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Original article

Impact of gut microbiota on plasma oxylipins profile under healthy and obesogenic conditions

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SUMMARY

Background & aims: Oxylipins (OXLs) are bioactive lipid metabolites derived from polyunsaturated fatty acids (PUFAs) which act as signaling molecules and are involved in inflammatory processes such as those that occur in obesity. On the other hand, gut microbiota plays an essential role in regulating inflammatory responses. However, little is known about the potential impact of gut bacteria on OXLs metabolism. Thus, the objective of this study was to investigate the effect of gut microbiota dysbiosis on plasma oxylipins profile in healthy and diet-induced obese animals.

Methods: Eight-week-old male Wistar rats were fed with either a standard or cafeteria diet (CAF) for 5 weeks and administered an antibiotic cocktail (ABX) in the drinking water (Ampicillin: 1 g/ml, Vancomycin: 0.5 g/ml, Imipenem: 0.25 g/ml) for the last 2 weeks in order to induce gut microbiota dysbiosis. Metabolomics analysis of OXLs in plasma was performed by HPLC–MS analysis. No antibiotic treated animals were included as controls.

Results: Plasma OXLs profile was significantly altered due to both CAF feeding and ABX administration. ABX effect was more pronounced under obesogenic conditions. Several significant correlations between different bacteria taxa and these lipid mediators were observed. Among these, the positive correlation of Proteobacteria with LTB4, a proinflammatory OXL involved in obesity-related disorders, was especially remarkable.

Conclusions: Gut microbiota plays a key role in regulating these lipid metabolites and, therefore, affecting oxylipins-mediated inflammatory processes. These results are the first evidence to our knowledge of gut microbiota impact on OXLs metabolism. Moreover, this can set the basis for developing new obesity markers based on OXLs and gut microbiota profiles.

1. Introduction

Oxylipins (OXLs) are bioactive lipid mediators, mainly generated by the oxidation of PUFAs by lipoxygenases (LOXs), which can lead to the activation of inflammatory response as well as to the resolution and prevention of acute [1] and chronic inflammatory processes [2,3] respectively. The initial phase of inflammation is characterized by the production of arachidonic acid (ARA)-derived lipid mediators also called eicosanoids, which have been shown to be involved in several physiological and pathological processes [4]. These ARA-derived OXLs can be both proinflammatory, such as prostaglandins, leukotrienes and thromboxanes, and anti-inflammatory such as lipoxins. When a certain level of proinflammatory OXLs is reached, lipoxins are synthetized from ARA by the lipoxigenase-15 (LOX-15), cyclooxygenase 2 and cytochrome P450, mediating the resolution of inflammation [5]. Moreover, different anti-inflammatory OXLs can be synthetized from dietary eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) [6]. The resolution of inflammatory response involves both the decrease of proinflammatory mediators (anti-inflammatory

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activity) and the removal of immune cells from the inflamed site (pro-resolving activity) [7–9]. In this pro-resolving environment, OXLs are subsequently transformed giving rise to a great variety of oxylipin metabolites such as lipoxins, resolvins, protectins and maresins. These lipid mediators have pivotal biological functions promoting the resolution of inflammatory processes and homeostasis [10]. In autoimmune diseases, low-grade inflammatory diseases, or recurrent inflammatory diseases, the inflammation is maintained over time. This non-resolving inflammatory environment leads to chronic inflammation, which is linked to the pathogenesis and progression of several prevalent disorders such as inflammatory bowel disease, arthritis or Alzheimer's disease among others [11–13] and can worsen obesity-associated conditions.

Obesity is of particular interest as it is a multifactorial disease with a major negative impact on human health that has reached epidemic proportions worldwide [14,15]. Obesity and obesity-related chronic diseases, such as type 2 diabetes, hypertension, cardiovascular disease and certain types of cancer, are linked to low-grade chronic inflammation [16,17]. This inflammation is influenced by the activation of the innate immune system in tissues involved in metabolic processes such as adipose tissue, liver, pancreas and muscle [18]. This inflammatory response leads to increased production and release of proinflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and resistin [19–21]. Evidence suggests that the excess of nutrients consumption and obesity are associated with elevated levels of free fatty acids, which can induce both insulin resistance in peripheral tissues and activation of innate immunity [21,22]. In this regard, the current Western pattern diet consumed in modern societies, characterized by high fat and carbohydrates contents, significantly contributes to the development of obesity and obesity-mediated inflammation due to its high energy density [17].

On the other hand, the gut microbiota has been shown to play a crucial role in the pathophysiology of both obesity and related metabolic disorders as well as in inflammatory responses, and is becoming increasingly recognized as a key regulator of host physiology and metabolism [14,23,24]. These microorganisms protect from pathogen infection, produce bioactive metabolites with anti-inflammatory function, such as short chain fatty acids (SCFAs), and shape the development of the immune system [25]. Gut microbiota dysbiosis can lead to dysregulation of the functions listed above triggering inflammatory processes [26]. Thus, gut bacteria may contribute to proinflammatory signaling via toll-like receptors (TLRs) and Nod-like receptors (NLRs) activation, which recognize several broadly conserved microbial components allowing the innate immune system to sense a wide variety of microorganisms. Moreover, under metabolic syndrome conditions, the loss of epithelial barrier leads to increased translocation of bacterial products such as lipopolysaccharide (LPS), which binds to TLR4 leading to the release of cytokines that promote insulin resistance and low-grade inflammation [27]. Moreover, gut bacteria are involved in PUFAs metabolism [28]. Thus, supplementation with 10-hydroxy-cis-12-octadecenoic acid, an initial linoleic acid-related gut-microbial metabolite, attenuated high-fat diet (HFD)-induced obesity in mice by improving metabolic condition via free fatty acid receptors. This illustrates the interplay between gut microbiota and host energy metabolism via the metabolites of dietary omega-6-FAs and sheds light on the prevention and treatment of metabolic disorders by targeting gut microbial metabolites. However, little is known about the link between gut microbiota and PUFA-derived OXLs metabolites and its implication in the pathophysiology of obesity.

In this study we investigated how alterations in gut microbiota composition induced by a broad-spectrum activity antibiotic cocktail administration may impact on the plasmatic oxylipins profile in Wistar rats under healthy and obesogenic conditions induced by a cafeteria diet (CAF). This diet is a hypercaloric diet, highly palatable, that induces hyperphagia and obesity and has widely been used in the literature. Thus, CAF-fed rats are a robust animal model for simulating diet-induced human obesity associated with muscle, adipose tissue and liver inflammation that leads to the development of metabolic syndrome, including hypertriglyceridemia, hyperglycemia, hypertension and insulin resistance [29,30]. Our findings show, for the first time to our knowledge, that gut microbiota dysbiosis significantly alter plasmatic OXLs levels in healthy and obesity conditions induced by CAF, suggesting a key role for gut bacteria in the metabolism of these inflammatory lipid mediators.

2. Materials and methods

2.1. Animal procedures

Twenty-four 8-weeks-old male Wistar rats (Charles River Laboratories, Barcelona, Spain) were housed by pairs under standard laboratory conditions (temperature 22 °C, 12 h light/dark cycle) with *ad libitum* access to food and drinking water. After one week of acclimatization period, rats were weighted and randomly divided into four groups ($n = 6$) depending on the treatments administered for 5 weeks: (1) a standard chow diet (STD) (kcal/100 g: 72.4% carbohydrate, 8.4% lipid, and 19.3% protein; Safe-A04c, Germany), (2) a STD and an antibiotics cocktail (ABX) administered in drinking water for the last two weeks (weeks 3–5), (3) a CAF composed of highly palatable and energy-dense human foods (kcal/100 g: 58.2% carbohydrate, 31.1% lipid, and 10.7% protein) and (4) a CAF and an ABX administered in drinking water for the last two weeks (weeks 3–5) (Fig. 1). As mentioned above, CAF is a widely used model for diet-induced obesity in rats and allows for the development of metabolic syndrome. Each component of the diet was freshly prepared every day accordingly to previous studies [30–32]. The ABX consisted of 1 g/l ampicillin, 0.5 g/l vancomycin and 0.25 g/l imipenem (Discovery fine chemicals, UK) and was freshly prepared every day. This mixture of antibiotics has a broad-spectrum activity, acting against Gram-positive and Gram-negative bacteria, and is known to have an antimicrobial effect in the rat intestinal microbiome [33]. CAF diet included the following (grams per rat): biscuits with pâté and cheese (14–15 g), bacon (5–7 g), *ensaimada* (pastry) (6–8 g), carrot (6–8 g), standard chow (10–12 g) and milk containing 22% sucrose (w/v). Body weight and food intake were recorded weekly during the whole experimental procedure. At the end of the study both blood and fecal samples were collected for microbiota and oxylipins analysis, respectively. Blood samples were collected from the saphenous vein using capillary action blood collection EDTA-tubes and plasma samples were obtained by centrifugation (5000 *g*, 5 min, 4 °C). Fecal samples were freshly collected and immediately frozen in liquid nitrogen. All the samples were stored at −80 °C until further analyses.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) and the Generalitat de Catalunya approved all the procedures (Code 9495) and in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2. Metabolomics analysis

A volume of 100 μL of plasma was mixed with 100 μL of internal standard prepared in methanol and incubated for 30 min at −20 °C after adding 750 μL of methanol. Then, the samples were centrifugated and supernatants were diluted with 6.5 mL of 0.1% formic acid aqueous solution. Thereupon, a cleanup was applied using

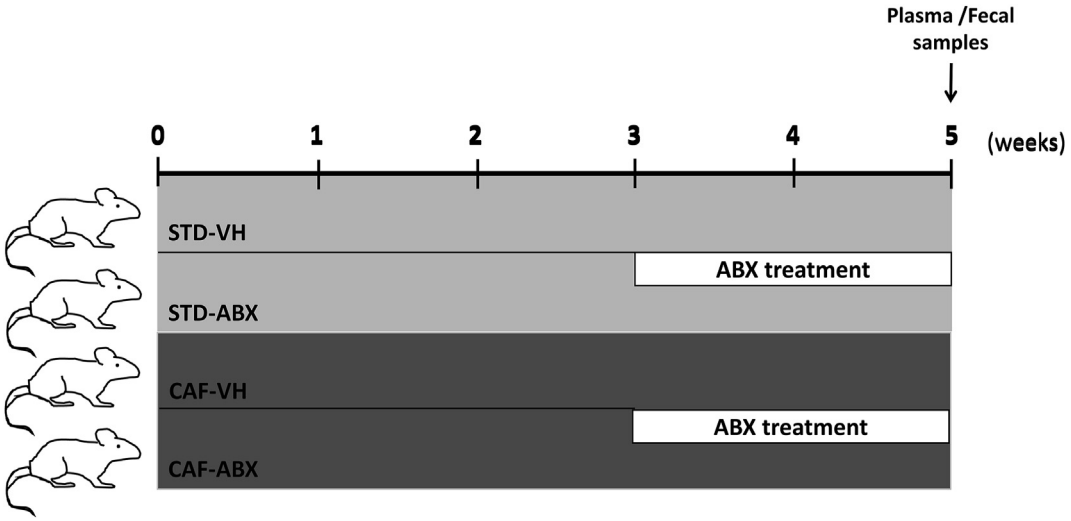

Fig. 1. Experimental model. Rats were randomly divided into four groups ($n = 6$) depending on the treatments administered for 5 weeks: (1) rats fed a standard chow diet and vehicle (STD-VH), (2) rats fed a standard chow diet and antibiotics cocktail (ABX) administered in drinking water for the last two weeks (weeks 3–5) (STD-ABX), (3) rats fed a cafeteria diet and vehicle (CAF-VH) and (4) rats fed a CAF and an ABX administered in drinking water for the last two weeks (weeks 3–5) (CAF-ABX). Plasma and fecal samples were collected at week 5.

OASIS HLB eluting with 600 μL of acetonitrile:methanol (9:1 v/v) twice. The elute was evaporated to dryness in nitrogen and reconstituted in 100 μL of Milli-Q water:methanol (1:1 v/v). All extraction processes were carried out in a dark room.

Plasma OXLs levels were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an UHPLC 1290 Series coupled to a QqQ/MS 6490 series instrument (Agilent Technologies, Santa Clara, California, USA). Separation of oxylipins was carried out onto an analytical column Eclipse XDB C18 1.8 um, 2.1 × 10 mm from Agilent Technologies. The chromatographic separation was performed using an acetonitrile:methanol (85:15, v/v) gradient in 0.01% acetic acid aqueous solution (90% acetonitrile–methanol for 4 min, 65% acetonitrile-methanol from 4 to 6 min, 60% acetonitrile–methanol from 6 to 8 min, 58% acetonitrile–methanol from 8 to 10 min, 50% acetonitrile–methanol from 10 to 16 min, 35% acetonitrile–methanol from 16 to 19 min, 25% acetonitrile–methanol from 19 to 21 min, 15% acetonitrile–methanol from 21 to 22, 5% acetonitrile–methanol from 22 to 25 min and finally back to 90% acetonitrile–methanol from 25 to 30 min for column equilibration). The flow rate was 0.4 mL/min, the column temperature was 45 °C and the injection volume was 10 μL at 4 °C. The electrospray source ionization for MS detection was in negative mode, and the capillary voltage was set to 2500 V. The gas source and desolvation temperatures were set at 150 °C and 300 °C, respectively.

2.3. Fecal microbiota analysis

Fecal DNA was isolated using a QiAamp Fast DNA Stool mini kit (Qiagen Inc., Hilden, Germany) and kept at −20 °C until further analysis. Isolated DNA was quantified using a NanoDrop ND2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and used for 16S ribosomal RNA sequencing by Ion S5 system (Life Technologies, California, USA) according to the manufacturer's instructions [34]. Briefly, the primer pairs 341F-532R (5′-CCTACGGGRSGCAGCAG-3′; 5′-ATTACCGCGGCTGCT-3′) and 15F-806R (5′-GTGCCAGCMGCCGCGGTAA-3′; 5′-GGACTACHVGGGTWTCTAAT-3′), were used to PCR-amplified the 16S rRNA variable regions V3 and V4 respectively and libraries prepared as previously described [34]. Specific Ion Torrent compatible adapters were ligated onto the 5′ of each primer. Moreover, a barcode sequence of ten bases was also added to allow for sequencing multiple samples simultaneously. Final barcoded amplicons were visualized using 2% agarose gel electrophoresis and further DNA band purification was performed with NucleoSpin (Macherey-Nagel, Berlin, Germany). The Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and the associated Agilent DNA 7500 Reagent kit (Agilent Technologies) were used to determine the quality, length, and concentration of the libraries needed for the sequencing procedure. Once individual libraries were created, they were diluted to 30 pM and pooled. The Ion 520 & Ion 530 Kit-Chef (Life Technologies, California, USA) was employed for template preparation and sequencing according to the manufacturer's instructions. Prepared samples were loaded on a 530 chip (Ion 530TM Chip Kit − 4 Reactions) and sequenced using the Ion S5 system (Life Technologies, California, USA). After sequencing, the reads were filtered by PGM software to remove low-quality and polyclonal sequences, producing a total of 6.615.201 reads. Filtered sequences were subsequently analyzed by QIIME and GreenGenes database. An OTU (operational taxonomic unit) table in BIOM format, which represents the taxa abundance profiles, was obtained and used in the subsequent analysis. The analysis included OTUs clustering, alpha-diversity analysis, and beta-diversity analysis.

2.4. Statistical analysis

Data were plotted using Graphpad Prism 8.0 software (Graphpad software Inc, San Diego, CA, USA) showing mean ± standard error of mean (SEM) of each group for body weight gain graph. Statistical analysis was performed using Statgraphics Centurion 18 (The Plains, VA, USA). For the body weight gain and glucose tolerance test data, normality as well as homogeneity of variance were tested by Shapiro–Wilk test and Levene test respectively, and differences between groups were assessed by one-way ANOVA with repeated measures followed by LSD post hoc test at each individual time point. Principal component analysis (PCA) and Heat map involving plasma OXLs levels and fecal microbiota relative abundance data were analyzed and plotted using MetaboAnalyst v.4.0 (McGuill University, Montreal, Canada) and MicrobiomeAnalyst [35] respectively. Comparisons between groups were performed by Kruskal–Wallis test followed by Dunn's multiple comparison and Bonferroni adjustment of p values. When considering only two groups data were analyzed by Mann–Whitney U test. For the gut

microbiota analysis, the data were filtered to remove those features with very low counts across samples were excluded, establishing 2 as the minimum count with a prevalence in samples of 10%, meaning that a feature could not be maintained unless there were 2 count in 10% of the samples. The number of features remaining after the data filtering step was 7779. Alpha diversity was calculated through Chao1 index, and the mean alpha diversity for each treatment were compared using Kruskal–Wallis test. The β-diversity was measured calculating the Bray–Curtis distances and permutational multivariate analysis of variance (PERMANOVA) was performed to assess the dissimilarity of fecal microbiota composition. The correlation analysis between fecal microbiota and oxylipins composition based on the Spearman's rank correlation method was carried out using Python script (developed by authors). Data and rho indexes were plotted using Seaborn (v.0.10.1), Pandas (v.1.0.3) and Matplotlib (v.3.2.1) libraries. The regression analysis was based on the Lowess method (Locally weighted linear regression) for non-parametric data. The script was developed using PyCharm software (v.2018.2.4, JetBrains s.r.o., Prague, Czech Republic) and Python version 3.7.7. Multiple hypothesis correction for p values was performed using the Benjamini–Hochberg method of False Discovery Rate (FDR) control. Statistical significances are subsequently depicted as follows: *indicating $p \leq 0.05$, **indicating $p \leq 0.01$ or ***indicating $p \leq 0.001$. The statistical test used for individual analysis is provided in the figure legends.

3. Results

3.1. Fecal microbiota is altered by cafeteria diet and antibiotics treatment

To elucidate the role of gut microbiota on OXLs metabolism, the plasma profile of these metabolites was analyzed under dysbiosis conditions induced by antibiotic cocktail administration in both healthy and obesogenic contexts.

Firstly, rats were fed a CAF for 5 weeks to induce obesity. Significant higher body weight gain and corresponding area under curve (AUC) were observed in CAF groups compared to STD groups, independently of ABX administration (Fig. S1a and b). Moreover, an oral glucose tolerance test (OGTT) was carried out in the last week of the experiment. CAF-fed rats showed significant higher blood glucose levels after 15 min of glucose administration and then decreased reaching similar levels to those observed in STD-fed rats (Fig. S1c). Interestingly, ABX administration led to a slower glucose levels decrease in CAF-fed rats showing significant increased blood glucose levels up to 60 min after glucose administration (Fig. S1c). Moreover, ABX administration significantly decreased the AUC derived from the OGTT in STD-fed rats (Fig. S1d). These findings collectively confirmed that the administration of this high-fat high-sugar diet for 5 weeks was enough to induce both obesity and glucose intolerance, and that gut microbiota dysbiosis induced by ABX administration altered glucose tolerance response in these animals.

ABX treatment significantly reduced gut microbiota diversity in both STD- and CAF-fed rats, as revealed by Chao1 alpha-diversity index, while CAF feeding did not significantly affect this diversity (Fig. 2a).

Moreover, overall changes in gut microbiota communities were analyzed by assessing microbial beta-diversity using a Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics (Fig. 2b). ABX- and vehicle-treated animals showed a separation through the first principal coordinate (PC1), which explained 45.8% of the overall variation. Moreover, CAF-fed rats clustered separately from STD-fed rats along the PC2 axis, explaining 16.9% of the overall variation. Therefore, ABX treatment exerted a stronger effect on microbiota composition than CAF feeding.

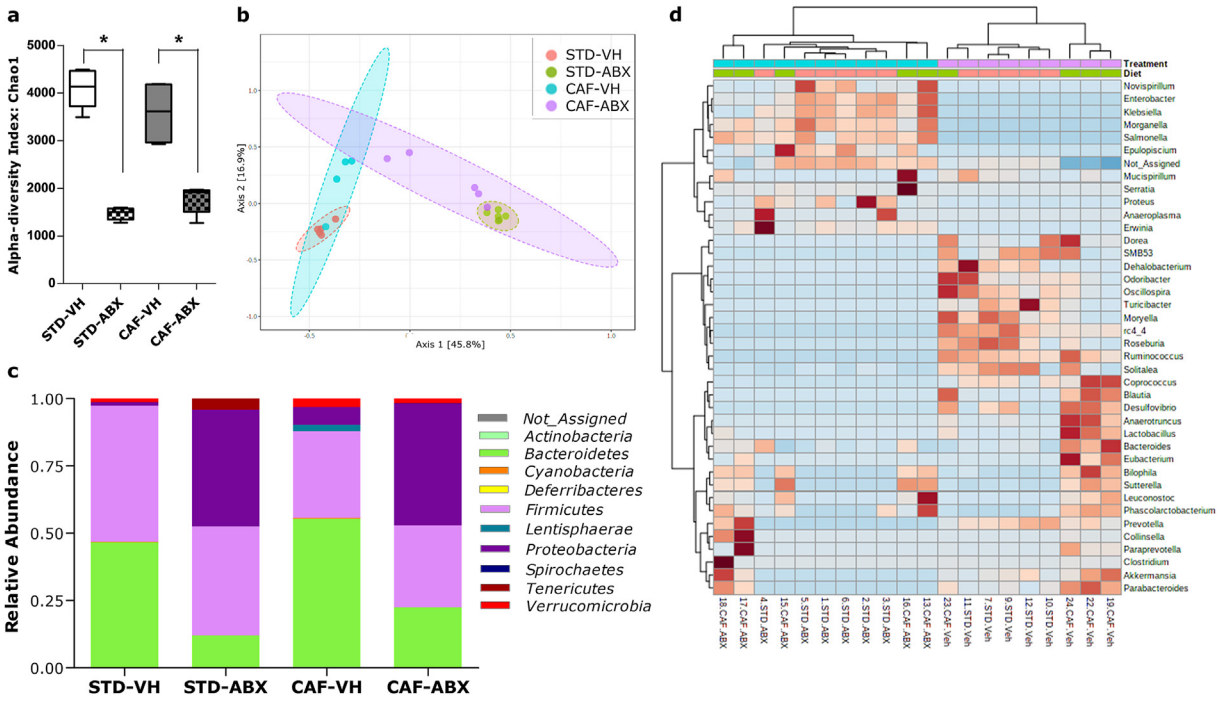

Fig. 2. Fecal microbiota is altered by antibiotics cocktail (ABX) treatment and cafeteria diet (CAF) administration. (**a**) Alpha diversity calculated by Chao1-index. * indicates significant antibiotic cocktail (ABX) effect analyzed by Kruskal–Wallis followed by Bonferroni correction for multiple comparisons ($p < 0.05$); (**b**) β-diversity calculated by Bray–Curtis distances and visualized by a principal coordinates analysis (PCoA) 2D plot (PERMANOVA test ($p < 0.001$)); (**c**) Stacked bar plots showing the average relative abundance of each taxa at phylum level; (**d**) Heat map showing hierarchical clustering of the most abundant genus taxa. STD-VH (standard diet-fed rats); STD-ABX (standard diet-fed and ABX-treated rats); CAF-VH (cafeteria diet-fed rats); CAF-ABX (cafeteria diet-fed and ABX-treated rats).

A significant effect of ABX treatment on phyla relative abundance was also observed in both STD- and CAF-fed rats (Fig. 2c; Table S1). Thus, ABX administration led to both a significant increase in Proteobacteria and a decrease in Bacteroidetes in both STD- and CAF-fed rats. Moreover, ABX did also significantly decrease Actinobacteria, Deferribacteraceae and Verrucomicrobia in STD-fed rats, and Spirochaetes in CAF-fed rats. In addition, CAF feeding also led to changes at phyla level including an increase in Proteobacteria, Lentisphaerae and Tenericutes in vehicle-treated rats only. However, Firmicutes and Bacteroidetes were not significantly altered by diet and, although a decrease in Firmicutes-to-Bacteroidetes ratio was observed, this did not result in significant changes.

When looking at genera level, a hierarchical clustering of bacterial genera was observed by ABX treatment but not by CAF feeding (Fig. 2d, Table S1). Indeed, ABX treatment significantly altered several bacterial genera in both STD- and CAF-fed rats. Although no overall diet clustering effect was observed at genera level, CAF feeding significantly increased the relative abundance of several genera when considering only vehicle-treated rats.

3.2. Oxylipins profile is modified due to cafeteria diet and antibiotic treatment

Overall CAF and ABX treatments effects on plasma OXLs profile were analyzed using a principal component analysis (PCA) (Fig. 3a). As expected, samples clustered based on the type of diet. Interestingly, ABX only showed an effect in CAF-fed rats while STD-fed rats clustered together independently of ABX treatment. Heat map analysis also revealed diet as the main factor affecting plasma OXLs profile and a stronger effect of ABX treatment in CAF-fed rats compared to STD-fed rats (Fig. 3b). Interestingly, although ABX treatment did not change the overall OXLs profile in STD-fed rats, the heat map analysis did show specific changes in some of them such as an increase in EPA, 4-HDHA and 8-HEPE as well as a decrease in 12, 13-DiHODE, Resolvin E2/E3, 910-EpODE, PGF2a, PGJ2, ARA, 9-oxoODE, 15R-Lipoxin A4/L and 18-HEPE, among others. Statistical analysis between the different treatment groups found 35 metabolites whose concentration was significantly decreased due to CAF feeding. However, ABX treatment did not show any significant effect other than increased 4-HDHA levels in STD-fed rats (Table 1). Moreover, when considering only STD-fed

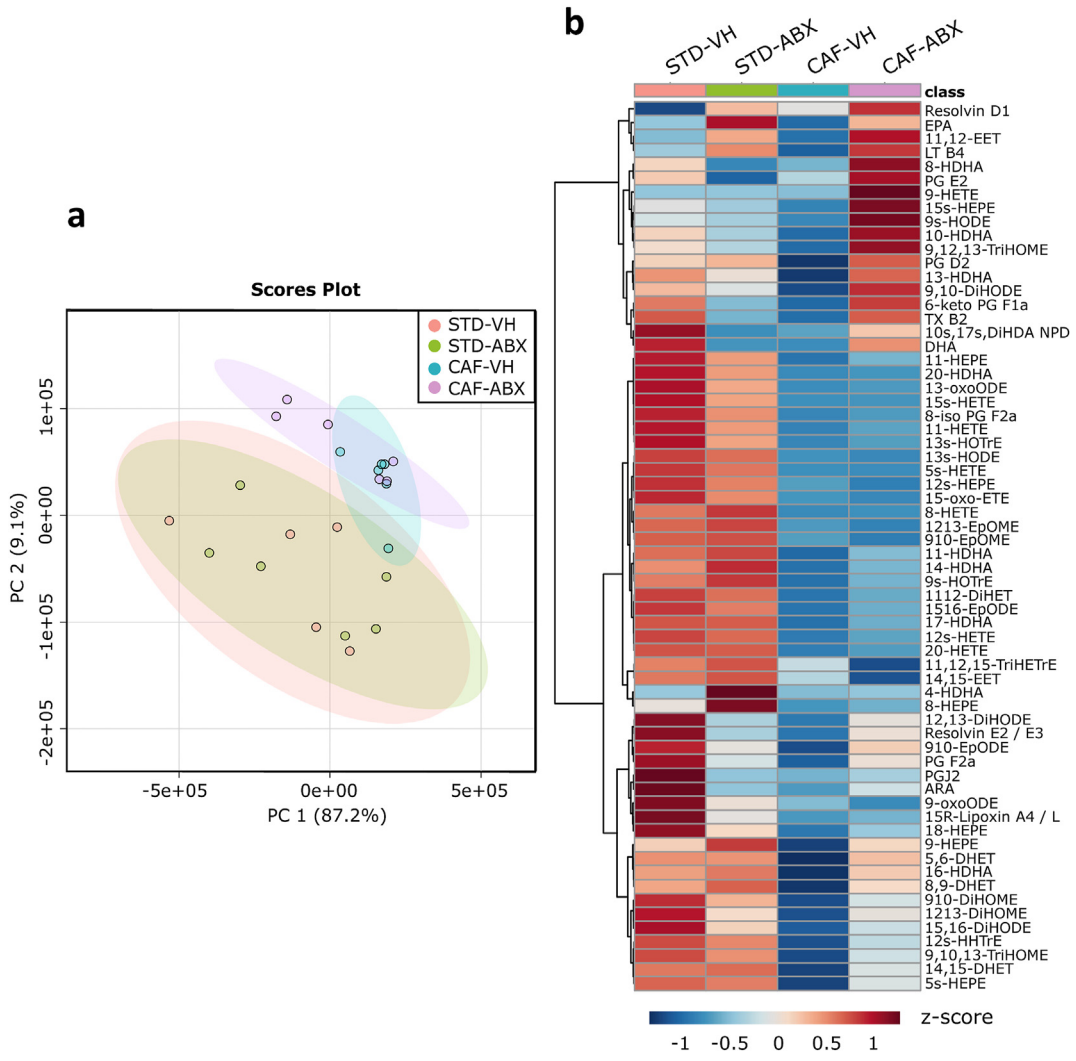

Fig. 3. Cafeteria diet (CAF) feeding and antibiotic cocktail administration led to changes in plasma oxylipins (OXLs) profile. (**a**) Relative OXL composition throughout Principal Component Analysis (PCA) of plasma samples of each group ($n = 5–6$). (**b**) Abundance of plasmatic OXL per experimental group ($n = 5–6$). STD-VH (standard diet-fed rats); STD-ABX (standard diet-fed and ABX-treated rats); CAF-VH (cafeteria diet-fed rats); CAF-ABX (cafeteria diet-fed and ABX-treated rats).

rats, in addition to the increase in 4-HDHA levels, significant increased 8-HEPE and decreased 15(R)-Lipoxin A4/A5 levels were observed in rats treated with ABX. On the other hand, when considering only CAF-fed rats, a significant increase in proinflammatory OXLs (11(12)-DiHET, 9-HETE, LT B4 and PG D2), as well as in anti-inflammatory OXLs (11-HEPE, 15(*S*)-HEPE, 10-HDHA and 13-HDHA) were observed in ABX-treated rats (Table 1).

3.3. Correlation between fecal microbiota and oxylipin composition

In order to further investigate the influence of gut microbiota on OXLs, we investigated the association between plasma OXLs levels and the relative abundance of different bacterial taxonomic groups using the Spearman's rank-order correlation coefficient (rho). To this aim, we selected only those OXLs and bacteria taxa that were significant altered by either CAF feeding or ABX administration. First, we established correlations between those OXLs and fecal microbiota at phylum level. Two main clusters were observed: a first cluster involving Firmicutes and Tenericutes phyla resulting in positive correlations with most of the OXLs, and a second cluster involving the rest of the phyla and resulting mostly in negative correlations with the majority of OXLs (Fig. 4a). In the first cluster, Tenericutes presented the highest number of strong or moderate positive significant correlations (rho > 0.5, Benjamini-Hochberg-FDR adjusted *p* value (FDR) < 0.05) with 13(*S*)-HODE, 12(13)-EpOME, 8-HETE, 15(*S*)-HETE, 15-oxo-ETE, 11-HETE, 20-HDHA, 5(*S*)-HETE, 9, (10)-EpOME, 8-iso PG F2a and 12(S)-HEPE (see Excel supplementary file for rho, *p*-value and FDR details). Among the second cluster, Lentisphaerae showed the highest number of strong or moderate negative significant correlations (rho < −0.5, FDR < 0.05), with 16-HDHA, 4-HDHA, 8-HEPE, PGD2, 13-HDHA, 14,15-DHET, 9,10,13-TriHOME, 11(12)-DiHET, 5,6-DHET, 20-HETE, 17-HDHA, 18-HEPE, LT B4 and 8-iso PGF2a (see Excel supplementary file for rho, *p*-value and FDR details). Bacteroidetes, one of the most abundant phyla, presented strong or moderated negative significant correlation (rho < −0.5 and FDR < 0.05) with 16-HDHA, LT B4, 8-HEPE and PG D2 (Fig. S2). A remarkable phylum on this cluster was Proteobacteria, which showed a strong positive significant correlation with LTB4 (rho = 0.693, FDR = 0.003) throughout the regression analysis based on Lowess method (Fig. 4b). Further, 4-HDHA, which was one of the most affected by CAF feeding and ABX administration, showed significant negative correlation (rho < −0.5 and FDR < 0.05) with Verrucomicrobia, Actinobacteria, Lentisphaerae and Deferribacteres (Fig. S3).

Table 1
Plasma oxylipins levels (pM) in standard (STD)- and cafeteria (CAF)-fed rats treated with vehicle (VH) or an antibiotic cocktail (ABX).

Oxylipins	STD-VH ($n = 5$)	STD-ABX ($n = 6$)	CAF-VH ($n = 6$)	CAF-ABX ($n = 6$)	*P*-value[b]
	Median (IQR)[a]	Median (IQR)[a]	Median (IQR)[a]	Median (IQR)[a]	
11(12)-DiHET	1661.328 (2481.107)	2296.983 (2017.148)	294.622 (39.417)**	660.850 (426.466)$	0.001
11-HEPE	1872.769 (2627.593)	1833.696 (2814.373)	204.662 (167.795)**	534.526 (750.543)$	0.005
11-HETE	1250.555 (835.022)	1031.219 (619.790)	211.392 (147.614)**	309.462 (334.725)	0.001
12(S)-HEPE	26032.015 (10087.113)	25427.630 (2876.542)	16198.007 (5732.323)*	14133.039 (5748.145)**	0.001
12(S)-HETE	28475.604 (46362.692)	34149.279 (53087.197)	3136.320 (5360.185)*	7101.068 (13314.490)	0.014
13(S)-HODE	3001.244 (1268.442)	2578.309 (1457.377)	430.743 (394.688)*	370.671 (257.892)*	0.001
13(S)-HOTrE	116149.483 (47566.768)	77012.056 (33452.778)	17372.566 (16705.742)**	26027.303 (19786.494)	0.001
13-HDHA	2903.827 (2336.121)	2888.441 (1227.410)	939.750 (846.127)*	2569.495 (2552.049)$	0.022
13-oxoODE	3409.196 (2699.442)	2581.990 (2401.890)	673.179 (703.098)*	874.794 (468.636)	0.004
14,15-DHET	19887.447 (7317.751)	18870.004 (9424.917)	7936.933 (2895.549)*	13345.837 (9884.030)	0.006
15(16)-EpODE	7090.270 (9360.003)	7720.084 (12104.890)	800.617 (657.243)**	1858.605 (3187.772)	0.005
15(R)-Lipoxin A4/A5	41085.385 (31264.763)	20452.980 (9789.600)$	8376.811 (7442.714)***	11410.731 (6685.493)	0.000
15(S)-HETE	6105.271 (2844.963)	4334.876 (2137.168)	641.062 (302.835)**	641.736 (559.584)	0.001
15,16-DiHODE	21834.899 (12962.440)	19566.222 (13094.552)	10538.166 (5580.299)*	14385.504 (12769.142)	0.033
15-oxo-ETE	1140.083 (621.224)	1117.735 (517.926)	263.048 (199.126)*	179.738 (157.443)*	0.001
16-HDHA	706.739 (416.855)	868.403 (432.046)	456.349 (176.794)*	701.705 (560.134)	0.021
17-HDHA	1640.338 (1435.356)	1759.262 (1829.995)	392.480 (213.871)*	759.386 (533.688)	0.004
18-HEPE	2259.847 (2467.270)	1380.655 (920.761)	275.811 (403.476)**	503.614 (1019.271)	0.003
20-HDHA	1039.531 (723.020)	818.587 (341.198)	182.281 (82.493)**	214.435 (215.961)*	0.001
20-HETE	1159.873 (567.339)	1257.256 (619.087)	312.673 (102.115)*	391.056 (481.532)*	0.003
5(S)-HETE	5228.429 (2613.529)	4429.005 (2005.372)	801.928 (864.557)**	759.953 (703.549)*	0.001
5,6-DHET	4074.785 (2043.074)	4111.513 (1743.315)	2138.077 (1022.006)*	3563.027 (3010.726)	0.018
8,9-DHET	413.134 (56.597)	402.772 (149.835)	222.518 (70.389)*	342.914 (331.134)	0.013
8-HETE	944.334 (484.475)	937.591 (733.541)	146.088 (54.901)*	125.512 (145.044)**	0.001
8-iso PG F2a	967.042 (796.685)	654.503 (332.533)	123.343 (120.371)**	159.190 (187.765)	0.001
9(10)-EpODE	11066.711 (9853.434)	9368.940 (3869.267)	1555.549 (8472.890)*	8460.025 (11840.393)	0.048
9,10,13-TriHOME	29459.970 (15567.949)	27155.737 (17584.558)	8983.110 (7341.700)*	15334.936 (19536.854)	0.015
PG D2	19571.772 (10361.085)	20808.024 (10588.786)	8849.061 (2427.149)*	17225.502 (19429.022)$	0.009
11,12,15-TriHETrE	5834.522 (3037.580)	5453.101 (3008.983)	4068.687 (3492.481)	3248.543 (1267.808)*	0.029
12(13)-EpOME	174438.699 (71101.345)	170527.002 (68000.594)	35278.717 (26083.618)	29497.559 (12961.138)**	0.001
14,15-EET	10753.041 (3955.561)	9786.236 (3429.102)	7993.635 (6182.779)	6542.202 (1540.205)*	0.018
8-HEPE	349.973 (141.130)	492.613 (316.706)$	262.575 (103.446)	306.150 (64.980)*	0.002
9(10)-EpOME	37363.885 (13854.464)	31401.418 (10580.993)	12067.085 (12172.395)	10955.152 (5886.456)**	0.001
9-oxoODE	1463.233 (1397.373)	728.186 (260.434)	406.493 (248.825)	319.122 (231.936)*	0.013
4-HDHA	3320.832 (1571.543)	6241.716 (4052.207)#$	3365.791 (1256.646)	3884.618 (2741.782)	0.018
10-HDHA	21.102 (20.023)	12.141 (12.356)	3.987 (5.724)	10.161 (69.078)$	0.037
15(S)-HEPE	271.334 (323.040)	211.027 (203.592)	157.825 (166.257)	335.025 (368.186)$	0.111
9-HETE	2359.320 (1605.371)	2751.398 (1371.556)	2181.102 (667.472)	3545.175 (72324.902)$	0.064
LT B4	97.835 (180.721)	191.328 (95.090)	75.717 (118.565)	174.530 (137.621)$	0.081

* and # indicate CAF feeding and ABX effects respectively by Dunn's multiple comparison test followed by Bonferroni *p* values adjustment: $p < 0.0125$, $^{**}p < 0.0025$, $^{***}p < 0.00025$; $\#p < 0.0125$; $ indicates ABX effects when comparing by Mann Whitney test. $ $P < 0.05$.

[a] Data shown as Median ± Interquartile Range (IQR) in pM units.

[b] *p*-value by Kruskal–Wallis test.

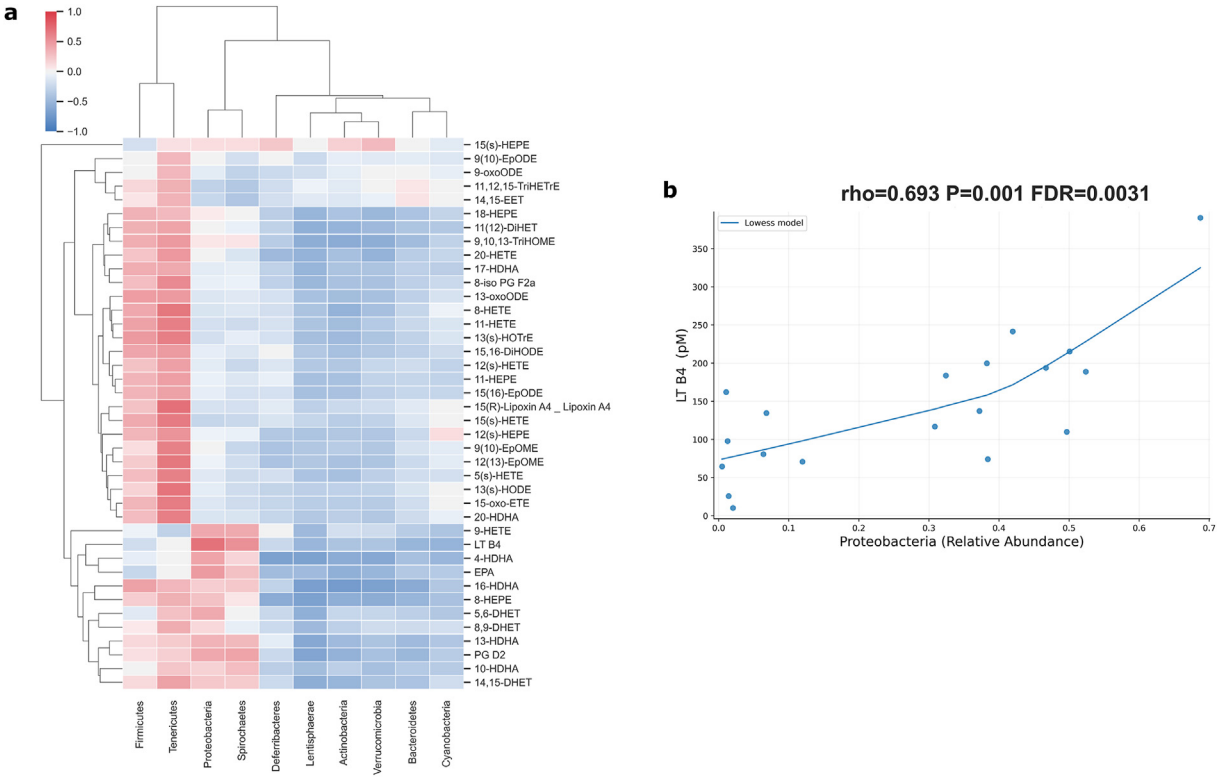

Fig. 4. Correlation analysis between fecal microbiota at phylum level and plasma oxylipin (OXL) composition evaluated by Spearman's rank correlation coefficient (rho). (**a**) Heat map showing the hierarchical clustering of the degree of correlation between phylum and OXL. Positive correlation coefficients are in red and negative correlation coefficients are in blue. The color intensity represents the degree of correlation. (**b**) Locally weighted linear regression (Lowess model) analysis between LT B4 concentration and relative abundance of Proteobacteria showing positive correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Since the assessment of these OXLs at a phyla level revealed widespread differences, we further investigated these associations at other taxonomical levels. Two clusters were observed when looking at family level. The first cluster is characterized by bacteria families belonging mostly to Firmicutes and Bacteroidetes phyla showing negative correlations (rho < -0.5, $p < 0.05$) with most of the OXLs included in this analysis (Fig. 5). These correlations were mostly moderate, with the exception of *Leuconostocaceae*, which showed a high number of moderate to strong significant negative correlations (rho < -0.6, FDR < 0.004) (see Excel supplementary file for details). The second cluster is characterized by bacteria families showing positive correlations (rho > 0.5, $p < 0.05$) with most of the OXLs. Although a high number of families in this cluster are from the Firmicutes and Bacteroidetes phyla, it is worth mentioning that, in contrast with the first cluster mentioned above, in this group appeared a significant number of families from the Proteobacteria phylum. Unfortunately, most of these correlations in this second cluster did not pass the multiple test corrections (FDR < 0.5) (see Excel supplementary file for details).

Regarding genus level, a first remarkable cluster involving bacteria belonging to the Proteobacteria, Tenericutes and Firmicutes phyla was identified (Fig. 6). This cluster was characterized by strong positive correlations (rho > 0.5, $p < 0.05$) with LTB4, 4-HDHA, EPA, 9-HETE, 8-HEPE, 10-HDHA, 14,15-DHET, 5,6-DHET, 16-HDHA, 13-HDHA and PGD2. From all these correlations, 14 passed the FDR correction (see Excel supplementary file for details). The second cluster was characterized by bacteria genera belonging to Actinobacteria, Bacteroidetes, Deferribacteres, Lentisphaerae, Firmicutes and Verrucomicrobia phyla showing negative correlations (rho < -0.5, $p < 0.05$) with the same group of OXLs. From all these correlations, 79 passed the FDR correction (see Excel supplementary file for details).

In addition to these taxonomical levels, we also looked at class (Fig. S4) and order levels (Fig. S5). Again, two clusters were observed at these levels, a first one characterized by bacteria showing negative correlations (rho < -0.5, $p < 0.05$) and a second cluster characterized by bacteria showing positive correlations (rho > 0.5, $p < 0.05$). However, only two correlations with 4-HDHA passed the FDR correction: strong negative correlations with both the Lentisphaeria class (rho $= -0.8312$, FDR $= 0.0006$) and the Victivallales order (rho $= -0.831$, FDR $= 0.0002$) (see Excel supplementary file for details).

4. Discussion

OXLs are PUFAs-derived bioactive metabolites and include prostaglandins, thromboxanes, mono-, di-, and tri-hydroxy fatty acids (FAs), epoxy FAs, lipoxins, eoxins, hepoxilins, resolvins, protectins, and maresins [36]. They are involved in different physiological processes including inflammation, immune actions, pain, apoptosis, blood clotting, cell proliferation, blood vessel permeability, blood pressure regulation and tissue repair [37]. Among these, it is worth highlighting their role as potent modulators of inflammatory processes since they can act as activators as well as pro-resolving mediators of inflammation through their binding to both peroxisome proliferator-activated receptors (PPARs) and G protein-coupled receptors (GPCRs) [38–40]. Indeed, they are emerging as potential new biomarkers of chronic low-grade inflammation [41]. In general, OXLs derived from ω3-PUFAs exert anti-inflammatory effects while those derived from ω6-PUFAs are more proinflammatory, which is in accordance with overall

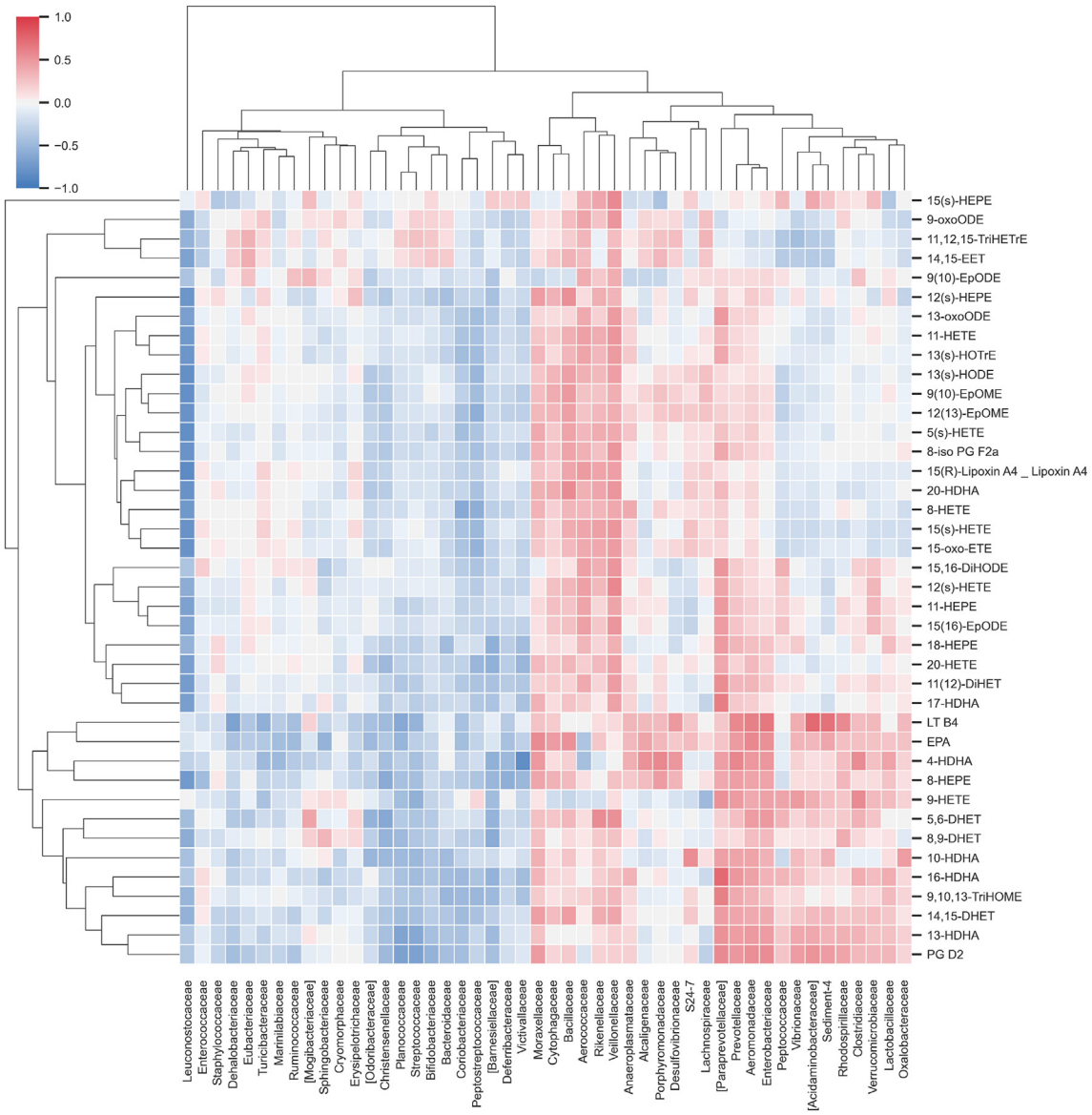

Fig. 5. Correlation analysis between fecal microbiota at family level and plasma oxylipins (OXL) evaluated by Spearman's rank correlation coefficient (rho). Heat map showing the hierarchical clustering of the degree of correlation between bacteria family and OXLs. Positive correlation coefficients are in red and negative correlation coefficients are in blue. The color intensity represents the degree of correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

beneficial and detrimental effects of ω3 and ω6-PUFAs respectively [42]. On the other hand, gut microbiota has been proven to be involved in PUFAs metabolic pathways, but its effects on these metabolites remains poorly understood [28,43]. In the present study, we found that gut microbiota dysbiosis altered plasma OXLs profile, suggesting that intestinal bacteria may indeed influence their metabolism and, ultimately, host inflammatory response and other physiological functions.

In healthy rats, gut microbiota dysbiosis caused by ABX administration did not change the overall plasma OXLs profile, suggesting that intestinal bacteria do not significantly contribute to the metabolism of these bioactive lipid mediators under healthy conditions. However, some specific changes were observed, including a significant increase in 4-HDHA and 8-HEPE, which are related to anti-inflammatory effects, and a significant decrease in 15(R)-Lipoxin A4/A5 levels, which are related to pro-resolving effects. These changes in OXLs may be in response to the known inflammation mediated by the ABX treatment due to both the translocation of gut microbiota [44] and to the long-term disturbances of microbiota composition [45,46]. Indeed, the ABX administration led to a significant increase in the relative abundance of Proteobacteria, which has been extensively linked to inflammation. Thus, bacteria from this phylum are able to utilize inflammatory by-products for their survival and, therefore, during chronic inflammation there is an increase in their relative abundance due to their advantage over other intestinal bacteria that lack this metabolic capacity [47]. Moreover, ABX administration led to significant decreased Bacteroidetes relative abundance, which has also been associated to inflammation [48]. This bacterial taxon, together with Firmicutes, is one of the dominant phyla in healthy vertebrate hosts and play a variety of roles, including the generation of metabolites and immune system maturation [49,50]. Moreover, ABX administration did also significantly affected other less abundant phyla such as Actinobacteria, linked to gut barrier impairment and proinflammatory cytokines [51], Deferribacteraceae, which involves harmful bacteria and has been associated to

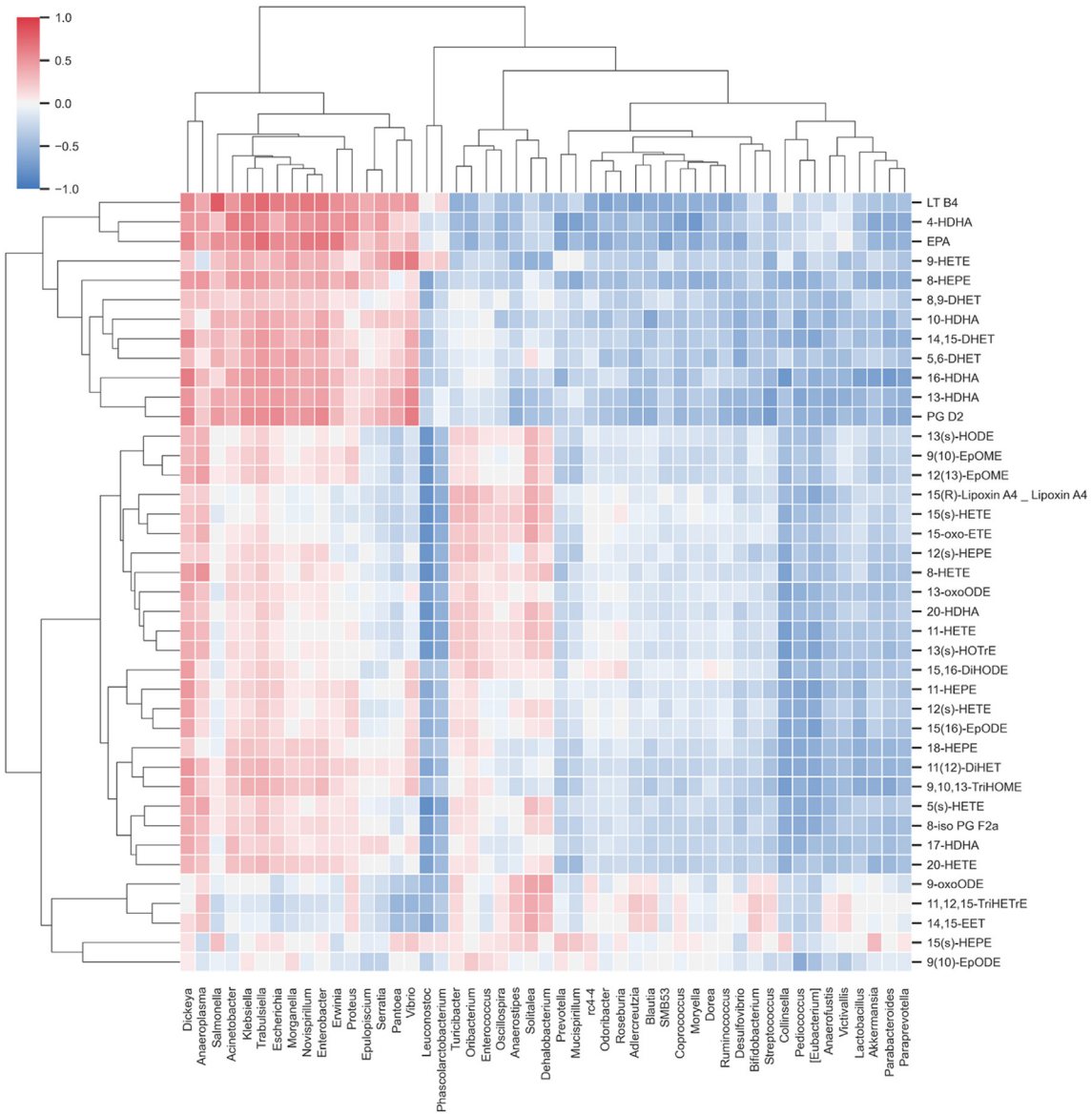

Fig. 6. Correlation analysis between fecal microbiota at genus level and plasma oxylipins (OXL) evaluated by Spearman's rank correlation coefficient (rho). Heat map showing the hierarchical clustering of the degree of correlation between bacteria genus and OXLs. Positive correlation coefficients are in red and negative correlation coefficients are in blue. The color intensity represents the degree of correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inflammatory diseases such as colitis [52], and Verrucomicrobia, also related to increased inflammation as their abundance bloomed in a mouse model of mucositis [53]. Therefore, ABX treatment leads to changes in gut bacteria that affect inflammatory responses which may ultimately impact on OXLs metabolism.

On the other hand, obesity and obesity-related disorders are linked to low-grade chronic inflammation [54] and gut microbiota dysbiosis [23]. Therefore, the establishment of obesogenic conditions by CAF administration allowed us for a better understanding of the potential role that gut bacteria may have on OXLs metabolism under pathologic conditions associated to chronic low-grade inflammation. Indeed, CAF feeding has been shown to effectively induce obesity, diabetes and other disorders associated to the metabolic syndrome, increasing inflammatory markers and significantly altering gut microbiota composition [29–31]. Moreover, CAF includes food regularly consumed by humans, leading to a more robust model for human obesity in comparison with others high fat and/or high sugar diets traditionally used [29]. We observed a completely different overall plasma OXLs profile in CAF-fed rats compared to STD-fed rats. These differences may be explained by the different nutritional composition of these diets. Indeed, OXLs profile is determined not only by the relative abundance of the enzymes that are involved in their synthesis and metabolization but also by the availability of PUFAs precursors, which is mainly influenced by the composition of dietary fatty acids [36,55–57]. Hence, CAF is enriched in lipid content, including higher levels of PUFAs, in comparison with STD [58]. In addition, the effect of CAF feeding on the OXLs profile may be related to the increased inflammation caused as a result of the chronic administration of this type of high fat high sugar diet and to the obesity development [29]. Interestingly, besides the high lipid content and the increased inflammation, CAF feeding led to an overall reduction in plasma OXLs levels, involving both pro- and anti-inflammatory metabolites. However, this is consistent with other diet induce obesity (DIO) study that show decreased plasma OXLs levels in HFD-fed animals [59]. Moreover, CAF feeding induced changes in

gut microbiota composition that may ultimately impact on the OXLs profile. Thus, CAF-fed rats showed an increased abundance in proinflammatory bacteria such as the above mentioned Proteobacteria as well as Lentisphaerae, which has been linked to patients with inflammation [60], and Tenericutes, which has been shown to positively correlate with proinflammatory cytokines such as IL-6, TNF-α or IL-17A [61]. Among these, it is worth mentioning the increased relative abundance in Proteobacteria, which is consistent with others studies using CAF [30,31] and has been reported to be highly associated to dysbiosis during metabolic disorders [62]. In fact, the mono-association of germ-free mice with *Enterobacter cloacae*, a strain belonging to this phylum, induced obesity and insulin resistance when mice were fed a HFD, which was not observed in control HFD-fed germ-free mice, supporting the relation between metabolic disorders and the expansion of Proteobacteria [63]. Hence, this also supports the potential link of Proteobacteria relative abundance with metabolic disorders associated to chronic low-grade inflammation and corroborates that plasma OXLs profile changes are associated with changes in the abundance of this bacteria taxa. Moreover, the ABX administration did change the overall gut microbiota composition in CAF-fed rats, having a higher impact than that produced by the diet. Indeed, rats administered with ABX showed similar overall gut bacteria composition independently of the diet. This is in agreement with previous studies which reported that the effects of oral antibiotics on gut bacteria composition and function were greater than those mediated by HFD administration [64]. Interestingly, the plasma OXLs profile in CAF-fed rats treated with ABX showed a significant increase in both pro- and anti-inflammatory plasma OXLs metabolites compared to non-ABX treated CAF-fed rats, indicating that gut microbiota may play a key role in the metabolism of these lipid metabolites under obesity conditions. As observed in STD-fed rats, the main change induced by the ABX treatment at phylum level was an increase in Proteobacteria and a decrease in Bacteroidetes relative abundance. Therefore, this corroborates the potential relationship between these bacterial taxa and the OXLs metabolism. The proportion between these two phyla may be a good marker for gut microbiota dysbiosis as ABX administration induced an increase in the Proteobacteria-to-Bacteroidetes ratio, reaching significance in STD-fed rats. Moreover, increase in this ratio has been associated to HFD administration, supporting its relation with gut microbiota alterations [65]. Indeed, decrease in this ratio has been linked to decrease in both LPS production and NF-κB activation in the colon of HFD-fed mice, ameliorating inflammation [66]. When analyzing gut bacteria relative abundance correlation with OXLs levels, there were several associations observed. Among these, it is worth highlighting those that correlated with Proteobacteria and Bacteroidetes, as they were the main phyla changed by the ABX treatment. Regarding Bacteroidetes, they showed negative correlations with most of the plasma OXLs, being significant for 16-HDHA, 8-HEPE, LT B4 and PG D2. Regarding 16-HDHA and 8-HEPE are derived from the ω3-PUFAs DHA and EPA respectively, which have been linked to anti-inflammatory effects. LT B4 and PG D2 are derived from ARA, ω6-PUFA linked to proinflammatory effects. Regarding Proteobacteria, both negative and positive correlations were observed. Among these, the significant positive correlation with LT B4 was especially remarkable. This oxylipin is a chemoattractant for neutrophils that has been demonstrated to initiate pathological inflammation in various tissues as well as to have a key role in the progression of chronic diseases related to inflammation such as infectious diseases, allergy, autoimmune diseases, and metabolic disease [67].

These findings are consistent with the essential role that gut bacteria play in lipid metabolism and absorption. Thus, germ-free animals are resistant to develop DIO and metabolize fat with specifically impaired lipid digestion and absorption [68,69]. Hence, gut microbes regulate gut epithelial processes involved in lipid digestion and assimilation which are crucial for host adaptation to dietary lipid changes [69]. Moreover, gut microbiota has been shown to produce PUFA-derived metabolites, including conjugated linoleic (CLA) and conjugated linolenic acids (CLnA) [43,70]. In addition, it was recently shown that gut microbiota confers host resistance to obesity by metabolizing dietary PUFAs [28]. Hence, it seems evident that OXL metabolites derived by dietary PUFAs such as LA, ALA and ARA, may be influenced by gut bacteria.

On the other hand, the correlations observed may contribute to elucidate specific profiles of OXLs-gut microbiota interactions in an obesity context, which may be useful as novel biomarkers for metabolic profiling. Indeed, OXLs and gut microbiota are emerging as new candidates to define new hallmarks of particular physiological conditions [71,72].

Finally, it is important to mention that ABX treatment also altered glucose tolerance response in both healthy and obese rats, indicating that the above discussed changes in gut microbiota and OXLs plasma profile mediated by the antibiotics intake may be linked to metabolic alterations such as glucose intolerance. Further studies are needed in order to elucidate the mechanisms involved and the effects in different tissues.

5. Conclusions

In conclusion, we have observed a clear association between gut bacteria changes and plasma OXLs profile under healthy and obesogenic conditions induced by CAF feeding in rats. These findings support a potential link between gut microbiota and the metabolism of these lipid mediators, which may be linked to the key role that gut microbiota has in inflammatory response. Moreover, this can set the basis for developing new obesity markers based on OXLs and gut microbiota profiles. Further studies are needed to elucidate the specific mechanisms involved.

Author contributions

Conceptualization: J.A.R., L.A. and C.T.F; Methodology: J.A.R, V.A.G., AJ.C.E., JR.S.R. and C.T.F. Investigation: J.A.R., M.M., B.M., A.A.A., L.A. and C.T.F. Validation: J.A.R., L.A. and C.T.F. Writing the original draft: J.A.R, V.A.G., AJ.C.E., JR.S.R. and C.T.F. Writing, review, and editing: J.A.R. and C.T.F. Funding acquisition: B.M. and L.A.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2021.02.035.

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