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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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20   <u>Summary:</u>

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   <u>Introduction:</u>

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

2

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   <u>Material and Methods:</u>

86   **Chemicals**

4

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.

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.

***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

7

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) =*   <u>ferritin formed by cells (ng per well)</u>

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

201

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

<u>Results and Discussion:</u>

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

330   Acknowledgments:

335

336   <u>Conflict of Interest:</u>

337   The authors declare no conflict of interest.

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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

492

493

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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