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In vitro evaluation of the effect of yogurt acid whey fractions on iron bioavailability.

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ABSTRACT

A side effect of the raised consumption of Greek yogurt is the generation of massive amounts of yogurt acid whey (**YAW**). The dairy industry has tried several methods for handling these quantities which constitute an environmental problem. Although the protein content of YAW is relatively low, given the huge amounts of produced YAW, the final protein content of the produced YAW should not be underestimated. Taking into consideration the increased interest for bioactive peptides and the increased demand for dietary proteins, combined with YAW's protein and peptides content, efforts should be made toward reintroducing the latter in the food supply chain. In this context and in view of the prevalent dietary iron deficiency problem, the objective of the present study was the investigation of YAW fractions' effect on Fe bioavailability. With this purpose, an in vitro digest approach, following the INFOGEST protocol, was coupled with the Caco2 cell model. To evaluate whether YAW digest fractions exert positive, negative or neutral effect on Fe bioavailability, they were compared with the ones derived from milk, a well-studied food in this context. YAW and milk showed the same effectiveness on both Fe bioavailability and the expression of relative genes (*DCYTB*, *DMT1*, *FPN1* and *HEPH*). Focusing further on YAW's fractions, by comparison with their blank digest control counterparts, it resulted that YAW 3–10 kDa digests fraction had a superior effect over the 0–3 kDa fraction on Fe-uptake, which was accompanied by a similar effect on the expression of Fe metabolism-related genes (*DCYTB*, *FPN1* and *HEPH*). Finally, although the 3–10 kDa fraction of bovine YAW digests resulted in a statistically non-significant increased Fe uptake, compared with the ovine and caprine YAWs, the expression of *DCYTB* and *FPN1* genes underlined this difference by showing a similar pattern with statistically significant higher expression of bovine compared with ovine and bovine compared with both ovine and caprine, respectively. The present study deals with the novel concept that YAW may contain factors affecting Fe bioavailability. The results show that it does not exercise any negative effect and support the extensive investigation for specific peptides with positive effect as well as that YAW proteins should be further assessed on the prospect that they can be used in human nutrition.

Keywords: In vitro digestion, iron bioavailability, acid whey upscale

INTRODUCTION

During the production of Greek-type strained yogurt there is a straining step in which the excess of aqueous serum is removed. This serum has low pH (4.21 - 4.60) (Menchik et al., 2019; Karastamatis et al., 2022) and is called yogurt acid whey (**YAW**). On average, for every unit of produced yogurt, 2 units of YAW are generated (Erickson 2017). YAW has, among others, considerable amount of lactose, high mineral content as well as small amounts of protein (Menchik et al., 2019). However, both yield and composition of YAW can vary depending on several factors, e.g., manufacturing conditions, method of straining and characteristics of milk (Lievore et al., 2015; Karastamatis et al., 2022).

The disposal of YAW as waste can present a huge environmental concern, rendering its handling problematic (Menchik et al., 2019). The increasing demand and production of Greek yogurt worldwide results in huge amounts of YAW, necessitating the development of new upcycling options on top of adopting the already existing. Presently, one of the most common ways for handling YAW is its use for biofuel production (Rocha-

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Mendoza et al., 2021). In parallel, several alternative strategies to cope with the problem (like production of YAW with higher pH or decreasing YAW yield) have been tried by several industries (Rocha-Mendoza et al., 2021) while new uses of YAW have been suggested by research groups (Camacho Flinois et al. 2019a; Camacho Flinois et al. 2019b; Simitzis et al., 2021; Andreou et al., 2022; Cetin et al., 2023).

As already mentioned, YAW, among others, contains protein. Specifically, 100 mL of YAW has been reported to contain protein in a range between 0.171 and 0.68 g (Menchik et al., 2019; Karastamatis et al., 2022). This protein concentration could be considered relatively low, but in view of the massive amounts of the produced YAW, the total amount of protein contained in YAW is substantial. Furthermore, the fraction of proteins that are present in YAW is considered to have higher biological value compared with the total milk proteins. This is because YAW proteins have higher ratios of essential amino acids and are more efficiently absorbed by the digestive system (Bozanic et al., 2014). Furthermore, whey proteins are an excellent source for bioactive peptides (Bozanic et al., 2014; Rocha-Mendoza et al., 2021; Karimi et al., 2022). Thus, there is a contradiction; on the one hand there is an increasing demand for quality dietary proteins and on the other hand a significant part of the YAW proteins is not reintroduced in the food supply chain.

Iron is involved in many essential biological processes like delivery of oxygen to tissues, energy metabolism and many more. However, the fine regulation of its absorption is fundamental because it can be toxic when present in excess (Fuqua et al., 2012). During post-natal life, iron physiologically enters the organism through the small intestine as a component of the diet. It is mainly found in 2 forms, heme iron, which is found in meat, and non-heme iron. The absorption of non-heme iron by the enterocytes starts with the reduction of ferric iron to ferrous, a process which is done by duodenal Cytochrome *b* (DCYTB) and possibly other reductases. Next, it is transported into cells by the divalent metal-ion transporter 1 (DMT1), where it can be stored within ferritin. The export to circulation is done across the basolateral membrane via ferroportin (FPN1) and is coupled with iron oxidization by hephaestin (HEPH), since the ferric form of iron is required for binding by transferrin (Fuqua et al., 2012; Gulec et al., 2014).

Iron deficiency, which is the depletion of iron stores, is associated with anemia (Camaschella 2019). Iron deficiency anemia is more common in women, children, people from low-income and middle-income countries and disadvantaged subpopulations of developed countries (e.g., immigrants, refugees, people of low income, indigenous population) (Pasricha et al., 2021). In 2016, 41.7% of young children and 40.1% of pregnant women worldwide were anemic. WHO and several meta-analysis studies, although they differ in their exact estimations, agree that a significant part of the above cases is attributed to iron deficiency (Pasricha et al., 2021). Iron deficiency anemia accounts for adverse outcomes of pregnancy, impairment of cognitive performance in young children, decreased physical condition in adults and cognitive decline in elder people (Camaschella 2019).

There is an ongoing worldwide effort for global control of anemia. For example, WHO aims to reduce anemia prevalence by 50% in women by 2025. Iron food fortification is considered effective, economically attractive, safe and socially acceptable, rendering it as the strategy of choice for reducing iron deficiency in many countries. As vehicles for iron fortification several industrially manufactured foods have been used, like sugar, salt, margarine, cereals, milk and other dairy products (Quintaes et al., 2017). In parallel, ongoing research efforts focus on the identification of food-derived bioactive peptides that promote the bioavailability of Fe (Caetano-Silva et al., 2015; Eckert et al., 2016; Ma et al., 2019; Yuanqing et al., 2021).

Given the raised demand for quality dietary protein along with the high biological value of whey proteins and the underuse of YAW as protein source, our group is involved in an effort to evaluate the advantages of reimporting YAW-derived proteins and peptides in the food chain supply. The objective of the present study was to evaluate the effect of YAW in iron absorption by enterocytes. We have used an established protocol for in vitro digestion and have assessed the effect of YAW fractions in iron absorption by Caco2 cells and the expression of the relative genes.

MATERIALS AND METHODS

Samples and experimental design

After a thorough search for available YAW samples, 33 YAW samples were obtained from 12 Greek dairy products enterprises. Twenty of the YAW samples were derived from bovine milk, 7 from ovine milk and 6 from caprine. Concerning the method of straining, 21 samples were produced by the traditional method with bag cloth/cheesecloth straining method, 6 by centrifugation, 4 by double drainage and 2 by ultrafiltration. A mix of YAWs, mentioned as "YAW-mix" hereafter, was made by mixing equal amounts of 10 bovine, 3 ovine and 4 caprine YAWs to obtain a sample with average composition. The YAWs' protein content was assessed by the Kjeldahl method in duplicates. For comparative purpose, commercial bovine milk was obtained from a

local supermarket and the protein content which was indicated in the label was validated on a Milkoscan FT120 (Foss).

For the comparison of YAW with milk (see paragraph 3.2), the YAW-mix and the commercial milk sample were fortified with $FeSO_4$, then digested and the 0–10 kDa fractions were checked on the Caco2 system for their effect on Fe bioavailability. For studying the effect of YAW fractions on Fe bioavailability (see paragraph 3.3), 8 YAW samples of cow origin that were manufactured with the traditional way (i.e., with cloth bags) as well as 6 control digest samples (digestion products using water instead of food – see paragraph 2.2), were digested and tested in the Caco2 system in the presence of 100 μM $FeSO_4$. For the case of the species effect on Fe bioavailability (see paragraph 3.4), the 3–10 kDa fractions of all 33 YAWs digests were tested in Caco2 in the presence of 100 μM $FeSO_4$.

In vitro digestion

YAW samples and YAW-mix were 5- and 4.5-fold concentrated by lyophilization, respectively. All experiments were done in the basis of equal protein amounts between the tested samples. Thus, during the in vitro digestion, the amounts of YAW or milk which were used corresponded to similar protein concentrations; namely 0.113825%wt/vol and 0.275%wt/vol for the comparisons within YAWs and between milk and YAW, respectively. For the comparative experiments between milk and YAW-mix (paragraph 3.2), before digestion both foods were fortified with $FeSO_4$ to a final concentration of 100 mg/L Fe and next were subjected to in vitro digestion. For the comparisons between different YAW samples (paragraphs 3.3 and 3.4), no Fe fortification was done before digestion since the $FeSO_4$ was added along with the digest fractions on the Caco2 cells as described in 2.4. For the in vitro digestion of the foods, the *INFOGEST 2.0* protocol (Brodkorb et al., 2019) was used with some modifications. Briefly, for the oral phase, each food was diluted 1:1 (vol/vol) with simulated salivary fluid without salivary amylase, the pH was adjusted to 7.0 and the total fluid was incubated for 2 min at 37°C while mixing. For the blank digest controls, which were used in the paragraph 3.3, pure water was diluted 1:1 with simulated salivary fluid and all further process was identical to that for experimental samples. For the gastric phase, the fluid was diluted 1:1 (vol/vol) with simulated gastric fluid, the pH was adjusted to 3.0, porcine pepsin (P7012, Sigma) was added at a final activity 2,000 U/ml and the mixture was incubated for 2 h at 37°C rotating. For the intestinal phase, the gastric mixture was diluted 1:1 (vol/vol) with simulated intestinal fluid, the pH was adjusted to 7.0, pancreatin (P3292, Sigma) was added so as the final activity of trypsin in pancreatin to be 100 U/ml, bile salts (B8631) were added at a final concentration of 4.8 mM and the mixture was incubated for 2 h at 37°C rotating. The specific activities of the digestive enzymes and bile salts concentration were determined according to the standardized assays in the *INFOGEST 2.0* protocol. After the completion of the intestinal phase, the digests were incubated for 10 min at 85°C for enzyme deactivation. Next, the samples were centrifuged for 15 min at 4,800 g and the supernatants were passed through 0.22 μM sterile filters (Merck). The samples were passed through 10 kDa molecular weight cutoff filters (Millipore and Thermo) and the 0–10 kDa fractions were used in the subsequent analyses both for protecting the Caco2 monolayer from harmful remaining parts of the digestion (Fairweather-Tait et al., 2007) and for focusing on lower peptide size ranges closer to the size of typical bioactive peptides (Liang et al., 2023). For the experiments in which the fraction effects were studied, the 0–10 kDa fractions were further passed through 3 kDa cutoff filters (Millipore and Thermo) for obtaining the 0–3 kDa and 3–10 kDa fractions. The fractionation was done also for the blank digest controls of the paragraph 3.3.

Caco-2 cell model

Caco2 cells were kindly gifted by Dr. Dimitris Kletsas (National Center for Scientific Research "Demokritos," Greece). The cells were maintained in high glucose Dulbecco′s Modified Eagle Medium (**DMEM**) (Pan Biotech) supplemented with 10% fetal bovine serum (**FBS**) (Gibco), 1x non-essential amino acids (Pan Biotech) and 1% Penicillin/Streptomycin (Pan Biotech) in a humidified incubator at 37°C in a 5% CO_2 atmosphere. Cells were passaged with trypsin (Pan Biotech) before reaching confluency. When used in assays, cells with passage number between 9 and 15 (passage was defined as 1 when arrived in the lab), were cultured for 14 d after seeding, changing the medium every 2 or 3 d.

Iron uptake assays

For assessing the Fe levels in Caco2, 14 d after seeding, the cells were incubated for 24 h in DMEM without FBS in the presence of 100 μM $FeSO_4$ and 15% of the tested fraction of YAW or milk digests. For the comparisons of YAW samples (section 3.3 and 3.4), in addition to the tested digestion fraction, 100 μM $FeSO_4$ were added to the Caco2 cells. For the comparative experiments between milk and YAW-mix, which had both been fortified with Fe before digestion, extra $FeSO_4$ was not added in the Caco2 cells. Since during

fractionation the initial volume is shared within fractions, the fraction volumes are smaller than the initial non-fractionated volume. The concentration of 15% that was used for incubation with the cells, refers to fractions after correcting their resulting volume to the initial non-fractionated volume. Next, the protocol of Riemer et al. (2014) for assessing Fe levels, was used with modifications. Briefly, the cells were washed twice with ice-cold PBS and then eluted in 50 mM NaOH by shaking for 2 h. The lysates were mixed at 1:1:1 (vol:vol:vol) with 10 mM HCl and freshly prepared iron releasing reagent (0.7 M HCl and 2.25% (wt/vol) $KMnO_4$) and incubated for 2 h at 60°C. After cooling in room temperature, freshly prepared iron detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, 1 M ascorbic acid) was added to the mixtures at 10:1 (vol:vol). The absorbance was measured in duplicate at 550 nM within 30 min. For the standard curve, known concentrations of $FeSO_4$ solutions in 50 mM NaOH were in parallel subjected to the above protocol. The final iron concentrations were normalized to the amount of cell protein per well (see below).

For the indirect estimation of the iron uptake by the cells via assessment of ferritin protein levels, a human ferritin enzyme-linked immunosorbent assay (**ELISA**) kit was used (AssayGenie, HUFI00311). The cells were treated with food digest fractions and $FeSO_4$ as described above for the colorimetric assay and the procedure was continued as described in the kit manual. Caco2 ferritin levels were normalized to the amount of Caco2 protein levels per well (see below).

The cell protein levels were estimated with the Lowry method. Briefly, 5 μL of cell lysates sample were diluted with 95 μL H_2O, 200 μL of freshly made reagent A (1% wt/vol $CuSO_4$, 2% wt/vol sodium potassium tartrate and 2% wt/vol Na_2CO_3 at 1:1:100 mix) were added and incubated for 10 min at room temperature. Next, 20 μL of 1 N Folin & Ciocalteu's reagent was added and the absorbance was measured at 750 nm after 30 min. For the standard curve, serial dilutions of bovine serum albumin of known concentration were used.

Quantification of gene expression

For quantifying the transcript levels of genes related to iron absorption, Caco2 cells were treated with food digest fractions as described in paragraph 2.4. Next, cells were lysed with NucleoZOL (Macherey-Nagel) and the RNA was extracted according to the manufacturers' instruction. RNAs were treated with DNase (NEB) for removal of remaining DNA and pure RNA was recovered by ethanol precipitation in the presence of ammonium acetate and glycogen. The quantity and purity of RNAs were calculated using a spectrophotometer (Q5000, Quawell). The reverse transcription was done with the PrimeScrip RT reagent Kit (Takara) using both oligo-dT and random hexamers for priming the reaction. qPCR reactions were done using the FastGene IC Green 2X qPCR Universal Mix (Nippon Genetics) and the primers shown in Table 1 in a SA cycler 96 (Sacace). Crossing points (Cp) were calculated using the instrument's software. Concerning the genes that were quantified, 2 of them code for proteins which are associated with the uptake of non-hemic Fe by the cells, DCYTB and DMT1. While 2 more genes are implicated in the efflux of Fe from the enterocytes, FPN1 and HEPH (Sucru et al., 2014). GAPDH and HPRT1 were used as housekeeping genes, with the geometric mean of both being used for normalizing gene expression (Vandosempele et al., 2001).

Statistical analysis

Statistical analyses were done in R or Microsoft Excel. Groups statistical comparisons were done by one-factor ANOVA (**ANOVA**), with Tukey's test for pairwise multiple comparisons. Comparison of 2 means were done by Student's *t*-test. All samples were measured in 2 technical replicates. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Protein level of YAW samples

Given that all comparisons in the present study were done in a protein content basis, we first quantified the proteins/peptides in all YAW samples. As shown in Figure 1, it ranged between 0.09106 and 1.24344%(wt/vol). Menchik et al. (2019) quantified the protein levels of YAW samples collected from 3 companies located in New York State and found that it ranged between 0.171 – 0.371%(wt/vol) (converted from mg/g with an assumed relative density of YAW equal to 1). Karastamatis et al. (2022), testing several conditions of yogurt manufacturing in the laboratory, reported that the protein content of YAW ranged between 0.41 – 0.68%(v/w). The protein contents in both of the above studies were fully within the range reported here. The wider range in our study may result from the much higher number of tested samples. The protein content of the commercial milk was 3.31%(wt/vol), which was very close to the values reported in the literature (Lin et al., 2021).

Comparison of YAW's and milk's effects on Fe bioavailability

Due to total lack of prior research concerning the effect of YAW on Fe uptake, our initial approach involved a comparative analysis of YAW and milk. The selection of milk as a reference material was done given the sufficient investigation that has been conducted regarding its effect on Fe bioavailability (Kapsokefalou et al., 2009; Palika et al., 2013; Stewart et al., 2018; Man et al., 2021), which make it a suitable benchmark for evaluating the as yet unexplored properties of YAW. An additional reason for choosing milk was that although YAW and milk digests do not necessarily contain the same peptides, their source proteins are, at least in part, common. The latter means that milk and YAW may share some common properties and accordingly outcomes of the research in milk may be the basis for further investigation for the case of YAW.

The comparison of YAW to milk was done on a protein content basis, meaning that the starting amounts of YAW-mix (see materials and methods) and milk in each experiment corresponded to same amount of protein. Figure 2A shows that concerning the effect of the 0–10 kDa digest fractions on Fe uptake by Caco2 cells, YAW-mix did not significantly differ from milk. Next, the transcriptional levels of the Fe-uptake associated genes, *DCYTB* and *DMT1,* were assessed and, as shown in Figure 2B, they did not differ between the 2 digests, in line with the results of Figure 2A. *HEPH* and *FPN1*, the genes which are implicated in the efflux of Fe from the enterocytes, were also checked. Figure 2C shows that neither these genes differ between YAW-mix and milk.

In literature there are several studies confirming the positive effect of milk components on iron absorption upon fortification. For example, Argyri et al. (2007) show that almost all fractions of cow milk digests have a positive effect on Fe uptake by Caco2, as assessed by ferritin levels. Palika et al. (2013) report a positive effect of human milk in both Fe solubility upon digestion and the actual uptake of Fe by Caco2 cells. Our procedure was a comparison on the basis of equal protein amounts. However, we should take into consideration ingredients

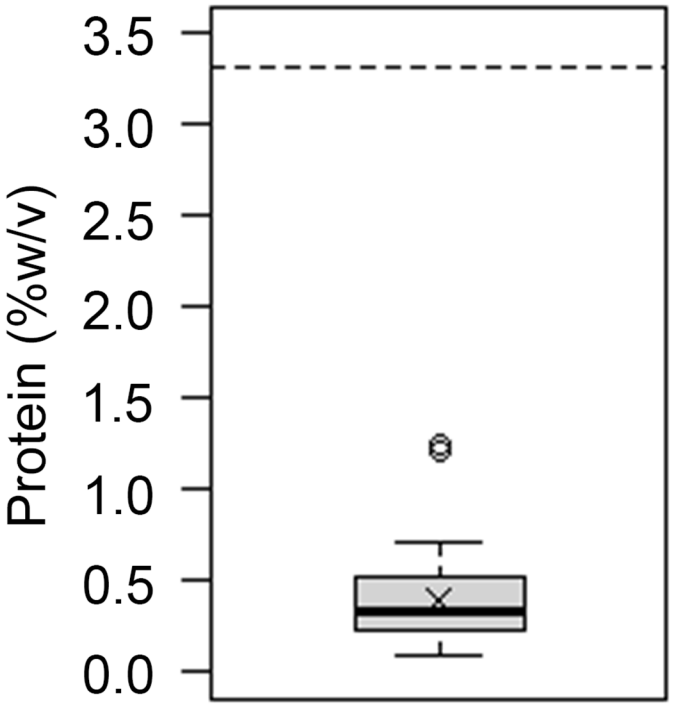

Figure 1. Protein concentration of the 33 yogurt acid whey (**YAW**) samples used in the study. The main box shows the 2nd and 3rd quartiles, the upper and lower whiskers show the 1st and 4th quartiles, the horizontal line within the box shows the median, X shows the mean value, circles show the outliers and the dashed line shows the protein concentration of the commercial milk sample.

of milk/YAW other than peptides that may affect Fe absorption. Such an ingredient is lactose, which according to the literature (Manchik et al., 2019; Karastamatis et al., 2022) should be around 5-fold higher in the protein-balanced YAW-mix compared with bovine milk. Although lactose is considered to have a rather positive effect on Fe absorption in rodents (Amine and Hegsted 1975; Minotti et al., 1993), in Caco2 cells it is reported to have no effect (Kongkachuichai et al., 1997). Our experiments cannot clarify whether the high lactose content of YAW-mix has no effect on Fe absorption or it counteracts the potential negative effect of other YAW ingredients, resulting this way in Fe absorption which is comparable with that of milk. Another YAW ingredient which is high in the YAW-mix

Table 1. qPCR primers sequences

Gene	Sequence forward	Sequence reverse
DCYTB	AACAGCACTTATGGGATTGAC	GATGAAGAATGGTAGAATTTGGCTC
HEPH	TCTCGAACAGAACACTTAAGCC	GATGGACCTCCTATTGCGTC
FPN1	GATGGGTTCTCACTTCCTGC	AGTTTGCTTCTGTCTTCTCCTG
DMT1	TATGTCACCGTCAGTATCCCA	CTTCCGCAAGCCATATTTGTC
GAPDH	CTCATTTCCTGGTATGACAACG	GGGAGATTCAGTGTGGTGG
HPRT1	CTTTGCTTTCCTTGGTCAGG	CAAATCCAACAAAGTCTGGCT

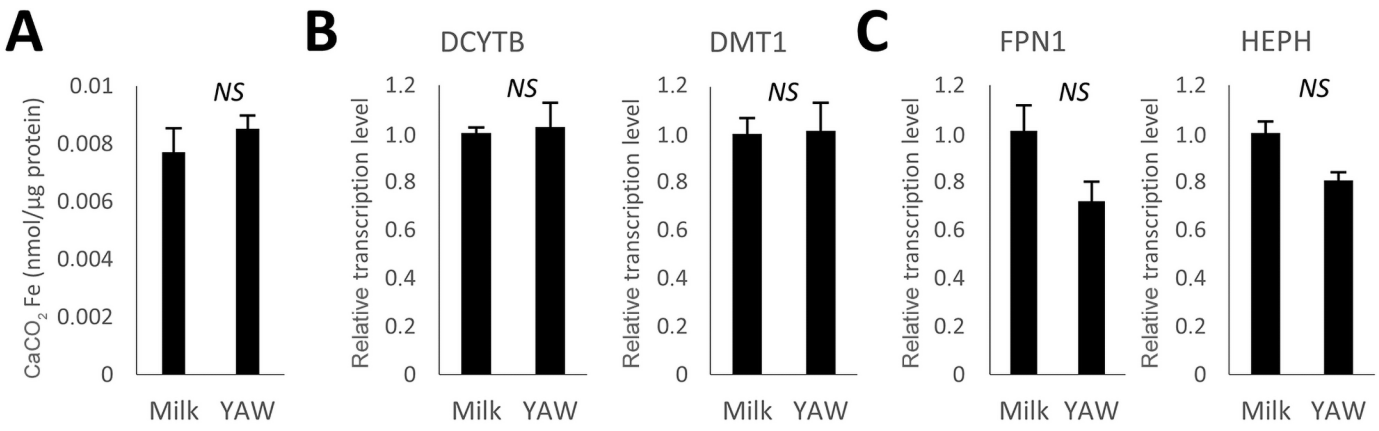

Figure 2. Comparison of the effects of Fe-fortified yogurt acid whey (**YAW**)-mix and milk on Fe uptake by Caco2. The 0–10 kDa fraction has been used in all plots. **(A)** Relative Fe concentration of Caco2 cells quantified by ferrozine-based colorimetric assay. **(B)** Relative transcription levels of genes related with import of Fe in the cells. **(C)** Relative transcription levels of genes related with export of Fe from the cells. The plots show mean values and standard errors resulted by 3 repeated experiments and duplicate technical replicates. Significance was tested by Student's t-test. Statistical significance was set at $p < 0.05$.

compared with milk is Calcium. Although it is reported to have an inhibitory effect on iron absorption (Harris 2002; Lynch 2010), the fact that the calcium content of the 4.5-fold concentrated YAW-mix is within the range of 475–560 mg/100g (Karastamatis et al., 2022), while milk contains 120 mg/100mL according to its labeling, did not seem to exert any negative effect on iron absorption. Our observations that the effect of YAW, which is rich in calcium, on iron uptake by Caco2 cells does not differ from that of milk, a food which has been widely studied and used in iron fortification schemes, provides sufficient evidence that YAW's effect on iron uptake is worth further investigation.

Effect of YAW fractions on Fe bioavailability

Having in mind that there is a fraction effect on Fe bioavailability for the case of milk (Argyri et al., 2007), next, this fraction effect was investigated also for YAW. Figure 3A shows that concerning the effect of the widest (0–10 kDa) fraction on Fe uptake, YAW digests do not differ from their control counterparts. The same is observed also for the 3–10 kDa fraction (Figure 3A). In contrast, for the 0–3 kDa fraction, YAW digests seem to have a negative effect on Fe uptake compared with their controls counterparts ($P = 0.018$) (Figure 3A). Next, for having a deeper insight into the mechanisms that may govern this differential effect of the YAW fractions, the effects of the 0–3 kDa and 3–10 kDa fractions on the transcription levels of genes related to Fe uptake, were investigated. Figure 3B shows that for the case of DMT1, for both fractions the effect of YAW and controls do not differ. In contrast, when considering the transcription of *DCYTB* gene, the fractions effects match those of Fe uptake, which means that while there is no difference between YAW and control for the 3–10 kDa fraction, for the 0–3 kDa fraction the YAW digests result in lower *DCYTB* levels ($P = 0.001$). The results were similar to that of Fe uptake also when considering expression of *FPN1* ($P = 0.02$) and *HEPH* ($P = 0.004$) which are related to the export of Fe from the enterocytes (Figure 3C).

One interpretation of the above results could be that the 0–3 kDa fraction contains factors that inhibit the uptake of Fe, with the potential mechanism including negative regulation of DCYTB expression. The 3–10 kDa fraction could either lack these inhibitory factors or could have extra factors that neutralize the inhibitory effect. The hypothesis of neutralizing factors could further be supported by the observation that the 0–10 kDa fraction, which should also contain the inhibitory factors, does not have any significant negative effect on Fe uptake.

This differential effect of digest fractions on Fe bioavailability has not been only observed in milk as already mentioned, but has been reported also for other foods. For example, Swain et al. (2002) report the differential fraction effect of beef digests on non-heme Fe, while Ying et al. (2014) observed this effect in soybean proteins. Although the fraction effect for other foods has been suggested to be associated with carbohydrates (Huh et al., 2004; Rodriguez-Ramiro et al., 2019), in milk, the fraction effect has been attributed mainly to peptides. Certain peptides exert their positive effect on Fe bioaccessibility through their increased chelating activities, which are connected with the structure of their backbones (Sun et al., 2020).

Although milk and YAW are 2 distinct food matrices and each of them has specific features, given that YAW digests' peptide content result from proteins present in milk, it is reasonable to focus on comparing our results with the relative literature regarding milk. It should be noted that for most of the studies found in the literature, including the studies that are referred here (concerning milk or other foods), the fraction effect has been shown either by direct comparison between the tested fractions or by comparison with Caco2 which are not treated with digests. However, we followed a slightly different procedure to filter out the effects of the factors derived from the digestion procedure per se which may affect iron bioavailability. Thus, as mentioned in materials and methods, we evaluated the effect of the digest fractions on iron bioavailability using blank digests as controls. Following this procedure, these big differences between the fractions which are shown in other studies, here (although still present) have been decreased due to the comparison with actual aqueous digests (blank digest controls).

Focusing on milk, Etcheverry et al. (2004) report the existence of a fraction effect in human milk whey. They show that the 0–10 kDa peptide fraction result in higher Fe uptake by Caco2 in comparison to the fraction bigger than 10 kDa. Argyri et al. (2007) show that 2 sharp fractions (within 1–1.5 kDa and 1.5–5 kDa) of bovine milk digests significantly enhance Fe uptake. Although the existence of peptides with positive effect between 1.5 and 5 kDa is in line with our results, the existence in YAW of peptides between 1 and 1.5 kDa with highly positive effect is not supported by our data. The latter implies that in the smallest fractions of milk digests, peptides with positive effect exist but are absent in YAW digests. However, it should be noted that cutoff membranes allow a small "leakage" between fractions that has to do mainly with the properties of the peptides. Since Argyri et al. used whole milk in their study, the mentioned peptide fractions (between 1 and 1.5 kDa) could have resulted from digestion of proteins absent in YAW, like casein, or from proteins/peptides which are absent in YAW due to proteolysis resulting from fermentation. Argyri et al. also show that among all peptidic fractions that were tested, the lower ferritin formation in Caco2 cells was resulted by the fraction having the smallest molecular weight. This is in line with the abovementioned hypothesis, about the existence of a factor/peptide in the 0–3 kDa fraction with negative effect which is neutralized by the 3–10 kDa fraction. Argyri et al. (2009), in a follow-up study, trying to investigate the mechanism underlying the enhanced effect of the above-mentioned fractions, show that they do not affect the expression of DMT1 (Argyri et al., 2009). This observation of altered Fe uptake which is not associated to DMT1, is in line with what we show here for YAW and suggests the existence of possible common mechanisms for the enhancing effect of the specific peptide fractions of milk and YAW.

It is known that there are casein-derived peptides with positive effect on Fe bioavailability. In this context, Argyri et al. (2007) have also checked the effect of a synthetic casein-derived peptide (PGPIPN) which they show to have a positive effect. However, there are a couple of studies supporting the idea that whey protein-derived peptides can also exert such positive effects. Caetano-Silva et al. (2015), subjected whey protein isolates to a hydrolysis protocol alternative to those followed in the in vitro digestions and identified specific peptides with increased Fe-binding capacity. The vast majority of these peptides results from β-lactoglobulin and α-lactalbumin. Although the major whey proteins may have different physio-chemical characteristics when present in acid, sweet or native whey (Nishanthi et al., 2017), given that both proteins are contained in considerable amounts in YAW (Smithers 2015; Karastamatis et al., 2022), the YAW peptides resulting from β-lactoglobulin and α-lactalbumin may warrant further investigation. Similarly, Ou et al. (2010) followed an alternative digestion protocol for sweet whey and also found peptide fractions with positive effect on Fe bioavailability.

Our data show a clear fraction effect of YAW on Fe bioavailability, as shown for milk and milk whey before. A positive effect in comparison to blank control samples was not possible to be shown with the procedure followed here. However, taking into consideration the literature regarding milk, it is reasonable to suggest the existence also in YAW digests of fractions/peptides with highly positive impact, the effect of which is masked by "negative" ones found in the same wide fractions. A more extensive fractionation could possibly help revealing these fractions.

Effect of species of YAW origin on Fe bioavailability

Next, we focused on the effect of the species of YAW origin on Fe bioavailability. We used the 3–10 kDa fractions of the YAWs because they exerted higher Fe bioavailability when compared with the 0–3 kDa fractions (see paragraph 3.3). As shown in Figure 4A, for both ferritin and Fe cell levels, cow YAW samples showed higher values than the other 2 species but this difference was not significant (ANOVA $P = 0.104$; cow-sheep post hoc $P = 0.088$). Interestingly, when the expression of the genes associated with Fe uptake was assessed, the higher value for cow was also the case for both genes (Figure 4B). Furthermore, this difference for the case of DCYTB was statistically significant ($P = 0.012$). The

same pattern was also observed for both genes associated with Fe release and it was significant for the case of FPN1 ($P = 0.001$) (Figure 4C).

As already mentioned, among the milk components which are associated to its Fe bioavailability, peptides are often mentioned for their central role. Concerning

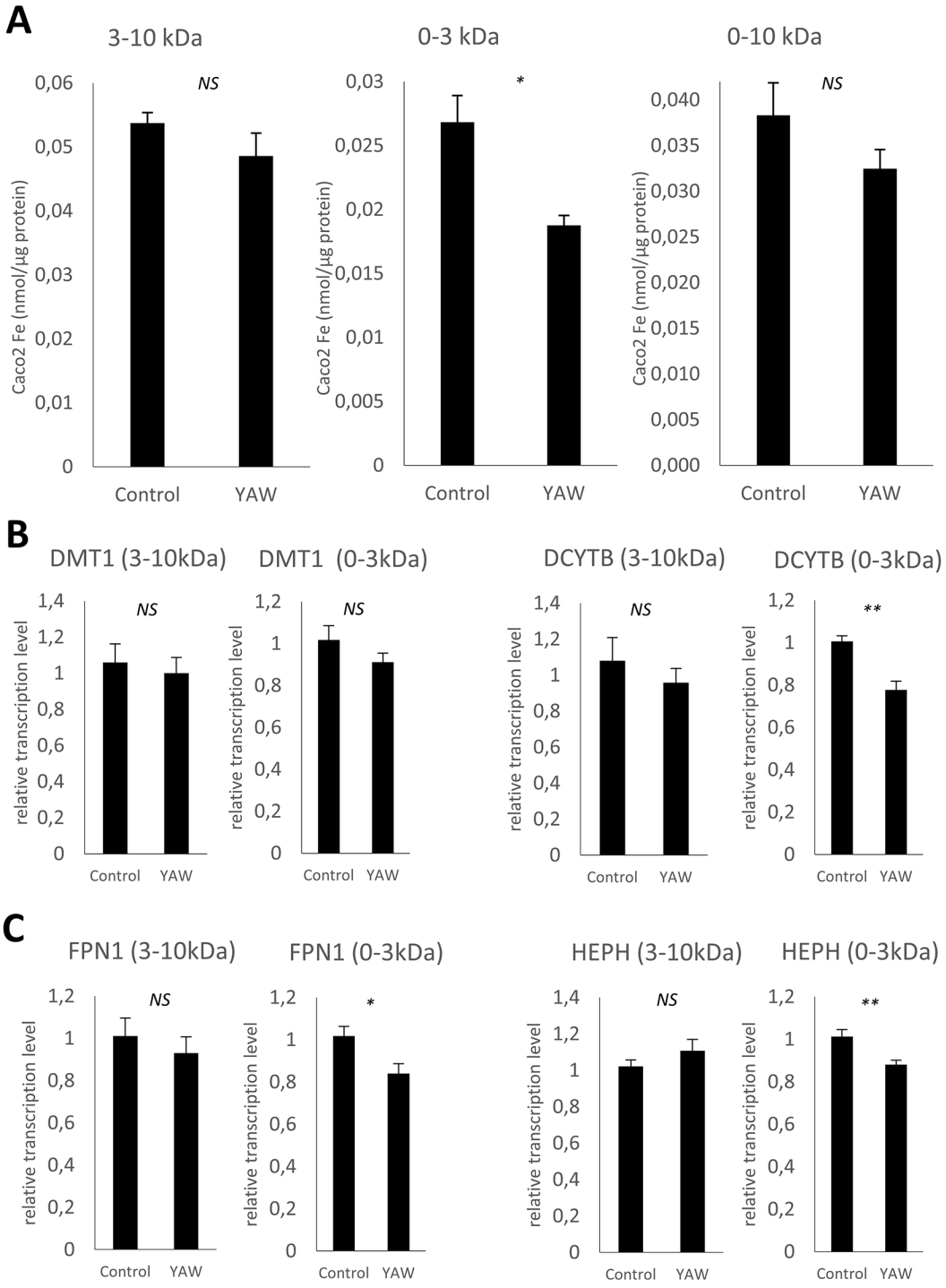

Figure 3. Fraction effect of yogurt acid whey (**YAW**) samples of bovine origin made by the traditional method on Fe uptake by Caco2. **(A)** Relative Fe concentration of Caco2 cells quantified by ferrozine-based colorimetric assay. **(B)** Relative transcription levels of genes related with import of Fe in the cells. **(C)** Relative transcription levels of genes related with export of Fe from the cells. The plots show mean values and standard errors of 8 YAW and 6 water-digests control samples run in duplicates. Significance was tested by Student's t-test. *, $p < 0.05$; **, $p < 0.01$.

milk, species differences in protein content and proteins' aminoacid composition (Jandal 1996; Nayak et al., 2020) are retained even after in vitro digestion with *INFOGEST* (Tagliazucchi et al., 2018), the protocol which was also used in the present study. Thus, it is reasonable to suggest that such differences exist also in YAW and could be a possible factor contributing to the YAW species effect of Figure 4. Data regarding species differences in YAW, to the best of our knowledge, do not exist but there is a single study reporting species effect on α-lactalbumin and β-lactoglobulin ratios in acid whey which is obtained by milk filtration under acidic conditions (Moatsou et al., 2005). Although the authors used a type of acid whey which differs from the acid whey generated during yogurt production, their results support the idea of different protein/peptide ratios in YAWs originated from different animal species.

The species effect on Fe bioavailability has been mentioned in several studies about milk and milk whey. Etcheverry et al. (1996) mention that human and bovine milk whey have different effect on Fe uptake by Caco2 cells. They show that while the 0–10 kDa fraction of human milk whey enhances Fe uptake, this fraction has no effect in the case of bovine milk whey. When the difference in Fe bioavailability between human and bovine milk was tested in human volunteers,

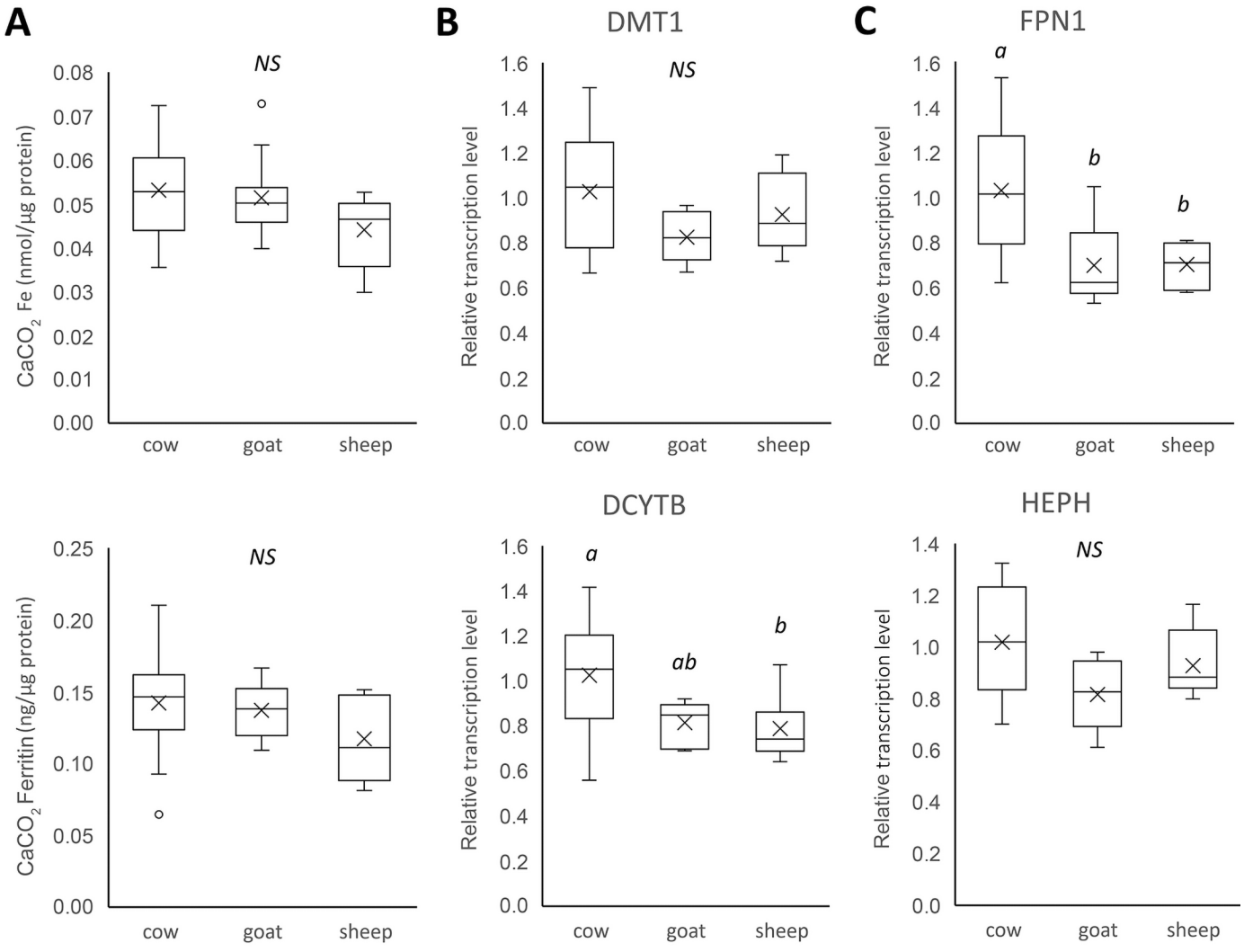

Figure 4. Yogurt acid whey (**YAW**) species effect on Fe uptake by Caco2. **(A)** Relative Fe uptake by Caco2 estimated by ferrozine-based colorimetric assay (*upper panel*) or ferritin levels with ELISA (*lower panel*). **(B)** Relative transcription levels of genes related with import of Fe in the cells. **(C)** Relative transcription levels of genes related with export of Fe from the cells. The plots show the effect of 33 YAW fractions (3-10 kDA) in duplicates. The main boxes show the 2nd and 3rd quartiles, the upper and lower whiskers show the 1st and 4th quartiles, the horizontal lines within the boxes show the median, circles show the outliers and the Xs show the mean values. Significances were checked by one-factor analysis of variance (ANOVA) with Tukey's test for pairwise multiple comparisons. NS and the presence of the same letter above groups indicate that there is no significance. $p < 0.05$.

it resulted that the former led to higher absorption ratio of Fe than the latter (Hallberg et al., 1992). The authors suggest as a possible explanation the lower Ca concentration of human milk, which inhibits uptake of Fe. Stewart et al. (2018) using the Caco2 system, concluded that the Fe-fortification of bovine milk resulted in higher Fe-bioavailability compared with the fortification of caprine milk. The authors suggest that this difference could have result from the higher level of cow milk in αs1-casein, which after digestion gives rise to Fe-binding phosphopeptides. Although the results of this work are in line with our data concerning YAW, peptides derived from casein's digestion cannot be responsible for the species effect of YAW and thus other factors should be further studied for this role.

Given that a) for YAW there is a significant species effect on the regulation of the genes implicated in Fe uptake and b) for the case of milk the differential effect of species on the Fe uptake per se by Caco2 cells is documented, we find that there are enough data to suggest that the trend observed in the Fe uptake by the Caco2 (Figure 4A) could reflect a real difference. Thus, we find that a deeper investigation of this potential species effect of YAW is interesting. Among the things that go beyond our study and could be helpful in this direction, is the inclusion of other experimental conditions like different Fe concentrations or incubation time with the Caco2 cells. Another improvement could be the use of semi-dynamic protocols for the in vitro digestion, which are increasingly used in food studies during the last years (Xavier et al., 2021). Furthermore, given that the discovery of new bioactive peptides is of considerable importance, as already suggested for the case of revealing potential effects of digest fractions, using methodologies for more extensive fractionation, could also help toward unraveling potential species-specific peptides with significantly enhancing effect on Fe bioavailability.

CONCLUSION

In the present study, the effect of YAW on Fe bioavailability was studied for the first time. Given that YAW is a potential source of dietary proteins and bioactive peptides, the whole study experimental design was done in a protein content basis. Our data show that Fe-fortification of YAW is not associated with any inhibitory effect regarding Fe-bioavailability. Furthermore, there is a clear fraction effect of YAW and a potential species effect on Fe absorption. The above help toward understanding and therefore manipulating those factors regarding yogurt production which impact the properties of YAW. Furthermore, our results put the basis for discovering bioactive peptides related to Fe-absorption in YAW. Although, the present study supports that YAW, concerning its role in Fe bioavailability, is acceptable for being used in food industry, it must be subjected to several in vitro and in vivo studies for characterizing a series of properties that will ensure its beneficial and safe use as a food or food ingredient.

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