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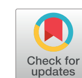
Fortifying diet with rapeseed oil instead of butterfat attenuates the progression of diet-induced non-alcoholic fatty liver disease (NAFLD) and impairment of glucose tolerance



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ABSTRACT

Background: Absolute dietary fat intake but even more so fatty acid pattern is discussed to be critical in the development of non-alcoholic fatty liver disease (NAFLD). Here, we determined if switching a butterfat enriched diet to a rapeseed oil (RO) enriched diet affects progression of an existing NAFLD and glucose intolerance in mice.
Methods: For eight weeks, female C57Bl/6J mice were either fed a liquid control (C) or a butterfat-, fructose- and cholesterol-rich diet (BFC, 25E% butterfat) to induce early signs of steatohepatitis and glucose intolerance in mice. For additional five weeks mice received either BFC or C or a fat-, fructose- and cholesterol-rich and control diet, in which butterfat was replaced with RO (ROFC and CRO). Markers of glucose metabolism, liver damage and intestinal barrier were assessed.
Results: Exchanging butterfat with RO attenuated the progression of BFC diet-induced NAFLD and glucose intolerance. Beneficial effects of RO were associated with lower portal endotoxin levels and an attenuation of the induction of the toll-like receptor-4-dependent signaling cascades in liver. Peroxisome proliferator-activated receptor γ activity was induced in small intestine of ROFC-fed mice.
Conclusion: Taken together, exchanging butterfat with RO attenuated the progression of diet-induced steatohepatitis and glucose intolerance in mice.
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1. Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) has increased within the last 20 years affecting by now ~25% of the global population [1]. NAFLD encompasses a broad spectrum of liver diseases ranging from simple steatosis to inflammatory steatohepatitis (NASH) and even cirrhosis as well as hepatocellular carcinoma [2]. As NAFLD is considered to be a slowly progressing disease with the time of the development of liver phenotype in most patients ranging from 7 to 14 years per stage of hepatic fibrosis, there seems to be a large window of opportunity to provide care for afflicted patients [3]. However, to date, universally accepted therapeutic approaches are still lacking.

Genetic predisposition and overnutrition along with overweight and insulin resistance are among the main factors discussed to contribute to the development of NAFLD (for overview see [4]). Furthermore, certain dietary patterns like a so called `Western-type dietary pattern´ composed of highly processed foods, sweets and sugar-sweetened beverages as well as red meat and refined grains, all adding to a low fiber but high sugar and saturated fat intake, are also discussed to be critical in the onset and progression of NAFLD [5–7]. Indeed, it has been shown that compared to isocalorically nourished healthy individuals, patients with NAFLD not only consume less fiber but also more saturated fatty acids (SFA) while intake of polyunsaturated fatty acids (PUFA) was lower [8]. Results of several meta-analyses further suggest that fortifying diets with n3 PUFA resulting in an improved ratio of n6 to n3 fatty acids in diet, respectively, may improve the liver function of NAFLD patients [9,10].

Abbreviations: 4-HNE, 4-hydroxynonenal; Acc, acetyl-CoA carboxylase; ALT, alanine transaminase; ANOVA, analysis of variance; AST, aspartate transaminase; AUC, area under the curve; BFC, butterfat-, fructose- and cholesterol-rich diet; C, control diet; CRO, control diet with rapeseed oil; Cramp, cathelicidin-related antimicrobial peptide; Fas, fatty acid synthase; GTT, glucose-tolerance-test; Il, interleukin; Ir, insulin receptor; Irs, insulin receptor substrate; Muc, mucin; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NOS, nitric oxide synthase; NS, not significant; PAI-1, plasminogen activator inhibitor-1; PPARγ, peroxisome proliferator-activated receptor γ; PUFA, polyunsaturated fatty acids; ROFC, rapeseed oil-, fructose- and cholesterol-rich diet; RO, rapeseed oil; Scd1, stearoyl-CoA desaturase-1; SFA, saturated fatty acids; SEM, standard error of mean; Srebp1c, sterol regulatory element-binding protein-1c; Tlr4, toll-like receptor-4; TNFα, tumor necrosis factor α.

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Starting from this background the aim of the present study was to determine if exchanging fat sources e.g. from butterfat being rich in SFA to rapeseed oil (RO) attenuates the progression of early signs of NASH and associated glucose intolerance in mice.

2. Experimental section

2.1. Animals and treatment

Female C57Bl/6J mice (8 weeks old) purchased from Janvier (SAS, Le Genest-Saint-Isle, France) were used to perform mouse experiments. Female mice were shown to be more susceptible to fructose-induced NAFLD [11] and results of the meta-analysis of Prendergast et al. further suggest that variability of traits and parameters is similar between male and female mice [12]. All procedures were approved by the local institutional animal care and use committee ('Landesamt für Verbraucherschutz', reference number: 02-004/16, Thuringia, Germany). Animals were handled in accordance to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Mice were housed in groups under controlled conditions in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and had free access to tap water at all times (12 h/12 h light/dark cycle, ~23 °C, ~65% rel. humidity). Calculation of number of cases was based upon previous findings [13,14]. After acclimatization to the facility, mice ($n = 6$–8/group, total of 4 groups) were either fed a liquid control diet (C; 69E% carbohydrates, 12E% fat (soybean oil), 19E% protein; Ssniff, Soest, Germany) or a liquid butterfat-, fructose- and cholesterol-rich diet (BFC; 60E% carbohydrates, 25E% fat derived from butterfat, 15E% protein with 50% wt/wt fructose and 0.16% wt/wt cholesterol; Ssniff, Soest, Germany) for 8 weeks, respectively. In week 9 of feeding, mice were randomly assigned to the following groups: mice continued to be fed control diet, mice continued to be fed BFC, mice being fed control diet in which fat source was exchanged to rapeseed oil (12E%) (CRO) and mice being fed a fat-, fructose- and cholesterol-rich diet in which fat source was exchanged to rapeseed oil (25E%) (ROFC) (study design see Fig. 1). Energy and nutritional composition of the diets is summarized in Supplementary Table A1 and was previously described in detail [13,14]. Mice fed control diets and those fed fat-, fructose- and cholesterol-rich diets, respectively, were pair-fed to ensure equal caloric intake as described in detail previously [14]. Dietary intake was assessed daily and body weight was assessed weekly. After 7 and 12 weeks of feeding and a 6 h fasting, mice were anesthetized with ketamine/xylazine solution and a glucose-tolerance-test (GTT) was performed as described before [14]. After 13 weeks of feeding, mice were again anesthetized (i.p.) with ketamine/xylazine in the morning, blood was collected from portal vein and animals were killed. Liver, muscle and intestinal tissue was collected to assess markers of glucose metabolism, liver damage and intestinal barrier. All further measurements as well as sacrifice were carried out in randomized order.

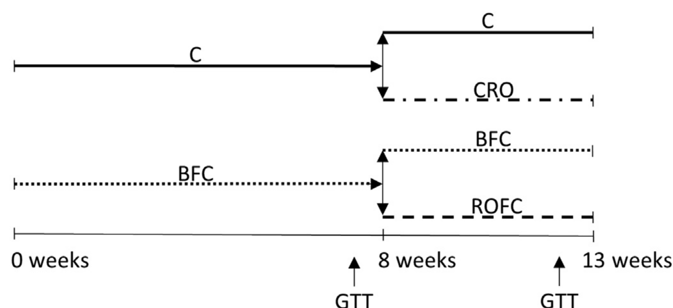

Fig. 1. Summary of study design. BFC: butterfat-, fructose- and cholesterol-rich diet, C: control diet; CRO: control diet with rapeseed oil; ROFC: rapeseed oil-, fructose- and cholesterol-rich diet; GTT: glucose-tolerance-test.

2.2. Evaluation of liver damage and inflammation

Alanine and aspartate transaminase (ALT, AST) activity in plasma was measured in a routine laboratory (University Hospital Jena, Jena, Germany). Hematoxylin and eosin staining was used to assess status of liver damage in paraffin-embedded liver sections (4 μm) using NAFLD activity score (NAS) [15,16]. Triglycerides from hepatic liver tissue were extracted and measured as described previously [17]. Neutrophil granulocytes were stained in liver sections using a commercially available staining kit (Naphthol AS-D Chloroacetate kit, Sigma-Aldrich, Steinheim, Germany) [17]. Immunohistochemical staining was used to assess F4/80 positive cells and 4-hydroxynonenal (4-HNE) protein adducts in liver sections as previously described [18,19]. Numbers of neutrophil granulocytes and F4/80 positive cells were counted per microscopic field using a camera integrated in a microscope (Leica DM4000 B LED, Leica, Wetzlar, Germany), while staining of 4-HNE protein adducts was evaluated with an analysis system (Leica Application Suite, Leica, Wetzlar, Germany) as detailed previously [17,19].

2.3. Endotoxin, NOS activity and free fatty acid measurement as well as ELISA measurements

Bacterial endotoxin concentration was determined using limulus amebocyte lysate assay as described previously [20] with recovery rates ranging from ~80% to ~120%. All measurements were within the detection range and the standard curve of the assay. Plasminogen activator inhibitor-1 (PAI-1) and tumor necrosis factor α (TNFα) concentration in liver homogenate were measured using commercially available ELISA kits (PAI:1: LOXO GmbH, Dossenheim, Germany, TNFα: Assaypro, St Charles, MO, USA). Peroxisome proliferator-activated receptor γ (PPARγ) activity was measured in whole-cell extract (nuclear extract kit, Active motif, La Hulpe, Belgium) of proximal small intestine using a commercially available kit (TransAM PPAR γ Transcription Factor Assay Kit, Active Motif, La Hulpe, Belgium) as detailed previously [21]. Nitric oxide synthase (NOS) activity in homogenate of intestinal tissue was assessed using a commercially available kit (abcam, Cambridge, UK). Free fatty acids levels in plasma were quantified using a commercially available kit (Free Fatty Acid Quantitation Kit, Sigma-Aldrich, Steinheim, Germany).

2.4. Arginase activity

Arginase activity was measured as previously described [22]. In brief, liver tissue was homogenized in a lysis buffer (0.1% Triton X-100, protease inhibitor cocktail, 50 mM Tris-HCl (pH 7.5) and 10 mM $MnCl_2$), 50 μl of 0.5 M arginine (pH 9.7) was added to 50 μl supernatant and was incubated at 37 °C. Reaction was stopped with 800 μl of H_2SO_4:H_3PO_4:H_2O (1:3:7), 50 μl of 9% α-isonitrosopropiophenone was added and samples were incubated at 100 °C followed by an incubation at 60 °C. Concentration of urea was measured at 540 nm using a plate reader (SpectraMax, Molecular Devices, San Jose, CA, USA).

2.5. Goblet cell staining

Goblet cells were stained with an Alcian Blue and periodic acid-Schiff staining in paraffin-embedded proximal small intestine (4 μm) as previously described [23]. Number of total goblet cells per 100 μm villus was counted using a camera integrated in a microscope (microscope: Axio Lab, camera: Axiocam, Carl Zeiss Microscopy GmbH, Jena, Germany).

2.6. RNA isolation and real-time RT-PCR

RNA from liver, muscle and intestinal tissue was extracted using peqGOLD Trifast (Peqlab, Erlangen, Germany). After assessing the concentration of RNA, cDNA was synthetized (Reverse Transcription

System, Promega GmbH, Madison, WI, USA). Using primers listed in Supplementary Table A2, real-time polymerase chain reaction (PCR) was performed to evaluate expression of respective genes normalized to 18S as previously described in detail [13].

2.7. Statistical analysis

Statistical evaluation was performed using PRISM (version 7.03, GraphPad Prism Software, San Diego, CA, USA). A Grubb's outlier test was performed before a Mann-Whitney test for the comparison of two groups or a two-factorial analysis of variance (ANOVA) to assess significant differences ($p < 0.05$) followed by Tukey's post hoc test were performed. In case of inhomogeneity of variances data were log-transformed. Data are presented as means $\pm$ standard error of mean (SEM).

3. Results

3.1. Effect of RO on markers of glucose metabolism

While food intake was significantly higher in BFC-fed mice compared to C-fed mice, body weight gain and absolute body weight did not differ between groups after eight weeks of feeding (see also Fig. 2, Supplementary Tables A3 & A4). Still, BFC-fed mice showed signs of glucose intolerance with fasting glucose levels and area under the curve (AUC) of GTT been all significantly higher in BFC-fed mice than in controls (Fig. 2, Supplementary Table A4). This effect of the BFC progressed over time. Indeed, after 12 weeks of feeding, fasting glucose levels and AUC of GTT were both significantly higher than in controls (Fig. 2) ($p < 0.05$ for all parameters). After 13 weeks of feeding, body weight and body weight gain were also significantly higher in BFC-fed mice than in controls (~ +2.2 g, $p < 0.05$). Caloric intake, body weight gain and absolute body weight were similar between BFC and ROFC groups (Fig. 2, Table 1, Supplementary Table A4). Fasting glucose levels were alike between BFC and ROFC-fed groups but significantly higher than in controls ($p < 0.05$ for all comparisons). In contrast, AUC of GTT was significantly lower in ROFC-fed mice when compared to BFC-fed mice continuously fed the BFC ($p < 0.05$). However, AUC of GTT of ROFC-fed mice were still higher than in both C-fed groups (Fig. 2). In line with these findings, mRNA expression of *insulin receptor substrate-1* (*Irs1*) was significantly and by trend lower in muscle tissue obtained from BFC-fed mice compared to both C-fed groups and ROFC-fed animals ($p < 0.05$ for C and CRO vs BFC, $p = 0.06$ for BFC vs ROFC). No differences were found between ROFC- and C-fed groups. Expression of *insulin receptor* (*Ir*) and *Irs2* was similar between all groups (Fig. 2).

3.2. Effect of RO on liver damage

In previous studies we showed that within 6–8 weeks of pair-feeding of mice with diets like the ones used in the present study, animals develop manifest steatosis and early signs of inflammation, progressing to early steatohepatitis after 13 weeks of feeding [14,24]. In the present study, when comparing steatosis score of C- and CRO-fed mice, CRO-fed mice were found to have significantly higher steatosis scores than C-fed mice ($p < 0.05$ for C vs CRO, Table 1). Indeed, despite isocaloric feeding and similar energy derived from fat, microvesicular fat accumulation was present in livers of CRO-fed mice. In line with these findings, hepatic triglyceride levels were also significantly higher in CRO-fed mice than in C-fed animals (Table 1). However, neither absolute body weight or weight gain, nor absolute liver weight, liver to body weight ratio or ALT and AST activity in plasma differed between control groups (Figs. 2, 3, Table 1, Supplementary Table A4). In livers of ROFC-fed mice macrovesicular fat accumulation, triglyceride concentration and number of inflammatory foci were significantly lower than in livers of BFC-fed animals (NAS: $p < 0.05$ for BFC vs ROFC). Still, in ROFC-fed mice signs of liver damage were significantly higher than in controls (Fig. 3, Table 1). Number of neutrophils and PAI-1 protein levels in liver tissue were similar between BFC and ROFC-fed groups and significantly higher than in both C-fed groups (Fig. 4, Table 2 and Supplementary Fig. A1). In contrast, number of F4/80 positive cells was only significantly higher in livers of BFC-fed mice compared to C-fed mice, while being at the levels of control groups in livers of ROFC-fed mice (Table 2). TNFα protein levels in liver tissue did not differ between groups; however, measurements varied considerable within groups (Table 2). Also, arginase activity, being an indication of macrophage polarization [25] was significantly lower in liver of BFC-fed mice compared to all other groups. However, arginase activity was also significantly lower in livers of CRO-fed and ROFC-fed mice than in C-fed animals, suggesting that RO per se affects hepatic arginase activity in mice, regardless of additional treatments (Table 2). Activities of transaminases in plasma were also significantly higher in BFC-fed animals when compared to both control groups, whereas in ROFC-fed mice, activities of ALT and AST in plasma were only significantly higher than in C-fed mice (Fig. 3).

3.3. Effect of RO on markers of lipogenesis in liver tissue

Expressions of *acetyl-CoA carboxylase* (*Acc*) and *stearoyl-CoA desaturase-1* (*Scd1*) mRNA in liver tissue were markedly higher in CRO-fed mice compared to C-fed mice ($p < 0.05$ for *Scd1*, $p = 0.09$ for *Acc*) while *fatty acid synthase* (*Fas*) mRNA expression was similar between C-fed groups (Table 2). *Acc* and *Fas* mRNA expressions were significantly higher in livers of BFC-fed mice than in both C-fed groups, while mRNA expression of these genes in livers of ROFC-fed mice was only significantly different from C-fed mice. Expression of *Scd1* mRNA in livers of BFC-fed mice was also significantly higher than in livers of C-fed animals and in those of ROFC-fed mice. Hepatic *Scd1* mRNA expression was also higher in ROFC-fed mice than in livers of C-fed animals. In contrast, *sterol regulatory element-binding protein-1c (Srebp1c)* mRNA expression in liver tissue was similar between groups (Table 2). Also, concentration of free fatty acids in plasma was similar between groups (Table 2).

3.4. Effect of RO on endotoxin levels in portal vein and the hepatic TLR4 signaling cascade

While bacterial endotoxin levels were similar between the two C-fed groups and ROFC-fed animals, bacterial endotoxin levels in portal plasma of BFC-fed mice were significantly higher than in CRO- and ROFC-fed animals. In line with these findings, expression of *Tlr4* mRNA was significantly higher in hepatic tissue of BFC-fed mice compared to C-fed mice, whereas *Tlr4* mRNA expression in livers of the ROFC group was similar to both control groups. Concentration of 4-HNE protein adducts was also significantly higher in livers of BFC-fed mice than in all other groups but was similar between C-, CRO- and ROFC-fed mice (Fig. 4, Supplementary Fig. A1).

3.5. Effect of RO on small intestine

Occludin mRNA expression was significantly lower in proximal small intestine of CRO-, BFC- and ROFC-fed mice when compared to C-fed animals. Total number of goblet cells in proximal small intestine was significantly lower in BFC-fed mice compared to both control groups, whereas number of goblet cells in ROFC-fed mice was similar to C and CRO-fed groups (Fig. 5, Supplementary Fig. A1). *Mucin-3 (Muc3)* mRNA expression was also significantly lower in small intestinal tissue of BFC-fed mice compared to C-fed mice. However, surprisingly, *Muc3* mRNA expression was also lower in small intestinal tissue of CRO-fed mice when compared to C-fed animals, while *Muc3* mRNA expression was similar between ROFC- and C-fed mice (Fig. 5). In contrast, *Mucin-2* mRNA expression in small intestine was similar between groups (data not shown). *Cathelicidin related antimicrobial peptide*

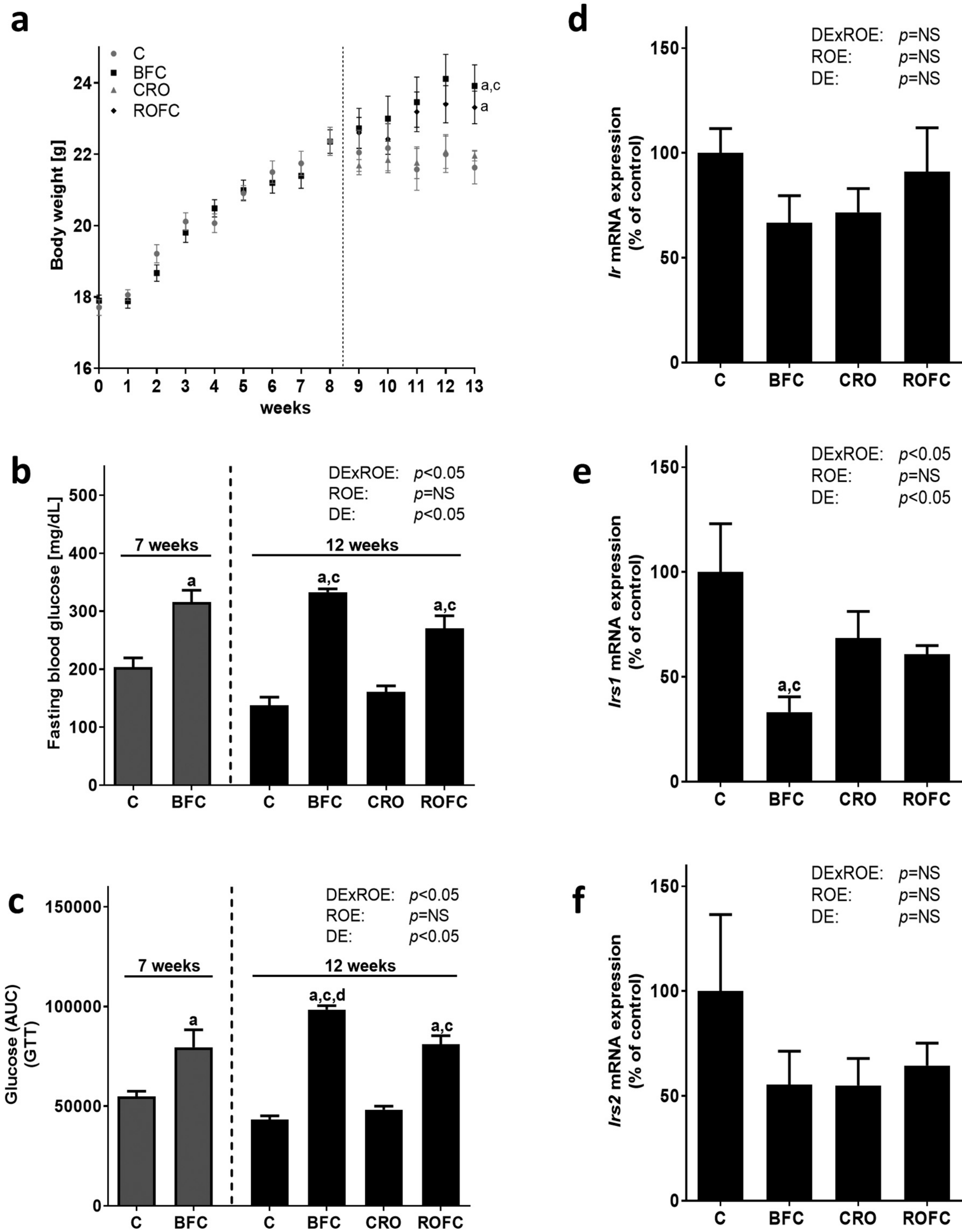

Fig. 2. Effect of exchanging the fat source in a fat-, fructose- and cholesterol-rich diet on body weight and markers of glucose metabolism. (a) Body weight during dietary treatment, (b) fasting blood glucose levels, (c) glucose levels during glucose-tolerance-test (GTT), mRNA expression of (d) *insulin receptor* (*Ir*), (e) *insulin receptor substrate-1* (*Irs1*) and (f) *Irs2* in muscle tissue. Data presented as means ± SEM, $n = 6$–8. BFC: butterfat-, fructose- and cholesterol-rich diet; C: control diet; CRO: control diet with rapeseed oil; DE: diet effect; DExROE: interaction between diet and rapeseed oil; NS: not significant; ROE: rapeseed oil effect; ROFC: rapeseed oil-, fructose- and cholesterol-rich diet. $^{a}p < 0.05$ compared with mice fed C; $^{c}p < 0.05$ compared with mice fed CRO; $^{d}p < 0.05$ compared with mice fed ROFC.

(Cramp) mRNA expression in proximal small intestine was significantly lower in BFC-fed and both RO-treated groups compared to C-fed mice (Fig. 5).

It has recently been shown that PPARγ may be a modulator of intestinal barrier function [26] and activated by orally ingested n3 fatty acids [27]. Indeed, while *PPARg1* mRNA expression was similar between groups, activity of PPARγ in small intestinal tissue was significantly higher in ROFC-fed mice than in all other groups (Fig. 6). *Interleukin 1b (Il1b)* mRNA expression was significantly and by trend higher in small intestinal tissue of BFC-fed mice than in all other groups (BFC vs C: $p = 0.06$, BFC vs CRO: $p < 0.05$, BFC vs ROFC: $p < 0.05$). In contrast, expression of *Il1b* in small intestinal tissue of ROFC-fed animals was at the

Table 1
Effect of exchanging the fat source in a fat-, fructose- and cholesterol-rich diet on body weight gain, food intake and markers of liver damage.

Parameter	Groups				*p*-Value		
	C	BFC	CRO	ROFC	DEx ROE	ROE	DE
Caloric intake [kcal/g BW/d]	0.39 ± 0.01	0.44 ± 0.01[a,c]	0.39 ± 0.01	0.43 ± 0.01[a,c]	NS	NS	<0.05
Weight gain [g]	3.6 ± 0.3	5.8 ± 0.4[a,c]	3.9 ± 0.2	5.7 ± 0.4[a,c]	NS	NS	<0.05
Liver weight [g]	0.9 ± 0.0	1.7 ± 0.1[a,c,d]	1.0 ± 0.0	1.3 ± 0.0[a,c]	<0.05	<0.05	<0.05
Liver:body weight ratio [%]	4.1 ± 0.1	7.1 ± 0.4[a,c,d]	4.5 ± 0.1	5.4 ± 0.1[a,c]	<0.05	<0.05	<0.05
Steatosis (NAS)	0.3 ± 0.1	2.4 ± 0.2[a,c,d]	1.1 ± 0.1[a]	1.6 ± 0.2[a]	<0.05	NS	<0.05
Inflammation (NAS)	0 ± 0	1.6 ± 0.1[a,c,d]	0 ± 0	1.0 ± 0.2[a,c]	<0.05	<0.05	<0.05
Triglycerides [μg/mg protein]	20.6 ± 3.1	111 ± 8.6[a,c,d]	52.0 ± 8.2[a]	81.5 ± 6.8[a,c]	<0.05	NS	<0.05

Values are means ± SEM. n = 6–8, [a]$p < 0.05$ compared with mice fed C; [c]$p < 0.05$ compared with mice fed CRO; [d]$p < 0.05$ compared with mice fed ROFC. BFC: butterfat-, fructose- and cholesterol-rich diet, C: control diet, CRO: control diet with rapeseed oil, DE: diet effect, DExROE: interaction between diet and rapeseed oil, NS: not significant, RO: rapeseed oil, ROE: rapeseed oil effect, ROFC: rapeseed oil-, fructose- and cholesterol-rich diet, NAS: NAFLD activity score.

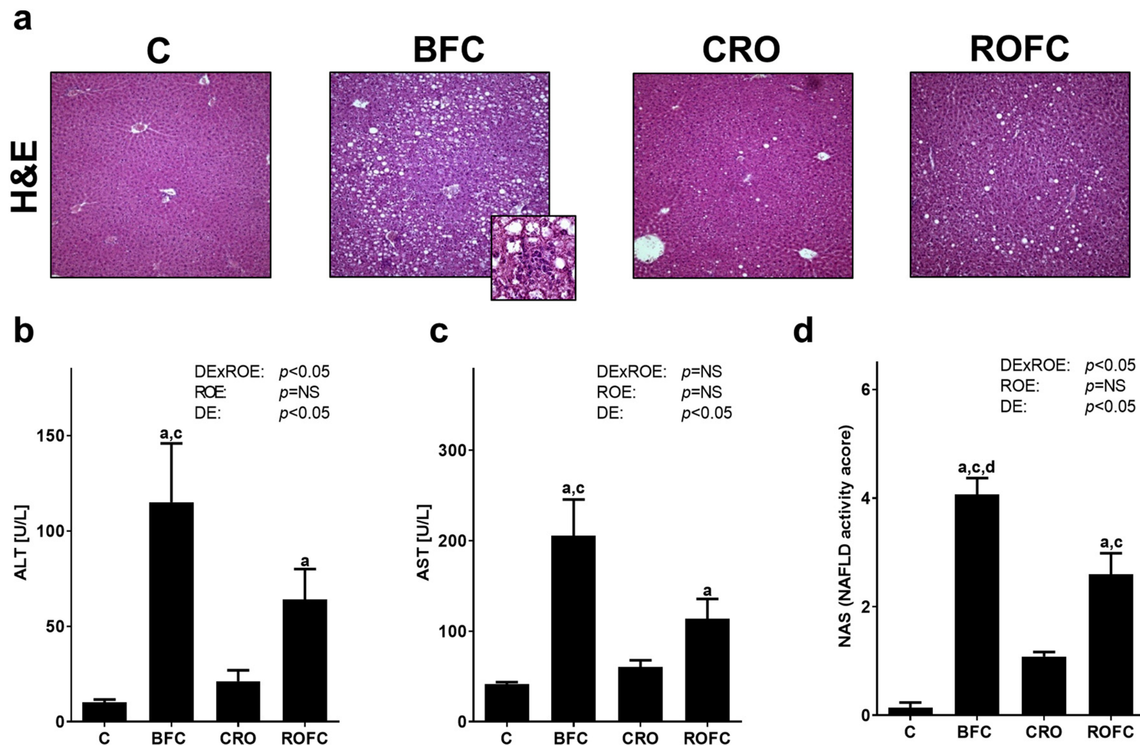

Fig. 3. Effect of exchanging the fat source in a fat-, fructose- and cholesterol-rich diet on markers of liver damage. (a) Representative pictures (200×, 630×) and (d) evaluation of hematoxylin and eosin (H&E) staining via NAFLD activity score of liver sections. (b) Alanine transaminase (ALT) and (c) aspartate transaminase (AST) activity in plasma. Data presented as means ± SEM, n = 6–8 (C group ALT only $n = 4$ as values were in part below the level of detection). BFC: butterfat-, fructose- and cholesterol-rich diet; C: control diet; CRO: control diet with rapeseed oil; DE: diet effect; DExROE: interaction between diet and rapeseed oil; NS: not significant; ROE: rapeseed oil effect; ROFC: rapeseed oil-, fructose- and cholesterol-rich diet. [a]$p < 0.05$ compared with mice fed C; [c]$p < 0.05$ compared with mice fed CRO; [d]$p < 0.05$ compared with mice fed ROFC.

level of controls. Moreover, activity of NOS in small intestinal tissue was increased by trend in BFC-fed mice (BFC vs C: $p = 0.09$), while NOS activity of ROFC-fed mice was at the level of controls; however, due to high interindividual variations it did not reach significance (Fig. 6).

4. Discussion

While being the most prevalent liver disease in the world by now [1], understanding of molecular mechanisms underlying the development of NAFLD and especially the impact of macronutrients like fat herein is still limited. In the present study, using a pair-feeding model, we assessed, if simply by exchanging the source of fat, e.g. exchanging butterfat being rich in SFA such as palmitic acid with equivalent amounts of RO being rich in oleic acids (18:1, ~62%), linoleic acid (18:2, ~22%) and α-linolenic acid (18:3, ~10%) [28], the progression of NAFLD and glucose intolerance could be attenuated in mice. Employing this model, we showed before that despite similar caloric intake, BFC-fed mice develop manifest steatosis with early signs of inflammation after 6–8 weeks of feeding and progresses to early steatohepatitis after 13 weeks [14,24]. Here, exchanging butterfat with RO attenuated the progression of glucose intolerance in ROFC-fed mice being also associated with lower indices of liver damage. Still, markers of liver damage, like NAS, hepatic triglyceride concentration and activity of transaminases but also of glucose tolerance were not all at the level of controls. Rather, the exchange of the fat source seemed to have attenuated the progression of both, glucose intolerance and liver damage. In line with these findings, studies in humans demonstrated that enriching the diet of NAFLD patients with RO while undergoing a life-style intervention improved liver scores, markers of insulin resistance and blood lipids [29]. Furthermore, in the present study, marker of lipid peroxidation like 4-HNE protein adducts and arginase activity were both markedly altered in livers of BFC-fed mice, too. However, due to the

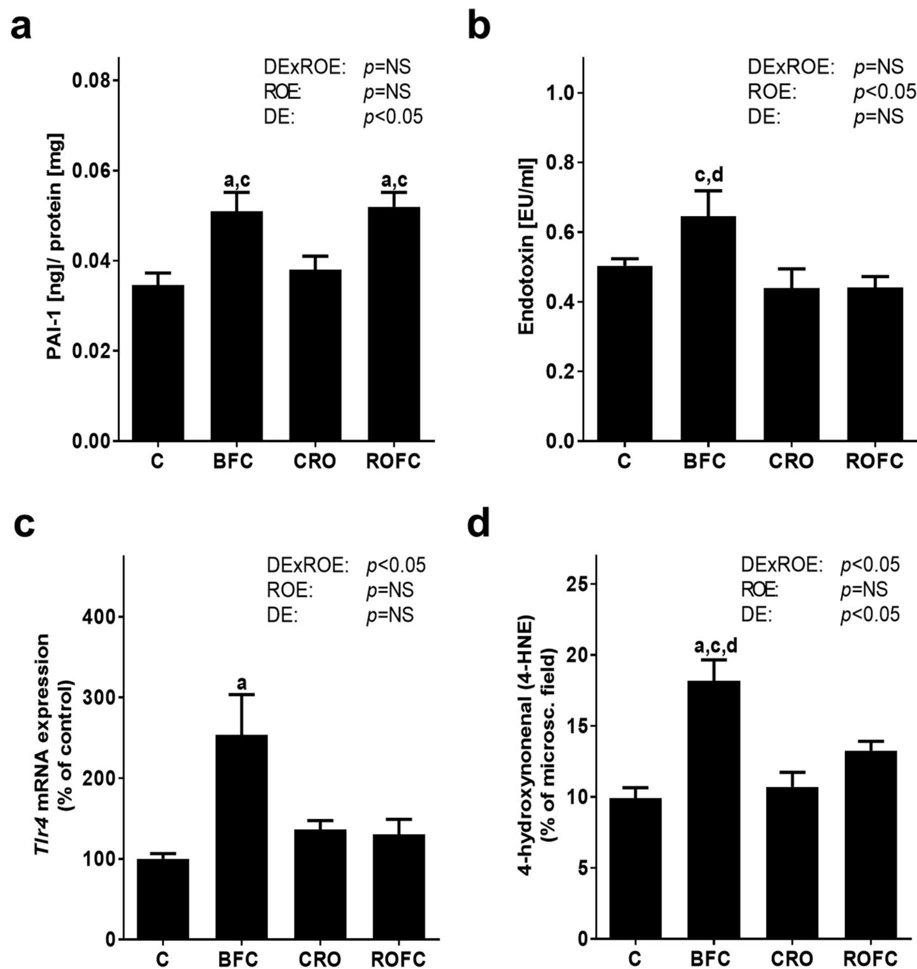

Fig. 4. Effect of exchanging the fat source in a fat-, fructose- and cholesterol-rich diet on portal endotoxin levels and markers of hepatic TLR4 signaling cascade. (a) Plasminogen activator inhibitor-1 (PAI-1) concentration in liver, (b) portal plasma endotoxin concentration, (c) hepatic mRNA expression of *toll-like receptor-4* (*Tlr4*) and (d) densitometric analysis of 4-hydroxynonenal (4-HNE) protein adduct staining in liver sections. Data presented as means ± SEM, n = 6–8. BFC: butterfat-, fructose- and cholesterol-rich diet; C: control diet; CRO: control diet with rapeseed oil; DE: diet effect; DExROE: interaction between diet and rapeseed oil; NS: not significant; ROE: rapeseed oil effect; ROFC: rapeseed oil-, fructose- and cholesterol-rich diet. [a]$p < 0.05$ compared with mice fed C; [c]$p < 0.05$ compared with mice fed CRO; [d]$p < 0.05$ compared with mice fed ROFC.

large interindividual variability, TNFα protein levels were similar between groups and PAI-1 concentration did not differ between BFC- and ROFC-fed groups. These data suggest that the development of steatohepatitis was not totally abolished by the exchange of dietary fat sources. Indeed, it has been shown before by others that PAI-1 protein levels may still be altered weeks after interventions like a fat-rich diet followed by energy-restriction [30]. Taken together, our results further bolster the hypothesis that changing the dietary source of fat in settings of NAFLD from SFA to PUFA may at least in part attenuate the progression of NALFD and glucose intolerance. However, further studies are needed to determine if these effects are also found long-term and when treatment is started at more progressed stages of the disease.

Table 2
Effect of exchanging the fat source in a fat-, fructose- and cholesterol-rich diet on marker of inflammation and of fatty acid metabolism.

Parameter	Groups				*p*-Value		
	C	BFC	CRO	ROFC	DExROE	ROE	DE
Neutrophil granulocytes (number/microsc. field)	1.1 ± 0.2	3.5 ± 0.5[a,c]	1.1 ± 0.2	2.1 ± 0.3[a,c]	NS	NS	<0.05
F4/80 positive cells (number/microsc. field)	8.1 ± 0.7	10.9 ± 0.6[a]	9.2 ± 0.3	9.0 ± 0.6	<0.05	NS	<0.05
TNFα [pg/mg protein]	28.8 ± 3.7	39.5 ± 4.0	24.0 ± 3.7	32.1 ± 4.3	NS	NS	<0.05
Arginase activity (% of control)	100 ± 2.3	29.3 ± 1.9[a,c,d]	63.6 ± 8.8[a]	48.1 ± 5.8[a]	<0.05	NS	<0.05
Acc mRNA	100 ± 18	446 ± 75[a,c]	182 ± 30	320 ± 44[a]	<0.05	NS	<0.05
Fas mRNA	100 ± 15	617 ± 65[a,c]	216 ± 49	423 ± 90[a]	<0.05	NS	<0.05
Scd1 mRNA	100 ± 9.8	393 ± 67[a,d]	247 ± 25[a]	198 ± 31[a]	<0.05	NS	<0.05
Srebp1c mRNA	100 ± 9.5	130 ± 17	93.6 ± 13	96.0 ± 10	NS	NS	NS
Free fatty acids [nmol/μl]	0.47 ± 0.06	0.33 ± 0.03	0.59 ± 0.11	0.52 ± 0.08	NS	NS	NS

Values are means ± SEM. mRNA expression presented as % of controls. n = 6–8. [a]$p < 0.05$ compared with mice fed C; [c]$p < 0.05$ compared with mice fed CRO; [d]$p < 0.05$ compared with mice fed ROFC. Acc: acetyl-CoA carboxylase, BFC: butterfat-, fructose- and cholesterol-rich diet, C: control diet, CRO: control diet with rapeseed oil, DE: diet effect, DExROE: interaction between diet and rapeseed oil, Fas: fatty acid synthase, NS: not significant, RO: rapeseed oil, ROE: rapeseed oil effect, ROFC: rapeseed oil-, fructose- and cholesterol-rich diet, Scd1: stearoyl-CoA desaturase-1, Srebp1c: sterol regulatory element-binding protein-1c, TNFα - tumor necrosis factor α.

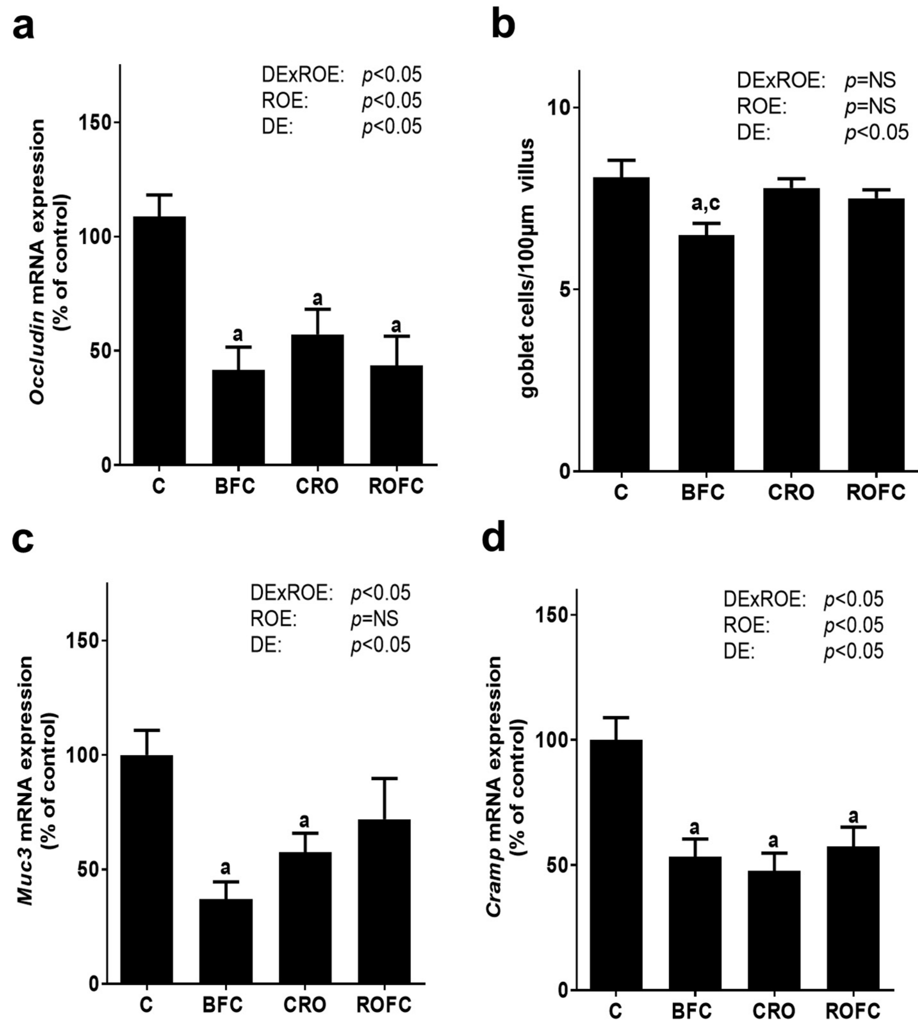

Fig. 5. Effect of exchanging the fat source in a fat-, fructose- and cholesterol-rich diet on markers of intestinal barrier function. (a) *Occludin* mRNA expression, (b) number of goblet cells per 100 μm villus, (c) *mucin-3* (*Muc3*) and (d) *cathelicidin-related antimicrobial peptide (Cramp)* mRNA expression of proximal small intestine. Data presented as means ± SEM, n = 6–8. BFC: butterfat-, fructose- and cholesterol-rich diet; C: control diet; CRO: control diet with rapeseed oil; DE: diet effect; DExROE: interaction between diet and rapeseed oil; NS: not significant; ROE: rapeseed oil effect; ROFC: rapeseed oil-, fructose- and cholesterol-rich diet. $^{a}p < 0.05$ compared with mice fed C; $^{c}p < 0.05$ compared with mice fed CRO.

Also, to determine effects of RO on glucose intolerance in more depth further studies including hyperinsulinemic euglycemic clamp measurements or at least fasting insulin levels are needed. The latter was not measured at the time of experimentation in the present study due to a lack of blood. Also, when interpreting the data regarding glucose tolerance of the present study one needs to keep in mind that an IPGTT was performed and not an oral GTT, the latter imitating the physiological route of glucose. Still, in mice IPGTT is more often used [31] as adverse effects afflicted with the oral administration of glucose by gavage are often observed [32]. Contrasting the finding in ROFC-fed mice, addition of RO to the control diet resulted in marked microvesicular fat accumulation in livers. The latter findings are in line with those of others, reporting that adding RO as sole dietary fat source to a control diet (10% wt/wt) resulted in increased plasma lipids in rats [33]. If these alterations are related to the induction of mRNA expression of enzymes involved in lipid metabolism found in the present study or resulted from other changes induced by the altered dietary fatty acid pattern remains to be determined. Furthermore, whether the beneficial effects found in the present study were related to the better n6:n3 ratio found in RO or to other compounds of the plant oil like tocopherols remains to be determined. It has been suggested that RO possesses an antioxidative capacity [34]. However, if the antioxidative capacity of RO contributed to the positive effects of RO found in the present study needs to be clarified in future investigations. Still, results of our study suggest, that exchanging fat source e.g. from a fat rich in SFA to a commercially available plant oil rich in PUFA may have beneficial effects on the progression of NAFLD.

Results of several studies suggest that n3 PUFA may through SREBP-dependent mechanisms, regulate enzymes involved in de novo lipogenesis (for overview see [35]). In the present study, expression of *Srebp1c* mRNA was similar between groups, while *Acc* and *Fas* mRNA expressions were induced to an almost similar extend in livers of BFC- and ROFC-fed mice compared to C-fed animals. In contrast, and as already discussed above in livers of CRO-fed mice compared to C-fed mice, expression of *Scd1* mRNA was induced in both RO-fed groups as well as in BFC-fed mice. Moreover, plasma free fatty acids were not induced in mice fed the fat-, fructose- and cholesterol-rich diet(s) in the present study. This could have been at least in part due to the lack of a marked weight gain of BFC-fed mice in our study. Studies of others also found no changes in free fatty acid concentration in plasma of mice fed a fat- and sugar-rich diet in the absence of the development of overweight, despite increased hepatic triglyceride levels [36]. Interestingly, in the same study, free fatty acid concentration of overweight mice fed a high fat diet was increased [36], further suggesting that body weight gain might indeed be critical in the regulation of free fatty acid levels in settings of a fat- (and sugar-) rich diet. The apparent ´lack´ of effect

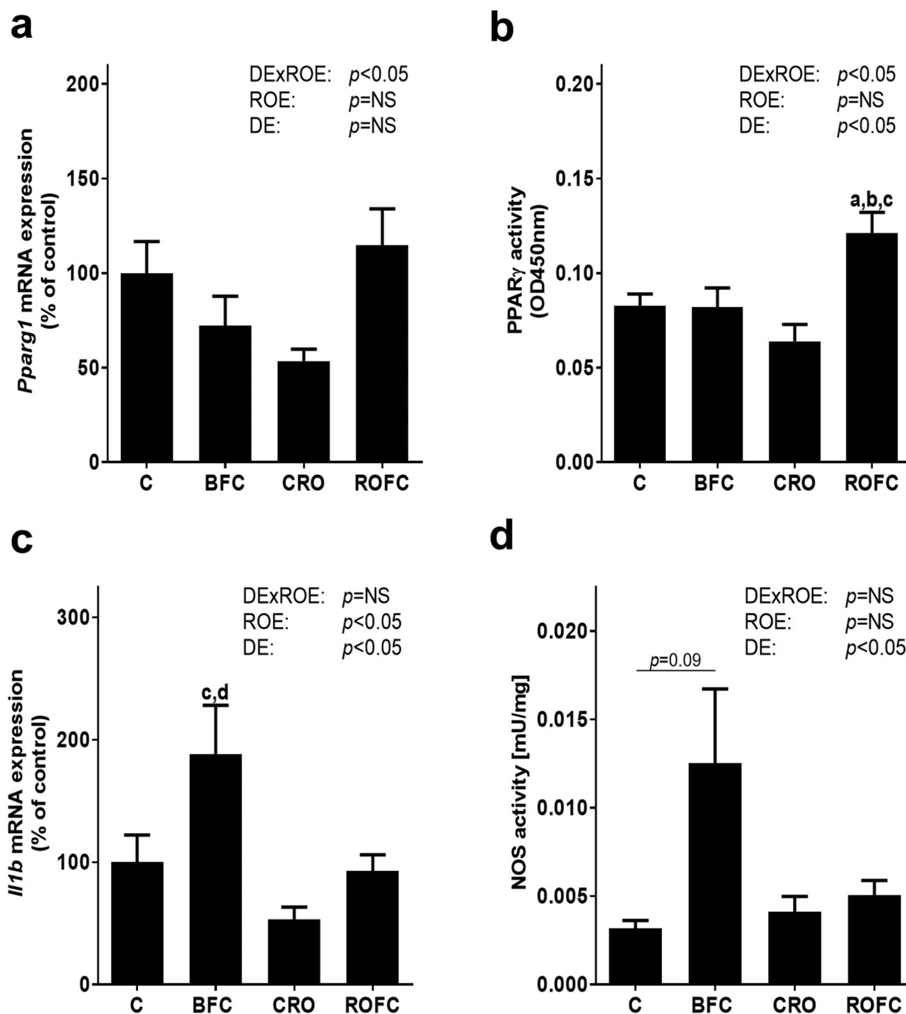

Fig. 6. Effect of exchanging the fat source in a fat-, fructose- and cholesterol-rich diet on PPARγ and markers of inflammation in proximal small intestine. (a) *Peroxisome proliferator-activated receptor gamma (Pparg)* mRNA expression, (b) PPARγ activity, (c) *interleukin 1β* (*Il1b*) mRNA expression and (d) nitric oxide synthase (NOS) activity of proximal small intestine. Data presented as means ± SEM, n = 6–8 (C group NOS only n = 4 as values were in part below the level of detection). BFC: butterfat-, fructose- and cholesterol-rich diet; C: control diet; CRO: control diet with rapeseed oil; DE: diet effect; DExROE: interaction between diet and rapeseed oil; NS: not significant; ROE: rapeseed oil effect; ROFC: rapeseed oil-, fructose- and cholesterol-rich diet. $^{a}p < 0.05$ compared with mice fed C; $^{b}p < 0.05$ compared with mice fed BFC; $^{c}p < 0.05$ compared with mice fed CRO; $^{d}p < 0.05$ compared with mice fed ROFC.

of RO to modulate genes involved in lipogenesis might have resulted from the composition of the oil. Studies reporting effects of PUFA on genes involved in lipogenesis mostly either used isolated PUFA or markedly higher concentrations/different oils [37–39]. The discrepancy between the findings for *Srebp1c* mRNA expression and those for *Scd1* mRNA expression might have resulted from the fact that Scd1 is not exclusively regulated through Srebp1c-dependent mechanisms. For instance, regulation of *Scd1* mRNA expression has been shown to be regulated by nuclear receptor TR4 [40]. Also, results of our own group suggest that hepatic VLDL export in settings of a fructose-rich diet is also altered through PAI-1, urokinase-type plasminogen activator- and hepatocyte growth factor-dependent signaling cascades thereby contributing to hepatic steatosis [41]. Moreover, results of others suggest that vegetable oils may affect fatty acid composition of hepatic triacylglycerols in rats [42]. It could very well be that supplementing the diet with 25E% RO may have affected fatty acid composition in small intestine or liver tissue. This needs to be determined in future studies. Taken together, results of the present study suggest that the beneficial effect of RO regarding the progression of NAFLD and glucose intolerance were not predominantly related to its effects on lipogenesis, but rather other factors may have been involved (see below). However, these findings by no means preclude that other concentrations or composition of PUFA may alter expression of genes involved in lipogenesis thereby also influencing the development and progression of NAFLD.

4.1. Exchanging butterfat with RO lowers portal bacterial endotoxin levels and attenuates induction of Tlr4-dependent signaling cascades in liver

Studies suggest that the development of NAFLD is associated with alterations of intestinal microbiota composition and intestinal barrier dysfunction along with an increased translocation of bacterial endotoxins and an induction of TLR4-dependent signaling cascade in liver tissue (for overview see [43]). Results of our own and other groups suggest that dietary sugar and fat content may be critical in mediating these effects [16,24,44,45]. In the present study, beneficial effects of exchanging dietary fat sources in the fat-, fructose- and cholesterol-rich diet for only 5 weeks while still feeding animals a fructose-rich diet were associated with a protection of animals against the translocation of bacterial endotoxin into portal vein and the subsequent induction of *Tlr4* and progression of liver damage. Indeed, disrupting endotoxin/TLR4 signaling, be it through improving intestinal barrier function or genetic deletion of lipopolysaccharide binding protein and TLR4, respectively, has been shown to be associated with a protection against the progression of NAFLD [17,46–48]. Contrasting the findings of the present study,

Laugerette et al. reported increased plasma endotoxin levels in mice exposed to a RO rich diet. However, in this study markers of inflammation and TLR4 signaling cascade were similar to controls [49]. Differences between results of this study and our study may have resulted from oil doses used (e.g. 25E% vs 38E% in the study of Laugerette et al.). Results of animal studies further suggest that in settings of NAFLD, the increased translocation of bacterial endotoxin may result from a loss of tight junction proteins in the small intestine [16,50]. In the present study, *occludin* mRNA expression was lower in both BFC- and ROFC-fed groups but also in CRO-fed animals than in C-fed mice. These data suggest that the addition of RO per se may affect expression of tight junctions in small intestine. Results of *in vitro* studies suggest that while n3 fatty acids may only have limited effects on tight junction expression, n6 fatty acids have been reported to decrease expression of tight junctions [51,52]. Also, it has been shown that effects may even differ between different tight junctions [51]. Tight junctions are not the only part of the intestinal barrier. Indeed, mucus composed of different mucins as well as antimicrobial peptides is thought to be the ´first´ line of defense and disturbances are discussed to be critical in the development of several disease (for overview see [53]). Here, number of goblet cells was significantly lower in small intestine of BFC-fed mice whereas being at the level of control in ROFC-fed animals. In line with these findings, *Muc3* expression, being lower in BFC-fed mice was at the level of C-fed animals in ROFC-fed animals. Surprisingly, in CRO-fed mice *Muc3* expression was also lower than in C-fed mice further suggesting that fatty acids may alter gene expression involved in intestinal barrier function. In line with this hypothesis expression of the antimicrobial peptide *Cramp* was not only lower in small intestine of BFC-fed mice but also in both RO-fed groups. Implications of these changes for intestinal homeostasis need to be determined in future studies. Also, it cannot be ruled out that changes of intestinal microbiota composition might also have contributed to the above discussed findings. Indeed, it has been suggested by the results of Lam et al. that dietary fat profile may alter intestinal microbiota composition being also associated with changes of intestinal barrier function [54]. If changes alike are also involved in the findings in the present study needs to be determined in future studies.

The protective effects of RO in ROFC-fed mice were associated with an increased PPARγ activity, a protection against the increase in *Il1b* expression and NOS activity, the latter two being at the level of controls in small intestine. Zhao et al. reported, that PPARγ may regulate number of goblet cells and mucin expression [26]. Studies also demonstrated that an induction of iNOS and NO oxidation intermediates, as well as intestinal inflammation may be crucial in the development of intestinal barrier dysfunction (for overview see [55]). Furthermore, in settings of Crohn's disease, it was recently shown that the beneficial effects of supplementing n3 PUFA on colonic inflammation were related to an activation of PPARγ [56]. Moreover, Il1b levels were reported being lower in intestine of dextran sulfate sodium-induced colitis in rats receiving a diet with an n6:n3 ratio of 2:1, while higher amounts of n6 fatty acids (10:1, 50:1 or 215:1) resulted in higher Il1b protein concentrations of colonic tissue [57]. However, further studies are needed to determine molecular mechanisms involved in the RO-dependent induction of PPARγ as well as its relation to intestinal barrier function.

5. Conclusion

Taken together, results of the present study suggest that exchanging the fat source, e.g. from a fat rich in SFA like butterfat to a PUFA rich fat like RO may attenuate the progression of NAFLD and glucose intolerance in mice even in the absence of any other changes in dietary composition. These results further bolster earlier findings of others suggesting that dietary fat source and herein probably especially the content of n3 and the ratio of n6 to n3 fatty acids may be critical in the development of NAFLD and insulin resistance (for overview see [58,59]). Results of the present study also suggest that RO may affect intestinal barrier function through PPARγ-dependent mechanisms, resulting in a lessening of bacterial endotoxin translocation. However, if these beneficial effects of RO are still present when animals are treated for a longer period of time and are also found in humans, as well as molecular mechanisms involved remains to be determined.

CRediT authorship contribution statement

Annette Brandt: Formal analysis, Investigation, Writing - original draft. **Dragana Rajcic:** Investigation. **Cheng Jun Jin:** Investigation. **Victor Sánchez:** Investigation. **Anna Janina Engstler:** Investigation. **Finn Jung:** Investigation. **Anika Nier:** Investigation. **Anja Baumann:** Investigation. **Ina Bergheim:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

I.B. received financial support from Yakult for another, unrelated research project. All other 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.metabol.2020.154283.

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