Title

Effect of virgin olive oil and thyme phenolic compounds on blood lipid profile: implications of human gut microbiota.

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Abbreviations

CVD: Cardiovascular diseases

FC: Flow cytometry

FISH: Fluorescence in situ hybridization

FSC: Forward scatter detector

FVOO: phenolic compounds-enriched virgin olive oil containing 500 mg phenolic compounds/kg, from olive oil

FVOOT: phenolic compounds-enriched virgin olive oil containing a mixture of 500 mg phenolic compounds/kg, from olive oil and thyme, 1:1.

MD: Mediterranean diet

PC: Phenolic compounds

PCA: Protocatechuic acid

SCFA: Short chain fatty acids

SSC: Side scatter detector

VOO: Virgin olive oil naturally containing 80 mg of phenolic compounds/kg

Abstract

Purpose To investigate the effect of virgin olive oil phenolic compounds (PC) alone or in combination with thyme PC on blood lipid profile from hypercholesterolemic humans, and whether the changes generated are related with changes in gut microbiota populations and activities.

Methods A randomized, controlled, double-blind, crossover human trial (n=12) was carried out. Participants ingested 25 mL/day for 3 weeks, preceded by 2-week washout periods, of three raw virgin olive oils differing in the concentration and origin of PC: (1) a virgin olive oil naturally containing 80 mg PC/kg, (VOO), (2) a PC-enriched virgin olive oil containing 500 mg PC/kg, from OO (FVOO), and (3) a PC-enriched virgin olive oil containing a mixture of 500 mg PC/kg from OO and thyme, 1:1 (FVOOT). Blood lipid values and fecal quantitative changes in microbial populations, short chain fatty acids, cholesterol microbial metabolites, bile acids, and phenolic metabolites were analyzed.

Results FVOOT decreased seric ox-LDL concentrations compared with pre-FVOOT, and increased numbers of bifidobacteria and the levels of the phenolic metabolite protocatechuic acid compared to VOO ($P<0.05$). FVOO did not lead to changes in blood lipid profile nor quantitative changes in the microbial populations analyzed, but increased the coprostanone compared to FVOOT ($P<0.05$), and the levels of the fecal hydroxytyrosol and dihydroxyphenilacetic acids, compared with pre-intervention values and to VOO respectively ($P<0.05$).

Conclusion The ingestion of a PC-enriched virgin olive oil, containing a mixture of olive oil and thyme PC for three weeks, decreases blood ox-LDL in hypercholesterolemic humans. This cardio-protective effect could be mediated by the increases in populations of bifidobacteria together with increases in anti-oxidant PC microbial metabolites.

Introduction

Adherence to the Mediterranean Diet (MD) has shown to be cardio-protective [1]. Consumption of virgin olive oil (OO), the main fat of the MD, has demonstrated a relevant influence on its beneficial effects [2]. Besides oleic acid, the phenolic fraction of virgin OO also contributes to the health effects associated with virgin OO consumption [3]. Its antioxidant and anti-inflammatory activities, acting as pathway and gene expression modulators [4], may explain such health properties. However, the mechanisms by which virgin olive oil PC influence cardiovascular disease (CVD) risk factors are not fully understood. Recent insights indicate that gut microbiota plays an important role in CVD, and represents a realistic therapeutic target [5]. Some of reported gut

microbiota-related mechanisms by which gut bacteria could influence CVD risk factors such as the presence of abnormal levels of blood lipids [6], could be one or a combination of the following: (1) involvement in cholesterol synthesis through generation of short chain fatty acids (SCFA) which are generated from microbial fermentation of undigested substrates, either increasing blood total cholesterol (i.e., acetic) or decreasing it (i.e., propionic, butyric) [7]; (2) reduction of the amount of cholesterol available for re-absorption from the intestine by either transforming gut cholesterol to insoluble metabolites and, thereby, its uptake from the gut [8] and/or by incorporating cholesterol into the microbial cellular membrane [9,10]; (3) deconjugation of bile salts in the gut, generating insoluble primary bile acids which are excreted in feces [11], which lead to cholesterol expenditure in the liver in order to synthesize new bile acids; (4) generation of bioactive metabolites in the gut with cardio-protective properties. Diet appears to critically in influence both the relative abundance of different gut microorganisms and their metabolic output. In this sense, high intake of PC from different sources appears to regulate some CVD risk factors [12, 13], through the modulation of microbial populations and activities [14], as many plants PC are not totally absorbed and become available for microbiota utilization as an energy source which has an impact on nutrient bioavailability and host metabolism. Regarding virgin olive oil PC, recent studies have demonstrated that they are able to reach the gut, being transformed by gut microbiota [15]. It has also been shown that the bioaccesibility of virgin olive oil PC can be increased by combining virgin olive oil PC with other PC sources (i.e, thyme) [16, 17]. Since the interaction of virgin olive oil phenolic compounds with gut microbiota and its involvement in CVD risk remains to be elucidated, the aim of our study was to investigate the effect of a sustained consumption of virgin olive oil PC, alone or in combination with thyme PC on blood lipid levels in hypercholesterolemic subjects, and whether this effect is mediated by gut microbiota-related mechanisms.

Material and methods.

Study subjects and design

The present study included a subsample of 12 hypercholesterolemic (total cholesterol>200 mg/dL) adults (5 females and 7 males) aged 46–67 y from the VOHF (Virgin Olive Oil and HDL Functionality) study. The VOHF study was a randomized, controlled, double-blind, crossover clinical trial with 33 hypercholesterolemic volunteers, aged 35 to 80 year. Exclusion criteria included the following: BMI>35 Kg/m2, smokers, athletes with high physical activity (>3000 Kcal/day), diabetes, multiple allergies, intestinal diseases, or other disease or condition that would worsen adherence to the measurements or treatments. The study was conducted at IMIM-

Hospital del Mar Medical Research Institute (Spain) from April 2012 to September 2012. Participants ingested 25 mL/day for 3 weeks, preceded by 2-week washout periods, of three raw virgin olive oils differing in the concentration and origin of phenolic compounds (PC): (1) a virgin olive oil naturally containing 80 mg PC/kg (VOO), (2) a PC-enriched virgin olive oil containing 500 mg PC/kg, from olive oil (FVOO), and (3) a PC-enriched virgin olive oil containing a mixture of 500 mg PC/kg from olive oil and thyme, 1:1 (FVOOT). Participants were randomized to one of 3 orders of administration (Order 1: FVOO, FVOOT and VOO, Order 2: FVOOT, VOO and FVOO, Order 3: VOO, FVOO and FVOOT). Elaboration of PC enriched olive oils (i.e. FVOO and FVOOT) is described by Rubió et al [18]. Full phenolic composition of the three oils is presented in Table 1. The random allocation sequence was generated by a statistician, participant enrolment was carried out by a researcher, and participants' assignment to interventions according to the random sequence was done by a physician. Due to the fact that all participants received each one of the three oils, restrictions such as blocking were unnecessary. In order to avoid an excessive intake of PC other than those provided by the intervention's oils, participants were asked to limit the consumption of rich-polyphenol food and dietary data was recorded by 3-day dietary record at baseline and before and after each intervention period. The corresponding 25 mL bottles of the corresponding oil for each day of consumption were provided to the participants at the beginning of each intervention period. The participants were instructed to return the bottles in order to register the amount of the intervention oil consumed. Subjects with less than 80% of treatment adherence (≥ 5 full oil containers returned) were considered non-compliant for the dietary intervention. Blood at fasting state (of at least 10 hours) and fecal samples were collected before and after each intervention period. All participants provided written informed consent, and the local institutional ethics committees approved the protocol (CEIC-IMAS 2009/3347/I). The trial was registered with the International Standard Randomized Controlled Trial register (www.controlled-trials.com; ISRCTN77500181).

Dietary adherence

Twenty-four-hour urine was collected at the start of the study and before and after each treatment. Urine samples were stored at –80°C prior to use. We measured urinary hydroxytyrosol sulfate and thymol sulfate as biomarkers of adherence to the type of OO ingested in urine by ultra-HPLC-ESI-MS/MS [19]. A 3-day dietary record was administered to the participants at baseline and before and after each intervention period. A nutritionist personally advised participants to replace all types of habitually consumed raw fats with the OOs, and to limit their polyphenol-rich food consumption.

Serum lipid profile analysis

Total and HDL cholesterol, and triglyceride concentrations were measured by using standard enzymatic automated methods. When triglyceride concentrations were <300 mg/dL, LDL cholesterol was calculated by using Friedewald's formula. Oxidized LDL was determined with an ELISA procedure that employed the murine monoclonal antibody mAb-4E65 (Mercodia AB).

Fecal sample collection and pre-analytical treatment

For fecal collection, participants were *g*iven a set containing: a sterile pot, a w-zip plastic pouch (AN0010W, Oxoid, Basingstoke, UK), two anaerobic sachets (AnaeroGen Compact AN0025, Oxoid), and one anaerobic indicator (BR0055, Oxoid). Freshly voided fecal samples were collected by the volunteers in the sterile pot and kept under anaerobic conditions by introducing them into the plastic pouch, together with the anaerobic sachets and the anaerobic indicator. In order to avoid changes in microbial populations, fecal samples were brought to the laboratory within 2 hours after defecation.

For quantitative analysis of gut microbiota, feces were diluted with sterile 0.1 M, pH 7.0, phosphate buffered saline (PBS, Sigma-Aldrich Co. LLC., St. Louis, United States) (1:10,w:w), mixed in a Stomacher 400 (Seward, Thetford, Norfolk, UK) for 2 min and fecal slurries homogenized. After centrifugation (1300 *g*, 3 min), hexane (Sigma-Aldrich, UK) was added to the fecal homogenate supernatant (4:1, v:v), mixed by inversion for 2 minutes and removed after centrifugation (15500 *g*, 5 min) and evaporation. Pellets were washed in 1mL of filtered sterile PBS and centrifuged (15500 *g*, 5 min). Afterwards, pellets were then diluted in 375 µL of PBS and fixed in ice-cold 4% (w:v) paraformaldehyde (PFA) (1:4, v:v) for 4 h at 4ºC. PFA was discarded after centrifugation (15500 *g*, 5 min) and washing twice in 1mL of sterile PBS. Cells containing pellets were homogenized in 150 µL of sterile PBS plus 150 µL of ethanol and stored at -20ºC until analysis.

For the rest of the fecal analytical determinations (phenolic metabolites, short chain fatty acids (SCFA), cholesterol microbial metabolites and bile acids) fresh feces were immediately frozen at -80ºC, freeze dried, weighed to determine the percentage of humidity, milled, desiccated, and stored at -20ºC.

Quantification of fecal microbiota by FISH-FC

Bacterial hybridizations were based on the method described by Massot-Cladera et al [20] with some modifications. Briefly, 5 µL of fixed cell suspensions were centrifuged at 15500 *g* for 5 minutes. 30 µL of a mixture (1:10, v:v) of synthetic oligonucleotide probes (50 ng/µL) targeting specific diagnostic regions of 16S rRNA and labeled with the fluorescent Cy3 dye (Ato291 [21], Bac303 [26], Bif164 [27], Chis150 [22], Erec482

[22], Fprau645 [23], Lab158 [24], Prop853 [25] and Rrec584 [25]), plus preheated hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl pH 8, 0.01% sodium dodecyl sulfate), were added to the pellets, homogenized and incubated in a thermocycler for 4 hours at each specific probe hybridization temperature, in the dark. After hybridization, samples were washed by adding 2 mL of a mixture of preheated wash buffer (0.9 M NaCl, 20 mM Tris–HCl pH 8) where 0.04% of 6-diamidino-2-phenylindole dihydrochloride (50 ng/ µL; Sigma-Aldrich) was added, for 15 minutes at each specific probe hybridization temperature in the dark. Washed samples were then centrifuged at 15500 *g* for 5 minutes. Pellets were homogenized in 200 µL of PBS solution and kept in the dark at 4ºC overnight. Immediately before the flow cytometry analysis, 30 µL of Commercial Flow CheckTM Fluorospheres (Beckman Coulter, Inc. FL, USA) were added to the samples in order to calculate total counts of bacteria.

A LSRFortessa flow cytometer (Becton Dickinson, New Jersey, United States) was used for bacteria quantifications. The flow cytometer parameters were adjusted for bacterial counts. Bacteria morphology was selected according to their FSC/SSC signal. For this purpose, selected bacteria (Leibniz-Institut DSMZ collection, Germany) representative of each bacterial *g*roup hybridized by each probe (*Collinsella aerofaciens* for Ato291; *Bacteroides caccae* for Bac303; *Bifidobacterium bifidum* for Bif164; *Clostridium acetobutylicum* for Chis150; *Clostridium saccharolyticum* for Erec482; *Faecalibacterium praustnizii* for Fprau645; *Lactobacillus plantarum* for Lab158; *Megasphaera elsdenii* for Prop853; *Roseburia intestinalis* for Rrec584) were *g*rown, fixed and hybridized as described above. Bacteria hybridized with Cy3-labeled probes were detected using a yellow and *g*reen laser (561 nm filter), and DAPI bacteria stained DNA was detected using violet one (405 nm filter). Both lasers worked at 50 mW power. An acquisition *g*ate of 2500 fluorospheres was established. Analysis was performed using the FACSDiva software version 6.1.2 (Becton Dickinson). Microbiota composition results were expressed as the log fecal cells/g dry feces for each sample.

Analysis of SCFA

For the analysis of SCFA, freeze-dried samples were diluted 10-fold with milliQ water and centrifuged, first at 1800 *g* for 5 minutes and afterwards at 8784 *g* for 4 minutes at 4ºC. Supernatants were filtered through a 0.22 µm filter and subjected to GC analysis (Agilent 7890A Series, Santa Clara, EEUU) using a capillary BP-21column (SGE, Cromlab SL, Barcelona, Spain) (30 m, 0.25 mm, 0.25 µm) coupled to a flame ionization detector (FID) [28]. 4-methyl valeric acid (Sigma-Aldrich) was used as internal standard. Concentrations of SCFA were calculated from calibration curves using standard solutions with known concentrations of acetic,

propionic, butyric, isobutyric, isovaleric and valeric acids (Sigma-Aldrich). Results were expressed as µmol/g dry feces.

Analysis of cholesterol microbial metabolites and bile acids.

Freeze dried milled feces were diluted in milliQ water (0.1:4, m:v) and homogenized. A volume of 400 µL of fecal homogenate was used for the extractions, following the method described Santas et al. [29] with some modifications. For cholesterol and its microbial metabolites, sterol mixtures were prepared as calibrators. 5-α-cholestane (10 µg, Sigma-Aldrich) was used as internal standard. Samples were hydrolyzed with 1 mL of NaOH (1N, in ethanol) for 1h at 70ºC. After cooling the tubes at room temperature, 0.5 mL of water was added and tubes were sonicated for 5 minutes. After 2 extractions with cyclohexane (3 mL each), mixed organic phases were evaporated under a 15 psi nitrogen stream at 30ºC.

For bile acid analysis, bile acid mixtures were prepared. 5-β-cholanic acid (Sigma-Aldrich) was used as internal standard. Samples were hydrolyzed with 1 mL NaOH 1N at 70ºC for 1h. After cooling the tubes, liquid-liquid extraction with 3 mL of *tert*-butylmethyl ether was done twice. Organic phases were further cleaned with 2 mL of NaCl 1%. The organic phase was evaporated under a 15 psi nitrogen stream at 30ºC.

Derivatization of both sterols and bile acids was carried out by addition of 50 µL of *N*-methyl-bis(trifluoroacetamide)/NH_4I/2-mercaptoethanol (1000/2/6) (Macherey-Nagel, Düren, Germany) and dry heated at 60ºC for 20 minutes. A gas chromatograph (6890 N; Agilent Technologies, Wilmington, DA, USA) equipped with a mass selective detector (5973 Network, AT) and an autosampler injector (7683 series, AT) was used for analysis and performed in a 100% methylsiloxane column (Agilent Ultra 1) in all cases. After derivatization, both neutral sterols and bile acids were quantified with their respective standards, expressed as µmol/g and µmol/10g dry feces, respectively.

Analysis of phenolic microbial metabolites

For PC metabolite analysis, freeze-dried feces (0.1 *g*) were mixed with 1 mL of milli-Q water. Samples were shaken for 30 min and centrifuged (13200 *g*, 10 min, 4ºC). Supernatants were centrifuged (13200 *g*, 10 min, 4ºC) and filtered through a membrane (0.22 µm pore size) and transferred to chromatographic vials. PC metabolites analysis was performed as previously described [15,17] and were quantified using the calibration curve of their respective standard. PC metabolites concentration results were expressed as µmol/100g dry feces.

Sample size

The sample size for this study was calculated with the free software GRANMO (http://www.imim.cat/ofertadeserveis/software-public/granmo/) by selecteing 80% power (5% α level) to detect a 0.4 log10/g dry feces difference among treatments in the primary outcome variable (Bif164 counts/g of dry feces), with a standard deviation of treatment differences less than 0.48 (log10 scale).

Statistical analysis

Normality of continuous variables was assessed with normal probability plots and the Shapiro-Wilk test. Non-normally distributed variables were log transformed previous to the analysis. Paired t test was used for intra-intervention comparisons. Adjusted general linear mixed models with a period-by-treatment interaction term were used for inter-intervention comparisons, given results as adjusted means. $P \leq 0.05$ was considered significant. Statistical analyses were performed with an R software version 2.11.1 (R Development Core Team, 2011; www.R-project.org).

Results

Compliance

Urine compliance markers (hydroxytyrosol sulfate and thymol sulfate) indicated good adherence to the oil interventions (Supplementary table 1). The three intervention oils were well tolerated by all participants and no adverse events were reported.

Blood lipid profile

Serum concentrations of cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol) did not present any statistical change with any of the dietary interventions (data not shown). Oxidized LDL concentrations decreased after FVOOT intervention compared to pre-FVOOT values (P=0.049) (Figure 1)

Analysis of quantitative changes in gut microbiota

Quantitative changes in fecal microbiota before and after interventions are presented in table 2. Consumption of virgin olive oil PC alone (FVOO) did not have a significant effect on bacterial counts. Only a decrease in numbers of *Clostridium cluster IX* (Prop853) compared to VOO intervention was observed, although this decrease did not reach statistical significance (P= 0.066). When virgin olive oil PC were combined with thyme PC (FVOOT) the numbers of the bacterial groups hybridized by Bif164 probe (most *Bifidobacterium spp* and

Parascardovia denticolens) significantly increased compared to VOO (P= 0.044). PC combination also increased the numbers of *Roseburia-Eubacterium rectale* group (Rrec584 probe) compared to FVOO intervention but this increment did not reach statistical significance (P= 0.085).

Analysis of changes in microbial activities.

The analysis of the main SCFA generated by gut microbial fermentation (acetic, butyric, propionic and branched acids) did not show significant changes with any of the interventions (table 3). Their relative amounts remained constant along the trial irrespective of the intervention (54-58%, 15-18%, 16-18% and 8-10%, respectively). From the fecal concentrations of cholesterol and microbial cholesterol metabolites (coprostanol, coprostanone, cholestanone) analysed, only coprostanone changed with the dietary interventions, by increasing with FVOO compared to FVOOT (P= 0.028) (table 3). Changes in concentrations of fecal bile acids after dietary interventions did not reach statistical significance (table 3). However, the relative proportion of isolithocholic acid, referred to the total of fecal bile acids analyzed, decreased after FVOO compared with pre-FVOO (from 6.29 % CI (5.81, 6.80) to 3.47 % CI (3.20, 3.75); P=0.020).

The analysis of fecal phenolic metabolites is presented in table 4. Hydroxytyrosol increased and tended to increase after FVOO (P=0.034) and FVOOT (P=0.064) respectively. FVOO increased dihydroxyphenylacetic acid compared to VOO (P=0.014). Protocatechuic acid increased with FVOOT compared to VOO (P=0.003). Two unknown phenolic metabolites increased after FVOO compared with VOO (P=0.027 and P=0.042, respectively).

Discussion

The aim of this study was to elucidate whether the possible cardio-protective effects of a sustained consumption of virgin olive oil phenolic compounds (PC) alone or in combination with thyme PC are mediated by changes in gut microbiota populations and metabolic activities, in hypercholesterolemic humans.

The combination of olive oil and thyme PC exerted a cardio-protective effect, by decreasing blood levels of ox-LDL. The anti-oxidant activity of some microbial phenolic metabolites such as hydroxytyrosol and protocatechuic acid, generated after gut microbial fermentation of phenolic compounds contained in FVOOT, could be involved in the observed effects on LDL. After oxidation, LDL becomes more toxic and plays a primary role in the development and progression of atherosclerosis [30]. It has been reported that oxidation of LDL decreases with increasing phenolic content of olive oil [3, 31,32]. In fact, the European Food Safety

Authority approved a claim concerning the benefits of olive oil polyphenols for the protection of LDL from oxidation [33]

We observed quantitative significant changes in gut microbiota only when virgin olive oil PC were ingested together with thyme PC, by increasing *Bifidobacterium* group numbers. The gut microbial usage of virgin olive oil and thyme PC has been recently reported in a linear non controlled study, by Mosele et al. [15,17]. They observed the generation of PC metabolites after microbial transformation of virgin olive oil and thyme parental PC, concluding that some of the investigated parental PC were able to reach the gut, afterwards being transformed by gut microbiota. Increases in gut bifidobacteria have been reported with other sources of phenolic compounds such as wine [34], wild blueberry [35], pomegranate peel [36] and cocoa [7]. However, this is the first time that a potential bifidogenic effect is reported for a combination of virgin olive oil and thyme PC. Since prebiotic is defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" [37] a potential prebiotic activity of the combination of PC in FVOOT is suggested, and should be further investigated. Since recent studies in animals and humans have shown improvements in blood lipid profile with the ingestion of bifidobacteria and lactobacilli mixtures [38, 39], the increase in *Bifidobacterium* could be responsible at least in part for the decrease in oxLDL levels observed with the ingestion of FVOOT.

Regarding blood cholesterol levels, hardly any effects were observed on the cholesterol lowering mechanisms investigated. This is in accordance with the lack of changes reported in blood cholesterol levels. First, we investigated whether microbial usage of PC was able to generate changes in SCFA production, related to cholesterol synthesis. In our study, neither fecal amounts of acetic, propionic and butyric acids nor their relative amounts changed with any of the interventions. Grapefruit PC have been shown to increase amounts of SCFA in rats [40] whereas black tea and red wine PC decreased them *in vitro* [41]. The lack of effects observed in our study could be due to the fact that, contrary to what occurs in an *in vitro* system, SCFA generated are also absorbed *in vivo*, making it difficult to observe differences at fecal level. Different results could also indicate that the influence on the microbial generation of SCFA depends on the PC source.

The second mechanism analyzed was the insolubilization of gut cholesterol by its transformation into non-soluble metabolites by gut microbiota. Gut bacteria are able to metabolize cholesterol by two pathways [11]. One of them transforms cholesterol directly in coprostanol. The other, implies the transformation of cholesterol to cholestanone, coprostanone and finally coprostanol. Few studies have investigated this gut microbiota-mediated blood cholesterol lowering mechanism after dietary interventions with PC [42], most of them focusing on

changes in total fecal cholesterol or total fecal lipids. In our study fecal concentrations of cholesterol remained constant with the dietary interventions besides the increase in coprostanone observed with FVOO. This could be due to the fact that we performed fecal analysis in the whole fecal residue, containing bacteria that could be also either assimilating cholesterol or including it in the bacterial cell wall or both. The absence of any impact on fecal cholesterol could be also related to the PC source. Whereas PC sources such as horseradish [42], apple skin [43] and sesame flour [44] have been shown to increase fecal cholesterol in murine models, other sources such as peanut skin [45] or oolong tea [46] decreased or have no effect on fecal cholesterol levels.

The third mechanism studied was the increase in liver cholesterol expenditure due to microbial generation of insoluble bile acids in the gut. Bile acids are synthesized in the liver from cholesterol, conjugated and excreted to the biliary system. From them, 200 to 800 mg/day passes to the colon. Probiotic bacteria, such as bifidobacteria, encode bile salt hydrolase (BSH) enzymes, which deconjugate bile salts [47]. Deconjugated bile acids are not absorbed and are excreted in faeces. As synthesis of new bile acids rises in compensation, blood cholesterol levels fall [14]. In our study, fecal concentrations of primary bile acids did not change with any of the interventions. This is in concurrence with other authors [48] who were not able to detect any effect of black tea PC on fecal bile acids in humans. Other PC sources such as peanuts, hazelnut skin and sesame flour [44, 49] have shown increases in bile acid excretion in murine models. Different sources of PC would have a different effect on BSH producing bacteria. In our study, the increase in populations of bifidobacteria with FVOOT, although significant, could have been not enough to significantly increase in the concentrations of deconjugated bile acids. A novel bile acid-related mechanism has been published recently [50], which suggest that *Lactobacillus*, could reduce blood cholesterol by lowering absorption of fat from the intestine via FXR activation by deconjugated bile acids absorbed from the intestine. Nevertheless, in the present work no decrease in the systemic cholesterol has been observed and further studies are needed to elucidate the mechanisms and their degree of involvement.

Besides the absence of changes in the amounts of fecal bile acids, we found a potential detoxificating effect of FVOO, which reduced the relative proportions of the toxic isolithocholic acid. Deconjugated forms are the substrate for 7-dehydroxylation, which generates toxic secondary bile acids. Consumption of common dietary polyphenols has shown to reduce toxic fecal deoxycholic acid and lithocholic acids in rats [51]. Probiotic bacteria are not capable of dehydroxylate deconjugated bile salts [52], and so the majority of the breakdown products of BSH activity by a probiotic strain may be precipitated and excreted in feces. Only certain strains of

Clostridium and *Eubacterium spp.* have been shown to possess dehydroxylating activity [53]. In our study, the decrease in the relative proportions of isolithocholic acid could be related to the decrease in populations of *Clostridium cluster IX* observed.

The last mechanism investigated in the present study was related to the microbial generation of bioactive PC metabolites in the gut. It has been demonstrated that bioactivity of some microbial metabolites from undigested phenolic compounds is physiologically more relevant on CVD risk than the native form present in the diet. Some representative examples are enterolignans from lignans [54] and protocatechuic acid (PCA) from flavonoids [55]. PCA promotes reverse cholesterol transport in mice [56] and inhibits LDL oxidation [57] suggesting a remarkable anti-atherogenic effect. These and other microbial phenolic metabolites with anti-oxidant activity, such as hydroxytyrosol, generated after gut microbial fermentation of phenolic compounds ingested with the oils can be further absorbed and enter into the blood stream. In our study, fecal PCA increased in FVOOT compared to VOO, which could be due to the microbial transformation of PCA precursors, vanillic and p-hydroxybenzoic acids, present in FVOOT and absent in VOO. We found also increase in hydroxytyrosol after FVOO and FVOOT, which could be due to microbial transformation of OO seicoiridoids, and demonstrates a high stability of hydroxytyrosol in the gut, which is considered to have the highest antioxidant power compared to other olive polyphenols [58]. The increase in the levels of free hydroxytyrosol in feces after FVOO and FVOOT ingestion confirms early observations suggesting that a fraction goes via feces [59] and recent observations in a parallel *in vivo* trial [15]. In this regard, although the increase in hydroxytyrosol with FVOOT did not reach statistical significance, it could be behind, in combination with the increase in PCA, the decrease in LDL oxidation observed after the FVOOT intervention.

Although effects on gut microbiota and ox-LDL were observed with the combination of both sources of PC (olive oil and thyme, FVOOT), it is not clear the relevance of each PC source in the results obtained. The lack of effects observed in FVOO would not necessary mean that these PC are not able to exert an effect on gut microbiota growth and metabolism. It would be probably due to the high amount used (500 ppm in FVOO compared to 250 ppm in FVOOT), which could exert an inhibitory effect on gut microbiota growth and metabolism. Furthermore, another advantage of the combination of both PC sources is that the pro-oxidant activity observed in with PC-rich foods containing unique PC sources could be eliminated with the use of complementary PC sources; a functional oil with complementary antioxidants (FVOOT), according to their

328 structure/activity relationship, could be a suitable option to obtain PC's beneficial effects avoiding these harmful
329 ones [60].
330 In conclusion, the ingestion of a PC-enriched virgin olive oil, containing a mixture of olive oil and thyme PC for
331 three weeks, decreases blood ox-LDL in hypercholesterolemic humans. This cardio-protective effect could be
332 mediated by the increases in populations of bifidobacteria together with increases in anti-oxidant PC microbial
333 metabolites such as protocatechuic acid and hydroxytyrosol. The specific growth stimulation of bifidobacteria in
334 human gut suggests for first time a potential prebiotic activity of an olive oil enriched in virgin olive oil and
335 thyme PC.

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Conflict of interest The authors have declared no conflict of interest.

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Table 1 Chemical characterization of the olive oils used in the study

Composition[b]	Olive oils[a]		
	VOO	FVOO	FVOOT
PHENOLIC COMPOUNDS (mg/25 mL)			
hydroxytyrosol	0.01 ± 0.00	0.21 ± 0.02	0.12 ± 0.00
3,4-DHPEA-AC[c)]	n.d.	0.84 ± 0.06	0.39 ± 0.04
3,4-DHPEA-EDA	0.04 ± 0.00	6.73 ± 0.37	3.43 ± 0.29
3,4-DHPEA-EA	0.26 ± 0.04	0.71 ± 0.06	0.36 ± 0.03
Total hydroxytyrosol derivates	0.30	8.49	4.30
p-hydroxybenzoic acid	n.d.	0.02 ± 0.00	0.06 ± 0.00
vanillic acid	n.d.	0.07 ± 0.00	0.13 ± 0.01
caffeic acid	n.d.	0.00 ± 0.00	0.06 ± 0.00
rosmarinic acid	n.d.	n.d.	0.41 ± 0.03
Total phenolic acids	-	0.09	0.65
thymol	n.d.	n.d.	0.64 ± 0.05
carvacrol	n.d.	n.d.	0.23 ± 0.02
Total monoterpenes	-	-	0.86
luteolin	0.04 ± 0.00	0.18 ± 0.02	0.21 ± 0.02
apigenin	0.02 ± 0.00	0.06 ± 0.00	0.10 ± 0.00
naringenin	n.d.	n.d.	0.20 ± 0.02
eriodictyol	n.d.	n.d.	0.17 ± 0.01
thymusin	n.d.	n.d.	1.22 ± 0.09
xanthomicrol	n.d.	n.d.	0.53 ± 0.06
7-methylsudachitin	n.d.	n.d.	0.53 ± 0.09
Total flavonoids	0.06	0.23	2.95
pinoresinol	0.05 ± 0.00	0.12 ± 0.00	0.10 ± 0.05
acetoxipinoresinol	2.47 ± 0.19	3.66 ± 0.31	3.24 ± 0.28
Total lignans	2.52	3.78	3.34
FAT SOLUBLE MICRONUTRIENTS (mg/25 mL)			
α-tocopherol	3.27 ± 0.01	3.40 ± 0.02	3.44 ± 0.01
lutein	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
β-cryptoxanthin	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
β-carotene	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
FATTY ACIDS (relative area %)			
Palmitic acid	11.21	11.20	11.21
Stearic acid	1.92	1.92	1.92
Araquidic acid	0.36	0.36	0.36
Behenic acid	0.11	0.11	0.11
Total saturated	13.75	13.74	13.75
Palmitoleic acid	0.70	0.70	0.69
Oleic acid	76.74	76.83	76.75
Gadoleic acid	0.27	0.27	0.27
Total monounsaturated	77.71	77.80	77.72
Linoleic acid	7.43	7.36	7.43
Timnodonic acid	0.36	0.36	0.35
Linolenic acid	0.43	0.43	0.43
Total polyunsaturated	8.22	8.15	8.22

PC and fat-soluble micronutrients are expressed as means ± SD of mg in 25 mL oil/day. The acidic composition is expressed as relative area percentage

[a] VOO, 80 mg/kg of phenolic compounds (PC) from olive oil; FVOO, 500mg/kg of PC from olive oil; FVOOT, 250 mg/kg of PC from olive oil and 250 mg/kg from thyme.

[b] 3,4-DHPEA-AC, 4-**(**acetoxyethyl**)**-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone

n.d., non detected

Table 2 Bacterial enumerations determined by FISH-flow cytometry in fecal samples collected before (B) and after (A) each olive oil intervention[a]

Probe		VOO	FVOO	FVOOT	*P*[b)] *VOO-FVOO*	*VOO-FVOOT*	*FVOO-FVOOT*
Ato291	B	8.80±0.09	8.86±0.11	8.72±0.10			
	A	8.78±0.09	8.64±0.09	8.72±0.09	0.319	0.947	0.293
Bac303	B	8.68±0.38	8.87±0.42	8.64±0.40			
	A	8.75±0.39	8.73±0.39	8.73±0.40	0.423	0.917	0.373
Bif164	B	8.33±0.25	8.29±0.27	8.14±0.26			
	A	8.10±0.25	8.11±0.26	8.32±0.26	0.818	0.044	0.073
Fprau645	B	8.90±0.06	9.03±0.07	8.96±0.06			
	A	8.90±0.06	8.92±0.06	8.93±0.06	0.420	0.831	0.558
Lab158	B	8.30±0.20	8.43±0.22	8.24±0.21			
	A	8.44±0.20	8.32±0.21	8.27±0.21	0.145	0.512	0.427
Prop853	B	8.81±0.08	8.99±0.10	8.81±0.09			
	A	8.98±0.08	8.79±0.08	8.80±0.08	0.066	0.338	0.364
Rrec584	B	8.74±0.08	8.81±0.10	8.63±0.09			
	A	8.76±0.09	8.61±0.09	8.79±0.09	0.303	0.461	0.085

Values are given as adjusted means of log10 bacteria/g dry feces ± SE; n=12 subjects.

[a] VOO, 80 mg/kg of phenolic compounds (PC) from olive oil; FVOO, 500mg/kg of PC from olive oil; FVOOT, 250 mg/kg of PC from olive oil and 250 mg/kg from thyme.

[b] P values for inter dietary intervention comparisons.

Table 3 Short chain fatty acids , neutral sterols and bile acids determined in fecal samples collected before (B) and after (A) each olive oil intervention[a]

		Olive oil interventions			P^{d}		
		VOO	FVOO	FVOOT	*VOO-FVOO*	*VOO-FVOOT*	*FVOO-FVOOT*
SCFA (µmol/g df [b]**)**							
Acetic	B	195.11 (102.26,372.27)	144.12 (73.80,281.43)	147.96 (80.50,271.96)			
	A	177.99 (94.02,336.97)	168.07 (94.50,298.93)	159.71 (74.71,341.41)	0.364	0.547	0.772
Butyric	B	64.25 (26.24,157.34)	42.40 (19.70,91.28)	44.38 (21.08,93.46)			
	A	57.97 (22.23,151.15)	52.64 (25.45,108.87)	42.27 (16.79,106.42)	0.264	0.854	0.347
Propionic	B	62.88 (32.56,121.45)	48.50 (24.03,97.87)	47.99 (23.89,96.39)			
	A	50.84 (26.72,96.71)	51.68 (26.89,99.32)	48.63 (21.00,112.64)	0.333	0.444	0.858
Branched[c]	B	28.18 (14.70,54.02)	25.78 (16.76,39.66)	25.26 (14.85,42.97)			
	A	24.63 (14.22,42.66)	29.95 (17.71,50.63)	23.42 (15.05,36.43)	0.117	0.751	0.207
Neutral sterols (µmol/g df **)**							
Cholesterol	B	4.18 (2.06,8.50)	2.17(0.29 16.05)	4.57 (1.72, 12.17)			
	A	3.61 (1.28,10.21)	3.33 (0.72, 15.44)	3.53 (1.28, 9.73)	0.176	0.798	0.103
Coprostanol	B	89.04 (38.77,204.48)	79.63 (17.92, 353.95)	109.58 (42.07, 285.43)			
	A	73.99 (26.96, 203.11)	111.49 (29.37, 423.25)	116.42 (38.99, 347.59)	0.266	0.622	0.559
Cholestanone	B	0.21 (0.01, 6.99)	0.07 (0.01, 0.33)	0.17 (0.02, 1.74)			
	A	0.28 (0.01, 6.74)	0.11 (0.03, 0.35)	0.21 (0.03, 1.37)	0.723	0.904	0.631
Coprostanone	B	0.73 (0.25, 2.16)	0.39 (0.04, 4.03)	1.02 (0.25, 4.12)			
	A	0.78 (0.17, 3.62)	0.82 (0.22, 2.97)	0.75 (0.23, 2.43)	0.155	0.440	0.028
Bile acids (mmol/10 g df)							
Cheno-deoxycholic	B	2.42 (0.14, 40.84)	2.39 (0.37, 15.29)	1.68 (0.18, 15.52)			
	A	2.78 (0.23, 33.32)	4.54 (0.99, 20.93)	1.55 (0.34, 7.15)	0.493	0.776	0.319
Cholic	B	1.10 (0.16, 7.68)	0.65 (0.14, 3.05)	0.64 (0.09, 4.35)			
	A	1.22 (0.24, 6.18)	1.46 (0.27, 7.76)	0.99 (0.17, 6.00)	0.356	0.666	0.621
Lithocholic	B	9.07 (1.73, 47.69)	11.00 (2.15, 56.20)	8.67 (1.47, 51.06)			
	A	9.81 (3.21, 30.02)	19.01 (7.09, 51.00)	11.58 (4.05, 33.14)	0.407	0.721	0.644
Isolithocholic	B	2.34 (0.52, 10.50)	3.98 (0.72, 21.90)	2.10 (0.25, 17.79)			
	A	2.17 (0.48, 9.81)	3.42 (1.18, 9.89)	2.80 (0.90, 8.79)	0.895	0.556	0.450
Deoxycholic	B	32.82 (11.37, 94.72)	34.37 (11.96, 98.75)	27.87 (3.37, 230.44)			
	A	39.97 (11.44,139.66)	54.67 (20.03, 149.23)	48.17 (8.15, 284.76)	0.604	0.519	0.870

Values are given as adjusted means and CI; *n*=12.

[a]25 mL/day extra virgin olive oil containing: VOO, 80 mg/kg of phenolic compounds (PC) from olive oil; FVOO, 500mg/kg of PC from olive oil; FVOOT, 250 mg/kg of PC from olive oil and 250 mg/kg from thyme.

[b] df, dry feces.

[c] Sum of isobutyric, isovaleric and valeric acids.

[d] *P* values for inter dietary intervention comparisons.

Table 4 Phenolic metabolites determined by LC in fecal samples collected before (B) and after (A) each olive oil intervention[a]

Phenolic metabolite		VOO	FVOO	FVOOT	P^b		
					VOO-FVOO	*VOO-FVOOT*	*FVOO FVOOT*
Hydroxytyrosol	B	0.19 (0.02, 2.28)	0.15 (0.02, 1.13)	0.08 (0.01, 1.16)			
	A	0.16 (0.02, 1.50)	0.74(0.10, 5.76)**	0.33 (0.01, 10.30)*	0.108	0.140	0.928
Dihydroxyphenyl-acetic acid	B	46.60 (39.05, 55.62)	30.07 (25.32, 35.72)	30.54 (25.29, 36.88)			
	A	23.25 (19.10, 28.30)**	40.93 (34.43, 48.66)	30.64 (25.53, 36.77)	0.014	0.092	0.435
Hydroxyphenylacetic acid	B	31.82 (24.61, 41.14)	18.67 (14.58, 23.92)	23.75 (17.93, 31.47)			
	A	14.32 (10.54, 19.45)*	17.95 (14.00, 23.03)	28.75 (21.98, 37.60)	0.226	0.129	0.708
Phenylacetic acid	B	248.64 (235.64, 262.35)	234.06 (222.17, 246.58)	232.18 (219.18, 245.96)			
	A	206.00 (193.89, 218.86)	262.72 (249.31, 276.86)	203.29 (192.30, 214.91)	0.194	0.818	0.281
Dihydroxyphenyl-propionic acid	B	0.01 (0.00, 0.02)	0.00 (0.00, 0.01)	0.00 (0.00, 0.02)			
	A	0.01 (0.00, 0.05)	0.00 (0.00, 0.01)	0.01 (0.00, 0.02)	0.690	0.617	0.913
Hydroxyphenyl-propionic acid	B	102.51 (56.87, 184.78)	42.20 (23.18, 76.82)	36.80 (19.52, 69.37)			
	A	65.56 (33.44, 128.56)	33.07 (18.61, 58.75)	39.08 (21.19, 72.07)	0.796	0.534	0.703
Phenylpropionic acid	B	165.40 (148.88, 183.75)	122.41 (110.50, 135.60)	127.89 (114.30, 143.11)			
	A	143.23 (127.36, 161.09)	138.01 (124.51, 152.97)	117.83 (105.70, 131.35)	0.394	0.847	0.510
Rosmarinic acid	B	2.60 (1.40, 4.83)	1.80 (1.01, 3.23)	2.76 (1.57, 4.87)			
	A	2.83 (1.41, 5.68)	1.85 (1.13, 3.04)	2.22 (1.29, 3.82)	0.938	0.684	0.733
Protocatechuic acid	B	0.55 (0.03, 9.15)	0.11 (0.00, 3.01)	0.07 (0.00, 1.45)			
	A	0.04 (0.00, 0.683)**	0,11 (0.00, 3.41)	0.10 (0.01, 1.80)	0.008	0.003	0.652
Coumaric sulfate acid	B	2.92 (1.74, 4.89)	3.96 (2.40, 6.53)	2.93 (1.64, 5.24)			
	A	1.50 (0.83, 2.70)	4.46 (2.70, 7.37)	3.81 (2.22, 6.54)	0.288	0.231	0.842
Caffeic acid	B	1.94 (1.68, 2.24)	2.18 (1.89, 2.51)	2.41 (2.07, 2.81)			
	A	2.74 (2.35, 3.19)	2.68 (2.32, 3.09)	2.16 (1.87, 2.49)	0.247	0.597	0.110
Ferulic acid	B	1.01 (0.61, 1.67)	0.58 (0.40, 0.85)	0.89 (0.52, 1.52)			
	A	1.81 (1.10, 2.97)	0.93 (0.65, 1.31)	1.30 (0.81, 2.09)	0.881	0.817	0.924
Hydroxyphenyl-valerolactone	B	2.52 (1.77, 3.60)	4.94 (3.48, 7.00)	3.93 (2.55, 6.06)			
	A	3.61 (2.32, 5.63)	3.42 (2.41, 4.84)	7.80 (5.39,11.28)	0.401	0.718	0.231
M1[c)]	B	141.53 (104.14, 192.33)	105.88 (78.05, 143.62)	126.55 (92.70, 172.76)			
	A	106.46 (77.81, 145.67)	134.85 (99.37, 183.01)	126.66 (93.00, 172.51)	0.027	0.236	0.295
M2	B	1.61 (1.18, 2.20)	1.00 (0.73, 1.37)	1.40 (1.01, 1.95)			
	A	0.88 (0.63, 1.25)*	1.50 (1.11, 2.04)	1.58 (1.15, 2.18)	0.042	0.152	0.559

Values are given as adjusted means of µmol/100g dry feces and CI; *n*=12 subjects.
[a] VOO, 80 mg/kg of phenolic compounds (PC) from olive oil; FVOO, 500mg/kg of PC from olive oil; FVOOT, 250 mg/kg of PC from olive oil and 250 mg/kg from thyme.
[b] *P* values for inter dietary intervention comparisons.
[c] M1: unknown metabolite. $(M-H)^-$ = 187 *m/z*. MS^2 fragments = 125, 117, 89 *m/z*; M2: unknown metabolite. $(M-H)^-$ = 243 *m/z*. MS^2 fragments = 227, 207, 119 *m/z*
*0.05<P<0.1;**P≤0.05 for intra dietary intervention differences.