Screening the impact of food co-digestion on lipolysis under sub-optimal intestinal conditions

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ABSTRACT

The scarce literature about the effect of meal-factors have on lipids digestibility encouraged the present study, in which olive oil was co-digested with naturally fat-free matrices that were rich in carbohydrate (potato and bread) or protein (degreased fresh cheese, hake and turkey) in single, binary and ternary combinations. Digestion was simulated in vitro, and the effect of co-digestion on the release of free fatty acid (FFA) from oil lipolysis were measured by gas chromatography-mass spectrometry. Regarding total FFA release, higher values were found in carbohydrate-rich systems, especially in potato, than in those with protein matrices. Thus, when co-digesting a carbohydrate matrix in addition to one or two protein matrices, lipolysis was reduced. This finding was explained by the carbohydrate and protein ratio of the resulting combinations, as the release of FFA increased with the carbohydrate/protein ratio (R^2=0.87, $p<0.001$ in potato; R^2=0.81, $p=0.04$ in bread systems). This study supposes the first approach towards characterisation of lipid digestion regarding food matrix nutritional composition.

KEYWORDS: in vitro digestion, food matrix, lipolysis, free fatty acids

1. INTRODUCTION

In the recent years, the study of lipid digestion has gained relevance for the direct implication of overconsumption in the development of type II diabetes and obesity (De Souza et al., 2015; Rolland-Cachera, et al., 2017). In this sense, research in food technology has focused on structuring foods towards controlling lipid release from the matrix and decreasing lipolysis (Guo et al., 2017). However, there are other situations, such as exocrine pancreatic insufficiency (EPI), in which maximising lipid digestion is targeted. In this scenario, the release of hydrolytic enzymes to the small intestine is impaired, impeding nutrient digestion, especially fat (Sikkens et al., 2010). In addition, reduced intestinal pH up to 5-6 and bile salts concentration up to 1 mmol/L in the intestinal fluid (ten times lower than in normal conditions) further compromise lipolysis (Gelfond et al, 2013; Humbert et al., 2018).

To palliate the insufficiency, adherence to pancreatic enzyme replacement therapy, consisting of the exogenous administration of pancreatic enzymes, is recommended in every meal. However, this therapy, normally adjusted to the lipid content of the meals, is not optimal, suggesting that food structure may be determinant in the efficacy of the enzyme supplements (Calvo-Lerma et al., 2019).

The food matrix is the spatial architecture resulting from the assembly of proteins, carbohydrates and lipids into a coordinated network. It plays a crucial role on how food interacts with the gastrointestinal tract and on the resulting release and digestion of nutrients (Guo et al., 2017). Up to date, in vitro digestion methods have enabled the study of several aspects related to lipolysis (Li & McClements, 2010; Ozturk et al., 2015). However, most of this research has been conducted on the basis of model foods or emulsions, limiting the generated knowledge to the molecular scale. More recently, the study of lipolysis in specific real food matrices such as egg, nuts or

cheese, have demonstrated that food structure determines subsequent lipolysis extent (Asensio-Grau et al., 2018; Asensio-Grau et al., 2019; Paz-Yépez et al., 2018). Therefore, food composition and foods co-digestion can be considered determinant factors in lipolysis extent. Despite of the progress in research, the combination of different foods, which is the normal pattern in dietary intake, could lead to an even more complex situation than that in a food individually digested. Lipolysis could be affected by new possible interactions between lipids and other macronutrients released from co-digested matrices to the digestion medium, which up to date has never been addressed.

To shed light on this situation, olive oil was digested with one, two or three free-fat matrices that were rich in carbohydrates or protein, and the impact of co-digestion on lipolysis extent was assessed.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

Five fat-free food matrices (<1% fat) were selected: two were rich in carbohydrates (bread and potato), and three were rich in protein of different types: casein (degreased fresh cheese) and fibrillar proteins (hake and turkey). Before in vitro digestion, hake and potato were cooked with a microwave (120 W/g food, 3 min); while bread, degreased fresh cheese and turkey were used in their raw form. Extra virgin olive oil was then added to these matrices as the common lipid substrate to all the experiments. The lipid substrate was incorporated to the food matrices prior mixing, and homogenisation was conducted jointly. Nutritional composition of the study foods was extracted from the official Spanish national food composition database (BEDCA, www.bedca.net).

For the preparation of the simulated digestive fluids, the following reagents were needed: human α-amylase (1000–3000 U/mg protein) pepsin from porcine gastric mucosa (≥ 2,500 U / g protein), bovine bile extract, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ $(H2O)_6$, $(NH_4)_2CO_3$ and $CaCl_2$, NaOH (1N) and HCl (1N). For the gas chromatography-mass spectrometry (GC-MS) analytical determinations, hexane, methanol, BF_3, H_2SO_4 and NaCl were required, as well as the following analytical standards: pentadecanoic acid, palmitic acid, stearic acid, oleic acid and linoleic acid (Sigma-Aldrich Chemical Company (St Louis, MO, USA)). Pancreatic enzyme supplements (Kreon 10,000 Lipase Units (LU)) were used as the source of lipase and colipase. Each capsule contains 0.15 g of porcine pancreatic enzyme (10,000 LU, 8,000 amylase units, and 600 protease units) in gastro-resistant microspheres form.

2.2. Experimental design

Extra virgin olive oil was co-digested with one, two or three of the five food matrices (bread, potato, degreased fresh cheese, turkey or hake) to assess the impact of its co-digestion on lipid digestibility (free fatty acid profile and total free fatty acid release). In all of the experimental sets, the food matrix/added fat ratio was 4.5 g/0.5 g (**Table 1)**. All the combinations were in vitro digested as described hereafter, and all the experiments were conducted in triplicate.

2.3. In vitro digestion simulation

Food samples were placed into 50 mL falcon tubes. Then, samples were subjected to the in vitro digestion process in which EPI conditions were simulated (lower intestinal pH = 6, and lower bile salts concentration =1 mmol/L) (Gelfond et al., 2013; Humbert et al., 2018), following the protocol first established by Asensio-Grau et

al. (2018), and using the recommended pancreatic enzyme supplement dose of 2000 LU/g fat (Turck et al., 2016). The digestion fluids were prepared fresh daily from stock solutions, following the guidelines established in the standardised protocol supported and applied by numerous research groups (Minekus et al., 2014; Brodkorb et al., 2019). The enzymatic activity was daily tested before starting the experiments (Carrière et al., 2000).

The in vitro digestion process consisted of three stages. In the oral stage (food sample in proportion with simulated salivary fluid pH 7 containing α-amylase 1:1 (w/v)), the food sample (fat-free matrix and extra virgin olive oil) was minced using a household mortar for 3 min in order to preserve the matrix effect, instead of using a blender for complete homogenisation as in previous in vitro digestion studies (Paz-Yépez et al. 2018). Following, in the gastric stage (oral bolus in proportion with simulated gastric fluid pH 3 1:1 v/v) pepsin was added in a concentration of 2000 U/mL of chyme and pH was adjusted to 3 with HCl (1N). Samples were rotated head-over-heels (55 rpm) for 2 h at 37 ºC (Intell-Mixer RM-2, Elmi Ltd, Riga, LV-1006, Latvia) in a thermostated chamber (JP Selecta SA, Barcelona). These mixing conditions provided constant mechanical energy to induce the breakdown of the food matrix occurring in stomach. Finally, in the intestinal stage (chyme in proportion with the simulated intestinal fluid pH 6 1:1 (v/v)) enzymatic supplements of pancreatin (2000 LU/g fat) and bile salts (1 mmol/L in the intestinal fluid) were added, and pH was adjusted to 6 with NaOH (1N). Samples were rotated as in the gastric stage and kept at 37 ºC in the interior of the chamber. During the process, pH was monitored and readjusted to prevent drops below 5.7 at which lipase activity might be inactivated (González-Bacerio et al. 2010). After 2 hours of intestinal stage lipolysis was immediately inactivated by

the addition of 4-bromophenylboronic acid and kept in ice for 15 minutes (Brodkorb et al. 2019).

2.4. Free fatty acid quantification

At the end of the intestinal stage, samples were ~~thieved~~ sieved and the freeze-dried drained phase was used for fatty acid release quantification by means of gas chromatography-mass spectrometry (GC-MS). Samples were first transesterified to methyl esters (FAMEs) with BF_3 and methanol at 20 °C according to the IUPAC standard method (IUPAC, 1992: Yaich et al., 2011). Fat extraction was done with 3 mL of hexane in 15 mL falcon tubes and by rotating head-over-heels at 55 rpm for 90 min using Intell-Mixer RM-2. Then, tubes were centrifuged for 5 min 5000 rpm and 1 mL of supernatant was dried with nitrogen flow. The residue was used for methylation. Following, 50 μL of internal standard (pentadecanoic, 1 mg/mL), 40 μL of hexane and 100 μL of BF_3 were added to the vial with the residue obtained, vortexed 15 seconds and heated at 70 °C during 90 minutes. Then 100 μL of NaCl (25 % w/v), 40 μL of H_2SO_4 (10 % w/v) and 700 μL of hexane were added to the mixture, vortexed 15 seconds and settled for 30 min. After that time 700 μL of upper layer was taken and transferred to the injection for analysis.

Samples were analysed with an Agilent 5977A system and an HP-5 MS UI (Agilent, 30 m x 0.25 mm, 0.25 μm film thickness) was used with helium. The oven was set at 90 °C for 2 min, increased to 222 °C at 5 °C/min for 5 min, and increased to 280 °C at 20 °C/min for 2 min; split flow was adjusted at 1 mL/min, and injector temperature was at 280 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 30 to 650. Identification of components done by matching against commercial libraries (Nis 11t, Nist_msms, mainlib, replib, wiley7n) and MS literature data.

Software 6890 was used for data acquisition and processing. FAMEs were identified by comparing retention times of the peaks with the pure standards (Supelco®37 Component FAMEs Mix, Sigma).

2.5. Statistical analysis

Data were summarised using mean and standard deviation (SD) in the case of continuous variables and with absolute ant relative frequencies in the case of categorical variables. Descriptive results were represented graphically (mean and SD). As for inferential analyses, linear mixed regression models were applied to study the association between: 1) FFA released and number of co-digested matrices; and 2) FFA released and carbohydrate/protein ratio. A random effect was included in the models to correct the effect of food combinations. For the models, potato and bread data were treated individually to evaluate the role of each CH matrix on lipolysis separately. The analyses were carried out using R software (version 3.5.0). P-values below 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

At the end of the simulated intestinal stage, individual FFA released during intestinal digestion were quantified in order to depict the FFA profile and to assess lipolysis extent in the five study matrices and their co-digestions. FFA profile was characterized by a high amount of free oleic acid (C18:1), followed by palmitic (C16:0), stearic (C18:0) and linolenic (C18:2) acids in lower quantities, regardless the food matrix which olive oil was co-digested with (**Figure 1**). Taking into account the fatty acid composition of olive oil, which is rich in monounsaturated fatty acids (80%) and has saturated (12%) and polyunsaturated fatty acids (8%) in lower proportions, the

obtained FFA profile in the intestinal medium was to be expected (Borges et al., 2017). This finding suggests that the food matrix did not have any effect on the pancreatic enzyme hydrolytic action on triglycerides, and that the resulting FFA profile was only dependant on the origin and structure of the type of fat. Indeed, the hydrolysis of a triglyceride molecule, composed by a glycerol backbone and three chains of fatty acids, is known to be a very selective process. Fatty acid chains are bonded the three stereospecific positions of the glycerol: sn-1, sn-2 or sn-3. Pancreatic lipase is very specific for the sn-1 and the sn-3 positions. Lipolysis reaction mediated by pancreatic lipase results in the formation of sn-2 mono-glycerides and two free fatty acids, which can be eventually absorbed (Hunter, 2001). In the case of olive oil, the sn-1 and sn-3 locations are bonded, in a high proportion, to oleic acid, and in minor frequency to palmitic acid (Brockerhoff & Yurkowski, 1966; Small, 1991), which is in accordance to the present study findings. According to the exposed biochemical foundations, the present results confirm the specificity and selectivity of pancreatic lipase as the FFA profile after digestion of olive oil with different food matrices followed the same pattern, regardless the structure characteristics of the digestion medium in which lipolysis occurred.

However, focusing on the extent to which FFA were released, the type of matrix did have an evident effect. The food matrices that were rich in carbohydrate (potato and bread) released higher amounts of all the analysed FFA compared with protein-rich matrices.

Within carbohydrate matrices, the total release of FFA was higher in potato than in bread. This finding could be related to the nutrient composition of both matrices. The high presence of dietetic fibre such as β-glycan in bread along with high carbohydrate content is related to viscous digestion medium (Kristensen & Jensen, 2011). It has been

200 previously shown that viscosity is a parameter that negatively affects lipolysis as it
201 hinders the accessibility of lipases to their substrate, the fat globules that are present in
202 the digestion medium (Sasaki & Kohyama, 2012). Another possible explanation could
203 be related to bread protein. In bread, starch granules are embedded in a continuous
204 protein network composed of gluten, which is a structural element reducing starch
205 digestibility (Jenkins et al. 1987). This reduction in starch digestibility contributes to
206 higher viscosity to the digestion medium, reducing lipolysis extent: viscosity decreases
207 as polysaccharide chains become hydrolysed (Bedford & Classen, 1992). Furthermore,
208 bread moisture is lower than in the rest of the assessed foods, which drastically
209 increases the viscosity and consistency of the digestion medium with the consequent
210 implications in enzymes accessibility to substrates (Gouseti, Bornhorst, Bakalis &
211 Makie, 2019). In potato, however, as a plant food, starch is naturally stored in the
212 endosperm of grains and tubers as granules. Potato was microwave-cooked, so starch
213 was in the amorphous rather than in the crystalline state, making it more susceptible to
214 amylases. Therefore, gelatinised starch could have been more hydrolysed, and thus
215 could have reduced consistency of the digestion medium, facilitating lipase accessibility
216 to fat, increasing lipolysis (Capuano, Oliviero, Fogliano & Pellegrini, 2018).

217 Conversely, degreased fresh cheese, hake and turkey, which are protein-rich
218 matrices in contrast to bread and potato, presented with a lower release of FFA. Proteins
219 are surface-active components that compete for occupying the oil-water interfaces at the
220 surface of fat globules during lipolysis, where about 80% of total lipolysis takes place
221 (Golding & Wooster, 2010). The lipase-colipase complex has to be adsorbed onto the
222 surface of fat droplets to hydrolyse the lipid substrate into FFA. However, there are
223 some factors that can prevent this reaction. For example, if proteins are located at the
224 interface, the access surface of lipases is limited. In this context, the role of bile salts

becomes crucial as they displace protein, easing the enzyme-substrate contact (Ye et al., 2018). However, in the simulated intestinal conditions of EPI, bile salts concentration was 10 times lower than in a normal physiological situation. The low concentration could probably explain that in these matrices FFA release was much lower, as bile salts were possibly not able to displace the protein of the interfaces (Pilosof, 2017). Additionally, some protein, such as soy-isolated protein, are known to be resistant to bile acid displacement from the fat globule surface, in contrast to lactoglobulins, for example, which are easily removed by the action of the bile (Bellesi, Pizones Ruiz-Henestrosa & Pilosof, 2014). However, most of these studies have been conducted in the context of emulsion stabilisation, and few studies address the role of dietary proteins (from fish, meat, etc.) at the interfacial level as determinants of lipid digestion in real food. As for concrete differences between protein-rich matrices in terms of total FFA release, proteins from fish seem to be more easily digested than those from other animals because of the lower collagen presence in fish muscle (Kong, Tang, Lin & Rasco, 2008). In addition, gastric pepsin has been suggested to have more affinity for myofibrillar and sarcoplasmic proteins than for connective ones, which are more fibrous and difficult to hydrolyse. So, the slight difference in lipolysis extent between hake and turkey could be attributed to the type of protein they are made of.

The results of total FFA released from olive oil co-digestion with individual fat-free matrices, and in binary and ternary combinations, are summarised in **Figure 2**. Given the noticeable differences between bread and potato, the study of association between FFA release and food matrix characteristics are presented separately.

Taking as a reference matrix either potato (**Figure 2a**) or bread (**Figure 2b**), total FFA release from protein-rich matrices was indeed significantly lower, $p<0.001$ and $p=0.047$ respectively. Then, when combining a carbohydrate with a protein matrix,

resulting total FFA release was an intermediate value. Thus, concerning the effect of matrices co-digestion, the fact of combining these carbohydrate-matrices with a rich-protein one (hake, turkey or degreased fresh cheese), diminished the major difference regarding the amount of olive oil-FFA released ($p<0.001$ in potato and $p=0.047$ in bread). Thus, both carbohydrate-matrices, bread and potato, showed similar amount of total FFA released from olive oil when they were digested with protein-rich food such as hake, turkey or degreased fresh cheese. In the case of co-digestion with bread, significant but small differences in FFA release were observed between the three protein matrices, while in potato, these were more noticeable. Finally, when additionally combined with degreased fresh cheese in a ternary system, the differences between both were minimised.

Overall, carbohydrate-rich matrices presented higher FFA release than protein-rich matrices. While hake, degreased fresh cheese and turkey co-digestion with olive oil showed similar results, in the case of potato and bread higher differences were found. Then, when combining one carbohydrate matrix with one or two protein matrices in co-digestion with olive oil, total FFA release decreased, reaching similar values than protein matrices digested alone. Thus, the ratio between carbohydrate and protein as a possible determinant of this finding was explored. As shown in **Figure 4c** and **4d**, this ratio was in fact significantly associated with the total release of FFA, both for bread and potato co-digestions with the other matrices ($p<0.001$ and $p=0.04$ respectively). Additionally there was a strong correlation between total FFA release and carbohydrate/protein ratio, R^2 being 0.87 for potato and 0.81 for bread.

Overall, the main finding of this study relates to the effect of the carbohydrate/protein ratio on FFA release, which should be taken into account when

establishing the criteria to adjust the dose of pancreatic enzyme supplements in the treatment of EPI.

4. CONCLUSIONS

Co-digestion of different food matrices has been explored regarding its effect on FFA release of olive oil as a model of high-fat food. Our results evidence that at the intestinal stage, FFA profile of olive oil is not affected by the foods that accompanied it along digestion, as expected. However, lipolysis is dependent on the type of food matrix which olive oil is co-digested with: it was higher when olive oil was co-digested with carbohydrate-rich matrices (potato and bread) than when it is ingested together with protein-rich matrices (hake, degreased fresh cheese and turkey). When combining matrices in the same digestion, lipolysis tends to decrease as the carbohydrate/protein ratio decreases by the addition of protein-rich matrices to bread and potato. In conclusion, this study supposes a first step towards characterisation of nutrient interactions and meal-factors of combined digestion of foods, guaranteeing further thorough research.

5. ACKNOWLEDGEMENTS

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FIGURES

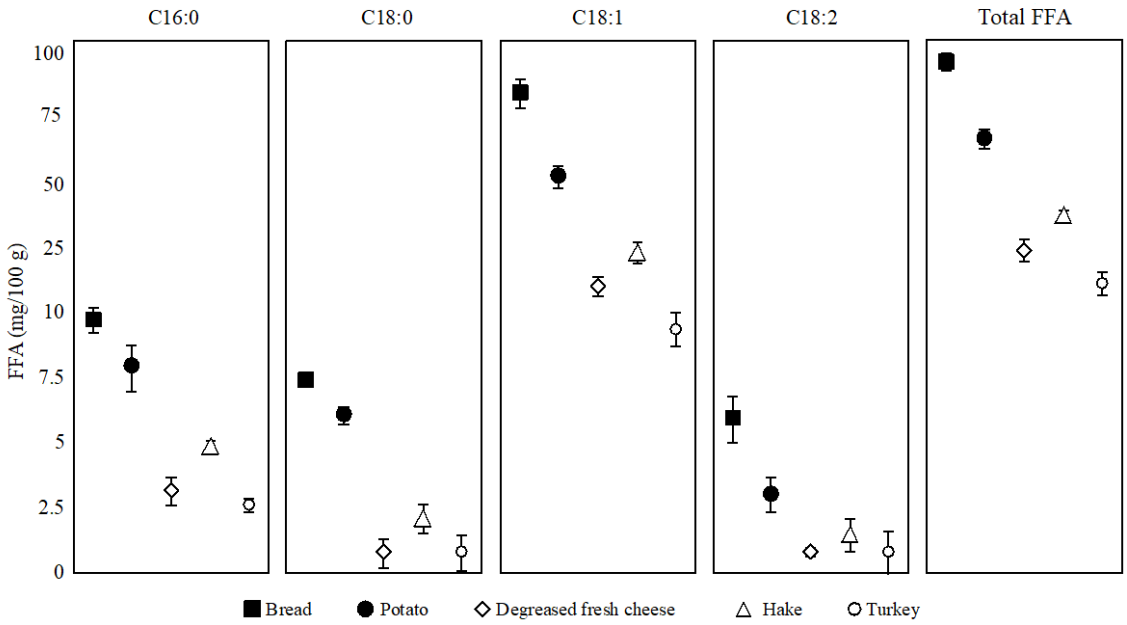
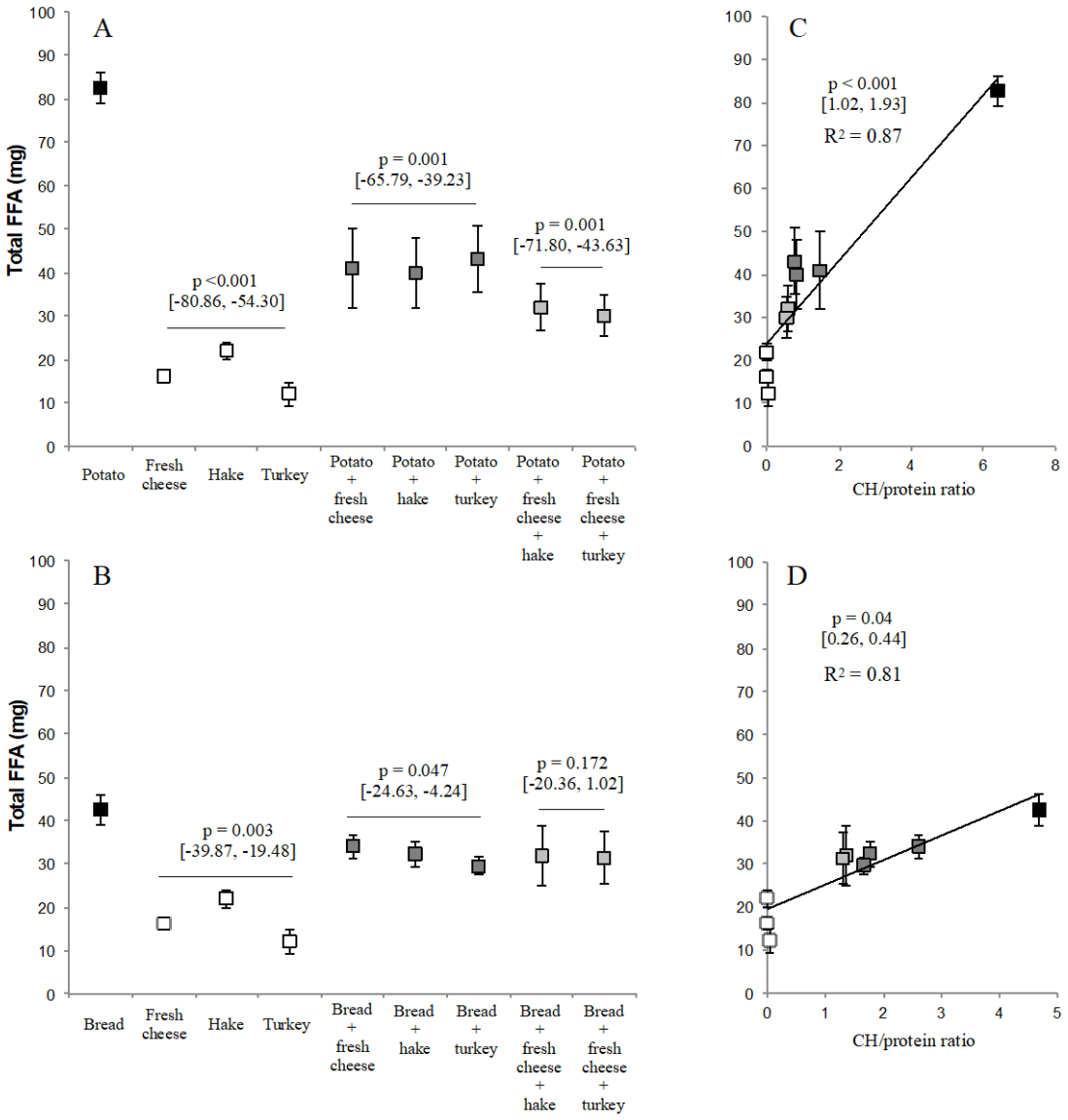


Figure 1. Free fatty acid (FFA) profile of olive oil when digested with bread, degreased fresh cheese, hake, potato and turkey. C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, lionleic acid. ■Bread, ● Potato, ◇ Degreased fresh cheese, Hake and Turkey



398

399 **Figure 2.** Effect of co-digestion of olive oil with carbohydrate (black) and protein
400 (white) rich fat-free matrices and in binary (dark grey) and ternary (light grey)
401 combinations. Total FFA release from co-digestion of olive oil with potato (A) and
402 bread (B) and combinations with the protein-rich matrices. Correlation between the
403 carbohydrate/protein ratio and total FFA release in the series of combinations with
404 potato (C) and bread (D). Predictive statistical parameters (95% Confidence Interval,
405 CI; and p-value) were obtained by means of linear mixed regression models, taking total
406 FFA release from potato and bread as reference. Linear correlations between total FFA
407 release and carbohydrate/protein ratio are expressed with the R^2.

Table 1. Experimental design. Combination of the lipid source (olive oil) with the naturally fat-free food matrices: mass proportions and resulting macronutrient profile.

	Fat-free food matrices			Nutrient composition g/100 g (g in the resulting system)					
	CH matrix (g)	Protein matrix (g)		CH	Protein	Fibre	Moisture	Lipid (from added 0.5 g olive oil)	CH/ protein ratio
Co-digestion of olive oil with 1 food matrix	bread 4.5	-	-	45 (2.03)	9.6 (0.43)	4.2 (0.19)	38.4 (1.73)	0 (11.1)	4.69
	potato 4.5	-	-	14.8 (0.67)	2.3 (0.1)	2.1 (0.09)	80.7 (3.63)	0 (11.1)	6.43
	-	degreased fresh cheese 4.5	-	0 (0)	7.7 (0.35)	0 (0)	92.2 (4.15)	0 (11.1)	0
	-	hake 4.5	-	0 (0)	15.8 (0.71)	0 (0)	82.7 (3.72)	0 (11.1)	0
	-	turkey 4.5	-	0.6 (0.03)	17.8 (0.8)	0 (0)	81.1 (3.65)	0 (11.1)	0.03
Co-digestion of olive oil with 2 food matrices	bread 2.25	degreased fresh cheese 2.25	-	22.5 (1.01)	8.65 (0.39)	2.1 (0.09)	65.3 (2.94)	0 (11.1)	2.60
	bread 2.25	hake 2.25	-	22.5 (1.01)	12.7 (0.57)	2.1 (0.09)	60.6 (2.72)	0 (11.1)	1.77
	bread 2.25	turkey 2.25	-	22.8 (1.03)	13.7 (0.62)	2.1 (0.09)	59.75 (2.69)	0 (11.1)	1.66
	potato 2.25	degreased fresh cheese 2.25	-	7.4 (0.33)	5.0 (0.23)	1.05 (0.05)	86.5 (3.89)	0 (11.1)	1.48
	potato 2.25	hake 2.25	-	7.4 (0.33)	9.05 (0.41)	1.05 (0.05)	81.7 (3.68)	0 (11.1)	0.82
	potato 2.25	turkey 2.25	-	7.7 (0.35)	10.05 (0.45)	1.05 (0.05)	90.9 (3.64)	0 (11.1)	0.77
Co-digestion of olive oil with 3 food matrices	bread 1.5	degreased fresh cheese 1.5	hake 1.5	15.0 (0.68)	11.0 (0.5)	1.4 (0.06)	71.1 (3.2)	0 (11.1)	1.36
	bread 1.5	degreased fresh cheese 1.5	turkey 1.5	15.2 (0.68)	11.7 (0.53)	1.4 (0.06)	70.6 (3.18)	0 (11.1)	1.30
	potato 1.5	degreased fresh cheese 1.5	hake 1.5	4.93 (0.22)	8.6 (0.39)	0.7 (0.03)	85.2 (3.83)	0 (11.1)	0.57
	potato 1.5	degreased fresh cheese 1.5	turkey 1.5	5.13 (0.23)	9.27 (0.42)	0.7 (0.03)	84.7 (3.81)	0 (11.1)	0.55

bread potato degreased fresh cheese hake turkey

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