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Polyphenol extracts interfere with bacterial lipopolysaccharide *in vitro* and decrease postprandial endotoxemia in human volunteers

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ABSTRACT

The intestinal absorption of bacterial lipopolysaccharide (LPS) and dietary fat has been implicated in the development of metabolic endotoxemia. This study first compared the ability of polyphenol extracts from grape, cranberry, avocado and apple to interfere with pancreatic lipase and LPS *in vitro*. The grape extract displayed a higher inhibitory activity of lipase (IC_{50} = 8.6 ± 1.1 mg/ml) and LPS binding (IC_{50} = 90 ± 1.1 μg/ml). Then, a study was carried out in 12 normal weight and 17 overweight/obese subjects to determine the effect of this extract on the postprandial changes in plasma triacylglycerols, LPS and IL-6. The presence of small intestine bacterial overgrowth (SIBO), in which higher levels of bacteria and eventually LPS are present in the upper intestine, i.e. where dietary fat absorption occurs, was also evaluated. Compared with placebo, the grape extract did not affect postprandial triacylglycerolemia but decreased plasma LPS, without affecting the IL-6-associated inflammatory response. SIBO did not affect these variables.

1. Introduction

Polyphenols are secondary metabolites, mainly including phenolic acids and flavonoids (flavanols, flavonols and anthocyanins), which are implicated in the protection of plants against diverse types of stress and injury. The chemical characteristic of these molecules allow them to act as an antioxidant, antibacterial, anti-inflammatory, anti-hypertensive, anti-proliferative, and regulator of the mitochondrial function, among others (Rodrigo, Libuy, Feliu, & Hasson, 2014). For this reason, dietary polyphenols are currently considered as non-nutrients with health-promoting properties and the intake of fruits and vegetables with high contents of these molecules

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is recommended to decrease the risk of non-communicable chronic diseases in humans (Woodside, Young, & McKinley, 2013).

Proanthocyanidins (PACs), or condensed tannins, are dimeric, oligomeric o polymeric forms of flavan-3-ols generally found in the insoluble fraction present in plant-derived foodstuffs such as grape seed and skin, red wine, cranberries, apples, avocados, green tea, cocoa, peanut husk, and cinnamon, among others (Gu et al., 2004; Lainas, Alasalvar, & Bolling, 2016). Type-B is the most abundant PAC whereas type-A PACs, whose constituent monomers are linked through an additional ether bond between C2 and C7, are only abundant in some vegetable species such as peanut skin, avocados and cranberries. PACs are widely consumed by the population and their daily intake in the U.S. population was estimated to be 224 mg (de Camargo, Regitano-d'Arce, Gallo, & Shahidi, 2015; Gu et al., 2004). PACs with a degree of polymerization >3 are poorly absorbed in the intestine and reach the colon where they are used as preferential substrates by specific bacterial populations of the resident microbiota (Etxeberria et al., 2013). This phenomenon probably contributes to the modulation of the composition of the colonic microbiota. In addition, the aromatic acids issued from PAC bacterial metabolism may be absorbed and exert biological activities on the colonic epithelium (Wong et al., 2016) and/or at the systemic level (Monagas et al., 2010). In the lumen of the small intestine, high molecular-weight PACs may also interfere with macronutrients, bile salts, mucosal α-glucosidase and pancreatic enzymes such as α-amylase, proteases, and lipase, resulting in decreased nutrient digestibility (Arimboor & Arumughan, 2011; Barros, Awika, & Rooney, 2012; de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2016; de la Garza, Milagro, Boque, Campión, & Martínez, 2011). For this reason, PACs have frequently been considered as antinutrients. However, the lipase inhibitory activity of PACs could also explain the improvement of lipid profile described in animal and humans fed with PAC-supplemented diets (de la Garza et al., 2011); an example of this is the administration of dealcoholized red wine that significantly decreases the plasma concentration of ApoB48, a marker of chylomicron secretion, after a mixed meal in dyslipidemic post-menopausal women (Pal, Naissides, & Mamo, 2004).

On the other hand, PACs also exert an antimicrobial activity by interfering with the adhesion of bacteria to their receptors on epithelial cells; this mechanism might support the protective effect of PAC-rich foodstuffs in some pathologic conditions involving pathogens (Ermel, Georgeault, Inisan, & Besnard, 2012; Gotteland et al., 2008; Koo et al., 2010). Another explanation for such antibacterial activity might be the ability of PACs to bind lipopolysaccharide (LPS), also known as endotoxin, the highly pro-inflammatory component present in the outer membrane of the gram-negative bacteria (Rhee, 2014). This property would be particularly interesting for human health, taking into account that LPS is currently considered as a crucial factor in the appearance of the low-grade inflammation occurring in patients with metabolic syndrome (Cani, Osto, Geurts, & Everard, 2012). The administration of high fat diet to mice has been shown to increase LPS-bearing gram negative bacteria in their gut microbiota and to impair gut barrier function, resulting in higher levels of plasma LPS (the so-called metabolic endotoxemia) and circulating markers of oxidative stress and inflammation (Cani et al., 2008, 2012). Such events may lead to the development of metabolic complications such as type-2 diabetes and hepatic steatosis (Cani et al., 2008, 2012). Another hypothesis suggests that the LPS present in the gut lumen might be absorbed by epithelial cells through a lipid-associated pathway, being subsequently bound to the chylomicrons which are released in the lymphatic circulation (Ghoshal, Witta, Zhong, de Villiers, & Eckhardt, 2009). These findings are supported by some studies describing an elevation of plasma LPS and IL-6 in human volunteers after the ingestion of a high fat meal (Erridge, Attina, Spickett, & Webb, 2007; Laugerette et al., 2011).

Based on these considerations, the aims of this study were (1) to determine the capacity of PAC-containing polyphenol extracts prepared from different fruits (apples, cranberries, grapes, and avocados) to inhibit pancreatic lipase activity and interfere with LPS *in vitro*, and (2) to determine the effect of the acute intake of the most powerful extract on plasma concentrations of triacylglycerols (TG) and LPS after the ingestion of a high fat breakfast in human volunteers. We hypothesized that the intake of the extract may decrease the postprandial elevation of plasma TG and LPS. As a human model of a situation in which a higher concentration of bacteria, and eventually LPS-bearing gram negative bacteria, is present in the proximal part of the intestine (i.e. where the absorption of dietary fat mainly occurs), the latter study was carried out in subjects with small intestine bacterial overgrowth (SIBO) and the results were compared with those obtained in subjects without bacterial overgrowth (Bouhnik et al., 1999). In this case, we hypothesized that the postprandial concentrations of plasma LPS are more elevated in the subjects with SIBO and that this phenomenon is attenuated by the concomitant intake of the PAC-containing polyphenol extract.

2. Material and methods

2.1. Chemicals

The limulus assay kit was purchased from Lonza (Basel, Switzerland), the enzyme-linked immunosorbent assay (ELISA) kit for interleukin-6 (IL-6) from ABCAM (Cambridge, UK) and the TG kit from Wiener Lab. (Riobamba, Argentina). All the other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Polyphenol extracts

The polyphenol extracts from apple peel, concentrated cranberry juice, grape skin and seeds and avocado peels were prepared by the Laboratory of Pharmacognosy of the University of Concepción (Concepción, Chile). We selected these fruits because apple and grape are known to contain only type B PAC while cranberry and avocado contained a combination of type A and type B PACs. The preparation and the polyphenolic composition of the extracts have been previously described in detail (Wong et al., 2016) and the corresponding data are summarized in Table 1. PAC contents of the grape, cranberry, avocado and apple extracts were 41.0%, 32.0%, 29.1% and 22.0%, respectively. In addition, PACs from apple and avocado were formed only by epicatechin monomers while those from cranberry and

Table 1 – Phenolic composition of the fruit extracts (Means ± SD).

Compounds (%)	Grape	Apple	Cranberry	Avocado
Flavan-3-ol monomers				
Epicatechin	3.66 ± 0.20	6.08 ± 0.98	1.02 ± 0.01	12.09 ± 0.38
Catechin	2.04 ± 0.13	0.03 ± 0.01	1.44 ± 0.03	1.92 ± 0.01
Epicatechin gallate	1.74 ± 0.09	ND	ND	ND
Catechin gallate	0.89 ± 0.02	ND	ND	ND
∑ flavan-3-ol monomers	8.33 ± 0.11	6.11 ± 0.50	2.46 ± 0.02	14.01 ± 0.20
∑ total procyanidins	41.01 ± 0.36	22.01 ± 2.88	32.01 ± 0.36	29.08 ± 1.01
Flavonoids				
Hyperoside	ND	22.12 ± 1.23	2.96 ± 0.06	ND
Isoquercitrin	ND	6.58 ± 0.98	3.88 ± 0.13	ND
Quercitrin	ND	8.23 ± 1.02	9.07 ± 0.11	ND
Quercetin	1.77 ± 0.07	0.09 ± 0.01	4.02 ± 0.18	1.23 ± 0.02
Quercetin-O-pentosides (≠of rutin)	4.79 ± 0.05	19.21 ± 2.66	1.03 ± 0.09	ND
Rutin	0.96 ± 0.01	4.01 ± 0.12	ND	1.09 ± 0.04
Apigenin	ND	ND	ND	12.80 ± 0.12
Kaempferol	1.81 ± 0.09	ND	ND	ND
Kaempferol derivatives	2.03 ± 0.05	ND	1.43 ± 0.01	ND
Myricetin	ND	ND	1.78 ± 0.03	ND
Myricetin hexosides	ND	ND	5.19 ± 0.09	ND
Isorhamnetin	ND	ND	0.68 ± 0.36	ND
Isorhamnetin derivatives	3.75 ± 0.05	ND	ND	ND
∑ Flavonoids	15.11 ± 0.05	60.24 ± 1.00	30.04 ± 0.12	15.12 ± 0.06
Anthocyanins				
Cyanidin -O-hexosides	1.23 ± 0.02	ND	5.23 ± 0.03	ND
Cyanidin-O-arabinoside	ND	ND	5.01 ± 0.03	ND
Cyanidin-3-O-glucoside	ND	ND	ND	3.00 ± 0.03
Peonidin-O-hexosides	1.01 ± 0.01	ND	8.46 ± 0.01	ND
Peonidin-O-arabinoside	ND	ND	4.97 ± 0.06	ND
Malvidin -O-hexosides	12.05 ± 0.01	ND	ND	ND
Delphinidin-O-hexosides	4.53 ± 0.08	ND	ND	ND
Petunidin-O-hexosides	3.01 ± 0.13	ND	ND	ND
∑ total anthocyanins	20.83 ± 0.03	ND	23.67 ± 0.03	3.00 ± 0.03
Phenolic acids				
Gallic acid	4.11 ± 0.01	ND	ND	ND
Syringic acid	0.63 ± 0.03	ND	ND	ND
Vanillic acid	3.94 ± 0.13	ND	ND	3.16 ± 0.01
Chlorogenic acid	ND	1.02 ± 0.05	ND	1.00 ± 0.02
Caffeic acid	1.96 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	8.01 ± 0.01
Ellagic acid	ND	ND	1.94 ± 0.11	ND
Sinapic acid	ND	ND	0.28 ± 0.09	0.25 ± 0.02
Ferulic acid	0.72 ± 0.06	ND	0.09 ± 0.01	6.54 ± 0.02
Anisic acid	ND	ND	ND	2.28 ± 0.02
p-coumaric acid	1.16 ± 0.03	ND	1.52 ± 0.18	ND
Cinnamic acid	ND	ND	0.38 ± 0.02	ND
5-caffeoyl quinic acid	ND	ND	3.83 ± 0.12	ND
4-caffeoyl quinic acid	ND	ND	0.23 ± 0.01	ND
Protocatechuic acid	2.20 ± 0.02	ND	0.78 ± 0.03	0.98 ± 0.09
p-hydroxybenzoic acid	ND	ND	0.98 ± 0.02	9.92 ± 0.08
m- hydroxybenzoic acid	ND	ND	ND	6.65 ± 0.10
Caffeoyl glucoside	ND	ND	0.89 ± 0.01	ND
Feruloyl glucoside	ND	ND	0.19 ± 0.01	ND
Coumaroyl glucoside	ND	ND	0.46 ± 0.02	ND
∑ phenolic acids	14.72 ± 0.04	1.08 ± 0.03	11.64 ± 0.05	38.79 ± 0.04
Dihydrochalcones				
Phloretin-2′-glucoside	ND	10.34 ± 3.88	0.18 ± 0.01	ND
Phloretin-2′-xyloglucoside	ND	0.22 ± 0.11	ND	ND
∑ dihydrochalcones	ND	10.56 ± 2.00	0.18 ± 0.01	ND
Total	**100 ± 0.12**	**100 ± 1.28**	**100 ± 0.10**	**100 ± 0.27**

ND: not detected.

grape also contained catechin and, in the case of grapes, epicatechin gallate and catechin gallate. All the extracts were freeze-dried and conserved at –80 °C until use. On the other hand, PACs were also characterized and quantified by HILIC-HPLC with fluorescent detection, according to Pastene, Troncoso, Figueroa, Alarcón, and Speisky (2009), using a 250 × 4.6 mm (5 μm) Kromasil Diol column (Bohus, Sweden). The solvent system was composed by solvent A (acetonitrile/acetic acid; 98:2, v/v) and solvent B (methanol/water/acetic acid; 95:3:2, v/v/v). PACs were separated with the following gradient program: 0–35 min: 0–40% B; 35–55 min; isocratic 40% B; 55–60 min; 40–0% B with 5 min of column re-conditioning at a flow rate of 0.8 ml/min. Elution was monitored by fluorescence detection with excitation at 230 nm and emission detection at 321 nm. Due to its low fluorescence, polymeric proanthocyanidins were analyzed at 280 nm. Monomers were determined as (+)– catechin equivalents. Dimers were quantified as procyanidin B2 equivalents (Sigma-Aldrich, St. Louis, MO, USA), except for cranberry where procyanidin A2 (Extrasynthese, France) was used. Trimers were expressed as procyanidin C2 equivalents (in house isolated from *Peumus boldus*) (Pastene, Parada, Avello, Ruiz, & García, 2014).

2.3. *Inhibition of pancreatic lipase activity*

The inhibitory effect of the extracts on pancreatic lipase activity was determined in white 96-well microplates according to Yuda et al. (2012) Type-2 porcine pancreatic lipase (100–400 U/mg of protein) was dissolved in dimethyl sulphoxide (DMSO). The reaction was based on the hydrolysis of the substrate 4-metilumbeliferil oleate (4-MUO) to the fluorescent 4-methylumbelliferone. Twenty-five microliters of lipase (2 mg/ml) and 25 μl of the PAC-containing polyphenol extracts at concentrations of 0, 0.065, 0.125, 0.25, 0.5 and 1 mg/ml were introduced in every well and the reaction was initiated by adding 50 μl of 4-MUO 0.1 mM in PBS buffer. After 2 h incubation at 23 °C, the reaction was stopped by adding 100 μl of 100 mM sodium citrate, pH 4.2. Fluorescence was measured in a Synergy HT multiplate reader (BioTek Instruments, Winooski, VT, USA) at 460/360 nm. The IC_{50}, defined as the concentration of the extracts needed to inhibit half of the maximal lipase activity, was determined for each extract using the GraphPad Prism software (La Jolla, CA, USA).

2.4. *Inhibition of LPS binding to polymyxin B*

The ability of the extracts to inhibit the binding of fluorescein isothiocyanate conjugated lipopolysaccharide (FITC-LPS) to polymyxin B coated agarose beads was determined fluorometrically, according to Delehanty, Johnson, Hickey, Pons, and Ligler (2007). The LPS used in the assay was from *Escherichia coli* 055:B5 (Sigma, St Louis, MI, USA). One hundred microliters of 1.2 mg/ml polymyxin B coated agarose beads (Sigma), previously washed with Tris buffer to eliminate glycerol and sodium azide, were mixed with 20 μl of LPS-FITC (1 mg/ml in sterile water), 30 μl of the extracts at different concentrations (0.03; 0.12; 0.24; 0.6; 1.2 mg/ml) and 100 μl Tris buffer. The reaction solution was incubated for 1 h at 25 °C in darkness under light stirring and subsequently centrifuged at 500 g for 1 min at room temperature. The supernatant was discarded and the pellet containing the agarose beads was re-suspended in 100 μl Tris. Therefore, 80 μl of the suspension were put in a 96-well microplate and the fluorescence corresponding to the LPS-FITC bound to the polymyxin B coated agarose beads was determined at 495/520 nm with a multiplate reader at room temperature. The IC_{50}, defined as the concentration of the extracts needed to inhibit half of the maximal binding of LPS to polymixyn B, was determined for each extract using GraphPad Prism.

2.5. *Effect of the grape polyphenol extract on post-prandial plasma triacylglycerols and LPS in human volunteers*

The study protocol was approved by the Ethics Committee for Research in Humans of the Faculty of Medicine and Hospital Clínico (HCUCH), University of Chile, in compliance with the Helsinki Declaration. All the subjects were informed about the aims and procedures of the study and those who agreed to participate and met the inclusion and exclusion criteria signed a written informed consent form. 31 volunteers of both genders, aged 20 to 60 years old, with or without SIBO, were recruited in this explorative study. Recruitment was carried out in the Gastroenterology Section of the HCUCH. Subjects with acute or chronic intake of proton-pump inhibitors, antibiotics, anti-inflammatory drugs, laxative, Orlistat or other drugs interfering with intestinal fat absorption as well as those with antecedent of digestive surgery (except appendectomy and cholecystectomy) or chronic digestive pathologies including chronic diarrhea, celiac disease, inflammatory bowel diseases, colorectal cancer, or gastroduodenal ulcer, with current radio- o chemotherapy, hepatic cirrhosis, diabetes mellitus, and pregnant women were excluded. The recruited subjects were classified according their nutritional status as normal weight (NW: body mass index (BMI) ≤ 24.9 kg/m^2) or overweight/obese (OW/Ob) (OW: BMI between 25 and 29.9 kg/m^2; Ob: BMI > 29.9 kg/m^2). A blood sample was obtained from each volunteer to carry out biochemical and lipid profiles. A nutritional survey of the volunteers was carried out by a registered dietician and the daily intake of PACs was calculated for each of them, based on national and international databases of polyphenol contents in foodstuff (Rothwell et al., 2013; USDA Database for the Proanthocyanidin Content of Selected Foods; PortalAntioxidantes, 2013).

A lactulose hydrogen breath test was also carried out at recruitment in the fasted subjects; alveolar breath samples were obtained before the ingestion of 25 g lactulose (basal) and thus every 10 minutes for 3 hours. The criterion used to define the presence of SIBO was an increase of the H_2 and/or CH_4 concentration of 10 ppm or more over basal values during the first 60 min, with an associated second peak caused by the colonic fermentation of lactulose (Madrid, Poniachik, Quera, & Defilippi, 2011). Orocecal transit time (OCTT) was determined as the time elapsed between lactulose ingestion and the initiation of the sustained increase in H_2 concentration corresponding to the colonic fermentation of lactulose (Madrid et al., 2011).

The post-prandial plasma concentrations of TG and LPS absorption was determined for each subject in two independent days separated by one week, one with the polyphenol extract and the other with the placebo, in a random, double blind form.

On the study day, an intravenous line was placed in the forearm vein of the fasted subjects and 2 basal samples were obtained at 10 min of interval. Then the subject had to ingest a capsule containing 1 g of the polyphenol extract or placebo (maltodextrin) and a breakfast consisting of bread (100 g), cheese (100 g), 2 eggs, and mayonnaise (8 g), representing a total of 63 g fat and 990 kcal. The polyphenol extract and the placebo were encapsulated by the Laboratory of Pharmacognosy (Faculty of Pharmacy, University of Concepción, Concepción, Chile) and the capsules subsequently packaged in different codified containers (placebo or extract) so that the investigators responsible of their administration to the subjects were blinded to their content. Blood samples were subsequently obtained at 30 min, 1 h and then every hour during the following 7 hours (8 h in total). Plasma concentrations of TG were determined in 8 samples by using a commercial kit, according to the manufacturer instructions. Plasma LPS was determined in duplicate in the samples 0, 30 min and in the following pooled samples: 1 + 2 h, 3 + 4 h, 5 + 6 h and 7 + 8 h, using a chromogenic Limulus amebocyte lysate (LAL) assay (Lonza, Basel, Switzerland). Exhaustive care was taken to avoid environmental endotoxin contamination and all material and reagents used for sample processing and the assay were pyrogen-free. The assay was performed after heating the samples at 70 °C for 10 min to inactivate endotoxin-neutralizing agents. Results were expressed as endotoxin units (EU) per ml. The incremental areas under the curve, i.e. the area above the baseline fasting value of triacylglycerolemia ($iAUC_{TG}$) and LPS ($iAUC_{LPS}$), were calculated by the trapezoidal method with GraphPad Prism 6.0. Plasma IL-6 concentrations in the same plasma samples were determined by using a commercial Elisa kit.

2.6. *Statistical analysis*

Results were processed and analyzed with the statistical package program Statistica 11.2 (StatSoft, Tulsa, OK, USA). Normality of the variable distributions was determined with the Shapiro–Wilk W-test. According their distribution, data were expressed as means ± SEM or as medians with their interquartile range [IQR]. Proportions (for the gender variable) between groups were compared by chi-square and quantitative variables by Student's t-test or the non-parametric Man–Whitney test according their distribution. The postprandial plasma concentrations of TG, LPS and IL-6 exhibited a skewed distribution and were logarithmically transformed for their posterior analysis. Corresponding data were expressed as geometric means and their 95% confidence interval ($CI_{95\%}$). The impact of the grouping variables (nutritional status, SIBO status) on the different parameters evaluated in the study was analyzed by three-way ANOVA or three-way ANOVA for repeated measurements (in the case of the postprandial changes of plasma TG, LPS and IL-6).

3. Results and discussion

3.1. *Characterization and quantification of PACs in the fruit extracts*

Results corresponding to the characterization of the PACs from the fruit extracts are described in Table 2. They show that grape PACs contained a higher proportion of pentamers and polymers than those from the other extracts. The mean degree of polymerization (DP) of the PACs from grape, cranberry, avocado and apple was 9.8, 7.3, 6.1 and 3.2, respectively.

It is important to consider, however, that a considerable part of the PACs from grape by-products is present in the fraction containing the insoluble phenolic compounds. Due to the method used in this study for the extraction of phenolic compounds, these PACs are not present in our extract that only contains the soluble phenolic compounds (de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2014).

3.2. *Inhibition of pancreatic lipase*

Nowadays, there is a high interest in developing new lipase inhibitors with less adverse effects than orlistat, the "gold standard" drug currently used for the management of patients with obesity and dyslipidemia. Accordingly, a great number of studies have evaluated the inhibitory activity of dietary polyphenols, either in pure form or present in complex extracts, and the corresponding results have been extensively reviewed (Buchholz & Melzig, 2015). In the first step of the present study, we determined the lipase inhibitory effect of PAC-containing polyphenol extracts from different fruits. Fig. 1A shows the half maximal inhibitory concentration (IC_{50}) of these extracts on pancreatic lipase activity. The grape extract was significantly more efficient than those from avocado and apple in inhibiting lipase

Table 2 – Quantification of proanthocyanidins in grape, apple, cranberry and avocado extracts by HILIC-HPLC with fluorescence detection (Mean ± SD).

	Grape	Apple	Cranberry	Avocado
Monomers	78.66 ± 1.07	43.12 ± 0.77	22.17 ± 0.09	100.08 ± 1.22
Dimers	113.11 ± 0.91	68.90 ± 0.13	160.34 ± 1.00[c]	82.34 ± 0.46
Trimers	80.12 ± 0.65	39.66 ± 0.56	70.56 ± 0.91	67.09 ± 0.55
Tetramers[a]	49.87 ± 0.82	34.54 ± 0.08	28.09 ± 0.07	20.65 ± 0.79
Pentamers[a]	23.44 ± 1.01	11.22 ± 0.23	3.60 ± 0.34	ND
Polymers (DP > 5)[a,b]	87.09 ± 1.10	29.92 ± 0.99	48.95 ± 0.61	35.59 ± 0.12
Total (mg/g)	433.10 ± 0.93	227.36 ± 0.46	333.71 ± 0.50	305.75 ± 0.52

[a] Tetramers, Pentamers and Polymers were expressed as trimers equivalents.
[b] Polymers were analyzed at 280 nm.
[c] Dimers were expressed as procyanidin A2 equivalents.

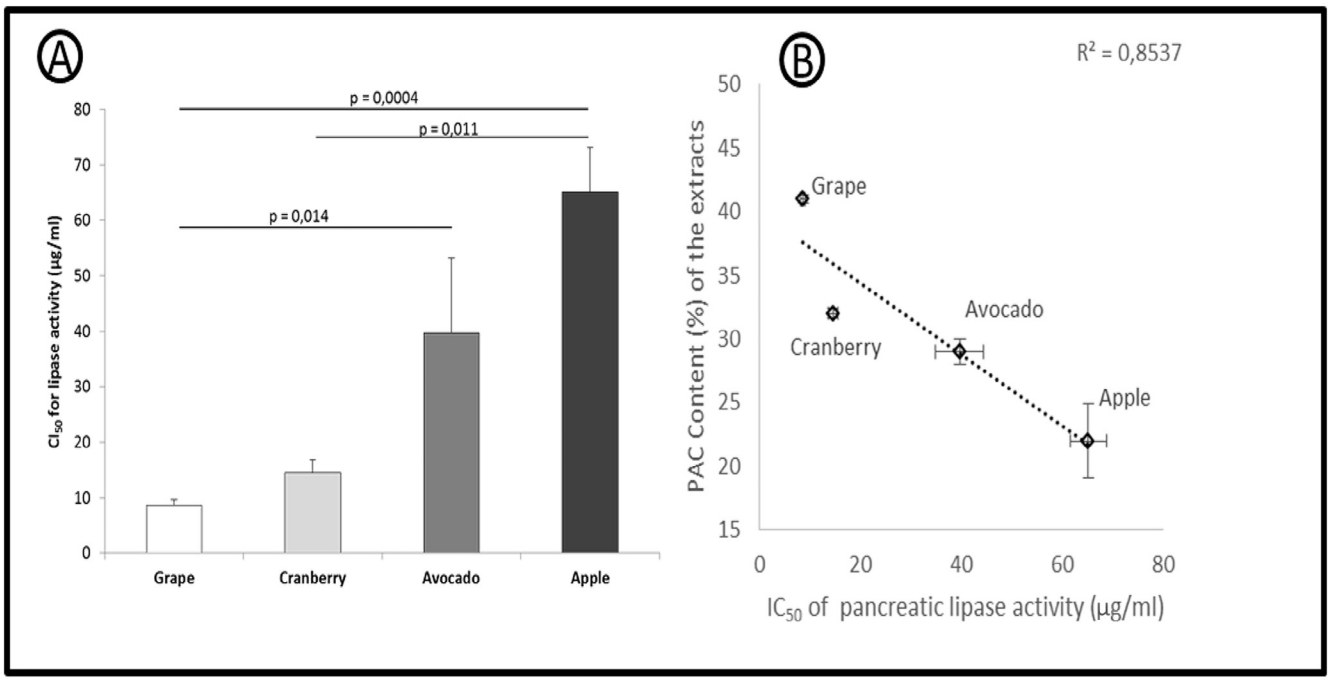

Fig. 1 – (A) Inhibition of pancreatic lipase by the polyphenol extracts from the different fruits (expressed as IC_{50}). (B) The IC_{50} of the pancreatic lipase activity correlates with the PAC content of the polyphenol extracts (r = 0.85; p < 0.001).

and the cranberry extract was more efficient than that from apple. Their ranking from the most to the less efficient, therefore, was grape > cranberry > avocado > apple. It has been reported that the DP of PACs and the presence of galloyl moieties in their molecular structure are important factors determining the ability of these compounds to inhibit lipase activity (Buchholz & Melzig, 2015; Gu, Hurst, Stuart, & Lambert, 2011; Sugiyama et al., 2007). Our results are in line with these findings as the strongest lipase inhibitory activity was exerted by the grape extract, i.e. that with the higher PAC content and higher DP (9.8) and the only ones containing galloyl moieties (Wong et al., 2016). Accordingly, we observed that the PAC content of the extracts correlated (r = 0.85; p < 0.001) with their inhibitory activity (Fig. 1B); a similar relation was also observed when considering the PAC degree of polymerization. However, the presence of type A-PACs did not affect the lipase inhibitory activity of the extracts in our study.

3.3. *Inhibition of LPS binding to polymyxin B*

Regarding the interaction between PACs and LPS, few data are available. Delehanty et al. (2007) showed that high molecular weight fraction from cranberry PACs efficiently bound LPS and that this activity was weaker with tea and grape PACs. These authors observed this interaction with LPS from different bacteria such as *E. coli*, *Salmonella*, *Shigella* and *Pseudomonas*, and they also identified the lipid A moiety as the LPS structure involved in PAC binding. Fig. 2 describes the IC_{50} for LPS binding to polymyxin B-coated agarose beads displayed by the extracts. Our results show that the grape extract was significantly more efficient than those from avocado and apple in inhibiting LPS binding and the cranberry and avocado extracts were more efficient than that from apple. Their ranking from the most to the less efficient was grape > cranberry > avocado > apple, similarly to that observed with lipase inhibition.

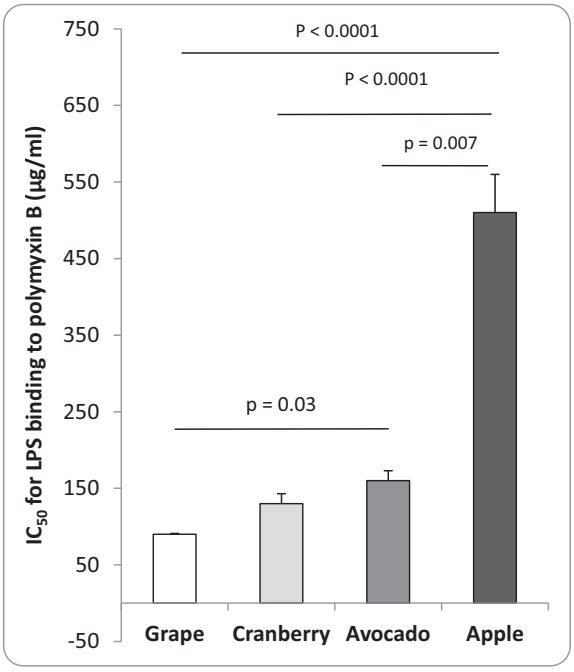

Fig. 2 – Inhibition of the binding of FITC-LPS to polymyxin B-coated agarose beads by the polyphenol extracts from the different fruits (expressed as IC_{50}; means ± SEM).

Table 3 – Anthropometric, biochemical and digestive characteristics of the subjects participating in the study (Mean ± SD).

	Normal weight (NW) (n = 12)	Overweight/Obese (OW/Ob) (n = 17)	All (n = 29)	p
%F	58.3 (7/12)	70.6 (12/17)	65.5 (19/29)	NS
Age (y)	26.9 ± 10.5	39.2 ± 15.5	34.1 ± 14.8	0.025
BMI (Kg/m2)	22.6 ± 1.7	28.8 ± 4.0	26.2 ± 4.4	0.0000
Glucose (mg/dl)	90.3 ± 10.7	91.1 ± 16.3	90.8 ± 14.0	NS
Urea nitrogen (mg/dl)	12.4 ± 2.4	12.2 ± 2.9	12.3 ± 2.7	NS
Uric acid (mg/dl)	3.76 ± 0.86	4.05 ± 1.29	3.93 ± 1.12	NS
Total bilirubin (mg/dl)	0.29 ± 0.16	0.26 ± 0.11	0.28 ± 0.13	NS
Alkaline phosphatase (U/l)	150 ± 40	193 ± 61	175 ± 57	0.04
Aspartate aminotransferase) (U/l)	21.4 ± 17.2	17.6 ± 6.0	19.2 ± 11.9	NS
Lactate dehydrogenase (U/l)	320 ± 70	341 ± 63	332 ± 65	NS
Calcium (mg/dl)	8.65 ± 1.02	8.96 ± 0.76	8.83 ± 0.88	NS
Phosphorous (mg/dl)	3.56 ± 0.62	3.64 ± 0.57	3.60 ± 0.58	NS
Total protein (g/dl)	5.91 ± 0.89	5.97 ± 0.56	5.95 ± 0.70	NS
Albumin (g/dl)	3.77 ± 0.41	3.81 ± 0.30	3.79 ± 0.35	NS
Total Cholesterol (mg/dl)	143 ± 36	157 ± 37	151 ± 37	NS
HDL-Cholesterol (mg/dl)	42.9 ± 12.4	38.5 ± 8.0	40.3 ± 10.1	NS
LDL-Colesterol (mg/dl)	84.7 ± 29.9	90.3 ± 27.9	88.0 ± 28.4	NS
TG (mg/dl)	78.7 ± 29.1	140 ± 77	115 ± 69	0.015
VLDL-cholesterol (mg/dl)	15.7 ± 5.8	28.0 ± 15.5	22.9 ± 13.7	0.015
Cholesterol/HDL	3.44 ± 0.90	4.14 ± 0.97	3.85 ± 0.99	0.057
Orocecal transit time (OCTT) (min)	83.3 ± 12.3	91.8 ± 34.5	88.3 ± 27.5	NS
Basal H2 (ppm)	13.1 ± 20.9	7.06 ± 9.00	9.5 ± 15.1	NS
Cumulate H2 at 60 min (ppm)	193.5 ± 293	132 ± 177	158 ± 229	NS
% SIBO	66.7 (8/12)	41.1 (7/17)	51.7 (15/29)	NS $_{(0.17)}$

P values from Student's t-test or Chi-square test, comparing NW and OW/Ob subjects.

Based on these observations, we selected the grape extract to determine its impact on the post-prandial changes of TG and LPS in human volunteers.

3.4. *Effect of the grape extract on the postprandial plasma concentrations of triacylglycerols in human volunteers*

Thirty one subjects were initially recruited and 29 finished the study. Twelve of them were normal weight (41.3%) and 17 (58.7%) overweight or obese. Anthropometric and biochemical data of the subjects are shown in Table 3. The age of the OW/Ob subjects was significantly higher than that of the NW group and, as expected, they also displayed higher BMI and plasma alkaline phosphatase, TG, VLDL and cholesterol/HDL ratio. The daily intake of dietary PACs by the subjects was 182 mg/d [125–239 mg/d] (mean [$CI_{95\%}$]), without differences according to their nutritional status or presence of SIBO. SIBO was detected in 15 subjects (51.7%); the proportion of subjects with SIBO did not differ between normal weight and overweight/obese groups and the biochemical parameters of the subjects were not affected by the presence of SIBO (data not shown). As shown in Table 4, the basal concentrations of breath H_2 and CH_4 and the cumulative levels of breath H_2 and CH_4 at 60 min were significantly higher in the subjects with SIBO than in those without SIBO ($p = 0.03$, $p = 0.0011$, $p = 0.009$ and $p < 0.0001$, respectively).

The concentrations of plasma TG were determined at baseline and during 8 hours after the intake of a high fat breakfast with placebo or grape extract, in the NW and OW/Ob subjects. Baseline concentrations of plasma TG (before breakfast) did not differ between the two periods of study. Changes in plasma TG (normalized to baseline values) are shown in Fig. 3A; the increase in plasma TG was significantly more elevated in the OW/Ob group that in the NW (ANOVA, $p = 0.0058$) but remained unaffected by the administration of the grape polyphenol extract ($p = 0.75$) or by the SIBO status of the subjects ($p = 0.44$, data not shown). The corresponding $iAUC_{TG}$ are shown in Fig. 3B; they indicate that the absorption of dietary TG was 2 times higher in the OW/Ob than in the NW subjects. No significant interactions [grape extract X SIBO] or [grape extract X nutritional status] were observed. These findings are in line with previous studies showing that abdominal obesity

Table 4 – Characteristics of the subjects according to their SIBO status (Median [IQR]).

	Without SIBO n = 14	With SIBO n = 15	p
Age (y)	26.5 [22–56.5]	25 [21–47]	NS
BMI (kg/m2)	27.5 [25.5–31.6]	25 [23.8–27.1]	NS
% Female	66.6	62.5	NS
Basal Breath H_2 (ppm)	0 [0–0]	17 [2–27]	0.03
60 min breath H_2 (ppm)	5 [4–17]	209 [113–295]	0.009
Basal breath CH_4 (ppm)	3.0 [1.0–6.0]	10 [6–21]	0.0011
60 min breath CH_4 (ppm)	25 [19–66]	148 [99–204]	0.0001
Orocecal transit time (min)	100 [70–120]	90 [70–90]	NS

p values from Man and Whitney U test.
SIBO: Small intestine bacterial overgrowth.

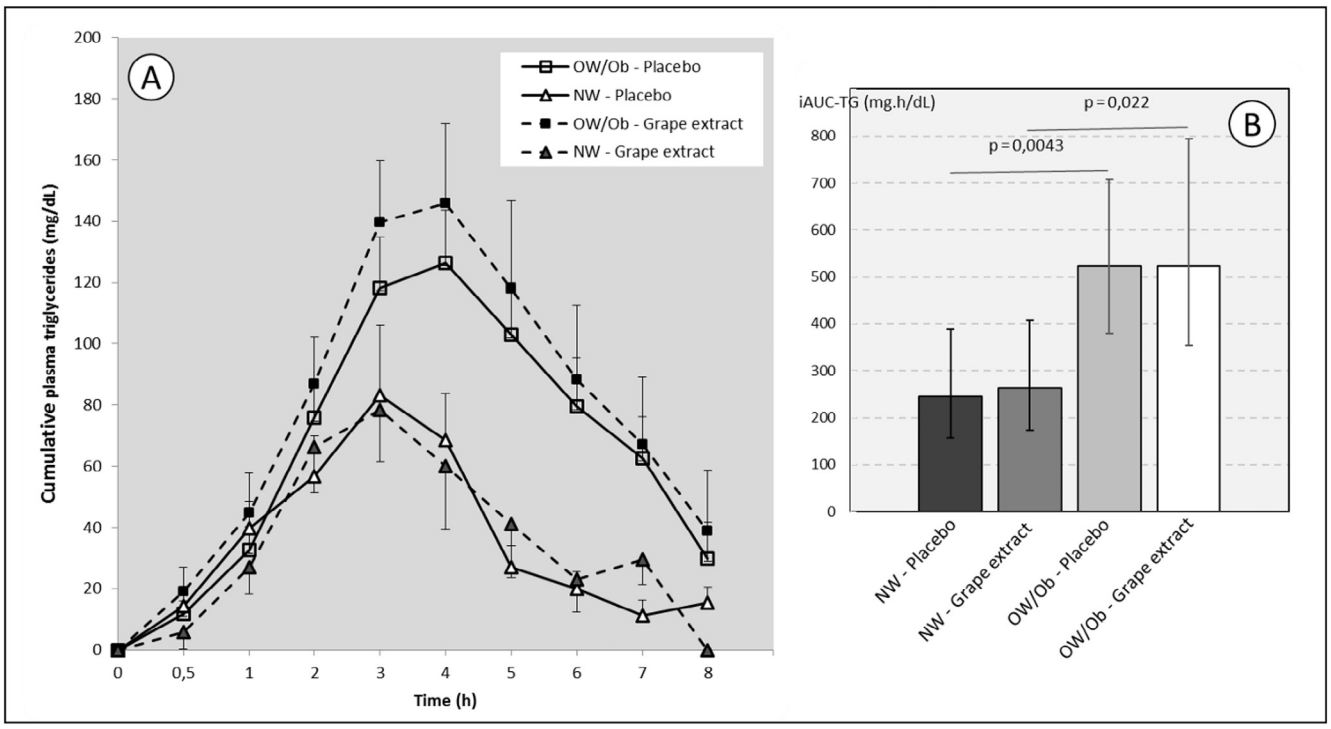

Fig. 3 – Impact of the intake of grape extract on post-prandial triacylglycerolemia in normal weight (NW) and overweight/obese (OW/Ob) subjects. (A) Normalized concentrations of plasma triacylglycerols after intake of high-fat breakfast with placebo (full lines) or PAC-rich grape extract (dashed line) in NW (triangle) and overweight/obese (square) subjects. (means ± SEM). (B) Changes in the $iAUC_{TG}$ between the grape extract period and the placebo period in the normal weight and overweight/obese subjects (Geometric means and $CI_{95\%}$).

is an important factor determining the post-prandial elevation of plasma TG (Couillard et al., 1998; Mekki et al., 1999; Nogaroto et al., 2015). It is interesting that the grape extract was able to inhibit pancreatic lipase *in vitro* but had no effect on the post-prandial elevation of plasma TG in the volunteers (Fig. 3A and B). It is noteworthy that most of the studies exploring the impact of dietary polyphenols on blood lipids have evaluated the long-term impact of polyphenol supplementation on lipid absorption and that only few studies have determined the acute effect of polyphenol on postprandial TG metabolism (Annuzzi et al., 2014; Bladé, Arola, & Salvadó, 2010; Burton-Freeman, 2010). An explanation for the lack of effect of the grape extract in our study could be that the amount of the extract administered with the test meal was insufficient to inhibit the lipase, considering that this enzyme is normally released in excess by the pancreas in the gut lumen.

3.5. *Effect of the grape extract on the postprandial plasma concentrations of LPS in human volunteers*

Plasma concentrations of LPS were determined at baseline and during the 8-hour period posterior to the high-fat breakfast ingestion. Baseline plasma concentrations of LPS in the placebo period (2.12 ± 1.26 EU/ml) did not differ from that observed in the grape extract period and were not affected by the nutritional or SIBO status of the subjects (ANOVA, $p = 0.59$ and $p = 0.71$, respectively). Postprandial changes in plasma LPS concentrations (normalized to baseline values) for all the subjects in the placebo and grape extract periods are shown in Figs. 4 and 5. Our results indicate that plasma LPS significantly increased after the high fat test meal, confirming the results of other studies suggesting that LPS handling in plasma depends

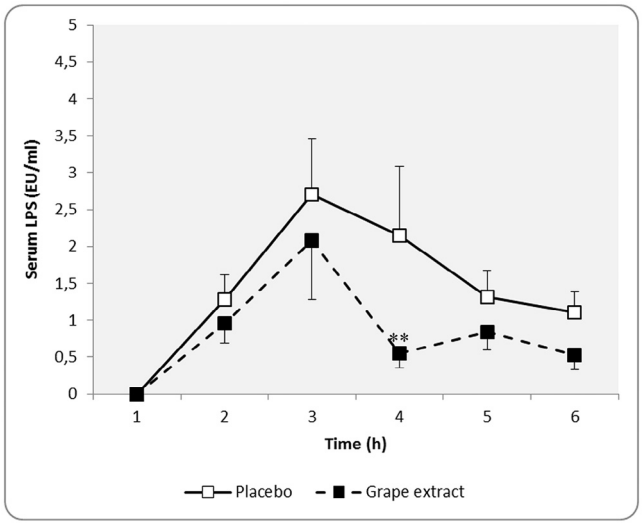

Fig. 4 – Changes in plasma LPS in all subjects after intake of a high-fat breakfast with placebo (solid line) or grape PAC-rich extract (dashed line). The post-prandial plasma LPS was significantly lower at 4 h when consuming the grape extract (p = 0.0039).**

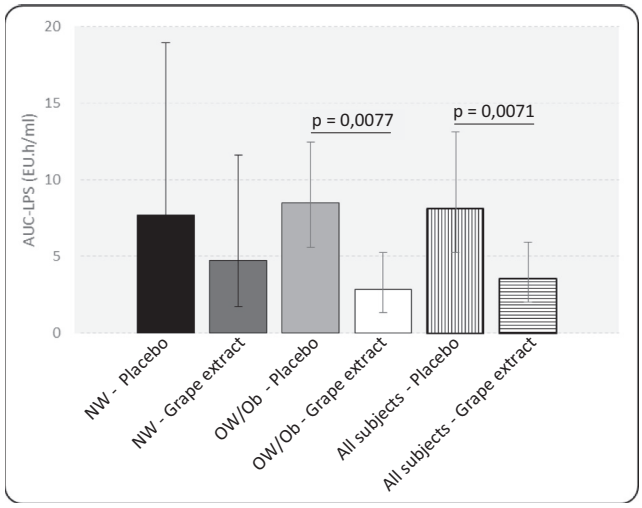

Fig. 5 – Effect of grape extract administration on $iAUC_{LPS}$ in normal weight (NW) and overweight/obese (OW/Ob) subjects and in all subjects (geometric means and $IC_{95\%}$).

on the intestinal absorption of dietary fat (Clemente-Postigo et al., 2012; Erridge et al., 2007; Ghoshal et al., 2009; Laugerette et al., 2011; Vors et al., 2015). As a more elevated post-prandial triacylglycerolemia was reported in the OW/Ob subjects, we therefore expected that these subjects would also exhibit higher postprandial plasma LPS compared with the NW. However, the higher amounts of dietary fat absorbed by the OW/Ob subjects in our study were not associated with higher postprandial plasma LPS, compared with the NW. Accordingly, it is probable that other factors, such as the degree of fat emulsification, are more important in determining increased plasma LPS (Laugerette et al., 2011).

Our results also show that the postprandial increase in plasma LPS was significantly prevented by the grape extract at 4 h ($p = 0.0039$), compared with the placebo. These results are opposed to those from Clemente-Postigo et al. (2013) who did not observe any effect of red wine or dealcoholized red wine (both containing polyphenols including PACs) intake on postprandial LPS. When considering $iAUC_{LPS}$ (Fig. 6), we observed that this parameter was not affected by the nutritional status of the subjects ($p = 0.54$) nor by the presence of SIBO ($p = 0.52$, data not shown). $iAUC_{LPS}$ was significantly decreased by the administration of grape extract in the OW/Ob subjects ($p = 0.0077$), not in the NW. The reasons for such finding are unclear. As it has been described that intestinal motility is frequently slower in obese subjects (Madrid et al., 2011), it is possible that PACs have more time to bind luminal LPS and therefore prevent its absorption than in the NW. Such observation is interesting as it supports the use of PAC-containing foodstuffs in the management of low-grade inflammation in the obese population.

On the other hand, we did not observe any significant interaction [grape extract X SIBO] on $iAUC_{LPS}$. Subjects with SIBO were recruited to determine whether the elevation in postprandial plasma LPS might be higher in subjects with higher LPS contents in their intestinal lumen. SIBO is a condition frequently associated with obesity and its complications (Sabaté et al., 2008; Shanab et al., 2011) and which is characterized by an abnormally high count of bacteria in the upper gut (>10^5 CFU/ml compared with ~10^3CFU/ml in the healthy subjects), more particularly gram-negative bacteria such *as E. coli, Klebsiella, Proteus, Bacteroides, Veillonella* and *Fusobacterium* (Bouhnik et al.,

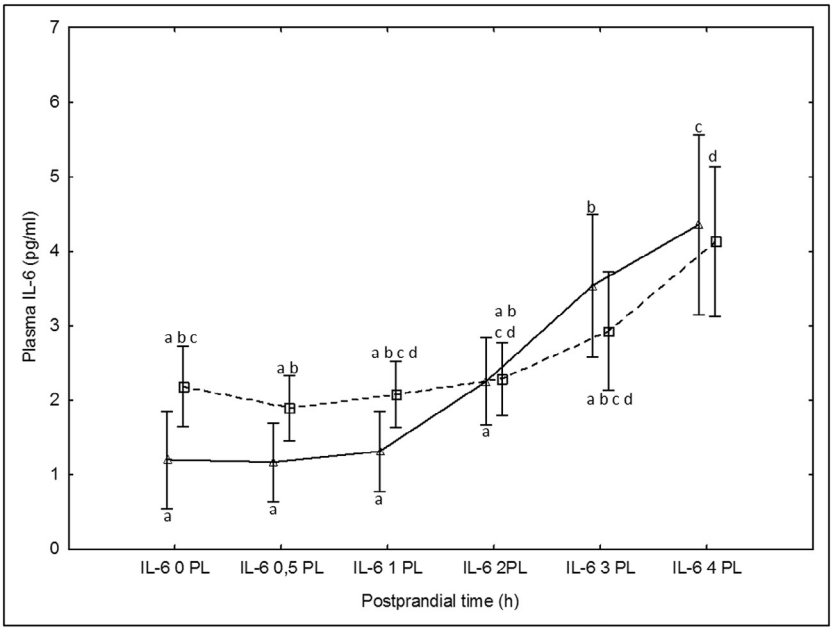

Fig. 6 – Changes in plasma IL-6 after intake of a high-fat breakfast in normal weight (solid line) and overweight/obese subjects (dashed line). (Geometric means [$CI_{95\%}$]). Post-prandial plasma IL-6 significantly increased in both groups of subjects (ANOVA, $p = 0.0001$). This increase significantly differed between both groups (ANOVA, Interaction Time × Nutritional Status: $p = 0.0029$). Values with different letters significantly differ ($p < 0.05$).

1999). Interestingly, SIBO occurs in the upper gut, i.e. where dietary fats are mainly absorbed. We therefore expected that the subjects with SIBO would display higher fasting and postprandial plasma LPS concentrations. However, our results indicate that these parameters are not affected by the presence of SIBO in the subjects, independently of their nutritional status. A limitation of this approach is that SIBO was detected only indirectly through the lactulose breath-test and that it may not be excluded that, in some subjects, it was due to the proliferation of gram-positive bacteria.

Finally, we also studied the impact of the grape extract on the changes in post-prandial plasma concentrations of IL-6, a biomarker of inflammation. We observed baseline concentrations of plasma IL-6 of 1.39 pg/ml [0.99–1.85 pg/ml] (geometric means [$CI_{95\%}$]) in the subjects, without differences between the placebo and grape extract periods. Baseline IL-6 concentrations were not affected by the SIBO status of the subjects ($p = 0.89$) or by their nutritional status (1.01 pg/ml [0.48–1.74 pg/ml] vs 1.69 pg/ml [1.14–2.39 pg/ml] in the NW and OW/Ob groups, respectively, $p = 0.11$). After the intake of the test meal, plasma IL-6 significantly increased ($p < 0.0001$) along the 8-hour period (Fig. 6), being significantly more important this increase in the NW than in the OW/Ob subjects (ANOVA, $p = 0.0026$). This finding probably reflects the fact that the plasma concentrations of IL-6 at baseline were (not significantly) higher in the OW/Ob than in the NW group. These results confirm previous studies reporting that the elevation of plasma LPS is accompanied by an inflammatory response reflected by changes in plasma IL-6 (Erridge et al., 2007; Laugerette et al., 2011; Vors et al., 2015). However, neither the administration of the grape extract nor the SIBO status of the subjects affected the increasing evolution of plasma IL-6 ($p = 0.23$ and $p = 0.37$, respectively), independent of the nutritional status of the individuals. Considering that the grape extract decreased the absorption of LPS without affecting that of TG in the OW/Ob subjects, this finding could mean that the postprandial increase of IL-6 is due to the circulating TG rather than to LPS (Santos, Oliveira, & Lopes, 2013; Youssef-Elabd et al., 2012).

4. Conclusions

Our results indicate that the grape extract was the most efficient compared to cranberry, avocado and apple extracts in inhibiting pancreatic lipase and interfering with LPS *in vitro*. In NW and OW/Ob subjects, the simultaneous intake of the grape extract with a high fat test meal did not affect the postprandial changes of triacylglycerolemia but decreased those of plasma LPS in the OW/Ob subjects without affecting the IL-6-associated inflammatory response. The presence of SIBO in the subjects did not affect the studied variables.

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