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Supplementation of essential fatty acids to Holstein calves during late uterine life and first month of life alters hepatic fatty acid profile and gene expression

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ABSTRACT

Linoleic acid is an essential dietary fatty acid (FA). However, how the supplementation of linoleic acid during uterine and early life may modify the FA profile and transcriptome regulation of the liver, and performance of preweaned dairy calves is unknown. Our objective was to evaluate the effect of supplementation of essential FA to Holstein calves during late uterine and early life on their hepatic FA profile and global gene expression at 30 d of age. During the last 8 wk of pregnancy, Holstein cattle (n = 96) were fed either no fat supplement (control), a saturated FA supplement enriched with C18:0, or an unsaturated FA supplement enriched with linoleic acid. Male calves (n = 40) born from these dams were fed a milk replacer (MR) with either low (LLA) or high linoleic acid (HLA) concentration as the sole feedstuff during the first 30 d. Liver biopsy was performed at 30 d of age, and microarray analysis was performed on 18 liver samples. Total concentration of FA in liver were greater in calves fed LLA compared with those fed HLA MR (8.2 vs. 7.1%), but plasma concentrations of total FA did not differ due to MR diets. The FA profiles of plasma and liver of calves were affected differently by the prepartum diets. Specifically, the FA profile in liver was affected moderately by the feeding of fat prepartum, but the profiles did not differ due to the type of FA fed prepartum. The type of MR fed during the first 30 d of life had major effects on both plasma and liver FA profiles, resembling the type of fat fed. Plasma and liver of calves fed LLA MR had greater percentage of medium-chain FA (C12:0 and C14:0), whereas plasma and liver from calves fed HLA MR had greater percentages of linoleic and α-linolenic acids. Dams fed fat or a specific type of FA modified the expression of some genes in liver of calves, particularly those genes involved in biological functions and pathways related to upregulation of lipid metabolism and downregulation of inflammatory responses. Feeding HLA instead of LLA MR modified the expression of hepatic genes, including genes predicted to decrease infections and to increase lipid utilization and protein synthesis. Research evaluating the effect of FA supplementation during uterine and neonatal life on the future productivity of the neonate is warranted.
Key words: gene expression, Holstein calf, linoleic acid, epigenetics

INTRODUCTION

Essentiality of linoleic acid was discovered by Burr and Burr (1930) when rats fed fat-free diets developed dermatitis, poor growth, poor reproduction, and death. Years later, α-linolenic acid also was recognized as an essential fatty acid (**FA**) due to the role of its derivative, C22:6n-3, in brain development and function (Neuringer et al., 1988; Anderson and Connor, 1989). Supplementation of essential FA during uterine and early life to improve performance and health of preweaned calves has been a research focus of our laboratory (Garcia et al., 2014a,b, 2015). Previously, we reported that linoleic and α-linolenic acids fed to preweaned calves improved their performance and enhanced a proinflammatory response by their immune cells. Moreover, as previously reported (Jenkins and Kramer, 1986), percentages of essential FA in plasma and liver reflected the FA composition of the milk replacer (**MR**) fed to preweaned calves (Garcia et al., 2014b, 2015), as well as affecting the percentage of other longer-chain FA. Therefore, strategic supplementation of FA was expected to modify the FA profile of different tissues and, by either direct or indirect effects, alter cell function.

The liver plays a key role in nutrient metabolism and utilization due to its strategic position of integration within the circulatory system. At the cellular level, the first recognized role of FA, stored as structural components of plasma phospholipids, has been to serve as precursors to signaling molecules such as eicosanoids

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(Wolfe, 1982). The discovery of the ability of FA to bind nuclear receptors and to suppress the activity of several transcription factors improved the understanding of the mechanisms by which FA have a direct role to alter the expression of target genes (Jump et al., 2013). Later, human and mouse studies revealed that the transcription factor class, peroxisome proliferator activated receptor (**PPAR**), is a master regulator of metabolism and inflammation, and that PPAR-α is the predominant PPAR subtype present in liver (Varga et al., 2011). In nonruminants, linoleic, α-linolenic, C20:4n-6, C20:5n-3, and C22:6n-3 were reported as the most potent ligands and activators of PPAR-α (Forman et al., 1997); however, C16:0 and C18:1 were reported as the most potent activators of PPAR-α in ruminants based upon indirect measures of gene expression (Bionaz et al., 2012).

Supplementation of PUFA has become an important dietary strategy to reduce hepatic lipogenesis in rodents and humans by inducing the regulation of gene expression. Clarke (2000) and Sampath and Ntambi (2005) reviewed studies supplementing n-3 and n-6 FA and concluded that n-3 FA were more potent activators of genes involved in lipid oxidation, more potent reducers of genes encoding enzymes for lipid synthesis, and important regulators of immune responses. Therefore, our objective was to evaluate the supplementation of essential FA to male Holstein calves during late uterine and early life on their hepatic FA profile and global gene expression.

MATERIALS AND METHODS

Prepartum Management

The present study was part of a larger research project, with portions of the results presented elsewhere (Garcia et al., 2014a,b). The experiment was approved by the University of Florida Institutional Animal Care and Use Committee. Briefly, a weekly cohort of pregnant nulliparous (n = 35) and previously parous (n = 61) Holstein cattle were blocked by parity and BCS and enrolled in the study starting at 8 wk before their calculated parturition date. The basal prepartum diet was formulated to have low concentrations of total FA (1.87% of DM), linoleic acid (0.38% of DM), and α-linolenic acid (0.20% of DM). Prepartum cattle were fed 1 of the following 3 supplements: (1) no-fat supplement (control), (2) a high-SFA supplement fed at 1.7% of dietary DM (Energy Booster 100, Milk Specialties Co., Dundee, IL), or (3) Ca salts of FA enriched with essential FA fed at 2.0% of dietary DM (**EFA**; Megalac-R, Church & Dwight Co. Inc., Princeton, NJ). The diets containing SFA and EFA supplements were isocaloric and greater in energy concentration than that of the control diet. All diets were isonitrogenous. Ingredient and chemical composition of the prepartum diets and specific details on prepartum feeding and management were reported previously (Garcia et al., 2014a).

Preweaning Diets and Management of Calves

Birth, housing, and feeding management of calves are described in Garcia et al. (2014b). Briefly, male calves (n = 40) were blocked by dam diet and assigned randomly to receive a MR (Land O'Lakes, Webster City, IA) containing concentrations of low linoleic acid (**LLA**; 0.48% linoleic acid, dietary DM basis or 2.9% linoleic acid, FA basis; Table 1) or high linoleic acid (**HLA**; 1.54% linoleic acid, dietary DM basis or 9.0%, FA basis) for 30 d starting at birth. Hydrogenated coconut oil (**CCO**) was the sole fat source in the LLA MR, whereas a mixture of CCO and porcine lard were the fat sources in the HLA MR. Milk replacer was the only feed for the first 30 d of calf life and was fed twice daily (0600 and 1230 h). Details of MR composition were described in Garcia et al. (2014b). Likewise, procedures for hormonal, metabolic, and immune assays for calves were reported previously (Garcia et al. 2014b).

Liver Biopsy and FA Profile Determination

Liver biopsies were performed in all male calves (n = 40) at 30 ± 2 d, but adequate amounts of liver samples were only obtained from 31 calves. Liver biopsies were not taken earlier than 30 d of life to minimize risks to calf health and to prevent trauma caused by the procedure from influencing the effect of dietary treatments on the calf immune system. Biopsy procedure was reported previously (Garcia et al., 2015); biopsied calves were monitored for postsurgical behavior for the first 12 h after biopsy. The sampled liver was stored at −80°C. A liver sample (~250 mg) per each calf was freeze-dried for 48 h (Labconco, Kansas City, MO) and delivered to Michigan State University for analysis of FA profile. Liver lipids were extracted using the method of Bligh and Dyer (1959). One milligram of *cis*-10 C17:1 (1:1 mg/mL of toluene) was added as the internal standard to freeze-dried liver samples. Fatty acid methyl esters were prepared by a modification of the 2-step procedure of Nuernberg et al. (2007) using a combination of the base-catalyzed (sodium methoxide) followed by acid-catalyzed (BF_3 in MeOH). Fatty acid methyl esters were quantified using a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a split injector (1:100 split ratio) and a flame

Table 1. Fatty acid (FA) profile of prepartum diets and milk replacers

FA, g/100 g of total FA	Prepartum diets[1]			Milk replacer[2]	
	Control	SFA	EFA	LLA	HLA
C8:0	ND[3]	0.4	ND	8.5	6.2
C10:0	ND	0.2	ND	6.1	4.5
C12:0	ND	0.3	0.2	42.5	29.9
C14:0	0.4	2.4	0.5	15.9	11.9
C16:0	19.9	27.6	26.9	10.5	14.6
C16:1	0.3	0.1	0.2	0.3	0.7
C17:0	0.3	1.0	0.2	ND	ND
C18:0	3.4	25.1	3.9	4.4	6.7
C18:1	37.7	19.9	32.4	8.8	15.7
C18:2n-6	20.5	10.9	23.8	2.9	9.0
C18:3n-3	11.0	6.0	7.0	ND	0.8
C20:0	1.0	0.9	0.7	ND	ND
Others	5.5	5.2	4.2	0	0

[1]Control = no fat supplement; SFA = saturated fatty acid supplement (Energy Booster 100, Milk Specialties, Dundee, IL); EFA = essential fatty acid supplement (Megalac-R, Church & Dwight, Princeton, NJ).

[2]Milk replacers are defined as low linoleic acid (LLA) and high linoleic acid (HLA).

[3]ND = not detected.

ionization detector using a CP-Sil 88 wall-coated open tubular-fused silica column (100 m × 0.25-mm i.d. × 0.2-μm film thickness; Varian Inc., Lake Forest, CA). Analytical conditions and FAME standards were the same as described previously (Lock et al., 2013).

RNA Isolation, Array Hybridization, Washing, Staining, and Scanning

Total RNA was isolated from liver samples (n = 31), previously stored at −80°C, using Qiazol reagent (Qiagen, Valencia, CA) and purified according to the manufacturer's recommendation (RNA MIDI isolation kit, Qiagen). A subset of 18 calves, whose liver RNA was verified to be of high purity (absorbance 280/260 ratio of 1.96; NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA) and had high-quality RNA (>7.5 RNA Integrity Number; Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA), was selected for microarray analysis. This subset included 3 calves from each of the 6 treatments. Calves that deviated from the mean ± 1 standard deviation of their respective group based on ADG and plasma concentrations of glucose and IGF-I were not included in the subset to minimize the risk of potential outliers and to accommodate the number of available budgeted microarrays. The RNA samples were submitted for fragmentation and hybridization following Affymetrix's protocol (Affymetrix GeneChip Bovine Genome Array, Affymetrix Inc., Santa Clara, CA). Arrays were washed and stained on a fluidics station 450 (Affymetrix Inc.); fluorescent signals were measured with the Affymetrix GeneChip scanner 3000 7G.

Affymetrix Data Analysis and Mining

The Affymetrix CEL files were loaded into an AffyBatch object using R Bioconductor environment (Gentleman et al., 2004). Array quality was confirmed by evaluation of quality control using the Array Analysis (www.arrayanalysis.org) pipeline. Data normalization and background correction were performed using guanine-cytosine robust multichip average function as described by Wu et al. (2004). Before analysis, all Affymetrix control probes (AFFX prefix) were removed and sets with an absent call (Affimetrix Microarray Suite Version 5.0) in at least 1 of the 18 samples were excluded. Differentially expressed genes (**DEG**) were identified using linear models for microarray (Smyth, 2005) using the method of Benjamini and Hochberg (1995) to adjust for multiple tests and to control for false discovery rate using an adjusted $P \leq 0.05$ as a cutoff. An additional cutoff was a log2 ratio $\geq|0.485|$ (equal to fold change $\geq$1.4). Treatments were arranged in a 3 (dam diets) × 2 (MR) factorial. The statistical model included the main effect of dam diet, MR, and their interactions. Three contrasts were evaluated: (1) contrast fat (**FAT**): dam diet (SFA + EFA)/2 ÷ control (reference); (2) contrast FA source (**FAS**): dam diet EFA ÷ SFA (reference); and (3) contrast MR: HLA ÷ LLA (reference).

Qiagen's Ingenuity Pathway Analysis (**IPA**, Qiagen; www.qiagen.com/ingenuity) was used to identify biological functions and canonical pathways within the list of DEG for each of the evaluated contrasts. A given biological function was considered to be enriched by a set of DEG associated with a particular biological

function (Abatangelo et al., 2009) if the Fisher's exact test *P*-value was ≤0.05 and the activation z-score was ≥0.5. A few biological functions with a z-score of <0.5 or without an assigned z-score, as recommended by IPA specialists, were discussed if considered of biological importance. Canonical pathways were considered to be enriched if the Fisher *P*-value was ≤0.05 and had a minimum ratio (number of DEG over the total number of genes involved in a given pathway) of 0.10 or if included at least 3 DEG. Regulator effect networks were selected using the criteria of a cutoff *P*-value of ≤0.05 and a z-score cutoff of ≥0.5 to identify upstream regulators and downstream diseases and functions, based on the set of DEG (See Supplemental Material for a more detailed criteria used for IPA analysis; http://dx.doi.org/10.3168/jds.2015-10472). A combined regulator network (including all regulators) was designed using IPA tools for the contrast of MR, as it was the only contrast involved in regulation of immune and metabolic functions.

Quantitative Reverse Transcription PCR Analysis and Validation of Microarray Results

Quantitative reverse transcription PCR (**qPCR**) was performed to verify the relative mRNA abundance of selected DEG from the microarray data analysis. Five genes were selected for qPCR validation and *GAPDH* was used as a reference gene. See Supplemental Tables S1 and S2 (http://dx.doi.org/10.3168/jds.2015-10472) for further details. The qPCR results were analyzed using the $2^{-(\Delta\Delta)Cq}$ method (Livak and Schmittgen, 2001).

Statistical Analyses

Total FA and FA composition of liver and plasma, as well as performance data of calves, were analyzed using the PROC MIXED procedure of SAS (release 9.2; SAS Institute, 2009) according to the following model: $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the observation; μ is overall mean, α_i is the fixed effect of dam diet (control, SFA, and EFA); β_j is the fixed effect of MR (LLA and HLA); $(\alpha\beta)_{ij}$ is the interaction of dam diet and MR; and ε_{ijk} is the residual error. Calf nested within dam diet and MR were used as random terms. For repeated measures, the model included the effect of age and its interactions.

The following orthogonal contrasts were performed: (1) FAT, dam diet of fat (SFA + EFA) versus no fat (control); (2) FAS, dam diet EFA versus SFA; and (3) MR, HLA versus LLA. These contrasts contained a minimum of 12 observations, or 6 per treatment, for the microarray data set. A *P*-value ≤0.05 was considered significant and a trend was considered when *P*-values were >0.05 but ≤0.10.

RESULTS

Plasma FA Profile

The FA profile of plasma for males calves (n = 37) at ~30 d of age is presented in Table 2. Percentage of C20:4n-6 increased ($P = 0.04$) in calves born from dams supplemented with fat compared with those born from dams fed the control diet (3.5 vs. 3.1%). Percentage of medium-chain FA (C14:0 and C16:0) tended to be greater ($P \leq 0.08$) in plasma of calves born from dams fed SFA instead of EFA supplement, which resulted in a greater percentage of total SFA in plasma of calves born from dams fed SFA instead of EFA supplement (35.4 vs. 33.6%). Percentage of total n-6 FA tended to increase ($P = 0.07$) in plasma of calves born from dams supplemented with EFA (51.1 vs. 48.7%).

The type of MR had the most substantial effect on plasma FA profile. Medium-chain SFA (C12:0, $P = 0.06$ and C14:0, $P < 0.01$; Table 2) and total SFA (35.9 vs. 33.5%, $P < 0.01$) were present in greater percentage in plasma of calves fed the LLA MR compared with calves fed HLA MR. Linoleic acid accounted for ~44% of the total FA in plasma, with calves fed HLA having a greater ($P = 0.01$) linoleic acid concentration compared with those fed LLA MR (46.1 vs. 42.7%). Percentage of α-linolenic acid was also greater ($P = 0.03$) in calves fed HLA instead of LLA MR (0.80 vs. 0.66%). Unexpectedly, FA derivatives from the n-6 family (C18:3n-6, C20:3n-6, and C20:4-n6) and the n-3 family (C20:5n-3) were or tended to be lower in calves fed HLA than LLA MR.

Liver FA Profile

The FA profile of liver from all enrolled male calves (n = 31) is presented in Table 3. Mean percentage of nearly every FA in calf liver was affected appreciably by MR, with minimal effects due to dam diet or the interaction of dam diet with MR. Liver of calves born from fat-supplemented dams instead of control dams had greater percentage of FA within the n-6 family (C20:4n-6, 10.8 vs. 10.0% of total FA, $P = 0.05$) and the n-3 family (C20:5n-3, 0.23 vs. 0.18%, $P < 0.01$; C22:5n-3, 1.87 vs. 1.70%, $P = 0.02$; and total n-3 FA, 4.75 vs. 4.38% of total FA, $P = 0.07$). However, percentage of C18:3n-3 was lower in liver of calves born from dams supplemented with fat (0.83 vs. 0.89%, $P = 0.05$). Calves fed the HLA MR had a lower ($P = 0.02$) mean concentration of total FA in liver (7.6 vs. 8.5% of total

DM; Table 3). Overall, feeding the HLA MR increased the total percentage of n-6 (37.9 vs. 31.1% of total FA, $P < 0.01$) and n-3 FA (5.1 vs. 4.2%, $P < 0.01$) and, as expected due to the FA in the MR, specifically increased linoleic (22.1 vs.15.9%, $P < 0.01$) and α-linolenic acids (1.0 vs. 0.7%, $P < 0.01$). Some FA derivatives (C20:2n-6, C22:5n-3, C20:4n-6 and C22:4n-6) also were increased, whereas percentage of other FA derivatives were decreased (C18:3n-6, C20:3n-6, and C20:5n-3) when more EFA were consumed. The increases in n-6 and n-3 FA were accompanied by decreases ($P \leq 0.02$) in medium-chain SFA and some MUFA (i.e., C12:0, C14:0, C16:0, C16:1 *cis*-9, and C18:1 *cis*-9). The sum of the SFA decreased ($P < 0.01$) from 45.1 to 40.0% and that of the MUFA decreased ($P < 0.01$) from 16.4 to 14.3%, with increasing intake of the longer-chain FA provided with the HLA MR.

Differential Expression of Genes in Liver

All DEG presented and discussed in this manuscript are listed in corresponding tables for each analyzed contrast. For further detail on the effect (log ratio) and probability (adjusted *P*-value) of the response of DEG to the effect of diets, the reader is referred to respective tables in the Supplemental Tables S2 to S11 (http://dx.doi.org/10.3168/jds.2015-10472). A total of 71, 143, and 57 genes were differentially expressed as tested by contrasts of FAT (SFA + EFA vs. control), FAS (EFA vs. SFA), and MR (HLA vs. LLA; Figure 1). These 3 treatment contrasts had 2 DEG in common, *IRF7* and *RRBP1*; these genes were downregulated in calves born from dams supplemented with fat compared with control (Supplemental Table S3; http://dx.doi.org/10.3168/jds.2015-10472) or with EFA compared with SFA (Supplemental Table S6; http://dx.doi.org/10.3168/jds.2015-10472) but were upregulated when calves were fed HLA compared with LLA MR (Supplemental Table S9; http://dx.doi.org/10.3168/jds.2015-10472). In addition, the contrasts of FAT and FAS had 4 DEG in common (Figure 1): *ANTXR2*, *IL8*, and *TMEM150C* were upregulated and *PSMF1* was downregulated (Supplemental Tables S3 and S6). The contrasts of FAT and MR shared 6

Table 2. Total fatty acids (FA; g/mL of plasma) and percentages of fatty acid (g of FA/100 g of total FA) in plasma of 30-d-old Holstein male calves (n = 37) fed milk replacer (MR) containing low linoleic acid (LLA) or high linoleic acid (HLA)

	Prepartum diets (DD)[1]				Milk replacer[2]			*P*-value[3]			
Item	Control	SFA	EFA	SEM	HLA	LLA	SEM	FAT	FAS	MR	DD × MR
No. of calves	12	9	16		18	19					
Total FA	2.13	1.99	2.03	0.12	1.98	2.12	0.10	0.43	0.78	0.36	0.68
C10:0	0.03	0.06	0.03	0.04	0.02	0.06	0.03	0.83	0.50	0.29	0.13
C12:0	0.72	0.73	0.55	0.11	0.54	0.79	0.09	0.55	0.26	0.06	0.97
C14:0	3.35	3.55	3.02	0.20	2.15	4.45	0.17	0.79	0.07	<0.01	0.56
C16:0	17.2	17.1	16.0	0.46	16.9	16.6	0.38	0.29	0.08	0.66	0.72
C16:1 *cis*-9	0.59	0.67	0.60	0.06	0.58	0.66	0.05	0.61	0.42	0.28	0.50
C17:0	0.30	0.30	0.32	0.03	0.32	0.30	0.02	0.78	0.63	0.54	0.33
C18:0	13.0	13.3	13.3	0.32	13.2	13.1	0.26	0.57	0.97	0.83	0.05
C18:1*cis*	11.0	11.0	10.3	0.52	10.5	11.0	0.43	0.60	0.33	0.46	0.44
C18:2n-6	44.0	43.7	45.7	1.00	46.1	42.7	0.82	0.60	0.16	0.01	0.45
C18:3n-6	0.20	0.20	0.20	0.03	0.13	0.28	0.03	0.94	0.99	<0.01	0.54
C18:3n-3	0.74	0.72	0.73	0.05	0.80	0.66	0.04	0.78	0.83	0.03	0.83
C20:2n-6	0.23	0.21	0.28	0.04	0.24	0.24	0.04	0.75	0.28	0.93	0.88
C20:3n-6	1.03	1.11	1.12	0.09	0.85	1.33	0.08	0.45	0.93	<0.01	0.36
C20:4n-6	3.11	3.34	3.60	0.13	3.21	3.49	0.11	0.04	0.18	0.08	0.42
C20:5n-3	0.09	0.09	0.11	0.02	0.06	0.13	0.02	0.78	0.57	0.01	0.59
C22:4n-6	0.18	0.18	0.27	0.04	0.19	0.22	0.03	0.33	0.07	0.45	0.78
C22:5n-3	0.33	0.25	0.37	0.02	0.32	0.31	0.02	0.47	<0.01	0.77	0.09
C22:6n-3	0.26	0.28	0.25	0.02	0.26	0.27	0.02	0.84	0.39	0.70	0.34
Σ Others	3.02	2.70	2.68	0.24	3.01	2.59	0.20	0.28	0.94	0.14	0.59
Σ SFA	35.1	35.4	33.6	0.50	33.5	35.9	0.41	0.39	0.02	<0.01	0.43
Σ MUFA *cis*	11.7	11.8	11.1	0.56	11.3	11.9	0.46	0.70	0.36	0.36	0.44
Σ n-3	1.42	1.34	1.46	0.05	1.44	1.37	0.04	0.72	0.11	0.31	0.52
Σ n-6	48.7	48.7	51.1	0.93	50.8	48.3	0.76	0.32	0.07	0.03	0.50

[1]Control = no fat supplement; SFA = SFA supplement, Energy Booster 100 (Milk Specialties, Dundee, IL); EFA = essential FA supplement, Megalac-R (Church & Dwight, Princeton, NJ).

[2]Milk replacer was the only feed for the first 30 d of life. Daily intakes of C18:2 from MR were 0.14 and 0.45 g of C18:2/kg of metabolic BW for LLA and HLA MR, respectively.

[3]*P*-values for orthogonal contrasts and interaction: FAT = control vs. (SFA + EFA); FA source (FAS) = EFA vs. SFA; MR = LLA vs. HLA; DD × MR = interaction of dam diet prepartum by milk replacer.

DEG (Figure 1; *AIM1*, *FAM189A2*, *CMTR2*, *GPT2*, *IRF3*, and *ZNFX1*), with only *AIM1* being regulated in the same direction (upregulated) for both contrasts (Supplemental Tables S3 and S9). Contrasts FA and MR had 4 DEG in common (Figure 1; i.e., *ARHGEF2*, *IFI35*, *PPM1K*, and *SLC51B*). Direction of regulation for each gene differed between contrasts (Supplemental Tables S6 and S9).

Effect of Fat Supplementation During the Prepartum Period

The top 5 upregulated genes in liver of calves born from dams supplemented with fat (SFA + EFA) compared with calves born from dams fed the control diet were *SFXN1*, *ACE2*, *PTPRD*, *UPB1*, and *ACADL* based upon the log ratio in descending order from 1.37 to 0.87 (Supplemental Table S3; http://dx.doi.org/10.3168/jds.2015-10472). From these 5 genes, only *ACADL* was part of an enriched biological function (accumulation of lipid; Table 4). The top 5 downregulated genes, in descending order of log ratio from −1.06 to −0.76, were *ZNFX1*, *CMTR2*, *IRF7*, *GNB4*, and *DTX3L* (Supplemental Table S3). Only *GNB4* was part of an enriched canonical pathway (Table 4).

Top Biological Functions. Table 4 summarizes the enriched biological functions according to the criteria described in Materials and Methods. Additional information such as category of each biological function and number of genes identified by IPA, such as predicting their effect in a given function, can be found in Supplemental Table S4 (http://dx.doi.org/10.3168/jds.2015-10472). Among the 10 listed biological functions, 8 are related to immune function and 2 are related to lipid metabolism (metabolism of terpenoids and accumulation of lipid). The top predicted biological effects

Table 3. Total fatty acids (FA; % of DM) and percentages of FA (g of FA/100 g of total FA) in liver of 30-d-old Holstein male calves (n = 31) fed milk replacer (MR) containing low linoleic acid (LLA) or high linoleic acid (HLA)

	Prepartum diets (DD)[1]				Milk replacer[2]			*P*-value[3]			
Item	Control	SFA	EFA	SEM	HLA	LLA	SEM	FAT	FAS	MR	DD × MR
No. of calves	11	9	11		14	17					
Total FA	7.63	8.23	8.19		7.56	8.47	0.25	0.13	0.94	0.02	0.23
C12:0	0.80	0.75	0.74	0.14	0.29	1.23	0.12	0.74	0.94	<0.01	0.96
C14:0	3.40	3.11	3.26	0.50	1.30	5.22	0.41	0.72	0.84	<0.01	0.98
C16:0	15.4	15.2	14.9	0.40	13.9	16.5	0.33	0.51	0.63	<0.01	0.90
C16:1 *cis*-9	0.46	0.44	0.41	0.03	0.39	0.48	0.02	0.26	0.53	0.02	0.99
C17:0	0.39	0.42	0.44	0.02	0.41	0.42	0.01	0.62	0.40	0.05	0.45
C18:0	21.6	22.8	22.9	0.67	23.5	21.3	0.54	0.13	0.95	0.01	0.96
C18:1 *cis*-9	11.7	11.2	10.7	0.42	10.3	12.1	0.35	0.17	0.48	<0.01	0.72
C18:1 *cis*-11	2.64	2.59	2.48	0.06	2.62	2.52	0.05	0.17	0.21	0.21	0.62
C18:2n-6	19.4	18.6	18.9	0.53	22.1	15.9	0.43	0.30	0.70	<0.01	0.21
C18:3n-6	0.05	0.04	0.04	0.01	0.03	0.07	0.01	0.62	0.97	<0.01	0.15
C18:3n-3	0.89	0.84	0.81	0.03	0.99	0.70	0.02	0.05	0.41	<0.01	0.11
C18:2 *cis*-9,*trans*-11	0.03	0.02	0.03	0.005	0.02	0.03	0.004	0.45	0.13	0.15	0.89
C20:2n-6	0.81	0.75	0.77	0.03	1.01	0.54	0.03	0.23	0.58	<0.01	0.75
C20:3n-6	2.83	3.13	3.13	0.20	2.70	3.36	0.16	0.22	1.00	0.01	0.12
C20:4n-6	10.0	10.5	11.0	0.30	10.8	10.2	0.25	0.05	0.24	0.09	0.52
C20:5n-3	0.18	0.24	0.22	0.01	0.19	0.24	0.01	<0.01	0.28	<0.01	<0.01
C22:4n-6	1.22	1.12	1.27	0.05	1.27	1.13	0.04	0.68	0.07	0.03	0.18
C22:5n-3	1.70	1.84	1.90	0.06	2.06	1.57	0.05	0.02	0.52	<0.01	0.89
C22:6n-3	1.56	1.95	1.62	0.12	1.78	1.63	0.09	0.13	0.06	0.27	0.08
Σ Others	4.50	3.92	3.92	0.34	3.78	4.46	0.27	0.13	0.98	0.07	0.38
Σ SFA	42.2	42.9	42.7	0.47	40.0	45.1	0.38	0.29	0.80	<0.01	0.95
Σ MUFA *cis*	16.0	15.3	14.7	0.56	14.3	16.4	0.46	0.14	0.46	<0.01	0.81
Σ C18:1 *trans*[4]	0.47	0.59	0.62	0.09	0.54	0.58	0.08	0.24	0.80	0.77	0.65
Σ n-3	4.38	4.91	4.58	0.16	5.08	4.17	0.13	0.07	0.17	<0.01	0.30
Σ n-6	34.3	34.1	35.1	0.73	37.9	31.1	0.60	0.73	0.36	<0.01	0.87

[1]Control = no fat supplement; SFA = SFA supplement, Energy Booster 100 (Milk Specialties, Dundee, IL); EFA = essential FA supplement, Megalac-R (Church & Dwight, Princeton, NJ).

[2]Milk replacer was the only feed for the first 30 d of life. Daily intakes of C18:2 from MR were 0.14 and 0.45 g of C18:2/kg of metabolic BW for LLA and HLA MR, respectively.

[3]*P*-values for orthogonal contrasts and interaction: FAT = control vs. (SFA + EFA); FA source (FAS) = EFA vs. SFA; MR = LLA vs. HLA; DD × MR = interaction of dam diet prepartum by milk replacer.

[4]Includes C18:1 *trans*-6–8, C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, and C18:1 *trans*-12. Each one was not affected by treatments.

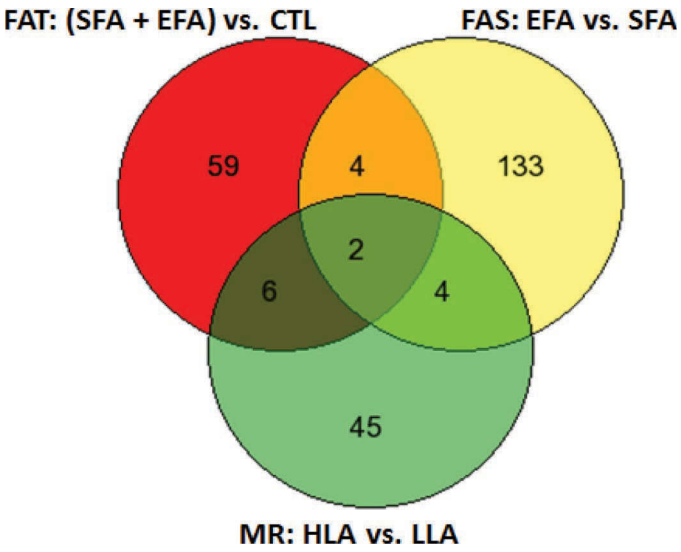

Figure 1. Venn diagram representing the number of differentially expressed genes, as identified by the Ingenuity pathway analysis (Qiagen, Valencia, CA), in liver of 30-d-old male calves fed milk replacer (MR) containing low (LLA) or high linoleic acid (HLA) and born from dams fed diets supplemented with no fat (CTL), SFA, or essential fatty acids (EFA) starting at 8 wk before expected calving date. FAS = fatty acid source. Color version available online.

(based on z-score values) were an increased infection of mammalia and increased metabolism of terpenoids for calves born to dams fed fat prepartum.

Top Canonical Pathways. Table 4 summarizes the most enriched canonical pathways considering the threshold criteria of Fisher's exact test P-value ≤ 0.05 and gene ratio ≥ 0.10 or minimum of 3 DEG. See Supplemental Table S5 (http://dx.doi.org/10.3168/jds.2015-10472) for details in ratio values. Six of the 11 listed canonical pathways are related to immune function, whereas the other 5 are related to metabolism of AA and FA. Based on the ratio value, the top pathways predicted to be enhanced were alanine degradation and biosynthesis and 4-hydroxyproline degradation, followed by predicted increase in L-carnitine biosynthesis. Contrarily, myo-inositol biosynthesis, activation of interferon regulatory factor (IRF) by cytosolic pattern of recognition receptors, and the role of retinoic acid-inducible gene-1 (RIG-I)-like receptors in antiviral innate immunity were predicted to be diminished.

Effect of Supplementing Essential FA Prepartum

Based upon the log ratio value, the top 5 upregulated genes in liver of calves born from dams supplemented with EFA compared with liver of calves born from dams supplemented with SFA were *RGS5*, *DUSP12*, *SFR1*, *KBTBD6*, and *UPP2*, in descending order of log ratio from 2.05 to 1.27 (Supplemental Table S6; http://dx.doi.org/10.3168/jds.2015-10472). The top 5 downregulated genes were *ISG15*, *IFI27*, *OAS1*, *ID1*, and *IRF7*, in descending order of log ratio from −3.02 to −1.04.

Top Biological Functions. Table 5 and Supplemental Table S7 (http://dx.doi.org/10.3168/jds.2015-10472) summarize the most enriched biological functions. Among the 5 listed biological functions, 3 fall into to the category of cell death and survival, 1 within the category of cardiovascular system development, and 1 in reproductive system development. The top predicted effects based upon activated z-score were decreases in death of brain cells and sperm disorder of calves born from dams supplemented with EFA compared with those born from SFA-supplemented dams.

Top Canonical Pathways. Table 5 and Supplemental Table S8 (http://dx.doi.org/10.3168/jds.2015-10472) summarize the most enriched canonical pathways. Out of the 8 enriched pathways, 3 are involved in decreased AA degradation (proline, 4-hydroxyproline, and threonine), 3 in vitamin metabolism (choline, flavin, and thiamin), 1 in the synthesis of nicotinamide adenine dinucleotide, and 1 in immunity [role of pattern recognition receptor (PRR) in recognition of bacteria and viruses]. Based on the ratio value, choline degradation and flavin biosynthesis were predicted to be enhanced in liver of calves born from dams supplemented with EFA compared with those calves born from dams supplemented with SFA. Contrarily, degradation of proline and hydroxyproline were the pathways with highest prediction to be diminished.

Effect of Feeding HLA MR

The top 5 upregulated genes in calves fed a HLA compared with calves fed a LLA MR were *ZNFX1*, *USP18*, *IRF7*, *CYP26A1*, and *ZFP36*, in descending order of log ratio from 1.22 to 1.02 (Supplemental Table S9; http://dx.doi.org/10.3168/jds.2015-10472). From these genes, all but *ZNFX1* were part of several enriched biological functions (Table 6). The top 5 downregulated genes were *ACMSD*, *SLC7A9*, *AAED1*, *PPM1K*, and *FAM189A2*, in descending order from −1.19 to −0.74. From these 5 genes, none was part of an enriched biological function and only *ACMSD* was part of an enriched canonical pathway (Table 6).

Top Biological Functions. Table 6 and Supplemental Table S10 (http://dx.doi.org/10.3168/jds.2015-10472) summarize the most enriched biological functions. Among the 14 listed biological functions, 7 are related to immune function, 3 to lipid metabolism, 3 to tissue and protein synthesis, and 1 is related to gene

Table 4. Biological functions and canonical pathways enriched in liver of Holstein male calves born from dams supplemented with saturated (n = 6) or essential fatty acids (n = 6) compared with those born from dams not supplemented with fat (n = 6)

Item	*P*-value[1]	Activation z-score[2]	Ratio[3]	Predicted effect[4]	No. of genes	Genes[5]
Biological function						
Infection of mammalia	0.04	2.17		↑	5	↑*ANTXR2*, ↓*IRF3*, ↓*IRF7*, ↓*MAVS*, ↓*STAT2*
Metabolism of terpenoids	0.03	1.94		↑	5	↑*ADH1C*, ↑*IL8*, ↑*HSD11B1*, ↑*LIPC*, ↑*PGRMC1*
Bacterial infection	0.02	1.00		↑	6	↑*ANTXR2*, ↓*CD22*, ↑*IL8*, ↓*ICAM1*, ↓*IRF3*, ↓*IRF7*
Morbidity or mortality	<0.01	0.94		↑	7	↑*ANTXR2*, ↓*ICAM1*, ↓*IRF3*, ↓*IRF7*, ↑*KCNK5*, ↓*MAVS*, ↓*STAT2*
Replication of RNA virus	0.04	0.84		↑	7	↓*APBB1IP*, ↑*IL8*, ↓*IRF3*, ↓*IRF7*, ↓*MAVS*, ↓*NMI*, ↓*STAT2*
Recruitment of antigen presenting cells	<0.01	−0.76		↓	4	↓*ICAM1*, ↑*IL8*, ↓*IRF3*, ↓*IRF7*
Immune response of cells	0.04	−0.58		↓	7	↓*CD22*, ↑*IL8*, ↓*ICAM1*, ↓*IRF3*, ↓*IRF7*, ↓*MAVS*, ↑*POSTN*
Recruitment of myeloid cells	0.03	−0.54		↓	4	↓*ICAM1*, ↑*IL8*, ↓*IRF3*, ↓*IRF7*
Accumulation of lipid	0.03	−0.39		↓	5	↑*ACADL*, ↑*FH*, ↓*ICAM1*, ↑*IL8*, ↑*SLC10A1*
Response of lymphocytes	0.02	−0.19		↓	4	↓*CD22*, ↑*IL8*, ↓*ICAM1*, ↓*POSTN*
Canonical pathway						
Alanine degradation III	0.01		0.50	↑	1	↑*GPT2*
Alanine biosynthesis II	0.01		0.50	↑	1	↑*GPT2*
4-Hydroxyproline degradation I	0.01		0.50	↑	1	↑*HOGA1*
L-Carnitine biosynthesis	0.02		0.33	↑	1	↑*ALDH9A1*
Myo-inositol biosynthesis	0.02		0.33	↓	1	↓*IMPA2*
Role of RIG1-like receptors in antiviral innate immunity[6]	<0.01		0.12	↓	3	↓*IRF7*, ↓*MAVS*, ↓*IRF3*
Activation of IRF by cytosolic PRR[7]	<0.01		0.09	↓	4	↓*IRF7*, ↓*MAVS*, ↓*STAT2*, ↓*IRF3*
Role of PRR in recognition of bacteria and viruses	<0.01		0.05	↓	3	↓*IRF7*, ↓*MAVS*, ↓*IRF3*
IL-8 Signaling	0.02		0.03	↓	4	↑*IL8*, ↓*GNB4*, ↓*ICAM1*, ↓*RHOJ*
Tec kinase signaling	0.04		0.03	↓	3	↓*GNB4*, ↓*RHOJ*, ↓*STAT2*
Dendritic cell maturation	0.04		0.03	↓	3	↓*ICAM1*, ↑*HLA-B*, ↓*STAT2*

[1]Calculated by the Ingenuity pathway system (Qiagen, Valencia, CA) using the tailed Fisher's exact test.

[2]Positive and negative values predict occurrence and not occurrence of the biological function, respectively. Missing value represents biological functions with a z-score not calculated by Ingenuity pathway system.

[3]Number of genes in a given pathway with respect to the total of genes in that pathway.

[4]Predicted effect for biological functions without z-score value were calculated using the number of genes expected to increase or decrease a given biological function.

[5]Upregulated (↑) and downregulated genes (↓).

[6]RIG-1 = retinoic acid-inducible gene-1.

[7]IRF = interferon regulatory factor; PRR = pattern recognition receptor.

Table 5. Biological functions and canonical pathways enriched in liver of Holstein male calves born from dams fed essential fatty acids (n = 6) compared with those born from dams supplemented with SFA (n = 6)

Item	*P*-value[1]	Activation z-score[2]	Ratio[3]	Predicted effect[4]	No. of genes	Genes[5]
Biological function						
Neovascularization of organ	0.03	0.70		↑	4	↓*IRS1*, ↑*KIT*, ↑*NRP1*, ↑*PLAT*
Cell death of brain cells	<0.01	−1.02		↓	9	↑*CDK1*, ↑*IL8*, ↑*GCLM*, ↑*HMGB2*, ↑*PAWR*, ↑*PIK3CA*, ↑*PLAT*, ↓*RASSF5*, ↑*RIT1*
Cell death of cerebral cortex cells	<0.01	−1.01		↓	8	↑*CDK1*, ↑*IL8*, ↑*GCLM*, ↑*HMGB2*, ↑*PAWR*, ↑*PIK3CA*, ↑*PLAT*, ↑*RIT1*
Cell death of cortical neurons	0.04	−0.95		↓	5	↑*CDK1*, ↑*GCLM*, ↑*HMGB2*, ↑*PIK3CA*, ↑*PLAT*
Sperm disorder	0.03	−0.95		↓	6	↓*EHD1*, ↑*FANCM*, ↑*GMCL1*, ↑*LRAT*, ↓*MAD2L2*, ↑*PPM1D*
Canonical pathway						
Choline degradation I	0.01		1.00	↑	1	↑*ALDH7A1*
Flavin biosynthesis IV	0.03		0.50	↑	1	↑*RFK*
NAD salvage pathway II	0.01		0.17	↑	2	↑*NMRK1*, ↑*NUDT12*
Role of PRR in recognition of bacteria and viruses[6]	0.02		0.06	↑	5	↑*IL8*, ↓*OAS1*, ↑*PIK3CA*, ↓*IRF7*, ↑*MBL2*
Proline degradation	0.03		1.00	↓	1	↓*ALDH4A1*
4-Hydroxyproline degradation I	0.03		0.50	↓	1	↓*ALDH4A1*
Threonine degradation II	0.03		0.50	↓	1	↓*GCAT*
Thiamin salvage III	0.01		0.20	↓	1	↓*TPK1*

[1]Calculated by the Ingenuity pathway system (Qiagen, Valencia, CA) using the tailed Fisher's exact test.

[2]Positive and negative values predict occurrence and not occurrence of the biological function, respectively. Missing value represents biological functions with a z-score not calculated by Ingenuity pathway system.

[3]Number of genes in a given pathway with respect to the total of genes in that pathway.

[4]Predicted effect for biological functions without z-score value were calculated using the number of genes expected to increase or decrease a given biological function.

[5]Upregulated (↑) and downregulated genes (↓).

[6]PRR = pattern recognition receptor.

Table 6. Biological functions and canonical pathways enriched in liver of Holstein male calves fed high linoleic acid (n = 9) compared with low linoleic acid (n = 9) milk replacer

Item	*P*-value[1]	Activation z-score[2]	Ratio[3]	Predicted effect[4]	No. of genes	Genes[5]
Biological function						
Metabolism of protein	0.05	1.98		↑	9	↓*APOM*, ↑*ARH*, ↓*GEF2*, ↓*ERRFI1*, ↓*GIMAP1-GIMAP5*, ↑*PPARA*, ↑*SLC51B*, ↓*SOD2*, ↑*USP18*, ↑*ZFP36*
Binding of DNA	0.05	1.57		↑	6	↑*CYP2E1*, ↑*HIVEP2*, ↑*IRF3*, ↑*IRF7*, ↑*PPARA*, ↓*SOD2*
Apoptosis of leukocytes	0.02	1.10		↑	6	↓*GIMAP1-GIMAP5*, ↑*IRF3*, ↓*MSH2*, ↑*PPARA*, ↓*SOD2*, ↑*THBS2*
Synthesis of protein	0.02	1.07		↑	6	↑*ARHGEF2*, ↓*ERRFI1*, ↓*GIMAP1-GIMAP5*, ↑*PPARA*, ↓*SOD2*, ↑*ZFP36*
Apoptosis of blood cells	0.01	0.69		↑	7	↓*GIMAP1-GIMAP5*, ↑*IRF3*, ↓*MSH2*, ↑*PALLD*, ↑*PPARA*, ↓*SOD2*, ↑*THBS2*
Formation of filaments	0.02	0.56		↑	6	↑*ARHGEF2*, ↑*FHL1*, ↑*PALLD*, ↓*SOD2*, ↑*TPM2*, ↑*TPPP*
Beta-oxidation of long chain fatty acid	<0.01			↑	2	↑*CYP2E1*, ↑*PPARA*
Generation of superoxide	<0.01			↑	3	↑*CYP2E1*, ↑*PPARA*, ↓*SOD2*
Peroxidation of lipid	<0.01			↑	3	↑*CYP2E1*, ↑*PPARA*, ↓*SOD2*
Bacterial infection	0.03	−2.00		↓	5	↑*IRF3*, ↑*IRF7*, ↑*SIGLEC1*, ↑*USP18*, ↑*ZFP36*
Quantity of blood cells	0.03	−1.08		↓	10	↓*GIMAP1-GIMAP5*, ↑*HIVEP2*, ↓*MSH2*, ↑*NUP98*, ↑*PER2*, ↓*PKLR*, ↑*SIGLEC1*, ↑*STEAP3*, ↑*THBS2*, ↑*ZFP36*
Organismal death	0.04	−1.04		↓	18	↑*CYP26A1*, ↑*CYP2E1*, ↓*DNAJC3*, ↓*ERRFI1*, ↑*FAT1*, ↑*GIMAP1-GIMAP5*, ↑*IRF3*, ↑*IRF7*, ↓*MSH2*, ↑*NUP98*, ↑*PALLD*, ↑*PER2*, ↑*PPARA*, ↑*RAD54B*, ↑*SOD2*, ↑*THBS2*, ↑*USP18*, ↑*ZFP36*
Differentiation of leukocytes	0.04	−0.69		↓	7	↓*GIMAP1-GIMAP5*, ↑*HIVEP2*, ↑*IRF7*, ↓*MSH2*, ↑*NUP98*, ↑*USP18*, ↑*ZFP36*
Inflammation of heart	<0.01			↓	3	↑*PPARA*, ↑*THBS2*, ↑*ZFP36*
Canonical pathway						
2-Ketoglutarate dehydrogenase complex	0.02		0.25	↑	1	↑*DHTKD1*
Thyroid hormone metabolism II	0.04		0.14	↑	1	↑*UGT2B17*
Histidine degradation VI	0.04		0.14	↑	1	↑*CYP2E1*
FXR/RXR activation	0.02		0.04	↑	3	↑*PPARA*, ↓*PKLR*, ↑*SLC51B*, ↓*APOM*
Alanine degradation III	0.01		0.50	↓	1	↓*GPT2*
Alanine biosynthesis II	0.01		0.50	↓	1	↓*GPT2*
Tryptophan degradation III	<0.01		0.13	↓	2	↓*ACMSD*, ↓*HAAO*

[1]Calculated by the Ingenuity pathway system using the tailed Fisher's exact test.

[2]Positive and negative values predict occurrence and not occurrence of the biological function, respectively. Missing value represents biological functions with a z-score not calculated by Ingenuity pathway system.

[3]Number of genes in a given pathway with respect to the total of genes in that pathway.

[4]Predicted effect for biological functions without z-score value were calculated using the number of genes expected to increase or decrease a given biological function.

[5]Upregulated (↑) and downregulated genes (↓).

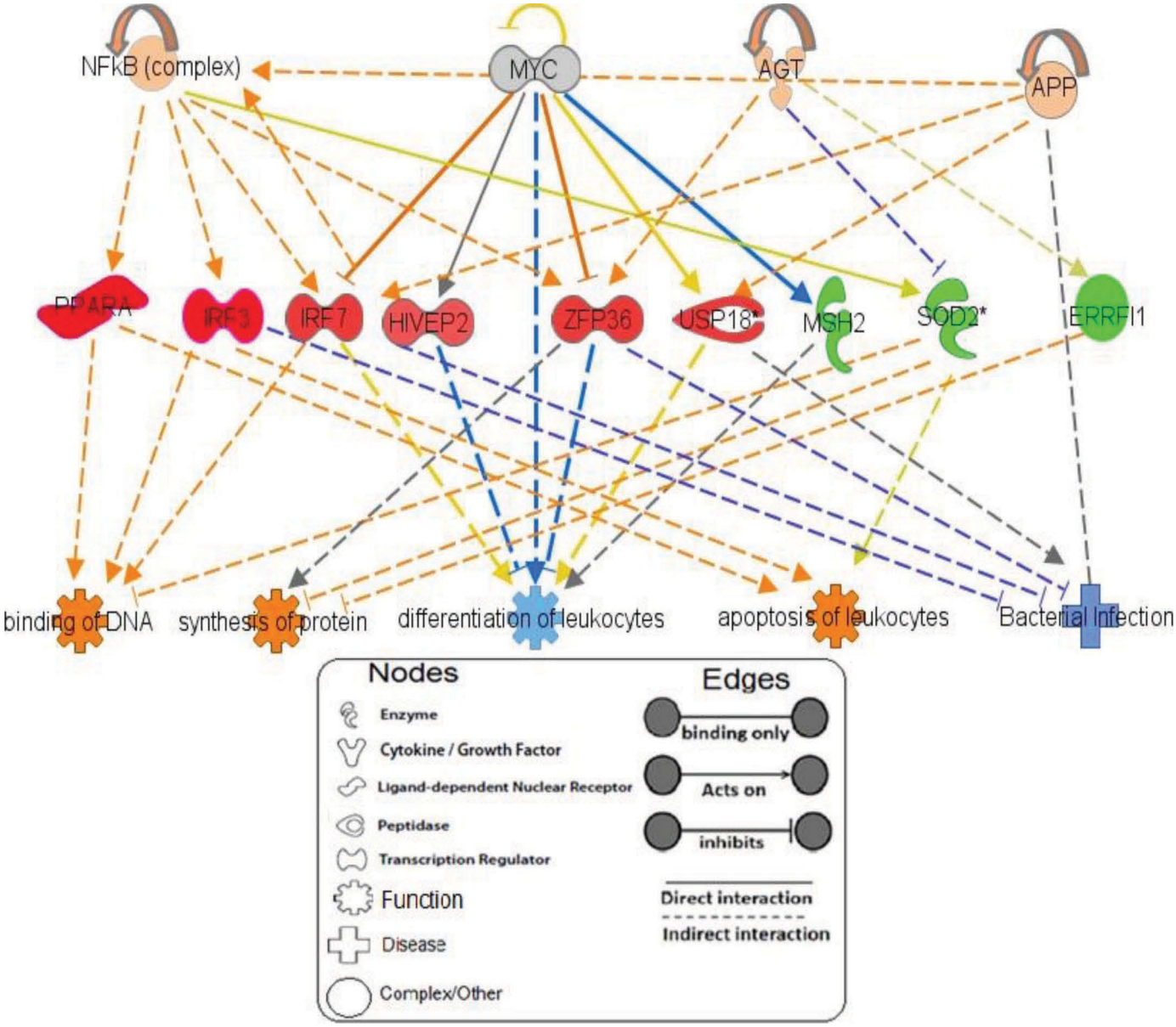

Figure 2. The upstream regulatory network of the top predicted biological functions and disease in liver of 30-d-old male calves fed milk replacer (MR) containing low (LLA) or high linoleic acid (HLA). NFkB = nuclear factor κ-B; MYC = Myc proto-oncogene protein; AGT = angiotensinogen; APP = amyloid β(A4) protein; PPARA = peroxisome proliferator-activated receptor α; IRF3 = interferon regulatory factor 3; IRF7 = interferon regulatory factor 7; HIVEP2 = human immunodeficiency virus type 1 enhancer binding protein 2; ZFP36 = zinc finger protein 36; USP18 = ubiquitin-specific peptidase 18; MSH2 = MutS homolog 2; SOD2 = superoxide dismutase 2; ERRFI1 = ERBB receptor feedback inhibitor 1. Color version available online.

expression. The most strongly predicted effects were increase in metabolism and synthesis of protein, binding of DNA, and apoptosis of leukocytes and decrease in bacterial infections, quantity of blood cells, and organismal death. In Figure 2, the top predicted biological functions and disease are depicted with their upstream regulatory networks. The upstream regulator nuclear factor-kB complex, growth factor angiotensinogen (AGT), and cell surface receptor amyloid β(a4) protein (APP) were predicted to be increased, all based on the observed pattern of gene expression and predicted downstream biological functions and disease.

Top Canonical Pathways. Table 6 and Supplemental Table S11 (http://dx.doi.org/10.3168/jds.2015-10472) summarize the most enriched canonical pathways. Seven pathways were enriched due to type of

Table 7. Measures of productive and immune performance of Holstein male calves (n = 40) fed milk replacer (MR) containing low linoleic acid (LLA) or high linoleic acid (HLA) from 1 to 30 d of age[1]

	Prepartum diets (DD)[2]				Milk replacer[3]			*P*-value[4]			
Measure	Control	SFA	EFA	SEM	HLA	LLA	SEM	FAT	FAS	MR	DD × MR
No. of calves	14	10	16		20	20					
Birth BW, kg	42.6	45.0	43.6	1.64	45.4	42.0	1.34	0.42	0.56	0.09	0.60
MR intake, kg/d	0.53	0.56	0.53	0.02	0.55	0.52	0.01	0.44	0.22	0.11	0.39
ADG, kg/d	0.30	0.36	0.30	0.03	0.34	0.29	0.02	0.38	0.08	0.07	0.24
Feed efficiency	0.56	0.65	0.56	0.05	0.62	0.55	0.04	0.47	0.19	0.22	0.45
Glucose, mg/dL	91.1	86.6	88.7	1.83	89.6	88.0	1.50	0.13	0.44	0.47	0.30
Insulin, ng/mL	1.11	1.17	1.26	0.13	1.16	1.19	0.12	0.56	0.68	0.92	0.12
IGF-I, ng/mL	56.1	43.8	44.9	4.2	50.4	46.2	3.5	0.03	0.80	0.40	0.32
Plasma urea N, mg/dL	8.27	9.57	9.17	0.51	8.96	9.04	0.42	0.08	0.59	0.89	0.80
BHB, mg/dL	0.78	1.07	1.19	0.18	0.78	1.25	0.14	0.11	0.64	0.03	0.53
NEFA, μEq/L	211	188	199	11	192	207	9	0.19	0.43	0.23	0.32
Cholesterol, mg/dL	76.5	76.6	83.8	3.53	75.7	82.2	2.90	0.41	0.19	0.13	0.04
Haptoglobin,[5] OD × 100	0.91	0.97	0.93	0.05	0.94	0.93	0.04	0.51	0.50	0.85	0.67
Acid soluble protein, mg/L	106	95	113	7.7	97	113	6.4	0.87	0.10	0.08	0.66
IFN-γ at 30 d, pg/mL	30.0	46.5	29.4	9.7	45.2	25.3	8.0	0.50	0.23	0.08	0.91
Hematocrit, %	35.2	33.3	35.2	1.2	35.6	33.5	1.0	0.51	0.29	0.15	0.52

[1]MR intake, ADG, and feed efficiency (kg of gain/kg of MR intake) for the period from 1 to 30 d of calf age. All metabolites but IFN-γ were analyzed in all available samples from 0 to 30 d of calf age.

[2]Control = no fat supplement; SFA = SFA supplement, Energy Booster 100 (Milk Specialties, Dundee, IL); EFA = essential fatty acid supplement, Megalac-R (Church & Dwight, Princeton, NJ).

[3]Milk replacer was the only feed offered during the first 30 d of life. Daily intakes of C18:2 from MR were 0.14 and 0.45 g of C18:2/kg of metabolic BW for LLA and HLA MR, respectively.

[4]*P* values for orthogonal contrasts: FAT = control vs. (SFA + EFA); fatty acid source (FAS) = essential FA vs. SFA; MR = LLA vs. HLA; DD × MR = interaction of prepartum dam diet by milk replacer. Effect of age was significant ($P < 0.01$) for all repeated measures with the exception of insulin that was not significant.

[5]OD = optical density.

MR fed. Based on the ratio value, the 2-ketoglutarate dehydrogenase complex, thyroid hormone metabolism II, histidine degradation VI, and the farnesoid X receptor/retinoid X receptor (FXR/RXR) activation pathways were the top pathways predicted to be enhanced. The alanine degradation III, alanine biosynthesis II, and tryptophan degradation III pathways were the top predicted to be diminished.

Performance and Blood Measurements of Preweaned Male Calves

Performance data for all enrolled calves (n = 40) is presented in Table 7. Calves born from dams supplemented with fat had lower plasma concentrations of IGF-I (44.4 vs. 56.1 ng/mL, $P = 0.03$), but greater concentrations of urea N (9.37 vs. 8.27 mg/dL, $P = 0.08$) compared with calves born from control dams. Calves born from dams fed SFA tended to have better ($P = 0.08$) ADG than calves born from dams fed EFA (0.36 vs. 0.30 kg/d), although differences in MR intake or feed efficiency were not statistically significant. However, concentration of acid-soluble protein tended to be less ($P = 0.10$) in calves born from dams fed SFA (95 vs. 113 mg/L). By random chance, BW at birth (45.4 vs. 42.0 kg, $P = 0.09$) tended to be greater for calves enrolled in the HLA MR compared with those fed the LLA MR. These calves also tended to have greater ADG (0.34 vs. 0.29 kg/d, $P = 0.07$), compared with calves fed LLA MR. Feeding the HLA MR decreased mean plasma concentrations of BHB (0.78 vs. 1.25 mg/dL, $P = 0.03$) and tended to decrease concentrations of acid-soluble protein (97 vs. 113 mg/L, $P = 0.10$), whereas concentrations of IFN-γ tended to be greater (45.2 vs. 25.3 pg/mL, $P = 0.08$) compared with calves fed LLA MR.

DISCUSSION

Plasma and Liver FA Profile

Although prepartum supplementation of FA had tremendous effect on the FA profile of calf plasma at birth (Garcia et al., 2014a), prepartum supplementation of fat or specific FA had lesser effects on plasma and liver FA profile of calves at 30 d of age. Supplementing fat had a greater effect on liver FA profile than supplementing a specific type of FA. In contrast feeding a specific type of FA (i.e., EFA vs. SFA) had a greater effect on the FA profile of plasma than did fat feeding. The type

of MR fed was the major contributor to changes in FA profile of liver and plasma with a greater diversity of FA responses in liver.

Although the MR were isolipidic, concentration of fat in liver but not in plasma was 11% less in calves fed HLA versus LLA MR. Calves fed a 100% CCO-based MR had 48% more total lipids in fresh liver and had a better growth rate compared with calves fed a 95% CCO and 5% corn oil-based MR (Jenkins and Kramer, 1986). Faster availability of medium-chain FA present in CCO that are rapidly oxidized (i.e., C12:0) and then preferentially (i.e., C6:0 and C8:0) elongated to synthesize longer-chain FA may result in liver accumulation of fat (Piot et al., 1999) due to a greater rate of synthesis than export, the latter due to limited ability to secrete triglyceride-very low density lipoprotein. Indeed, calves fed LLA MR rich in CCO had greater percentage of medium-chain SFA in plasma (C12:0 and C14:0) and in liver (C12:0, C14:0, and C16:0) as compared with calves fed HLA MR.

The FA profile of the liver and plasma closely reflected the FA profile of the type of MR fed. The low percentage of total *trans* C18:1 in liver (0.6%) may be a result of limited substrate available in the rumen, considering that calves were fed no solid feed and milk may be bypassing the rumen through the esophageal groove. Furthermore, total *trans* C18:1was not influenced by either MR or prepartum maternal diets. In the adult confined cow, linoleic acid often makes up only about 12 and 33% of total FA in the liver (Bilby et al., 2006) and plasma (Moallem and Zachut, 2012), respectively. However, linoleic acid comprised about 20 and 46% of total FA in the liver and plasma of the preweaned calf, respectively. Likewise, the concentration of C20:4n-6 as synthesized from linoleic acid comprised ~10% of the FA in calf liver, whereas it made up ~7% of hepatic FA in the adult bovine liver (Bilby et al., 2006).

Calves fed HLA MR had a total greater percentage of n-6 and n-3 FA in liver, but only a total greater percentage of n-6 FA in plasma. These FA are reported to be potent inducers of FA oxidation in liver by enhancing PPAR-α activation (Sampath and Ntambi, 2005), and this effect might prevent the calves fed the HLA MR from having greater concentrations of total FA in liver. Unlike the placenta in fetal life, the liver appears to be the primary location for postpartum desaturation and elongation of essential FA in preruminants and ruminants (Shand et al., 1978; Shand and Noble, 1979). In the present study, the greater linoleic acid derivative in liver was C20:4n-6 (~70% of the total linoleic acid derivatives), whereas C22:5n-3 and C22:6n-3 were the greater α-linolenic acid derivatives (53 and 45% of the total α-linolenic acid derivatives, respectively). As percentages of C20:4n-6 and C22:5n-3 were greater in liver of calves fed HLA instead of LLA MR, it is likely that the activities of Δ^5- and Δ^6-desaturases and elongases 2 and 5 were enhanced in liver of calves fed HLA MR, as previously reported by others working with supplementation of linoleic acid to pregnant ewes (Shand et al., 1978); others (Goyens et al., 2006; Liou et al., 2007) reported a preferential desaturase or elongase activity of a parent FA when provided in greater concentrations in the diet. However, in the current study, preferential pattern of synthesis of essential FA derivatives was not apparent (i.e., some n-3 and n-6 derivatives were increased and others were decreased due to feeding of HLA MR).

Prepartum Feeding of Fat May Reduce Exacerbated Proinflammatory Response

The number of enriched biological functions (8/10) and canonical pathways (6/11) with immune focus indicate that supplementation of fat, regardless of the type of FA, highly regulates the expression of immune-related genes (Table 4). Calves born from dams fed fat compared with calves born from dams not supplemented with fat had a predicted diminished effect on immune functions, such as recruitment and response of different immune cells, predicted reduction of pathways such as activation of immune response by receptors and signaling molecules, and reduction of dendritic cell maturation (Table 4). This coordinated response appears to be driven by the downregulation of several proinflammatory genes. Based upon the percentage of days with diarrhea and poor attitude, the concentrations of acute phase proteins in plasma, and the production of IFN-γ by stimulated blood mononuclear cells, the health status and immune responsiveness of male calves during the first 30 d of life were not affected by the type of diet fed to their dams. The lower expression of proinflammatory genes in calves born from dams supplemented with fat may indicate either (1) a better ability by these calves to minimize an excessive proinflammatory state, thus preventing a negative effect of chronic inflammation on calf productivity (e.g., exacerbated response of immune cells), or (2) a less proximal provocation of the immune system.

Calves born from dams fed supplemental fat had a high confidence (z-score = 1.94) of having an increased metabolism of terpenoids due to the upregulation of *ADH1C*, *IL8*, *HSD11B1*, *LIPC*, and *PGRMC1*. The potential increased activity of hepatic triglyceride lipase, encoded by *LIPC*, may indicate an enhanced uptake of lipoproteins from plasma and enhanced triglyceride hydrolysis. Similarly, increased cortisol metabolism

(*HSD11B1*), lipid peroxidation (*ADH1C*), and sterol regulation (*PGRMC1*) are all indicative of an enhanced lipid oxidation. Indeed, accumulation of lipids in calves born from dams supplemented with fat was predicted to be diminished by the primary upregulation of *ACADL* and *FH*. A reduction in lipid accumulation was predicted with very low confidence (z-score of −0.39). The increase of L-carnitine biosynthesis in liver of calves born from dams fed fat supports the predicted reduction in lipid accumulation due to the role of carnitine on mitochondrial β-oxidation. However, the increased expression of *GPT2*, which codes for the enzyme alanine aminotransferase 2, a serum biomarker of hepatitis and fatty liver (Liu et al., 2009), contradicts the expected increased lipid oxidation (due to increased metabolism of terpenoids and reduced accumulation of lipids) in liver of calves born from dams fed fat. This apparent contradictory effect may be due to a regulation of lipid metabolism in liver of calves that, in their late uterine life, were nurtured by dams fed diets with more fat (1.87 vs. 3.45% of total DM) and energy concentrations (1.50 vs. 1.42 Mcal of NE_L/kg of total DM). Total FA concentration in plasma of newborn calves (Garcia et al., 2014a) and in plasma and liver of the 30-d-old male calves did not differ between those born from dams fed fat or control diets. However, some saturated (C18:0) and elongated FA (C20:4n-6, C20:5n-3) were increased by the feeding of fat in late uterine life.

Feeding of EFA May Reduce Nutrient Catabolism and Enhance Brain Cell Survival

No biological function related to metabolism of nutrients was enriched when feeding a specific type of FA during uterine life (Table 5). This does not imply a lack of effect of prepartum nutrient intake on nutrient metabolism by the liver, considering that many studies using rodents have reported big maternal effects on metabolism of nutrients in liver with a consequent effect on growth and health of the offspring (Mennitti et al., 2015). Calves born from dams supplemented with EFA as compared with SFA may not catabolize AA and glucose as extensively for further entrance into the Krebs cycle, as pathways involved in proline or hydroxyproline degradation, thiamin salvage III, and threonine degradation II were predicted to be decreased due to the downregulation of genes (*ALDH4A1*, *TPK1*, and *GCAT*). These proteins are known to enhance the degradation of AA and sugars (Lee et al., 2011; Pemberton and Tanner, 2013; Zastre et al., 2013). This sparing of AA and sugars may indicate a preferential use of FA for energy through the Krebs cycle in calves born from dams fed EFA. The potential sparing of anabolic substrates did not alter the concentration of anabolic metabolites in plasma of calves (i.e., IGF-I, urea N, and cholesterol) but changed the percentage of some FA in plasma. The trend for a beneficial effect of prepartum SFA on ADG does not appear to be reflected in the gene expression for enhanced AA sparing for protein synthesis in liver tissue at 30 d of life after differential feeding of MR.

We recognize the limitation of applying a potential effect of dietary treatments on brain cells using liver tissue. However, the pattern of change in expression of some genes tended to cluster together for brain and liver tissues (Shyamsundar et al., 2005). In the current study, calves born from dams fed EFA instead of SFA had a predicted reduction in brain cell death due to the primary upregulation of 5 hepatic genes (Table 5) whose mRNA or protein activity have been reported as reducers of neuronal death (*HMGB2*, Qi et al., 2007; *IL8*, Watson and Fan, 2005; *PIK3CA*, Hetman et al., 2002; *RIT1*, Cai et al., 2012; *GCLM*, Diaz-Hernandez et al., 2005). The developing brain of neonates requires primarily C22:6n-3 and C20:4n-6 FA; indeed, brain is the tissue with the highest concentrations of these FA (~24 and 35% of total FA are C20:4n-6 and C22:6n-3 in bulls, respectively; Adeyeye, 2012). Regulation of these critical FA starts in utero and continues during first 2 yr of life in humans (Koletzko et al., 2008). Based upon the different percentage of elongated FA in colostrum and plasma of newborn calves born from dams fed SFA or EFA diets, it appears that cows tightly regulate placental transfer and fetal liver synthesis of critical FA for brain development but this regulation is influenced by the type of FA fed prepartum (Garcia et al., 2014a).

HLA MR May Improve Overall Inflammatory Responses and Enhance Nutrient Utilization

Type of MR fed was the major contributor to changes in the FA profile in liver and plasma and in total FA in liver of calves. These changes can potentially alter hepatic metabolism as suggested by the transcriptome regulation. The biological functions with greater likelihood to be regulated by feeding HLA instead of LLA MR were reductions in bacterial infection (z-score of −2.00), in quantity of blood cells (z-score of −1.08), in organismal death (z-score of −1.04), and in differentiation of leukocytes (z-score of −0.69). These responses, coupled with a predicted increase of apoptosis of blood cells (e.g., leukocytes such as neutrophils) and generation of superoxide without formation of reactive oxygen species (i.e., due to downregulation of *SOD2*, which is only enhanced by activated neutrophils; Olsson et al., 2011), may indicate that the immune system of calves

fed HLA MR either was not being challenged or were able to mount a proper inflammatory response, avoiding exacerbated inflammation. Unchanged concentrations of plasma haptoglobin and trend for reduced acid-soluble protein also are indicative that calves fed HLA were not undergoing sickness events (Table 7). Moreover, the trend for increased IFN-γ by stimulated immune cells in vitro (Table 7) may support the hypothesis that calves fed HLA MR had an enhanced ability to mount a proinflammatory response (Garcia et al., 2014b, 2015).

The most commonly identified gene in various biological functions was *PPARA* (10 of 14 biological functions; Table 6), coding for the transcription factor PPAR-α that upregulates the expression of several genes involved in nutrient metabolism (Calder, 2012). The predicted increased binding of DNA, within the category of gene expression, included the upregulated genes *PPARA*, *IRF7*, and *IRF3*, which regulate the expression of immune-related (i.e., *PPARA*, *IRF3*, and *IRF7*; Figure 2; Taniguchi et al., 2001) and metabolic-related (i.e., *PPARA*; Calder, 2012) genes. The regulation of metabolic- and immune-related genes is further confirmed by the upregulation of genes involved in AA, protein, and lipid metabolism and function of immune cells.

Linoleic acid is a potent ligand of PPAR-α allowing its activation (Forman et al., 1997). Therefore, feeding greater amounts of linoleic acid in the HLA MR, which also was present in greater percentage in liver and plasma of these calves, may have enhanced the upregulation of *PPARA* gene expression to regulate the expression of *PPARA* target genes, inducing primarily a greater uptake and utilization of lipids. Indeed, downregulation of *GPT2*, coding for the enzyme alanine aminotransferase 2, which is a known biomarker of hepatitis and fatty liver (Liu et al., 2009), was further confirmed by upregulation of genes associated with β-oxidation of FA and peroxidation of lipids, possibly contributing to the reduced concentrations of total FA in liver of calves fed HLA compared with LLA MR. Moreover, genes *CYP2E1* and *CYP26A1*, coding for cytochrome P450 enzymes, were also upregulated in calves fed HLA MR. These P450 enzymes induce the bioactivation of linoleic acid and C20:4n-6 to their intermediary bioactive products having key functions as second messengers of hormones and growth factors (Konkel and Schunck, 2011).

Average daily gain the first 30 d of life tended to be greater for calves fed HLA MR. Feeding HLA MR increased the likelihood for increased synthesis of hepatic proteins (Z-score of 1.07), which may also indicate an enhancement of body protein accretion.

Potential Regulation of FA Fed on the Response of the Offspring to Supplemental FA

In the larger research project from which the present study was generated, we aimed to investigate the adaptive metabolism of calves fed high or low amounts of linoleic acid in response to the amount and type of FA to which they were exposed during their last trimester of fetal life. However, because of the limited number of calves in each dam diet-MR group (3 calves), we restrained from analyzing the interaction contrasts of dam diets by MR. The possibility that a fetal programing effect of maternal diets can regulate response of the neonate to the type of diets fed in early life warrants further investigation on development, health, and productivity of the offspring.

CONCLUSIONS

The FA profile of liver and plasma of 30-d-old calves was minimally influenced by the diet fed to their dams during late uterine life. However, hepatic transcriptome was more sensitive to the type of diets fed prepartum. Expression of genes in liver of calves born from dams fed fat or a specific type of FA resulted in the enrichment of several biological functions involved in immune responses and nutrient metabolism. The FA profile of the MR had the most influence on the FA profiles of liver and plasma of 30-d-old male calves. Linoleic acid and some elongated derivatives were greater in the liver of calves fed HLA MR, which may be PPAR-α ligands promoting activation and further regulation of PPAR-α target genes. Feeding a HLA MR modified the expression of hepatic genes, including genes predicted to decrease infections and to increase lipid utilization and protein synthesis. Future studies evaluating the effect of fat and FA supplementation, at different physiological stages, on future productive and reproductive efficiency of heifers are warranted.

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