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Supplementation with grape pomace in healthy women: Changes in biochemical parameters, gut microbiota and related metabolic biomarkers

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ABSTRACT

This paper reports a comprehensive pilot study of the effects of dietary supplementation with grape pomace (GP) in humans. After an initial washout period, the diet of 10 healthy women was supplemented with a dairy amount of 1.4 g of a red Grape Pomace (GP) extract (Eminol®) for 21 days. Among the different biochemical and immune parameters measured in plasma, a significant decrease ($p < 0.05$) was observed in blood fasting glucose levels after the 21-day supplementation. Overall, the GP supplementation did not lead to significant changes in faecal bacterial populations or, in general, in the content of faecal and urine phenolic metabolites. Nevertheless, significant changes ($p < 0.05$) were observed in the short-chain and medium-chain fatty acid profiles (SCFAs and MCFAs). Due to observed inter-individual differences it was not possible to establish a pattern on the microRNA expression profile associated to GP supplementation, however modulation of the expression of miRNA related to glucose metabolism was perceived after the intervention period.

1. Introduction

Grape pomace (GP) is a winery by-product composed basically of grape seeds, skin and stems. It is rich in dietary fibre and polyphenols including anthocyanins (in red GP), flavan-3-ols, flavonols, phenolic acids and stilbenes (Fontana, Antoniolli, & Bottini, 2013), which provides a wide variety of potential biological activities (Yu & Ahmedna, 2013). In recent years, there has been an increased interest in the use of GP to develop functional ingredients and in other applications for the food industry (Charalampia & Koutelidakis, 2016). But the majority of scientific evidence concerning GP benefits is derived from experiments performed *in vitro* and in animal models, often using a concentration much higher than that contained in biological fluids and in diet, respectively. Moreover, in some cases, GP parent compounds are tested instead of their derived metabolites, which would be the responsible for beneficial effects *in vivo* (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010). Therefore, it is essential to conduct human intervention studies for evaluating the biological properties of GP.

To our knowledge, only two human intervention studies have been published concerning the effects of the inclusion of GP in the human diet. Yubero et al. (2013) evaluated the effects of the supplementation with a GP extract (700 mg/day, 56 days) on cardiovascular risk and oxidative stress indicators in healthy volunteers (n = 60). The results of this study showed that the GP was able to modulate the lipid profile, lowering total blood cholesterol and LDL cholesterol levels (Yubero et al., 2013). Later on, Urquiaga et al. (2015) investigated the effect of the supplementation with a GP flour (20 g/day, 16 weeks) in patients (n = 38) who suffered at least from one component of metabolic syndrome. The results of this study indicated a significant improvement in blood pressure, fasting glucose levels and protein damage after supplementation with GP flour (Urquiaga et al., 2015). On the other hand, some *in vitro* studies have assessed the metabolism and further bioavailability of GP polyphenols. The majority of GP phenolic compounds are present in the form of esters, glycosides or polymers and suffer

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multiple degradation reactions by intestinal enzymes and/or by the colonic microbiota, prior to be absorbed (Cueva et al., 2013). Data currently available on the digestibility and metabolism of GP polyphenols suggest a relatively great bioavailability of their metabolites (Motilva et al., 2016; Sasot et al., 2017).

Another interesting issue about GP functionality is its potential ability to modulate the intestinal microbiota. In this line, the study by Viveros et al. (2011) revealed that broiler chicks fed with GP extracts had higher intestinal populations *Lactobacillus, Clostridium* and *Enterococcus* species than the animals from the untreated control group. Low counts of *Streptococcus* spp. and *Clostridium* cluster XIVa were reported in the faecal microbiota from weaned pigs fed with a GP meal extract (Fiesel, Gessner, Most, & Eder, 2014). Another animal study showed that lambs fed with a diet supplemented with GP presented lower growth of potential pathogenic bacteria, in particular *Enterobacteriaceae* and *E. coli* (Kafantaris et al., 2016). Some of our previous *in vitro* studies indicated changes in some bacterial groups after fermentation of a red GP extract (Eminol®) with faecal microbiota, with increments for *Lactobacillus* and *Bacteroides* groups (Gil-Sánchez et al., 2017; Gil-Sánchez, in press). However, to the best of our knowledge, there are no studies available concerning the impact of GP supplementation on the intestinal microbiota (composition and/or functionality) in humans.

Therefore, the aim of this study was to assess the possible effect of the supplementation with a red GP extract (Eminol®), on different biochemical and molecular biomarkers as well as in the composition and activity of the human gut microbiota. The study was focussed on healthy women to limit inter-individual variability and strengthen further conclusions. A complete biochemical study was performed in blood samples. Populations of the major groups of the intestinal microbiota, as well as short-chain and medium-chain fatty acids (SCFAs and MCFAs) were quantified in faecal samples, and microbial phenolic metabolites were determined in urine and faecal samples. In addition, modulation of 734 miRNAs expression by GP extract intake and validation of five miRNAs related to glucose metabolism was performed in serum.

2. Materials and methods

2.1. Grape pomace extract

The GP extract used in this study, trademarked as Eminol® (ABROBIOTEC S.L., Valbuena de Duero, Valladolid, Spain), was obtained according to previous-reported procedures (Gil-Sánchez et al., 2017). Briefly, fresh grape pomace was submitted to a distillation process to remove alcohol and aromatic compounds. Later, the residue was extracted through traditional solid-liquid extraction by diffusion using a hydroalcoholic solution (water:ethanol) as solvent. The resulting product was centrifuged and stabilized in order to delete solid residues. Finally, the final solution was concentrated and dried by a spray-drying process.

The red grapes used in the winemaking process were all of the Tempranillo variety, harvested from vineyards located in the Ribera de Duero Designation of Origin (Spain). The extract contained high amounts of dietary fibre (659.7 mg/g extract) (Table 1), as determined by the methodology reported in Martin-Cabrejas, Waldron, Selvendran, Parker, and Moates (1994). The concentration of total polyphenols was moderate (37.44 mg gallic acid/g extract) (Table 1), as determined by the Folin-Ciocalteu reagent (Singleton and Rossi, (1965). Among phenolic compounds, different phenolic acids, flavonols and flavanols were previously identified in this extract (Gil-Sánchez et al., 2017) (Table 1). In addition, other chemical parameters were previously analysed in the extract such as moisture (4.6%), total protein (8.7%), fat (0.8%) and ash (20.7%) (Gil-Sánchez et al., 2017).

The extract, disposed in capsules containing 700 mg of extract/capsule, was supplied by Grupo Matarromera (Valbuena de Duero, Valladolid, Spain).

Table 1
Content in polyphenols and dietary fibre of Eminol® extract.

	GP Extract
PHENOLIC COMPOSITION (mg/g extract)	
Total polyphenols (mg gallic acid/g)	37.44
Phenolic acids	
Ellagic acid	5.64 ± 0.65
Flavanols	
Catechin	0.28 ± 0.07
Epicatechin	1.03 ± 0.25
Proanthocyanidin	4.62
Flavonols	
Myricetin	0.26 ± 0.01
Quercetin	0.34 ± 0.03
Kaempferol	0.16 ± 0.00
ALCOHOL-INSOLUBLE RESIDUE COMPOSITION (mg/g AIR)	
Total AIR yield	660
Neutral sugars	294.7
Uronic acids	32.3 ± 1.0
Total sugars	328.2
Klason lignin	84.3 ± 1.1

All values are means of three replicates (n = 3).

2.2. Human intervention study design

The intervention study was conducted in accordance with the Helsinki Declaration and was approved by the Ethics Committee from CSIC (Madrid, Spain) (Approval number (23_08_2012). All volunteers gave their written informed consent prior to participate. The study involved 10 healthy women (age ranged 25–65 years, BMI $< 25\ kg/m^2$). The participants were not suffering from diabetes, hypertension, or dyslipidemia, acute or chronic inflammatory disease, infectious disease, cancer, or a previous cardiovascular event at study entry. They had not received any antibiotic therapy, prebiotics, probiotics, synbiotics, or vitamin supplements or any other medical treatment influencing intestinal microbiota during the 6 months before the start of the study or during the study (including the washout period). Volunteers followed an initial washout period of 10 days (baseline) during which they maintained a low-polyphenol diet. After this period, the participants were instructed to take two capsules per day of the GP extract (1400 mg of extract/day) at breakfast over 21 days. Thus, the average daily consumption of fibre and phenolic compounds, in the present study, was 923.58 mg and 54.42 mg respectively. During the intervention period, they also maintained the restrictions of polyphenol rich foods in the diet. Volunteers filled in a questionnaire on their dietary habits to verify the diet.

Blood samples were extracted from volunteers after an overnight fast, by skilled professionals, at three points: (a) after the run-in washout period, (b) after 14 days of consumption of the GP extract and (c) at the end of the GP intervention (21 days). The serum was separated into aliquots and immediately frozen at −80 °C.

At the same time as blood samples were collected, participants provided samples of faeces and 24 h-urine. Faecal samples were immediately frozen and stored at −80 °C awaiting analysis. Urine samples were measured (the total urine volume in 24 h), homogenized, acidified with HCl to achieve a final concentration of 0.2 M, aliquoted, and then aliquots were frozen and stored at −80 °C.

2.3. Serum biochemical and immune parameters measurement

Serum biochemical parameters were measured using an automated biochemical auto-analyser in an accredited external laboratory (Unilabs, Madrid). The tests included the measure of glucose, uric acid, albumin, hepatic enzymes (glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and gamma-glutamyl transferase

(γ-GT), lipid metabolism (Total cholesterol, HDL cholesterol, total cholesterol/HDL cholesterol ratio, LDL cholesterol, LDL cholesterol/HDL cholesterol ratio, and triglycerides), proteins (C-reactive protein), basal hormones (insulin and serum leptin) and tumoral necrosis factor (TNF-α). The levels of TNF-α were determined using a human TNF-α ultrasensitive ELISA kit (Invitrogen, Barcelona, Spain) following the manufacturer's recommended protocol. All determinations were carried out at least in duplicate.

2.4. Microbial community analysis in faecal samples

2.4.1. Bacterial DNA extraction

Faecal samples collected were thawed, weighed, diluted in sterile PBS solution (1:10, w/v), and homogenized in a LabBlender 400 stomacher (Seward Medical, London, UK) at full-speed for 4 min. Later on, samples were centrifuged at 14000 rpm for 15 min at 5 °C in order to remove the faecal supernatant. Microbial DNA extraction from pellets of faecal samples or standard bacteria was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's recommended protocol. The extracted DNA was kept frozen at -80 °C until analysis.

2.4.2. Determination of faecal microbiota composition by qPCR

Quantification of the different bacterial populations was carried out by qPCR using specific primers targeting different bacterial groups (Table 2), as described by Arboleya et al. (2012). Briefly, all reactions were performed on MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems, Foster City, CA) in a 7500 Fast Real Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). Each amplification reaction was done in duplicate in two independent PCR runs. The final volume of each reaction was 25 μL, containing 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μL of each primer (10 μM), 10.5 μL of nuclease-free water purified for PCR (Sigma-Aldrich) and 1 μL of DNA template. Thermal cycling consisted of an initial cycle of 95 °C 10 min, followed by 40 cycles of 95 °C 15 s, and 1 min at the appropriate primer-pair temperature (Table 2). Microorganisms used for performing standard curves in order to assign microbial counts to Ct values for each of the microbial groups analysed, were grown overnight under anaerobic conditions in GAM medium (Nissui Pharmaceutical Co, Tokyo, Japan). These standard strains were obtained from different culture collections (Table 2).

2.5. Targeted analysis of phenolic metabolites in faecal and urine samples

2.5.1. Sample preparation

For preparation of faecal solutions, samples were thawed at room temperature. One gram of faecal sample was spiked with 10 mL of sterile saline solution (NaCl 0.9%, Fresenius Kabi, Spain), and centrifuged twice (10 min, 10,000 rpm, 4 °C). Supernatants (faecal solutions) were collected, filtered (0.22 μm) and an internal standard 4-hydroxybenzoic-2,3,5,6-d4 acid solution (Sigma-Aldrich, St. Louis, MO) [1250 μg/mL in formic acid/acetonitrile (1:200, v/v)] was added to the samples in a proportion 1:5 (v/v).

To obtain total aglycon metabolites in urine, samples were hydrolyzed by incubating with β-glucuronidase from Helix Pomatia (Sigma, St, Louis, MO, USA) in sodium acetate buffer (100 mM, pH 5.2), in a proportion 1:3 (v/v), at 37 °C for 18 h. Then, hydrolyzed samples were passed through a0.22-μm pore size filter and mixed with the internal standard solution.

2.5.2. Analysis of phenolic metabolites by UPLC–ESI–MS/MS

Phenolic metabolites were analysed by UPLC–ESI–MS/MS, as described previously (Jimenez-Giron et al., 2013; Muñoz-Gonzalez, Jimenez-Giron, Martin-Alvarez, Bartolome, & Moreno-Arribas, 2013) with some modifications. The liquid chromatographic system was a Waters Acquity UPLC (Milford, MA) equipped with a binary pump, an auto sampler thermos tatted at 10 °C, and a heated column compartment (40 °C). The column employed was a BEH-C18 model (2.1 × 100 mm and 1.7 μm particle size) from Waters (Milford, MA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min, resulting in a total runtime of 18 min. The flow rate was set constant at 0.5 mL/min and the injection volume was 2 μL. The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in both negative and positive ion modes. For quantification, data were collected in multiple reactions monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The 60 phenolic compounds targeted in the study were quantified using the calibration curves of their corresponding standards as described

Table 2
Bacterial groups, standard cultures, primers sequences and melting temperature (T°m) used to qPCR.

Target group	Standard strain	Oligonucleotide sequence (5′–3′)	T°m (°C)	References
Bacteroides group *Bacteroides-Prevotella-Porphyromonas*	*Bacteroides thetaiotaomicron* (DSMZ 2079)	F: GAGAGGAAGGTCCCCCAC R: CGCKACTTGGCTGGTTCAG	60	Echarri et al. (2011)
Bifidobacterium	*Bifidobacterium longum* (NCIMB8809)	F: GATTCTGGCTCAGGATGAACGC R: CTGATAGGACGCGACCCCAT	60	Gueimonde, Tolkko, Korpimaki, and Salminen (2004)
Clostridia XIVa *Blautia cocccoides-Eubacterium rectale*	*Blautia coccoides* (DSMZ 935)	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG	55	Rinttila, Kassinen, Malinen, Krogius, and Palva (2004)
Faecalibacterium	*Faecalibacterium Prausnitzii* (DSMZ 17677)	F: GGAGGAAGAAGGTCTTCGG R: AATTCCGCCTACCTCTGCACT	60	Ramirez-Farias et al. (2009)
Enterobacteriaceae	*Escherichia coli* (LMG2092)	F: TGCCGTAACTTCGGGAGAAGGCA R: TCAAGGACCAGTGTTCAGTGTC	60	Matsuda, Tsuji, Asahara, Kado, and Nomoto (2007)
Enterococcaceae	*Enterococcus faecalis* (IPLA IF3/1)	F: CCCATCAGAAGGGGATAACACTT R: ACCGCGGGTCCATCCATC	60	Matsuda et al. (2007)
Lactobacillus group *Lactobacillus-Weisella*	*Lactobacillus gasseri* (IPLA IF7/5)	F: AGCAGTAGGGAATCTTCCA R: CATGGAGTTCCACTGTCCTC	60	Echarri et al. (2011)
Total Bacteria	*Bacteroides thetaiotaomicron* (DSMZ 2079)	F:CGGTGAATACGTTCCCGG R: TACGGCTACCTTGTTACGACTT	60	Furet et al. (2009)

DSMZ: Leibniz Institute-DMZ German collection of microorganisms and cell cultures; BCCM-LMG: Belgian coordinated collection of microrganisms; IPLA: collection of microorganisms from IPLA; NCIMB: National Collection of Industrial, Food and Marine Bacteria from UK.

Table 3
Characteristics of the five miRNA related to glucose metabolism analysed in the volunteers and selected after miRNA screening on volunteers #8 and #10.

miRNA	Sequence	Implication on glucose metabolism	References
hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG	↑: Implicated on metabolic syndrome and type-2-diabetes ↑: low glucose intake on breast cancer ↓: hepatic insulin resistance	Willeit et al. (2016) and Fong et al. (2015)
hsa-miR-34a-5p	UGGCAGUGUCUUAGCUGGUUGU	It acts over targets related with insulin signaling and pancreas development ↑: on pancreatic islands on type-2 diabetes patients ↑: Impaired glucose metabolism	Rottiers and Naar (2012), Kong et al. (2011), and Rokavec et al. (2014)
hsa-miR-342-3p	UCUCACACAGAAAUCGCACCCGU	↓: type-1 diabetes, type-2 diabetes and gestational diabetes	Fernandez-Valverde et al. (2011), Collares et al. (2013)
hsa-miR-130a-3p	CAGUGCAAUGUUAAAAGGGCAU	↓: type-2 diabetes ↑: rise in insuling signaling	Ye et al. (2015), Jiao et al. (2015), and Xiao et al. (2014)
hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG	↓: type-1 diabetes	Zampetaki et al. (2010)

previously by Sánchez-Patán et al. (2011). Data acquisition and processing were realized with MassLynx 4.1 software. All analyses were performed in duplicate.

2.6. Short and medium fatty acids analysis in faeces

The volatile fatty acid composition was analysed by SPME-GCMS following the method described by Cueva et al. (2015). Two hundred and ninety microliters of faecal solutions (1:10 w/v, faeces/saline solution) or calibration stock solutions were added with 10 µL of internal standard solution of 2-methylvaleric acid (1 g/L) and 30 µL of a 0.9 M H_2SO_4 solution (pH 2). Then, 100 µL of the acidified sample were transferred to a 20 mL hermetically closed vial. The extraction procedure was automatically performed by using a CombiPAL system (CTC Analytics AG, Zwingen, Switzerland) with a 50/30 µm DVB/CAR/PDMS SPME fibre of 2 cm length (Supelco, Bellefonte, PA). The chromatographic separation was performed in a DB-FFAP capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) (J&W, Agilent). Compounds identification was carried out by comparison of retention times and mass spectra of the reference compounds with those reported in the mass spectrum library NIST 2.0. Quantitative data were obtained by calculating the peak area of each compound in relation to that of the internal standard (2-methylvaleric acid). Calibration curves of each compound (acetic acid, propionic acid, butyric acid, pentanoic acid, hexanoic acid, octanoic acid and decanoic acid) were obtained by diluting the original stock standard solution (5000 mg/L of all volatile fatty acids) in deionized water. Analyses were performed in duplicate.

2.7. Analysis of circulating serum miRNAs

2.7.1. RNA extraction

Total RNA (including miRNA) was extracted from serum samples corresponding to initial and end time (21 days) of the GP extract intervention period, following the instructions of the miRCURY™ RNA Isolation kit for Biofluids (Exiqon, Vedbaek, Denmark). After the extraction, the final concentration of total RNA enriched in miRNAs was quantified using a NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Spain) and purity was assessed by measuring the 260/280 nm and 260/230 nm ratios. Samples were stored at −20 °C until further analysis. The absence of haemolysis in serum was verified, by amplification of miR23a and miR454 as controls.

2.7.2. Screening of miRNA using panels

Initially, miRNA expression in serum of volunteers #8 and #10 were analysed. 200 ng of total RNA per panel was reverse transcribed using the miRCURY LNA™ Universal RT microRNA PCR and polyadenylation and cDNA synthesis kit (Exiqon, Vedbaek, Denmark). For each sample, the cDNA was diluted, and a mix with 45 µL of cDNA, 4455 µL of nuclease-free sterile water and 4500 µL of SYBR Green probe (Exiqon, Vedbaek, Denmark) was prepared and added to the Ready-to-Use PCR panels (10 µL/well). Human miRNA Panels I and II v4.0 hold 743 different miRNA targets and six reference gene assays (mirBase13) (Exiqon, Vedbaek, Denmark) were used for the miRNA screening. Temperature protocol was performed as follows: enzyme activation at 95 °C for 10 min; 45 cycles of 95 °C for 15 s. and 60 °C for 60 s. All reactions were carried out in a LightCycler® 480 real-time PCR equipment (Roche, Basel, Switzerland). Melting curves were analysed after amplification and Cq values were calculated using 2nd derivative method (LightCycler® 480 Software 1.5, Roche). All reactions were run in triplicate UniSp5 and UniSp3 IPC were used for normalization of miRNAs Panel I and II, respectively. Data were calculated as fold change, according to the $2^{-\Delta\Delta Cq}$ method, where ΔΔCq = (CqmiRNA-CqUniSP) miRNA after the intake - (CqmiRNA-CqUniSP)ctrl miRNA before the intake.

Final results express the difference between post-intake and pre-intake folds (variance).

2.7.3. miRNA validation

After the screening described above, we selected five modulated human circulating miRNAs (hsa-miR-122-5p, hsa-miR-34a-5p, hsa-miR-342-3p, hsa-miR-130a-3p and hsa-miR-191-5p) related to glucose metabolism, insulin resistance status and biomarkers of type 2 diabetes (T2D) (Hernandez-Alonso, Giardina, Salas-Salvado, Arcelin, & Bullo, 2016; Jiao et al., 2015; Tome-Carneiro et al., 2013; Xiao et al., 2014; Ye et al., 2015) (Table 3). Real time PCR detection was performed using SYBR Green and locked nucleic acid (LNA™) specific probes (Exiqon, Vedbaek, Denmark), following manufacturer's instructions in a LightCycler® 480 Instrument (Roche). Temperature protocol was performed as follows: enzyme activation at 95 °C for 10 min; 45 cycles of 95 °C for 15 s. and 60 °C for 60 s. All reactions were carried out in triplicate and Cq values and fold-changes were calculated as stated previously.

2.8. Statistical analysis

A one-way repeated measures ANOVA was conducted to compare the data obtained from volunteers at the three times (wash-out period and after 14 and 21 consuming GP). One-way between-groups analysis of variance was performed to study the different miRNA profiles among subjects. The least significant differences in both cases were calculated by Tukey's test ($p < 0.05$). The relationship between bacterial population and their metabolites as well as the expression of miRNA and the blood fasting glucose levels were investigated using the Pearson correlation coefficient. The STADISTICA program 7.1 was used for processing these data. Finally, Principal Component Analysis (PCA) was performed to summarize changes in the miRNA profiles of volunteers. These tests were done using the IBM SPSS Statistics for Windows, Version 23.0; Armonk, NT: IBM Corp).

Table 4
Endogenous biochemical and immune parameters analysed in blood samples during the intervention study.

		Before GP extract intake	After 14 days GP extract intake	After 21 days GP extract intake
Biochemistry	Glucose (mg/dl)	89.00 ± 1.84[b]	87.10 ± 1.66[a,b]	84.50 ± 2.61[a]
	Uric acid (mg/dl)	4.48 ± 0.35	4.49 ± 0.30	4.24 ± 0.29
	Albumin (mg/dl)	4.46 ± 0.05	4.34 ± 0.04	4.49 ± 0.07
Enzymes	ASAT GOT (UI/l)	19.10 ± 0.62	20.70 ± 2.55	19.90 ± 1.73
	ALAT GOT (UI/l)	17.00 ± 2.21	21.80 ± 6.82	20.30 ± 2.76
	Gamma-GT (UI/l)	13.30 ± 1.13	11.10 ± 1.31	11.60 ± 1.56
Lipidic metabolism	Total Cholesterol (mg/dl)	201.60 ± 7.70	196.90 ± 8.21	198.50 ± 9.00
	Triglycerides (mg/dl)	82.50 ± 18.36	87.2 ± 19.22	91.10 ± 19.93
	HDL Cholesterol (mg/dl)	59.40 ± 4.30	59.20 ± 3.55	58.60 ± 4.00
	Tot. Chol./HDL-Chol	3.57 ± 0.34	3.49 ± 0.37	3.56 ± 0.36
	LDL Cholesterol (mg/dl)	125.40 ± 6.19	120.10 ± 6.63	121.60 ± 7.22
	LDL-Chol/HDL-Chol	2.25 ± 0.24	2.15 ± 0.26	2.20 ± 0.26
Proteins	C-reactive protein (mg/l)	3.82 ± 1.79	2.85 ± 1.55	2.60 ± 1.57
Basal hormones	Insulin (μUI/ml)	9.44 ± 1.88	10.15 ± 1.93	10.52 ± 2.30
	Serum leptin (ng/ml)	12.12 ± 2.61	11.05 ± 19.93	11.47 ± 2.27
Inmune markers	TNF-α (pg/ml)	7.20 ± 0.68	8.01 ± 0.65	7.73 ± 0.33

[a] Means ± Error standard were calculated (n = 10).
[b] Values followed with different code letters are significantly different ($p < 0.05$) according to LSD test. The letter "a" was assigned to the lowest value.

3. Results

3.1. Intervention compliance

The recruitment of volunteers was carried out at the Institute of Food Science Research (CIAL), Madrid (Spain), and the study was approved by the Ethics Committee of CSIC. Initially, eleven healthy women gave their written informed consent, however one participant dropped out the study after the initial 14-days intervention period because of individual reasons. In general, GP extract was well tolerated by the volunteers, however two participants reported slight episodes of increased intestinal gas and dyspepsia.

3.2. Effect of GP extract intake on serum biochemical and immune parameters

Data of biochemical parameters through the intervention study, including levels of glucose, uric acid, albumin, hepatic metabolism enzymes, cholesterol and triglycerides, proteins and basal hormones and the immune marker TNF-α, are shown in Table 4. A significant decrease ($p < 0.05$) was observed in blood fasting glucose levels (from 89.00 ± 1.84 to 84.50 ± 2.61 mg/dL) after the 21-days period of supplementation with 1.4 g/day of GP extract. As Fig. 1a shows, the decline in the blood glucose levels was evident in almost all participants. Then, results were studied carefully by separate, and it was found that this decrease was especially pronounced in the case of volunteers #8 and #10 (who reduced around a 15% their glucose values), followed by volunteers #3 and #9 (who experienced a reduction of 7%) (data not shown).

Uric acid, albumin, hepatic enzymes, proteins and basal hormones (including insulin) were not significantly affected by the GP supplementation. With regard to analytical parameters related to lipid metabolism, although non-significant, a trend towards lower levels of total cholesterol and LDL-cholesterol at the end of the intervention period was observed (from 201.60 ± 7.70 to 198.50 ± 9.00 mg/dL for total cholesterol and from 125.40 ± 6.19 to 121.60 ± 7.22 mg/dL for LDL-cholesterol). Moreover the number of subjects with cholesterol values lower than < 200 mg/dL, and LDL cholesterol < 120 mg/dL at the end of the intervention period increased from 3 to 7, and from 4 to 6, respectively. Finally, it is worth nothing that significant differences were not observed in the analysed safety variables: creatine and transaminases (GPT, GGT and GOT), before and after GP supplementation (Table 4).

3.3. Impact of GP supplementation on faecal microbiota: composition and SCFAs production

Quantification of the intestinal microbial groups and total bacteria before and after 14 and 21 days of GP supplementation, are shown in Table 5. The coefficients of variation for qPCR data were lower than 5%. Overall, all faecal microbial groups analysed remained without noticeable variations along the study period, although, as expected, large inter individual differences were observed among subjects.

In contrast, the levels of total SCFAs (μmol/g faeces) significantly increased during the intervention (Table 5). Propionic acid concentration increased ($p < 0.05$) all along the intake period, whereas significantly higher levels of acetic acid ($p < 0.05$) were evidenced after 14 days but not at the end of the intervention. No remarkable changes were found in the faecal concentrations of butyric acid.

Pearson's correlations were calculated between the levels of the different bacterial groups and the concentrations of SCFAs. Remarkably, *Bifidobacterium* correlated positively with the concentrations of propionic acid at the end of the intervention period (21 days) ($r > 0.7$, $p < 0.05$), but not in the samples taken before the intervention ($r < 0.7$, $p > 0.05$) (Fig. 2).

3.4. Effect of GP supplementation on faecal medium chain fatty acids concentration

Non-microbial origin fatty acids such as pentanoic (C5), hexanoic (C6), octanoic (C8) and decanoic (C10) acid were also determined in faecal samples. Data corresponding to the analysis of these medium chain fatty acids (MCFAs) are shown in Table 6. Notably, a significant decrease ($p < 0.05$) was observed in the concentration of pentanoic, hexanoic and octanoic acids during the GP consumption period, unlike decanoic acid that became constant along intervention (Table 6). In Fig. 1b, 1c and 1d can appreciated the decline of MCFAs previously mentioned. However, it is important remark that there was wide variability in the data.

In addition, Pearson's correlations were calculated between data of bacteria populations and concentrations of MCFAs. *Faecalibacterium* genus was correlated positively with the concentration of all MCFAs at the end of the supplementation period (21 days) ($r > 0.7$, $p < 0.05$), but not in the samples collected before the intervention ($r < 0.7$, $p > 0.05$) (Supplementary Fig. S1).

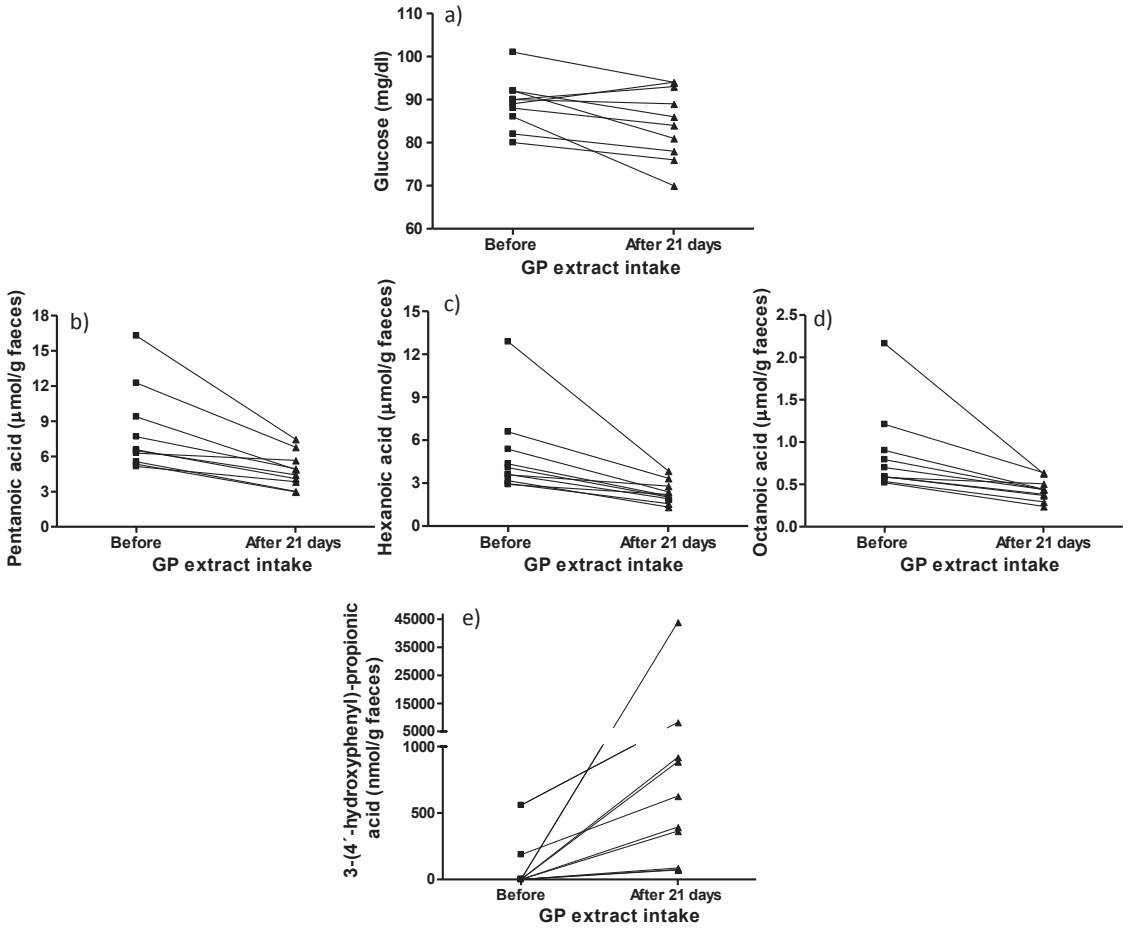

Fig. 1. Individual concentrations (n = 10) of glucose (mg/dl) (a), pentanoic acid (µmol/g faeces) (b), hexanoic acid (µmol/g faeces) (c), octanoic acid (µmol/g faeces) (d) and 3-(4′-hydroxyphenyl)-propionic acid (nmol/ g faeces) (e) before and after 21 days of GP supplementation.

Table 5
Mean values (Standard deviation) of the qPCR data (log cells/g) for the main bacterial groups of intestinal microbiota and the short chain fatty acids (SCFA) (µmol g^{-1} faeces) analysed in the faecal samples from 10 subjects before, during and after Eminol® intake.

	Log_{10} cells/g		
	Before GP extract Intake	After 14 days of GP extract intake	After 21 days of GP extract intake
Bacterial groups (Log_{10} cells/g)			
Enterobacteriaceae	6.43 ± 1.04	6.43 ± 1.11	6.69 ± 1.23
Enterococcaceae	6.33 ± 0.46	6.05 ± 0.15	6.46 ± 0.65
Bifidobacterium	7.90 ± 1.14	8.07 ± 1.32	8.46 ± 0.65
Lactobacillus group	4.91 ± 0.90	5.06 ± 1.08	4.99 ± 0.72
Bacteroides group	8.64 ± 0.76	8.43 ± 1.63	8.96 ± 0.90
Faecalibacterium	6.63 ± 1.51	7.01 ± 1.63	6.56 ± 1.43
Clostridia XIVa	7.28 ± 1.12	7.36 ± 1.18	7.61 ± 1.10
Total bacteria	9.95 ± 0.45	9.88 ± 0.84	9.98 ± 0.54
Short chain fatty acids (µmol/ g faeces)			
Acetic acid	51.10 [a] ± 12.48	71.63[b] ± 15.48	50.11[a] ± 8.01
Propionic acid	18.41[a] ± 4.59	32.75[b] ± 9.86	25.94[b] ± 4.22
Butyric acid	13.83 ± 4.40	16.14 ± 4.36	12.41 ± 2.66

[a] Means ± standard deviations were calculated (n = 10).
[b] Values followed with different code letters are significantly different ($p < 0.05$) according to LSD test. The letter "a" was assigned to the lowest value.

3.5. Phenolic metabolic profiles in faeces and urine after GP supplementation

Main phenolic metabolites determined by UPLC-ESI-MS/MS in urine and faeces were benzoic acids, mandelic acids, phenylacetic acids, phenylpropionic acids, valeric acids, valerolactones, cinnamic acids and hippuric acids (Tables 7 and 8). As expected, large inter-individual variations were observed for the concentrations of these compounds. In the case of faecal samples, the concentration of some metabolites such as 4-hydroxyphenylacetic acid, 3-(3′-hydroxyphenyl)-propionic acid, 4-hydroxy-5-phenyl-valeric acid, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-dihydroxyphenyl)-γ-valerolactone seemed to increase after 14 and 21 days with GP supplementation, but significant differences ($p < 0.05$) were only found for 3-(4′-hydroxyphenyl)-propionic acid (Table 7). This phenolic acid increased mainly in the volunteers #5 and #7, reaching concentrations of 43,935 and 8275 nmol g^{-1} faeces, respectively (Fig. 1e). Supplementary Fig. S2 displays phenolic metabolite profiles evolution from volunteers #5, #6 and #7, who among others, showed progressive increase of faecal phenolic-derived compounds after GP intervention. In these women, the amounts of 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3-hydroxyphenylacetic acid, 3-(3′-hydroxyphenyl)-propionic acid, phenylpropionic acid and 4-hydroxy-5-phenyl-valeric acid were higher than those obtained at the baseline.

As it expected, a similar trend was observed in the case of urine samples. No significant differences in the concentrations (mean values) of phenolic metabolite were found after GP supplementation (Table 8).

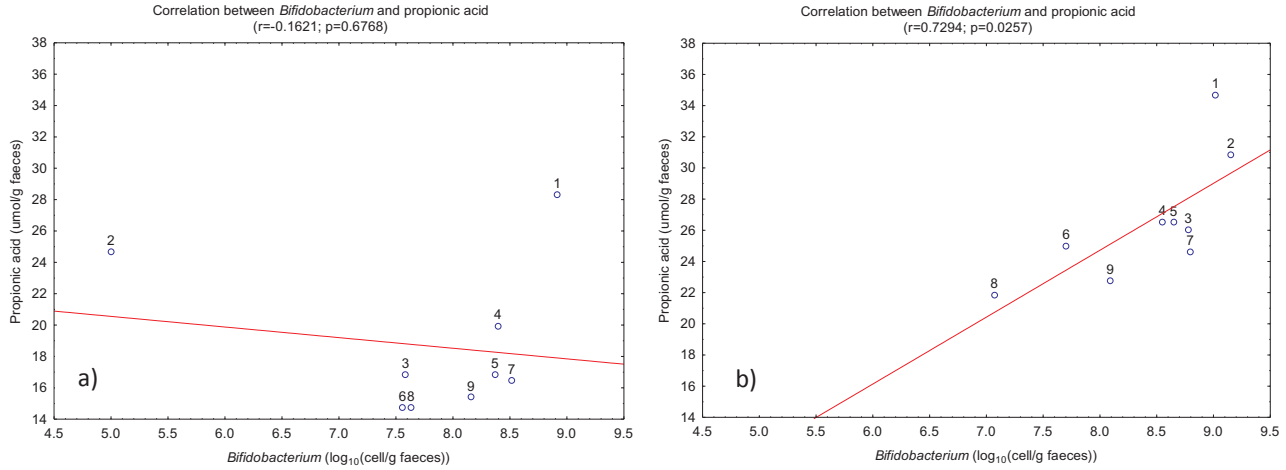

Fig. 2. Pearson's correlations among *Bifidobacterium* genus and propionic acid (n = 9) before (a) and after (b) the GP supplementation for 21 days.

Table 6
Faecal concentrations of medium chain fatty acids (MCFA) (μmol g^{-1} faeces) in 10 subjects before and after Eminol® intake.

		Concentration (μmol/ g faeces)		
		Before GP extract intake	After 14 days GP extract intake	After 21 days GP extract intake
Medium chain fatty acids	Pentanoic acid	8.09[b] ± 3.61	8.22[b] ± 2.53	4.82[a] ± 1.49
	Hexanoic acid	4.95[b] ± 3.01	4.19[a,b] ± 1.81	2.36[a] ± 0.78
	Octanoic acid	0.86[b] ± 0.51	0.58[a,b] ± 0.20	0.44[a] ± 0.13
	Decanoic acid	0.33 ± 0.15	0.32 ± 0.11	0.28 ± 0.07

[a] Means ± standard deviations were calculated (n = 10).
[b] Values followed with different code letters are significantly different ($p < 0.05$) according to LSD test. The letter "a" was assigned to the lowest value.

3.6. miRNA modulation by grape pomace extract supplementation

Modulation of miRNAs expression profiles after GP supplementation in the serum samples of the two volunteers who showed the highest decrease on glucose levels (volunteers #8 and #10) was analysed. MicroRNAs screening exhibited some changes in the expression of several miRNAs of the 734 different miRNA targets (data not shown). Based on these preliminary results, five miRNA related to glucose metabolism (miRNA-130a-3p, miRNA-122-5p, miRNA-34a-5p, miRNA-191-5p and miRNA-342-3p) were selected for further quantitative analysis using LNA™ probes in samples of all the participants in the study (Fig. 2). Table 3 shows the main basic information about the miRNA studied in the sample set.

Overall, a great variability on expression of the five miRNAs was observed (Fig. 2), thus revealing large inter individual differences. The miRNA-130a-3p was significantly down-regulated after GP supplementation in five out of ten volunteers (#6, #7, #8, #9 and #10), three of which displayed the highest serum glucose decrease after intervention, whereas it was significantly up-regulated in the case of volunteer #4 who displayed the lowest hypoglycaemic effect. The miRNA-122-5p expression level increased in three volunteers (#5, #6 and #8) while it was significantly diminished in other four volunteers (#2, #3, #7 and #10). In the case of miRNA-34a-5p its expression was significantly modified for volunteer #9, in which it suffered a significant decrease, and for volunteers #8 and #10, whereby an increase was observed. A significant modification in the miRNA-191-5p expression pattern was clearly observed for all the volunteers. Specifically, the general trend observed was a significant decrease in the expression of this miRNA (7 out of 10 volunteers). However, the expression significantly increased in volunteers #8 and #10, together with volunteer #5. Finally, in the case of miRNA-342-3p, the volunteers #1 and #3 showed significant antagonist effect on its expression compared to volunteers #8 and #10.

In spite of the great inter individual differences observed, the volunteers #8 and #10 (who shared a similar decrease in glucose blood levels) showed the same trend in miRNA expression pattern for four of the five analysed miRNA (miRNA-130a-3p, miRNA-34a-5p, miRNA-191-5p and miRNA-342-3p) (Fig. 3).

A principal component analysis (PCA) was performed to group the subjects in relation to their miRNA expression pattern and glucose values. PCA analysis (Supplementary Fig. S3) grouped together volunteers #8 and #10 which were directly related to miRNA191-5p, this miRNA also correlating negatively with glucose variance. The volunteer #2 patterns seem to be totally different to the rest of volunteers, keeping a direct link with miRNA-342-3p and miRNA-130-3p. Finally, the rest of volunteers grouped very closely.

4. Discussion

GP has shown attractive health-promoting activities *in vitro*, but little efforts have been made until now to translate this work into humans (Urquiaga et al., 2015; Yubero et al., 2013). In the present work we conducted in human volunteers, for the first time, a pilot intervention study with the aim to assess not only the effects of GP supplementation in general host biological and physiological parameters, but its impact on the composition and metabolic activity of the gut microbiota, a physiological target that has been suggested from several animal trials (Fiesel et al., 2014; Kafantaris et al., 2016; Viveros et al., 2011) and from *in vitro* studies (Gil-Sánchez et al., 2017; Gil-Sánchez, in press). In the present work, a significant decrease ($p < 0.05$) in blood fasting glucose levels was observed after GP supplementation. This hypoglycaemic effect has been previously reported in animal studies (Cho et al., 2014; Hogan et al., 2010). Even, a human intervention study showed a reduction in the blood glucose levels after the inclusion of grape pomace flour in the human diet (Urquiaga et al., 2015). Overall, the capacity to lower glucose levels has been mainly attributed to the dietary fibre present in GP. However, the mechanism associated with this benefit remains poorly understood. Previous studies have suggested that it could be related to the action of the intestinal SCFAs on blood glucose regulatory system (Puddu, Sanguineti, Montecucco, & Viviani, 2014), and other researchers also attribute this effect to the presence of undigested dietary fibre itself (independently of the gut microbiota action), which may be capable of creating viscous solutions that reduce the glucose absorption at the intestinal level (Bindels et al., 2017; Repin, Cui, & Goff, 2016). In turn, there are a few studies that attributed the hipoglycaemic effect to grape polyphenols, which although do not

Table 7
Faecal concentrations of phenolic metabolites (nmol g^{-1} faeces) in 10 subjects before and after Eminol® consumption.

	Concentration (nmol/g faeces) Before GP extract intake	After 14 days GP extract intake	After 21 days GP extract intake
Benzoic acids			
3-O-methylgallic acid	0.98 ± 0.01 (n = 2)	nd	0.79 (n = 1)
Gallic acid	1.19 ± 0.02 (n = 2)	1.30 ± 0.47 (n = 4)	1.05 (n = 1)
Protocatechuic acid	2.79 ± 1.75 (n = 10)	2.04 ± 0.1.27 (n = 10)	1.70 ± 0.69 (n = 9)
3-Hydroxybenzoic acid	7.47 ± 4.44 (n = 2)	4.86 ± 1.36 (n = 3)	3.77 ± 0.90 (n = 5)
4-Hydroxybenzoic acid	4.99 ± 6.99 (n = 5)	1.64 ± 1.11 (n = 6)	1.30 ± 1.09 (n = 7)
3,5-Dihydroxybenzoic acid	0.57 ± 0.16 (n = 7)	0.62 ± 0.49 (n = 6)	0.52 ± 0.38 (n = 4)
Benzoic acid	11.01 ± 4.98 (n = 2)	11.66 ± 5.04 (n = 3)	10.12 ± 5.37 (n = 3)
Salicylic acid	2.92 (n = 1)	1.43 (n = 1)	1.01 ± 0.15 (n = 2)
Syringic acid	1.86 ± 0.42 (n = 2)	nd	1.09 (n = 1)
Mandelic acids			
3,4-Dihydroxymandelic acid	7.30 ± 0.07 (n = 2)	7.48 (n = 1)	nd d
3-Hydroxymandelic acid	8.25 ± 0.10 (n = 6)	8.22 ± 0.02 (n = 3)	8.23 ± 0.01(n = 2)
4-Hydroxy-3-methoxymandelic acid	1.42 (n = 1)	nd	1.22 ± 0.00 (n = 2)
Phenols			
3,4-Dihydroxyphenylacetic acid	2.78 ± 1.81 (n = 2)	1.42 ± 0.14 (n = 2)	nd
4-Hydroxyphenylacetic acid	9.55 ± 11.49 (n = 9)	14.11 ± 16.71 (n = 10)	7.00 ± 5.04 (n = 10)
3-Hydroxyphenylacetic acid	55.13 ± 81.06 (n = 6)	57.87 ± 56.66 (n = 3)	31.04 ± 28.13 (n = 6)
Phenylacetic acid	520.29 ± 227.90 (n = 10)	390.06 ± 103.52 (n = 10)	380.05 ± 192.72 (n = 10)
Phenylpropionic acids			
3-(3′,4′-Dihydroxyphenyl)-propionic acid	10.62 ± 1.85 (n = 6)	7.92 ± 1.07 (n = 3)	9.90 ± 2.97 (n = 6)
3-(4′-Hydroxyphenyl)-propionic acid	372.98[a] ± 263.23 (n = 2)	559.12[a] (n = 1)	*5563.69[b] ± 13709.74 (n = 10)
3-(3′-Hydroxyphenyl)-propionic acid	19.97 ± 14.81 (n = 9)	144.84 ± 366.23 (n = 10)	128.94 ± 316.10 (n = 10)
Phenylpropionic acid	232.91 ± 146.61 (n = 9)	405.34 ± 485.64 (n = 10)	215.43 ± 159.90 (n = 10)
Valeric acids			
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid	6.82 ± 2.65 (n = 2)	5.58 ± 0.67 (n = 3)	6.34 ± 2.47 (n = 5)
4-Hydroxy-5-(4′-hydroxyphenyl)-valeric acid	0.24 (n = 1)	0.47 ± 0.19 (n = 3)	0.85 ± 0.30 (n = 2)
4-Hydroxy-5-phenyl-valeric acid	71.46 ± 89.03 (n = 9)	82.95 ± 94.50 (n = 9)	97.70 ± 126.24 (n = 8)
Valerolactones			
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	0.53 ± 0.01 (n = 2)	1.44 (n = 1)	1.48 ± 1.91 (n = 3)
5-(4′-Hydroxyphenyl)-γ-valerolactone	nd	15.78 ± 8.72 (n = 2)	nd
5-(3″-Hydroxyphenyl)-γ-valerolactone	20.80 ± 4.92 (n = 2)	36.63 ± 22.60 (n = 2)	39.36 ± 35.42 (n = 3)
Cinnamic acids			
Caffeic acid	8.90 ± 0.60 (n = 10)	8.61 ± 0.23 (n = 9)	8.74 ± 0.54 (n = 9)
p-Coumaric acid	6.25 ± 0.25 (n = 9)	6.11 ± 0.12 (n = 10)	6.16 ± 0.28 (n = 8)
m-Coumaric acid	nd	2.77 (n = 1)	1.50 (n = 1)
Ferulic acid	6.61 ± 2.70 (n = 9)	5.84 ± 0.24 (n = 8)	6.33 ± 1.46 (n = 9)

[c]nd, not detected.
[d]Values followed with different code letters are significantly different ($p < 0.05$) according to LSD test. The letter "a" was assigned to the lowest value.
[a] Means ± standard deviations were calculated excluding cases with values below the limit of quantification.
[b] In parentheses, number of cases considered for each of the compounds.
* Outline value.

have a direct effect on glycaemia they could affect other related parameters (Hogan et al., 2010; Pérez-López & Merkoçi, 2011). Accumulating evidence suggests that circulating miRNAs are useful biomarkers of type 2 diabetes, and glucose metabolism (Hernandez-Alonso et al., 2016; Yan et al., 2016). However, the mechanisms regulating the profile of circulating miRNA are still under debate, and little is known about the potential modulatory role exerted by diet. To go deeper into the effect of GP extract on glucose metabolism, the expression of five literature based targeted miRNA related to glucose metabolism (including miRNA-130a-3p, miRNA-122-5p, miRNA-34a-5p, miRNA-191-5p and miRNA-342-3p) was analysed on serum samples by RT-PCR. The results obtained after GP supplementation showed upregulation of miRNA191-5p and miRNA-342-3p levels correlating negatively with glucose variance. In this way, miR-191 and miR-342were significantly downregulated in adult peripheral T-reg cells of diabetic patients compared with healthy individuals (Hezova et al., 2010).

Significant changes ($p < 0.05$) on the profile of circulating miRNA after GP supplementation suggests that GP may modulate miRNAS expression, regulation of gene expression at posttranscriptional level, and thus be involved in glucose metabolism. As well as, the statistical analysis confirmed high interindividual differences ($p < 0.001$) (data not shown). A decrease in miRNA-342-3p expression levels has been previously found in diabetes type 1 and type 2 patients (Collares et al., 2013; Fernandez-Valverde, Taft, & Mattick, 2011; Hezova et al., 2010). In agreement, an increase in miRNA-342-3p levels was found for volunteers #8 and #10. In a similar manner, miRNA-191-5p expression was found to be diminished in type 1 diabetes patients, as previously reported by Zampetaki et al. (2010). The volunteers #8 and #10 showed a negative correlation between serum miRNA-191-5p and glucose levels (Pearson test, $p = 0.003$). An antagonist effect for miRNA-34a-5p has been reported in the literature regarding glucose metabolism, and miRNA-34a-5p was suggested to differentiate between nondiabetic and early T2D patients (Kong et al., 2011; Rokavec, Li, Jiang, & Hermeking, 2014). In our study a possible protective effect was observed in the case of volunteer #9, for which a significant reduction of miRNA-34a-5p was described, in accordance with the blood glucose level decrease in this volunteer after consumption of GP. In contrast, the volunteers #8 and #10, whose glucose blood levels decrease more, showed upregulation of miRNA-34a-5p. In the same way, these antagonic effects and inter individual differences were also detected in miRNA-122-5p expression (Fong et al., 2015). Inter individual differences on miRNA profiles have been widely reported in the bibliography

Table 8
Urinary concentrations of phenolic metabolites (μmol, 24 h) in 10 subjects before and after Eminol® consumption.

	Concentration (μmol, 24 h)		
	Before GP extract intake	After 14 days GP extract intake	After 21 days GP extract intake
Benzoic acids			
3-O-methylgallic acid	0.59 ± 0.81 (n = 6)	0.76 ± 0.80 (n = 10)	0.37 ± 0.26 (n = 10)
4-O-methylgallic acid	1.19 ± 2.52 (n = 5)	2.23 ± 3.43 (n = 10)	0.86 ± 0.53 (n = 10)
Gallic acid	5.34 ± 7.03 (n = 3)	6.45 ± 8.25 (n = 3)	1.96 ± 0.71 (n = 2)
Protocatechuic acid	19.78 ± 10.24 (n = 10)	20.55 ± 9.40 (n = 10)	21.29 ± 11.60 (n = 10)
3-Hydroxybenzoic acid	6.14 ± 6.02 (n = 7)	3.60 ± 1.75 (n = 7)	3.25 ± 4.06 (n = 7)
4-Hydroxybenzoic acid	31.51 ± 21.39 (n = 10)	27.44 ± 11.12 (n = 10)	23.54 ± 9.35 (n = 10)
Benzoic acid	28.59 (n = 1)	65.12 (n = 1)	53.90 ± 29.36 (n = 3)
Salicylic acid	2.22 ± 4.39 (n = 10)	1.85 ± 1.55 (n = 10)	1.46 ± 0.83 (n = 10)
Vanillic acid	23.86 ± 8.91 (n = 10)	23.19 ± 7.93 (n = 8)	22.16 ± 8.85 (n = 10)
Syringic acid	2.08 ± 1.39 (n = 2)	2.22 ± 0.61 (n = 4)	1.70 ± 0.30 (n = 5)
Phthalic acid	0.0034 ± 0.0022 (n = 10)	0.0034 ± 0.0023 (n = 10)	0.0026 ± 0.0012 (n = 10)
Mandelic acids			
3-Hydroxymandelic acid	1.18 ± 0.61 (n = 3)	1.09 (n = 1)	0.89 (n = 3)
4-Hydroxymandelic acid	32.45 ± 19.61 (n = 7)	38.84 ± 25.46 (n = 10)	30.35 ± 17.10 (n = 10)
4-Hydroxy-3-methoxymandelic acid	39.33 ± 10.41 (n = 10)	39.01 ± 10.27 (n = 10)	37.42 ± 11.59 (n = 10)
Mandelic acid	4.01 ± 2.38 (n = 7)	3.89 ± 0.83 (n = 6)	3.00 ± 0.58 (n = 4)
Phenols			
Catechol/Pyrocatechol	23.36 ± 27.81 (n = 9)	17.31 ± 10.70 (n = 10)	21.16 ± 18.62 (n = 10)
4-Methylcatechol	13.37 ± 4.03 (n = 5)	12.80 ± 4.52 (n = 6)	13.34 ± 6.61 (n = 4)
4-Ethylcatechol	1.90 ± 1.08 (n = 8)	1.74 ± 1.78 (n = 9)	2.03 ± 1.30 (n = 7)
3-Hydroxyphenylacetic acid	81.37 ± 51.07 (n = 10)	116.25 ± 112.60 (n = 10)	59.06 ± 44.57 (n = 10)
4-Hydroxyphenylacetic acid	69.43 ± 23.02 (n = 10)	86.05 ± 31.11 (n = 10)	70.08 ± 26.56 (n = 10)
3,4-Dihydroxyphenylacetic acid	30.44 ± 21.41 (n = 10)	18.45 ± 5.27 (n = 10)	26.39 ± 22.99 (n = 10)
4-Hydroxy-3-methoxyphenylacetic acid	46.38 ± 23.85 (n = 10)	38.68 ± 9.63 (n = 10)	42.62 ± 18.14 (n = 10)
Pyrogallol	2.01 (n = 1)	6.22 ± 6.55 (n = 2)	3.74 ± 0.90 (n = 2)
Phenylpropionic acids			
3-(3′-Hydroxyphenyl)-propionic acid	69.63 ± 133.28 (n = 10)	36.90 ± 26.46 (n = 10)	46.57 ± 75.33 (n = 10)
3-(3′,4′-Dihydroxyphenyl)-propionic acid	7.67 ± 8.31 (n = 6)	7.42 ± 3.53 (n = 6)	8.98 ± 5.73 (n = 5)
Valeric acids			
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid	1.52 ± 1.83 (n = 6)	1.41 ± 0.92 (n = 3)	0.38 ± 0.25 (n = 4)
4-Hydroxy-5-(4′-hydroxyphenyl)-valeric acid	0.22 (n = 1)	1.21 (n = 1)	0.12 (n = 1)
4-Hydroxy-5-phenyl-valeric acid	275.86 ± 91.44 (n = 10)	285.09 ± 40.48 (n = 10)	295.14 ± 59.71 (n = 10)
Valerolactones			
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	34.57 ± 39.25 (n = 6)	33.59 ± 18.27 (n = 3)	9.73 ± 9.69 (n = 4)
5-(3′-Hydroxyphenyl)-γ-Valerolactone	57.71 ± 21.64 (n = 3)	53.43 ± 16.34 (n = 2)	46.90 ± 17.28 (n = 3)
Cinnamic acids			
Caffeic acid	0.0017 ± 0.0013 (n = 10)	0.0018 ± 0.0010 (n = 10)	0.0016 ± 0.0001 (n = 10)
p-Coumaric acid	5.20 ± 3.61 (n = 8)	4.55 ± 2.98 (n = 8)	4.02 ± 2.94 (n = 8)
m-Coumaric acid	0.82 ± 1.54 (n = 8)	0.29 ± 0.21 (n = 8)	0.49 ± 0.72 (n = 7)
Ferulic acid	23.53 ± 9.98 (n = 10)	28.06 ± 15.54 (n = 10)	23.86 ± 14.86 (n = 10)
Isoferulic acid	15.78 ± 8.80 (n = 10)	16.36 ± 9.53 (n = 10)	12.60 ± 10.99 (n = 10)
Hippuric acids			
4-Hydroxyhippuric acid	155.87 ± 109.11 (n = 10)	159.73 ± 67.46 (n = 10)	132.96 ± 51.60 (n = 10)
Hippuric acid	2644.65 ± 1867.11 (n = 10)	2419.34 ± 963.12 (n = 10)	2459.34 ± 1412.49 (n = 10)

[a]Means and standard deviations were calculated excluding cases with values below the limit of quantification.
[b]In parentheses, number of cases considered for each of the compounds.

(Flowers, Gadgil, Aouizerat, & Kanaya, 2015; Geeleher, Huang, Gamazon, Golden, & Seoighe, 2012; Ludwig et al., 2016; Lukiw, 2013) since miRNAs are specific fine-tune regulators of body homeostasis. However, the above described miRNAs differences associated to GP supplementation could indicate that GP effects might be mediated by miRNAs modulation, although additional functional experiments should be done to prove it.

In contrast to that previously found in animal or human studies with GP supplementation (Tebib, Bitri, Besançon, & Rouanet, 1994; Yubero et al., 2013), the extract tested in the present study did not promote any significantly decrease of total cholesterol and LDL cholesterol levels. These results are in line with other studies reporting no changes in these parameters after the intake of this by-product (Urquiaga et al., 2015). Discrepancies of results between studies may be related with variations on the intervention design, the duration of the intervention, the composition of GP and doses administered to volunteers, among other factors.

Some evidences arisen in recent years suggest that GP can exert a modulatory effect on the gut microbiota composition; however all the information available is based on *in vitro* studies or animal assays (Gil-Sánchez et al., 2017; Viveros et al., 2011). In the present human intervention study, no significant differences were detected on the intestinal microbiota composition of healthy women after the supplementation period with GP. This fact would indicate that the GP intake does not alter the equilibrium state in healthy adult's intestinal microbiome.

In addition, the bacterial metagenome contributes to the production of metabolites that modulate human health in many ways (Sharon et al., 2014). In this vein, diet plays a key role in the bacterial metabolome, since it is the major source of precursors for healthy metabolite production, as it would be the case for SCFA and phenolic compounds produced through colonic fermentation of our GP extract.

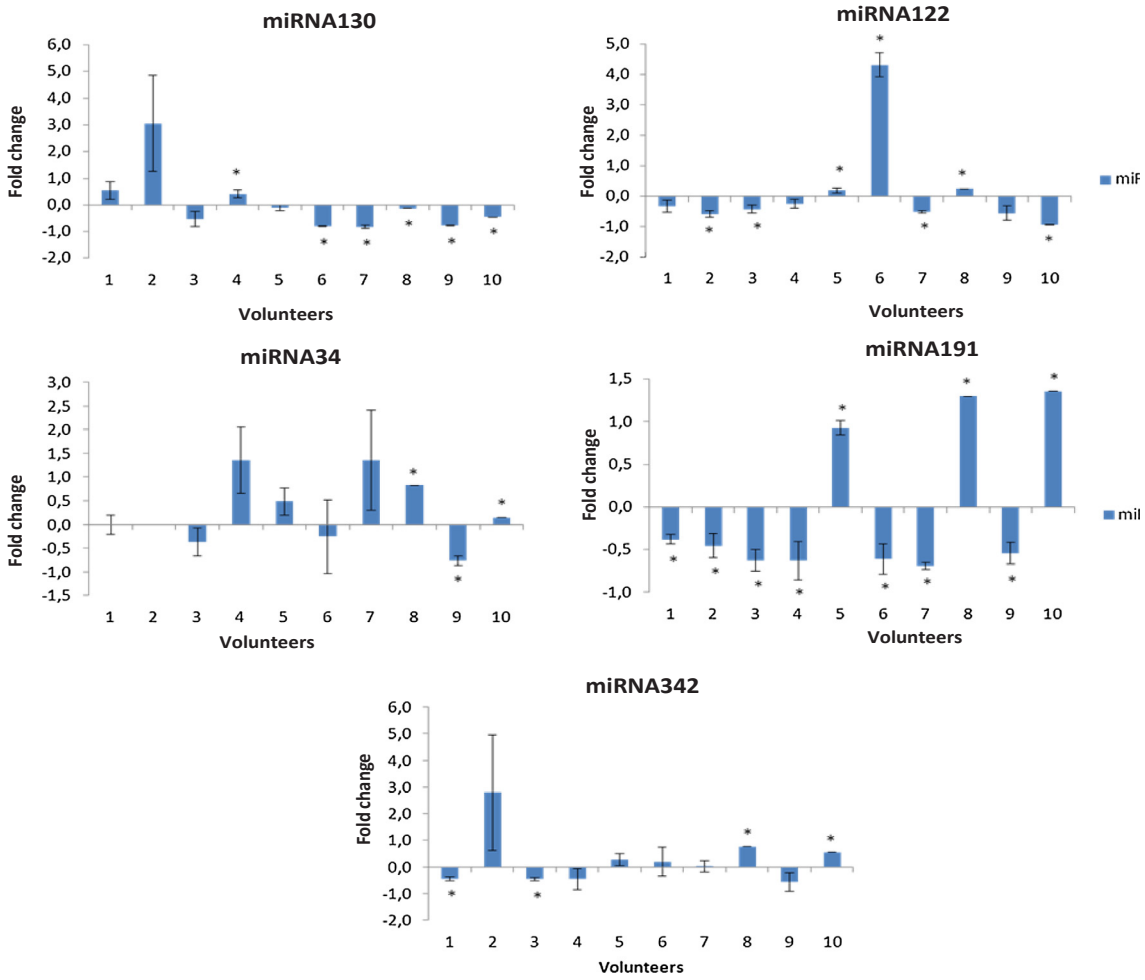

Fig. 3. Changes in the expression levels of five miRNAs related to glucose metabolism in all volunteers after the GP intake. Fold Changes ($2^{-\Delta\Delta Cq}$) expressed the MiRNA differences after and before consuming the grape pomace extract capsules. Samples were analysed by triplicates. * indicates values significantly different from zero ($p < 0.05$) as analysed by *t*-test for single samples.

The amount and relative abundance of SCFAs have been related with the human health status (Ríos-Covián et al., Ruas-Madiedo, Margolles, Gueimonde, & De los Reyes-Gavilán, & Salazar, 2016). In accordance with other studies, the faecal levels of total SCFAs in our human sample ranged from 60 to 90 μmol/g faeces (Fernando et al., 2010; McOrist et al., 2011). This amount varies between individuals (Marchesi et al., 2016) and it has been described as depending, on the type and amount of colonic fermentable substrates available (mainly complex carbohydrates) (Verbeke et al., 2015), among other factors. We have obtained a significant increase of total SCFAs during GP supplementation. This may be attributed to the fibre contained in the GP, which is a rich carbon source metabolizable by intestinal bacteria. In this context, previous studies have described GP as an important source of dietary fibre for functional foods development (Valiente, Arrigoni, Esteban, & Amado, 1995). In addition, the observed increase of SCFAs may have also been influenced by the degradation of phenolic compounds present in the GP extract (Sadeghi Ekbatan et al., 2016).

Fecal acetic and propionic acids concentrations varied significantly during the intervention. SCFAs are involved in the control of appetite and satiety mechanisms (Byrne, Chambers, Morrison, & Frost, 2015). Both, acetate and propionate, participate in the lipid and glucose metabolism in the host (Ríos-Covián et al., 2016). Acetate is used by the liver as a precursor for the synthesis of cholesterol and long-chain fatty acids; on the other hand, propionic acid is gluconeogenic and plays an important role in the competitive regulation of lipogenesis. Moreover, SCFA can regulate the lipid and energetic metabolism in the liver and adipose tissue by switching lipogenesis and fat oxidation through a PPARγ-dependent mechanism (den Besten et al., 2015). Some of these recognized biological effects of SCFAs may be on the basis of the lowering of blood fasting glucose promoted by GP during the intervention study. However, more research is still needed to clarify the physiological mechanisms participating in this effect.

We have found a strong positive correlation between the intestinal bifidobacteria and propionic acid at the end of the intervention period, in spite that bifidobacteria cannot synthesize this SCFA. The lack of this correlation during the wash-out period suggests that the GP is directly involved in this association. Similar results were observed in studies carried out in dynamical gastrointestinal simulators after feeding with GP (Campos Freire, 2016; Gil-Sánchez, in press). The fibre contained in GP and polyphenols associated to them could be exerting a prebiotic effect and promoting some interactions among members of the intestinal microbiota (Pozuelo et al., 2012). In this regard, a recent cross-sectional study with Spanish healthy adults following a regular diet, Klason lignin (fibre) and lignans (polyphenols) were identified as directly associated with intestinal levels of *Bacteroides* and/or *Bifidobacterium*, as well as anthocyanidins as associated with faecal propionate concentrations (Fernandez-Navarro et al., 2016). It has been shown that the synthesis of propionate in faecal batch cultures or in pure cultures of *Bacteroides* is enhanced by the addition of bifidobacteria (Salazar et al., 2009) and by the presence of slowly fermentable carbohydrates (Ríos-Covián, Salazar, Gueimonde, & de los Reyes-Gavilan, 2017; Salazar, Gueimonde, Hernández-Barranco, Ruas-

Madiedo, & de los Reyes-Gavilán, 2008). Thus, probably the high content of dietary fibre together with the proanthocyanidins and other polyphenols present at high concentration in the GP extract could be modulating the intestinal microbiota and its metabolic activity towards the production of SCFAs through favouring particular ways of interactions between specific dietary components and some members of the intestinal microbiota.

Regarding MCFAs, few reports related to their benefits on human have been published. MCFAs are mainly provided by diet. Milk fat, coconut oil and palm oil are among the food providers (Takeuchi, Sekine, Kojima, & Aoyama, 2008). MCFAs influence lipid metabolism by increasing mitochondrial oxidation of fatty acids without increasing oxidative stress (Ishizawa, Masuda, Sakata, & Nakatani, 2015); they have been related with reduced adiposity whereas exerting few deleterious effects on insulin action (Montgomery et al., 2013), then pointing to a potential action in obesity and glucose metabolic regulation of these compounds. On the other hand, some findings indicate that polyphenols can modify the energy uptake by influencing mitochondrial activity in the adipose tissue, skeletal muscle and liver (Lama et al., 2017; Serrano et al., 2013). We have found a marked decrease of MCFAs levels in faeces of the participants in the study after the GP intervention. Although we cannot provide any physiological explanation to this fact, the results obtained suggest that MCFAs from diet may be involved in some way in the biological effect found for GP in the present study.

The analysis of phenolic metabolites in faeces and urine samples is considered a reliable method for detecting biomarkers of polyphenol intake (Mennen et al., 2006; Urpi-Sarda et al., 2015). In our work, a wide variety of phenolic compounds were analysed. Due to their metabolism, a greater content of these compounds was excreted in the urine (Monagas et al., 2010). The concentration of phenolic compounds detected in the biological samples during the wash-out period was similar to those reported in other human-intervention studies (Muñoz-Gonzalez et al., 2013; Urpi-Sarda, Garrido, et al., 2009; Urpi-Sarda, Monagas, et al., 2009).

To date, there are several human studies that have demonstrated significant changes in the profile of phenolic metabolites after the intake of rich polyphenols sources such as red wine (450 mg of total polyphenols/day) (Muñoz-Gonzalez et al., 2013), cocoa (495.2 mg of total polyphenols/day) (Urpi-Sarda, Monagas, et al., 2009), oregano extract (500 mg polyphenols/day) (Nurmi, Nurmi, Mursu, Hiltunen, & Voutilainen, 2006) or almond polyphenol extract (884.4 mg of total polyphenols/day) (Urpi-Sarda, Garrido, et al., 2009). In addition, some studies have revealed significant changes in the urinary excretion of phenolic metabolites after the intake of GP. For instance, Motilva et al. (2016) showed a significant increase in the flavan-3-ols group (i.e. catechin, epicatechin and their respective conjugated forms) after a diet enriched with GP, what is in line with the high content in procyanidins of the GP extract. In the same way, Sasot et al. (2017) reported a wide range of phase-II metabolites (sulphated and glucuronidated) after the intake of a functional food made from grape pomace. In contrast, we did not find significant variations in the content of faecal and urine phenolic metabolites after Eminol® supplementation (52.42 mg polyphenols/day), what could suggest that the excretion of these compounds varies noticeably according to the consumed substrate and the amount of total polyphenols consumed per day.

In our case, the content of total phenolic metabolites remained stable during the study, with a slightly increasing trend in the case of faecal samples, in particular to 4-hydroxyphenylacetic acid, 3-(3′-hydroxyphenyl)-propionic acid, 4-hydroxy-5-phenyl-valeric acid, 5-(3′,4′-dihydroxyohenyl)-γ-valerolactone or 5-(3′-dihydroxyphenyl)-γ-valerolactone. The increment of these phenolic metabolites has been observed in previous *in vitro* incubations of Eminol® with colonic microbiota (Gil-Sánchez et al., 2017). Furthermore, 3-(4′-hydroxyphenyl)-propionic acid, usual microbial metabolite derived from grape products (Aura et al., 2013; Cueva et al., 2013), was the only phenolic compound that clearly increased following GP supplementation. Inter-individual differences detected in the phenolic metabolite profiles could be related to variances in the dynamics of gastrointestinal time, diverse absorption efficiencies, and particularly to individual microbiota composition (Mosele, Macià, & Motilva, 2016). This is in line with some studies that have reported certain stratification of human populations by their polyphenol-metabolizing phenotypes, as a result of their different gut microbiota compositions (Muñoz-Gonzalez et al., 2013; Tomás-Barberán, Selma, & Espín, 2016).

5. Conclusion

In conclusion, the results of the present work suggest that the supplementation with grape pomace extract (Eminol®) (2 capsules/day) over three weeks led to significant decrease the blood fasting glucose levels. Although further studies with more volunteers are needed, the *in vivo* regulation of targeted miRNAs related to glucose metabolism observed following GP extract consumption suggests a possible role of GP on glucose metabolism, which would contribute to decrease the risk of type 2 diabetes. Overall, no statistically significant changes were observed in the intestinal microbiota composition or in the content of phenolic metabolites detected in faeces and urine. Nevertheless, significant increases in the formation of some SCFAs as well as significant decreases in the MCFA were observed during the GP supplementation. In summary, Eminol® can be considered a promising functional ingredient for health promotion and disease prevention. Future studies are needed if these results are to be extended to subjects with diabetes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2018.03.031.

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