ÍCARO RAINYER RODRIGUES DE CASTRO

FEEDING STRATEGIES TO IMPROVE THE DEVELOPMENT AND HEALTH OF DAIRY CALVES

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Marcos Inácio Marcondes

Co-adviser: Juana Catarina Cariri Chagas

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RESUMO

CASTRO, Ícaro Rainyer Rodrigues de, D.Sc., Universidade Federal de Viçosa, julho, 2024. **Estratégias Alimentares para Melhorar o Desenvolvimento e a Saúde de Bezerros Leiteiros**. Orientador: Marcos Inácio Marcondes. Co-orientadora: Juana Catarina Cariri Chagas.

Esta tese foi desenvolvida a partir de três estudos. Os objetivos do primeiro estudo foram investigar os efeitos da forma física da ração inicial sobre o consumo, desempenho, metabólitos sanguíneos e saúde dos bezerros. Vinte e quatro bezerras Holstein (5 dias de vida; 40,4 ± 3,86 kg de peso inicial; média ± DP) foram utilizadas em um delineamento em blocos completamente casualizados. Os bezerros foram alojados individualmente e, em seguida, distribuídos aleatoriamente para os tratamentos (n = 12 bezerros/tratamento): 1) ração inicial texturizada (RIT, com milho em grão inteiro) e 2) ração inicial peletizada (RIP). Os resultados indicam que rações iniciais contendo milho em grão inteiro como texturizador não melhoraram o consumo e o desempenho das bezerras em comparação com dietas contendo ração inicial peletizada; no entanto, melhorias foram observadas nos escores de saúde, na probabilidade de não ocorrência de doenças e no perfil bioquímico dos bezerros alimentados com ração inicial texturizada. Os objetivos do segundo estudo foram investigar como a incorporação de diferentes níveis de tributirina (TB4) e tricaproina (TC6) no sucedâneo afeta o consumo, desenvolvimento e a saúde digestiva de bezerros recém-nascidos (experimento 1) e bezerros de 18 dias de vida (experimento 2). No experimento 1, 24 bezerros machos (0,90 ± 0,35 dias de idade; 42,8 ± 4,43 kg de peso ao chegar; média ± DP) foram blocados por dia de chegada e idade. Dentro de cada bloco, os bezerros foram distribuídos aleatoriamente para 1 de 4 tratamentos com sucedâneo experimental contendo uma mistura de TB4 com TC6 (TRI) em 4 níveis de inclusão de 0, 33, 67 e 100% (TRI0, TRI33, TRI67 e TRI100, respectivamente). No experimento 2, 60 bezerros machos (18,4 ± 2,4 dias de idade; 47,5 ± 1,46 kg de peso; média ± DP) foram blocados por peso corporal e distribuídos aleatoriamente para 1 de 4 tratamentos com sucedâneo, incluindo 1) o mesmo CON fornecido no experimento 1, 2) CON com TB4 a 6% do total de gordura (TB4), 3) CON com TC6 a 4% do total de gordura (TC6), e 4) o mesmo sucedâneo fornecido no experimento 1 no nível TRI100 (TBTC). Constatou-se que a inclusão de TC6 no sucedâneo reduziu a necessidade de assistência na alimentação quando administrado a bezerros recém-nascidos e diminuiu a ingestão em bezerros com mais de 18 dias. No entanto, a suplementação de substitutos do leite com TB4, TC6 e TBTC melhorou a consistência fecal dos bezerros. O terceiro estudo teve como objetivo investigar o metaboloma hepático de bezerros de leite. Quarenta e cinco bezerros machos (46,1 ± 4,6 kg de peso; 2,1 ± 0,63 dias de idade; média ± DP) foram blocados por ordem de chegada à instalação de pesquisa. Dentro de cada bloco, os bezerros foram distribuídos aleatoriamente para 3 tratamentos com sucedâneo (n = 15 por grupo): (1) um controle positivo contendo creme de leite (CL), (2) um controle negativo (CN) contendo uma mistura de gorduras vegetais, e (3) um sucedâneo (TRI) contendo a mesma mistura de gordura vegetal que o CN, com a adição de TB4 e TC6. O tratamento CL serviu como uma referência biológica para a composição de gordura. No 35º dia, os bezerros foram sacrificados e amostras de tecido hepático foram coletadas e analisadas usando uma abordagem de metabolômica direcionada. Não foram encontradas diferenças significativas entre bezerros alimentados com CN e TRI, indicando nenhum efeito da incorporação de TB4 e TC6 no metaboloma hepático. Esses achados mostram que a inclusão de gordura do leite no sucedâneo modula significativamente o metaboloma hepático de bezerros jovens em comparação com um sucedâneo à base de gordura vegetal, destacando a importância de considerar a composição de gordura no sucedâneo para otimizar o desenvolvimento metabólico no início da vida.

Palavras-chave: Ácido butírico. Ácido caproico. Análise de sobrevivência. Forma física da ração inicial. Metaboloma hepático. Milho em grão inteiro. Sucedâneo.

ABSTRACT

CASTRO, Ícaro Rainyer Rodrigues de, D.Sc., Universidade Federal de Vicosa, July, 2024. **Feeding Strategies to Improve the Development and Health of Dairy Calves.** Adviser: Marcos Inacio Marcondes. Co-adviser: Juana Catarina Cariri Chagas.

This thesis was developed from three studies. The objectives of the first study were to investigate the effects of the physical form of starter feed (PFSF) on feed intake, growth performance, blood metabolites, and the health of dairy calves. Twenty-four female Holstein calves (5 d old; 40.4 ± 3.86 kg of initial BW; mean ± SD) were used in a completely randomized block design. Calves were individually housed and then randomly assigned to the treatments (n = 12 calves/treatment): 1) textured starter feed (TSF, with whole-kernel corn) and 2) pelleted starter feed (PSF). The findings indicate that starter feeds containing whole-kernel corn as a texturizer did not improve the intake and performance of dairy calves compared with diets containing pelleted starter feed; however, improvements were observed in health scores, non-disease probability and biochemical profile of calves fed textured starter feed. The objectives of the second study were to investigate how the incorporation of different levels of TB4 and TC6 affects the milk intake, growth, and digestive health of newborns (experiment 1) and 18-d-old calves (experiment 2). In experiment 1, 24 male calves (0.90 ± 0.35 d old; 42.8 ± 4.43 of arrival BW; mean ± SD) were blocked by arrival day and age. Within each block, calves were randomly assigned to 1 of 4 MR treatments of an experimental MR containing both TB4 and TC6 (TRI) in 4 inclusion levels of 0, 33, 67, and 100% (TRI0, TRI33, TRI67, and TRI100, respectively. In experiment 2, 60 male calves (18.4 ± 2.4 d old; 47.5 ± 1.46 of BW; mean ± SD) were blocked by BW and randomly assigned to 1 of 4 MR treatments, including 1) the same CON MR fed in experiment 1, 2) CON-MR with TB4 at 6% of total fat (TB4), 3) CON MR with TC6 at 4% of total fat (TC6), and 4) the same MR fed in experiment 1 in the TRI100 level (TBTC). It was found that the inclusion of TC6 into MR reduced the need for drinking assistance when fed to newborn calves and decreased intake in calves older than 18 d. However, supplementing milk replacers with TB4, TC6, and TBTC improved the fecal consistency of calves. The third study aimed to investigate the liver metabolome of dairy calves. Forty-five male dairy calves (46.1 ± 4.6 kg BW; 2.1 ± 0.63 d of age; mean ± SD) were blocked by arrival sequence to the research facility. Within a block, calves were randomly assigned to 3 MR treatments (n = 15 per group): (1) a positive control containing dairy cream (MF), (2) a negative control (NC) containing a blend of vegetable fats, and (3) an MR (TRI) containing the same mixture of vegetable fat as NC to which TB and TC were incorporated. The MF treatment served as a biological reference for fat composition. On d 35 calves were euthanized and liver tissue samples were collected and analyzed using a targeted metabolomics approach. No significant differences were found between calves fed NC and TRI, indicating no effect of TB and TC incorporation on liver metabolome. These findings show that including milk fat in MR significantly modulates the hepatic metabolome of young calves compared to a vegetable fat-based MR, highlighting the importance of considering fat composition in the MR composition to optimize metabolic development in early life.

Keywords: Butyric acid. Caproic acid. Liver metabolome. Milk replacer. Physical form of starter feed. Whole kernel corn. Survival analysis.

SUMMARY

GENERAL INTRODUCTION

Achieving a functional rumen and transforming calves into ruminant animals is a primary goal in commercial calf nutrition. Proper rumen development is essential for nutrient intake, efficient feed digestion, successful weaning, and optimal growth of replacement heifers before and after weaning (Van Niekerk et al., 2021). The provision of a liquid diet, either as whole milk or milk replacer (**MR**), along with an early intake of solid feed, influences rumen development and can have long-term effects on the productivity and longevity of dairy cows (Diao et al., 2019). Both the metabolic and physical aspects of rumen development should be considered in a way that a smooth transition from liquid to solid feed is critical for post-weaning performance (Khan et al., 2011).

The physicochemical properties of calf starter feed play a crucial role in initiating solid feed intake, ruminal development, and growth performance of calves by weaning (Pazoki et al., 2017). The processing methods of cereal grains, such as grinding, flaking, and rolling, as well as the feed manufacturing techniques, significantly affect the ruminal and intestinal digestibility of feed and starch (Hu et al., 2018) and overall performance (Mirzaei et al., 2016) of both young and adult cattle.

Grinding was found to be involved in rumen parakeratosis (Beharka et al. 1998). Calves fed ground starter feed had shorter papillae and decreased surface area than calves fed chopped hay and rolled grains (Beharka et al., 1998). According to Porter et al. (2007), starter particle size exceeding 1190 µm was suggested to reduce mashed diets' negative effects. Ghassemi Nejad et al. (2012) found that compared with mashed starters, pelleted or texturized starters could enhance neonatal Brown Swiss calves' performance. In contrast, in a recent study, authors found no effects of starter particle size (pelleted *vs.* ground starter) with or without hay inclusion on rumen pH, feed intake, and weight gain of calves during 63 d of the experimental period (Leão et al., 2020).

Many commercial calf starters available today are textured and include steam-flaked, steam-rolled, coarsely rolled, or whole grains combined with a pelleted supplement (Jafari et al., 2020). However, studies have reported conflicting results regarding the effects of starter physical form on the performance and ruminal development of dairy calves (Bach et al., 2007; Porter et al., 2007; Mirzaei et al., 2016; Pazoki et al., 2017; Omidi-Mirzaei et al., 2018). Therefore, scientific evidence is lacking to suggest a preferable starter form to be fed to calves. The inconsistencies observed between studies can be partially described by variations in processing methods, grain source, milk feeding programs, weaning age, source and particle size of forage, and other management practices, highlighting the need for further investigation.

In addition to the physical form of starter feed, milk replacer feeding is also a major factor in calf development. There is a shift in dairy calf-raising practices towards more natural methods, mimicking how calves are raised by their mothers and weaned gradually. In this approach, calves receive about 10-12 liters of whole milk per day from their dams, significantly more than the traditional rearing systems where calves are artificially raised in individual pens or hutches, fed from a bucket twice a day, and receive about 4 liters per day (Khan et al., 2011)

Bovine milk composition consists of approximately 87% water, 4.6% lactose, 3.4% protein, 4.2% fat, 0.8% minerals, and 0.1% vitamins (Månsson, 2008). However, milk composition depends on multiple factors, such as breed, lactation stage, management of the cow, and feeding strategies (Lindmark-Månsson et al., 2003). Moreover, milk fat contains in total more than 400 different fatty acids (**FA**), consisting of short-chain (**SCFA**), medium-chain (**MCFA**), and long-chain fatty acids (**LCFA**) (Jensen et al., 1990; Lindmark-Månsson et al., 2003).

Milk replacer composition largely differs from bovine whole milk (WM) in macronutrient and micronutrient composition. Conventional MR for calves contains approximately 16 to 22% fat (DM basis) compared to 30 to 40% fat in the WM (Berends et al., 2020). Bovine WM provides approximately 50% of the total dietary energy as well as essential FA and bioactive compounds critical for calf development and health (Delplanque et al., 2015; van Niekerk et al., 2021b). However, MRs have a different triglyceride (**TG**) structure than WM. These differences are linked to the type of fat source used in MR: mostly animal fats in North America (Esselburn et al., 2013), and mostly vegetable oils in Europe and other parts of the world (Welboren et al., 2021a; b; Yohe et al., 2021). Animal fats and vegetable oils are cheaper than milk fat (**MF**) and therefore used in the current MR. Although C4:0 and C6:0 are naturally present in WM, dependent on the ingredients used, MR amounts of C4:0 and C6:0 are completely absent or only present in small quantities (Kato et al., 2011; Esselburn et al., 2013), and consequently, they may be supplemented in MR.

Besides the role of C4:0 as a primary nutrient (Venegas et al., 2019) , C4:0 was shown to be an important stimulator of calf development by improving nutrient digestibility (Guilloteau et al., 2010a; b; Sun et al., 2019b), and improving intestinal development and health in the preweaning (Liu et al., 2022), increasing ADG (Guilloteau et al., 2009; Liu et al., 2021) and promoting epithelial cell proliferation, and a regulating cell differentiation and apoptosis in the gastrointestinal tract development (Guilloteau et al., 2009; Górka et al., 2011). In contrast, The roles of C6:0 in newborn development have been less studied but may also contribute to energy metabolism and have antimicrobial properties (Aurousseau, 1984; Castro et al., 2024 in press). Wilms (2024) reported lower postprandial TG, weekly serum NEFA, and weekly plasma cholesterol in calves fed MR containing TB and TC compared to calves fed an MR that did not contain these FA. Consistently, Kato et al. (2011) reported a trend toward lower postprandial NEFA concentrations in calves fed MR supplemented with sodium butyrate (NaB). In addition, butyrate treatment has significantly reduced total cholesterol and TG concentrations (Kato et al., 2011). Moreover, in obese rodent models (Li et al., 2013, 2018; Arnoldussen et al., 2017; Adeyanju et al., 2021) and individuals with metabolic syndrome (Jocken and Blaak, 2008), butyrate treatment significantly reduces total cholesterol and TG concentrations. These so-called "enhanced lipid markers" suggest increased storage capacity for adipose tissue. However, this could also be due to improved liver function or a combination (van Deuren et al., 2022).

Extensive data regarding the function of C4:0 in calves is available. Some studies showed the importance of this FA in the metabolic development and performance of calves in the pre-weaning and weaning phases when supplemented in the MR (Górka et al., 2011; Nazari et al., 2012). In contrast, C6:0 has been less studied, but its presence in WM may also be relevant for calves. Therefore, it is important to consider fat composition when increasing the fat fraction in MR. Developing knowledge regarding the effects of tributyrin and tricaproin on health, metabolism, and development for newborn calves is relevant since it may contribute to developing the optimal MR and improve their health and development.

This thesis provides a comprehensive overview of the current understanding of how different physical forms of starter feed affect calves' intake, performance, and health. It also evaluates how various compositions of milk replacers affect calf development, with a focus on the changes in macronutrient levels in liquid feed and their impact on calf metabolism.

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CHAPTER 1

Under Review in Animal – R2

EFFECTS OF THE PHYSICAL FORM OF STARTER FEED ON THE INTAKE, PERFORMANCE, AND HEALTH OF FEMALE HOLSTEIN CALVES

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ABSTRACT

Available literature on the effect of various physical forms of starter feed (PFSF) on calf performance is conflicting. Thus, this study aimed to investigate the effect of the PFSF on feed intake, growth performance, blood metabolites, and the health of dairy calves. Twenty-four female Holstein calves (5 d old; 40.4 ± 3.86 kg of initial BW; mean ± SD) were used in a completely randomized block design. Calves were individually housed and randomly assigned to the treatments (n = 12 calves/treatment): 1) textured starter feed (TSF, a mix of pelleted ingredients and whole-kernel corn) and 2) pelleted starter feed (PSF). Both starter feeds had the same ingredients and nutrient compositions. Calves were fed the same milk replacer and weaned in a step-down scheme at 67 d. Health was evaluated daily until weaning. Treatments did not affect starter feed intake, water intake, BW, ADG, withers height, and clinical signs of disease (loss of appetite, ear position, and cough incidence). Nonetheless, scores for abnormal attitude ($P = 0.01$), presence of ocular discharge ($P < 0.01$), total respiratory disease ($P = 0.02$), and fecal consistency ($P = 0.04$) of PSF-fed calves were higher than those fed the TSF. Based on that, TSF-fed calves exhibited a higher non-disease probability compared to the PSF over time. Calves fed TSF sorted or tended to sort against small particles (0.425-mm sieve; $P = 0.01$) and 0.3-mm sieve; $P = 0.06$), respectively. Coincidently, PSF-fed animals sorted for small particles in the same sieve sizes. Creatinine ($P = 0.06$, total protein ($P = 0.06$), and globulin ($P = 0.07$) tended to be higher for TSF-fed calves, while albumin:globulin ratio ($P = 0.09$), tended to be higher in the PSF-fed calves. In conclusion, starter feeds containing whole-kernel corn as a texturizer did not improve the intake and performance of dairy calves compared with pelleted starter feed; however, improvements were observed in health scores, non-disease probability and biochemical profile of calves fed textured starter feed.

Keywords: Fecal composition, particle size distribution, sorting behavior, survival analysis, whole grain

IMPLICATIONS

Feeding dairy calves with either textured starter feed or pellets results in similar performance outcomes. However, textured starter feeds may offer additional health benefits. Further investigation is needed to understand the impact of textured feed on calf welfare and rumen development, as this study did not thoroughly examine these aspects.

INTRODUCTION

Early weaning and restricted milk feeding programs are strategies widely used to reduce the feeding cost of rearing young calves and encourage starter feed consumption in dairy calves. Typically, commercial starter feeds are textured and contain coarsely rolled or ground grains, whole grains, protein, mineral, and vitamin supplements in their composition (Jones and Heinrichs, 2022). However, many commercial starter feeds are formulated with inadequate fiber levels and large amounts of fine particles, which fall below the recommended standards for a good-textured diet. Ideally, NDF levels should range between 15% and 25% (Davis and Drackley, 1998), and a good-textured diet should have a minimum of 45% grains, whether whole, steam-flaked, dry-rolled, or containing more than 75% of particles >1,190 µm in length (Khan et al., 2016; Ghaffari and Kertz, 2021). This inadequacy in the formulation of these feeds decreases solid feed intake and negatively impacts calf ruminal health (Bateman et al., 2009). As shown by Rezapour et al. (2016), dry-rolling compared with grinding, regardless of the grain source, improved dairy calves' performance during the pre- and post-weaning periods.

Improvement in average daily gain (ADG), starter feed intake, digestibility, and earlier rumination were already observed in neonatal calves fed a coarse meal *versus* a pelleted starter feed (Porter et al., 2007). Moreover, these authors noticed that the physical form of starter feed (PFSF) and particle size distribution are more important than fiber inclusion levels in improving rumen fermentation, digestibility, and initiating rumination. All these factors work together to prepare calves for the weaning period allowing them to transition from a pre-ruminant to a mature ruminant state, improving ADG and BW.

The impact of feed abrasiveness, particularly when incorporating whole grains into calf starter feeds, has been investigated over the years. Since the 1950s, when foundational studies identified butyrate as the principal volatile fatty acid (VFA) influencing papillae development (Flatt et al., 1959), research has continued to explore the interaction between diet texture and rumen physiology. Subsequent research further elucidated that the keratinization of papillae exhibited an inverse relationship with the abrasiveness of the diet, particularly when employing a finely ground and pelletized diet (Greenwood et al., 1997) and the physical effects of starter on rumen anatomical, microbial, and fermentative development (Beharka et al., 1998). Recent studies have shown that feeding calves texturized starter feed leads to several benefits. These include improved morphological development of the reticulorumen due to increased nutrient digestion (Quigley et al., 2018), higher rumen VFA proportion and production (Diao et al., 2019), greater DM intake and ADG (Terré et al., 2016), and enhanced immune status (Jahani-Moghadam et al., 2015).

There is evidence that PFSF plays a role in the calf's performance before and after weaning and might also influence rumen development and health. However, due to conflicting data in the literature, the mechanism behind it is not fully comprehended. Thus, it was hypothesized that feeding a textured pellet concentrate (a mix of a protein pellet with whole-kernel corn) during the preweaning period of female dairy calves would improve intake and growth with no changes in their health status. Therefore, this study aimed to evaluate dairy calves' nutrient intake, performance, and health under different PFSF diets.

MATERIAL AND METHODS

Calves and housing

The experiment was conducted at the Knot Dairy Farm (KDC), an experimental dairy station at Washington State University in Pullman, WA, USA. The trial was conducted between April and September of 2022. Meteorological data of the period is presented in Table 1.

Twenty-four 5-d-old female Holstein dairy calves (40.4 ± 3.86 kg BW; mean ± SD) were used in the trial. A power analysis was conducted, and a difference from the control of 15% was expected in DM intake and starter intake within each timepoint, with a coefficient of variation of 14.5%, a *P-value* of 0.05, and a Power (1 – Beta) of 90%. The calculation resulted in 12 calves per treatment for a total of 24 calves. Calves were treated according to the farm's procedures for the five first days of life (navel care, identification, and housing). All calves receive 1 gallon of the first colostrum of cows within 3 h of life and another 1 gallon of the second milking of cows (second colostrum) 8 h after. The following meals consist of 3 qt of milk twice a day at 0500 and 1330 h. Before entering the experiment, calves had their total circulatory protein level measured using a refractometer (HHTEC®, model RHB-32ATC, Heidelberg, Germany) to evaluate the success in the colostrum feeding, with 5.5 g/dL as the threshold (McGuirk and Collins, 2004). The colostrum provided to the calves was obtained from the farm's colostrum bank, and to ensure the transfer of passive immunity, only colostrum with a quality rating of good or excellent was fed (>50 mg/mL of Immunoglobulins; Puppel et al., 2019). Calves were blocked using a randomized complete block design based on their date of birth as blocking criteria, being block 1 – born in April (n = 7); block 2 – born in May (n = 10), and block 3 – born in June (n = 7).

The calves were individually housed in a naturally ventilated calf barn. Each stall (3.05 × 1.12 m) had specific spots for feeders and freshwater buckets. During the experiment, calves had access to water *ad libitum* and were fed milk replacer (MR) and starter feed. On a 2-d basis, manure removal and replacement of bedding with wheat straw were done to keep the stalls visibly dry and clean.

Experimental treatments and feeding schedule

Calves were randomly assigned to one of the following treatments fed as a starter feed mixture:

- Pelleted starter feed (PSF): 12 calves (fed exclusively a protein pellet containing ground corn in its composition, with the size of the die on the pelleting machine being 11/64 inch).
- Textured starter feed (TSF): 12 calves (fed a mix of a protein pellet with whole-kernel corn, 60:40% as fed matter). USDA #2 grade dent corn, the predominant type grown in the United States, was used. This corn has 84.5% dry matter and 71% starch (on a dry matter basis). It was sourced from a mixed collection of fields to ensure consistent quality and meet USDA grading standards and harvested at a stage of full physiological maturity (maximum dry weight).

Forage was not offered, but alfalfa meal was included in the pellet composition independently of the treatment since both starter feeds had the same ingredient and chemical composition (Table 2).

Regarding the feeding scheme, MR (Calva®, Optimum, Acampo, CA, USA; Table 2) was mixed according to the manufacturer's guidelines and fed at 15% of their birth weight at a concentration of 140 g/L (14% DM) in a step-down scheme, in which calves received 6 L/d from d 5 to 30; then, it was fed 10% of their birth weight (4 L from d 31 to 60). Weaning started at d 61 of life by decreasing 1 L every other day until the calves were completely weaned at d 67. The MR allowance was fed twice daily (0600 and 1400 h) until reaching 3 L of milk allowance during weaning (two meals of 1.5 L). When the feeding scheme reached the amount of only 2 L a day, it was fed in a single meal daily, in the afternoon (1400 h) up to total weaning.

After weaning on d 67, the calves were moved into the farm's herd and grouped into pens of five calves each, fed the experimental diets and hay *ad libitum*, and provided free choice water.

Then, calves were monitored daily to assess for any abnormalities until the last measurement of post-weaning weight to evaluate any treatment carry-over effect.

Sampling procedures

Intake and feed composition

Starter feed and water intake offered and refused were weighed daily at 1400 h. Water intake was corrected for evaporative loss using the Penman-Monteith equation (Penman, 1948). Starter feed was offered at a rate that allowed about 10% refusals. Calf starters and orts were sampled every other week and analyzed as follows. All samples were pre-dried at 60 °C for 48 to 72 h in a forced-air oven (until present constant weight), ground, and sifted through a 1-mm screen in a Wiley mill (Thomas Wiley®, Model 4, Philadelphia, PA, USA) and analyzed for DM [method 934.01; Association of Official Analytical Chemists (AOAC) 2006], OM (method 942.05; AOAC, 2006), CP (method 990.03; AOAC, 2006), ether extract (EE; method 2003.05; AOAC, 2006), NDF (Van Soest et al. (1991) method using ANKOM® system with the addition of heat-stable α-amylase (Mertens, 2002), and starch (method 996.11; AOAC, 2006). All analyses were performed at the Department of Animal Sciences (WSU).

Performance

Body measurements were conducted weekly, before the afternoon feeding, for 9 consecutive weeks. The BW was measured with an electronic scale (Metter Toledo®, model IND236, Columbus, OH, USA), whilst withers height (WH) was measured using a graduated ruler. Calves were weighed and measured at 30 and 90 d after weaning to determine the postweaning performance. Pre-weaning, postweaning, and overall means of ADG were calculated as the difference between BW taken in a 7-d interval divided by 7 was considered as the ADG (g of BW/d) (Khan et al., 2007).

Fecal composition

Once a week, after the fourth week of the experiment, a spot sample of feces was collected from each calf, frozen immediately after sampling to stop fermentation, and stored at -20 °C until further analysis. Fecal samples were chemically analyzed for DM, NDF, CP, and starch.

Particle size and sorting behavior evaluation

The particle size distribution and the geometric distribution of particles (GDP) were determined by a particle analyzer, according to the American Society of Agricultural and Biological Engineers (ASABE, 2006). A representative sample (~100 g) was sieved for 10 min through a series of 6 screen sieves with nominal aperture sizes of 4, 2, 1.18, 0.71, 0.425, and 0.3 millimeters (mm) using a coarse sieve shaker (W.S. Tyler®, RX-812 model, Mentor, OH, USA). A bottom pan was included as a 7th screen to retain particles smaller than 0.125 mm. Each sieve was individually weighed before and after each sieve to obtain the weight of the samples on each sieve, thus determining particle size distribution and the geometric mean particle size. One run per sample was done, and sieves were cleaned thoroughly with an air compressor prior to each run.

The sorting index was computed as the ratio of actual intake to expected intake for particles retained on each sieve (Leonardi and Armentano, 2003). The predicted intake of an individual fraction was computed as the product of the DM intake of the total diet multiplied by the DM percentage of that fraction in the fed TMR. Values equal to 1.0 indicate no sorting, < 1.0 indicate selective refusals (sorting against), and > 1.0 indicate preferential consumption (sorting for). One sorting value was generated per calf every 14 d until the end of the experimental period to test whether calves were sorting the diet for each particle size fraction.

Health evaluation

A daily health evaluation of the calves was conducted during the MR-feeding period (d-5 to 67) using the Calf Health Score App® (McGuirk and Peek, 2014). A trained handler evaluated all enrolled calves in a blinded way to the treatment groups. Calves were also examined daily for respiratory disease incidence during the experimental period. Calves were categorized based on the clinical signs of disease exhibited, with a scoring system ranging from 0- normal, 1- variation of or slightly abnormal, 2- abnormal, to 3- severely abnormal for nasal discharge, cough, appetite, attitude, ocular discharge, and ear position. The ocular and ear scores were used to obtain a sum of the clinical scores. Calves with a sum score of ≥ 4 or that have two or more clinical parameters with scores of 2 or 3 were classified as having respiratory disease, and the remaining calves (those with scores of 3 or less) were considered healthy calves.

Fecal score

A daily fecal score assessment was conducted using the Calf Health Score App®. The fecal consistency scores ranged from 0 to 3 (0 - normal; 1 - semi-formed, pasty feces; 2 - loose feces, but feces stayed on the top of the bedding; 3 - meant watery feces sifted through the bedding). Five calves under 1 month of age identified with a score of 3 were treated according to the farm's protocol for scours. The treatment was initiated as soon as a calf was identified as depressed, with eyes slightly sunken, did not finish a bottle or refused to drink the liquid diet, had signs of liquid manure, wet tail, or presence of fever. If the calf refused to drink milk, 2 quarts of electrolytes were offered instead. If skipping a meal, an esophageal feeder ensured the calf could get at least 2 quarts of milk or electrolytes daily. For the severe cases (three in total), calves under antibiotic treatment received Polyflex® (>1 mL/45.4 kg of BW intra-muscular) 4-5 days once a day and Banamine (> 1 mL/45.4 kg of BW intra-venous 1-3 days once a day). A reassessment was performed on d 4. If the scouring was severe, a 3-d treatment with Albon bolus® was added (Sulfadimethoxine – Zoetis Inc., Kalamazoo, MI, U.S.A.), as per label instructions.

Survival analysis

Non-disease probability curves were built according to the Kaplan-Meier estimator function method to better illustrate the health results by describing the probability (with a 95% confidence interval; Kaplan and Meier, 1958) of survival for the calves during the days of the experiment receiving the different types of starter feed. The survival was modeled as a function of calf age, with day five as the date of the calf's entrance in the trial up to weaning (d 67).

Blood sampling

Two blood samples (per calf) were collected by venipuncture of the jugular vein into vacutainer tubes, one at 31 d of life and another at 61 d of life. Samples were centrifuged at 1,500 × *g* for 15 min (Geyer®, Premiere model XC-2415, Cincinnati, OH, USA), and serum was aliquoted and stored at -80 °C until used. Samples were submitted to Catalytic One Chemistry Analyzer (IDEXX®, Westbrook, ME, U.S.A.) using its specific kits (Chem-15 CLIP) and the following parameters were analyzed according to the manufacturer's instructions: Glucose, creatinine, BUN, phosphorus, calcium, total protein, albumin, globulin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), bilirubin, and cholesterol.

Statistical Analysis

Data analysis was done using the GLIMMIX procedure of SAS (Statistical Analysis System, version 9.4). All variables measured over time were analyzed as a randomized block design, and the week was included as a repeated measure in the model:

$$Y_{ijkl} = \mu + T_i + \delta_{ij} + P_k + (T \text{ x } P)_{ik} + B_l + iBW_{ijkl} + \varepsilon_{ijkl}$$

where μ = general mean; T_i = fixed effect of the treatment I; δ_{ij} = random error with a mean of zero and variance of σ^2, the variance among calves within treatment, equal to the covariance among repeated measures within calves; P_k = fixed effect of the period; $(T \text{ x } P)_{ik}$ = fixed effect of the interaction between treatment I and period e; B_l = random effect of the block (entry date); iBW_{ijkl} = initial BW as a covariate and ε_{ijkl} = random error with a mean of zero and variance of σ^2, the variance among measures between calves. It was excluded from the model whenever the covariate did not demonstrate statistical significance. All variance-covariance structures available in the GLIMMIX procedure were tested, and the one that provided the best fit based on the Akaike information criterion was used.

Since health scores typically do not conform to a normal distribution, weekly averages for the daily scores were calculated. However, it is worth noting that the residual scores did follow a normal distribution (with a *P-value* > 0.05), and the Gaussian distribution exhibited the lowest AIC for all the scores. Cox proportional hazard models (PROC PHREG, SAS 9.4) were also created to evaluate the proportional association between treatment and health scores over time. Then, non-disease probability curves were estimated by the Kaplan-Meier function method (PROC LIFETEST, SAS 9.4) for all significant health scores.

RESULTS

There were no interactions between time and treatment ($P > 0.05$; Table 3). Similarly, no significant differences were detected in the calf's starter feed intake ($P > 0.05$) and water intake ($P > 0.05$), irrespective of the week. Furthermore, BW, ADG, and WH ($P > 0.05$) were not affected by treatment in the pre and postweaning periods of evaluation.

Orts from TSF-fed calves were lower in NFC content ($P = 0.001$; Table 4) than those from the PSF-fed calves. Additionally, for fecal chemical composition, treatments did not affect the percentage of fecal NDF nor starch ($P > 0.05$; Table 4), however, a trend for interaction between time and treatment was found in the fecal protein content ($P = 0.069$).

Regarding the sorting behavior, it was noticed that TSF-fed calves highly sorted against small particle sizes in the sieve of 0.425 mm (*P = 0.011*; Table 5), and a trend with the same pattern was also found in the sieve of 0.3 mm (*P = 0.060;* Table 5); however, no sorting behavior was observed in the sieves of 4, 3, 1.18, 0.71, and 0.125 (Pan) mm (*P > 0.05*). Conversely, calves receiving the PSF diet sorted for these particle sizes in the same sieves.

There was only one observation regarding nasal discharge in one PSF-fed calf throughout the entire evaluation period. Clinical signs of disease based on health scores, such as loss of appetite, ear position, and cough incidence, were not affected by treatments (*P > 0.05;* Table 6), whereas calves fed PSF presented higher/worse scores in the variables regarding abnormal attitude (*P = 0.010*), presence of ocular discharge (*P = 0.004*), total respiratory score (*P = 0.019*), and fecal score (*P = 0.040*) than those fed the TSF. Furthermore, non-respiratory scores had a similar pattern (trend, *P = 0.087*).

Both treatment groups initially exhibited similar health performance trends during the early stages of the trial (Figure 1). However, a noticeable divergence emerges between the two groups around the very first week of evaluation. The TSF treatment group shows a distinct improvement in health performance compared to the PSF group, which became more pronounced as the trial progresses.

The PFSF did not affect glucose, BUN, BUN:creatinine ratio, phosphorus, calcium, albumin, ALT, ALP, GGT, bilirubin, and cholesterol (*P > 0.05*, Table 7). A trend was found in creatinine (*P = 0.061*), total protein (*P = 0.058*), globulin (*P = 0.067*) with higher means for TSF-fed calves, and albumin:globulin ratio (*P = 0.093)*, higher in the PSF-fed calves. The age at sampling affected the levels of creatinine (*P = 0.003*), GGT *(P = 0.001*), and bilirubin *(P = 0.022*), all were higher in the calves with 30 d of age, while calcium *(P = 0.015*) had a higher mean at 60 d of age. Conversely, there was no age-related effect on the variables glucose, BUN:Creatinine ratio, phosphorus, total protein, albumin, Globulin, Albumin:Globulin ratio, ALT, ALP, and cholesterol (*P > 0.05*), and only a trend was observed in the variable BUN *(P = 0.071*).

DISCUSSION

Feed intake was not affected by diet, contradicting the expectation of a higher intake of starter feed with TSF-fed calves. It is commonly known that pelleting may reduce the particle size of starter feeds and might, consequently, negatively influence rumen fermentation and feed intake, accompanied either with or without impacts on growth performance (Bach et al., 2007; Porter et al., 2007). Calves usually avoid finely ground feeds (e.g., meal), frequently associated with reduced palatability and DM intake (Bateman et al., 2009). Further, PFSF can affect rumen development because of its effects on ruminal fermentation (greater pH and lower NH_3-N; Pazoki et al., 2017), which is also linked to DM intake. However, similar to studies of Mirzaei et al. (2016) and Omidi-Mirzaei et al. (2018), there was no difference between the PFSF diets concerning starter feed intake (textured *vs.* pelleted forms). Visually, no corn kernels were found in the calves' feces during the samplings, possibly attributed to the coarser nature of the TSF. Strappini et al. (2021), evaluating calves' behavior and oral interactions, observed that the calves spent more time manipulating objects with their mouth than doing other activities, which might be expressed by chewing the whole-kernel corn of the TSF in the present study. Nevertheless, it is acknowledged that certain researchers may perceive the observed phenomenon as indicative of rumination.

Miller-Cushon and DeVries (2017) suggested that feed sorting behavior in calves is most likely to initiate when they are: 1) first given a diet that they are physically able to sort (e.g., a diet comprising of a mixture of components that can be sorted) and 2) driven to sort (e.g., a diet that diverges in chemical composition from the ratio of constituents they would sort themselves). Despite anticipating different sorting behaviors based on feeding preferences due to visual differences in the PFSP, it was insufficient to stimulate the sorting among all 7 sieves evaluated; noticeable effects were only observed in the smallest sieves (0.425 and 0.3 mm). The present study findings for the PSF treatment were contrary to Costa et al. (2016), in which calves could not sort for specific components within the calf starter feed. Although, to the best of the authors' knowledge, there has been no research specifically investigating this matter, PSF, in contrast to texturized varieties, has been suggested to reduce sorting behavior (Moran, 2012; Costa et al., 2016).

According to Bateman et al. (2009), calves tend to eat less starter feeds that are finely ground compared to those with larger particles. Thus, consistent with this finding, calves in the present study preferred coarser particles, while calves in the TSF group sorted the starter feed against the smaller particles. Similarly, Webb et al. (2014) evaluated that 2–to 5-month-old calves fed MR and concentrate that were trained to work for roughage rewards from two simultaneously available panels. The authors noticed that the calves already choose to consume longer/bigger particles at this age. Moreover, it supports the authors’ statement that ruminants can make choices based on rumen function/health and possibly also based on their motivation to chew and ruminate.

While existing studies suggest a link between sorting and rumen metabolism and development, the literature contains conflicting data (Pezhveh et al., 2014; Mirzaei et al., 2016; Pazoki et al., 2017). The sorting preference observed in the present study indicates that differences in rumen development may have affected calves' sorting behavior, highlighting distinctions between the two evaluated groups. In previous studies, the focus often centered on the PFSF, particularly grain fineness. However, despite influencing starter feed intake, particle size primarily determines the feedstuff's abrasive values, impacting papillae development and overall health rather than the chemical composition (Yavuz et al., 2015). The reduction in episodes of loose feces for TSF compared with PSF may have contributed to a lower likelihood of disease

No direct measurements of gastrointestinal tract development and function were performed in the current study, but daily monitoring of fecal scores permitted a good determination of the digestive health status of each calf individually. It was possible to observe with the survival analysis that along with higher average scores, PSF-fed calves had a higher incidence of loosened feces throughout the experiment when compared to the TSF-fed calves, which might indicate better digestive and intestinal health of the TSF-fed calves. Moreover, existing literature provides evidence that when calves are fed diets rich in easily digestible corn (processed corn such as ground corn, steam-flaked corn, etc.), their fecal consistency is affected, resulting in looser and softer stools in comparison to calves that consume conventional corn-based diets (Casper et al., 2017). Nevertheless, despite the similar challenges faced by both groups, TSF-fed calves could express better health scores when compared to the PSF-fed group.

Data were also gathered regarding the metabolic profile of the calves under study. Creatinine, GGT, and bilirubin, and a trend in BUN exhibit a consistent decreasing pattern as the calves mature, and effects were observed in blood calcium levels increasing as calves aged. Overall, diets did not impact the metabolic status of the calves or influence the blood concentrations of key enzyme markers. This suggests that regardless of the treatment, there was no impairment in the healthy functioning of the liver, consequently posing no threat to the overall health status of the calves.

The findings reported in this study warrant further investigation through slaughter analysis that is scarce in the literature, focusing on specific aspects of structural development like papillae development, variations in volatile fatty acids production, and changes in the microbial population in calves receiving different types of PFSF. Expanding this research in this direction could unveil the underlying mechanisms and provide valuable insights for optimizing calf nutrition, health, and overall performance. These combined findings underscore the importance of adopting an integrated approach that considers sorting behavior and feed particle size to optimize intake and performance in young calves.

CONCLUSIONS

Contrary to the initial hypothesis, incorporating whole-kernel corn as a texturizer in calf starter feed did not improve starter feed intake and performance in young dairy calves when compared to pelleted starter feed. However, the results pointed to an improvement in calf health considering the enhancements in scores of general attitude, reduced ocular discharge, better fecal score, and lower total respiratory disease. These findings imply a potential association between the use of textured starter feed and improved gastrointestinal development, suggesting a positive impact on calf health when fed with coarser starter feed diets since early life.

ETHICS APPROVAL

The Institutional Animal Care and Use Committee of Washington State approved the study under protocol #7069.

DATA AND MODEL AVAILABILITY STATEMENT

None of the data were deposited in an official repository. The authors' data supporting the study findings are available upon request.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

The authors did not use any artificial intelligence-assisted technologies in the writing process.

DECLARATION OF INTEREST

The authors have no conflicts of interest to report.

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TABLES

Table 1. The average monthly temperature, humidity, temperature and humidity index (THI), and their respective standard errors of the experimental period.

Month	Weather data		
	Air Temperature (ºC)	Relative Humidity (%)	THI[1]
April	4.7 ± 3.3	69 ± 9.8	43.4 ± 5.7
May	9.4 ± 2.8	71 ± 8.4	50.4 ± 4.2
June	14.9 ± 3.1	71 ± 11.1	58.7 ± 4.7
July	19.8 ± 2.8	62 ± 7.8	65.6 ± 3.6
August	21.6 ± 2.6	45 ± 7.3	66.9 ± 3.2

Adapted from (AgWeatherNet, 2022)

[1] Calculated using the equation: $THI = 0.8 \times T + RH \times (T - 14.4) + 46.6$ where, T is temperature in (ºC), and RH is relative humidity in decimal form (Epstein and Moran, 2006).

Table 2. Ingredients, chemical composition (% of DM unless otherwise noted), and particle size distribution in the basal starter feed diet (Measured data).

Ingredient composition	Starter feed		MR[1]
Alfalfa meal	15.1		-
Corn grain, ground	56.7		-
Soybean meal	23.3		-
Molasses	2.5		-
Limestone	0.8		-
Mineral mixture[2]	0.5		-
Dicalcium phosphate	0.3		-
Salt	0.2		-
Magnesium oxide	0.2		-
Chemical composition	TSF[3]	PSF[3]	
DM	93.5	93.8	95.6
OM	93.5	93.6	90.1
CP	18.8	18.6	26.8
NDF	15.2	15.4	-
Ether-extract (EE)[5]	2.9	1.3	20.9*
Ash	6.5	6.4	8.90
Starch	35.2	36.7	-
NFC[4]	21.4	21.6	-
Particle size distribution (% of DM retained on sieves)			
4.00 mm	93.93	84.71	-
2.00 mm	3.07	3.44	-
1.18 mm	0.66	2.63	-
0.71 mm	0.87	3.86	-
0.425 mm	0.39	2.14	-
0.3 mm	0.29	1.32	-
Pan	0.80	1.89	-

[1] Milk replacer; Mineral composition: 1.3 g of Calcium, 0.8 g of Phosphorus, 0.1 g of Magnesium, 1.7 g of Potassium, 0.4 g of Sulfur, 64.4 PPM of Iron, 59 PPM of Zinc, 11.4 PPM of Copper, 34.4 PPM of Manganese, 0.9 PPM of Molybdenum.0.69 % of Sodium. Soluble Protein % CP = 97.4 (%DM), NEL = 2.89 Mcal/Kg. *Crude fat. Pool from 5 samples. Data from Dairy One Laboratory Analyses (*n = 5).*

[2] Contained per kilogram of the supplement: 975,000 IU of vitamin A, 750,000 IU of vitamin D, 1,800 IU of vitamin E, 143 g of Zn, 76 g of Mn, 48.6 g of Cu, 19.5 g of Se, 18.4 g of Fe, 8 g of Ca, and 1.3 g of Co + 0.02 g of Vitamin A and 0.40 g of Vitamin E.

[3] TSF = Textured feed and PSF = Pelleted starter feed (*n = 7*).

[4] NFC = 100 - (starter CP + NDF + EE + Ash; NRC, 2001).

[5] Means obtained from analysis at WSU Feed Mill Facility.

Table 3. Nutrient intake and performance influenced by feeding textured starter feed (TSF) or pelleted starter feed (PSF) of young calves (n = 24; 12 per treatment).

Item	*Treatment (T)*		*SEM*	*P-value*		
	TSF	PSF		T	Week (W)	T×W
Intake						
Starter, g/d	611	581	41.6	0.564	0.001	0.921
Water, g/d	1859	1973	353.7	0.595	0.001	0.729
Performance						
ADG, kg/d	0.76	0.78	0.029	0.664	0.001	0.252
Initial BW, kg	39.7	41.2	1.11	0.363	-	-
BW[1], kg	77.1	79.6	1.34	0.109	0.001	0.706
Final BW, kg	191.2	196.0	8.09	0.520	-	-
Withers height[1], cm	85	84	0.67	0.257	0.001	0.853

[1] Average over the experiment.

Table 4. Orts and fecal chemical composition influenced by feeding textured starter feed (TSF) or pelleted starter feed (PSF) of young calves (Measured data, n = 24; 12 per treatment).

Item, % of DM	*Treatment (T)*		*SEM*	*P-value*		
	TSF	PSF		T	Week (W)	T×W
Orts						
DM	93.4	93.7	0.06	0.001	0.294	0.431
OM	93.5	93.5	0.06	0.719	0.663	0.901
CP	18.6	18.4	0.13	0.319	0.088	0.727
NFC	55.7	58.0	0.36	0.001	0.511	0.883
NDF	16.3	15.8	0.25	0.152	0.590	0.814
Starch	15.2	14.3	0.37	0.122	0.531	0.688
Fecal						
NDF	39.6	38.3	13.93	0.987	0.589	0.090
Starch	2.84	2.67	0.400	0.768	0.005	0.277
Protein	22.5	22.9	0.49	0.567	0.004	0.069

Table 5. The sorting index of the diet (DM-based) influenced by feeding textured starter feed (TSF) or pelleted starter feed (PSF) by particle size distribution (n = 24; 12 per treatment).

Particle size distribution, mm	*Treatment (T)*		*SEM*	*P-value*		
	TSF	PSF		T	Week (W)	T×W
4.00	0.982	0.971	0.2674	0.456	0.473	0.464
2.00	1.100	1.085	0.0410	0.831	0.471	0.687
1.18	1.001	0.911	0.4961	0.701	0.603	0.461
0.71	1.167	1.129	0.2011	0.897	0.008	0.436
0.425	0.688	1.183	0.1332	0.011	0.927	0.402
0.3	0.753	1.228	0.1596	0.060	0.482	0.383
Pan	1.245	1.447	0.6677	0.511	0.028	0.564

Table 6. Health scores influenced by feeding textured starter feed (TSF) or pelleted starter feed (PSF) of young calves (n = 24; 12 per treatment).

Item*	*Treatment (T)*		*SEM*	*P-value*		
	TSF	PSF		T	Week (W)	T×W
Attitude	0.005	0.028	0.0007	0.010	0.001	0.114
Appetite	0.007	0.024	0.0085	0.122	0.001	0.786
Ear	0.002	0.005	0.0020	0.103	0.603	0.603
Cough	0.053	0.044	0.0263	0.672	0.001	0.577
Ocular	0.070	0.160	0.0219	0.004	0.438	0.998
Fecal	0.151	0.237	0.0325	0.040	0.001	0.279
TRS	0.121	0.206	0.0430	0.019	0.068	0.967
NRS	0.071	0.126	0.0278	0.087	0.001	0.143

*TRS = Total respiratory score; NRS: Non-respiratory score

Table 7. Blood metabolites in mg/dL (unless otherwise stated) influenced by feeding textured starter feed (TSF) or pelleted starter feed (PSF) of young calves (n = 24; 12 per treatment).

Item*	*Treatment (T)*		*Age, in days*		*SEM*	*P-value*		
	TSF	PSF	30	60		T	Age (A)	T×A
Glucose	96.9	94.6	98.3	93.1	2.45	0.564	0.166	0.899
Creatinine	0.9	0.8	0.9	0.8	0.03	0.061	0.003	0.827
BUN	7.5	7.2	7.9	6.8	0.43	0.592	0.071	0.423
BUN:Creatinine	9.2	9.4	9.3	9.3	0.46	0.705	0.899	0.264
Phosphorus	7.8	7.5	7.5	7.8	0.26	0.382	0.336	0.667
Calcium	7.9	7.9	7.0	8.8	0.49	0.933	0.015	0.783
Total protein, g/dL	5.8	5.4	5.5	5.7	0.14	0.058	0.318	0.967
Albumin, g/dL	2.4	2.3	2.3	2.4	0.05	0.235	0.157	0.999
Globulin, g/dL	3.4	3.1	3.2	3.3	0.10	0.067	0.318	0.910
Alb:Glob	0.7	0.8	0.7	0.7	0.02	0.093	0.563	0.999
ALT, U/L	31.5	29.1	31.3	29.3	1.01	0.118	0.177	0.818
ALP, U/L	259	235	244	250	17.4	0.331	0.804	0.602
GGT, U/L	33.1	33.0	42.0	24.1	2.35	0.981	0.001	0.797
Bilirubin	0.2	0.2	0.3	0.1	0.03	0.350	0.022	0.850
Cholesterol	98.8	102	104	96.3	5.65	0.719	0.324	0.518

*BUN = Blood Urea Nitrogen; BUN:Creatinine = ratio of Blood Urea Nitrogen (BUN) to Creatinine; Alb:Glob = ratio albumin (Alb) to globulin (Glob); ALT = Alanine Aminotransferase; ALP = Alkaline Phosphatase; GGT = Gamma-Glutamyl Transferase; g/dL = grams per deciliter; U/L = units per liter.

FIGURE

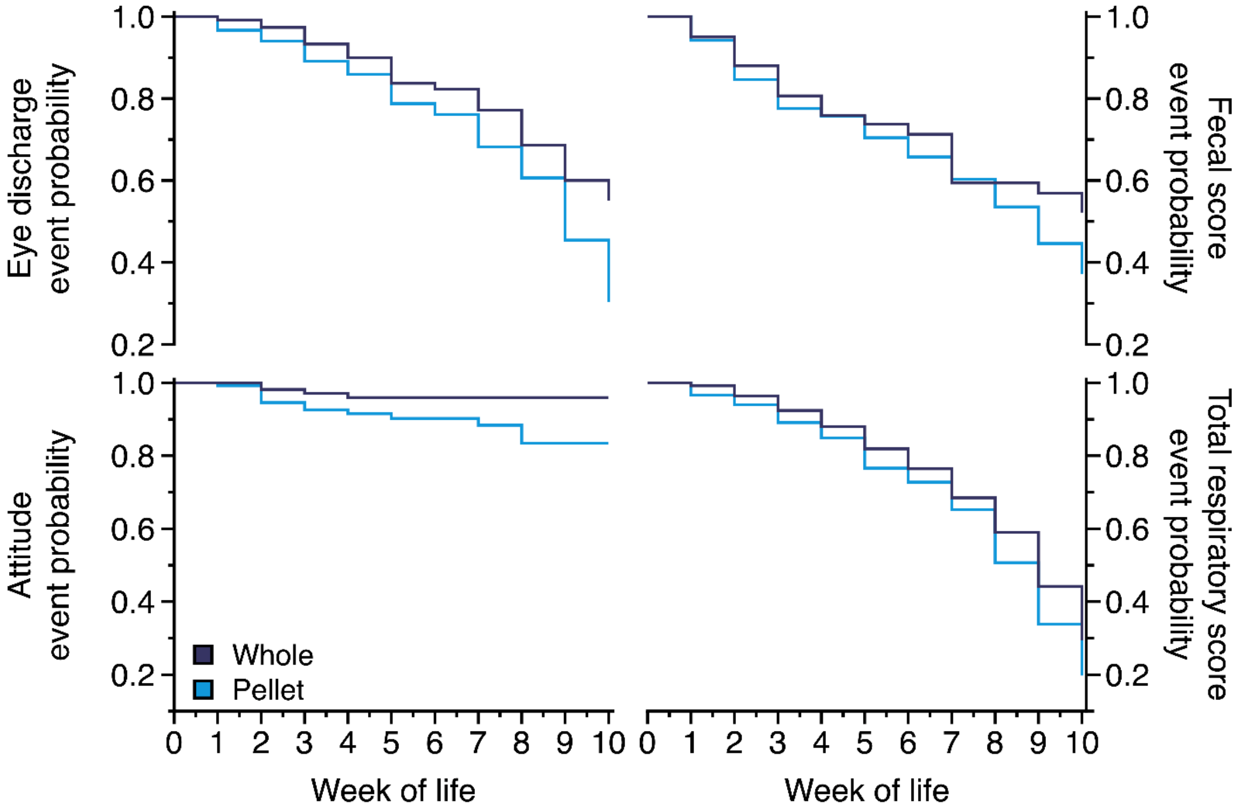

Figure 1. Kaplan-Meier survival curves influenced by the physical form of starter feed provided to Holstein's calves

CHAPTER 2

Under Review in Journal of Dairy Science – R2

INCORPORATION OF TRIBUTYRIN AND TRICAPROIN ACIDS INTO MILK REPLACER ON INTAKE, GROWTH, AND HEALTH OF HOLSTEIN CALVES FED TWICE DAILY

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ABSTRACT

The fat composition of milk replacer (MR) for calves differs from milk fat because of the use of alternative fat sources resulting in low levels of butyric and caproic acids. This study investigated how the incorporation of different levels of tributyrin (TB4) and tricaproin (TC6) in MR affects feed intakes, growth, and health of newborns (experiment 1) and 18-d-old calves (experiment 2). In experiment 1, 24 male calves (0.90 ± 0.35 d; 42.8 ± 4.43 BW; mean ± SD) were blocked by arrival day and age. Within each block, calves were randomly assigned to treatments consisting of a mixture of a control MR (CON) and an experimental MR containing TB4 and TC6 (TRI) at different percentages being TRI0 (0% TRI MR), TRI33, TRI67, and TRI100. The TRI100 MR contained 3.36% C4:0 and 2.49% C6:0 (as % total FA). This resulted in isoenergetic MR diets containing 23.7% CP, 25.8% fat, and 37.5% lactose (DM basis). Calves were housed individually for 21 d. The daily MR allowance was 7.0 L/d (15% solids) fed in 2 equal meals at 0600h and 1700h. Water and straw were fed ad libitum. Daily MR intake was measured by weighing refusals at 10 and 30 min after the start of the meal. Fecal consistency was scored after the morning meal over the first 21 d. In experiment 2, 60 male calves (18.4 ± 2.4 d; 47.5 ± 1.46 BW) were blocked by BW at arrival and randomly assigned to MR treatments including 1) a control MR (CON) that did not include TB4 and TC6 1, 2) an MR in which TB4 was incorporated resulting in 3.36% of total fatty acid (FA), 3) an MR to which TC6 was incorporated resulting in 2.49% of total FA, and 4) an MR with both TB4 and TC6 called TBTC with 2.41% C4:0 and 2.10% C6:0 (% total FA). All MR were isoenergetic and isonitrogenous with 29.2% fat, 24.3% CP, and 33.9% lactose (DM basis). Calves were individually housed for 28 d and then grouped in pairs or trios by merging adjacent pens. The daily MR allowance was 7.0 L/d (13.5% solids) fed in 2 equal meals at 0600h and 1700h. Weaning was initiated on d 36 after arrival by feeding 4.0 L from d 36-49 and calves were weaned at d 50 after arrival and monitored until d 63. From arrival onwards, calves had ad libitum access to starter feed, straw, and water. Weights and body dimensions were measured at arrival and weekly after that. Feed intake was recorded daily for 63 d, while fecal consistency was evaluated until d 28 after arrival. In experiment 1, the inclusion of TB4 and TC6 did not affect growth and MR refusal. Calves fed TRI0 and TRI33 tended to have higher abnormal fecal scores in wk 2 and 3 than other treatment groups. In addition, higher TB4 and TC6 inclusion levels in MR reduced the need for drinking assistance. In experiment 2, a shorter wither height at 8 wk in calves fed TBTC and a smaller body barrel at 7 and 8 wk was observed in calves fed TB4 and TBTC.

Milk replacer refusals were higher in calves fed TC6 and TBTC than in other treatments between wk 1-3 after arrival. Calves fed CON had a higher percentage of days with loose feces. In conclusion, the inclusion of TC6 into MR reduced the need for drinking assistance when fed to newborn calves and decreased MR intake in 18-d-old calves. However, supplementing MR with TB4, TC6, or their combination improved the fecal consistency of calves.

Keywords: Dairy calf. Diarrhea. Fatty acid. Macronutrient

INTRODUCTION

The variations in fat composition between milk replacer (**MR**) and bovine whole milk (**WM**) are due to the type of fat used in MR formulations (Mellors et al., 2023; Wilms et al., 2023b). Blends of animal and vegetable fats such as lard, tallow, and coconut are prevalent in North America (Esselburn et al., 2013). In contrast, in Europe and other geographical areas, vegetable fats, such as palm and coconut fats, are the primary fat sources (Welboren et al., 2021a; b; Yohe et al., 2021), contributing to these variations because of their unique fatty acid (**FA**) profiles and triglyceride (**TG**) structure (Welboren et al., 2021b, 2023). These differences directly affect fat absorption and utilization in neonates (Fischer et al., 2019; Hageman et al., 2019; Sun et al., 2019b). It is not possible to fully mimic the FA profile of bovine milk fat because some FA only exist in milk fat (Mellors et al., 2023; Wilms et al., 2023a). Consequently, MR contains low levels of butyric (**C4:0**) and caproic (**C6:0**) acids which are natural substances in mammalian milk (Pietrzak-Fiecko and Kamelska-Sadowska, 2020).

Both C4:0 and C6:0 can be incorporated into MR in various forms such as free FA, salts, or esters (Górka et al., 2018; Guilloteau et al., 2010a). As sodium butyrate (**NaB**) has an unpleasant smell to humans, esters such as butyrins (mono-, di-, and tributyrin [**TB4**]) may be an interesting alternative for inclusion in MR for calves. However, the strong astringent taste of butyrins can negatively affect MR palatability (Araujo et al., 2016; Gerbert et al., 2018). Several studies have found positive effects in calves receiving C4:0 supplementation in MR, such as higher MR intake and growth (Nazari et al., 2012), improved intestinal development (Sun et al., 2019; Liu et al., 2022), and improved digestive health (Liu et al., 2022; Dell'Anno et al., 2023). Nevertheless, discrepancies exist in the literature, as calf growth has also been reported to be adversely affected by TB4 supplementation in the MR (Araujo et al., 2016).

Caproic acid can be obtained from coconut and palm kernel fats; however, it accounts for less than 1% of the total FA in both fats (Cavalcante et al., 2017) and can be supplemented in MR as salt or as ester form such as tricaproin (**TC6**) in MR. Although the physiological role of C6:0 has been less studied, it could be relevant for newborn animals, particularly in species such as ruminants, whose milk contains a significant amount of C6:0 (2-3%; Mollica et al., 2021). In lambs, Aurousseau (1984) observed that supplementing TC6 improved feed and energy efficiency, regardless of the protein content in MR. The enhanced feed efficiency was partially attributed to increased MR digestibility with TC6 supplementation in MR (Aurousseau et al., 1983) compared to a control MR with tallow and coconut fat, resulting in higher growth rates in lambs (Aurousseau, 1984) and calves (Aurousseau et al., 1984). In addition, Wu et al. (2018) studied TC6 liquid supplementation (0.5% of the total diet) in piglet feed and reported a decrease in diarrhea rates up to 21 d of life.

Both C4:0 and C6:0 appear to decrease the incidence of diarrhea, treatment frequency, and duration in calves and rodents (Liu et al., 2022; Dell'Anno et al., 2023; Yang et al., 2023). Therefore, exploring compounds such as TB4 and TC6 supplementation in MR could provide alternatives to antimicrobials for treating calves with diarrhea (Dell'Anno et al., 2023). However, comprehensive studies on the effects of supplementing calves with TB4 and TC6 at different ages, particularly regarding feed acceptance, are lacking. In Wilms (Chapter 8, 2024), the percentage of calves refusing 20% or more of their daily allowance was higher in newborns fed twice daily an MR containing TB4 and TC6 on d 3 and 4 after arrival, but total intake was not affected. Thus, it was hypothesized that feeding MR, including TB4 and TC6, would negatively affect MR intake in old calves, but not in newborns. A secondary hypothesis was that incorporating TB4 and TC6 into MR would reduce the incidence of diarrhea and the number of calves treated for disease. Therefore, this study aimed to evaluate how incorporating TB4 and TC6 affects feed intakes, health, and growth of calves of different ages.

MATERIAL AND METHODS

The 2 experiments presented in this paper were conducted at the Research Facility of Trouw Nutrition Research & Development (Sint Anthonis, Netherlands). All procedures complied with the Dutch law on Experimental Animals, which follows the principles of ETS123 (Council of Europe, 1985 and the 86/609/EEC Directive), and were approved by the animal welfare authority (Centrale Commissie Dierproeven, CCD, the Netherlands). The project application code is AVD2040020173425.

Experimental Design, Treatments, and Feeding - Experiment 1

In the first experiment, 24 Holstein-Friesian male newborn calves (0.90 ± 0.35 d old; 42.8 ± 4.43 of BW; mean ± SD) were enrolled in the experiment from November 14th, 2022 until December 19th, 2022. The calves originated from a neighboring dairy farm and followed a standardized colostrum management protocol. The colostrum was pooled from all cows calving on a given day, and quality was measured with a Brix refractometer and required a value above 23%

[indicating an immunoglobulin G (**IgG**) content of 50 g/L or greater, NAHMS, 2007]. Calves were fed colostrum (4.0 L) within 30 min after birth using an esophageal tube, followed by 5 more meals (2.0 L) of first and second milking colostrum in 3 meals per day, for 2 d. Calves were then transported to the research facility after birth and on weekdays. Successful application of the colostrum protocol was evaluated upon arrival to the research farm, and between 36 to 72 h after birth, by measuring serum IgG concentrations using a portable Multi-Test Analyzer (DVM Rapid Test II, Vetlab, Palmetto Bay, FL, USA) and requiring a minimum of 10 g/L (Lombard et al., 2020). Calves had an average blood IgG of 1670 ± 477.8 mg/dL. Subsequently, the calves were blocked by arrival day and age at arrival (6 blocks of 4 calves each). Within each block, calves were randomly assigned to 1 of 4 dietary treatments using the random function [RANDBETWEEN (0,100000)] in Microsoft Excel (Microsoft 365 MSO, version 2212, build 16.0.15928.20278). Treatments consisted of a mixture of a control MR (**CON**) and an experimental MR containing TB4 and TC6 (**TRI**) at different percentages being TRI0 (0% TRI MR), TRI33 (33% TRI MR), TRI67 (67% TRI MR), and TRI100 (100% TRI MR). The CON MR (Sprayfo Delta, Trouw Nutrition, Deventer, the Netherlands) included a blend of vegetable fats (65% palm and 35% coconut as % total fat). The TRI MR contained the same fat blend as CON to which TB and TC were incorporated by reducing the inclusion of palm fat. The inclusion levels of TB4 and TC6 were based on the natural levels of C4:0 (3.25% total FA) and C6:0 (2.14% total FA) measured in dairy cream (Wilms, Chapter 8, 2024). The incorporation of TB4 and TC6 resulted in 3.36% C4:0 and 2.49% C6:0 when expressed as % total FA in the TRI100 MR. Milk replacer treatments were isoenergetic and isonitrogenous diets that contained 23.7% CP, 25.8% fat, and 37.5% lactose (DM basis). For all MR treatments in both experiments, the fats were spray-dried and subsequently encapsulated in a protein matrix. The analyzed chemical composition of the MR diets (CON and TRI) used in Experiment 1 are presented in Table 1 and the FA profile of CON and TRI100 can be found in Wilms (Chapter 8, 2024). Calves were fed their respective MR diets from arrival until d 21 after arrival in 2 meals of 3.5 L at 0600 h and 1700 h using individual nipple buckets. All MR treatments were fed at 150 g/L (15.0% solids). To focus solely on the effect of MR, no starter feed was fed during this study. The calves had *ad libitum* access to water provided by plain buckets and chopped wheat straw. Chopped straw was provided to ensure the welfare of calves and to prevent them from consuming excessive bedding material.

Measurements – Experiment 1

Individual water and straw intakes were recorded daily throughout the experimental period by weighing leftovers. To measure MR intake, the bucket was removed, and the volume of leftovers was recorded by weighing refusals at 10 min relative to the start of the meal. Subsequently, calves that did not consume the entire milk allowance were given an additional 20 min to complete their MR meal. During this 20 min timeframe, calves received assistance from caretakers when needed and this was classified into 3 categories, according to the number of times that the caretaker had to guide the calf to the teat; 1) the calves did not need assistance, 2) small assistance was provided by guiding the calf to the bucket only once, and 3) a high level of assistance was required, and the calf was guided to the bucket by the caretaker multiple times. Thirty min after the initiation of the MR meal, all nipple buckets were removed, and the volume of refusals was weighed. Body weight was recorded using a digital scale (model W2000, Welvaart Elektro BV, Vught, Netherlands) upon arrival and weekly on d 7, 14, and 21 at 1200 h. Fecal scores were evaluated by visual assessment of photos of feces taken after the morning meal (around 0900 h) from arrival until 21 d later. This was performed according to Renaud et al. (2020), in which scores 0 and 1 were considered the absence of diarrhea, and scores 2 and 3 were considered visible diarrhea.

Experimental Design, Treatments, and Feeding - Experiment 2

In the second experiment, 60 Holstein-Friesian male calves (18.4 ± 2.4 d old; 47.5 ± 1.46 of BW; mean ± SD) were enrolled in the experiment from December 27th, 2022 until February 28th, 2023. All calves originated from a collection center and arrived at the research facility in a single day and were initially housed in individual pens. The assignment to individual pens considered future grouping of calves (of 2 to 3 calves from the same treatment group) formed by removing the gates between adjacent pens. Calves were blocked by BW at arrival (6 blocks of 8 calves and 1 block of 12 calves). Within each block, 2 calves (blocks of 8 calves) and 3 calves (blocks of 12 calves) were randomly assigned to 1 of 4 dietary treatments using the random function in Microsoft Excel. Dietary treatments (15 calves per treatment) consisted of 1) a control MR (**CON**) that did not include TB4 and TC6, 2) an MR to which tributyrin (**TB4**) was incorporated resulting in 2.40% C4:0 (% total FA), 3) an MR to which tricaproin (**TC6**) was incorporated resulting in 2.34% C6:0 (% total FA), and 4) an MR to which both TB4 (2.41% C4:0, % total FA) and TC6 (2.10% C6:0) were incorporated (**TBTC**). The CON MR was the same as in Wilms (Chapter 8, 2024) and contained a blend of vegetable fats (62.7% palm, 35% coconut, and 2.3 % linseed), expressed as

% total fat. The inclusion of TB4, TB6, and their combination in the fat fraction was performed by reducing the inclusion level of palm fat accordingly. All MR were isoenergetic and isonitrogenous with 29.2% fat, 24.3% CP, and 33.9% lactose (DM basis). All MR comprised 62.5% skimmed milk powder, 29.5% of a fat blend, 6.0% sweet whey powder, and 2.0% premix (Trouw Nutrition, Deventer, the Netherlands). The premix contained vitamins, minerals, probiotics and prebiotics, stabilizers, AA, as well as flavoring and stability agents (Trouw Nutrition, Putten, the Netherlands). The analyzed chemical compositions of the diets used in Experiment 2 are presented in Table 2. One week after arrival calves were vaccinated with a Bovilis Intranasal RSP™ Live (MSD©, Walton, Milton Keynes, UK). On the day of vaccination, the temperature of each calf was recorded and in case of a body temperature superior to 39.3°C, vaccination was delayed by one week. One month after the first vaccination, the calves were vaccinated a second time with Bovalto Respi Intranasal (Boehringer Ingelheim©, Amsterdam, Netherlands). Calves were treated for respiratory disease when, at normal environmental conditions, exhibited clinical signs of disease including a fever over 39.3°C, rapid breathing with more than 30 breaths per minute, and at least one supportive symptom such as coughing (observed when the calf was standing), nasal discharge (either watery or thick), or changes in eyelid mucosa coloration (white vs. red). In experiment 2, calves were initially housed individually to ensure proper milk intake, monitor health, and assess fecal scores individually. To facilitate future grouping, calves intended to be in the same group pen were placed in adjacent pens, separated by panels that allowed for visual and physical interactions. From 28 d after arrival onwards, the separation panels were removed, and calves were grouped in pens; with either 2 calves (6 group pens per treatment) or 3 calves (1 group pen per treatment) in each pen. The group was then the experimental unit. Calves were fed their respective MR diets in 2 meals of 3.5 L at 0600 h and 1700 h using individual nipple buckets. Weaning was initiated on d 36 by feeding 4.0 L from d 36 to 49 in 2 equal meals. Calves were completely weaned at d 50 after arrival and monitored until d 63. Milk replacers were fed at a concentration of 135 g/L (13.5% solids). Starter feed, chopped wheat straw, and water were provided *ad libitum* from arrival onwards in plain buckets.

Measurements – Experiment 2

Individual water and straw intakes were recorded daily throughout the experimental period by weighing leftovers. Body measurements (hip height, wither height, chest girth, and body barrel) and BW were measured on the day of arrival and weekly at 1200 h after that until d 63, comprising pre- and post-weaning. Wither and hip height measurements were taken from the floor (base of hook bone) to the highest part of the back of the calf. The chest girth was measured by placing the measuring tape immediately behind the front shoulders and the body barrel was measured by placing the measuring tape around the belly area. For all the measurements, the calf stood straight on a flat surface. Health status was monitored daily, and a standardized protocol was followed in cases of illness. Records were kept for any medical treatment, including oral rehydration solution (Sprayfo OsmoFit®, Trouw Nutrition, Germany). Fecal scores were recorded as described in Experiment 1.

Housing

In experiment 1 and in the first phase of experiment 2, calves were housed indoors in individual pens (2.34 m × 1.16 m) separated by galvanized bar fences and equipped with rubber-slatted floors in the front area (50% of the total pen area) and a lying area in the back, which contained a mattress covered with flax straw (Flax Farm®). The temperature in the calf pen was maintained at a minimum of 12°C and a maximum of 28°C. Relative humidity was maintained between 60 and 85% The regulation was controlled by sensors placed in the barn which recorded temperature and humidity and adjusted it accordingly. Calves were exposed to daylight and artificial light controlled with a timer from 0530 to 2130 h.

Statistical Analysis

A power analysis was conducted in both experiments to determine the number of experimental units required. The power ($1 - \beta$) was chosen as 80% and the α-level was 0.05. In the first experiment, milk refusal was considered the most reliable parameter for determining statistical power. Based on a previous study conducted at the same research facility (Wilms, Chapter 8, 2024) for 21 d after arrival at 0 to 3 d of age, a standard deviation of 754 mL/d was assumed. The minimal meaningful difference in milk refusal rate was 20% of the daily allowance. The minimal sample size was 5 calves per treatment. All variables measured over time were analyzed using a randomized block design, and the week was included as a repeated measure in the model:

$$Y_{ijkl} = \mu+T_i+\delta_{ij}+P_k+(T \text{ x } P)_{ik}+B_l+\varepsilon_{ijkl}$$

where μ = general mean; T_i = fixed effect of treatment i; δ_{ij} = random error with a mean of zero and variance of σ^2, the variance among animals within treatment, equal to the covariance among repeated measures within the experimental unit; P_k = fixed effect of the period; $(T \times P)_{ik}$ = fixed effect of the interaction between treatment i and period e; B_l = random effect of the block (entry date); $\varepsilon ijkl$ is the random error with a mean of zero and variance of σ^2, the variance among measures between experimental unit.

For the second experiment, growth was considered the most reliable parameter for determining statistical power. Based on a previous study conducted at the same research facility (Amado et al., 2019), a standard deviation of 98 g/d was assumed for 70 d after arrival at 0 to 3 d of age. The minimal meaningful difference in the ADG was an increase of 10%. The minimum sample size was 12 calves per treatment group. Continuous variables were analyzed using a mixed-effects model with PROC MIXED in SAS (SAS 9.4M6, SAS Institute Inc., Cary, NC, USA), with the pair or trio (as per d 28) as the experimental unit. Experiment 2 was designed as a randomized block design with a 2×2 factorial arrangement, which included 2 factors, the inclusion of TB4 and that of TB6. All variables were measured over time and the week was included as a repeated measure in the model:

$$Y_{ijklm} = \mu + TB4_i + TC6j + (TB4 \text{ x } TC6)ij + \delta_{ijk} + P_l + (TB4 \text{ x } P)_{il} + (TC6 \text{ x } P)_{jl} + (TB4 \text{ x } TC6 \text{ x } P)_{ijl} + B_m + \varepsilon_{ijklm}$$

Where TB4i is the fixed effect of TB4 treatment i; $TC6_j$ is the fixed effect of TC6 treatment j; $(TB4\times TC6)_{ij}$ is the fixed effect of the interaction between TB4 treatment i and TC6 treatment j; δ_{ijk} is the random error with a mean of zero and variance σ^2, representing the variance among animals within the combination of TB4 and TC6 treatments, equal to the covariance among repeated measures within the experimental unit; P_l is the fixed effect of the period; $(TB4\times P)_{il}$ is the fixed effect of the interaction between TB4 treatment i and period l; $(TC6\times P)_{jl}$ is the fixed effect of the interaction between TC6 treatment j and period l; $(TB4\times TC6\times P)_{ijl}$ is the fixed effect of the interaction between TB4 treatment i, TC6 treatment j, and period l; B_m is the random effect of block (entry date) m; ϵ_{ijklm} is the random error with a mean of zero and variance σ^2, representing the variance among measures between experimental units.

Comparisons of means across treatments were conducted using the PDIFF option of the LSMEANS statement in the MIXED procedure of SAS. Significant interactions between treatment and time were explored with the SLICE option of the LSMEANS statement, using the PDIFF option of the MIXED procedure in SAS. Discrete variables (e.g., scours, calves treated, days treated, and delayed vaccination) were analyzed with mixed-effects logistic regressions using PROC GENMOD in SAS. The results in tables and figures are presented as the least squares mean (**LSM**) with standard error of the mean (**SEM**). Significance was declared at $P \leq 0.05$. Disease probabilities (e.g., fever and lungs) were analyzed using Cox's proportional hazard regression with the PHREG procedure in SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). Calves that did not receive medical treatments for fever or lungs during the entire study period were right-censored.

RESULTS

Experiment 1

The different levels of TB4 and TC6 in MR did not affect growth (Table 3). Similarly, there was no observed effect on MR refusals evaluated at 10 and 30 min after the start of an MR meal. However, calves fed TRI0 required the most assistance in drinking their MR meal. With increasing inclusion of TB4 and TC6 inclusion in MR, the amount of assistance needed for drinking reduced ($P < 0.01$). Throughout the 21-d monitoring period, no significant effect was observed on the fecal scores of calves in any of the levels evaluated. However, on a weekly basis, calves receiving the control (TRI0) and TRI33 tended to have a higher percentage of abnormal fecal scores in wk 2 ($P = 0.06$) and wk 3 ($P = 0.10$) when compared to other treatment groups.

Experiment 2

Solid feed intake was not affected by treatments. Wither height was the shortest in calves fed MR with TBTC at 8 wk after arrival compared to the other treatment groups, as shown by the significant treatment by time interaction ($P = 0.01$*;* Figure 1A, Table 4). At 7 and 8 wk after arrival, calves fed MR with only TB4 had a higher body barrel than the other treatment groups, as shown by the significant interaction between treatment and time ($P = 0.02$*;* Figure 1B). In contrast, growth, hip height, and chest girth were not affected by dietary treatments. Furthermore, there was an interaction between treatment and time in calves fed MR with TBTC, which had decreased MR intake ($P < 0.01$, Figure 2), as well as calves fed MR with only TC6 ($P = 0.05$). Both treatment groups fed the experimental MR containing solely TC6 or TBTC had elevated refusal rates, particularly in the first 3 wk after arrival.

Calves fed MR with only TB4 ($P = 0.02$) and only TC6 ($P = 0.04$) tended to have a lower number of animals treated for fever than those fed CON and TBTC. The percentage of calves treated for fever tended to be lower in those fed only TB4 ($P = 0.06$) compared to other treatments. Additionally, there was a trend for a lower percentage of calves treated for respiratory disease in calves fed MR with only TC6 ($P = 0.09$) and TBTC ($P = 0.09$) compared to the CON group. Calves fed MR with only TB4 ($P = 0.02$) and only TC6 ($P = 0.04$) also presented a lower percentage of calves treated for fever than those fed CON and TBTC. Consequently, calves fed only TB4 ($P =$

0.06) and TBTC ($P = 0.06$) tended to experience fewer delays in vaccination due to fever compared to calves fed CON and TC6 only. Considering the hazard ratio of the non-disease probability analysis (Supplemental material S1), calves fed MR with only TB4 demonstrated a decreased likelihood of fever ($P = 0.02$; Figure 3A) than calves fed CON. Calves fed MR with only TC6 exhibited a lower probability of respiratory disease ($P = 0.02$; Figure 3B) than calves fed CON. Calves fed TBTC had a lower probability of abnormal fecal scores ($P = 0.05$; Figure 3C), while those fed MR with only TC6 showed a lower number of total medical treatments ($P = 0.01$; Figure 3D), both in comparison to calves fed CON.

DISCUSSION

This study evaluated the response of calves fed MR containing TB4, TC6, or their combination. The first experiment evaluated whether feeding MR containing both TB4 and TC6 at varying levels would affect MR acceptance and total MR intake in newborn calves. The second experiment evaluated whether the inclusion of TB4 and TC6 in MR, individually or in combination, would affect feed intake, health, and growth of 18-d-old calves. Although newborn calves did not experience increased refusals with increasing levels of TB4 and TC6 in MR fed from birth onwards, the presence of solely TC6, as well as in combination with TB4 in MR, reduced MR acceptance in older calves. Although growth was not affected by MR treatments, the inclusion of TB4 and TC6 seemed to positively affect calf health.

The comparable growth and lack of differences in MR refusals in the first experiment suggested that feeding an MR containing TB4 and TC6 from birth did not adversely affect its palatability or acceptance by calves. The calves consumed their daily MR allowances without any signs of rejection across all dietary treatments. Additionally, upon initial exposure to the liquid diet, calves did not exhibit rejection behaviors, indicating their inability to detect the presence of TB4 and TC6, likely for being the first MR they were being exposed to. The MR refusals observed in experiment 2 indicate that as early as 18 d of age, calves can already identify changes in their diet, in contrast to the observations in the first experiment where high levels of TB4 and TC6 in MR were fed to newborn calves without adverse effect on palatability. This shows that when introducing an MR with TB4 and TC6 later in life, after calves have become accustomed to an MR that does not contain these compounds: feed rejection behavior can be observed. In experiment 2, this was particularly the case with MR containing solely TC6 and both TB4 and TC6 (TBTC), where an increase in MR refusals was observed among older calves in the first 3 wk following arrival. These findings show that age may be an important factor for the acceptability of calves fed MR containing these TG compounds, particularly TC6. This behavior could be attributed to food neophobia in calves, characterized by avoidance and reluctance to taste unfamiliar food. Neophobia refers to an aversion to or avoidance of novelty, a phenomenon commonly observed in ruminants (Costa et al., 2020). The sensory properties of food, such as smell, texture, and taste, play a significant role in modulating their preferences or aversions, impacting hedonic behavior, and influencing feed intake positively or negatively (Terré et al., 2022).

The increased incidence of diarrhea in the CON (TRI0) and TRI33 in experiment 1 likely resulted in an increased need for drinking assistance, which was probably due to the detrimental effects associated with digestive disorders faced by the animal receiving TRI 0 and TRI33 (Foster and Smith, 2009; Trefz et al., 2017; Wilms et al., 2024). Consistently, calves fed TBTC in the second experiment had a lower probability of abnormal fecal scores. Studies have consistently demonstrated a significant decrease in days with abnormal fecal score incidence with the incorporation of NaB (Hill et al., 2007; Górka et al., 2011) and TB4 (Liu et al., 2021, 2022; Dell'Anno et al., 2023) when supplemented in waste milk or MR, compared with control liquid feeds that did not include NaB nor TB4. However, there are discrepancies in the literature, in which C4:0 supplementation in MR did not reduce the incidence of diarrhea (Araujo et al., 2016; Murayama et al., 2023). These contradictory outcomes may be related to the timing of C4:0 introduction, as Araujo et al. (2016) supplemented MR with TB4 from d 12 to 42 of age, whereas Liu et al. (2022) initiated TB4 supplementation as early as d 2 after birth. Liu et al. (2022) observed reduced diarrhea frequencies throughout the entire experimental period from d 1 to 56 in calves. This reduced diarrhea incidence may be attributed to the favorable effect of C4:0 on enhancing gut development and gut integrity (Górka et al., 2018; Liu et al., 2022), which may provide enhanced resistance against pathogens causing diarrhea (Dell'Anno et al., 2023). This was observed in Wilms (Chapter 8, 2024), in which calves fed an MR including TB4 and TC6, had increased villus length at wk 5 of age in the ileum and had improved fecal consistency at wk 3 of age. Consistently, the positive effects of TB4 on gut health were also observed in broilers, with Wu et al. (2018b) reporting enhanced intestinal development following dietary NaB supplementation. Moreover, including NaB at higher doses in the basal diet (800 mg NaB/kg), improved the microbial community and diversity in the intestine, potentially leading to improved intestinal structure and function in broilers (Wu et al., 2018b). Likewise, Liu et al. (2022) found the same positive effects of improved intestinal development and health of pre-weaned dairy calves by stimulating SCFA-producing bacteria colonization, enhancing intestinal barrier functions, and suppressing inflammatory responses.

Regarding the potential effect of TC6 in improving digestive health, Wu et al. (2018a) also observed a significant decrease in diarrhea rates from d 0 to 10 (1.3% *vs.* 15.0%) and d 0 to 21 (1.19% *vs.* 8.33%) in piglets fed diets supplemented with TC6. Furthermore, the authors reported increased jejunal and ileal villus height, surface area, and villus height-to-crypt depth ratio in the jejunum and ileum (Wu et al., 2018a). Although this study focused on piglets, its applicability to other mammalian species remains to be explored. In addition, C6:0 exhibited antimicrobial properties within the intestinal lumen of rabbits (Skřivanová and Marounek, 2006) and *in vitro* (Kabara and Vrable, 1977; Hristov et al., 2004; Huang et al., 2011; Zentek et al., 2011). Characterized as inhibitors of bacterial growth, MCFA act through various biological mechanisms such as targeting the bacterial cell membrane, inducing cell lysis, inhibiting enzyme activity, and interfering with nutrient uptake (Yoon et al., 2018). It has also been suggested that C4:0 possesses antimicrobial properties (Hansen et al., 2021; Miragoli et al., 2021), potentially mitigating diarrhea severity, given that enteric pathogens are active in the intestines (Ma et al., 2023). Thus, the synergistic effects of TB4 and TC6 in both experiments might have promoted the development of the gastrointestinal tract (**GIT**) and provided antimicrobial functions, thereby enhancing the digestive health of calves. However, C4:0 and C6:0 supplementation in the MR is dependent on the compounds used, duration of supplementation, age of calves at initiation, and inclusion levels of C4:0 and C6:0.

Furthermore, it is worth mentioning that improvements in fecal consistency associated with C4:0 supplementation may be associated with enhanced fat digestibility. Fatty acids with a chain length shorter than 10 carbon atoms are more efficiently digested than FA with a longer chain length (Toullec et al., 1969), mostly because of the increased activity of pancreatic lipase within the C2:0 – C8:0 range of FA (Benito-Gallo et al., 2015). Moreover, the addition of C4:0 to MR also enhances the secretion of pancreatic enzymes, such as chymotrypsin and lipase, thus further enhancing fat digestibility (Guilloteau et al., 2010b). In addition, Górka et al. (2014) noted increased activity of brush border enzymes, such as lactase, maltase, aminopeptidase A, and N, in the jejunum and ileum of calves fed MR supplemented with NaB. This is consistent with results from Wilms (Chapter 8, 2024) where calves fed an MR with TB4 and TC6 tended to have enhanced apparent total-tract fat digestibility compared to an MR that did not include these compounds.

The reduction of abnormal fecal scores, along with the trends for lower respiratory disease incidence linked with TB4 and TC6 incorporation in MR, likely explains the reduction in fever and respiratory treatments. Although the mechanisms linking physiological characteristics and gut microbiota are not fully understood, the gut microbiota is an indicator of animal health, as the GIT is the largest immune organ (Chen et al., 2022). The gut microbiota plays a crucial role in educating the immune system during early life (Vientós-Plotts et al., 2023). Recent studies suggest that changes in the gut microbiome affect lung immunity and may influence susceptibility or resistance to respiratory infections (Welch et al., 2022; Eicher et al., 2023; McDaneld et al., 2023) due to the gut-lung axis. Lymphocytes from the immune system can migrate between mucosal-associated lymphoid tissues, such as those present in the GIT and respiratory system, through a mechanism known as the "common mucosal immune system" (Chase, 2018). As a result, Vientós-Plotts et al. (2023) indicated that disruptions in the microbiota of either the GIT or respiratory tract can lead to inflammation and microbial imbalances at other sites, and vice versa.

Finally, it is important to acknowledge the limitations of the study. In Experiment 1, the sample size was relatively small, with 6 calves per treatment. A more comprehensive and detailed experiment with a larger sample size could provide additional insights that were not described in the present study. Furthermore, in experiment 1, one limitation of the simultaneous incorporation of both TB4 and TC6 in the MR posed a challenge in isolating the individual effects of each FA or assessing their potential synergistic impacts. This distinction is particularly significant for C6:0, which has been less studied, and its relevance to calf health and development remains to be further evaluated.

CONCLUSIONS

Refusals of milk replacer containing a high inclusion of tributyrin and tricaproin occur only when fed for the first time to calves older than 18 d of age. In contrast, incorporating both tributyrin and tricaproin into the MR reduced the need for drinking assistance when fed to newborn calves. A consistent outcome between both experiments was a notable improvement in fecal consistency, which may indicate a reduction in diarrhea incidence and the need for medical treatment for fever and respiratory disease associated with the incorporation of tributyrin and tricaproin in MR.

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CONFLICT OF INTEREST

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TABLES

TABLE 1. Nutrient composition of milk replacers fed twice daily to newborn calves in experiment 1.

Item[1]	Treatments[2,3]	
	CON	TRI
Nutrient composition		
DM	96.8	97.1
Crude protein	23.5	23.8
Crude fat	25.0	26.6
Crude ash	6.3	6.2
Lactose	38.6	36.3
Glucose	0.5	0.2
Mineral composition		
Sodium	0.45	0.40
Potassium	1.20	1.23
Chloride	0.83	0.78
Calcium	0.80	0.95
Phosphorous	0.66	0.65
Magnesium	0.12	0.11
ME (MJ/kg DM)[4]	21.3	21.5
CP:ME ratio	1.10	1.11
Osmolality[5] (mOsm/kg)	326	320
SID[6] (mEq/L)	44	40

Abbreviations: ME, metabolizable energy; CP:ME ratio, ratio of crude protein to metabolizable energy; SID, strong ion difference

[1] Expressed as % DM unless specified otherwise.

[2] Milk replacers were fed from arrival to 21 d after arrival. Water and straw were provided *ad libitum* throughout the experiment.

[3] Treatments (n = 6 calves per treatment group) consisted of a mixture of a control MR (CON) and an experimental MR containing TB4 and TC6 (TRI) at different percentages being TRI0 (0% TRI MR), TRI33 (33% TRI MR), TRI67 (67% TRI MR), and TRI100 (100% TRI MR). The CON MR (Sprayfo Delta, Trouw Nutrition, Deventer, the Netherlands) included a blend of vegetable fats (65% palm and 35% coconut as % total fat). The TRI MR contained the same fat blend as CON to which TB and TC were incorporated by reducing the inclusion of palm fat.

[4] For MR based on NRC (2001).

[5] Osmolality (in moles per kg of solvent, expressed in mOsm/kg) was calculated by adding the osmolality of carbohydrates and minerals, as described previously (Wilms et al., 2020), at a concentration of 150 g/L.

[6] Strong ion difference (SID) = sodium + potassium – chloride considering a concentration of 150 g/L

TABLE 2. Nutrient composition of milk replacers and solid feeds fed twice daily to 18-d old calves in experiment 2.

Item[1]	Treatments[2,3]				Starter	Straw
	CON	TB4	TC6	TBTC		
Nutrient composition						
DM	96.7	96.7	96.7	96.8	90.0	91.4
Crude protein	24.3	24.3	24.2	24.2	20.1	3.1
Crude fat	29.2	28.9	29.4	29.2	3.1	-
Crude ash	6.46	6.33	6.37	6.32	7.5	4.5
Lactose	33.6	33.6	34.1	34.1	-	-
Starch	-	-	-	-	22.4	-
ME (MJ/kg DM)[4]	21.0	20.9	21.1	21.1	5.6	0.2
CP:ME ratio	1.2	1.2	1.1	1.1	3.6	4.5
NDF	-	-	-	-	19.2	77.2
ADF	-	-	-	-	11.3	48.1
ADL	-	-	-	-	0.7	8.0

Abbreviations: ME, metabolizable energy; CP:ME ratio, ratio of crude protein to metabolizable energy; SID, strong ion difference; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin.

Formulated values of mineral composition for all treatments (% product): MR = Sodium (0.48), Potassium (1.05), Chloride (0.80), Calcium (0.90), Phosphorous (0.70), Magnesium (0.12); Starter = Sodium (0.27), Potassium (1.40), Chloride (0.35), Calcium (1.29), Phosphorous (0.49); Straw = Sodium (0.49).

[1] Expressed as % DM unless specified otherwise.

[2] Milk replacers were fed from arrival to 63 d of age, Water, straw, and starter feed were available *ad libitum* throughout the experiment.

[3] Dietary treatments (15 calves per treatment) consisted of 1) a control MR (CON) with a blend of vegetable fats that did not include TB4 and TC6, 2) an MR to which tributyrin (TB4) was incorporated, 3) an MR to which tricaproin (TC6) was incorporated, and 4) an MR to which TB4 and TC6 were incorporated (TBTC).

[4] For MR based on NRC (2001).

TABLE 3. Growth and drinking refusals measured in newborn calves fed milk replacers with different inclusion levels of tributyrin and tricaproin (Experiment 1, n = 24).

Item	Replacement level (RL)[1]				Pooled	*P*-value				
	TRI0	TRI33	TRI67	TRI100	SEM	Linear (L)	Quadratic (Q)	Time (T)	L×T	Q×T
Serum IgG, mg/dL[2]	1692.7	1607.3	1615.0	1768.7	139.37	0.563	0.684	-	-	-
Growth										
IBW, kg	42.38	41.48	42.97	44.40	1.969	0.801	0.434	-	-	-
BW, kg	48.9	48.2	50.3	51.8	1.872	0.716	0.260	<0.001	0.997	0.992
ADG, kg/d	0.61	0.63	0.69	0.70	0.054	0.739	0.366	-	-	-
Refusals										
10 min, mL	1322	879	988	572	300	0.557	0.957	0.037	0.987	0.845
Total 30 min, mL	823	471	603	405	186	0.375	0.638	0.065	0.881	0.663
Drinking scores, %[3]	71.7	79.2	76.0	84.9	-	0.003	-	-	-	-
Fecal scores, %[4]	64.3	62.7	70.6	72.2	-	0.288	-	-	-	-
Week 1	81.0	80.0	81.0	76.0		0.602	-	-	-	-
Week 2	38.0	29.0	50.0	52.0		0.059	-	-	-	-
Week 3	74.0	79.0	81.0	88.0		0.097	-	-	-	-

[1] Treatments (n = 6 calves per treatment group) consisted of a mixture of a control MR (CON) and an experimental MR containing TB4 and TC6 (TRI) at different percentages being TRI0 (0% TRI MR), TRI33 (33% TRI MR), TRI67 (67% TRI MR), and TRI100 (100% TRI MR). The CON MR (Sprayfo Delta, Trouw Nutrition, Deventer, the Netherlands) included a blend of vegetable fats (65% palm and 35% coconut as % total fat). The TRI MR contained the same fat blend as CON to which TB and TC were incorporated by reducing the inclusion of palm fat.

[2] Blood concentration of Immunoglobulin G at arrival.

[3] Percentage of days with a drinking score of 0 (no assistance) within the first 21 d after arrival.

[4] Percentage of days with a fecal score of 0 (normal) within the first 21 d after arrival.

TABLE 4. Weekly BW and body measurements (least square means) measured at 1300 h in calves fed restricted amounts of milk replacer for 9 wk after arrival (Experiment 2, n = 60).

Item	Treatment (T)[1]				Pooled	*P*-value						
	CON	TB4	TC6	TBTC	SEM	TB4	TC6	TBTC	Week (W)	TB4×W	TC6×W	TBTC×W
Initial BW (kg)	47.4	47.5	47.4	47.9	1.76	0.865	0.894	0.996	-	-	-	-
Final BW, kg	73.6	74.1	72.2	72.8	1.63	0.727	0.427	0.965	<0.01	0.989	0.574	0.223
Wither height, cm	88.1	87.4	87.7	87.5	0.43	0.164	0.603	0.498	<0.01	0.474	0.886	0.002
Hip height, cm	92.5	92.0	92.7	91.9	0.64	0.295	0.975	0.805	<0.01	0.362	0.614	0.121
Chest girth, cm	96.0	95.0	95.3	94.9	0.54	0.154	0.435	0.576	<0.01	0.415	0.250	0.837
Body Barrel, cm	104.9	105.5	104.4	104.2	0.81	0.838	0.237	0.591	<0.01	0.017	0.242	0.266

[1] Dietary treatments (15 calves per treatment) consisted of 1) a control MR (CON) with a blend of vegetable fats that did not include TB4 and TC6, 2) an MR to which tributyrin (TB4) was incorporated, 3) an MR to which tricaproin (TC6) was incorporated, and 4) an MR to which TB4 and TC6 were incorporated (TBTC).

TABLE 5. Intakes of milk replacer, solid feeds, and water (least square means) measured daily in calves fed restricted amounts of milk replacer for 9 wk after arrival (Experiment 2, n = 60).

Intake	Treatment (T)[1]				Pooled	*P*-value						
	CON	TB4	TC6	TBTC	SEM	TB4	TC6	TBTC	Week (W)	TB4×W	TC6×W	TBTC×W
Milk replacer (L/d)	5.86	5.77	5.58	5.60	0.073	0.389	0.007	0.010	<0.01	0.637	0.054	0.004
Starter (kg/d)	0.99	1.06	1.02	0.95	0.094	0.607	0.851	0.770	<0.01	0.996	0.359	0.516
Straw (g/d)	59	72	72	55	13.2	0.470	0.471	0.844	<0.01	0.679	0.998	0.088
Water (L/d)	3.02	3.31	3.34	3.09	0.287	0.476	0.426	0.871	<0.01	0.961	0.772	0.268

[1] Dietary treatments (15 calves per treatment) consisted of 1) a control MR (CON) with a blend of vegetable fats that did not include TB4 and TC6, 2) an MR to which tributyrin (TB4) was incorporated, 3) an MR to which tricaproin (TC6) was incorporated, and 4) an MR to which TB4 and TC6 were incorporated (TBTC).

TABLE 6. Parameters describing calves at arrival and proportion of calves receiving a therapeutic intervention (Experiment 2, n = 60).

Item	Treatment (T)[1]				Pooled SEM	*P*-value		
	CON	TB4	TC6	TBTC		TB4	TC6	TBTC
Calves treated (%)[2]					-			
Fever (%)[3]	73.3	40.0	46.7	46.7	-	0.063	0.133	0.133
Respiratory (%)[4]	86.7	80.0	60.0	60.0	-	0.623	0.093	0.093
Other (%)[5]	7.7	13.3	13.3	13.3	-	0.539	0.539	0.539
Therapeutic interventions (n)[6]	3.5	2.3	2.4	2.5	0.69	0.200	0.250	0.279
Fever (n)	1.6	0.5	0.7	1.1	0.31	0.019	0.038	0.231
Respiratory (n)	1.9	1.5	1.4	1.1	0.39	0.552	0.405	0.156
Other (n)	0.1	0.2	0.3	0.3	0.21	0.651	0.366	0.366
Delayed vaccination (%)[7]	33.3	6.7	13.3	6.7	-	0.059	0.190	0.059

[1] Dietary treatments (15 calves per treatment) consisted of 1) a control MR (CON) with a blend of vegetable fats that did not include TB4 and TC6, 2) an MR to which tributyrin (TB4) was incorporated, 3) an MR to which tricaproin (TC6) was incorporated, and 4) an MR to which TB4 and TC6 were incorporated (TBTC).

[2] Percentage of calves that received a therapeutic intervention, which included oral rehydration solutions (Osmofit) and medicines (Novem, Resflor, Tulissin, Dexaject, Milbosin, Florkem, Dofatrim, and Ampicillin).

[3] Percentage of calves that received a therapeutic intervention for fever, including Novem.

[4] Percentage of calves that received a therapeutic intervention for respiratory disease, which included Resflor, Tulissin, Dexaject, Milbosin, Florkem, and Dofatrim.

[5] Percentage of calves that received a therapeutic intervention for other complications, including oral rehydration solutions (Osmofit), Dofatrim, and Ampicillin.

[6] Number of therapeutical interventions received by each calf, including intravenous fluids and medicines (Dofatrim, Dexaject, Novem, Florkem, Engemycine, and Micotil).

[7] Calf had a body temperature >39.3°C, and the vaccination was delayed by 3 days.

Legend of the Figures

Figure 1. Withers height and body barrel of calves fed restricted amounts of milk replacer for 9 wk after arrival (Experiment 2; n = 15 per treatment). Dietary treatments (15 calves per treatment) consisted of 1) a control MR (CON) that did not include TB4 and TC6, 2) an MR containing TB4, 3) an MR containing TC6, and 4) an MR (TBTC) containing both TB4 and TC6.

Figure 2. Milk intake refusals of calves fed restricted amounts of milk replacer for 9 wk after arrival (Experiment 2; n = 15 per treatment). Dietary treatments (15 calves per treatment) consisted of 1) a control MR (CON) that did not include TB4 and TC6, 2) an MR containing TB4, 3) an MR containing TC6, and 4) an MR (TBTC) containing both TB4 and TC6.

Figure 3. Non-disease probability for fever and respiratory disease in calves fed a milk replacer with different amounts of tributyrin and tricaproin (Experiment 2; n = 15 per treatment). Dietary treatments (15 calves per treatment) consisted of 1) a control MR (CON) that did not include TB4 and TC6, 2) an MR containing TB4, 3) an MR containing TC6, and 4) an MR (TBTC) containing both TB4 and TC6

FIGURES

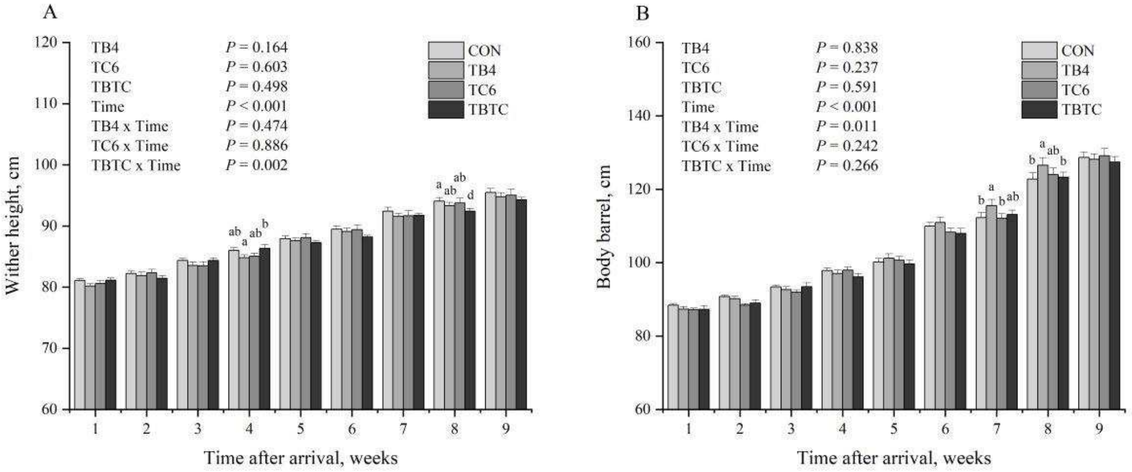

Figure 1.

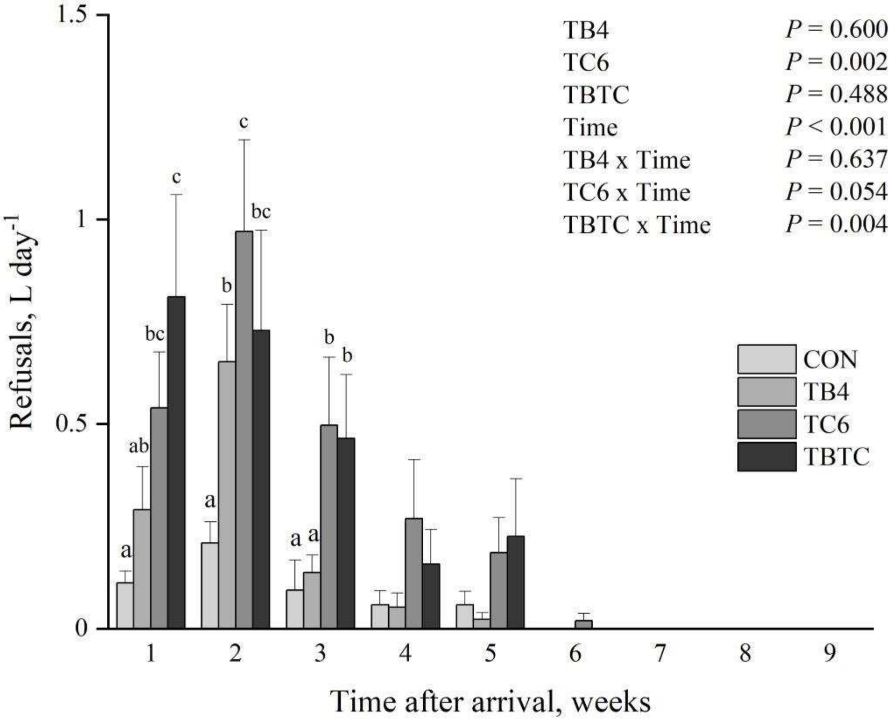
1.5
1
0.5
0
Refusals, L day-1
TB4 P = 0.600
TC6 P = 0.002
TBTC P = 0.488
Time P < 0.001
TB4 x Time P = 0.637
TC6 x Time P = 0.054
TBTC x Time P = 0.004
CON
TB4
TC6
TBTC
a
ab
bc
c
a
b
c
bc
a
a
b
b
1
2
3
4
5
6
7
8
9
Time after arrival, weeks

Figure 2.

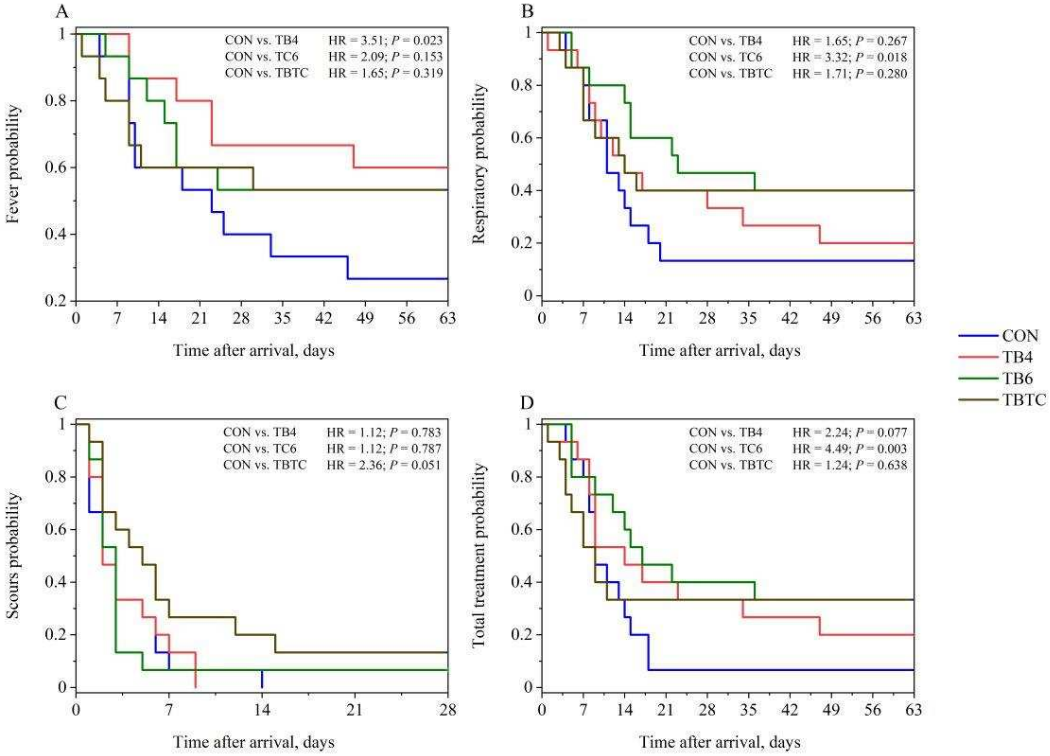
A
CON vs. TB4 HR = 3.51; P = 0.023
CON vs. TC6 HR = 2.09; P = 0.153
CON vs. TBTC HR = 1.65; P = 0.319
Fever probability
1
0.8
0.6
0.4
0.2
0 7 14 21 28 35 42 49 56 63
Time after arrival, days
B
CON vs. TB4 HR = 1.65; P = 0.267
CON vs. TC6 HR = 3.32; P = 0.018
CON vs. TBTC HR = 1.71; P = 0.280
Respiratory probability
1
0.8
0.6
0.4
0.2
0
0 7 14 21 28 35 42 49 56 63
Time after arrival, days
CON
TB4
TB6
TBTC
C
CON vs. TB4 HR = 1.12; P = 0.783
CON vs. TC6 HR = 1.12; P = 0.787
CON vs. TBTC HR = 2.36; P = 0.051
Scours probability
1
0.8
0.6
0.4
0.2
0
0 7 14 21 28
Time after arrival, days
D
CON vs. TB4 HR = 2.24; P = 0.077
CON vs. TC6 HR = 4.49; P = 0.003
CON vs. TBTC HR = 1.24; P = 0.638
Total treatment probability
1
0.8
0.6
0.4
0.2
0
0 7 14 21 28 35 42 49 56 63
Time after arrival, days

Figure 3.

Supplementary table 1. Hazard ratio of fever and respiratory disease in calves fed a milk replacer with different amounts of tributyrin and tricaproin (Experiment 2, n = 60).

Variable and comparison	Coefficient	SEM	HR[5]	95% CI[6]	*P*-value
Fever[1]					
CON vs. TB4	1.256	0.555	3.51	1.18, 10.42	0.023
CON vs. TC6	0.737	0.516	2.09	0.76, 5.75	0.153
CON vs. TBTC	0.503	0.505	1.65	0.61, 4.45	0.319
TB4 vs. TC6	-0.451	0.582	0.64	0.20, 1.99	0.438
TB4 vs. TBTC	-0.671	0.589	0.51	0.16, 1.62	0.254
TC6 vs. TBTC	-0.311	0.561	0.73	0.24, 2.20	0.579
Respiratory disease[2]					
CON vs. TB4	0.499	0.451	1.65	0.68, 3.99	0.267
CON vs. TC6	1.199	0.510	3.32	1.22, 9.02	0.018
CON vs. TBTC	0.534	0.495	1.71	0.65, 4.51	0.280
TB4 vs. TC6	0.731	0.493	2.08	0.79, 5.46	0.138
TB4 vs. TBTC	0.402	0.497	1.49	0.56, 3.96	0.419
TC6 vs. TBTC	-0.431	0.496	0.65	0.25, 1.72	0.384
Scours[3]					
CON vs. TB4	0.111	0.404	1.12	0.51, 2.47	0.783
CON vs. TC6	0.109	0.406	1.12	0.50, 2.47	0.787
CON vs. TBTC	0.859	0.439	2.36	1.00, 5.58	0.051
TB4 vs. TC6	-0.073	0.406	0.93	0.42, 2.06	0.857
TB4 vs. TBTC	0.670	0.443	1.95	0.82, 4.65	0.130
TC6 vs. TBTC	0.728	0.429	2.07	0.89, 4.80	0.089
Total treatments[4]					
CON vs. TB4	0.805	0.455	2.24	0.92, 5.45	0.077
CON vs. TC6	1.503	0.508	4.49	1.66, 12.17	0.003
CON vs. TBTC	0.214	0.456	1.24	0.50, 3.03	0.638
TB4 vs. TC6	0.491	0.462	1.64	0.66, 4.05	0.288
TB4 vs. TBTC	-0.032	0.464	0.99	0.39, 2.40	0.946
TC6 vs. TBTC	-0.438	0.470	0.65	0.26, 1.62	0.351

Dietary treatments (15 calves per treatment) consisted of 1) a control MR (CON) with a blend of vegetable fats that did not include TB4 and TC6, 2) an MR to which tributyrin (TB4) was incorporated at 3.75% of total fat, 3) an MR to which tricaproin (TC6) was incorporated at 2.5% of total fat, and 4) an MR to which TB4 and TC6 were incorporated (TBTC).

[1] Calf had a body temperature >39.3°C.

[2] Calf showed both indicative signs of fever (>39.3°C) and rapid breathing (>30 breaths/min), and at least one supportive symptom such as coughing (observed when the calf was standing), nasal discharge (either watery or thick), or changes in eyelid mucosa coloration (white vs. red).

[3] Days with a fecal score of 2 (watery feces) within the first 28 days after arrival.

[4] Days in which calves received therapeutic intervention over the entire study period.

[5] The hazard ratio indicates the probability of having elevated fever, respiratory disease, scours or a treatment. If the HR is >1, a given MR in the comparison is more likely to have elevated fever, respiratory disease, scours or a treatment incidence than the other MR by a factor of the difference above 1. If the HR is <1, a given MR has a lower probability of occurrence than the other MR.

[6] Confidence interval.

CHAPTER 3

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EFFECT OF INCORPORATING TRIBUTYRIN AND TRICAPROIN IN MILK REPLACER ON THE HEPATIC METABOLOME OF CALVES

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ABSTRACT

Incorporating tributyrin (TB) and tricaproin (TC) into milk replacers (MR) can affect lipid metabolism in calves, but their effects on liver function have not yet been investigated. In this study, the effects of incorporating of TB and TC in MR on the liver metabolome of dairy calves were investigated. Forty-five male dairy calves (46.1 ± 4.6 kg BW; 2.1 ± 0.63 d of age; mean ± SD) were blocked in order of arrival at the research facility. Within a block, calves were randomly assigned to 3 MR treatments (n = 15 per group): (1) a positive control containing dairy cream as milk fat (MF), (2) a negative control (NC) containing a blend of vegetable fats, and (3) a MR (TRI) containing the same mixture of vegetable fats as NC, to which TB and TC were incorporated. The MF treatment served as a biological reference for fat composition. All MR were isoenergetic with 36% lactose, 27% fat, and 24% protein on a DM basis. Calves were housed individually and received MR (13.5% solids) via teat buckets twice daily at 0630 and 1730 h. Daily milk allowance was 6.0 L from d 1-5, 7.0 L on d 6-9 and 8.0 L from d 10-35. Calves had ad libitum access to water and chopped straw but no starter feed. On day 35, the calves were euthanized and liver tissue samples were collected and analyzed using a targeted metabolomics approach. Liquid chromatography and flow injection with electrospray ionization triple quadrupole mass spectrometry using an MxP® Quant 500 kit was used. The analysis identified 83 significant metabolites, including various phospholipids, bile acids, AA, and fatty acids. Principal component analysis (PCA) revealed different metabolic profiles between calves fed the MF and other treatment groups, accounting for almost 50% of the total variation. Partial Least Squares Discriminant Analysis (PLS-DA) confirmed significant differences between the liver metabolomes of calves fed the MF and other treatments. Volcano plot analysis showed that compared to calves fed NC, 51 metabolites were higher in calves fed MF, including 34 phosphatidylcholines, 8 sphingomyelins, 3 lysophosphatidylcholines, 1 ceramide, 3 hexosylceramides, eicosapentaenoic acid (EPA) and glycochenodeoxycholic acid (GUDCA), while 8 metabolites were lower, including 2 phosphatidylcholines, 1 sphingomyelin (SM C22:3), 1 diacylglycerol (DG 16:0_18:2), 1 lysophosphatidylcholine (lysoPC a C18:2), 2 nitrogen-containing compounds (putrescine and serine) and C5 acylcarnitine. In addition, when comparing calves fed MF to calves fed TRI, 51 metabolites were higher in calves fed MF, including 37 phosphatidylcholines, 8 sphingomyelins, 4 lysophosphatidylcholines, 3 ceramides, 3 hexosylceramides, EPA and GUDCA, while 7 metabolites were lower, including 2 phosphatidylcholines, 1 sphingomyelin (SM C22:3), 1 diacylglycerol (DG 16:0_18:2), 1 lysophosphatidylcholine (lysoPC a C18:2), putrescine and valerylcarnitine (C5). However, no significant differences were found between calves fed NC and TRI, indicating that TB and TC incorporation had no effect on the liver metabolome. These results showed that the inclusion of MF in MR significantly altered the liver metabolome in young calves compared to MR based on vegetable fat, emphasizing the need to consider fat composition for optimal early-life metabolic development.

Keywords: Calf, liver metabolome, butyric acid, caproic acid

INTRODUCTION

Fat inclusion and fat composition in milk replacers (**MR**) significantly impact metabolism and development of calves (Berends et al., 2020; Welboren et al., 2021; Wilms et al., 2023b). Milk replacers for calves contain alternative fat sources to milk fat (MF; Wilms et al., 2023a; b), leading to variations in the fatty acid (**FA**) profile and triglyceride (**TG**) structure compared with those in MF. This can influence lipid metabolism, oxidative stress, gut microbiota composition, and inflammation in neonates (Tan and Norhaizan, 2019; Basak et al., 2022). Butyric acid (**C4:0**) and caproic acid (**C6:0**) are often missing or only present in small amounts in MR for calves, as they are natural substances from mammalian milk (Esselburn et al., 2013; Mellors et al., 2023). The inclusion of tributyrin (**TB**) and tricaproin (**TC**) into MR allows for the restoration of physiological levels of C4:0 and C6:0, as observed in bovine whole milk (Castro et al., 2024, in press; Wilms, 2024). These artificial TG provide health benefits to dairy calves, including enhanced gut development, reduced abnormal fecal scores, and reduced medical interventions (Liu et al., 2022; Wilms, 2024; Castro et al., 2024, in press).

The metabolism of C4:0 is essential for neonatal development (Guilloteau et al., 2009, 2010b; a). Butyric acid is rapidly absorbed in the gastrointestinal tract (**GIT**), serving as a vital energy source for colonocytes and promoting intestinal health and development (Venegas et al., 2019). Butyric acid improves nutrient absorption (Guilloteau et al., 2010b) and gut barrier function (Zhou et al., 2017), which in turn support the overall growth and health of calves (Wilms, 2024; Castro et al., 2024, in press). The role of C6:0 in newborn development have been less studied, but it may also contribute to energy metabolism and have antimicrobial properties (Aurousseau, 1984; Castro et al., 2024 in press). Wilms (2024) reported lower postprandial TG, weekly serum NEFA, and weekly plasma cholesterol in calves fed MR containing TB and TC compared to calves fed MR that did not contain these FA. Consistently, Kato et al. (2011) reported a trend toward lower postprandial NEFA concentrations in calves fed MR supplemented with sodium butyrate (**NaB**). In addition, butyrate treatment significantly reduced total cholesterol and TG concentrations (Kato et al., 2011). In obese rodent models (Li et al., 2013, 2018; Arnoldussen et al., 2017; Adeyanju et al., 2021), butyrate treatment significantly reduces total cholesterol and TG concentrations. These so-called “enhanced lipid markers” suggest increased storage capacity for adipose tissue. However, this could also be due to improved liver function or a combination of these factors (van Deuren et al., 2022).

Investigating the hepatic metabolome provides insights into the dynamic interactions between diet, metabolism, and physiological responses in cattle (Hao et al., 2021). The liver plays a central role in regulating metabolic processes, including nutrient metabolism, detoxification, and the synthesis of essential biomolecules in multiple species (Rui, 2014; Alamri, 2018; Kalra et al., 2023). In rabbits, exogenous administration of C4:0 affects lipid metabolism by modulating lipid synthesis and decomposition in the adipose tissue and liver (Zhang et al., 2022). Additionally, NaB supplementation has alleviated hepatic steatosis in rats (Khan and Jena, 2016; Chang et al., 2018; Sun et al., 2019). Zhou et al. (2017) reported that dietary supplementation with NaB decreased hepatic lipid deposition, which was related to improved gut microbiota and gastrointestinal health and improved whole-body metabolism in mice. Although less studied, caproic acid is a medium-chain fatty acid (**MCFA**) that plays a crucial role as a substrate in mammalian energy metabolism and anabolic processes (Schönfeld and Wojtczak, 2016). Like other MCFA, C6:0 is rapidly absorbed and directly transported from the intestine to the liver through the portal vein (Bach and Babayan, 1982). In addition, C6:0 actively participates in energy-dependent mitochondrial processes that are fundamental for ATP production, cell energy currency, and various metabolic reactions essential for cellular function and survival (Wojtczak and Schönfeld, 1993).

Metabolomic profiling of the liver in response to the incorporation of TB and TC into MR is lacking, despite studies on the effects of C4:0 and C6:0 on calf growth and health. Therefore, it was hypothesized that incorporating TB and TC into MR would induce a distinct metabolic profile in the liver, reflecting differences in nutrient utilization, energy metabolism, and the metabolic health of calves. The objective of this study was to evaluate the liver metabolome profile of calves fed different MR, with a focus on comparing these profiles to a group of calves fed milk MR containing MF.

MATERIAL AND METHODS

This study was conducted at the Research Facility of Trouw Nutrition Research & Development (Sint Anthonis, the Netherlands) between November 15th, 2021 and March 31st, 2022. All procedures complied with the Dutch law on Experimental Animals, which follows the principles of ETS123 (Council of Europe, 1985 and the 86/609/EEC Directive), and were approved by the animal welfare authority (Centrale Commissie Dierproeven, CCD, the Netherlands). The project application code was AVD2040020173425.

Animals, Experimental Design, and Feeding Scheme

This study is part of a companion study by Wilms (2024), which provides detailed insights into the effects of incorporating TB and TC into MR on outcomes related to digestibility, development of the GIT, postprandial metabolism, and health of dairy calves. This study employed a randomized complete block design with 45 male Holstein-Friesian calves (46.1 ± 4.6 kg arrival BW; 2.1 ± 0.63 age at arrival; mean ± SD). The calves originated from collaborating dairy farms and followed a standardized colostrum management protocol. Colostrum was pooled from all cows calving on a given day, and colostrum quality was evaluated using a portable refractometer (model RHB-32ATC; Westover Scientific, Mill Creek, WA) to measure soluble solids. A quality of 22% Brix or greater was required, indicating an Immunoglobulin G (**IgG**) content of 50 g/L or greater. The initial colostrum feeding of 3.0 to 4.0 L was administered within 1 h after birth, followed by 2 additional feedings of 2.0 L within the first 24 h after birth. Following colostrum feeding, calves were fed 6.0 L/d of bulk tank whole milk (**WM**) in 2 equal meals of 3.0 L. Both the colostrum and bulk tank WM were fed at 40°C using nipple buckets. Successful application of the colostrum protocol was evaluated upon arrival at the research farm and between 36 and 72 h after birth by measuring Serum IgG concentrations using a portable Multi-Test Analyzer (DVM Rapid Test II, Vetlab, Palmetto Bay, FL, USA) and required a minimum of 10 g/L (Lombard et al., 2020).

Upon arrival at the research facility, calves were assigned to 1 of 15 blocks based on the arrival sequence and age at arrival. Within each block, calves were randomly assigned to dietary MR treatments using the random function [RANDBETWEEN (0,100000)] in Microsoft® Excel® (Microsoft 365 MSO, Version 2212, Build 16.0.15928.20278). Dietary MR treatments were anonymized to animal caretakers by randomly assigning a letter (A, B, or C) to each treatment. All MR used in this study were manufactured by Trouw Nutrition (Deventer, the Netherlands). Ingredients included 62.5% skim milk powder, 29% oils and fats, 6% whey powder, and 2.5% premix (vitamins, minerals, and additives; Trouw Nutrition, Deventer, the Netherlands). This led to isoenergetic and isonitrogenous MR treatments with 36% lactose (DM basis), 27% fat, and 24% protein. During MR production, the fats were sprayed and then encapsulated in a protein matrix. For refined vegetable oils, a maximum of 0.1% free FA and 1 meq/kg peroxide were defined as quality parameters. In addition, the strong ion difference was 40 mEq/L and the osmolality was 310 mOsm/kg considering a concentration of 135 g/L (13.5% solids). Experimental MR diets included: (1) an MR with dairy cream as milk fat (MF), (2) an MR serving as vegetive control (**NC**) with a blend of vegetable fats

(62.7% palm, 35% coconut, and 2.3 % linseed) that did not include C4:0 and C6:0, and (3) an MR with the same fat blend as NC, to which TB and TC were incorporated by reducing the inclusion of palm fat (56.5% palm, 35% coconut, 2.3 % linseed, 3.75% TB, and 2.5% TC, **TRI**). Tributyrin and TC were incorporated at the same level of C4:0 and C6:0 as in the MF treatment. The MF treatment served as a biological reference for MF composition, expected from its natural FA profile and TG structure. The FA profile of each MR diet is presented in Table 1.

The MR was fed in 2 equal meals daily at 0630 h and 1700 h. The concentration of 135 g/L (13.5% solids) was based on the industry recommendation for MR high in fat. The MR treatments were prepared using a milk shuttle (Urban MS100 Wüsting Germany), reconstituted with water, and administered via individual nipple buckets at 40°C. Calves were fed 6.0 L/d on d 1-5, 7.0 L/d on d 6-9, and 8.0 L from d 10-35. From arrival onwards, each calf had ad libitum access to water and chopped wheat straw (3 to 5 mm; JL-Agro, Volkel, the Netherlands) in separate plain buckets placed in front of each calf. No starter feed was available to focus solely on the metabolic and physiological effects of the MR treatments. The provision of chopped straw was intended to ensure the welfare of the calves and prevent them from overeating their bedding.

Housing

Calves were housed indoors in individual pens (2.34 m × 1.16 m) separated by galvanized bar fences and equipped with rubber-slatted floors at the front area (50% of the total pen area) and a lying area at the rear containing a mattress covered with flax straw (Flax Farm®). The temperature in the calf pen was maintained at a minimum of 12°C and a maximum of 28°C. Ventilation was automated and relative humidity was maintained between 60 and 85%. The calves were exposed to daylight and artificial light from 0530 to 2130 h.

Post-Mortem Analysis

On d 35 after arrival (35.4 ± 0.3 d of age), the calves were prepared for euthanasia and tissue sampling. The procedure started 3 h after morning feeding of 4.0 L of the corresponding MR treatment of the calf. Sedation was initiated 2.5 h after feeding by an intramuscular injection of Sedamun (xylazine 20 mg/mL, 23.32 mg xylazine hydrochloride; Eurovet Animal Health BV/Dechra, Bladel, the Netherlands). After 20 min, intravenous ketamine (Ketalin, Ketamine 10%; A.A.-Vet Diergenesmiddelen N.V., Biddinghuizen, the Netherlands) was administered. After surgical anesthesia was achieved and the calves were rendered insensible to pain, they were exsanguinated by severing the jugular vein. A post-mortem ventral incision was performed in the midline from the sternum to the anus to gain access to the abdominal cavity. The entire GIT was excised from the esophagus to the rectum. Liver samples (1 cm × 2 cm) were taken, placed in tissue bags, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent laboratory analysis.

Metabolomics Analysis and Data Processing

For the metabolomic analysis of liver tissue, a refined protocol from Biocrates Life Sciences AG (Innsbruck, Austria) was used, adapted from the methodology described by Zukunft et al. (2018). In brief, 100 mg of liver tissue was homogenized in an ethanol/phosphate buffer solution (85:15) at a tissue to solvent ratio of 1:3 (w/v). This was performed in a 2 mL Precellys CK14 tube containing 1.4 mm ceramic beads (zirconium oxide) using a Precellys 24 tissue homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France). Homogenization was performed in three consecutive 20-second cycles at 5,500 rpm with 30-sec pauses. The samples were cooled on ice for 20 min and inverted every 5 min to ensure uniform homogeneity. Finally, samples were centrifuged at 10,000 × *g* for 5 min at 4°C, resulting in a uniform supernatant.

To quantify metabolite concentrations, targeted metabolomic analysis was performed according to the manufacturer's protocol described on the Biocrates website (https://biocrates.com/mxp-quant-500-kit, accessed February 14, 2024). This analysis was performed using the MxP® Quant 500 kit from Biocrates Life Sciences AG (Innsbruck, Austria) at the Institute of Clinical Chemistry and Laboratory Medicine of the University Medicine Greifswald (Greifswald, Germany), and lipid concentrations were quantified using Flow Injection Analysis Tandem Mass Spectrometry (**FIA-MS/MS**) on a 5500 QTRAP® instrument (AB SCIEX, Darmstadt, Germany) equipped with an electrospray ionization source. This instrument was used with an HPLC system (Agilent 1260 Infinity Binary LC, Santa Clara, United States) comprising a degasser unit, column oven, autosampler, and binary pump. In the FIA-MS/MS system, acylcarnitines, phospholipids, and sphingolipids were measured in the positive ionization mode, while the sum of hexoses was measured in the negative ionization mode. Concurrently, small molecules were analyzed using liquid chromatography-mass spectrometry (**LC-MS/MS**) in conjunction with a tandem mass spectrometer. Multiple reaction monitoring was used to detect analytes using the FIA-MS/MS and LC-MS/MS methods. Sample preparation was performed in 96-well plates, with each well pre-labeled with internal standards, and filled with a predetermined number of samples. For the derivatization of specific analytes, such as AA, a phenyl isothiocyanate solution was used, followed by the extraction of the target analytes with an organic solvent and their subsequent dilution.

Data quantification was performed using SCIEX Analyst® MS software (version 1.7.2 Build 8287) and subsequently integrated with Biocrates MetIDQ™ software (Oxygen-DB110-3023) to improve analyte identification, concentration calculations, and data compilation. For LC-MS/MS assays, stable isotope dilution and seven-point calibration curves were used to quantify the metabolites. In contrast, in the FIA-MS/MS assays, metabolite concentrations were determined using a one-point calibration based on the internal standard, incorporating isotopic corrections. All metabolite quantifications were performed in strict compliance with the manufacturer's guidelines using MetIDQ Boron software, which is specifically developed for the processing and management of targeted metabolomic data.

To account for the daily performance variations of the LC-MS/MS platform, we included up to two quality control samples on each plate, that is, for each series of measurements. Quality control sample concentrations were used for normalization. For each plate, we calculated the median concentration of the metabolites and divided the measured concentrations of the metabolites by this median. We then recalculated the median of these adjusted values across all plates for each metabolite, returning the concentration to its original scale (expressed in µmol/L). In addition, the coefficient of variation (**CV**) was determined for each metabolite based on the quality control samples. Only metabolites with a mean CV of less than 30% and at least five measurements above the limit of detection (**LOD**) were included in the final data for subsequent analysis. The MxP® Quant 500 kit enabled the quantitative analysis of a broad range of metabolites from 12 biochemical lipid classes and 14 classes of small molecules.

Statistical Analysis

Statistical analysis of liver metabolite data was performed using the web-based metabolomic data processing tool MetaboAnalyst 6.0 (https://dev.metaboanalyst.ca/). Prior to analysis, the integrity of the data was ensured by carefully checking the CSV file to confirm that the data matrix contained only numerical values and conformed to standard formatting protocols. As part of the data filtering process, variables were subjected to interquartile range (**IQR**) assessment to identify and eliminate outliers to refine the data set for subsequent analysis. According to the “80% rule” proposed by Bijlsma et al. (2006), metabolites with more than 20% of missing values were not considered for the statistical analysis. To address issues related to heteroscedasticity and skewness, the data were subjected to a generalized log transformation (base 10) and Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable). After data preparation, the dataset was processed using MetaboAnalyst 6.0, which allowed various multivariate data analyses to be performed, including principal component analysis (**PCA**), partial least squares discriminant analysis (**PLS-DA**), variable importance in projection (**VIP**), and heat map and hierarchical clustering, with heat map clustering using Euclidean distance and Ward's minimum variance method (ward.D). Volcano plots were generated to identify significant metabolic differences between the different treatments (MF vs. CON, MF vs. TRI, and NC vs. TRI). These plots depict the relationship between biological significance, represented by fold changes, and statistical significance, indicated by False Discovery Rate (**FDR**)-adjusted *P*-values. Metabolites were identified as significant if they had a fold change ≥ 1.5 and an FDR ≤ 0.05.

RESULTS

In this study, 83 significant metabolites were identified (Figure 1). These included phosphatidylethanolamines (n = 28), phosphatidylcholines (n = 22), lysophosphatidylcholines (n = 7), sphingomyelins (n = 5), sphingosines (n = 5), ceramides (n = 4), hexosylceramides (n = 2), other phospholipids (n = 1), bile acids (n = 2), AA (n = 3), and FA (n = 4). A list of significantly different hepatic metabolites between treatments is presented in Table 2 for calves fed MF and NC and Table 3 for calves fed MF and TRI. Interestingly, there were no significant differences in hepatic metabolites between calves fed NC and TRI.

A PCA of the liver metabolome (Figure 2) revealed distinct clustering between calves fed MF compared to those fed CON and TRI. The first 3 principal components represented 56.1% of the total variance, with PC1 explaining 37.5%, PC2 10.7%, and PC3 7.9%. Calves fed MF formed a separate cluster, indicating significant differences in hepatic metabolic profiles compared with calves fed CON and TRI. In contrast, calves fed the CON and TRI diets completely overlapped, indicating no significant differences in their metabolic profiles. To further investigate these differences, a PLS-DA analysis was conducted. This supervised method maximizes the separation between predefined groups by identifying variables that contribute to class discrimination. The PLS-DA results (Figure 3A) showed significant clustering between the treatment groups. Component 1 explained 36.7%, component 2 explained 8.8%, and component 3 explained 5.3% of the variance, for a total of 50.8% of the variation explained. The permutation test, which assessed the reliability of the model by comparing it with randomly permuted models, confirmed the robustness of the model (Figure 3B). The VIP scores identified important metabolites, such as phosphatidylcholines (PC ae C34:0, PC aa C36:6, PC ae C36:1), sphingomyelins (SM OH C16:1, SM OH C14:1, SM OH C22:2), and eicosapentaenoic acid (**EPA**), which were higher in calves fed MF than in calves fed CON and TRI (Figure 3C).

Volcano plot analysis between calves fed MF and NC revealed significant differences in liver metabolite levels (Figure 4; Table 2). Of the 173 metabolites analyzed, 59 differed significantly, with 51 metabolites showing increased levels in calves fed MF compared to NC, and 8 metabolites showing decreased levels, while 114 metabolites were not significantly different (Figure 4). Among the increased metabolites were 34 phosphatidylcholines, 8 sphingomyelins, 4 lysophosphatidylcholines, 4 ceramides, EPA, and glycochenodeoxycholic acid (**GUDCA**). In contrast, the metabolites with reduced levels included 2 phosphatidylcholines (PC aa C34:2, PC ae C42:1), diacylglycerol (DG 16:0/18:2), valerylcarnitine (C5), lysophosphatidylcholine (lysoPC a C18:2), 1 sphingomyelin (SM C22:3), putrescine and serine.

The volcano plot analysis between calves fed MF and NC revealed significant differences in liver metabolite levels (Figure 5; Table 3). Of the 173 metabolites analyzed, 64 differed significantly, with 57 metabolites showing increased levels in calves fed MF compared to TRI, and 7 metabolites showing decreased levels, while 109 metabolites were not significantly different (Figure 5). Among the increased metabolites were 37 phosphatidylcholines, 8 sphingomyelins, 6 ceramides, 4 lysophosphatidylcholines, EPA, and GUDCA. In contrast, metabolites with reduced levels included 2 phosphatidylcholines (PC aa C34:2, PC ae C42:1), diacylglycerol (DG 16:0/18:2), C5, lysophosphatidylcholine (lysoPC a C18:2), 1 sphingomyelin (SM C22:3), and putrescine. Finally, no significant metabolite alterations were observed between calves fed TRI and NC diets (Figure. S1).

The hierarchical cluster map of the 50 most important metabolites showed different metabolic profiles between treatment groups (Figure 6). Calves fed the MF diet had higher levels of several of the top 50 metabolites, including PC, EPA, and lysophosphatidylcholines, compared to the NC and TRI groups. In contrast, calves fed NC and TRI groups had similar metabolic profiles with lower levels of these metabolites than those fed MF (Figure 6), suggesting that including dairy cream in the MF diet significantly increased lipid-related metabolites.

DISCUSSION

This study aimed to characterize the hepatic metabolome of 5-wk-old dairy calves fed MR containing TB and TC at the same level as bovine MF, in the presence of a positive control consisting of MF. Incorporating TB and TC did not affect the liver metabolome of calves compared to NC MR, which did not contain these FA. In contrast, clear distinctions were observed in phosphatidylcholines, sphingomyelins, lysophosphatidylcholines, and EPA as the main groups of metabolites present in the hepatic metabolism of calves fed MR containing vegetable fats from palm, coconut, and linseed and MR containing MF. To the best of our knowledge, this study is the first to report hepatic metabolic changes in calves based on the fat composition in MR.

Identified with high VIP scores, EPA, phosphatidylcholines, lysophosphatidylcholines, and sphingomyelins are the major components of very low-density lipoproteins (**VLDL**), which are essential for calves and other young animals due to its role in lipid transport and metabolism (Bauchart, 1993). The significance of these metabolites in calves fed MF suggests a response to the unique composition of MF. Previous work has shown that feeding WM powder leads to a higher postprandial TG response compared to calves fed an MR with the same macronutrient profile containing a blend of palm and coconut (Wilms et al., 2024a). Consistently, feeding MR with a blend of lard and dairy cream also led to increased postprandial TG levels (Wilms et al., 2024b). In response to feeding MR with varying fat composition, large differences in plasma cholesterol and FA profiles in the blood and tissues were observed (Mellors et al., 2023; Welboren et al., 2023; Wilms et al., 2024c). The function of VLDL particles is to transport TG from the liver to peripheral tissues as it releases TG into the bloodstream, resulting in an increase in circulating TG levels (Bauchart, 1993). Therefore, the current results are consistent with the differences in postprandial TG observed in calves fed liquid feeds with MF against those fed vegetable fats-based MR. These changes suggest a shift in cholesterol and TG metabolism, potentially influenced by liver function and inflammatory responses in calves (Razavi et al. 2012).

The findings of the current study show that the fat composition in MR significantly influences the hepatic phosphatidylcholine profile of calves. The significant increase in phosphatidylcholines in calves fed MF suggests enhanced synthesis or reduced degradation of these lipids, which could be attributed to the specific FA composition of MF modulating the activities of key enzymes involved in phospholipid metabolism. The MF treatment was characterized by lower levels of C12:0, C18:1 *cis*-9, and ALA, but higher levels of C14:0 and C18:0 than other MR treatments. Additionally, the saturation level of the MF MR was slightly higher (68% vs. 65%), while the PUFA content was lower (3.4% vs. 7.8%) than that of the MR with vegetable fats. The primary pathway for phosphatidylcholines synthesis in mammalian cells is the CDP-choline pathway, also known as the Kennedy pathway, which involves the phosphorylation of choline by choline kinase, followed by the conversion of phosphocholine to CDP-choline. Finally, the phosphocholine group is transferred to diacylglycerol to form phosphatidylcholines (Kennedy and Weisst, 1956; Vance, 1990). The FA composition of MF can enhance the activity of enzymes in this pathway, leading to increased phosphatidylcholines synthesis. Additionally, phospholipase D (**PLD**) catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid and choline, which can be recycled for the synthesis of new phosphatidylcholines via the CDP-choline pathway (Dennis et al., 2011). The specific FA in MF may enhance PLD activity, thereby increasing the availability of choline for phosphatidylcholines synthesis. In addition, phosphatidylcholines are crucial for biological membranes to maintain their integrity and support cellular functions (van der Veen et al., 2017). Therefore, differences between MF and vegetable fats may have a more pronounced effect on membrane fluidity than the incorporation of TB and TC in MR. This is consistent with the findings of Sunshine and Iruela-Arispe (2017), who highlighted the importance of the types and saturation of phospholipids and their fatty acyl chains in dictating membrane fluidity, along with lower quantities of MUFA, such as oleic acid, and minimal PUFA.

Differences in fat composition between MF and vegetable fats in MR also significantly influenced the hepatic lysophosphatidylcholine profile of the 12 lysophosphatidylcholine profiles examined, 5 were significantly affected by fat composition in MR, with higher levels of lysoPC, C16:1, C17:0, C28:1, and C20:4 in calves fed MF and lower levels of lysoPC and C18:2 in calves fed NC and TRI. These changes in the lysophosphatidylcholine profile could be due to modulation of the activities of key enzymes involved in lysophospholipid metabolism in response to fat composition. This was particularly marked for phospholipase A2 (**PLA2**; Murakami et al., 2020) and lecithin-cholesterol acyltransferase (**LCAT**; Jonas, 2000). The PLA2 enzymes play a crucial role in the hydrolysis of phospholipids to generate lysophospholipids and free FA (Murakami et al., 2020). The activity of PLA2 can be influenced by the fat composition of the diet, with *cis*-FA known to inhibit calcium-dependent PLA2 *in vitro* (Raghupathi and Franson, 1992). This suggests that the differences in FA profiles between MF and other MR treatments can selectively activate or inhibit different PLA2 isoforms, leading to changes in lysophosphatidylcholine production and, consequently, oxidative stress and vascular metabolic disorders by releasing unsaturated fatty acids (Schwenke, 1998). In addition, LCAT catalyzes the transfer of FA from phosphatidylcholine to free cholesterol, forming cholesterol esters and lysophosphatidylcholines (Jonas, 2000). The LCAT plays an essential role in high-density lipoprotein (**HDL**) metabolism and reverse cholesterol transport, and its activity can be modulated by the FA composition of lipoproteins (Santamarina-Fojo et al., 2000). Furthermore, the interaction between dietary lipid composition and lysophosphatidylcholine metabolism may have broader effects on lipid metabolism and immune function.

Lysophosphatidylcholines are known to play a role in various physiological processes, including cell signaling and inflammation (Liu et al., 2020). In a preliminary report, Tate and McFadden (2021) established that saturated lysophosphatidylcholines enhanced the oxidative burst, potentiating the ability of endotoxins to promote tumor necrosis factor-α (**TNFα**) and interleukin-6 secretion, and accelerate the killing of *Escherichia coli* in neutrophils isolated from the neonatal calf, suggesting that lysophosphatidylcholine could activate bovine neutrophils, which are "first responder" innate immune cells that migrate to invading pathogens and neutralize them using bactericidal mechanisms such as oxidative burst and phagocytosis (Yan et al., 2023). Subcutaneous administration of lysophosphatidylcholine to dairy calves has demonstrated immunomodulatory effects by inducing a febrile response and altering physiological parameters related to liver function, immune response, metabolic health (Tate et al., 2023) , cellular signaling, lipid metabolism, and membrane dynamics (Cole et al., 2012; Shin et al., 2012).

Sphingomyelins are pivotal for structural integrity and stability, are involved in vital processes such as cell signaling and apoptosis, and interact with various lipid components (Yang and Chen, 2022). Consistent with previous findings from this study, calves fed MF differed from calves fed NC and TRI in terms of sphingomyelin metabolism and function. The higher expression of metabolites in calves fed MF suggests that large differences in the fat composition of MR, rather than the incorporation of TB and TC, modulate sphingomyelin activity (Yang and Chen, 2022). This suggests that some FA present in bovine MF and absent from the vegetable fat sources used in this study are important for optimizing the functionality of sphingomyelins. Emerging research has underscored the significance of sphingomyelins in dairy cattle physiology with implications for health and disease (McFadden and Rico, 2019; Rico et al., 2021; Kenéz et al., 2022). However, the functional consequences of altered sphingomyelin profiles in calves remain unclear. Lower concentrations of blood sphingomyelins (SM OH C14:1 and SM OH C16:1), similar to those observed in the present study, were associated with metabolic stress in periparturient cows (Kenéz et al., 2016). Amin et al. (2023) evaluated plasma metabolome alterations in calves in stressful developmental stages of life and observed reduced plasma sphingomyelin levels in early-weaned calves. These findings highlight the potential relevance of sphingomyelins in the metabolic health of dairy calves, warranting further investigation into their role in calf physiology and pathology, as sphingomyelins are either obtained from dietary sources or synthesized by the liver or other tissue cells (Nilsson and Duan, 2006).

In the present study, some specific AA were significantly downregulated in the hepatic metabolome of MF-fed calves. Notably, putrescine, a polyamine derived from ornithine, plays a crucial role in cell proliferation, protein synthesis, and the maintenance of cellular integrity (Sagar et al., 2021). Serine, classified as a non-essential AA, is involved in protein synthesis and is a pivotal precursor for biosynthetic pathways that are integral to cellular function and survival (Banh et al., 2020). It also produces phosphatidylserine, ceramide, and glucose (Li et al., 2007). Serine is vital for the biosynthesis of phosphatidylserine and ceramides, which are crucial for cell membrane composition and signaling (Li et al., 2007). The reduced serine levels observed in calves fed MF in the present study might indicate impairment of these processes, disrupting membrane integrity and lipid signaling pathways. Additionally, serine is essential for glucose production (Bismut and Plas, 1991) and is a primary energy source for immune cells such as lymphocytes and macrophages (Newsholme et al., 1999). Hepatic serine concentrations reflect the connection between biosynthetic demand, dietary input, and metabolic flux (Wu et al., 2020). Feeding MF alters hepatic lipid metabolism, interacts with serine metabolism, and affects ceramide levels. The observed changes in ceramide levels (Cer_d18_1_16_0, Cer_d18_1_24_0, Cer_d18_1_24_1, and Cer_d18_2_23_0) further indicated complex interactions between diet, lipid metabolism, and overall health in these animals. Symmetric dimethylarginine (**SDMA**), a methylated derivative of arginine, is a surrogate marker of renal function and methyl group balance. The presence of SDMA in the hepatic metabolome suggests an intricate relationship between organ systems and metabolic pathways (Tain and Hsu, 2017).

In the current study, the concentrations of EPA in the liver were higher in calves fed MF than in calves fed NC and TRI, consistent with the EPA levels in the diet. Eicosapentaenoic acid (EPA) is an omega-3 FA known for its anti-inflammatory properties, reduction in the oxidant status index in the first week of life, and the possibility of improving metabolism, health, and performance of calves (Opgenorth et al., 2020a; b; Śpitalniak-Bajerska et al., 2020). In hepatic metabolism, EPA is enzymatically converted into bioactive lipid mediators, such as prostaglandins and leukotrienes, which influence inflammatory responses and lipid metabolism (Dyall, 2015). Li et al. (2008) have shown that n-3 polyunsaturated fatty acids prevent disruption of epithelial barrier function induced by proinflammatory cytokines. Additionally, Karcher et al. (2014) found that feeding fish oil diets reduced TNF-α expression in blood cells following in vitro LPS stimulation. These cytokines play a crucial role in mediating and regulating the inflammatory response, potentially affecting the ability of calves to manage disease challenges.

It is unlikely that differences in the hepatic metabolome between calves fed MF and TRI are related to C4:0 and C6:0, as both treatments contained the same levels of these two FA. However, it cannot be excluded that synthetic C4:0 and C6:0 in the TRI MR lead to different metabolic effects than natural C4:0 and C6:0, as TB and TC are metabolized differently because of their unique TG structure that does not occur in nature (Blasi et al., 2008). Indeed, C4:0 and C6:0 are almost exclusively found in the sn-3 position on the TG backbone in milk from ruminant species (Blasi et al., 2008) and are almost exclusively released in the abomasum and upper intestines (Carlier et al.; Edwards-Webb A N and Thompson, 1978). In contrast, C4:0 and C6:0 at the sn-2 position on the glycerol backbone are likely incorporated into chylomicrons and absorbed in the lower gut, which may affect the metabolism of these FA. Nevertheless, the absence of differences between calves fed NC and TRI suggests that the incorporation of TB and TC into MR has no effect on the hepatic metabolome of calves. Therefore, the large differences observed in the current study were caused by the use of dairy cream in the MF treatment and a blend of vegetable fats (palm, coconut, and linseed) in the NC and TRI MR. However, it is difficult to identify which FA caused the observed differences, as the 2 fat blends differed largely in terms of FA profile and TG structure. A limitation of dietary fat research is the challenge of isolating the effects of individual FA, accounting for potential interactions between different FAs, and considering TG structure. When interpreting these results, it is essential to account for variations in the FA profiles of dietary fats.

CONCLUSIONS

Through a comprehensive analysis, the current study showed that large variations in dietary milk replacer fat composition between bovine milk fat and vegetable fat blends led to a distinct hepatic metabolome in calves. This was characterized by the elevation of specific metabolite groups, such as phosphatidylcholines, sphingomyelins, lysophosphatidylcholines, and eicosapentaenoic acid, in calves fed MF compared to calves fed CON and TRI. In contrast, the incorporation of tributyrin and tricaproin into milk replacers did not lead to differences in the hepatic metabolome profiles of calves.

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CONFLICT OF INTEREST

Trouw Nutrition funded this study. Several authors (J. N. Wilms and L. N. Leal) are employed by the company, which has commercial interests in milk replacers for calves. Trouw Nutrition R&D adheres to the principles of the European Code of Conduct for Research Integrity (Drenth, 2012).

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TABLES

Table 1: Descriptive summary of the fatty acid profile from milk replacers fed to calves.

Fatty acid (% total FA)	Treatment[1]		
	MF	NC	TRI
C4:0	3.25	0.38	3.36
C6:0	2.14	0.46	2.49
C8:0	1.28	2.56	2.67
C10:0	2.81	2.13	2.00
C12:0	4.77	15.08	15.46
C14:0	11.17	7.21	6.53
C15:0	1.02	0.13	0.04
C16:0	29.83	31.26	28.18
C17:0	0.55	0.10	0.07
C18:0	9.67	4.55	3.85
C20:0	0.14	0.20	0.22
trans-6 C18:1	0.20	-	-
trans-9 C18:1	0.16	-	-
trans-10 C18:1	0.24	-	-
trans-11 C18:1	1.04	0.07	-
Total 18:1 *trans*	1.64	-	-
cis-9 C18:1	20.63	26.63	26.70
cis-9, *trans*-11 C18:2	0.47	0.08	-
C18:2 n-6	2.06	6.79	6.19
C20:4 n-6	0.05	-	-
C18:3 n-3	0.58	1.13	1.45
C20:5 n-3	0.06	-	-
C22:5 n-3	0.08	-	-
Total SFA[2]	68.5	64.1	64.9
Total MUFA[4]	27.3	27.7	27.3
Total PUFA[5]	3.4	8.0	7.6
Total n-6 FA[6]	2.2	6.8	6.2
Total n-3 FA[7]	0.8	1.1	1.5
n-6:n-3 ratio	2.75	6.2	4.1

[1] Treatments included an MR with dairy cream (MF), an MR with a blend of vegetable oils (palm, coconut, and linseed, NC), and an MR with the same oil blend as NC to which tributyrin and tricaproin were incorporated (TRI).

[2] Sum of saturated fatty (SFA) acids from 4 to 24 carbons in length.

[3] Sum of monounsaturated fatty acids (MUFA) from 14 to 22 carbons in length.

[4] Sum of PUFA from 18 to 22 carbons in length.

[5] Sum of omega 3 PUFA (n-3) from 18 to 22 carbons in length.

[6] Sum of omega 6 PUFA (n-6) from 18 to 22 carbons in length.

Table 2. Significantly different liver metabolites identified through volcano plot analysis between calves fed dairy cream (MF) and vegetable fats (NC).

Metabolite	Fold change (FC)	log2(FC)	FDR
PC ae C32:1	1.52	0.61	<0.001
Cer (d18:1/24:1)	1.58	0.66	0.01
EPA	3.13	1.65	<0.001
GUDCA	2.13	1.09	0.01
Hex2Cer (d18:1/16:0)	1.6	0.68	<0.001
Hex3Cer (d18:1/18:0)	2.31	1.21	<0.001
HexCer (d18:1/18:0)	1.61	0.68	0.05
lysoPC a C16:1	2.44	1.29	<0.001
lysoPC a C17:0	1.96	0.97	<0.001
lysoPC a C28:1	1.68	0.75	<0.001
PC aa C32:1	2.53	1.34	<0.001
PC aa C34:4	1.74	0.8	<0.001
PC aa C36:1	2.14	1.1	<0.001
PC aa C36:5	2.71	1.44	<0.001
PC aa C36:6	3.45	1.79	<0.001
PC aa C38:0	1.63	0.71	<0.001
PC aa C38:5	1.89	0.91	<0.001
PC aa C40:4	1.55	0.64	<0.001
PC aa C40:5	2.33	1.22	<0.001
PC aa C40:6	1.91	0.93	<0.001
PC aa C42:5	1.74	0.8	<0.001
PC ae C30:0	1.86	0.9	<0.001
PC ae C30:2	2.09	1.06	<0.001
PC ae C32:2	1.77	0.82	<0.001
PC ae C34:0	4.72	2.24	<0.001
PC ae C34:1	2.76	1.47	<0.001
PC ae C36:0	2.55	1.35	<0.001
PC ae C36:1	4.03	2.01	<0.001
PC ae C36:3	1.72	0.78	<0.001
PC ae C38:0	2.43	1.28	<0.001
PC ae C38:1	3.45	1.79	<0.001
PC ae C38:2	1.89	0.92	<0.001
PC ae C38:3	1.77	0.83	<0.001
PC ae C38:4	2.54	1.34	<0.001
PC ae C38:5	1.96	0.97	<0.001
PC ae C40:1	1.65	0.72	<0.001
PC ae C40:2	2.34	1.23	<0.001
PC ae C40:3	2.37	1.25	<0.001
PC ae C40:4	2.06	1.04	<0.001
PC ae C40:5	3.49	1.8	<0.001

PC ae C40:6	2.37	1.24	<0.001
PC ae C42:2	1.58	0.66	<0.001
PC ae C42:3	1.71	0.77	<0.001
SM (OH) C14:1	2.4	1.26	<0.001
SM (OH) C16:1	3.29	1.72	<0.001
SM (OH) C22:1	1.79	0.84	<0.001
SM (OH) C22:2	2.46	1.3	<0.001
SM (OH) C24:1	1.53	0.61	0.01
SM C18:0	1.53	0.62	<0.001
SM C18:1	1.57	0.65	<0.001
SM C26:1	1.9	0.92	0.01
DG (16:0/18:2)	0.44	-1.18	<0.001
C5	0.46	-1.11	0.01
lysoPC a C18:2	0.53	-0.92	<0.001
PC aa C34:2	0.54	-0.89	<0.001
PC ae C42:1	0.54	-0.88	<0.001
SM C22:3	0.55	-0.85	<0.001
Putrescine	0.61	-0.71	0.01
Ser	0.64	-0.63	0.01

PC aa: Diacyl phosphatidylcholine; PC ae: Acyl-alkyl phosphatidylcholine; lysoPC: Lysophosphatidylcholine; SM: Sphingomyelin; Cer: Ceramide; HexCer: Hexosylceramide; Hex2Cer: Dihexosylceramide; Hex3Cer: Trihexosylceramide; DG: Diglyceride; EPA: Eicosapentaenoic acid; GUDCA: Glycochenodeoxycholic acid; C5: valerylcarnitine; log2(FC): Log base 2 of the Fold Change; FDR: False Discovery Rate.

Table 3. Significantly different liver metabolites identified through volcano plot analysis between calves fed milk replacer with dairy cream (MF) and milk replacer with vegetable fats plus tributyrin and tricaproin (TRI).

Metabolite	Fold change (FC)	log2(FC)	FDR
Cer (d18:1/16:0)	1.63	0.71	<0.001
Cer (d18:1/24:1)	1.59	0.67	0.05
Cer (d18:2/23:0)	2.09	1.07	0.01
EPA	2.85	1.51	<0.001
GUDCA	1.92	0.94	0.02
Hex3Cer (d18:1/18:0)	1.99	0.99	<0.001
HexCer (d18:1/18:0)	1.66	0.73	0.05
HexCer (d18:1/24:0)	1.66	0.73	<0.001
lysoPC a C16:1	2.29	1.2	<0.001
lysoPC a C17:0	2.4	1.26	<0.001
lysoPC a C20:4	1.55	0.64	0.01
lysoPC a C28:1	1.86	0.89	<0.001
PC aa C32:1	2.38	1.25	<0.001
PC aa C36:0	1.72	0.78	<0.001
PC aa C36:1	2.23	1.16	<0.001
PC aa C36:5	2.27	1.18	<0.001
PC aa C36:6	3.04	1.6	<0.001
PC aa C38:0	1.77	0.83	<0.001
PC aa C38:4	1.6	0.68	<0.001
PC aa C38:5	1.86	0.9	<0.001
PC aa C40:4	1.79	0.84	<0.001
PC aa C40:5	2.71	1.44	<0.001
PC aa C40:6	2.15	1.11	<0.001
PC aa C42:5	1.82	0.87	<0.001
PC ae C30:0	2.07	1.05	<0.001
PC ae C30:2	2.56	1.36	<0.001
PC ae C32:1	1.63	0.7	<0.001
PC ae C32:2	1.95	0.97	<0.001
PC ae C34:0	7.47	2.9	<0.001
PC ae C34:1	2.86	1.52	<0.001
PC ae C34:2	1.61	0.68	<0.001
PC ae C36:0	3.14	1.65	<0.001
PC ae C36:1	5.19	2.38	<0.001
PC ae C36:2	2.01	1.01	<0.001
PC ae C36:3	1.85	0.89	<0.001
PC ae C38:0	2.24	1.16	<0.001
PC ae C38:1	3.92	1.97	<0.001
PC ae C38:2	2.79	1.48	<0.001

PC ae C38:3	2.46	1.3	<0.001
PC ae C38:4	3.19	1.67	<0.001
PC ae C38:5	2	1	<0.001
PC ae C38:6	1.57	0.65	<0.001
PC ae C40:1	1.67	0.74	<0.001
PC ae C40:2	2.63	1.39	<0.001
PC ae C40:3	2.95	1.56	<0.001
PC ae C40:4	2.59	1.37	<0.001
PC ae C40:5	4.69	2.23	<0.001
PC ae C40:6	2.83	1.5	<0.001
PC ae C42:3	1.69	0.76	<0.001
SM (OH) C14:1	3.13	1.65	<0.001
SM (OH) C16:1	4.43	2.15	<0.001
SM (OH) C22:1	2.09	1.06	<0.001
SM (OH) C22:2	3.45	1.79	<0.001
SM (OH) C24:1	1.56	0.64	0.01
SM C18:0	1.66	0.73	<0.001
SM C18:1	1.8	0.85	<0.001
SM C26:1	2.41	1.27	<0.001
lysoPC a C18:2	0.59	-0.76	<0.001
SM C22:3	0.58	-0.79	<0.001
C5	0.39	-1.35	0.01
DG (16:0/18:2)	0.58	-0.8	0.01
PC aa C34:2	0.57	-0.8	<0.001
PC ae C42:1	0.54	-0.88	<0.001
Putrescine	0.54	-0.89	<0.001

PC aa: Diacyl phosphatidylcholine; PC ae: Acyl-alkyl phosphatidylcholine; lysoPC: Lysophosphatidylcholine; SM: Sphingomyelin; Cer: Ceramide; HexCer: Hexosylceramide; Hex2Cer: Dihexosylceramide; Hex3Cer: Trihexosylceramide; DG: Diglyceride; EPA: Eicosapentaenoic acid; GUDCA: Glycochenodeoxycholic acid; C5: Valerylcarnitine; log2(FC): Log base 2 of the Fold Change; FDR: False Discovery Rate.

Titles of the Figures

Figure 1. 83 significant metabolites revealed by ANOVA analysis in the hepatic metabolome of dairy calves (n = 15 per treatment).

Figure 2. Principal component analysis (PCA) scores plot showing the relationship between principal components (PC) 1 and 2 applied to the longitudinal hepatic metabolome of dairy calves. Each symbol represents the PC values of a calf within each treatment. Symbols of different colors represent different treatments (n = 15 per treatment).

Figure 3. (A) Three-dimensional scores plot of Partial Least Squares-Discriminant Analysis (PLS-DA) showcasing the separation of the longitudinal hepatic metabolome of dairy calves. PLS-DA is a statistical method used for classification and distinguishing samples based on their hepatic metabolomic profiles (B) Permutation test results, indicating the prediction accuracy during training. The permutation test is a statistical technique used to validate the significance of a model's predictive performance by randomly shuffling the data labels and assessing the resulting accuracy. (C) Variable Importance in Projection (VIP) scores plots revealing the key metabolites driving the separation of longitudinal hepatic metabolome profiles in dairy calves. VIP scores measure metabolite's importance in contributing to the observed separation between sample groups in multivariate analysis, such as PLS-DA (n = 15 per treatment).

Figure 4. Volcano plot picturing hepatic metabolites showing a difference between calves fed MF vs. NC treatments. The x-axis represents the mean of the $\log^2$ fold-change (FC) values, while the y-axis corresponds to the negative logarithm of the P-values (FDR <0.05). Each circle represents a single metabolite. The vertical lines show the fold change threshold (1.5). Liver samples were taken from each calf after harvest at 35 d of age (n = 15 per treatment).

Figure 5. Volcano plot picturing hepatic metabolites showing a difference between calves fed MF vs. TRI treatments. The x-axis represents the mean of the $\log^2$ fold-change (FC) values, while the y-axis corresponds to the negative logarithm of the P-values (FDR <0.05). Each circle represents a single metabolite. The vertical lines show the fold change threshold (1.5). Liver samples were taken from each calf after harvest at 35 d of age (n = 15 per treatment).

Figure 6. Longitudinal changes of the hepatic metabolome profile of calves across treatments: heatmap with hierarchical cluster analysis using the top 50 metabolites ranked by P-value of one-way ANOVA (effect of treatment). The colors in the heatmap reflect the hepatic metabolite abundance within each treatment (n = 15 per treatment).

Supplemental Figure S1. Volcano plot picturing hepatic metabolites showing a difference between calves fed TRI vs. NC treatments. The x-axis represents the mean of the $\log^2$ fold-change (FC) values, while the y-axis corresponds to the negative logarithm of the p-values (FDR <0.05). Each circle represents a single metabolite. The vertical lines show the fold change threshold (1.5). Liver samples were taken from each calf after harvest at 35 d of age (n = 15 per treatment).

FIGURES

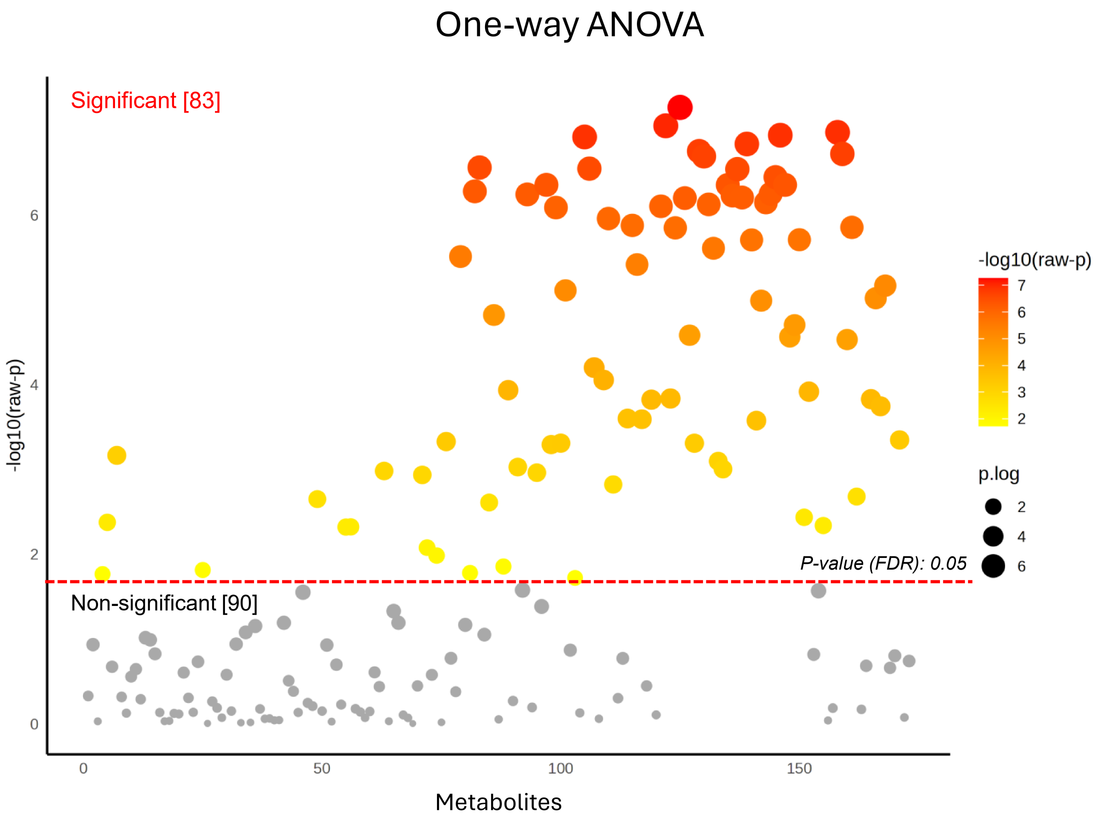


Figure 1.

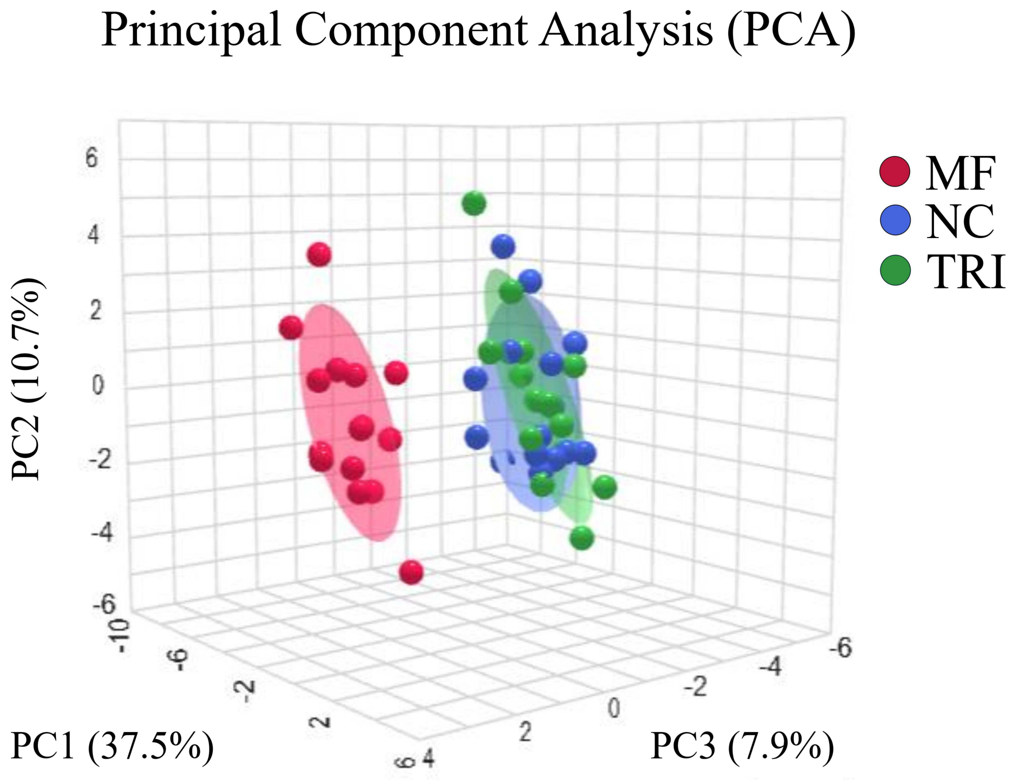

Principal Component Analysis (PCA)
MF
NC
TRI
PC2 (10.7%)
PC1 (37.5%)
PC3 (7.9%)
6
4
2
0
-2
-4
-6
-10
-6
-2
2
6
4
2
0
-2
-4
-6

Figure 2.

Partial Least Squares-Discriminant Analysis (PLS-DA)

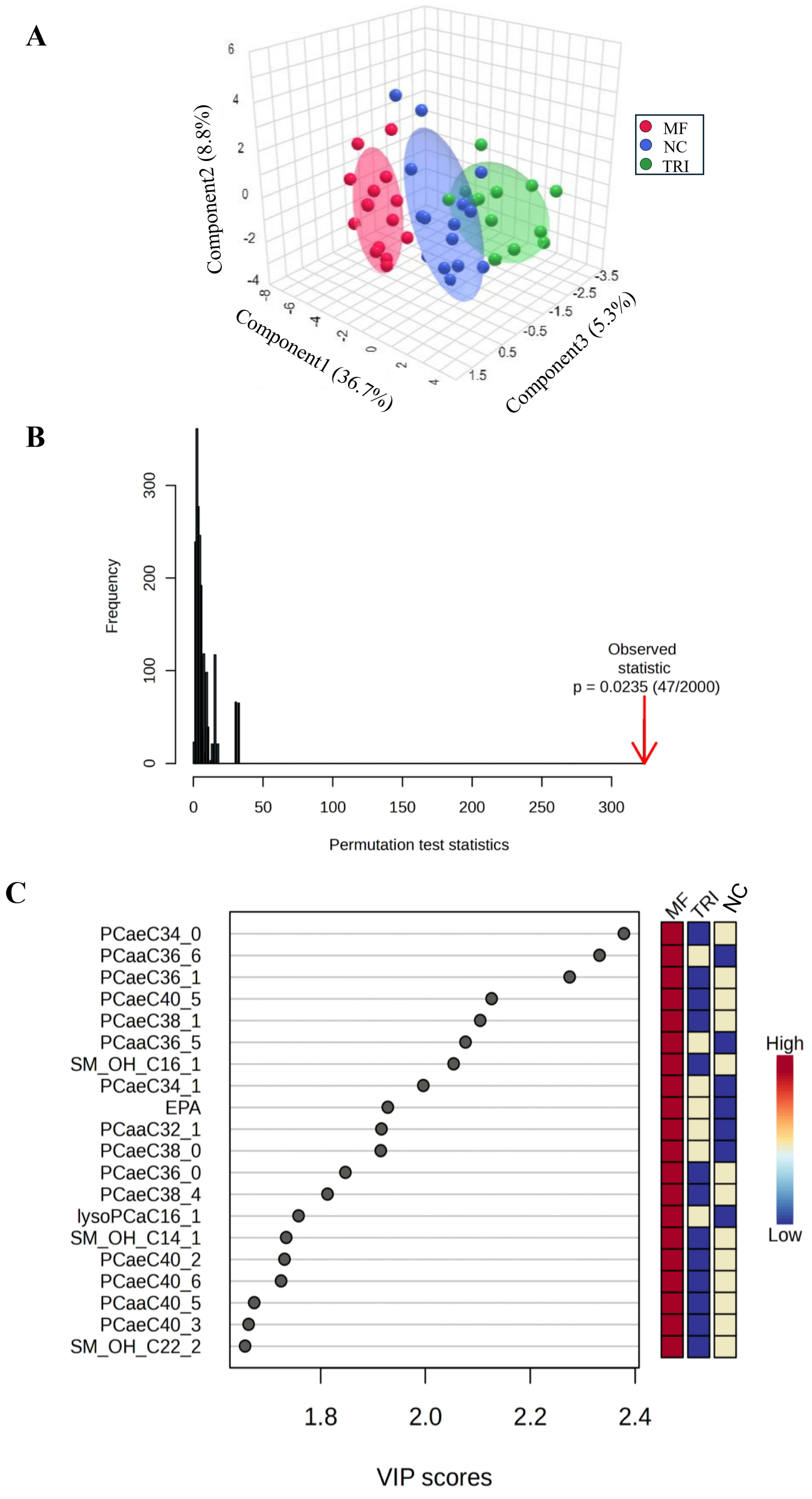
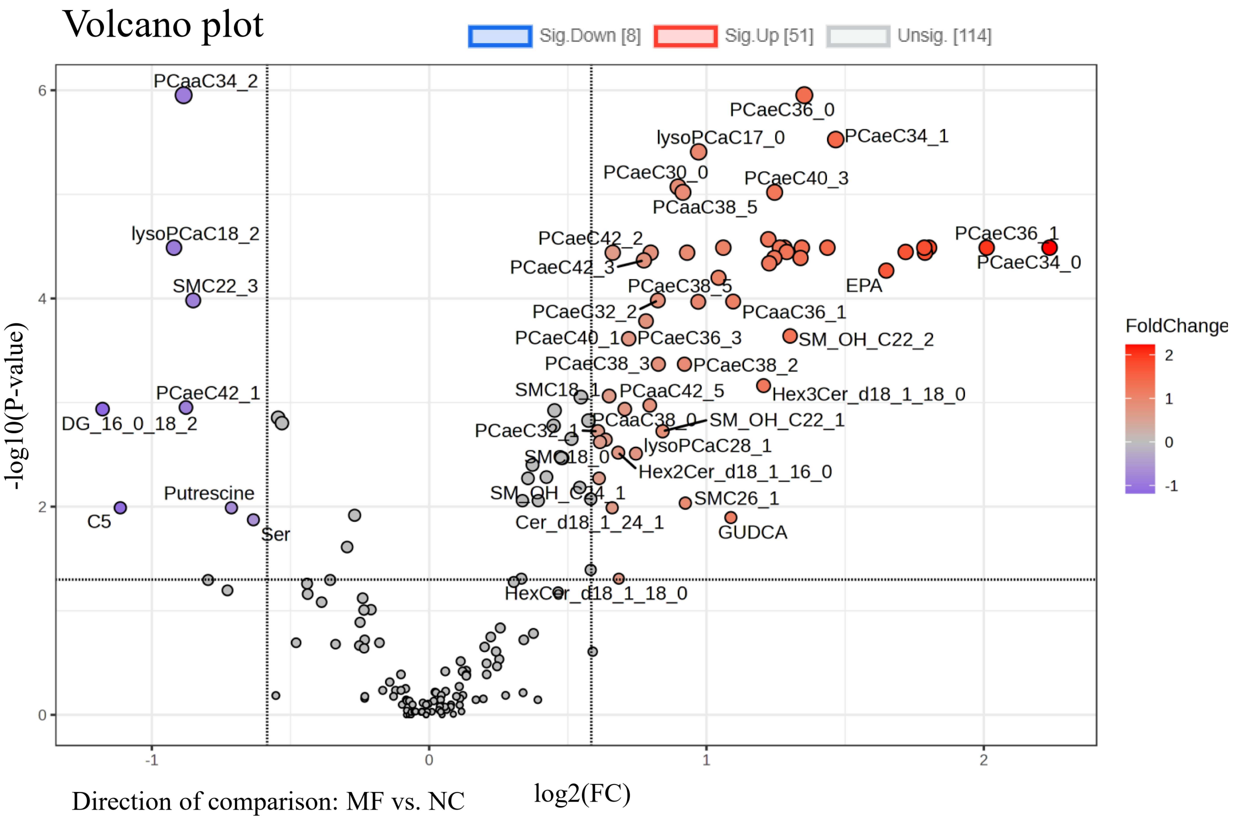


Figure 3.

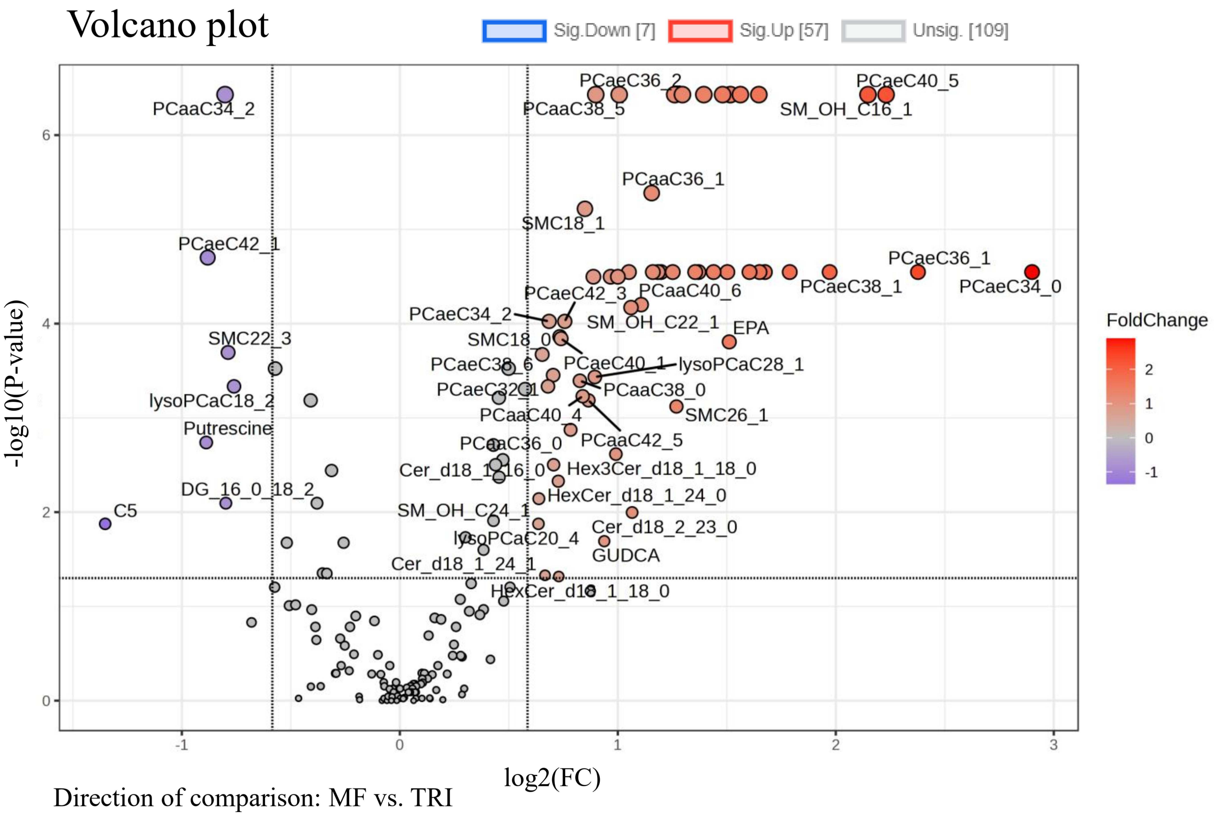


Direction of comparison: MF vs. NC

P-value (FDR): 0.05

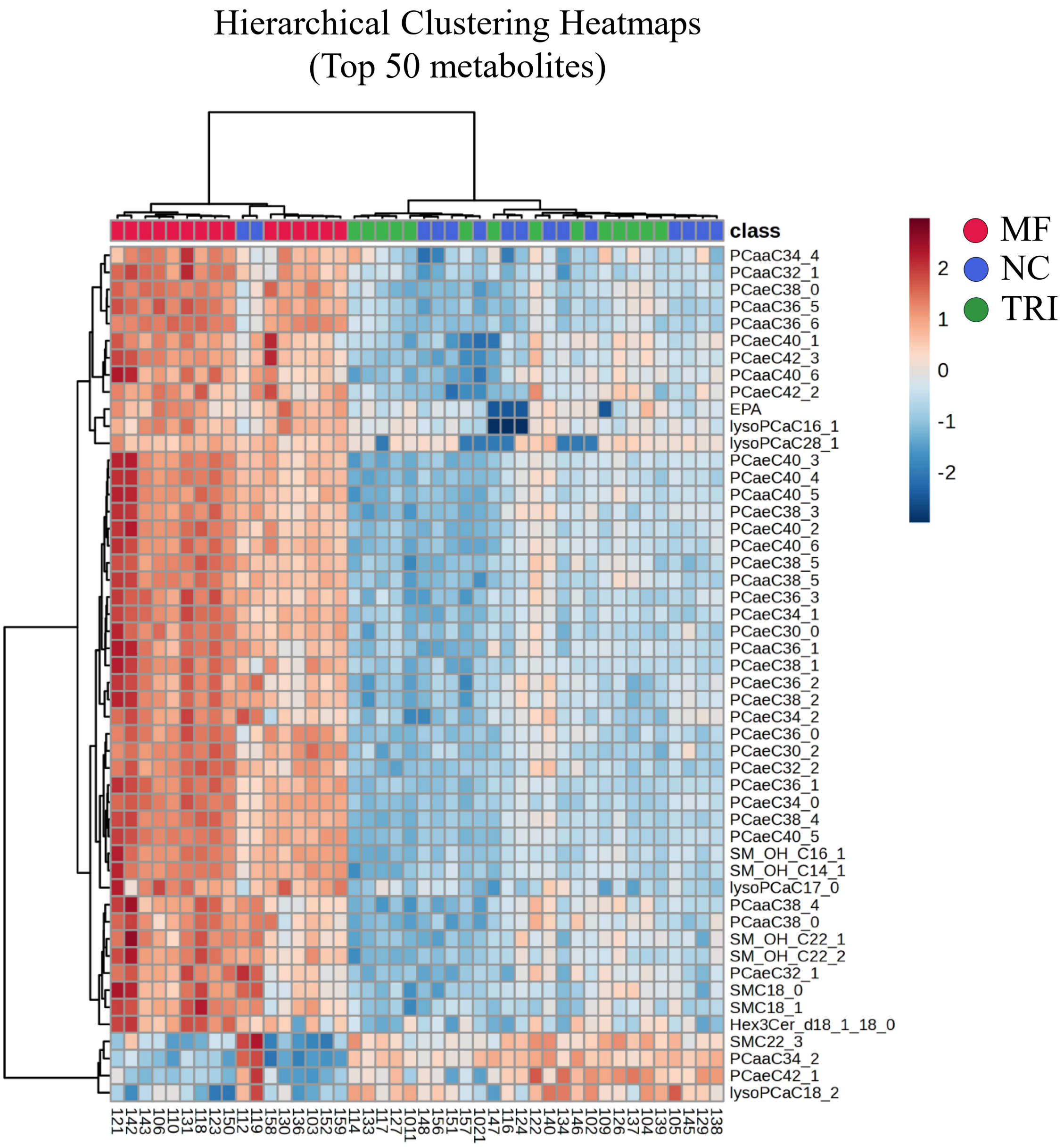
Fold change (FC): 1.5

Figure 4.



Direction of comparison: MF vs. TRI
P-value (FDR): 0.05
Fold change (FC): 1.5

Figure 5.


Hierarchical Clustering Heatmaps
(Top 50 metabolites)
class
MF
NC
TRI
2
1
0
-1
-2
PCaaC34_4
PCaaC32_1
PCaeC38_0
PCaaC36_5
PCaaC36_6
PCaeC40_1
PCaeC42_3
PCaaC40_6
PCaeC42_2
EPA
lysoPCaC16_1
lysoPCaC28_1
PCaeC40_3
PCaeC40_4
PCaaC40_5
PCaeC38_3
PCaeC40_2
PCaeC40_6
PCaeC38_5
PCaaC38_5
PCaeC36_3
PCaeC34_1
PCaeC30_0
PCaaC36_1
PCaeC38_1
PCaeC36_2
PCaeC38_2
PCaeC34_2
PCaeC36_0
PCaeC30_2
PCaeC32_2
PCaeC36_1
PCaeC34_0
PCaeC38_4
PCaeC40_5
SM_OH_C16_1
SM_OH_C14_1
lysoPCaC17_0
PCaaC38_4
PCaaC38_0
SM_OH_C22_1
SM_OH_C22_2
PCaeC32_1
SMC18_0
SMC18_1
Hex3Cer_d18_1_18_0
SMC22_3
PCaaC34_2
PCaeC42_1
lysoPCaC18_2
121
142
143
106
110
131
118
123
150
112
119
158
130
136
103
152
159
114
133
117
127
1011
148
156
151
157
1021
147
116
124
122
140
134
146
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126
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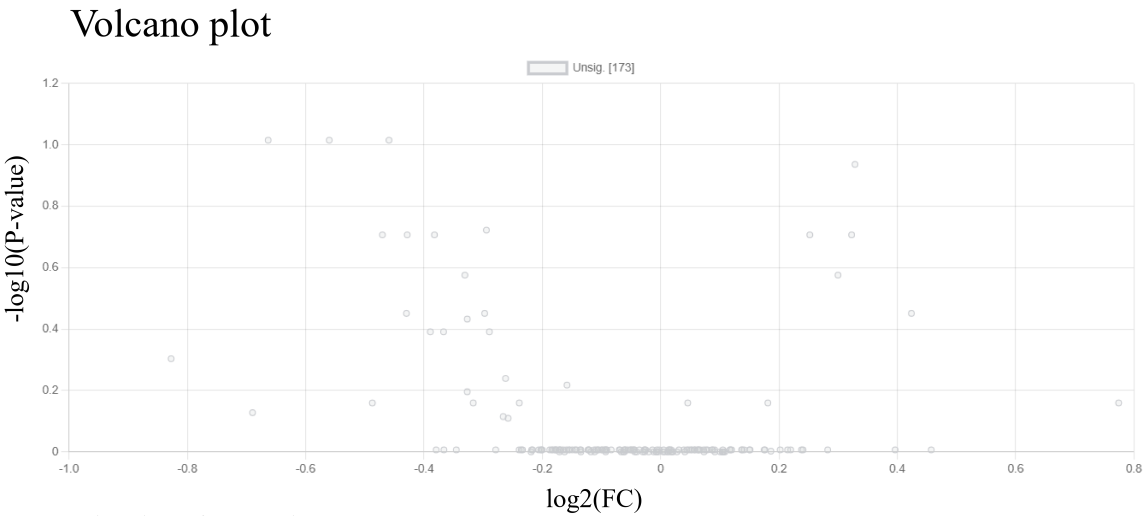
105
145
129
138

Figure 6.



Direction of comparison: TRI vs. NC

P-value (FDR): 0.05

Fold change (FC): 1.5

Supplemental Figure S1.