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An *in vitro* study to assess bioaccessibility and bioavailability of calcium from blue whiting (*Micromesistius poutassou*) fish bone powder

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Abstract

The aim of this study was to determine how well calcium-rich mineral extracts derived from blue whiting fish bone powders compare with existing calcium sources (commercially available fish bone supplement, calcium carbonate and milk powder) in terms of physicochemical properties, in vitro ***bioaccessibility and bioavailability using simulated gastrointestinal tract treatment and a Caco-2 cell culture model. Blue whiting calcium-rich fish bone powders (A to E) were supplied by Bio-marine Ingredients Ireland (BII) and a commercial calcium-rich fish bone powder was used as the positive control F. The BII calcium-rich fish bone powders analysed through atomic emission spectrometry were shown to have similar levels of mineral content in comparison with powder F. Solubility and rheology tests were performed on the rehydrated powders. The pH of BII calcium-rich fish bone powders in water solution (10% w/v) ranged from 6.96 to 9.09 compared to control F (pH 7.33). Following simulated oral, gastric and duodenal*** in vitro ***digestion using the COST INFOGEST standardised static adult digestion method, the fish powders A, E and F showed higher values of soluble ionic calcium than rehydrated milk powder. We compared*** in vitro ***bioavailability of the powders using the Caco-2 cell line to test the effects of calcium on human colonic epithelial cells, which confirmed that calcium from blue whiting fish bone was more bioavailable than calcium from milk and calcium carbonate. These data indicate that calcium-rich blue whiting fish bone powder compares well with existing calcium sources, in terms of physicochemical properties, bioaccessibility and bioavailability.***

Keywords

Calcium source • *fish bone* • in vitro *bioaccessibility and bioavailability*

Introduction

In the current global scenario, large amounts of agri-food waste and by-products are generated. The Food and Agriculture Organization (FAO, 2020) has estimated that globally one-third of all food produced is either wasted or lost, among which fruit, vegetables and the seafood sector constitute a major share. The fishing industry is a key contributor to the economy of numerous regions globally with annual production of over 178.5 million tonnes in 2018 as fish or shellfish from fisheries and aquaculture (FAO, 2020). Food waste disposal and by-product management are issues of concern for aquatic and terrestrial environments along with the economic and food security implications (Ghosh *et al*., 2016; Torres-León *et al*., 2018).

The increase in annual fish production also generates large amounts of fish-processing by-products, which can account for up to three-quarters of the total fish weight (Rustad *et al*., 2011; Karayannakidis & Zotos, 2016). Most of those fish-processing by-products are usually dumped in landfills or into the oceans having potentially harmful environmental effects or end up as low commercial value products – such as fish meal, fish silage and fertiliser (Rustad *et al*., 2011; Karayannakidis & Zotos, 2016). Despite this, seafoods are inherently functional and possess many components that are beneficial for human health. These by-products contain valuable protein and lipid fractions as well as a variety of bioactive compounds with potential health benefits (Shahidi *et al*., 2019; Nguyen *et al*., 2020). In recent years, research is focused on utilising the ingredients originating from by-products of the seafood industry in diverse sectors such as food, nutraceutical/functional food, pharmaceutical/biomedical and fine chemicals (Shahidi *et al*., 2019; Nguyen *et al*., 2020).

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Fish and fish products have a central position in the functional foods arena as sources of calcium from fish bones and shells, anti-hypertensive proteins/peptides, antioxidants, selenium, chitin, omega-3 polyunsaturated fatty acids (PUFAs), taurine and other bioactive components (Ben-Othman *et al.*, 2020). Fishing industry by-products such as fish bones and shells can be used as sources of value-added minerals such as calcium phosphates (Terzioglu *et al.*, 2018). Extensive supplies of fish bones and crab shells exist in Ireland, most of which are classified as waste. Apart from their culinary value, these marine bivalves are regarded as a potential source of proteins, lipids, fatty acids (ω-3 PUFAs) and carbohydrates, which may have beneficial effects on human health (Gosling *et al.*, 2008; Fernández *et al.*, 2015).

Furthermore, previous studies reported that fishbone-derived peptides from hoki (*Johnius belengerii* (Cuvier, 1830)) and Pacific cod (*Gadus macrocephalus* (Tilesius, 1810)) also possess high calcium-binding activity which can improve calcium bioavailability and serve as a potential pharmacological substance for controlling bone metabolism, calcium absorption and anti-osteoporosis activities (Jung & Kim, 2007; Peng *et al.*, 2017; Heo *et al.*, 2018; Zhang *et al.*, 2018). Therefore, reutilisation of fish by-products in human nutrition could render positive health benefits and can also decrease disposal costs and risk of environmental pollution. Hence, the exploitation of aquaculture by-products for the extraction and investigation of high-quality proteins, lipids and carbohydrates might be of economic value. As there are potential health benefits reported for fish by-products, they might find an application as promising functional food ingredients in the future.

Calcium together with vitamin D play a crucial role in bone health for achieving and maintaining optimal bone mass (Ross *et al.*, 2011; Flammini *et al.*, 2016). Many dietary sources of calcium exist, although they may present problems with assimilation of the mineral. For example, dairy products are high in calcium, but many people are lactose intolerant, particularly those of Asian descent and people can also be allergic to dairy products. However, there is increasing public awareness of the importance of calcium in the diet and this has created a demand for dedicated calcium supplements. Many endogenous (parathyroid hormone [PTH], calcitonin and vitamin D_3) and exogenous (food ingredients and specific diets) factors affect the bioavailability of calcium (Wawrzyniak & Sulibursk a, 2021). Some food ingredients and dietary modifications can increase bone density and prevent osteoporosis by maintaining adequate calcium levels through endogenous pathways, thus it is important to include appropriate supplements that increase the bioavailability of calcium (Wawrzyniak & Sulibursk a, 2021).

Calcium from fish bones may be highly bioavailable to humans, thus fish bone could be useful as a high-quality food ingredient or supplement that can help avoid calcium deficiency and reduce the risk of osteoporosis (Flammini *et al.*, 2016; Zhang *et al.*, 2018). A recent study found that calcified skeletal remains of the red marine algae species *Lithothamnion* (Heydrich, 1897), found off the coast of Ireland and Iceland, is a rich source of calcium and is available commercially as Aquamin in a number of forms, which have calcium concentrations ranging up to 31 weight percent (wt%) (Brennan *et al.*, 2017).

The fish bone powder used in this study is produced from low-value but highly sustainable whole fish biomass called blue whiting (*Micromesistius poutassou* (A. Risso, 1827)). Most of the fish bone powders available on the market are emulsified, not produced from whole fish but from fish off cuts or scales, skin and bones. However, the blue whiting fish bone powder used in this study is produced from whole fish which are non-emulsified, neutral in taste and odour through enzymatic hydrolysis, and hence has a huge potential as a functional food ingredient (Geirsdottir *et al.*, 2011). In addition, the important seafood potential–recovered ingredients from blue whiting fish bone powder have 25% protein which is rich in collagen and is suitable for use in food, cosmetics and the pharmaceutical industries. This feature distinguishes it from existing calcium-rich supplements such as hydroxyapatite and calcium carbonate ($CaCO_3$) which have no protein content. This study investigated the bone fraction of blue whiting as a potential source of calcium given that bone fractions are currently considered as by-products of enzymatic hydrolysis with limited value. The fish bone samples used in this study were sourced from an Irish marine ingredients manufacturer (Bio-marine Ingredients Ireland, Co. Monaghan, Ireland), where five different bone powders A, B, C, D and E were produced from whole blue whiting fish and were compared and screened for their physicochemical properties and bioaccessibility of ionic calcium. The present study is first of its kind where the bioaccessibility and bioavailability of calcium from blue whiting bone powder was investigated.

Materials and methods

Materials

Five fish bone powders derived from blue whiting were provided by Bio-marine Ingredients Ltd (BII, Ireland). Original (A) blue whiting powder is a free-flowing, enzymatically hydrolysed powder with high calcium content and is fat free. The powder is white in colour and is an excellent source of calcium, phosphorous and other minerals (http://www.biomarine.ie/our-products/fish-bone-powder.221.html, BII, Ireland). After developing a further optimisation process of milled twice and dried from the Original B-1 (A) powder, the other powders B-2 (B), C-60 (C), C-33 (D) and C-25 (E) were made. This material was clean, with no residual organic

material having undergone enzymatic treatment. The BII fish bone powder A was crude, while powders B, C, D and E underwent processing, with powder E being most processed. A commercially available bone fish powder F (NutriZing Calcium Fishbone, https://nutrizing.co.nz/product/calcium-fish-bone-powder/) was used as control for comparative purposes. The process of blue whiting bone powder production is robust where approximately 500 kg/day bone powder is produced industrially. Each experiment was performed using replicate samples of each powder (A, B, C, D, E and F) from their individual bulk sample obtained from BII.

Compositional analysis of minerals

Powders A (less optimised powder) and E (the most optimised powder) are selected to find differences between the optimisation process taking the most different powders A and E with the positive control F. The samples were analysed for mineral content and the data compared with the positive control (F). Samples were sent to an external company (Testing Laboratory No. 1163, Accredited by Czech Accreditation Institute [CAI] according to CSN EN ISO/IEC 17025:2005) for the following mineral analysis: calcium, phosphorous, magnesium, sulphur, potassium, sodium, zinc, manganese, iron, selenium, copper, boron and chloride. Samples of powders A and E were homogenised and mineralised by acids and hydrogen peroxide prior to analysis. Determination of elements was done by atomic emission spectrometry with inductively coupled plasma and stoichiometric calculations of compound concentration from measured values.

Physicochemical properties of fish powders

Particle size

Particle size of the powders (A, B, C, D, E and F) was determined using the Mastersizer 3,000 (Malvern Instruments Ltd, Worcestershire, UK). Measurements were conducted in duplicate samples and data were presented as mean ± standard deviation.

Microstructure

Scanning electron microscopy (Carl Zeiss SMT Ltd., Cambridge, UK) was utilised for observing the surface and microstructure of powder samples, performing magnification 200× and 10,000× under 2 kV. Representative pictures of powders are provided in Figure 1.

Solubility

The solubility of the powders (A, B, C, D, E and F) was assessed at room temperature (20°C) and 50°C with one sample tested for each powder. The pH value was considered for rehydration of the fish powders, and for this 0.5 g powder was added to 25 mL buffer (0.1M citric acid/ 0.2M Na_2HPO_4) at pH 3, 4, 5, 6 and 7, while water was used as control. The pH of buffers was determined by a pH meter (S220 Seven Compact, Mettler Toledo). Then, the solution of fish powder was stirred at 450 rpm by a magnetic stirrer for 10 min. The samples were centrifuged at 600 g for 10 min. The supernatant was stored at 4°C for further experiments and the pellet was used to calculate the solubility of the powders. The pellet was incubated at different temperatures (room temperature and 50°C) and the % of solubility was calculated as follows: [weight of powder (wp-sediment weight (sw)]/weight of powder (wp] * 100. The supernatant generated above was used to measure the soluble calcium ion and conductivity using a pH/ION Meter kit (Mettler Toledo Ltd., Switzerland). The experiment was conducted with duplicate samples of each powder (A, B, C, D, E and F).

Determination of* in vitro *bioaccessibility using simulated gastrointestinal tract digestion

To mimic the human gastrointestinal digestion conditions, a simulated *in vitro* static digestion was used based on a COST INFOGEST standardised method with some modifications (http://www.costinfogest.eu/) (Minekus *et al*., 2014; Egger *et al*., 2016). In order to determine the influence of optimisation process in the bioaccessibility, powder samples A and E were selected based on the difference in optimisation. The comparison was made with commercially available bone fish powder (sample F), $CaCO_3$ and milk powder as controls. The powders (A, E and F) were subjected to simulated oral, gastric and intestinal *in vitro* digestion processes, which was performed in duplicate.

The initial oral phase involved mixing of 10 g samples (powders A, E, F and $CaCO_3$) with salivary simulant fluid (SSF) electrolyte stock solution. Then salivary α-amylase solution (1,500 U/mL), 0.3 M $CaCl_2$ and water were added and each sample was mixed by incubating at 37°C for 2 min in a shaking water bath at 220 rpm. Briefly, each 20-mL sample mixtures from the oral phase was treated with simulated gastric fluid (SGF), 0.3M $CaCl_2$, porcine pepsin stock solution (25,000 U/mL), 1 M HCl and water. The gastric pH was adjusted at 3.0. The gastric digestion phase was performed on each sample for 2 h. After gastric digestion, 40 mL digestate of each sample was mixed with simulated intestinal fluid (SIF) containing pancreatin solution (800 U/mL), 10 mM fresh bile, 0.3 M $CaCl_2$, 1 M NaOH to reach pH 7.0. All treatments were performed in duplicate by incubating for 2 h at 37°C in the shaker at 220 rpm. After simulated digestion and enzymatic inactivation (100°C for 10 min), samples were centrifuged (3,500 g for 1 h at 4°C) and the resultant supernatant was used for measurement of the final soluble calcium concentration using the pH/ION Meter kit (Mettler Toledo Ltd., Switzerland).

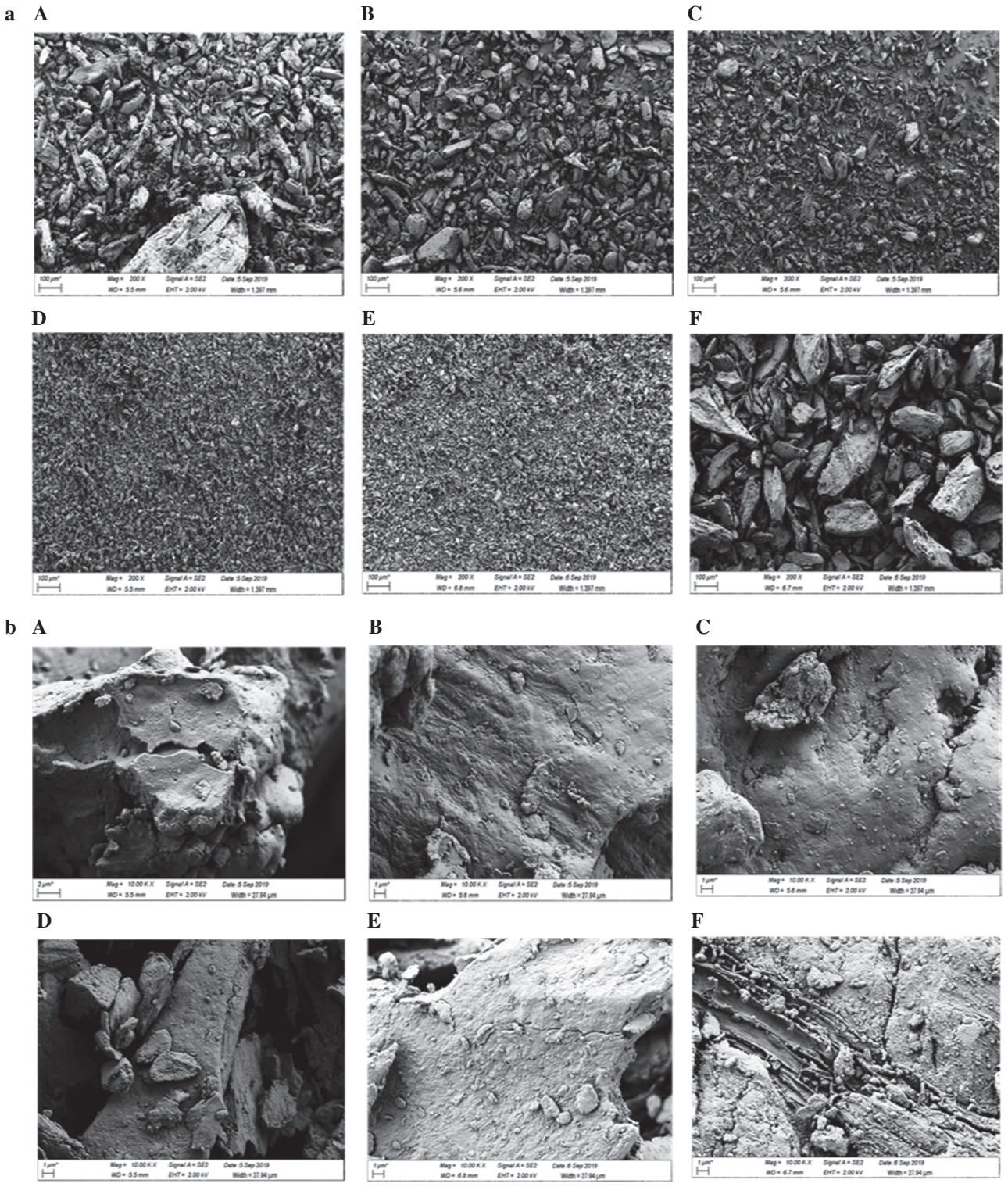


Figure 1. Microstructure of the fish powders (A, B, C, D, E) and the positive control (F) by scanning electron microscope observation (200× [a] and 10,000× [b]).

Determination of* in vitro *bioavailability using a cytotoxicity MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and Caco-2 (human colorectal adenocarcinoma cells) cell line model

The Caco-2 human colon cancer cell line was obtained from the American Type Cell Collection (ATCC® HTB-37™). Cells were cultured in 75 cm^2 tissue culture flasks (Sarstedt, SARSTEDT AG & Co. KG, Sarstedtstraße 1, Nümbrecht, Germany) and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and a mixture of antibiotics to give a final concentration of 50 μg/mL penicillin, 50 μg/mL streptomycin and maintained in a humidified incubator (Sanyo™, MCO-20AIC, CO_2 Incubator, Sanyo, Osaka, Japan) with 5% CO_2 at 37°C. Cells were sub-cultured every 2 days or when the cells reached a confluency of 80–90% and were detached from the flasks using 0.25% trypsin-ethylene diamine tetraacetic acid solution following the procedure described by Glahn *et al.* (1998). All reagents and media were purchased from Sigma-Aldrich, Inc., St. Louis, MO, USA.

To assess cytotoxicity, samples E and F were selected and $CaCO_3$ was included as the calcium positive control. The cytotoxicity was measured by MTT assay (Twentyman & Luscombe, 1987; Hansen *et al*., 1989). Prior to the uptake experiment, Caco-2 cells were seeded in 96-well plates (Sarstedt, SARSTEDT AG & Co. KG, Sarstedtstraße 1, Nümbrecht, Germany) at a concentration of 8 × 10^4 cells per well. The cells were incubated at 37°C in 5% CO_2 for 24 h to allow them to become fully differentiated. Then the medium was discarded, and digested samples in duplicates were added to the wells at different ratios (v/v) of digested samples/medium (50/50, 25/75, 10/90, 5/95, 4/96, 3/97, 2/98 and 1/99) at 37°C for 2 h. The results were based on the colorimetric data obtained using spectrophotometer (Biotek brand Synergy HT model) and represented as the % of viable cells. After the selection of the non-toxic sample concentration, cells were seeded on transpermeable polyester inserts of 12-well plates (0.4 μm pore size and 12 mm diameter, Transwells® Permeable Support plates, Costar®, Costar/Corning, One Riverfront Plaza, Corning, NY, USA). The culture media were added into both apical and basolateral chambers of the transwells and the cells were incubated at 37°C in 5% CO_2. Then the cells were maintained for 21 days and the medium was changed every 2 days until they were fully differentiated as a monolayer. During the incubation time, the monolayer integrity was measured by transepithelial electrical resistance (TEER-Millicell-ERS, MERSStX01 electrode). When TEER values reached to 1,000 Ω cm^2, the cells were assumed to be fully differentiated as a monolayer and suitable for nutrient or calcium transport studies.

After 21 days, and prior to adding the digested samples, apical and basolateral compartments were washed with phosphate buffered saline (Sigma-Aldrich, USA) to remove non-attached cells and 445 μL and 1,500 μL of Hank's medium (Sigma-Aldrich, USA) was added on the apical and basolateral compartments, respectively. Plates were incubated at 37°C for 30 min in a 5% CO_2 humidified atmosphere to reach equilibration of the monolayer. A final ratio of 10/90 of digested sample/media in triplicate, based on cytotoxicity experiments, was added in the apical compartment and transport buffer was added to the basolateral chamber. Plates were incubated for another 2 h, after which the samples E, F and $CaCO_3$ from the basolateral chamber were collected for the determination of total calcium content. Measurements were conducted in triplicate samples of powders E, F and $CaCO_3$. The % calcium absorption was calculated as: (calcium collected/calcium added) * 100. The quantification of the total calcium was carried out using the inductively coupled plasma-mass spectrometry technique from an external company (FBA Laboratories, Co. Waterford, Ireland).

Statistical method

For comparison of the particle size of the powders E and F, and for comparison of solubility of the fish powders at different temperatures (room temperature [RT] and 50°C): two-sample t-test was performed at the level of significance 0.05. For comparison between the samples (powders A, E and F) and control (milk and $CaCO_3$), in the soluble calcium concentration and total calcium absorption measurements, one-way analysis of variance single-factor analysis method was used at the level of significance 0.05.

Results

Mineral analysis

The composition of fish powders A and E is provided in Table 1. Regarding the calcium content, sample A contained 195 g/kg calcium, while sample E had 290 g/kg calcium. Therefore, the calcium level in sample E was closer to that of the control powder (commercially available bone fish powder, sample F), which was 251 g/kg (Table 1). Moreover, sample E was also comparable with powder F for the other minerals tested, including phosphorous, magnesium, zinc, manganese, copper and iron.

Particle size and microstructure of fish powder

All the samples provided by BII (fish powders A, B, C, D, E) exhibited smaller particle size than the commercially available bone fish powder F (Table 2). Meanwhile, sample E had the smallest particle size and was approximately 10 times smaller than powder F (*P*-value = 0.02 ≤ 0.05). The results confirmed that the particles of BII powders were smaller and more uniform than powder F. In terms of microstructure, powder A was most similar to powder F (Figure 1A and B).

Table 1: Composition of fish powders A, E and F (g/kg)

Composition (g/kg)	Samples		
	B-1 (1909-B-original) A	C-25 (C-3718-8) E	Positive control F
Protein	363	248	250
Collagen	90.4	97.6	142.7
Calcium (Ca)	194.7	290	251.3
Phosphorous (P)	94.7	132	127.5
Magnesium (Mg)	5.1	6.77	4.3
Sulphur (S)	NA	1.82	2.5
Potassium (K)	19.8	143[1]	0.3
Sodium (Na)	31.6	117	NA
Zinc (Zn)	77[1]	76.1[1]	90[1]
Manganese (Mn)	12.5[1]	16.1[1]	17.2[1]
Iron (Fe)	15[1]	6.2[1]	6.2[1]
Selenium (Se)	NA	<0.50[1]	0.69[1]
Copper (Cu)	0.85[1]	0.26[1]	0.158[1]
Boron (B)	10.6[1]	2.3[1]	<5[1]

[1](mg/kg).

Physicochemical properties: solubility and rheology analysis

All samples exhibited highest solubility at pH 3, and only marginal differences in solubility were observed at RT compared with 50°C at different pH among all powders (Figure 2). The soluble calcium concentration correlated with total solubility, with the highest soluble calcium found at pH 3 and lowest at pH 7 (Table 3). At higher pH, calcium solubility was very low in all cases. Powder B exhibited the highest soluble ion concentration (158.76 mg/L) at pH 3 at RT compared to other powders A, C, D, E and F (Table 3). Consequently, smaller particle size did not correlate with higher solubility (Tables 2 and 3). Table 4 shows the conductivity (millisiemens/centimetre or mS/cm) of powder samples A–F, which increased in parallel with increasing pH for all powders. The pH of the powders in solution (10% w/v) in water ranged from 6.96 to 9.09, with sample A having the lowest value and samples D and E showing the highest pH values, while reconstituted powder F had a pH of 7.33 in water (Table 5).

Comparative in vitro *gastrointestinal tract treatment for determination of soluble ionic calcium concentration*

After simulated gastric digestion, $CaCO_3$ (positive control) showed the highest concentration of soluble ionic calcium (24,400 and 66,400 mg/L) in both replicates, while powders A, E and F showed higher values of soluble ionic calcium compared to milk powder (Figure 3). Among the samples tested, sample E showed the highest soluble calcium compared to A, F and milk samples with significant difference observed ($P < 0.01$).

Comparative in vitro *calcium absorption study using the Caco-2 cell line model*

All ratios of the digested samples added to the cell monolayer showed that the percentage of cell viability did not fall by more than 1%, thus these solutions were not toxic to the cell line (Figure 4). The ratio of 10/90 (sample/buffer) was selected to carry out the calcium bioavailability experiment of the digested samples as it was the highest amount of calcium that showed no toxic effects during the preliminary experiments. The initial amount of calcium measured in samples E, F and $CaCO_3$ were at an average of 740 mg/kg, 460 mg/kg and 2,088 mg/kg, respectively, before they were added to the cells.

After 21 days of incubation, the TEER values were over 1,000 Ω cm^2, indicating that the monolayer was intact and healthy and confirmed the integrity of the cell membrane for calcium absorption experiments. In all cases, the cell monolayer maintained the integrity (TEER values >1,000 Ω cm) after treatment (Figure 5). Treatment with sample E resulted in the highest average absorption (28.15%), while sample F and CaCO3 showed 22.71 and 6.7% solubility, respectively (Figure 6A). Therefore, fish powder E presented higher Ca absorption in comparison with commercial fish powder F. The total calcium from powders E and F absorbed was 197.3 ± 3.5 and 101.5 ± 21.1 mg/kg, respectively, meanwhile the total calcium from $CaCO_3$ was 140.64 ± 43.66 mg/kg. Treatment with fish powder E resulted in significantly higher ($P < 0.05$) calcium absorption compared with sample F (Figure 6B).

Table 2: Particle size of the powders analysed using Mastersizer 3,000 (± SD)

Code	Sample ID	Dx (90 µm)	Dx (50 µm)	Dx (10 µm)	D [4,3] (µm)	D [3,2] (µm)
B-1	A	182 (±1.41)	69.05 (±0.92)	23.25 (±1.06)	89.95 (±1.06)	46.4 (±1.41)
B-2	B	106.5 (±0.71)	50.6 (±0.42)	18.7 (±0.28)	57.7 (±0.57)	36.2 (±0.42)
C-60	C	84.35 (±0.07)	32.7 (±0.14)	10.03 (±0.10)	43.6 (±0.00)	19.05 (±0.21)
C-33	D	31.85 (±0.21)	14.9 (±0.14)	4.15 (±0.07)	16.75 (±0.07)	6.725 (±0.06)
C-25	E	26.6 (±0.00)	12.65 (±0.07)	3.68 (±0.04)	14.15 (±0.07)	6.03 (±0.04)
Control (positive)	F	293 (±2.83)	140.5 (±3.54)	45.2 (±1.41)	157 (±2.83)	78.1 (±1.70)

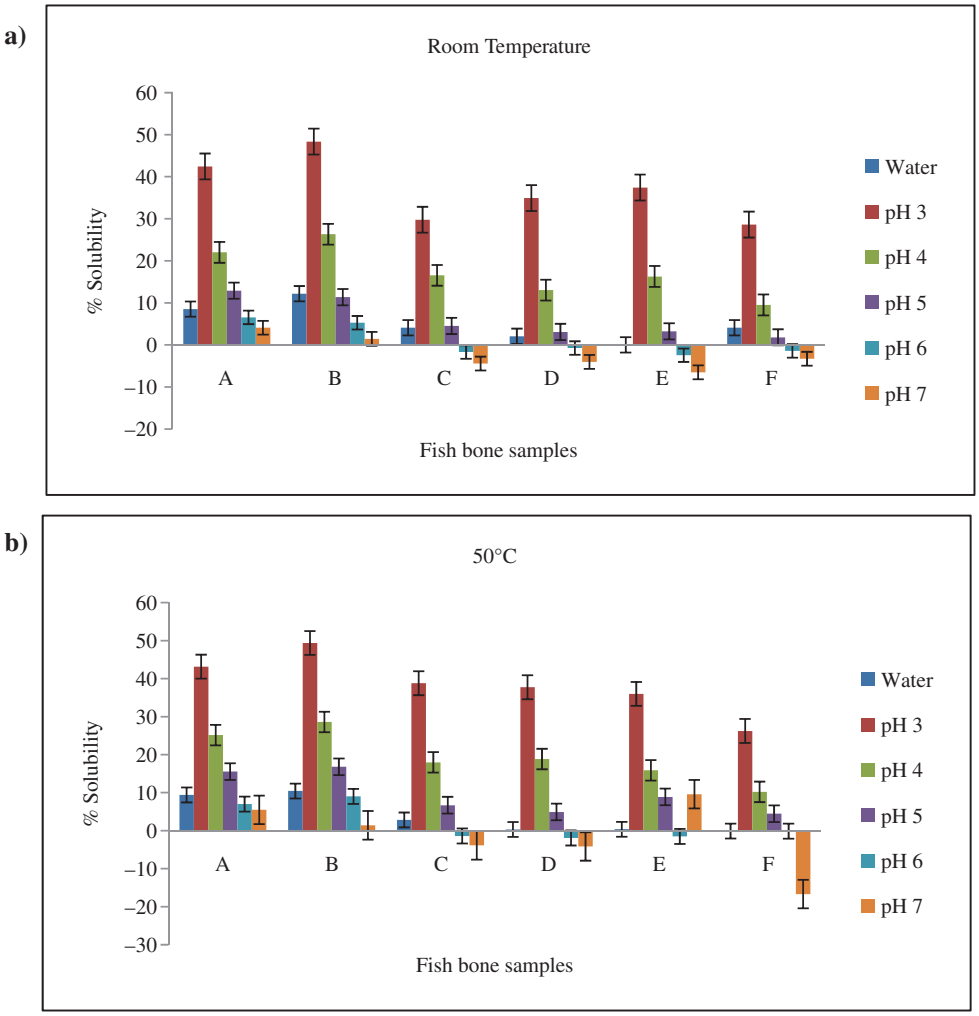


Figure 2. Solubility of the fish powders (A, B, C, D, E and F) at room temperature (a) and 50°C (b) at different pH values. Data were presented as mean ± standard deviation.

Table 3: Soluble calcium ion concentration (mg/L) of fish powders at different pH tested at room temperature (RT) and 50°C

	Calcium (mg/L)											
Powders	**A**		**B**		**C**		**D**		**E**		**F (Positive control)**	
pH tested	**RT**	**50°C**	**RT**	**50°C**	**RT**	**50°C**	**RT**	**50°C**	**RT**	**50°C**	**RT**	**50°C**
Water	34.802	18.649	38.042	20.06	6.687	6.133	3.228	3.622	2.689	3.709	41.414	20.905
pH 3	139.699	101.323	158.762	108.203	140.281	111.176	127.395	112.185	120.99	114.889	135.134	103.85
pH 4	42.092	31.626	53.334	33.107	54.679	30.871	50.913	30.902	55.616	30.578	54.37	29.743
pH 5	9.966	10.343	11.013	10.416	9.947	8.975	9.557	9.134	10.448	9.376	9.309	8.979
pH 6	4.596	4.315	4.868	4.467	4.412	3.82	4.44	3.906	4.518	4.048	3.83	3.502
pH 7	2.166	2.014	2.237	1.99	1.758	1.543	1.89	1.638	1.982	1.676	1.644	1.507

Table 4: Conductivity (mS/cm) of fish powder samples at different pH

Conductivity (mS/cm)	A	B	C	D	E	F
Water	708[1]	694[1]	374[1]	405[1]	407[1]	90.7[1]
pH 3	8.12	8.16	7.88	8.05	8.15	7.74
pH 4	9.83	9.88	9.59	9.75	9.76	9.46
pH 5	11.67	11.78	11.61	11.58	11.5	11.52
pH 6	13.72	13.65	13.54	13.67	13.69	13.41
pH 7	17.48	17.41	17.22	17.33	17.2	17.16

[1](microsiemens/centimetre or µS/cm).
Note: Single sample tested for each powder for conductivity.

Table 5: pH of the powders in water (10% w/v)

Powders	pH measured
A	6.96 (± 0.01)
B	6.97 (± 0.33)
C	8.97 (± 0.93)
D	9.13 (± 1.11)
E	9.09 (± 1.02)
F	7.33 (± 0.51)

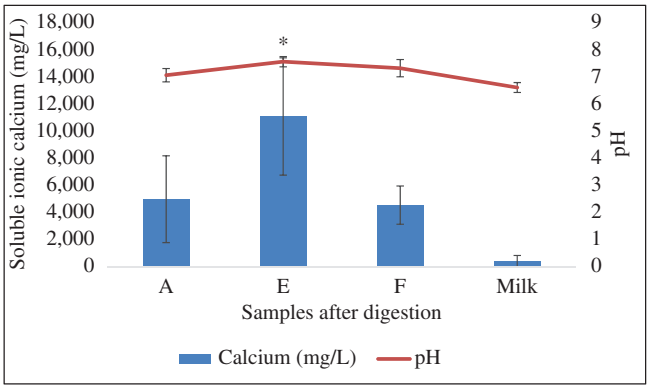


Figure 3. Soluble ionic calcium concentration (mg/L) and pH of samples A, E, F and milk (control), after simulated gastric digestion protocol. Among the samples tested, there was a significance difference observed with $P < 0.01$.

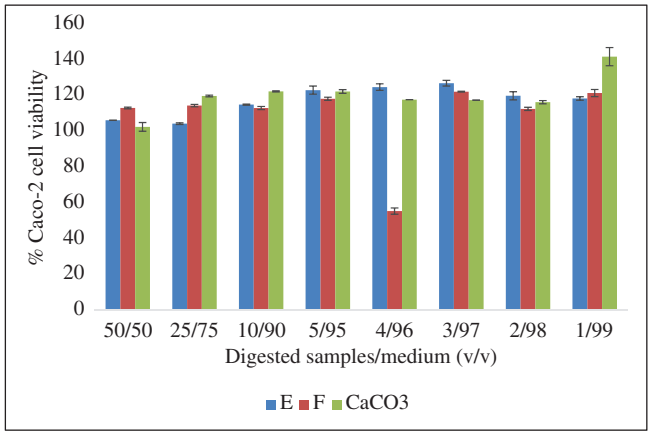


Figure 4. Cell viability (%) Caco-2 cells in the presence of powders E, F and $CaCO_3$ at different ratios (v/v) of digested samples/medium using the cytotoxicity test (MTT). Powders E and F were selected to assess cytotoxicity by MTT assay at different ratios (v/v) of digested samples/medium (50/50, 25/75, 10/90, 5/95, 4/96, 3/97, 2/98 and 1/99), and $CaCO_3$ was included as the positive control.

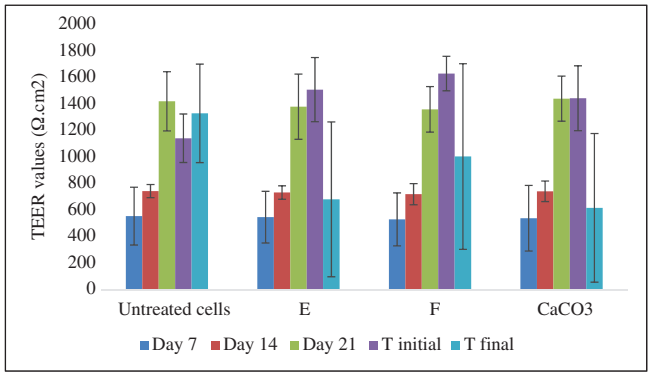


Figure 5. Transepithelial/transendothelial electrical resistance (TEER) values (Ω cm^2) during differentiation of the Caco-2 cells with powders E, F, $CaCO_3$ and untreated cells after 7, 14 and 21 days of incubation (Day 21) until they were fully differentiated as a monolayer. A final ratio of 10/90 of digested sample/media in triplicates was added in the apical compartment as treatment and the TEER values at Time 0 h (T initial) and after 2 h (T final) of incubation were calculated. Reduction in TEER values before (T0) and after (T2) treatment was observed in all Caco-2 cells with treated samples, but sample F showed less toxicity to the cells compared to sample E and $CaCo_3$.

Discussion

Powder E, the most processed of the samples analysed, proved comparable to powder F in terms of mineral content, especially with regard to calcium and phosphorous. The crude powder A had a higher protein content than powder F but similar to levels reported for salmon (350 g/kg) and cod (324 g/kg) bone powders (Malde *et al.*, 2010a). The mineral composition analysis hence revealed that the BII fish bone powders A (crude) or E (highly processed) are potential sources of important minerals for human nutrition.

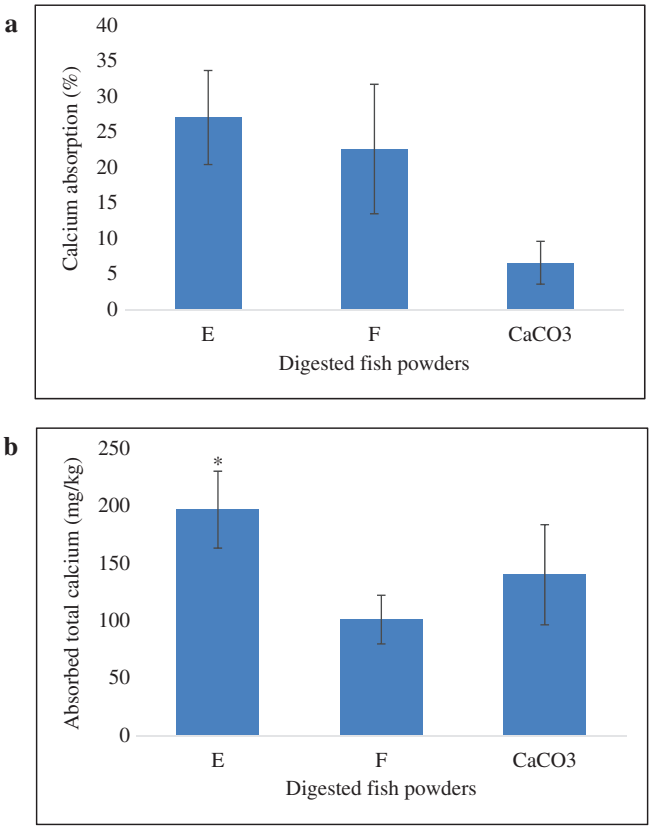


Figure 6. *In vitro* calcium absorption study using Caco-2 cell line model. (a) Calcium absorption (%) and (b) total calcium absorbed (mg/kg) of powders E, F and $CaCO_3$. There was a significance difference observed among samples with total calcium absorbed with $P < 0.05$. Data are presented as mean ± standard deviation.

Fish bone is composed of approximately 30% collagen and 60–70% inorganic compounds such as calcium, phosphorus, magnesium, iodine and selenium (Välimaa *et al*., 2019). Fish bone naturally contains calcium and phosphorus in the right ratio of approximately 2:1 to form hydroxyapatite, which is considered the most bioavailable form of calcium (Venkatesan *et al*., 2015; Välimaa *et al*., 2019). A similar calcium (194.7, 290, 251.3 g/kg) and phosphorus (94.7, 132 and 127.5 g/kg) ratio of 2:1 was identified in the fish bone powders A, E and F analysed. In addition to calcium, phosphorus used by the fertiliser and chemical industries can be isolated and recovered from fish bones (Välimaa *et al*., 2019).

Solubility of the fish powders was investigated as solubilisation determines the release of nutrients in the powder. The solubility was inversely associated with pH, with all powders exhibiting not more than 10% solubility at pH 7 and approximately 40% solubility at pH3. However, temperature did not play a significant role in the solubility profile of fish powders. Marginal differences were found at RT compared with 50°C. Calcium solubility was very low in all cases, which followed a similar trend to total solubility at pH 7, whereas that with the highest soluble calcium was found at pH 3. Therefore, the soluble nature of fish powder positively correlates with calcium release from the powder. Meanwhile, it was noted that as pH increased, the conductivity and calcium activity decreased among all powders tested. In addition, it was noted that particle size of powders was not a critical factor for solubilisation of the fish powders. Although with 10 times smaller particle size than powder F, powder E did not show higher soluble calcium compared to powder F at RT or 50°C.

The solubility of calcium in the gastrointestinal tract is important before it can be absorbed, but certain dietary factors can impact calcium solubility, thereby affecting calcium bioavailability at the absorptive surface of intestinal cells. *In vitro* bioaccessibility/bioavailability methods are useful to provide knowledge on either micronutrient absorbability (a component of bioavailability) or on the potential for a nutrient to be absorbed, that is, bioaccessibility (Etcheverry *et al*., 2012; Mackie *et al*., 2020). The principal *in vitro* methods for measuring bioaccessibility and/or bioavailability includes the gastrointestinal model (e.g. INFOGEST static Egger *et al*., 2016; Brodkorb *et al*., 2019; Minekus, 2015) for bioaccessibility and the Caco-2 models for bioavailability. There are several types of *in vitro* digestion methods that are commonly used to study the bioaccessibility of food ingredients, which can be divided into static and dynamic methods. These models aim to simulate the physiological conditions of the upper gastrointestinal tract, namely the oral, gastric and small intestinal phases. Recently, an international consensus of the digestion conditions was reached within the European COST Action INFOGEST (http://www.cost-infogest.eu/) (Minekus *et al*., 2014; Brodkorb *et al*., 2019).

The calcium solubility level, obtained by applying the *in vitro* digestion method, makes it possible to establish trends in the bioavailability of calcium. Fish bone powders A, E and F were compared to milk powder and $CaCO_3$ (as positive controls) for *in vitro* bioaccessibility using simulated gastrointestinal tract treatment. Milk and calcium carbonate are used as the standard reference calcium sources for digestibility. Following simulated oral, gastric and duodenal *in vitro* digestion processes, fish powders A, E and F showed higher values of soluble ionic calcium than milk powder. Also, both powders A and E showed higher soluble calcium (mg/L) than powder F. Similarly, a study conducted by Yin *et al.* (2016) reported that the calcium solubility of ultrafine fish bone powder (UFBP) significantly increased as the particle size decreased, which might be due to the increase of specific surface area and the destruction of the collagen matrix. It was also suggested that a decrease of fish bone particle size could facilitate the absorption of calcium (Yin *et al*., 2016). Comparatively, our finding indicated that blue whiting fish bone powders (A and

E) not only have smaller particle size, similar soluble calcium ion concentration but also higher bio-accessible calcium compared with the commercial fish powder F.

In vitro bioavailability of calcium in the commercial powder F was previously reported (Beldman *et al*., 1996; https://nutrizing.co.nz/product/calcium-fish-bone-powder/) using mouse osteoblast cell lines. The results identified that powder F matched $CaCO_3$ in bioavailability. They also identified a small but significant effect of powder F on osteoblast mineralisation assisting bone-making osteoblast cells to produce bone mineral. This indicates positive effect of powder F on bone cell function.

Previous studies demonstrated that calcium from commercial fish like salmon and cod is absorbed as efficiently as $CaCO_3$ in both growing pigs and humans (Malde *et al*., 2010a and 2010b). Malde *et al.* (2010a) demonstrated calcium absorption rates of 21.9% and 22.5% for cod and salmon bones, respectively, and 27.4% for $CaCO_3$ in humans. In our study using the Caco-2 cell line, powder E showed the highest absorption (28.15%), compared to powder F (22.71%) and CaCO3 (6.7%). The TEER assay also confirmed that powder E presented higher % and total calcium absorption compared with powder F and $CaCO_3$. Moreover, the results demonstrated that the composition of blue whiting fish bone powder E is extremely bioavailable and did not exhibit a cytotoxic effect on the Caco-2 cell line. Therefore, we can speculate that the difference in mineral composition, particle size and bioavailability might have influenced the difference in absorption of calcium between powders E and F. A study by Suntornsaratoon *et al.* (2018) demonstrated that lactating rats given either tuna bone or purified $CaCO_3$ produced milk with higher calcium concentration, which, in turn, increased bone mineral density in the offspring. Therefore, the blue whiting fish bone powder E with comparable calcium absorption ratios to other fish bone powders from cod and salmon (Malde *et al.*, 2010a) can also be utilised as an alternative calcium source to test for bioavailability and absorption in future animal and human studies.

Overall, our data indicate the potential of BII fish powder E as a source of highly bio-accessible and bioavailable calcium. The calcium-rich blue whiting fish bone powder E compares well with existing calcium sources, in terms of physicochemical properties, bioaccessibility and bioavailability, based on *in vitro* assay and cell culture studies. Thus, blue whiting fish bone powder has the potential to be an alternative source of calcium especially in promoting bone formation and as a value-added product that can be used in a wide range of industrial applications. Future preclinical and human studies will be required to confirm the potential of fish powder E as a calcium-rich nutritional supplement for human health. In this regard, the blue whiting fishery represents a significant opportunity for the Irish seafood sector and related industries due to its relatively large quota and global availability.

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