Dual-fortified lentil products – a sustainable new approach to provide additional bioavailable iron and zinc in humans

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Short Running Head: Fe and Zn fortification of lentil

Links to supporting data deposited in repositories: Supplementary Table 1: 10.6084/m9.figshare.13574888; Supplementary Table 2: 10.6084/m9.figshare.13517201; Supplementary Table 3: 10.6084/m9.figshare.13517204; Supplementary Table 4: 10.6084/m9.figshare.13517207; Supplementary Table 5: 0.6084/m9.figshare.13517213; Supplementary Table 6: doi:10.6084/m9.figshare.13517216; Supplementary Text: 10.6084/m9.figshare.13574900.

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A list of abbreviations and their definitions:

Fe - Iron

a* - Redness
b* - Yellowness
L – Lightness
LPT - Lentil product types
PA – Phytic acid
RDAs - Recommended Daily Allowances
RF - Red-football
RFeB% - Relative iron bioavailability
RS – Red-split
YS – Yellow-split
Zn - Zinc

Conflicts of Interest:
All the three authors, Rajib Podder, Raymond P. Glahn, and Albert Vandenberg declare no conflict of interest.

Abstract:

Background: Iron (Fe) and zinc (Zn) deficiencies are global health problems affecting 20% and 33% of the world’s population, respectively. Lentil (Lens culinaris Medik.), part of the staple food supply in many countries, can be a potential vehicle for Fe and Zn fortification.
Objective: We developed a dual-fortification protocol to fortify three milled lentil product types (LPT) [red-football (RF), red-split (RS), and yellow-split (YS)], with NaFeEDTA and ZnSO$_4$.H$_2$O to increase the bioavailable content of Fe and Zn.

Methods: Appropriate Fe and Zn doses were determined to fortify lentils based on Recommended Daily Allowances (RDAs). Relative Fe bioavailability (RFeB%) and phytic acid (PA) content were assessed using an in-vitro Caco-2 cell-bioassay and PA analysis, respectively. One-way analysis of variance determined the differences in colorimetric score, concentration of Fe, Zn and PA concentration, and RFeB% among samples. The LSD was calculated with significance level set at P<0.05.

Results: Fe and Zn concentration, and RFeB% increased, and PA concentration decreased significantly in dual-fortified lentils. Dual-fortified lentil samples had higher RFeB% compared to Fe-fortified (single) samples in all three LPT. Whereas RFeB% decreased in Zn-fortified (single) RF and YS sample by 43.4% and 36%, respectively. The RF, RS, and YS samples, fortified with 16 mg Fe and 8 mg Zn/100 g of lentil provided 27 mg Fe and 14 mg Zn, 28 mg Fe and 13.4 mg Zn, 29.9 mg Fe and 12.1 mg Zn, respectively. RFeB% of RF, RS, and YS lentil samples increased by 91-307, 114-522 and 122-520%, respectively. Again, PA concentrations of RF, RS, and YS lentils were reduced by 0.63-0.53, 0.83-0.71, and 0.96-0.79 mg/g, respectively.

Conclusions: Dual-fortified lentil consumption can cost-effectively provide a significant part of the daily bioavailable Fe and Zn requirements of people with these two globally important micronutrient deficiencies.

Keywords: Iron; Zinc; Fortification; Bioavailability; Phytic acid
**Teaser Text:** Fortified lentils with iron and zinc fortificants can provide a significant part of the daily bioavailable Fe and Zn requirements of people with these two globally important micronutrient deficiencies.

**Introduction**

Micronutrient deficiency or “hidden hunger” is a worldwide public health problem. Iron (Fe) and zinc (Zn) are physiologically essential for all forms of life on the planet (1). Nearly 30% and 17.3% of the world’s population is Fe- and Zn-deficient, respectively (1). In humans, Fe deficiency is a condition in which an insufficient amount of Fe causes Fe deficiency anemia (2), the most common micronutrient deficiency in the world. Zn is also an essential micronutrient many enzyme’s activity of and plays a central role in cellular growth, tissue differentiation, protein, and DNA synthesis (1,2).

Recent evidence showed that plant-based diet intake is increasing due to its significant impact on reducing heart disease, high blood pressure, stroke, and type-2 diabetes (3). Lentil (*Lens culinaris* Medik.) is becoming a popular ingredient in plant-based diets because it is a relatively inexpensive protein source compared to animal protein, and it cooks quickly (4), saving fuel consumption and time. Unlike other pulses, lentil consumption over the past 30 years has grown at a much higher rate than the growth in human population (4). Lentil is produced in more than 50 countries (5), and some non-producing countries consume lentils as a staple food – they are obligate importers. Lentil is considered an excellent source of crude protein (25.8-27.1%), Fe (73–90 mg/kg), Zn (44–54 mg/kg), Se (425-673 µg/kg) etc. (6–8). Of the total Fe content in lentil, ~10% is ferrous
(0.31±0.01 mg/100g of dry matter), and ~90% is ferric (2.69 ± 0.15 mg/100g of dry matter) (9). However, the bioavailability of the micronutrient minerals can be compromised due to the presence of antinutritional factors (e.g. phytate, polyphenols, protein, etc.) in lentil seeds (10).

To overcome these limitations, several effective strategies have been used to improve micronutrient content in crop or food production, such as biofortification, food-fortification, public health intervention, supplementation, nutrition education, dietary diversification and food safety measures (11,12). Among these, biofortification is a commonly used strategy to improve Fe and Zn concentration and bioavailability in several food crops, including lentil (13,14). But considering the lower bioavailability of Fe and Zn in lentil, recommended daily allowances of Fe and Zn, existing consumption rate (12g/day/person) compared to recommended consumption rate (50g/person/day) of pulses (15), improvement of micronutrient concentration using other approaches may improve the concentration of these two micronutrients in lentil.

Among all the approaches to improve micronutrient concentration in foods, food fortification is a cost-effective intervention due to its sustainability for consistently improving the dietary quality of a targeted group or population without compromising dietary habits (11). Fortification of staple foods at the industrial level with multiple micronutrients, including essential minerals and vitamins, has been a common practice in both developed and developing countries to improve micronutrient intake at the population level. There are no recommendations for lentil fortification, but WHO has recommended some Fe and Zn fortificants for different food products in different countries (16). Globally,
84 countries now have legislation to mandate fortification of at least one industry milled cereal grain (17). Wheat flour fortification with B vitamins is mandatory in 14 countries (18). FDA (U.S. Food and Drug Administration) established a requirement to fortify an appropriate food vehicle for fortification in 1995 (19). Fortified rice is mandatory in six countries, and several sub-national efforts are ongoing around the world to combat micronutrient deficiencies by mandating fortified rice in diets (20).

The possibility of fortifying lentils with more bioavailable Fe and Zn was the focus of our current study to improve Fe and Zn bioavailability in lentils to reduce both Fe- and Zn-deficiency. Several food vehicles are used in fortification programs, including staple foods, such as rice and flour, dairy products (milk and yogurt), non-dairy beverages, biscuits, edible oil and salt using different technologies (21,22). Unlike other food product fortification strategies, lentil fortification is a whole food approach that requires an application of fortificant solution to the surface of the dal. Several Fe and Zn fortificants, such as NaFeEDTA, ferrous sulfate, zinc sulfate, and zinc oxide are recommended by WHO and FAO for fortifying food products. In this study, we identified suitable Fe and Zn fortificants to fortify selected dehulled red and yellow cotyledon lentil product types, and modified a previous technique developed for Fe-fortification (23), based on current commercial lentil processing practices.

We also quantified colorimetric changes in fortified lentils after adding both Fe and Zn. In addition, Fe, Zn and phytic acid concentration, and relative Fe bioavailability of dual-fortified lentils were determined.

Several in-vitro screening methods are available to measure the bioaccessibility or bioavailability of micronutrients. Caco-2 cell method is a widely used method that allows
the study of nutrient or food component competition at the side of absorption (24). In this study, the Caco-2 cell bioassay was used to measure Fe absorption as this model mimics conditions in the small intestine, and ferritin formation in the Caco-2 cell monolayers is considered a marker for iron uptake (25). The most commonly used in-vitro method for assessing Zn bioaccessibility is dializability (26). A study reported that in-vitro dializability data had a strong correlation coefficient (0.93; P<0.0001) with in-vivo human absorption data (26). Phytic acid (PA) content was measured using a colorimetric assay kit, which is widely used as it provides accurate and reliable data (27). This method often provides more accurate results than HPLC, and quality controls are easier than using HPLC methods if the person running the system is less experienced (27,28).

In this study, we hypothesized that it would be possible to increase the amount of bioavailable Fe and Zn in dehulled lentil, in a biologically and culturally meaningful way, to a level that could meet a major part of the RDA for humans. We also expect that the dual-fortified lentil can supplement a significant amount of Fe and Zn to populations at risk of Fe and Zn deficiency.

Methods

The protocol used to produce dual-fortified lentil is shown in Figure 1.

Selection of appropriate red and yellow genotypes and three milled lentil product types for dual fortification

Several red and yellow lentil cultivars/genotypes were analyzed to estimate the Fe and Zn concentration (μg/g) in seeds (Supplemental Table 1). The widely grown red cotyledon lentil cultivar, ‘CDC Maxim’ which has high intrinsic Fe (70-85 μg/g) and Zn (35-45 μg/g)
concentration was selected for fortification. For red cotyledon lentils, both football (unsplit-cotyledon) and split (split-cotyledon) lentil product types (LPT) were used for dual fortification. For yellow cotyledon lentil, the cultivar ‘CDC Greenstar’ (yellow split product) with an intrinsic Fe (50-65 μg/g) and Zn (28-35 μg/g) concentration was selected. Both lentil varieties were developed at the Crop Development Centre, University of Saskatchewan, Saskatoon, SK, Canada and they are widely grown in Canada due to their high yield potential and resistance to biotic and abiotic stresses. All LPT [red football (RF), red split (RS) and yellow split (YS)] (Figure 1) were selected based on their intrinsic Fe and Zn concentration, cultivation status, commercial availability, and main type for consumer demand in lentil consuming regions around the world. All three LPT are manufactured as two sub-types, polished and unpolished (Supplemental Table 2). The polished subtype typically receives a light coating of water and/or canola oil after milling. Both subtypes of the three LPT were evaluated to assess the differences in intrinsic Fe and Zn concentration before fortifying them with Fe and Zn fortificants. Fe and Zn concentration were assessed using an ICP-Mass Spectrometer (iCAP 6500 series, Thermo Jarrell Ash Corp., Franklin, MA, USA).

Selection of appropriate Zn and Fe fortificants for dual fortification

The fortification method (spraying, coating, shaking and drying) that was used in this study, required fortificants with high water solubility. Consideration was given to potential interaction with the food vehicle, availability and cost of fortificants, and relative bioavailability compared to other bioavailable fortificants. In general, fortificants added to food products are mostly in dry powder forms or directly added to liquid food or beverages.
For lentil fortification, fortificants need to be coated onto or absorbed into the exterior surface of the dehulled lentil (23,29). NaFeEDTA was previously found to be the most suitable Fe fortificant for lentils based on ease of fortification, consumer acceptability, bioavailability, and changes of organoleptic characteristics compared to the unfortified lentil (29–31). NaFeEDTA was food grade with a solubility of 120 g/L and 300 g/L water at 30°C and 70°C, respectively. Selection of a Zn fortificant was a critical consideration because the fortificant needs to be suitable to both fortify dehulled lentils and to have compatibility with selected Fe fortificants. A number of WHO and FAO approved Zn fortificants are available to fortify food products (2). Initially, two food-grade Zn fortificants, zinc-sulfate monohydrate (ZnSO$_4$.H$_2$O) and zinc-oxide (ZnO) were selected on the basis of cost, active ingredient, water solubility or insolubility, compatibility with Fe fortificants and bioavailability of Zn. Two food-grade ZnSO$_4$.H$_2$O and ZnO were compared, and the ZnSO$_4$.H$_2$O was selected because it is water soluble, whereas ZnO is nearly insoluble in water. NaFeEDTA and Zn fortificants were supplied by Akzo Nobel Functional Chemicals, LLC, Chicago, IL, USA and Spectrum Chemical, Gandora, CA, USA, respectively.

**Modification of previous fortification strategy for dual-fortification**

For dual-fortification with both Fe and Zn fortificants, the previous protocol (23) was modified to ensure compatibility with existing commercial-scale processing practices. A stand mixer (Kitchen-Aid, Artisan series 5-Quart Tilt-head, Century Avenue, Mississauga, ON, Canada) was used to mix the fortificant instead of spraying the fortificant solution over the lentils. Both Fe and Zn fortificants were mixed in a similar amount of water to prepare
the fortificant solution, thereby reducing the amount of fortificant solution to help maintain the acceptable moisture content of the fortified lentil. Unpolished dehulled lentils were fortified with fortificants for 10 min followed by polishing with 0.5% canola oil for five min. After 15 min of mixing in the bowl, fortified lentils were poured into a round aluminum foil tray placed over a Barnstead Thermolyne M49235 Bigger Bill Orbital Shaker (Sigma-Aldrich Corp., St. Louis, MO, USA) for another 10 min. A 250-watt electric heat lamp (NOMA incandescent, clear, 130 V heat lamp, Trileaf Distributors, Toronto, ON, Canada) and a mini portable desk fan (model 043-5498-4, Trileaf Distributors, Toronto, ON, Canada) were used to provide both heat and air to achieve the desirable moisture content (< 14%) of the lentil products. The fortified samples were checked for moisture content and water activity at the Saskatchewan Food Industry Development Centre (32).

The Fe and Zn concentrations in each dual-fortified lentil sample and controls were averaged over three replications with two repeats. The fortification protocol was repeated ten times and samples were analyzed to determine the Fe and Zn concentration.

**Selection of appropriate dose of iron and zinc fortificants**

Seven samples from each of the three LPT (Table 1) were prepared using either ZnSO₄·H₂O (single) or NaFeEDTA (single) or both ZnSO₄·H₂O and NaFeEDTA (dual). The amounts of Fe and Zn concentration to fortify lentils were selected based on the RDA of Fe and Zn recommended by WHO and FAO (2). Two control samples (unpolished and polished with 0.5% canola oil) from each of the three LPT were used to compare the Fe and
Zn concentration with fortified samples. Fe and Zn concentration for different samples were quantified using an ICP-Mass Spectrometer.

**HunterLab colorimetric measurements of dual-fortified lentil samples**

The initial color (CIELAB color score, L, a* and b*) of Fe, Zn and dual-fortified lentil samples from all three LPT were measured using a HunterLab (Hunter Associates Laboratory Inc., Reston, VA, USA) instrument, and compared the changes with two unfortified control lentil samples (12,33). The Hunterlab L*, a* and b* scales were measured three times per sample. L* indicates the darkness to lightness, ranging from 0 to 100; a* indicates greenness to redness, ranging from -80 to +80 and b* indicates blueness to yellowness, ranging from -80 to +80 (34).

**Effect of storage time on colorimetric changes of stored dual-fortified lentil samples**

All fortified samples used for colorimetric analysis were assessed for colorimetric changes initially, after 8 months, and after 12 months of storage in room temperature (18-25 °C) and relative humidity (45-60%) to determine if color change had occurred. Each sample was stored separately in a clear plastic bag (Ronco, Toronto, ON, Canada), similar to methods typically used to store dal products. The one-year storage period was considered an approximate maximum storage period from processing to consumption by dal consumers.
Assessment of relative Fe bioavailability and phytic acid concentration of dual-fortified lentil

Five samples from each of the three LPT, including a control (unfortified and polished with 0.5% canola oil) were cooked with 18 MΩ de-ionized water in stainless steel cookware. The cooked samples were cooled to room temperature for 2h followed by frozen at -80°C for 24h. Frozen samples were freeze-dried using a Freezone 12 L Console Freeze Dry System with Stoppering rays (Labconco, Model 7759040, Kansas City, MO, USA) for 72h and then stored at room temperature (25,31). Fifteen grams of freeze dried lentil from each sample was finely ground and sent to the USDA-ARS Robert Holley Centre for Agriculture and Health (Ithaca, New York, NY, USA) to assess Fe and Zn concentration and Fe-bioavailability using an in-vitro digestion/Caco-2 cell culture bioassay which mimics conditions in the small intestine (35). Ferritin formation in the Caco-2 cell monolayers is considered a marker for Fe uptake (26). Caco-2 cell monolayers (American Type Culture Collection, Rockville, MD) were seeded at a density of 50000 cells/cm$^2$ in collagen-treated six-well plates (Costar Corp., Cambridge, MA). The cells were then grown for 13 d in Dulbecco’s Modified Eagle Medium (GIBCO, Grand Island, NY) with 10% v/v fetal calf serum (GIBCO), 25 mmol/L HEPES, and 1% antibiotic antimitotic solution (GIBCO) after placement in an incubator, then used in the Fe uptake experiments.

Each lentil sample (0.5 g) was digested in an in-vitro digestion system to extract the “intestinal digest” using a digestion solution (pepsin, pancreatin, and bile extract) at pH 7.0. Before the intestinal digestion, growth medium was removed from each culture well, and
the cell culture was washed twice with 37 °C Minimum Essential Media (MEM, no. 41500; GIBCO; Inc.) at pH 7.0. Then the Six-Well Culture Plates with Cell Monolayers were prepared to complete the intestinal digestion. The intestinal digest cell monolayers were then harvested for ferritin analysis at 24 h after the start of the intestinal digestion period. The medium covering the cells was removed, and the cells were harvested and washed once with a 2 mL volume of a “rinse” solution containing 140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol of PIPES, at pH 7. After rinsing, 2 mL of deionized water was placed on each monolayer. The plates were then placed on a rack with the bottom of each plate in contact with the water of a benchtop sonicator (Lab-Line Instruments, Melrose Park, IL), which was kept in a cold room at 4 °C. The cells were sonicated for 15 min and then scraped from the plate surface and harvested along with the 2 mL volume of water in each well. The samples were immediately frozen and stored at -20 °C. Caco-2 cell protein was measured from samples that had been solubilized in 0.5 mol/L NaOH, using a semi-micro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Ferritin Assay, RAMCO Laboratories, Houston, TX). A 10 μL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each ferritin measurement. Analysis of the Fe in solutions and digested biological samples was determined by inductively coupled argon plasma emission spectrometry (ICAP model 61E trace analyzer, Thermo Jarrell Ash Corp., Franklin, MA).

Ferritin values from all fortified samples of the three LPT were compared with the control lentil (unfortified and unpolished) to calculate the relative Fe bioavailability.
(RFeB%) using the equation: relative Fe bioavailability (RFeB %) = ((ng ferritin of the lentil sample/mg protein of the lentil sample)/(ng ferritin/mg protein of the control lentil)) × 100 (13,31). The calculated RFeB% was used to assess the % of increase or decrease of bioavailability compared to the control. Sample one (unfortified lentil) from each of the three groups of LPT was used to calculate the RFeB% of other four samples from each of the group. Zn bioaccesibility was assessed using in-vitro dializability (26). Phytic acid (PA) content was measured for all samples used for bioavailability assessment, using the PA (total phosphorus) test kit (Megazyme International, Country Wicklow, Ireland), a simple, quantitative, colorimetric and high throughput method (25,36).

Statistical Methods

One-way analysis of variance (ANOVA) in SAS 9.4 (SAS Inc. Cary, NC, USA) was used to determine differences in Fe and Zn concentration of fortified red and yellow lentil genotypes of each of the three LPT at concentrations ranging from 6 to 24 mg/100 g of lentil. The initial HunterLab colorimetric L*, a* and b* and effect of storage time on colorimetric changes of stored control and fortified lentil samples were analyzed using ANOVA in SAS 9.4. Similarly, ANOVA was used to analyse RFeB% and PA concentration differences among the samples. In all analyses, the Fisher’s least significant difference (LSD) was calculated with level of significance set at P<0.05.

Results

Selection of red and yellow lentil genotypes and product types for dual-fortification

The Supplemental Table 2 shows Fe and Zn concentration of the two subtypes of three LPT. Significant differences were observed among three LPT for both Fe and Zn
concentration but there was no significant difference between the two subtypes of all three LPT. RF had significantly higher Fe concentration than RS and YS lentil. Unlike Fe, RF and RS lentil had significantly similar Zn concentration, and was higher than YS lentil.

**Modifications of previous fortification strategy for dual-fortification**

The modifications to the fortification protocol for lentils proved that the modified method was easier to use than the previous method (23) for fortifying all LPT by using 50% less solvent, which helped to maintain an acceptable moisture content of the fortified lentil. Coating with 0.5% canola oil after fortification helps to protect the fortificants from washing out during rinsing of fortified lentil before cooking. Moreover, non-significant differences were observed for Fe and Zn concentration for all the three LPT samples, indicating that the protocol was repeatable and reproducible ([Supplemental Table 3](https://academic.oup.com/cdn/advance-article/doi/10.1093/cdn/nzab004/6124628)).

Moisture content and water activity of unfortified lentils (control) were 9.86% and 0.45, respectively, which was statistically similar to the dual-fortified lentils, (10.41% and 0.44).

**Selection of appropriate dose of iron and zinc fortificants**

Iron and zinc concentration in three LPT samples that were either single – or dual-fortified at different concentrations are shown in [Table 2](https://academic.oup.com/cdn/advance-article/doi/10.1093/cdn/nzab004/6124628). Fe concentration ranged from 7.5-28.6 mg, 7.1-31.6 mg and 5.9-32.9 mg/100 g of lentil for RF, RS and YS lentil samples, respectively. Zn concentration ranges were 4.3-15.7, 4.3-15.3 and 3.9-14.1 mg/100 g of lentil in RF, RS and YS lentils samples, respectively. No significant differences were found for Fe concentration within two control and two Zn-fortified samples (samples 3 and 4) of each of the three LPT. These four samples had significantly different Fe concentration when Fe was added in either the Fe-fortified (samples 5 and 6) or in three dual-fortified
samples (samples 7-9) for all the three LPT. Similarly, non-significant differences were observed for Zn concentration among two controls and two Fe-fortified samples (samples 5 and 6) in each of the three LPT. These four samples had significant differences for Zn concentration compared with two Zn-fortified (samples 3 and 4) and all three dual-fortified samples for all three LPT. Overall, among the three dual-fortified lentil samples, RS had significantly higher Fe concentration than RF and YS, when similar amounts of Fe and Zn (12 mg) were used to fortify (sample 7). In samples 8 and 9, YS had significantly higher Fe concentration than RS and RF lentil samples. Unlike Fe concentration, all the nine RF lentil samples had higher Zn concentration followed by RS and YS lentil samples.

**HunterLab colorimetric measurements of dual-fortified lentil samples**

The CIELAB score from HunterLab measurements for initial samples (after fortification) showed significant variation for all three scales (L, a* & b*) in all three LPT; RF (Supplemental Table 4), RS (Supplemental Table 5) and YS (Supplemental Table 6). In all three LPT, significantly higher and lower L value was observed for Zn-fortified samples (sample-3) and in both Fe-fortified lentil samples (samples 5 & 6), respectively. The range of L value in RF, RS and YS samples were 50.8-53.2, 53.2-54.1 and 59.2-62.1, respectively.

Among all three LPT, a* value was significantly higher in unpolished control samples and was also significantly different from the polished control samples (sample-2). The range of a* value for RF, RS and YS samples were 27.8-32.2, 27.0-33.1 and 10.6-12.7, respectively.

Among the three LPT, significantly higher and lower b* values were observed in unpolished control (sample-1), and in dual-fortified lentil sample fortified with 12 mg of Zn
and 24 mg of Fe/100 g (sample-9), respectively. The b* values of RF, RS and YS samples ranged from 39.7-47.5, 40.4-47.5 and 45.8-51.7, respectively. More details of the colorimetric results have showed in Supplemental Text 1.

**Effect of storage time on colorimetric changes of stored dual-fortified lentil**

Results of changes of L, a* and b* values of three LPT with storage time is shown in Supplemental Tables 4 to 6. L value increased, and a* and b* values decreased for all the three LPT over time. The ranges of L, a* and b* values were wider in initial samples than the 8 months and one-year stored samples in all the three LPT. A similar trend of increment or decrement of L, a* and b* scores with changes of Fe and Zn doses were observed for all 8 months and one-year stored samples.

**Assessment of relative Fe bioavailability and phytic acid concentration of dual-fortified lentil**

The average Fe and Zn concentration, ng ferritin/(mg protein), and PA concentration of five samples from each of the three LPT are shown in Table 3. Significant differences were observed among fortified and unfortified lentil samples for all the four attributes. Non-significant difference for Fe concentration was observed between control and Zn-fortified sample (sample-2) in all LPT indicating that there was no influence on Fe concentration from Zn-fortified lentil. Zn concentration was similar between control (sample-1) and the Fe-fortified sample (sample-3). These two samples had significantly different Zn concentrations when compared with the other three samples (2, 4 and 5) in all three LPT. Significant differences were found for ferritin ["ng ferritin/(mg protein)" concentration between control (sample-1) and fortified lentil samples (samples 2-5) for all three LPT.
Ferritin concentration increased with the increase of Fe concentration and the highest ferritin concentration was found in dual-fortified sample-5 in all three LPT. Dual-fortified samples had higher ferritin concentration compared to single fortified samples (samples 2 & 3). Comparing samples 3, 4 and 5 from all three LPT, both dual-fortified samples had higher RFeB% than that of Fe-fortified sample (sample-3). RFeB% decreased in Zn-fortified RF and YS sample (sample-2) by 43.4% and 36%, respectively. Only 2% RFeB% increase was observed in the RS sample 2. PA concentration was reduced significantly from the control in all the fortified lentil samples of the three LPT. The two dual-fortified samples had similar PA concentration, compared to other 3 samples in all three LPT.

Discussion

The aim of the dual-fortification investigations with Fe and Zn was to improve of the bioavailable Fe and Zn intake of the human population that consumes lentil as a major or partially staple food in their diets and are also deficient for these two micronutrients. Among the two market classes of lentil used in this study, red lentil has wide acceptability in South Asia and the Middle East (37). Yellow cotyledon lentils are mostly consumed in Europe and are also used in several value added or processed food products (e.g., snacks) around the world. In our previous study (23), only RF lentil was fortified with Fe. In this study, the modified method encouraged us to fortify both football and split types at a relatively low fortification cost compared to all other probable fortification techniques (23). This protocol can be easily integrated into existing medium- or large-scale processing plants to commercially produce dual-fortified lentils. A similar fortification protocol was implemented at the Saskatchewan Food Industry Development Centre, Saskatchewan,
Canada to produce 200 kg fortified lentil/hour for use in a double blind community-based randomized controlled trial with adolescent girls in Bangladesh (38). A feasibility study was also done at a local lentil processing plant (Prairie Pulse Inc. 700 Campbell Dr., Vanscoy, Saskatchewan, Canada) using a similar protocol to evaluate whether the protocol can be merged with the large-scale fortified lentil production. This technology is flexible and will help to accommodate the preferences for different LPTs by consumers in most lentil consuming regions.

In favor of selecting NaFeEDTA and ZnSO$_4$H$_2$O as Fe and Zn fortificants, respectively, recommendations from WHO were used as a reference (2). The combination of Fe and Zn doses for dual-fortification were selected based on the RDAs of micronutrients, mentioned in the WHO fortification guide (2). The Fe concentration used was higher than that of Zn concentration based on the RDAs for these two minerals in human diets. Both Fe and Zn fortificants used in this study are water soluble, allowing mixing in the same water solution. This helped us to reduce the risk of adding excess moisture during fortification and to avoid microbial contamination and oxidation. Polished football lentil with either 0.5-1% water or edible oil has demand in many lentil consuming countries around the world because the dehulled unsplit product has a clear and shiny appearance that is attractive to consumers.

Polishing after fortification has also a significant effect on mixing the fortification solution uniformly and drying on the shaker helps to move and agitate fortified lentils more quickly in the mixing trays (23). Dual-fortified lentils were polished with 0.5% canola oil that acts as a coating material to protect the fortificants after first or second rinse of the product before cooking.
In the bioavailability study (Table 3), although Fe concentration did not significantly differ between Fe-fortified and dual-fortified samples, RFeB% was significantly higher in dual-fortified samples. This could be due to a positive effect of Zn fortificants on the absorption of the Fe fortificant. On the other hand, using a similar amount of Zn (12 mg/100 g of lentil) in sample-2 and sample-5, Zn concentration was significantly lower in the dual-fortified sample. It might be due to the influence of Fe fortificants on Zn availability.

Again, in Zn-fortified lentils (sample-2) of RF and YS types, RFeB% was decreased by 43.4% and 36%, respectively and was increased only 2% in RS lentils. It could be due to the Fe and Zn homeostasis and interaction, and competition between Fe and Zn for a shared absorption pathway (39). A reduction of Fe absorption from ZnSO$_4$$\cdot$H$_2$O fortified wheat flour dumplings was also reported in a previous study with Indonesian children (40). This result indicated that dual-fortified lentil can provide more bioavailable Fe than single fortified Fe- or Zn-fortified lentil. Some ingredients may help to increase the bioavailability of minerals (e.g. EDTA, some polyphenols) (11,41). NaFeEDTA with zinc sulfate or zinc methionine can improve iron and zinc absorption for food production (42) compared to sole use of these fortificants. Inclusion of EDTA with Zn salt increased Zn bioaccessibility in three-fold from fortified millet flour (43). Since in vitro bioaccessibility was not measured in this study, it will be used to estimate zinc absorption in future studies. PA concentration was significantly higher in control lentil than in the fortified lentil samples of the three LPT. It could be due to the dephytinization that can activate phytases largely (44). The PA:Fe molar ratio was also reduced in fortified lentils compared to the control lentil. A previous study with Fe fortified lentil showed that phytic acid concentration was reduced
due to the fortification process. Another study also reported that for Fe-fortified fonio porridge, dephytinization and fortification reduced the PA:Fe molar ratio from 24:1 to 0.3:1 (45).

Fortificants have not only the desired components (e.g. Fe and Zn) but also some ingredients that may react with the food vehicle resulting in development of off-color, rancidity, degradation of vitamins and decrease in bioavailability (46,47). The expectation for the fortification is to reduce the off-color development to the minimum possible. In this study, the L*, a* and b* values of two control samples of three LPT were significantly different, indicating that polishing has a significant effect on colorimetric changes. Again, the three dual-fortified samples, even with the highest dose (24 mg of Fe and 12 mg Zn/100 g of lentil) of Fe and Zn, showed significantly lower L value than the Fe-fortified (single) samples. This result indicated that NaFeEDTA had the most influence on off-color development or darkening of the lentil samples compared to the Zn-fortificant. The previous study with Fe-fortified lentil showed that off-color development was increased with an increase in the Fe concentration of the fortificant in lentil samples (23). Stability of Fe and Zn may alter the storage time due to presence of humidity, temperature fluctuation, light. Lentil has a small amount of lipid (1.52-2.95%) (48) and lipid oxidation may influence the colorimetric changes of fortified samples. More discussion on colorimetric results have showed in Supplemental Text 1.

Lentil is primarily consumed in dehulled form and has the potential to be used as a vehicle for micronutrient fortification. Globally, lentil is consumed in 120 countries and consumption rates is vary from region to region. For example, in Bangladesh, lentil is
considered a staple to partially staple food. The FAO recommended consumption rate of lentil in Bangladesh is 50 g/person/day, but the actual consumption rate is only 12 g/person/day (15,49). Results from this dual fortification study show that lentil can be used as a potential vehicle for dual fortification, and NaFeEDTA and ZnSO$_4$$\cdot$H$_2$O were found as the most suitable Fe and Zn fortificants, respectively. The amount of Fe and Zn doses selected in this study was based on the RDAs referred by WHO and FAO (2). A 50 g RF lentil sample fortified with 12 mg of Zn and 24 mg of Fe/100 g, can provide approximately 13.5 mg and 7.0 mg of Fe and Zn, respectively. This amount of Fe and Zn is safe for human consumption considering the tolerable upper intake level of Fe (45 mg/person/day) and Zn (40 mg/person/day) for adults (50,51).

**Conclusion**

High consumption of foods with low bioavailable Fe and Zn is one of the major causes of Fe and Zn deficiency globally. Overall results from this study showed increased Fe, Zn, and RFeB%, and decreased PA concentration in dual-fortified lentils compared to the unfortified lentil. Results also revealed that the dual-fortification protocol could merge with the existing medium or large-scale commercial production of dual-fortified lentils in a biologically and culturally meaningful way. We conducted a consumer study with dual-fortified lentil recently, and results were published in a manuscript (12) that showed that the consumers widely accepted dual-fortified lentil. Stability of added micronutrients and changes of bioavailability of Fe over time are also important considerations that have been assessed and will be reported in a subsequent manuscript. We have not yet investigated the influence of the storage period on the stability and bioavailability of Fe and Zn of dual-
fortified lentils under retail storage conditions with high temperature (>35°C) and high relative humidity (>85%) of tropical and sub-tropical regions. Additional research to choose a suitable packaging system for dual-fortified lentil considering various retail market conditions will need to be considered. Community-based efficacy trials with dual-fortified lentils in different lentil consuming regions of the world can provide an empirical estimate of the Fe and Zn requirement to meet a major part of the RDAs of Fe and Zn to deficient populations, especially in regions where lentil is frequently consumed as a staple or partial staple food.

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Table 1. Nine dehulled lentil samples from each of the three lentil product types (red football, red split and yellow split) used for single (either Fe or Zn fortified) and dual (both Fe and Zn) fortification with different doses of Fe and Zn from NaFeEDTA and ZnSO$_4$.H$_2$O, respectively.

| Samples with their Fortification status | Fortificant dose/s added 100$^{-1}$ g of lentil | Fe (mg) from NaFeEDTA | Zn (mg) from ZnSO$_4$.H$_2$O |
|----------------------------------------|------------------------------------------------|----------------------|-----------------------------|
| Sample 1 - Control                      | Unfortified and unpolished                      |                      |                             |
| Sample 2 - Control                      | Unfortified and polished with 0.5% canola oil   |                      |                             |
| Sample 3 - Zn fortified                 | --                                              |                      | 6                           |
| Sample 4 - Zn fortified                 | --                                              |                      | 12                          |
| Sample 5 - Fe fortified                 | 16                                              |                      | --                          |
| Sample 6 - Fe fortified                 | 24                                              |                      | --                          |
| Sample 7 - Fe and Zn fortified          | 12                                              | 12                   |                             |
| Sample 8 - Fe and Zn fortified          | 16                                              | 8                    |                             |
| Sample 9 - Fe and Zn fortified          | 24                                              | 12                   |                             |
Table 2: Iron and zinc concentration in three milled lentil product types (red football, red split and yellow split) samples (n=3), fortified with either NaFeEDTA (single fortified) or ZnSO$_4$.H$_2$O (single fortified) or both NaFeEDTA and ZnSO$_4$.H$_2$O (dual-fortified) at different concentrations.

| Sample # | Fe (mg/100 g lentil)* | Zn (mg/100 g lentil)* |
|----------|-----------------------|-----------------------|
|          | Red football | Red split | Yellow split | Red football | Red split | Yellow split |
|          | Mean (CI 95%) | Mean (CI 95%) | Mean (CI 95%) | Mean (CI 95%) | Mean (CI 95%) | Mean (CI 95%) |
| Sample 1 | 7.5 (0.1)$^a$    | 7.1 (0.3)$^a$    | 5.9 (0.1)$^a$    | 4.3 (0.1)$^a$    | 4.4 (0.2)$^a$    | 3.9 (0.1)$^a$    |
| Sample 2 | 7.6 (0.6)$^a$    | 7.3 (0.2)$^a$    | 5.9 (0.3)$^a$    | 4.3 (0.2)$^a$    | 4.3 (0.1)$^a$    | 3.9 (0.1)$^a$    |
| Sample 3 | 7.5 (0.2)$^a$    | 7.4 (0.2)$^a$    | 6.0 (0.2)$^a$    | 9.9 (0.2)$^c$    | 9.8 (0.5)$^b$    | 8.8 (0.1)$^b$    |
| Sample 4 | 7.7 (0.3)$^a$    | 7.4 (0.2)$^a$    | 6.1 (0.3)$^a$    | 15.5 (0.3)$^f$   | 15.2 (0.2)$^e$   | 13.9 (0.3)$^e$   |
| Sample 5 | 25.7 (2.9)$^c$   | 20.5 (0.2)$^b$   | 19.6 (1.1)$^b$   | 4.4 (0.1)$^{ab}$ | 4.3 (0.1)$^a$    | 3.9 (0.1)$^a$    |
| Sample 6 | 27.5 (0.3)$^d$   | 31.1 (1.5)$^d$   | 21.6 (0.5)$^c$   | 4.5 (0.1)$^b$    | 4.4 (0.1)$^a$    | 3.9 (0.1)$^a$    |
| Sample 7 | 20.5 (2.1)$^b$   | 20.7 (1.3)$^b$   | 19.4 (0.1)$^b$   | 15.7 (0.3)$^g$   | 15.3 (0.3)$^e$   | 14.1 (0.6)$^f$   |
| Sample 8 | 27.1 (1.9)$^d$   | 28.0 (2.0)$^c$   | 29.9 (1.1)$^d$   | 13.9 (0.2)$^d$   | 13.4 (0.6)$^c$   | 12.1 (0.4)$^c$   |
| Sample 9 | 28.6 (0.3)$^e$   | 31.6 (1.2)$^d$   | 32.9 (1.0)$^d$   | 15.1 (0.2)$^e$   | 14.6 (0.1)$^d$   | 13.2 (0.3)$^d$   |

* Mean scores (95% CI) for iron and zinc concentration (mg/100 g of lentil) in three milled lentil product types (red football, red split and yellow split) samples (n=3) followed by different Roman letters within columns are significantly different (P<0.0001).
Table 3. Mean iron (Fe) and zinc (Zn) concentration, ng ferritin/(mg protein), relative iron bioavailability and phytic acid concentration of five cooked freeze-dried lentil samples (n=3) from each of the three milled lentil product types (red football, red split and yellow split).

| Fortified and unfortified cooked lentil samples | Fe (mg/100 g of lentil) | Zn (mg/100 g of lentil) | ng ferritin/(mg protein) | RFeB% | %RFeB increase/decrease than control | Phytic acid (mg g⁻¹) |
|-----------------------------------------------|-------------------------|-------------------------|--------------------------|--------|-------------------------------------|---------------------|
| Sample No.                                    | Fe added (mg) from NaFeEDTA | Zn (mg) added from ZnSO₄H₂O | Mean (CI 95%)            | Mean (CI 95%) |
| Red Football                                  |                         |                         |                          |         |
| Oneᵇ                                           | Unfortified control     |                         | 7.6 (0.6)a                    | 4.3 (0.2)a                      | 10.8 (1.4)b | 91.3                   | 0.0                  | 0.63 (0.08)ab         |
| Twoᶜ                                           | 0.0                    | 12                      | 7.7 (0.3)a                    | 15.5 (0.3)c                      | 6.1 (0.5)a | 51.7                   | -43.4                | 0.62 (0.09)b          |
| Threeᵈ                                         | 24                     | 0.0                     | 27.5 (0.3)b                   | 4.5 (0.1)b                       | 26.0 (2.4)c | 220.1                  | 141.1                | 0.55 (0.01)c          |
| Fourᵉ                                          | 16                     | 8                       | 27.1 (1.9)b                   | 13.9 (0.2)c                      | 27.6 (9.4)d | 233.1                  | 155.4                | 0.51 (0.11)d          |
| Fiveᵉ                                          | 24                     | 12                      | 28.6 (0.3)c                   | 15.1 (0.2)d                      | 36.4 (4.0)e | 307.3                  | 236.6                | 0.53 (0.03)d          |
| Red Split                                     |                         |                         |                          |         |
| Oneᵇ                                           | Unfortified control     |                         | 7.3 (0.2)a                    | 4.3 (0.1)a                      | 7.9 (2.1)a | 113.6                  | 0.0                  | 0.83 (0.15)a          |
| Twoᶜ                                           | 0.0                    | 12                      | 7.4 (0.2)a                    | 15.2 (0.2)d                      | 8.2 (3.7)a | 116.1                  | 2.1                  | 0.80 (0.14)b          |
| Threeᵈ                                         | 24                     | 0.0                     | 31.1 (1.5)c                   | 4.4 (0.1)a                       | 28.9 (1.8)b | 411.1                  | 261.7                | 0.71 (0.02)c          |
| Fourᵉ                                          | 16                     | 8                       | 28.0 (2.0)b                   | 13.4 (0.6)b                      | 32.3 (7.0)bc | 459.4                  | 304.3                | 0.73 (0.07)c          |
|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| Five | 24 | 12 | 31.6 (1.2) | 14.6 (0.1) | 36.7 (10.1) | 521.8 | 359.2 | 0.71 (0.18) |
| Yellow split |   |   |   |   |   |   |
| One | Unfortified control | 5.9 (0.3) | 3.9 (0.1) | 19.9 (7.5) | 122.0 | 0.00 | 0.96 (0.16) |
| Two | 0.0 | 12 | 6.1 (0.3) | 13.9 (0.3) | 12.8 (8.8) | 78.1 | -36.00 | 0.92 (0.04) |
| Three | 24 | 0.0 | 21.6 (0.5) | 3.9 (0.1) | 40.1 (12.1) | 245.5 | 101.16 | 0.87 (0.19) |
| Four | 16 | 8 | 29.9 (1.1) | 12.1 (0.4) | 70.0 (31.7) | 428.9 | 251.47 | 0.79 (0.11) |
| Five | 24 | 12 | 32.9 (1.0) | 13.2 (0.3) | 84.8 (19.0) | 519.5 | 325.67 | 0.79 (0.11) |

*Mean scores (95% CI) for iron (Fe) and zinc (Zn) concentration, ng ferritin/(mg protein), relative iron bioavailability and phytic acid concentration followed by different Roman letters within columns are significantly different (P<0.0001). b Unfortified control lentil polished with 0.5% canola oil; c Zn-fortified lentil with ZnSO₄H₂O, d Fe-fortified lentil with NaFeEDTA; e Dual-fortified lentil with NaFeEDTA and ZnSO₄H₂O.*
Figure 1. Dual-fortification protocol to fortify red and yellow cotyledon dehulled lentil products with both Fe and Zn fortificants.