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High-Fat Diet Consumption Induces Microbiota Dysbiosis and Intestinal Inflammation in Zebrafish

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Abstract

Energy-dense foods and overnutrition represent major starting points altering lipid metabolism, systemic inflammation and gut microbiota. The aim of this work was to investigate the effects of a high-fat diet (HFD) over a period of 25 days on intestinal microbiota and inflammation in zebrafish. Microbial composition of HFD-fed animals was analysed and compared to controls by 16S rRNA sequencing and quantitative PCR. The expression level on several genes related to inflammation was tested. Furthermore, microscopic assessment of the intestine was performed in both conditions. The consumption of the HFD resulted in microbial dysbiosis, characterised by an increase in the relative abundance of the phylum Bacteroidetes. Moreover, an emerging intestinal inflammation via NF-κβ activation was confirmed by the overexpression of several genes related to signalling receptors, antimicrobial metabolism and the inflammatory cascade. The intestinal barrier was also damaged, with an increase of goblet cell mucin production. This is the first study performed in zebrafish which suggests that the consumption of a diet enriched with 10% fat changes the intestinal microbial community composition, which was correlated with low-grade inflammation.

Keywords Zebrafish · Microbiota · Inflammation · High-fat diet

Introduction

Overweight and obesity are defined as abnormal or excessive fat accumulation and a low-grade systemic inflammatory tone in the presence of a positive energy balance, representing a

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major risk factor for a number of chronic diseases including cancer, cardiovascular diseases, diabetes and premature mortality [1]. The consumption of a high-fat diet (HFD) is one of the main factors contributing to the development of obesity [2]. Human and animal studies have shown that both HFD and obesity are associated with changes in the gut microbiota, reducing the abundance and diversity of microorganisms [3, 4] and impacting both immunological and metabolic functions of the host [5, 6]. To date, studies performed in zebrafish describe microbial community changes or inflammation developed by the consumption of a high-fat, high-protein diet [7–9] or a high-cholesterol diet (HCD) [10].

Studies performed in rodents have shown that the prolonged exposure to a high-fat diet (HFD) can alter the intestinal microbiota and perturb immune homeostasis, inducing intestinal inflammation [11]. These alterations lead to the activation of an innate immunity-mediated chronic low-grade inflammation known as meta-inflammation (metabolically triggered inflammation) [12], suspected to be chronically activated and modulated by pro-inflammatory cytokines. These molecules are likely to play a key role in metabolic disease pathogenesis which develops locally, but becomes systemic

through the release of numerous pro-inflammatory mediators into the blood stream [13].

Diet is an important modulator of the intestinal microbiota in humans and other animals [2, 14, 15] and associated with impairments in epithelial integrity and barrier function [16]. The intestinal mucosa is the first barrier where fat is absorbed and metabolised and might therefore be involved in responses triggered by dietary lipids [10]. Both resident antigen-presenting cells and intestinal mucosal epithelial cells are equipped with pattern recognition receptors (PRR), such as toll-like receptors (TLR) and NOD-like receptors (NLR). They detect pathogen-associated molecular patterns (PAMPs), for instance lipopolysaccharides (LPS), flagellin or peptidoglycans [17], and protect the organism from harmful pathogens, thereby promoting repair, regeneration and immune homeostasis of the intestine [18]. Recent findings have demonstrated that fatty acids and cholesterol are able to attach to those receptors, leading to inflammation [19] via stimulating inflammatory-signalling cascades, such as the $I\kappa B\alpha$ kinase/nuclear factor-KB (IKK/NF-$\kappa\beta$), the endoplasmic reticulum (ER), the stress-induced unfolded protein response (UPR) and the NOD-like receptor P3 (NLRP3) inflammasome pathway [20].

The zebrafish (*Danio rerio*) has an increasing recognition as an excellent animal model for studying human metabolic or inflammatory diseases due to its similarity to humans in terms of organs and genomic content, its highly conserved biochemical and physiological pathways and the availability of a complete genome sequence. Despite the differences between the zebrafish and mammal microbiota composition, dominated by Proteobacteria in zebrafish and by Firmicutes and Bacteroidetes in mice and humans, the responses to microbial colonisation are similar [21]. The microbiota also play a crucial role in immunity and host response to pathogens in zebrafish [22–24].

Moreover, pathways regulating microbial recognition and activation of the innate immune response are also greatly conserved [25]. In addition, zebrafish maintenance and manipulation are cheaper than in rodent models [26–28].

In the present work, we described for the first time the correlation between intestinal microbiota dysbiosis and inflammation in zebrafish, induced by the consumption of a high-fat diet over the period of 25 days.

Materials and Methods

Zebrafish Husbandry and Experimental Diets

Zebrafish embryos were obtained from wild-type adult zebrafish (*D. rerio*, Hamilton 1822) bred in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain), following standard conditions. The fish were maintained at 27 °C in 60-L tanks with aerated freshwater, according to standard protocols [29]. They were fed daily with a pellet-formulated diet (Gemma Micro 300; Skretting) and reared on a 12-h light/12-h dark cycle. Zebrafish embryos were collected directly from the breeding tanks immediately after fertilisation and transferred to 2-L fish tanks. At 5 days post-fertilisation (dpf), larvae were equally separated into two tanks and subjected to the control and the high-fat diet (HFD). Both were maintained in 500 mL of isowater (CaCl$_2$ 294 mg L^{-1}, MgSO$_4$ 123 mg L^{-1}, NaHCO$_3$ 64.7 mg L^{-1}, LCl 5.7 mg L^{-1}) at 27 °C for 1 month (30 dpf). Concentrations of nitrate, nitrite and ammonium were tested weekly (data not shown). All experimental procedures were approved by the regional animal welfare body (NEIKER-OEBA-AZTI14–005).

The control diet consisted of a commercial diet for zebrafish larvae (ZF Biolabs), prepared in Milli-Q water (1 g in 100 mL water) [30] and autoclaved (Supplementary material, Table 1). The HFD consisted of the autoclaved control diet supplemented with 10% (*w/w*) of cocoa butter to enrich the fat content [31]. The cocoa butter was melted and added to the liquid control diet previously to the sterilisation. This ingredient was concretely chosen for fat enrichment because is composed of saturated fatty acids. Dietary lipid profiles are compiled in supplementary material, Table 2. Zebrafish larvae were fed three times per day during the experimental period, increasing the amount of food over time to facilitate normal zebrafish development [32]. At 30 min after feeding, the remaining food was removed. Additionally, at the end of the day, half of the medium was replaced by fresh isowater.

Bacterial DNA Extraction and Quantification

After 30 dpf, five control larvae and five HFD larvae were immersed in a bath of sterile 0.01% Tween20 (Merck). The container was stirred for few seconds to ensure that the solution reached all the larvae surface and subsequently in two consecutive baths of sterile isowater in order to remove any bacteria from the skin [33]. Then, each larva was placed in a sterile Eppendorf vial and immediately frozen in liquid nitrogen. Samples were stored at − 80 °C until further use. Genomic DNA was extracted from frozen samples using a QIAamp DNA Mini Kit (Qiagen LTD, West Sussex, UK), following the manufacturer's instructions. The DNA concentration and purity were analysed using a Nanodrop (Thermo), measuring the absorbance at 260 nm and the A260/A280 ratio, respectively. This experiment was conducted once ($n = 10$).

Analysis of the Microbial Community Composition

Characterisation of the microbial community composition was performed on an Illumina Miseq Platform by sequencing the V3-V4 16S rRNA region, using the primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 [34]. Primer

sequences were removed with Trimmomatic and paired reads were merged using Flash [35]. The rest of the bioinformatics analysis was performed using the Mothur platform [36] (1.37.2). The merged reads were quality-trimmed (with a minimum Phred average quality score of 35 over a 50 bp window) and aligned against a reference SILVA alignment. The resulting sequences were denoised by a pre-clustering method allowing one mismatch [37], as recommended previously [38], and Uchime was used to remove chimeras. Reads were clustered at the genetic distance cut-offs 0.01 and 0.03 substitution per nucleotide for OTU (operational taxonomic unit) construction, using the average linkage method. The Wang [39] approach was used for the taxonomic assignment of the OTUs by classification with the SILVA taxonomy (version 128). Furthermore, the total number of bacteria per sample was quantified by qPCR, as previously described by [40].

All sequences from this study are available from the European Nucleotide Archive (ENA) (PRJEB23882).

Gene Expression Analysis by Real-Time PCR

To study gene expression levels, groups of 20 larvae were sampled at 30 dpf. Three replicates per diet were analysed. The gene expression was conducted in two independent experiments ($n = 240$). At the end of the experimental period, zebrafish larvae were immediately frozen in liquid nitrogen and stored at $-80°C$ until being processed. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The quantity and quality of RNA samples were determined by capillary electrophoresis, using an Agilent 2100 Bioanalyzer (Agilent Technologies); only RNA samples with an RNA integrity number (RIN) of at least 8.5 were used [41].

A reverse transcription reaction was performed with the Taqman Reverse Transcription Kit (Applied Biosystems), using synthesised cDNAs from the RNA samples containing 20 ng of RNA per assay. The mixture was incubated at 25 °C for 10 min and at 48 °C for 30 min, and the enzyme was inactivated at 95 °C for 10 min. Changes in mRNA expression of the genes related to the innate immune system (*ASC*, *CASP1A*, *IL1*, *IL1β*, *IL22 TNF*, *NFKB* and *MyD88*), host-microbe interactions (*TLR4*, *TLR5*, *TLR2*, *MMP9*, *MPO*, *NOD1* and *Defensin 1*) and metabolic activity (*iap*) were monitored by real-time PCR (qPCR), using β-*actin* (*ACT*) and *elongation factor 1* (*EF1*) as reference genes to normalise the results. The primer sequences are listed in the Supplementary material, Table 2. Quantitative PCR was carried out with a Light Cycler 480 sequence detection system (Roche Diagnostics). Each reaction was performed in a 10 μL solution containing 300 nM primers, 5 μL 2× Brilliant III SYBR Green qPCR Master Mix (Agilent) and 10 ng of cDNA template, as previously described [42]. The qPCR

efficiency was maintained between 90 and 110%. RNA expression levels were calculated by the 2-DDCt method [43].

Evaluation of Intestinal Mucus Secretion and Intestinal Track Histology

Intestinal mucus secretion was visualised with a Nikon SMZ1000 stereomicroscope, staining whole larvae with alcian blue as previously described [44]. Briefly, at 30 dpf, five larvae per treatment were analysed and processed with "Colour Deconvolution" plug of the ImageJ software v1.47 (National Institutes of Health, NIH) to obtain the staining area (mm^2) [45]. The alcian blue evaluation was conducted in two independent experiments ($n = 20$).

In addition, five larvae of each treatment were fixed in 10% (*v/v*) neutral buffered formalin, dehydrated and paraffin-embedded. Subsequently, 4-μm sections were stained with haematoxylin and eosin as well as with alcian blue ($n = 10$). Slides were analysed under a microscope by a blind pathologist. Histological damage was calculated using the following criteria: For mucopolysaccharide secretion, every goblet cell per intestinal crest was counted. Mucosal architecture and cellular infiltration were scored as follows: normal, moderate or extensive damage, respectively 0, 1 or 2; and normal, moderate and transmural infiltration, respectively, 0, 1 or 2. The scores for the last two criteria were then summed with a maximum possible score of 4, as previously described [46, 47].

Statistics

The *p* values were calculated with a *t* test, using the software package Statgraphics v16.1.17 (StatPoint Technologies, Inc.). For taxa comparisons non-parametric Kolmogorov-Smirnov test was used. All *p* values were adjusted via Benjamini Hochberg correction [48]. A *p* value < 0.05 was considered statistically significant. Alpha diversity measures were calculated in Mothur and R (3.4.0): number of observed species (SOBS), species richness (Chao1), community evenness (Simpson's evenness) and diversity (Shannon). Beta diversity was analysed from a Bray Curtis distance matrix in a three-dimensional non-multimetric scaling plot (NMDS). Differences between treatments were determined with AMOVA (Mothur). Comparisons at different taxonomical levels were compared using the Kolmogorov-Smirnov test. Discriminatory analysis between treatments at the OTU level was performed with the LEfSe command implemented in Mothur. Spearman correlation among genes, phylotypes and goblet cell counts was accomplished using the Corrplot and Hmisc R packages.

Results

The Consumption of a High-Fat Diet Affects Intestinal Microbial Composition

To evaluate the effect of the diet on zebrafish microbiota, the microbial community composition of larvae fed each experimental diet was analysed and described by metabarcoding at 30 days post-fertilisation (dpf). Compared to the control samples, HFD did not affect the microbial community alpha-diversity, calculated by different diversity indices such as community richness (measured by observed OTUs (SOBS) and Chao1 (Chao)) (Fig. 1a). Community evenness and diversity, tested by Simpson's evenness and Shannon indices, were not affected by the HFD. Beta diversity analysis revealed that the overall composition of the microbial community did not show significant differences due to the HFD, as control and HFD samples were clustered together in the NMDS (AMOVA test, $p = 0.31$; Fig. 1b).

However, there were significant changes in community phylotypes. Overall, 31 phyla were characterised in both diet conditions, of which Proteobacteria was the most abundant one (59 and 66% of the sequences in control and HFD groups, respectively). However, Bacteroidetes were significantly

increased ($p < 0.01$) in HFD samples (from 2 to 16% of the total sequences; Fig. 2a, b). The increase of Bacteroidetes due to the HFD was reflected at the family level in the enrichment of the *Cytophagaceae* family ($p < 0.01$), *Flectobacillus*, the *NS11-12 marine group* ($p < 0.01$) and the genus *Runella* ($p < 0.05$; Fig. 2c). Furthermore, a tendency to increase in the Proteobacteria phylum was observed. In contrast, Firmicutes and Actinobacteria tended to decrease. Moreover, the number of total bacteria from the same larvae extracted DNA was quantified by qPCR, showing that the consumption of a HFD over the period of 25 days did not significantly modify the amount of total bacteria ($p = 0.60$; Fig. 1c).

In addition, Linear Discriminant Analysis (LDA) effect size (LEfSe) was used to identify microbial OTUs that differed significantly between control and HFD groups. Twenty-one OTUs were significantly enriched or decreased in HFD samples. The OTUs affiliated to *Flectobacillus*, *Runella*, *Flavobacterium* and the *NS11-12 marine group*, Bacteroidetes phylotypes and to *Acidovorax*, *Rhizobiales* and *Acinetobacter*, Proteobacteria phylotypes, increased in HFD samples. In turn, OTUs affiliated to Proteobacteria, *Rhodobacteraceae*, *Meganema* and *Rhizobiales* phylotypes, Actinobacteria, *Mycobacterium* phylotype, and to Firmicutes, *Finegoldia* phylotype, were depleted (Fig. 3).
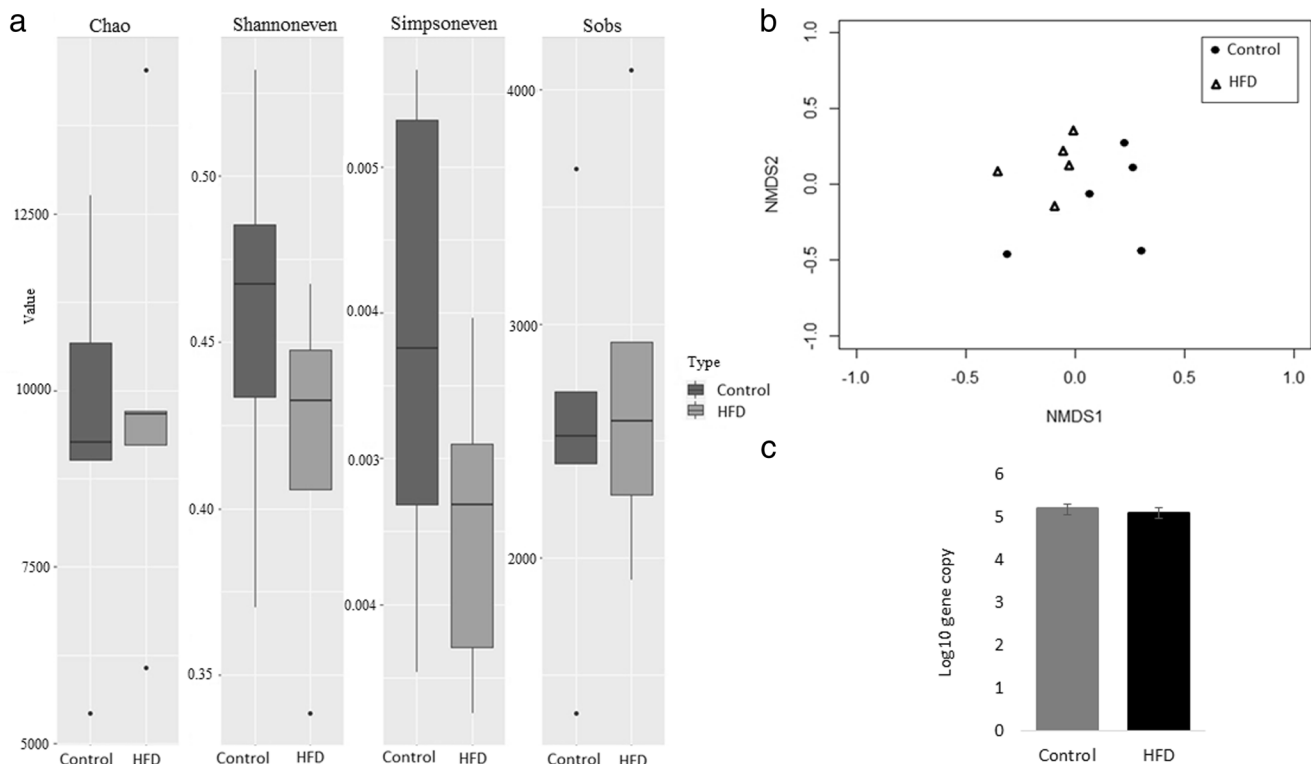
Fig. 1 **a** Estimates of alpha-diversity represented in boxplots. **b** Non-metric multidimensional scaling plot (using Bray Curtis dissimilarity) of Control and HFD samples. Lower stress = 0.115; $R^2 = 0.894$; $p = 0.317$, calculated via the AMOVA test. **c** Total number of bacteria (log 16S rRNA gene copies/larva) in control and HFD zebrafish larvae ($n = 10$). Results represent the mean values of each condition in the vertical bars. The error bars indicate the standard error of the mean
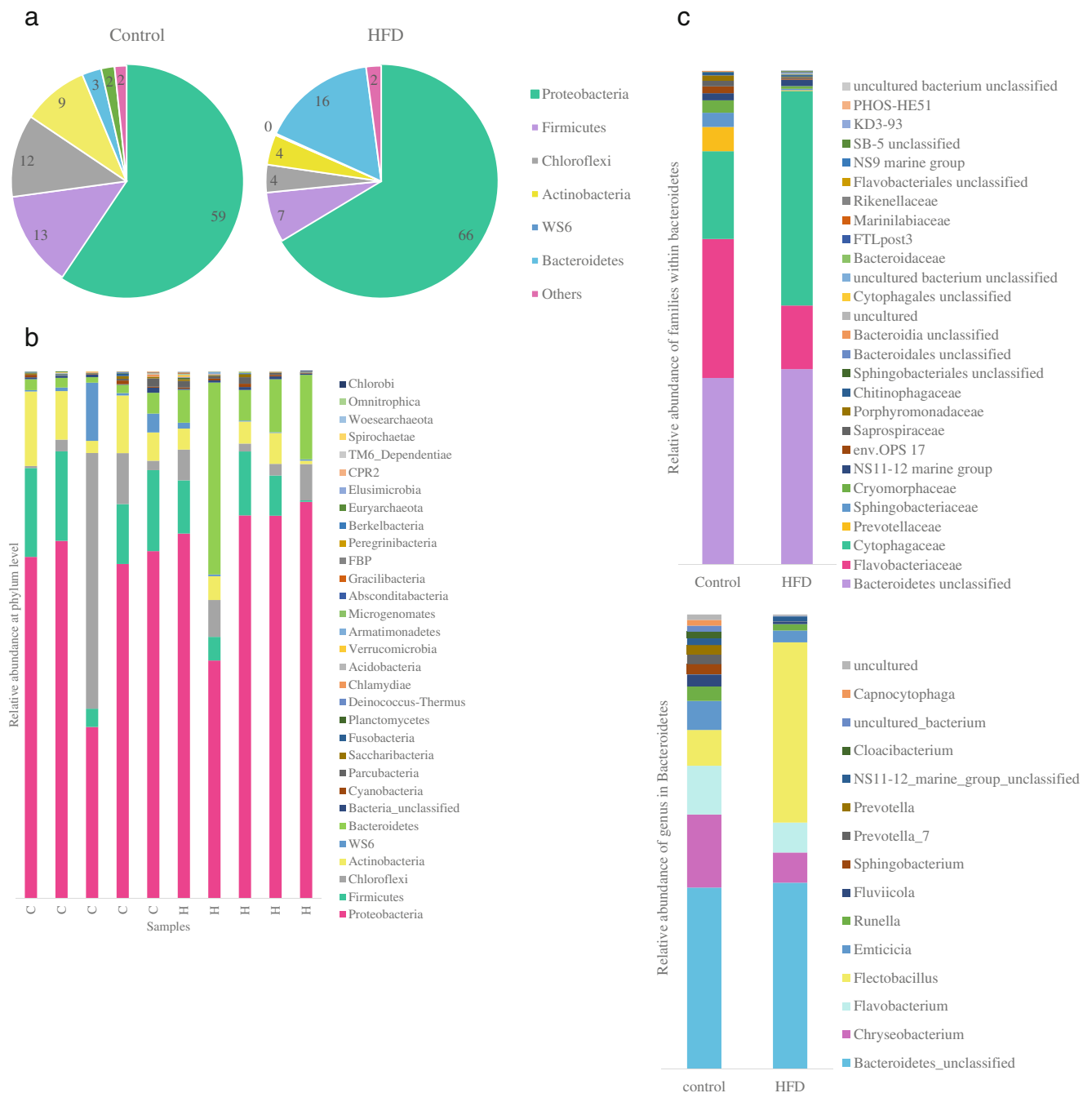
Fig. 2 **a** Relative abundance at the phylum level in larvae fed a control diet and the HFD, represented in a stacked bar chart. **b** Pie chart representing the mean of control and HFD phyla. **c** Relative abundance of Bacteroidetes at family and genus level

The OTU 16, related to the genus *Flectobacillus*, a member of the phylum Bacteroidetes, was the most enriched OTU in HFD samples.

The Consumption of a High-Fat Diet Induces the Overexpression of Immunity-Related Genes

We assessed the expression of 16 genes related to the zebrafish immune system by qPCR at 30 dpf to test the effect of the HFD on the immune system. Specifically, *NOD1* and

TLR2, *TLR4* and *TLR5* are host receptors involved in the first contact and interaction with microbiota; *ASC* and *CASP1a* are essential parts of the NPLR3 inflammasome; *NFKB*, *IL10*, *IL22* and *MyD88* take part in cellular signalling and the triggering of inflammation; *IL1β* and *TNF* are cytokines associated to the inflammatory cascade; finally, *iap*, *Defensin 1*, *MMP9* and *MPO* are antimicrobial peptides secreted by the host in response to microbial attacks.

Compared with the control, the expression levels of 12 of the genes studied were markedly upregulated in HFD
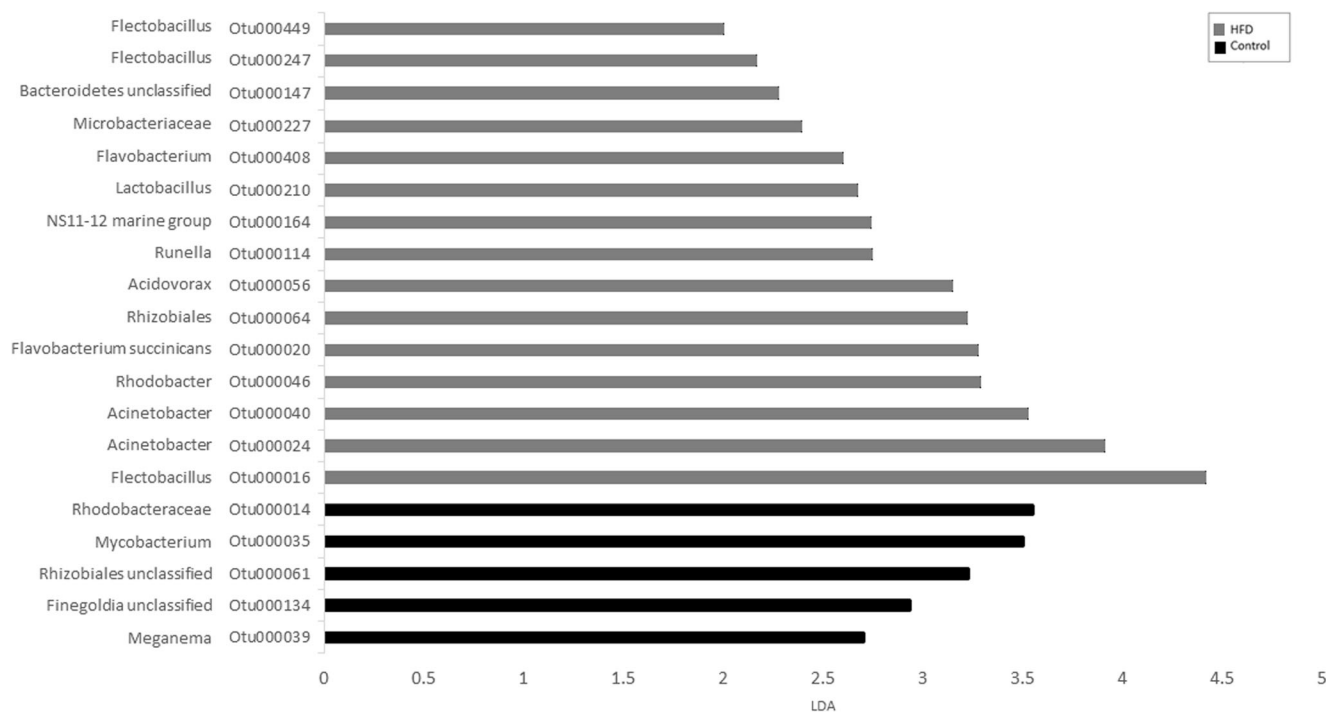
Fig. 3 Linear Discriminant Analysis (LDA) effect size (LEfSe). OTUs and associated OTU taxonomy significantly enriched in HFD are plotted in black, whereas grey bars correspond to OTUs enriched in the control samples. Differences were considered statistically significant at $p < 0.05$

samples. The HFD consumption induced an immune response mediated by $IL1\beta$ activation by the canonical NF-κβ pathway, as $MyD88$, $NFKB$ and finally $IL1\beta$ were significantly upregulated in HFD samples. Conversely, the NLRP3 inflammasome was not affected in HFD-fed animals, as $CASPa$ and ASC gene expressions did not change. Moreover, TNF and $IL22$, pro-inflammatory cytokines, were also enhanced in HFD samples. Changes in the microbial composition, induced by HFD consumption, also increased the expression of $TRL2$, $TLR5$ and $NOD1$ genes, receptors implicated in host-microbe interactions, and iap, MPO, $MMP9$ and $Defensin\ 1$, genes related to the secretion of anti-microbial peptides as a defence strategy (Fig. 4).

Alcian Blue Staining and Histology Confirm an Emerging Inflammation in the Gut Induced by High-Fat Diet Consumption

Alcian blue staining of whole-mount larvae at 3 dpf revealed a statistically significant increase in mucus production due to HFD consumption ($p < 0.05$; Fig. 5a, b). Furthermore, to re-inforce these observations, alcian blue was also used to stain histological preparations of larvae intestines in order to observe mucus-producing goblet cells. A statistically significant increase in goblet cells was observed in HFD-fed animals at 50 μm, based on histological preparations ($p < 0.01$; Fig. 5c), counting an average of 8.6 compared to 1.8 goblet cells per crest in HFD and control animals, respectively (Fig. 5d, e). Moreover, a moderate loss of mucosal architecture was found

in the intestinal epithelium of two out of five HFD-fed larvae with a score of 1 (Fig. 5h), observing apical brush border cell fusion (Fig. 5f, g). We observed no infiltration of inflammatory cells, irrespective of the diet.

The High-Fat Diet-Induced Microbiota Changes Are Correlated to Inflammation

We found a positive correlation among microbial groups, which shifted between diets, gene expression and goblet cell count (Fig. 6). The major correlations were identified among TNF, $MyD88$, $IL1\beta$ genes and goblet cell count with Bacteroidetes. The increase in TNF and $MyD88$ gene expression levels was positively correlated to the enrichment of Bacteroidetes (genus $Cytophaga$ and OTUs affiliated to $Flectobacillus$ and $Flavobacterium$ (16, 20, 408, 247 and 449); $R = .66$–0.79; $p < 0.05$) and Proteobacteria (OTUs 40, 46 and 64 affiliated to $Acinetobacter$, $Rhodobacter$ and $Rhizobiales$; $R = 0.65$–0.88; $p < 0.05$). The upregulation of the $NFKB$ gene was correlated to the increase of Bacteroidetes-affiliated taxa, the genus $Cytophaga$, $Flectobacillus$ OTU 247 and Bacteroidetes unclassified OTU 147. The most correlated gene, $IL1\beta$, was strongly associated to the Bacteroidetes genera $Flectobacillus$, $Emitricia$, $Runella$, $Cytophagaceae$ unclassified, $UKL\ 13.3$, the $NS11\text{-}12$ $marine\ group$ and the associated OTUs 16, 449, 114, 164, 480 and 147 ($R = 0.70$–0.93; $p < 0.05$). In addition, the overexpression of $IL1\beta$ was also correlated to the Proteobacteria-affiliated OTU 40 ($R = 0.40$–0.66; $p < 0.05$). The OTU 24,
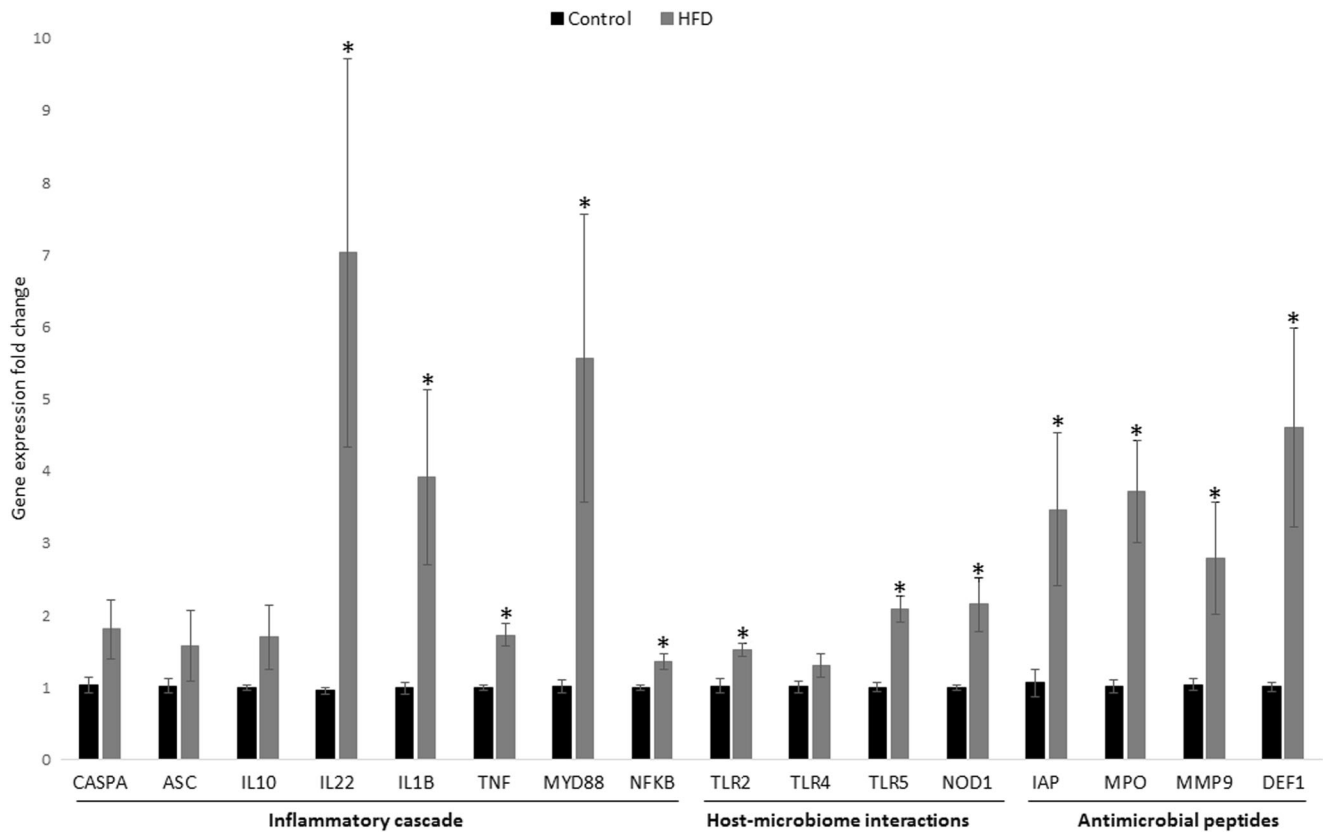
Fig. 4 Gene expression fold change analysis after 25 days of diet exposure (30 dpf) in control and HFD larvae ($n = 240$) (highlighted with different grey gradients). Error bars indicate the standard error of the mean. Differences were considered statistically significant at $p < 0.05$ (*)

related to *Acinetobacter*, was the most correlated OTU in the experiment, with the major correlation coefficients being related to the genes *IL22*, *CASPA*, *IL10* and *ASC* ($R = 0.90$–0.96; $p < 0.05$).

Furthermore, the increase in goblet cells in HFD samples was significantly positively related with the enrichment of Bacteroidetes *(Runella, Pseudarcicella* and the *NS11-12 marine group*, OTUs 20, 114 and 164; $R = 0.65$–0.93; $p < 0.05$) and to Proteobacteria (OTUs 40, 46, 64 and 56; $R = 0.65$–0.81; $p < 0.05$). Cellular receptors *TLR 2, 5* and *NOD1* were positively correlated to *Cytophagaceae* unclassified (TLR5) and *Flectobacillus* genus, and OTUs 24 (NOD1 and TLR2) and 247 (TLR2 and TLR5) related to *Acinetobacter* and *Flectobacillus* ($R = 0.58$–0.93; $p < 0.05$). No significant negative correlations were found. Significant correlations are represented in Fig. 6.

Discussion

In the present study, we report that the consumption of a 10% fat diet, enriched with cocoa butter (HFD), induces intestinal microbiota dysbiosis and inflammation in zebrafish larvae. Cocoa butter-enriched diet, which is mainly composed by

saturated fatty acids, has been described to induce body fat accumulation in zebrafish [31]. In addition, there is a strong evidence supporting that an overconsumption of nutrients initiates and triggers meta-inflammation, altering metabolic homeostasis [49]. Our results are in accordance with studies carried out in rodent models which showed that HFD consumption leads to intestinal inflammation, related to microbial dysbiosis [4]. Up to date, diet studies performed in zebrafish have focused on the effect of diets on other physiological aspects [8, 50]. Furthermore, most of these experiments were performed in adults [31].

In this work, we used zebrafish larvae to study the effects of a HFD on the intestinal microbiota and inflammation, taking into consideration that the larval adaptive immune system is not fully mature until 4–6 weeks post-fertilisation, and therefore does not interfere with the innate [51]; in addition, the transparency of the larvae allows whole-mount microscopy. The zebrafish anterior-mid and posterior gut segments are functional analogues of the mammalian small and large intestines. In addition, most of the differentiated epithelial cell types found in mammals and the architectural organisation of the intestines are also conserved [52].

As previously shown, the gut microbiota can be directly or indirectly affected through diet in humans [53], mice [54] and

Fig. 5 **a** and **b** Alcian blue-stained control larva and HFD larva. Arrows indicate goblet cells stained blue. **c** Percentage of alcian blue-stained area in control and HFD larvae, represented as the mean of three replicates; error bars indicate the standard error of the mean ($n = 20$). Differences were considered statistically significant at $p < 0.05$ (*). **d** and **e** Goblet cells stained with alcian blue in control and HFD intestine histological preparations at 50 μm. Arrows show goblet cells. **f** and **g** Histological damage in the HFD zebrafish intestine, haematoxylin- and eosin-stained at 50 μm. Arrows show enterocyte degeneration points. **h** Histological assessment of intestinal damage. Values represent means ± SEM, with n being the number of larvae ($n = 10$). Differences were considered statistically significant at $p < 0.01$ (**)
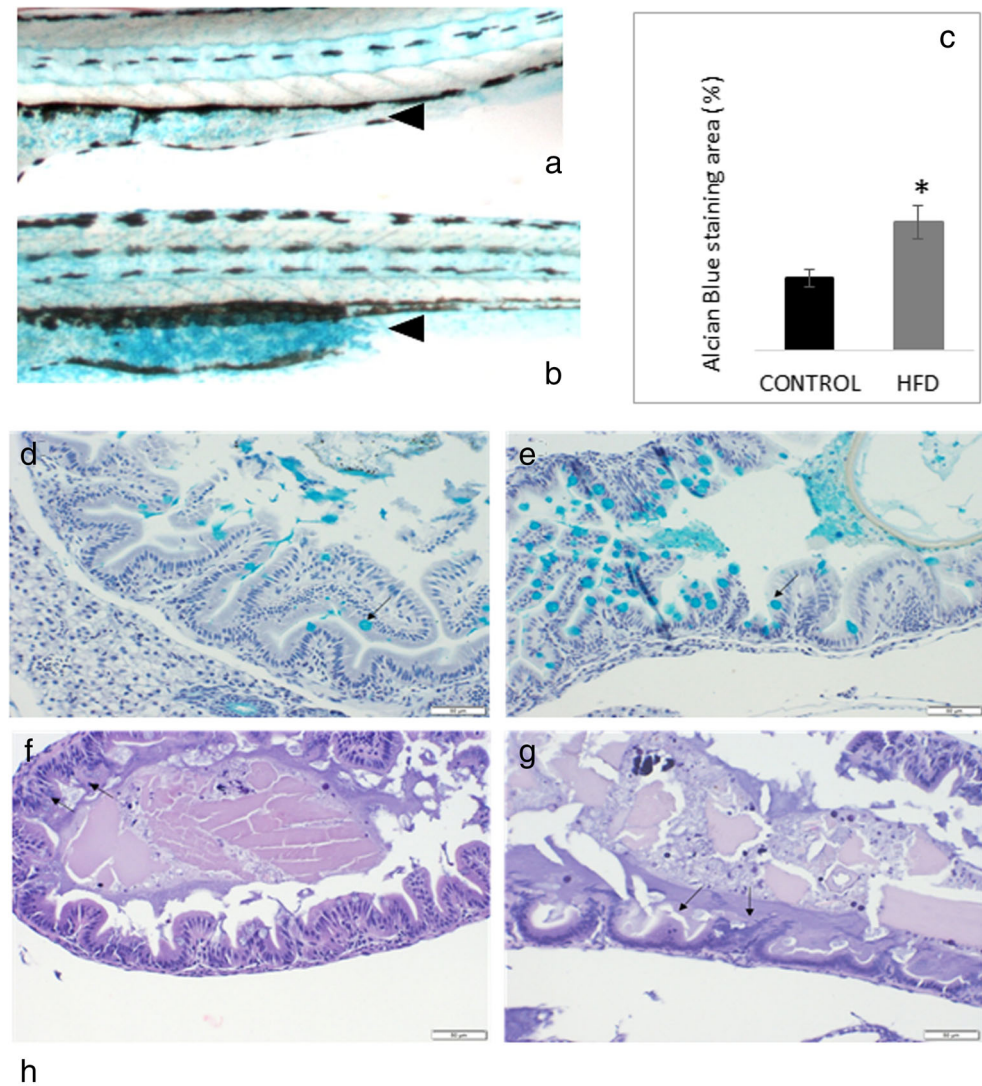

Histological assesment of intestinal damage	Control	HFD
Loss of mucosal architecture	0	0.4
Cellular infiltration	0	0
Average of goblet cells per crest	1.80±0.20	8.60±0.51**

zebrafish [8], which can in turn influence host physiology [55]. In this report, the consumption of a high-fat diet did not affect the total number of bacterial copies and the alpha and beta community diversity of 30 dpf zebrafish larvae, as previously described in reports studying the relationship between the intestinal microbiota and a high-fat diet or chemically-induced inflammation [8, 10, 56]. Although Wong et al. [8] did not report alpha diversity changes with diets, they detected a significant dietary fat effect on the beta diversity at 35 and 75 dpf when HFD-fed larvae (28% fat)

were compared to larvae fed a low-fat diet (10% fat). In this work, the enrichment of the diet with 10% of cocoa butter did not affect the diversity of the community. However, the microbial composition changed at different taxonomic levels, which correlated with an acute inflammation. Thus, nutritional intervention from an early stage could lead to a difference in the gut microbiota assembly, with potential consequences on host physiology in the adult stage [8]. The phylum Bacteroidetes was increased via the HFD. At the OTU level, the majority of the OTUs significantly enriched in HFD

Fig. 6 Correlation plot showing Spearman's correlations between gene expression fold changes, goblet cell count and significantly different genera within Bacteroidetes and LEfSe; OTUs are represented by coloured dots. Positive correlations are in blue. Only significant correlations are represented ($p < 0.05$)
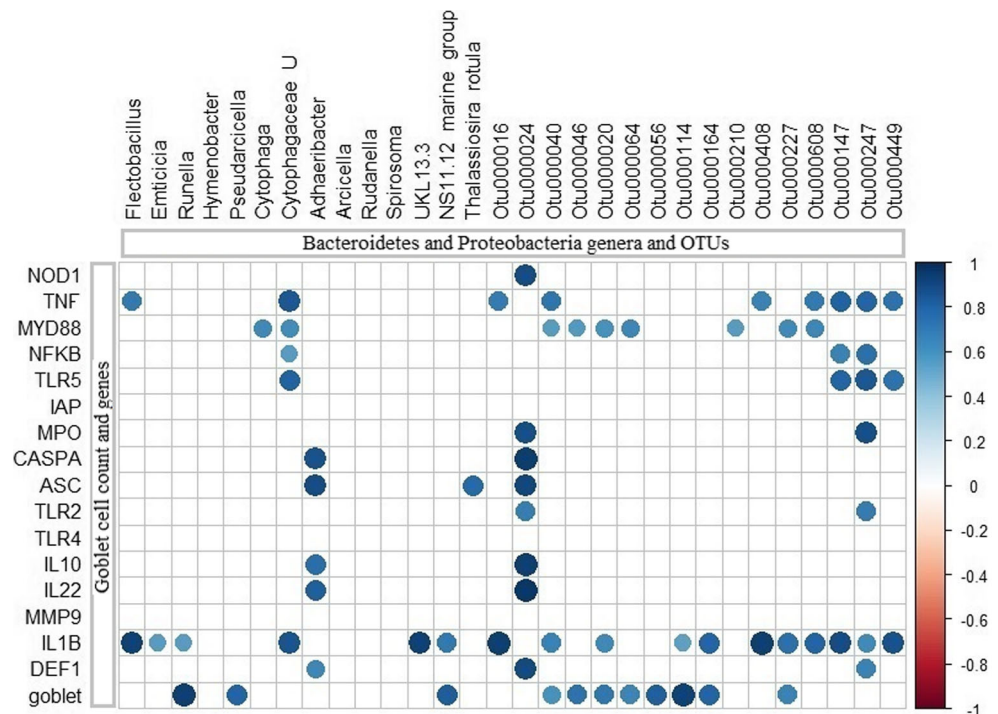
samples were affiliated to Bacteroidetes of the genera *Flectobacillus*, *Flavobacterium* and *Runella*. Some other increased OTUs were members of Proteobacteria, namely of the genera *Acinetobacter*, *Rhizobiales*, *Acidovorax* and *Rhodobacter*, the most abundant phyla in all zebrafish developmental stages [7]. Proteobacteria and Firmicutes tended to increase and reduce, respectively, as described by Qi He et al. [56]. They also related the overrepresentation of Proteobacteria and the lack of Firmicutes to the upregulation of TNF and, finally, to intestinal inflammation induced by chemicals [56]. As expected, the diet-induced inflammation only slightly affected the microbiota compared to chemicals.

Obesity-induced inflammatory mechanisms in human involve the activation of genes encoding for cytokines, chemokines and other immune inflammatory mediators through the activated transcription factors *NFKB* and inflammasome, resulting in the proteolytic conversion of pro-IL1β to activated IL1β [57]. Zebrafish possess both innate and adaptive immunity. In addition, key mediator-signalling proteins and cytokine [58] as well as the major mammalian blood cell lineages [25] have been identified in the zebrafish. The canonical NF-κβ pathway and NLRP3 inflammasome are also conserved between zebrafish and mammals [10, 59].

In the present study, we observed the activation of the *IL1β* gene by the canonical NF-κβ pathway, suggesting that in zebrafish larvae, IL1β might be activated by the consumption of HFD at least by this pathway. This is in agreement with Landgraf et al. [6], who observed HFD-induced metabolic alterations in adult zebrafish (e.g. hyperglycaemia and ectopic

lipid accumulation in the liver and a metabolically unhealthy adipose tissue phenotype with adipocyte hypertrophy), accompanied by changes in the expression of marker genes such as *IL1β* [6]. It would be very interesting to corroborate this information with inflammatory metabolite measures. Our results suggest that HFD consumption induced changes in the relative abundance of Bacteroidetes. Specifically, these changes enriched the genera *Flectobacillus*, *Cytophaga* and *Runella*, which may activate an inflammatory response mediated by *IL1β* via the NF-κβ pathway. Furthermore, shifts in Proteobacteria in HFD samples were correlated to the overexpression of the *TNF* gene, as previously described in a chemically induced inflammation [56]. The activation of *IL1β* via the NPLR3 inflammasome was not upregulated. However, there was a strong correlation between Proteobacteria OTU 24, affiliated with *Acinetobacter*, ASC and *CASP1a*; NPLR3 components needed to activate this pathway. A major increase of Proteobacteria might correlate with the activation of the inflammasome.

In addition, to initiate the inflammation in human and mice, the binding to cellular receptors of endogenous danger-associated molecular patterns (DAMPs) is required [60]. Orthologs of mammalian TLR and NLR have been identified in zebrafish [58]. Microbial signals such as LPS and lipid compounds are able to bind to these receptors, inducing expression [61, 62]. Dietary fatty acids affect the development of many human chronic diseases, in part mediated through the modulation of TLR [63]. Dietary saturated fatty acids such as palmitate, stearate and oleate, the main components of cocoa butter, used to enrich the diet in the current study (HFD), can

activate *TLR2* and *TLR4*, stimulating the expression of *IL1β* via NF-κβ [64]. As a result, other molecules involved in the inflammatory process, such as *MyD88* or *MMP9* [65], are also activated, as observed in our experiment. These interactions are well described in diabetes [66] or obesity [64] in human. In the present study, the genes *TLR5*, *TLR2* and *NOD1* were overexpressed, correlating to the increase of Bacteroidetes and Acinetobacter. Altogether, these results suggest that the consumption of a high-fat diet activates the host immune response via the NF-κβ pathway, initiated by cellular receptor stimulation through changes in the microbial community.

The inflammation process also triggers the secretion of a vast number of molecules by the host, which are required for the maintenance of the intestinal barrier function, a viscoelastic protective layer composed of mucins secreted by goblet cells [67], defensins (including Defensin 1) [68, 69], matrix metalloproteinases (MMPs) [70] and enzymes such as iap [71]. The increase in antimicrobial peptides (AMP) (*MMP9*, *MPO*, *iap* and *Defensin 1)* gene expression levels, as occurred in chemically and microbiologically induced intestinal inflammation [23, 69, 72], together with the increase in mucus production by goblet cells, correlated with the increase in Bacteroidetes and Proteobacteria. Il-22, a cytokine with protective effect, regulates the mucus production by goblet cells in mice [73]. The gene encoding for this cytokine was the most upregulated in the experiment. These results suggest that the host defence mechanisms are active and regulate the extracellular matrix, protecting the epithelium from the inflammation induced by HFD consumption. In contrast, intestinal tissue damage was not observed in histological preparations, as reported by Falcinelli et al. [74]. Changes exerted by the diet are not as aggressive as alterations induced by chemicals or antibiotics [75], as a detectable injury in the intestinal barrier has not yet developed.

Host physiological and morphological changes during development have significant effects on microbiota [8]. We suggest that the maturation of the innate immune system at 4–5 weeks post-fertilisation, together with the microbial colonisation and evolution in zebrafish, might be related to some of these changes and powerfully affected by the diet. Therefore, the use of this diet-induced inflammation larvae model might be highly recommended to understand how dietary compounds interact with the microbiota and the host innate immune system, with the aim to mitigate the inflammation process. In the present work, we have achieved a dietary-induced inflammatory state, correlated to microbial changes, in 30 days, whereas dietary experiments in adults generally last 6–8 weeks [31, 76]. In contrast, it is of tough to remove and analyse each organ due to the size of the larvae, being complex to separate local from global effects. Performing the experiment with zebrafish adults might solve this limitation. Nevertheless, the easy and cheap maintenance, the reproducibility and the high number of individuals achieved per spawning confer the high potential of the larvae model.

In conclusion, the present study supports the assumption that in zebrafish, the consumption of a HFD over the period of 1 month leads to microbiota dysbiosis and inflammation. We suggest that the fatty acids present in a 10% fat diet, enriched with cocoa butter, could modify the intestinal ecosystem, changing the microbial composition and favouring the increase of Bacteroidetes and Proteobacteria. These bacterial changes activated the innate immune system, inducing an inflammation via NF-κβ activation and the secretion of protective molecules by the host. We therefore demonstrated that zebrafish larvae are a reproducible and adequate model to test nutritional interventions in early stages; this model allows us to study the effects of a particular diet on microbiota composition and the innate immune system.

Compliance with Ethical Standards

Conflict of Interest   The authors declare that they have no conflicts of interest.

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