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Authors	O'Flaherty, Elisabeth A. A.;Tsermoula, Paraskevi;O'Neill, Eileen E.;O'Brien, Nora M.
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Co-products of beef processing enhance non-heme iron absorption in an *in vitro* digestion/caco-2 cell model

Elisabeth A. A. O'Flaherty, Paraskevi Tsermoula, Eileen E. O'Neill and Nora M. O'Brien*

School of Food and Nutritional Sciences, College of Science, Engineering and Food Science (SEFS), University College, Cork, Ireland

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

* To whom correspondence should be sent: Professor Nora O'Brien, School of Food and Nutritional Sciences, College of Science, Engineering and Food Science (SEFS), University College Cork, Ireland. E-mail: nob@ucc.ie

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Summary:

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

Introduction:

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

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

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

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

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

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

Material and Methods:

Chemicals

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

Raw materials

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

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

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

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

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

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

Protein, cholesterol, lipid and iron measurements.

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

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

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

Cell culture

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

***In vitro* digestion**

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

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

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

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

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

Iron uptake and ferritin measurement

Iron uptake was determined by cellular formation and storage of ferritin, an established model first described by Glahn *et al.* (1998). An adapted model, where digests are applied directly to caco-2 cells (Andre *et al*., 2015; Pongrac *et al*., 2016; Perfecto *et al*., 2017) 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.

Absorption efficiency (ng/µg) = $\frac{\text{ferritin formed by cells (ng per well)}}{\text{Fe in 2ml of 1:2 diluted digest applied to cells (µg per well)}}$

Statistical methods

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

Results and Discussion:

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

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

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

Intrinsic iron content of test materials

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

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

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

To further distinguish the "meat factor" component of the test materials from their 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).

Intrinsic iron content of bovine co-products

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

Conclusion:

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

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Conflict of Interest:

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)

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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)* = $\frac{\text{ferritin formed by cells (ng per well)}}{\text{Fe in 2ml of 1:2 diluted digest applied to cells (µg per well)}}$

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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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