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In vitro digestion of salmon: influence of processing and intestinal conditions on macronutrients digestibility

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Abstract

Salmon is the main dietary source of omega-3 lipids and contains high-biological value protein. However, processing techniques could affect macronutrient digestibility. Also, altered intestinal conditions, particularly given in pancreatic insufficiency, could threaten digestibility. This study tested both hypotheses by subjecting raw, marinated and microwave-cooked salmon to static in vitro digestion under healthy (pH 7, bile concentration 10 mM) and altered (pH 6, bile 1 or 10 mM) intestinal conditions with different pancreatin concentrations. In the standard conditions, proteolysis was not affected by processing, but lipolysis decreased in marinated (46%) and raw salmon (57%) compared to the cooked matrix (67%). In altered conditions, proteolysis and lipolysis decreased to different extents depending on the treatment. Overall, processing affected proteolysis the most (f-ratio=5.86), while intestinal conditions were the major determinants of lipolysis (f-ratio=58.01). This study could set the ground to establish dietary recommendations of salmon for specific population groups.

Keywords: pancreatic insufficiency; lipolysis; proteolysis; salmon, processing

1. INTRODUCTION

In the last decade, oily fish consumption has increased due to the awareness raised on their beneficial effects in health. Oily fish contains high biological value protein and polyunsaturated fatty acids (PUFA), which contribute to reduced risk of obesity, inflammatory and cardiovascular diseases and hypertension (Cohen et al., 2005; Hosomi, Yoshida & Fukunaga, 2012). PUFAs are the most important fatty acids (FA) since their dietary sources are limited to not so frequently consumed foods, and they exert relevant for physiological functions. For these reasons, the World Health Organisation recommends that PUFA intake should be 10% of the daily energy intake. Additionally, a balanced omega-6:omega-3 ratio (close to 1:1) is advised in order to prevent from inflammatory status and development of diseases such as obesity and cancer. However, current dietary patterns situate the ratio in 20:1 due to the rare intake of fish among other sources of omega-3 (Simopoulos, 2016). In this sense, salmon is of special interest as compared to other fishery products, contains higher contents of omega-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). When absorbed, these fatty acids are transformed in bioactive compounds with cytoprotective and anti-inflammatory activities, either via generation of anti-inflammatory products (e.g. resolvins) or by blocking inflammatory agents. Thus, EPA and DHA contribute to the prevention and treatment of numerous diseases, especially those in which inflammation plays a relevant role (Calder, 2006). Altogether, regular consumption of salmon contributes to reach the recommended PUFA intake and to balance the desirable ratio.

Although salmon can be consumed raw in some culinary preparations, it is usually subjected to processing, such as–thermal treatments (baking, boiling, frying, etc.), smoking, marinating or salting. Foods subjected to certain heat treatments (boiling, baking, etc.) result in different sensorial and structural properties and a loss of water,

which finally concentrates other components such as fat, proteins or minerals. In addition, the reduction or degradation of thermolabile compounds is accounted, including vitamins and carotenoids (Gladyshev, Sushchik, Gubanenko, Demirchieva & Kalachova, 2006). Marinating and salting, on the other hand, imply salt and sugar penetration into the raw muscle, causing salt-driven protein denaturalisation together with considerable loss of muscle water, resulting in decreased fillet ~~loin~~ weight (Laub-Ekgreen, Martinez-Lopez, Frosch & Jessen, 2018). Marinating is usually an artisanal process~~,~~ and the conditions of the technique vary regionally. For example, Larrazábal-Fuentes, Escriche-Roberto & Camacho-Vidal (2009) described marinating as the application of a mix of salt and sugar for 5-72 hours at 5 ºC. In turn, gastronomy globalisation has triggered the consumption of raw fish beyond Japan or Peru, the countries where raw fish, such as sushi or ceviche, have been traditionally consumed for centuries. Therefore, assessing nutrient digestibility in different salmon preparations raises as a relevant research question, as background knowledge suggests that protein and lipid digestibility could be affected by processing (Asensio-Grau, Peinado, Heredia & Andrés, 2018; Asensio-Grau, Calvo-Lerma, Heredia & Andrés, 2019 (a); Asensio-Grau, Peinado, Heredia & Andrés (b)).

On the other hand, apart from the possible impact of food processing on nutrient digestibility, the luminal gastrointestinal alterations (such as digestive fluids composition, intestinal pH, etc.) could play an additional relevant role on digestibility (Calvo-Lerma, Fornés-Ferrer, Heredia & Andrés, 2019). In this sense, lipid digestion is the most compromised hydrolytic phenomenon when pancreatic function alterations are present (such as in exocrine pancreatic insufficiency (EPI)), as the main lipase activity comes from pancreatin secreted by this organ. Altered pancreatic function causes decreased pancreatin and bicarbonate secretions, which results in reduced pH in the intestinal medium. This fact, together with a reduced bile secretion, which is also possible in some

74 individuals suffering EPI, results in suboptimal intestinal conditions (Sarkar, Ye & Singh,
75 2016; Maldonado-Valderrama, Wilde, Macierzanka & Mackie, 2011). In this scenario,
76 compositional and/or structural changes in food resulting from processing can be
77 determinants of the lipolysis extent during digestion, some structures having proved to be
78 more accessible to lipase than others (Calvo-Lerma, Fornés-Ferrer, Heredia & Andrés,
79 2018). Individuals suffering EPI need pancreatin oral supplements to enable lipolysis
80 (namely pancreatic enzyme replacement therapy). Up to now, there is low evidence to
81 establish a dosing criterion of the supplements (Calvo-Lerma, Martínez-Barona, Masip,
82 Fornés, & Ribes-Koninckx, 2017), but recent studies suggest that food processing can be
83 used for modulating intestinal lipolysis (Guo, Ye, Bellissimo, Singh & Rousseau, 2017).
84 In this context, the aim of the present study was to assess, by means of a static in
85 vitro digestion model, the impact of salmon processing (raw, marinating and microwave-
86 cooking) against different settings of intestinal conditions and pancreatin concentration,
87 on lipid and protein digestibility.
88
89 **2. MATERIALS AND METHODS**
90 **2.1. Sample preparation**
91 Three salmon specimens were purchased at a local supermarket from the same batch.
92 On the same day of the acquisition, head, spines and tail were removed, leaving aside the
93 completely clean fillets. Then, from each salmon specimen, fillets were separated in three
94 sets and frozen (-20 ºC) in order to preserve the same starting batch of salmon for all the
95 subsequent determinations. One set was cooked using a microwave oven (Samsung
96 GW72N) at 2.25 W/g for 4 minutes. The second set was marinated according to
97 Larrazábal-Fuentes et al., (2009). For this purpose, a mixture of 50 g of salt and 50 g of
98 sugar was added to 100 g of salmon in 1:1 (w/w) ratio. The fillets were covered with the

mixture and wrapped with parafilm during 9 hours in refrigeration (5 ºC). Then, salmon was rinsed with water and dried with a paper towel to remove the excess water. The third set was not processed to assess digestibility in thawed raw salmon.

2.2. Materials

For the preparation of the simulated digestive fluids the following reagents were used: KCl, KH_2PO_4, NaCl, $NaHCO_3$, $MgCl_2$ $(H_2O)_6$, $(NH_4)_2CO_3$, $CaCl_2$, pepsin from porcine gastric mucosa (≥2500 U/mg protein) and bovine bile extract all of them from Sigma-Aldrich Chemical Company (St Louis, MO, USA).

A commercial pancreatic enzyme supplement (Kreon 10000 LU, Mylan) was used to study the impact of the pancreatin concentration. Each capsule contains 150 mg of gastro-resistant microspheres that include porcine pancreatic enzyme equivalent to 10,000 lipase units, 8,000 amylase units, and 600 protease units.

For the analytical determinations, the following products were required: Triton-X 100 %, trichloroacetic acid (TCA), dichloromethane and deuterated chloroform. All were acquired from Sigma-Aldrich Chemical Company (St Louis, MO, USA), while ethanol (96 % v/v for analysis), NaOH and HCl, were from AppliChemPanreac.

2.3. Experimental design

The experimental design consisted in two sets of experiments. In the first one, the influence of different combinations of intestinal pH and bile salts concentration (pH 6 – 1 mM, pH 6 – 10 mM and pH 7 – 10 mM) were simulated to evaluate lipolysis and proteolysis under a fixed pancreatin concentration (2000 LU/ g lipid). The intestinal conditions of pH 7 and bile salts concentration 10 mM was selected as control, or standard conditions, as they have been agreed as the standard intestinal conditions of a healthy

adult (Humbert et al., 2018), whereas the conditions of pH 6 and bile salts concentration 1 or 10 mM would represent two possible altered or suboptimal situations typically present in EPI (Humbert et al., 2018; Gelfond 2013). In the second set of experiments, different concentration of pancreatin (2000, 4000 and 6000 LU/ g lipid) were used to assess the influence of this concentration in the intestinal stage of digestion on lipolysis and proteolysis under two suboptimal intestinal scenarios (pH 6 – 1 mM and pH 6 – 10 mM). The selection of the studied range of pancreatin concentration was based on the current clinical recommendations of pancreatic enzyme supplementation in pancreatic insufficiency (Turck et al., 2016) which recommend a dose range between 2000 and 4000 LU/g fat. In addition, we assessed the 6000 LU/g fat as complete lipid hydrolysis was not reached with the referred pancreatin concentrations. In both experimental sets, the three preparations of salmon were tested. All experiments were performed at least in triplicate.

2.4. In vitro digestion

To assess the impact of altered intestinal conditions, *in vitro* digestion was carried out according to Asensio-Grau, Peinado, Heredia, & Andrés, 2018. Simulated digestion fluids (SSF, salivary; SGF, gastric; and SIF, intestinal) were prepared from the corresponding stock solutions according to Brodkorb et al., 2019. The lipase (29.2 ± 3.9 U/mg) and protease (trypsin (0.96 ± 0.08 U/mg) and chymotrypsin (0.24 ± 0.05 U/mg)) activities in pancreatin were tested prior ~~to~~ digestion in each experiment according to the protocol published by Brodkorb et al., 2019. The *in vitro* digestion process was simulated in three stages: For the oral stage, SSF (pH 7) was added in ~~a~~ 1:1 proportion (w/v) and properly homogenized using a kitchen blender for 30 seconds (Vario Mixer, Ufesa 600 W) until obtaining an equivalent consistency to tomato paste (Minekus et al., 2014). The gastric stage continued by the incorporation of the SGF (pH 3), containing gastric pepsin

149 (2000 U/mL), to the oral bolus in 1:1 proportion (v/v), and tubes were head-over-heels
150 rotated at 55 rpm for 2 hours at 37 ºC in an incubator chamber (JP Selecta SA, Barcelona).
151 Afterwards, the pancreatic enzyme supplement in the concentration of 0, 2000, 4000 or
152 6000 LU/g lipid, was added to mimic the *in vivo* intake. The corresponding protease
153 concentrations were 0, 44, 88 and 132 PU/ g protein in raw salmon, 0, 70, 140 and 210
154 PU/ g protein in marinated salmon and 0, 43, 87 and 130 PU/ g protein in cooked salmon
155 The supplement is directly swallowed without mastication and its gastroresistant coating
156 is thereafter degraded at the intestinal stage due to the pH increase. Finally, the intestinal
157 stage was simulated by adding the SIF (pH 6 or 7) and bile salts solution (bovine bile, 1
158 or 10 mM) to the gastric chyme in a 1:1 proportion (v/v), and tubes remained in agitation
159 during 2 h at 37 ºC as in the gastric stage. During all the *in vitro* digestion process, pH
160 was controlled to keep the experimental conditions of each set.
161
162 **2.5. Analytical determinations**
163 *2.4.1 Sample characterization*
164 Fat, water and protein content were determined in raw, marinated and cooked
165 salmon by following the official methods (AOAC, 2000). Water activity (a_w) was
166 measured for raw, marinated and cooked salmon by CX-2 AQUALab (Decagon Devices,
167 Inc., Pullman, WA). All determinations were performed in triplicate.
168 *2.4.2 Matrix Degradation Index (MDI (%))*
169 Matrix degradation Index (%) was estimated by considering the proportion of
170 dispersed solids in the digested fluid at the end of the intestinal stage Lamothe, Azimy
171 & Bazinet, 2014). The total content of the digestion tubes was centrifuged (4000 x g-force
172 20 minutes, 4 ºC) and then filtered on a metallic sieve (1.6 mm x 1.6 mm mesh) to separate
173 the solid fraction. The liquid fraction was used for lipolysis and proteolysis extent

174 determinations. To determine the mass of the solid large particles, the solid fraction of
175 digesta was placed in a force-air oven at 60 °C for 48 hours until reaching constant weight.
176 MDI (%) was expressed as grams of solid large particles in 100 grams of total solid
177 fraction in salmon.

178 *2.4.3 Proteolysis extent*

179 The extent of proteolysis was determined by measuring the soluble protein
180 fraction in TCA (Lamothe et al., 2014). TCA was added to the liquid fraction from
181 digested samples to a final concentration of 12% (w/w). The mixture was vortexed,
182 incubated for 15 min and filtered using a Whatman nº. 40 filter paper. The soluble fraction
183 in 12% TCA is composed of small peptides and amino acid residues. The filtrate was
184 diluted in buffer (50 mM EDTA, 8 M urea, pH 10) and protein was determined by
185 measuring absorbance at 280 nm against a prepared blank with appropriate digestion
186 fluids. Bovine Serum Albumin (BSA) was used for the quantification and proteolysis
187 extent (%), expressed as grams of soluble TCA protein in 100 grams of initial protein in
188 raw, marinated or cooked salmon.

189 *2.4.4 Lipolysis extent*

190 *Enzymatic kit assay*

191 Lipolysis was determined as free fatty acids at the end of intestinal stage in all the
192 simulated sets of intestinal conditions. Aliquots from the liquid fraction of digested
193 samples were 100-fold diluted with a solution made with 5.6% Triton X-100 and 6%
194 ethanol in water. This solution was used to both solubilize the free fatty acids and to stop
195 lipolysis reaction. The amount of free fatty acids at the end of digestion was quantified
196 using a free fatty acid colorimetric assay kit (Roche Diagnostics, Indianapolis, IN, USA)
197 and the absorbance was measured with a spectrophotometer (UV/vis, Beckman Coulter)
198 at wavelength of 546 nm (Lamothe et al., 2014). Docosahexaenoic acid standard was used

for quantitative determination of free fatty acids (FFA) in salmon. Lipolysis extent (%) was expressed as grams of hydrolysed TG in 100 grams of initial TG in raw, marinated or cooked salmon. For calculations, it was considered that one molecule of triacylglycerol results into the release of two fatty acids molecules and one monoacylglycerol (Hunter, 2001).

Nuclear Magnetic Resonance (NMR)

Lipidic fraction of undigested (raw, marinated and cooked salmon) and digested salmon in the different intestinal scenarios were analysed by H^1 NMR. Lipid extraction, spectra acquisition and quantification of lipolytic products were conducted according to Nieva-Echevarría, Goicoechea, Manzanos & Guillén (2015). The number of moles of each molecule was calculated considering acyl groups by the previously validated equations by Nieva-Echevarría, Goicoechea, Manzanos & Guillén (2014). The NMR technique allows for quantifying triglycerides, partial triglycerides (monoglycerides and diglycerides) and free fatty acids. From ~~a~~ the physiological point of view, the lipid bioaccessible fraction can be estimated considering fatty acids and monoglycerides.

2.6. Statistical analyses

In order to study the significance of the differences of the factors (processing, intestinal conditions and pancreatin concentration) on each study variable (MDI, proteolysis and lipolysis), an unifactorial analysis of variance (ANOVA) was performed using Statgraphics Centurion XVII software with a confidence level of 95 % (p-value ≤0.05). Moreover, a multifactor analysis of variance (multivariate ANOVA) was also performed with a confident interval of 99 % ($p<0.001$) and 95% ($p<0.05$) to know which factor (intestinal conditions or processing) affected the response variables (MDI, proteolysis and lipolysis) the most (F-ratio). The ~~higher~~ F-ratio value is directly

proportional to the statistical effect of each factor on the response variables. The multifactor ANOVA was only applied to the results obtained at a fixed dose of pancreatin concentration (2000 LU/ g lipid).

3. RESULTS AND DISCUSSION

3.1.Impact of food processing on salmon composition

Marinated and cooked salmon exhibited different nutrient composition compared to raw salmon as a result of processing (**Table 1**). Marinating was the method affecting water and protein contents the most, with a reduction of 28 and 13%, respectively. Salt addition is known to cause muscle dehydration, along with washing away of hydrosoluble protein, resulting in a significant protein loss (Hao, Dong, Li & Lin, 2016). In contrast, cooking imparted in salmon a higher change in lipid content, which decreased in 5%. High temperatures reached in microwaving caused lipid melting and its subsequent exudation, along with a partial loss of 6.5% of the soluble protein fraction (Farmer, McConnell & Kilpatrick, 2000). Water activity was also affected by processing, which particularly decreased in the marinated salmon, by the incorporation of sucrose and salt.

Complementarily to the quantification of total lipid content, the different lipid species of salmon were determined by NMR. Concretely, NMR allows to specifically quantify triglycerides, partial triglycerides (monoglycerides and diglycerides) and free fatty acids. Looking into lipids more thoroughly, the NMR analysis (**Figure 1**) depicted that the majority of lipid species in all three undigested salmon samples were triglycerides, while free fatty acids and monoglycerides represented a small percentage (less than 5%), and 1,2 and 1,3 diglycerides were in marginal proportions (≈ 0). This result was expected, as the majority of dietary lipids are known to be triglyceride structures, which are thereafter the main substrate for lipases during intestinal digestion, as shown in the coming sections

249 (Hunter, 2001). However, small differences in monoglycerides and fatty acids were
250 detected between the three preparations, raw salmon resulting with the highest amount of
251 fatty acids. As above-commented, part of the water content was lost during cooking and
252 marinating. Both processes led to a decrease in fatty acids, since this chemical structure
253 is simpler and present lower molecular weight and higher solubility in the aqueous
254 fraction than other lipidic species. On the other hand, marinated salmon showed higher
255 monoglyceride content than the raw and cooked samples. During the post-mortem
256 storage, some endogenous enzymes are activated and account for different biochemical
257 changes, their activity being determined by the amount of water in the muscle.
258 Particularly and according to Motilva & Toldrá, (1993), acid lipase and acid esterase
259 enzymes, which participate in muscle lipolysis, are activated when the water activity is
260 decreased. Thus, the explanation behind this finding could rely on the loss of water
261 occurring in marinated salmon, and also on the presence of salt and sugar, which leads to
262 decreased water activity (Hao et al.,2016). Furthermore, lower water activity increases
263 the hydrolytic activity of lipases as well, since these enzymes exert their action on the
264 hydrophobic surface of lipids (Toldrá, 2003).
265
266 **3.2. Influence of food processing on salmon macronutrient digestibility**
267 Results of matrix degradation index (MDI), lipolysis and proteolysis of raw,
268 marinated and cooked salmon when digested in simulated standard in vitro digestion
269 conditions (intestinal pH of 7, bile salts concentration of 10 mM and pancreatic
270 concentration of 2000 LU/ g lipid) are shown in **Figure 2A**.
271 During digestion, several key factors contribute to the progressive disruption of the
272 food matrix, including the enzymes taking part throughout the gastrointestinal tract, the
273 acidic conditions in each stage, and the peristaltic movements. Previous food processing
274 also affects the mechanical changes experimented by the food matrix during digestion.

Thus, cooked salmon presented the least MDI after gastrointestinal digestion, while marinated process did not show differences compared to raw salmon (**Figure 2A**). This result could be probably related to protein denaturation induced by the high temperatures reached during microwave-cooking (Asensio-Grau et al., 2018). Overall, MDI provides a relevant insight to figure out complex solid matrices' disruption during digestion. In most of the cases, the release and digestibility of nutrients are influenced by the complexity of the food matrix (Guo et al., 2017). In general, the MDI (%) is directly proportional to macronutrient digestibility (Asensio-Grau et al., 2018; Asensio-Grau et al., 2019 (a)). However, the lower degradation achieved by cooked salmon was not directly related to the nutrient digestibility thereafter, as later on discussed.

Concerning protein digestibility (**Figure 2A**), marinated and cooked salmon showed a slight (but not statistically significant) decrease in proteolysis compared to the raw counterpart. Cooking temperatures and times seem to be the main factors affecting protein structure during cooking process, due to underlying mechanisms such as aggregation and oxidation (Asensio-Grau et al, 2018; Bax et al., 2012; Promeyrat, Gatellier, Lebret, Kajak-Siemaszko, Aubry & Sante-Lhoutellier, 2010). Similarly, marinating promotes protein oxidation, affecting functionality due to cleavage of protein bounds or modifications in aminoacids side chains (Zhang, Xiao, & Ahn, 2013; Estévez, Ventanas, & Cava, 2005). However, the relevance of the changes imparted by processing on proteolysis depends on luminal gastrointestinal conditions as well, as explained in the next section.

Concerning lipolysis, the structural changes imparted by the different processing techniques showed an impact, but the accounted effects in the three salmon types were different than that in proteolysis (**Figure 2A**). Focusing on total lipolysis extent at the end of the intestinal stage, 57% was registered in raw salmon. Compared to this one, cooked

300 salmon showed improved lipolysis (68%), while marinating led to a decrease to 45%.
301 Increased lipolysis in the cooked sample can be explained by higher lipid release during
302 cooking (exudation), thus allowing for higher extractability during digestion (Larsen,
303 Quek & Eyres, 2010) and eventual lipolysis. In contrast, lipids in raw salmon seem to be
304 more strongly bound to the matrix tissue, becoming difficult to release during digestion.
305 Indeed, despite the literature reports that 95% of dietary lipids digestion is generally
306 achieved, recent studies in specific foods reveal that some food characteristics, such as
307 the matrix structure or the nature of lipids can prevent from complete hydrolysis (Guo et
308 al., 2017; Calvo-Lerma et al., 2018). In this sense, the relatively low lipolysis extents
309 obtained in this study, could be explained by the complex food matrix of salmon in which
310 lipid fraction is entrapped in a protein fibres structure. Similarly, Grundy et al. (2016)
311 found that in almonds, the lignin structure of the plant cell walls in which lipids are
312 contained, prevented from being release to the digestion medium, resulting in lipolysis
313 extents lower than 60%.

314 Conversely, the explanation for lower lipolysis as a consequence of marinating could
315 be supported by the presence of NaCl in the digestive medium. During marinating, salt
316 and sugar were solubilised into sample muscle water by osmosis (Rastogi, 2020), and
317 thereafter released to the digestion medium. According to Chaparro, Gil & Aristizábal
318 (2011), a concentration of salt above 0.4 M increases the ionic strength in the liquid
319 medium and reduces the interfacial activity of emulsifiers. Of note, effective lipid
320 digestion depends on the presence of emulsifiers, such as bile salts, at the surface of fat
321 droplets. Considering that in marinated salmon 25% of total weight was salt (1.07 M in
322 the intestinal medium), the formation of lipid micelles could have been affected by the
323 high ionic strength.

In addition to the quantification of total lipolysis extent, the different lipid species (triglycerides, diglycerides, monoglycerides and fatty acids) coming from the hydrolytic process on lipids were determined by NMR. The quantification of these lipolytic products allows for estimation of lipolysis extent, and the bioaccessible fraction by considering monoglycerides and free fatty acids. In **Figure 2B**, results obtained from the NMR spectra are presented for the three salmon products. They are coherent with previous literature (Hunter, 2001) because, as expected, total triglycerides decreased while free fatty acids increased during digestion. The higher amount of 1,2-DG released during digestion could be explained by the pancreatic lipase stereopreference for the sn-3 position in the triglyceride (Carrière et al., 1997). Moreover, pancreatic lipase has less affinity for hydrolysing the ester bond in the sn-2 position in the triglyceride. Therefore, the low amount of 1,3-DG presented in the digested sample could be mainly due to the 1,2-DG isomerization reaction, which is catalysed by the free fatty acid released during digestion (Nieva-Echevarría et al., 2015; Spyros, Philippidis, & Dais, 2004). On the other hand, as the figure shows, processing also had a determinant role in the resulting lipolysis species. Concretely, cooking led to a higher amount of 1,2-DG, 1,3- DG and FA release after digestion, while marinated process favoured the most a decrease of diglycerides and fatty acids species.

Therefore, comparing **Figure 2A** and **2B**, the differences in lipolysis among the three samples depicted the same pattern as in the results obtained by means of the free fatty acid kit: cooked salmon resulted in the highest molar percentage of free fatty acids, followed by raw and marinated salmon. When assessing the correlation between both methods (NMR and free fatty acid kit), a correlation coefficient of 0.97 was obtained ($p<0.001$). Thus, in this study a comprehensive assessment of lipolysis was conducted, which was also validated against two methods.

Overall, the present results evidence that different processing techniques applied to salmon have an impact on nutrient digestibility. However, as before commented, intestinal conditions can further modulate the fate of protein and lipid digestibility. These are addressed and discussed in the coming section.

3.3. Impact of intestinal pH and bile salts concentration on macronutrients digestibility in salmon products

Digestive fluids are mainly composed by salts, enzymes and amphiphilic molecules that facilitate macronutrient breakdown and absorption. Digestive fluids secretion and composition are commonly altered in some pathologies, especially in EPI and diseases affecting the biliary tract. In order to understand digestibility mechanisms in altered intestinal conditions, the study of these variables should be addressed, besides the sole objective of assessing the food matrix effect. Previous studies have focused on characterising the role of gastrointestinal conditions on macronutrient digestibility, in the context of EPI, pointing out that amongst them, intestinal pH and bile salts concentration are the major determinants (Calvo-Lerma et al., 2019). However, both factors entail variable effects on digestibility depending on the type of food (Asensio-Grau et al., 2019 (b); Calvo-Lerma et al., 2018). The intestinal environment is therefore a cornerstone and should be considered in lipid digestibility studies.

Consequently, in the present study, two different sets of altered intestinal conditions were simulated: reduced pH with normal bile salts concentration (pH 6, bile salts 10 mM) which represent a standard EPI situation due to reduced sodium bicarbonate pancreatic secretion (Humbert et al 2018); and the worst-case scenario with reduced pH and bile concentration (pH 6, bile salts 1 mM) which is likely to occur when EPI is combined with reduced biliary secretion (Humbert et al 2018; Carrier et al., 2005). Results of MDI,

proteolysis and lipolysis under the mentioned simulated altered conditions and fixed pancreatic concentration of 2000 LU/g lipid are presented in **Figure 3 (A** and **B)**.

Matrix degradation was conditioned by the intestinal scenario, either by reduced pH or by both low pH with low bile salts concentration. As the main constituents of salmon are protein and lipid, the MDI mainly depends on the protein and lipid structural changes occurring during digestion. In the framework of altered intestinal conditions, these showed to compromise protein hydrolysis, which could be mainly attributed to the low concentration of bile salts. According to Gasset, Vora, Hofmann, Gray & Khosla (2007), bile salts presence is proportionally related to the pancreatic proteases-effected (trypsin and chymotrypsin) protein digestion. Besides, surfactants as bile salts, are known to affect the protein structure making it more accessible to the proteolytic enzymes (Mackie & Macierzanka, 2010). Low intestinal pH also led to decreasing proteolysis, since pH 8.1 is the optimal for trypsin activity, and pH 7.8 is for chymotrypsin (Minekus et al., 2014). Thus, the main changes in MDI during digestion in altered conditions could be explained by the proteolysis extent in salmon. Apart from differences in proteolysis compared to the standard intestinal conditions, salmon processing techniques led to different patterns, as above anticipated. In normal intestinal conditions protein changes resulting from processing did not exhibit a significant effect on proteolysis. However, conformational alterations of proteins affecting molecule solubility seems to have a noticeable effect on proteolysis under suboptimal intestinal conditions. Both marinating and cooking processes seemed to lead to protein hydrophobicity, aggregation, interfering in protein hydrolysis during digestion, as previously described by Sun, Zhou, Zhao, Yang & Cui (2011).

As expected, compared to the standard healthy conditions (**Figure 2**), total lipolysis extent significantly decreased in all fish preparations. The optimal pH for the pancreatin

399 activity ranges between 7 and 9 (Li & Somerset, 2014). On the other hand, the bile salts
400 help in the emulsification of fat droplets, contributing to decrease particle size, thus
401 enhancing lipase access lipolysis (Maldonado-Valderrama et al., 2011). With regard to
402 the resulting lipidic species (NMR) (**Figure 3 C** and **D**), the amounts of generated
403 monoglycerides (1-MG or 2-MG) were insignificant in all cases. The concentration of
404 diglycerides (1,2-DG or 1,3-DG) were, however, dependent on the intestinal conditions,
405 particularly in raw and marinated salmon. Standard pH (7) and bile salts concentration
406 (10 mM) led to slightly higher release of 1,2-DG. Despite pancreatic lipases rarely deliver
407 1,3-DG, their presence in low proportions indicate the possible 1,2-DG isomerization
408 reactions during digestion (Nieva-Echevarría et al., 2015). Conversely, the presence of
409 1,2-DG on favourable intestinal conditions suggests the adequacy of the intestinal
410 environment to promote lipid digestibility, specifically regarding the role of bile salts. For
411 their part, low pH and also low bile salts concentration contribute to decreased 1,2-DG
412 and FA release (**Figure 3 C** and **D)**.

413 Altogether, results confirm that processing and intestinal environment conditions
414 are determinants in the process of digestion. Taking the advantage this multi-variable
415 study offered, the relative effect that each variable on proteolysis and lipolysis was
416 assessed by means of estimating the variance. **Table 2** shows a multivariate ANOVA to
417 evaluate the factor (intestinal conditions or processing) that affected (F-ratio) the response
418 variables (MDI, proteolysis extent, lipolysis extent and molar percentage of FA) the most.
419 The standard intestinal conditions and the both altered intestinal conditions were taken
420 into account for the multifactor analysis of variance. As presented in **Table 2**, food
421 processing and also intestinal conditions affected matrix degradation during digestion and
422 had a similar and significant effect on lipid and protein digestibility. The intestinal
423 conditions were the factors affecting lipolysis the most, both in terms of lipolysis extent

and molar percentage of fatty acids, while the processing method presented a higher impact on proteolysis in both unfavourable EPI intestinal scenarios.

3.4. Influence of pancreatin concentration on lipid and protein digestibility in salmon products

As shown in the previous section, the intestinal conditions are crucial for the optimal digestibility of salmon macronutrients, particularly lipids. Shedding light on this situation is of special relevance in EPI, in which energy and lipid dietary intake requirements are increased (40% from total daily energy intake). In addition, the lipid supply should be represented by healthy sources, i.e. with polyunsaturated fatty acid profile (Turck et al., 2016). Hence, salmon presents as an ideal food to support the nutritional treatment of EPI, as it contains considerable amount of fat, which is mainly omega-3. As a consequence of EPI, patients have to take oral supplements of pancreatic enzymes to enable digestion. So, optimising the dose of this supplements to maximise lipolysis and eventual energy uptake, would be a worthwhile purpose.

In this sense, the present study addressed lipolysis and proteolysis extents obtained with different pancreatin concentration (0, 2000, 4000 and 6000 LU/g lipid) under both possible intestinal scenarios in EPI subjects (pH 6 - bile salts concentration 1 mM and pH 6 – bile salts concentration 10 mM). **Figure 4** shows MDI, proteolysis and lipolysis extents obtained in all salmon samples at the different enzyme concentrations. The effect of increasing pancreatin concentration on MDI was noticed in the worst-case set of conditions, while it showed no effect in the case of normal bile salt concentration (10 mM). Regarding proteolysis, the pancreatin concentration seemed to have only minor effects, except in the case of cooked salmon, in which protein were denatured as a consequence of the high temperatures. The subsequent conformational changes imparted

by this processing seem to have negatively affected the role of proteases contained in the enzymatic supplement in breaking down the protein molecules. Moreover, denatured proteins suggest to have influenced the interaction between fish protein and bile salts, as discussed in the previous section.

Concerning lipid hydrolysis (the main nutrient of interest in this framework) cooked salmon exhibited the highest extent, compared to raw and marinated samples, regardless the pancreatic concentration; while similar results were achieved in marinated and raw salmon, in both sets of simulated conditions. Focusing on cooked salmon, lipid digestibility showed an increasing tendency with the pancreatin concentration in the frame of worst-case conditions (pH 6 – bile salts concentration 1 mM), while no improvement was shown when the conditions included normal bile salts concentration (pH 6 – bile salts concentration 10 mM). In contrast, raw salmon resulted in slight increases of lipolysis as the pancreatic concentration increased in both situations. Finally, marinated salmon, in the two scenarios, allowed for increased lipolysis when moving from 2000 to 4000 LU/g lipid, but remaining stable if the pancreatin concentration increased to 6000 LU/g lipid. None of the experiments led to lipolysis extents values above 60%. So, despite the registered increasing tendency with the pancreatin concentration in some cases could suggest further increments in total lipolysis, this result would prevent from expecting complete fat hydrolysis.

Considering the maximum pancreatin concentration, either with normal or reduced bile salts concentration, salmon fat would not be optimally digested. At most, around 60 % of lipolysis extent could be expected in the case of cooked salmon at the maximum pancreatin concentration. In practical terms, and considering the increase from 4000 to 6000 LU/g fat only accounts for minor increase of lipolysis, 4000 LU/g lipid would be the optimal pancreatin concentration for salmon intake in the context of EPI intestinal

474 conditions. So, a recommendation for pancreatic enzyme replacement therapy in EPI
475 should be supplying 4000 LU/g of lipid when salmon is consumed.

476 Despite satisfactory levels of salmon fat digestion should not be expected in patients
477 coursing with EPI, the simulation of the healthy conditions, according to the present
478 study, would not either fulfil complete lipolysis, and around 80% would be the expected
479 extent. Incomplete lipid digestion may have several implications. Recent studies suggest
480 that undigested fat reach the colon carrying away bile salts (Louis et al., 2014). The
481 microbiota in the colon is able to metabolise the bile salts in secondary bile acids, which
482 can accumulate to high levels in the enterohepatic circulation of some individuals and
483 may contribute to the pathogenesis of colon cancer, gallstones, and other gastrointestinal
484 (GI) diseases (Ridlon et al., 2006). More generally, incomplete lipid digestion is directly
485 associated with lipid malabsorption and excretion in faeces, which is a regular condition
486 in cystic fibrosis and pancreatic insufficiency. Thus, the energy value of a food with
487 incomplete lipid digestion should be considered to be lowered, rather than assuming the
488 value of a complete nutrient digestion. The elimination of fat with faeces also implies that
489 liposoluble vitamins are carried away too (Domínguez-Muñoz, 2011).
490

491 **4. CONCLUSIONS**

492 The results of the present study provide a thorough characterisation of digestibility of
493 salmon macronutrients (lipids and protein) as conditioned by processing and intestinal
494 conditions. In the situation of standard healthy intestinal conditions, proteolysis was not
495 largely affected by processing. Lipid digestibility, however, improved when salmon was
496 cooked, marinated process resulting in the lowest lipolysis extent. Nonetheless, the
497 intestinal conditions negatively affected both salmon proteolysis and lipolysis,
498 specifically the combination of low pH (6) and low bile salts concentration (1 mM)

corresponding to the most adverse intestinal scenario of EPI. The reduced pH and bile salts concentration also promoted decreased 1,2-DG and FA release, thus representing a drawback for lipolysis.

The results also confirmed that the concentration of pancreatin did not have an effect on proteolysis, being 2000 LU/g lipid the optimal dose to reach the highest proteolysis under both EPI intestinal conditions. Only marinated salmon increased lipolysis when moving from 2000 to 4000 LU/g fat, but not changing if the dose increased to 6000 LU/g fat. Despite of increasing pancreatin concentration under both EPI intestinal scenarios, lipolysis extent did not reach values above of 60% regardless the processing technique.

Overall, salmon preparations affect macronutrients digestibility, lipolysis being compromised in any intestinal scenario. The consumption of cooked salmon instead of other preparations would be recommendable in order to enhance lipolysis, especially in EPI patients. The results of this study can provide evidence to establish dietary recommendations regarding salmon consumption and support to explain results in further in vivo studies.

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662 **Figure caption**

663 **Figure 1.** Distribution of lipid species present in raw, marinated and cooked salmon,
664 assessed by nuclear magnetic resonance. The small graphic shows a zoom of the 2-MG, 1-
665 MG and FA. *Different letters mean statistically significant ($p<0.05$) differences between
666 salmon preparations.

667 **Figure 2.** Matrix degradation index (%), proteolysis (%) and lipolysis extent (%)
668 achieved in raw, marinated and cooked salmon under standard intestinal conditions (pH
669 7, bile salts concentration 10 mM) and pancreatin concentration 2000 LU/ g lipid **(A).**
670 Distribution of lipid species (molar percentage) in terms of triglycerides (TG), 1,3-
671 diglycerides (1,3-DG), 1,2-diglycerides (1,2-DG), 2-monoglycerides (2-MG), 1-
672 monoglycerides (1-MG) and fatty acids (FA) under standard intestinal conditions and
673 pancreatin concentration 2000 LU/ g lipid **(B)**. The small graphic shows a zoom of the
674 1,3-DG, 1,2-DG, 2-MG and 1-MG. *Different letters mean statistically significant
675 ($p<0.05$) differentces between salmon preparations. Results correspond to 120 min of
676 intestinal digestion.

677 **Figure 3.** Matrix degradation index (%), proteolysis (%) and lipolysis extent (%) in the
678 salmon samples after in vitro digestion under both altered intestinal scenarios and
679 pancreatin concentration of 2000 LU/ g lipid: reduced pH and bile salts concentration (pH
680 6, bile salts concentration 1 mM) **(A)** and reduced pH and normal bile salts concentration
681 (pH 6, bile salts concentration10 mM) **(B)** . Distribution of lipid species (molar
682 percentage) under both intestinal scenarios and pancreatin concentration 2000 LU/ g
683 lipid: reduced pH and bile salts concentration **(C)** and reduced pH and normal bile salts
684 concentration **(D)**. The small graphic shows a zoom of the 1,3-DG, 1,2-DG, 2-MG and 1-
685 MG. *Different letters mean statistically significant ($p<0.05$) differences between salmon
686 preparations. Results correspond to 120 min of intestinal digestion.

687

688 **Figure 4.** Matrix degradation index (%), proteolysis (%) and lipolysis extent (%)
689 achieved in raw, marinated and cooked salmon at different pancreatin concentration (0,
690 2000, 4000 and 6000 LU/g lipid. The corresponding protease activities were 0, 44, 88 and
691 132 PU/ g protein in raw salmon, 0, 70, 140 and 210 PU/ g protein in marinated salmon
692 and 0, 43, 87 and 130 PU/ g protein in cooked salmon. *Different letters mean statistically
693 significant ($p<0.05$) differences between the pancreatin concentration. Results
694 correspond to 120 min of intestinal digestion.

695