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Fecal metabolomics to understand intestinal dysfunction in male dairy beef calves at arrival to the rearing farm

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Fecal biomarkers are becoming an important analytical tool since feces are in direct contact with the inflamed intestine and site for the gut microbiome. The objective of this study was the identification of differential fecal metabolites by means of ^{1}H-NMR to evaluate the management of male dairy beef calves, and which could become potential biomarkers of gastrointestinal disorders. Holstein calves were subjected to a protocol aimed to simulate real conditions of the dairy beef market. Three groups were studied: Control (CTR: high colostrum, no transport, milk replacer), LCMR (low colostrum, transport, milk replacer) and LCRS (low colostrum, transport, rehydrating solution). Fecal lactoferrin was determined as marker of intestinal inflammation, and metabolomic profiling was performed in feces collected the day after arrival to the farm. 41 polar and 10 non-polar metabolites were identified, of which proline, formate and creatine increased in the LCRS group, whereas butyrate and uracil decreased. Less differences were found in non-polar metabolites. Multivariate analysis indicated that most differences are found between the LCRS group and the others. In conclusion, this study indicates that feed restriction has a more important effect at this age than colostrum uptake and transport. These results should help to identify robust fecal biomarkers to assess calf intestinal health and improve management protocols.

Keywords ^{1}H-NMR, Dairy beef calves, Feces, Inflammation, Lactoferrin, Metabolomics

The identification and development of novel biomarkers would help to improve animal health and welfare and reduce both disease incidence and antibiotic use[1]. In relation to gut inflammatory disorders, the analysis of fecal biomarkers is becoming an important tool since feces are in direct contact with the inflamed intestinal area and site for the gut microbiome, from which potential compounds are likely to originate[2,3]. This has been extensively studied in humans, especially in the case of inflammatory bowel disease (IBD), that includes ulcerative colitis and Crohn's disease[4], and other intestinal pathologies[2,5–7]. One of the best characterized fecal biomarkers associated to gut inflammation is lactoferrin, a neutrophil-derived glycoprotein with iron-binding activity and with antimicrobial potential due to its capacity to limit iron uptake by pathogenic organisms[8].

Much less information exists in veterinary medicine, but gastrointestinal disorders frequently affect health, welfare and productive performance[9]. In the field of calf management, biomarkers at the veal farm might provide information about an ongoing or future disease process, and help calf management[10]. One specific problem in the cattle industry is the management of male dairy calves, which have become an important supply of beef in many countries. These calves receive lower volumes of colostrum than females, and the process of marketing and transportation from their original dairy farm to the rearing facilities often includes mixing at auction markets or assembly centers, where calves are normally fed only rehydrating solutions, exposure to new pathogens and stress associated to truck transport, leading to a higher risk of mortality and morbidity[11]. Feed-restricted and fasted unweaned calves take approximately 21 days to recover their concentrate intake after arrival to the farm[12]

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compared to non-feed restricted and non-transported calves, and feed restriction can cause modifications in the normal functioning of the intestinal barrier[12–16]. Loss of integrity of the gut mucosa affects metabolic and inflammatory parameters, facilitates the entrance of pathogens into the bloodstream, and increases susceptibility to infection[13,14].

In the search of new biomarkers, -*omics* technologies offer a high throughput approach and metabolomics is a useful tool for the identification of new parameters, and to study the biology of physiological and pathological conditions. In particular, ^{1}H-NMR based metabolomics offers the advantage compared to MS-based techniques of being a robust technology able to detect multi-parametric compounds from a single sample with little sample processing, and being quantitative. When comparing data in the literature, the technology used has to be taken into account since different metabolites are more easily targeted depending on the technique[17,18]. In human medicine, metabolomics has been applied to IBD and other human intestinal pathologies[4,7,19]. In animal science, metabolomics has been applied to several sample types and animal species, with cattle being one of the most studied[20–23]. In calves, plasma metabolomics has been used to study the physiological changes associated with weaning due to the diet change[24,25]. Recently, particular attention has been paid to feces which, besides being non-invasive, may serve as a functional readout of the gut microbiota and be influenced by nutrition and disease[26,27]. In bovine, metabolomic analysis has been carried out in feces of dairy cows with mastitis[28], in relation with the rumen fluid[29] and in relation with short-term stress[30].

Our research groups have previously analyzed the effects of colostrum consumption and feed restriction in male dairy calves subjected to marketing (assembly center simulation) and long road transport. Our results showed that feed restriction negatively affects parameters related to body weight, intake recovery, energy balance, gut permeability and behavior, and specifically worsens the fecal score[15]. In the present work, we have extended this study by selecting a group of these calves and using metabolomics on fecal samples to identify candidate metabolites that could be used to improve management strategies at the rearing facilities. In farm animals, the use of feces as sample matrices has the advantage of being non-invasive, easy to perform on the farm and more specific for gastrointestinal disorders.

The main goal of the present study was to identify differences in fecal metabolite composition by using ^{1}H-NMR to evaluate the effects of colostrum consumption and feed restriction during marketing and transportation at the arrival to the rearing farm, using the same individuals used in Pisoni et al.[15].

Results

Fecal concentration of lactoferrin in male Holstein calves

Lactoferrin concentration at d −4 was similar in the three groups (Fig. 1), indicating that intake of colostrum did not affect fecal lactoferrin at this age. After three days at the assembly center (d −1), the concentration of fecal lactoferrin was higher in the LCRS group ($P < 0.05$), whereas there was no difference among the CTR group and the LCMR group. One day after the long transport and arrival to the farm (d 1), the concentration of lactoferrin was again higher in the LCRS group compared with the other treatments ($P < 0.01$).

Characterization of the calf fecal metabolome

The compounds identified by ^{1}H-NMR in fecal samples were classified into two groups depending on the behavior during the extraction procedure: polar metabolites and non-polar metabolites (Supplementary Table S1). A total

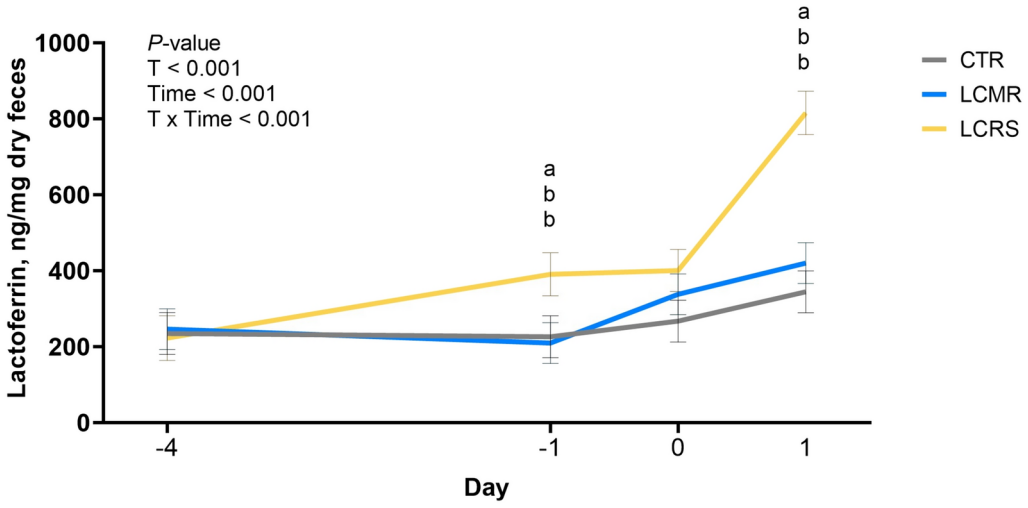


Fig. 1. Fecal concentration (mean ± SE) of lactoferrin in male Holstein calves in the following groups: (CTR) fed 10 L of colostrum at birth, MR at the assembly center simulation period, and not transported; (LCMR), fed 2 L of colostrum at birth, MR at the assembly center simulation period and transported during 19 h; (LCRS), fed 2 L of colostrum at birth, RS at the assembly center simulation period and transported during 19 h. Fecal samples were collected at d −4, −1, 0 and 1. Different letters within a time point denote differences among treatments ($P < 0.05$); the order of the letters denotes the treatment with the highest value. T: treatment.

of 41 polar metabolites were identified, belonging to several groups (Fig. 2A). Amongst these groups, organic acids including the five more relevant short chain fatty acids (SCFA) (formate, acetate, propionate, butyrate and valerate) were the most representative. Besides these compounds, protein amino acids, nitrogenous bases, amino acid derivatives and amines were the most abundant groups. Referring to non-polar compound types, there was a majority of fatty acids, saturated fatty acids and different types of unsaturated fatty acids: poly unsaturated fatty acids (PUFA), omega-6 and omega-7 fatty acids (w6 + w7), omega-9 fatty acids (w9) and specific polyunsaturated as Linoleic Acid (LA), and Arachidonic acid + Eicosapentaenoic acid (ARA + EPA) (Fig. 2B).

A correlation analysis indicated some highly positively correlated groups of compounds: butyrate, isobutyrate and valerate; acetate, propionate and 4-hydroxybutyrate (all SCFAs); the branched chain amino acids (BCAAs) Leu, Ile and Val; and the amines creatine, cadaverine and 4-aminobutyrate. The strongest negative correlations were found between formate/proline *versus* butyrate and between formate/proline *versus* hypoxanthine/uracil (Fig. 2C).

Identification of differential polar fecal compounds in calves with different nutritional treatments by using metabolomics

Metabolomic analysis considering the three groups

The quantification of polar metabolites for the three treatment groups (9 samples from the CTR group, 9 samples from the LCMR group, and 8 samples from the LCRS group), is shown in Tables 1 and 2. The global appreciation of the results indicated that the CTR and the LCMR groups were similar, whereas LCRS showed more differences.

Eight polar metabolites were identified which presented significant differences among groups, namely, the amino acid proline, the amino acid derivatives creatine and desaminotyrosine, the organic acids formate, butyrate, lactate and valproate, and the nitrogenous base uracil. Glycine, leucine (amino acids), hypoxanthine (nitrogenous base) and N-methylhydantoin (bacterial metabolite) showed statistical tendencies. The three amino acids and creatine were found at higher ($P \leq 0.05$) concentrations in the severely restricted group (LCRS). Formate and lactate were also found at higher ($P < 0.05$) concentration in feces of the LCRS group, as well as creatine and N-methylhydantoin. On the contrary, concentrations of hypoxanthine, uracil and valproate were lower ($P < 0.05$) in this group.

Regarding the non-polar metabolites (Tables 3 and 4), two of them were found to be significantly different among groups: sphingomyelin (SM) and arachidonic acid and eicosapentaenoic acid (ARA + EPA). The results show that sphingomyelin (SM) was at higher ($P < 0.01$) concentration in LCMR individuals, whereas ARA + EPA were higher ($P = 0.03$) in the CTR group and their concentration decreased in LCMR and LCRS groups.

Differences between the fecal metabolite profile of calves from the CTR, LCMR and LCRS groups were visualized by a supervised PLS-DA, that resulted in a clear discrimination between the CTR and the LCRS groups whereas the LCMR groups partially overlapped with the other two (Fig. 3A). Metabolites showing significant differences were selected based on the variable importance in the projection (VIP) threshold > 1 and a false discovery rate (FDR) < 0.001 (ANOVA), and the best one was the amino acid proline (Fig. 3B).

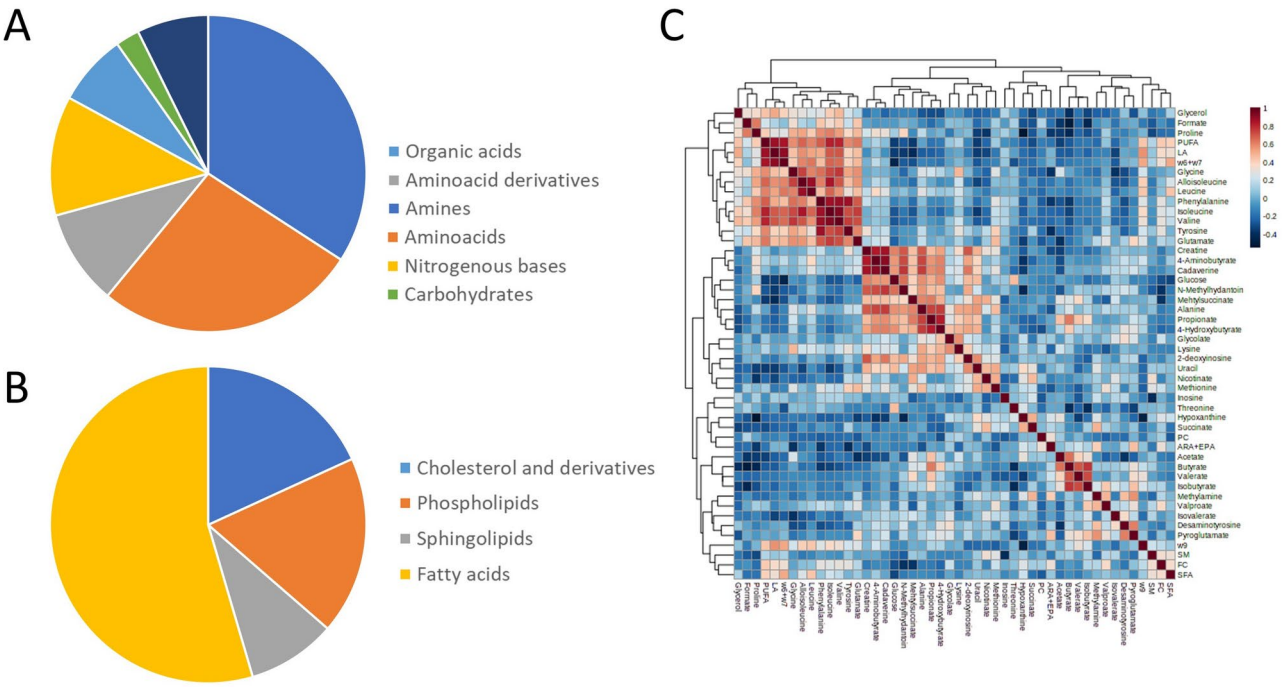


Fig. 2. Types of identified compounds in calf fecal samples. (**A**) Polar compounds. (**B**) Non-polar compounds. (**C**) Correlation heatmap of polar metabolites identified in calf fecal samples.

Chemical group	Compound	CTR	LCMR	LCRS	p-value
Amine	Cadaverine	1.45	2.75	5.17	0.39
Amino acid	Tyrosine	0.35	0.49	0.36	0.54
	Glutamate	7.27	5.43	12.21	0.51
	Phenylalanine	0.31	0.63	0.93	0.12
	Methionine	0.29	0.47	0.64	0.63
	Lysine	4.94	6.10	5.06	0.51
	Proline	2.76[b]	1.55[b]	13.97[a]	**< 0.01**
	Glycine	0.87	1.51	4.25	**0.10**
	Threonine	1.03	1.43	1.96	0.12
	Alanine	3.69	5.25	4.12	0.35
	Isoleucine	0.67	0.89	1.74	0.50
	Valine	0.89	0.89	1.82	0.60
	Leucine	1.31	0.88	2.26	**0.09**
Amino acid derivative	**Creatine**	0.40[b]	1.47[ab]	2.93[a]	**< 0.01**
	Pyroglutamate	1.19	1.29	1.16	0.66
	Alloisoleucine	0.26	0.21	0.67	0.55
Carbohydrate	Glucose	4.87	7.25	9.01	0.51
Nitrogenous base	2-deoxyinosine	0.10	0.08	0.04	0.95
	Hypoxanthine	1.83	1.65	0.89	**0.08**
	Nicotine	0.44	0.61	0.24	0.27
	Uracil	2.62[a]	2.83[a]	1.12[b]	**0.02**
Organic acid	Glycolate	3.11	2.44	1.93	0.20
	Methylsuccinate	3.29	6.05	4.38	0.41
	4-Aminobutyrate	0.13	1.23	3.37	0.24
	4-Hydroxybutyrate	4.91	4.70	4.30	0.59
	Succinate	2.33	2.60	2.10	0.69
	Isobutyrate	3.54	3.22	2.73	0.38
	Isovalerate	8.58	7.11	5.89	0.38
Organic acid SCFA 1C	**Formate**	0.80[b]	5.04[ab]	11.14[a]	**0.04**
Organic acid SCFA 2C	Acetate	144	135	106	0.47
Organic acid SCFA 3C	Propionate	50.54	59.14	44.59	0.57
Organic acid SCFA 4C	**Butyrate**	18.75[a]	18.56[a]	4.93[b]	**0.05**
Others (Alcohol)	Glycerol	1.36	0.83	1.38	0.64
Others (Heterocyclic organic compound)	***N*-Methylhydantoin**	0.33	0.52	0.85	**0.06**

Table 1. Median differences of polar fecal metabolites identified in dairy beef calves corresponding to groups CTR, LCMR and LCRS. Units are in µmol/g dry feces. Significant differences ($p \leq 0.05$) and tendencies ($p \leq 0.10$) are labeled in bold.

Chemical group	Compound	CTR	LCMR	LCRS	p-value
Amine	Methylamine,%	44.4	22.2	25.0	0.66
Amino acid derivative	**Desaminotyrosine,%**	88.9	11.1	50.0	**< 0.01**
Nitrogenous base	Inosine,%	33.3	22.2	12.5	0.84
Organic acid	**Lactate,%**	11.1	0.00	50.0	**0.02**
	Valproate,%	77.8	77.8	25.0	**0.04**
Organic acid SCFA 5C	Valerate,%	55.6	55.6	25.0	0.42
Others	Phenylacetate,%	33.3	11.1	12.5	0.56

Table 2. Chi-square of percentage of calves with polar fecal metabolites identified in dairy beef calves corresponding to groups CTR, LCMR and LCRS. Units are in µmol/g dry feces. Significant differences ($p \leq 0.05$) are labeled in bold.

	CTR	LCMR	LCRS	p-value
Cholesterol and derivatives				
Free cholesterol (FC)	240	236	174	0.28
Phospholipids				
Phosphatidylcholine (PC)	1.52	2.07	2.08	0.75
Sphingomyelin (SM)	6.66[b]	13.62[a]	8.91[ab]	**< 0.01**
Fatty acids				
Polyunsaturated fatty acids (PUFA)	56.9	65.7	82.5	0.36
Linoleic Acid (LA)	39.2	35.8	28.4	0.38
Saturated fatty acids (SFA)	708	895	815.0	0.90
Omega-6 & omega-7 fatty acids (w6 + w7)	296	270	274	0.45
Omega-9 fatty acids (w9)	865	637	745	0.41
Arachidonic acid + Eicosapentaenoic acid (ARA + EPA)	40.2[a]	16.5[b]	16.5[b]	**0.03**

Table 3. Median differences of non-polar fecal metabolites identified in dairy beef calves corresponding to groups CTR, LCMR and LCRS. Units are in arbitrary units/mg. Significant differences ($p \leq 0.05$) are labeled in bold.

	CTR	LCMR	LCRS	p-value
Cholesterol and derivatives				
Esterified cholesterol (EC), %	11.1	22.2	37.5	0.44
Phospholipids				
Lysophosphatidylcholine (LPC), %	100	100	100	0.99

Table 4. Chi-square of percentage of calves with non-polar fecal metabolites identified in dairy beef calves corresponding to groups CTR, LCMR and LCRS. Units are in arbitrary units/mg.

The hierarchical clustering heatmap correctly grouped most of the individuals corresponding to the LCRS group, whereas samples from the CTR and LCMR groups were not discriminated, indicating that colostrum provision did not have important consequences at this time period (calves around 19 days old) whereas nutritional restriction was an influential factor affecting the fecal metabolome (Fig. 3C). Furthermore, the analysis indicated a high variability between the metabolic profiling of individual samples, especially in the LCRS group.

Metabolomic analysis considering pairwise comparisons

Since the quantitative results and the discriminant heatmap analysis both indicated a distinct pattern of the LCRS group, which was different from the other two groups, the analysis with MetaboAnalyst was performed comparing LCRS and CTR groups. The Volcano plot identified seven upregulated metabolites (proline, formate, phenylalanine, glycine, methylhydantoin, isoleucine, alloisoleucine) and two downregulated metabolites (butyrate and ARA + EPA) in the LCRS group compared to the CTR group (Fig. 4A,B). PLS-DA analysis indicated a clear separation between both groups. Likewise, the hierarchical clustering heatmap discriminated between groups, with the exception of one individual corresponding to the CTR group and two individuals from the LCRS group (Fig. 4C,D).

Instead, the Volcano plot from the comparison between LCMR and CTR groups showed less differences and less relevant between both groups, and the PLS-DA analysis and the hierarchical clustering heatmap did not discriminate so well between groups (Fig. 5).

Furthermore, when assessing the quality of the PLS-DA models using Q2 as performance measure, Q2 was positive for the PLS-DA between groups CTR and LCRS, whereas it was negative for the PLS-DA between CTR and LCMR. Besides, the metabolites with VIP > 1.0 corresponding to component 1, and the weighted sum of absolute regression coefficients were different for both comparisons: Proline, ARA + EPA, methylhydantoin, hypoxanthine and butyrate rated highest in the comparison between CTR and LCRS groups, whereas desaminotyrosine, phenylalanine, inosine and SM rated highest in the comparison between CTR and LCMR groups (Supplementary Fig. S1).

Discussion

The concentration of fecal lactoferrin, which was determined as gut inflammatory marker, was similar in all groups at d −4, indicating that colostrum uptake does not have a significant effect at this age (around 15 days old). This is important since this protein is abundant in colostrum[31] and in consequence colostrum uptake could interfere in its utility as a disease biomarker. Whether colostrum intake may modify fecal lactoferrin in very young calves (few days old) is a question to be addressed in the future.

In LCRS calves, fecal lactoferrin increased at d −1 (after 3 days of feed restriction at the auction market simulation) and at d 1 (first day on the facilities after 19 h of fasting due to transport). This indicates that

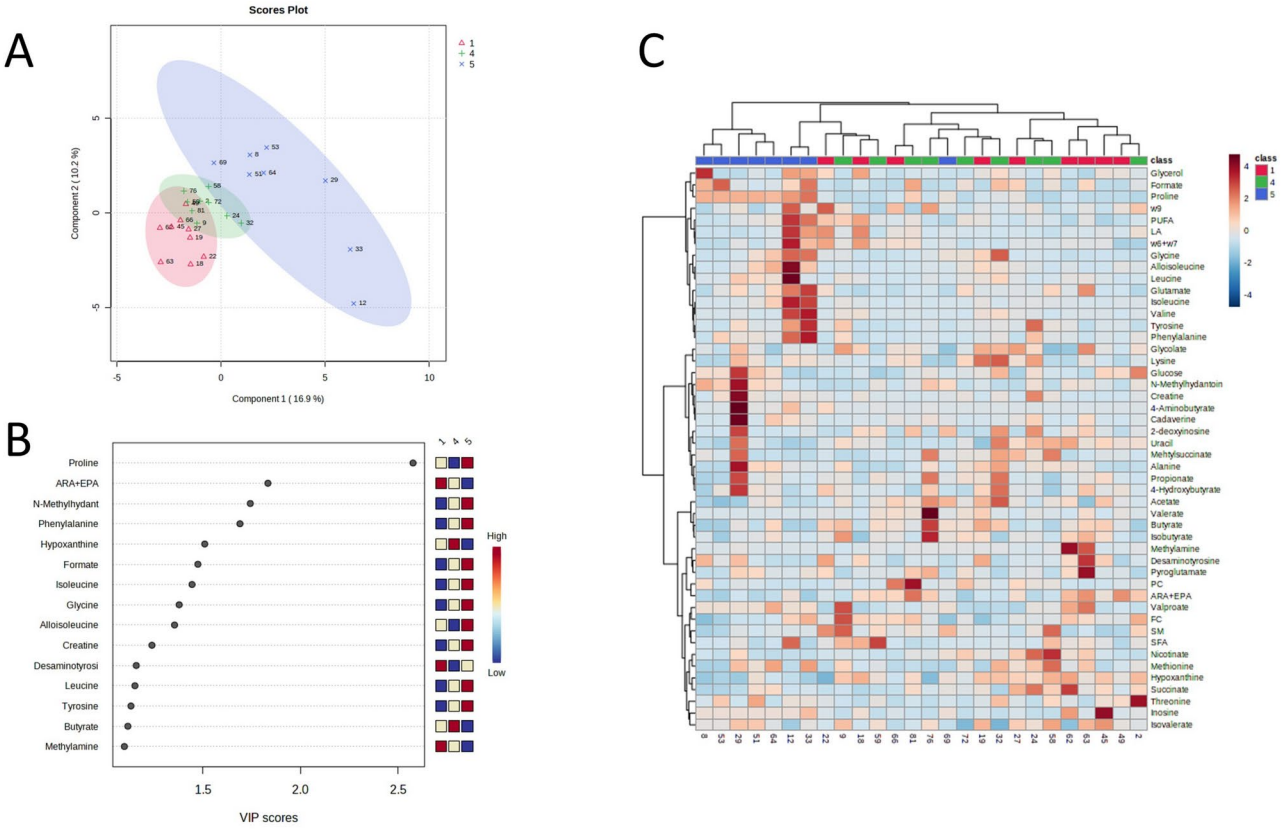


Fig. 3. Metabolomic analysis considering the three groups CTR, LCMR and LCRS. (**A**) Metabolic profiles of the CTR (group 1, red), LCMR (group 4, green) and LCRS (group 5, blue) fecal samples using PLS-DA score plots. Each shape indicates one sample colored according to the group with ellipse indicating the 95% confidence region. (**B**) Metabolites with VIP > 1.0 corresponding to component 1. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study. (**C**) Hierarchical clustering heatmap from groups CTR (group 1, red), LCMR (group 4, green) and LCRS (group 5, blue).

intestinal inflammation is progressive and gets aggravated after several days in the market and during transport. Our previous results[15] have shown that Cr-EDTA (Chromium-EDTA) test was higher in LCRS group at d 0, just at the arrival to the farm, whereas suddenly decreased at d 1. This indicates that damage of the enterocyte layer precedes the appearance of lactoferrin in feces. Likewise, serum citrulline was lower in the LCRS group affecting the enterocyte mass. These two procedures are widely accepted as measures of intestinal integrity[9,32]. In that study, health scores were analyzed in 2 periods, period 1 from d −4 to d −1 (assembly center simulation period) and period 2 from d 0 to d 7 (rearing farm). Results from the fecal score and the Wisconsin calf health score showed period by treatment differences with an increase in both scores for the LCRS calves during period 2. Period 2 was later than our experimental time, suggesting that an increase in fecal lactoferrin may be a prognostic indicator of future health problems.

Although fecal lactoferrin may vary under other circumstances, it can be concluded that calves under more severe nutritional management (RS) showed higher lactoferrin concentration, indicating that these calves, probably due to their poor condition, suffer from intestinal inflammation, predisposing them to intestinal pathologies.

To characterize the calf fecal metabolome, an ^{1}H-NMR based approach was used. Metabolites found in feces can have two main different origins. One of them is the gut microbiota, which produce a wide variety of metabolites either through direct fermentation of dietary substrates or through utilization of endogenous compounds produced by other gut microbes and the host[24]. On the other side, compounds coming from the host can be released into feces especially during intestinal disorders, since the intestinal mucosa is altered, the epithelial barrier damaged and the inflammatory cells of the mucosa proliferate and are activated producing inflammation-related substances[19].

Our results indicate that the polar fecal metabolome is represented mainly by organic acids, including SCFAs, with acetate, propionate and butyrate the most abundant reaching the amount of 15–150 µmol/g dry feces. Similar results have been described in neonatal calves[33]. These acids are produced by the transformation of complex and simple carbohydrates from the diet which are fermented by intestinal microorganisms into SCFAs, especially in the hindgut[34,35]. A second group of very abundant compounds are amino acids, of which 11 proteinogenic amino acids have been identified, in agreement with previous reports on the fecal metabolome

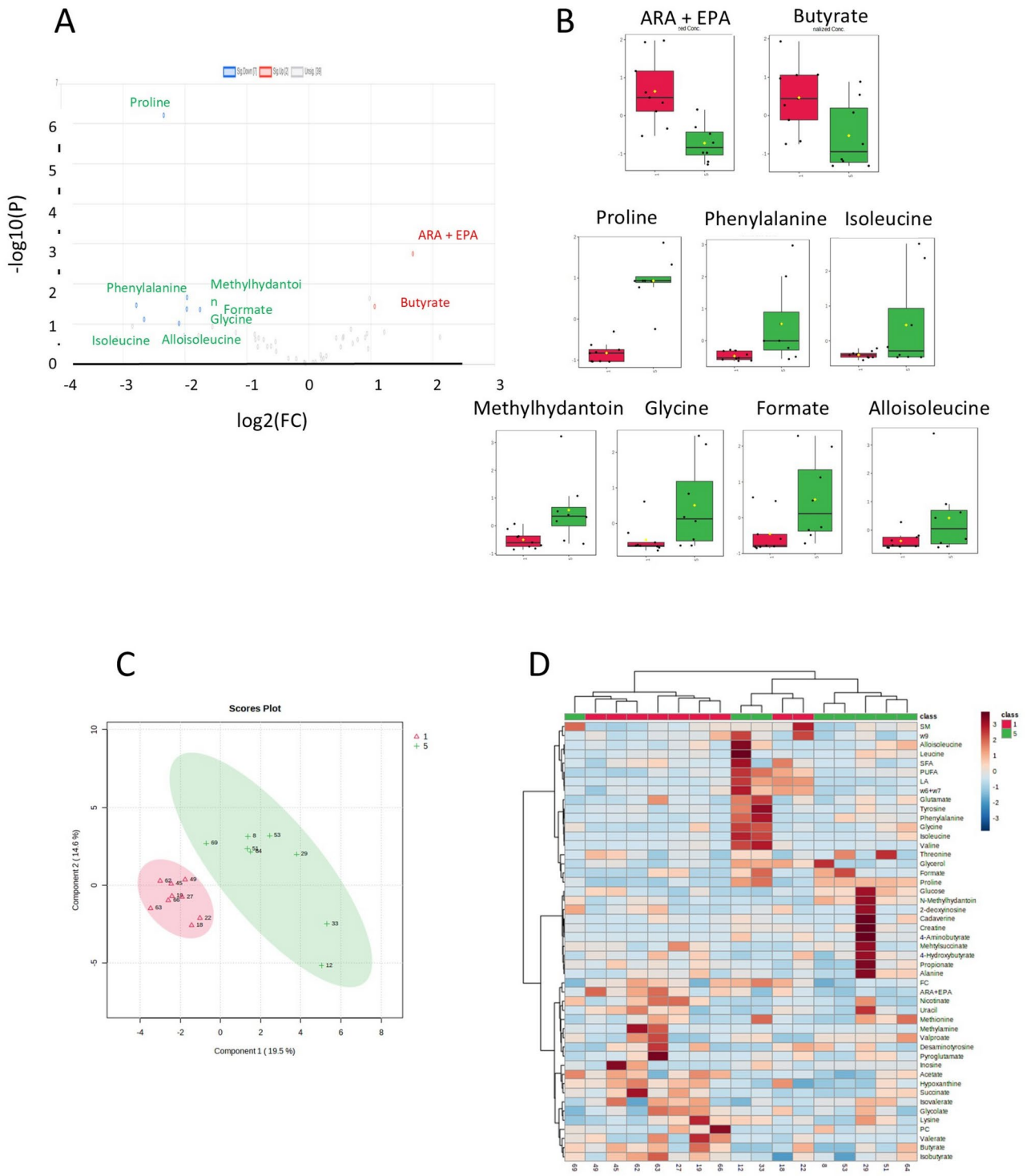


Fig. 4. Metabolomic analysis considering groups CTR and LCRS. (**A**) Volcano plot indicating the upregulated and downregulated metabolites in the comparison between CTR and LCRS groups. (**B**) Box plots showing the distribution of normalized values between CTR (red) and LCRS (green) groups. (**C**) PLS-DA showing the separation between the CTR (red) and LCRS (green) groups. (**D**) Hierarchical cluster heatmap indicating the clustering between samples corresponding to CTR (red) and LCRS (green) groups.

of neonatal calves[33,36]. Although the prevalent notion is that amino acids from the diet are mainly absorbed in the small intestine in mammals, there are robust evidences that they may arrive to the hindgut and be absorbed there[36]. Indeed, a pathway enrichment analysis of polar compounds identified that the main pathways were related to amino acid metabolism (Val/Leu/Ile, Ala/Asp/Glu, Arg/Pro, Phe, Gly/Ser/Thr, Gluthathione

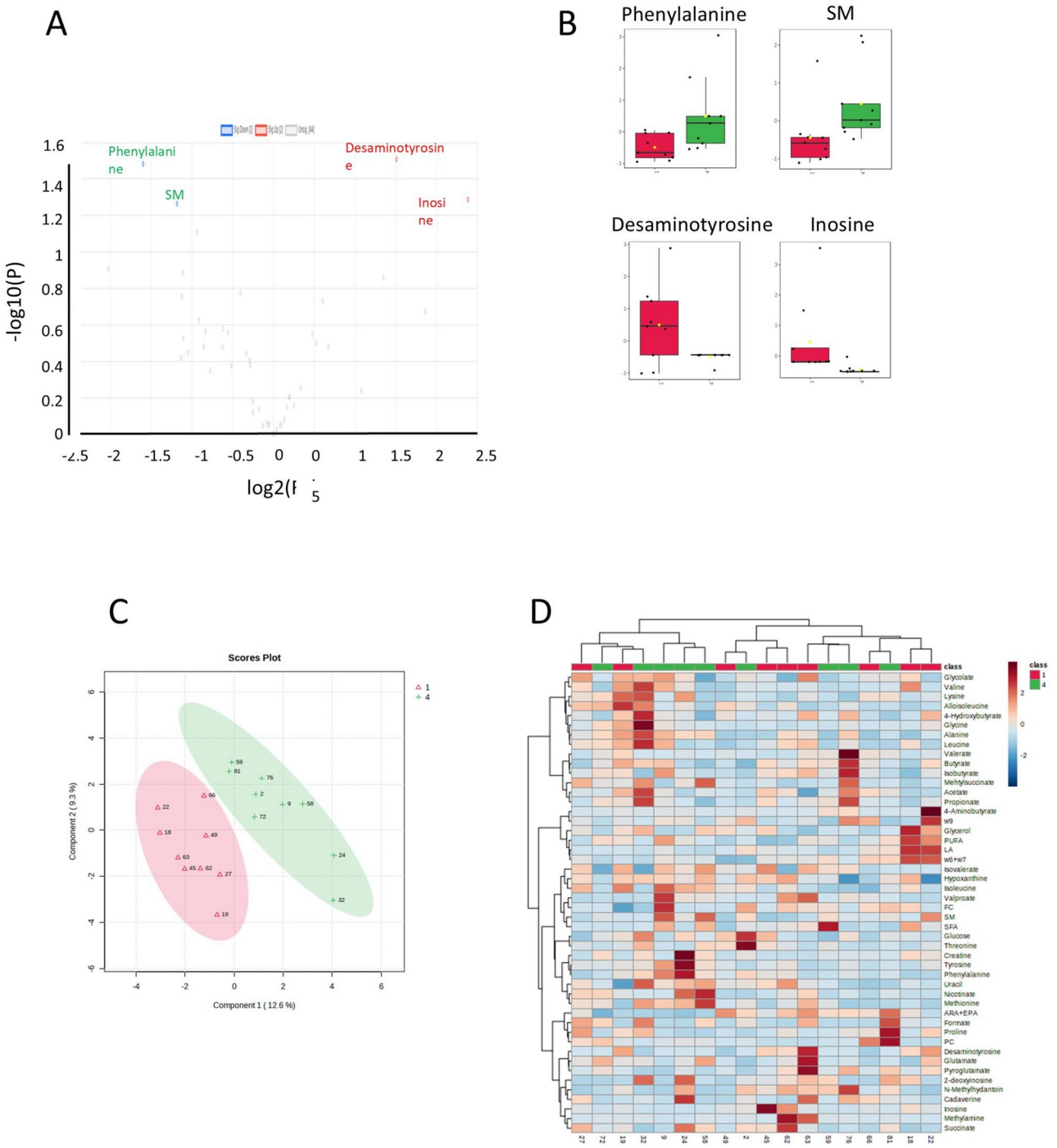


Fig. 5. Metabolomic analysis considering groups CTR and LCMR. (**A**) Volcano plot indicating the upregulated and downregulated metabolites in the comparison between CTR and LCMR groups. (**B**) Box plots showing the distribution of normalized values between the CTR (red) and LCMR (green) groups. (**C**) PLS-DA showing the separation between the CTR (red) and LCMR (green) groups. (**D**) Hierarchical cluster heatmap indicating the clustering between samples corresponding to CTR (red) and LCMR (green) groups.

metabolism) and organic acid metabolism (Butanoate metabolism, Glyoxylate and dicarboxylate metabolism, Pyruvate metabolism, Propanoate metabolism) (Supplementary Fig. S2).

It is interesting to note that the calf fecal metabolome seems to have some differences from the adult metabolome. In fecal samples from adult dairy cows, Kim et al.[37] described abundance of several organic acids (formate, acetate, propionate, butyrate and valerate, and also isobutyrate and isovalerate) similar to our results in calves, but also the presence of many monosaccharides and derivatives, and almost no amino acids and no nitrogenous bases amongst the 30 most abundant polar metabolites. These differences may be explained because

the ruminal function and metabolism of adult cows is very different from neonatal calves: first, the calf rumen is not yet developed and its microbiota would be different; secondly, the main feed in unweaned calves is based on liquid feeding, directly transferred from the abomasum to the intestine, where it is digested in a way similar to monogastric species[38].

The differential analysis of the fecal metabolome between groups can provide potential biomarkers to assess health and management. The calf stage is the most critical period for cattle growth and development, with the survival and growth rates of calves directly influencing economic benefits. The gut microbiota is established during the first 7 weeks of life and this period, characterized by rapid structural and microbial changes in the digestive system, is crucial for calf health and weight gain[39].

In our study, the comparison of the CTR, LCMR and LCRS groups allowed us to assess the effect of colostrum/transport (CTR versus LC groups) as well as the effect of nutritional deficiency on the fecal metabolome (CTR and LCMR groups *versus* RS). Our results have shown that most of the differences are found between the LCRS group and the other two (CTR and LCMR), indicating that the severity of feed restriction has a more important effect at this age (around 19 days-old when fecal samples were collected) than transport or colostrum uptake, as also indicated by fecal lactoferrin. It is interesting to note that interindividual variability was higher in the treated groups. This is not uncommon since individuals may vary in their response to a particular challenge. All potential factors that may affect the results of our work (diet, housing, genetics, weather season, etc.) have been kept constant for all calves to reduce this variability.

The most affected group of metabolites were amino acids. Three of them were increased in the LCRS group (proline, glycine, leucine), and others were also found at higher concentrations in this group (glutamate, threonine, valine and isoleucine). Glycine and proline are the main amino acids in collagen I, one of the main components of the connective tissue, and in collagen IV, one of the main components of the intestine basal membrane which separates the epithelial layer from the *lamina propia*, and which may function as an intestinal barrier to absorption[40]. In collagen I and IV, one third of the amino acids are glycine and there is a great abundance of proline and hydroxyproline[41]. On the other hand, leucine, valine and isoleucine are the three BCAAs, and they are metabolically related to threonine, which is the precursor of isoleucine. Other compounds are derived from amino acids: Creatine is formed from glycine and arginine and intracellular creatine has been recently related to intestinal gut health[42]. Cadaverine, which is also increased in the LCRS group (not significant), is also related to amino acid metabolism since it is produced by decarboxylation of lysine (https://metacyc.org/).

The origin of these amino acids, which are increased in the LCRS group compared to CTR and LCMR groups, could be the microbiota metabolism, which would synthesize more amino acids to compensate the lack of milk proteins in the diet (since these animals were fed only RS). Another possibility to explain the higher concentration of amino acids in the LCRS group could be the damage of the intestinal mucosa, which could then release tissue components to the intestinal content. As described above, our previous results[15] indicated that the LCRS group showed higher serum Cr-EDTA concentration and lower concentration of serum citrulline than the other two groups and, in consequence, a damage of the mucosa could explain the presence of increased concentrations of Gly and Pro in the LCRS group. The possibility of both sources (microbial and from the host) being true is also feasible. Proteolytic degradation of microbial or host proteins may also contribute to the origin of amino acids.

The results described herein agree with the study performed in young female Holstein dairy calves comparing the fecal microbiome and the fecal metabolome between two groups: healthy and "diarrhea" groups. The altered metabolites were similar to those described herein: amino acids (35%), SCFAs (23%), fatty acids (22%), and carbohydrates (11%) and could separate the groups in a PLS-DA analysis. According to the markedly altered metabolites, enriched KEGG pathway analyses were also similar to the ones described here: pyruvate metabolism; valine, leucine, and isoleucine biosynthesis; tricarboxylic acid (TCA) cycle; glycolysis or gluconeogenesis; propanoate metabolism, and a-linoleic acid metabolism. Acetate, butyrate and isovalerate were more prevalent in the healthy fecal metabolome of neonatal calves, while lactic acid was more prevalent in the diarrheal metabolome[33].

The results presented here are also in concordance with the study on fecal microbiota transplantation (FMT), which has been attempted for ameliorating calf diarrhea[43]. In this study, the fecal metabolome was analyzed before and after 54 days of treatment. The authors found a marked abundance of fecal amino acids (alanine, BCAAs, glycine, arginine, ornithine, and glutamic acid) in calves with diarrhea. FMT treatment causes a reduction in fecal amino acids that strongly correlate with the remission of diarrhea. The conclusion was that the remission of diarrhea in FMT-calves is accompanied by changes in their metabolomes, and in particular by decreases in amino acid concentrations, which are concomitant with changes in the gut microbiome.

SCFAs are the other group of compounds clearly present in the metabolomic analysis. As stated before, acetate, propionate and butyrate are amongst the most abundant metabolites in feces in our work as in others[33,34]. In our experiment, butyrate is lower in the feces of the LCRS group. On the contrary, formate, the one-carbon organic acid, is greatly increased in feces of the LCRS group. Acetate, propionate and butyrate are absorbed from the gut into the plasma and used as energy substrates. Especially butyrate exerts beneficial effects in maintaining intestinal and immune homeostasis since it inhibits the genotoxic activity of nitrosamides and hydrogen peroxide, it has immunoregulatory effects, and it plays a role in the prevention and treatment of inflammatory diseases as ulcerative colitis and Crohn's disease[34]. On the contrary, formate does not seem to have any beneficial effect: it is not an energy substrate and although relatively little is known about its function in the intestine, there are indications that its concentration may be elevated during inflammation[44]. In consequence, high amounts of formate in the LCRS group compared to CTR and LCMR cows may indicate a higher degree of inflammation in food-deprived individuals. A special mention is deserved to lactate: only four fecal samples had detectable amounts of lactate, but all of them corresponded to the LCRS group, whereas this metabolite was undetectable in all the individuals from the CTR and the LCMR groups (the individual in the CTR group shown

in Table 2 was actually a non-accessible value). In the same study described above in calves with diarrhea[33], lactate was abundant in the diarrheal groups, which can be attributed to the prominent contribution of hindgut fermentation in metabolizable energy supply during the first weeks after birth.

Finally, the nitrogenous bases uracil and hypoxanthine were found at lower concentrations in the LCRS group. Uracil is one of the pyrimidine bases components of RNA and hypoxanthine is an intermediate in the degradation pathway of purines (nucleic acid components) to uric acid. The lower amount of these compounds in the LCRS group may indicate a lower proliferation rate of microorganisms and/or cells from the host.

Regarding the differential non-polar metabolites, SM was at higher concentration in the LCMR group whereas and ARA + EPA were higher in the CTR group. Many alterations in non-polar metabolites have been identified in feces of patients with IBD, including arachidonic acid, phospholipids and long-chain fatty acids derivatives[19]. Arachidonic acid-derived eicosanoids are a complex family of lipid mediators that can regulate inflammatory responses[45]. Eicosapentanoic acid is an ω3-fatty acid with anti-inflammatory properties. The impossibility to separate these compounds in the spectral analysis makes it difficult to interpret the results. The source of these compounds in feces is unknown, but these metabolites may be obtained from food or by denaturation and elongation of essential fatty acids[46]. In calves, there is very scarce information about fecal non-polar metabolites, although it has been described that the plasma concentrations of most of the sphingomyelins (SMs) were lower in early weaned calves compared to late weaning[24].

The present study is one of the first to study the fecal calf metabolome. As far as to our knowledge, there are three other reports addressed to analyze changes in the metabolome, in all cases associated with weaning[36,39,47].

It is also interesting to compare our results with those obtained from the fecal metabolomic analysis in IBD. There are some similarities between human IBD and gut inflammation in calves, which deserve to be further explored in the future. Amino acids have been found to be increased in IBD[19]. It is suggested that, due to inflammation, the epithelial barrier protection is disrupted leading to the malabsorption of nutrients in the gut and resulting in increased levels of amino acids in feces of IBD patients. As stated above, a similar mechanism may happen in young calves suffering from intestinal disorders associated to management and transport. SCFAs (butyrate, propionate, acetate) are decreased in IBD- derived fecal samples[7,19]. Likewise, changes in plasma lipids with a dysregulation of arachidonic acid, glycerophospholipid and sphingolipid pathways have been reported in patients with IBD[7,48]. It is interesting to note that the pathway enrichment analysis regarding the disease signature specific for polar fecal metabolites gave an enrichment of compounds related to intestinal disorders: irritable bowel syndrome, diverticulosis, Crohn's disease, ulcerative colitis (both diseases usually included in IBD), and diseases with an immune/infectious component, such as Myalgic Encephalomyelitis (Supplementary Fig. S3).

In conclusion, our results show that fecal lactoferrin could be a good biomarker for intestinal inflammatory processes, although further studies should be performed to analyze the relationship with the degree of inflammation and the influence of colostrum uptake. Furthermore, the study of the metabolome allows to identify specific changes in the fecal composition due to the calf management and nutrition. In our experimental design performed in young calves (≈ 14–19 days old), colostrum uptake and road transport did not have major effects on the fecal metabolome, whereas feeding MR or RS had important consequences in several compounds. These differential compounds were mainly butyrate, which was downregulated, and formate and proline (with other amino acids and amino acid derivatives), which were upregulated in food-restricted calves. The probable sources of these metabolites are the gut microbiota and/or the release from the damaged intestinal mucosa. This is a small exploratory study that provided preliminary but interesting results, which deserve to be validated in future studies with larger sample sizes, in order to propose robust fecal biomarkers to assess calf intestinal health. An extension of the present study is underway in our laboratory to correlate these changes with modifications in the gut microbiota and in gut morphology and functionality. Taken together, all these data will provide insight into the biological relevance of our findings.

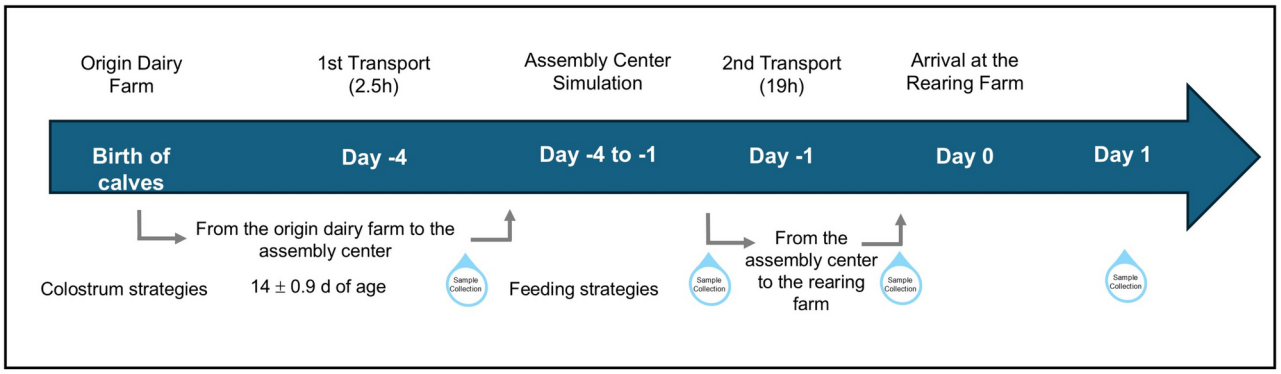
Methods

Experimental design and animal management

This study was approved by the Animal Care Committee of Generalitat de Catalunya (Barcelona, Spain; RD 53/2013; project no. 11211).

For this study, a total of 82 male Holstein calves (42 ± 1.2 kg of BW and 14 ± 0.9 d of age; mean ± standard error) born at a commercial dairy farm (Granja Selergan S.A., Lleida, Spain) were used. These animals were previously studied[15], and calves corresponding to the control group (CTR) and the two groups that received low-colostrum treatment were selected for the study since dietary treatment appeared to be more important than colostrum uptake at birth (n = 49). The experimental design is shown in Fig. 6. Calves in the CTR treatment received 4 L of colostrum within the first 2 h after birth, and 2 L of colostrum in the next 3 feedings (a total of 10 L) within the first 24 h after birth. Calves in the low-colostrum (LC) treatment received only 2 L of colostrum within the first 2 h after birth[49]. Calves were balanced by birth body weight, cow parity (primiparous or multiparous), and birth time (day or night). Only high-quality colostrum (Supplementary Table S2) was used and administered via esophageal tube.

After colostrum consumption, calves were allocated in individual hutches and received 2 L of milk replacer (MR) at a concentration of 125 g/L twice daily. Approximately two weeks after birth, calves were transported during 2.5 h to an experimental research unit located at IRTA (Torre Marimon, Caldes de Montbui, Spain). This trip was intended to mimic the typical transportation of calves from their origin dairy farms to the assembly centers or auction markets. At arrival, the effect of the feed restriction normally suffered at an assembly center was applied during 3 days (from d − 4 to d − 1 of the study). During this period calves were divided into three groups: the control group (CTR) did not suffer any nutritional or transportation challenge and was fed 2.5 L of MR at a concentration of 125 g/L twice daily and had ad-libitum access to a pellet starter feed and water;



Treatment	Colostrum Strategies		Feeding Strategies (d -4 to -1)			19h transportation (d -1)		Lactoferrin fecal sample	Metabolomics fecal sample
	LC (2L)	HC (10L)	Milk Replacer (MR)	Rehydrate solution (RS)	Concentrate	Yes	No		
CTR		X	X		X		X	X	X
LCMR	X		X			X		X	X
LCRS	X			X		X		X	X

Fig. 6. Experimental design describing the timeline of the calf management process and treatments. *CTR* control group, *LCMR* low-colostrum/milk replacer, *LCRS* low-colostrum/rehydrate solution.

moderately restricted calves (LCMR) were only fed 2.5 L of MR at a concentration of 125 g/L twice daily (MR); and severely restricted calves (LCRS) were only fed 2.5 L of a rehydrating solution (RS) at a concentration of 60 g/L twice daily (RS). Treatment compositions are shown in Supplementary Table S2.

Therefore, the final treatments were as follows: (a) control group (CTR): high-colostrum, no transport, MR (n = 16); (b) LCRM group: low-colostrum, transport, MR (n = 17); and (c) LCRS group: low-colostrum, transport, RS (n = 16). After the 3-days at the assembly center simulation (d − 1), LCMR and LCRS were transported by road for 19 h. CTR calves stayed at the experimental research unit with access to MR, feed, and water. The long-distance transportation was intended to simulate an international purchase of male dairy calves. During the 19 h trip, calves had access to water inside the truck during the 1 h rest stop after 9 h of transport, following the regulations from the European Commission for the transport of unweaned calves (European Council, 2005). After transportation, all calves were fed 2.5 L of MR at a concentration of 125 g/L as fed twice daily and had ad-libitum access to the same pellet starter feed used for the CTR calves during the assembly center simulation period in addition to straw and water.

The effects of colostrum consumption, feed restriction and long distance transport on performance, gut functionality, health status and behavior have been previously described[15].

Lactoferrin: sample preparation and quantification by ELISA

Fecal samples were collected on 4 different days: 3 days before transport to the rearing facilities (d − 4); the day of transport (d − 1); the day of the arrival to the rearing facilities (d 0); and the first day in the rearing facilities (d 1). Calf feces were collected via rectal stimulation and stored at − 80 °C.

On the day of extraction, samples were thawed at room temperature, and a 1:100 dilution was prepared by weighting 50 mg of feces in 5 ml of Buffer A (0.37 M NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, pH 7.4) and protease inhibitors (cOmplete, EDTA-free Protease Inhibitor Cocktail 50x, Roche, Germany). Tubes were vigorously vortexed, centrifuged and a second extraction was performed with the same conditions. The extracts were combined and stored at − 80 °C. To obtain the fecal dry matter weight, the organic samples were placed in aluminium molds, weighed, placed in an oven at 105 °C for 24 h and weighed again.

Lactoferrin was measured with the commercial Bovine Lactoferrin ELISA Kit, a sandwich-type ELISA for the detection of bovine lactoferrin in milk (E11-126, Bethyl Laboratories) validated for its use in bovine feces[50] and optimized for calf samples in our laboratory.

Sample preparation for metabolomics

Fecal samples from CTR (n = 9), LCMR (n = 9) and LCRS (n = 8) groups collected at d 1 were randomly selected for metabolomic profiling since the main differences for lactoferrin were observed at this day. Fecal material was shipped on dry ice to Biosfer Teslab (Reus, Spain) for the whole NMR analysis.

Lipophilic and aqueous extracts were obtained from 25 mg (dry weight) of fecal material using the BUME method[51] with slight modifications[52]. Three extraction cycles were performed in order to maximize lipid extraction: In the first cycle, 500 µL of a butanol:methanol (3:1) mix, 500 µL of a di-isopropyl ether:ethyl acetate (3:1) mix and 500 µL of D_2O were added. In the second and the third cycle, only 500 µL of a di-isopropyl ether:ethyl acetate (3:1) mix was added. The lipophilic and aqueous extracts were recovered and completely dried in Speedvac until evaporation of organic solvents and frozen at − 80 °C until ^{1}H-NMR analysis. Aqueous and lipid extracts were reconstituted in deuterated solutions and transferred into 5-mm NMR glass tubes: 45 mM PBS containing 2.32 mM of Trimethylsilylpropanoic acid (TSP) for aqueous extracts, and a solution of $CDCl_3$:CD_3OD:D_2O (16:7:1, v/v/v) containing Tetramethylsilane (TMS) for lipid extracts.

Nuclear Magnetic Resonance (^{1}H-NMR)

^{1}H-NMR spectra were recorded operating at a proton frequency of 600.20 MHz using an Avance III-600 Bruker spectrometer. One-dimensional ^{1}H pulse experiments were carried out at 300 K using the nuclear Overhauser effect spectroscopy (NOESY)-pre-saturation sequence to characterize small molecules such as amino acids and sugars from the aqueous extracts as previously reported[53]; and a 90° pulse operating at 286 K with water pre-saturation sequence (ZGPR) was used for lipid extracts analysis[54]. The acquired spectra were phased, baseline-corrected and referenced before performing the automatic metabolite profiling of the spectra dataset[52].

Quantification of lipid signals in ^{1}H-NMR spectra was carried out with LipSpin[55], an in-house software based on Matlab (MATLAB 2014a, The MathWorks, Natick, 2014).

Statistical analysis

Lactoferrin

Fecal lactoferrin was analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc.) with repeated measures; treatment, time and its interaction were the main effects and pen was a random effect. Fecal lactoferrin concentration was log-transformed to achieve normal distribution. The compound symmetry covariance structure and the first-order autoregressive covariance structure were tested according to the time points. Kenward–Roger degrees of freedom were used based on the lower Bayesian information criterion value[15].

Metabolomics

Polar and non-polar fecal metabolites with more than 50% of under detection limit values or non-accessible values were categorized to a binary classification according to concentration detection or not detection and analyzed using a Chi-square test using the FREQ procedure of SAS (version 9.4, SAS Institute Inc.). Otherwise, concentration of polar and non-polar fecal metabolite values under the detection limit were calculated based on half of the minimum value of their treatment group; and concentration of polar and non-polar fecal metabolite values that were not accessible were calculated based on the average of their treatment group. Therefore, polar and non-polar fecal metabolites with less than 50% of under detection limit values or non-accessible values were analyzed with a non-parametric Kruskal–Wallis test using the NPAR1WAY procedure of SAS (version 9.4, SAS Institute Inc.).

The multivariate and statistical analysis of the metabolome data was performed in MetaboAnalyst 5.0, an opensource R-based program for metabolomics[56]. The data containing the absolute concentrations of compounds was normalized before analysis using the auto scaling method. The maximum separation between groups was explained based on supervised partial least squares-discriminant analysis (PLS-DA). The quality of the PLS-DA models was assessed using Q2 as performance measure and five fold cross-validation method. Q2 indicates the predictive ability of the model, with high Q2 means good prediction and negative Q2 means overfitting of the model[57]. To visualize differences between groups, a hierarchical clustering heatmap was generated from normalized metabolite concentrations. Pairwise distances between metabolic compounds were calculated based on Pearson distance measure and ward clustering method.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

AB: conception and design, data interpretation, manuscript writing; NA: work design, data acquisition and analysis, software development, manuscript revision; MPR: data acquisition; YS: data acquisition; RP: data acquisition; RP: data acquisition; LP: data acquisition and analysis; MD: conception and design; data interpretation, manuscript revision; SM: conception and design, data interpretation, manuscript revision.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics declaration

All calves used in this study were managed following the principles and guidelines of the Animal Care Committee of Institut de Recerca i Tecnologia Agroalimentàries (Barcelona, Spain; RD 53/2013; project no. 11211).

Additional information

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