milk (Sprong et al., 2001). However, an excess (40% of the fat content of the MR) of coconut oil supplementation (in which lauric acid (C12:0) is the predominant fatty acid), resulting in a daily increase of 17.5 g of lauric acid compared with control MR, increased the incidence of diarrhea in pre-weaned calves (Bowen Yoho et al., 2013). In contrast, Mills et al. (2010) reported no effects on fecal scores after increasing the amounts of C8:0 and C12:0 fatty acids up to 32% of the MR fatty acids in suckling calves.

Table 7.2. Daily intake of fatty acids from the MR of calves in the enhanced feeding study.

	Amount of MR offered		
	4 L/d	6 L/d	Difference[1]
Fatty acid intake, g/d			
butyrate (C4:0)	0.46	0.67	0.21
caprylic (C8:0)	2.11	3.08	0.97
caproic (C10:0)	0.12	0.18	0.06
capric (C10:0)	2.41	3.52	1.11
lauric (C12:0)	14.47	21.10	6.64
myristic (C14:0)	5.58	8.13	2.56
palmitic (C16:0)	26.07	38.03	11.96
palmitoleic (C16:1)	0.03	0.04	0.01
stearic (C18:0)	3.32	4.84	1.52
oleic (C18:1)	29.09	42.43	13.34
linoleic (C18:2)	4.97	7.25	2.28
arachidic (C20:0)	0.03	0.04	0.01

[1]Aritmethical difference of the daily intake, in g/d, of the different fatty acids between calves fed 4 and 6 L/d of MR.

Therefore, the daily fatty acid intake of calves in the enhanced feeding study of the present thesis may have negatively affected the incidence of diarrhea when calves were fed 6 L/d of MR.

7.2. Effects of diet on performance of young Holstein calves

In the present thesis, the effects of diet on performance were evaluated in all four studies. However, in the butyrate and the enhanced feeding studies, performance parameters were evaluated more exhaustively because the hypothesis included effects of treatments on intake and growth. On the permeability and probiotic studies, performance parameters were assessed to provide complementary information. Therefore, discussion about the effects of diet on performance will mainly focus on the butyrate and enhanced feeding studies. Factors affecting intake and growth in calves are numerous including health, GIT development, feed characteristics and glucose metabolism among the most influencing. The effects of GIT development, feed characteristics and glucose metabolism will be discussed in the current section whereas the effects of health on performance will be discussed in the following section.

In the permeability and probiotic studies, growth was recorded for a 3-wk period and no differences were found among treatments in both studies probably due to the short duration of the study. Even though in the permeability study some calves presented diarrhea, growth was similar for diarrheic compared with healthy calves, which was probably due to the mild diarrhea episodes found for calves that had diarrhea (Woode, 1978). Moreover, recently weaned calves in the probiotic study showed similar growths despite the probiotic supplementation, which was probably due to the similar total DMI found between treatments. Changes in diet during weaning may have affected the GIT microbiota (Malmuthuge et al., 2013) to a greater extent than the probiotic supplementation masking the possible effects on performance of the *L. rhamnosus* on recently weaned calves. Therefore, it seems that either increased intestinal permeability (resulting in mild diarrhea episodes) in newborn calves or *L. rhamnosus* supplementation in weaned calves had no effects on short-term performance parameters, because sample size of both studies was sufficient to achieve a statistical power analysis over 90%.

In the butyrate study, the GIT development was measured indirectly by measuring the concentration of BHBA in blood (Quigley et al., 1991; Quigley and Bernard, 1992).

Even though butyrate has been shown to improve rumen development (Sander et al., 1959), no changes in BHBA plasma concentrations were found by the addition of butyrate in the MR, which has been suggested to be due to the fact that orally provided butyrate may be metabolized in the GIT wall and thus not detected in plasma (Manzanilla et al., 2006; Guilloteau et al., 2009). However, calves supplemented with tributyrin showed a reduced growth compared with control calves at the end of study, although all calves had similar total DM intakes. This may have been due to the numerically lower starter feed intake of calves supplemented with tributyrin compared with control. For instance, butyrate is naturally present in the rumen of calves as a fermentation product of solid feeds; thus, the butyrate produced in the rumen as a result of starter intake may have influenced the GIT development and consequent performance parameters rather than the butyrate supplemented in the MR. Therefore, similar than in the probiotic study, effects of starter intake may have influenced rumen development and consequent performance parameters rather than the butyrate supplementation via MR (Ferreira and Bittar, 2011).

In the enhanced feeding study, GIT development was not evaluated, but rumen development is known to be strongly dependent on solid feed intake (Tamate et al., 1962) which was indeed evaluated. For instance, increasing milk or MR allowances to more than 0.8 kg/d has reduced starter feed intake (Cowles et al., 2006; Hill et al., 2010; Davis Rincker et al., 2011) and delay rumen development (Terré et al., 2007; Suárez-Mena et al., 2011) mainly due to an insufficient starter feed intake, which depressed feed efficiency and digestibility. However, starter feed intake was found to be similar for calves fed 4 and 6 L/d of MR and apparent digestibility was similar for all calves. Therefore, it may be assumed that in the enhanced feeding study of the present thesis, rumen development was similar for all calves. Thus, the increased growths before weaning found for calves fed 6 L/d of MR as well as the improved growths after weaning when offered a high-fat starter feed may have been influenced by factors other than rumen development, such as energy content of the experimental diets.

As stated in section 1.2.2.2, feed characteristics are known to influence intake and growth in young calves. Several dietary factors, such as nutrient content and palatability have been shown to modulate voluntary intake (Montoro and Bach, 2012). In the butyrate study, calves supplemented with tributyrin and sodium butyrate refused more MR during the first week of study which may have been due either to the increase in

diarrhea incidence for these calves (diarrheic calves usually refuse milk) or to a modification of the MR taste caused by the addition of butyrate. However, no differences in growth were found during the first 2 weeks of study among treatments and all calves consumed the entire daily MR allowance for the rest of study. In the enhanced feeding study, the increase in fat content of the starter feed was achieved by including soybean full-fat, which has been shown to be a preferred ingredient for young calves (Montoro and Bach, 2012) but no differences were found on starter intake before weaning. However, after weaning, calves fed the low fat starter feed showed increased intakes compared with calves fed the high-fat starter feed probably due to satiety signals modulating intake. Therefore, effects of palatability of diets in both studies had no major effects on performance. Nevertheless, the daily nutrient and ME intake seemed to have had major impact on growth. For instance, in the enhanced feeding study, before weaning, CP, fat and ME intake were increased for calves fed 6 L/d of MR and, thus, performance was improved. Also, until 1 week after weaning, calves offered a high-fat starter feed also had greater fat and ME daily intakes than calves offered a low fat starter feed, and calves fed 6 L/d and a high-fat starter feed had increased BW than the rest after weaning. In the butyrate study, the energy metabolism may have been modulated by the butyrate supplementation. For instance, an increase in energy expenditure after supplementation with butyrate (Gao et al., 2009) and tributyrin (Vinolo et al., 2012) has been described in mice fed a high-fat diet; and Vinolo et al. (2012) suggested that tributyrin may enhance energy expenditure by mechanisms different from those of butyrate, which has been suggested to stimulate PGC-1α activity (Gao et al., 2009). This may explain the lower BW of calves supplemented with tributyrin found at the end of study compared with control calves. In summary, it seems that energy intake and metabolism in the butyrate and enhanced feeding studies played a crucial role on performance parameters.

On the other hand, in both the butyrate and enhanced feeding studies, diet was modified in some aspects susceptible to affect glucose metabolism, which in turn may affect intake (Houpt and Hance, 1971; Landsberg and Young, 1985). Moreover, several authors have reported an improvement in glucose metabolism after butyrate supplementation in both calves and mice (Guilloteau et al., 2010b; Kato et al., 2011; Vinolo et al., 2012) and providing calves with increased amounts of MR (Terré et al., 2009; Bach et al., 2013a) or feeding mice with high-fat diets (Vinolo et al., 2012) were

found to induce insulin resistance. Moreover, feeding calves large MR allowances (i.e., 8 L/d) has been reported to induce insulin resistance in calves (Bach et al., 2013a), but providing 6 L/d of MR in the enhanced feeding study resulted in no effects on insulin sensitivity. In addition, the trend to increased GCR of calves fed the low-fat starter feed found before weaning had no effect on intake or growth. Nevertheless, effects of the different diets on performance in both studies were notably different. Whereas butyrate supplementation had no beneficial effects on intake and growth, calves fed 6 L/d of MR in the enhanced feeding study had improved performance before weaning and when offered a high-fat starter feed they also had increased postweaning BW and growth. Therefore, it seems that, in both studies, diet did not modulate glucose metabolism and, thus, it had no major effect on performance parameters.

7.3. Effects of health on performance of young Holstein calves

In the four studies of the present thesis, performance and health parameters were recorded. For instance, in the permeability study, daily fecal scores and weekly BW was measured for the first 21 d of life; in the probiotic study, immune parameters were measured along with daily starter feed and straw intake and weekly BW for 3 wk after weaning; in the butyrate and in the enhanced feeding studies, daily fecal scores were measured throughout the preweaning period. Then, the effects of health on performance of young Holstein calves will be discussed considering results of the four studies. However, discussion will mostly focus on the butyrate and enhanced feeding studies because performance parameters of the permeability and probiotic studies were measured for a very short period of time (3 wk).

In the permeability study, calves that presented diarrhea during the first 7-14 d of life showed no retarded growth until day 21 of life compared with healthy calves. This is consistent with previous findings reporting no effects of mild diarrhea on performance (Woode, 1978), and calves with diarrhea showed mean fecal scores of 2.2 in a 1-5 scale (Lesmeister and Heinrichs, 2004). Even though no information on later performance parameters was recorded, it is likely that performance parameters among diarrheic and non diarrheic calves were similar throughout the preweaning period unless severe diarrheic episodes occurred after the first 21 d of life. Similarly, in the probiotic study performance was measured for 3 wk after weaning and was similar for all calves. Also, immune parameters measured were similar for unsupplemented and supplemented

calves with *L. rhamnosus*. Therefore, the lack of differences in immunity among treatments may explain the similar performance results among calves.

On the other hand, on the butyrate and enhanced feeding studies, performance was assessed during the diarrhea episodes until weaning and two weeks after weaning, respectively, thus that longer term effects on performance of the diarrhea incidences can be evaluated. All diarrhea incidences in both studies appeared during the first 2 wk of study. In the butyrate study, calves supplemented with either sodium butyrate or tributyrin tended to have increased incidence of diarrhea and also had reduced MR intake during the first week of life, probably because diarrheic calves usually refuse milk. However, even though BW during the first 14 d of study was similar for all calves, ADG gain was increased for unsupplemented calves until the end of study, suggesting that diarrhea incidence may have impaired growth rates until weaning. In fact, BW at the end of study was greater for unsupplemented calves. However, it should be considered that diarrheic calves coincide with calves supplemented with sodium butyrate or tributyrin, so that factors affecting performance throughout the preweaning period may have been more related to the butyrate supplementation itself than to the effects of the diarrhea incidences during the first days of study. For instance, mild diarrhea has been found to have no detrimental effects on growth (Woode, 1978). Therefore, in the butyrate study, it is unlikely that diarrhea incidences during the first days of study may have affected performance.

On the enhanced feeding study, even though calves fed 6 L/d of MR had increased probability to suffer diarrhea than calves fed 4 L/d, ADG was greater for calves fed 6 L/d of MR from weeks 1 to 5 of study. Moreover, BW was greater for calves fed 6 L/d from day 14 of study until weaning. It seems that in this study, incidence of diarrhea also had no effect on preweaning and 2 wk postweaning performance parameters.

Differences related to the severity of diarrhea between studies that may explain the controversial effects of scouring on performance among both studies are not considered, because mean fecal scores found for calves that did and calves that did not incur diarrhea were relatively low (according to the 1-5 scale considered; Lesmeister and Heinrichs, 2004) either in the butyrate (1.9 vs 1.2 ± 0.06; respectively) and the enhanced feeding studies (1.7 vs 1.2 ± 0.04; respectively) and similar between both studies. Therefore, reasons modulating the controversial effects of diarrhea incidences on

performance parameters between both studies may be related to other factors, such as nutrient intake. For instance, the main difference between both studies concerning diarrhea and growth is that calves in the butyrate study had reduced MR intake during the diarrhea episodes and all calves had similar total DM intake throughout the study, whereas calves fed 6 L/d of MR in the enhanced feeding study had greater MR intake than calves fed 4 L/d until weaning. Then, nutrient intake may have played a role on the performance parameters of calves suffering diarrhea. Indeed, daily protein, fat, and ME intakes were greater for calves consuming 6 than for those consuming 4 L/d from weeks 1 to 5 of study (when the full amount of MR was offered), coinciding with the increased ADG of calves fed 6 L/d of MR, and it has been demonstrated that plane of nutrition affects immunity in calves (Pollock et al., 1993 and 1994; Nonnecke et al., 2003; Foote et al., 2005b; Ballou, 2012). In fact, during an early immune response, the metabolic response is characterized by increased energy resting expenditure, extensive protein and fat catabolism and hyperglycemia, along with changes in the metabolic priorities of the liver to the production of acute phase proteins, resulting in reduced growth (Chiolero et al., 1997; Gifford et al., 2012). Then, calves suffering diarrhea in the enhanced feeding program (fed 6 L/d of MR) may have been able to compensate the possible detrimental effects of diarrhea on growth due to the increased energy intake, whereas supplemented calves in the butyrate study showed decreased growth. Indeed, calves in the butyrate study were fed 4 L/d of the same MR as the enhanced feeding study and during the first week of study, coinciding with diarrhea episodes, supplemented calves had reduced nutrient and energy intake compared with control due to the often MR refusals. Therefore, it may be speculated that diarrhea incidence may not have resulted in detrimental performance parameters during scouring when calves received a high nutrient supply.

Chapter 8

CONCLUSIONS

8. CONCLUSIONS

The results obtained in this thesis allow to conclude that:

1. Calves with a correct passive transfer that develop diarrhea during the first 21 days of life were born with an increased intestinal permeability within the first 3h of life compared with calves that did not. Therefore, calves that experience diarrhea may be born more susceptible to suffer scours.

2. Supplementing calves with the probiotic *L. rhamnosus* at doses of 10^7 or 10^9 CFU/50kg of body weight in the milk replacer for 14 consecutive days around weaning had no effects on immunity.

3. Supplementing calves with butyrate in the form of sodium butyrate or tributyrin in the milk replacer at doses of 3 g/kg of dry matter for 42 days during the preweaning period did not improve performance parameters or glucose metabolism before weaning and tended to increase the incidence of diarrhea.

4. Supplementing calves with butyrate in the form of tributyrin in the milk replacer at doses of 3 g/kg of dry matter for 42 days during the preweaning period resulted in retarded growth.

5. Calves fed 6 L/d of milk replacer showed increased growth before weaning compared with calves fed 4 L/d.

6. Feeding calves with 6 L/d of milk replacer had no effects on insulin sensitivity, but tended to increase the incidence of diarrhea.

7. Calves fed a starter feed containing 11.2% of fat showed reduced starter intake after weaning. However, calves fed 6 L/d of milk replacer had increased body weight before and after weaning when offered a starter feed containing 11.2% of fat.

Chapter 9

LITERATURE CITED

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