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Original Research

Does Yogurt Enriched with Platelet-Activating Factor Inhibitors from Olive Oil By-Products Affect Gut Microbiota and Faecal Metabolites in Healthy Overweight Subjects? (A randomized, parallel, three arm trial.)

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Abstract

Objective: The effect of the daily consumption of a low-fat yogurt (150 g) enriched with Platelet-Activating Factor receptor (PAF-R) antagonists, or the plain one, on gut microbiota and faecal metabolites was investigated in healthy overweight subjects. **Methods**: A randomized, three-arm, double-blind, placebo-controlled, parallel-group study was performed that lasted 8 weeks. Blood and stools were collected and analyzed before and after the intervention. **Results**: Our findings revealed that the intake of the enriched yogurt resulted in a significant increase in the levels of *Bifidobacterium* spp., *Clostridium perfringens* group and Firmicutes-to-Bacteroidetes (F/B) ratio. On the other hand, a significant increase in the levels of *Lactobacillus* and *C. perfringens* group was detected after the intake of the plain yogurt. The increase in the levels of *C. perfringens* group was inversely associated with the plasma catabolic enzyme of PAF, namely LpPLA$_2$ (lipoprotein-associated phospholipase A$_2$), a cardiovascular risk marker that has been linked with inflammation and atherosclerosis. Moreover, in the enriched with PAF-R antagonists yogurt group, the increased levels of *C. perfringens* group were also associated with lower PAF action assessed as *ex vivo* human platelet-rich plasma (PRP) aggregation. Additionally, a higher % increase in molar ratio of Branched Short Chain Fatty Acids (BSCFAs) was detected for both yogurt groups after the 8 week-intervention compared to control. The consumption of the enriched yogurt also resulted in a significant drop in faecal caproic levels and a trend for lower ratio of butyrate to total volatile fatty acids (VFAs) compared to baseline levels. **Conclusion**: Yogurt consumption seems to favorably affect gut microbiota while its enrichment with PAF-R antagonists from olive oil by-products, may provide further benefits in healthy overweight subjects. **Clinical Trial Registration**: ClinicalTrials.gov (NCT02259205).

Keywords: yogurt; PAF; olive oil by-products; platelet-rich plasma aggregation; *C. perfringens* group; *Bifidobacterium* spp.; lipoprotein-associated phospholipase A$_2$; *Lactobacillus*; caproic levels; branched-chain short chain fatty acids

1. Introduction

During the last decades, a significant number of studies have explored the role of gut microbiota in several pathophysiological conditions including obesity, cancer, inflammatory bowel diseases, metabolic syndrome and neurological diseases [1,2]. Diet components as well as dietary habits have been implicated in the modulation of the gut microbiota composition and functionality [3,4]. Fermented foods and especially dairy fermented products containing high populations of live microorganisms have been tested for their efficacy in the modulation of the gut microbiota composition [5,6].

Yogurt, a well-known fermented dairy product, is included in almost all the heart-healthy dietary patterns, while it should be noted that the benefits of low-fat and fat-free compared to the full-fat dairy products are still under discussion [7,8]. Reviews and meta-analyses have concluded that yogurt consumption has an inverse association with the risk of type 2 diabetes (T2D), whilst no significant association seems to exist between yogurt consumption and the risk of stroke, hypertension, coronary artery disease (CAD) and cardiovascular diseases (CVDs). Meanwhile, yogurt consumption and risk of all-cause and CVD mortality has been inversely associated [9–11]. The results from a recent meta-analysis suggest that dairy intake might improve inflammatory biomarkers but without taking into account the yogurt intake per se as the real cause of the observed changes [12].

Yogurt is a fermented food containing 10^7 colony-forming unit (CFU)/g viable cells from *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* bacteria [13]. The frequent yogurt consumption impacts significantly the composition of the gut microbiota resulting in increased bifidobacterial and lactobacilli populations [14,15]. Yogurt can also be used as a food matrix for other functional ingredients such as other bacterial cultures, fla-

voring ingredients, nutritive carbohydrate sweeteners, vitamins etc. [16], while any unwelcome organoleptic properties of the active ingredients can be concealed when it is flavored [17].

We have previously demonstrated that the daily consumption for 8 weeks of a yogurt, fortified with an olive oil pomace extract (OOPLE), attenuated *ex vivo* platelet-rich plasma (PRP) aggregation induced by PAF, decreased IL-6 and IL-10 levels, and also favorably modulated Platelet-activating factor (PAF) metabolic enzymes, in healthy mainly overweight adults [18,19]. It has been demonstrated that the OOPLE enrichment contains PAF receptor (PAF-R) inhibitors [20] and has the capacity to prevent the development of atherosclerotic plaques in rabbits fed a high cholesterol diet and also to regress the formed plaques in the same animal model [21]. PAF, chemically identified as 1-O-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine [22] is a potent endogenous lipid mediator that induces platelet activation, aggregation and secretion as well as mobilization of intracellular calcium, while its intravenous administration in experimental animals leads to anaphylactic shock and even in death in higher concentrations [23]. PAF is implicated in a variety of pathological conditions where inflammation, thrombosis and immune activation has a recognized role [24–27]. More specifically, PAF binding to its receptor (PAF-R), a G-protein-coupled receptor expressed in many cell types, triggers signaling pathways that result in the activation of a variety of kinases, in the production of nitric oxide and in the production of arachidonic acid metabolites through the activation of cytoplasmic phospholipase A_2 [28]. Additionally, the role of PAF in inflammatory bowel diseases has been described [29–32]. PAF concentration in plasma, cells and tissues is mainly controlled via its biosynthetic and catabolic pathways. Two distinct pathways are responsible for PAF biosynthesis, the *de novo* and the remodeling pathway. The *de novo* pathway utilizes alkylacetylglycerols as substrates and with the action of 1-alkyl-2-acetyl-*sn*-glycerol choline phosphotransferase (PAF-CPT) is producing the elementary PAF levels [33], while the remodeling pathway uses Lyso-PAF as substrate that is subsequently acetylated by the action of the acetyl-CoA:lyso–platelet-activating factor acetyltransferase (Lyso-PAF AT), is mainly activated under inflammatory conditions [34]. PAF biological action is contributed to the presence of the acetyl group in the *sn*-2 position of the glycerol backbone and thus is inactivated when this group is hydrolyzed by intracellular PAF-specific acetylhydrolase (PAF-AH) and its plasma isoform lipoprotein-associated phospholipase A_2 ($LpPLA_2$) [35].

The role of PAF in the development of inflammatory bowel diseases has been well-established in necrotizing enterocolitis (NEC) where it has been reported that PAF-AH reduces the incidence of NEC [36]. In addition, PAF-R is constitutively expressed in human intestinal epithelium [37]. This data has led the authors to suggest that PAF is produced by the human intestinal epithelium and exerts autocrine and/or paracrine action [37]. Additionally, PAF upregulates both Toll-Like Receptors 4 (TLR4) mRNA and protein expression in intestinal epithelial cell lines and the PAF-PAF-R complex interacts with and subsequently activates TLR4 [38,39]. These receptors recognize specific repetitive patterns associated with bacterial products such as lipopolysaccharide (LPS) and mediate cellular responses that lead also to inflammation [40]. It has also been reported that PAF-R also acts as a recognition receptor for the phosphorylcholine group of Gram-positive lipoteichoic acid (LTA) as well as for the Gram-negative LPS [41,42].

According to the above, the aim of the present study was to investigate the impact of the daily consumption of a low-fat yogurt fortified with PAF-R antagonists, or the plain one, on gut microbiota and faecal metabolites. Additionally, potential associations between inflammatory indices, hemostatic markers and PAF metabolic enzymes with gut microbiota characteristics and faecal metabolites in humans were investigated. For this purpose, a randomized, three-arm, double-blind, placebo-controlled, parallel-group trial was performed in apparently healthy mainly overweight adults.

2. Materials and Methods

2.1 Study Design

The study protocol, the intervention as well as the inclusion and the exclusion criteria have been presented elsewhere [18,19,43]. Briefly, the trial included 92 apparently healthy participants aged 35–65 years old that were randomly assigned into the three arms, 31 in Group A, 30 in Group B and 31 in Group C. Group A was advised to consume at most one yogurt every 14 days, Group B consumed one serving of plain yogurt every day (150 g) and Group C consumed one serving of yogurt enriched with OOPLE on a daily base (150 g). Both yogurts had the same composition apart from the OOPLE enrichment. The intervention lasted 8 weeks and 4 participants did not complete the 8-week intervention. In addition, stool samples were provided before and after the intervention from 51 adults (58% participation rate), specifically from 11 participants in Group A, 17 in Group B and 23 in Group C. The study took place at the Department of Nutrition and Dietetics of Harokopio University in Athens, Greece, followed the ethical guidelines of the Declaration of Helsinki (1989) of the World Medical Association, was approved by the Bioethics Committee of Harokopio University (40/30-10-2013) and was registered in ClinicalTrials.gov (NCT02259205). The CONSORT guidelines for parallel group randomized trials have been adopted [44].

2.2 Production of the Enriched Yogurt

The extraction method for OOPLE as well as the method for the production of the low-fat enriched yogurt have been previously described [19]. The Greek dairy in-

dustry (MEVGAL SA) manufactured both yogurts flavored with strawberry (16% w/w) in a pilot scale line during the whole duration of the trial and physiochemically tested their concentration in macronutrients and microbial parameters. Lactic acid bacteria were measured at 5.1×10^6 CFU/g product. Both yogurts had identical caloric content (85 kcal per serving) and final composition, containing 1.0% fat (saturated fat 0.6%), 14% carbohydrate (sugars 13%), 5% proteins and 0.1% sodium. In addition, approximately 0.5% w/w OOPLE extract was added to the low-fat enriched yogurt. The OOPLE extract after the addition of maltodextrin (30% w/w), contained 56.9% carbohydrates, 2.35% proteins, 7.66% polar lipids, 9.57% dietary fibers and less than 0.01% phenolic compounds expressed as gallic acid. The fatty acid (FA) analysis of the OOPLE extract showed that the predominant FAs were the monounsaturated ones (71.8%), specifically oleic acid (C18:1n9 *cis*, 71.2%), followed by the saturated FAs (16.5%) with the palmitic acid (C16:0, 12.5%) as the main representative one and the polyunsaturated FAs (11.7%) with the linoleic acid (C18:2n6 *cis*, 10.5%) having the highest concentration.

2.3 Hematological and Biochemical Measurements

The methodology for all the measurements has been previously presented [18]. Briefly, a Mindray BC-3000 hematology analyzer (Mindray, Shenzhen, China) was used for the blood count in whole blood with ethylenediaminetetraacetic acid (EDTA obtained from Sigma-Aldrich, St. Louis, MO, USA) as anticoagulant. Serum glucose, triacylglycerols, total cholesterol, and high-density lipoprotein cholesterol (HDL-C) were assessed enzymatically with an ACE biochemical analyzer (Schiapparelli Biosystems, Fairfield, NJ, USA). An immunoenzymometric assay was used for the estimation of serum insulin (Invitrogen, Thermo Fisher Scientific, Vienna, Austria). The calculation of low-density lipoprotein (LDL) cholesterol was performed by the Friedewald formula. C-reactive protein (CRP), IL-6, adiponectin and IL-10 were evaluated in serum by commercially available ELISA kits (CRP and adiponectin from Invitrogen, Thermo Fisher Scientific, Vienna, Austria; IL-6 from Tecan Trading AG, Männedorf, Switzerland; and IL-10 from BioLegend, San Diego, CA, USA). A Chromogenic Activity Kit (Assaypro LLC, St. Charles, MO, USA) was used to determine the activity of Plasminogen Activator Inhibitor-1 (PAI-1) and tissue Plasminogen Activator (tPA) in citrate plasma.

2.4 Ex Vivo Human Platelet-Rich Plasma Aggregation

The procedure used has been already reported [18]. Briefly, trisodium citrate (Sigma-Aldrich, St. Louis, MO, USA) was used as anticoagulant during blood collection and subsequently platelet-rich plasma (PRP) was obtained by centrifugation followed by further centrifugation of the residue to obtain platelet-poor plasma (PPP). Various concentrations of PAF were tested for their ability to induce human PRP aggregation presenting different heights of reversible and irreversible curves by light transmission aggregometry in a Chrono-Log (Havertown, PA, USA) aggregometer (model 440VS). The height of the aggregation curve of minimum-irreversible platelet aggregation of human PRP was defined as the 100% aggregation. The concentration of PAF that induces platelet aggregation equal to 50% (EC_{50} or half maximal effective concentration) was calculated from the aggregation curve. The PAF-induced platelet aggregation is an internationally accepted technique to evaluate the *ex vivo* and *in vitro* action of PAF.

2.5 Measurement of PAF Metabolic Enzymes

The procedure used as well as the methods of PAF metabolic enzymes determination, have been already reported [19]. Briefly, the activities of the PAF metabolic enzymes namely, two isoforms of Lyso-PAF acetyltransferase (Lyso-PAF AT), the activity of PAF choline phosphotransferase (PAF-CPT) and the activity of PAF intracellular catabolic enzyme, namely PAF-acetylhydrolase (PAF-AH), were determined in isolated leucocyte homogenates. The two isoforms of Lyso-PAF AT were the one that is activated under inflammatory conditions in the presence of calcium (Lyso-PAF ATC), while the other one is calcium independent and the assay was performed in the presence of ethylenediaminetetraacetic acid (EDTA) (Lyso-PAF ATE). Lastly, the activity of the plasma isoform of PAF-AH, lipoprotein-associated phospholipase A_2 ($LpPLA_2$) was measured in serum.

2.6 Gut Microbiota Analysis

Stool collection procedure and gut microbiota analysis using both plate count techniques and real-time quantitative polymerase chain reaction (qPCR) were performed as previously described [4]. Baseline culturable gut microbiota members were expressed as a $\log_{10}$ colony-forming units (CFUs)/g wet faeces, whereas detection frequencies were further estimated. For molecular analysis, genomic DNA was extracted from faecal samples at baseline and after intervention [45] using QIAamp® DNA Mini Kit (ref. 51306, QIAGEN GmbH, Hilden, Germany). Quantification of selected members of gut microbiota (Firmicutes, Bacteroidetes, *Bifidobacterium* spp., *Lactobacillus* group, *Clostridium perfringens* group) was performed by quantitative real-time PCR based on SYBR Green I detection chemistry using group- and genus-specific primers (**Supplementary Table 1**). PCR amplification and detection were performed in a LightCycler® 2.0 Real-Time PCR System (Product No. 03531414001, Roche Diagnostics GmbH, Mannheim, Germany), whereas bacterial quantification was based on the LightCycler® software version 4.1 (Roche Diagnostics GmbH, Mannheim, Germany) using standard curves from reference bacterial strains. Melting curves analysis was performed in each run for the validation of assay specificity. Data were expressed as $\log_{10}$ copies of 16S rRNA gene/g wet faeces [4].

Table 1. Baseline characteristics and hematologic markers of the participants according to their allocation to study groups.

	Group A	Group B	Group C	*p*
	(Control group, n = 11)	(Plain yogurt, n = 17)	(Enriched yogurt, n = 23)	
Men/Women	5/6	9/8	10/13	0.82
Age (years)	49.7 ± 8.6	49.6 ± 10.3	47.4 ± 9.1	0.70
Smoking (Yes/No)	6/5	7/10	7/16	0.53
BMI (kg/m^2)	25.8 ± 4.1	29.1 ± 4.2	27.0 ± 3.6	0.08
Systolic blood pressure (mmHg)	119.4 ± 13.1	131.8 ± 19.0	121.0 ± 13.2	0.06
Diastolic blood pressure (mmHg)	76.6 ± 9.6	77.8 ± 10.2	74.2 ± 8.5	0.48
Glucose (mg/dL)	87.9 ± 11.6	94.7 ± 10.2	95.3 ± 28.8	0.61
Triacylglycerols (mg/dL)	106.9 ± 59.3	131.3 ± 56.0	115.9 ± 50.7	0.48
Total-cholesterol (mg/dL)	200.8 ± 26.6	214.4 ± 33.2	214.2 ± 42.5	0.56
HDL-cholesterol (mg/dL)	53.4 ± 14.6	53.8 ± 14.4	54.1 ± 13.3	0.99
LDL- cholesterol (mg/dL)	126.1 ± 20.5	134.3 ± 26.8	136.9 ± 36.8	0.63
Insulin (µU/mL)	9.7 ± 3.9	12.0 ± 6.7	9.5 ± 5.0	0.33
WBC (10^3/µL)	6.9 ± 1.8	6.0 ± 1.6	5.7 ± 1.4	0.10
LYMPH (10^3/µL)	1.9 ± 0.4	1.9 ± 0.4	1.9 ± 0.6	0.96
MID (10^3/µL)	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.35
GRAN (10^3/µL)	4.6 ± 1.4^a	3.7 ± 1.3	3.4 ± 1.0^b	**0.03**
RBC (10^6/µL)	4.7 ± 0.5	4.7 ± 0.4	4.6 ± 0.5	0.78
Hct (%)	39.8 ± 4.8	41.3 ± 5.0	41.0 ± 5.7	0.74
Hgb (g/dL)	13.3 ± 1.7	14.4 ± 1.7	14.1 ± 2.0	0.35
MCV (fL)	82.5 ± 9.8	84.2 ± 6.7	84.9 ± 7.4	0.68
RDW-CV (%)	14.0 ± 1.1	13.5 ± 0.9	13.5 ± 1.2	0.32
RDW-SD (fL)	40.4 ± 3.0	39.6 ± 3.0	39.8 ± 3.5	0.82
Platelets (10^3/µL)	265 ± 76	253 ± 82	243 ± 52	0.68
MPV (fL)	7.9 ± 0.9	7.8 ± 0.8	8.1 ± 0.8	0.32
PDW (fL)	15.8 ± 1.0	15.5 ± 0.9	15.4 ± 1.1	0.68
PCT (%)	0.2 ± 0.04	0.2 ± 0.05	0.2 ± 0.04	0.76
PLT/LYMPH	144 ± 30	134 ± 36	137 ± 45	0.82

Data are presented as means ± standard deviations for normally distributed variables. Categorical variables are presented as absolute values. Comparison of means was performed by one-way analysis of variance (ANOVA) for normally distributed variables. Associations between categorical variables were evaluated using the chi-square test. ^{a,b}Different letters indicate significant difference $p < 0.05$ based on *t*-test. Bold numbers indicate significant difference $p < 0.05$.

BMI, Body Mass Index; Hct, Hematocrit; HDL, High density lipoprotein; Hgb, Hemoglobin; GRAN, Granulocytes; LDL, Low density lipoprotein; LYMPH, Lymphocytes; MCV, Mean Corpuscular Volume; MID, Mid-range cells; MPV, Mean Platelet Volume; PCT, Plateletcrit; PDW, Platelet distribution Width; PLT, Platelets; RBC, Red Blood Cells; RDW-CV, Red Distribution Width-Coefficient Variation; RDW-SD, Red Distribution Width-Standard Deviation; WBC, White Blood Cells.

2.7 Measurement of Faecal SCFAs – Stool pH and Moisture Determination

Frozen faecal samples were 1:3 diluted with 0.9% saline and faecal SCFAs concentrations were then assessed with the use of capillary gas chromatography as previously described in detail [46]. Total volatile fatty acids (VFAs) and individual SCFAs concentrations were expressed as µmol/g of sample and molar ratios (% of VFAs) of acetate, propionate, butyrate, branched-chain SCFAs (BSCFAs; iso-butyrate, iso-valerate, iso-caproic acid) and other SCFAs (valerate, caproic acid and heptanoic acid) were further calculated [46]. Moisture and pH of fresh samples were also determined as previously described [47].

2.8 Statistical Analysis

The Kolmogorov–Smirnov criterion was used to test normal distribution of data. Normally distributed continuous variables were displayed as means ± SD, whereas continuous skewed variables as medians (25th–75th quartiles). Categorical variables were displayed as absolute values or relative frequencies (%) and associations between categorical variables were evaluated using the chi-square test. Comparisons of the baseline characteristics of our population as well as comparisons of the % changes of tested parameters were based on one-way analysis of variance (ANOVA) analysis for normally distributed variables, whereas the Kruskal-Wallis test was applied for skewed variables. The independent samples *t* test and the Mann-Whitney U test for normally distributed variables and for

Table 2. The inflammation indices, the hemostatic markers and platelet aggregation against PAF expressed as EC_{50} value, at baseline.

	Group A	Group B	Group C	*p*
	(Control group, n = 11)	(Plain yogurt, n = 17)	(Enriched yogurt, n = 23)	
CRP (mg/L)	2.6 ± 2.9	2.2 ± 1.6	1.6 ± 1.1	0.24
Adiponectin (µg/mL)	5.4 ± 3.5	4.6 ± 2.7	5.6 ± 3.8	0.62
IL-6 (pg/mL)	0.6 ± 0.4^a	1.2 ± 0.6^b	1.0 ± 0.6	**0.02**
IL-10 (pg/mL)	2.7 ± 0.9	2.3 ± 0.6	2.6 ± 0.8	0.28
PAI-1 (mAU/mL)	103.1 ± 75.6	120.8 ± 67.8	100.7 ± 37.8	0.54
tPA (mIU/mL)	0.063 (0.051, 0.092)	0.067 (0.043, 0.085)	0.072 (0.059, 0.092)	0.48
EC_{50} PAF (nM)	33.5 (23.4, 156.5)	42.7 (24.8, 161.0)	33.3 (26.4, 68.2)	0.71

Data are presented as means ± standard deviations for normally distributed variables and as medians (25th, 75th percentiles) for skewed variables. Comparison of means was performed by one-way analysis of variance (ANOVA) for normally distributed variables. Comparison was performed by Kruskal-Wallis for skewed variables. ^{a,b}Different letters indicate significant difference $p < 0.05$ based on *t*-test for normally distributed variables and on the Mann-Whitney U test for skewed variables. Bold numbers indicate significant difference $p < 0.05$.
CRP, C-reactive protein; EC_{50} PAF, Half maximal effective concentration of Platelet-activating factor; PAI-1, Plasminogen activator inhibitor-1; tPA, Tissue plasminogen activator; IL-6, Interleukin-6.

skewed variables, respectively, were also performed to estimate the difference between the intervention groups, i.e., "Group C versus Group B", "Group C versus Group A" and "Group B versus Group A". The paired *t*-test for normally distributed variables and the Wilcoxon test for paired samples for skewed variables were also performed for testing the time effect within each group in comparison to baseline values.

The % change was calculated by the following formula: Final value-Baseline value / Baseline value × 100. Linear regression models were applied to the data in order to uncover the relationship of tested parameters (e.g., PAF action and its metabolic enzymes activity, inflammatory and hemostatic parameters) with gut microbiota and faecal SCFAs, the latest were considered as the independent variables. Adjustments were made for sex, age and Body Mass Index (BMI). Results from linear regression models are presented as β-coefficients ± standard error with p-values. Scatterplots with linear predictions were also created to visually inspect these associations. All reported *p*-values were two-sided (significance level 5%). STATA version 15 statistical software was used for the statistical analysis (STATA Corp., College Station, TX, USA).

3. Results

3.1 Anthropometric Characteristics and Classical Biomarkers of the Participants

The baseline characteristics as well as the hematologic markers of all the participants that provided stool samples according to their intervention groups are shown in Table 1. Biochemical markers and blood count parameters did not differ among groups at baseline with the exception of granulocytes that were higher in the control group (Group A) than in the enriched yogurt group (Group C) ($p = 0.02$). It should note that participants in Group B had higher BMI, systolic blood pressure, and triacylglycerols, even though no statistically significant difference was detected. During the 8 weeks of intervention, the participants retained their dietary macronutrient intake and physical activity as well (data not shown). Furthermore, the majority of the classical biochemical markers and blood count parameters did not present any significant change. A slight decrease was recorded in red blood cells ($p = 0.04$) and in their distribution width (RDW-SD, $p = 0.02$) in Group A as well as in the mean corpuscular volume (MCV, $p = 0.03$) in Group B compared to baseline levels, all of them remaining into the normal range after the intervention. The intake of the plain yogurt (Group B) also led to a significant decrease of systolic (SBP) and diastolic blood pressure (DBP) by approximately 5–6 mmHg ($p = 0.004$, $p = 0.045$, respectively) at 8 weeks, resulting in blood pressure values closer to the baseline ones of the other groups. Comparison of the % changes of evaluated parameters revealed that the participants in Group A resulted in decreased number of granulocytes compared to Group B ($p = 0.016$) and C ($p = 0.016$). Additionally, lower RDW-SD values were detected in Group A compared to the yogurt groups and especially compared to Group C ($p = 0.012$) after the intervention. However, all values were in normal range (**Supplementary Table 2**).

3.2 Inflammation Indices, Hemostatic Markers and Platelet Aggregation against PAF

The inflammation and hemostatic markers as well as *ex vivo* platelet aggregation against PAF expressed as PAF EC_{50} values at baseline, are presented in Table 2. No differences were detected among groups with the exception of IL-6 that was higher in Group B compared to Group A ($p = 0.02$). After the intervention, a trend for a decrease in IL-10 was only observed in Group C ($p = 0.05$) and also a decre-

Table 3. Specific activities of PAF metabolism enzymes, at baseline and after the 8 weeks of intervention.

	Group A (Control group, n = 11)			Group B (Plain yogurt, n = 17)			Group C (Enriched yogurt, n = 23)			*p* Among groups	
	Baseline	End	%Δ	Baseline	End	%Δ	Baseline	End	%Δ	Baseline	%Δ
PAF-CPT (pmol/mg/min)	240.8 ± 136.6	255.3 ± 160.2	6.5 ± 26.1	167.6 ± 101.5	173.3 ± 118.4	0.4 ± 28.8	185.0 ± 132.2	166.2 ± 87.3	8.53 ± 78.0	0.309	0.907
Lyso-PAF ATC (pmol/mg/min)	64.5 (35.5, 123.8)	85.3* (40.3, 156.6)	16.1 (–1.9, 26.5)	98.3 (65.9, 105.6)	74.1 (55.4, 111.5)	–19.4 (–36.5, 25.5)	63.8 (33.3, 93.0)	70.6 (37.6, 108.0)	–1.0 (–20.6, 29.0)	0.229	0.095
Lyso-PAF ATE (pmol/mg/min)	29.3 ± 8.3^{a}	31.5 ± 10.0	8.6 ± 28.9	65.5 ± 24.6^{b}	61.7 ± 22.7	–4.2 ± 18.3	59.8 ± 26.5^{b}	57.0 ± 18.9	4.25 ± 34.9	**0.001**	0.480
PAF-AH (pmol/mg/min)	64.7 ± 23.1	62.0 ± 22.3	7.7 ± 43.1	54.6 ± 26.4	52.8 ± 27.9	3.6 ± 53.1	57.2 ± 31.1	58.3 ± 34.4	10.1 ± 43.7	0.644	0.911
$LpPLA_2$ (nmol/mL/min)	24.6 ± 7.5	25.4 ± 8.7	1.5 (–6.9, 12.1)	30.0 ± 6.6	30.7 ± 7.2	0.8 (–6.1, 4.4)	27.7 ± 5.7	26.4 ± 6.1*	–3.3 (–7.8, 0.0)	0.101	0.710
$LpPLA_2$-to-LDL ratio	0.2 ± 0.06	0.2 ± 0.07	1.03 (–5.86, 3.67)	0.2 ± 0.05	0.2 ± 0.05	–3.46 (–9.59 5.69)	0.2 ± 0.07	0.2 ± 0.14	–4.29 (–8.58, 3.42)	0.433	0.707

Data are presented as means ± standard deviations for normally distributed variables and as medians (25th, 75th percentiles) for skewed variables. Comparison of baseline means as well as comparisons of the % changes of tested parameters was performed by one-way analysis of variance (ANOVA) for normally distributed variables and by Kruskal-Wallis for skewed variables. ^{a,b}Different letters indicate significant difference $p < 0.05$ based on *t*-test for normally distributed variables and on the Mann-Whitney U test for skewed variables. The paired *t*-test for normally distributed variables and the Wilcoxon test for paired samples for skewed variables, were also performed for testing the time effect within each group in comparison to baseline values (* for $p \leq 0.05$). Bold numbers indicate significant difference $p < 0.05$.

%Δ, % change calculated by the formula, Final value-Baseline value / Baseline value × 100; LDL, Low-density lipoprotein; $LpPLA_2$, Lipoprotein-associated phospholipase A_2; Lyso-PAF ATC, Lyso-platelet-activating factor acetyltransferase in the presence of Ca^{2+}; Lyso-PAF ATE, Lyso-platelet-activating factor acetyltransferase in the presence of EDTA; PAF-AH, Platelet-activating acetylhydrolase; PAF-CPT, Platelet-activating factor-choline phosphotransferase.

Table 4. Stool characteristics and gut microbiota qPCR-based analysis among study groups at baseline and after 8 weeks of intervention.

	Group A			Group B			Group C			*p*
	(Control group, n = 11)			(Plain yogurt, n = 17)			(Enriched yogurt, n = 23)			Among Groups
	Baseline	End of study	%Δ	Baseline	End of study	%Δ	Baseline	End of study	%Δ	
Stool characteristics										
Faecal pH	6.9 ± 0.5	7.1 ± 0.6	0.3 (0.3, 1.6)	6.9 ± 0.8	6.9 ± 0.6	2.3 (–4.4, 9.2)	6.8 ± 0.6	7.0. ± 0.6	6.1 (–6.5, 9.7)	0.661
Faecal moisture (%)	69.0 ± 6.4	70.2 ± 5.8	1.8 ± 4.3	71.6 ± 5.3	72.7 ± 5.9	1.9 ± 8.3	73.6 ± 7.7	72.1 ± 5.2	–1.2 ± 10.1	0.451
qPCR-based gut microbiota analysis (log_{10} copies of 16S rRNA gene/g wet faeces)										
Firmicutes	11.7 ± 0.2	11.7 ± 0.2	0.1 ± 0.6	11.6 ± 0.2	11.7 ± 0.2	0.5 ± 1.1	11.7 ± 0.2	11.8 ± 0.2	0.7 ± 2.0	0.318
Bacteroidetes	11.1 ± 0.2	11.1 ± 0.21	–0.9 (–1.5, –0.1)	11.1 ± 0.3	11.1 ± 0.2	–0.2 (–1.2, 1.0)	11.1 ± 0.25	11.1 ± 0.3	–0.4 (–1.1, 1.9)	0.556
Firmicutes-to-Bacteroidetes ratio	1.05 ± 0.02	1.06 ± 0.03	1.08 (0.0, 1.96)	1.05 (1.04, 1.07)	1.06 (1.05, 1.07)	0.95 (0.0, 1.45)	1.06 (1.05, 1.07)	1.06* (1.06, 1.08)	0.95 (0.0, 1.89)	0.905
Bifidobacterium spp.	10.3 (8.8, 10.9)	10.2 (8.9, 10.9)	–0.7 (–1.7, 2.6)	10.6 (10.2, 10.8)	10.4 (9.9, 10.9)	–0.6 (–3.1, 3.3)	10.6 (10.0, 10.8)	10.5* (10.2, 11.0)	2.4 (–0.9, 4.3)	0.185
Lactobacillus group	7.7 ± 0.5	7.8 ± 0.4	1.0 (0.7, 1.2)	7.8 ± 0.9	8.1 ± 0.8*	2.1 (–1.7, 8.3)	7.6 ± 0.6	7.8 ± 0.7	1.2 (–6.4, 6.8)	0.616
C. perfringens group	8.1 ± 0.5	8.0 ± 0.5	–1.2 ± 1.9^{a}	8.2 ± 0.8	8.6 ± 0.5*	5.9 ± 10.2^{b}	8.0 (7.6–8.9)	8.9* (8.1–9.1)	4.7 ± 8.0^{b}	**0.001**

Data are presented as means ± standard deviations for normally distributed variables and as medians (25th, 75th percentiles) for skewed variables. The paired *t*-test for normally distributed variables and the Wilcoxon test for paired samples for skewed variables, were also performed for testing the time effect within each group in comparison to baseline values (* for $p < 0.05$). ^{a,b}Comparisons of the % changes of tested parameters were based on ANOVA analysis for normally distributed variables and on the Kruskal-Wallis test for skewed variables (different letters indicate significant difference for $p < 0.05$ based on test). Bold numbers indicate significant difference $p < 0.05$.

%Δ: % change calculated by the formula, Final value-Baseline value / Baseline value × 100. qPCR, quantitative polymerase chain reaction; rRNA, ribosomal RNA.

Table 5. SCFAs among study groups at baseline and after 8 weeks of intervention.

	Group A (Control group, n = 11)			Group B (Plain yogurt, n = 17)			Group C (Enriched yogurt, n = 23)			*p* Among Groups	
	Baseline	End of study	%Δ	Baseline	End of study	%Δ	Baseline	End of study	%Δ	Baseline	%Δ
SCFAs (concentration, µmol/g of wet faeces)											
Total Volatile Fatty Acids (VFAs)	68.9 ± 45.1	72.7 ± 42.5	11.5 (0.6, 17.4)	76.3 ± 31.0	80.2 ± 43.6	0.6 (–32.5, 46.9)	82.6 ± 31.3	71.5 ± 36.9	–27.6 (–44.4, 46.1)	0.552	0.119
Acetate	32.1 ± 18.7	35.9 ± 17.5	23.8 (4.1, 35.6)	35.9 ± 12.9	37.9 ± 19.8	–0.92 (–28.5, 44.5)	38.4 ± 14.7	35.4 ± 20.2	–22.0 (–38.9, 44.6)	0.553	0.122
Propionate	9.9 (5.1, 18.2)	11.3 (7.2, 15.5)	2.7 (–4.5, 14.0)	14.3 ± 6.4	13.9 ± 7.1	1.6 (–29.5, 21.5)	15.2 (11.6, 18.1)	11.9 (7.1, 14.6)	–27.7 (–51.1, 23.2)	0.849	0.199
Butyrate	18.2 ± 13.1	18.2 ± 12.3	2.3 (–1.3, 11.1)	21.8 ± 14.4	23.4 ± 18.0	–23.6 (–35.9, 67.9)	21.4 (15.1, 27.0)	18.2 (8.0, 22.8)	–26.3 (–59.8, 29.8)	0.569	0.212
BSCFAs	2.1 (1.9, 3.3)	2.5 (2.0, 2.8)	–0.2 (–1.3, 15.5)	2.0 (1.4, 2.9)	1.8 (1.5, 3.4)	12.4 (–22.6, 57.0)	2.1 (1.7, 3.0)	2.1 (1.7, 3.5)	16.5 (–31.3, 43.8)	0.462	0.743
Iso-butyrate	1.2^{c} (0.7, 1.5)	1.0 (0.8, 1.3)	–1.5 (–7.5, 7.5)	0.5^{d} (0.4, 0.7)	0.6 (0.4, 1.3)	18.9 (–13.0, 90.2)	0.6^{d} (0.5, 1.0)	0.6 (0.4, 1.3)	15.7 (–31.3, 43.7)	**0.005**	0.316
Iso-valerate	1.1 (1.1, 1.7)	1.2 (0.9, 1.7)	–3.0 (–21.0, 31.2)	1.6 (0.8, 1.7)	1.3 (0.8, 1.3)	1.8 (–53.1, 29.9)	1.6 ± 0.9	1.6 ± 0.7	5.9 (–33.5, 47.7)	0.972	0.598
Iso-caproic	0.0 (0.0–0.07)	0.0 (0.0–0.07)	–	0.0 (0.0–0.07)	0.0 (0.0–0.03)	–	0.0 (0.0–0.06)	0.0 (0.0–0.06)	–	0.962	ND
Other SCFAs	1.3 (0.9, 3.4)	1.3 (0.9, 2.8)	–2.3 (–17.0, 14.6)	2.2 (1.4, 3.0)	2.0 (1.1, 3.1)	–21.9 (–33.9, 52.9)	2.7 (2.2, 3.3)	2.1 (1.5, 3.3)	–11.0 (–47.6, 19.2)	0.107	0.456
Valerate	0.9 (0.8, 2.3)	1.1 (0.6, 2.0)	–4.0 (–14.3, 16.7)	1.5 (1.1, 1.9)	1.3 (0.9, 2.2)	–21.1 (–42.3, 49.2)	1.8 ± 0.8	1.6 ± 0.9	–10.6 (–45.6, 35.9)	0.256	0.900
Caproic (hexanoic)	0.2^{c} (0.1, 1.0)	0.3 (0.1, 0.8)	–10.8 (–25.4, 23.5)	0.3^{c} (0.2, 1.1)	0.5 (0.2, 1.1)	–1.5 (–53.9, 110.9)	1.1^{d} (0.4, 1.3)	0.5* (0.3, 1.0)	–41.1 (–68.5, 13.1)	**0.013**	0.290
Heptanoic	0.04^{c} (0.0, 0.1)	0.06 (0.0, 0.1)	–	0.06^{c} (0.0, 0.1)	0.06 (0.0, 0.2)	–	0.1^{d} (0.1, 0.2)	0.1 (0.0, 0.2)	–	**0.046**	ND
SCFAs (Molar ratios, % total VFAs)											
Acetate	49.2 ± 5.7	51.9 ± 6.1	5.9 ± 9.1	48.2 ± 5.7	49.0 ± 7.2	2.1 ± 13.9	46.3 ± 5.8	48.6 ± 5.2	5.9 ± 14.0	0.358	0.623
Propionate	18.5 ± 4.2	17.4 ± 4.1	–5.3 ± 11.0	18.9 ± 4.8	17.9 ± 3.5	–0.7 ± 24.3	18.9 ± 3.4	18.6 ± 3.9	–0.1 ± 21.5	0.953	0.780
Butyrate	24.2 ± 7.4	23.2 ± 6.2	–2.9 ± 13.9	26.6 ± 7.0	25.9 ± 8.4	–2.7 ± 22.8	27.2 ± 5.4	24.8 ± 4.8	–6.9 ± 18.8	0.446	0.748
BSCFAs	5.0 ± 2.0^{c}	4.6 ± 2.2	-12.0 (–15.2, 2.7)^{a}	3.2^{d} (2.3, 3.8)	4.2 (2.0, 5.2)	23.3 (–7.3, 104.4)^{b}	3.3^{d} (1.6, 4.4)	3.9 (2.4, 5.3)	45.2 (–29.8, 121.2)^{b}	0.052	0.101
Iso-butyrate	2.1 ± 1.0^{c}	2.0 ± 1.0	–9.7 (–17.2, 1.5)	0.8^{d} (0.6, 1.0)	1.1 (0.5, 2.2)	14.9 (–21.7, 102.7)	0.9^{d} (0.6, 1.0)	1.0 (0.7, 1.8)	29.4 (–20.0, 123.8)	<**0.001**	0.276
Iso-valerate	2.8 ± 1.5	2.6 ± 1.7	–13.2 (–21.3, 4.3)	2.2 ± 1.3	2.4 ± 1.5	11.2 (–37.9, 66.6)	2.3 ± 1.6	2.7 ± 1.5	50.8 (–30.4, 111.3)	0.587	0.120
Iso-caproic	0.0 (0.0–0.2)	0.0 (0.0–0.1)	–	0.0 (0.0–0.1)	0.0 (0.0–0.0)	–	0.0 (0.0–0.1)	0.0 (0.0–0.1)	–	0.882	ND
Other SCFAs	3.1 ± 1.5	2.9 ± 1.5*	–3.2 (–17.4, 1.0)	3.1 ± 1.0	3.3 ± 1.4	0.5 (–17.8, 30.6)	3.9 ± 2.0	3.9 ± 1.8	–6.7 (–23.7, 44.5)	0.242	0.716
Valerate	2.2 ± 0.8	2.0 ± 0.7	–8.9 (–20.4, 2.4)	2.1 ± 0.6	2.2 ± 0.9	1.3 (–16.9, 25.3)	2.3 ± 1.1	2.5 ± 1.0	1.0 (–12.7, 40.3)	0.745	0.226
Caproic (hexanoic)	0.6^{c} (0.2, 1.0)	0.7 (0.1, 0.8)	–14.3 (–26.8, 6.0)	0.9 ± 0.7^{c}	1.0 ± 0.8	18.5 (–28.8, 89.1)	1.4 ± 1.0^{d}	1.1 ± 0.8	–32.9 (–45.6, 55.0)	**0.047**	0.366
Heptanoic	0.1 ± 0.2	0.1 ± 0.2	–	0.1 (0.0–0.1)	0.1 (0.0–0.3)	–	0.2 ± 0.2	0.2 ± 0.2	–	0.121	ND

Data are presented as means ± standard deviations for normally distributed variables and as medians (25th, 75th percentiles) for skewed variables. The paired *t*-test for normally distributed variables and the Wilcoxon test for paired samples for skewed variables, were also performed for testing the time effect within each group in comparison to baseline values (* for $p < 0.05$). Comparisons of the baseline values^{c,d} as well as comparisons of the % changes^{a,b} of tested parameters were based on ANOVA analysis for normally distributed variables and on the Kruskal-Wallis test for skewed variables (different letters indicate significant difference for $p < 0.05$ based on test). Bold numbers indicate significant difference $p < 0.05$.

%Δ, % change calculated by the formula, Final value-Baseline value / Baseline value × 100; BSCFAs, Branched short chain fatty acids; ND, Not determined; SCFAs, Short chain fatty acids; VFAs, Volatile fatty acids.

ase in the PAF EC_{50} value was observed in Group A (p = 0.03), compared to baseline. Comparison of the % changes of the above markers revealed that the participants in Group C resulted in higher PAF EC_{50} values compared to Group A (p = 0.045) (**Supplementary Table 2**).

3.3 PAF Metabolic Enzymes

The specific activities of PAF biosynthetic as well as catabolic enzymes at baseline, are presented in Table 3. Besides the difference in the specific activity of Lyso-PAF ATE that was significant lower in Group A compared to Group B (p = 0.001) and C (p = 0.002), no other difference was detected. After the 8 weeks of intervention, Lyso-PAF ATC activity was higher in Group A (p = 0.04) and Lp-PLA_2 activity was lower in Group C (p = 0.04) compared to baseline. Comparison of % changes of the specific activity of PAF metabolic enzymes presented a borderline difference in Lyso-PAF ATC activity between Groups A and B (p = 0.07) (Table 3).

3.4 Stool Characteristics, Gut Microbiota qPCR-Based Analysis and SCFAs

The participants allocated in yogurt groups (Groups B and C) did not report any severe gastrointestinal side effects or significant changes in stool frequency and consistency, although increase of abdominal bloating and/or borborygmi were the most frequently reported side effects, as it has been reported elsewhere [18]. All participants reported full compliance with the intervention. In addition, stool characteristics, gut microbiota qPCR-based analysis and major SCFAs (acetate, propionate, butyrate) did not present any difference among study groups at baseline (Tables 4,5). Culture-based analysis revealed higher baseline levels of *Clostridium perfringens* in plain yogurt compared to control group (p = 0.010) and enriched yogurt group (p = 0.035) (**Supplementary Table 3**). Significant baseline differences were detected in the concentration and molar ratios of iso-butyrate acid with Group A presenting the highest values from all groups ($p_{A\text{-}B}$ = 0.002 and $p_{A\text{-}C}$ = 0.011) and in the concentration ($p_{B\text{-}C}$ = 0.030 and $p_{A\text{-}C}$ = 0.010) and molar ratio of caproic ($p_{B\text{-}C}$ = 0.044 and $p_{A\text{-}C}$ = 0.043) and in the concentration of heptanoic acid ($p_{B\text{-}C}$ = 0.044 and $p_{A\text{-}C}$ = 0.042) with Group C showing significant higher values in both cases (Table 5).

In Group A, a significant drop in molar ratio of other SCFAs was detected after 8 weeks of intervention, which could be rather attributed to the decrease of molar ratio of valerate (p = 0.051). A trend for increased molar ratio of acetate (p = 0.065) and decreased levels of *C. perfringens* group (p = 0.059) were further detected. In Group B, a significant increase in baseline levels of *Lactobacillus* (p = 0.034) and *C. perfringens* (p = 0.032) group was observed after 8 weeks of intervention while no significant differences were detected in the concentrations and molar ratio of SCFAs. In Group C, a significant increase in baseline levels of *C. perfringens* group (p = 0.014), *Bifidobacterium* spp. (p = 0.021) and Firmicutes-to-Bacteroidetes ratio (F/B ratio, p = 0.035) (mean F/B ratio increase of 0.01/g faeces) was observed after the intervention. In the case of bifidobacteria, four subjects exhibited a rather extreme $\geq$20.0% positive change in initial levels (one in each Group A and B, two in Group C) – further analysis without these cases confirmed the significant increase in initial bifidobacterial levels in Group C (10.43 $\pm$ 0.46 vs. 10.59 $\pm$ 0.50, p = 0.049), with no further change in the other two study groups. SCFAs analysis indicated a significant drop in baseline faecal caproic levels (p = 0.025) and a trend for lower molar ratio of butyrate (p = 0.058) after the consumption of enriched yogurt (Tables 4,5).

Comparison of % changes of tested parameters revealed a significant higher % increase in *C. perfringens* group levels in both enriched (p = 0.002) and plain yogurt (p = 0.012) groups after the 8 week-intervention compared to control. Likewise, a higher % increase in molar ratio of BSCFAs was detected for both enriched (p = 0.007) and plain yogurt (p = 0.057) groups after the 8 week-intervention compared to control. Moreover, plain yogurt had a trend for higher % change in iso-butyrate concentration compared to control (p = 0.086), whereas enriched yogurt group had a trend for higher % increase in iso-valerate concentration (p = 0.074), but also for greater % decrease of TVFAs (p = 0.058), acetate (p = 0.080) and propionate (p = 0.074) levels compared to control. Based on analysis, no significant difference was detected in % changes of tested parameters between plain and enriched yogurt groups (Tables 4,5). In 'control vs. overall yogurt analysis', a significant higher % increase in *C. perfringens* group levels (p < 0.001) and molar ratio of BSCFAs (p = 0.035) was also detected in the case of yogurt consumption, with a trend for higher molar ratios of iso-valerate (p = 0.090) and valerate (p = 0.099) and lower TVFAs (p = 0.099) and acetate concentrations (p = 0.078).

3.5 Associations between the % Change of the Specific Activities of PAF Metabolic Enzymes and Platelet Aggregation against PAF and the % Change of Gut Microbiota qPCR-Based Analysis in Yogurt Groups

Results from linear regression analysis after sex, age and BMI adjustment are presented as β-coefficients $\pm$ standard error with p-values in Table 6.

In Group C, the augmentation of *C. perfringens* group had a trend for inverse correlation with the specific activity of the plasma catabolic enzyme, $LpPLA_2$ (–0.511 $\pm$ 0.282, p = 0.087) and for positive correlation with PAF EC_{50} values (5.976 $\pm$ 2.880, p = 0.053). Additionally, the increased F/B ratio tended to positively correlate with the ratio Lp-PLA_2 activity/LDL (5.022 $\pm$ 2.709, p = 0.080).

In Group B, the increased levels of *C. perfringens* group had also a trend for inverse correlation with the specific activity of the plasma catabolic enzyme, $LpPLA_2$ (–0.484 $\pm$ 0.238, p = 0.067), as well as an inverse correlation

Table 6. Linear regression analysis regarding the association between the % change of the specific activities of PAF metabolic enzymes and platelet aggregation against PAF and the % change of gut microbiota qPCR-based analysis in yogurt groups.

Group B				
(Plain yogurt, n = 17)				
	C. perfringens group	*Lactobacillus* group	*Bifidobacterium* spp.	F/B
Beta-Coefficient (standard error)				
EC_{50} PAF	ns	ns	–8.319*** (2.492)	ns
Lyso-PAF ATE	–1.099*** (0.337)	ns	ns	ns
PAF-AH	ns	–3.343** (1.442)	ns	ns
$LpPLA_2$	–0.484* (0.238)	ns	ns	ns
Group C				
(Enriched yogurt, n = 23)				
EC_{50} PAF	5.976* (2.880)	ns	ns	ns
$LpPLA_2$	–0.511* (0.282)	ns	ns	ns
$LpPLA_2$/LDL	ns	ns	ns	5.022* (2.709)

Beta-Coefficients and their corresponding standard errors were obtained from linear regression analysis after adjusting for age, sex and body mass index. Gut microbiota was considered the independent variable.
EC_{50} PAF, Half maximal effective concentration of PAF; F/B, Firmicutes-to-Bacteroidetes ratio; $LpPLA_2$, Lipoprotein-associated phospholipase A_2; Lyso-PAF ATE, Lyso-platelet-activating factor acetyltransferase in the presence of EDTA; PAF-AH, Platelet-activating acetylhydrolase; PAF-CPT, Platelet-activating factor-choline phosphotransferase.
***$p < 0.01$, **$p < 0.05$, *$p < 0.10$, ns: no significant.

with the specific activity of the biosynthetic enzyme, Lyso-PAF ATE (–1.099 ± 0.337, $p = 0.007$) while the increase in *Lactobacillus* group was inversely correlated with the specific activity of the intracellular catabolic enzyme, PAF-AH (–3.343 ± 1.442, $p = 0.039$). In addition, the levels of *Bifidobacterium* spp. were inversely correlated with PAF EC_{50} values (–8.319 ± 2.492, $p = 0.006$).

3.6 Associations between the % Change of Faecal SCFAs and the % Change in PAF Metabolic Enzymes in Yogurt Groups

The specific activity of PAF-AH was inversely correlated with the levels of butyrate (–0.366 ± 0.173, $p = 0.049$) in Group C and with BSCFAs levels (–0.201 ± 0.100, $p = 0.068$) and their molar ratio (–0.270 ± 0.107, $p = 0.027$) in Group B, after sex, age and BMI adjustment.

3.7 Associations between the % Change of Faecal SCFAs and the % Change in the Inflammation and Hemostatic Markers in Enriched Yogurt Group

The decrease of IL-10 in Group C was positively correlated with the reduction of the concentration of butyrate (0.175 ± 0.082, $p = 0.045$) and an inverse correlation was observed between the % change in the molar ratio of iso-butyrate and PAI-1 activity (–0.060 ± 0.027, $p = 0.039$), both in unadjusted model. Nevertheless, connections of butyrate with IL-10 (0.157 ± 0.101, $p = 0.138$) and molar ratio of iso-butyrate with PAI-1 (–0.048 ± 0.031, $p = 0.140$) in Group C were not significant in the final regression model after adjustment for sex, age and BMI.

4. Discussion

The present clinical trial aimed to investigate whether the intake of a yogurt on a daily basis, fortified with a polar lipid extract of olive-oil by-products that contains PAF-R antagonists, could affect gut microbiota and faecal metabolites in apparently healthy, mainly overweight, participants. According to the results, the consumption of the enriched yogurt resulted in a significant increase in the levels of *Bifidobacterium* spp., *C. perfringens* group and F/B ratio. On the other hand, a significant increase in the levels of *Lactobacillus* and *C. perfringens* group was observed after the intake of the plain yogurt. Additionally, a higher % increase in molar ratio of BSCFAs was detected for both yogurt groups after the 8 week-intervention compared to control. The consumption of the enriched yogurt also resulted in a significant drop in faecal caproic levels and a trend for lower ratio of butyrate to total VFAs compared to baseline levels.

Previous intervention trials in adults have reported increased levels of bifidobacteria and in some cases lactobacilli populations after daily yogurt intake [15,48,49] even though no effect in faecal bifidobacteria has also been stated [50,51]. No differences were detected in the faecal levels of clostridia and *E. coli* in previous studies [48,51], while in a study that included over 65 years old Irish subjects, the presence of *C. perfringens* was negatively correlated with the number of *Bifidobacterium* spp. recovered [52]. Similar bacterial changes, with a concomitant decrease in *Bacteroides vulgatus* levels, after yogurt consumption have been previously attributed to the normalization of the bacte-

rial density groups, probably reflecting a more balanced gut microbiota profile [53,54]. Furthermore, it should be noted that despite the increase of *C. perfringens* group levels after yogurt consumption in our study, no diarrheic episodes or severe gastrointestinal side effects were reported during the intervention period. The increase in the levels of *Bifidobacterium* spp. observed after the intake of the enriched yogurt may be also due to the existence of olive pomace carbohydrates and polysaccharides since it has been documented that fructo-oligosaccharides promote the growth of endogenous beneficial organisms such as bifidobacteria [55].

It should note that the OOPLE extract contained 30% w/w maltodextrin (MDX) as a protective colloid for emulsions as well as an emulsion polymerization material, resulting in an intake of approximately 0.23 g MDX/day. It has been reported that MDX dosages ranging between 0.5 and 15 g/d for 1–16 weeks resulted in a significant increase of various Firmicutes members, in increased acetate and propionate levels, in decreased BSCFAs while the effect of MDX on butyrate levels varied between studies [56,57]. The significant increase in F/B ratio after the consumption of the enriched yogurt was probably due to the raise of baseline Firmicutes levels and could be merely attributed to the presence of MDX in the food matrix, though daily intake of MDX in our study was much lower compared to the previous reported. Furthermore, elevated F/B ratio may be resulted by components present in the OOPLE extract of the yogurt, since a similar rise in F/B ratio has already been documented in mice fed with a virgin olive oil enriched diet [58].

In our study, SCFAs analysis indicated a significant drop in faecal caproic levels and a trend for lower ratio of butyrate to total VFAs after the consumption of the enriched yogurt. Caproic acid, also characterized as medium chain fatty acid (MCFA), seems to exert pro-inflammatory properties through the activation of p38 MAPK signaling and also its serum concentration is increased in multiple sclerosis patients, although its role in other pathological conditions is still under consideration [59,60]. It has been suggested that caproic acid is produced from the elongation pathway of acetyl-CoA, -the product of lactate oxidation-, resulting in the formation of butyryl-CoA and hexanoyl-CoA [61]. The decrease of both butyrate and caproic acid detected after the intake of the enriched yogurt is consistent with the above biosynthetic pathway. Additionally, a significant higher % change in molar ratio of BSCFAs was detected for both enriched and plain yogurt group after the 8 week-intervention compared to control. Even though, no significant differences were detected between yogurt groups in the % changes of the gut microbiota and faecal metabolites, plain yogurt had a trend for higher % change in iso-butyrate concentration whereas enriched yogurt group had a trend for higher % increase in iso-valerate concentration. The elevated BSCFAs and specifically the increase in iso-butyrate and iso-valerate levels after yogurt consumption was somewhat expected since it has been documented that they are mainly produced during protein fermentation and especially from valine and leucine degradation by the intestinal microbiota carried out mainly by genera *Bacteroides* and *Clostridium* [62–64].

The relation between gut microbiota and PAF metabolism has not been studied with the exception of one study that revealed no connection between *Akkermansia muciniphila* and $LpPLA_2$ activity [65]. Regarding PAF action, it has been reported that *L. acidophilus*, as a probiotic, counteracts PAF-induced inflammation in cell line derived from normal human colon and Caco-2 cells by reducing nuclear factor kappa B activation and IL-8 production [66]. Lyso-PAF that is the precursor molecule for the remodeling PAF biosynthesis, as well as the product of PAF-acetylhydrolases action, has been detected in faecal samples of healthy donors and also PAF and lyso-PAF levels have been augmented in ileoanal anastomosis and pouchitis [67]. As far as yogurt intake is considered, the microbial species present in yogurts and especially *S. thermophilus* and *L. acidophilus* have the metabolic capacity to synthesize phospholipids among them glyceryl-ether analogs [66] and lyso-PAF plasma levels were directly associated with full-fat dairy intake including yogurt [68]. Additionally, the existence of PAF inhibitors in the lipid fraction of yogurts has been documented [66,69–71]. In the plain yogurt group, the increase in *C. perfringens* seems to reduce both PAF catabolism through $LpPLA_2$ action and the calcium-independent PAF remodeling biosynthetic pathway, while the increase in *Lactobacillus* group appears also to reduce intracellular PAF catabolism (PAF-AH activity), indicating that PAF levels may be increased. Similar to our results, phospholipase C from *C. perfringens* had been documented to rise PAF levels by stimulating PAF remodeling biosynthetic pathway in cultured intestinal epithelial cells, but no data presented whether this stimulation was observed in the presence or absence of calcium [72]. In the same group, *Bifidobacterium* spp. reinforced PAF action in platelets as lower concentration of PAF was needed to induce *ex vivo* PRP aggregation implying the secretion of a PAF analog that is in accordance with previous data [73]. Interestingly, in the enriched yogurt group, the increase of *C. perfringens* group was also inversely correlated with the plasma catabolic enzyme, $LpPLA_2$, but the increased F/B ratio was positively correlated with the $LpPLA_2$ activity taking under consideration the LDL levels, the main carrier of $LpPLA_2$. In addition, we have previously reported that the intake of the enriched yogurt resulted in lower $LpPLA_2$-to-LDL ratio at 8 weeks compared to the plain yogurt [19]. Moreover, *C. perfringens* group seems to inhibit PAF action as a positively correlation with PAF EC_{50} values, lower response of platelets against PAF, was detected. Since, this correlation was not observed in the plain yogurt group, the implication of PAF-R antagonists present in the OOPLE ex-

tract cannot be excluded and the reduced PAF action has already been documented in this group [18]. Additionally, it was recently reported that PAF is a weak activating agonist and an allosteric inducer of FFAR2 (free fatty acid receptor 2) also known as GPR43, a G protein-coupled receptor that responds to short-chain fatty acids, especially acetate and propionate, produced by gut microbiota fermentation, through a receptor cross-talk mechanism and the PAF induced transactivation of FFAR2 was inhibited by the PAFR specific antagonist WEB in human neutrophils [74]. Since the OOPLE extract contains PAF-R antagonists, it may also be implicated in the inhibition of the PAF induced transactivation of FFAR2.

5. Conclusion

Daily low-fat yogurt consumption for 8 weeks led to a significant increase in the levels of *C. perfringens* group that was inversely associated with the plasma catabolic enzyme of PAF, namely $LpPLA_2$, that is considered a cardiovascular risk marker and has been associated with inflammation and atherosclerosis. Moreover, in the enriched with PAF-R antagonists yogurt group, the increased levels of *C. perfringens* group were also associated with lower PAF action. An increase in the levels of *Bifidobacterium* spp. and a significant drop in faecal caproic levels was also detected in the enriched yogurt group indicating a favorably effect of PAF antagonists on gut microbiota in healthy overweight subjects although further exploration is needed to elucidate the mechanism.

6. Limitations

The participants were apparently healthy overweight adults of Greek ethnicity with medium adherence to Mediterranean diet, thus the findings may not apply to different population groups. Even though the study protocol included 92 participants, stool samples were provided before and after the intervention from only 51 adults (58% participation rate). Additionally, the participants of Group B that provided stool samples presented a metabolically slightly less healthy profile that may have influenced their response to the intervention. PAF levels were not evaluated that would provide a direct information regarding its relation with specific microbial species. Gut microbiota diversity could be further analyzed in-depth by e.g., 16S rRNA sequencing. Also, we did not measure other microbial-derived metabolites such as trimethylamine, a precursor of trimethylamine N-oxide that has been associated with platelet activity. On the other hand, the study design (i.e., randomized, double-blind) eliminated any potential sources of bias (i.e., selection, performance and detection bias). Lastly, subjective tools and methods, i.e., self-administered records, phone calls were used to evaluate the compliance of participants to the study protocol.

To our knowledge, this is the first study that has investigated the impact of two yogurts, a plain one and an one enriched with PAF inhibitors on gut microbiota and faecal metabolites in healthy overweight subjects and report significant associations between gut microbiota and PAF metabolism and action.

Availability of Data and Materials

Data are available from the corresponding author upon reasonable request.

Author Contributions

SA designed the research study, supervised its implementation and drafted the manuscript; EKM performed the evaluation of stool characteristics, gut microbiota qPCR-based analysis and SCFAs, performed the statistical analyses and critically revised the manuscript; AK designed and supervised the gut microbiota analysis and SCFAs and critically revised the manuscript; EF supervised the biochemical analyses, performed the statistical analyses and critically revised the manuscript; MD performed the biochemical analyses. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All participants were informed about the objectives and procedures of the study and provided their written consent before enrollment. The study took place at the Department of Nutrition and Dietetics of Harokopio University in Athens, Greece, from October 2014 to June 2016. The trial adhered to the guidelines of the Declaration of Helsinki (1989) of the World Medical Association, was approved by the Bioethics Committee of Harokopio University (40/30-10-2013) and was registered in ClinicalTrials.gov (NCT02259205).

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Conflict of Interest

Smaragdi Antonopoulou states that given her role as Guest Editor, she had no involvement in the peer-review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Dr. Amedeo Amedei and Dr. Eugene Rosenberg. Smaragdi Antonopoulou has a relevant patent (Hellenic Industrial Property Organisation, 1008550—25/08/2015 issued). The other authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2904159.

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