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Essential oil supplementation in milk replacers: short- and long-term impacts on feed efficiency, the faecal microbiota and the plasma metabolome in dairy calves

Sonia Andrés[1], Chiara Gini[2], Fabrizio Ceciliani[2], Daniel Gutiérrez-Expósito[1,3], Noive Arteche-Villasol[1,3], Alba Martín[1], Paola Cremonesi[4], Fiorenza Faré[5], Morteza Hosseini Ghaffari[6], F. Javier Giráldez[1] and Latifa Abdennebi-Najar[7,8]

[1]Instituto de Ganadería de Montaña (CSIC-Universidad de León), León, Spain; [2]Department of Veterinary Medicine and Animal Sciences, Università degli Studi di Milano, Lodi, Italy; [3]Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de León, León, Spain; [4]Department of Bioinformatics, Biostatistics, Genomics, Institute of Agricultural Biology and Biotechnology, IBBA-CNR, Milano, Italy; [5]Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milano, Italy; [6]Institute for Animal Science Physiology Unit, Rheinische Friedrich-Wilhelms-Universität Bonn, Bonn, Germany; [7]Quality and Health Department, IDELE Institute, Paris, France and [8]Centre de Recherche Saint-Antoine (CRSA), Sorbonne University, Paris, France

Original Article

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Corresponding author:
S. Andrés; Email: sonia.andres@eae.csic.es

Abstract

Early supplementation with oregano essential oil (EO) in milk replacer (MR) may improve growth, immune responses, the microbiota and the metabolome in dairy calves during pre-weaning and in adulthood. Sixteen female dairy calves (3 days of age) were divided in two groups ($n = 8$/group): the control group (no EO) and the EO group (0.23 ml of EO in MR during 45 days). After weaning, calves were kept in a feedlot and fed *ad libitum*. The animals were weighed, and blood and faecal samples were collected on days 3 (T0), 45 (T1) and 370 (T2) to measure the biochemical profile and characterise peripheral blood mononuclear cells (PBMCs; $CD4^+$, $CD8^+$, $CD14^+$, $CD21^+$ and $WC1^+$), the metabolome and microbiota composition. The EO group only had greater average daily weight gain during the suckling (EO supplementation) period ($P = 0.030$). The EO group showed higher average $CD14^+$ population (monocytes) values, a lower abundance of *Ruminococcaceae UCG-014*, *Faecalibacterium*, *Blautia* and *Alloprevotella* and increased abundances of *Allistipes* and *Akkermansia*. The modification of some metabolites in plasma, such as butyric acid, 3-indole-propionic acid and succinic acid, particularly at T1, are consistent with intestinal microbiota changes. The data suggest that early EO supplementation increases feed efficiency only during the suckling period with notable changes in the microbiota and plasma metabolome; however, not all of these changes can be considered desirable from a gut health point of view. Additional research studies is required to demonstrate that EOs are a viable natural alternative to antibiotics for improving calf growth performance and health.

Introduction

Developmental programming refers to the influence of pre- and postnatal factors that affect growth and development and result in long-term consequences for the health and productivity of the animals. Early life is a susceptible window in which different factors may affect the developmental plasticity of the organism, with consequences throughout the lifespan[1,2] In the past decades, nutritional events have been considered the most relevant factors driving metabolic programming and phenotypic expression in dairy cattle; thus, it has been demonstrated that milk yield potential can be negatively affected by malnutrition during the preweaning period.[3] It has recently been hypothesised that one of the factors involved in this long-term effect is microbial gut colonisation during early life, which shapes the immune response throughout life by providing stimulatory signals that activate innate and adaptative immune responses.[4] Once established, the microbiota composition often results in permanent effects on the host' phenotype. Therefore, the microbiota can be envisaged as a key participant in the Developmental Origins of Health and Disease.[5] Accordingly, manipulating the gut microbiome of livestock animals during early life may be an effective way to achieve long-term health benefits, thus improving feed efficiency traits.[6,7]

Dietary administration of essential oil (EO) has shown an impact on rumen fermentation processes, including less methane production,[8] decreased degradation of feed protein, or increased feed efficiency via selection for or against specific groups of microorganisms in the rumen.[9,10] The potential of EOs to modulate the gut microbiome during early life (when

included in milk replacer for dairy calves) is a less-explored strategy to 'program' long-term effects. Pieces of evidence have revealed a promising impact on the growth, feed efficiency, nutrient digestibility and immunity of neonatal calves when supplementing a blend of EO 70 days from birth,[11] together with increased concentrations of propionate and a higher abundance of *Prevotella ruminicola*.[12] Targeting calves' in early life by supplementing them with the appropriate amount of EO to increase feed efficiency and/or improve the animal's health status would reduce antibiotic use and allow them to boost their immunity, thus preventing diseases in the adult stage.[13,14] Moreover, this strategy may provide farmers with opportunities to enhance future cattle dairy performance by paying attention to appropriate early intervention, guaranteeing efficient neonate nutrition and profitable cattle production.

The present study hypothesises that EO administration during the first 45 days of life could promote short and long-term effects on feed efficiency traits, immune status, the microbiota and the plasma metabolome when included in milk replacer (MR) for newborn dairy calves.

Material and methods

Care and use of animals

All handling practices followed the recommendations of Directive 2010/63/EU of the European Parliament, the Council on Protecting Animals used for scientific purposes, and the IGM-CSIC Animal Experimentation Committee (protocol 736/2018). The study reported in the manuscript follows the recommendations in the ARRIVE guidelines.[15]

Animals, diets and feed efficiency measurement

CSIC conducted feeding trials using 16 female dairy calves born in the same commercial farm and selected to minimise the range of live body weight (LBW) and day of birth. These animals were separated from the cows in the first 24 hours of life and fed 3 L of colostrum daily until day 3 of life. Starting at 3 days, once housed in individual straw-bedded hutches, they were allocated to one of two groups ($n = 8$ per group; control and EO) balanced according to the body weight at birth. Calves were fed MR (145 g of Novilac Turbostart, Schils/L) twice a day using a bucket with a nipple (allowing the reflex closure of the oesophageal groove) until day 45. During this phase, these animals were fed daily a fixed amount of reconstituted MR (divided into two feedings, at 09:00 a.m. and 6:00 p.m.) that was gradually increased from 3 to 7 L at the same rate for all the individuals.

Moreover, the animals in the EO group were supplied daily with 0.23 ml of oregano EO, accounting for 200 mg of carvacrol (Zane Hellas 100%) diluted in the first 100 mL of MR for 45 days. No concentrates were offered, and no leftovers of MR remained during this phase, so dry matter intake was similar for all calves. All the animals were weighed at birth and on days 4, 10, 25 and 45 of life (e.g., at the end of the EO administration period) to estimate the average daily gain (ADG) and then calculate the feed-to-gain conversion rate during the suckling period.

Animals were managed in a feedlot once they reached 45 days of age using an automatic feeding machine until weaning (70 days of life) programmed to control the total amount of MR (without EO) consumed by each animal [recognised by radio frequency identification (RFID) ear tags]. Thus, the automatic feeding systems stopped delivering MR when each animal had reached a total accumulated dry matter intake of 54 kg MR. During this phase, animals were allowed access to a starter feed compound (*ad libitum*).

Table 1. Ingredients and chemical composition (g/kg DM unless otherwise stated) of the total mixed ration (TMR-2) consumed by the heifers during the replacement phase

Ingredients, g/kg	
Barley straw	114
Grass forage	114
Vetch silage	343
Maize silage	343
Concentrate	86
Chemical composition, g/kg DM	
DM, g/kg	436
[a]aNDF	481
ADF	290
CP	142
Fat	33
Ash	104
Crude energy, kcal/kg DM	4342

[a]Amylase-treated neutral detergent fibre.

After weaning (70 days of life), all the calves were managed in the feedlot and fed with a total mixed ration (TMR-1; Barley straw, 120 g/kg; dehydrated alfalfa, 140 g/kg; concentrate, 740 g/kg) formulated (with no EO) to cover their nutritional requirements during the first post-weaning period (up to 6 months of life) and a second TMR (TMR-2, with no EO) with a higher amount of fibre during the replacement phase (6-12 months of life). Voluntary feed intake was recorded individually daily using control feed intake devices (Agrolaval S.L., Gijón, Asturias, Spain) and RFID ear tags for 70 days, starting when animals were, on average, 322 days old. Daily sampling of TMR-2 after mixing (before feeding) and sufficient mixing and subsampling to minimise sampling error was conducted. Weekly analysis of daily composited feed samples of TMR-2 supplied to dairy cows was performed for DM (ISO 6496:1999), ash (ISO 5984:2002), CP (ISO 5983:2009), amylase-treated neutral detergent fibre [(aNDF), NDF assayed with a heat-stable amylase and expressed inclusive of residual ash; Ankom Technology Corp., Macedon, NY, USA] and ADF (ADF expressed inclusive of residual ash; Ankom Technology Corp., Macedon, NY, USA). The ingredients and chemical composition of TMR-2 administered during the feed intake control period are summarised in Table 1.

Animals were weighed monthly during the replacement phase to measure feed efficiency traits regarding the feed-to-gain ratio and residual feed intake (RFI). Average daily weight gain (ADG, g/d) was estimated as the regression coefficient (slope) of LBW against time using the REG procedure of the SAS package (SAS Institute Inc., Cary, NC). The feed-to-gain ratio was obtained by dividing daily feed intake by the ADG (g/d). RFI was calculated using a multiple linear regression model. The DMI, ADG and mid-test metabolic body weight data (MBW, as $LBW^{0.75}$) of all the calves were introduced. The statistical model used was $Y_i = \beta_0 + \beta_1\ MBW_i + \beta_2\ ADG_i + e_i$, where Y_i represents the predicted feed intake of the i_{th} animal; β_0 is the equation intercept; β_1, the regression coefficient on

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MBW; β_2, the regression coefficient on ADG and e_i, the residual of the i_{th} animal. Thus, this prediction may be considered the 'average' or expected value for animals of similar weights and rates of gain. The actual daily feed intake minus the predicted feed intake of each individual corresponds to the RFI, so the RFI value is inversely related to the feed efficiency.

Blood and faecal sampling

Three blood sampling times, 3 (T0), 45 (T1) days and 13 months old (T2), were planned. Blood samples were collected into glass tubes by venipuncture (tail or jugular). Tubes with no anti-coagulant were allowed to clot at room temperature and then centrifuged at 3.520 × *g* for 16 min at 4 °C. After that, the obtained serum samples were stored at −80 °C until use in biochemical profiling. A second blood sample was collected into tubes containing lithium-heparin, placed in iced water and centrifuged at 3.520 × *g* for 16 min at 4 °C. Then, plasma samples were stored at −80 °C until use in metabolomics. As explained below, a third blood sample collected into a lithium-heparin tube was used for flow cytometry analyses to measure several peripheral blood mononuclear cells (PBMCs) markers such as $CD4^+$, $CD8^+$, $CD21^+$, $WC1^+$ and $CD14^+$.

Two sampling times for faeces [45 days (T1) and 13 months old (T2)] were planned. Faecal samples (rectal grab samples) were collected before the morning meal and stored at −80 °C until freeze-drying and use in either total apparent digestibility (T2) determination according to Keulen and Young (1997)[16] or total DNA (T1 and T2) extraction as explained below.

Biochemical profile and antioxidant status

Serum samples were stored at −80 °C until used for the analysis of the biochemical profile (albumin, aspartate aminotransferase [AST/GOT], gamma-glutamyl transpeptidase [GGT], beta-hydroxybutyrate [BHB], total bilirubin, Ca, Zn, Mg, ceruloplasmin, creatine kinase, creatinine, high-density lipoprotein [HDL], low-density lipoprotein [LDL], cholesterol, glucose, insulin, non-esterified fatty acids [NEFA], triglycerides, urea, protein and globulin). The biochemical profile was measured using clinical chemistry and the turbidimetry analyser Biosystems BA400 (Biosystems S.A., Barcelona, Spain). According to the manufacturer's instructions, superoxide dismutase activity (SOD) was assayed using a Sigma Aldrich kit (Ref. 19160).

Flow cytometry analyses on the mononuclear cell population

PBMCs were isolated from 30 mL of heparinised blood by density-gradient centrifugation with Lymphoprep™ (STEMCELL Technologies Cologne, Germany) as previously described elsewhere.[17] The resultant PBMCs were washed three times with PBS, pelleted and resuspended in supplemented RPMI1640 medium + GlutaMAX™ with phenol red (Gibco, Paisley, UK). PBMCs were counted in a Neubauer chamber and adjusted to a final concentration of 10^6 cells mL^{-1}. Cell viability (> 90%) was assessed by Trypan blue dye exclusion.

Single-colour flow cytometry analyses were performed as previously described.[18] Briefly, a total of 2×10^5 cells per well were seeded in different wells in a 96-well plate (Thermo Fisher Scientific, Roskilde, Denmark) and individually incubated with non-conjugated anti-bovine CD4 (MCA834GA, Bio-Rad®), CD8 (MCA837GA, Bio-Rad®) and CD21 (MCA1424GA, Bio-Rad®) as primary antibodies at a 1:400 dilution for 1 h at 4 °C. Cells were washed twice with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (F0313, Dako®) as a secondary antibody at a dilution of 1:50 for 1 h at 4 °C in the dark. At the same time, cells from two wells were also individually incubated with FITC-conjugated anti-bovine CD14 (MCA2678F, Bio-Rad®) and WC1 (MCA383F, Bio-Rad®) antibodies at a 1:400 dilution for 1 h in the darkness at 4 °C. Finally, the wells were washed twice with PBS, and the cells were fixed with 1% CellFIX™ (Becton Dickinson and Company, Erembodegem, Belgium) and kept in the dark until flow cytometric analysis.

Sample acquisition and data analysis were performed as previously described by Arteche-Villasol *et al.* (2020).[17] At least 10,000 events were acquired using a MACSQuant flow cytometer (Miltenyi Biotec®) and gated to discard the presence of air and doublets. Then, the data were analysed using MACSQuantify10 Software™ (Miltenyi Biotec®), and the results were expressed as a percentage of positive cells for each lymphocyte surface marker.

Microbiota characterisation of faecal samples

DNA was extracted from each faecal sample using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany), according to the manufacturer's protocol. DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated DNA was then stored at −20 °C until use. Then, bacterial DNA was amplified using primers to target the V3–V4 hypervariable regions of the 16S rRNA gene, as previously described.[19] DNA Sequencing, bioinformatic processing and statistical analysis were performed as described in Ranilla et al. (2023).[20]

Metabolomics analysis

A volume of 400 µL of ice-cold methanol (LC-MS grade) was added to 100 µL of plasma sample containing 5 µL of internal standard (15N L-Leucine, 98%, Sigma –Aldrich, 1 mg/mL), vigorously vortexed and then stored for 30 min at − 20°C. Samples were centrifuged at 14,800 r.p.m. for 10 min at + 4°C. 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 analysed by LC-QTOF-MS. A blank sample (100 µL of water LC-MS grade) and a quality control sample (QC, a pool of all samples analysed) were also prepared.

Five microlitres of each sample were injected in duplicate. The autosampler and column temperatures were set at 10°C and 40°C, respectively. The analytes were eluted using a CORTECS UPLC T3 C18 (2.1 × 150 mm, 1.6 µm) (Waters™) connected to an ExionLC™ AD system (Sciex™) using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The gradient was from 99% A to 5% A in 8 min, with a total run of 20 min at a flow rate of 0.4 mL/min. The separated metabolites were then ionised using a Turbo V™ Ion Source with an ESI Probe source (Sciex™) and analysed in a ZenoTOF 7600 System equipped with a Zeno trap (Sciex™). Mass spectra were collected, in both positive- and negative ion modes, in Full-Mass Scan from 50 to 1500Da (250 ms accumulation time) and in IDA® mode (Information Dependent Acquisition) from 40 to 1500Da (50 ms accumulation time). For both methods, ion source gas 1 (GS1) and 2 (GS2) were set to 40 and 55 psi, respectively; the curtain gas to 25 psi, CAD gas to 7 and the source temperature to 550°C. The Spray Voltage was fixed at 4.5 kV (−4.5 kV in negative

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mode), the declustering potential (DP) was 80 eV and the collision energy was 30 eV with a collision energy spread of 15 eV.

The data were analysed using SCIEX OS 2.2 software (SCIEX™), in two processing functions: (1) FormulaFinder, to identify possible compound formulas based on TOF-MS spectra (compound molecular weight); (2) LibraryView™ (ver 1.0) to search MS/MS spectra with the built-in accurate mass spectral library (Metabolite High-Resolution MS/MS Spectral Library). Data were expressed as the ratio of the analytes versus the internal standard. The average corresponding area' was normalised to the estimated protein concentration in each extracted sample. Identifications (ID) were obtained based on the value m/z (parent ion) achieved and determined in high resolution.

Statistical analyses

Feed efficiency traits and digestibility were analysed by one-way ANOVA, whereas the biochemical profile, SOD and PBMCs were assessed by repeated measurements using the MIXED procedure of SAS. In the repeated measurement analysis, data from the first sampling day (day 3, before including EO in the MR of the EO group) was used as a covariate, and adjusted mean values were estimated when the effect was significant.

Regarding the faecal microbiota, differences between groups (EO vs. control) along time points in terms of OTU abundances and diversity indices were evaluated with the three following linear models: linear model A: $Y_{ik} = \mu + timepoint_k + e_{ik}$, or linear model B: $Y_{ij} = \mu + treatment_j + e_{ij}$, or linear model C: $Y_{ikj} = \mu + timepoint_k + treatment_j + e_{ikj}$, where Y_{kj} is the abundance (counts) or index value for each taxonomy (OTU) and diversity metric per sample $_i$ in timepoint $_k$ and treatment $_j$; $timepoint_k$ is the effect of the categorical variable time point (2 classes); $treatment_j$ is the effect of the categorical variable treatment (2 classes); e_{kj} are the residuals of the model. Faecal microbial diversity was assessed within- (alpha-diversity) and across- (beta-diversity) samples. All indices (alpha- and beta-diversity, previously listed in Ranilla *et al.* 2023)[20] were estimated from the complete OTU table and filtered for OTUs with more than 10 total counts distributed in at least two samples. Details about the indices used can be found in Appendix S2 of Biscarini *et al.* (2018).[21] Beta-diversity significances were tested using PERMANOVA.

MetaboAnalyst 5.0[22] was used to perform multivariate data analyses, including principal component analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), heat maps, hierarchical clustering and pathway analysis[22] Volcano plots were drawn at T0 (3 days), T1 (45 days) and T2 (13 months) to identify significant metabolites between treatments. Plots used a threshold of 1.5 for fold change (FC) on the × axis and a threshold of 0.05 for the false-discovery rate (adjusted *P*-value) on the *y* axis.

Results

Feed efficiency, biochemical profile and antioxidant status

According to the results obtained (Table 2), the ADG of the EO group was higher (100 vs. 172 g/d; $P = 0.003$) during the first 45 days of life. Thus, these animals were heavier (44.0 vs. 47.4 kg; $P = 0.030$) and more efficient when they received supplementation with EO in the MR. However, no long-term effects of EO supplementation during the milk-fed period on RFI (−0.118 vs. 0.119, $P = 0.308$) nor digestibility (51.9 *vs.* 51.7%; $P = 0.965$) were observed at the adult stage when animals were fed the TMR-2 diet. In addition, the biochemical profile (Table 3) and SOD inhibition rate measurement (181.8 *vs.* 79.5% for the control and EO groups, respectively; $P = 0.795$) were similar between the two groups at all sampling times ($P > 0.05$).

Table 2. Effects of essential oil supplementation during the first 45 days of life on the pre-weaning and replacement performance of dairy calves

	CTRL	EO	SED[a]	*P*-value
Birth LBW[b], kg	39.42	39.09	1.620	0.838
Suckling period (from day 4–45)				
ADG[c], g/d	100	172	20.3	0.003
Final LBW, kg	44.0	47.4	1.40	0.030
Replacement period (from day 322–392)				
ADG, g/d	497	502	52.6	0.926
Final LBW, kg	351.9	341.6	11.6	0.394
DMI, g DM/animal day^{-1}	6.30	6.52	0.10	0.403
DM digestibility, %	51.94	51.71	4.819	0.308
RFI[d], g DM animal^{-1} day^{-1}	−0.118	0.119	0.222	0.308
Feed-to-gain ratio, g DMI/g ADG	13.05	13.50	1.287	0.728

[a]Standard error of the difference.
[b]LBW = live body weight.
[c]Average daily gain.
[d]Residual feed intake (RFI) calculated using ADG estimated by regression.

Flow cytometry analyses of the mononuclear cell population

As far as the mononuclear cell population is concerned, none of the markers studied ($CD4^+$, $CD8^+$, $CD21^+$, $WC1^+$ and $CD14^+$) presented significant differences between treatments (Table 4; $P > 0.05$). It is remarkable, however, that a statistical interaction existed between diet and day for $CD21^+$; accordingly, the reduction of this parameter over time was lower for the EO group (Figure 1). Interestingly, higher significant values ($P = 0.006$) for monocytes ($CD14^+$) were obtained in the EO group, indicating a direct effect of EO treatment on this population.

Microbiome analysis

Sequencing results

Sequencing the V3-V4 regions of the bacterial 16S rRNA gene of the 32 feces samples produced 2,184,670 assembled reads (joined R1-R2 paired-end reads). After quality filtering, 354,387 sequences were removed, leaving 1,830,283 sequences for subsequent analyses (84% average retention rate, maximum 94.5%, minimum 79.2%). On average, there were 55,694 (± 22,788) sequences per sample in the control group and 58,698 ± 25,898 in the EO group. The initial number of OTUs identified was 6,466; after filtering out OTUs with less than 10 counts in at least 2 samples, 2,531 distinct OTUs were left.

Alpha- and beta-diversity indexes

The estimated alpha-diversity indices are reported in Supplementary Table S1. Considering the effect of the treatment alone, several indices (Fisher, Chao1, Shannon, ACE, Observed OTUs and Simpson) were statistically significant at T1, while none were significant at T2 ($P \leq 0.05$). Still, three indices (Simpson, InvSimspon and Shannon) were positive in the 0.05–0.10 area of significance at T2 (Figure 2A). On the other hand, considering the time effect alone, three indices (Observed_OTUs, Chao1 and ACE)

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Table 3. Biochemical profile the day 45 (end of EO administration) and day 370 (replacement phase) of dairy calves being supplied essential oil (EO group) or not (CTRL group) during the suckling period

	Diet		Sampling day				*P*-value		
	CTRL	EO	45 d	370 d	SED[1]	SED[2]	Diet	Day	Diet × Day
Albumin (g/l)	32.7	31.7	30.9	33.5	0.776	0.767	0.224	0.005	0.659
AST[a] (U/l)	92.4	99.2	99.3	92.3	15.18	15.17	0.660	0.650	0.997
βHB[b] (mg/dl)	2.85	2.82	1.64	4.04	0.231	0.216	0.928	<0.000	0.371
Bilirubin (mg/l)	0.43	0.38	0.603	0.199	0.050	0.050	0.356	<0.001	0.371
Ca (mg/dl)	9.62	9.54	9.82	9.33	0.179	0.171	0.658	0.014	0.232
Ceruloplasmin (mg/dl)	3.60	3.35	1.98	4.96	0.315	0.315	0.435	<0.001	0.145
Creatine kinase (U/l)	232	335	225	342	95.5	90.3	0.300	0.218	0.311
Creatinine (mg/dl)	0.83	0.82	0.967	0.686	0.052	0.033	0.895	<0.001	0.655
HDL (mg/dl)	61.3	61.4	57.8	64.9	3.828	2.732	0.967	0.024	0.892
LDL (mg/dl)	9.3	9.57	10.3	8.63	1.586	1.200	0.880	0.197	0.513
Cholesterol (mg/dl)	93.9	100	103	91.2	12.1	5.8	0.615	0.069	0.099
GGT[c] (U/l)	23.4	25.8	28.6	20.6	3.359	2.366	0.484	0.005	0.136
Glucose (mg/dl)	59.2	56.0	84.5	30.7	4.434	3.951	0.494	<0.001	0.459
Insulin (μUI/ml)	3.96	3.92	5.58	2.3	1.684	1.405	0.979	0.038	0.327
Mg (mg/dl)	1.94	1.86	1.64	2.15	0.076	0.076	0.335	<0.001	0.058
NEFA[d] (mmol/l)	0.23	0.18	0.225	0.188	0.038	0.038	0.231	0.347	0.969
Protein (g/l)	64.6	63.1	58.8	68.9	1.864	1.554	0.415	<0.001	0.028
Triglycerides (mg/dl)	24.0	25.6	28.6	21.0	5.133	4.783	0.770	0.136	0.878
Urea (mg/dl)	18.0	17.4	18.5	16.9	2.213	1.226	0.802	0.224	0.984
Zn (mg/dl)	45.2	42.1	65.1	22.2	4.078	4.078	0.469	<0.001	0.571

[a]AST, aspartate aminotransferase.
[b]Beta-hidroxybutirate.
[c]GGT, gamma-glutamyl transpeptidase.
[d]NEFA, non-esterified fatty acid.

Table 4. Peripheral blood mononuclear cell (PBMC, % positive cells) measured by flow cytometry the day 45 (end of EO administration) and day 370 (replacement phase) of dairy calves being supplied essential oil (EO group) or not (CTRL group) during the suckling period

	Diet		Sampling day				*P*-value		
	CTRL	EO	Day 45	Day 370	SED[a]	SED[b]	Diet	Day	Diet × Day
$CD4^+$	18.1	17.4	15.7	19.8	2.21	2.14	0.760	0.039	0.445
$CD8^+$	13.7	13.0	7.87	18.9	1.05	1.06	0.517	<0.001	0.118
$CD14^+$	29.0	35.1	34.2	29.9	1.86	1.84	0.006	0.072	0.106
$CD21^+$	24.6	22.9	28.1	19.3	2.87	1.58	0.551	<0.001	0.048
$WC1^+$	18.3	21.2	18.2	21.3	3.02	1.97	0.361	0.134	0.295

[a]Standard error of the difference to compare experimental groups.
[b]Standard error of the difference to compare sampling days.

were significant in the control group. By contrast, the EO group had no index with a significant *P*-value (Figure 2B). Still, the time effect did not show the same behaviour (only three indices −Observed_OTUs, Chao1 and ACE − in the 0.05 − 0.10 range) (Figure 2C). The relationships between samples were assessed using the weighted Unifrac distances between samples.

The distribution of samples (PCA) showed no clustering for samples grouped by treatment ($P = 0.905$). Clear clusters were only detected when considering samples grouped by time point ($P = 0.001$).

Description of the core microbiome in faeces

The core faecal microbiota at the family and phylum level comprised 87 and 21 OTUs shared within 100% of the samples (Supplementary Table S2). Eight main phyla were detected in the faecal microbiota, with a relative abundance larger than 1%, in particular, *Firmicutes* (49.21%), *Bacteroidetes* (32.19%), *Proteobacteria* (4.15%), *Verrucomicrobia* (3.1%), *Cyanobacteria* (2.9%), *Euryarchaeota* (2.26%), *Spirochaetes* (1.54%) and *Actinobacteria* (1.29%). The core faecal microbiota at the genus level was composed of 228 OTUs shared within 100% of

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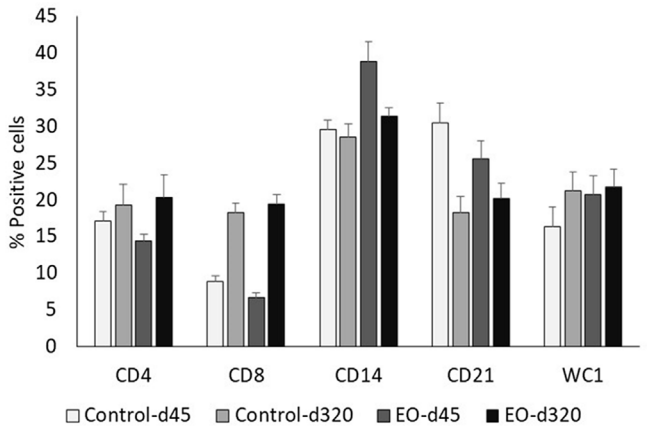

Figure 1. Peripheral blood mononuclear cell (PBMC) measured by flow cytometry the day 45 (end of EO administration) and day 370 (replacement phase) of dairy calves being supplied essential oil (EO group) or not (CTRL group) during the suckling period.

the samples, plus uncultured or unknown genera (Supplementary Table S3, Figure 3). Fourteen main genera were detected in the faecal microbiota, with a relative abundance larger than 2%, in particular, *Bacteroides* (13.4%), *Ruminococcaceae* UCG-005 (12%), *Alloprevotella* (9.63%), *Ruminococcaceae* UCG-010 (6.7%), *Faecalibacterium* (5.9%), *Ruminococcaceae UCG-014* (4.8%), *Prevotella 9* (4.49%), *Akkermansia* (4.45%), *Blautia* (3.75%), *Alistipes* (3.24%), *Rikenellaceae RC9 gut group* (3.16%), *Prevotella 2* (2.69%), *Escherichia-Shigella* (2.49%) and *Methanobrevibacter* (2.38%).

Effects of treatments on microbiota at the genus level

Thirty-eight genera differed significantly when the treatment groups were compared over time: 34 genera were differentially expressed at T1, of which 18 decreased in the EO group compared with the control, the most significant being that 16 increased. The remaining four genera were differentially expressed at T2, of which 2 (*Prevotella 2* and *Howardella*) decreased in the EO group compared with the control, and in the same group, two (*Caldicoprobacter* and *Ruminiclostridium 1*) increased (Supplementary Table S4, Figure 3) as compared to the control, although the increase was limited.

Plasma metabolome analysis

Untargeted metabolomic analyses identified 109 metabolites (Supplementary Table S5). The supervised PCA (Figure 3A) showed separation of the three age-related group samples. The heat map in Figure 3B, generated by hierarchical clustering, also presented evidence that three age-related groups can be distinguished by their metabolomes. Figure 3C shows the Volcano plot score of the changes, proving the significant metabolites that change along the time course.

When the plasma metabolomes from control calves and those fed with EO were compared, no statistical changes were evidenced between the groups regarding the plasma metabolome at T0 (Figure 4, Panels A and B). However, at T1, we observed significant statistical changes ($P > 0.05$) for indole, propionic acid, succinic acid and butyric acid (Figure 4, Panel C and D), whereas T2 differed significantly for 10 metabolites (Figure 4, Panel E and F).

The major metabolic pathways that changed with age in control calves were aminoacyl-tRNA biosynthesis and valine, leucine and isoleucine biosynthesis, followed by the biosynthesis of several amino acid pathways (Figure 5, Panel A). The significant pathways modified by EO feeding at T2 were arginine biosynthesis and alanine, aspartate and glutamate metabolism (Figure 5, Panel B).

Discussion

Calves are born as functional monogastric animals that rely on nutrients from milk or MR in early life.[23] This initial period of life is essential and could affect the welfare, growth and development of newborn calves as well as drive their future performance. The present study investigated the effects of neonatal feeding with EO on feed efficiency and related performance parameters, immune status, the gut microbiome and the plasma metabolome in both the short and the long term.

According to the results, calves receiving EO in the MR improved their feed efficiency during this phase. Thus, these EO calves gained more weight during the suckling phase consuming the same amount of dry matter as the control group. Our results agree with previous studies,[24] showing that EO (carvacrol, caryophyllene, -cymene, cineole, terpinene and thymol) enhance the growth rate and stimulate appetite in newborn dairy calves. Moreover, Liu *et al.*[11] demonstrated that calves fed commercial EO from three days of age through the end of the 70-day experimental period showed greater apparent total-tract nutrient digestibility of DM, ADF, NDF, starch and minerals (Na, Mg, *P*, S, *K*, Ca, Cl, Mn, Fe and Cu) as compared with calves fed the control diet. Moreover, they also indicated that EO given during the early life may consistently challenge gut health and prevent diarrhoea. Other authors have suggested that the positive effects of EO might be related to the inhibition of pernicious bacteria exerted by carvacrol and thymol lipophilic components at the gut level.[25] Nevertheless, in the present study, this positive effect on feed efficiency was not observed later on during the replacement phase.

Early supplementation with EO did not modify most PBMC populations, including $CD4^+$ (T helper lymphocytes), $CD8^+$ (T cytotoxic lymphocytes), $CD21^+$ (B cells) and $WC1^+$ (γδ T lymphocytes) at 3 and 45 days and 13 months. However, an interaction between diet and day for $CD21^+$ was observed ($P = 0.048$; Table 4), which may indicate a persistent decrease of $CD21^+$ in the EO group compared to the control group that did not receive EO. By contrast, $CD14^+$ monocytes presented statistically significant increased average values in the EO group, and this effect persisted throughout the replacement phase. It must be considered that $CD14^+$ monocytes are a different lineage from intestinal macrophages, cells which are key regulators of inflammatory responses to the gut microbiome and play a central role in maintaining gastrointestinal homoeostasis, epithelial integrity and protecting against specific mucosal pathogens;[26] however, if a similar effect of EO on the intestine were assumed, the changes observed in $CD14^+$ and the smaller decrease in $CD21^+$ over time observed in the EO group might have influenced or be related to changes in the gut microbiome.

To clarify this hypothesis, the effects of EO on the uncultured faecal microbiota were studied. Diversity significance indices described the impact of time and treatment, underlying that, for differences between groups, treatment was crucial, while for diversity within groups (beta-diversity) the time effect was critical. Moreover, feeding EO during early life increased the abundance of two genera, namely *Akkermansia* and *Allistipes,* at T1. Bacteria belonging to the genus *Akkermansia* are included in the intestinal mucus and regulate the integrity of the intestinal barrier by producing both anti-inflammatory cytokines (e.g., IL-10)[27] and

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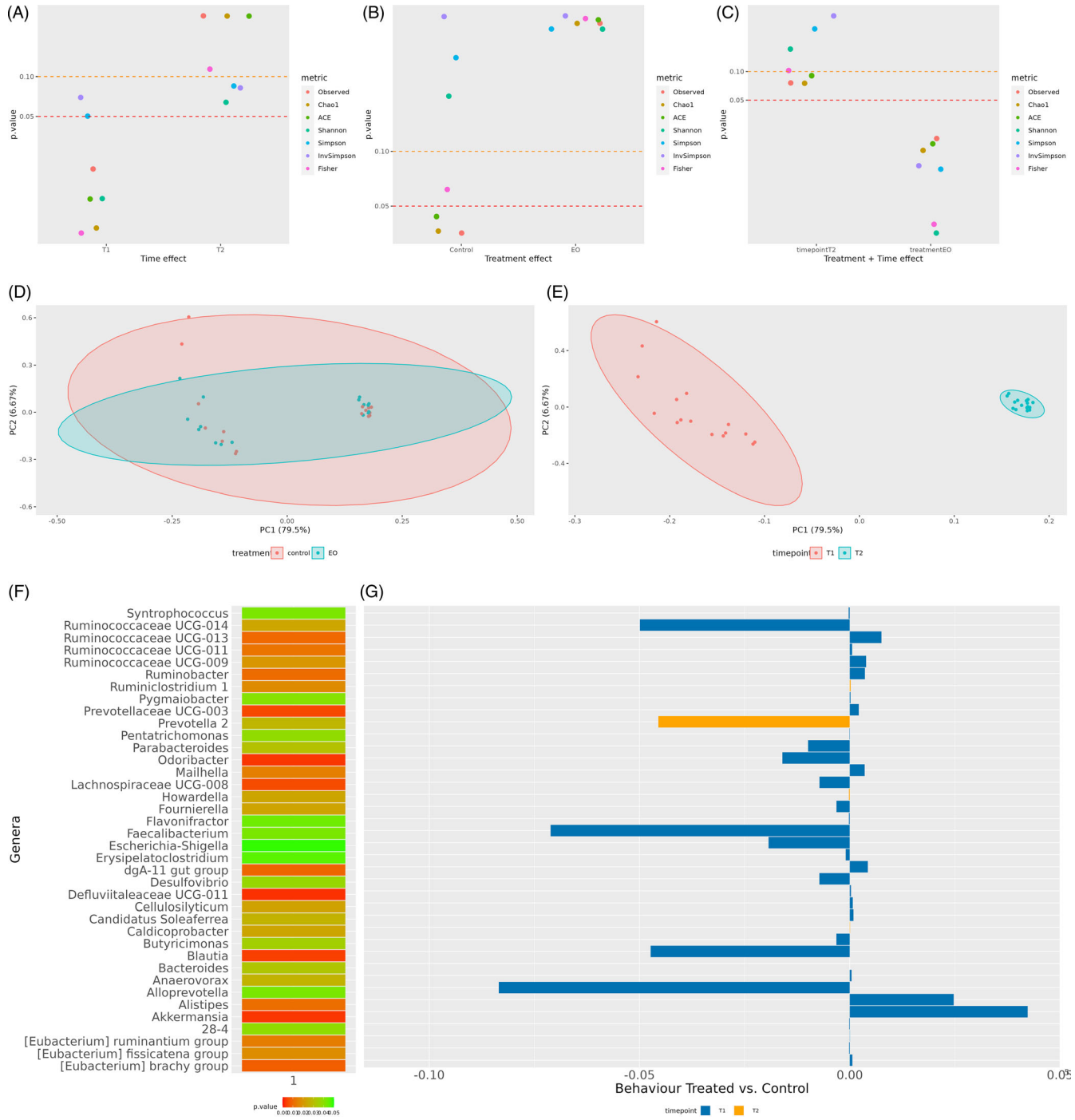

Figure 2. The fecal microbiota of calves after EO feeding. Panel A, B, and C are scatterplots reporting the significance of the alpha diversity indices in treatments and time points. P-values were obtained from a linear regression model with the Control group as the benchmark. The dashed lines identify the suggestive-significance area. Panel D and E present the Principal Component Analysis (PCA) plot of unweighted Unifrac distances of fecal microbiota in this study. Panel D represents the clustering per treatment, while Panel E represents the clustering per time point. Panels F and G show significantly abundant taxa in fecal microbiota at the genera level. Panel F is the heatmap of the significance of taxa relative abundance. Panel G Bar plot presents the behavior of the significantly different abundant genera along time points: the scale in the x-axis is the ratio of Treated against Controls.

SCFA to further support gut health.[28] In addition, when abundant, *Akkermansia* fulfils protective roles against several diseases in humans[29] as well as in calves[30] and is regarded as a third-generation probiotic.[29] Regarding *Allistipes*, previous studies have demonstrated in humans that this genus includes bacteria positively related to healthy anaerobes in the host.[31]

Of the four bacteria genera whose abundance was decreased in EO calves at T1 (e.g., *Ruminococcaceae UCG-014, Faecalibacterium, Blautia and Alloprevotella*), bacteria belonging to genera such as *Faecalibacterium* and *Blautia* dominate the faeces of pre-weaned calves.[32] A higher abundance of *Faecalibacterium* has been associated with a lower sensitivity to neonatal

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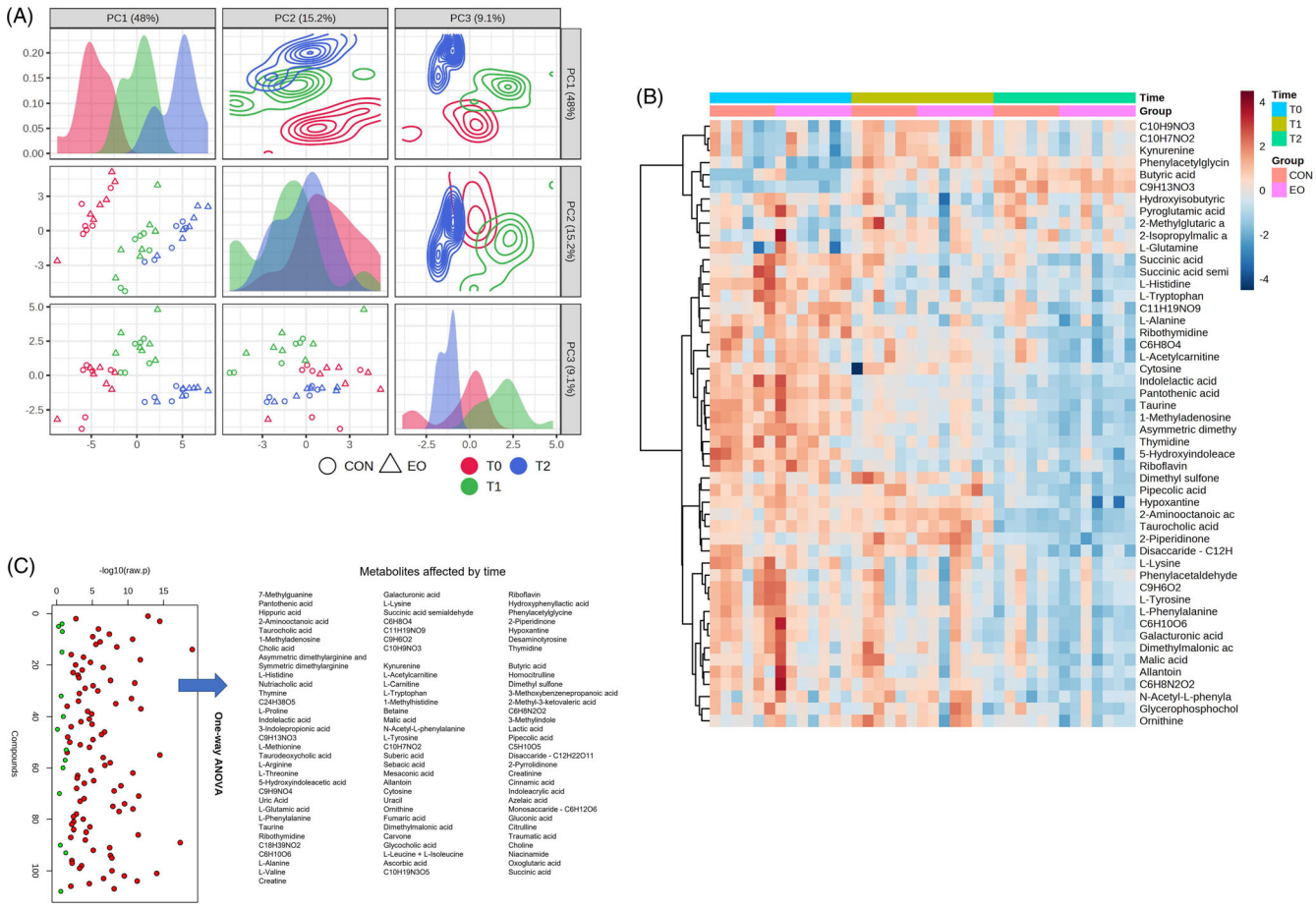

Figure 3. The plasma metabolome changes in calves not fed with EO. Panel A: Pairwise PCA with density plots outlines the different times of sampling (T0, T1, and T2). Panel B: Presentation of the 50 most significant metabolites determined by one-way ANOVA analysis. Panel C: Clustering results are shown as a heatmap (distance measured using Euclidean and clustering algorithm using ward).

diarrhoea.[33,34] We may then speculate that decreasing the abundance of *Faecalibacterium* genus when feeding EO might expose the calves to an increased risk of developing gut alterations related to body weight and ADG.[35] However, we observed an increase of ADG at T1, probably because the magnitude of the decreased abundance of *Blautia* in EO animals was insufficient (by itself) to induce changes in the calves' ADG. The other two genera that were decreased at T1 in the group of calves being fed EO are *Ruminococcaceae UCG-014* and *Alloprevotella*. *Alloprevotella* produces succinic acid as the end-product of plant fibre.[36] Interestingly, as discussed below, the decrease in the abundance of the genus *Alloprevotella* corresponds to the decrease of succinic acid in the plasma metabolome. In calves, both *Ruminococcaceae UCG-014* and *Alloprevotella* are critical biomarkers of gut health, and their abundance correlates with a healthy phenotype.[37,38] Again, reducing the abundance of both genera might increase the risk of developing intestinal diseases in calves fed EO. Finally, the effect of EO at T2 was limited to a decrease in the genus *Prevotella-2*. The bacteria belonging to this genus, like other *Prevotellaceae*, are reduced during calf diarrhoea,[38] so again, this change promoted by EO might indicate gut dysbiosis.

The last part of the study also revealed changes induced by treatment on the untargeted metabolome of calf' plasma. No changes were found at T0, confirming that the two groups were homogeneous concerning the plasma metabolome at 3 days of life. At T1 (45 days old), only three metabolites changed: 3-indole propionic acid, butyric acid and succinic acid. The descent of butyric acid (a metabolite produced by intestinal bacteria) agrees with the decrease in *Blautia*, *Ruminococcaceae* and *Faecalibacterium*, three bacterial genera associated with butyric acid production. Moreover, 3-indole-propionic acid is a bacterial metabolite derived from tryptophan metabolism and produced by the genus *Faecalibacterium*. As for *Blautia*, there was a correlation between the decrease of *Faecalibacterium* at T1 and the decrease of 3-indole propionic acid,[39] an inhibitor of gut dysbiosis and endotoxin leakage and a potent scavenger of hydroxyl radicals.[40] Butyric acid also shows a protective effect against the inflammatory response,[41] so the decrease of both metabolites at T1 seems to corroborate the increased risk of gut disease when feeding EOs during early life.

At T2, 340 days after birth, the plasma abundance of 14 metabolites changed. Pathway analysis of the changes occurring at T2 suggests that most changes were related to a reduction of energy and protein metabolism and a potential decrease of the antioxidant capacity. In particular, metabolites associated with the citric acid cycle, such as fumaric acid and malic acid, and carnitines, such as L-acetyl carnitine and L-carnitine, were decreased in the EO group. Biogenic amines, such as taurine (a sulphur-containing non-essential amino acid whose low level may reduce antioxidant activity) and citrulline, were also decreased. Additionally, two metabolites related to decreased protein catabolism (e.g., creatinine and 1-methylhistidine) were also decreased, which may indicate

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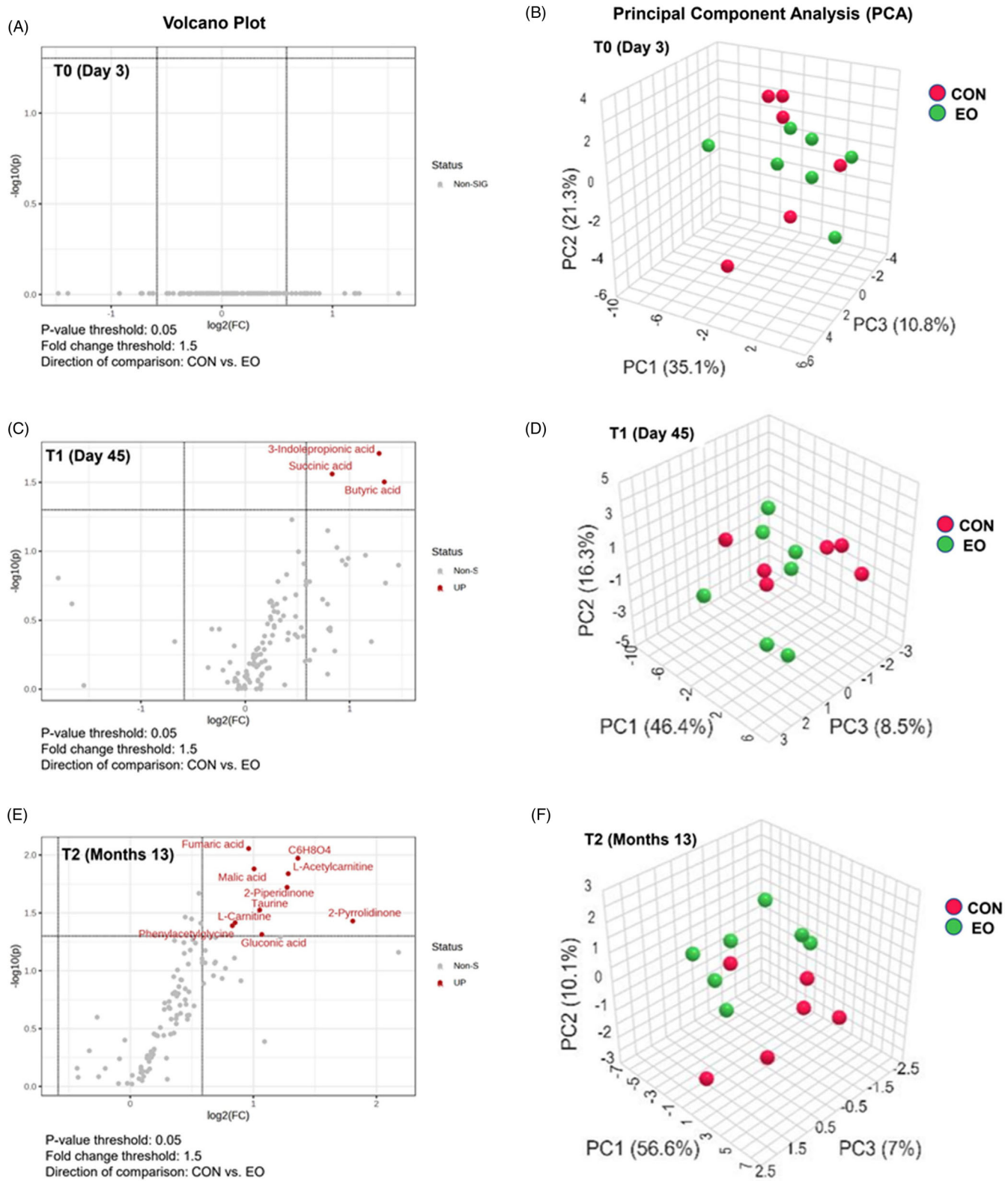

Figure 4. The changes in the plasma metabolomes of calves fed with EO-enriched diets. The plasma metabolome changes between in calves fed (EO) and not fed (CON) with EO at T0 (Panels A and B), T1 (Panels C and D), and T2 (Panel E and F). Panels A, C, and E show the Volcano plot of changes at T0, T1, and T2, respectively. Important features selected by the volcano plot with fold change threshold (x) 2 and t-tests threshold (y) 0.1. The red circles represent features above the threshold. Note that both the fold changes and the p-values are log-transformed. The further its position away from the (0,0), the more significant the feature is. Panel B, D, and F present the 3D scores plot between the selected PCs at T0 (B), T1 (D), and T2 (F), respectively. The explained variances are shown in brackets.

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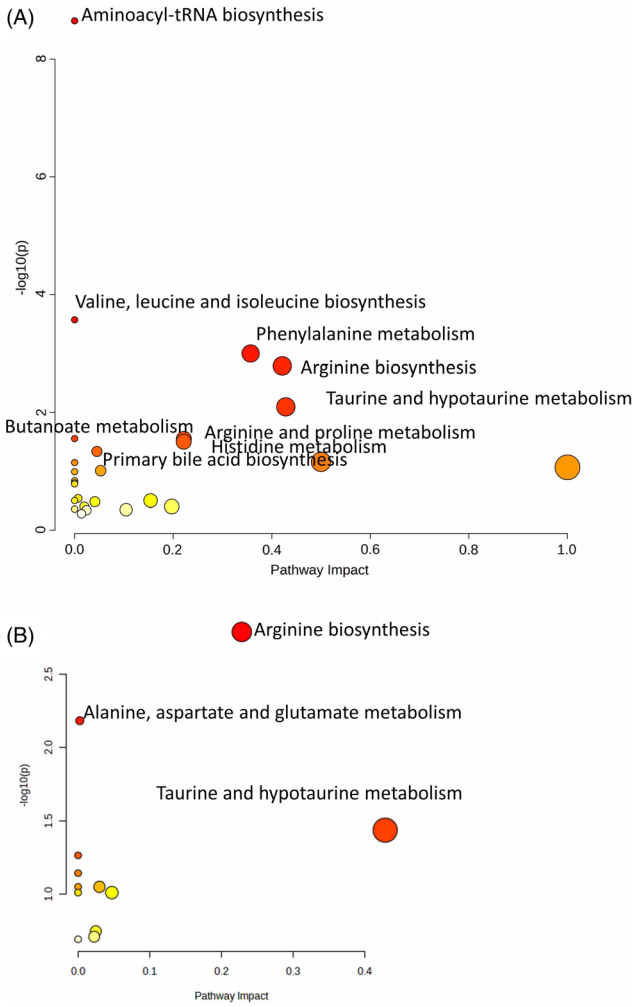

Figure 5. The metabolic pathway changes. Summary of Pathway Analysis: the circle's size indicates the pathway's impact, while the color represents the significance (the more intense the red color, the lower the significance). Panel A: The significant pathway modified in the plasma metabolome in control calves not fed with EO. All the changed metabolites are included. Panel B: The significant pathway modified in the plasma metabolome changes in calves at T2.

reduced proteolytic processes.[42] In any case, the magnitude of all these changes was not enough to promote variations in ADG during the replacement phase. In conclusion, according to the results of the present study, daily administration of 0.23 ml of oregano EO (accounting for 200 mg of carvacrol) to newborn dairy calves promotes a positive transitory effect on body growth and feed efficiency during the suckling period but no long-term effects during the replacement phase. This early supply of EO also induces early changes in the gut microbial population consistent with changes in the plasma metabolome, but not all of them are considered desirable from a gut health point of view. Future work should be focused on determining the optimum dosage and duration of EO administration to determine if EOs may provide a viable natural alternative to antibiotics to minimise health challenges while improving calf growth performance and economic returns to the dairy calf raiser. Therefore, additional research studies is warranted to evaluate different mechanisms of action of EO on the interaction between the microbiota, immunity and health.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S2040174424000084.

Data availability statement. The 16S rRNA gene sequences obtained from this study were deposited in the EMBL-EBI European Nucleotide Archive (ENA) repository with the accession number PRJEB57631. The rest of the data are available upon request. The 16S rRNA gene sequences obtained from this study and the OTU table derived as described in the test were deposited and available in the Università degli Studi di Milano Dataverse repository. A private URL for reviewers is hereby provided, while datasets will be disclosed upon article acceptance (https://dataverse.unimi.it/privateurl.xhtml?token=8b1dab61-e1e3-49a2-a4a2-aa1e969c6eb7).

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Author contribution. SA, LA-N and FC contributed to the conception and design of the study. SA, FG, AM and IM conducted the in vivo study. DGE, NA and AM carried out the flow cytometry analyses. PC sequenced the DNA microbiome samples, and CG conducted the bioinformatics analyses. FG performed the statistical analysis of the in vivo study. FF carried out the metabolome analysis. MG and FC carried out the metabolomic statistical analysis. SA and LA-N wrote the first draft of the manuscript. All authors contributed to writing, revising and reading the manuscript before submission.

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Competing interests. None.

Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (Directive 2010/63/EU) and has been approved by the institutional committee (IGM-CSIC Animal Experimentation Committee).

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