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Abstract

Synthetic emulsifiers promote metabolic syndrome and considerably alter gut microbiota. Data is lacking regarding natural emulsifiers like plant lecithins, a polar lipid-rich source of 18:3 n-3 PUFA (ALA). For 13 weeks, male Swiss mice were fed ALA-replete high-fat diet (HFD) including lecithin from rapeseed (RL) or soy, *vs* 2 HFD-controls devoid of lecithin (ALA-replete; low-ALA), *vs* Chow. Lecithins did not enhance HFD-induced adiposity nor increased inflammation, did not alter gut barrier markers and caecal bile acids, and contributed to n-3 PUFA status. Lecithins improved gut microbiota diversity. RL (10% in fat) even restored α-diversity similar to Chow, increased *Lachnospiraceae* NK4A136, *Lactobacillus* and *Ruminococcaceae* UCG-014 groups, and decreased *Blautia* genus bacteria. The abundance of most beneficial lecithin-enhanced bacteria was positively correlated to the amount of faecal polar lipid-bound ALA. These findings show that lecithins can beneficially affect the gut microbiota in association with changes in lipid residues in the distal gut.

Keywords: nutrition; food additive; phospholipid; intestine; absorption; microbiota

Abbreviations: α-linolenic acid (ALA), bile acid (BA), cholic acid (CA), chylomicron (CM), deoxycholic acid (DCA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), European Food Safety Agency (EFSA), fatty acid (FA), linoleic acid (LA), oleic acid (OA), palmitic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phospholipid (PL), polysorbate 80 (PS80), polyunsaturated fatty acid (PUFA), rapeseed lecithin (RL), saturated fatty acid (SFA), soy lecithin (SL), triacylglycerol (TAG).

1. Introduction

The last decades have been characterized by a dramatic increase in obesity and associated metabolic disorders, which now constitute one of the major burdens for public health globally. The increased consumption of ultra-processed foods rich in food additives undoubtedly plays a major role in this phenomenon. Recently, evidence implicating synthetic emulsifiers in the development of such obesity-related metabolic disorders and inflammatory bowel disease (IBD) has been accumulating [1–3]. Two synthetic emulsifiers in particular, carboxymethylcellulose (CMC) and polysorbate 80 (PS80), have been shown to induce metabolic syndrome and promote gut inflammation together with a dysbiotic state of the gut microbiota [1,4].

Conversely, lecithins appear as a good alternative to such emulsifiers [5]. They are natural lipid mixtures, commonly used by the food industry for their emulsion stabilizing properties and obtained as a by-product during the degumming processing of oils. We have previously shown in mice that short term consumption of nutritional doses of lecithin exerted beneficial effects on lipid metabolism, bile acid (BA) profile and the gut microbiota [6]. A recent study using *in vitro* incubation of human gut microbiota showed that lecithins were among the only few emulsifiers to have no major deleterious impact on microbiota composition [7]. However, their long term impact on overall intestinal health, such as intestinal barrier integrity, metabolic inflammation and the gut microbiota, remains to be determined *in vivo*. Such data is crucial, as intestinal health and low-grade inflammation are at the heart of obesity and associated metabolic disorders. The gut microbiota is now recognized as a central actor and mediator in both intestinal and metabolic health [8] and it is now well-known that individual food components may interact with the gut microbiota and induce changes in microbial function and composition [9]. Particularly, an association between total fat intake and the reduction of the microbiota richness has been repeatedly found. Indeed, whereas the majority of consumed lipids are absorbed in the small intestine, the amount of total lipids that reach the colon under physiological conditions has been evaluated at between 5 and 8 g per day in humans [10]. Interestingly, different types of fat exert different or even opposite effects on the microbiota. Non-absorbed or excreted fatty acid residues from dietary lipids may thus reach the colon where they can interact and impact gut microbial populations, leading to various health effects [11]: such effect thus cannot be ruled out for lecithins.

As such, certain lecithins, namely soy and rapeseed lecithins, are of particular nutritional interest as they are rich in alpha-linolenic acid (ALA, 18:3 n-3), an essential omega 3 fatty acid. By providing ALA in the form of phospholipids (PL) comparatively to its vectorization as triacylglycerols (TAG) in oils, they can induce a faster appearance of ALA in lymph [12]. ALA offers many health benefits, such as anti-inflammatory and anti-thrombotic effects, and may hence serve as a preventive and protective agent in the development of HFD-induced metabolic and inflammatory disorders.

Nevertheless, the effects of lecithin on gut health remain controversial. As such, previous work has reported that, when added to a semi-synthetic Western diet rich in saturated fatty acids (SFA), soy lecithin was associated with pro-inflammatory and adipogenic effects in mice [13]. Moreover, as lecithins are extracted from natural sources and using different processes, their biochemical and fatty acid compositions vary considerably [5], making it difficult to conclude on the effects of lecithins on metabolic health. As highlighted in a recent review on the health effects of emulsifiers, it is therefore essential to study the effects of individual lecithins within different diets of known and controlled composition [2]. The aim of this study was thus to assess the long-term effects of ALA-rich lecithins from two different sources, soy and rapeseed, on HFD-induced metabolic and inflammatory outcomes and on the gut microbiota, within diets of similar nutritional composition. This approach may help assessing the effects of two different lecithins on gut and metabolic health and hence better understand how lecithins interact with the intestinal barrier and gut microbiota.

2. Experimental Section

2.1. Preparation and characterization of the experimental diets

Soybean and rapeseed lecithin (SL and RL) were kindly provided by Novastell (Etrepagny, France) (typical chemical structures within are shown in **Supplementary Figure S1**). Grapeseed, rapeseed and palm oils were provided by ITERG (Canéjan, France). The experimental semi-synthetic high-fat diets (HFD) were prepared by SAFE (Augy, France) by mixing the aforementioned vegetable oils with varying doses of RL or SL and delipidated powdered diets, so as to obtain diet pellets containing an identical amount of 25% total lipids, as well as similar macronutrient repartition and caloric input (**Supplementary Table S1**). The proportions of the vegetable oils in experimental HFD were defined in order to obtain (i) a control HFD poor in ALA and (ii) four other HFD of similar FA profiles, including ~4.5% ALA, but differing in lecithin content and/or type (as further explained in section 2.2). The

FA and phospholipid compositions of the two lecithins (**Supplementary Table S2**) and diet lipids (**Table 1**) were verified as described in section 2.4. As reported in the literature [5], RL and SL have similar phospholipid class profile and ALA content (~4%) but the major FA in RL is oleic acid (C18:1 n-9, 47.7%) while it is linoleic acid in SL (C18:2 n-6, 51.9%) (**Supplementary Table S2**). The FA profile and PL composition of experimental HFD lipids are consistent with the designed formulation. The thermal properties of lipids in the different diets were analyzed as described in section 2.5: HF_Cont diet presented the highest solid fat content at room temperature (represented by the partial melting enthalpy from 20°C till the end of melting, $\Delta H_{20°C}$), namely $\Delta H_{20°C}$ >30% *vs* <20% in other groups, and the highest melting temperature: 38°C *vs* 35-37°C in other groups.

2.2. Animal ethics statement, animals and diet groups

All experiments were performed in accordance with the EU Council Directive for the Care and Use of Laboratory Animals (n° 2010/63/EU). The protocol was approved by the Animal Ethics Committee of National Institute of Applied Sciences of Lyon (INSA-Lyon, France) and registered under the number 2019021315358441_v4 APAFIS #19141. Experiments were performed using male Swiss mice (Elevage Janvier), obtained at 4 weeks old and weighing 30-40 g. Of note, Swiss mice are known to express all isoforms of PLA2, the enzyme required to completely digest and metabolize phospholipids [14]. The mice were acclimatized for 2 weeks prior to the experiment, during which they were housed 6 per cage and kept in a controlled environment (temperature 24±1°C; 12h light – 12h dark cycle) with free access to water and food (standard Chow, A04 diet, SAFE, Augis, France).

After acclimatization, the mice were randomly assigned to one of six experimental groups (n=12/group) for 13 weeks (**Figure 1)**. One group was fed a standard Chow diet (A04), to serve as healthy control (typical nutrient composition: starch 33.8 wt%, sugars 3.2 wt%, proteins 16.1 wt%, fibers 3.9 wt%, minerals ash 4.6 wt%, lipids 3.1 wt% among which 0.27 wt% polar lipids, [15]). Another group consumed a semi-synthetic HFD (25 wt% lipids, detailed composition in **Supplementary Table S1**) rich in palm oil and n-6 PUFAs, depleted in ALA (**Table 1**), to serve as a Western diet control group (HF_Cont). The 4 remaining groups consumed HFD (25 wt% lipids) differing only in the amount of lecithin: no lecithin (HF_0L), RL or SL at 10% of total lipids (ie 2.5 wt% in the diet; HF_10RL and HF_10SL), or RL at 20% of total lipids (ie 5 wt% in the diet; HF_20RL). These 4 HFD presented similar FA profiles and were enriched in ALA (4.7%) in order to reach a recommended n-6/n-3 ratio of 5 [16] (**Table 1**). Because both rapeseed and soybean lecithins are vectors of ALA, the HF_0L

group thereby served as another high-fat control group to distinguish potential lecithin effect from a simple ALA effect.

The feed supply was changed twice per week. Body weight was recorded weekly. Feces were collected before, after 2 weeks and at the end of the 13 week-diet. After 13 weeks of dietary intervention, the mice were euthanized by inhalation of isoflurane. Death was induced by cardiac puncture, followed by cervical dislocation. Blood was collected directly in heparin-containing tubes and placed immediately in ice. Plasma was obtained by centrifugation (4600rpm at 4°C for 5 minutes). The plasma, liver, intestinal segments, caecum and fat depots were weighed, frozen in liquid nitrogen and stored at -80°C until analysis.

2.3. Dosage information/dosage regimen

On average, the mice weighed 37g at the beginning of dietary intervention and consumed 8.8g of diet per day. According to the FDA's Human Equivalent Dose calculation, the daily doses of lecithin consumed by the mice are equivalent to 97 $mg.kg^{-1}$ body weight for both 10% lecithin groups [17]. According to the EFSA Panel on Food Additives, the estimated average exposure to lecithins as food additives in Europe ranges from 32 to 177 $mg.kg^{-1}$ body weight per day in adolescents, and 70 to 118 $mg.kg^{-1}$ body weight per day in adults according to the regulatory maximum level exposure assessment scenario [18], consistent with another estimation in the US population of 60 $mg.kg^{-1}$ body weight per day on average (~115 $mg.kg^{-1}$ body weight per day as 90^{th} percentile) [3]. The doses used in this study are hence representative of the daily intakes of the Western population (10% in HFD lipids as a nutritional dose, 20% in HFD lipids may be a supplementation dose).

2.4. Histological analysis

Tissues were collected and paraffin embedded after 24h of 3.7% formaldehyde fixation. For adipocyte area measurement, 4µm-sections of epididymal adipose tissue (EAT) were generated and acquired for autofluorescence with a DP74 camera and 200x magnification (Olympus, France). An average of 20 non-overlapped fields were considered and analyzed for each sample.

Regarding the quantitative analysis of colonic crypt goblet cells, serial sections were deparaffinised and rehydrated before a blue alcian staining (pH=2.5) for 30min followed by 2 minutes of running tap water then distilled water. Nuclei were stained by aluminum sulfate-nuclear fast red incubation for 5 minutes (Sigma-Aldrich). Slides were then washed in tap

water for 1 minute before dehydration and mounting. For each sample, a percentage of positive area was measured, using at least 15 well-oriented crypts. All histology analyses were performed using Fiji software.

2.5. Lipid characterization of diets and ingredients

Lipids were extracted from diets and ingredients by the Folch method [19]. The lipid content was estimated by gravimetry according to the French regulation NF EN ISO 3727-2, and FA profile determined after FA derivatization into FAME by transmethylation method [20] and adapted from [21,22]. The resulting FA methyl esters (FAME) were analyzed by gas chromatography (GC, TRACE GC, Thermo Scientific, Waltham, Massachusetts, USA), equipped with a flame ionization detector (FID) and a split injector. A fused-silica capillary column (BPX 70, 60 m × 0.25 mm i.d., 0.25 µm film; SGE, France) was used with hydrogen as a carrier gas (inlet pressure: 120 kPa). GC peaks were integrated using Chromquest software (Thermofinnigan, Courtaboeuf, France). FA were quantified using tri-heptadecaenoic acid as an internal standard, and were added at 10% of the lipid weight before the (trans) methylation procedure.

The phospholipid fractions were evaluated therefrom by high-performance liquid chromatography (HPLC) coupled to an evaporative light-scattering detector (SEDEX LT-ELSD SOLT, HPLC DDL Sedere, Thermo Fisher Scientific) [23,24], using an HILIC column (ACQUITY UPLC BEH HILIC 100 X 2,1 mm, 130 Å ; 1.7 µm, waters). The chromatographic separation was carried out using a linear binary gradient consisting of acetonitrile (eluent A) and acetonitrile/water with formic acid (0.1 %) and ammonium formiate (5 mM) (50:50, v/v) (eluent B). The flow rate of the eluent was 1 mL/min. Identification of phospholipids and lysophospholipids was carried out by comparison with the retention time of pure standards (Avanti polar Lipids). Calibration curves for each compound were calculated from the area values of stock solution of pure standards. Results were analyzed using Chromeleon software (Thermo Fisher Scientific).

The melting curves of the extracted lipids (about 5mg) were obtained using a Q-2000 DSC (TA Instruments, New Castle, USA), using Tzero hermetic pans. Calibration was made with indium and eicosane standards; nitrogen was used as purge gas and a similar Tzero hermetic empty pan was used as a reference. The melting profiles were recorded using AOCS cj1-94

method [25]. The integration and peak temperature measurements were performed using the Universal Analysis Software version 4.2 (TA Instruments, New Castle, USA).

2.6. Lipid characterization of tissues

Liver, adipose tissue and faeces samples were thawed to room temperature. Total lipids were extracted according to the Folch method [19]: an equivalent amount of 100 mg of adipose tissue, 200 mg of liver or 200 mg of feces was extracted with 20-fold volume (weight/volume) of a $CHCl_3$/MeOH mixture (2/1 ; vol/vol).

Lipid fractions were separated by thin layer chromatography (TLC) (glass plates 20x20 cm pre-coated with silica gel 60H) and migration was performed using a solvent mixture composed of Heptan, diethyl ether, and formic acid, 55 :45 :1, respectively. After vaporization of 2,7-DCF and visualization under UV-light, spots corresponding of PL, TAG (liver, WAT) and FFA (faeces) were scrapped-off to be then derived into FAME by transmethylation method [20][21,22]. The resulting FA methyl esters (FAME) were analyzed by GC-FID as above-described.

2.7. Real-time quantitative RT-PCR analysis

Total RNA was extracted from the jejunum, ileum, colon and epididymal AT using the TRI Reagent (Ambion/Applied Biosystems). RNA quality and concentration were measured using the Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using 1µg of RNA and the PrimeScript RT reagent kit (Ozyme, Saint Quentin-en-Yvellines, France). Real-time PCR assays were then performed using a Rotor-Gene Q (Qiagen, Hilden, Germany) and SYBR qPCR Premix Ex Taq (Tli RNaseH Plus) reagents. PCR primers are available upon request (emmanuelle.meugnier@univ-lyon1.fr). The results were normalized using the expression of the *Tbp* (TATA-box-binding protein) gene as a reference in all tissues but the EAT, where *Hprt* (Hypoxanthine Guanine Phosphoribosyltransferase) was used instead.

2.8. Faecal microbiota analysis

Extraction of total bacterial DNA from fecal pellets was performed using the QIAamp PowerFecal DNA Kit (Qiagen) and the mechanical bead-beating disruption method as previously described [26]. The V3-V4 region of the 16S rRNA genes was amplified using Taq KAPA2G Robust KK5005 (CliniSciences, Nanterre, France) and primers V3F: TACGGRAGGCAGCAG and V4R: ATCTTACCAGGGTATCTAATCCT. The purified

amplicons were sequenced using Miseq sequencing technology (Illumina) at the @BRIDGe sequencing facility (GABI, INRAE, AgroParisTech, Paris-Saclay University, Paris, France). Paired-end reads obtained from MiSeq sequencing were analyzed using the Galaxy-supported FROGS (Find, Rapidly, OTUs (Operational Taxonomic Units) with Galaxy Solution) pipeline [27,28]. For preprocessing, reads with length ≥ 380 bp were kept. Clustering and chimera removal steps followed the FROGS guidelines. Assignation was performed using SILVA 16S. OTUs with abundances lower than 0.005% of the total read set were removed prior to analysis. Then, 16S sequencing data were analyzed using the Phyloseq, and ggplot2 R packages in addition to custom scripts as previously described [28].

2.9. Caecal bile acid analysis

Caecal BA molecular species concentrations were measured by HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) as previously described [29] with slight modification using a 5500 Q-trap (SCIEX). Taking into account the concentration and the retention time of different BA on a C18 column with a gradient of methanol; lithocholic acid has the highest retention time, and tauroursodeoxycholic acid-3S the lowest.

2.10. Statistical analysis

All statistical analyses were performed using the GraphPad Prism software (version 8). Data normality and homogeneity of variance were verified using a Shapiro-Wilk test and Bartlett test respectively before performing comparisons across groups by one-way ANOVA. For non-normal data, or in the case of a non-normal distribution of ANOVA residuals (tested by Anderson-Darling, D'Agostino-Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests), a Kruskal-Wallis test was performed instead. When the analysis revealed a significant diet effect, a Tukey post hoc test was used after ANOVA, or a Dunn's post hoc test after Kruskall-Wallis, to identify significant differences between groups. For microbiota analysis, differential abundance of bacteria was tested using negative binomial model implemented in DESeq2 and p-values corrected with False Discovery Rate (FDR) procedure. Diversity indices were compared between groups using one-way ANOVA. Principal Coordinate Analysis was performed on Jaccard dissimilarities to obtain a 2D representation of the samples. To evaluate possible relationships among outcomes, Spearman correlations were performed. Conventional values of $p<0.05$ were considered statistically significant. Data in tables and figures is presented as mean±SEM.

3. Results

3.1. Lecithins do not exacerbate HFD-induced metabolic and inflammatory outcomes and contribute to tissue omega 3 profile

Because synthetic emulsifiers have been shown to mitigate metabolic health, we first assessed the impact of the natural lecithins on physiological and metabolic changes induced by HFD in mice. During the 13-week dietary intervention, all HFD-fed mice gained more weight than those fed the control Chow diet, independently of changes in energy intake (**Fig. 2A**). After 13 weeks, all HFD fed mice presented a statistically similar final body weight gain, associated with similar energy intake (**Supplementary Table S3**). All HFD displayed significantly higher total white adipose tissue (WAT) mass and epididymal (EAT) adiposity vs Chow (**Fig. 2B**, **Supplementary Fig. S2D**), which was not further enhanced by the presence of lecithin. Likewise, the weight of other fat depots, namely mesenteric (MAT) and subcutaneous adipose tissue (ScAT), was similar across all HFD (**Supplementary Fig. S2B-C**). At the structural level, histological analysis of the EAT revealed an increase in adipocyte size with HF_10SL and HF_20RL vs Chow, which was however not significantly different from HF_Cont (**Supplementary Fig. S2E**). Liver weight (**Supplementary Fig. S2A**), fasting glycaemia, plasma TAG and total cholesterol (**Supplementary Table S3**) were not affected by the consumption of lecithin. Plasma NEFA were decreased in HF_Cont vs Chow but not in HF_0L nor in lecithin-enriched groups (**Supplementary Table S3**).

The mRNA expression of genes involved in inflammation and macrophage infiltration in the EAT was analysed. We targeted specifically immune cell populations that play a key role in the lipid response of WAT, namely *Adgre* (encoding for macrophage marker F4/80, to track this cell population), *Cd11c* (or *Integrin alpha X*), which is characteristic of pro-inflammatory macrophages (M1 type, that play an important role in WAT inflammation and insulin resistance development) and *Tumor necrosis factor alpha* (*Tnfα*, a major cytokine produced by M1 macrophages). In line with the known induction of metabolic inflammation by HFD, their expression was increased in HF_Cont vs Chow (**Fig. 2C-D-E**, $p \leq 0.01$). The expression of these genes was statistically similar to Chow in all ALA-rich HFD but HF_10SL, and the presence of lecithins did not modify HFD impact on these inflammatory markers. The gene expression in the EAT of leptin, an adipokine involved in energy balance, was not different among high-fat groups (**Supplementary Fig. S2F**).

As RL and SL are rich in an essential plant n-3 PUFA, namely ALA, we evaluated the capacity of these lecithins to affect n-3 PUFA status in tissues of metabolic interest. As expected, given the low level of ALA in HF_Cont Diet, HF_Cont group presented decreased n-3 PUFA compared to Chow in both PL (structural lipids) of EAT and liver (**Fig. 2F-G**) and TAG (storage lipids) of these tissues (**Supplementary Fig. S3**) ($p<0.001$). In turn, a higher accretion of ALA and long-chain n-3 PUFA in liver PL was observed in HF_0L and in the groups which consumed RL or SL compared to HF_Cont ($p<0.0001$). This was accompanied with decreased levels of total n-6 PUFAs, notably driven by a decrease in arachidonic acid.

3.2. Lecithins do not alter important features of the intestinal barrier

We next assessed the impact of lecithin on intestinal barrier integrity, which is known to be altered in HFD-induced metabolic disturbances and further degraded in the presence of synthetic emulsifiers. Histological analysis of colon revealed that the mice having consumed the HF_Cont and HF_0L displayed a diminished number of mucin-producing cells (**Fig. 3A-B**; $p=0.0036$ and $p=0.027$ vs Chow, respectively). This effect was not worsened by lecithins. In line with this, the mRNA expression of *Tight junction protein 1* (*Tjp1),* also known as *Zonula occludens 1* (*Zo-1*) and of *Occludin* in colon was significantly reduced in the HF_Cont compared to Chow (**Fig. 3C-D**, $p=0.030$ and $p=0.032$, respectively), but not in any other HFD and the presence of lecithin had no specific impact.

3.3. Rapeseed lecithin improves gut microbial profiles

In light of the adverse effects of synthetic emulsifiers on the gut microbiota, we investigated by 16S rRNA gene sequencing whether the presence of RL and SL in the diet also induced such effects. Principle coordinate analysis (PCoA) of Jaccard β-diversity revealed that, while all groups clustered similarly at baseline, all HFD assembled separately from Chow after 2 weeks of dietary intervention and those containing lecithin further drifted to form a partially separate cluster after 13 weeks (**Fig. 4A**). Both HF_Cont and HF_0L significantly decreased the microbial α-diversity (observed richness) compared to Chow (**Fig. 4B**, $p<0.0001$) but the presence of RL, especially HF_10RL, partially preserved microbiota α-diversity (**Fig. 4B**, $p\leq0.01$ HF_10RL vs HF_Cont and HF_0L). This was not the case with HF_10SL, suggesting that this effect was specific to RL. At the phylum taxonomic level, both control HFD (HF_Cont and HF_0L), compared to chow, were characterized by a concomitant decrease in *Bacteroidetes* and increase in the levels of *Firmicutes* and mucosa-associated inflammation-promoting *Proteobacteria*, while HF_10RL and HF_20RL presented less altered phyla

profiles (**Fig. 4C**). Pairwise DESeq analysis revealed that, at the genus level, RL increased the abundance of several operational taxonomic units (OTUs) associated with health, notably those belonging to the *Lachnospiraceae* NK4A136 (Cluster 25), *Lactobacillus* and *Ruminococcaceae* UCG-014 groups (Clusters 52 and 46), and simultaneously reduced an OTU belonging to the *Blautia* genus (Cluster 23) (**Fig. 4D**). This effect was also observed with SL (**Supplementary Fig. S4**), but not in the HF_0L group devoid of lecithin (as no differential OTUs were identified between HF_0L and HF_Cont), suggesting that these changes at the genus level were specific of lecithins.

3.4. Lecithins do not alter caecal bile acid profiles

As bile acids are strongly implicated in lipid digestion but also in host-gut microbiota interactions and in intestinal homeostasis, we next assessed whether the observed shifts in gut microbiota were linked to changes in the caecal BA pool. The size of the caecal BA pool was significantly increased in HF_Cont vs Chow (**Fig. 5A**, p=0.0008) but not in HF_0L nor in lecithin groups. Consistent with the literature, this altogether tended to be associated with higher caecal concentrations of total primary and taurine-conjugated BA, alongside an increase in β-muricholic acid (βMCA) and its tauro-conjugate, a known farnesoid X receptor (FXR)-antagonist, in HF_Cont compared to Chow (**Fig. 5A-E**). This was not observed in the HFD containing lecithins nor in HF_0L.

3.5. Lecithins can modify faecal lipid amount and composition

Lastly, we determined the profile of excreted lipids in faeces, as previous studies showed they can be correlated with diet-induced changes in the gut microbiota [15]. The majority of excreted FFA was represented by the SFA palmitic acid (16:0) (**Table 2**). We found that the amount of total free fatty acids (FFA, ie unesterified FA) was significantly elevated in the HF_Cont compared to Chow (**Fig. 5F**, p=0.0054), as well as in HF_0L to a lesser extent (p=0.056 vs Chow), but not in lecithin groups. This increase in FFA excretion was mostly attributed to the loss of SFA (**Fig. 5F**, p=0.0003), which was significantly higher in HF_Cont and HF_0L vs Chow ($p \leq 0.01$), but not in lecithin groups. HF_10RL and HF_20 RL even presented a lower 16:0 proportion in FFA compared to HF_Cont (both $p<0.0001$, **Table 2**) and to HF_0L ($p<0.05$ and $p<0.0001$, respectively). The faecal loss of free MUFA was also impacted (**Fig. 5F**, p=0.007), only due to an increased excretion in HF_Cont vs Chow ($p<0.05$) but no significant difference among HFD groups. MUFA consisted mostly in 18:1

(oleic acid, **Table 2**), whose proportion in lecithin groups was not different from both control HFD (HF_Cont and HF_0L, **Table 2**). The faecal amount of free *trans* fatty acids was similar among all groups (**Fig. 5F**, p=0.65), mainly consisting of 18:1 *trans* isomers (**Table 2**). The amount of n-6 PUFA FFA in faeces and the proportion of linoleic acid (18:2 n-6) were not different among groups (**Fig. 5F**, p=0.44, **Table 2**), however, the proportion of conjugated 18:2 (CLA) was blunted by both control HFD but not in lecithin groups (**Table 2**). The amount of n-3 PUFA FFA excreted in faeces was also similar among all groups (**Fig. 5F**, p=0.08) albeit we observed a lower proportion of ALA within faecal FFA of HF_Control vs Chow (**Table 2**, $p<0.01$) that was altogether restored in lecithin groups ($p<0.05$ vs HF_Control).

As previously observed in human faeces [30], PL-bound FA represented a lower amount in faeces (~1 µg FA/g faeces) than FFA (2-3 µg FA/g) (**Fig. 5F-G**). No significant diet impact on total faecal PL content was observed (p=0.089) even if it tended to be higher in HF_20RL vs HF_Cont (**Fig. 5G**, p=0.057). However, more specifically, there was a significant diet impact on the faecal contents of PL-bound MUFA, n-6 PUFA and n-3 PUFA (p=0.011, 0.0064 and 0.0051, respectively, **Fig. 5G**), altogether with higher contents in HF_20RL vs HF_Cont (MUFA: p=0.004; n-6 PUFA: p=0.012; n-3 PUFA: p=0.0046). The essential n-3 PUFA ALA bound to PL was notably higher in faeces of HF_20RL vs both HF_Cont and HF_0L (p=0.0009 and p=0.003). The fatty acid profile of faecal PL (**Table 2**) further shows that fewer were impacted by the diet compared to faecal FFA. Palmitic acid proportion in faecal PL was increased in HF_Cont vs Chow, and was lower in both RL groups than in HF_Cont and HF_0L groups (p=0.0001 to 0.045). Oleic acid proportion in faecal PL was altogether similar among high-fat groups, noting that in HF_20RL it was higher than HF_Cont (p=0.045) but not different from other ALA-rich groups. The proportion of ALA (18:3 n-3) in faecal PL in high-fat groups presented a dose-response pattern from 0.1% to 1.3% according to lecithin content in the diet (**Table 2**) ($p<0.001$ HF_20RL *vs* HF_Cont and HF_0L).

3.6. Faecal loss of polar lipids typical of lecithins are associated with the observed changes in gut microbial species

As gut microbiota can be impacted by lipids and BA, we assessed in the entire mice cohort whether the abundance of microbiota OTUs that were significantly impacted by lecithins (**Fig. 4D**) was correlated with the faecal amount of lipids and BA of interest (FFA: total, 16:0, ALA; PL-bound FA: total, 16:0, ALA; Total, primary and secondary BA) (**Fig. 5H**). The

most significant correlations for most OTUs of interest (8/10) were observed with faecal PL-bound ALA that was positively correlated with Clusters 8, 12 (*Muribaculaceae*, **Fig. 5I**), 25, 159 (*Lachnospiraceae* NK4A136 group and other *Lachnospiraceae*), 52 (*Lactobacillus* group), 62, 103 (*Clostridiales vadinBB60* group) and inversely correlated to Cluster 23 (an OTU belonging to *Blautia* genus). Conversely, as a mirror effect, Clusters 8, 12, 25, 52 and 62 were inversely correlated with the amount of faecal 16:0 FFA, while Cluster 23 was positively correlated with 16:0 FFA (**Fig. 5J**). Almost no correlation was observed between OTUs of interest and faecal ALA in free form, nor faecal 16:0 bound to PL. Some associations were observed with secondary BA but that were less significant than with the faecal lipids of interest (**Fig. 5I**).

4. Discussion

The Western diet is high in sugar, fat, processed red meats, refined grains and processed foods that contain multiple food additives added for preservation, flavor and/or texture enhancement. The increased prevalence of chronic metabolic disorders and the concomitant steady increase in the use of food additives have recently led to a growing interest and need to understand the specific impact of such ingredients on metabolic health [2,3,31–33]. Pioneering work revealed that certain synthetic or semi-synthetic emulsifiers and texture promoters used in processed foods, namely Polysorbate 80 (PS80) and Carboxymethylcellulose (CMC), exert deleterious effects in mice, notably alterations of the gut microbiota and the gut barrier, associated with low-grade inflammation, obesity and metabolic abnormalities [4]. A recent study in mice has also suggested that CMC and PS80 may promote intestinal carcinogenesis [34]. Their detrimental impact on human gut microbiota, by reducing diversity, altering composition and inducing expression of pro-inflammatory molecules, was confirmed *in vitro* [7,35] and more recently in a human dietary intervention using CMC [1]. Natural dietary emulsifiers, such as lecithins, may represent an attractive alternative to synthetic compounds. However, data on their metabolic and intestinal impacts are still scarce, despite the relevance of lecithin in human nutrition. Indeed, 17% of manufactured food products in France contain lecithin [31] and 87% of the population within the NutriNet cohort (n=106,000) was shown to consume lecithins [32]. In the US, exposure appears even higher : it is estimated that 100% of individuals are exposed to lecithins [3]. Interestingly, this ingredient category, rich in phospholipids, can also be an interesting source

of essential n-3 PUFA, if ALA-rich sources such as rapeseed or soy lecithin are used [5]. Here, we demonstrate that, in mice, the incorporation of 10 to 20% of rapeseed lecithin, or 10% of soy lecithin in fat within a semi-synthetic HFD (25% fat), (i) does not alter certain important parameters of adiposity, low-grade inflammation, nor gut barrier markers, both compared to a HFD of similar fatty acid composition and to a more SFA-rich HFD low in ALA, (ii) contributes to improved n-3 PUFA status in the liver and adipose tissue as efficiently as lecithin-free HFD containing ALA and (iii) modifies β-diversity and induces beneficial changes in gut microbiota. Rapeseed lecithin even partially prevents HFD-associated loss of microbial α-diversity.

Although it is difficult to precisely evaluate daily exposure data in the overall population, intakes in the present study are estimated to be consistent with average consumption at nutritional and supplementation doses in human adults [18]. While semi-synthetic emulsifiers can worsen weight gain and metabolic inflammation associated with HFD [1–3], we observed here that rapeseed and soybean lecithins do not induce such alterations when incorporated at both nutritional (10 wt% of lipids, 2.5% in diet) and higher supplementation levels (20 wt% of lipids for RL, 5% in diet). This is of particular relevance, as lecithins have been attributed the GRAS (generally regarded as safe) status, meaning they can be added to most foods with no restriction of amount. Regarding metabolic inflammation, our results appear consistent with recent data on the use of lecithin-based formulation (PC-enriched, delayed release) as anti-inflammatory therapeutic agent for treatment of ulcerative colitis [36]. Soy lecithin was also recently shown to have no effect on the inflammatory functional profile of human gut microbiota *in vitro*, conversely to sunflower lecithin, that is richer in n-6 PUFA [7]. However, the present neutral effect of soy lecithin on adiposity and inflammation remains controversial and may depend notably on the dose, background diet formulation and fatty acid profile. Indeed, in a previous study, we observed that 1.2% of soy lecithin in a palm-oil (~40% palmitic acid)-based or in a flaxseed-oil (~32% ALA)-based semi-synthetic HFD (i.e. 6 wt% lecithin in diet lipids) increased adiposity and metabolic inflammation in C57Bl/6J mice after 8 weeks compared to the respective control HFD [37]. Adipose tissue inflammation enhancement by soybean lecithin was even higher when associated with flaxseed oil than palm oil [37], ie with diet having a n-6/n-3 ratio ~15 in palm groups and ~2 in flaxseed groups, vs a recommended ratio of ~5 in HF_0L and lecithin groups in the present study. Differences with the present results may also be partly due to the different mice model used.

The mucus layer and goblet cells involved in its secretion play an extremely important role in gut physiology and homeostasis, as the mucus layer as a protective barrier between the external and internal environments [38,39]. HFD are reported to alter gut barrier notably by inducing goblet cell depletion [38]. Consistently here, the HFD devoid of lecithin decreased goblet cell mucin in colon, indicating that this effect occurred regardless of the n-6/n-3 ratio in the diet. Synthetic emulsifiers, such as CMC and PS80, have also been shown to deplete the mucus layer, both in mice and humans [1,4]. We found here that RL and SL do not further mitigate this important marker of the gut barrier compared to HFD. This is consistent with a previous study where 1.2% soy lecithin in a palm-based HFD did not modify the number of colonic goblet cells *vs* HFD devoid of lecithin [37]. Moreover, RL and SL did not affect the gene expression of tight-junction proteins otherwise downregulated by SFA-rich HFD in this study and in the literature [40,41].

Adequate tissue status in n-3 PUFA, notably in its PL fraction, is another important feature of metabolic health and contributes to limit inflammation [42]. As expected, ALA-rich diets induced higher proportion of n-3 PUFA in the structural PL of liver and WAT, both central organs of lipid metabolism and homeostasis, including both ALA and its long-chain derivatives EPA and DHA. This is consistent with our previous studies both in the short term in mice (5 day diet) [6] and in rats, where only 3 wt% ALA was sufficient to promote the maximal bioconversion of ALA in DHA in both PL and TAG fractions [43]. As also observed and discussed in the latter, because n-3 and n-6 PUFA share the same enzymes (Δ6, Δ5 desaturases and elongases) for the conversion of their precursors to their long-chain derivatives, it appears consistent that when the proportion of EPA and DHA is increased in livers lipids with ALA-rich diets, the proportion of AA is decreased [43]. Here, we show that for the same 4%-ALA content in the diet, lecithin-enriched oils appear to be as efficient as lecithin-devoid oils to improve n-3 PUFA status in the liver and adipose tissue. This contribution of lecithins to tissue n-3 PUFA status could contribute to their neutral/non-deleterious effect on HFD-induced adiposity and metabolic inflammation in this study.

The emulsifier-induced metabolic perturbations and inflammation described in previous studies has been shown to be mediated by the gut microbiota [1,4]. Recent data assessing 18 commonly used emulsifiers has revealed that soy lecithin appeared as the only rare emulsifier to exert a neutral effect on human gut microbiota *in vitro* in acute conditions (up to 6 days) [7]. The present study corroborates these findings *in vivo*, as we report here that, in high-fat fed mice, soy and rapeseed lecithins do not induce detrimental effects on gut microbiota

composition. On the contrary, they limit the HFD-induced shifts in some microbial populations and limit the loss of diversity brought about by HFD. Although its relevance as a marker of obesity is now debated [44], the ratio of Bacteroidetes/Firmicutes was favorably increased by lecithins. Moreover, lecithins induced specific changes, as several OTUs were differentially impacted by RL and SL, notably those belonging to *Lachnospiraceae* NK4A136, *Ruminococcaceae* UCG-014 group (increased) and *Blautia* genus (decreased). Interestingly, *Lachnospiraceae* NK4A136 has been associated with a favorable metabolic profile and adherence to healthy diet in humans [45,46]. *Ruminococcaceae* UCG-014, a SCFA-producing bacteria, has been found to be depleted in HFD-fed mice [47] and increased after a healthy whole-grain diet in humans [48]. While the overall impact of the genus *Blautia* on health is controversial, it has been repeatedly associated with obesity and fat accumulation [49–51], suggesting that the decrease observed in response to lecithins may be favorable in the context of metabolic disorders. Altogether, the present study in mice reveals that consumption of SL and RL in a HFD for 13 weeks induces limited but rather beneficial shifts in gut microbiota populations.

Bile acids are now recognized as strongly implicated in both lipid digestion/metabolism and host-microbiota interactions [52,53]. Their pool is reported to be increased by HFD [54] as also observed here. Interestingly, the ALA-enriched HFD did not alter bile acid pool and profile compared to chow, and irrespective of their lecithin content. This is consistent with our previous 5 day-study in mice where 0.5% RL in the diet did not modify total caecal BA content [6]. Among the bacteria phyla that were specifically modified by lecithins, a few were correlated with the amount of total and secondary bile acids. However, most of these clusters were much more strongly correlated with some of specific faecal lipids.

Indeed, interestingly, we hereby demonstrate that observed effects of lecithins on the gut microbiota may be attributed to their specific PL-rich molecular composition. In order to grasp a better insight on how lecithin-induced microbiota changes may be related to lipids in the gut, we assessed the FFA and PL fraction in faeces. The amount of FFA in faeces is known to be strongly related to residual unabsorbed fatty acids originating from the diet [10]. This phenomenon occurs mainly for SFA, such as palmitic acid, because of their ability to partly form calcium salts that cannot be absorbed, and the solid state of SFA-rich fats, which somewhat limits their digestion and absorption [55,56]. Consistently, we observe in the present study that HF_Cont diet, higher in SFA and also presenting the highest solid fat content and melting temperature (38°C vs 35-37°C in other HFD), results in the highest faecal

FFA. Moreover, biliary PC is known to be high in palmitic acid both in human and mice [57,58], hence the increased loss of palmitic acid in faeces in the control HFD is consistent with the observed increased amount of total caecal BA and implies that a SFA-rich HFD induces an increase in bile production to allow for increased fat absorption. Interestingly, lecithin groups did not induce increased faecal FFA and their proportion of SFA in faecal FFA was even lower than that of the HF_0L matched control, which could have contributed to observed lecithin-specific changes in gut microbiota.

Importantly, we also gathered data on the fatty acids bound to the PL fraction in faeces, mainly consisting of phospholipids and lyso-phospholipids. Faecal PLs are indeed derived from both host (notably the bile and shed epithelial cells) and microbial cell membranes [10]. They may also include potential dietary PL residues, as lecithin PL digestion results in FFA, lyso-PL and small amounts of potential undigested PL. Here, although total faecal PL were not greatly increased in the HFD groups containing lecithin vs HF_0L, certain faecal PL-bound fatty acids specific of RL and SL, namely PL-bound ALA, increased dose-dependently according to amount of lecithin in the diet. This suggests that specific PL and lyso-PL from lecithins have reached the colon, where they may have contributed to impacts on the gut barrier and gut microbiota as previously described using polar lipids of different composition, i.e. milk sphingomyelin [13,15].

The observed diet impact on free and PL-bound faecal fatty acids appeared to contribute to changes in gut microbiota, notably regarding residual lipids typical of the diet: free 16:0 regarding HFD and ALA-PL regarding lecithin. Indeed, most health-promoting clusters enhanced by lecithins were (i) positively correlated with ALA-PL that increased dose-dependently in faeces according to lecithin amount (but not with free ALA, which was not modulated by lecithin) and (ii) negatively correlated with free palmitic acid that was higher in HFD devoid of lecithin (but not with PL-bound palmitic acid). In turn, cluster 23, a bacterial species belonging to the *Blautia* genus, was positively correlated with free palmitic acid in faeces. This highlights the importance of dietary lipid residues in modulating the gut microbiota in a way that can promote diversity and health-promoting species. This is consistent with a previous study with milk polar lipids, where their typical faecal residues typical were correlated with the differential abundance of *Lactobacillus* spp. and *Lactobacillus reuteri* [15]. Similarly, Davies et al. described that the diet, notably a HFD, may generate a characteristic PL profile in faeces, which may be correlated to particular bacterial groups [59].

Differences in faecal lipids may also arise from the generation of so-called postbiotics obtained from the metabolization of lumen lipids by microbiota microorganisms [60]. In the present study, certain specific fatty acids were present in greater proportion in faecal lipids than in the diet. For example, trans-vaccenic acid (18:1 n-7 *trans*) is a bacterial metabolite that can be produced both from 18:2 n-6 and ALA, notably in bovine rumen, whose dietary intake (notably via milkfat) has recently been associated with health benefits [61,62]. Interestingly, trans-vaccenic acid has also been shown to increase in the caecum and colon of mice after gavage with sunflower oil (63% 18:2 n-6, linoleic acid), suggesting it can be produced endogenously by commensal bacteria from dietary PUFA [60]. Here, even if the total faecal amount was not impacted by the diet, the proportion of 18:1 *trans* FFA was lower in faecal FFA of HF_Control vs Chow, and was altogether restored in lecithin groups (despite similar amounts of TFA in all HFD). Druart et al. (2014) also reported a significant increase of different conjugated linolenic acids (CLA), derived from both linoleic acid and ALA, in mice colon. CLA have been reported to reduce colitis and reverse gut barrier alteration and metabolic impairments in obese mice [63–65]. In this study, faecal free CLA were reduced by both high fat diets, but restored by lecithins. Overall, this suggests that lecithins may be able to restore some microbiota abilities to generate lipid metabolites that may be involved in beneficial effects notably in the intestine. Moreover, such metabolites (22:5 n-6, 18:1 *trans* and CLA) were less observed within the faecal PL, and unaffected by the diet therein. Therefore, the observed correlations between PL-bound ALA typical of lecithins and the favorably modified gut bacterial strains support a direct role of dietary residues of lecithins as potential modulators of gut microbiota diversity and functions.

Altogether, the present study demonstrates that two common lecithins are capable of beneficially affecting the gut microbiota in association with changes in lipid residues in the distal gut. This conclusion may be made in light of the specific design of the study, which used precisely controlled lipid formulations with matched fatty acid profiles and two different control HFD, in order to differentiate the specific impacts of lecithins from that of their ALA content. Moreover, lecithins encompass a wide range of differently sourced ingredients (eg from soy, rapeseed, sunflower, or egg) with different molecular compositions which raises the question of a differential impact [2]: the study design addressed this important issue by testing two different widely used plant sources of lecithins. Nevertheless, it must be taken into consideration that more research is warranted to clearly conclude on the metabolic effects of lecithin on overall health: the observed impacts of lecithins on gut microbiota and metabolism

should now be confirmed using other background diets (ie semi-synthetic vs chow-based), formulations (different fatty acid profiles, fiber content, presence of other food additives), in a sex-dependent manner and must be confirmed in clinical trials.

5. Conclusion

The modern Western diet is rich in ultra-transformed foods, which contain multiple additives, often used simultaneously within one product. Assessing the synergic effects of these ingredients is nearby impossible and further research in humans is required to conclude on their effects on human health. Nevertheless, the results of the current study, alongside those recently described in the literature, offer some reassuring data on the use of lecithins. As natural lipids and a source of plant n-3 PUFA, soy lecithin and rapeseed lecithin seem to contribute to tissue n-3 status but also to beneficially impact both intestinal health and the gut microbiota in mice, which may be due to the bioactive lipid species they engender in the distal gut. As consumer awareness and fondness for natural products is evermore increasing, these preliminary data on lecithins supports perspectives of further research for their use in healthy food formulation based on natural ingredients. Moreover, they support the use of rapeseed lecithin as a potential local sustainable alternative to soy lecithin.

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CrediT authorship contribution statement

CR, CV and M-CM designed the research; CR, AP, LC, DR, EM, SB, HA, EL, MM, PK, SC, AVM, FC, SD, NG, PG, CV and M-CM conducted the research; CR, MM, PG and M-CM analyzed data and performed statistical analyses; HV provided useful scientific insight; NG, CR and AP participated in the *in vivo* experiments; SC and AVM performed histological analyses; EM and EL performed qPCR analysis; CR, LC, EM and EL performed lipid analyses; MM and PG performed faecal microbiota analysis; PK and DR performed bile acid analysis; SD performed fat structure analysis; CR and M-CM wrote the paper; CR, CV and M-CM had primary responsibility for final content. All authors read and approved the final manuscript.

Data availability statement

The data supporting this study's findings are available from the corresponding authors upon reasonable request.

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Declaration of competing interest

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Figure Captions

Figure 1. Study design including experimental semi-synthetic diets. The lecithin-enriched diets contained 10 to 20 wt% of lecithin within the fat fraction, corresponding to 2.5 to 5 wt% of lecithin in the diet.

Figure 2. (A) Mice body weight gain along the 13 weeks of diets. (B) Total WAT mass at the end of dietary intervention. (C-E) relative mRNA expression in EAT of *TNFalpha* (C), *Adgre* (coding for F4/80, D) and *CD11c* (E), (F) Fatty acid profile of EAT PL, (G) Fatty acid profile of liver PL. Values are mean ±SEM (A-B : n=12 ; C-E : n=7-8 ; F-G : n=10-12), data were analyzed by (A) two-way ANOVA, (B-G) one-way ANOVA followed by Tukey test, or Kruskall-Wallis test followed by Dunn's test in case of non-normal distribution of the data or of non-normal distribution of ANOVA residues. Means that do not share a common letter are significantly different ($p<0.05$).

Figure 3. (A) Abundance of mucin in colon by histological analysis. (B) Colon sections stained with alcian blue highlighting mucins (point examples of Chow, HF_Cont and HF_SL groups). (C-D) relative mRNA expression in colon of *Zo-1* (C) and *Occludin* (D). Values are mean ±SEM (n=8), data were analyzed by one-way ANOVA followed by Tukey test. Means that do not share a common letter are significantly different ($p<0.05$).

Figure 4. (A) Jaccard plot of microbiota profile in mice faeces before dietary intervention, after 2 weeks and at the end of dietary intervention. (B) Observed diversity of microbiota in mice faeces at 2 weeks and at the end of dietary intervention. (C) Phylum analysis of microbiota in mice faeces at the end of dietary intervention. (D) Genus analysis of differentially observed OTUs in faeces of HF_10RL vs HF_Cont groups at the end of dietary intervention.

Figure 5. (A) Amount of caecal bile acids at the end of dietary intervention. (B-E) Amount of caecal primary BA (B), secondary BA (C), beta-MCA (D) and Tauro-conjugated BA (E). (F-G) Amount of fatty acids in faeces, either in free form (F) or bound to PL (G). (H) Heat map of correlations between gut microbiota OTUs of interest and faecal lipids and bile acids of interest. (I-J) Examples of significant correlations revealed by the heat map : Cluster 12 (*Muribaculaceae*) with α-linolenic acid (ALA) in PL (ie esterified to phospholipids and lysophospholipids), (I) and Cluster 23 (*Lachnospiraceae Blautia*) with unesterified palmitic acid, 16:0-FFA (J). (A-G) Values are mean ±SEM (A-E : n=9 ; F-G : n=6), data were analyzed by one-way ANOVA followed by Tukey test, or Kruskall-Wallis test followed by Dunn's test in case of non-normal distribution of the data or of non-normal distribution of

ANOVA residues. Means that do not share a common letter are significantly different ($p<0.05$). (H) Significant Spearman correlations are shown: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Tables

Table 1. Main fatty acid and phospholipid profiles of the experimental diets.

	Chow	HF_cont	HF_0L	HF_10RL	HF_20RL	HF_10SL
Fatty acid composition (g/100g of total fatty acids)[a]						
12:0	-	2.2	0.12	0.09	0.1	0.1
14:0	0.4	1.6	0.4	0.4	0.4	0.4
15:0	0.1	<0.05	<0.05	<0.05	<0.05	<0.05
16:0	15.8	32.0	17.6	17.4	17.0	17.7
18:0	2.0	4.7	3.1	3.0	2.9	3.0
18:1 n-9	21.9	32.2	47.3	48.1	48.9	48.0
18:2 n-6	50.0	24.9	23.8	23.6	23.4	23.3
18:3 n-3	4.3	0.2	4.7	4.6	4.6	4.7
20:0	0.3	0.3	0.5	0.5	0.4	0.5
20:5 n-3[b]	0.6	-	-	-	-	-
22:0	0.3	0.1	0.5	0.5	0.4	0.4
22:6 n-3[b]	1.0	-	-	-	-	-
24:0	0.2	0.09	0.2	0.2	0.2	0.2
Σ(SFA)[c]	19.2	41.7	22.5	22.1	21.5	22.4
Σ(MUFA)[c]	24.3	32.6	48.3	49.1	50.0	49.0
Σ(PUFA)[c]	56.5	25.1	28.6	28.1	28.0	28.0
Σ(n-6 PUFA)[c]	50.2	24.9	23.8	23.6	23.4	23.3
Σ(n-3 PUFA)[c]	6.2	0.2	4.7	4.6	4.6	4.7
n-6/n-3 ratio	8.0	113.1	5.0	5.1	5.1	5.0
Σ(TFA)[d]	0.2	0.5	0.6	0.6	0.6	0.5
Phospholipid composition (g/100g of total lipids) [a]						
PC	3.2 [e]	0.08	0.06	2.6	5.3	2.1
PE	0.6 [e]	0.08	0.06	1.6	3.3	1.1
PI	1.0 [e]	0.03	0.02	1.6	3.2	1.4
LPC	1.9 [e]	0.06	0.05	0.2	0.3	0.2
LPE+PS+PAc	1.6 [e]	0.09	0.08	0.4	0.8	0.5
SM	<0.2 [e]	0.1	0.08	0.1	0.1	0.1
Total PL	8.7 [e]	0.4	0.4	6.6	13.4	5.4

a) Data was obtained by gas chromatography coupled to a flame ionization detector (GC-FID) and high-performance thin layer chromatography (HPTLC), respectively.

b) Of note, the lipid formulations were devoid of LC n-3 PUFAs.

c) MUFA=mono-unsaturated fatty acids; PUFA=poly-unsaturated fatty acids; RL=rapeseed lecithin; SFA=saturated fatty acids; SL=soybean lecithin; TFA=trans-fatty acids; PC=phosphatidylcholine; PE=phosphatidylethanolamine;

PI=phosphatidylinositol; SM=sphingomyelin; LPC=Lyso-PC; LPE=Lyso-PE; PS=phosphatidylserine; PAc=phosphatidic acid.

d) among which in HFD fatty acids: 18:1 trans (<0.05-0.08%), 18:2 trans (0.21-0.34%), 18:3 trans (0.08-0.31%).

e) Phospholipid composition of the Chow diet was performed by HPTLC in a previous study (Milard et al., 2019).

Table 2. Free fatty acid and phospholipid-bound fatty acid profile in mice faeces.
Values represent mean ±SEM, n=6 for all groups.

	Chow	HF_Cont	HF_0L	HF_10RL	HF_20RL	HF_10SL	p value
Faecal FFA (wt% of total FA)							
14:0	0.9 ±0.3 [c]	1.3 ±0.2 [b,c]	0.4 ±0.05 [a,c]	0.5 ±0.08 [a,b,c]	0.3 ±0.02 [a]	0.3 ±0.03 [a,c]	***0.0002***
15:0	1.0 ±0.5 [a,b]	0.2 ±0.02 [a]	0.6 ±0.3 [a,b]	0.7 ±0.2 [a,b]	0.8 ±0.1 [b]	0.9 ±0.1 [b]	***0.0158***
16:0	23.2 ±1.3 [b,c,d]	57.2 ±3.7 [e]	30.9 ±3.4 [a]	23.8 ±4.5 [b,c,d]	19.0 ±2.3 [b,d]	26.6 ±2.9 [a,c]	**< 0.0001**
18:0	15.7 ±1.7 [a,b]	13.0 ±1.4 [a]	19.1 ±2.3 [a,b]	17.6 ±0.6 [a,b]	14.7 ±0.7 [a,b]	21.0 ±1.4 [b]	**0.0095**
18:1 trans*	6.7 ±1.4 [d]	0.8 ±0.1 [c]	3.1 ±0.5 [a,c,d]	7.5 ±1.2 [a,b,d]	12.5 ±1.1 [b]	8.9 ±2.0 [b,d]	**< 0.0001**
18:1 n-9	9.6 ±1.4 [c]	13.9 ±1.6 [a,b,c]	16.2 ±4.1 [a,b,c]	20.3 ±2.2 [a]	22.8 ±1.2 [a]	10.4 ±0.8 [b,c]	**0.0008**
18:2 n-6	16.2 ±4.1 [a]	6.2 ±0.8 [a,b]	3.2 ± 0.8 [b]	5.1 ±0.7 [a,b]	6.0 ±0.9 [a,b]	4.4 ±0.5 [a,b]	***0.049***
18:3 n-3	0.9 ±0.2 [b]	0.02 ±0.02 [a]	0.2 ±0.05 [a,b]	0.3 ±0.06 [a,b £]	0.4 ±0.07 [b]	0.2 ±0.02 [a,b]	***0.0043***
Conj. 18:2	1.5 ±0.1 [a]	0.3 ±0.08 [b]	0.5 ±0.06 [b]	1.7 ±0.3 [a]	1.3 ±0.2 [a,b]	1.2 ±0.1 [a,b]	***0.0003***
20:0	4.5 ±1.8 [a,b]	1.6 ±0.3 [a]	5.9 ±1.0 [b]	4.2 ±0.6 [a,b]	3.6 ±0.8 [a,b]	5.3 ±0.8 [a,b]	***0.0266***
20:4 n-6	0.7 ±0.2 [a,b]	0.3 ±0.07 [a]	0.5 ±0.1 [a]	0.9 ±0.2 [a,b]	1.3 ±0.2 [b]	1.0 ±0.2 [a,b]	**0.0019**
22:0	3.1 ±1.2 [a,b]	0.7 ±0.2 [a]	6.1 ±1.1 [b]	4.5 ±0.6 [a,b]	3.7 ±0.4 [a,b]	5.8 ±0.8 [b]	***0.0024***
22:5 n-6	1.8 ±0.5 [a,b]	0.5 ±0.1 [a]	3.6 ±0.7 [b]	3.1 ±0.5 [a,b]	2.6 ±0.1 [a,b]	4.0 ±0.4 [b]	***0.0012***
22:6 n-3	0.9 ±0.3 [a,c]	0.06 ±0.02 [b]	0.3 ±0.03 [b,c]	0.3 ±0.04 [a,b,c]	0.4 ±0.04 [a,b,c]	0.5 ±0.06 [c]	***0.0002***
Total FFA (µg FA/mg feces)	1.7 ±0.4 [a]	8.6 ±2.0 [b]	5.4 ±1.5 [a,b &]	3.2 ±0.3 [a,b]	2.6 ±0.1 [a,b]	3.0 ±0.5 [a,b]	***0.0052***
Faecal PL (wt% of total FA)							
14:0	3.4 ±0.7 [a]	2.6 ±0.2 [a,b]	2.4 ±0.2 [a,b]	2.2 ±0.2 [a,b]	1.2 ±0.1 [b]	1.3 ±0.1 [b]	***0.0014***
15:0	2.4 ±0.4	2.4 ±0.2	3.3 ±0.6	3.4 ±0.5	2.5 ±0.2	2.8 ±0.3	0.33
16:0	27 ±1.0 [d]	32.9 ±1.9 [a]	30.1 ±1.1 [a,d]	24.2 ±1.5 [b,c,d]	20.9 ±0.9 [b]	29.1 ±0.7 [a,c,d]	**< 0.0001**
18:0	12.4 ±2.8	13.8 ±0.6	10.5 ±1.2	11.6 ±1.3	9.2 ±0.3	11.3 ±0.6	*0.0787*
18:1 trans*	3.0 ±0.8 [a]	0.9 ±0.2 [b]	1.5 ±0.3 [a,b]	1.6 ±0.3 [a,b]	1.1 ±0.07 [b]	1.4 ±0.1 [a,b]	***0.0085***
18:1 n-9	7.5 ±0.9 [c]	10.3 ±0.2 [b,c]	14.6 ±0.9 [a,b]	19.4 ±3.5 [a,b]	25.1 ±1.5 [a]	12.3 ±0.4 [a,b,c]	***< 0.0001***
18:2 n-6	11.0 ±2.7 [a,b]	5.1 ±0.6 [a,b]	3.8 ±0.6 [a]	7.0 ±1.3 [a,b]	11.7 ±0.9 [b]	10.9 ±0.8 [b]	***0.0017***
18:3 n-3	0.6 ±0.2 [a,b]	0.08 ±0.02 [a]	0.1 ±0.03 [a]	0.6 ±0.2 [a,b]	1.3 ±0.1 [b]	0.7 ±0.07 [a,b]	***0.0001***
Conj. 18:2	1.5 ±0.3	1.9 ±0.2	2.0 ±0.2	1.4 ±0.2	1.6 ±0.2	1.5 ±0.04	*0.178*
20:0	1.6 ±1.2	0.9 ±0.1	1.1 ±0.2	0.8 ±0.05	0.9 ±0.06	1.0 ±0.1	*0.136*
20:4 n-6	0.9 ±0.1 [a]	2.8 ±0.4 [b]	1.7 ±0.3 [a]	1.2 ±0.2 [a]	1.5 ±0.08 [a]	1.6 ±0.2 [a]	**0.0002**
22:0	1.4 ±0.9 [a]	1.7 ±0.3 [a,b]	1.5 ±0.3 [a,b]	1.1 ±0.1 [a,b]	1.0 ±0.04 [a,b]	1.7 ±0.1 [b]	***0.0206***
22:5 n-6	1.0 ±0.5	1.1 ±0.2	1.0 ±0.2	0.7 ±0.08	0.9 ±0.1	1.3 ±0.1 [#]	*0.0724*
22:6 n-3	2.0 ±0.4 [a]	0.3 ±0.08 [b]	1.1 ±0.6 [a,b]	2.0 ±0.8 [a,b]	1.6 ±0.2 [a]	1.0 ±0.1 [a,b]	***0.0067***
Total PL (µg FA/mg feces)	0.9 ±0.1	0.4 ±0.07	0.8 ±0.2	1.0 ±0.2	1.1 ±0.1 [$]	0.9 ±0.2	0.089

P values in regular font were obtained by ANOVA; p value in italic font were obtained by Kruskall-Wallis.
[a,b,c,d] Means that do not share a common superscript letter are significantly different ($p<0.05$). [£]$p=0.058$ HF_10RL vs HF_Cont. [&]$p=0.056$ HF_0L vs Chow. [#]$p=0.053$ HF_10SL *vs* Chow. [$]$p=0.057$ HF_20RL *vs* HF_Cont.
FFA = free fatty acid; PL = phospholipid; Conj. = conjugated.
* 18:1 Δ9-trans, 18:1 Δ11-trans, 18:1 Δ12-trans, and 18:1 Δ15-trans isomers.

Figure 1.

Male SWISS mice
n=12/group

3 wt% lipids

Chow control (4% ALA)

Semi-synthetic, 25 wt% lipids

1st HF control ALA-poor, SFA-rich (HF_Cont)

2nd HF control enriched in ALA, without lecithin (0% lecithin) (HF_0L)

HF enriched in ALA including 10 wt% rapeseed lecithin within fat (HF_10RL)

HF enriched in ALA including 20 wt% rapeseed lecithin within fat (HF_20RL)

HF enriched in ALA including 10 wt% soy lecithin within fat (HF_10SL)

0

13

Weeks

Similar balanced FA profile (~4.7% ALA)

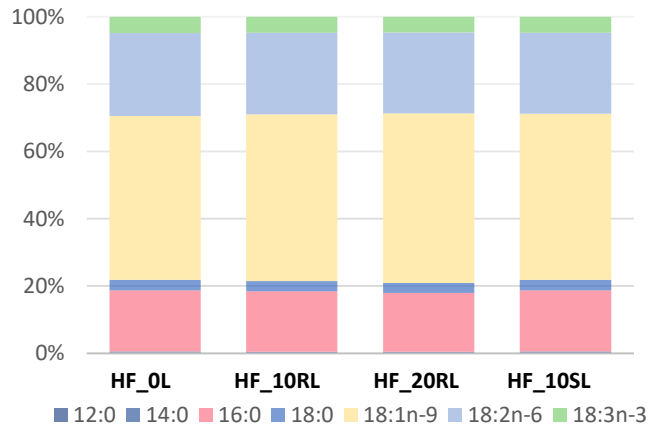
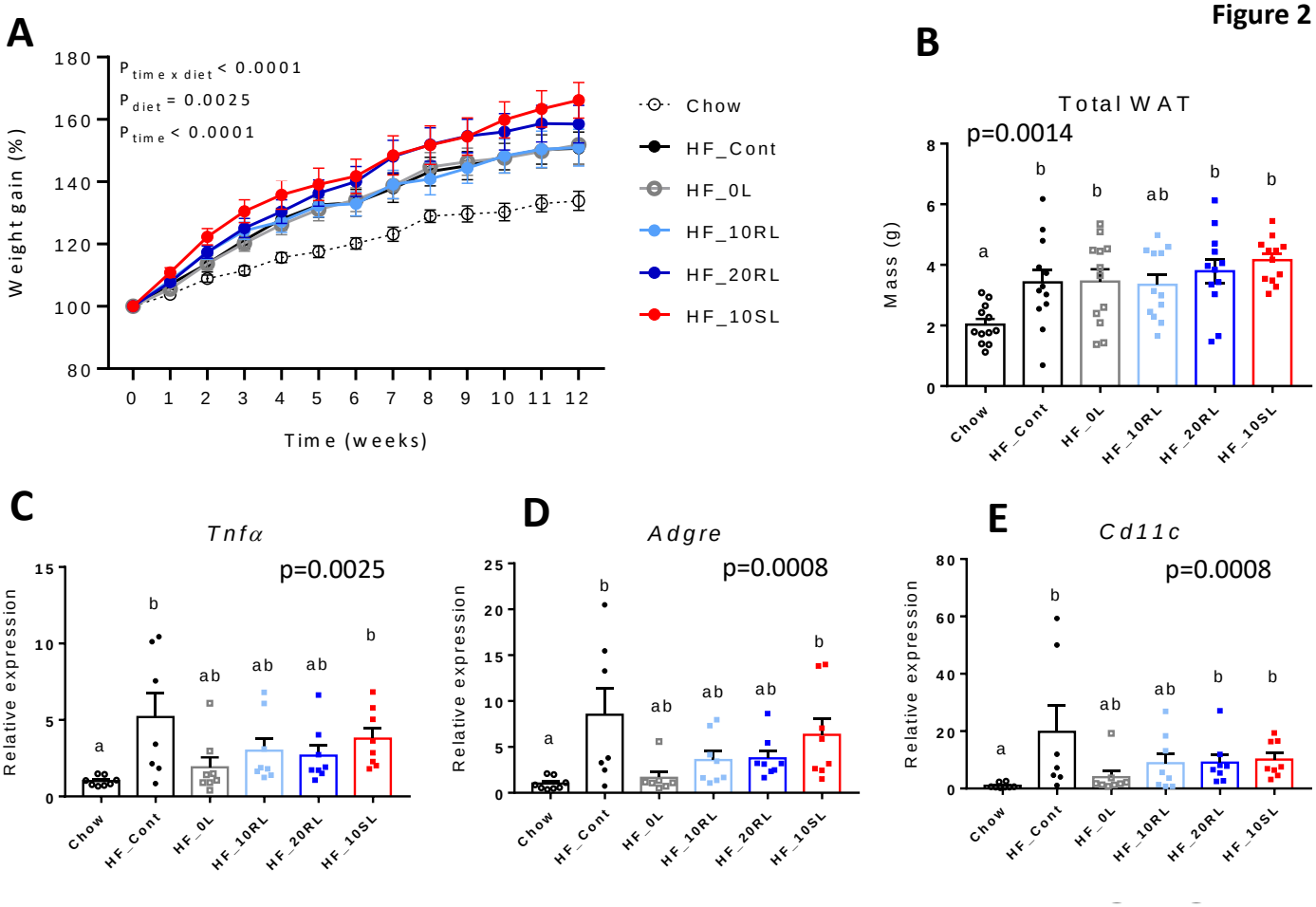
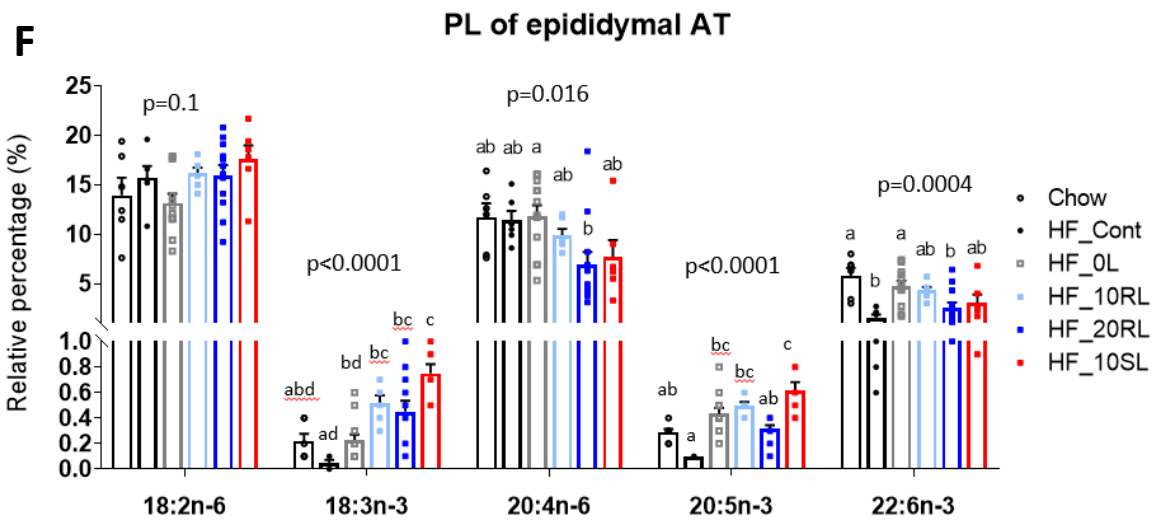
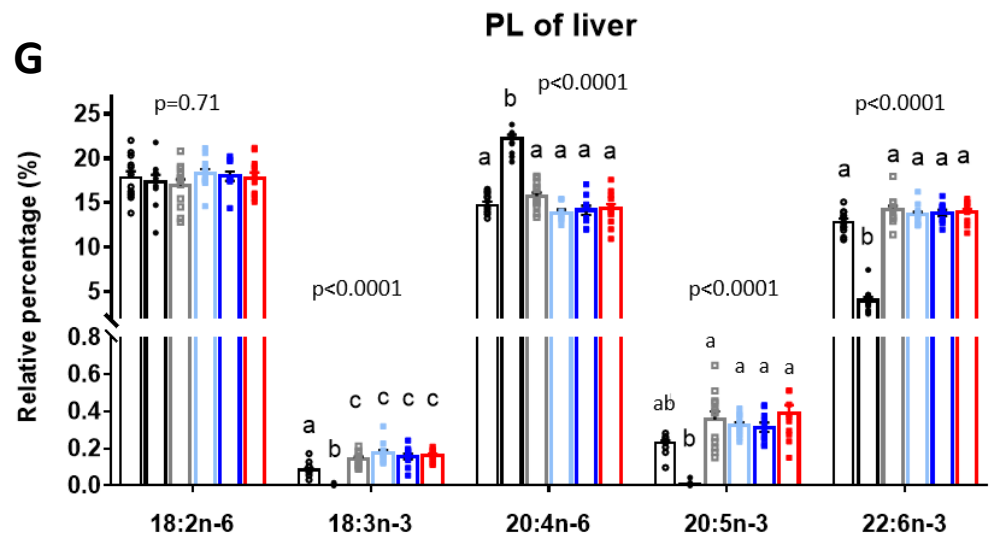
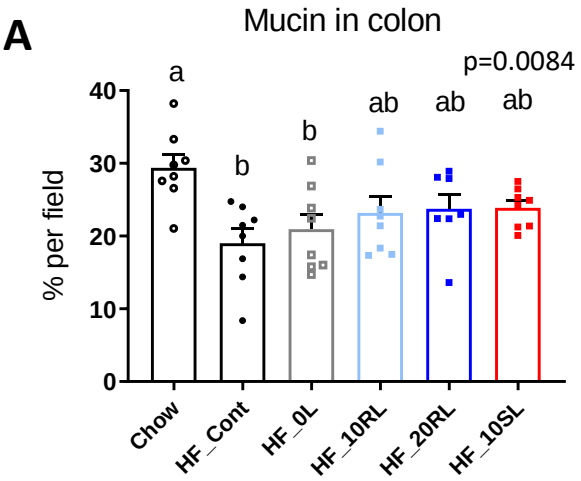


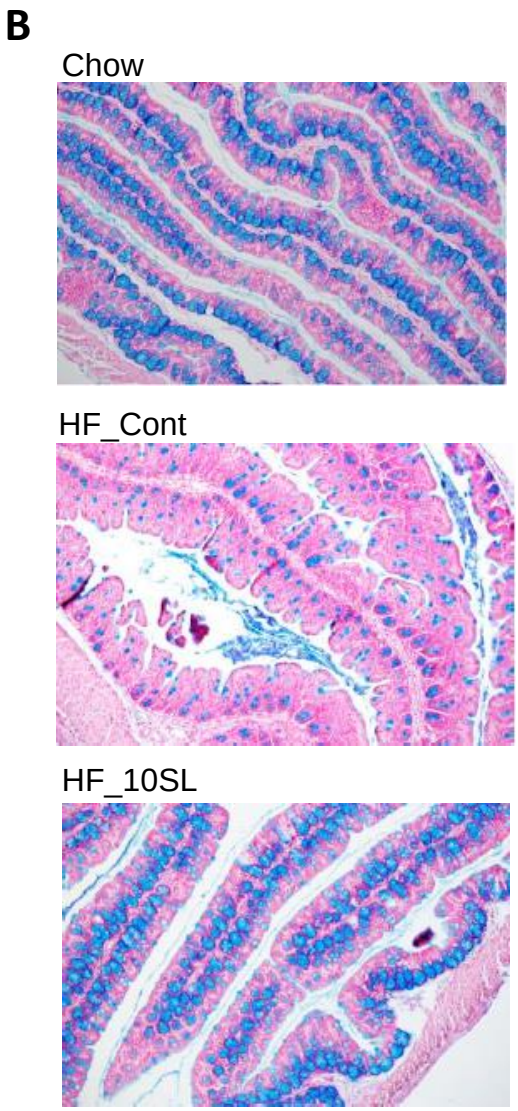
Figure 2

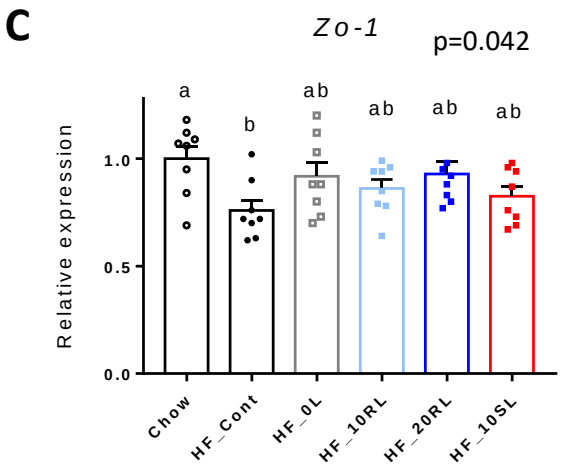

A
$P_{time\ x\ diet}$ < 0.0001
P_{diet} = 0.0025
P_{time} < 0.0001
Weight gain (%)
Time (weeks)
Chow
HF_Cont
HF_0L
HF_10RL
HF_20RL
HF_10SL
B
Total WAT
p=0.0014
Mass (g)
C
Tnfα
p=0.0025
Relative expression
D
Adgre
p=0.0008
Relative expression
E
Cd11c
p=0.0008
Relative expression

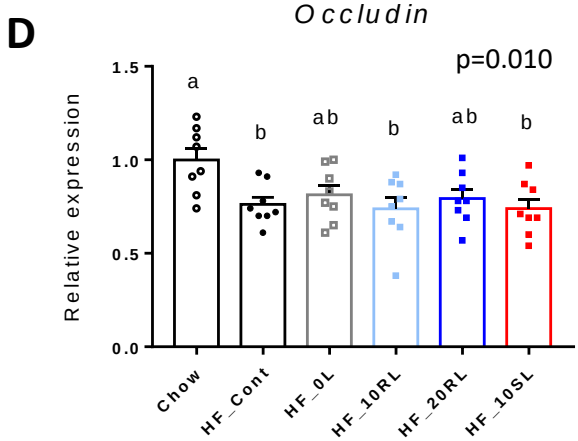

F
PL of epididymal AT
Relative percentage (%)
p=0.1
p<0.0001
p=0.016
p<0.0001
p=0.0004
18:2n-6
18:3n-3
20:4n-6
20:5n-3
22:6n-3
Chow
HF_Cont
HF_0L
HF_10RL
HF_20RL
HF_10SL

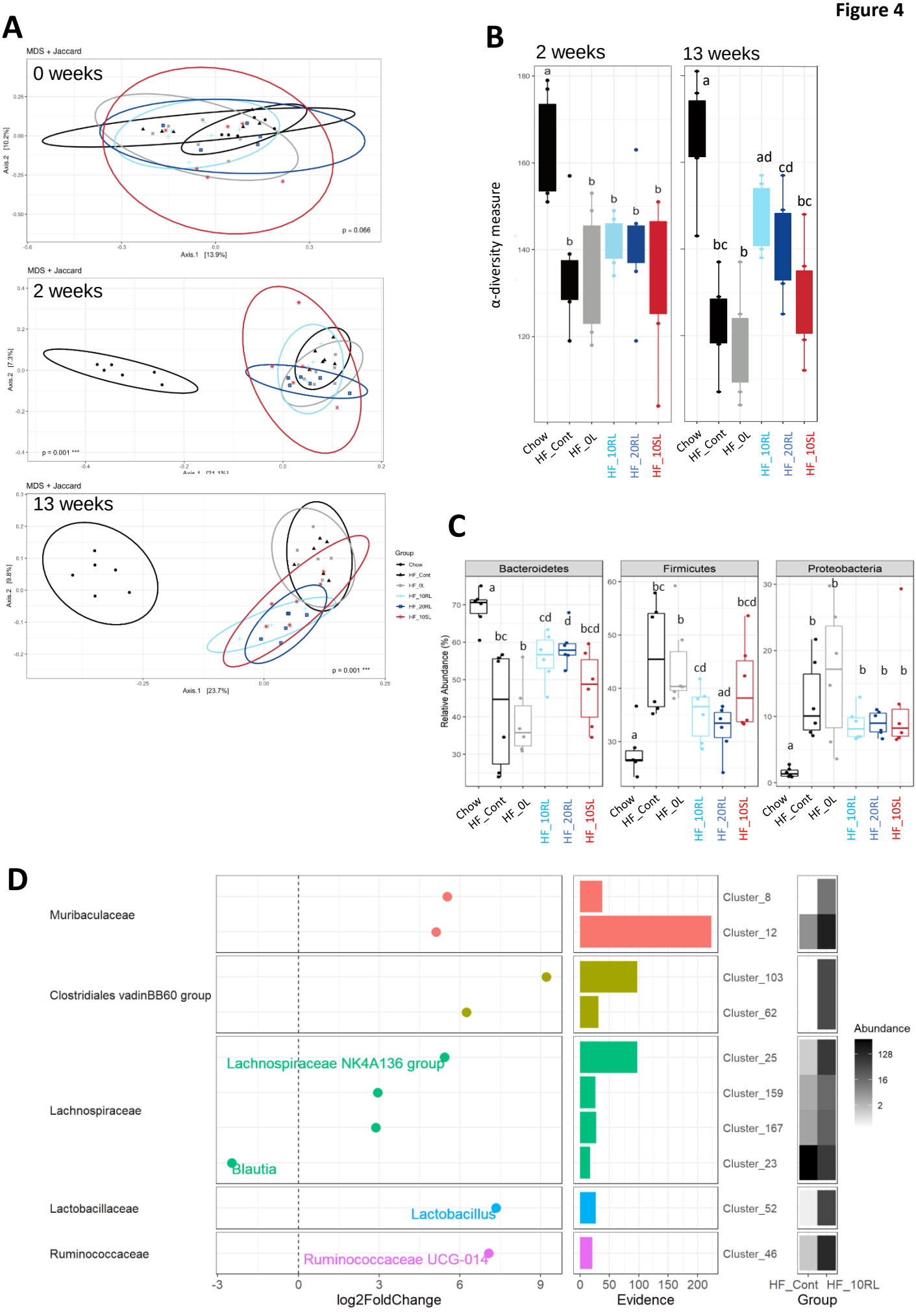

G
PL of liver
Relative percentage (%)
p=0.71
p<0.0001
p<0.0001
p<0.0001
p<0.0001
18:2n-6
18:3n-3
20:4n-6
20:5n-3
22:6n-3

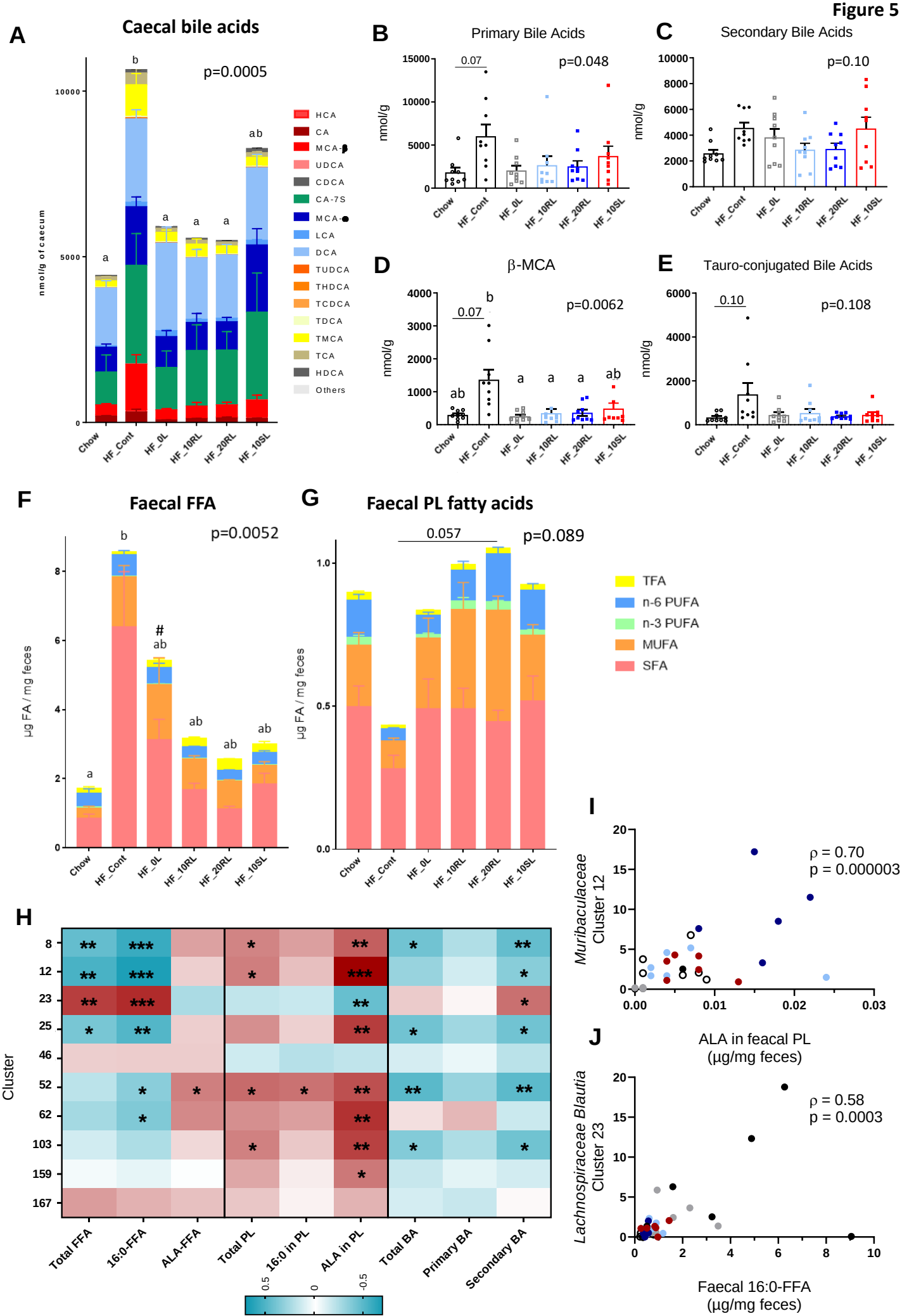

A
Mucin in colon
p=0.0084
% per field
40
30
20
10
0
a
b
b
ab
ab
ab
Chow
HF_Cont
HF_0L
HF_10RL
HF_20RL
HF_10SL


B
Chow
HF_Cont
HF_10SL


C
Zo-1
p=0.042
Relative expression
1.0
0.5
0.0
a
b
ab
ab
ab
ab
Chow
HF_Cont
HF_0L
HF_10RL
HF_20RL
HF_10SL


D
Occludin
p=0.010
Relative expression
1.5
1.0
0.5
0.0
a
b
ab
b
ab
b
Chow
HF_Cont
HF_0L
HF_10RL
HF_20RL
HF_10SL

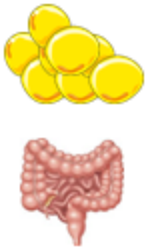

A
MDS + Jaccard
0 weeks
p = 0.066
Axis.1 [13.9%]
Axis.2 [10.2%]
MDS + Jaccard
2 weeks
p = 0.001 ***
Axis.2 [7.3%]
MDS + Jaccard
13 weeks
p = 0.001 ***
Axis.1 [23.7%]
Axis.2 [9.8%]
Group
Chow
HF_Cont
HF_0L
HF_10RL
HF_20RL
HF_10SL
B
2 weeks
13 weeks
α-diversity measure
Chow
HF_Cont
HF_0L
HF_10RL
HF_20RL
HF_10SL
C
Bacteroidetes
Firmicutes
Proteobacteria
Relative Abundance (%)
D
Muribaculaceae
Clostridiales vadinBB60 group
Lachnospiraceae
Lactobacillaceae
Ruminococcaceae
Lachnospiraceae NK4A136 group
Blautia
Lactobacillus
Ruminococcaceae UCG-014
Cluster_8
Cluster_12
Cluster_103
Cluster_62
Cluster_25
Cluster_159
Cluster_167
Cluster_23
Cluster_52
Cluster_46
log2FoldChange
Evidence
Abundance
128
16
2
HF_Cont
HF_10RL
Group


A
Caecal bile acids
p=0.0005
nmol/g of caecum
HCA
CA
MCA-β
UDCA
CDCA
CA-7S
MCA-ω
LCA
DCA
TUDCA
THDCA
TCDCA
TDCA
TMCA
TCA
HDCA
Others
Chow
HF_Cont
HF_0L
HF_10RL
HF_20RL
HF_10SL
B
Primary Bile Acids
p=0.048
0.07
nmol/g
C
Secondary Bile Acids
p=0.10
D
β-MCA
p=0.0062
0.07
E
Tauro-conjugated Bile Acids
p=0.108
0.10
F
Faecal FFA
p=0.0052
µg FA / mg feces
G
Faecal PL fatty acids
p=0.089
0.057
TFA
n-6 PUFA
n-3 PUFA
MUFA
SFA
H
Cluster
Total FFA
16:0-FFA
ALA-FFA
Total PL
16:0 in PL
ALA in PL
Total BA
Primary BA
Secondary BA
I
Muribaculaceae
Cluster 12
ρ = 0.70
p = 0.000003
J
ALA in feacal PL
(µg/mg feces)
Lachnospiraceae Blautia
Cluster 23
ρ = 0.58
p = 0.0003
Faecal 16:0-FFA
(µg/mg feces)

ALA-rich lecithins in high-fat diets

Lecithin in oils vs. oils only

13 weeks



Neutral effect on adiposity, gut barrier and metabolic inflammation

Contribute to tissue omega 3

↗ Microbiota diversity in link with faecal polar lipid residues