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1 **Co-products of beef processing enhance non-heme iron absorption in an *in vitro***
2 **digestion/caco-2 cell model**

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9 Running title: Bovine co-products enhance non-heme iron uptake

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16 Keywords: Valorisation, bovine, offal, kidney, lung, heart, meat-factor, ferritin, non-heme,
17 iron bioavailability

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19

20 Summary:

21 Beef processing produces high volumes of protein rich, low value, "waste" co-products such
22 as offal. Beef improves uptake of low bioavailable non-heme iron (found in vegetables,
23 fortificants, supplements) and this effect is dubbed the "meat-factor", although the
24 underlying mechanism is not fully understood. Here, we investigate whether bovine co-
25 products (kidney, lung, heart) not previously studied share this enhancing potential. This was
26 determined by coupled *in vitro* digestion of co-products and subsequent caco-2 cell ferritin
27 formation (an intracellular iron storage protein). In this study we show that bovine co-
28 products significantly increase caco-2 cells' response to non-heme iron from infant rice cereal.
29 The presence of these co-products, (kidney, lung and heart), increased relative uptake (by
30 207.13%, 171.21%, 265.28%, respectively), to a greater extent than beef (30.23%). Our
31 findings present a novel function for co-products of beef processing that may have potential
32 as food ingredients to improve non-heme iron bioavailability, thus adding value.

33

34 Introduction:

35 Iron deficiency anaemia persists worldwide (Stevens *et al*., 2013) and in Europe, where
36 10-32% of reproductive age women are iron deficient (Milman *et al*., 2017). Dietary iron
37 comprises two forms: heme iron, from animal sources like meat, fish and blood; and non-
38 heme iron, an inorganic form, found in vegetables and supplements/fortificants (López and
39 Martos, 2004). Heme iron absorption is higher than non-heme (>15% vs <5%) and is resistant
40 to dietary factors which inhibit non-heme iron absorption (López and Martos, 2004). To
41 circumvent the low bioavailability of non-heme iron supplements, these are produced with

42 very high iron contents to compensate for their poor uptake. This leads to unabsorbed
43 quantities of iron in the intestinal lumen which may participate in Fenton reactions, the
44 formation of free radicals, lipid peroxidation and negatively affect the gut microbiome (Babbs,
45 1990; Knutson *et al*., 2000; King *et al*., 2008; Jaeggi *et al*., 2015). High dose iron supplements
46 and fortificants are therefore not wholly innocuous in preventing or treating iron deficiency
47 as they can contribute to these adverse intestinal events.

48 Vitamin C (ascorbic acid) and meat are known to potentiate non-heme iron uptake
49 and while the role of ascorbic acid is understood (López and Martos, 2004), the exact
50 mechanism for meat, dubbed the "meat factor" has remained elusive. Although some studies
51 suggest phospholipids (Armah *et al*., 2008) or carbohydrates (Huh *et al*., 2004; Wang and
52 Betti, 2017) as this factor, the majority of support is for the protein fraction of meat as the
53 enhancer of non-heme iron bioavailability (Hurell *et al*., 2006; Storcksdieck *et al*., 2007; Wu
54 *et al*., 2014; Zhao *et al.,* 2017). This "meat factor" is capable of increasing non-heme iron
55 uptake up to five-fold, as described in human studies up to 50 years ago (Layrisse *et al*., 1968;
56 Cook and Monsen, 1975). Populations with no or restricted access to meat may therefore
57 suffer nutritional anaemias due to the low bioavailability of plant-based iron, however, this
58 can be improved by including enhancers such as vitamin C in the diet while avoiding uptake
59 inhibitors such as tea and coffee, as recommended by the World Health Organization (WHO,
60 2017).

61 In recent years the growing importance of sustainability has increased the focus of the
62 food industry on adding or recovering value from by-product streams produced during
63 processing (Toldrá *et al*., 2012). In the Irish beef industry, non meat products, now commonly
64 referred to as meat co-products, represent approximately 54-56% of the animal live weight

65 and include blood, offal, etc. Many meat co-products are in fact protein rich material,
66 including edible offal. Due to consumer trends and culture, this "fifth quarter" is often
67 diverted to pet food, animal feed and export markets (Mullen *et al*., 2017) often having a
68 lower commercial value and an increased carbon footprint. To reduce the environmental
69 impact and improve local economic performance, there is a new impetus to search for novel
70 functional roles for these co-products (Toldrá *et al.*, 2016: Lynch *et al.,* 2018), one of which
71 may be as potential sources of the "meat factor". While offal remains part of the diet in many
72 countries (Toldrá *et al*., 2012), acceptability of these co-products by Irish consumers varies
73 demographically (Henchion *et al.*, 2016), presenting potential difficulties to their return to
74 regular consumption.

75 A model of iron uptake established by Glahn *et al*. (1998) which couples *in vitro*
76 digestion of foods with caco-2 cells has been further developed over the last 20 years and
77 validated as a tool to study iron bioavailability from a wide variety of food sources (Jin *et al*.,
78 2009; Kim *et al*., 2011; Rodriguez-Ramiro *et al*., 2017) and circumvents the need for
79 radiolabelling. The objective of this study was to investigate the potential of co-products of
80 the Irish beef industry to act as enhancers of non-heme iron bioavailability. To accomplish
81 this, we screened food combinations, namely a non-heme iron fortified rice cereal combined
82 with bovine offal, using *in vitro* digestion and subsequent ferritin formation as a marker of
83 iron uptake in caco-2 cells.

84

85 Material and Methods:

86 **Chemicals**

87 All chemicals were purchased from Sigma-Aldrich Ireland Limited, Co. Wicklow,
88 Ireland unless otherwise stated. $FeCl_3$ source was a 1g/L solution of Fe in 2% HCl (12923155,
89 Fisher Scientific UK, Loughborough, Leicestershire, UK). Human ferritin enzyme-linked
90 immunosorbent assay (ELISA) kits were obtained from Thermo Scientific™, Frederick, MD,
91 USA.

92 **Raw materials**

93 Bovine skeletal muscle (Longissmus dorsi) (beef), hearts, kidney and lung were
94 delivered to University College Cork 48 h *post mortem* from a local abattoir. Tissues were
95 trimmed of external fat and cut into cubes and minced through a 5mm plate using a Sirman
96 Meat Grinder, Model TC22 Dakota, Sirman SPA. The minced beef and co-products were then
97 vacuum packed in polyethylene bags and stored at -20 °C.

98 Single grain infant rice cereal fortified with iron as ferrous fumarate was purchased
99 from Gerber Products Company, Freemont, MI, USA.

100 **Preparation of acid and alkaline extracts of bovine heart by acid or alkaline solubilisation**
101 **and isoelectric point precipitation**

102 Researchers have attributed the meat factor to the myofibrillar protein fraction
103 (Mulvihill *et al.,* 1998; Storcksdieck *et al.,* 2007). Of the tissues used in this study, heart is the
104 only tissue which contains the normal profile of myofibrillar proteins. Several studies have
105 shown that acid and alkaline solubilisation is a very effective strategy to obtain extracts rich
106 in myofibrillar proteins from meat sources (Mireles de Witt *et al.,* 2002; Hrynets *et al.,* 2010;
107 Nurkhoeriyati *et al.,* 2011). Therefore, we investigated if acid solubilized- or alkaline

108 solubilized-fractions, which are rich in myofibrillar proteins, would enhance iron uptake to a
109 greater degree than heart tissue.

110 Acid and alkaline solubilized extracts were prepared from bovine heart using the
111 procedure outlined by Mireles DeWitt *et al*. (2002) with some modifications. Minced bovine
112 heart was thawed overnight at 4 °C. Minced heart was mixed with dH_2O (at 4 °C) (ratio of 1:4
113 w/w) and homogenised at high speed using a Waring blender for 30 secs × 4. In between each
114 homogenisation the slurry was placed into an ice bath for 10 min. Further dH_2O was added
115 to the slurry to give a final ratio of 1:9 (w/w). The pH was adjusted at 2.0 and 11.0 with 2N
116 HCl for acid extraction and 2N NaOH for alkali extraction, respectively. The slurry was stirred
117 slowly for 30 min and then centrifuged at 4000 g for 20 min at 4°C. The supernatant was
118 poured through cheese cloth prior to isoelectric precipitation of proteins by adjusting the pH
119 to pH 5.5 using 2N HCl or 2N NaOH. Precipitated protein was collected by centrifugation at
120 4000 g for 20 min at 4 °C. The excess water in the recovered protein extract was removed by
121 centrifuging at 10000 g for 15 min at 4 °C. Protein extracts were stored at -20 °C until use.

122 **Protein, cholesterol, lipid and iron measurements.**

123 Protein content of each meat product was determined by Kjeldahl method (N x 6.25)
124 (AOCS, 2012). A 2055 Soxtec™ System (Tecator, Eden Prairie, Minn., USA) was used for fat
125 analysis in heart and heart extracts according to AOAC (1995). Cholesterol analysis in heart
126 and heart extracts was performed using an enzymatic colorimetric method (Boehringer
127 Mannheim, R-Biopharm).

128 Iron content of the food products was determined by flame atomic absorption
129 spectroscopy (AAS) of dry ashed samples. Prior to ashing, crucibles and glassware were

130 soaked overnight in 4% nitric acid. Briefly, 1 g of each food sample was charred in a crucible
131 over a bunsen and then transferred to a muffle furnace at 500°C for 4 h. Resulting ash was
132 boiled in 25% HCl and diluted in dH_2O before AAS analysis. Iron content of digests (diluted in
133 dH_2O) were determined by AAS analysis.

134 **Cell culture**

135 Caco-2 cells were maintained in Dulbecco Modified Eagle Medium (DMEM, D5796)
136 with 10% foetal bovine serum (FBS) and 1% non-essential amino acids (NEAA) at 37°C in a 5%
137 CO_2 incubator. Cells were used for experiments between passages 66 and 75. Cells were
138 seeded at a density of 50,000 cells/cm^2 in 6-well plates. Once seeded, medium was changed
139 every 2-3 days. Cells were used in uptake experiments between 13 and 15 days post-seeding
140 at which point the caco-2 cells had differentiated to represent a monolayer of absorptive
141 enterocytes like cells with protein levels per well of 1.19 mg ± 0.04.

142 ***In vitro* digestion**

143 Each test material (infant rice cereal, beef, kidney, lung, heart, acid and alkaline heart
144 extracts) was sampled 3 times and subjected to a simulated gastrointestinal digestion. In each
145 digestion a negative control, a digest containing no food, was included. A positive control,
146 $FeCl_3$ with ascorbic acid (A0278) was subjected to digestion at concentrations which resulted
147 in cell exposure to 1 mM ascorbic acid and 41.7 µM $FeCl_3$, as described as optimal by Huh *et*
148 *al*. (2004). Iron fortified infant rice cereal (1.5 g) was digested as an additional control.

149 To have similar levels of protein in each digest, and from the protein contents
150 measured (table 1), the following amounts of the test materials were sampled: beef (0.5 g,
151 control); kidney (0.62 g); lung (0.46 g); heart (0.53 g); acid heart extract (0.83 g) and alkaline

152 heart extract (1.31 g). As beef has been shown to contain the "meat factor" required to
153 increase bioavailability of iron from fortified foods in this *in vitro* model when combined in a
154 3:1 fortified food:beef ratio (Pachón *et al*., 2008;2009) infant rice cereal (1.5 g) was added in
155 relevant digests.

156 In preparation for digestion, test materials were defrosted overnight at 4°C and
157 cooked by microwave energy (800W) for 20 secs. These test materials were then
158 homogenised for 90 secs at 13500 rpm (T 25 Ultra-turrax, IKA®-Werke GmbH & Co., Staufen,
159 Germany) in 2.5 ml Earle's Balanced Salt Solution (EBSS, E3024).

160 *In vitro* digestions were carried out in nitric acid washed amber bottles on a 95rpm
161 shaking platform in a 37°C water bath (Grant OLS 200, Grant Instruments, Cambridge, UK)
162 and comprised 1 h gastric phase at pH 2 with 500 µl porcine pepsin (P6887) (0.02 g/ml in 0.1M
163 HCl) at a final volume of 10 ml, made up with EBSS. pH was then increased to 7.4 using 1N
164 NaOH and 100 µl porcine pancreatin (P3292) (0.08 g/ml) and 200 µl bile salts (0.2 g
165 glycodeoxycholate, 0.125 g taurodeoxycholate and 0.2 g taurocholate in 5 ml EBSS) were
166 added, volume brought to 20 ml with EBSS and incubated for 2 h. After the total 3 h digests
167 were heat treated for 10 mins in a 90°C water bath, centrifuged for 20 mins at 25000 g and
168 supernatants filtered (pore size 0.2 µm) prior to storage at -80°C.

169 **Iron uptake and ferritin measurement**

170 Iron uptake was determined by cellular formation and storage of ferritin, an
171 established model first described by Glahn *et al.* (1998). An adapted model, where digests are
172 applied directly to caco-2 cells (Andre *et al*., 2015; Pongrac *et al*., 2016; Perfecto *et al*., 2017)
173 was used in our experiments. Twenty-four h before each uptake experiment, DMEM was

174 replaced with Minimum Essential Medium (MEM, M4655) with 1% NEAA but without FBS.
175 Digests were defrosted and combined with MEM at a ratio of 1:2 and warmed to 37°C. Digests
176 at this concentration were non-lethal to cells as determined by a neutral red assay (Babich
177 and Borenfreund, 1992). Cells were washed with warmed MEM and 2 ml of each independent
178 test digestions was applied directly to duplicate wells. Baseline cellular ferritin was
179 determined by incubation with fresh MEM. Cells were incubated for 3 h at 37°C after which
180 the digests were aspirated and plates were incubated with 2 ml MEM for a further 21 h to
181 allow ferritin formation. Cells were then lysed by addition of 375 µl of ice-cold CelLytic™M
182 (C2978) with 1% protease inhibitor cocktail (P8340). Lysed cells were scraped, centrifuged at
183 25000 g for 20 mins at 4°C and supernatants stored at -80°C for subsequent analysis.

184 Cellular ferritin was measured using a Ferritin Human ELISA Kit (EHFTL, Invitrogen) and
185 expressed as ng/mg of cellular protein, determined by a bicinchoninic acid assay (BCA) (Smith
186 *et al.,* 1985).

187 **Calculation of "increase in ferritin response" and "absorption efficiency"**

188 Data were adjusted as follows to correct for the intrinsic iron content of the bovine
189 test materials. To isolate the "meat factor" mediated increased bioavailability of the non-
190 heme iron the following calculation was carried out.

191 *Increase in ferritin response of cells to iron in digest (ng/mg) =*

192 Cellular ferritin response (ng/mg) to rice cereal + test material combination digest – (cellular
193 ferritin response (ng/mg) to rice cereal digest + cellular ferritin response (ng/mg) to test
194 material digest)

195

196 To determine overall absorption efficiency of the iron from digests of the test
197 materials combined with infant cereal compared to the test materials or infant cereal alone,
198 caco-2 cell ferritin formation was expressed per unit iron in each corresponding digest.

199 *Absorption efficiency (ng/µg)* = ferritin formed by cells (ng per well) / Fe in 2ml of 1:2 diluted digest applied to cells (µg per well)

200

201

202 **Statistical methods**

203 All data represent the mean ± standard error (SE) of three independent experiments 204 unless otherwise stated. Statistical analysis was carried out using GraphPad Prism 5 205 (GraphPad software, La Jolla, CA). Means were considered statistically significantly different 206 if $p < 0.05$. In accordance with this * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

207

208 Results and Discussion:

209 Protein levels of test materials studied (table 1) are similar to ranges reported for most 210 of the test materials; 21% for beef, 17.4% for kidney, and 17.7% for heart with the exception 211 of the lung 16.2% (USDA, 2018), lower than the 21.12 ± 0.56% determined in our sample. Protein, 212 cholesterol and fat contents of acid and alkaline heart extracts are lower than heart (table 1) 213 as the extraction processes cause sedimentation of insoluble proteins (such as collagen) with 214 membrane lipids (including cholesterol) leading to their removal by centrifugation while 215 retaining functional proteins, i.e. myofibrillar proteins (Hultin *et al.* 1997; Mireles Dewitt *et* 216 *al.* 2002).

217 Despite differing iron contents of the bovine test materials prior to digestion (table 1) 218 no statistical difference in the concentration of soluble iron present after digestion and 219 centrifugation was seen between any infant rice cereal – test material digest (figure 1). 220 Ferritin formation by caco-2 cells in response to incubation with these digests is shown in

221 figure 2. A significant increase in iron uptake and storage by the cells was seen between the
222 infant rice cereal control and the cereal-test material combinations including kidney, lung and
223 both heart extracts. No significant increase was seen between cereal and cereal-beef or
224 cereal-heart digests.

225 **Intrinsic iron content of test materials**

226 Although the soluble iron contents of the cereal-test material digests did not differ
227 significantly, we could not exclude the possibility that the increase in ferritin formation seen
228 in figure 2 was due to the intrinsic iron content of the test materials. To account for this
229 intrinsic iron, we adjusted each cereal-test material ferritin formation values for the response
230 that could be attributed to the iron from the materials themselves. This was done by
231 subtracting the ferritin formation ng/mg value for the test material alone from the cereal-test
232 material combination value for each independent experiment. This value was compared to
233 the cell's response to infant rice cereal and is shown in table 2 with the difference displayed
234 as "calculated increased response".

235 These results suggest that bovine kidney, lung and the acid extract of heart protein
236 have the ability to significantly increase the uptake of the non-heme iron from the infant rice
237 cereal (by 8.05 ng/mg, 17.78 ng/mg and 10.54 ng/mg respectively) and that this increase is
238 not due to the iron content of the test materials themselves. This adjustment also revealed
239 an enhancing role of beef (of 3.30 ng/mg), not previously seen in figure 2.

240 **Absorption efficiency of fortified rice cereal – bovine co-product digests**

241 To further distinguish the "meat factor" component of the test materials from their
242 intrinsic iron contents and standardise results to reduce inter-experiment variation, a

243 calculation of *in vitro* bioavailability per unit iron in the digest applied, (figure 1), was carried
244 out and results are presented in figure 3. This gives an “absorption efficiency” of the cereal-
245 test material combinations and has been used in previous studies (Pachón *et al*., 2008; 2009)
246 where intrinsic iron content of foods presented a problem when studying the iron uptake
247 enhancing properties of meat. When compared to the infant cereal reference, the cereal:test
248 material food combinations that had more bioavailable iron were infant rice cereal with:
249 kidney, lung or heart, represented by a statistically significant increase in relative uptake of
250 207.13%, 171.21% and 265.28%, respectively, all significant at $p < 0.05$.

251 Despite meat’s recognised role as an enhancer of both heme and non-heme iron
252 absorption (López and Martos, 2004), in the present study beef did not significantly increase
253 absorption efficiency (figure 3) with fortified infant rice cereal, consistent with findings
254 reported by Pachón *et al*. (2008). Particle size of meat has been linked to its iron enhancing
255 potential, with increasing homogenisation times increasing the “meat factor” effect (Pachón
256 *et al*., 2009). These authors reported a homogenisation time of 360 seconds for bovine
257 tissues, beef and liver as required to see a significant increase in ferritin formed per unit iron
258 when compared to less homogenous or lyophilised meats products. In the present study, an
259 increase in homogenisation time may have revealed a definite enhancing effect for the beef-
260 cereal food combination, as an increase in the ferritin response of caco-2 cells to a non-heme
261 iron source was detected (3.30 ng/mg), table 2, and should be considered in future studies.

262 **Bovine heart extracts**

263 Acid and alkaline extracts of heart increased overall ferritin formation when digested
264 in combination with the infant rice cereal (figure 2E, F). Adjusting for intrinsic iron content of
265 these preparations revealed only an increased response to non-heme iron in combination

266 with the acid extract, an increase of 10.54 ng/mg compared to 3.67 ng/mg for the alkaline
267 extract (table 2). Acid extractions of meat have been previously shown to increase iron uptake
268 from a non-heme iron source. These include extracts of haddock (Huh *et al*, 2004) and chicken
269 (Pachón *et al*., 2009) in caco-2 cells and chicken and beef, used in a human feeding trial
270 (Hurrell *et al*., 2006). While all these extracts were prepared from lyophilised meat, they differ
271 in composition: The Haddock extract was reported to be essentially protein free and
272 comprising mainly carbohydrates; whereas the meat extracts produced by Hurrell *et al*.
273 (2006) were between 94-98% protein; and the composition of chicken liver extract were not
274 stated (Pachón *et al*., 2009). In this study acid extraction followed by isoelectric precipitation
275 was used to prepare the acid extracts. Extracts prepared using this pH shift technology have
276 been shown to be dominated by myofibrillar proteins and have the advantage of having lower
277 fat and cholesterol levels than the starting materials (Mireles DeWitt *et al*., 2002). In this study
278 the heart and acid extract of heart had cholesterol contents of 122.85 +/- 3.29 mg/100 g and
279 52.15 +/- 1.6mg/100 g, respectively and fat levels of 1.05 +/-0.385 and 0.28+/-0.4%,
280 respectively (table 1). Mean absorption efficiencies were similar for bovine heart and the acid
281 extract of the same when compared to the infant cereal reference, 365% and 349% (figure 3).
282 Although only the heart's initiated increase was found to be statistically significant, these
283 values suggest a potentially similar enhancing effect for the acid heart extract, or acid extracts
284 of other co-products, warranting further elucidation. This is supported by the *in vitro* and
285 human studies of Pachon *et al.* (2009) and Hurrell *et al*. (2006), who reported iron uptake
286 potentiating effects of their acid extracts were not statistically significantly different from
287 lyophilised tissues from which they were prepared. Additionally, acid extractions of meats
288 and co-products improve some functional properties such as gelation, emulsification and

289 water holding capacity (Matak *et al.,* 2015), meaning that this preparation of our tissues could
290 have a multi-functional effect as a novel food ingredient.

291 **The "meat factor"**

292 Many studies have concluded that low-molecular-weight peptides generated during
293 digestion of meat protein are major contributors to the "meat factor" and implicate cysteine
294 or histidine rich sites in these as vital to this role (Taylor *et al*., 1986; Mulvihill *et al*., 1998;
295 Seth and Mahoney, 2000; Swain *et al*., 2002). However, isolation of the particular peptides
296 responsible (as a means of generating a food additive) has proven unsuccessful as of yet
297 (Storcksdieck *et al.,* 2007). On the other hand, in support of a non-peptide originating "meat
298 factor", an oligosaccharide extracted from chicken and a phospholipid, L-α-
299 glycerophosphocholine identified in beef were shown to enhance iron absorption in a caco-2
300 *in vitro* model (Wang and Betti, 2017; Armah *et al*., 2008). These difficulties in isolating
301 particular functional peptides as well as the evidence that other meat constituents,
302 carbohydrates and lipids, can improve non-heme iron uptake suggests that the "meat factor"
303 is more likely a group of factors, each with their own role and mechanism for binding non-
304 heme iron to improve solubility and bioavailability.

305 This study presents novel findings on the potential of co-products of the Irish beef
306 industry as sources of the "meat factor". To the best of our knowledge, only two groups have
307 previously investigated the non-heme iron bioavailability enhancing capacities of meat co-
308 products in a similar *in vitro* model, focussing on tissues of chicken origin: liver, blood, spleen
309 (Pachón *et al*., 2008) liver (Pachón *et al.,* 2009); and connective tissue derivatives (Wang and
310 Betti, 2017).

311 **Intrinsic iron content of bovine co-products**

312 It is important to note that while we have expressed our results to account for the
313 intrinsic iron contents of the bovine co-products, there is a potential for this to have affected
314 our results. Future preparation of a heme-free extract from these co-product test materials
315 might be useful in further validating our findings. The caco-2 model used is useful in providing
316 hypotheses for testing in human as it generally can predict the direction of response, despite
317 using ferritin as an indirect measure of iron uptake (Sandberg, 2010) and further *in vitro*
318 studies are therefore warranted which may lead to future investigations in humans.

319 Conclusion:

320 Our results present a potential new role for "fifth quarter" waste streams of the beef
321 industry, both in Ireland and worldwide, with offal such as heart, lung and kidney as possible
322 higher sources of the "meat factor" than beef itself. Further studies examining protein,
323 carbohydrate or lipid fractions of these co-products may reveal where this "meat factor" is
324 concentrated to allow for specific processing and better application in foods. Conversely, it
325 may reveal that these fractions are required in cooperation for an enhancing effect on iron
326 uptake from non-heme sources. Processing these tissues as food ingredients that can be used
327 to improve iron uptake presents a novel potential to add value to these co-products.

328

329

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335

336 Conflict of Interest:

337 The authors declare no conflict of interest.

338 References:

339 Andre, C. M., Evers, D., Ziebel, J., Guignard, C., Hausman, J.F., Bonierbale, M., Zum Felde, T. &
340 Burgos, G. (2015). In Vitro Bioaccessibility and Bioavailability of Iron from Potatoes with
341 Varying Vitamin C, Carotenoid, and Phenolic Concentrations. *Journal of Agricultural and Food*
342 *Chemistry*. **21**, 9012-21.

343 AOAC. (1995). Official methods of analysis. Association of official analytical chemists 16th
344 edition. Washington D.C., USA.

345 AOAC. (2012). Official methods of analysis. Association of official analytical chemists 19th
346 edition, Washington D.C., USA.

347 Armah, C.N., Sharp, P., Mellon, F.A., Pariagh, S., Lund, E.K., Dainty, J.R., Teucher, B. &
348 Fairweather-Tait, S.J. (2008). L-alpha-glycerophosphocholine contributes to meat's
349 enhancement of non-heme iron absorption. *Journal of Nutrition*, **138**, 873-7.

350 Babbs, C.F. (1990). Free radicals and the etiology of colon cancer. *Free Radical Biology and*
351 *Medicine*, **8**, 191-200.

352 Babich, H. & Borenfreund, E. (1992). Neutral Red Assay for Toxicology in vitro. In R.R. Watson
353 (ed) In Vitro Methods for Toxicology, Chapter 17, pp238-51. CRC Press, Inc., Boca Raton,
354 Florida, US

355 Cook J.D. & Monsen, E.R. (1975). Food iron absorption. I. Use of semisynthetic diet to study
356 absorption of non-heme iron. *The American Journal of Clinical Nutrition*, **28,** 1289-95.

357 Glahn, R.P., Lee, O.A., Yeung, A., Goldman, M.I. & Miller, D.D. (1998). Caco-2 cell ferritin
358 formation predicts non-radiolabeled food iron availability in an in vitro digestion/Caco-2 cell
359 culture model. *Journal of Nutrition*, **128,** 1555-61.

360 Henchion, M., McCarthy, M., & O'Callaghan, J. (2016). Transforming beef by-products into
361 valuable ingredients: Which spell/recipe to use? *Frontiers in Nutrition*, **3:** 53.

362 Hrynets Y., Dileep, A., Omana, Y.X. & Betti, M. (2010). Effect of acid and alkaline aided extractions on
363 functional and rheological properties of proteins recovered from mechanically separated turkey meat.

364 Huh, E.C., Hotchkiss, A., Brouillette, J. & Glahn, R.P. (2004). Carbohydrate fractions from
365 cooked fish promote iron uptake by Caco-2 cells. *Journal of Nutrition*, **134**, 1681-9.

366 Hultin, H. O. & Kelleher, S. D. (1997). Process for isolating a protein composition from a muscle
367 source and protein composition. Patent nr 6005073 (Feb. 12)

368 Hurrell, R.F., Reddy, M.B., Juillerat, M. & Cook, J.D. (2006). Meat protein fractions enhance
369 non-heme iron absorption in humans. *Journal of Nutrition*, **136,** 2808-12.

370 Jaeggi, T., Kortman, G.A., Moretti, D., Chassard, C., Holding, P., Dostal, A., Boekhorst, J.,
371 Timmerman, H.M., Swinkels, D.W., Tjalsma, H., Njenga, J., Mwangi, A., Kvalsvig, J., Lacroix, C.
372 & Zimmermann, M.B. (2015). Iron fortification adversely affects the gut microbiome,
373 increases pathogen abundance and induces intestinal inflammation in Kenyan infants. *Gut*,
374 **64,** 731-42.

375 Jin, F., Frohman, C., Thannhauser, T.W., Welch, R.M. & Glahn, R.P. (2009). Effects of ascorbic
376 acid, phytic acid and tannic acid on iron bioavailability from reconstituted ferritin measured
377 by an in vitro digestion-Caco-2 cell model. *British Journal of Nutrition*, **101,** 972-81.

378 King, S.M., Donangelo, C.M., Knutson, M.D., Walter, P.B., Ames, B.N., Viteri, F.E. & King, J.C.
379 (2008). Daily supplementation with iron increases lipid peroxidation in young women with
380 low iron stores. *Experimental Biology and Medicine*, **233,** 701-7.

381 Kim, E.Y., Ham, S.K., Bradke, D., Ma,Q. & Han, O. (2011). Ascorbic acid offsets the inhibitory
382 effect of bioactive dietary polyphenolic compounds on transepithelial iron transport in Caco-
383 2 intestinal cells. *Journal of Nutrition,* **141,** 828-34.

384 Knutson, M.D., Walter, P.B., Ames, B.N. & Viteri, F.E. (2000). Both iron deficiency and daily
385 iron supplements increase lipid peroxidation in rats. *Journal of Nutrition*, **130**, 621-8.

386 Layrisse, M., Martínez-Torres, C. & Roche M. (1968). Effect of interaction of various foods on
387 iron absorption. *The American Journal of Clinical Nutrition*, **21,** 1175-83.

388 Lynch, S.A., Mullen, A.m., O' Neill, E., Drummond, L. & Álvarez, C. (2018). Opportunities and
389 perspectives for utilisation of co-products in the meat industry. *Meat Science* **144**, 62-73.

390 López, M.A. & Martos, F.C. (2004). Iron availability: An updated review. *International Journal*
391 *of Food Sciences and Nutrition*, **55**, 597-606.

392 Matak, K. E., Tahergorabi, R. & Jaczynski, J. (2015). A review: Protein isolates recovered by
393 isoelectric solubilization/precipitation processing from muscle food by-products as a
394 component of nutraceutical foods. *Food Research International*, **77**, 697-703.

395 Milman, N., Taylor, C.L., Merkel, J. & Brannon, P.M. (2017). Iron status in pregnant women
396 and women of reproductive age in Europe. *The American Journal of Clinical Nutrition,* **106,**
397 1655S-1662S.

398 Mireles DeWitt, C.A., Gomez, G & James, J.M. (2002). Protein extraction from beef heart using
399 acid solubilisation. *Journal of Food Science* **67**, 3335-3341.

400 Mullen, A.M., Álvarez, C., Zeugolis, D.I., Henchion, M., O'Neill, E. & Drummond, L. (2017).
401 Alternative uses for co-products: Harnessing the potential of valuable compounds from meat
402 processing chains. *Meat Science*, **132**, 90-8.

403 Mulvihill, B., Kirwan, F.M., Morrissey, P.A. & Flynn, A. (1998). Effect of myofibrillar muscle
404 proteins on the in vitro bioavailability of non-haem iron. *International Journal of Food Sciences*
405 *and Nutrition*, **49**, 187-92.

406 Nurkhoeriyati, T. Huda, N. & Ahmad, R. (2010). Gelation properties of spent duck meat surimi
407 like material produced using acid- alkaline solubilisation methods. *Journal of Food Science* **76**,
408 S48-S55.

409 Pachón, H., Stoltzfus, R.J., & Glahn, R.P. (2008). Chicken thigh, chicken liver, and iron-fortified
410 wheat flour increase iron uptake in an in vitro digestion/Caco-2 cell model. *Nutrition*
411 *Research,* **28**, 851-8.

412 Pachón, H., Stoltzfus, R.J. & Glahn, R.P. (2009). Homogenization, lyophilization or acid-
413 extraction of meat products improves iron uptake from cereal-meat product combinations in
414 an in vitro digestion/Caco-2 cell model. *British Journal of Nutrition,* **101,** 816-21.

415 Perfecto, A., Elgy, C., Valsami-Jones, E., Sharp, P., Hilty, F. & Fairweather-Tait, S. (2017).
416 Mechanisms of iron uptake from ferric phosphate nanoparticles in human intestinal Caco-2
417 Cells. *Nutrients*, **9,** E359. doi: 10.3390/nu9040359.

418 Pongrac, P., Scheers, N., Sandberg, A.S., Potisek, M., Arčon, I., Kreft, I., Kump, P. & Vogel-
419 Mikuš, K. (2016). The effects of hydrothermal processing and germination on Fe speciation
420 and Fe bioaccessibility to human intestinal Caco-2 cells in Tartary buckwheat. *Food Chem*istry,
421 **15**, 782-90.

422 Rodriguez-Ramiro, I., Perfecto, A. & Fairweather-Tait, S.J. (2017). Dietary factors modulate
423 iron uptake in Caco-2 Cells from an iron ingot used as a home fortificant to prevent iron
424 deficiency. *Nutrients*, **12,** E1005. doi: 10.3390/nu9091005.

425 Sandberg, A.S. (2010). The use of caco-2 cells to estimate Fe absorption in humans--a critical
426 appraisal. *International Journal for Vitamin and Nutrition Research*. **80,** 307-13.

427 Seth, A. & Mahoney, R.R. (2000). Iron chelation by digests of insoluble chicken muscle protein:
428 the role of histidine residues. *Journal of the Science of Food and Agriculture,* **81**, 183–187.

429 Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D.,
430 Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk D.C. (1985). Measurement of protein using
431 bicinchoninic acid. *Analytical Biochemistry,* **150**, 76–85.

432 Stevens G. A., Finucane M. M., De-Regil L. M., Paciorek C. J., Flaxman S. R., Branca F., Peña-
433 Rosas, J.P., Bhutta, Z.A. & Ezzati M. (2013). Global, regional, and national trends in
434 haemoglobin concentration and prevalence of total and severe anaemia in children and
435 pregnant and non-pregnant women for 1995–2011: A systematic analysis of population-
436 representative data. *Lancet Globalization and Health*, **1**, e16–e25. doi: 10.1016/S2214-
437 109X(13)70001-9

438 Storcksdieck, G., Bonsmann, S. & Hurrell, R.F. (2007). Iron-binding properties, amino acid
439 composition, and structure of muscle tissue peptides from in vitro digestion of different meat
440 sources. *Journal of Food Science*, **72**, S019-29.

441 Swain, J.H., Tabatabai, L.B. & Reddy, M.B. (2002). Histidine content of low-molecular-weight
442 beef proteins influences non-heme iron bioavailability in Caco-2 cells. *Journal of Nutrition,*
443 **132,** 245-51.

444 Taylor, P.G., Martínez-Torres, C., Romano, E.L. & Layrisse, M. (1986). The effect of cysteine-
445 containing peptides released during meat digestion on iron absorption in humans. *The*
446 *American Journal of Clinical Nutrition*, **43,** 68-71.

447 Toldrá, F., Aristoy, M.C., Mora, L. & Reig, M. (2012). Innovations in value addition of edible
448 meat by- products. *Meat Science* **92**, 290-296.

449 Toldrá, F., Mora, L. & Reig, M. (2016). New insights into meat by-product utilization. *Meat*
450 *Science* **120**, 54-59.

451 USDA (United States Department of Agriculture). (2018). Food Composition Databases. URL:
452 https://ndb.nal.usda.gov/ndb/. Accessed 10/9/2018

453 Wang, H. & Betti, M. (2017). Sulfated glycosaminoglycan-derived oligosaccharides produced
454 from chicken connective tissue promote iron uptake in a human intestinal Caco-2 cell line.
455 *Food Chemistry*, **220,** 460-469.

456 World Health Organization (WHO). (2017). Nutritional Anaemias: Tools for Effective
457 Prevention and Control. *Geneva: World Health Organization, 2017*. [ISBN 978-92-4-151306-
458 7;http://apps.who.int/iris/bitstream/handle/10665/259425/9789241513067-
459 eng.pdf?sequence=1]

460 Wu, H., Zhu, S., Zeng, M., Liu, Z., Dong, S., Zhao, Y., Huang, H. & Lo, Y.M. (2014). Enhancement
461 of non-heme iron absorption by anchovy (Engraulis japonicus) muscle protein hydrolysate
462 involves a nanoparticle-mediated mechanism. *Journal of Agricultural and Food Chemistry*, **27,**
463 8632-9.

464 Zhao, L., Wu, H., Zeng, M. & Huang, H. (2017). Non-heme iron loading capacities of anchovy
465 (Engraulis japonicus) meat fractions under simulated gastrointestinal digestion. *Journal of*
466 *Agricultural and Food Chemistry*, **65,** 174-81.

467

468 **Table 1.** Protein, cholesterol, fat and iron content of test materials. Protein content of beef,
469 kidney, lung, heart, acid and alkaline extracts of heart expressed as mean ± SD n=3. Protein
470 content of infant rice cereal from nutritional information on packaging. Cholesterol and fat
471 contents of heart and acid and alkaline extracts of heart expressed as mean ± SE n=3.

472

Test Material	Protein content (%)	Cholesterol content (mg/100g)	Fat content (%)	Fe content (μg/g)
Infant rice cereal	6.60	nd	nd	415.00
Bovine skeletal muscle (beef)	19.32 ± 0.09	nd	nd	48.26
Bovine kidney	15.50 ± 0.27	nd	nd	107.45
Bovine lung	21.12 ± 0.56	nd	nd	201.40
Bovine heart	18.20 ± 0.48	122.85 ± 3.29	1.05 ± 0.38	101.60
Acid extract of bovine heart	11.60 ± 0.12	52.15 ± 0.92	0.31 ± 0.04	73.05
Alkaline extract of bovine heart	7.40 ± 0.21	73.98 ± 3.79	0.27 ± 0.04	58.15

473 nd = not determined

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482 **Table 2**. Calculated increase in caco-2 cell ferritin formed in response to non-heme iron from
483 infant rice cereal in the presence of bovine test materials. Data from at least three
484 independent experiments for all samples and expressed as mean ± SE. Statistical significance
485 was determined by a one tailed paired t-test, * p <0.05, ** p<0.01 and *** p<0.001.

486

Infant rice cereal in the presence of:	Calculated increased response (ng / mg)[#]	SE	Significance
Bovine skeletal muscle (beef)	3.30	1.77	*
Bovine kidney	8.05	0.97	**
Bovine lung	17.78	4.11	*
Bovine heart	14.65	7.46	ns
Acid extract of bovine heart	10.54	0.15	***
Alkaline extract of bovine heart	3.67	3.22	ns

487 # *Increase in ferritin response of cells to iron in digest (ng/mg) =*

488 Calculated from data in figure 2. Cellular ferritin response (ng / mg) to rice cereal **+** test
489 material combination digest **–** (cellular ferritin response (ng / mg) to rice cereal digest **+**
490 cellular ferritin response (ng / mg) to test material digest)

491

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494 Figure legends

495 **Figure 1.** Soluble iron (µg) in 2ml of diluted test digest after centrifugation (1:2
496 digest:Minimum Essential Medium) applied to caco-2 cells. Digests were prepared using iron
497 fortified infant rice cereal (1.5 g) alone or with bovine test materials as follows: beef (0.5 g);
498 kidney (0.62 g); lung (0.46 g); heart (0.53 g); acid heart extract (0.83 g) and alkaline heart
499 extract (1.31 g). Data are expressed as mean ± SE with a minimum n=3 for each digest.
500 Statistical analysis by a one way ANOVA with Dunnet's post hoc test shows no difference
501 between any test material digest.

502

503 **Figure 2.** Caco-2 cell ferritin formation (ng / mg) in response to incubation with test
504 material digests (1:2 digest:Minimum Essential Medium). Data represent at least three
505 independent experiments for all samples, and are shown as mean ± SE. A: digests prepared
506 from an infant rice cereal (1.5 g), beef (0.5 g), and infant rice cereal (1.5 g) beef (0.5 g)
507 combination. B: digests prepared from an infant rice cereal (1.5 g), bovine kidney (0.62 g),
508 and infant rice cereal (1.5 g) bovine kidney (0.62 g) combination. C: digests prepared from
509 an infant rice cereal (1.5 g), bovine lung (0.46 g), and infant rice cereal (1.5 g) bovine lung
510 (0.46 g) combination. D: digests prepared from an infant rice cereal (1.5 g), bovine heart
511 (0.53 g), and infant rice cereal (1.5 g) bovine heart (0.53 g) combination. E: digests prepared
512 from an infant rice cereal (1.5 g), acid bovine heart extract (0.83 g), and infant rice cereal
513 (1.5 g) acid bovine heart extract (0.83 g) combination. F: digests prepared from an infant
514 rice cereal (1.5 g), alkaline bovine heart extract (1.31 g), and infant rice cereal (1.5 g)
515 alkaline bovine heart extract (1.31 g) combination. Statistical significance as determined by

516 a two-tailed unpaired t test with Welch's correction, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

517 IRC = infant rice cereal.

518

519 **Figure 3.** *In vitro* bioavailability efficiency of the cereal-test material combinations.

520 Digests were prepared using infant rice cereal (1.5 g) alone or in combination with bovine

521 test materials as follows: beef (0.5 g); kidney (0.62 g); lung (0.46 g); heart (0.53 g); acid heart

522 extract (0.83 g) and alkaline heart extract (1.31 g). Soluble iron content of digests after

523 centrifugation as in fig. 1 were used in preparing data. “Absorption efficiency” for infant

524 rice cereal digests with bovine test materials is expressed as a % of the reference, infant rice

525 cereal (100%). Absorption efficiency determined as follows:

526 *Absorption efficiency (ng/µg)* = ferritin formed by cells (ng per well)

527 Fe in 2ml of 1:2 diluted digest applied to cells (µg per well)

528

529 Data shown are from at least three independent experiments and are expressed as mean ±

530 SE. For statistical analysis data were log transformed and analysed using a one sample t-test

531 comparing each condition to the reference, infant rice cereal, * $p < 0.05$.

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