Nicotianamine-chelated iron positively affects iron status, intestinal morphology and microbial populations \textit{in vivo} (\textit{Gallus gallus})

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Wheat flour iron (Fe) fortification is mandatory in 75 countries worldwide yet many Fe fortificants, such as Fe-ethylenediaminetetraacetate (EDTA), result in unwanted sensory properties and/or gastrointestinal dysfunction and dysbiosis. Nicotianamine (NA) is a natural chelator of Fe, zinc (Zn) and other metals in higher plants and NA-chelated Fe is highly bioavailable \textit{in vitro}. In graminaceous plants NA serves as the biosynthetic precursor to 2$^\prime$-deoxymugineic acid (DMA), a related Fe chelator and enhancer of Fe bioavailability, and increased NA/DMA biosynthesis has proved an effective Fe biofortification strategy in several cereal crops. Here we utilized the chicken (\textit{Gallus gallus}) model to investigate impacts of NA-chelated Fe on Fe status and gastrointestinal health when delivered to chickens through intraamniotic administration (short-term exposure) or over a period of six weeks as part of a biofortified wheat diet containing increased NA, Fe, Zn and DMA (long-term exposure). Striking similarities in host Fe status, intestinal functionality and gut microbiome were observed between the short-term and long-term treatments, suggesting that the effects were largely if not entirely due to consumption of NA-chelated Fe. These results provide strong support for wheat with increased NA-chelated Fe as an effective biofortification strategy and uncover novel impacts of NA-chelated Fe on gastrointestinal health and functionality.

Iron (Fe) supplementation and fortification are the two most widely used strategies to combat human Fe deficiencies that affect over 2 billion people worldwide\cite{1,4}. Iron supplementation involves large dose delivery of highly absorbable (bioavailable) Fe to humans and is effective in treating severe cases of Fe deficiency anemia\cite{2,5}. Iron fortification involves low dose delivery of bioavailable Fe fortificants to food products during manufacture (or point-of-use) and is an effective population-based strategy to boost Fe intakes. Iron fortification of wheat flour is now mandatory in 75 countries worldwide (Flour Fortification Initiative; \url{https://fortificationdata.org/}), however, the tendency of Fe fortificants such as ferrous sulfate (FeSO$_4$) to oxidize and cause undesired organoleptic and sensory properties pose significant challenges\cite{6,7}. Almost 90% of countries utilize fortificants with poor bioavailability or fortify at sub-optimal concentrations, although recent evidence suggests that Fe fortification can effectively reduce symptoms of Fe-deficiency anemia when correctly implemented\cite{8,9}. Iron chelated by ethylenediaminetetraacetate (EDTA) is a commonly recommended fortificant for cereal flour to minimize sensory alterations while providing Fe in a bioavailable form\cite{10,11,12}. Fortificants that utilize micro- and/or nanoencapsulation can further improve bioavailability\cite{13,14} although the cost of using appropriately chelated and/or encapsulated Fe fortificants ($2 USD per ton to fortify wheat flour with EDTA-chelated Fe alone), and the requirement for centralized cereal processing and industrial milling limits flour fortification programs in less developed countries\cite{11,15}. Furthermore, and perhaps more importantly, both supplementation and fortification frequently deliver excess...
dietary Fe to the human intestinal lumen which can cause severe gastrointestinal disruption, dysbiosis and the proliferation of non-beneficial gut bacteria.17–21.

Nicotianamine (NA) is a non-protein amino acid that functions as an endogenous chelator of Fe, zinc (Zn) and other transition metals in higher plants. In graminaceous cereals NA serves as the biosynthetic precursor to 2′-deoxymugenic acid (DMA), a related Fe chelator in plant tissues that also functions as a root-secreted phyto- siderophore to chelate ferric Fe in the rhizosphere.23 Both NA and/or DMA are major Fe chelators in white wheat (Triticum aestivum L.) flour and enhancers of in vitro Fe bioavailability24–26 and increased NA/DMA biosynthesis has been employed to biofertilize wheat25,27 and rice (Oryza sativa L.)28–30 with Fe and Zn. While both NA and DMA chelate ferric (Fe3+) ions, only NA is capable of chelating highly-bioavailable Fe2+ ions.31,32 Iron biofortified rice with increased NA biosynthesis has also reversed anemia symptoms in mice, suggesting that NA-chelated Fe is bioavailable in vivo.33,34 Taken together these results reveal NA-chelated Fe as a natural and highly bioavailable Fe fortificant that improves host Fe status.

The chicken (Gallus gallus) model is physiologically relevant for estimating dietary micronutrient absorption in humans due to similarities in intestinal morphology and enteric microbiota, and has been used in numerous studies to evaluate Fe and Zn bioavailability in staple foods.35–44 Here we utilized the chicken model to investigate the impact of NA-chelated and EDTA-chelated Fe on Fe status and gastrointestinal health when delivered alongside extracts of control and biofortified white wheat flour containing increased NA, Fe, Zn and DMA through intraamniotic administration four days prior to hatch (short-term exposure). Short-term exposure to NA-chelated Fe and extracts of biofortified wheat flour had similar effects on gastrointestinal health; we therefore conducted a separate feeding trial study of control and biofortified wheat-based diets over a period of six weeks (long-term exposure). Together this study highlights the versatility of the chicken model and demonstrates novel positive effects of NA-chelated Fe on host Fe status and gastrointestinal health when administered as an Fe fortificant or as part of a biofortified diet.

Results

Experiment 1 – Intraamniotic administration of EDTA-chelated and NA-chelated Fe fortificants

Intraamniotic administration of NA-chelated Fe improves Fe status and alters expression of Fe homeostasis/hypertension genes. Blood serum Fe concentration was significantly elevated in chickens that received intraamniotic administration of EDTA-chelated Fe (‘Fe EDTA’) and NA-chelated Fe (‘Fe NA’) relative to unchelated Fe (‘Fe’) and non-injected (‘NI’) treatment groups (Fig. 1A). Blood serum Zn, liver Fe and liver Zn concentrations were not significantly different between treatment groups (Fig. 1B–D).

Duodenal cytochrome B (DcytB), divalent metal transporter 1 (DMT1) and Zn transporter (ZIP4) expression was significantly upregulated in intestinal tissue of chickens that received ‘Fe’ relative to all treatment groups, except for DcytB and DMT1 expression in control white flour extract (‘C WF’) and biofortified white flour extract (‘B WF’) treatment groups (Fig. 1E). Both alkaline phosphatase (AKP) and Zn transporter 1 (Znt1) expression were significantly upregulated in chickens that received intraamniotic administration of H2O (‘H2O’) relative to ‘Fe’, ‘C WF’ and ‘B WF’ treatment groups (Fig. 1E). No differences in heart gene expression were observed between treatment groups (Fig. 1F).

Intraamniotic administration of NA-chelated Fe positively affects intestinal morphology and microbiota density. Goblet cell number increased significantly in ‘Fe NA’ intestinal villi relative to all treatment groups and in ‘B WF’ relative to all groups except for ‘Fe NA’ (Fig. 2A). Goblet cell number decreased significantly in ‘Fe EDTA’ intestinal villi relative to all treatment groups. Intestinal villi length increased significantly in ‘Fe EDTA’ relative to all treatment groups except for ‘H2O’ and in ‘H2O’, ‘Fe NA’ and ‘B WF’ treatment groups relative to ‘NI’, ‘Fe’ and ‘B WF’ treatment groups (Fig. 2B). Intestinal villi width increased significantly in ‘Fe EDTA’ relative to all treatment groups, and in ‘H2O’ relative to ‘Fe NA’ (Fig. 2C). Intestinal villi width decreased significantly in ‘C WF’ relative to all treatment groups.

The abundance of Bifidobacterium significantly increased in ‘Fe NA’ cecum relative to all treatment groups apart from ‘Fe EDTA’ (Fig. 2D). The abundance of both Escherichia significantly increased in ‘Fe EDTA’ cecum relative to ‘Fe NA’ and Clostridium significantly increased in ‘H2O’ and ‘Fe EDTA’ relative to ‘C WF’ (Fig. 2D).

Experiment 2 – Feeding trial of control and biofortified white wheat flour

Biofortified white wheat flour increases total body hemoglobin and hemoglobin maintenance efficiency with lower feed intake and feed conversion ratio. The concentrations of Fe, Zn, NA and DMA were significantly higher in white flour derived from field-grown bread wheat expressing the rice nicotianamine synthase (OsNAS2) gene compared to control white flour (Fig. S1, Table S1) and significantly increased 1.1- to 1.2-fold (Fe and Zn) and 1.7- to 1.8-fold (NA and DMA) in diet containing 80% biofortified white flour (‘Biofortified’) relative to diet containing 80% control white flour (‘Control’) (Table 1). Caco-2 cell ferritin significantly increased after exposure to biofortified white flour relative to control white flour (Fig. S2). At week 2, hemoglobin (Hb), total body Hb and hemoglobin maintenance efficiency (HME) decreased significantly in ‘Biofortified’ relative to ‘Control’ chickens (Table 2). From week 4 onwards, a trend of lower cumulative feed intake (g) and cumulative feed conversion ratio (FCR) was present in ‘Biofortified’ relative to ‘Control’ chickens. No differences in body weight between ‘Biofortified’ and ‘Control’ chickens were observed throughout the study (Table 2).

Biofortified white wheat flour improves iron status and glycogen storage. No differences in blood serum Fe and Zn concentrations were observed between ‘Biofortified’ and ‘Control’ chickens throughout the study (Fig. 3A,B). At week 2, blood linoleic acid:dihomo-γ-linolenic acid ratio (LA:DGLA) was significantly decreased in ‘Biofortified’ relative to ‘Control’ chickens (Fig. 3C). At the conclusion of the study, liver Fe concentration and glycogen
storage in both liver and pectoral tissue was significantly elevated in ‘Biofortified’ relative to ‘Control’ chickens (Fig. 3D,E). No differences in nail or feather Fe and Zn concentrations were observed throughout the study (Fig. S3). Expression of cytochrome c oxidase (COX) was significantly upregulated in ‘Biofortified’ heart tissue relative to ‘Control’ (Fig. 3F).
Biofortified white wheat flour increases goblet cell number and positively alters gut health and the microbiome. The number of intestinal goblet cells significantly increased, the number of acidic/neutral goblet cells significantly increased, and the diameter of intestinal goblet cells significantly decreased in ‘Biofortified’ relative to ‘Control’ chickens (Figs. 4A, S4). No difference in intestinal villi length and width was detected (Fig. 4B). Short-chain fatty acid (SCFA) production significantly increased for acetic acid, propionic acid and valeric acid and decreased for butanoic acid in ‘Biofortified’ relative to ‘Control’ chickens (Fig. 4C). For major bacteria phyla the proportion of Actinobacteria increased 1.9-fold while the proportion of Firmicutes and Proteobacteria decreased 1.2- and 2.0-fold, respectively in ‘Biofortified’ ceca relative to ‘Control’ (Fig. 4D). For major bacterial genera the proportion of Bifidobacterium and Lactobacillus increased 1.9- and 1.5-fold, respectively while the proportion of Streptococcus (1.7-fold), Coprococcus (1.4-fold), Ruminococcus (1.2-fold), Faecalibacterium (2-fold), and Escherichia (2-fold) decreased in ‘Biofortified’ relative to ‘Control’ (Fig. 4D). The proportion of family Lachnospiraceae decreased 1.7-fold and was significantly (p = 0.045) lower in ‘Biofortified’ relative to ‘Control’ (Fig. 4D). Only one genus, Enterococcus, was significantly (p = 0.010) more abundant in ‘Biofortified’ (3.5%) relative to ‘Control’ (>1.0%). The abundance of all families and genera detected decreased 1.5-fold in ‘Biofortified’ cecum relative to ‘Control’.
goblet cell number (Fig. 2A) and a mucosal habitat that supports probiotic highly bioavailable NA-chelated Fe is readily absorbed by the intestinal epithelia, leading to significantly increased relative to unchelated 'Fe' is evidence that NA delivers relatively more Fe. NA-chelated Fe is readily absorbed by the host and not available to Fe-responsive pathogenic bacteria. Bifidobacterium tauri is linked with a positive gut microbiome and that 'Fe NA' administration resulted in proliferation of intestinal DMT1 (a major Fe transporter) relative to administration of unchelated 'Fe' (Fig. 1A,E) suggesting that both EDTA-chelated and NA-chelated Fe are readily absorbed into the small intestine before export into the blood stream. Decreased expression of DCTB (which catalyzes the reduction of Fe3+ to Fe2+) in 'Fe NA' relative to unchelated 'Fe' is evidence that NA delivers relatively more Fe2+ ions, and that administration of an unchelated Fe solution delivers relatively more oxidized Fe3+ ions to the intestine. The expression of Ferroportin (the only known intestinal Fe exporter) was similar between treatment groups, and determining whether these NA-chelated Fe2+ ions would be preferentially absorbed into intestinal enterocytes or transferred paracellularly into the blood stream requires further investigation. Given that low expression of duodenal DMT1/DCTB relative to Ferroportin is linked with a positive gut microbiome and that 'Fe NA' administration resulted in proliferation of probiotic Bifidobacterium in the ceca relative to Escherichia coli and Clostridium (Fig. 2D), we hypothesize that NA-chelated Fe is readily absorbed by the host and not available to Fe-responsive pathogenic bacteria. Increased cecal Escherichia abundance in 'Fe EDTA' relative to Escherichia coli instead suggests that EDTA-chelated Fe persists in the intestinal lumen and contributes to the proliferation of non-beneficial bacteria (Fig. 2D). Within the intestine, goblet cells are responsible for the synthesis and secretion of mucus, a polysaccharide/protein rich layer that physically protects epithelial cells, provides microbial habitat and facilitates nutrient exchange. We hypothesize that highly bioavailable NA-chelated Fe is readily absorbed by the intestinal epithelia, leading to significantly increased goblet cell number (Fig. 2A) and a mucosal habitat that supports probiotic Bifidobacterium. By contrast, reduced intestinal goblet cell numbers (and mucus production) coupled with increased villi surface area in 'Fe EDTA' may amplify the risk of bacterial infection due an increased proportion of potentially pathogenic Escherichia coli and Clostridium relative to probiotic Bifidobacterium. Together these results suggest NA-chelated Fe is highly bioavailable to the host and improves intestinal functionality without causing dysbiosis and proliferation of pathogenic bacteria as commonly seen in traditional Fe supplements and fortificants.

Biofortification is a cost-effective strategy to combat human micronutrient deficiencies by improving the density and/or bioavailability of micronutrients in staple crops through agronomic practices, conventional breeding, or modern biotechnology. Biofortification efforts in pearl millet (Pennisetum glaucum L.) and common bean (Phaseolus vulgaris L.) have increased seed Fe concentration (up to 3.9-fold and 1.7-fold, respectively), and

| Diet Ingredient | Control | Biofortified |
|-----------------|---------|-------------|
| g/Kg (by formulation) | | |
| Control white wheat flour | 800 | — |
| Biofortified white wheat flour | — | 800 |
| skim milk, dry | 99.75 | 99.75 |
| DL-methionine | 2.5 | 2.5 |
| corn oil | 27 | 27 |
| choline chloride | 0.75 | 0.75 |
| vitamin/mineral premix (no Fe/Zn) | 70 | 70 |
| **Selected Components** | | |
| Dietary Fe (µg/g) | 25.9 ± 0.12 | 28.9 ± 0.13*** |
| Dietary Zn (µg/g) | 16.6 ± 0.06 | 19.2 ± 0.03*** |
| Dietary NA (µmol/g) | 18.1 ± 0.32 | 33.0 ± 0.21*** |
| Dietary DMA (µmol/g) | 19.5 ± 0.16 | 34.1 ± 0.74*** |
| Dietary Phytate (mg/g) | 0.5 ± 0.09 | 0.5 ± 0.08 |
| Total Fiber (µg/g) | 19.9 ± 0.18 | 23.8 ± 1.12 |
| Total Protein (%) | 13.47 ± 0.08 | 13.67 ± 0.08 |
| Total Carbon (%) | 41.90 ± 0.13 | 41.30 ± 0.13 |
| Phytate: Fe molar ratio | 1.63 | 1.46 |

Table 1. Composition of the experimental diets. Component values represent mean ± SEM of at least four technical replicates. Asterisks denote significant differences for p < 0.001 (***).
Table 2. Body weight, biomarkers of Fe status and feed consumption throughout the study. Values represent mean ± SEM of at least nine biological replicates. Asterisks denote significant differences between diet treatments for p ≤ 0.001 (***) as determined by Student's t-test. Hb: hemoglobin, HME: hemoglobin maintenance efficiency, FCR: feed conversion ratio.

| Variable               | Diet          | Baseline          | Week 1          | Week 2          | Week 3          | Week 4          | Week 5          | Week 6          |
|------------------------|---------------|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Body Weight (kg)       | Control       | 0.125 ± 0.007     | 0.158 ± 0.004   | 0.195 ± 0.007   | 0.236 ± 0.010   | 0.286 ± 0.013   | 0.355 ± 0.016   | 0.365 ± 0.029   |
|                        | Biofortified  | 0.122 ± 0.006     | 0.160 ± 0.009   | 0.191 ± 0.007   | 0.222 ± 0.010   | 0.260 ± 0.014   | 0.318 ± 0.018   | 0.353 ± 0.029   |
| Hb (g/L)               | Control       | 72.7 ± 2.3        | 96.3 ± 1.6      | 112.1 ± 1.2***  | 103.5 ± 3.5     | 99.6 ± 3.7      | 82.4 ± 3.7      | 94.9 ± 3.6      |
|                        | Biofortified  | 72.7 ± 2.3        | 93.2 ± 1.6      | 92.5 ± 1.5      | 97.8 ± 3.7      | 104.7 ± 4.0     | 91.4 ± 3.4      | 101.2 ± 3.5     |
| Total Body Hb (mg)     | Control       | 2.59 ± 0.14       | 4.26 ± 0.124    | 6.15 ± 0.17***  | 7.09 ± 0.35     | 7.87 ± 0.33     | 8.48 ± 0.67     | 9.83 ± 1.01     |
|                        | Biofortified  | 2.52 ± 0.13       | 4.20 ± 0.124    | 4.72 ± 0.17     | 6.21 ± 0.39     | 7.57 ± 0.36     | 7.74 ± 0.70     | 10.06 ± 0.98    |
| HME (%)                | Control       | 12.16 ± 0.879     | 13.80 ± 0.67*** | 10.21 ± 0.77    | 8.32 ± 0.50     | 4.36 ± 0.76     | 3.14 ± 1.02     |                  |
|                        | Biofortified  | 11.7 ± 0.879      | 7.14 ± 0.71     | 8.56 ± 0.85     | 8.41 ± 0.45     | 4.65 ± 0.76     | 5.84 ± 1.00     |                  |
| FCR                    | Control       | 5.85 ± 0.738      | 4.82 ± 0.55     | 6.10 ± 0.52     | 7.94 ± 0.51     | 4.09 ± 0.24     | 22.30 ± 3.19    |                  |
|                        | Biofortified  | 4.86 ± 0.736      | 5.98 ± 0.55     | 6.14 ± 0.57     | 6.89 ± 0.53     | 3.63 ± 0.25     | 19.81 ± 2.99    |                  |
| Feed Intake (g)        | Control       | 180.6 ± 20.0      | 157.8 ± 13.3    | 251.0 ± 27.4    | 299.9 ± 41.9    | 284.5 ± 29.8    | 243.7 ± 25.2    |                  |
|                        | Biofortified  | 171.0 ± 20.0      | 152.6 ± 13.3    | 201.3 ± 27.4    | 244.8 ± 41.9    | 190.8 ± 29.8    | 185.6 ± 21.8    |                  |
| Cumulative Feed Intake (g) | Control     | 338.5 ± 31.6      | 589.5 ± 50.7    | 889.4 ± 91.2    | 1174.0 ± 119.0  | 1333.0 ± 153.0  |                  |                  |
|                        | Biofortified  | 323.6 ± 31.6      | 524.9 ± 50.7    | 767.9 ± 91.2    | 960.0 ± 119.0   | 1096.0 ± 153.0  |                  |                  |
| Cumulative FCR         | Control       | 4.56 ± 0.38       | 5.00 ± 0.39     | 5.45 ± 0.63     | 4.92 ± 0.52     | 6.12 ± 0.85     |                  |                  |
|                        | Biofortified  | 5.15 ± 0.38       | 5.25 ± 0.39     | 4.73 ± 0.63     | 4.16 ± 0.52     | 4.66 ± 0.73     |                  |                  |

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neutral and provide mucin with an appropriate chemical composition to support these populations. We hypothesize that additional *Bifidobacterium* and *Lactobacilli* in the mucosal layer upregulate glycolysis/gluconeogenesis enzymes and increase the production of acetic, propionic and valeric SCFAs (Figs. 4C, 5C), leading to improved host Fe absorption and carbohydrate metabolism in 'Biofortified' chickens relative to 'Control'. Improved metabolic capacity in 'Biofortified' chickens manifested as reduced cumulative FCR (consuming ~20% less for the same weight gain) and increased glycogen storage in both liver and pectoral tissue. Bars represent mean ± SEM of nine biological replicates. (f) Transcript quantification relative to 18S in chicken duodenal and heart tissue. Bars represent mean ± SEM of at least eight biological replicates, each with two technical replicates of quantitative RT-PCR. Asterisks denote significant differences for *p* < 0.05, **p** ≤ 0.001 as determined by Student's t-test.

Figure 3. Biomarkers of Fe and Zn status and glycogen storage following consumption of experimental diets. (a,b) Fe and Zn concentration (µg/g) in chicken blood serum, respectively. (c) Ratio of LA:DGLA in chicken blood cells. Measurements were taken at the beginning (Baseline) and in the 2nd, 4th and 6th week of the study. (d) Fe and Zn concentration (µg/g) in chicken liver. (e) Glycogen (mg/mL) concentration in chicken liver and pectoral tissue. Bars represent mean ± SEM of nine biological replicates. (f) Transcript quantification relative to 18S in chicken duodenal and heart tissue. Bars represent mean ± SEM of at least eight biological replicates, each with two technical replicates of quantitative RT-PCR. Asterisks denote significant differences for *p* < 0.05, **p** ≤ 0.001 as determined by Student's t-test.

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Traditional biomarkers of Zn status such as ZIP4 and ZnT1 gene expression and Zn concentration in blood serum, nails, and feathers were unchanged in 'Biofortified' chickens relative to 'Control', suggesting that Zn status was also unchanged (Figs. 3B,F, S3). Given the small differences in dietary Zn concentration (<3 ppm), 'Biofortified' chickens had lower Zn consumption than 'Control' chickens over the course of the study (21.0 mg compared to 22.1 mg Zn, respectively). Together these results suggest that 'Biofortified' chickens had improved Zn bioavailability likely due to consumption of increased dietary NA and/or DMA, although whether NA and/or

Figure 4. Intestinal functionality, short-chain fatty acid production and cecal microbial composition following consumption of experimental diets. (a) Chicken intestinal goblet cell number and diameter (µm). (b) Chicken intestinal villi length and width (µm). (c) Cecal short-chain fatty acid (SCFA) composition. Bars represent mean ± SEM of nine biological replicates. Relative abundance of microbial populations at the levels of (d) phyla; and (e) families and genera. Asterisks denote significant differences for *p < 0.05, **p ≤ 0.001 as determined by Student’s t-test. AU: arbitrary units.
DMA increase Zn bioavailability requires further investigation. We observed significantly decreased LA:DGLA at week 2 and a trend of decreased LA:DGLA from week 4 onwards in ‘Biofortified’ relative to ‘Control’ (Fig. 3C). As the LA:DGLA is a sensitive novel biomarker for evaluating Zn status74, these results suggest that longer-term (6 months) exposure to ‘Biofortified’ diet may demonstrate clearer improvements to Zn status and is warranted. Zinc deficiency in chickens is known to negatively alter the gut microbiome, and improved microbial composition in ‘Biofortified’ chickens may be an additional symptom of improved Zn status75. Given NA and DMA enhance Fe bioavailability we have previously argued these natural metal chelators function as phytonutrients25,26,76. It is well established that NA exhibits anti-hypertensive effects in vivo34,77 although we did not detect differences in heart angiotensin-converting enzyme (ACE) and angiotensin II receptor type 1 (AT1R) gene expression throughout our study (Figs. 1F, 3F). We suspect similar heart ACE and AT1R expression between treatment groups in both the intraamniotic administration and feeding trial experiments is due to the relatively short exposure time to Fe solutions (4 days) or experimental diets (6 weeks) and it is worth investigating whether longer-term (6 months) exposure to increased dietary NA reduces hypertension. Nevertheless, the improved Fe status, gastrointestinal health and microbial composition in chickens following short and long-term exposure to NA-chelated Fe reinforces the idea of NA as an important phytonutrient in plant foods. Furthermore, utilization of NA-chelated Fe in food fortification and crop biofortification programs shows great potential to improve human health.

Materials and Methods

Plant material and white flour production. Vector construction, plant transformation and the initial selection of biofortified wheat material is described in25. In brief, the full-length coding sequence of OsNAS2 (LOC_Os03g19420) was PCR amplified from rice (Orzya sativa L.) cv. Nipponbare and recombined into a modified pMDC32 vector under transcriptional control of the maize (Zea mays L.) ubiquitin 1 (UBI-1) promoter with a hygromycin phosphotransferase plant-selectable marker (Fig. S1). Bombardment of the construct into immature wheat (Triticum aestivum L.) cv. Bobwhite embryos was performed at the University of Adelaide (Adelaide, Australia). One double-insert event and corresponding null segregant (termed ‘Biofortified’ and ‘Control’, respectively) were grown were grown for two seasons for use in intra-amniotic administration (2016 field season) and feeding trial (2017 field season) in New Genes for New Environment facilities located in Merredin, Western Australia (Fig. S1, Table S1). Whole grain samples from Merredin were conditioned to 15% moisture content and milled (70–75% extraction) using a Quadrumat Junior laboratory mill (Brabender, Duisburg, Germany) for intraamniotic administration or a Buhler MLU-202 laboratory mill at The Commonwealth Scientific and Industrial Research Organisation (CSIRO, ACT, Australia) for the feeding trial. All break and reduction fractions of ‘Biofortified’ or ‘Control’ grain were combined to form either ‘Biofortified’ white flour or ‘Control’ white flour (Table 1).
Preparation of extracts, solutions and diets. Wheat extracts were generated as described in 26. In brief, 'Biofortified' white flour or 'Control' white flour was mixed in dH2O (50 g/L), filtered (600 µm) and centrifuged, and the resulting supernatant was dialyzed (MWCO 12–14 kDa, Medicell International Ltd., London, UK) exhaustively against dH2O (48 hrs.). The dialysate was lyophilized, and the resulting powder dissolved in 18MΩ H2O (0.05 g/mL) forming the white wheat flour extracts for intra-amniotic administration. Iron solutions were prepared by combining an Fe standard (1000 µg/mL, 2% HCl High-Purity Standards, Charleston, SC, USA) with either 18MΩ H2O ('Fe'), or 1.6 mM Na (Toronto Research Chemicals Inc., Toronto, Canada) dissolved in 18MΩ H2O ('Fe NA'). The ('Fe EDTA') solution was achieved by combining ferric nitrate (Fe(NO3)3, 9H2O, Sigma, St. Louis, MO, USA) with hydroxyethyl ethylenediamine triacetic acid (H3HEDTA, Sigma, St. Louis, MO, USA) dissolved in sodium hydroxide (NaOH, Sigma, St. Louis, MO, USA) to represent an anionic chelate of dissolved NaFeEDTA45 with final Fe concentration of 77 µM. Osmolarity and final Fe concentration of extracts/Fe solutions for intra-amniotic administration is provided (Table S2).

Dietary analysis (phytate, protein, carbon, fiber, NA, DMA). Dietary phytate was calculated relative to total phosphorus released from diet and flour samples by phytase and alkaline phosphatase enzymes according to manufacturer's instructions (K-PHYT 11/15. Megazyme International, Bray, Ireland). Total dietary carbon (%) and nitrogen (%) was measured via Dumas combustion using a TruMac® CN (LECO Corporation, St. Joseph, MI, USA) with total protein (%) for wheat diet samples equal to 5.7 ± total nitrogen (%). Total dietary fiber was measured via enzymatic digestion using heat-resistant amylase, protease and amyloglucosidase according to manufacturer's instructions (Total Dietary Fiber Assay Kit, Sigma, St. Louis, MO, USA). Quantification of NA and DMA in diet and flour samples was performed as described in35. Briefly, sequential MeOH (100%) and 18MΩ H2O sample were derivatized by 9-fluorenylmethoxycarboxyl chloride (FMOC-Cl) and quantified via RP LC-MS on a 1290 Infinity II and 6490 Triple Quadrupole LC/MS system (Agilent Technologies Inc., Santa Clara, CA, USA).

Caco-2 Fe bioavailability bioassay. Diet and flour samples were subjected to the Caco-2 cell bioassay as previously described26. Briefly, gastric-digested samples (1.5 mL) were added to cylindrical Transwell inserts (Corning Life Sciences, Corning, NY) fitted with a semipermeable (15 000 Da MWCO) basal membrane (Spectra/ Por 2.1, Spectrum Medical, Gardena, CA) and placed within wells containing Caco-2 cell monolayers. Following overnight incubation, cells were washed, harvested, and analyzed for ferritin (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and total protein contents (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA). As Caco-2 cells synthesize ferritin in response to intracellular Fe, we used the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) as an index of cellular Fe uptake.

Micronutrient analysis. Micronutrient concentration in white flour, diets and extracts, Fe solutions, blood serum, and all animal tissues was determined by nitric/perchloric acid digestion as previously described38 followed by inductively coupled plasma-optical emission spectrometry (ICP-OES) using a Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA).

Animals and study design. Cornish-cross fertile broiler eggs (n = 70) were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA) and incubated at the Cornell University Animal Science poultry farm until hatching. All animal protocols were approved by Cornell University Institutional Animal Care and Use committee (protocol number: 2007–0129). All methods were performed in accordance with the relevant guidelines and regulations. For intraamniotic administration, eggs (n = 5) containing viable embryos were weighed and randomly assigned to seven groups (n ≥ 5) based on weight distribution. At day 17 of incubation, extracts/Fe solutions (1 mL) were injected into the amniotic fluid via a 21-gauge needle for the seven treatment groups as follows: (1) non-injected (NI); (2) 18MΩ H2O (H2O); (3) Fe solution (Fe); (4) Fe-EDTA solution (Fe-EDTA); (5) Fe-NA solution (Fe-NA); (6) 'Control' white flour extract (C WF) and (7) 'Biofortified' white flour extract (B WF) and eggs were subsequently incubated for four days until hatch as described in39,40. Chicks were euthanized by CO2 exposure after hatching and all tissues were collected. The remaining hatchlings (n = 30) were allocated based on body weight into two treatment groups: (1) 80% 'Control' white flour diet ('Control') and (2) 80% 'Biofortified' white flour diet ('Biofortified') as described in38. All chickens received a commercial diet (Nutrena® Chick Starter Poultry (NRC Poultry reference) excluding Fe and Zn. Chickens (n = 3) were housed in cages (1 m2) and provided ad libitum access to food and H2O. Feed intakes were measured daily, and body weight and blood samples were obtained weekly. Feed conversion ratio (FCR) represents weekly feed intake (g) proportional to the weekly increase in body weight (g). Chickens were euthanized by CO2 exposure seven weeks post-hatch and tissues collected.

Blood measurements. Wing-vein blood samples (100 µL) were collected using micro-hematocrit heparinized capillary tubes (Fisher, Pittsburgh, PA, USA). Blood plasma Hb concentrations were determined spectrophotometrically using the Triton® NaOH method according to manufacturer's instructions (Hemoglobin Assay Kit, Sigma, St. Louis, MO, USA). The Hb maintenance efficiency (HME) was calculated as previously described38. Blood serum Linoleic Acid:Dihomo-γ-Linolenic Acid ratio (LA:DGLA) was determined as previously described39.

Gene expression analysis (Tissue harvesting, RNA isolation, cDNA synthesis, primer design). Total RNA extraction from duodenal and heart tissue (30 mg) using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA), cDNA synthesis and real time-polymerase chain reaction (RT-PCR) analysis were performed as previously described36,41 with minor adjustments. In brief, the cycle product (Cp) of each gene
was quantified using a seven-point standard curve in duplicate. Gene expression was obtained relative to 18S (Cp), primer pair efficiency, and control treatments: ‘NI’ for intraamniotic administration and ‘Control’ for feeding trial. Alkaline phosphatase (AKP) and sucrase isomaltase (SI) acted as intestinal reference genes following intraamniotic administration (Fig. 1E). All primers used for gene expression analysis are provided in Table S3.

**Ferritin and glycogen analysis.** Liver ferritin was determined as previously described. In brief, samples (1 g) were homogenized in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 mM) and heat treated (75°C, 10 min) before centrifugation. Native polyacrylamide gel electrophoresis (PAGE) gels were stained with Coomassie blue G-250 stain or potassium ferricyanide \([K_2Fe(CN)_6]\) and quantified using the Quantity-One 1-D analysis program (Bio-Rad, Hercules, CA). Liver and pectoral glycogen was determined colorimetrically as described in with minor adjustments. After centrifugation and mixing with petroleum ether, homogenized tissue was mixed with color reagent (300 µL) and total glycogen determined on an ELISA plate reader (450 nm) according to a standard curve.

**Intestinal functionality and short-chain fatty acid (SCFA) analysis.** Duodenal samples were fixed in fresh 4% (v/v) buffered formaldehyde, dehydrated, and embedded in paraffin as previously described. Serial sections (5 μm) were deparaffinized in xylene and stained with hematoxin and eosin before goblet cell number and villi surface area examination under light microscopy using EPIX XCAP software (Standard version, Olympus, Waltham, MA, USA). Cecal samples were homogenized in HCl (2 mL, 3%, 1 M), centrifuged and combined with ethyl acetate (100 µL) and acetic acid-d4 (1 µg/mL) before collecting the organic phase to determine short chain fatty acid (SCFA) composition. Samples were quantified via GC-MS using a TRACE™ 1310 gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) and a TraceGOLD™ TG-WaxMS A column (Thermo Fisher Scientific, Waltham, MA, USA).

**Microbial population analysis.** Lactobacillus, Bifdobacterium, Escherichia, and Clostridium density in intraamniotic administration treatment groups was determined as previously described. In brief, cecal contents were homogenized with phosphate-buffered saline (PBS, 9 ml), centrifuged and the pellet resuspended in 50 mM ethylenediaminetetraacetic acid (EDTA, 50 mM) and treated (37°C, 45 min) with lysozyme (10 mg/mL, Sigma Aldrich CO., St. Louis, MO, USA). Bacterial genomic DNA was isolated according to manufacturer’s instructions (Wizard® Genomic DNA Purification Kit, Promega Corp., Madison, WI, USA) and bacterial genera are presented in relative proportions. All primers used for microbial population analysis are provided in Table S4.

**16S rRNA gene sequencing and analysis.** Microbial genomic DNA extraction from ‘Control’ and ‘Biofortified’ cecal samples, gene sequencing and analysis was conducted as previously described. In brief, 16S rRNA gene sequences were amplified from the V4 hypervariable region of microbial genomic DNA (Powersoil DNA isolation kit, MoBio Laboratories Ltd., Carlsbad, CA, USA, purified (AMPure, Beckman Coulter, Atlanta, GA, USA) and quantified according to manufacturer’s instructions (QuantiT™ PicoGreen™ dsDNA Assay Kit, Invitrogen, Carlsbad, CA, USA). Samples were sequenced at Bar Ilan University (Safed, Israel) using an Illumina MiSeq Sequencer (Illumina, Inc., Madison, WI, USA). Amplicon reads were analyzed using ‘Divisive Amplicon Denosing Algorithm’ (DADA2) and ‘quantitative insights into microbial ecology’ (QIME) software before taxonomic classification using Greengenes database (http://greengenes.lbl.gov/) and Faith’s phylogenetic diversity (PD) was used to assess α-diversity and principal component (PC) analysis of weighted UniFrac distances was used to assess β-diversity. Relative abundance was determined using linear discriminant analysis effect size (LEfSe) and metabolic capacity was determined using ‘phylogenetic investigation of communities by reconstruction of unobserved states’ (PICRUSt) software compared to known pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg) and permutational multivariate analysis of variance (PERMANOVA) tests, respectively and LEfSe significant differences were corrected for false discovery rate (FDR).

**Data availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**References**

1. Beal, T., Massiot, E., Arsenault, J. E., Smith, M. R. & Hijmans, R. J. Global trends in dietary micronutrient supplies and estimated prevalence of inadequate intakes. PLoS One 12 (2017).
2. WHO. The global prevalence of anaemia in 2011. World Heal. Organ. (2015).
3. Prentice, A. M. et al. Dietary strategies for improving iron status: Balancing safety and efficacy. Nutr. Rev. 75, 49–60 (2017).
4. Hurrell, R. & Egli, I. Iron bioavailability and dietary reference values. Am. J. Clin. Nutr. 91, 1461–1467 (2010).
5. Pasricha, S.-R. S. et al. Diagnosis and management of iron deficiency anaemia: a clinical update. Med. J. Aust. 193, 525–532 (2010).
42. Tako, E., Beebe, S. E., Reed, S., Hart, J. J. & Glahn, R. P. Polyphenolic compounds appear to limit the nutritional benefit of biofortified

41. Tako, E., Rutzke, M. A. & Glahn, R. P. Using the domestic chicken (Gallus gallus) as an

46. Cherrier, M. V. 

45. Świetlik, R., Trojanowska, M. & Dębska, P. Modeling the chemical speciation of iron released from commercially available oral iron

37. Tako, E., Bar, H. & Glahn, R. P. The combined application of the Caco-2 cell bioassay coupled with

35. Patterson, J. K., Lei, X. G. & Miller, D. D. The Pig as an Experimental Model for Elucidating the Mechanisms Governing Dietary

39. Knez, M. 

48. Lane, D. J. R., Bae, D. H., Merlot, A. M., Sahni, S. & Richardson, D. R. Duodenal cytochrome b (DCYTB) in Iron metabolism: An

22. Zimmermann, M. B. 

27. Singh, S. P., Keller, B., Gruissem, W. & Bhullar, N. K. Rice NICOTIANAMINE SYNTHASE 2 expression improves dietary iron and

26. Beasley, J. T., Hart, J. J., Tako, E., Glahn, R. P. & Johnson, A. A. T. Investigation of Nicotianamine and 2

28. Johnson, A. A. T. 

15. Gregory, P. J. 

13. Bryszewska, M. A. Comparison study of iron bioaccessibility from dietary supplements and microencapsulated preparations. 

Nutrients 11 (2019).

4. Blanco-Rojo, R., Vaquero, M. P. Iron bioavailability from food fortification to precision nutrition. A review. In "Theoretical and Applied Genetics". 168, 127–141 (2018).

3. Blanco-Rojo, R., Vaquero, M. P. Iron bioavailability from food fortification to precision nutrition. A review. In "Theoretical and Applied Genetics". 168, 127–141 (2018).

2. Blanco-Rojo, R., Vaquero, M. P. Iron bioavailability from food fortification to precision nutrition. A review. In "Theoretical and Applied Genetics". 168, 127–141 (2018).

1. Blanco-Rojo, R., Vaquero, M. P. Iron bioavailability from food fortification to precision nutrition. A review. In "Theoretical and Applied Genetics". 168, 127–141 (2018).

www.nature.com/scientificreports/
50. Lin, F. et al. Probiotic/prebiotic correction for adverse effects of iron fortification on intestinal resistance to Salmonella infection in weaning mice. Food Funct. 9, 1070–1078 (2018).
51. Kortman, G. A. M., Boelej, A., Swinkels, D. W. & Tjalsma, H. Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface. PLoS One 7, 1–7 (2012).
52. Ho, T. D. & Ellermeier, C. D. Ferric Uptake Regulator Fur Control of Putative Iron Acquisition Systems in Clostridium difficile. J. Bacteriol. 197, 2930–2940 (2015).
53. Birchenough, G. M. H., Johansson, M. E. V., Gustafsson, J. K., Bergström, J. H. & Hansson, G. C. New developments in goblet cell mucus secretion and function. Mucosal Immunol. 8, 712–719 (2015).
54. Sonnenburg, J. L., Angenent, L. T. & Gordon, J. I. Getting a grip on things: How do communities of bacterial symbionts become established in our intestine? Nat. Immunol. 5, 569–573 (2004).
55. Hansson, G. C. Role of mucus layers in gut infection and inflammation. Curr. Opin. Microbiol. 15, 57–62 (2012).
56. Conrad, M. E. & Umbreit, J. N. Pathways of Iron Absorption. Blood Cells, Mol. Dis. 29, 336–355 (2002).
57. Bosu, H. E., Hotz, C., McClafferty, B., Meenakshi, J. V. & Pfeiffer, W. H. Biofortification: A new tool to reduce micronutrient malnutrition. Food Nutr. Bull. 32 (2011).
58. Bosu, H. E. & Saltzman, A. Improving nutrition through biofortification: A review of evidence from HarvestPlus, 2003 through 2016. Glob. Food Sec. 12, 49–58 (2017).
59. Haan, J. D. et al. Consuming Iron Biofortified Beans Increases Iron Status in Rwandan Women after 128 Days in a Randomized Controlled Feeding Trial. J. Nutr. 1–7, https://doi.org/10.3945/jn.115.224741.1 (2016).
60. Finkelstein, J. L. et al. A Randomized Trial of Iron-Biofortified Pearl Millet in School Children in India. J. Nutr. 145, 1576–1581 (2015).
61. Weger, M. J. et al. Changes in Iron Status Are Related to Changes in Brain Activity and Behavior in Rwandan Female University Students: Results from a Randomized Controlled Efficacy Trial Involving Iron-Biofortified Beans. J. Nutr. 149, 687–697 (2019).
62. Scott, N. P. et al. Cognitive performance in Indian school-going adolescents is positively affected by consumption of iron-biofortified pearl millet: A 6-month randomized controlled efficacy trial. J. Nutr. 148. 1462–1471 (2018).
63. Liao, X., Ma, C., Lu, L., Zhang, L. & Luo, X. Determination of dietary iron requirements by full expression of iron-containing cytochrome c oxidase in the heart of broilers from 22 to 42 d of age. Br. J. Nutr., https://doi.org/10.1017/S0007114517002458 (2017).
64. Qin, J. et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65 (2010).
65. Thomas, M. et al. Gut Microbial Dynamics during Conventionalization of Germfree Chicken, mSphere 4, e0035–19 (2019).
66. Oakley, B. B. et al. The chicken gastrointestinal microbiome. FEMS Microbiol. Lett. 360, 100–112 (2014).
67. Wei, S., Morrison, M. & Yu, Z. Bacterial census of poultry intestinal microbiome. Poult. Sci. 92, 671–683 (2013).
68. Turnbaugh, P. J. et al. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1031 (2006).
69. Vaughan, E. E., Heilig, H. G. H., Ben-Amor, K. & De Vos, W. M. Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. FEMS Microbiol. Rev. 29, 477–490 (2005).
70. Forder, R. E. A., Howarth, G. S., Tivey, D. R. & Hughes, R. J. Bacterial modulation of small intestinal goblet cells and mucin composition during early posthatch development of poultry. Poult. Sci. 86, 2396–2403 (2007).
71. Jozefiak, D., Rutkowski, A. & Martin, S. A. Carbohydrate fermentation in the avian ceca: A review. Anim. Feed Sci. Technol. 113, 1–15 (2004).
72. Xu, Z. R. et al. Effects of Dietary Fructooligosaccharide on Digestive Enzyme Activities, Intestinal Microflora and Morphology of Growing Pigs. Asian-Australasian J. Anim. Sci. 15, 1784–1789 (2012).
73. King, J. C. Yet Again, Serum Zinc Concentrations Are Unrelated to Zinc Intakes. J. Nutr. 148, 1399–1401 (2018).
74. Knež, M., Stangouilis, J. C. R., Gilbitisc, M. & Tako, E. The Ileneic acid: Dihomo-γ-Ileneic acid ratio (LA:DGLA)—An emerging biomarker of Zn status. Nutrients 9 (2017).
75. Reed, S. et al. Chronic zinc deficiency alters chick gut microbiota composition and function. Nutrients 7, 9768–9784 (2015).
76. Martin, C. & Li, J. Medicine is not health care, food is health care: plant metabolic engineering, diet and human health. New Phytol. 216, 699–719 (2017).
77. Kimashita, E., Yamakoshi, J. & Kikuchi, M. Purification and Identification of an Angiotensin I-converting Enzyme Inhibitor from Soy Sauce. Biosci. Biotechnol. Biochem. 57, 1107–1110 (1993).
78. Tako, E., Glahn, R. P., Knez, M. & Stangouilis, J. C. R. The effect of wheat prebiotics on the gut bacterial population and iron status of iron deficient broiler chickens. Nutr. J. 13, 1–10 (2014).
79. Hou, T., Kolba, N., Glahn, R. P. & Tako, E. Intra-amniotic administration (Gallus gallus) of cicer arietinum and lens culinaris prebiotics extracts and duck egg white peptides affects gut bacterial status and intestinal functionality. Nutrients 9 (2017).
80. Hou, T. & Tako, E. The in ovo feeding administration (Gallus gallus)—An emerging in vivo approach to assess bioactive compounds with potential nutritional benefits. Nutrients 10 (2018).
81. Reed, S. et al. Alterations in the Gut (Gallus gallus) Microbiota Following the Consumption of Zinc Biofortified Wheat (Triticum aestivum)-Based Diet. J. Agric. Food Chem. 66, 6291–6299 (2018).
82. Pfiă, M. W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45 (2001).
83. Kornasio, R., Haly, O., Kedar, O. & Uni, Z. Effect of in ovo feeding and its interaction with timing of first feed on glycogen reserves, muscle growth, and body weight. Poult. Sci. 90, 1467–1477 (2011).
84. Callahan, B. J. et al. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583 (2016).
85. Caporaso, J. G. et al. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336 (2010).
86. DeSantis, T. Z. et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72, 5069–72 (2006).
87. Faith, D. P. Conservation evaluation and phylogenetic diversity. Biol. Conserv. 61, 1–10 (1992).
88. Langille, M. G. I. et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31, 814–821 (2013).
89. Lozupone, C. A. & Knight, R. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. Appl. Environ. Microbiol. 71, 8228–8235 (2005).
90. Segata, N. et al. Metagenomic biomarker discovery and explanation. Genome Biol. 12, 1–18 (2011).

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Author contributions
J.T.B., A.A.T.J., R.P.G., O.K. and E.T. contributed to the conception and design of the work; J.T.B., N.K., J.P.B., L.O., O.K. and E.T. contributed to the acquisition, analysis and interpretation of data; J.T.B. drafted the work; J.P.B., A.A.T.J. and E.T. substantively revised the work. All authors have read and approved the submitted manuscript.

Competing interests
The authors declare no competing interests.

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