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Impact of plant sterols enrichment dose on gut microbiota from lean and obese subjects using TIM-2 *in vitro* fermentation model

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ABSTRACT

There are scarce data on plant sterols (PS) and gut microbiota relationship. The purpose of this study is to compare the interaction between PS and gut microbiota through *in vitro* colonic fermentation studies using a validated system (TIM-2) with a PS-enriched dose (~2 g/day) from two sources (food PS-source ingredient and commercial standard) using microbiota from lean and obese populations. Fecal sterols, short chain fatty acids (SCFA) and microbiota composition were determined by GC/MS, IEC, and 16S-sequencing, respectively.

PS-feeding decreased coprostanol and ethylcoprostanol concentration and increased the production of acetate and butyrate (mainly with lean microbiota). In addition, the PS-enrichment dose increased the proportion of some genera from phylum *Firmicutes* with lean and obese microbiota.

The results obtained suggest that the gut microbiota preferably use PS as a substrate. In addition, PS-enrichment dose had no effect on the production of SCFA but modified the microbial profile of lean and obese populations.

1. Introduction

The daily dietary intake of PS (160–400 mg/day) (Gylling et al., 2014; Lagarda, García-Llatas, & Farré, 2006) does not reach the established levels to have a hypocholesterolemic effect (1.5–3.0 g/day) (Commission Regulation (EU), 2014). Therefore, the mentioned levels can only be achieved by incorporating PS-enriched foods to the usual diet (such as, yellow fat spreads, salad dressings, milk type products, fermented milk products, soya drinks, cheese type products, rye bread and rice drinks).

While dietary cholesterol is absorbed between 20 and 80% (de Boer, Kuipers, & Groen, 2018; Stellaard & Lütjohann, 2015), the intestinal absorption rate of total PS is only 2–3% (García-Llatas & Rodríguez-Estrada, 2011). Non-absorbed sterols reach the colon and can be biotransformed by the gut microbiota. Although cholesterol biotransformation mediated by gut microbiota has been widely studied using *in vitro* assays (Wong, 2014), less information is available on the biotransformation of PS and their possible impact (in the case of high intake levels) on colonic microbial metabolization of other substrates.

There is an important association between colonic microbiota and host, and the microbiota has been acknowledged as a metabolic organ (Bäckhed et al., 2004). A well-balanced intestinal microbiota has been shown to be important for the health of the host (e.g. increased colonization resistance, stimulation of the immune system, support in digestion, synthesis of nutrients and production of butyrate for enterocytes) (Portune, Benítez-Páez, Del Pulgar, Cerrudo, & Sanz, 2017). The metabolic function of the microbiota highly depends on the type of substrates available to be fermented. The major fermentation processes in the colon are saccharolytic and proteolytic fermentation. However, there are many more dietary components that make it to the colon and are fermented, but there are few studies on the lipolytic fermentation process, and specifically on PS (Bernalier-Donadille, 2010).

On the other hand, overweight and obesity constitute the main risk factors for a wide range of chronic disorders, including diabetes (with a 44% increased risk in obese subjects), cardiovascular disease (23%), and cancer (41%) (Bray, 2004; WHO, 2018). Some studies in humans (Ley, Turnbaugh, Klein, & Gordon, 2006; Turnbaugh et al., 2009) and animals (Bäckhed et al., 2004; Ley et al., 2005; Turnbaugh et al., 2006)

Abbreviations: AS, animal sterols; BMI, body mass index; BSFTA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; IEC, ion exclusion chromatography; GC/MS, gas chromatography mass spectrometry; KEGG, Kyoto encyclopedia of genes and genomes; LEfSe, linear discriminant analysis effect size; OTUs, operational taxonomic units; PCoA, principal coordinate analysis; PS, plant sterols; QIIME, Quantitative Insights Into Microbial Ecology; SCFA, short chain fatty acids; SIEM, standard ileal efflux medium; TIM-2, TNO intestinal model-2; TMCSL, trimethylchlorosilane; TMSE, trimethylsilyl ether

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have associated the intestinal microbial dysbiosis with obesity. In general, obese individuals show a decrease in bacterial diversity (Kasai et al., 2015; Lau, Carvalho, Pina-Vaz, Barbosa, & Freitas, 2015; Mishra, Dubey, & Ghosh, 2016; Turnbaugh et al., 2009) characterized by an increase in the *Firmicutes-Bacteroidetes* ratio (Boulangé, Neves, Chilloux, Nicholson, & Dumas, 2016; Gerard, 2016; Kasai et al., 2015; Lau et al., 2015; Lecomte et al., 2015).

In the present study, the validated dynamic *in vitro* proximal colon model developed by The Netherlands Organization for Applied Scientific Research (TNO) was used to evaluate the effect of PS-enrichment dose (2 g/day) on composition and metabolic functions of microbiota from lean and obese subjects, since there are no *in vitro* fermentation studies by a dynamic system and with PS-enriched medium and microbiota from lean and obese population.

2. Material & methods

2.1. Test compounds

A PS-source ingredient used in enrichment of foods by industry (Lipophytol® 146ME Dispersible), which present free microcrystalline PS from tall oil in powder form (Lipofoods SLU, Barcelona, Spain) were used. In addition, a standard of β-sitosterol ≥70% obtained from Sigma-Aldrich® (Munich, Germany) has also been used. Both test compounds were previously analyzed according to González-Larena et al. (2011). The purity of PS-source ingredient was 54% (w/w) (sitosterol: 82%, sitostanol: 10%, campesterol: 6.2%, stigmasterol: 1.5% and campestanol: 0.2%), the rest of its composition (46%, w/w) corresponds to maltodextrin, inulin and sucrose esters. In the analysis of β-sitosterol ≥70%, 80% purity was obtained (sitosterol: 84%, sitostanol: 6.4% and campesterol 9.1%) and the remaining 20% corresponded to other unidentified steroid compounds. Therefore, this standard is called "PS-mixture standard" throughout the manuscript. Besides, the PS-mixture standard was chosen because their PS composition was similar to the PS-source ingredient and thus know the behaviour of PS without matrix interference.

PS from the PS-source ingredient were micro-encapsulated with maltodextrin, inulin and sucrose esters, thus it was necessary to carry out an acidification (pH 2, 37 °C with a water bath) for 2 h on the same day prior to introduction of the test compounds in the system, to degrade the carbohydrates capsule. It was considered appropriate to apply this acidification also to the PS-mixture standard and control (without PS and commented below in Section 2.4), so all experiments were treated under the same conditions. Taking into account the purity of test compounds, the acidification was carried out on 4 g of PS-source ingredient and 2.5 g of PS-mixture standard in glass jars (100 mL) and in the case of control experiment, the glass jar was empty. Then, at each experiment, NaCl 0.85% (20 mL), Tween 80 (0.5 g) and HCl 1 M (160 μL for PS-source ingredient, 140 μL for PS-mixture standard and 120 μL for control experiment) were added. After acidification the pH was adjusted to 5.8, the final volume was 30 mL, which was collected with a sterile plastic syringe.

2.2. Microbiota: source, collection and processing

Homogenates of human feces were made from subgroups of healthy volunteers who were selected according to their body mass index (BMI). The group of participants had not used prebiotics or probiotics in the 2 weeks prior to the donation and had not taken antibiotics and PS-supplements or enriched foods for at least 3 months.

Lean participants: five donors (3 women and 2 men) with a mean age of 27.2 ± 4.3 years, an average weight of 61.6 ± 3.9 kg and a mean BMI of 21.7 ± 0.8. Obese participants: thirteen donors (7 women and 6 men) with a mean age of 55.1 ± 9.8 years, an average weight of 100.0 ± 12.9 kg and mean BMI of 33.2 ± 3.5.

All the donors collected a fresh fecal sample in a gastight bag and placed it immediately into a plastic jar containing an anaerobic strip (AnaeroGen™, Cambridge, UK). Samples were transported in a period not longer than 5 h to the laboratory, were homogenized and mixed under strict anaerobic conditions (Sheldom Lab-Bactron IV, Cornelius, OR, USA) to create a standardized microbiota stock according to Aguirre, Jonkers, Troost, Roeselers, and Venema (2014). Mixing was done with a dialysate solution (content per litre: 2.5 g $K_2HPO_4{\cdot}3H_2O$, 4.5 g NaCl, 0.005 g $FeSO_4{\cdot}7H_2O$, 0.5 g $MgSO_4{\cdot}H_2O$, 0.45 g $CaCl_2{\cdot}2H_2O$, 0.05 g ox bile, 0.4 g cysteine hydrochloride; pH 5.8), with 1 mL of a vitamins mixture (content per litre: 1 mg menadiona, 2 mg D-biotin, 0.5 mg vitamin B12, 10 mg pantothenate, 5 mg nicotinamide, 5 mg *p*-animobenzoic acid and 4 mg thiamine), and glycerol (14% w/w) as cryoprotective agent. Fecal suspension was aliquoted (35 mL), frozen in liquid nitrogen, and stored at −80 °C until inoculation in TIM-2. Before being introduced into the system, the inoculum was thawed during 1 h in a 37 °C in a water bath (Aguirre et al., 2015). Subsequently, in the anaerobic chamber the aliquot (35 mL) of fecal suspension was diluted with dialysate (1:1, v/v) and 60 mL transferred to a sterile plastic syringe. This was used as the inoculum of the TIM-2 unit.

2.3. Standard ileal efflux medium (SIEM)

SIEM contained the following components: 100 g CHO medium (per litre: 12 g pectine, 12 g xylan, 12 g arabinogalactan, 12 g amylopectine, 100 g starch), 25 g TBCO 6.25x (per litre: 270 g Tween 80, 375 g bactopepton, 375 g casein, 6.25 g ox-bile), 2 g $MgSO_4$ (50 g/L), 2 g cysteine (20 g/L), 0.2 mL vitamin mixture (mentioned above), 4 mL salts solution (4.7 g $K_2HPO_4{\cdot}3H_2O$, 8.4 g NaCl, 0.8 g $CaCl_2{\cdot}2H_2O$, 0.009 g $FeSO_4{\cdot}7H_2O$, 0.02 g haemin) and antifoam B emulsion (Sigma-Aldrich®, Munich, Germany). The pH was adjusted to 5.8. SIEM simulates material passing the ileocecal valve in humans, or in other words material reaching the colon, and was used during the period of microbiota standardization and to feed the system during fermentation period for three experiments (control, PS-source ingredient and PS-mixture standard) (Fig. 1).

2.4. TIM-2 fermentation assays

The TIM-2 system is a validated dynamic, computer-controlled model which simulates the human proximal colon, mimicking body temperature (37 °C), lumen pH (5.8), absorption of water and microbial metabolites through a semipermeable membrane inside de model, mixing and transporting the intestinal contents with peristaltic movements, anaerobic conditions by flushing with gaseous N_2, and using an microbiota from human origin (Maathuis, Hoffman, Evans, Sanders, & Venema, 2009; Venema, Vermunt, & Brink, 2005).

All experiments (PS-source ingredient, PS-mixture standard and control) were performed in duplicate. At the start of each experiment, the model was inoculated with approximately 60 mL of the standardized microbiota from lean or obese donors and 60 mL of dialysate (see Section 2.2). SIEM was fed at a rate of 2.5 mL/h. The microbiota was allowed to adapt to the model conditions and SIEM for 16 h and after that, a 4 h starvation period allowed the bacteria to ferment all available carbohydrates in the system prior to the addition of the test compounds (Fig. 1). After the starvation period, samples of lumen and dialysate were collected at time-point zero (t0), and SIEM was fed in all units of the system (feeding rate: 60 mL/day – 2.5 mL/h). Acidified samples of PS-source ingredient, PS-mixture standard (2 g/day) and control (without PS) (see Section 2.1) collected in the syringe were introduced through the sampling port each day. After 24, 48 and 72 h, samples of the lumen and dialysate were collected, as is shown in Fig. 1. A total lumen sample of 30 mL was removed from the system to simulate passage of material from the proximal to the distal colon.

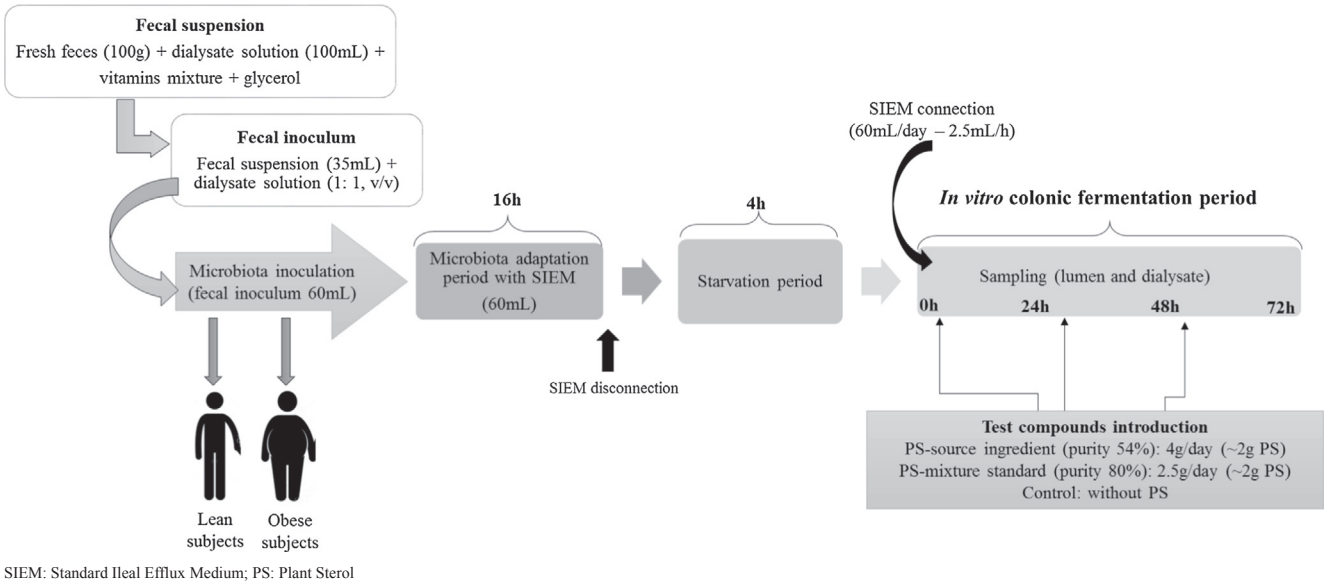

Fig. 1. Experimental design.

2.5. Analytical methods

2.5.1. Fecal sterols

Fecal sterols and their metabolites were determined according to Cuevas-Tena, Alegría, and Lagarda (2017). Briefly, the samples were diluted with MilliQ water (1:100) and volumes from 25 µL to 200 µL were needed for those samples with large amounts of PS (PS-source ingredient and PS-mixture standard 24, 48 or 72 h), and volumes between 1 and 3 mL were taken mainly for the analysis of samples at time zero and control. The analysis was performed in triplicate using 5α-cholestane (20 µg) as internal standard. The saponification step was carried out with 1 mL of ethanolic potassium hydroxide solution 0.71 M (65 °C/1 h) using a block heater. The unsaponifiable fraction was extracted with 0.5 mL of Milli-Q water and 2 mL of n-hexane (centrifuged at 18 °C/10 min/3600 rpm). The n-hexane extraction step was performed twice under the same conditions as described above. The organic extracts were evaporated to dryness under nitrogen. In order to obtain the trimethylsilyl ether (TMSE) derivatives, 200 µL of BSTFA + 1% TMCS:pyridine 10:3 (v/v) were added (65 °C/1 h). The TMSE derivatives obtained were dissolved with 3 mL of n-hexane, filtered (Millex-FH filter unit, 0.45 µm Millipore, Milford, MA, USA), evaporated under nitrogen, and dissolved in 40 µL of n-hexane. One µL of this solution was injected into a GC/MS (Thermo Science Trace® GC-Ultra with ion trap ITQ 900, Waltham, MA, USA) with a CP-Sil8 CB low bleed/MS (50 m × 25 mm × 0.25 µm) column (Agilent Technologies®, CA, USA). Hydrogen was used as carrier gas, operating at a constant flow of 1 mL/min. The mass spectrometer operated at −70 eV, and a mass range from 50 to 650 *m/z* was scanned.

2.5.2. SCFA, lactate and succinate

Both lumen (1.5 mL) and dialysate (2 mL) were centrifuged at 14,000 rpm for 10 min, filtered through a 0.45 µm PFTE filter, and diluted in the mobile phase (1.5 mM aqueous sulfuric acid). Ten microliters were loaded into the column with the help of an automatic sampler 730 (Metrohm, Herisa, Switzerland). The acids were eluted according to their p*K*a. The analysis was carried out by ion exclusion chromatography (IEC) using an 883 chromatograph (IC, Metrohm,) equipped with a Transgenomic IC Sep ICE-ION-300 column (30 cm × 7.8 mm × 7 µm) and a MetroSep RP2 Guard. A column flow of 0.4 mL/min with a column temperature of 65 °C was used. The acids were detected using suppressed conductivity detection. The company Brightlabs (Venlo, The Netherlands) carried out these analyses.

2.5.3. Gut microbiota

The isolation of genomic DNA from the fecal samples (3 mL lumen) was performed using standard molecular biology kits from ZYMO Research provided by BaseClear (Leiden, The Netherlands). The PCR amplification of the 16S rRNA gene (V3 and V4 regions), the barcoding and the library preparation were carried out by BaseClear. The sequencing was carried out using the Illumina MiSeq system and later the sequences were converted into FASTQ files using BCL2FASTQ pipeline version 1.8.3. The quality cut was applied based on the quality level of Phred (Phred quality score). Quantitative Insights Into Microbial Ecology (QIIME) software package (1.9.0) was used for microbial analyses (Caporaso et al., 2010). The sequences were classified using Greengenes (version 13.8) as a reference 16S rRNA gene database. Linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) was used to find biomarkers between groups using relative abundances from the operational taxonomic unit (OTU) tables generated in QIIME. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) was used to predict the gene families contributing to the metagenomes of the samples identified using 16S rRNA sequencing. This data was visualized using statistical analysis of taxonomic and functional profiles (STAMP) (Parks, Tyson, Hugenholtz, & Beiko, 2014).

2.6. Statistical analysis

Changes in fecal sterols, SCFA and lactate after *in vitro* colonic fermentation assays during 3 days were evaluated by ANOVA and means were compared by an LSD test (confidence level 95.0%) using Statgraphics Centurion XVI.I package. The software package R (3.5.0) (R Core Team, 2013) was used to determine correlations between OTUs and metabolites. Statistical analyses were performed with RStudio. Spearman correlation was calculated between the relative abundance of OTUs and continuous variables (fecal sterols and SCFA). Multiple comparison was corrected using the false discovery rate (FDR), and q-values (adjusted p-values) were considered significantly different at < 0.05. Correlation between OTUs and metabolites are indicated by the rho-value.

Table 1
Cholesterol and metabolites concentration (μg/mL fermentation liquid) after *in vitro* colonic fermentation. Data are means of two independent *in vitro* assays with range (n = 2). For each fermentation time (0, 24, 48 and 72 h), different letter indicate statistical differences (confidence level 95.0%) between experiments (control *vs.* PS-source ingredient *vs.* PS-mixture standard) for lean (a-c) and obese (x-z) microbiota.

	Lean			Obese		
	Control	PS-source ingredient	PS-mixture standard	Control	PS-source ingredient	PS-source standard
Cholesterol						
0 h	123.1 (94.0–152.2)a	133.2 (113.9–152.5)a	159.2 (114.8–203.5)a	106.6 (84.1–129.0)x	241.6 (172.2–311.0)x	106.9 (74.4–139.4)x
24 h	43.8 (40.3–47.3)a	173.2 (157.3–189.2)b	94.0 (89.4–98.5)c	52.8 (46.4–59.2)x	132.2 (130.6–133.7)y	112.7 (110.2–115.6)z
48 h	39.2 (22.5–55.9)a	93.2 (80.9–105.5)a	63.1 (52.1–74.1)a	46.9 (34.9–59.0)x	102.4 (94.6–110.2)y	116.3 (105.8–126.7)y
72 h	27.4 (19.4–35.4)a	106.5 (95.4–117.5)b	75.4 (71.0–79.8)b	57.7 (55.8–59.6)x	74.9 (71.0–78.8)xy	79.0 (73.8–84.1)y
Coprostanol						
0 h	116.6 (91.6–141.5)a	112.1 (102.9–121.3)a	124.2 (89.1–159.2)a	283.7 (221.4–346.0)x	500.7 (401.9–599.6)x	447.8 (299.8–595.9)x
24 h	127.7 (108.1–147.3)a	115.5 (114.7–116.3)ab	66.3 (66.2–66.4)b	102.0 (99.5–104.6)x	241.2 (221.0–261.3)y	163.1 (151.5–174.8)z
48 h	39.3 (29.2–49.4)a	59.8 (47.9–71.7)a	46.2 (42.9–49.5)a	111.6 (84.3–138.8)x	277.2 (275.3–278.6)y	228.8 (207.6–250.0)y
72 h	100.6 (92.5–108.8)a	66.7 (57.3–76.0)b	48.3 (47.0–49.8)b	134.3 (129.5–139.0)x	225.9 (217.6–234.2)y	178.9 (178.1–179.6)z
Coprostanone						
0 h	31.4 (30.5–32.4)a	32.0 (31.2–32.8)a	36.8 (34.8–38.9)a	169.1 (168.3–169.9)x	248.6 (203.4–293.8)x	330.4 (242.8–418.1)x
24 h	51.8 (51.2–52.5)a	39.3 (38.5–40.1)b	39.8 (37.9–41.6)b	61.3 (58.4–64.1)x	60.7 (55.2–66.3)x	55.0 (54.8–55.2)x
48 h	40.6 (38.2–43.0)a	39.5 (39.4–39.6)a	45.4 (44.9–46.0)a	72.9 (54.2–91.6)x	53.4 (51.4–55.4)x	65.3 (57.0–73.6)x
72 h	74.3 (44.5–104.1)a	75.6 (75.5–75.7)a	77.0 (76.8–77.2)a	61.1 (57.0–65.2)x	54.1 (51.9–56.3)x	51.0 (49.5–52.5)x
Cholestanol						
0 h	7.6 (5.9–9.2)a	8.0 (7.1–8.9)a	7.2 (6.5–7.8)a	14.7 (13.1–16.2)x	23.9 (20.2–27.6)x	21.5 (18.4–24.6)x
24 h	8.5 (7.8–9.3)a	9.6 (8.7–10.5)a	7.0 (6.6–7.4)a	8.2 (7.8–8.5)x	16.4 (15.1–17.7)y	12.9 (12.4–13.4)y
48 h	6.3 (4.8–7.7)a	7.4 (7.2–7.5)a	0.01 (0.0005–0.02)a	8.7 (6.9–10.4)x	14.4 (10.1–18.7)x	17.1 (16.1–18.2)x
72 h	7.3 (7.0–7.6)a	10.2 (10.1–10.2)b	9.2 (8.3–9.5)b	10.6 (10.3–10.8)x	16.6 (16.4–16.9)y	13.0 (12.7–13.4)y

3. Results

3.1. Microbiota effect on sterols

3.1.1. Fecal sterols

Tables from 1 to 3 show the mean concentrations (μg/mL) of fecal sterols and metabolites present in control, PS-source ingredient and PS-mixture standard experiments, during the fermentation period (0–72 h) using microbiota from lean and obese population.

A significant increase ($p < 0.05$) in cholesterol concentration (μg/mL) was observed in the experiments with PS-source ingredient (24 and 72 h) or PS-mixture standard (24, 48 and 72 h) with respect to the control experiment and independently of the microbiota used (Table 1). In the assays with lean population microbiota there was a significant decrease in coprostanol (24 and 72 h) and coprostanone (24 h) concentrations in experiments with PS-source ingredient or PS-mixture standard compared to control. In addition, a significant increase ($p < 0.05$) in cholestanol (72 h) concentration is also observed between experiments with a medium enriched in PS and control (Table 1). When microbiota of obese population were used, there was a significant increase in concentrations of coprostanol (24, 48 and 72 h) and cholestanol (24 and 72 h) in the experiments with PS-source ingredient or PS-mixture standard compared to control (Table1).

Taking into account the entire fermentation period (0–72 h), cholesterol concentration decrease in control (77% for lean microbiota and 45% for obese microbiota), PS-source ingredient (20% and 69%) and PS-mixture standard (32 and 26%) when using lean and obese microbiota, respectively. It should be noted that all metabolites (coprostanol, coprostanone and cholestanol) were higher in obese than in lean microbiota assays. In addition, the coprostanol concentration throughout the fermentation period (0–72 h) decreased in control (13% for lean and 52% for obese), PS-source ingredient (41 and 55%) and PS-mixture standard (61 and 60%). However, coprostanone concentration at the end of fermentation period (72 h) increased in experiments with PS-source ingredient (136%) and PS-mixture standard (109%) when lean microbiota was used. In the assays with obese microbiota, the coprostanone concentration decreased from time zero to 24 h (control: 64%; PS-source ingredient: 76%; PS-mixture standard: 83%) and then the concentration remained more or less equal until 72 h for all experiments. In the case of cholestanol concentrations, at the end of fermentation period using lean microbiota, their concentration increased in experiments with PS-source ingredient (27%) and PS-mixture standard (29%), while in the assays with obese microbiota, their concentration decreased in presence of PS-source ingredient (30%) and PS-mixture standard (40%).

The mean concentration of sitosterol and campesterol and their metabolites in control, PS-source ingredient and PS-mixture standard experiments, during the fermentation period (0–72 h) using microbiota from lean and obese population are show in Table 2 and Table 3, respectively.

A significant increase ($p < 0.05$) in the concentration of sitosterol, sitostanol, campesterol and campestanol in presence of PS-source ingredient and PS-mixture standard compared to control was observed in all fermentation times (24, 48 and 72 h) (Tables 2 and 3).

Ethylcoprostanol decreased significantly ($p < 0.05$) during all fermentation period (24–72 h) in experiments with PS-source ingredient (32% for lean and 88% for obese) or PS-mixture standard (35% for lean and 87% for obese) compared to control. A significant increase ($p < 0.05$) of ethylcoprostanone was observed with PS-source ingredient (46 and 35 fold more) and PS-mixture standard (21 and 33 fold more) respect to control when microbiota of lean and obese subjects was used, respectively (Table 2). In the case of methylcoprostanone, a significant increase ($p < 0.05$) of their concentration was observed from 24 to 72 h with PS-source ingredient (8 fold more) and PS-mixture standard (25 fold more) (Table 3) using lean microbiota. When obese microbiota was used, the methylcoprostanone concentration increased significantly only between control and PS-mixture standard after 24 h of fermentation.

3.2. Production of microbial metabolites

3.2.1. SCFA, succinate and lactate

The average cumulative amount of individual SCFA (acetate, propionate and butyrate), succinate and lactate produced during the fermentation period (24, 48 and 72 h) in control, PS-source ingredient and PS-mixture standard experiments using microbiota from lean or obese subjects is shown in Fig. 2.

Production of SCFA (acetate, propionate and butyrate) was higher

Table 2
Sitosterol and metabolites concentration (μg/mL fermentation liquid) after *in vitro* colonic fermentation. Data are means of two independent *in vitro* assays with range (n = 2). For each fermentation time (0, 24, 48 and 72 h), different letter indicate statistical differences (confidence level 95.0%) between experiments (control *vs*. PS-source ingredient *vs*. PS-mixture standard) for lean (a-c) and obese (x-z) microbiota.

	Lean			Obese		
	Control	PS-source ingredient	PS-mixture standard	Control	PS-source ingredient	PS-mixture standard
Sitosterol						
0	36.4 (34.5–38.3)a	35.3 (35.1–35.6)a	59.3 (45.0–73.6)a	36.4 (33.8–39.9)x	57.0 (38.6–75.3)x	42.6 (36.3–48.9)x
24	45.8 (43.7–48.0)a	15760.7 (15664.6–15856.8)b	7658.0 (6573.6–8742.4)c	41.3 (39.9–42.8)x	11382.0 (10362.0–12401.9)y	8579.8 (8495.3–8664.3)z
48	55.3 (51.1–59.4)a	18976.3 (18767.1–19185.6)b	18552.8 (17783.6–19321.9)b	42.3 (41.4–43.3)x	20492.4 (19171.1–21813.6)y	17403.5 (11228.2–23578.7)y
72	52.6 (43.5–61.7)a	49928.7 (47600.8–52256.6)b	63621.0 (57496.0–69746.0)b	51.0 (49.7–52.3)x	34395.0 (31676.7–37113.3)y	32691.7 (27207.6–38175.8)y
Sitostanol						
0	14.8 (14.3–15.3)a	14.3 (14.1–14.6)a	18.3 (15.9–20.8)a	14.9 (14.4–15.4)x	17.6 (14.9–20.3)x	15.6 (14.8–16.3)x
24	21.3 (20.8–21.8)a	2116.2 (2085.1–2147.2)b	1022.5 (858.3–1186.7)c	20.6 (20.3–20.9)x	1762.0 (1650.8–1873.1)y	1218.3 (1103.3–1333.2)z
48	25.3 (21.6–29.0)a	4458.65 (4343.2–4574.1)b	4825.5 (4444.0–5207.1)b	21.0 (20.7–21.4)x	3016.8 (2849–7–3183.7)y	2664.8 (1868.0–3461.6)y
72	21.9 (21.3–22.5)a	6908.6 (5893.0–7924.2)b	6021.8 (4557.2–7486.3)b	22.0 (21.8–22.1)x	5816.3 (5287.2–6345.4)y	6022.1 (4565.2–7478.9)y
Ethylcoprostanol						
0	19.0 (16.2–21.8)a	20.1 (17.8–22.3)a	23.1 (20.0–26.2)a	35.9 (30.3–41.5)x	62.3 (48.6–75.9)x	57.1 (41.3–72.8)x
24	25.6 (25.3–25.8)a	7.8 (7.5–8.1)b	9.3 (8.2–10.5)b	16.2 (14.8–17.6)x	10.2 (9.8–10.6)y	8.0 (7.1–8.8)y
48	15.1 (12.9–17.2)a	7.1 (7.0–7.1)b	7.4 (7.3–7.4)b	18.1 (15.9–20.3)x	7.9 (7.7–8.1)y	8.0 (7.9–8.1)y
72	24.8 (22.0–27.5)a	13.5 (13.4–13.6)b	15.0 (14.4–15.5)b	25.1 (24.9–25.3)x	7.3 (7.2–7.4)y	7.1 (7.1–7.2)y
Ethylcoprostanone						
0	29.4 (28.0–30.8)a	26.3 (25.3–27.3)a	32.2 (30.6–33.9)a	33.7 (33.3–34.1)x	41.1 (36.9–45.2)x	35.9 (35.4–36.3)x
24	39.6 (38.7–40.4)a	743.2 (743.0–743.5)b	341.6 (311.7–371.5)c	39.6 (38.9–40.2)x	805.4 (751.6–859.2)y	484.4 (431.2–537.5)y
48	79.6 (39.8–119.5)a	1046.4 (889.5–1203.3)9b	1708.2 (1472.9–1943.4)b	38.9 (38.0–39.9)x	1555.5 (1475.2–1635.8)y	1243.3 (1113.4–1373.31)y
72	41.8 (40.0–43.6)a	1252.4 (1210.0–1294.9)b	732.9 (633.3–832.6)b	40.5 (41.1–40.0)x	1469.7 (1375.9–1563.5)y	1217.5 (1051.4–1383.5)y

in the experiments with microbiota of lean population than obese (Fig. 2). In fact, only statistically significant differences have been detected in assays with lean microbiota. Specifically, a significant increase ($p < 0.05$) of acetate after 24 and 48 h of fermentation occurred in the experiments with PS-enriched medium compared to control, being highest under presence of PS-source ingredient. In the case of butyrate, a significant increase ($p < 0.05$) was only found after 24 h of fermentation with PS-mixture standard. Lactate only showed a significant increase ($p < 0.05$) in experiments with PS-source ingredient (net increment: 0.50 mmol) after 24 h of fermentation. For propionate and succinate, greater production was observed when PS-source ingredient and PS-mixture standard were used, but no significant differences were found between these two and control.

3.3. Plant sterol modulation of the microbiota

Sequencing of the V3-V4 region of 16S rRNA gene was performed and reads were analysed using QIIME. Principal Coordinate Analysis (PCoA) and Linear discriminant analysis effect size (LEfSe) were performed to determine OTUs that were modulated by the interventions. Neither weighted nor unweighted PCoA showed specific clustering by substrate (not shown). Yet LEfSe showed some OTUs that were specific for the treatments. For the lean microbiota, LEfSe only showed OTUs that were more prevalent upon addition of PS-source ingredient (Fig. 3A), which belonged to the genus *Catenibacterium*. When PS-source ingredient and PS-mixture standard were combined (indicated as Soy) and compared to control, a few more discriminative OTUs were observed (Fig. 3B). Apart from *Catenibacterium*, *Coprococcus* was increased upon addition of PS, while *Collinsella* and *Slackia* of the *Coriobacteriaceae* family were increased in control.

Table 3
Campesterol and metabolites concentration (μg/mL fermentation liquid) after *in vitro* colonic fermentation. Data are means of two independent *in vitro* assays with range (n = 2). For each fermentation time (0, 24, 48 and 72 h), different letter indicate statistical differences (confidence level 95.0%) between experiments (control *vs*. PS-source ingredient *vs*. PS-mixture standard) for lean (a-c) and obese (x-z) microbiota.

	Lean			Obese		
	Control	PS-source ingredient	PS-mixture standard	Control	PS-source ingredient	PS-mixture standard
Campesterol						
0	45.6 (44.8–46.4)a	47.0 (45.3–48.8)a	48.3 (46.7–49.9)a	45.8 (45.1–46.6)x	51.5 (48.2–54.8)x	46.6 (44.9–48.3)x
24	62.7 (62.3–63.1)a	1649.6 (1623.4–1675.8)b	925.1 (883.4–966.8)c	62.6 (62.3–62.8)x	1329.1 (1187.0–1471.1)y	961.2 (942.0–980.5)y
48	62.6 (60.1–65.2)a	3372.9 (3353.7–3392.0)b	3894.4 (3812.4–3976.4)c	62.5 (61.3–63.1)x	2392.9 (2310.2–2475.6)y	2257.3 (1839.7–2674.8)y
72	62.1 (61.7–62.6)a	5008.4 (4253.6–5763.2)b	4466.9 (3353.6–5581.3)b	63.0 (62.9–63.1)x	4039.4 (3662.8–4415.9)y	4344.6 (3330.2–5358.9)y
Campestanol						
0	14.4 (14.1–14.7)a	15.1 (14.6–15.6)a	15.5 (14.7–16.2)a	16.3 (15.6–17.1)x	18.7 (17.6–19.9)x	18.5 (17.0–20.0)x
24	21.2 (20.7–21.7)a	255.4 (234.1–276.7)b	160.7 (156.9–164.6)c	20.4 (20.3–20.4)x	284.6 (270.8–298.4)y	252.5 (250.1–254.9)y
48	20.4 (19.6–21.3)a	678.0 (672.3–683.7)b	729.4 (724.5–734.2)c	20.6 (20.5–20.8)x	552.1 (544.9–559.2)y	523.5 (476.2–570.8)y
72	22.9 (22.0–23.8)a	826.0 (787.0–865.0)b	773.8 (650.3–897.2)b	20.7 (20.5–20.9)x	761.4 (714.2–808.6)y	793.6 (663.3–923.9)y
Methylcoprostanone						
0	–	–	–	32.3 (30.5–34.1)x	34.9 (33.5–36.3)x	38.8 (37.4–40.2)x
24	–	10.0 (7.9–12.0)a	8.5 (8.5–8.6)a	37.3 (36.9–37.7)x	42.2 (40.9–43.4)xy	43.5 (41.7–45.4)y
48	–	49.6 (44.3–54.9)a	50.0 (49.9–50.1)a	37.2 (36.3–38.0)x	44.0 (43.5–44.6)x	48.9 (44.4–53.3)x
72	–	87.6 (83.7–91.4)a	226.1 (171.7–280.5)a	18.4 (0.00–36.7)x	23.4 (0.00–46.7)x	42.9 (42.7–43.2)x

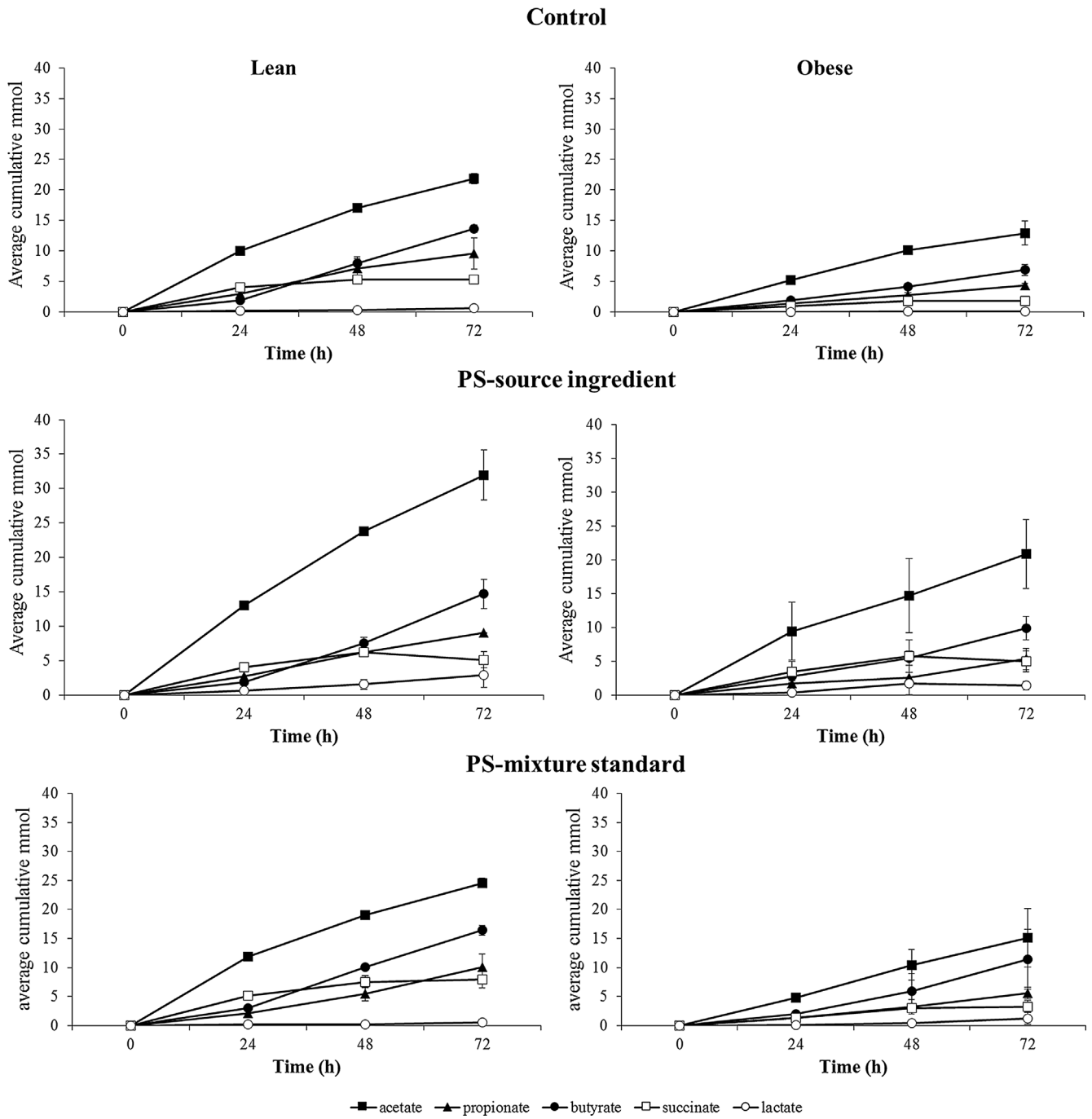

Fig. 2. Average cumulative production of the short chain fatty acids acetate, propionate and butyrate and the organic acids succinate and lactate (mmol) in the experiments with control, ingredient and standard using lean and obese microbiota. The concentrations at t0 was artificially set to zero.

For the obese microbiota, the genus *Clostridium* was increased for PS-source ingredient and the kingdom *Bacteria* for PS-mixture standard, while a few OTUs (including *Christensenellaceae* and other OTUs in the order *Clostridiales*) were higher in control (Fig. 3C). Also here, when PS-source ingredient and PS-mixture standard were combined and compared to control, more discriminative OTUs were observed (Fig. 3D).

Using Spearman correlation with false-discovery rate correction, the presence of OTUs was correlated to microbial metabolites produced (Fig. 4, only shown for strong correlations with a rho > 0.666 or $-0.666 >$ rho). For the lean microbiota negative correlations between the genus *Dorea* and propionate acid, and between an uncharacterized OTU in the order *Clostridiales* and methylcoprostanone was observed. In addition, positive correlations were found between propionic acid and the genus *Atopobium* and *Peptostreptococcus*. In the case of experiments with obese microbiota, there were negative correlations between the genus *Klebsiella* and butyric acid and methylcoprostanone. The latter metabolite (methylcoprostanone) has also been shown to be negatively correlated with the genus *Methanobrevibacter*. Other negative correlations recorded were between propionic acid and the *Rikenellaceae* family, and between butyrate acid and the *Ruminococcaceae* family. Finally, a positive correlation was observed between the genus *Bacteroides* and methylcoprostanone (Fig. 4).

PICRUSt was used to predict the microbial pathways contributing to the metagenomes of the samples identified using 16S rRNA sequencing, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the data was visualized using STAMP. Using Welsh's two-sided *t*-

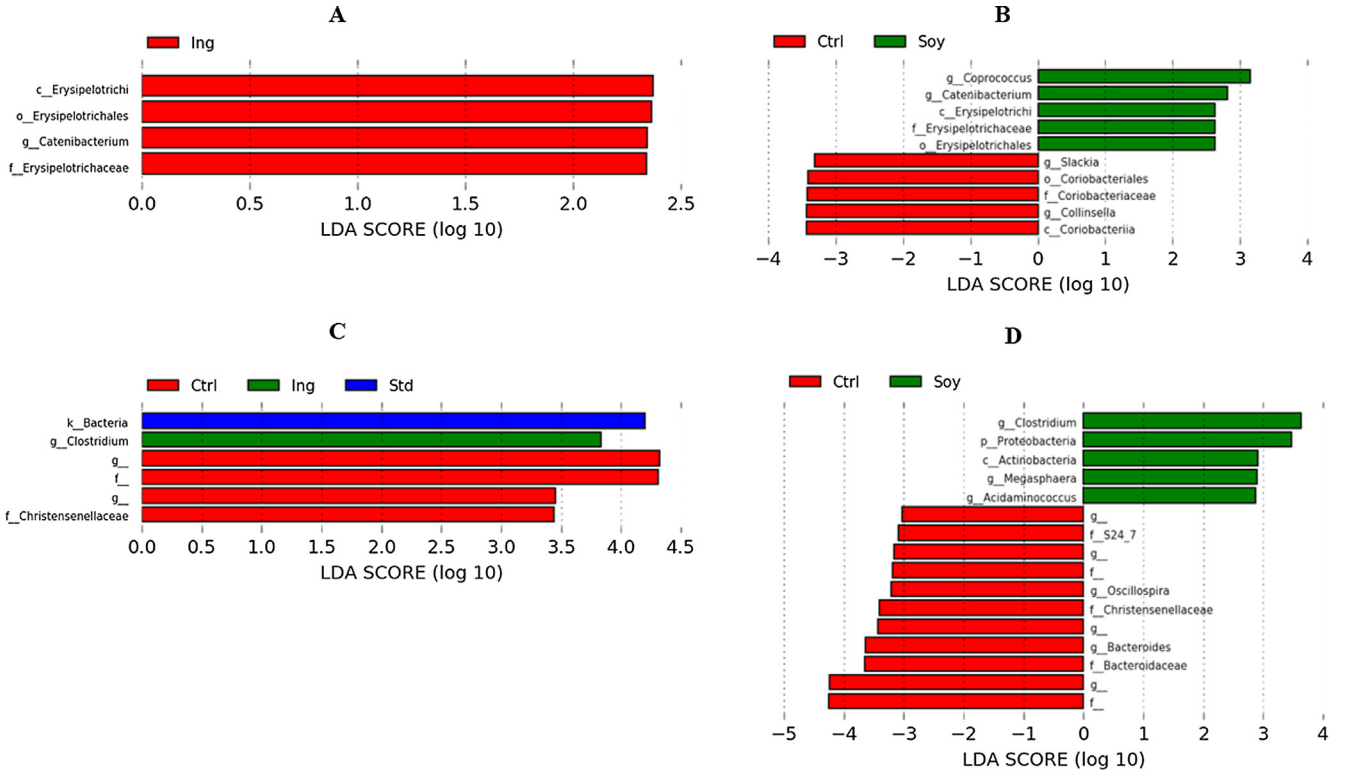

Fig. 3. Linear discriminant analysis effect size for microbiota composition of the lean (A, B) and obese (C, D) microbiota. (Ing: PS-source ingredient; Ctrl: control; Soy: PS-source ingredient + PS-mixture standard; Std: PS-mixture standard).

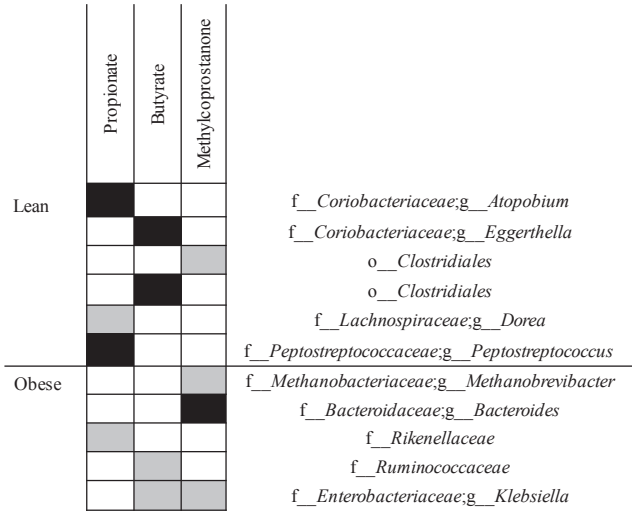

Fig. 4. Spearman correlation with false-discovery rate (FDR) correction between OTUs and some microbial metabolites produced (short chain fatty acids and fecal sterols).

test, pathways predicted for each test product were compared to the other experiments. For the lean microbiota three pathways were significantly different (Table 4A). For the obese microbiota a total of 47 pathways were predicted to be differentially present (Table 4B), including those for flavone and flavonol biosynthesis (Fig. 5A), flavonoid biosynthesis (Fig. 5B). The box-plots in Fig. 5C shows the predicted pathway presence and the heatmap shows the abundance of the pathways in the individual samples.

4. Discussion

Given that, coprostanol is the most abundant animal sterol in feces, followed by coprostanone and cholesterol, which can be transformed to a lesser extent into cholestanol (Cuevas-Tena, Alegría, & Lagarda, 2018), the decrease of coprostanol and coprostanone and increase of cholestanol concentration in PS-source ingredient and PS-mixture standard experiments using microbiota from lean population, suggest that the enrichment of the culture medium with PS has been able to modify the route of cholesterol biotransformation in this assays. In addition, the results obtained also are in concordance with other *in vivo* studies carried out by Weststrate, Ayesh, Bauer-Plank, and Drewitt (1999), where after the intake of margarine enriched with PS (8.6 g/day) by healthy subjects, an increase in the concentrations of neutral sterols was found and the amount of cholesterol metabolized into coprostanol was reduced. Cuevas-Tena, Bermúdez, Silvestre, Alegría, and Lagarda (2018) evaluated the impact of intake of a PS-enriched milk based fruit beverage (2 g PS/day) by postmenopausal women on fecal sterols excretion, and found a lower tendency in the production of coprostanol in presence of PS-enrichment dose. In a previous study in rats, following a diet with sitosterol (0.8%, w/w) also a decrease in the coprostanol production compared to a diet containing cholesterol (1.2%, w/w) was observed (Cohen, Raicht, & Mosbach, 1974). However, in the assays with obese population microbiota, the increase in the concentration of coprostanol and cholestanol indicate that the cholesterol could have been biotransformed by two different routes (Cuevas-Tena, Alegría, et al., 2018). These facts could also be due to the effect of the diet of the fecal samples donors, since the real sterol intake has not been controlled, and it was not previously consider whether the donors of the fecal samples were high or low sterol converters (Wilkins & Hackman, 1974), which are limitations in the study.

PS are also transformed by the gut microbiota through biochemical reactions similar to those occurring for cholesterol (Keller & Jahreis, 2004; Wong, 2014). In this study, the decrease of ethylcoprostanol and the increase of ethylcoprostanone observed in both assays (lean and obese microbiota) under presence of PS-enriched dose suggests that, the capacity of the gut microbiota from both populations was not sufficient

Table 4
Pathways significantly different between control, PS-source ingredient and PS-mixture standard after projecting the OTU abundance on the pathways in KEGG (Kyoto Encyclopaedia of Genes and Genomes) for the lean (A) and obese (B) microbiota.

Observations Ids	p-values
Lean (A)	
Phenylpropanoid biosynthesis	0.03
Starch and sucrose metabolism	0.04
Transcription related proteins	0.03
Obese (B)	
Alpha-Linolenic acid metabolism	0.01
Alzheimer's disease	0.02
Amytrophic lateral sclerosis (ALS)	0.02
Apoptosis	0.01
Bacterial toxins	0.00
Benzoate degradation	0.04
Beta-Alanine metabolism	0.01
Butanoate metabolism	0.03
Carbohydrate metabolism	0.04
Chaperones and folding catalysts	0.004
Chromosome	0.05
D-Alanine metabolism	0.02
Dioxin degradation	0.05
Drug metabolism – other enzymes	0.03
Electron transfer carriers	0.01
Fatty acid biosynthesis	0.02
Flavanone and flavonol biosynthesis	0.01
Flavonoid biosynthesis	0.04
Galactose metabolism	0.01
Glyoxylate and dicarboxylate metabolism	0.02
Inositol phosphate metabolism	0.05
Isoquinoline alkaloid biosynthesis	0.003
Lipoic acid metabolism	0.03
Membrane and intracellular structural molecules	0.02
Novobiocin biosynthesis	0.04
One carbon pool by folate	0.001
Other glycan degradation	0.02
Other ion-coupled transporters	0.04
Other transporters	0.03
Others	0.02
Pentose and glucoronate interconversions	0.02
Pentose phosphate pathway	0.01
Phosphonate and phosphinate metabolism	0.02
Polycyclic aromatic hydrocarbon degradation	0.01
Prenytransferases	0.02
Pyruvate metabolism	0.01
Riboflavin metabolism	0.00
Ribosome biogenesis	0.02
RNA degradation	0.05
Sphingolipid metabolism	0.01
Terpenoid backbone biosynthesis	0.01
Transcription machinery	0.01
Transcription related proteins	0.02
Transporters	0.03
Tuberculosis	0.03
Vibrio cholerate pathogenic cycle	0.03

to transform sitosterol into ethylcoprostanol. This fact is perhaps due to the large amounts of PS present in culture medium (Cohen et al., 1974), causing a lower production of ethylcoprostanol. Similarly, an increase of methylcoprostanone has also been observed with lean microbiota, mainly after 72 h and in presence of PS-source ingredient and PS-mixture standard (Table 3). Generally, the results suggest that gut microbiota from lean and obese populations would prefer PS as a substrate, because they were present in greater proportion than cholesterol. In the same way, in other *in vitro* static colon fermentation study of our research group, lower microbial metabolism for cholesterol and higher for sitosterol and campesterol were found (Cuevas-Tena, del Pulgar, et al., 2018). In addition, some studies have suggested that cholesterol metabolites could act as carcinogenic compounds (Hill & Aries, 1971; Korpela, 1982; Perogambros, Papavassiliou, & Legakis, 1982; Reddy & Wynder, 1977).

SCFA are produced in the proximal colon through the fermentation of mainly indigestible carbohydrates. As far as we know, this is the first time that microbial SCFA production is reported after *in vitro* fermentations assays with PS-enrichment dose with gut microbiota from lean and obese population. In this sense, a greater production of SCFA was observed in experiments with microbiota from lean *versus* obese subjects, perhaps this fact is due to the different microbial profile of these two populations. However, it has not been possible to establish an association between the genera identified in both trials (lean and obese) and their activity as SCFA producers.

Acetate was the main SCFA produced independently of the type of microbiota (lean or obese) and substrates (control, PS-source ingredient or PS-mixture standard) used. However, this SCFA showed a significantly higher production in presence of PS-enrichment dose using lean microbiota. It has been suggest that acetate stimulates cholesterol and long chain fatty acids synthesis in the liver (Delzenne, Neyrinck, Bäckhed, & Cani, 2011) and a study using mice showed that acetate from colonic fermentation can cross the blood-brain barrier and suppress appetite in the hypothalamus (Frost et al., 2014). Butyrate is another main SCFA with higher production in experiments with microbiota from lean than obese population. Butyrate functions as the major energy source for colonic epithelial cells (Koenen, Rubio, Mueller, & Venema, 2016). In addition, this SCFA is considered to be especially important, as it may also play a major role in the prevention of colon cancer (Leonel & Alvarez-Leite, 2012; Mortensen & Clausen, 1996; Perrin et al., 2001) and other colonic diseases (Hamer et al., 2008). Besides, butyrate protects against diet-induced obesity and suppresses food intake (Lin et al., 2012). Propionate was also shown to be produced at greater amounts in experiments with microbiota from lean subjects. In the case of this SCFA, it has been suggested that it reduces liponeogenesis, cholesterol synthesis and recently has been demonstrated to be involved in the activation of G-protein coupled receptors (GPR-41 and GPR-43) releasing satiety hormones, thus reducing food intake (Kimura et al., 2011). Succinate and lactate showed to be the minority compounds and a greater production was observed in the experiments with microbiota from lean subjects *versus* obese. These acids serve as intermediates in the metabolism of SCFA and generally they do not accumulate in the colon (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). However, a significant increase in lactate production was observed in the presence of PS-source ingredient only in lean microbiota experiment. In this context, lactate only accumulates when there is a fast fermentation of a substrate. If substrates are fermented slowly, lactate is mostly converted into butyrate (Morrison et al., 2006).

Besides, our results indicate that PS influences *Catenibacterium* and *Coprococcus* genera (*Erysipelotrichaceae* family) in experiments with lean microbiota and *Clostridium* genus in obese microbiota, with an increase in the proportion of these bacteria when PS was added to the culture medium. Regarding to the increase of the *Erysipelotrichaceae* family members, these results were not coincident with those found in a previous study by our group (Cuevas-Tena, del Pulgar, et al., 2018). In a clinical trial (Baumgartner et al., 2017), where 13 healthy subjects received during three weeks a control or plant stanol ester enriched margarine (3 g/day plant stanols), no differences in gut microbiota composition were recorded between the two target groups. However, van Faassen et al. (1987) found slight changes for *Bifidobacterium*, *Bacteroides* and *Clostridium* genera in subjects with a vegan diet, which was rich in PS and dietary fiber, which affect gut microbiota composition.

Previously were not established a similar age range between the donors of the lean and obese population and not to considered also the enterotypes (Arumugam et al., 2011) of the microbial profile for fecal samples used as inoculum, which constitute limitations for the study.

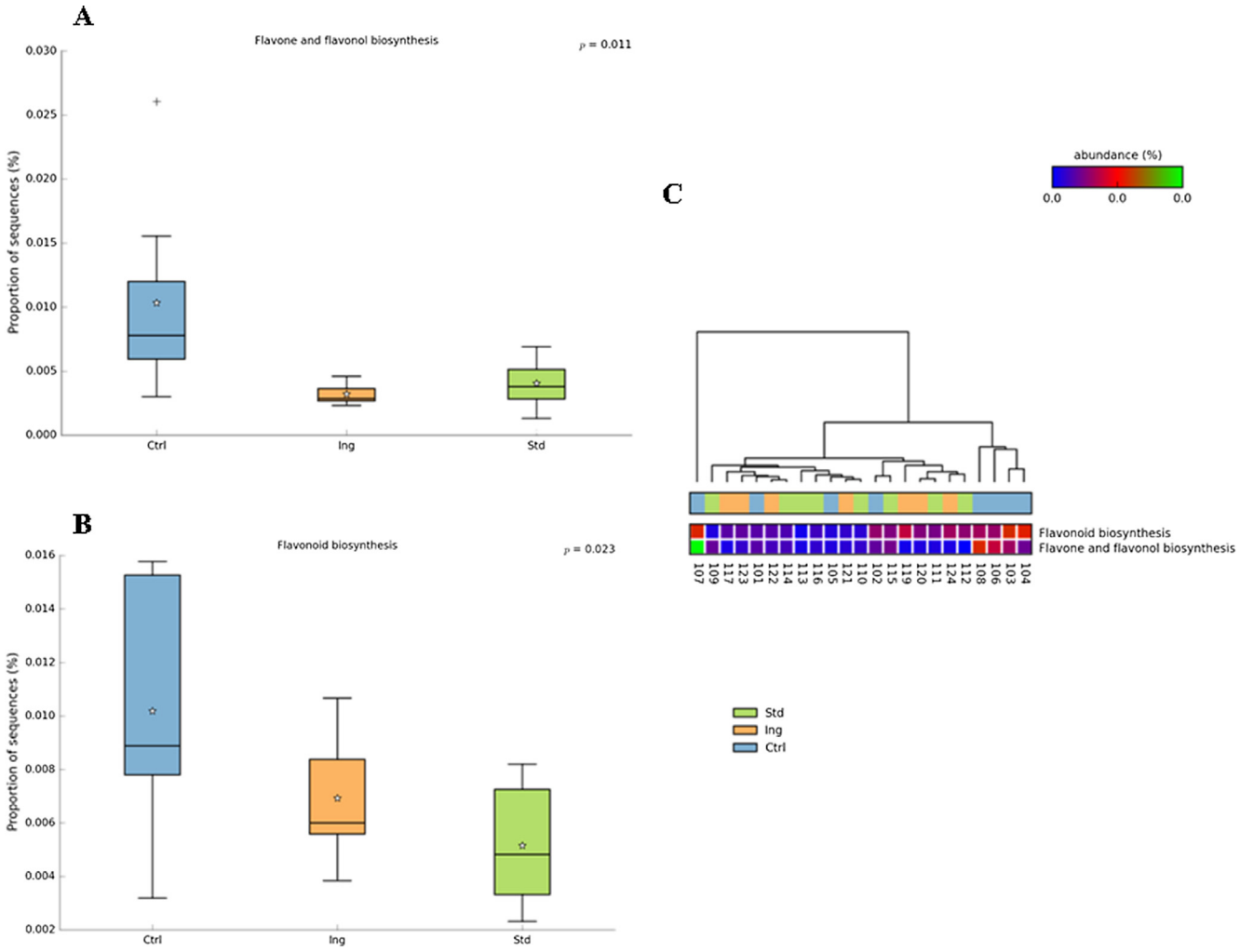

Fig. 5. Boxplot of flavone and flavonol biosynthesis (A) and flavonoid biosynthesis (B) pathways and heatmap (C) predicted to be differentially present in the different experiments by using PICRUSt, visualized using STAMP.

5. Conclusions

The concentration of animal and PS metabolites (coprostanol, coprostanone, cholestanol, ethylcoprostanol and methylcoprostanone) was higher in experiments with microbiota of obese *versus* lean, suggesting a different microbial activity between these two population groups. In addition, the presence of PS-enrichment dose (2 g/day) there has been a modification of the cholesterol biotransformation route with a lower production of their main metabolites (coprostanol and coprostanone) fundamentally using lean population microbiota. Besides, PS-source ingredient and PS-mixture standard have been able to saturate the intestinal microbial metabolism of sitosterol for lean and obese population, which produces a decrease in the concentration of ethylcoprostanol (main sitosterol metabolite) and increase of ethylcoprostanone.

The total SCFA production was different between experiments with microbiota from lean and obese subjects, being higher when lean microbiota was used. This fact would support the hypothesis that the microbial profile of these population groups is different, which was corroborated by PCoA in QIIME (not shown). In presence of PS-enrichment dose, the production of acetate and butyrate increased significantly using mainly microbiota of lean population. Therefore, it could be confirmed that the supplementation of the diet with PS-enrichment dose would not modify the carbohydrates metabolic activity of the gut microbiota, and therefore SCFA continues to be produced. The enrichment of the culture medium with PS-source ingredient or PS-mixture standard increased the proportion of the genera belonging to the *Firmicutes* phylum. While in the control trials, the proportion of the genera that increase are those belonging to the *Actinobacteria*, *Bacteroidetes* and *Firmicutes*.

The present work is an *in vitro* study and although the obtained results showed be confirmed in human studies with daily intake of PS (2 g/day) over several weeks, the different fecal inocula used may lead to different effects on gut microbiota composition.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics statements

Our research did not include any human subjects and animal experiments.

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