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IMPACT OF ELDERLY GASTROINTESTINAL ALTERATIONS ON IN VITRO DIGESTION OF SALMON, SARDINE, SEA BASS AND HAKE: PROTEOLYSIS, LIPOLYSIS AND BIOACCESIBILITY OF CALCIUM AND VITAMINS

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Abstract

This study aimed to analyze the effect of elderly gastrointestinal (GI) conditions on proteolysis, lipolysis and calcium and vitamins A and D3 bioaccessibility in salmon, sardine, sea bass and hake. For this purpose, cooked fishes were in vitro subjected to three elderly in vitro digestion models: E1 (oral elderly conditions), E2 (oral and gastric elderly conditions) and E3 (oral, gastric and intestinal elderly conditions)). In parallel, samples were digested under standardized GI conditions of a healthy adult as a control. Proteolysis was highly affected by elderly GI alterations ($p<0.05$) (50% of reduction compared to control), being salmon and sea bass proteolysis extent (40 and 33%, respectively) the most affected with an important descend in leucine release. Calcium and vitamins bioaccessibility seemed to be also compromised for elders; however, the extent of the reduction highly depends on the fish type. Finally, these GI disorders did not negatively influence the bioabsorbable lipids of the fishes.

Key words: elderly; in vitro digestion; fish; macronutrients, micronutrients

1. INTRODUCTION

The world population is aging rapidly, considering that the population of "advanced age" is over 65 years old in developed countries (WHO, 2017). It is expected that in the first five decades of the 21st century, the proportion of the world's population over 65 will double from 11 to 22%. In addition, the population aged 80 or older will be the fastest growing and expected to triple by 2060 (Agarwal, Miller, Yaxley, & Isenring, 2013). This is why the life quality while aging is a growing global concern, identified as one of humanity's next challenges (United Nations. Department of International Economic and Social Affairs. Population

Division, 2015). Life quality and the prevalence of chronic diseases depend on diet, among other factors. Nevertheless, a deterioration of certain gastrointestinal (GI) functions (i.e. reduction or alteration of enzyme secretions, luminal electrolyte composition, motility and bile secretion, among others) could lead to macronutrient maldigestion and malabsorption, among which sarcopenia or protein deficit, stands out (Shani-Levi et al., 2017). Similarly, the bioaccessibility of certain micronutrients, such as vitamins and/or minerals, could also be compromised in the elderly (Rahme et al., 2017; Sales et al., 2018). Thus, a state of malnutrition can trigger a progressive worsening of health status, increasing the risk of falls, anemia, immune dysregulation, deterioration of cognitive status or reduction of muscle function, among others (Rashid, Tiwari, & Lehl, 2019). From a sensorial point of view, studies also indicate that elderly people experience food in a different way, due to the reduction of sensory perceptions, changes in salivation and poor oral health (Shani-Levi et al., 2017). In order to minimize nutritional deficiencies in senior population, the European Society for Clinical Nutrition and Metabolism (ESPEN) recommends rich-protein foods with a daily protein intake of 1.0–1.2 g protein per kg body weight and healthy lipids to individuals over 65 years (Volkert et al., 2018).Preferably, this protein should be leucine-enriched essential amino acid based (Morley, 2016). Meat and fish meet these characteristics due to their biological value of proteins, but also legumes, dairy or eggs. Thus, fish consumption for elderly is advisable due to its high nutritional quality given by the appropriate balance of amino acids and healthy unsaturated fatty acids.

However, these recommendations consider neither that dietary proteins may be digestible differently depending on their origin, chemical properties or their interactions with other macronutrients into the food matrix (food-inherent factors) nor the influence of the different elderly GI alterations (host-related factors) on protein digestibility. The study of the influence of food-inherent and host-related factors on protein digestibility in different food matrices might generate useful scientific knowledge for health professionals in order to provide accurate dietetic recommendations for elderly, as well as for the food industry in charge of supplying functional products addressed to elderly. In this sense, in vitro digestion models could be considered a useful tool to screen food matrices behavior along digestion under specific and controlled GI conditions of elderly, since they are faster, less expensive and laborious and with significantly lower bioethical restrictions than in vivo studies. In addition to their easy reproducibility, the possibility of easily sampling, make in vitro models very suitable for digestibility studies. Thus, host-related factors such as number of chewing cycles to achieve the physical characteristics of bolus in oral stage, pH and pepsin concentration in gastric stage, and transit time, bile and pancreatic enzymes concentrations in intestinal stages can be in vitro modulated to mimic luminal digestion of different population targets such as elderly people.

In this context, the objective of the present study is to evaluate, using a static in vitro digestion system based on Shani-Levi et al. (2017), the impact of the GI alterations commonly observed in the elderly, on the luminal digestion of macronutrients (proteins and lipids) and the bioaccessibility of micronutrients (calcium and vitamins A and D3) in four different fishes (Hake, Sea bass, Salmon and Sardine).

2. MATERIAL AND METHODS

2.1. Chemicals

Pepsin from the porcine gastric mucosa (3200–4500 U/mg), porcine pancreatin (8 x USP), bovine bile (dried and unfractionated), analytical grade salts (potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, magnesium chloride, ammonium carbonate, calcium chloride and potassium sulfate), boric acid (4%), hydrochloric acid (ACS reagent grade, 37%), sulfuric acid (ACS reagent grade, 95-97%), sodium hydroxide (ACS reagent grade, ≥97.0%), methanol (HPLC grade, ≥99.9%), tetrahydrofuran (HPLC grade, ≥99.9%) and retinol (99%, 3100U/mg) and cholecalciferol (≥98%) as vitamin A and D3 HPLC analytical standards. All reagents were obtained from Sigma-Aldrich.

Also, nitric acid (70%), lanthanum (III) chloride heptahydrate (analytical grade) and dichloromethane (HPLC grade >99.8%) were purchased from Honeywell Fluka; petroleum ether (40-60°C, VWR CHEMICALS), sodium chloride (PanReac AppliChem), anhydrous sodium sulfate (PanReac AppliChem), EZ-Faast amino acid kit (Phenomenex) and acetonitrile (HPLC grade, JT-Baker) were used.

Fishes (salmon, sardine, sea bass and hake) were purchased the same day and from the same lot in order to avoid differences in fishes of the same specie due to seasonality, diet or cultivation methods, at a local store in Valencia (Spain). Fishes were bought fresh, cleaned and eviscerated and were frozen at -20 ºC until its posterior cooking and analysis.

2.2. Sample Preparation

High-consumed species in Spain were selected. Salmon and sardine are commonly considered as oily fish, and sea bass and hake as white fish. The fish were thawed at refrigeration temperature (5 °C) for 8 hours. Subsequently, 400 g of each type of fish were cooked in batches of 200 g by microwave heating (SAMSUNG brand, model GW72N) at 600 W for 4 min (2 min each side) on an extended plate with a lid without additional fat. After cooking, fishes were cooled at room temperature and the skin and bones were removed.

2.3. Compositional analysis

After cooking, moisture, ashes, fat and protein contents were determined according to the official methods 934.01, 942.05, 920.39 and 960.52 (AOAC, 2000), respectively. Moreover, calcium content in cooked samples of the four types of fish was determined. Ashed samples were used to determine the free calcium usinga flame atomic absorption spectrometer (Thermo Scientific, iCE 3000 Series) and calcium was detected at 422.7 nm(Noël, Carl, Vastel, & Guérin, 2008).

Samples were subjected to saponification and extraction of vitamins A (retinol) and D3 (cholecalciferol) according to the protocol of Castaneda & Lee, (2019). To quantify the liposoluble vitamins, aRP-HPLC (Waters e2695 Separation Module, Waters, Milford, MA, USA) with a Kinetex™C18 column 5µm, 100 Å, 150 x 4.6 mm (Phenomenex, Torrance, CA, USA) was used. Vitamins were detected using a photo diode array detector (Waters PDA 2996) at 265 and 325 nm for vitamin D3 and vitamin A, respectively. An isocratic separation was performed with 15% acetonitrile, 7% water and 78% methanol:tetrahydrofuran (90:10 v/v) during 10 min using a flow rate of 1 mL/min and an injection volume of 20 µL. Retinol (99%, 3100U/mg) , and cholecalciferol (≥98%) were used as standards for vitamin A and D3, respectively.

Additionally, in salmon and sea bass (samples with the highest lipidic concentration), a cold lipid extraction was performed in order to study the lipid profile using Proton Nuclear Magnetic Resonance (^{1}H NMR)

(Bruker, model 400 / R), according to the published protocol by (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2016).

2.4. Static in vitro simulation of GI digestion

Four in vitro models were defined to evaluate the contribution of the different GI alterations appearing with ageing on the digestibility and bioaccessibility on nutrients present in fish meat. Concretely, the control model (C) corresponding to the standard GI conditions of a healthy adult (Minekus et al., 2014), and three Elderly models mimicking the accumulative alterations possibly given in elderly (Elderly 1 (oral stage altered (E1), Elderly 2 (oral and gastric stages altered (E2)) and Elderly 3 (oral, gastric and intestinal stages altered (E3)) (Table 1). Specific digestion conditions of elderly (>65 years old) were established according to Shani-Levi et al. (2017), with except of transit time of gastric and intestinal stages (Denis et al., 2016). Chewing (number of mastication cycles) was established according to Jalabert-Malbos, Mishellany-Dutour, Woda, & Peyron (2007) and to reach a bolus consistency similar to a tomato or mustard paste (Minekus et al., 2014). Of note, all cooked fish samples required a similar number of mastication cycles of 20. For elderly mastication, chewing cycles number were reduced at 50%, i.e. 10, in order to achieve the most critical oral elderly scenery, generating a bolus with larger particle size and difficult to swallow (Lee et al., 2004; O'Keeffe et al., 2019).Oral stage was *in vivo* performed by a healthy volunteer with normal dentition under informed consent. Specific conditions of each model are summarized in Table 1. Stock solutions of simulated digestive fluids of gastric and intestinal stages were weekly formulated according to Minekus et al. (2014) and stored at 4 ºC. Simulated gastric and simulated intestinal fluids (SGF and SIS, respectively) were daily prepared from the respective stock solutions and taking into account the pH value, digestive enzymes and bile salts concentrations of each model.

In vitro digestion was performed as follows:

Oral stage: 5 g of cooked fish were subjected to an*in vivo* chewing by the volunteer with normal dentition. 20 and 10 chewing cycles for healthy adult and elderly were performed, respectively. After chewing, food boluses were transferred to the falcon tubes to continue gastrointestinal digestion.

Gastric stage: Simulated was added to food boluses, adjusting the pH and the pepsin concentration, depending on the conditions to be tested (Table 1). Subsequently, the samples were flipped from top to bottom at 55 rpm at 37 °C using an Intelli-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia) and incubated for 2 h in a chamber Selecta (JP Selecta SA, Barcelona).

Intestinal stage: After the gastric stage, SIF was incorporated in a proportion 1:1 (v/w) to each tube containing the gastric chime depending on the conditions of the models (Table 1). Samples were then being flipped from top to bottom at 55 rpm for another 2 or 4 h, depending on the model tested, at 37 °C. pH was monitored during the digestion process and readjusted if necessary to keep it constant.

Digested samples were kept in ice bath for 10 min to lessen the enzymatic reactions before phase separation and analytical determinations. Where needed, separation of the liquid phase (referred as "micellar phase") of solid phase resulting from the digestion process was performed by centrifuging at 4000 g-force during 5 min at 10 °C and the supernatant was collected.

2.5. Analytical determinations in the digesta

2.5.1. Free amino acids profile

Free amino acids resulting of proteins digestion were determined following the protocol published by Peinado, Koutsidis, & Ames (2016) with some amendments. Briefly, 100 µL of micellar phase were derivatized using the EZ-Faast amino acid kit and analyzed using a GC-MS (Agilent Technologies, Injector 7683B series, Network GC System 6890N series, Inert Mass Selective Detector 5975 series).The chromatograms obtained were analyzed by integrating the areas under the curve (MSDChemStation software), according to the retention times given by the kit standards and Norvaline as internal standard. The extent of proteolysis was calculated according to the equation 1:

$$\boldsymbol{Proteolysis\ extent}(\%) = \frac{(g\ \Sigma\ free\ amino\ acids\ in\ micellar\ phase)}{(g\ initial\ protein)} \times \mathbf{100} \qquad \textbf{(1)}$$

2.5.2. Lipid extraction and ^{1}H NMR analysis

Digesta were subjected to a liquid-liquid extraction using dichloromethane according to Nieva-Echevarría, Goicoechea, Manzanos, & Guillén (2016). Subsequently, the lipid profile of the fat extracted from the digested was analyzed by Proton Nuclear Magnetic Resonance ([1]H NMR) using a BRUKER 400/R operating at 400 MHz. The lipid profile obtained reveals the proportion of 1-monoglycerides (1-MG), 1, 2-diglycerides (1,2-DG), 1,3-diglycerides (1,3-DG), 2-monoglycerides (2-MG), glycerol and fatty acids (FA) of the samples.

2.5.3. Calcium bioaccessibility

4 mL of the micellar phase were used free calcium determination by flame atomic absorption spectroscopy following the same protocol as for total calcium determination in undigested samples. The bioaccessibility of calcium was estimated based on the equation 4:

$$\boldsymbol{Calcium\ bioaccessibility}\ (\%) = \frac{(mg\ Ca^{2+} free\ in\ micellar\ phase)}{(mg\ Ca^{2+} total\ in\ undigested\ food)} \times \mathbf{100}\ \textbf{(2)}$$

Where the free calcium was estimated in the micellar phase of the digested and the total calcium estimated in the cooked samples before digestion.

2.5.4. Vitamin A and D3 bioaccessibility

The micellar phase was used to determine the bioaccessibility of vitamin Aand D3 following the same protocol as for total vitamin content in undigested cooked fish. The bioaccessibility of vitamins was calculated according to equation 5:

$$\boldsymbol{Vitamin\ bioaccessibility}\ (\%) = \frac{(\mu g\ of\ released\ vitamin)}{(\mu g\ of\ total\ vitamin)} \times \mathbf{100} \qquad \textbf{(3)}$$

Where the amount of released vitamin represents the recovered part in the micellar phase after in vitro digestion and the total amount of vitamin found in the cooked fish before in vitro digestion.

2.6. Statistical analysis

The results obtained were evaluated by means of an analysis of Variance (one-way ANOVA). In addition, Multiple Range Tests was determined by the LSD (Less Significant Difference) of Fisher test were applied to identify homogeneous groups between models and fish species. Statgraphics Centurion XVII software was used with a confidence level of 95% (p-value <0.05).

3. RESULTS AND DISCUSSION

3.1. Nutritional composition of the samples

The nutritional characterization of the four cooked fish species are gathered in Table 2. In general, protein, total fat and ashes contents were similar to those reported in literature for the same food matrices(U.S. Department of Agriculture, 2019). As expected, all fishes presented high protein content, between 22.74 and 27.1%, salmon being the most and hake the least. The seasonality and the type of production influence the lipid content in fishes. Of note, the total fat content of sardine (12 ± 1%) was lower than expected according to scientific literature. In fact, sardines used were wildlife and caught in winter, explaining the lower fat content than those that are bred in captivity (Bandarra, Marçalo, Cordeiro, & Pousão-Ferreira, 2018). With regard to calcium content of the different fishes, results were consistent with those reported in the literature (Lopez, 2008; U.S. Department of Agriculture, 2019), being the calcium content of sardine much more higher than in the other fishes, because bones were not totally removed in this fish specie remaining as part of the edible part of the sample. Vitamins A and D3 contents were also in agreement with data reported (U.S. Department of Agriculture, 2019), with exception of sardine. Thus, sea bass presents remarkable high vitamin D3 content; while salmon has the highest content in vitamin D3. Vitamins A and D3 were, however, not detected by chromatography in hake. According to these results, sea bass can be considered as the major source of vitamin A and salmon of vitamin D3 among the studied fishes.

3.2. Influence of Elderly GI conditions and fish species on protein digestibility

Figure 1A shows proteolysis extent (g of free amino acids/ 100 g of initial protein) at the end of intestinal stage in the different fish species (sea bass, hake, salmon and sardine) digested under standardized (C) and elderly GI conditions (E1, E2 and E3).Firstly, it can be noted that the extent of fish protein hydrolysis to amino acids under standardized GI conditions (C) ranged from 50 to 70% depending of the fish species, hake proteins being less digestible than the other fish protein. Dielectric properties are dependent on polar molecules in the food matrix, and mainly of water content. An increase in water content results in higher values of dielectric constant and dielectric loss factors, and therefore a higher depth penetration of microwave energy. Regularly, low fat content is coupled with high moisture content in fishes. Therefore, it could be expected a higher microwave energy penetration, and microwave heating, into leans fishes, e.g.

hake, than in oily ones. This fact has been also associated to a greater level of protein denaturalization than by other cooking techniques (Liu, Fukuoka, & Sakai, 2012). On the other hand, it is important to point out that the fish species were frozen since their acquisition and until their posterior cooked. Changes in protein muscle have been reported during storage because of the lipid oxidation during frozen storage. Consequently, the resulted free radicals can react with protein side chains and the carbonyl groups of the oxidized lipids, participating in more form stable protein-lipid aggregates by means of covalent bonding, and thus reducing protein digestibility (Saeed & Howell, 2002; Tejada, Mohamed, Huidobro, & García, 2003). Paradoxally, the effect of lipid oxidation on protein changes is most significant in lean species, such as hake, than in oily ones. In the lean fish muscle, the lipids are limited to the physiologically necessary membrane lipids, that is, they are comprised of phospholipids almost solely and a little amount of sterol esters. Hydrolysis and oxidation of these lipids may result in membrane damage and increased membrane permeability. This, in consequence, may lead to increased activity enzymes directly or indirectly, such as those responsible of oxidative reactions, involved in protein changes (Sikorski & Kolakowska, 1994).

Concerning the effect of elderly GI conditions on proteolysis, protein hydrolysis was negatively affected under any of the simulated elderly alterations (E1, E2 or E3 models). An exception to this event was found in hake for which neither oral (E1) or gastric (E2) alterations affected its protein digestibility. Thus, a reduction of proteolysis extent of 42 ± 4, 40 ± 1, 33 ± 2, 39 ± 2 % for hake, sea bass, salmon and sardine were registered under the worst scenario of digestion for elderly people (E3). Salmon and sea bass presented the highest protein digestibility under standard conditions and the lowest under the most affected elderly conditions (E3), being these species of higher fat content than the others.

The presence of high fat content in these fishes, and the interactions between proteins and lipids or proteins and lipid oxidation derivatives may occur, limiting or impeding the hydrolytic action of proteases, being this fact more relevant under suboptimal conditions (Desai, Brennan, Guo, Zeng, & Brennan, 2019).

Therefore, the impact level of elderly GI conditions on protein digestibility might depend on fish matrix-inherent properties. C and E1 models differ in oral stage conditions, pretending that the breakdown of the food structure is superior in C than E1. The main objective of chewing is to reduce the particle size of ingested food particle and mix them with saliva to form a bolus with optimal characteristics to swallow. In this way, smaller particles maximize the protein surface exposure, facilitating better the accessibility of enzymes to cleavage sites (Paz-Yépez, Peinado, Heredia, & Andrés, 2019). Figure 1A shows proteolysis achieved at the end of digestion depends on the level of mastication of fish matrix, excepting in hake. The moisture content defines texture of fish meat, resulting in a softer matrix when the moisture is higher. Hake presented the greater moisture content of four cooked fishes. Beside this, hake is well-known to be poor to keep the quality in fresh and frozen storage. The flesh is characteristically soft and, that quality attribute get worse with time life(Santos, Saldanha, Gaspar, & Monteiro, 2003).

On the other hand, the comparison between models E1 and E2 aimed to find out the contribution of gastric stage alteration to proteolysis. However, it is necessary to point out that proteolysis is estimated by means of free amino acids quantification at the end of luminal digestion, i.e. after intestinal stage. Consequently, the products of gastric proteolysis are peptides of low molecular weight that cannot be seen by the used method. Hence, the results show that an increase the pH to 6and pepsin concentration reduction to 75% (1500 U/mL) during gastric stage would not affect protein digestibility measured after luminal simulation. Thus, if a decrease of protein hydrolysis into peptides during gastric stage due to a lower pepsin activity and higher pH in stomach would occur, the analytical method will not register it. Moreover, taking in account that close to pH 6 protein aggregates could be generated due to the isoelectric point of some proteins ($4.5 < pH < 5.5$) and, hindering hydrolysis could occur (Levi & Lesmes, 2014).

In any case, the similar proteolysis extent achieved E1 and E2 indicates that the activity of pancreatic proteases might compensate the suboptimal conditions of the gastric stage (E2) with the hydrolysis of proteins into peptides and free amino acids. Finally, a decrease in the pancreatin concentration can lead to poor digestion and therefore to protein malabsorption causing nutritional deficiencies (Rémond et al., 2015). This fact is in concordance to proteolysis extent obtained under suboptimal intestinal conditions (E3) compared with optimal ones (E2). Statistical significant differences ($p<0.05$) exist between results obtained for all cooked fishes digested under E2 and E3 GI conditions, even when the transit time is longer.

Tables 3 and 4 show free amino acids profile resulting of the proteolysis occurring under the four in vitro digestion models (C, E1, E2 and E3) and are consistent with that reported (Özyurt & Polat, 2006; U.S. Department of Agriculture, 2019; Usydus, Szlinder-Richert, & Adamczyk, 2009) for the same fish species. As it can be observed, major free amino acids correspond to leucine, lysine, phenylalanine and valine, all of them essential ones. Specifically, leucine is an amino acid of interest in the elderly, since it is a key-nutrient for the stimulation of muscle protein synthesis (Rémond et al., 2015). However, free leucine content decreased in the digesta under altered GI conditions, and significantly ($p<0.05$) under Elderly model 3 (E3). Of note, free leucine was reduced closed to 40% in salmon, sardine and sea bass digested under E3, while the release of this amino acid from hake proteins does not seem to be affected.

3.3. Influence of GI conditions in elderly on the lipid digestibility of salmon and sea bass

Fat digestibility was evaluated in salmon and sea bass, two species with high fat content, after in vitro digestion under control and altered conditions. This analysis was carried out through the evaluation of the spectral data obtained from ^{1}H NMR. The spectra obtained were analyzed according to Nieva-Echevarría et al. (2016)for the quantification of the main products derived from triglyceride hydrolysis (TG) after digestion. Table 5gathers molar percentages of acyl groups (AG) supported on the different glyceryl backbone structures (TG, 1,2-DG, 1,3-DG, 2-MG, 1-MG) and fatty acids (FA), present in the non-digested (ND) and digesta (C, E1, E2, E3) of salmon and sea bass. As expected, almost all fat was present as TG, with 99.3% in salmon and 98.6% in sea bass before digestion. These results are consistent with those obtained by Nieva-Echevarría et al.(2015)in fish oil samples.After digestion under healthy standard GI conditions (C), a total lipolysis extent of 76% in salmon and 84.6% in sea bass occur because of the hydrolytic action of pancreatic lipase, with a conversion of TG mainly into FA (55 and 70% for salmon and sea bass, respectively), followed by 1,2-DG, 2-MG 1,3-DG and 1-MG. Considering that fat content in salmon is higher than in sea bass (33 and 21 g fat/ g dry matter, respectively, the amount of hydrolyzed fat at the end of the digestion is higher in salmon than in sea bass. Both FA and MG structures could be absorbed by the intestinal epithelium, after undergoing a micellization process thanks to the presence of bile salts (Salvia-

Trujillo et al., 2017). Thus, the absorbable fraction was slightly superior as FA molar percentage, in sea bass than in digested salmon.

Figure 1B shows the lipolysis extent, the absorbable (bioaccessible) and non-absorbable fractions generated after in-vitro digestion. With respect to the elderly GI conditions and their effect on fat digestibility, similar total lipolysis extent (around 80%) were obtained regardless the GI models under both fishes were digested. Therefore, there would not be a significant ($p<0.05$) negative effect of elderly GI conditions on fish fat digestion. Since fat digestion seems not to be affected by elderly gastrointestinal conditions, health problems like dyslipidemia could be associated to an imbalance between the recruitment of lipid substrates and the capacity of their subsequent oxidation by lipid metabolism (Toth & Tchernof, 2000). This condition is well common in older individuals and is characterized by increased triglyceride levels, small high dense LDL, and a low concentration of HDL is being noted in older adults (Choudhury, Tuncel, & Levi, 2009).

Moreover, the lower pancreatic enzymes and bile concentration, and alterations in the oral and gastric phase, may not be sufficient to cause an alteration over the extent of lipolysis. Calvo-Lerma, Fornés-Ferrer, Heredia, & Andrés, (2019) reports that a gastric pH variation from 3 to 5 does not modify lipid digestibility. In fact, they found that the maximum lipolysis extent occurs at gastric pH 5 and intestinal pH 7. These conditions are quite similar to those simulated in E2 model (gastric pH 6 and intestinal pH 7). Thus, comparing E1 and E2, it could be suggested that the gastric pH, together with the altered concentration of pepsin (1500 U/mL), only affect the lipid digestibility in sea bass but not in salmon.

Finally, and related to the intestinal alterations represented by model E3, the longer intestinal transit time with respect to control conditions (4 h instead of 2h) turns out to be a favorable factor for the digestion of lipids. In fact, lipolysis extent under E3 conditions was similar in salmon, and even higher in sea bass, than under C conditions. The bioaccessible fraction was, however, slightly lower than under control conditions even if sea bass digested under E3 model presented the greatest percentage of FA. Differences found in fat digestibility between the two fish species at the same GI conditions are attributed to the inherent-food characteristics (the structural matrix, the type of fat, nutrients, among others) (Shani Levi, Goldstein, Portmann, & Lesmes, 2017), being similar the ratio of monounsaturated fatty acids, polyunsaturated fatty acids and saturated fatty acids (Eroldoğan et al., 2013; Peng, Larondelle, Pham, Ackman, & Rollin, 2003; U.S. Department of Agriculture, 2019), but the different composition (moisture and fat) defines the texture and structure and so, the degree of enzymatic breakdown.

3.4. Influence of elderly GI conditions and fish species on calcium mineral, vitamins A, D2 and D3 bioaccessibility

A reduced digestion of macronutrients, such as proteins and lipids, could be coupled to a deficient release and solubilization of micronutrients leading to a decrease of the bioabsorbable fraction. Table 6 presents the bioaccessibility (%) of calcium, vitamin A and D3 for the four fish species digested under the different in vitro models. Vitamin D2 was not found in samples due that it is found only in vegetables (Etcheverry, Grusak, & Fleige, 2012).Calcium bioaccessibility values ranged from 94% in sea bass to 20% in sardine under standard conditions of digestion (C). Despite having sardine the highest calcium content, this mineral was less bioaccessible in this fish than in others, due to a bone matrix non-broken by the chewing process. Within the remaining three, the protein content could have a negative effect on the calcium bioaccessibility, due to the salting-out effect that exert the free amino acids when are present in salt form with a negative or positive charge promoting less solubility of calcium species (Moreda-Piñeiro et al., 2013). In the opposite, sea bass can be considered a good source of bioaccessible calcium despite its low calcium content. The results obtained in sardine agree with that published by Titchenal & Dobbs (2007), which analyzed calcium bioaccessibility in canned sardines in oil and concluded that this mineral is mainly found in the fish bones, which are ingested but not entirely digested. The obtained results showed that the suboptimal intestinal conditions given in elderly (E3) lead to a statistical significant ($p<0.05$) reduction of calcium bioaccessibility in all fishes (values from 66 to 8 % in sea bass and sardines, respectively). However, alterations occurring at oral and gastric stages (E1 and E2) did not seem to affect the release and solubilization of this mineral, excepting from sardines. Diet recommendations addressed to elderly advice an increase of calcium intake, since bone density decreases with ageing, which can lead to osteopenia and, in extreme cases, osteoporosis, which is partly related to the consumption of dietary calcium. The latter is a significant health problem that contributes to disability and premature mortality among women and older men. Although genetic factors influence maximum bone mass, diet together with an active life style are clearly two of the modifiable risk factors for osteoporosis (Rémond et al., 2015). In addition, vitamins A and D3 bioaccessibility were analyzed as the percentage of vitamin recovered in the micellar phase after in vitro digestion compared to the amount of vitamin found in the cooked samples before digestion. As it can be observed (Table 6), vitamin A bioaccessibility ranged from 14 to 50% under control GI conditions (C); while vitamin D3 bioaccessibility did from 19 and 66% under the same GI model. The differences in terms of release, solubilization and micellar incorporation of these vitamins among fish species could be attributed to the lipid content. Thus, it is found the higher the fat content the greater the fat-soluble vitamins bioaccessibility (Etcheverry et al., 2012). In fact, vitamins A and D3 exhibited the highest bioaccessibility in salmon that has the highest fat and achieved the highest lipolysis extent.

The digestion and absorption of the fat-soluble vitamins basically follow the same path as lipids (Rémond et al., 2015). This behavior is shown when no statistical differences ($p<0.05$) were found among values of bioaccessibility achieved under Elderly models of digestion (E1, E2 and E3) in sea bass and sardines. Moreover, in salmon does not occur of this way, and the vitamins bioaccessibility was strongly reduced when intestinal conditions were altered in the Elderly model (E3), even when the fat digestibility presented a contrary behavior. Liposoluble vitamins are dependent on solubilization by bile acids, and an alteration in bileflow results in malabsorption (Werner, Kuipers, & Verkade, 2013). Thus, the vitamin bioaccessibility decreased when the lipid content is greater, even that lipid bioaccessibility do not show alterations. This result indicates the importance of lipid concentration, showing low vitamins bioaccessibility when the lipid content is higher.

Therefore, the elders are advised to strengthen their skeletal health by following a diet rich in nutrients with adequate amounts of protein, vitamins and minerals. This is why the consumption of salmon could be recommended to this population group, since this fish is characterized by unsaturated fatty acids along with its high calcium content that is easily assimilated, due to the parallel supply of vitamin D offered by this food, which would favor intestinal absorption of this mineral (Michigami, 2019).

4. CONCLUSIONS

Results from this study evidenced that elderly GI conditions differently affected fish macronutrients and micronutrients depending on fish type. Thus, proteins fish-proteolysis extent ranged from 50 and 70% under healthy gastrointestinal conditions (control), being hake proteins the least digested. Elderly GI conditions highly affected proteolysis extent with an accumulative decreasing of extent as long as alterations in digestion stages were incorporated to the in vitro simulation. Thus, a 50% of reduction was reported for salmon and sea bass when oral, gastric and intestinal stages conditions mimicked elderly ones (proteolysis extent of 40 and 33% for salmon and sea bass, respectively). To note, leucine was among the amino acids whom release was affected the most under a total digestive disorder (E3) in all type of fish.

With respect of lipolysis, elderly GI alterations do not statistical significantly ($p<0.05$) affected the absorbable and non-absorbable fractions of lipids of salmon and sea bass. In fact, the longer intestinal transit time characteristic of elderly seems to be favorable to fat digestion. Finally, calcium and liposoluble vitamins A and D3 release were compromised under elderly GI conditions, however the extent of reduction seems to be very dependent of the fish type.

Thus, host-individual gastrointestinal conditions together with fish matrix and its inherent characteristics, influence macronutrients digestibility and micronutrients bioaccessibility. Therefore, this study provides relevant information to understand fish digestibility under altered gastrointestinal conditions on specific population-groups as elderly and depending on fish origin.

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Statement of Informed Consent, Human/Animal Rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Conflictofinterest

There are no conflicts to declare

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TABLE CAPTIONS

Table 1. Specific gastrointestinal conditions of the four in vitro digestion models of this study: control and eldely GI conditions.

Table 2. Total contents of moisture, protein, fat, ashes, calcium and vitamins A and D3 in the four types of microwaved cooked fish (salmon, sea bass, sardine and hake).

Table 3a. Amino acids profile (mg free amino acid / 100 g fish protein) of hake and sea bass achieved under different GI conditions (control (C), Elderly 1 (E1), Elderly 2 (E2), Elderly 3 (E3) models).

Table 3b. Amino acids profile (mg free amino acid / 100 g fish protein) and proteolysis extent of salmon and sardine achieved under different GI conditions (control (C), Elderly 1 (E1), Elderly 2 (E2), Elderly 3 (E3) models).

Table 4. Molar percentages of acyl groups (AG) supported on the different glyceryl backbone structures (TG, 1,2-DG, 1,3-DG, 2-MG, 1-MG) and fatty acids (FA), present in the lipidic phase of sea bass and salmon non digested (ND) and digested samples under different GI conditions (control (C), Elderly 1 (E1), Elderly 2 (E2), Elderly 3 (E3) models).

Table 5. Micronutrients (Calcium and vitamins A and D3) bioaccessibility in sea bass, salmon, sardine and hake under different GI conditions (control (C), Elderly 1 (E1), Elderly 2 (E2), Elderly 3 (E3) models).

FIGURE CAPTIONS

Figure 1. A) Proteolysis extent (g free amino acid (AA)/100 g total protein) of hake, sea bass, salmon and sardine under different in vitro digestion models (control (C), Elderly 1 (E1), Elderly 2 (E2), Elderly 3 (E3) models) B) Molar percentage (%) of the absorbable and non-absorbable lipid fractions of sea bass and salmon under the different in vitro digestion models. Absorbable fraction includes to AG2-MG% + AG1-MG% + FA%, non-absorbable fraction to AG1,2-DG% + AG1,3-DG% and lipolysis extent represent the summarize. a-c: different letters indicate significant differences of proteolysis/lipolysis extent between models. A-C: different letters indicate significant differences between foods ($p < 0.05$).

Table 1

Digestive stage	Model			
	Control (C)	**Elderly 1 (E1)**	**Elderly 2 (E2)**	**Elderly 3 (E3)**
Oral stage	Chewing until a consistency like a tomato or mustard paste (20 cycles)	**50% of reduction with respect to Control chewing (10 cycles)**	**50% of reduction with respect to Control chewing (10 cycles)**	**50% of reduction with respect to Control chewing (10 cycles)**
Gastric stage	pH 3 Pepsin (2000 U/mL) 2 h	pH 3 Pepsin (2000 U/mL) 2 h	**pH 6** **Pepsin (1500 U/mL)** **2 h**	**pH 6** **Pepsin (1500 U/mL)** **2 h**
Intestinal stage	pH 7 Bile(10mM) + Pancreatin (100 U/mL) 2 h	pH 7 Bile (10mM) + Pancreatin (100 U/mL) 2 h	pH 7 Bile (10 mM) + Pancreatin (100 U/mL) 2 h	**pH 7** **Bile (5 mM)** **+ Pancreatin** **(50 U/mL)** **4 h**

Table 2

Nutrient	Salmon	Sea Bass	Sardine	Hake
Moisture (g/100 g)	58.15 ± 0.10^{a}	67.57 ± 0.06^{b}	69.20 ± 0.15^{c}	76.059 ± 0.119^{d}
Protein (g/100 g)	27.1 ± 0.3^{c}	23.8 ± 0.9^{ab}	24.1± 0.4^{b}	22.74 ± 0.09^{a}
Fat (g/100 g)	14.0 ± 0.6^{d}	6.7 ± 0.3^{c}	3.6 ± 0.2^{b}	0.34 ± 0.05^{a}
Ashes (g/100 g)	1.33 ± 0.07^{a}	1.252 ± 0.017^{a}	2.22 ± 0.04^{c}	1.70 ± 0.12^{b}
Calcium (mg/100 g)	25 ± 4^{ab}	20.7 ± 0.8^{ab}	315 ± 36^{c}	50 ± 11^{b}
Vitamin A (µg/100 g)	14.6 ± 0.8^{b}	30 ± 1^{c}	9.7 ± 0.6^{a}	-
Vitamin D3 (µg/100 g)	14.3 ± 0.8^{c}	5.50 ± 0.08^{a}	7.6 ± 0.9^{b}	-

Data shown are mean values from triplicates and the standard deviation.

Table 3a

Amino acid	Hake				Sea bass			
	C	E1	E2	E3	C	E1	E2	E3
Alanine	27.1 ± 0.6^{b}	26 ± 2^{b}	28 ± 1^{b}	18.9 ± 0.8^{a}	37 ± 3^{c}	28 ± 1^{b}	28 ± 1^{b}	20 ± 2^{a}
Glycine	8 ± 1^{ab}	9 ± 2^{b}	8.1 ± 0.4^{ab}	4 ± 1^{a}	18 ± 2^{c}	13 ± 1^{b}	11.9 ± 0.7^{b}	8 ± 1^{a}
Valine	39 ± 1^{b}	40.0 ± 0.4^{b}	45.2 ± 0.9^{c}	35 ± 1^{a}	51 ± 3^{c}	42 ± 2^{b}	41.1 ± 0.8^{b}	33.6 ± 0.4^{a}
Leucine	78 ± 4^{b}	78 ± 4^{b}	88 ± 2^{c}	70 ± 3^{a}	105 ± 7^{c}	81 ± 5^{b}	76 ± 1^{b}	63.7 ± 0.3^{a}
Isoleucine	29 ± 1^{a}	29 ± 1^{a}	34.6 ± 0.4^{b}	27 ± 1^{a}	37 ± 2^{b}	32 ± 3^{a}	31.5 ± 0.8^{a}	27.29 ± 0.09^{a}
Threonine	21.0 ± 0.9^{b}	20.5 ± 0.5^{b}	24.0 ± 0.6^{c}	16.6 ± 0.5^{a}	27 ± 1^{b}	20.9 ± 0.7^{a}	21.9 ± 0.4^{a}	
Serine	18.2 ± 0.4^{b}	16.6 ± 0.5^{b}	18 ± 2^{b}	10.0 ± 0.6^{a}	20.3 ± 0.8^{c}	17.6 ± 0.6^{b}	17.9 ± 0.9^{b}	9.7 ± 0.2^{a}
Proline	4.7 ± 0.1^{b}	5.9 ± 0.4^{c}	5.5 ± 0.2^{bc}	2.8 ± 0.3^{a}	7.3 ± 0.4^{c}	4.7 ± 0.7^{b}	4.6 ± 0.1^{b}	2.61 ± 0.12^{a}
Asparagine	14.3 ± 0.8^{b}	13.9 ± 0.3^{b}	14.4 ± 0.5^{b}	5.5 ± 0.3^{a}	14.1 ± 0.1^{c}	14.5 ± 1.0^{bc}	12.8 ± 0.7^{b}	4.9 ± 0.2^{a}
Aspartic acid	14.0 ± 0.4^{c}	10.7 ± 0.5^{b}	13.8 ± 0.5^{c}	7 ± 1^{a}	16.1 ± 0.4^{c}	13 ± 1^{b}	13.3 ± 0.9^{b}	8.59 ± 0.04^{a}
Methionine	25 ± 1^{ab}	26 ± 2^{ab}	29 ± 1^{b}	22.5 ± 0.9^{a}	35 ± 2^{b}	24 ± 2^{a}	23.3 ± 0.2^{a}	20.0 ± 0.2^{a}
Glutamic acid	21.10 ± 0.10^{b}	19 ± 2^{b}	22.3 ± 0.6^{b}	14 ± 1^{a}	22 ± 1^{c}	20 ± 1 ^{bc}	19.6 ± 1.0^{b}	14.19 ± 0.02^{a}
Phenylalanine	38 ± 1^{ab}	39 ± 4^{ab}	46 ± 5^{b}	34 ± 2^{a}	67 ± 6^{b}	45 ± 4^{a}	42 ± 1^{a}	35.9 ± 0.5^{a}
Glutamine	44 ± 3^{a}	42.6 ± 0.9^{a}	31 ± 9^{ab}	30 ± 3^{a}	36 ± 4^{c}	41 ± 2^{bc}	42.5 ± 1.0^{b}	25 ± 2^{a}
Lysine	83 ± 4^{ab}	76 ± 5^{ab}	73 ± 7^{b}	71 ± 11^{a}	79 ± 3^{b}	84 ± 8^{b}	81 ± 4^{ab}	65.5 ± 0.9^{a}
Histidine	15.1 ± 0.8^{a}	16 ± 1^{ab}	19.4 ± 0.5^{b}	13 ± 1^{a}	22.0 ± 0.3^{c}	19 ± 1^{b}	18.9 ± 0.4^{b}	15.49 ± 0.19^{a}
Tyrosine	20.9 ± 0.5^{a}	24.1 ± 0.2^{ab}	39 ± 4^{b}	23 ± 6^{a}	43 ± 2^{a}	29 ± 2^{a}	41 ± 10^{a}	30 ± 2^{a}
Tryptophan	16.70 ± 0.18^{a}	18 ± 2^{ab}	22 ± 2^{b}	14 ± 1^{a}	25 ± 2^{b}	19 ± 2^{a}	18.8 ± 0.7^{a}	14.8 ± 0.5^{a}
Cystine	8 ± 2^{a}	9.89 ± 0.03^{a}	-	-	11.8 ± 0.4^{b}	8.8 ± 0.9^{a}	-	-

Data shown are mean values from triplicates and the standard deviation. ^{abc} Different lowercase letters indicate significant differences between models, with a significance level of 95% ($p<0.05$).

Table 3b

Amino acid	Salmon				Sardine			
	C	E1	E2	E3	C	E1	E2	E3
Alanine	34 ± 4^{c}	22 ± 2^{b}	25 ± 2^{b}	15.8 ± 0.8^{a}	31 ± 4^{c}	25 ± 2^{b}	24 ± 4^{b}	17.9 ± 0.7^{a}
Glycine	12 ± 1^{b}	9 ± 2^{b}	8 ± 2^{b}	3.4 ± 0.9^{a}	10 ± 2^{b}	11 ± 5^{b}	9 ± 2^{ab}	3.4 ± 0.2^{a}
Valine	46 ± 5^{c}	37 ± 4^{b}	33 ± 2^{ab}	26.7 ± 0.6^{a}	46 ± 5^{c}	35.6 ± 0.5^{b}	37 ± 2^{b}	31.24 ± 0.06^{a}
Leucine	96 ± 12^{b}	71 ± 9^{a}	69 ± 2^{a}	54 ± 1^{a}	93 ± 12^{c}	69.7 ± 0.9^{ab}	78.7 ± 0.9^{b}	62 ± 2^{a}
Isoleucine	34 ± 4^{c}	25.4 ± 0.6^{b}	25 ± 1^{ab}	21.1 ± 0.5^{a}	32 ± 4^{c}	26.3 ± 0.5^{ab}	28.5 ± 0.2^{b}	24.4 ± 0.5^{a}
Threonine	25 ± 3^{c}	17 ± 3^{b}	15.1 ± 0.3^{ab}	10.9 ± 0.7^{a}	22 ± 4^{c}	17.6 ± 0.4^{b}	17 ± 1^{ab}	13.6 ± 0.7^{a}
Serine	21 ± 4^{b}	14 ± 3^{a}	11.5 ± 0.3^{a}	-	14 ± 2^{b}	13.8 ± 0.8^{b}	12 ± 3^{b}	7 ± 0.8^{a}
Proline	10 ± 1^{c}	6.8 ± 0.7^{b}	5.33 ± 0.15^{b}	3.1 ± 0.15^{a}	6 ± 1^{c}	4.2 ± 0.2^{b}	4.69 ± 0.03^{a}	-
Asparagine	15 ± 2^{c}	10.2 ± 0.7^{b}	6.9 ± 0.3^{a}	-	9 ± 2^{a}	11 ± 1^{a}	8 ± 3^{a}	-
Aspartic acid	14 ± 2^{b}	11.5 ± 0.7^{b}	7.8 ± 0.7^{a}	-	12 ± 2^{a}	9.7 ± 0.8^{a}	16 ± 8^{a}	6.2 ± 0.2^{a}
Methionine	31 ± 5^{b}	20 ± 2^{a}	19 ± 1^{a}	16 ± 1^{a}	30 ± 4^{c}	19.7 ± 0.3^{a}	23.5 ± 0.02^{b}	19.2 ± 0.7^{a}
Glutamic acid	17.6 ± 0.7^{b}	16 ± 2^{b}	17 ± 2^{b}	11.0 ± 0.6^{a}	20 ± 2^{c}	18.3 ± 0.6^{b}	20.4 ± 0.7^{c}	13.5 ± 0.3^{a}
Phenylalanine	55 ± 11^{b}	44 ± 8^{a}	40 ± 2^{a}	32 ± 2^{a}	54 ± 8^{c}	35.8 ± 0.9^{ab}	43 ± 3^{b}	32 ± 2^{a}
Glutamine	45 ± 6^{c}	29 ± 4^{ab}	33.5 ± 0.2^{b}	23 ± 2^{a}	20 ± 5^{a}	27 ± 8^{a}	34.1 ± 0.7^{a}	30 ± 2^{a}
Lysine	72 ± 3^{b}	58 ± 8^{ab}	45 ± 11^{a}	57 ± 6^{ab}	73 ± 9^{b}	72 ± 5^{b}	40 ± 4^{a}	76 ± 5^{b}
Histidine	22 ± 2^{c}	17 ± 2^{b}	15 ± 1^{ab}	12.2 ± 0.3^{a}	25 ± 4^{b}	23 ± 1^{a}	21.2 ± 0.8^{a}	21.3 ± 0.7^{a}
Tyrosine	52 ± 5^{c}	29.7 ± 0.3^{ab}	35 ± 1^{b}	25.7 ± 0.7^{a}	38 ± 2^{b}	24 ± 1^{a}	35 ± 4^{b}	23 ± 2^{a}
Tryptophan	24 ± 2^{c}	17 ± 2^{b}	17.6 ± 0.7^{b}	12.9 ± 0.8^{a}	23 ± 3^{c}	15.8 ± 0.1^{ab}	19 ± 2^{b}	13.1 ± 0.2^{a}
Cystine	34 ± 4^{c}	22 ± 2^{b}	25 ± 2^{b}	15.8 ± 0.8^{a}	10.4 ± 0.6	-	-	-

Data shown are mean values from triplicates and the standard deviation. ^{abc} Different lowercase letters indicate significant differences between models, with a significance level of 95% (p<0.05).

Table 4

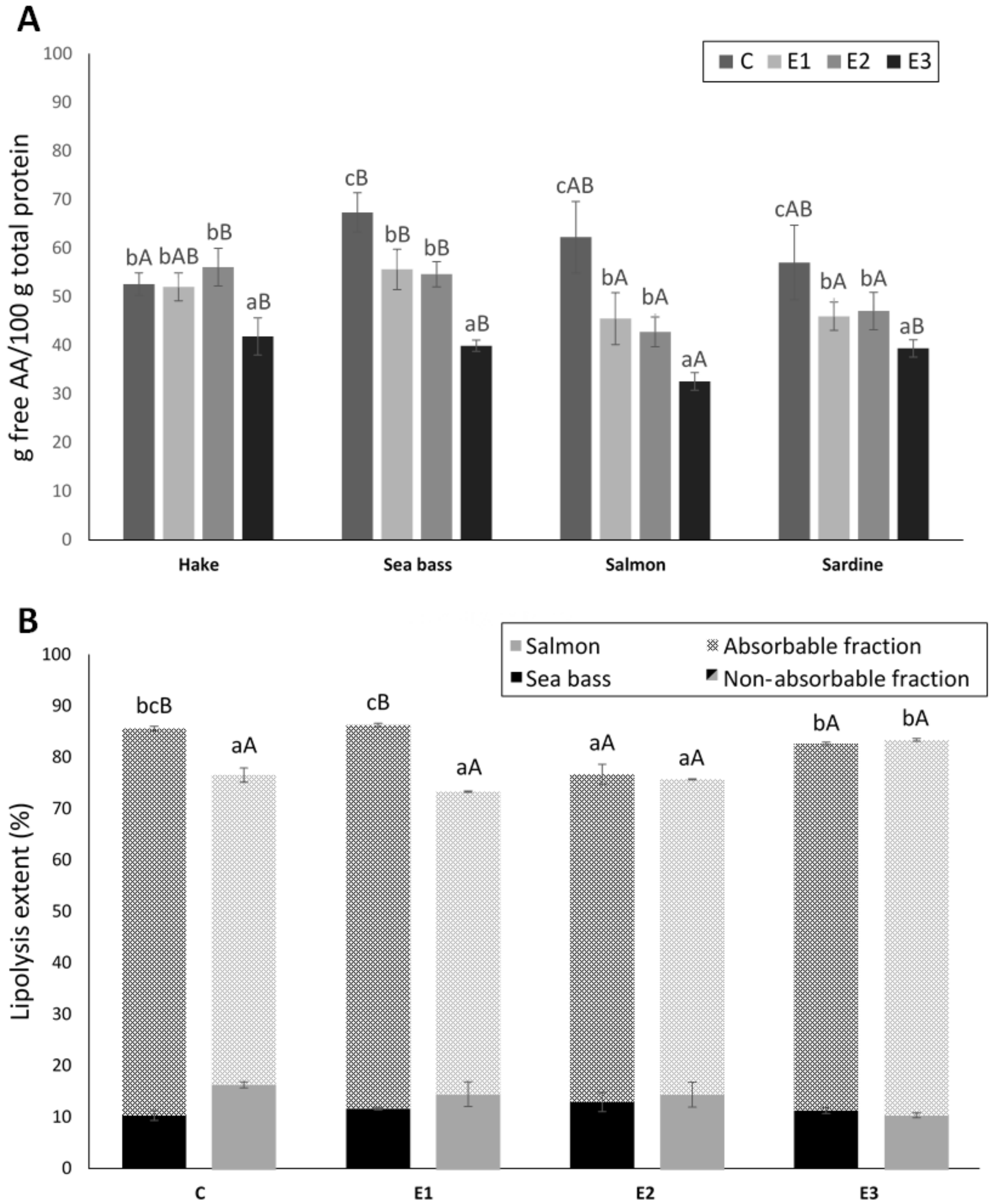
Cooked Fish	In vitro digestion model	AG_{TG}%	$AG_{1,2\text{-}DG}$%	$AG_{1,3\text{-}DG}$%	$AG_{2\text{-}MG}$%	$AG_{1\text{-}MG}$%	FA%
Salmon	Non digested	99.3 ± 0.7	-	0.43 ± 0.19	-	-	0.2 ± 0.9
	C	23.9 ± 0.5^{b}	14.91 ± 0.18^{d}	1.5 ± 0.2^{a}	3.495 ± 0.004^{b}	1.12 ± 0.14^{ab}	55.1 ± 0.4^{ab}
	E1	28.75 ± 0.08^{c}	13.8 ± 0.3^{c}	1.21 ± 0.13^{a}	3.6 ± 0.15^{b}	1.22 ± 0.15^{ab}	53 ± 2^{a}
	E2	23.14 ± 0.09^{b}	12.68 ± 0.03^{b}	1.38 ± 0.04^{a}	3.53 ± 0.19^{b}	1.4 ± 0.4^{b}	57 ± 1^{b}
	E3	17.1 ± 0.4^{a}	9.3 ± 0.6^{a}	1.2 ± 0.1^{a}	1.8 ± 0.1^{a}	0.73 ± 0.02^{a}	69.79 ± 1.16^{c}
Sea bass	Non digested	98.7 ± 0.9	-	0.08 ± 0.40	-	-	1.24 ± 0.5
	C	15.4 ± 0.9^{ab}	9.3 ± 1.3^{a}	0.95 ± 0.09^{a}	3.2 ± 0.4^{b}	0.5 ± 0.3^{a}	70.6 ± 0.5^{b}
	E1	14.8 ± 0.2^{a}	10.6 ± 0.2^{ab}	1.02 ± 0.05^{a}	3.24 ± 0.14^{b}	8 ± 10^{a}	69.7 ± 0.3^{b}
	E2	24.18 ± 1.18^{c}	11.8 ± 0.3^{b}	1.1± 0.3^{a}	3.6 ± 0.2^{b}	0.55 ± 0.03^{a}	59 ± 2^{a}
	E3	18.3 ± 0.6^{b}	9.6 ± 0.2^{a}	1.61 ± 0.06^{b}	2.1 ± 0.2^{a}	0.8 ± 0.6^{a}	67.6 ± 0.4^{b}

Data shown are mean values from triplicates and the standard deviation. *AG: acyl groups. a-d: different letters means significant difference between models ($p<0.05$).

Table 5

Fish	In vitro digestion model	Bioaccessibility (%)		
		Calcium	Vitamin A	Vitamin D3
Sea bass	**C**	94.3 ± 13.2^{bB}	21 ± 2^{bA}	34 ± 3^{aB}
	E1	81 ± 3^{bC}	19.0 ± 0.3^{bB}	28 ± 1^{aB}
	E2	99 ± 4^{bD}	12.6 ± 0.7^{aA}	26 ± 5^{aA}
	E3	66 ± 3^{aC}	13 ± 1^{aA}	25 ± 1^{aB}
Salmon	**C**	86 ± 8^{bB}	48 ± 4^{bB}	66 ± 2^{cC}
	E1	73 ± 7^{abC}	48 ± 1^{bC}	57 ± 2^{bcC}
	E2	68 ± 7^{aC}	51 ± 4^{bB}	50 ± 5^{abB}
	E3	60 ± 4^{aB}	30 ± 2^{aB}	42.5 ± 0.4^{aC}
Sardine	**C**	20.3 ± 0.8^{bA}	14 ± 2^{aA}	19 ± 3^{bA}
	E1	20 ± 1^{bA}	13.2 ± 0.5^{aA}	14.37 ± 0.17^{abA}
	E2	8 ± 1^{aA}	13.3 ± 0.2^{aA}	13.2 ± 0.3^{abA}
	E3	8 ± 1^{aA}	14.0 ± 0.4^{aA}	12.23 ± 0.04^{aA}
Hake	**C**	40 ± 4^{aA}	-	-
	E1	40 ± 10^{aB}	-	-
	E2	33.70 ± 0.10^{aB}	-	-
	E3	30 ± 2^{aA}	-	-

a-c: different letters indicate significant differences between models ($p<0.05$). A-C: different letters indicate significant differences between foods ($p<0.05$). Data shown are mean values from triplicates and the standard deviation.



557 **Figure 1.**

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