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1 Article

# 2 Maternal linoleic acid overconsumption alters 3 offspring gut and adipose tissue homeostasis in 4 young but not older adult rats.

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16 **Abstract:** Maternal *n*-6 polyunsaturated fatty acids (PUFA) consumption during gestation and  
17 lactation can predispose offspring to the development of metabolic diseases such as obesity later in  
18 life. However, the mechanisms underlying the potential programming effect of *n*-6 PUFA upon  
19 offspring physiology are not yet all established. Herein, we investigated the effects of maternal and  
20 weaning linoleic acid (LA)-rich diet interactions on gut intestinal and adipose tissue physiology in  
21 young (3-month-old) and older (6-month-old) adult offspring. Pregnant rats were fed a control diet  
22 (2%LA) or an LA-rich diet (12%LA) during gestation and lactation. At weaning, offspring were  
23 either maintained on the maternal diet or fed the other diet for 3 or 6 months. At 3 months of age,  
24 the maternal LA-diet favored low-grade inflammation and greater adiposity, while at 6 months of  
25 age, offspring intestinal barrier function, adipose tissue physiology and hepatic conjugated linoleic  
26 acids were strongly influenced by the weaning diet. The maternal LA-diet impacted offspring cecal  
27 microbiota diversity and composition at 3 months of age, but had only few remnant effects upon  
28 cecal microbiota composition at 6 months of age. Our study suggests that perinatal exposure to high  
29 LA levels induces a differential metabolic response to weaning diet exposure in adult life. This  
30 programming effect of a maternal LA-diet may be related to the alteration of offspring gut  
31 microbiota.

32 **Keywords:** *n*-6 PUFA; gut microbiota; gut permeability; adipose tissue; conjugated linoleic acids  
33

## 34 1. Introduction

35 In the past three decades, the overconsumption of vegetable oils rich in *n*-6 polyunsaturated  
36 fatty acids (PUFA) and the lower intake of *n*-3 PUFA has resulted in an imbalance in the *n*-6/*n*-3 ratio,  
37 rising from 1:1 to 20:1 nowadays [1]. Consequently, the levels of linoleic acid (LA), an essential *n*-6  
38 fatty acid, of women's milk also increased from 6% to a plateau at around 16% of total fatty acids [2].  
39 Strong evidence indicates that maternal nutrition during gestation and lactation can profoundly  
40 affect offspring health and disease risk later in life [3]. Specifically, LA, and especially its derivative,  
41 arachidonic acid (ARA), display pro-adipogenic properties that are related to a greater risk of obesity  
42 and related metabolic conditions [4,5]. A high intake of *n*-6 PUFA during this critical window of  
43 development results in a progressive accumulation of body fat across generations [2,6,7]. Similarly,  
44 Rudolph et al. reported that a low perinatal *n*-6/*n*-3 PUFA ratio exposure predisposed adult offspring

45 to a phenotype of resistance to diet-induced obesity [8]. In epidemiological studies, the level of LA,  
46 of its derivatives, or of the *n*-6/*n*-3 ratio in maternal or cord plasma was positively correlated to  
47 adiposity immediately at birth (GUSO cohort [9]), or in childhood at 6 or 7 years of age (Generation  
48 R cohort [10], MEFAB cohort [11], SWS cohort [12], US pregnant cohort [13]). However, this effect  
49 does not seem to be long-lasting, since no such correlation was found when offspring were 23 years  
50 old in the MEFAB cohort [14], or at 5 years in the GUSO cohort [9]. Breast milk PUFA levels have also  
51 been correlated to adiposity in two studies reporting higher fat mass at 4 months of age in the quartile  
52 with the greater ARA/ *n*-3 long chain (LC)-PUFA ratio in breast milk [8], and a negative correlation  
53 has been reported between fat mass at 7 years of age and breast milk docosahexaenoic acid (DHA)  
54 content [15]. Interestingly, in several studies, maternal dietary *n*-3 PUFA supplementation did not  
55 affect offspring adiposity [16,17], suggesting that lowering *n*-6 PUFA content may be more effective  
56 in reducing the risk of metabolic disease in offspring.

57 The mechanisms underlying the potential programming effect of *n*-6 PUFA status on adiposity  
58 are not all yet established. Epigenetic mechanisms have been shown to play a major role in neonatal  
59 programming by imprinting gene expression [18]. However, the microbiota may also be involved. It  
60 is well established now that host physiology, metabolism and behavior may be affected by the gut  
61 microbiota [19,20]. More specifically, altered gut homeostasis has been linked to adipose tissue  
62 expansion and inflammation through several mechanisms—increased intestinal permeability-  
63 promoting endotoxemia, modulation of energy harvested from diet by the microbiota, and altered  
64 production of bacterial metabolites (e.g. short chain fatty acids) interacting with adipocyte  
65 metabolism through GPR41/43 receptors [21,22]. This has been elegantly demonstrated by Cox et al.  
66 using antibiotic treatment—mice receiving antibiotics during the suckling period exhibited increased  
67 fat mass gain under a high-fat diet (HFD) later in life compared to vehicle-treated mice [25]. Thus,  
68 maternal nutrition and diet-modulated maternal microbiota [26] can affect offspring microbiota  
69 composition. Recent data have drawn attention to the effect of PUFA intake on gut homeostasis [27].  
70 Specifically, an enrichment of HFD with *n*-6 PUFA has been shown to cause increased intestinal  
71 inflammation and barrier dysfunction. These alterations were associated with intestinal dysbiosis led  
72 by a blooming of the Enterobacteriaceae and Firmicutes families [28–31]. Similarly, the capacity of  
73 Fat-2 mice to endogenously overproduce *n*-6 PUFA led to a higher risk of metabolic syndrome,  
74 inflammation, bacterial translocation and non-alcoholic fatty liver, likely mediated by altered host –  
75 microbiota interactions [31]. On the other hand, studies using transgenic Fat-1 mice demonstrated  
76 that inflammation and alteration of the gut microbiota induced by a high-*n*-6, high-fat diet could be  
77 restored by the transgenic conversion of tissue *n*-6 to *n*-3 PUFA levels, suggesting a strong  
78 involvement of *n*-6 PUFA levels in gut homeostasis [30–32]. These studies using Fat-1 and Fat-2 mice  
79 elucidated the role of *n*-6 PUFA status, but not of dietary *n*-6 PUFA consumption, which might have  
80 a different effect on gut barrier function and microbiota. Thus, and contrary to *n*-3 PUFA, where few  
81 studies evaluated their impact on gut microbiota when incorporated into a normal/moderate fat diet  
82 [33–36], the precise role of dietary *n*-6 PUFA in intestinal homeostasis is difficult to identify due to  
83 the confounding factor of high calorie intake, differences in type and source of PUFA, duration of  
84 intervention or age of individuals. This is even more true when examining the effect of maternal  
85 dietary *n*-6 PUFA in a moderate fat diet during gestation and lactation on host–microbiota  
86 interactions. In a recent study, Shrestha et al. demonstrated that a high consumption of LA (6.2% of  
87 energy) in a normal fat diet (9% of energy) does not significantly alter the gut microbiota composition  
88 of dams during pregnancy as compared to a lower intake of LA, but they did not investigate the  
89 offspring microbiota [37].

90 Thus, taking into account the possible impact of *n*-6 PUFA on adiposity, gut barrier function and  
91 the importance of the microbiota in neonatal programming, our study aimed at evaluating the impact  
92 of maternal *n*-6 PUFA intake in a moderate fat (21% energy) diet during gestation and lactation on  
93 offspring gut homeostasis and adipose tissue physiology, in young (3-month-old) and older (6-  
94 month-old) adult rats.

## 95 2. Materials and Methods

## 96 2.1. Animal protocol

97 All experiments were performed in accordance with the European Union Guidelines for animal  
98 care and use (2003/35/CEE) and approved by the Ethics & Animal Experimentation Committee of  
99 Rennes (MENESR under number: 01375.02).

100 The control diet (C-diet) and the LA-rich diet (LA-diet) were isocaloric and isolipidic. Both diets  
101 contained 10.0% fat (21% of total energy), 22.0% proteins, 70.3% carbohydrates, 2.0% fiber and 5.7%  
102 minerals and vitamins. We chose a moderate fat diet (21% of energy) in order to allow for high  
103 proportions of LA intake without reaching the fat percentage observed in high-fat diets. The lipid  
104 blends were made from a combination of commercial vegetable oils, C16:0 triglycerides and ethyl LA  
105 (TCI Europe, Zwijndrecht, Belgium) at the Unité de Production d'Aliments Expérimentaux (INRA,  
106 Jouy en Josas, France). The fatty acid composition of diets was confirmed by gas chromatography  
107 (Figure S1A). Experimental diets were stored at  $-20^{\circ}\text{C}$  and provided fresh every day.

108 Eight-week-old female Wistar rats from Janvier Labs (Le Genest-Saint-Isle, France) were fed  
109 either the C- or LA-diets during pregnancy and lactation ( $n=4-5$  per group). At birth, pups were  
110 culled to 8 per litter. At weaning, on postnatal day 21, male offspring were randomly separated into  
111 two groups (10 rats per group). Pups were maintained on the same maternal diet or fed the other  
112 experimental diet, thereby generating 4 groups that differed from maternal and/or weaning diets (C-  
113 C, C-LA, LA-C and LA-LA, Figure S1B). All animals were housed on a 12 h light/dark cycle and  
114 maintained at  $22 \pm 2^{\circ}\text{C}$  with free access to water and food ad libitum. They were fed their respective  
115 weaning diets for 3 ( $n=4$  rats/dietary group, except for C-LA,  $n=3$ ) or 6 ( $n=6$ /dietary group) months.  
116 Within a dietary group, rats were housed in 5 different cages with 2 rats/cage.

117 Body weight was measured every other day. At weaning, the dams and some of the remaining  
118 pups ( $n=3$  dams/dietary group and  $n=3$  pups/dietary dam group) were euthanized to collect liver and  
119 epididymal tissue as well as mammary glands from the dams. Tissues were immediately snap-frozen  
120 for later fatty acid composition determination. At the end of the 3- or 6-month period, fasted rats were  
121 anesthetized with an intraperitoneal injection of pentobarbital (140 mg/kg, Merial, Lyon, France) and  
122 blood samples were collected by cardiac puncture into EDTA K2-treated vacutainers (Dominique  
123 Dutscher, Brumath, France). Plasma was obtained by centrifugation (3000 g, 15 min,  $4^{\circ}\text{C}$ ) and stored  
124 at  $-20^{\circ}\text{C}$  until further analysis. Adipose tissues were dissected out and weighted. Epididymal  
125 adipose tissue samples were washed and fixed in 4% paraformaldehyde (1 week) for further  
126 histology analysis or snap-frozen for fatty acid composition determination. Other samples of  
127 epididymal adipose tissue were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA  
128 extraction. Cecal contents were collected, snap-frozen and stored at  $-80^{\circ}\text{C}$ . Cecal and colonic tissue  
129 were rinsed with ice-cold PBS. Colonic tissue was snap-frozen and stored at  $-80^{\circ}\text{C}$  until further  
130 analysis, while cecal tissue was stored in ice-cold DMEM for immediate mounting in Ussing  
131 chambers.

## 132 2.2. Microbiota analysis

133 Bacterial DNA was extracted from 100–150 mg of cecal luminal content samples using the ZR  
134 fecal DNA Miniprep kit (Zymo Research, USA). The V3-V4 region of 16S rRNA gene was amplified  
135 using the following primers:  
136 CTTTCCCTACACGACGCTCTTCCGATCTACTCCTACGGGAGGCAGCAG (V3F) and  
137 GGAGTTCAGACGTGTGCTCTTCCGATCT TACCAGGGTATCTAATCC (V4R), Taq Phusion (New  
138 England Biolabs) and dNTP (New England Biolabs) during 25 cycles (10 s at  $98^{\circ}\text{C}$ , 30 s at  $45^{\circ}\text{C}$ , 45 s  
139 at  $72^{\circ}\text{C}$ ). The purity of amplicons was checked on agarose gels before sequencing using Illumina  
140 Miseq technology, performed at the Genotoul Get-Plage facility (Toulouse, France). Briefly, single  
141 multiplexing was performed using a homemade 6 bp index, which was added to R784 during a  
142 second PCR in 12 cycles using forward primer  
143 (AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC) and reverse primer  
144 (CAAGCAGAAGACGGCATAACGAGAT-index-GTGACTGGAGTTCAGACGTGT). The resulting  
145 PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the  
146 manufacturer's instructions. The quality of the run was checked internally using PhiX, and then each

147 paired-end sequence was assigned to its sample with the help of the previously integrated index.  
148 Each paired-end sequence was assembled using Flash [38], using at least a 10 bp overlap between the  
149 forward and reverse sequences, allowing 10% mismatch. The lack of contamination was checked with  
150 a negative control during the PCR (water as template). The quality of the stitching procedure was  
151 controlled using 4 bacterial samples that ran routinely in the sequencing facility in parallel with the  
152 current samples. Raw sequences can be found at  
153 <https://data.inrae.fr/dataset.xhtml?persistentId=doi:10.15454/Y2AHF7>. Raw sequences were  
154 analyzed using the bioinformatic pipeline FROGS [39], based on several R software [40] packages.  
155 Briefly, quality control and trimming was performed using Cutadapt (version 1.18) [41] and Flash  
156 (version 1.2.11) [38] to keep sequences between 380 and 500 bp, sequences with ambiguous bases and  
157 sequences that do not contain good primers. Clustering was then performed using Swarm v2.1.2 [42]  
158 with an aggregation maximal distance of 3 bases. Chimera were removed using VSEARCH (v2.9.1)  
159 [43] with cross-sample validation. PhiX contamination was removed using VSEARCH. Affiliation  
160 was performed using the Silva 123 16S database [44] and NCBI Blastn++ [45]. A phylogenetic tree was  
161 constructed using FastTree (v2.1.10) [46] and sample depth was normalized using GMPR [47].

162 The phyloseq package [48] was used for biostatistical process. Alpha-diversity was estimated  
163 using two metrics: the number of observed species (richness index) and the Shannon index. A two-  
164 way ANOVA test was then performed on these two indexes to assess maternal and weaning diet  
165 effects and their interaction. Cage effect was not considered in the statistical analysis. Beta-diversity  
166 was evaluated by calculating the weighted Unifrac distance between samples. Ordination using  
167 principal coordinates analyses (PCoA) was performed to represent samples on a 2D plot. Multivariate  
168 ANOVA was performed using adonis function (Vegan Package, <https://cran.r-project.org>,  
169 <https://github.com/vegandevs/vegan>) with 9999 permutations. Relative abundances of the major  
170 phyla and of families were compared using two-way ANOVA to test maternal, dietary and their  
171 interaction effects.

### 172 2.3. Intestinal Alkaline Phosphatase activity (IAP) and Ussing chamber assay

173 IAP activity in the colon was determined using the Sensolyte p-nitrophenyl phosphate Alkaline  
174 Phosphatase Assay kit (Anaspec, Fremont, CA, USA) and expressed as specific activity (Arbitrary  
175 Unit/mg of protein) after protein quantification in tissue homogenates (Pierce Protein BCA Protein  
176 Assay kit, ThermoFisher Scientific). Cecal tissues were mounted into Ussing chambers to measure  
177 permeability to probes of various size (FITC-dextran 4000 (FD-4, 4 kDa) and horseradish peroxidase  
178 (HRP, 40 kDa) as well as tissue conductance as previously described [49].

### 179 2.4. Real-time PCR

180 Total RNA from colonic and epididymal adipose tissue was extracted via the Trizol method  
181 (15596-018; Fischer Scientific) and quantified using a spectrophotometer (Denovix). In total, 2 µg  
182 RNA was converted to cDNA using a High Capacity Complementary DNA Reverse Transcription  
183 Kit (Applied Biosystems) following the manufacturer's protocol. Real-time PCR was performed with  
184 a StepOnePlus real-time PCR machine using SyberGreen master mix (Fischer Scientific) for detection.  
185 Primers for selected genes (Table S1) were designed using Integrated DNA Technologies Primer  
186 Quest. HPRT-1, RPS18 and Actin were used as housekeeping genes. The standard curve method was  
187 assessed for each gene and the 2- $\Delta\Delta$ Ct method was applied for quantification. The reference and  
188 targeted gene primers had efficiencies between 95 and 101%, ( $R^2 \geq 0.98$ ). Data were normalized to the  
189 C-C group for each respective age as a reference group.

### 190 2.5. Adipose tissue histology

191 Epididymal fat pads were fixed in 4% paraformaldehyde (PAF), processed and embedded in  
192 paraffin using a tissue processor (Excelsior ES; ThermoFischer Scientific, Waltham, MA, USA) for  
193 histology. Sections of 5µm thickness were deparaffined and stained with hematoxylin and eosin for  
194 morphometric analysis, and then scanned with the NanoZoomer 2.0 RS (Hamamatsu, Tokyo, Japan).

195 Images were captured at 20x magnification for analysis. Five representative pictures of each section  
196 were selected for counting. A semi-automated custom image analysis protocol was developed using  
197 ImageJ software to quantify the area of individual adipocytes. Histological images were segmented,  
198 creating a binary mask and converting the image to 1-bit configuration. The adipocytes were counted  
199 if they met the following criteria: (1) the adipocyte has an equivalent sphere surface diameter between  
200 20 and 200  $\mu\text{m}$ ; (2) the adipocyte has a shape factor of 0.5–1 (circularity indicator) and (3) the  
201 adipocyte does not border the image frame. Over 10% of the adipocytes were included manually.  
202 Each adipocyte was subsequently labeled with a number to visualize the adipocytes more clearly and  
203 provide traceability for their respective quantified area. About 100 adipocytes were counted per  
204 picture. Morphometric data were then exported, and expressed as adipocyte frequency per mean  
205 diameter for statistical analysis.

## 206 2.6. Fatty acid analysis and conjugated linoleic acid quantification

207 Solvents used in the lipid analysis were purchased from Fischer Scientific (Elancourt, France).  
208 Lipids were extracted as previously described [50]. Briefly, lipids were extracted from adipose tissue,  
209 saponified and methylated. Fatty acid methyl esters (FAME) were extracted and analyzed by gas  
210 chromatography with a flame ionization detector (Agilent Technologies 6890N, Bios Analytique,  
211 France). Hydrogen was used as the carrier gas. The identification of FAME peaks was based on the  
212 retention times obtained from fatty acids standards. The area under peaks was determined using  
213 ChemStation software (Agilent), and results were expressed as the total fatty acid percentage.  
214 Conjugated linoleic acids (CLA) were quantified in the liver as already described [51].

## 215 2.7. Statistical analysis

216 Data are presented as box and whisker plots with means and min-max. At each age (3 and 6  
217 months), the data (body weight, adiposity, gut function, qPCR data) were analyzed by two-way  
218 ANOVA testing of the maternal diet, weaning diet and maternal x weaning diet effects using the  
219 GraphPad Prism software. For adipocyte distribution analysis, Gaussian curves were fit to each  
220 dataset and a comparison of fits was performed. At weaning (dams' and pupss tissue fatty acid  
221 composition), data were analyzed by Mann–Whitney tests. Microbiota data were analyzed as  
222 described in the corresponding session. Spearman rank correlation between data was performed  
223 using cor function in R and the corrplot R package of R.

## 224 3. Results

### 225 3.1. Maternal LA-diet impacted dam and offspring tissue composition at weaning

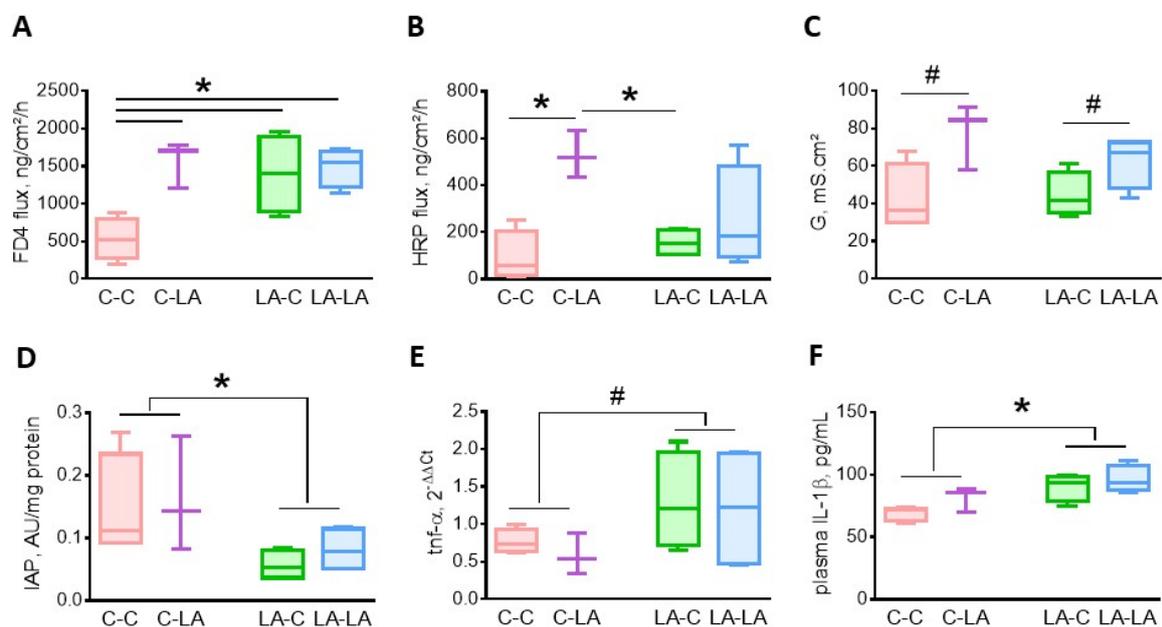
226 We verified that the maternal diet affected maternal tissue fatty acid composition as well as  
227 offspring tissue fatty acid composition at weaning. At the end of the gestation and lactation periods,  
228 although dam body weight was similar (C:  $329 \pm 7$  vs. LA:  $313 \pm 10$  g,  $p > 0.05$ ), the LA-diet impacted  
229 the fatty acid compositions of multiple tissues. Indeed, fatty acid analyses of dam liver, epididymal  
230 adipose tissue and the mammary gland indicated the maternal diet intake of greater proportions of  
231  $n$ -6 PUFA (LA and ARA) in all tissues at the expense of mono-unsaturated fatty acids (MUFA) in the  
232 LA-fed animals (Table S2). At weaning, offspring tissue fatty acid composition was also profoundly  
233 impacted by the maternal diet with greater proportions of  $n$ -6 PUFA and a lower proportion of MUFA  
234 in the liver and epididymal adipose tissue (Table S3) despite similar body weights (C:  $60 \pm 5$  vs. LA:  
235  $63 \pm 4$  g,  $p > 0.05$ ).

### 236 3.2. Maternal LA-diet impacted gut barrier function and adipose tissue in young adult offspring

#### 237 3.2.1. Cecal barrier function and inflammation

238 At 3 months of age, cecal barrier function was influenced by the maternal and/or the weaning  
239 diets. Compared to C-C rats, the cecal permeability to small molecules (FD-4) was greater in rats

240 exposed to the LA-diet either through the maternal or the weaning diet, i.e., C-LA, LA-C and LA-LA  
 241 groups, (maternal diet x weaning diet,  $p = 0.03$ , Figure 1A). The cecal permeability to large molecules  
 242 (HRP) was greater in C-LA rats compared to those fed the weaning C-diet (maternal diet x weaning  
 243 diet,  $p = 0.04$ , Figure 1B). Cecal conductance was greater in rats fed the weaning LA-diet (weaning  
 244 diet  $p = 0.01$ , Figure 1C). We also measured IAP activity. IAP is a brush border enzyme secreted along  
 245 the intestine with several biological roles, including LPS detoxification, the resolution of  
 246 inflammation and the maintenance of gut microbiota homeostasis. Several reports have suggested  
 247 that  $n-6$  PUFA can impact intestinal production and secretion of IAP [28,30,52,53]. IAP activity in the  
 248 colon was lower in rats born to LA mothers compared to those born to C-mothers, irrespective of  
 249 their weaning diet (maternal diet  $p = 0.02$ , Figure 1D). Finally, we evaluated local and systemic  
 250 inflammation. Colonic *tnf- $\alpha$*  mRNA levels tended to be greater in rats born to LA dams compared to  
 251 those born to C ones (maternal diet  $p = 0.08$ , Figure 1E); *il-1 $\beta$*  and *mcp-1* mRNA levels were not affected  
 252 by the maternal or the weaning diet (data not shown). The plasma IL-1 $\beta$  concentration was greater  
 253 in rats born to LA mothers compared to those born to C ones, irrespective of their weaning diet  
 254 (maternal diet  $p = 0.004$ , Figure 1F).



255

256 **Figure 1.** Impact of maternal and weaning LA-diets on large intestine barrier function and systemic  
 257 inflammation at 3 months of age. Permeability to small (A) or large probes (B), tissue conductance (C)  
 258 of the cecum, colonic IAP activity (D) and *tnf- $\alpha$*  mRNA levels (E) and plasma IL-1 $\beta$  concentrations (F)  
 259 were determined in C-C, C-LA, LA-C and LA-LA rats at 3 months of age. Two-way ANOVA were  
 260 performed to test the maternal, weaning and interaction effects. \*  $p \leq 0.05$ , #  $p \leq 0.08$ ,  $n = 4$ /group,  
 261 except C-LA, where  $n = 3$ . FD4: HRP: G:

### 262 3.2.2. Epididymal adipose tissue

263 Based on the concept of a “leaky gut” in the pathogenesis of several metabolic diseases [54], we  
 264 further analyzed the impact of LA-diet on adipose tissue physiology in young offspring. We first  
 265 confirmed the dietary fatty acid’s incorporation into the offspring tissue by analyzing the epididymal  
 266 adipose tissue fatty acid composition. The fatty acid composition of rat epididymal fat at 3 months of  
 267 age was affected by the weaning, but not the maternal diet, with greater proportions of  $n-6$  PUFA  
 268 at the expense of MUFA in rats fed the weaning LA-diet compared to the weaning C-diet, irrespective  
 269 of the maternal diet (Table 1).

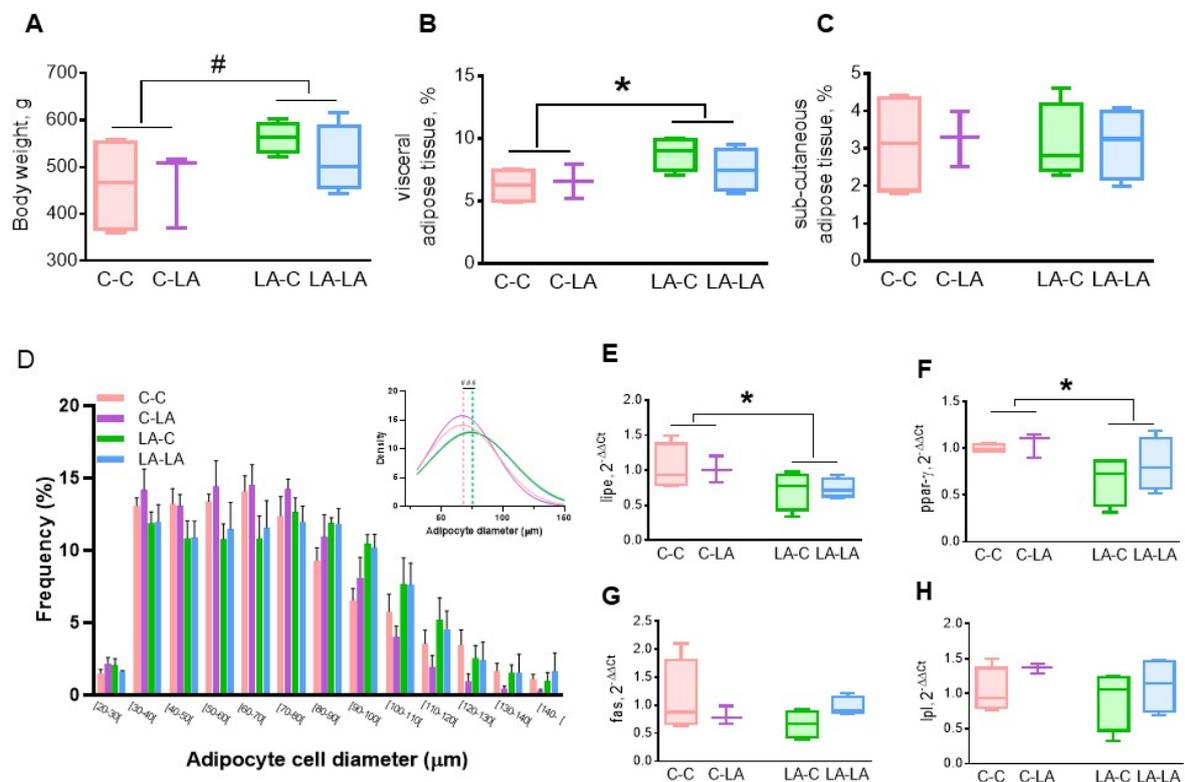
270

**Table 1.** Fatty acid composition of offspring epididymal adipose tissue at 3 months of age.

Maternal diet	C		LA		p-value			
	Weaning diet	C	LA	C	LA	Maternal diet	Weaning diet	x <sup>1</sup>
SFA		24.5 (0.7)	25.9 (1.3)	25.5 (0.4)	24.6 (1.3)	0.89	0.76	0.27
MUFA		67.5 (0.5)	31.2 (1.1)	66.4 (0.2)	30.7 (1.6)	0.44	<0.001	0.79
n-6 PUFA		6.4 (0.3)	41.6 (1.8)	6.7 (0.2)	43.2 (1.4)	0.39	<0.001	0.54
18:2 n-6		6.2 (0.2)	39.8 (2.0)	6.5 (0.2)	41.3 (1.4)	0.44	<0.001	0.59
20:4 n-6		0.1 (0.0)	0.8 (0.1)	0.1 (0.0)	0.9 (0.1)	0.33	<0.001	0.50
n-3 PUFA		1.6 (0.0) ab	1.6 (0.0) ab	1.4 (0.1) a	1.8 (0.0) b	0.95	0.03	0.01
n-6/n-3		4.0 (0.1) a	26.7 (0.6) b	4.9 (0.5) a	24.2 (0.3) c	0.07	<0.001	<0.01

271 <sup>1</sup>maternal diet x weaning diet interaction. Two-way ANOVAs were performed to test the maternal,  
 272 weaning and interaction effects. a,b,c:  $p < 0.05$ ,  $n = 4/\text{group}$ , except C-LA, where  $n = 3$ .

273 We next evaluated adipose tissue morphology and lipid metabolism. Rats born to LA mothers  
 274 tended to be heavier (maternal diet  $p = 0.08$ , Figure 2A) than those born to C mothers, irrespective of  
 275 their weaning diet. Adipose tissue physiology was altered mainly by the maternal diet, but not the  
 276 weaning diet, with enhanced visceral adiposity in rats born to LA mothers (maternal diet  $p = 0.04$ ,  
 277 Figure 2B) but no difference in the percentage of subcutaneous adipose tissue (Figure 2C). When  
 278 comparing the means of Gaussian curve fits of the adipocyte distribution of epididymal fat, the  
 279 offspring of LA mothers exhibited an upwards shift due to a higher frequency of larger adipocyte ( $p$   
 280  $= 0.02$ , Figure 2D). Adipose tissue metabolism was also affected by the maternal diet with lower *lipE*  
 281 (maternal diet  $p = 0.02$ , Figure 2E) and *ppar-γ* (maternal diet  $p = 0.04$ , Figure 2F) mRNA levels in rats  
 282 born to LA mothers compared to those born to C mothers, irrespective of their weaning diet.  
 283 Transcriptomic expressions of *fasn* and *lpl* were not altered by the diet (Figure 2G and H).



284

285 **Figure 2.** Impact of maternal and weaning LA-diets on adipose tissue physiology at 3 months of age.  
 286 Body weight (A), visceral adiposity (B) and sub-cutaneous adiposity (C) were determined in -C, C-  
 287 LA, LA-C and LA-LA rats at 3 months of age. Distribution of adipocyte diameters (D), insert represents

288 the mean adipocyte diameter), hormone-sensitive lipase (*lipe*, E), peroxisome proliferator-activated  
289 receptor- $\gamma$  (*ppar-\gamma*, F), fatty acid synthase (*fas*, G), and lipoprotein lipase (*lpl*, H) mRNA levels were  
290 determined in the epididymal adipose tissue. Two-way ANOVAs were performed to test the  
291 maternal, weaning and interaction effects. \*  $p \leq 0.05$ , #  $p \leq 0.08$ ,  $n = 4/\text{group}$ , except C-LA, where  $n = 3$ .

### 292 3.2.3. Conjugated-linoleic acids

293 There are several potential mediators of the crosstalk between the gut and adipose tissues,  
294 including the CLAs. CLAs are produced from LA by bacteria in the intestinal lumen, but can transfer  
295 to the inner milieu and impact host physiology [51]. CLA concentrations were measured in the liver  
296 as a reflection of CLA production and absorption through the intestine. At 3 months of age, hepatic  
297 CLA concentrations were mainly affected by the weaning diet. Rats fed the weaning LA-diet had  
298 increased hepatic levels of 18:2 *trans*-10, *cis*-12, but reduced levels of 18:2 *cis*-9, *cis*-11, ( $p = 0.04$  and  
299 0.01, respectively, Table 2). Rats born to LA dams displayed a tendency for greater levels of 18:2 *cis*-  
300 9, *trans*-11 and 18:2 *trans*-10, *cis*-12 ( $p = 0.08$  and 0.08, respectively, Table 2) in their liver compared to  
301 rats born to C dams. Higher hepatic levels of 18:1 *trans*-11 were observed in rats fed the weaning LA-  
302 diet when they were born to LA mothers (maternal diet  $\times$  weaning diet  $p = 0.02$ , Table 2).

**Table 2.** Hepatic conjugated linoleic acid concentrations at 3 months of age.

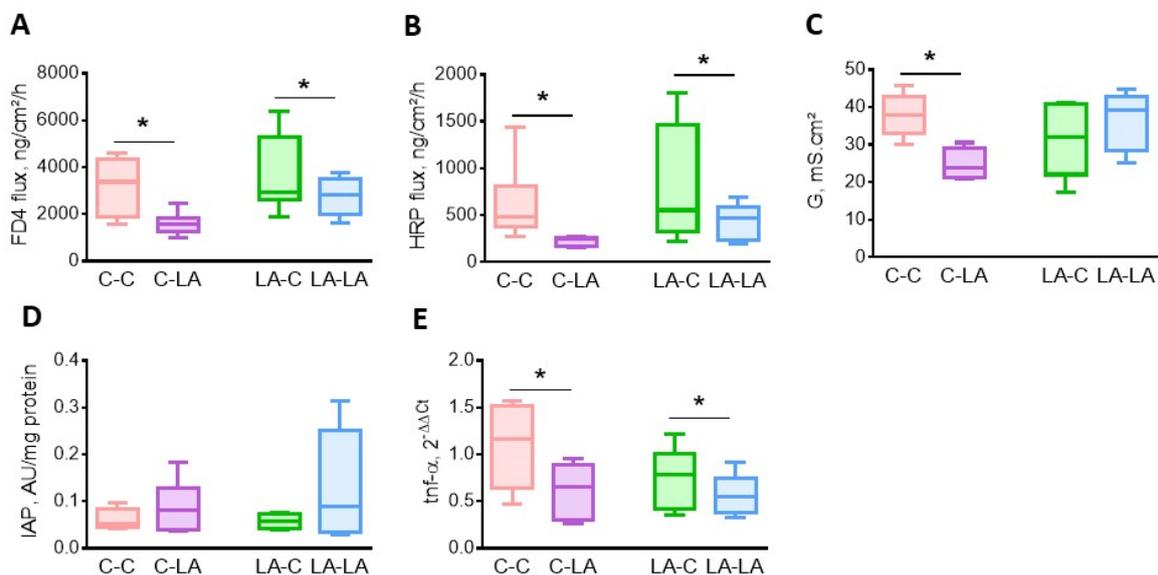
Maternal diet	C		LA		Maternal diet	<i>p</i> -value	
	Weaning diet	C	LA	C		LA	Weaning diet
18:2 <i>cis</i> -9, <i>trans</i> -11	0.012 (0.003) a	0.014 (0.002) a	0.010 (0.002) a	0.024 (0.002) b	0.08	0.1	0.02
18:2 <i>trans</i> -10, <i>cis</i> -12	0.008 (0.002)	0.057 (0.03)	0.006 (0.001)	0.013 (0.005)	0.08	0.04	0.11
18:2 <i>cis</i> -9, <i>cis</i> -11	0.026 (0.009)	0.012 (0.003)	0.039 (0.006)	0.013 (0.004)	0.30	0.01	0.40
18:2 <i>trans</i> -11, <i>trans</i> -13	0.011 (0.003)	0.009 (0.002)	0.006 (0.001)	0.007 (0.004)	0.35	0.80	0.61
18:2 <i>trans</i> -9, <i>trans</i> -11	0.011 (0.003)	0.004 (0.009)	0.008 (0.003)	0.015 (0.005)	0.30	0.87	0.08
18:1 <i>trans</i> -11	0.042 (0.009) ab	0.031 (0.005) ab	0.027 (0.005) a	0.050 (0.004) b	0.77	0.34	0.02

<sup>1</sup>maternal diet x weaning diet interaction, Two-way ANOVAs were performed to test the maternal, weaning and interaction effects. a,b,c:  $p < 0.05$ ,  $n = 4/\text{group}$ , except C-LA, where  $n = 3$ .

1 3.3. Maternal LA-diet had a limited impact on gut barrier function and adipose tissue in older adults  
 2 compared to the weaning diet itself

3 3.3.1. Cecal barrier function and inflammation

4 At 6 months of age, no maternal diet effect was observed on cecal barrier function or  
 5 inflammation. However, the weaning diet affected cecal homeostasis—FD-4 (Figure 3A) and HRP  
 6 (Figure 3B) fluxes across the cecal mucosa were decreased in weaning LA-diet-fed rats, irrespective  
 7 of the maternal diet (weaning diet  $p = 0.01$  and  $0.04$ , respectively). Cecal conductance was decreased  
 8 by the weaning LA-diet in rats born to C mothers only (weaning  $\times$  maternal diet  $p = 0.03$ , Figure 3C).  
 9 The activity of IAP tended to be greater (weaning diet  $p = 0.07$ , Figure 3D), while the colonic *tnf- $\alpha$*   
 10 mRNA level was reduced (weaning diet  $p = 0.03$ , respectively Figure 3E) in rats fed the weaning LA-  
 11 diet, irrespective of their maternal diet. Plasma IL-1 $\beta$  concentration was lower in rats fed the weaning  
 12 LA-diet, irrespective of the maternal diet (data not shown, published in Marchix et al., 2020 [50]).



13  
 14 **Figure 3.** Impact of maternal and weaning LA-diets on large intestine barrier function at 6 months of  
 15 age. Permeability to small (A) or large probes (B), tissue conductance (C) of the cecum, colonic IAP  
 16 activity (D) and *tnf- $\alpha$*  mRNA levels (E) were determined in C-C, C-LA, LA-C and LA-LA rats at 6  
 17 months of age. Two-way ANOVAs were performed to test the maternal, weaning and interaction  
 18 effects. \*  $p \leq 0.05$ ,  $n = 6$ /group. FD4: HRP: G:

19 3.3.2. Epididymal adipose tissue

20 As previously observed at 3 months of age, the fatty acid composition of rat epididymal fat at 6  
 21 months of age was mainly affected by the weaning diet, with greater proportions of *n-6* PUFA at the  
 22 expense of MUFA in rats fed the weaning LA-diet compared to the weaning C-diet, irrespective of  
 23 the maternal diet (Table 3). SFA proportions was greater in rats born to LA mothers, compared to  
 24 those born to C mothers ( $p = 0.05$ , Table 3).

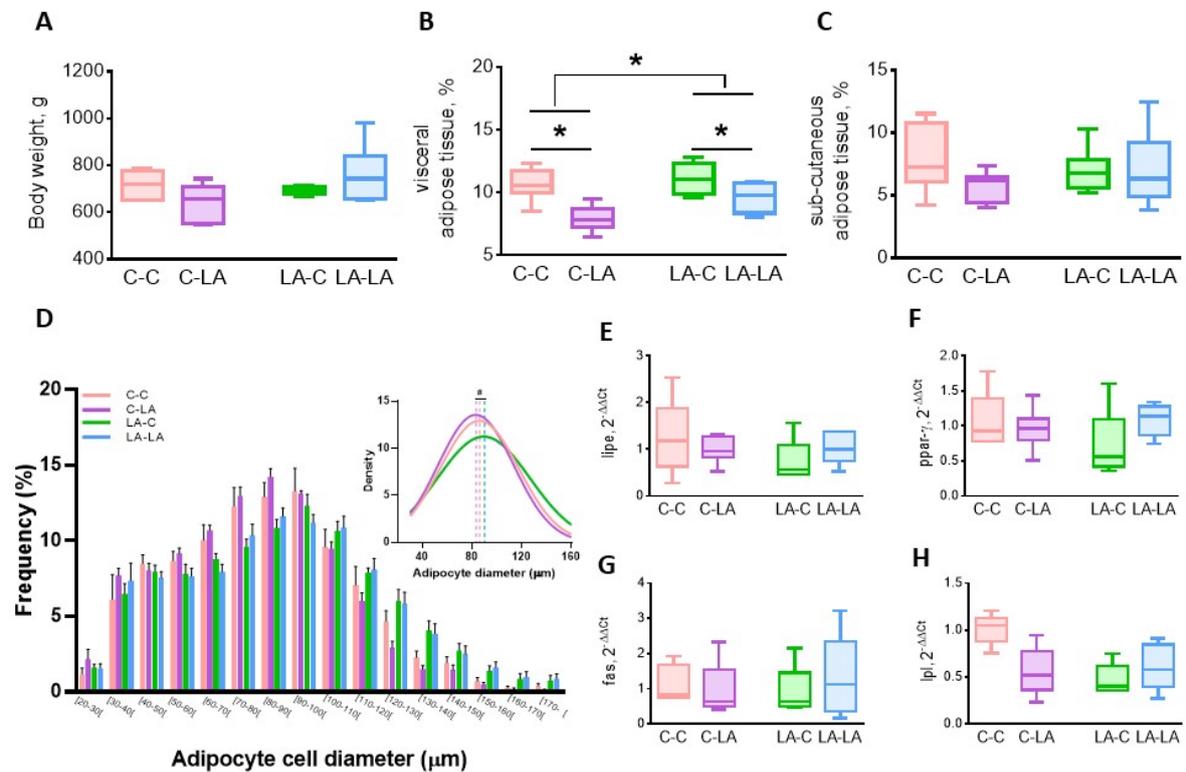
25 **Table 3.** Fatty acid composition of offspring epididymal adipose tissue at 6 months of age.

Maternal diet	C		LA		<i>p</i> -value		
	C	LA	C	LA	Maternal diet	Weaning diet	$\chi^2$
Weaning diet							
SFA	21.7 (0.3)	23.1 (0.3)	22.9 (0.4)	24.2 (0.9)	0.05	0.02	0.96

MUFA	70.8 (0.3)	30.4 (0.7)	69.2 (0.3)	29.8 (0.7)	0.08	<0.001	0.36
n-6 PUFA	6.4 (0.1)	44.9 (0.9)	6.9 (0.1)	44.5 (1.4)	0.99	<0.001	0.59
18:2 n-6	6.1 (0.1)	43.4 (0.8)	6.5 (0.1)	42.8 (1.5)	0.94	<0.001	0.60
20:4 n-6	0.2 (0.0)	0.7 (0.1)	0.1 (0.0)	0.8 (0.1)	0.27	<0.001	0.50
n-3 PUFA	1.0 (0.0)	1.3 (0.1)	1.0 (0.0)	1.3 (0.0)	0.08	0.02	0.66
n-6/n-3	6.6 (0.1)	34.9 (0.8)	6.6 (0.0)	33.2 (1.4)	0.31	<0.001	0.31

26 <sup>1</sup>maternal diet x weaning diet interaction. Two-way ANOVAs were performed to test the maternal, weaning  
 27 and interaction effects. *n* = 6/group.

28 Compared to 3-month-old rats, the weaning LA-diet rather than the maternal LA-diet affected  
 29 adipose tissue physiology in older offspring. The weaning LA-diet did not significantly impact rat  
 30 body weight (Figure 4A), however it decreased the visceral adiposity (weaning diet *p* = 0.003, Figure  
 31 4B) but not the subcutaneous one (Figure 4C). A slight maternal diet effect was still observed for  
 32 visceral adiposity, which was greater in rats born to LA mothers (maternal diet *p* = 0.04, Figure 4B).  
 33 Furthermore, the comparison of the means of the Gaussian curve fits of the adipocyte distribution  
 34 showed the long-lasting effect of maternal LA-diet, with a higher frequency of greater adipocyte in  
 35 LA-LA offspring compared to the C-LA ones (*p* < 0.01, Figure 4D). The lipid metabolism key enzyme  
 36 expression in the epididymal adipose tissue was not affected by the diet (Figure 4E-H).



37  
 38 **Figure 4.** Impact of maternal and weaning LA-diets on adipose tissue physiology at 6 months of age.  
 39 Body weight (A), visceral adiposity (B) and sub-cutaneous adiposity (C) were determined in C-C, C-  
 40 LA, LA-C and LA-LA rats at 6 months of age. Distribution of adipocyte diameters (D, insert represents  
 41 the mean adipocyte diameter), hormone-sensitive lipase (*lipo*, E), peroxisome proliferator-activated  
 42 receptor- $\gamma$  (*ppar*- $\gamma$ , F), fatty acid synthase (*fas*, G), and lipoprotein lipase (*lpl*, H) mRNA levels were  
 43 determined in the epididymal adipose tissue. Two-way ANOVAs were performed to test the  
 44 maternal, weaning and interaction effects. \* *p*  $\leq$  0.05, *n* = 6/group.

45 3.3.3. Conjugated-linoleic acids

46 At 6 months of age, the hepatic CLA concentrations were affected by the weaning diet with  
47 greater levels of 18:2 *cis*-9, *trans*-11 and 18:1 *trans*-11 (weaning diet  $p < 0.0001$  and  $p = 0.02$ , respectively,  
48 Table 4) and lower levels of 18:2 *cis*-9, *cis*-11 and 18:2 *trans*-11, *trans*-13 (weaning diet  $p < 0.01$  and  $p =$   
49 0.04, respectively, Table 4) in the liver of rats fed the weaning LA-diet, compared to the weaning C-  
50 diet-fed ones, irrespective of the maternal diet. Maternal diet affected the hepatic 18:2 *trans*-9, *trans*-  
51 11 levels, which were markedly lower in the livers of rats born to LA dams, irrespective of the  
52 weaning diet (maternal diet  $p < 0.01$ , Table 4). The hepatic level of 18:2 *trans*-10, *cis*-12 was not  
53 influenced by any of the diets at 6 months of age (Table 4).

**Table 4.** Hepatic conjugated linoleic acid concentrations at 6 months of age.

Maternal diet	C		LA		Maternal diet	<i>p</i> -value		
	Weaning diet	C	LA	C		LA	Weaning diet	$\chi^2$
18:2 <i>cis</i> -9, <i>trans</i> -11		0.015 (0.001)	0.033 (0.004)	0.011 (0.002)	0.029 (0.002)	0.21	< 0.001	0.98
18:2 <i>trans</i> -10, <i>cis</i> -12		0.025 (0.013)	0.016 (0.003)	0.020 (0.007)	0.016 (0.004)	0.75	0.37	0.78
18:2 <i>cis</i> -9, <i>cis</i> -11		0.033 (0.004)	0.013 (0.002)	0.024 (0.006)	0.014 (0.004)	0.34	< 0.01	0.25
18:2 <i>trans</i> -11, <i>trans</i> -13		0.013 (0.003)	0.006 (0.001)	0.012 (0.002)	0.010 (0.003)	0.64	0.04	0.35
18:2 <i>trans</i> -9, <i>trans</i> -11		0.022 (0.009)	0.026 (0.004)	0.008 (0.001)	0.010 (0.003)	< 0.01	0.54	0.89
18:1 <i>trans</i> -11		0.030 (0.005)	0.045 (0.006)	0.016 (0.004)	0.037 (0.01)	0.13	0.02	0.64

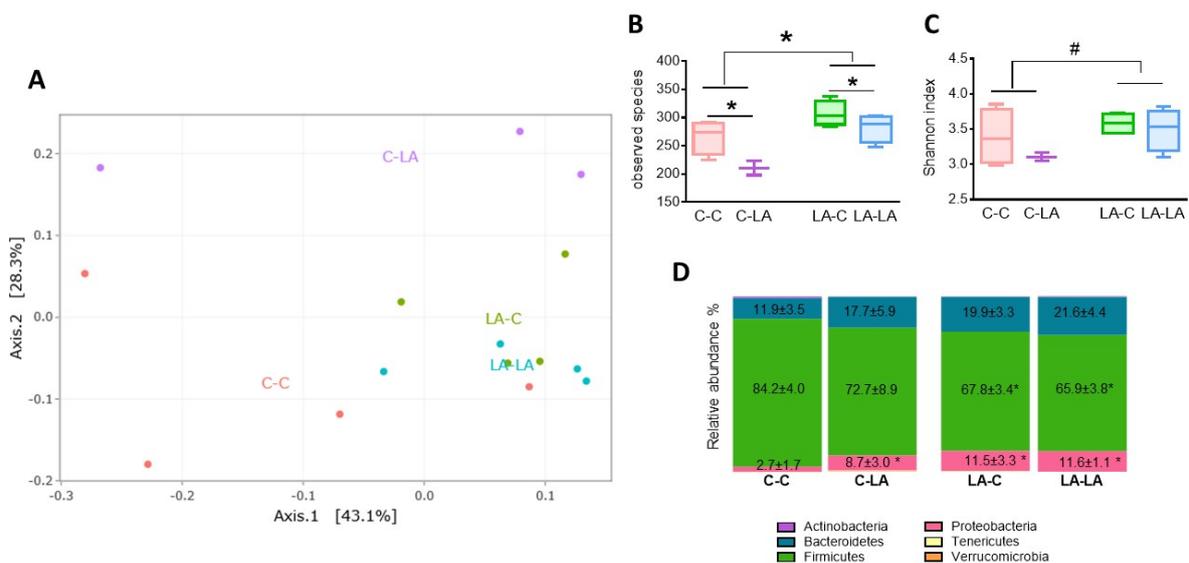
<sup>1</sup>maternal diet x weaning diet interaction. Two-way ANOVAs were performed to test the maternal, weaning and interaction effects.  $n = 6/\text{group}$ .

1 3.4. Cecal microbiota

2 Gut dysbiosis has often been associated with the development of metabolic diseases and  
 3 intestinal barrier dysfunction [30]. Moreover, while it is well established that diet can alter the gut  
 4 microbiota, evidence also suggests that maternal diet-induced changes in microbiota could be  
 5 transferred to offspring [55,56]. Therefore, we determined whether altered cecal gut microbiota was  
 6 related to changes in gut physiology in offspring at both ages. These microbiota data need, however,  
 7 to be taken with caution, due to the low n number, which is not optimal for microbiota analysis  
 8 [57,58].

9 3.4.1. Cecal microbiota at 3 months of age

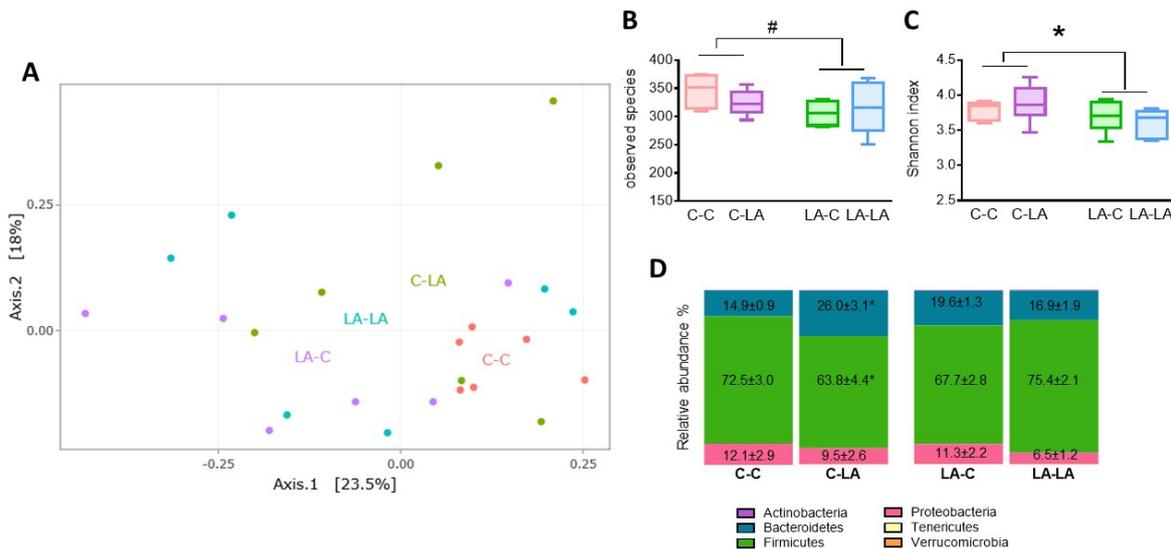
10 At 3 months of age, principal coordinates analysis (PCoA) using the weighted Unifrac distance  
 11 revealed the distinct clustering of cecal microbiota in the four dietary groups (PERMANOVA  $p =$   
 12 0.008, Figure 5A), with a significant effect of the maternal diet ( $p = 0.04$ ) but not of the weaning diet  
 13 ( $p = 0.30$ ). Indeed, the cecal microbiota of rats born to C and LA mothers were segregated, but only  
 14 the microbiota of rats born to C mothers were clustered according to the weaning diet (Figure 5A).  
 15 The cecal microbiota of rats born to LA mothers exhibited a greater richness (number of observed  
 16 species) than those born to C mothers ( $p = 0.001$ , Figure 5B). However, the weaning LA-diet had the  
 17 opposite effect, with reduced cecal microbiota richness ( $p = 0.009$ , Figure 5B). Microbial diversity  
 18 estimated by the Shannon index also tended to be greater in rats born to LA mothers compared to  
 19 those born to C mothers ( $p = 0.07$ , Figure 5C). At the phylum level, rats born to LA mothers had  
 20 greater relative abundances of Proteobacteria (maternal diet effect,  $p = 0.03$ ) and lower relative  
 21 abundances of Firmicutes (maternal diet effect,  $p = 0.04$ ) compared to rats born to C mothers (Figure  
 22 5D). No significant effect of the weaning diet was observed at the phylum level. To further  
 23 characterize the impact of the maternal diet on the cecal microbiota's response to the weaning diet,  
 24 we analyzed the difference in family abundances between dietary groups (Table S4). Most of the  
 25 differences were attributed to the maternal diet or to an interaction between the maternal and the  
 26 weaning diets. It is noteworthy that rats born to LA dams exhibited greater relative abundances of  
 27 Tannerellaceae, Ruminococcaceae, Rhodospirillaceae and Desulfovibrionaceae, and lower relative  
 28 abundances of Sporichthyaceae and Akkermansiaceae, irrespective of the weaning diet (Table S4).  
 29 Moreover, all the rats that had received LA (C-LA, LA-C and LA-LA groups) had reduced levels of  
 30 Lactobacillaceae and Clostridiaceae compared to the C-C group (Table S4). Moreover, C-LA rats had  
 31 increased levels of Bacteroidaceae, Christensenellaceae, Lachnospiraceae and Mollicutes (Table S4).



33 **Figure 5.** Impact of maternal and weaning LA-diets on microbiota composition at 3 months of age.  
 34 Cecal microbiota composition of C-C, C-LA, LA-C and LA-LA rats was determined at 3 months of  
 35 age through 16S rRNA gene sequencing. **A.** Principal coordinates analysis based on weighted Unifrac  
 36 distances. **B.** Microbiota richness (observed OTU). **C.** Microbiota diversity estimated by the Shannon  
 37 index. **D.** Main phyla relative abundance (mean ± SEM). Two-way ANOVAs were performed to test  
 38 the maternal, weaning and interaction effects. Panels B–C: \* indicates significant effect ( $p \leq 0.05$ ), #  
 39 indicates a trend for significance ( $p \leq 0.07$ ), Panel D: \* indicates maternal diet effect ( $p \leq 0.05$ )  $n =$   
 40 4/group, except C-LA, where  $n = 3$ .

41 3.4.2. Cecal microbiota at 6 months of age

42 While at 3 months of age the cecal microbiota were profoundly impacted by the maternal and/or  
 43 weaning diets, the clustering of rat cecal microbiota upon maternal diet and/or weaning diet was less  
 44 apparent on the weighted Unifrac distance PCoA at 6 months of age (Figure 6A). No significant  
 45 differences were noted when comparing the microbiota compositions associated with maternal or  
 46 weaning diet (PERMANOVA  $p = 0.07$  and  $0.21$ , respectively). As opposed to the 3-month-old rats, the  
 47 cecal microbiota richness (number of observed species) tended to be lower ( $p = 0.07$ , Figure 6B) and  
 48 the diversity (Shannon index) was significantly lower ( $p = 0.04$ , Figure 6C) in 6-month-old rats born  
 49 to LA mothers compared to those born to C mothers. At the phylum level, no maternal diet effect  
 50 was observed, but the relative abundances of Bacteroidetes (maternal x weaning diet  $p = 0.002$ ) and  
 51 of Firmicutes (maternal x weaning diet  $p = 0.02$ ) were respectively lower and greater in C-LA rats  
 52 compared to C-C ones (Figure 6D). At the family level (Table S5), the maternal LA-diet had some  
 53 slight yet significant effects on the relative abundances of several families (decreased abundance of  
 54 Muribaculaceae, increased abundances of Micrococcaceae, Lactobacillaceae and Erysipelotrichaceae  
 55 (Table S5)). The weaning LA-diet slightly reduced the abundance of Lachnospiraceae, and some  
 56 maternal x diet interactions were still observed, such as for Tannerellaceae abundance (Table S5).



57 **Figure 6.** Impact of maternal and weaning LA-diets on microbiota composition at 6 months of age.  
 58 The cecal microbiota composition of C-C, C-LA, LA-C and LA-LA rats was determined at 6 months  
 59 of age through 16S rRNA gene sequencing. **A.** Principal coordinates analysis based on weighted  
 60 Unifrac distances. **B.** Microbiota richness (observed OTU). **C.** Microbiota diversity estimated by the  
 61 Shannon index. **D.** Main phyla relative abundance (mean ± SEM). Two-way ANOVAs were  
 62 performed to test the maternal, weaning and interaction effects. Panels B–C: letters indicate significant  
 63 difference ( $p \leq 0.05$ ), #  $p \leq 0.07$ , Panel D: \* indicates difference between C-C and C-LA ( $p \leq 0.05$ ),  $n =$   
 64 6/group.  
 65

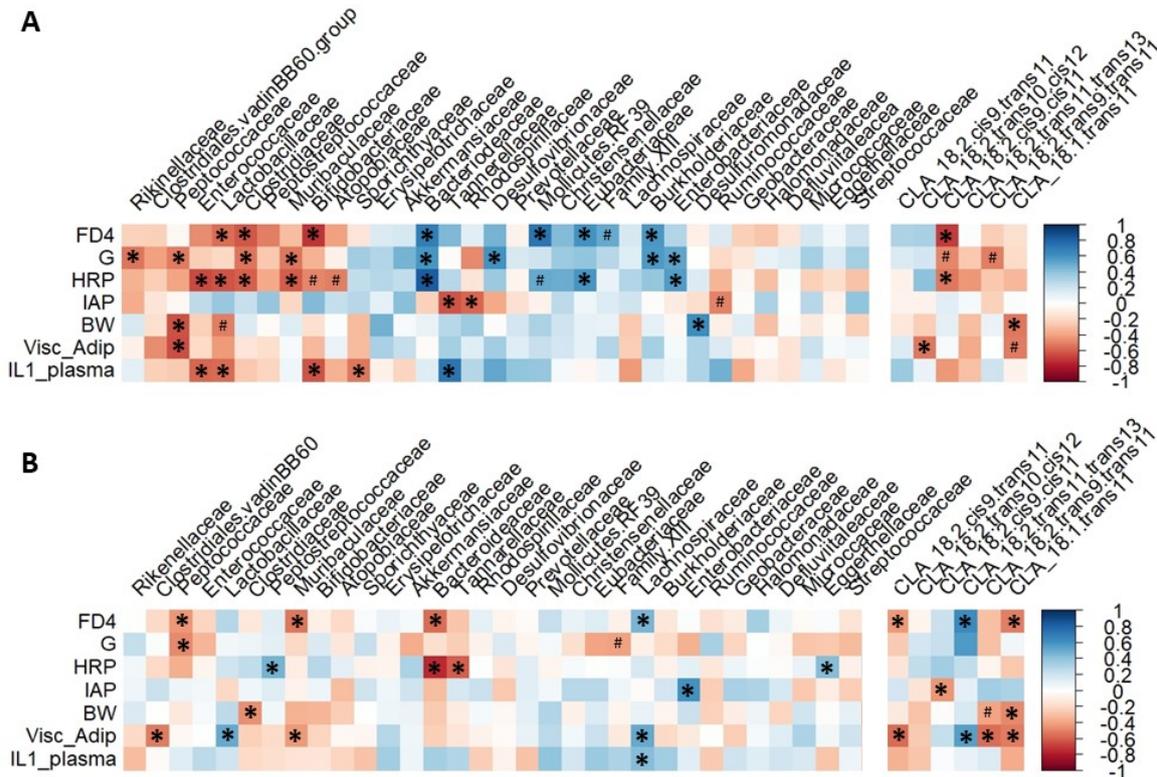
66 Interestingly, when we compared the cecal microbiota between 3- and 6-month-old rats, the  
67 cecal microbiota of rats born to LA mothers exhibited only a few changes between 3 and 6 months of  
68 age compared to rats born to C mothers, which exhibited marked changes with age. This was  
69 demonstrated by weighted Unifrac distance PCoA (Figure S2A) and the absence of increase in  
70 richness and diversity with age in rats born to LA mothers, irrespective of the weaning diet,  
71 compared to rats born to C mothers (Figure S2B and S2C). Likewise, the relative abundances at the  
72 family level did not changed markedly with age in rats born to LA mothers compared to those born  
73 to C mothers (Figure S2D).

74 3.4.3. Microbiota composition correlates with rat gut barrier and obesity phenotype at 3 but not 6  
75 months of age

76 Spearman rank correlations between microbiota family relative abundances, hepatic CLA  
77 concentrations, key gut barriers (FD4 and HRP fluxes, G, IAP activity) and obesity and low-grade  
78 inflammation (body weight, visceral adiposity index, plasma IL-1 $\beta$ ) parameters were performed to  
79 highlight possible links between changes in microbiota and rat phenotype.

80 At 3 months of age, a cluster of bacterial families, including Peptococcaceae, Enterococcaceae,  
81 Lactobacillaceae, Clostridiaceae, Bifidobacteriaceae, and to a lesser extent the Rikinellaceae and  
82 Clostridiaceae vadinBB60 groups, correlated negatively with both gut barrier parameters and  
83 obesity/inflammation markers (Figure 7A). Two other bacterial clusters correlated with gut barrier  
84 parameters: the relative abundances of Tannerellaceae and Rhodospirillaceae correlated negatively  
85 with IAP activity, and the relative abundances of Bacterioidaceae, Desulfovibrionaceae, Mollicutes  
86 RF39, Eubacteriaceae, Burkholderiaceae and Enterobacteriaceae correlated positively with gut  
87 permeability parameters. Hepatic 18:2 *cis*-9, *cis*-11 correlated negatively with gut permeability  
88 parameters, and 18:1 *trans*-11 with body weight and adiposity (Figure 7A).

89 At 6 months of age, less significant correlations were observed (Figure 7B). However, the relative  
90 abundances of Bacterioidaceae and Peptococcaceae correlated negatively with gut permeability  
91 parameters. Enterobacteriaceae relative abundances correlated positively with IAP activity. Visceral  
92 adiposity correlated with the hepatic levels of many CLAs—positively with 18:2 *trans*-11, *trans*-13  
93 levels and negatively with 18:2 *cis*-9, *trans*-11, 18:2 *trans*-9, *trans*-11 and 18:1 *trans*-11 levels. Cecal  
94 permeability correlated positively with hepatic 18:2 *trans*-11, *trans*-13 levels and negatively with 18:2  
95 *cis*-9, *trans*-11 and 18:1 *trans*-11 levels (Figure 7B).



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**Figure 7.** Correlations between family relative abundances and CLAs, and rat phenotype key parameters. Spearman rank correlations were performed at 3 months (A) and 6 months (B) of age between family relative abundances or CLAs and gut barrier, adiposity and systemic inflammation parameters, and represented as a correlation matrix between parameters. \*  $p < 0.05$ , #  $p < 0.06$ . FD4: G; HRP: IAP.

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#### 4. Discussion

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In this study, we investigated the interactive effects of high LA intake in the maternal diet during gestation and lactation and in the weaning diet on intestinal and adipose tissue and gut microbiota, in young (3-month-old) and older (6-month-old) adult offspring. To our knowledge, our report is the first to demonstrate the effects of such diet interaction on host physiology and its associated alterations of gut microbiota, in the context of moderate fat intake. Here, we found that exposure to LA during gestation and lactation had long-lasting effects on offspring gut functions at 3 months of age. Maternal high LA intake appeared to be responsible for the major changes in inflammation with increased colonic *tnf- $\alpha$*  mRNA and plasma IL-1 $\beta$ , and a decreased IAP activity. IAP has been well described for its role in LPS detoxification and in tissue resolution of inflammation, but a role in the maintenance of gut microbiota homeostasis has also been suggested [70,71]. The reduced IAP activity induced by maternal LA-diet in offspring gut at 3 months of age corresponds with changes in pro-inflammatory cytokines, and may be involved in gut barrier function and gut microbiota alterations. Our study supports a recent multi-omics study using transgenic mice able to overproduce *n*-6 PUFA, which suggested the potential harmful effects of *n*-6 PUFA excess, with increases in endotoxemia and inflammation. These *n*-6 PUFA effects likely resulted from an altered gut microbiota with a dominance of pro-inflammatory bacteria, such as Proteobacteria [31], as also observed in our study of rats born to LA mothers. According to our study, the authors observed a lower IAP activity in wild type mice with a high *n*-6 status compared to Fat-1 mice with high *n*-3 status, and suggested IAP to be a mediator of the effects of *n*-6/*n*-3 tissue composition on gut microbiota [30]. On the other hand, intestinal permeability was mainly altered by high LA in the weaning diet, in line with previous studies [31,72,73]. Using the intestinal cell line, Jiang et al, demonstrated that LA reduced

124 transepithelial cell resistance (TER), a measure of barrier integrity, and increased paracellular  
125 permeability [72]. Kirpich et al. also showed that a high intake of *n*-6 PUFA exacerbates the alcohol-  
126 induced increase in permeability in a model of alcoholic liver disease [73]. Thus, our results suggest  
127 that the inflammatory process in offspring might be imprinted by the maternal diet-induced gut  
128 microbiota alterations, while intestinal barrier function might be driven mostly by the weaning diet.

129 The mechanisms by which LA modulates intestinal barrier function remain to be fully  
130 determined, but several reports have suggested that CLAs could participate in these changes. CLAs  
131 naturally occur in food sources, but several in vitro and in vivo studies have demonstrated the ability  
132 of specific gut bacteria species to convert LA into diverse CLA isomers [51,74]. Here, hepatic CLA  
133 levels, which reflect gut CLA production and absorption, were mainly affected by the weaning diet.  
134 The levels of 18:2 *cis*-9, *cis*-11 were reduced in rats fed the weaning LA-diet, consistent with those of  
135 LA-rich oil force-fed mice [51]. Furthermore, 3 months of weaning LA-diet led to an increase in  
136 rumenic acid (RU, 18:2 *cis*-9, *trans*-11) and 18:2 *trans*-10, *cis*-12, the major isomers of CLAs also  
137 observed after gavage with LA rich oil [51]. The LA weaning diet led to an increase in the abundance  
138 of some genera of the Lachnospiraceae family, including the genera *Blautia* and *Roseburia*, in the  
139 offspring of C mothers. Lachnospiraceae and *Roseburia* bacterial genera have been previously  
140 described to participate in the biohydrogenation of LA into CLAs [51,74]. Thus, difference in  
141 microbiota composition could account for this difference in the major CLA isomers produced upon  
142 age and diet. We speculate that in addition to a direct effect of LA on intestinal barrier integrity, the  
143 higher levels of 18:2 *trans*-10, *cis*-12 in weaning LA-diet-fed offspring might also contribute to the  
144 alteration of the intestinal function at 3 months of age. Previous work already supports this  
145 hypothesis [75]. Specifically, the supplementation of Caco-2 cells with 18:2 *trans*-10,*cis*-12 alters the  
146 distribution of tight junction-associated proteins, occludin and ZO-1, involved in the maintenance  
147 and integrity of the epithelial barrier. This was accompanied with a greater paracellular permeability  
148 to [<sup>14</sup>C] mannitol and a delay in the increase of TER [75]. Negative correlations between gut  
149 permeability and specific bacterial families also suggest that the decrease in the abundance of some  
150 beneficial species, such as *Lactobacillus* [76], *Enterococcus* [77] or *Bifidobacterium* [76], may contribute  
151 to the weaning LA-diet-induced gut barrier dysfunction.

152 High LA intake can increase the levels of CLAs, which mainly accumulate in the cecum and  
153 colon and act locally [51,74], but some studies have also demonstrated a CLA enrichment in  
154 peripheral tissues following CLA supplementation [78]. This suggests that CLAs could exert both  
155 local and systemic effects on host physiology. At 3 months of age, we found that changes in gut  
156 barrier function and inflammation were associated with alterations of adipose tissue physiology in  
157 rat offspring. These changes were mainly driven by the maternal LA diet. The offspring of mothers  
158 fed the LA-diet during gestation and lactation exhibited higher visceral fat mass, larger adipocyte  
159 frequency and reduced hormone-sensitive lipase transcript level. This is in line with previous studies  
160 suggesting an effect of LA-enriched diet on greater adiposity in both animal and humans [6,7,10].  
161 However, the lower expression of PPAR $\gamma$  transcripts in the offspring of LA mothers contrasts with  
162 previous work showing that PPAR $\gamma$  is a key mediator of adipogenesis induced by LA-derivative  
163 fatty acid [2,6], and may suggest another adiposity-induced mechanism in our study. Furthermore,  
164 it was later determined that CLAs could alter adipose tissue physiology (for review [79]). While the  
165 18:2 *trans*-10, *cis*-12 isomer has been described as the main isomer responsible for reducing adiposity  
166 [80], the increased levels of 18:2 *trans*-10, *cis*-12 observed in 3-month-old offspring in the present study  
167 do not seem to corroborate the literature. In our study, CLA level variation appeared to depend  
168 mainly on the weaning diet at 3 months, while adiposity changes were related to maternal diet and,  
169 therefore, may suggest that adipose tissue changes might be related to other mechanisms or other LA  
170 derivatives. Furthermore, the negative correlations of some bacterial families with both gut barrier,  
171 plasma IL-1 $\beta$  and adipose tissue parameters suggests a role for gut-mediated systemic low-grade  
172 inflammation in the alteration of adipose tissue physiology [22], which warrants further  
173 investigation.

174 At 6 months of age, these alterations were not exacerbated as expected, but rather showed  
175 opposite results. Our data provide evidence that the maternal LA dietary level impacts gut function,

176 adipose tissue physiology and gut microbiota in young adult rats, but that this imprinting effect fades  
177 later in life in favor of a direct effect of the weaning diet, as previously suggested [65]. Microbiota  
178 composition seems to be more similar within the offspring groups, although microbiota richness and  
179 diversity and the abundance of some families were still altered by the maternal LA-diet. Unlike the  
180 3-month-old offspring, rats fed the weaning LA-diet for 6 months displayed reduced intestinal  
181 permeability and inflammation (IAP, colonic *tnf- $\alpha$*  mRNA, plasma IL-1 $\beta$ , etc.), as well as lower  
182 adiposity, compared to those fed the weaning C-diet. These alterations may result from the increased  
183 levels of RU and 18:2 *trans*-9, *trans*-11 and lower levels of 18:2 *cis*-9, *cis*-11 and 18:2 *trans*-11, *trans*-13  
184 CLA isomers, since visceral adiposity correlated significantly with these CLA isomer hepatic levels  
185 at 6 months. The anti-adiposity properties of CLAs have been mainly attributed to 18:2 *trans*-10, *cis*-  
186 12, but this CLA was no longer increased at 6 months of age. The beneficial effects of CLA isomers  
187 have been mainly described in relation to the CLA mixture of RU and 18:2 *trans*-10, *cis*-12, but the  
188 specific role of each isomer is still unclear [79]. In addition to CLAs, the biohydrogenation pathway  
189 leads to other intermediates, among which vaccenic acid (VA, *trans*-11 18:1) is the most important  
190 and could also be associated with beneficial effects on the host [81]. Here, we found that VA was  
191 significantly increased in offspring fed weaning LA-diets over 6 months. This is in line with previous  
192 studies showing that VA attenuates complications in metabolic syndrome, including low-grade  
193 inflammation, by acting directly in the intestine and adipose tissue [82,83]. Thus, physiological  
194 alterations of gut and adipose tissue may also result from the combined variations of the other CLAs,  
195 but the isomer-specificity of CLAs needs to be further elucidated. Interestingly, unlike the 3-month  
196 age, lower levels of the CLA isomer 18:2 *trans*-9, *trans*-11 were observed in the offspring of dams  
197 exposed to the LA-diet during gestation and lactation, but the biological meaning of this remains to  
198 be fully explored [84]. Thus, at 6 months of age, LA weaning diet-fed rats appeared to exhibit a  
199 healthier phenotype compared to C weaning diet-fed ones, with lower cecal permeability and colonic  
200 and plasma cytokine levels, as well as reduced adiposity. However, recent published data from our  
201 group revealed a hepatic steatosis in these animals [50], suggesting the strong alteration of lipid and  
202 glucose metabolism in these animals. Hepatic steatosis was associated with increased levels of the  
203 LA derivatives oxidized linoleic acid metabolites (OXLAMs) and pro-inflammatory eicosanoids.  
204 Further studies are required to identify the balance among LA derivatives, LA-induced changes in gut  
205 microbiota, and their specific effects on host physiology.

206 The gut microbiota are considered a major regulator of host physiology and metabolism.  
207 Alterations of intestinal microbiota have been described in several chronic metabolic diseases,  
208 including obesity, in which the concept of altered gut permeability and adipose tissue plasticity have  
209 been described (for review [54]). Although the number of animals studied was low and caution needs  
210 to be taken with these results [57,58], we observed a long-lasting effect of the maternal LA-diet on the  
211 gut microbiota's composition and diversity compared to the effects of a direct weaning LA-diet in  
212 young adult offspring. Indeed, microbiota composition and diversity at 3 months of age differed  
213 between rats born to C or LA mothers. Moreover, the weaning LA-diet strongly impacted the  
214 microbiota composition when rats were born to C mothers (C-LA group compared to C-C group),  
215 but not when born to LA mothers (LA-LA group compared to LA-LA group). Interestingly, all the 3-  
216 month-old rats exposed to LA exhibited reduced Lactobacillaceae and Clostridiaceae relative  
217 abundances when compared to the C-C group, suggesting that these two families are sensitive to LA.  
218 Both families were negatively correlated to gut permeability and obesity/inflammatory parameters.  
219 At 6 months of age, some remnant effects of the maternal diet were still observed, and the weaning  
220 diet had almost no impact on the gut microbiota. Our data are in line with previous studies showing  
221 the long-term impacts of maternal HFD on offspring gut microbiota composition in animal models  
222 [59,60] or in humans [61], and the long-term impact of maternal *n*-3 PUFA intake [34,62]. However,  
223 in these studies, the offspring were usually kept on a control diet at weaning. If offspring were  
224 challenged with an experimental diet, the maternal diet effect disappeared or faded [63–65]. For  
225 example, in macaques, an effect of maternal HFD on fecal microbiota was observed 4 months after  
226 weaning in animals kept on a control diet, but this maternal diet effect was no longer observed when  
227 offspring were fed an HFD after weaning [65]. Interestingly, the same group published later on that

228 maternal HFD impacted microbiota maturation throughout adult life—the offspring of HFD mothers  
229 reached a mature microbiota as early as 1 year of age, whereas the offspring of control mothers  
230 reached this stage 2 years later [66]. Our data also suggest a more ‘mature’ microbiota in rats born to  
231 LA mothers, with few changes in composition, richness and diversity between 3 and 6 months of age  
232 in these animals. The establishment and maturation of the gut microbiota are under dynamic  
233 processes, and can be influenced by various environmental factors, including the diet [67]. Our work  
234 suggests that high LA intake during gestation and lactation may account for the different colonization  
235 patterns of offspring microbiota. Interestingly, while high gut microbiota diversity is often associated  
236 with a healthy host phenotype in adults [68], this might not be the case in younger individuals. For  
237 example, breast-fed infants harbor lower fecal microbiota richness than formula-fed ones [69], yet  
238 breast-feeding is associated with lower risks of diseases. Further analysis will be required to fully  
239 determine the causal effects of maternal LA on precocious microbiota maturation and its impact on  
240 host phenotype.

241 Our study has several limitations that we need to acknowledge. As already mentioned, the  
242 number of animals per group may be too low for microbiota analysis. The sample size was initially  
243 calculated for the 6-month time point, according to physiological data and fatty acid composition  
244 variability based on preliminary data. The 3-month time point was considered as an intermediate  
245 time point, whereas the 6-month time point was considered as the “main” end time-point to complete  
246 a previous study. Although some differences in microbiota profiles were observed among groups,  
247 previous studies have suggested a sample size >10 for sufficient power based on diversity metrics  
248 and microbiota composition [57,58]. Thus, the lower sample size of our study could result in  
249 undetected small effects. Moreover, the cage effect was not corrected for in the microbiota analysis,  
250 while co-caging also impacts microbiota composition [85]. However, the highly controlled  
251 environment of the animal facility, and the standardization of sample collection, storage and  
252 extraction, helped in minimizing the technical variation and bias that could increase variability  
253 within samples and reduce statistical power. Further investigations are, however, necessary in order  
254 to confirm this maternal LA-diet effect upon offspring microbiota composition. Another limitation of  
255 the study is the fact that we investigated only one section of the rat intestine, i.e., the cecum and  
256 proximal colon, while *n*-6 PUFA could have also affected more proximal regions of the gut. Indeed,  
257 Druart et al. observed lower yet detectable amounts of CLA in the ileum of mice gavaged with LA-  
258 rich oil, suggesting the possible effect of *n*-6 enriched diet on the small intestine microbiota [51].  
259 Finally, while we observed significant effects of the maternal and weaning LA-diets on adipose tissue  
260 morphology and key lipid metabolism gene expression, we did not explore glucose homeostasis in  
261 our rats. A strong link between gut microbiota and glucose homeostasis has also been described [86],  
262 and would deserve investigations in this model.

## 263 5. Conclusions

264 We showed the differential and interactive effects of high LA intake in maternal and weaning  
265 diets on intestinal and adipose tissues, as well as on gut microbiota in young and older adult  
266 offspring. Despite a low *n* value, we observed that the gut microbiota of offspring at both 3 and 6  
267 months were altered by the early perinatal exposure to LA, suggesting that high maternal LA intake  
268 may shape the gut microbiota of offspring and induce differential metabolic responses to diet  
269 exposure. As such, this study highlights the need for additional work to investigate how the increased  
270 consumption of LA in females may affect offspring outcomes.

271 **Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Table 1: Fatty acid  
272 composition of the experimental diets and animal study design, Figure S2: Evolution of microbiota between 3  
273 and 6 months of age, Table S1: Primers used for PCR, Table S2: Fatty acid composition (% total fatty acids) of  
274 dam liver, mesenteric adipose tissue and mammary gland at weaning, Table S3: Fatty acid composition (% total  
275 fatty acids) of offspring liver and mesenteric adipose tissue at weaning, Table S4: Microbiota family relative  
276 abundances in the rat cecum at 3 months of age, Table S5: Cecal microbiota family relative abundances at 6  
277 months of age.

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544