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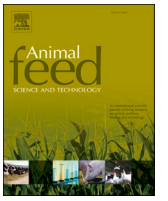

Methionine supplementation during early post-natal life of ewe lambs: Developmental programming of the female offspring and effects on the first lactation

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ABSTRACT

Methionine supplementation during the suckling period of ewe lambs may modify DNA methylation in the germline (F0) during this critical window period for the neonate. This circumstance may promote the intergenerational transmission of epigenetic marks to the offspring (F1), thus altering the expression of specific genes and physiological traits throughout F1 life. To test this hypothesis, 27 newborn ewe lambs (F1) born from either ewes being supplemented 0.1 % methionine (dry matter basis) during the suckling period (F0-MET) or not supplemented (F0-CTRL) were assigned to two different groups (F1-CTRL, n = 13 and F1-MET, n = 14), being the dietary treatment of their mothers (F0) the only source of variation. Thus, all the F1 animals (both groups) were raised exactly in the same way along the whole life (including lactation). In this study, we determined differences in the global blood methylation patterns, biochemical profile, and metabolome of female offspring (F1). Our data showed that functional categories such as those related to developmental process and anatomical structure development were significantly enriched in the F1-MET ewe lambs due to differentially methylated regions of genes in these categories. These F1-MET ewes also presented lower live body weight ($P < 0.05$) and reduced lipomobilization and milk yield ($P = 0.099$) during the lactation period, together with increased PUFAs content ($P = 0.075$) in the milk fatty acid profile when compared to F1-CTRL. Increased levels of insulin ($P = 0.031$) and β-hydroxybutyrate ($P = 0.043$), along with certain features of the metabolome profiles, revealed altered lipid metabolism when

Abbreviations: ADG, average daily gain; AIA, acid insoluble ash; AST, aspartate aminotransferase, BHB: beta-hydroxybutyrate; CPD, complete pelleted diet; DHA, docosahexanoic acid; DM, dry matter; DMI, dry matter intake; ECM, energy-corrected milk yield; FA, fatty acids: FCR: feed conversion rate; FDR, false discovery rate; GGT, gamma-glutamyl transferase; GO, Gene Ontology; HDL, high-density lipoprotein; KEGG, Kyoto Encyclopedia of Genes and Genomes; LBW, live body weight; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acids; NEFA, non-esterified fatty acids; OTUs, operational taxonomic units; PLS-DA, Partial Least Squares Discriminant Analysis; PUFA, polyunsaturated fatty acids; RDP, Ribosomal Data Project; RFI, residual feed intake; RRBS, reduced representation bisulfite sequencing; SFA, saturated fatty acids; SAM, S-adenosylmethionine; SCC, somatic cells count.

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compared to F1-CTRL animals. As far as fecal microbiota is concerned, no significant differences were found in alpha and beta diversity between the two groups. However, DESeq2 analysis performed on OTU-filtered data revealed that *Treponema* (genus) and *Spirochaetaceae* (family), both from the *Spirochaetes* phylum, were reduced in the F1-MET group compared to the F1-CTRL group (log2FC=-3.96, $p_{adj} < 0.05$). The results suggest that early methionine supplementation in F0 ewe lambs has an intergenerational impact on their F1 female offspring, with negative consequences on lipomobilization and milk production during F1 lactation.

1. Introduction

Postnatal nutritional events (e.g., early feed restriction) during the early life of ewe lambs can modulate DNA methylation in the germline during this critical window period for the neonate (F0), hence promoting intergenerational transmission of persistent epigenetic marks to the offspring (F1) that alter physiological traits throughout the whole life (Andrés et al., 2021). Improving knowledge about intergenerational effects caused by postnatal early nutritional events is crucial for refining strategies dedicated to animal breeding and replacement purposes. Accordingly, the supply of methyl group donors [e.g., precursors of S-adenosylmethionine (SAM), the universal methyl donor involved in the methylation of all biological molecules, including DNA] has attracted attention due to their potential to induce persistent epigenetic modifications in both, F0 and F1 (Zhang, 2018).

Particularly, methionine (MET) plays not only a key role in physiological intracellular processes as a methyl donor, but it is also one of the most limiting amino acids needed for ruminant muscle growth (Loest et al., 2002). Several studies have supplied rumen-protected methionine (0.09–0.1 % diet DM) to pregnant ewes (Rosa-Velazquez et al., 2022) and cows (Jacometo et al., 2016; Alharthi et al., 2018), trying to positively impact the number of myofibers of the fetus and the quality of the meat produced. Conversely, a limited number of studies in domestic animals have examined epigenetic intergenerational inheritance (Zhang, 2018; Thompson et al., 2020). And, to our knowledge, there are no studies focused on the intergeneration transmission of epigenetic marks caused by methionine supplementation of ewe lambs (F0), and the consequences on the phenotype of the offspring (F1). If positive effects on F1 could be achieved by methionine supplementation during the early life of F0 ewe lambs, this strategy might become decisive under intensive production systems, in which ewe lambs are raised on milk replacers formulated using powder cow milk, with a lower amount of methionine in the amino acid profile when compared to milk sheep (Dehnavi et al., 2025).This study hypothesized that methionine supplementation of ewe lambs during the early post-natal life (suckling period of F0) leads to detectable epigenetic and metabolic alterations in F1 offspring (F1), thus modifying feed efficiency, milk quality, and production of the F1 adult dairy ewes. Moreover, differences in the global blood methylation patterns, biochemical profile, metabolome and gut microbiota of female offspring (F1) have been studied using a multi-omics approach (e.g., epigenetic marks, metabolomics and gut microbiome) to understand the mechanisms behind the observed effects.

2. Materials and methods

2.1. Care and use of animals

Experimental conditions and handling of the animals followed the recommendations of Directive 2010/63/EU of the European Parliament on the protection of animals used for experimental and other scientific purposes and were approved by the CSIC Animal Experimentation Committee and the competent authority (protocol number 100102/2021–6).

2.2. Animals, experimental groups, sampling and analysis of feed and milk

All the details of the Assaf dairy ewes [F0, experimental flock of the *Instituto de Ganadería de Montaña* (CSIC, León)] giving birth to the lambs of the present study (F1, female lambs) are explained in Dehnavi et al. (2025). Briefly, 34 new-born ewe lambs (F0) were separated from the ewes within 36–48 h of birth, stratified based on live weight at birth and type of lambing (single or twin), and assigned to two balanced groups (n = 17 animals per group), being the twin female lambs evenly distributed between the two treatments. Thus, a control group of newborn Assaf ewe lambs (F0-CTRL, n = 17) was fed *ad libitum* with a commercial milk replacer (Cordevit Calostrado, Leches Maternizadas S.A., León, Spain). In contrast, the other group (F0-MET, n = 17) received the same milk replacer supplemented with 0.1 % D,L-methionine (Rhodimet® NP 99, Addiseo Commentry, France) on a DM basis. After weaning (approximately 45 days of age, after having received D,L-methionine supplementation during 43 days), all F0 ewe lambs were housed together in feedlot and reared under identical conditions, being offered *ad libitum* a formulated complete pelleted diet (CPD) according to their nutritional requirements (AFRC, 1993). Once they were 9 months old, all F0 ewe lambs were synchronised before being artificially inseminated to produce the progeny (F1).

The F1 female ewe lambs (n = 27) were assigned to two different groups (F1-CTRL and F1-MET) according to the dietary treatment received by the dams (F0). Then, all F1 female Assaf lambs were fed the same commercial milk replacer ad libitum (MR, Cordevit Calostrado, Leches Maternizadas S.A., León, Spain) until weaning. After approximately 7.5 weeks, 27 healthy female lambs (F1-CTRL, n = 13 and F1-MET, n = 14) were weaned, housed together (single feedlot on a floor covered by a sawdust bed and automatic drinkers available) and reared under identical conditions during the whole life being the dietary treatment of their dams (F0) the only difference

between both groups. All the management details were similar to those published for their mothers (F0) in Dehnavi et al. (2025). All ewe lambs (F1-CTRL= 13 animals; F1-MET=14 animals) were synchronised at 9 months before being artificially inseminated to produce the progeny (F2) and initiate the first lactation period of F1. Finally, 11 ewes from each group (F1) gave birth to F2, but two animals were excluded from the F1-CTRL group due to lambing problems or mastitis, which required antibiotic treatment. During the lactation period, all F1 animals (9 and 11 ewes in F1-CTRL and F1-MET groups, respectively) received a CPD ad libitum (Table 1) with fresh drinking water always available. Ingredients and chemical composition of the CPD administered to F1 (lactation period) are summarised in Table 1.

During the milking period, individual feed intake was recorded from week 7 (35 ± 1.0 days in milk) to week 10 (70 ± 1.0 days in milk) using automatic devices (Agrolaval S.L., Gijón, Asturias, Spain) and radio frequency identification (RFID) ear tags. Faeces were collected (rectal grab samples) from each animal for nine consecutive days at 08:00 am to estimate the apparent total tract digestibility of DM using acid insoluble ash (AIA) as an internal marker. In addition, the F1 ewes were milked once a day at 09:30 in a 1 × 10 Low-Line Casse system milking parlour with 10 milking units. Milk yield was recorded on days 1, 3, 7, 10, 14, 17, 21, 24, 28, 31 and 35. Milk samples (100 mL) were collected from each animal on days 1, 7, 14, 21, 28, and 35, preserved with an antimicrobial tablet (Bronopol, Broad Spectrum Micro-tables II, D&F Control Systems Inc., San Ramon, CA) and stored at 4°C until laboratory analysis [(e.g., milk's total solids, fat, protein, lactose concentrations and somatic cell counts (SCC)] according to Dehnavi et al. (2025). Energy-corrected milk yield (ECM) standardized the amount of energy in the milk to 5 MJ/L, as described by Bocquier et al. (1993): ECM (g/day) = milk yield (g/day) × (0.071 × % fat + 0.043 × % protein + 0.2224). Finally, the milk fatty acid (FA) profile on day 35 was analysed using the same protocol as described for F0-MET samples in Dehnavi et al. (2025).

2.3. Reduced Representation Bisulfite Sequencing (RRBS) Analyses (Before Weaning)

Before being weaned (approx. 45 days of life), a blood sample was collected for DNA extraction to compare DNA methylation by RRBS. The full description of this protocol can be found in Andrés et al. (2021) and Dehnavi et al. (2025), with some modifications. Briefly, blood samples were collected from five animals from each group (in total 10 samples; 5 F1-CTRL ewe lambs and 5 F1-MET ewe lambs, all 45 days old) by jugular venipuncture into tubes containing EDTA. This sample size is considered adequate to detect differentially methylated regions by RRBS (Liu et al., 2020). DNA extraction was performed using the QIAamp DNA Blood Mini Kit (Qiagen Iberia S.L., Barcelona, Spain; Cat# 51104). Then, the quantity and quality of the DNAs were evaluated with Qubit dsDNA HS DNA Kit (Thermo Fisher Scientific, Cat. # Q32854) and agarose gels, respectively. Sequencing libraries were prepared using

Table 1
Ingredients and chemical composition of the diet used during the lactation period.

Ingredients (g/kg as feed basis)	
Alfalfa	370
Barley straw	60
Wheat bran	20
Barley grain	75
Maize grain	145
Soybean meal	120
Soy hull	60
Sugar beet pulp	50
Molasses	40
Hydrogenated fat	18
Urea	5
Bicarbonate	20
Magnesium oxide	2
Corrector	15
Chemical composition (g/kg DM)	
DM (g/kg fresh matter)	904
CP	169
NDF	347
ADF	217
Ether extract	42
Ash	124
Crude energy (Mcal/kg DM)	4.12
ME (Mcal/kg)	2.61

Mineral and vitamin premix - vitamin A (7000 IU/kg), vitamin D3 (1400 IU/kg), copper (copper sulfate pentahydrate) 2 mg/kg, iron (iron sulfate monohydrate) 49 mg/kg, zinc (zinc oxide) 34 mg/kg, manganese (manganese oxide) 32 mg/kg, cobalt (cobalt carbonate monohydrate) 0.9 mg/kg, iodine (potassium iodide) 2 mg/kg; DM - dry matter (g/kg fresh matter); CP - crude protein (g/kg DM); NDF - neutral detergent fiber (g/kg DM); ADF – acid detergent fiber (g/kg DM); ME- estimated values for metabolizable energy.

NEXTflex™ Bisulfite-Seq Kit (Bioo Scientific Corporation, Austin, Texas) and EZ DNA Methylation-GoldTM Kit (Zymo Research, Irvine, California). A total of 1000 ng of DNA was digested with the *Msp*I enzyme (Biolabs, Paris, France; Cat#R0106S). Then, 664 ng of *Msp*I digested DNA was used for library preparation procedure with the NEXTflex bisulfite library preparation kit (Bioo Scientific Corporation, Cat#5119–01or5119–02) following the manufacturer's instructions. Bisulfite conversion of the DNA was performed with the EZ Methylation Gold kit (Zymo Research, Cat. # D5005) according to the manufacturer's instructions. Afterwards, libraries were quantified using Qubit dsDNA HS DNA Kit (Thermo Fisher Scientific, Cat. # Q32854) and visualized on an Agilent 2100 Bioanalyser using Agilent High Sensitivity DNA kit (Agilent Technologies, Cat. # 5067–4626). Sequencing of high-quality libraries was performed with paired-end 100 bp reads (PE100; 100 million total reads) on an Illumina NovaSeq 6000 platform (50 million for Read-1 & 50 million for Read-2).

Quality control and metric calculation of raw and processed sequence files were performed using FastQC (v0.11.6) and MultiQC (v1.13). Mapping of the reads to the reference *Ovis aries* genome assembly ARS-UI_Ramb_v3.0 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_016772045.2/) and adapter trimming were performed using BS_seeker2 version v2.1.7. Aligned BAM files were sorted by genomic position using Samtools version 1.16.1. Methylation information was extracted additional methylation stats were calculated, and differentially methylated regions (DMRs) were calculated from BAM files using CGmap Tools version 0.1.2 and MethylKit version 1.27.1 in R (version 4.3.1). Pathway over-representation (or enrichment) analyses was performed using STRING and gProfiler2 version 0.2.2 to identify significantly enriched biological functions and pathways obtained from the Human Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, applying an FDR < 0.05.

2.4. Blood biochemical parameters and progesterone

Blood samples were collected from all animals before weaning (T1, day 45), immediately before mating (T2, month 9), and during the mid-lactation period (T3, 35 days after lambing F2). The plasma samples were used to analyze the biochemical profile according to Dehnavi et al. (2025). Only biochemical data (T1, T2 and T3) from those ewes taking part of the lactation trial (F1-CTRL= 9; F1-MET= 11) were used for analysis. In addition, blood samples were collected weekly during the replacement period from four months of age to measure serum progesterone concentrations [sequential competitive immunoassay (Immulite®/Immulite® 1000 Progesterone, Siemens Healthineers, Madrid, Spain)] to determine the onset of puberty for each animal, considering that the first ovulation is reached when serum progesterone levels are above 0.4 ng/mL.

2.5. Metabolomics analysis (Mid-Lactation period)

Plasma samples collected during the mid-lactation period (F1-CTRL, n = 9 and F1-MET, n = 11) were used for metabolomic analysis according to the protocol described in Dehnavi et al. (2025). In brief, a volume of 400 μL of ice-cold methanol: ethanol (1:1, v: v) was added to 100 μL of plasma sample containing 5 μL of internal standard (hippuric acid 0.5 mg/mL, 98 %, Sigma – Aldrich), and the supernatant was transferred to a new tube and dried under a stream of nitrogen. The residue was reconstituted in 100 μL of mobile phase (1 % of 0.1 % formic acid in acetonitrile – FM B) and analyzed by LC-QTOF-MS. Five microliters of each sample were injected in duplicate. The analytes were eluted using a CORTECS UPLC T3 C18 column (2.1 × 150 mm, 1.6 μm) (Waters™) connected to an Agilent 1290 Infinity II LC system (Agilent). The separated metabolites were then ionized using a Turbo V™ Ion Source with an ESI Probe source (Sciex™) and analyzed in a ZenoTOF 7600 system with a Zeno trap (Sciex™). Mass spectra were collected in both positive- and negative ion modes in a full mass scan from 50 to 1500 Da (250 ms accumulation time) and IDA® mode (Information Dependent Acquisition) from 40 to 1500 Da (50 ms accumulation time).

Analysis of data was performed using MetaboAnalyst 6.0 (http://www.metaboanalyst.ca). After filtering, the data were subjected to a generalized log transformation (base 10) and Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable). Then, Partial Least Squares Discriminant Analysis (PLS-DA), heat maps, hierarchical clustering, KEGG enrichment and pathway analysis were obtained. Differentially accumulated metabolites between groups (F1-MET vs. F1-CTRL) were identified using volcano plots, with selection criteria based on a fold-change threshold of 1.3 on the x-axis and a P-value threshold of < 0.05 (adjusted P-value on the y-axis) to relate fold-change with statistical significance. Differential metabolites were selected and tentatively identified by comparing their *m/z*, neutral mass, mass error, and molecular weight, based on their elemental composition, with freely available databases including PubChem, METLIN, HMDB, KEGG, ChEBI, and LipidMaps.

2.6. Gut Microbiome (Mid-Lactation Period)

Sampling, Analytical Procedures, and Data Processing. Faeces collected during the mid-lactation period (F1-CTRL, n = 9 and F1-MET, n = 11) were freeze-dried, stored at −20°C, and then used for microbial DNA extraction using the QIAamp Fast DNA Stool Mini Kit (Qiagen). Library preparation was assessed according to Dehnavi et al. (2025), thus targeting the V3-V4 hypervariable regions of the 16S rRNA gene (Illumina protocol). The first amplification step was performed using an Applied Biosystems 2700 thermal cycler (ThermoFisher Scientific). Amplicons were purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA), and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The prepared libraries were quantified using Real-Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportions, and sequenced on a MiSeq (Illumina) platform with 2 × 250-base paired-end reads. Then, demultiplexed paired-end reads from 16S rRNA-gene sequencing were cleaned by removing primers and adapters (Cutadapt: Martin, 2011) and trimmed for quality (Phred threshold > 20; Sickle, Joshi and Fass, 2011). After cleaning, paired-end reads were joined using the

pipeline Micca (Microbial Community Analysis: Albanese et al., 2015) with default values (i.e., minimum overlap length = 32, maximum number of mismatches in the overlap region = 8). Joined reads with missing/uncalled bases or an expected error rate larger than 1 % were discarded. From the remaining reads, operational taxonomic units (OTUs) were identified the denoising approach (Rosen et al., 2012) and then classified by assigning taxa as annotated in the RDP reference database (Ribosomal Database Project, Wang et al., 2007: minimum confidence value to assign taxonomy was 0.8). Filtered OTU counts (fewer than 10 counts in less than 3 samples) were used to calculate the alpha (ACE, Chao1, Fisher's alpha, Inverse Simpson, Observed, Shannon, Simpson) and beta (Bray-Curtis distances) diversity indexes. Details on the alpha and beta diversity indices can be found in Biscarini et al. (2018), Appendix S2.

2.7. Statistical analysis

Average daily gain (ADG), feed conversion rate (FCR), residual feed intake (RFI) and feed efficiency index were calculated as described in Dehnavi et al. (2025). Normality of the data and homogeneity of variances were assayed using the Shapiro–Wilk test and Levene's test, respectively. Data corresponding to reproductive parameters (progesterone concentration and the age at the first peak of progesterone) and animal performance of F1 animals (LBW, LBW changes, FCR, RFI, feed efficiency index, and digestibility) were analysed by one-way analysis of variance (ANOVA) with the GLM procedure of Statistical Analysis System (SAS Institute Inc., Cary, NC, USA) and with dietary treatment of the mothers (F0-CTRL or F0-MET) as fixed effect. In all cases, the individual lamb was considered the experimental unit. Significance was declared at $P < 0.05$.

Data on blood biochemical parameters measured at different growth stages (at 45 days of age -T1-, before mating -T2-, and during the lactation period -T3-), DMI, and milk yield and composition were analysed as repeated measures using the MIXED procedure of SAS. For repeated measures, diet, time, and their interaction were included as fixed effects, with animal nested within diet as a random effect. Different covariance structures were evaluated using Bayesian and Akaike information criteria. LSMEANS and PDIFF statements were used to compare means. For the post hoc multiple comparisons among the three time points evaluated, the Tukey-Kramer (Honestly Significant Difference, HSD) method was used to adjust the p-values and control the Family-Wise Error Rate (FWER). Significance was declared at $P < 0.05$.

Table 2
Effect of methionine-supplementation of ewe lambs during the early life (F0) on the suckling (pre-weaning), replacement and milking periods of the Assaf female offspring (F1-CTRL vs. F1-MET).

	F1-CTRL	F1-MET	SED	P-value
Suckling period (n)	13	14	-	-
LBW at birth (kg)	4.84	3.81	0.334	0.005
LBW at weaning (kg)	19.2	17.3	1.34	0.165
ADG (g/day)	272	248	20.81	0.239
Age at weaning (days)	52.6	54.4	2.54	0.499
Replacement period (n)	13	14	-	-
LBW 120 days	40.5	36.8	2.39	0.133
LBW 180 days	56.2	51.7	2.75	0.117
LBW at first peak of progesterone (kg)	61.9	56.4	4.40	0.237
LBW at mating (kg)	68.0	62.4	2.60	0.040
ADG from weaning until mating (g/day)	224	207	8.99	0.069
Progesterone at first peak (ng/mL)	2.05	2.15	0.467	0.827
Age at first peak of progesterone (days)	214	231	18.2	0.366
Milking period (n)	9	11	-	-
Feed intake (kg DM/day)	2.29	2.35	0.112	0.607
DM digestibility (%)	74.4	73.5	1.011	0.344
Feed conversion rate (kg DM/kg milk)	1.41	1.83	0.199	0.047
INRA-Feed efficiency index (kg)	0.296	0.275	0.205	0.921
AFRC-Feed efficiency index (kg)	0.105	0.113	0.217	0.973
Residual Feed Intake (kg)	-0.094	0.071	0.122	0.194
LBW at beginning of lactation (kg)	79.9	69.8	3.05	0.004
LBW change during lactation (g/day)	-8.26	55.4	37.3	0.107
Milk yield (g/day)	1718	1404	179.8	0.099
Energy-corrected milk (g/day)	1438	1143	175.8	0.112
Total solids (g/kg)	16.4	16.1	0.28	0.331
Fat (g/kg)	59.8	58.6	2.52	0.608
Protein (g/kg)	45.8	46.5	0.79	0.367
Lactose (g/kg)	47.9	46.5	0.98	0.169
Urea (mg/kg)	464	459	29.8	0.861
Acetone (mmol/L)	0.176	0.129	0.045	0.306
BHB (mmol/L)	0.121	0.134	0.03	0.683
Log (SCC)	4.76	5.38	0.508	0.237

LBW- live body weight; ADG- average daily gain; DM- dry matter; INRA- Institut National de la Recherche Agronomique; AFRC- Agricultural and Food Research Council; BHB- beta-hydroxybutyrate; SCC- somatic cell count.

Table 3
Fatty acid profile (%) of milk samples obtained during the peak lactation period of F1 Assaf dairy sheep born from ewe lambs (F0) being fed the control or the methionine-supplemented diet during the early life (suckling period).

	F1-CTRL	F1-MET	SED	P-value
Total SFA	63.3	64.6	0.8413	0.166
C6:0	0.028	0.043	0.0050	0.009
C8:0	2.35	3.14	0.2001	0.001
C9:0	0.071	0.095	0.0146	0.119
C10:0	5.62	7.09	0.5211	0.012
C12:0	3.57	4.50	0.3617	0.021
C13:0	0.022	0.005	0.0076	0.037
C14:0	7.26	7.34	0.3391	0.783
C14:0 *iso*	0.126	0.150	0.0170	0.185
C14:0 *anteiso*	0.132	0.131	0.0089	0.906
C15:0	1.02	0.949	0.0865	0.453
C15:0 *iso*	0.233	0.205	0.0201	0.196
C15:0 *anteiso*	0.399	0.374	0.0392	0.536
C16:0	27.2	26.2	0.9646	0.319
C16:0 *iso*	0.285	0.316	0.0411	0.466
C17:0	0.798	0.738	0.0501	0.259
C17:0 *iso*	0.478	0.441	0.0630	0.573
C17:0 *anteiso*	0.571	0.526	0.0929	0.644
C18:0	12.4	11.6	0.7884	0.286
C18:0 *iso*	0.046	0.045	0.0177	0.937
C20:0	0.372	0.390	0.0316	0.584
C21:0	0.084	0.088	0.0152	0.792
C22:0	0.170	0.183	0.0178	0.495
C23:0	0.048	0.045	0.0129	0.797
C24:0	0.036	0.036	0.0115	0.976
Total MUFA	28.7	26.4	0.8506	0.015
C10:1*c*9	0.283	0.389	0.0329	0.005
C12:1*c*9	0.020	0.027	0.0085	0.416
C14:1*c*9	0.125	0.120	0.0113	0.670
C15:1*c*10	0.054	0.069	0.0105	0.177
C16:1*t*9	0.102	0.077	0.0323	0.465
C16:1*c*7	0.302	0.350	0.0420	0.279
C16:1*c*9	0.511	0.477	0.0423	0.446
C16:1*c*13	0.000	0.010	0.0103	0.354
C17:1*c*9	0.292	0.218	0.0306	0.030
C18:1*t*6 +*t*9 +*t*10	1.51	2.06	0.3091	0.096
C18:1*c*9	25.2	22.3	0.8818	0.004
C18:1*c*11 +*c*12	0.255	0.281	0.0513	0.628
Total PUFA	7.99	9.09	0.4548	0.029
C18:2*t*9,*t*12	0.204	0.250	0.0266	0.108
C18:2*n*6 (LA)	5.63	6.44	0.3551	0.039
C18:2*c*9,*t*11(CLA)	0.508	0.617	0.0870	0.235
C18:3*n3*	0.852	0.976	0.0538	0.036
C20:2*n*6	0.015	0.002	0.0044	0.009
C20:3*n*6	0.040	0.040	0.0081	1.000
C20:4*n6* (ARA)	0.407	0.463	0.0339	0.125
C20:5*n*3 (EPA)	0.072	0.065	0.0135	0.596
C22:4*n*6	0.065	0.062	0.0135	0.820
C22:4*n*3	0.021	0.022	0.0077	0.919
C22:5*n*3 (DPA)	0.131	0.108	0.0243	0.370
C22:6n3 (DHA)	0.047	0.046	0.0104	0.953
Total BCFA	2.27	2.18	0.1624	0.618
Total OCFA	4.07	3.76	0.2342	0.213
Total trans-FA	2.33	3.01	0.3709	0.089
Total *n*3	1.12	1.22	0.0718	0.182
Total *n*6	6.16	7.00	0.3695	0.037
PUFA/SFA	0.127	0.142	0.0077	0.075
n6/n3	5.52	5.81	0.3055	0.369

SFA- saturated fatty acid; MUFA- monounsaturated fatty acid; PUFA- poly unsaturated fatty acid; LA – linolenic acid; CLA - isomer of conjugated linoleic acid -rumenic acid-; ARA - arachidonic acid; EPA - eicosapentaenoic acid; DPA - docosapentaenoic acid; DHA - docosahexaenoic acid; Total BCFA - total branched chain fatty acids (C14:0 *iso*, C14:0 *anteiso*, C15:0 *iso*, C15:0 *anteiso*, C16:0 *iso*, C17:0 *iso*, C17:0 *anteiso*, C18:0 iso); Total OCFA - total odd-chain fatty acids (C9:0, C13:0, C15:0, C15:0 *iso*, C15:0 *anteiso*, C15:1*c*10, C17:0 *iso*, C17:0 *anteiso*, C17:0, C17:1*c*9; C21:0, C23:0).

3. Results

3.1. Animal performance, feed efficiency, milk composition and fatty acid profile

Animal performance data are summarized in Table 2. As can be observed, the F1-MET ewe lambs showed reduced live weight (LBW) at birth and mating ($p < 0.05$), thus gaining less weight and therefore being smaller in adulthood (lactation period) when compared to F1-CTRL animals. No differences were observed for indicators of reproductive performance such as the age of puberty ($P = 0.364$) or progesterone concentration ($P = 0.562$).

Regarding the milking period, no differences were found for feed intake ($P = 0.607$), dry matter digestibility ($P = 0.344$) and RFI ($P = 0.194$). However, feed conversion rate was reduced for F1-MET (1.41 vs. 1.83 kg DM/kg milk, $P = 0.047$), whereas milk yield (recorded on days 1, 3, 7, 10, 14, 17, 21, 24, 28, 31 and 35) presented a tendency to lower production when compared to F1-CTRL ewes (1718 *vs.* 1404 g/day, $P = 0.099$). No differences were observed in the chemical composition of milk (total solids, fat, protein, lactose, urea) or SCC. Nevertheless, the milk FA profile of F1 presented differences between FA groups (Table 3). The percentages of most of the shorter-chain (C6-C13) saturated FAs (SFAs) were higher in the F1-MET milk, but total SFA content was similar for both groups. Moreover, total monounsaturated FAs (MUFAs) and oleic acid (C18:1*c*9) percentages were lower in the milk of F1-MET animals when compared to F1-CTRL dams. In contrast, the percentages of total polyunsaturated FAs (PUFAs), total *n*3 PUFAs, and the two main PUFAs, linoleic (C18:2*n*6) and linolenic acids (C18:3*n*3), were higher in F1-MET milk. Consequently, the PUFA/SFA ratio tended to be higher in the F1-MET group ($P = 0.075$) when compared to F1-CTRL.

3.2. RRBS methylation analysis

The average level of methylation in the two groups varied between 60.0 % and 61.7 % [Supplementary Material 1 (QC)], and high methylation correlation was observed between all the samples (correlation 0.96–0.97, including F1-CTRL and F1-MET; Supplementary Figure S1), so methionine supplementation during the early life of dams (F0) did not promote detectable differences in global blood methylation patterns of F1. Differentially methylated regions (DMRs) were assessed at 62,585 and 406 sites using MethylKit (q value < 0.05) and CGmap Tools (FDR < 0.05), respectively. All this information can be found in detail in Supplementary Material 1.

According to STRING, the functional gene enrichment analysis of F1 (offspring) revealed differentially methylated regions (MethylKit) for genes involved in 49 biological processes (GO), 2 molecular functions, and 17 cellular components [Supplementary

Table 4
Thirty most significant gene ontology enrichment terms from the list of genes showing differentially methylated regions in the female progeny (F1-MET) of ewe lambs being supplemented methionine during the early life.

Functional Category	Enrichment FDR	Total genes	Genes in list[a]
Developmental process	3.01E-07	4531	401
Anatomical structure development	3.01E-07	4118	372
Multicellular organism development	1.60E-06	3345	308
System development	1.60E-06	3005	282
Generation of neurons	4.80E-05	933	109
Cell projection organization	1.00E-04	999	113
Neuron differentiation	1.00E-04	872	102
Plasma membrane bounded cell projection organization	1.00E-04	968	110
Neurogenesis	1.70E-04	1054	116
Cell morphogenesis	1.80E-04	609	77
Neuron development	1.80E-04	697	85
Cell morphogenesis involved in neuron differentiation	1.80E-04	375	55
Cell morphogenesis involved in differentiation	1.90E-04	483	65
Cellular developmental process	3.30E-04	2846	253
Cell differentiation	3.70E-04	2827	251
Cell projection morphogenesis	3.70E-04	400	56
Nervous system development	4.00E-04	1588	156
Regulation of cell projection organization	4.00E-04	477	63
Axon development	4.00E-04	331	49
Cell part morphogenesis	4.10E-04	416	57
Neuron projection development	4.30E-04	558	70
Plasma membrane bounded cell projection morphogenesis	4.30E-04	397	55
Axonogenesis	4.50E-04	306	46
Anatomical structure morphogenesis	4.50E-04	1817	173
Regulation of plasma membrane bounded cell projection organization	5.70E-04	467	61
Cell development	1.10E-03	1436	141
Animal organ development	1.10E-03	2524	224
Neuron projection morphogenesis	1.10E-03	393	53
Regulation of cellular component organization	1.50E-03	1955	180
Positive regulation of biological process	1.60E-03	5423	429

FDR- false discovery rate
[a] Number of genes showing differentially methylated regions in the functional category

Material 1 (MethylKit.STRING pathway)]. The 30 most significant terms are compiled in Table 4. Among them, 19 of the main functional categories significantly affected in F1-MET ewe lambs were related to anatomical development, cell morphogenesis and organization, whereas 10 terms were related to neurons and axons. The last GO term was associated with the positive regulation of other biological processes. All this information, together with the pathways identified by gProfiler2 (using DMRs detected by both MethylKit and CGmap Tools), is detailed in Supplementary Material 1.

3.3. Blood biochemical profile

The effects of methionine supplementation during the suckling period of ewe lambs (F0) on the biochemical profile of the female offspring (F1) are shown in Table 5. The female offspring (F1-MET) born from early supplemented methionine ewe lambs (F0) showed increased insulin concentrations ($P = 0.031$), particularly during the pre-weaning phase (T1), these differences being decreased during the replacement (T2) and lactation (T3) periods, but being still significantly higher when compared to those observed for F1-CTRL animals. Moreover, F1-MET animals presented greater blood concentrations of β-hydroxybutyrate (BHB, $P = 0.043$), particularly during the lactation period (T3), when compared to F1-CTRL animals. Additionally, creatine kinase ($P = 0.061$) and triglycerides ($P = 0.058$) also presented a tendency to increased values in F1-MET animals. Other blood parameters, including albumin, protein, urea, AST, GGT, total bilirubin, creatinine, glucose, cholesterol, HDL, LDL, NEFA, calcium, magnesium and zinc showed no significant differences between F1 groups.

3.4. Metabolomics analysis (Mid-Lactation Period)

The untargeted metabolomic analysis carried out according to the protocol described by Dehnavi et al. (2025) identified 148 metabolites shared by all 21 samples. The clustering of samples from each group in separate areas of the PLS-DA space shows the impact of methionine supplied to F0 on the offspring (F1), so both groups of F1 animals (F1-CTRL vs. F1-MET) can be separated according to their metabolome profile (Fig. 1A). The heat map illustrates the top 25 metabolites after hierarchical clustering of the samples, however, hierarchical clustering in the heat map indicated partial but not complete separation between the two groups (Fig. 1B). However, the volcano plot highlighted seven metabolites differentially accumulated in F1-MET ewes (Fig. 1C). Accordingly, tetradecanedioic acid, L-3-phenyllactic acid, taurocholic acid, and taurodeoxycholic acid were increased in the F1-MET relative to the F1-CTRL group, whereas phenylacetylglycine was reduced in the F1-MET. Although ursodeoxycholic acid and deoxycholic acid also decreased in F1-MET animals, the changes were not statistically significant (Fig. 1C and Table 6).

3.5. Gut microbiome (Mid-Lactation Period)

The gut microbiome diversity in F1 ewes (F1-CTRL and F1-MET groups) was studied using 16S rRNA sequencing. Alpha diversity

Table 5
Biochemical parameters of the suckling (T1, pre-weaning), replacement (T2) and lactation (T3) of the Assaf female offspring (F1-CTRL vs. F1-MET) born from Assaf dairy sheep being fed the control (F0-CTRL) or the methionine-supplemented diet (F0-MET) during the early life (suckling period).

	Group		Growth stage					P-value		
	F1-CTRL	F1-MET	T1	T2	T3	SED_a	SED_b	Group	Day	G*Day
Albumin (g/L)	39.2	38.7	33.2^a	41.2^b	42.4^b	0.805	1.033	0.542	0.000	0.584
Protein (g/L)	63.9	63.6	47.5^a	72.5^b	71.3^b	0.777	1.125	0.702	0.000	0.495
Urea (mg/dL)	38.8	39.1	23.9^a	44.6^b	48.3^b	2.150	2.196	0.897	0.000	0.872
AST (U/L)	114	123	75.7^a	123^b	157^c	12.9	13.9	0.481	0.000	0.767
GGT (U/L)	86.6	88.6	100.6^b	79.5^a	79.7^a	6.526	5.895	0.759	0.001	0.720
Creatine kinase (U/L)	195	260	344^b	181^b	159^b	32.42	41.11	0.061	0.001	0.212
Creatinine (mg/dL)	0.680	0.669	0.371^a	0.874^c	0.777^b	0.0375	0.0344	0.762	0.000	0.342
Glucose (mg/dL)	70.3	71.0	99.3^a	48.6^c	64.1^b	3.782	3.766	0.857	0.000	0.886
Insulin (uUI/mL)	33.5	45.1	75.1	18.5	24.4	4.934	5.454	0.031	0.000	0.892
Total bilirubin (mg/L)	0.206	0.228	0.318^a	0.179^b	0.153^b	0.0130	0.0181	0.106	0.000	0.575
Triglycerides (mg/dL)	24.3	32.5	42.3^b	18.0^a	25.0^a	4.024	4.985	0.058	0.000	0.118
Cholesterol (mg/dL)	85.3	82.0	93.1^b	72.5^a	85.4^b	5.809	4.673	0.579	0.001	0.599
HDL (mg/dL)	63.5	62.3	68.5^b	52.2^a	68.1^b	4.139	2.805	0.777	0.000	0.813
LDL (mg/dL)	17.9	16.6	18.8^a	17.7^{ab}	15.3^b	1.528	1.481	0.407	0.046	0.185
NEFA (mmol/L)	0.216	0.249	0.200	0.283	0.215	0.0306	0.0370	0.297	0.086	0.029
BHB (mg/dL)	2.21	2.84	1.31^a	2.14^a	4.14^b	0.288	0.396	0.043	0.000	0.919
Ca (mg/dL)	9.73	9.74	11.02^b	9.16^a	9.03^a	0.184	0.211	0.978	0.000	0.388
Mg (mg/dL)	3.12	3.12	3.39^b	2.80^a	3.17^b	0.098	0.078	0.960	0.001	0.720
Zn (ug/dL)	169	165	117^a	192^b	191^b	6.090	6.652	0.501	0.000	0.071

F1-MET – female offspring born from dams being supplemented 1 g of D,L-methionine (DM basis) in the milk replacer; Growth stage - T1 (end of the methionine supplementation period, 45 days of life); T2 -synchronization period before mating; T3 - mid-lactation period; AST - aspartate amino-transferase; BHB - beta-hydroxybutyrate; NEFA - non-esterified fatty acids; HDL - high-density lipoprotein; LDL - low-density lipoprotein; GGT - gamma-glutamyl transpeptidase; SED_a - standard error of the difference to compare experimental groups; SED_b - standard error of the difference to compare days; different subscripts in the different line indicate significant differences among sampling times

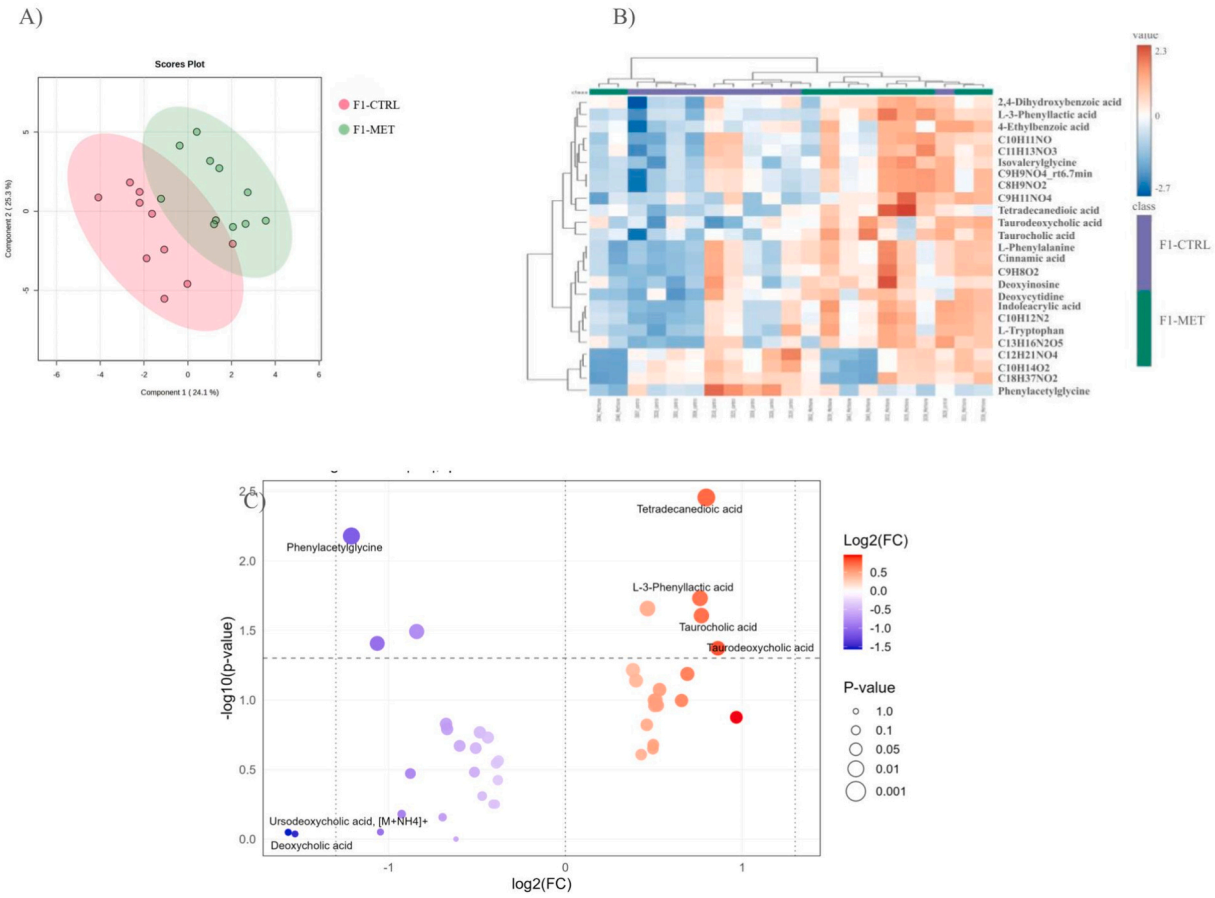

Fig. 1. Metabolome analysis of plasma samples collected during the mid-lactation period from the Assaf female offspring (F1-CTRL, n = 9 and F1-MET, n = 11). (A) Partial least-squares discriminant analysis (PLS-DA) of the plasma metabolome of F1-CTRL (red circles) and F1-MET (green circles) groups. Percentages of variation explained by each component are shown along the axes. (B) The top 25 discriminant metabolites are shown in a heatmap. The F1-MET and F1-CTRL groups are indicated by boxes above the sample numbers. The red shading indicates high abundances, while the blue shading indicates low abundances. (C) Volcano plot shows the ratio of blood metabolites in the F1-MET to the F1-CTRL groups. This graph displays the log2 fold change on the x-axis and the -log10 P value on the y-axis. The blue circles show significant reductions in metabolites in the F1-MET group compared to the F1-CTRL group, while the red circles indicate significant increases in metabolites.

comparisons (ACE, Chao1, Fisher, Inverse Simpson, Observed, Shannon, Simpson, Figure S2A and S2B) and beta diversity analysis (Bray-Curtis; Figure S3A and S3B) showed no significant differences between the two groups. Neither significant difference was found when DESeq2 analysis was performed on the filtered OTU data to identify differentially abundant microbial taxa in the F1-CTRL and F1-MET groups at both the genus and family taxonomic levels.

In the histogram of adjusted P-values (p_{adj}) most taxa did not show any difference between the two groups ($p_{adj} = 1.0$) (Fig. 2A). Moreover, among the top 20 families and genera ranked by absolute log2FC values only *Spirochaetaceae* family (Fig. 2B) and *Treponema* genus (Fig. 2C), both from the phylum *Spirochaetes*, were lower in the F1-MET group when compared to F1-CTRL (log2FC = –3.96, $p_{adj} < 0.05$).

4. Discussion

Nutritional programming events during early life (e.g., postnatal) elicit the establishment of epigenetic marks that can be transmitted through the germline across multiple generations. Thus, some of these marks can persist for up to F3 or later generations, potentially impacting the phenotype of subsequent offspring. In contrast, others are erased during early development due to epigenetic reprogramming events (Heard and Martienssen, 2014). These reprogramming episodes can explain why F1 offspring studied in this experiment presented phenotypic traits (Table 2) aligned with the differential methylation patterns observed in F0 mothers (Dehnavi et al., 2025), even though the epigenetic marks differed slightly between the two generations, as explained below. Nevertheless, the low body weight of F1-MET animals (Table 2) and the enriched pathways in anatomical structure development (GO:0048856) and morphogenesis (GO:0009653) [Table 4 and Supplementary Material 1 (MethylKit.STRING pathway) align with the significantly enriched pathways observed in F0-MET ewe lambs [e.g., bone cell development (GO:0098751) or osteoclast development

Table 6
List of metabolites up- or down-accumulated in plasma samples of Assaf female offspring (F1-MET, lactation period) born from ewe lambs being supplemented methionine during the early life.

Metabolites	log2FC[a]	Regulation
Ursodeoxycholic acid, $[M+NH_4]+$	-1.5696	down
Deoxycholic acid	-1.5311	down
Phenylacetylglycine	-1.2118	down
$C_{12}H_{21}NO_4$	-1.0655	down
Nutriacholic acid	-1.0475	down
Serotonin	-0.92731	down
Lithocholic acid	-0.87774	down
$C_{10}H_{14}O_2$	-0.84232	down
Cholic acid	-0.6958	down
Elaidic acid	-0.67598	down
1-Methylhistidine	-0.67003	down
$C_{12}H_{24}O_3$	-0.61939	down
Decanoylcarnitine (C_{10})	-0.59872	down
C_9H_9NO	-0.51524	down
$C_9H_9NO_4$	-0.51524	down
Undecanedioic acid	-0.50809	down
Octanoylcarnitine (C_8)	-0.48466	down
Homocitrulline	-0.47171	down
Indoleacetic acid	-0.4393	down
3-Cresotinic acid	-0.41102	down
$C_7H_{12}N_2O_3$	-0.397	down
Stearic acid	-0.39202	down
Octadecenoylcarnitine (C18:1)	-0.3829	down
Hexanoylcarnitine (C_6)	-0.37946	down
$C_{13}H_{16}N_2O_5$	0.38204	up
Isovalerylglycine	0.39934	up
Ascorbic acid	0.42828	up
Kynurenic Acid	0.45995	up
Deoxyinosine	0.46432	up
Glycocholic acid	0.49484	up
Cholesterol sulfate	0.49679	up
$C_{11}H_{13}NO_3$	0.5025	up
$C_9H_9NO_4$_rt6.7 min	0.50459	up
4-Ethylbenzoic acid	0.51267	up
$C_{10}H_{11}NO$	0.52068	up
$C_8H_9NO_2$	0.53245	up
$C_9H_{11}NO_4$	0.65693	up
2,4-Dihydroxybenzoic acid	0.68969	up
L-3-Phenyllactic acid	0.76152	up
Taurocholic acid	0.76945	up
Tetradecanedioic acid	0.79721	up
Taurodeoxycholic acid	0.86279	up
$C_9H_{10}O_3$	0.96703	up

[a] Positive values of log2FC refer to up-accumulated metabolites in F1-MET when compared to F1-CTRL ewes, whereas negative values mean that the metabolites were down-accumulated

(GO:0036035)] as a consequence of different methylation of the *ATP6AP1* gene (Dehnavi et al., 2025).

Furthermore, F1-MET animals exhibited variations in the milk FA profile (Table 3). The higher levels of short-chain fatty acids in F1-MET may have an impact on the flavor of the milk, as well as higher levels of PUFAs, which are considered desirable from a health standpoint. Consistently with the milk FA profile, some DMRs for the *CPT1B* and *CYP2J* genes [Supplementary Material 1 (MethylKit. DMR)] were identified in F1 animals, with the latter playing a role in the metabolism of various endogenous substrates, including PUFAs. These findings for F1-MET agree with the GO pathways significantly enriched in F0-MET animals [(e.g., long-chain fatty acid transport (GO:0015909) and fatty acid beta-oxidation (GO:0006635) due to differential methylation of the *CPT1B* gene (Dehnavi et al., 2025)]. Moreover, the milk FA profile of F0-MET also presented higher PUFA levels (Dehnavi et al., 2025) thus suggesting possible intergenerational transmission of both epigenetic marks (e.g., DMR via germline of F0) and phenotypic traits (milk FA profile) to the offspring (F1). The altered behavior exhibited by F0-MET dams (Dehnavi et al., 2025) together with the enriched pathways linked to neuron differentiation, neurogenesis and neural development observed in F1-MET (Table 4) point towards the same conclusion.

Similarly, F0-MET animals (Dehnavi et al., 2025) and F1-MET ewes presented common traits in the biochemical profile (Table 5), also suggesting intergenerational transmission of some traits related to altered energy and lipid metabolism. For example, F1-MET animals showed higher values of BHB and increased insulin levels, both parameters being indicators of insulin resistance (also observed in F0-MET ewes; Dehnavi et al., 2025). Accordingly, the LBW change during lactation (-8.26 vs. 55.4 g/day for F1 CTRL and F1-MET, respectively; $P = 0.107$; Table 2) suggests a trend towards accumulation of fat depots and limited lipo-mobilization in F1-MET ewes that might have hampered the ability to meet the energy demands required for milk synthesis (Contreras et al., 2010),

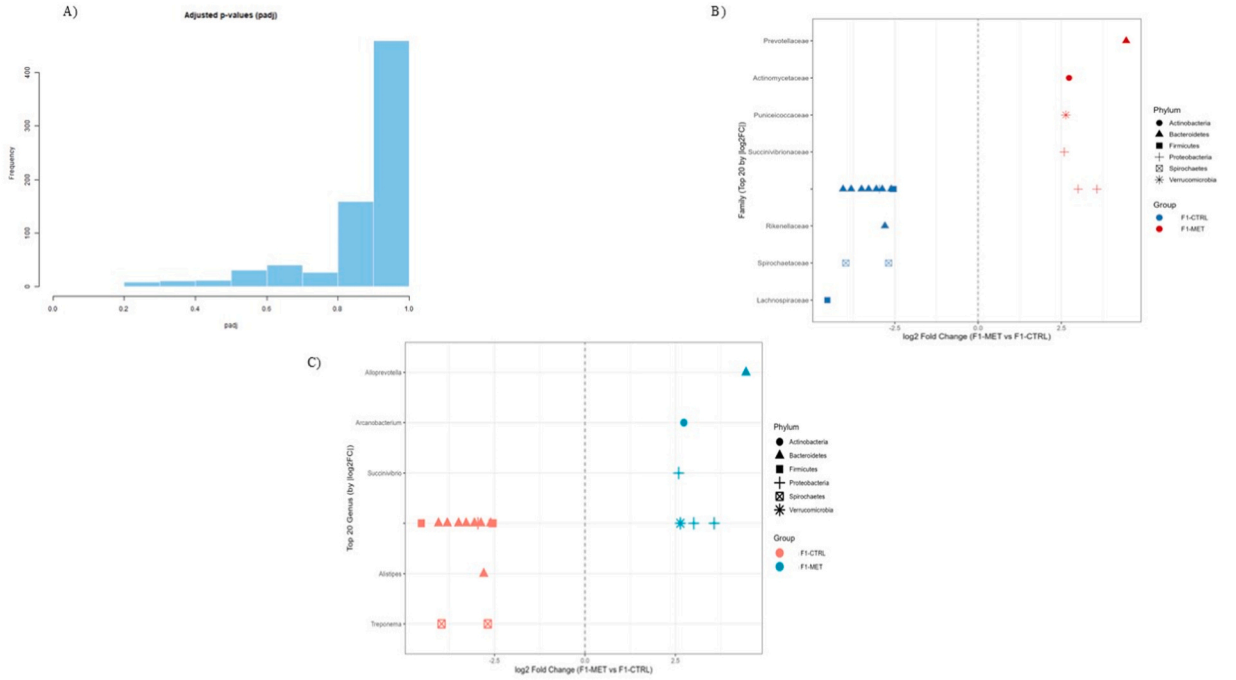

Fig. 2. Differential abundance analysis of microbial taxa between F1-MET and F1-CTRL groups using DESeq2. (A) Histogram of adjusted p-values (p_{adj}) showing the distribution of differential abundance test results for all taxa. Most taxa showed no significant group-level differences ($p_{adj} = 1.0$). (B) Dot plot of the top 20 microbial families by absolute log2FC. A greater abundance of the top 20 family is shown in the F1-MET group with positive log2FC value in blue, whereas a higher abundance is shown in the F1-CTRL group with a negative log2FC value in red. Several phyla are presented, including *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*, but only *Spirochaetaceae* reach statistical significance. (C) Dot plot of the top 20 genera by absolute log2FC. A greater abundance of the top 20 genus is shown in the F1-MET group with positive log2FC value in blue, whereas a higher abundance is shown in the F1-CTRL group with a negative log2FC value in red. *Treponema* was the only genus significantly reduced in F1-MET group.

thus explaining the lower production observed in this group of animals when compared to F1-CTRL ewes (Table 2).

Accumulation of fat depots or limited lipo-mobilization in F1-MET ewes during the lactation period might be also explained, at least partially, by the metabolomics analysis. In this case, tetradecanodioic acid was increased in the plasma of F1-MET ewes (Table 6). In agreement with the results described so far (e.g., small size, milk FA profile, enriched GO terms observed in the F1-MET ewes), this metabolite has been suggested as a biomarker of altered beta-oxidation pathway (e.g., altered lipid metabolism) and osteoporosis (Wu et al., 2024). Moreover, two KEGG pathways were enriched: 1) taurine and hypotaurine metabolism and 2) primary bile acid biosynthesis. Therefore, differences in biliary acids metabolism might be also at least partially responsible for the reduced body weight observed in this nutritionally programmed group of dams (Table 2). In this sense, it is well known that cholic acid and chenodeoxycholic acid are primary bile acids (synthesized from cholesterol in hepatocytes) which are conjugated with glycine or taurine to form bile salts (or conjugated bile acids) such as taurocholic acid. These bile salts are stored in the gallbladder and released into the intestine, where some of them are deconjugated and converted into secondary bile acids (e.g., deoxycholic acid and lithocholic acid) by gut bacteria. Finally, bile acids are reabsorbed and reintroduced into the enterohepatic circulation, so they can be conjugated again with glycine or taurine in the liver. In the present study (Table 6), taurocholic acid (a primary bile salt formed by the conjugation of the primary bile acid cholic acid with taurine; Kase et al., 1986) and taurodeoxycholic acid (a secondary bile salt formed by the conjugation of the secondary bile acid deoxycholic acid with taurine) were up-accumulated in the plasma of F1-MET ewes, whereas secondary bile acids produced by intestinal bacteria (e.g., deoxycholic acid and ursodeoxycholic acid) were decreased. All these compounds enable the emulsion and digestion of fats in the intestine, taking part of digestion and absorption of dietary fats and fat-soluble vitamins. Consequently, their differential accumulation in the plasma of F1-MET ewes may indicate hepatic dysfunction, altered bile acid metabolism, or even modified gut microbiota in these group of animals that might be at least partially responsible for modified lipid digestion, and therefore absorption and synthesis of fat depots. These features, together with the significantly enriched GO terms involved in cell differentiation and morphogenesis (Table 4) and the significantly increased levels of creatine kinase (an indicator of muscle damage, Table 5) seem to be pieces of the same puzzle explaining the lower body weight observed in F1-MET animals when compared to F1-CTRL ewes.

It is also remarkable that some microbial-derived metabolites (e.g., phenylacetylglycine and indole acetic acid) were significantly down-accumulated or up-accumulated (e.g., L-3-Phenyllactic acid) in F1-MET ewes (Table 6). These metabolites have been associated with the gut-brain axis (Swer et al., 2023; Toft et al., 2023; Krishnamoorthy et al., 2024), so these data corroborate the findings previously mentioned for both, F0-MET and F1-MET as far as neurological disorders is concerned. Consequently, the gut microbiome

may also have played a role in the phenotype of F1-MET ewes. Accordingly, the relative abundance of the family *Spirochaetaceae*, which is involved in the degradation of carbohydrates, was significantly reduced in the F1-MET group when compared to F1-CTRL ewes. The decrease in this part of the microbiota in the gut of the F1-MET animals may have impacted nutrient availability and live body weight, given the fact that as anaerobic fermenters they break-down glucose (glycolysis) to produce energy, with end-products like ethanol, acetate, lactate. Nevertheless, even though the dry matter digestibility presented numerically lower values for F1-MET ewes during the lactation period (Table 2), the differences between both groups did not reach the significance level ($P = 0.344$).

Finally, it must be stated that all the findings described in the present study are limited to the first lactation of the F1 ewe lambs, so conclusions about persistent effects on further lactations, or subsequent generations (F2, F3) cannot be inferred. Moreover, the intergeneration transmission of epigenetic marks was studied on peripheral blood samples (e.g., white blood cells), and similar patterns of differential methylation were assumed in other target tissues with a greater metabolic relevance (e.g., liver or mammary gland). Although this is a proxy to reduce pain according to the Three Rs principle in animal experimentation, it must be also stressed as a clear limitation of the study.

5. Conclusions

Under the conditions of the present study, it can be concluded that 0.1 % D,L-methionine included in the milk replacer (DM basis) during the early post-natal life of ewe lambs (F0) provokes permanent changes that are transferred to the progeny (F1, females) through the germline. These animals exhibit a reduced size and phenotypic traits compatible with limited lipomobilization, which explains the reduced milk production observed during the lactation phase. Therefore, the intergenerational effects observed in the offspring (F1) raises concerns about the convenience of supplementing methionine during the postnatal period of ewe lambs (F0) kept in the farm for replacement purposes. Further experiments will be required to confirm causality and molecular mechanisms observed.

Author contributions

SA and FG contributed to the conception and design of the study. SA, FG, MD and AM conducted the in vivo study. FG and SL conducted statistics of the in vivo study. SA and FG listed the differentially methylated genes and performed the statistical analysis of the in vivo study. JM and NS analysed the milk fatty acid profile. FC FB and PC sequenced the DNA microbiome samples and conducted the bioinformatics analyses. FF, and NS carried out the metabolome analysis and the metabolomic statistical analysis. SA and MD wrote the first draft of the manuscript. AM revised the previous literature and wrote sections of the manuscript. All authors contributed to writing, revising, and reading the manuscript before submission.

CRediT authorship contribution statement

Paola Cremonesi: Methodology, Investigation. **Filippo Biscarini:** Writing – review & editing, Software, Methodology, Investigation. **Fiorenza Faré:** Writing – review & editing, Methodology. **Secundino López:** Writing – review & editing, Investigation. **Nuria Santos:** Writing – review & editing, Software, Methodology. **Mahsa Dehnavi:** Writing – original draft, Investigation. **Andres Sonia:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Javier Mateo:** Writing – original draft, Supervision, Methodology, Formal analysis. **Alba Martín:** Methodology, Investigation. **Fabrizio Ceciliani:** Writing – review & editing, Methodology, Investigation. **Firáldez F. Javier:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2026.116640.

Data availability

The RRBS data used in this study are available from the following digital source. CSIC accession number 389409 (http://hdl.handle.net/10261/389409). The 16S rRNA gene sequences obtained from this study were deposited in the NCBI SRA repository under Bioproject ID PRJNA1273996 (SUB15375344). http://www.ncbi.nlm.nih.gov/bioproject/1273996.

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