Essential Oil Supplementation in Milk Replacers: Short- and Long-Term Impacts on Feed Efficiency, the Faecal Microbiota and the Plasma Metabolome in Dairy Calves

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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 characterize 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 hypothesized that one of the factors involved in this long-term effect is microbial gut colonization during early life, which shapes the immune response throughout life by providing stimulatory signals that activate innate and adaptive 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.

79 The present study hypothesizes that EO administration during the first 45 days of life could promote

80 short and long-term effects on feed efficiency traits, immune status, the microbiota and the plasma

81 metabolome when included in milk replacer (MR) for newborn dairy calves.

82 **Material and methods**

83 *Care and use of animals*

84 All handling practices followed the recommendations of Directive 2010/63/EU of the European

85 Parliament, the Council on Protecting Animals used for scientific purposes, and the IGM-CSIC Animal

86 Experimentation Committee (protocol 736/2018). The study reported in the manuscript follows the

87 recommendations in the ARRIVE guidelines[15].

88

89 *Animals, diets, and feed efficiency measurement*

90 CSIC conducted feeding trials using 16 female dairy calves born in the same commercial farm and

91 selected to minimize the range of live body weight (LBW) and day of birth. These animals were

92 separated from the cows in the first 24 hours of life and fed 3 L of colostrum daily until day 3 of life.

93 Starting at 3 days, once housed in individual straw-bedded hutches, they were allocated to one of two

94 groups (n = 8 per group; control and essential oil -EO-) balanced according to the body weight at birth.

95 Calves were fed milk replacer (MR, 145 g of Novilac Turbostart, Schils/L) twice a day using a bucket

96 with a nipple (allowing the reflex closure of the oesophageal groove) until day 45. During this phase,

97 these animals were fed daily a fixed amount of reconstituted MR (divided into two feedings, at 09:00

98 a.m. and 6:00 p.m.) that was gradually increased from 3 to 7 L at the same rate for all the individuals.

99 Moreover, the animals in the EO group were supplied daily with 0.23 ml of oregano EO, accounting

100 for 200 mg of carvacrol (Zane Hellas 100%) diluted in the first 100 mL of MR for 45 days. No

101 concentrates were offered, and no leftovers of MR remained during this phase, so dry matter intake was

102 similar for all calves. All the animals were weighed at birth and on days 4, 10, 25 and 45 of life (e.g. at

103 the end of the EO administration period) to estimate the average daily gain (ADG) and then calculate

104 the feed-to-gain conversion rate during the suckling period.

105 Animals were managed in a feedlot once they reached 45 days of age using an automatic feeding

106 machine until weaning (70 days of life) programmed to control the total amount of milk replacer

107 (without EO) consumed by each animal [recognized by radio frequency identification (RFID) ear tags].

108 Thus, the automatic feeding systems stopped delivering milk replacer when each animal had reached a

109 total accumulated dry matter intake of 54 kg MR. During this phase, animals were allowed access to a

110 starter feed compound (*ad libitum*).

111 After weaning (70 days of life), all the calves were managed in the feedlot and fed with a total mixed

112 ration (TMR-1; Barley straw, 120 g/kg; dehydrated alfalfa, 140 g/kg; concentrate, 740 g/kg) formulated

113 (with no EO) to cover their nutritional requirements during the first post-weaning period (up to 6 months

114 of life) and a second TMR (TMR-2, with no EO) with a higher amount of fibre during the replacement

115 phase (6-12 months of life). Voluntary feed intake was recorded individually daily using control feed

116 intake devices (Agrolaval S.L., Gijón, Asturias, Spain) and RFID ear tags for 70 days, starting when

117 animals were, on average, 322 days old. Daily sampling of TMR-2 after mixing (before feeding) and

118 sufficient mixing and subsampling to minimize sampling error was conducted. Weekly analysis of daily

119 composited feed samples of TMR-2 supplied to dairy cows was performed for DM (ISO 6496:1999),

120 ash (ISO 5984:2002), CP (ISO 5983:2009), amylase-treated neutral detergent fibre [(aNDF), NDF

121 assayed with a heat-stable amylase and expressed inclusive of residual ash; Ankom Technology Corp.,

122 Macedon, NY, USA] and ADF (ADF expressed inclusive of residual ash; Ankom Technology Corp.,

123 Macedon, NY, USA). The ingredients and chemical composition of TMR-2 administered during the

124 feed intake control period are summarized in Table 1.

125 Animals were weighed monthly during the replacement phase to measure feed efficiency traits

126 regarding the feed-to-gain ratio and residual feed intake (RFI). Average daily weight gain (ADG, g/d)

127 was estimated as the regression coefficient (slope) of LBW against time using the REG procedure of

128 the SAS package (SAS Institute Inc., Cary, NC). The feed-to-gain ratio was obtained by dividing daily

129 feed intake by the ADG (g/d). Residual feed intake was calculated using a multiple linear regression

130 model. The DMI, ADG, and mid-test metabolic body weight data (MBW, as $LBW^{0.75}$) of all the calves

131 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

132 the predicted feed intake of the i_{th} animal; β_0 is the equation intercept; β_1, the regression coefficient on

133 MBW; β_2, the regression coefficient on ADG and e_i, the residual of the i_{th} animal. Thus, this prediction

134 may be considered the "average" or expected value for animals of similar weights and rates of gain. The

135   actual daily feed intake minus the predicted feed intake of each individual corresponds to the residual

136   feed intake (RFI), so the RFI value is inversely related to the feed efficiency.

137   ***Blood and faecal sampling***

138   Three blood sampling times, 3 (T0), 45 (T1) days and 13 months old (T2), were planned. Blood samples

139   were collected into glass tubes by venipuncture (tail or jugular). Tubes with no anti-coagulant were

140   allowed to clot at room temperature and then centrifuged at $3.520 \times g$ for 16 min at 4 ℃. After that, the

141   obtained serum samples were stored at -80℃ until use in biochemical profiling. A second blood sample

142   was collected into tubes containing lithium-heparin, placed in iced water and centrifuged at $3.520 \times g$

143   for 16 min at 4 ℃. Then, plasma samples were stored at –80 ℃ until use in metabolomics. As explained

144   below, a third blood sample collected into a lithium-heparin tube was used for flow cytometry analyses

145   to measure several peripheral blood mononuclear cells (PBMCs) markers such as $CD4^+$, $CD8^+$, $CD21^+$,

146     $WC1^+$ and $CD14^+$.

147   Two sampling times for faeces [45 days (T1) and 13 months old (T2)] were planned. Faecal samples

148   (rectal grab samples) were collected before the morning meal and stored at –80℃ until freeze-drying

149   and use in either total apparent digestibility (T2) determination according to Keulen and Young (1997)[16]

150   or total DNA (T1 and T2) extraction as explained below.

151   ***Biochemical profile and antioxidant status***

152   Serum samples were stored at –80 ℃ until used for the analysis of the biochemical profile (albumin,

153   aspartate aminotransferase [AST/GOT], gamma-glutamyl transpeptidase [GGT], beta-hydroxybutyrate

154   [BHB], total bilirubin, Ca, Zn, Mg, ceruloplasmin, creatine kinase, creatinine, high-density lipoprotein

155   [HDL], low-density lipoprotein [LDL], cholesterol, glucose, insulin, non-esterified fatty acids [NEFA],

156   triglycerides, urea, protein and globulin). The biochemical profile was measured using clinical

157   chemistry and the turbidimetry analyser Biosystems BA400 (Biosystems S.A., Barcelona, Spain).

158   According to the manufacturer's instructions, superoxide dismutase activity (SOD) was assayed using

159   a Sigma Aldrich kit (Ref. 19160).

160   ***Flow cytometry analyses on the mononuclear cell population***

161   Peripheral blood mononuclear cells (PBMCs) were isolated from 30 mL of heparinized blood by

162   density-gradient centrifugation with Lymphoprep™ (STEMCELL Technologies Cologne, Germany) as

163　previously described elsewhere [17]. The resultant PBMCs were washed three times with PBS, pelleted

164　and resuspended in supplemented RPMI1640 medium + GlutaMAX™ with phenol red (Gibco, Paisley,

165　UK). PBMCs were counted in a Neubauer chamber and adjusted to a final concentration of 10^6 cells

166　mL^{-1}. Cell viability (> 90%) was assessed by Trypan blue dye exclusion.

167　Single-color flow cytometry analyses were performed as previously described[18]. Briefly, a total of $2 \times$

168　10^5 cells per well were seeded in different wells in a 96-well plate (Thermo Fisher Scientific, Roskilde,

169　Denmark) and individually incubated with non-conjugated anti-bovine CD4 (MCA834GA, Bio-Rad®),

170　CD8 (MCA837GA, Bio-Rad®) and CD21 (MCA1424GA, Bio-Rad®) as primary antibodies at a 1:400

171　dilution for 1 h at 4 ℃. Cells were washed twice with PBS and incubated with fluorescein isothiocyanate

172　(FITC)-conjugated anti-mouse IgG (F0313, Dako®) as a secondary antibody at a dilution of 1:50 for 1

173　h at 4 ℃ in the dark. At the same time, cells from two wells were also individually incubated with FITC-

174　conjugated anti-bovine CD14 (MCA2678F, Bio-Rad®) and WC1 (MCA383F, Bio-Rad®) antibodies

175　at a 1:400 dilution for 1 h in the darkness at 4 ℃. Finally, the wells were washed twice with PBS, and

176　the cells were fixed with 1% CellFIX™ (Becton Dickinson and Company, Erembodegem, Belgium) and

177　kept in the dark until flow cytometric analysis.

178　Sample acquisition and data analysis were performed as previously described by Arteche-Villasol et al.

179　(2020) [17]. At least 10,000 events were acquired using a MACSQuant flow cytometer (Miltenyi Biotec®)

180　and gated to discard the presence of air and doublets. Then, the data were analysed using

181　MACSQuantify10 Software™ (Miltenyi Biotec®), and the results were expressed as a percentage of

182　positive cells for each lymphocyte surface marker.

183

184　*Microbiota characterization of faecal samples*

185　DNA was extracted from each faecal sample using a QIAamp Fast DNA Stool Mini Kit (Qiagen,

186　Germany), according to the manufacturer's protocol. DNA quality and quantity were assessed using a

187　NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The

188　isolated DNA was then stored at −20 ℃ until use. Then, bacterial DNA was amplified using primers to

189　target the V3-V4 hypervariable regions of the 16S rRNA gene, as previously described[19]. DNA

190   Sequencing, bioinformatic processing, and statistical analysis were performed as described in Ranilla

191   et al. (2023)[20].

192

193   *Metabolomics analysis*

194   A volume of 400 µL of ice-cold methanol (LC-MS grade) was added to 100 µL of plasma sample

195   containing 5 µL of internal standard (15N L-Leucine, 98%, Sigma –Aldrich, 1 mg/mL), vigorously

196   vortexed and then stored for 30 min at −20 °C. Samples were centrifuged at 14,800 rpm for 10 min at

197   +4 °C. The supernatant was transferred to a new tube and dried under a stream of nitrogen. The residue

198   was reconstituted in 100 µL of mobile phase (1% of 0.1% formic acid in acetonitrile – FM B) and

199   analysed by LC-QTOF-MS. A blank sample (100 µL of water LC-MS grade) and a quality control

200   sample (QC, a pool of all samples analysed) were also prepared.

201   Five microliters of each sample were injected in duplicate. The autosampler and column temperatures

202   were set at 10 °C and 40 °C, respectively. The analytes were eluted using a CORTECS UPLC T3 C18

203   (2.1 × 150 mm, 1.6 µm) (Waters™) connected to an ExionLC™ AD system (Sciex™) using 0.1%

204   formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The

205   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.

206   The separated metabolites were then ionized using a Turbo V™ Ion Source with an ESI Probe source

207   (Sciex™) and analysed in a ZenoTOF 7600 System equipped with a Zeno trap (Sciex™). Mass spectra

208   were collected, in both positive- and negative ion modes, in Full-Mass Scan from 50 to 1500 Da (250

209   ms accumulation time) and in IDA® mode (Information Dependent Acquisition) from 40 to 1500 Da

210   (50 ms accumulation time). For both methods, ion source gas 1 (GS1) and 2 (GS2) were set to 40 and

211   55 psi, respectively; the curtain gas (CUR) to 25 psi, CAD gas to 7 and the source temperature to 550

212   °C. The Spray Voltage was fixed at 4.5 kV (−4.5 kV in negative mode), the declustering potential (DP)

213   was 80 eV and the collision energy was 30 eV with a collision energy spread (CES) of 15 eV.

214   The data were analysed using SCIEX OS 2.2 software (SCIEX™), in two processing functions: 1)

215   FormulaFinder, to identify possible compound formulas based on TOF-MS spectra (compound

216   molecular weight); 2) LibraryView™ (ver 1.0) to search MS/MS spectra with the built-in accurate mass

217   spectral library (Metabolite High-Resolution MS/MS Spectral Library). Data were expressed as the

218   ratio of the analytes versus the internal standard. The average corresponding area' was normalized to

219   the estimated protein concentration in each extracted sample. Identifications (ID) were obtained based

220   on the value m/z (parent ion) achieved and determined in high resolution.

221

222   *Statistical analyses*

223   Feed efficiency traits and digestibility were analysed by one-way ANOVA, whereas the biochemical

224   profile, SOD and PBMCs were assessed by repeated measurements using the MIXED procedure of

225   SAS. In the repeated measurement analysis, data from the first sampling day (day 3, before including

226   essential oil in the MR of the EO group) was used as a covariate, and adjusted mean values were

227   estimated when the effect was significant.

228   Regarding the faecal microbiota, differences between groups (EO vs. control) along time points in terms

229   of OTU abundances and diversity indices were evaluated with the three following linear models: linear

230   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}

231    $= \mu + timepoint_k + treatment_j + e_{ikj}$, where Y_{kj} is the abundance (counts) or index value for each

232   taxonomy (OTU) and diversity metric per sample $_i$ in timepoint $_k$ and treatment $_j$; timepoint$_k$ is the effect

233   of the categorical variable time point (2 classes); treatment$_j$ is the effect of the categorical variable

234   treatment (2 classes); e_{kj} are the residuals of the model. Faecal microbial diversity was assessed within-

235   (alpha diversity) and across- (beta diversity) samples. All indices (alpha and beta diversity, previously

236   listed in Ranilla et al. 2023 [20]) were estimated from the complete OTU table and filtered for OTUs with

237   more than 10 total counts distributed in at least two samples. Details about the indices used can be found

238   in Appendix S2 of Biscarini et al. (2018)[21]. Beta diversity significances were tested using

239   PERMANOVA.

240   MetaboAnalyst 5.0[22] was used to perform multivariate data analyses, including principal component

241   analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), heat maps, hierarchical

242   clustering, and pathway analysis [22] Volcano plots were drawn at T0 (3 days), T1 (45 days) and T2 (13

243   months) to identify significant metabolites between treatments. Plots used a threshold of 1.5 for fold

244   change (FC) on the x axis and a threshold of 0.05 for the false-discovery rate (adjusted P value) on the

245   y axis.

246

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 milk replacer. However, no long-term effects of EO supplementation during the milk-fed period on residual feed intake (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).

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.

10

274   *Alpha- and beta-diversity indexes.* The estimated alpha diversity indices are reported in Supplementary

275   Table S1. Considering the effect of the treatment alone, several indices (Fisher, Chao1, Shannon, ACE,

276   Observed OTUs, and Simpson) were statistically significant at T1, while none were significant at T2 (P

277   ≤ 0.05). Still, three indices (Simpson, InvSimspon and Shannon) were positive in the 0.05-0.10 area of

278   significance at T2 (Figure 2, A). On the other hand, considering the time effect alone, three indices

279   (Observed_OTUs, Chao1 and ACE) were significant in the control group. By contrast, the EO group

280   had no index with a significant P value (Figure 2, B). Still, the time effect did not show the same

281   behavior (only three indices −Observed_OTUs, Chao1, and ACE− in the 0.05−0.10 range) (Figure 2,

282   C). The relationships between samples were assessed using the weighted Unifrac distances between

283   samples.

284   The distribution of samples (PCA) showed no clustering for samples grouped by treatment (P = 0.905).

285   Clear clusters were only detected when considering samples grouped by time point (P = 0.001).

286

287   *Description of the core microbiome in faeces.* The core faecal microbiota at the family and phylum level

288   comprised 87 and 21 OTUs shared within 100% of the samples (Supplementary Table S2). Eight main

289   phyla were detected in the faecal microbiota, with a relative abundance larger than 1%, in particular,

290   *Firmicutes* (49.21%), *Bacteroidetes* (32.19%), *Proteobacteria* (4.15%), *Verrucomicrobia* (3.1%),

291   *Cyanobacteria* (2.9%), *Euryarchaeota* (2.26%), *Spirochaetes* (1.54%) and *Actinobacteria* (1.29%). The

292   core faecal microbiota at the genus level was composed of 228 OTUs shared within 100% of the

293   samples, plus uncultured or unknown genera (Supplementary Table S3, Figure 3). Fourteen main genera

294   were detected in the faecal microbiota, with a relative abundance larger than 2%, in particular,

295   *Bacteroides* (13.4%), *Ruminococcaceae* UCG-005 (12%), *Alloprevotella* (9.63%), *Ruminococcaceae*

296   UCG-010 (6.7%), *Faecalibacterium* (5.9%), *Ruminococcaceae UCG-014* (4.8%), *Prevotella 9*

297   (4.49%), *Akkermansia* (4.45%), *Blautia* (3.75%), *Alistipes* (3.24%), *Rikenellaceae RC9 gut group*

298   (3.16%), *Prevotella 2* (2.69%), *Escherichia-Shigella* (2.49%) and *Methanobrevibacter* (2.38%).

299   *Effects of treatments on microbiota at the genus level.* Thirty-eight genera differed significantly when

300   the treatment groups were compared over time: 34 genera were differentially expressed at T1, of which

301   18 decreased in the EO group compared with the control, the most significant being that 16 increased.

302   The remaining four genera were differentially expressed at T2, of which 2 (*Prevotella 2* and

303   *Howardella*) decreased in the EO group compared with the control, and in the same group, two

304   (*Caldicoprobacter* and *Ruminiclostridium 1*) increased (Supplementary Table S4, Figure 3) as

305   compared to the control, although the increase was limited.

306

307   *Plasma metabolome analysis*

308   Untargeted metabolomic analyses identified 109 metabolites (Supplementary Table S5). The supervised

309   PCA (Figure 3 (A)) showed separation of the three age-related group samples. The heat map in Figure

310   3 (B), generated by hierarchical clustering, also presented evidence that three age-related groups can be

311   distinguished by their metabolomes. Figure 3 (C) shows the Volcano plot score of the changes, proving

312   the significant metabolites that change along the time course.

313   When the plasma metabolomes from control calves and those fed with EO were compared, no statistical

314   changes were evidenced between the groups regarding the plasma metabolome at T0 (Figure 4, Panels

315   A and B). However, at T1, we observed significant statistical changes ($P > 0.05$) for indole, propionic

316   acid, succinic acid, and butyric acid (Figure 4, Panel C and D), whereas T2 differed significantly for 10

317   metabolites (Figure 4, Panel E and F).

318   The major metabolic pathways that changed with age in control calves were aminoacyl-tRNA

319   biosynthesis and valine, leucine, and isoleucine biosynthesis, followed by the biosynthesis of several

320   amino acid pathways (Figure 5, Panel A). The significant pathways modified by EO feeding at T2 were

321   arginine biosynthesis and alanine, aspartate, and glutamate metabolism (Figure 5, Panel B).

322

323   **Discussion**

324   Calves are born as functional monogastric animals that rely on nutrients from milk or milk replacer

325   (MR) in early life[23]. This initial period of life is essential and could affect the welfare, growth and

326   development of newborn calves as well as drive their future performance. The present study investigated

327   the effects of neonatal feeding with EO on feed efficiency and related performance parameters, immune

328   status, the gut microbiome, and the plasma metabolome in both the short and the long term.

329 According to the results, calves receiving EO in the MR improved their feed efficiency during this

330 phase. Thus, these EO calves gained more weight during the suckling phase consuming the same

331 amount of dry matter as the control group. Our results agree with previous studies[24], showing that EO

332 (carvacrol, caryophyllene, -cymene, cineole, terpinene, and thymol) enhance the growth rate and

333 stimulate appetite in newborn dairy calves. Moreover, Liu et al.[11] demonstrated that calves fed

334 commercial EO from three days of age through the end of the 70-day experimental period showed

335 greater apparent total-tract nutrient digestibility of DM, ADF, NDF, starch, and minerals (Na, Mg, P,

336 S, K, Ca, Cl, Mn, Fe and Cu) as compared with calves fed the control diet. Moreover, they also indicated

337 that EO given during the early life may consistently challenge gut health and prevent diarrhoea. Other

338 authors have suggested that the positive effects of EO might be related to the inhibition of pernicious

339 bacteria exerted by carvacrol and thymol lipophilic components at the gut level[25]. Nevertheless, in the

340 present study, this positive effect on feed efficiency was not observed later on during the replacement

341 phase.

342 Early supplementation with EO did not modify most PBMC populations, including CD4$^+$ (T helper

343 lymphocytes), CD8$^+$ (T cytotoxic lymphocytes), CD21$^+$ (B cells) and WC1$^+$ ($\gamma\delta$ T lymphocytes) at 3

344 and 45 days and 13 months. However, an interaction between diet and day for CD21$^+$ was observed (P

345 = 0.048; Table 4), which may indicate a persistent decrease of CD21$^+$ in the EO group compared to the

346 control group that did not receive EO. By contrast, CD14$^+$ monocytes presented statistically significant

347 increased average values in the EO group, and this effect persisted throughout the replacement phase.

348 It must be considered that CD14$^+$ monocytes are a different lineage from intestinal macrophages, cells

349 which are key regulators of inflammatory responses to the gut microbiome and play a central role in

350 maintaining gastrointestinal homeostasis, epithelial integrity and protecting against specific mucosal

351 pathogens[26] ; however, if a similar effect of EO on the intestine were assumed, the changes observed in

352 CD14$^+$ and the smaller decrease in CD21$^+$ over time observed in the EO group might have influenced

353 or be related to changes in the gut microbiome.

354 To clarify this hypothesis, the effects of EO on the uncultured faecal microbiota were studied. Diversity

355 significance indices described the impact of time and treatment, underlying that, for differences between

356 groups, treatment was crucial, while for diversity within groups (beta diversity) the time effect was

357   critical. Moreover, feeding EO during early life increased the abundance of two genera, namely

358   *Akkermansia* and *Allistipes,* at T1. Bacteria belonging to the genus *Akkermansia* are included in the

359   intestinal mucus and regulate the integrity of the intestinal barrier by producing both anti-inflammatory

360   cytokines (e.g., IL-10)[27] and SCFA to further support gut health[28]. In addition, when abundant,

361   *Akkermansia* fulfills protective roles against several diseases in humans[29] as well as in calves [30] and is

362   regarded as a third-generation probiotic [29]. Regarding *Allistipes,* previous studies have demonstrated in

363   humans that this genus includes bacteria positively related to healthy anaerobes in the host[31].

364   Of the four bacteria genera whose abundance was decreased in EO calves at T1 (e.g. *Ruminococcaceae*

365   *UCG-014, Faecalibacterium, Blautia, and Alloprevotella*), bacteria belonging to genera such as

366   *Faecalibacterium* and *Blautia* dominate the faeces of pre-weaned calves[32]. A higher abundance of

367   *Faecalibacterium* has been associated with a lower sensitivity to neonatal diarrhoea[33,34]. We may then

368   speculate that decreasing the abundance of *Faecalibacterium* genus when feeding EO might expose the

369   calves to an increased risk of developing gut alterations related to body weight and ADG[35] . However,

370   we observed an increase of ADG at T1, probably because the magnitude of the decreased abundance of

371   *Blautia* in EO animals was insufficient (by itself) to induce changes in the calves' ADG. The other two

372   genera that were decreased at T1 in the group of calves being fed EO are *Ruminococcaceae UCG-014*

373   and *Alloprevotella*. *Alloprevotella* produces succinic acid as the end-product of plant fibre[36].

374   Interestingly, as discussed below, the decrease in the abundance of the genus *Alloprevotella* corresponds

375   to the decrease of succinic acid in the plasma metabolome. In calves, both *Ruminococcaceae UCG-014*

376   and *Alloprevotella* are critical biomarkers of gut health, and their abundance correlates with a healthy

377   phenotype[37,38]. Again, reducing the abundance of both genera might increase the risk of developing

378   intestinal diseases in calves fed EO. Finally, the effect of EO at T2 was limited to a decrease in the

379   genus *Prevotella-2*. The bacteria belonging to this genus, like other *Prevotellaceae*, are reduced during

380   calf diarrhoea[38], so again, this change promoted by EO might indicate gut dysbiosis.

381   The last part of the study also revealed changes induced by treatment on the untargeted metabolome of

382   calf' plasma. No changes were found at T0, confirming that the two groups were homogeneous

383   concerning the plasma metabolome at 3 days of life. At T1 (45 days old), only three metabolites

384   changed: 3-indole propionic acid, butyric acid and succinic acid. The descent of butyric acid (a

385 metabolite produced by intestinal bacteria) agrees with the decrease in *Blautia*, *Ruminococcaceae* and

386 *Faecalibacterium*, three bacterial genera associated with butyric acid production[39]. Moreover, 3-indole-

387 propionic acid is a bacterial metabolite derived from tryptophan metabolism and produced by the genus

388 *Faecalibacterium*. As for *Blautia*, there was a correlation between the decrease of *Faecalibacterium* at

389 T1 and the decrease of 3-indole propionic acid[40] , an inhibitor of gut dysbiosis and endotoxin leakage

390 and a potent scavenger of hydroxyl radicals[41]. Butyric acid also shows a protective effect against the

391 inflammatory response[42], so the decrease of both metabolites at T1 seems to corroborate the increased

392 risk of gut disease when feeding EOs during early life.

393 At T2, 340 days after birth, the plasma abundance of 14 metabolites changed. Pathway analysis of the

394 changes occurring at T2 suggests that most changes were related to a reduction of energy and protein

395 metabolism and a potential decrease of the antioxidant capacity. In particular, metabolites associated

396 with the citric acid cycle, such as fumaric acid and malic acid, and carnitines, such as L-acetyl carnitine

397 and L-carnitine, were decreased in the EO group. Biogenic amines, such as taurine (a sulphur-containing

398 non-essential amino acid whose low level may reduce antioxidant activity) and citrulline, were also

399 decreased. Additionally, two metabolites related to decreased protein catabolism (e.g., creatinine and

400 1-methylhistidine) were also decreased, which may indicate reduced proteolytic processes[43]. In any

401 case, the magnitude of all these changes was not enough to promote variations in ADG during the

402 replacement phase. In conclusion, according to the results of the present study, daily administration of

403 0.23 ml of oregano EO (accounting for 200 mg of carvacrol) to newborn dairy calves promotes a

404 positive transitory effect on body growth and feed efficiency during the suckling period but no long-

405 term effects during the replacement phase. This early supply of EO also induces early changes in the

406 gut microbial population consistent with changes in the plasma metabolome, but not all of them are

407 considered desirable from a gut health point of view. Future work should be focused on determining

408 the optimum dosage and duration of EO administration to determine if EOs may provide a viable natural

409 alternative to antibiotics to minimize health challenges while improving calf growth performance and

410 economic returns to the dairy calf raiser. Therefore, additional research studies is warranted to evaluate

411 different mechanisms of action of EO on the interaction between the microbiota, immunity and health.

Author contributions

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.

Competing interest statement

The authors declare no competing interests.

Ethical standards

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)

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

440 derived as described in the test were deposited and available in the Università degli Studi di Milano

441 Dataverse repository. A private URL for reviewers is hereby provided, while datasets will be disclosed

442 upon article acceptance (https://dataverse.unimi.it/privateurl.xhtml?token=8b1dab61-e1e3-49a2-a4a2-

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558   Friesian cows. *J Dairy Sci*. 2013;96,6423-6439.
559

560   **Table 1.** Ingredients and chemical composition (g/kg DM unless otherwise stated) of the total
561   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
[1]aNDF	481
ADF	290
CP	142
Fat	33
Ash	104
Crude energy, kcal/kg DM	4342

562   [1] amylase-treated neutral detergent fibre

563

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

	CTRL	EO	SED[1]	*P*-value
Birth LBW[2], kg	39.42	39.09	1.620	0.838
Suckling period (from day 4 to 45)				
ADG[3], g/d	100	172	20.3	0.003
Final LBW, kg	44.0	47.4	1.40	0.030
Replacement period (from day 322 to 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[4], 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

566 [1] Standard error of the difference.
567 [2] LBW = live body weight.
568 [3] Average daily gain
569 [4] Residual feed intake (RFI) calculated using ADG estimated by regression
570

22

571   **Table 3.** Biochemical profile the day 45 (end of EO administration) and day 370 (replacement phase) of dairy calves being supplied essential oil
572   (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[1] (U/l)	92.4	99.2	99.3	92.3	15.18	15.17	0.660	0.650	0.997
βHB[2] (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[3] (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[4] (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

573   [1]AST, aspartate aminotransferase
574   [2]Beta-hidroxybutirate
575   [3]GGT, gamma-glutamyl transpeptidase
576   [4]NEFA, non-esterified fatty acid

1

577 **Table 4.** Peripheral blood mononuclear cell (PBMC) measured by flow cytometry the day 45 (end of EO administration) and day 370
578   (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[1]**	**SED[2]**	**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

579 [1] standard error of the difference to compare experimental groups

580 [2] standard error of the difference to compare sampling days

581

582 **Supplementary Table 1:** estimated alpha diversity indices of the fecal microbiota in the two experimental groups

583

Treatment	Timepoint	Observed	Chao1	Ace	Shannon	Simpson	Invsimpson	Fisher
Control	T1	1027,25	1152,918	1140,058	5,585	0,99	107,155	219,759
EO	T1	847,27	947,27	943,406	5,049	0,982	73,143	162,225
Control	T2	883,375	995,327	991,574	5,352	0,987	96,967	186,068
EO	T2	780,25	875,515	874,901	4,861	0,977	61,217	146,787

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586

587   **Supplementary Table 2:** The core fecal microbiota at the family and phylum level

588

Phylum	average abundance expressed in %
Firmicutes	49,21505799
Bacteroidetes	32,19819766
Proteobacteria	4,152545955
Verrucomicrobia	3,096955875
Cyanobacteria	2,917567392
Euryarchaeota	2,257383339
Spirochaetes	1,549456278
Actinobacteria	1,299001154
Elusimicrobia	0,9014819117
Fusobacteria	0,5712724842
Tenericutes	0,4261650468
Lentisphaerae	0,3998672588
Kiritimatiellaeota	0,3962669664
Patescibacteria	0,2589079844
Epsilonbacteraeota	0,2175828889
Planctomycetes	0,07310158929
Excavata	0,02856753757
Synergistetes	0,02700219305
Chloroflexi	0,01142701503
Chlamydiae	0,00125227562
Fibrobacteres	0,0009392067147

589

Family	Percentage
Ruminococcaceae	28,35032383
Prevotellaceae	13,63601855
Lachnospiraceae	11,58471442
Bacteroidaceae	9,283816307
Rikenellaceae	4,632555227
Akkermansiaceae	3,088891933
Erysipelotrichaceae	1,749114603
Methanobacteriaceae	1,734165574
Enterobacteriaceae	1,727356331

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Acidaminococcaceae	1,565186765
Spirochaetaceae	1,549455065
Christensenellaceae	1,237560412
Clostridiales vadinBB60 group	1,03610073
Elusimicrobiaceae	0,9014812061
Streptococcaceae	0,8865321776
Marinifilaceae	0,7816541765
Veillonellaceae	0,7555129434
Muribaculaceae	0,6984561802
Bifidobacteriaceae	0,64711292
Clostridium sp, K4410,MGS-306	0,6056313225
Barnesiellaceae	0,5913084313
Fusobacteriaceae	0,5712720371
Lactobacillaceae	0,5340951337
Bacteroidales RF16 group	0,5315123173
Desulfovibrionaceae	0,5156240828
Peptostreptococcaceae	0,4732815466
Family XIII	0,4552018315
Coriobacteriaceae	0,4410354746
Clostridiaceae 1	0,4302346058
Succinivibrionaceae	0,3716907664
Methanocorpusculaceae	0,3498542274
Tannerellaceae	0,3288786272
Burkholderiaceae	0,2664214295
Saccharimonadaceae	0,2589077817
Paludibacteraceae	0,2445848905
Victivallaceae	0,2179740544
Campylobacteraceae	0,2121822842
vadinBE97	0,1818928914
Methanomethylophilaceae	0,1645958479
p-251-o5	0,1592736807
Eggerthellaceae	0,1587258106
Peptococcaceae	0,1109045728
F082	0,09313792631
p-2534-18B5 gut group	0,09133778152

Flavobacteriaceae	0,08319799636
Pirellulaceae	0,07310153208
Clostridium sp, CAG:306	0,06699669321
Pasteurellaceae	0,0337331481
Trichomonadea	0,02856751521
Atopobiaceae	0,02778484356
Synergistaceae	0,02700217191
Mycoplasmataceae	0,02238440918
Defluviitaleaceae	0,02105386738
gut metagenome	0,01596650165
Corynebacteriaceae	0,01267928072
Acetobacteraceae	0,01166180758
Anaeroplasmataceae	0,01033126578
metagenome	0,009548594126
Puniceicoccaceae	0,008061517992
Anaerolineaceae	0,007983250827
Blattella germanica (German cockroach)	0,007122312012
Leuconostocaceae	0,006652709022
Methanosarcinaceae	0,006183106032
Candidatus Melainabacteria bacterium MEL,A1	0,005635235878
Eubacteriaceae	0,005087365723
Arcobacteraceae	0,004382961238
Caldicoprobacteraceae	0,004304694073
rumen bacterium YS2	0,003678556753
gir-aah93h0	0,003443755259
Staphylococcaceae	0,003443755259
JG30-KF-CM45	0,003365488094
Moraxellaceae	0,003052419434
Dietziaceae	0,002739350774
Methanomicrobiaceae	0,002582816444
Aerococcaceae	0,002582816444
Xanthomonadaceae	0,001878411959
Enterococcaceae	0,001643610464
Brevibacteriaceae	0,001643610464
Dysgonomonadaceae	0,001330541804

Chlamydiaceae	0,001252274639
Coriobacteriales Incertae Sedis	0,001174007475
Sphingobacteriaceae	0,001017473145
Helicobacteraceae	0,0009392059796
Fibrobacteraceae	0,0009392059796
Nocardiaceae	0,0007826716497
Rhizobiaceae	0,0007044044847
Uncultured or unknown	5,256814135

590

591 **Supplementary Table 3:** The core fecal microbiota at the genus level

592

Genus	average abundance expressed in %
Uncultured or unknown	14,46141372
Bacteroides	9,283859904
Ruminococcaceae UCG-005	8,341205743
Alloprevotella	6,674889975
Ruminococcaceae UCG-010	4,658483535
Faecalibacterium	4,103801532
Ruminococcaceae UCG-014	3,336779714
Prevotella 9	3,114969527
Akkermansia	3,088906438
Blautia	2,607091508
Alistipes	2,246747788
Rikenellaceae RC9 gut group	2,193369331
Prevotella 2	1,872863786
Escherichia-Shigella	1,725877359
Methanobrevibacter	1,653792962
Phascolarctobacterium	1,561593809
[Eubacterium] coprostanoligenes group	1,506650001
Christensenellaceae R-7 group	1,231148286
Treponema 2	1,206494014
Subdoligranulum	0,9208957876
Elusimicrobium	0,9014854395
Streptococcus	0,8855971304
Lachnospiraceae NK4A136 group	0,8747179434
Roseburia	0,6617519874

Bifidobacterium	0,6464898186
Anaerostipes	0,6437504549
[Ruminococcus] torques group	0,6273925406
Odoribacter	0,6261402601
Ruminococcaceae UCG-013	0,6022686627
Ruminococcaceae UCG-002	0,6021903952
Lachnoclostridium	0,5859107484
Fusobacterium	0,5712747198
Prevotellaceae UCG-004	0,5348020497
Lactobacillus	0,5340976419
Barnesiella	0,4805626496
Tyzzerella 4	0,469292125
Collinsella	0,4410375457
Lachnospiraceae AC2044 group	0,4390808574
Lachnospiraceae UCG-008	0,4363414938
Erysipelotrichaceae UCG-003	0,3834326418
Clostridium sensu stricto 1	0,3791279275
Ruminiclostridium 9	0,3692662184
Lachnospiraceae FCS020 group	0,368327008
Anaerovibrio	0,353925782
Methanocorpusculum	0,3498558703
Sphaerochaeta	0,3429683275
Parabacteroides	0,3288801716
Ruminococcaceae UCG-009	0,3198794054
Romboutsia	0,3149485508
GCA-900066575	0,3039128288
Oscillibacter	0,27088393
Mailhella	0,263526782
Faecalitalea	0,2629006417
Candidatus Saccharimonas	0,2589089976
Ruminococcus 1	0,2528823976
Desulfovibrio	0,2488124859
Erysipelotrichaceae UCG-004	0,2418466755
Megasphaera	0,2410640001
Ruminococcaceae NK4A214 group	0,2288542651

Agathobacter	0,2278367871
Family XIII AD3011 group	0,2271323794
Ruminobacter	0,2252539586
Lachnoclostridium 10	0,2193056261
Lachnospiraceae UCG-004	0,2136703638
Campylobacter	0,2121832806
[Ruminococcus] gauvreauii group	0,2089743118
Prevotellaceae UCG-003	0,19856473
dgA-11 gut group	0,1923815949
Prevotella 1	0,187685543
Sutterella	0,1851809819
Prevotellaceae NK3B31 group	0,1716406988
Ruminococcus 2	0,1698405456
Oscillospira	0,1687448001
Butyricicoccus	0,1573960079
[Eubacterium] nodatum group	0,1446384001
Butyricimonas	0,140803291
Megamonas	0,1400206157
Prevotellaceae UCG-001	0,1152880754
Dorea	0,1040958182
Intestinibacter	0,0957994598
Turicibacter	0,09564292473
Fournierella	0,095095052
Ruminiclostridium 5	0,08687696109
Marvinbryantia	0,08664215849
Tyzzerella	0,08656389096
Ruminococcaceae UCG-008	0,08484200525
Candidatus Soleaferrea	0,08124169875
Methanosphaera	0,08030248836
Parasutterella	0,07787619485
p-1088-a5 gut group	0,07310187537
[Eubacterium] oxidoreducens group	0,07106691952
Lachnospiraceae UCG-010	0,06793621822
Lachnospiraceae UCG-001	0,06785795069
Intestinimonas	0,06073560523

Sharpea	0,05564821562
Ruminococcaceae UCG-004	0,05517861042
[Eubacterium] hallii group	0,05290885198
Terrisporobacter	0,05251751432
RumEn M2	0,04860413769
Succinivibrio	0,04664744938
Sarcina	0,04641264678
Negativibacillus	0,04492556366
Lachnospiraceae NK3A20 group	0,0447690286
Cellulosilyticum	0,04124698964
Ruminococcaceae UCG-011	0,03952510392
Anaerovorax	0,03921203379
Ruminiclostridium	0,03882069613
Coprobacter	0,03772495067
[Anaerorhabdus] furcosa group	0,03709881041
Prevotellaceae Ga6A1 group	0,03568999483
Acetitomaculum	0,03435944677
Gallibacterium	0,03373330651
Coprococcus 3	0,0317766182
Erysipelatoclostridium	0,03169835067
[Eubacterium] eligens group	0,03146354807
Fretibacterium	0,02700229872
[Eubacterium] brachy group	0,02645442599
Butyrivibrio	0,02410640001
Anaerofilum	0,02410640001
Lachnospiraceae NC2004 group	0,02363679482
Mycoplasma	0,0223845143
Defluviitaleaceae UCG-011	0,02105396625
Holdemanella	0,02097569871
Simplicimonas	0,02066262858
Olsenella	0,02027129092
Anaerocolumna	0,02019302339
Dielma	0,01917554547
[Ruminococcus] gnavus group	0,01815806754
Lachnospiraceae UCG-006	0,01714058962

Veillonella	0,01682751949
Candidatus Methanomethylophilus	0,01643618183
Pseudobutyrivibrio	0,01596657663
Howardella	0,01494909871
Oribacterium	0,01471429611
[Acetivibrio] ethanolgignens group	0,01416642339
Ruminiclostridium 1	0,01408815585
Coprococcus 2	0,01314894546
Faecalicoccus	0,01307067793
Lachnospiraceae UCG-002	0,01260107273
Syntrophococcus	0,01205320001
Mogibacterium	0,01166186234
Acetobacter	0,01166186234
Holdemania	0,01095745455
Anaeroplasma	0,01033131429
Peptococcus	0,01025304676
Candidatus Stoquefichus	0,01009651169
Eisenbergiella	0,01001824416
Corynebacterium 1	0,009939976629
Fusicatenibacter	0,009861709097
[Eubacterium] xylanophilum group	0,009783441564
Ruminiclostridium 6	0,009705174032
Lachnospiraceae ND3007 group	0,009235568837
Papillibacter	0,008765963642
Pygmaiobacter	0,008609428577
Flexilinea	0,007983288316
Pentatrichomonas	0,007905020784
Ruminococcaceae UCG-003	0,007591950654
UBA1819	0,007435415589
Cerasicoccus	0,007357148056
Anaerosporobacter	0,007357148056
Leuconostoc	0,006652740264
Hydrogenoanaerobacterium	0,006652740264
Lachnoclostridium 5	0,006496205199
GCA-900066225	0,006417937666

Atopobium	0,006417937666
Saccharofermentans	0,006339670134
Victivallis	0,006261402601
Methanimicrococcus	0,006183135069
Family XIII UCG-001	0,006026600004
[Eubacterium] fissicatena group	0,005556994809
Flavonifractor	0,005400459743
Sanguibacteroides	0,005165657146
28-4	0,005165657146
Solobacterium	0,005009122081
Clostridium sensu stricto 6	0,004696051951
Moryella	0,004539516886
Enterorhabdus	0,004539516886
Caproiciproducens	0,004461249353
Arcobacter	0,004382981821
Caldicoprobacter	0,004304714288
Erysipelotrichaceae UCG-006	0,004226446756
Erysipelotrichaceae UCG-010	0,004148179223
Lachnospiraceae UCG-003	0,004069911691
Succiniclasticum	0,003600306496
Jeotgalicoccus	0,003443771431
Pelistega	0,003365503898
A2	0,003365503898
Coprococcus 1	0,003287236366
Bilophila	0,003287236366
Anaerofustis	0,003208968833
DNF00809	0,003130701301
Acinetobacter	0,003052433768
[Eubacterium] ruminantium group	0,002739363638
Dietzia	0,002739363638
Corynebacterium	0,002739363638
Methanomicrobium	0,002582828573
Gordonibacter	0,00250456104
[Eubacterium] ventriosum group	0,00219149091
Clostridioides	0,00219149091

Slackia	0,002113223378
Lachnospiraceae NK4B4 group	0,001956688313
Stenotrophomonas	0,00187842078
Murimonas	0,001800153248
Eubacterium	0,001800153248
Catenibacterium	0,001800153248
Aerosphaera	0,001721885715
Enterococcus	0,001643618183
Brevibacterium	0,001643618183
Serratia	0,001487083118
[Clostridium] innocuum group	0,001408815585
Candidatus Methanogranum	0,001408815585
Fermentimonas	0,001330548053
Candidatus Methanoplasma	0,001330548053
Erysipelotrichaceae UCG-008	0,00125228052
Chlamydia	0,00125228052
Ruminococcaceae UCG-001	0,001095745455
Coriobacteriaceae UCG-002	0,001095745455
Ruminococcaceae UCG-007	0,001017477923
Erysipelotrichaceae UCG-009	0,001017477923
Lactococcus	0,0009392103902
Helicobacter	0,0009392103902
Fibrobacter	0,0009392103902
Flavobacterium	0,0008609428577
Facklamia	0,0008609428577
Lachnospiraceae UCG-009	0,0007826753251
Gordonia	0,0007826753251
Pseudohoeflea	0,0007044077926
Lachnospiraceae XPB1014 group	0,0007044077926
Succinivibrionaceae UCG-002	0,0006261402601

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594
595   **Supplementary Table 4:** Differentially abundant genera by time point considering the treatment effect.
596

Genus	timepoint	p-value	The behavior of the Treated against the Control
Bacteroides	T1	0,032	-0,1082255713

Alloprevotella	T1	0,042	-0,083461477
Faecalibacterium	T1	0,042	-0,07114639098
Ruminococcaceae UCG-014	T1	0,024	-0,04990262563
Blautia	T1	0,004	-0,04734452235
Prevotella 2	T2	0,028	-0,04551684
Escherichia-Shigella	T1	0,049	-0,01930628431
Odoribacter	T1	0,003	-0,01601543762
Parabacteroides	T1	0,031	-0,00990739325
Desulfovibrio	T1	0,038	-0,007213628188
Lachnospiraceae UCG-008	T1	0,005	-0,007191420713
Butyricimonas	T1	0,035	-0,003175830801
Fournierella	T1	0,023	-0,003156335113
Erysipelatoclostridium	T1	0,046	-0,000958589825
Syntrophococcus	T1	0,042	-0,000258443715
Flavonifractor	T1	0,044	-0,0002002584138
Howardella	T2	0,023	-0,0001895123775
[Eubacterium] fissicatena group	T1	0,018	-0,0001574886075
28-4	T1	0,039	-0,000135367475
Pentatrichomonas	T1	0,039	-8,19E-05
[Eubacterium] ruminantium group	T1	0,014	5,14E-05
Caldicoprobacter	T2	0,023	7,55E-05
Pygmaiobacter	T1	0,041	0,0001902854863
Ruminiclostridium 1	T2	0,017	0,0002197081638
Defluviitaleaceae UCG-011	T1	0,002	0,0003010802013
Anaerovorax	T1	0,027	0,000436225175
Ruminococcaceae UCG-011	T1	0,012	0,000572855325
[Eubacterium] brachy group	T1	0,008	0,00063284201
Cellulosilyticum	T1	0,023	0,000726269475
Candidatus Soleaferrea	T1	0,027	0,0008924702675
Prevotellaceae UCG-003	T1	0,005	0,002153055325
Mailhella	T1	0,014	0,003556814265
Ruminobacter	T1	0,010	0,00361514429
Ruminococcaceae UCG-009	T1	0,020	0,003896232625
dgA-11 gut group	T1	0,009	0,004310734625
Ruminococcaceae UCG-013	T1	0,010	0,007547157838

Alistipes	T1	0,011	0,02473722013
Akkermansia	T1	0,003	0,04232093193

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598

599 **Supplementary Table 5:** the list of differentially abundant metabolites

600

601 Figure 1. Peripheral blood mononuclear cell (PBMC) measured by flow cytometry the day 45 (end of

602 EO administration) and day 370 (replacement phase) of dairy calves being supplied essential oil (EO

603 group) or not (CTRL group) during the suckling period.

604

605 Figure 2: the fecal microbiota of calves after EO feeding

606 Panel A, B, and C are scatterplots reporting the significance of the alpha diversity indices in

607 treatments and time points. P-values were obtained from a linear regression model with the Control

608 group as the benchmark. The dashed lines identify the suggestive-significance area. Panel D and E

609 present the Principal Component Analysis (PCA) plot of unweighted Unifrac distances of fecal

610 microbiota in this study. Panel D represents the clustering per treatment, while Panel E represents the

611 clustering per time point. Panels F and G show significantly abundant taxa in fecal microbiota at the

612 genera level. Panel F is the heatmap of the significance of taxa relative abundance. Panel G Bar plot

613 presents the behavior of the significantly different abundant genera along time points: the scale in the

614 x-axis is the ratio of Treated against Controls.

615

616 Figure 3. The plasma metabolome changes in calves not fed with EO

617 Panel A: Pairwise PCA with density plots outlines the different times of sampling (T0, T1, and T2).

618 Panel B: Presentation of the 50 most significant metabolites determined by one-way ANOVA

619 analysis.

620 Panel C: Clustering results are shown as a heatmap (distance measured using Euclidean and clustering

621 algorithm using ward.D).

622

623 Figure 4: the changes in the plasma metabolomes of calves fed with EO-enriched diets.

624 The plasma metabolome changes between in calves fed (EO) and not fed (CON) with EO at T0

625 (Panels A and B), T1 (Panels C and D), and T2 (Panel E and F).

626 Panels A, C, and E show the Volcano plot of changes at T0, T1, and T2, respectively. Important

627 features selected by the volcano plot with fold change threshold (x) 2 and t-tests threshold (y) 0.1. The

628 red circles represent features above the threshold. Note that both the fold changes and the p-values are

629 log-transformed. The further its position away from the (0,0), the more significant the feature is.

630 Panel B, D, and F present the 3D scores plot between the selected PCs at T0 (B), T1 (D), and T2 (F),

631 respectively. The explained variances are shown in brackets.

632

633 Figure 5. The metabolic pathway changes

634 Summary of Pathway Analysis: the circle's size indicates the pathway's impact, while the color

635 represents the significance (the more intense the red color, the lower the significance). Panel A: The

636 significant pathway modified in the plasma metabolome in control calves not fed with EO. All the

637 changed metabolites are included. Panel B: The significant pathway modified in the plasma
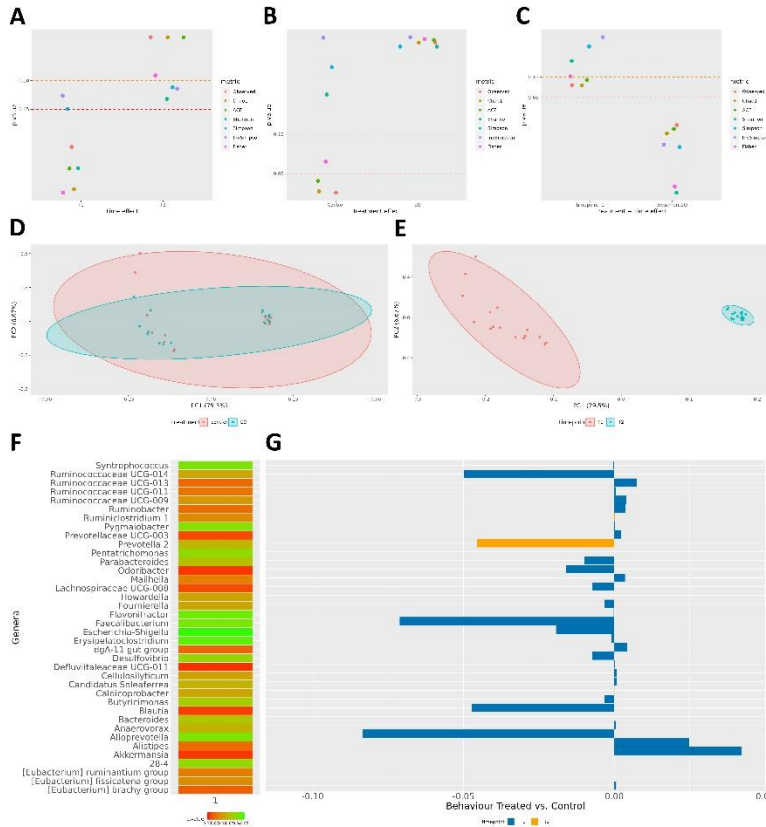
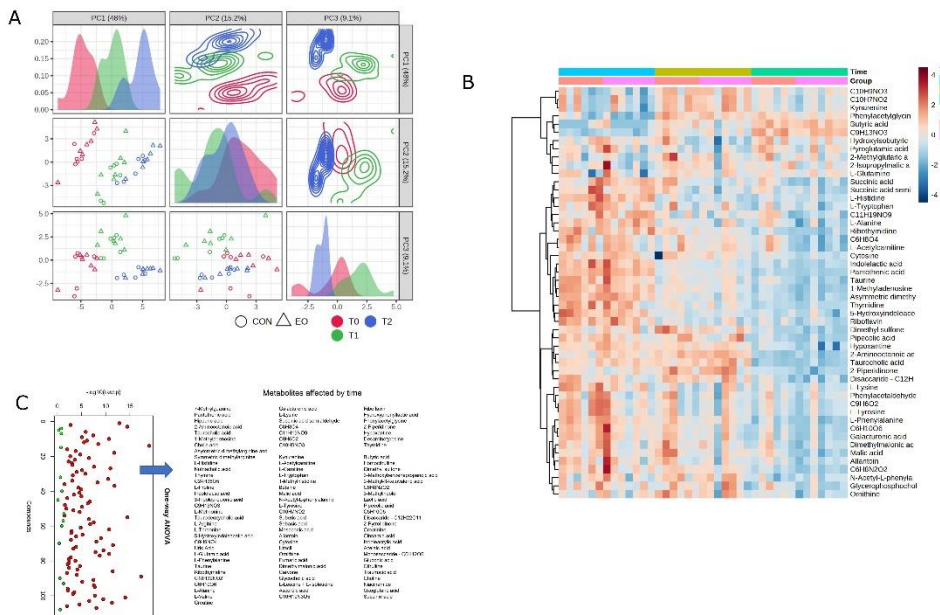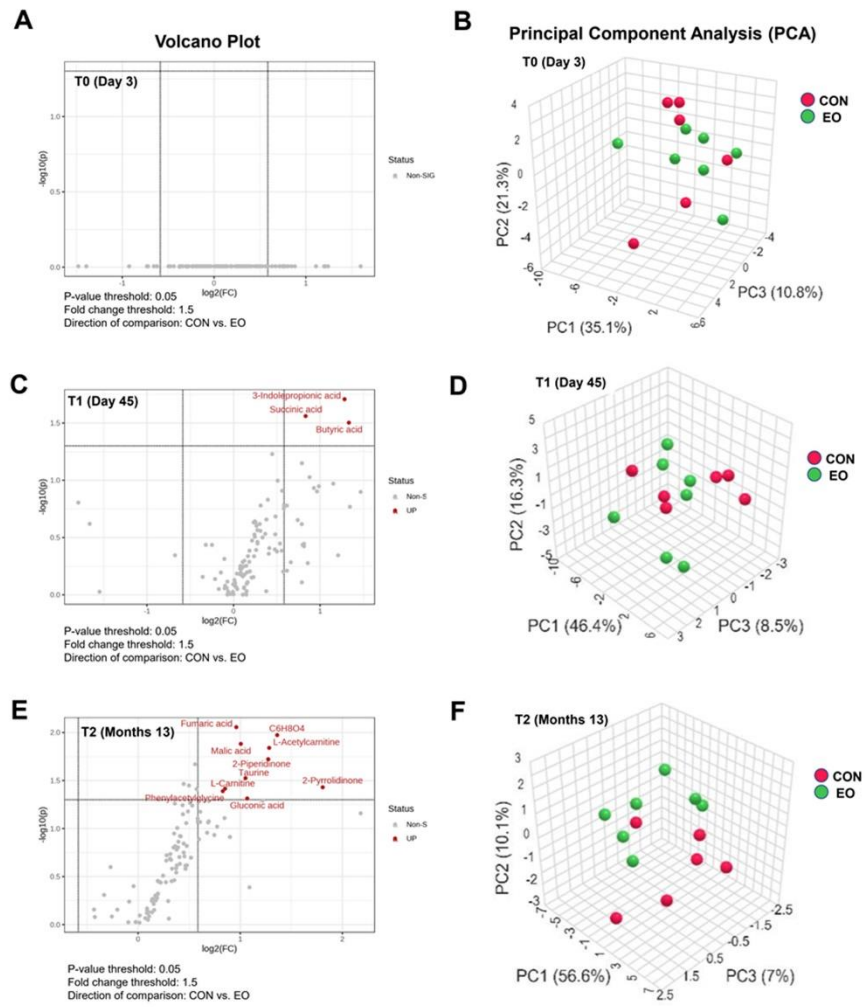638 metabolome changes in calves at T2

639
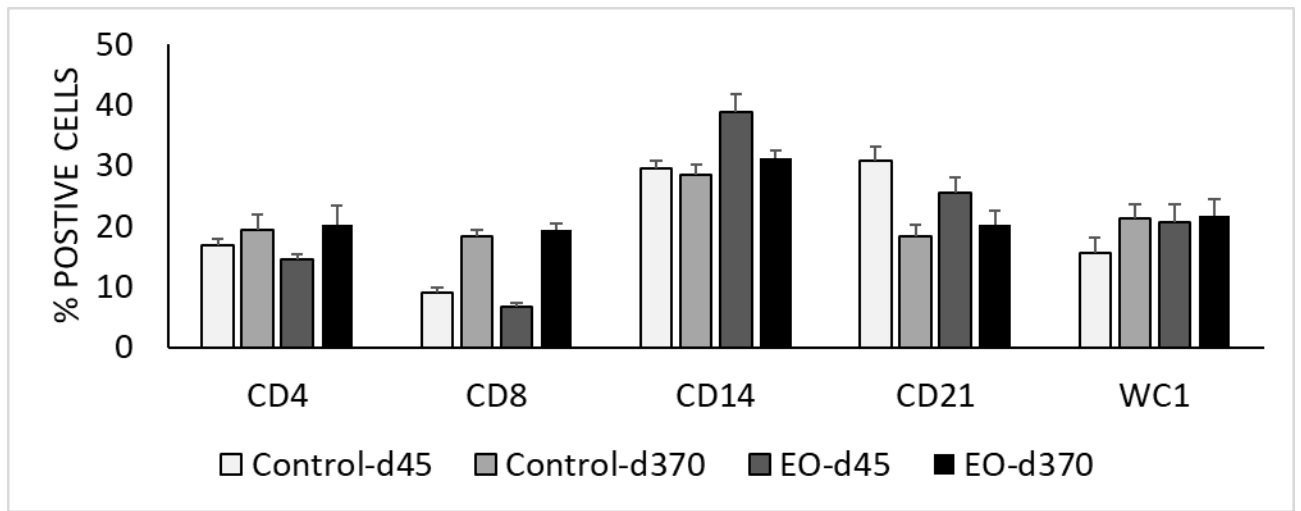
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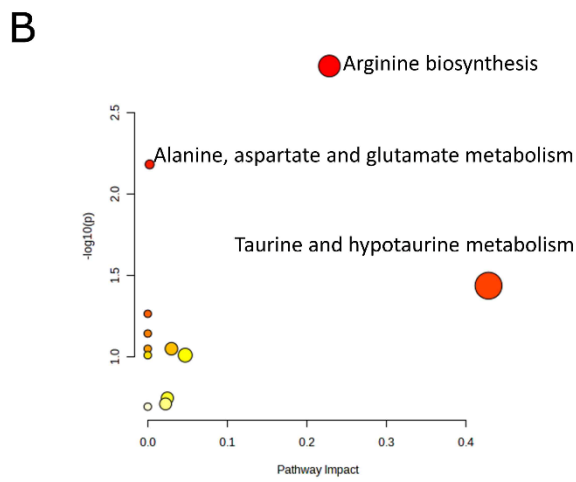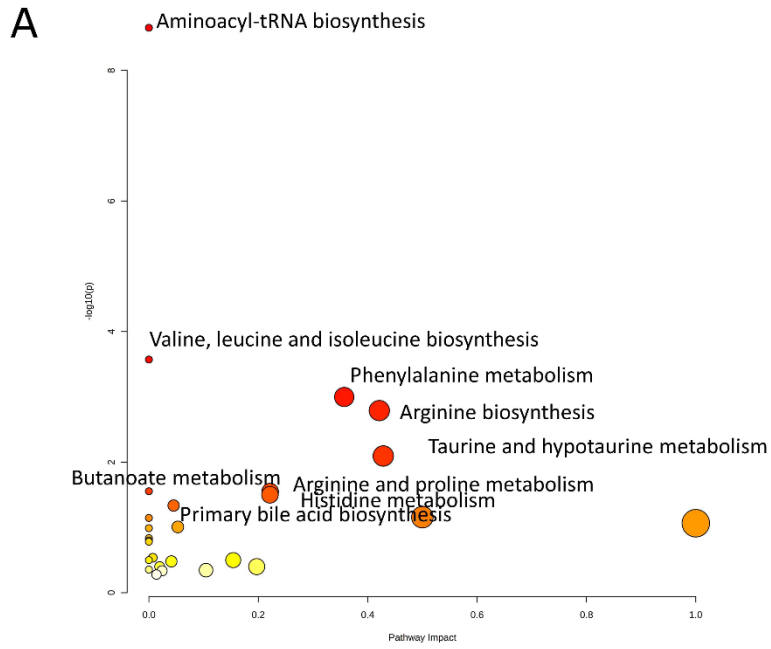641
642   Figure 1

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645   Figure 2
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648   Figure 3

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653   Figure 5