ORIGINAL RESEARCH

CURRENT DEVELOPMENTS IN NUTRITION

Community and Global Nutrition

Dual-Fortified Lentil Products—A Sustainable New Approach to Provide Additional Bioavailable Iron and Zinc in Humans

Rajib Podder,[1] Raymond P Glahn,[2] and Albert Vandenberg[1]

[1]Department of Plant Sciences, University of Saskatchewan, Saskatoon, Canada; and [2]Robert W Holley Center for Agriculture and Health, Agricultural Research Service, USDA, Ithaca, NY, USA

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 3 milled lentil product types (LPTs) [red-football (RF), red-split (RS), and yellow-split (YS)], with NaFeEDTA and $ZnSO_4.H_2O$ to increase the bioavailable content of Fe and Zn.

Methods: Appropriate Fe and Zn doses were determined to fortify lentils based on 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-factor ANOVA determined the differences in colorimetric score; concentrations of Fe, Zn, and PA; and RFeB% among samples. The least significant difference 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 with Fe-fortified (single) samples in all 3 LPTs, whereas RFeB% decreased in Zn-fortified (single) RF and YS samples by 43.4% and 36%, respectively. The RF, RS, and YS samples, fortified with 16 mg Fe and 8 mg Zn/100 g of lentils, provided 27 mg Fe and 14 mg Zn, 28 mg Fe and 13.4 mg Zn, and 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 2 globally important micronutrient deficiencies. *Curr Dev Nutr* 2021;5:nzab004.

Keywords: iron, zinc, fortification, bioavailability, phytic acid

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Address correspondence to RP (e-mail: rap039@mail.usask.ca).

Abbreviations used: a∗, redness; b∗, yellowness; L, lightness; LPT, lentil product type; PA, phytic acid; RF, red-football; RFeB%, relative iron bioavailability; RS, red-split; YS, yellow-split.

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 for the activity of many enzyme's 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 with animal protein, and it cooks quickly (4), saving fuel consumption and time. Unlike other pulses, lentil consumption over the past 30 y has grown at a much higher rate than the growth in the human population (4). Lentils are produced in >50 countries (5), and some non–producing countries consume lentils as a staple food—they are obligate importers. Lentils are considered an excellent source of crude protein (25.8–27.1%), Fe (73–90 mg/kg), Zn (44–54 mg/kg), selenium (Se; 425–673 μg/kg), etc. (6–8). Of the total Fe content in lentils, ∼10% is ferrous (0.31 ± 0.01 mg/100g of dry matter) and ∼90% is ferric (2.69 ± 0.15 mg/100 g 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 lentils (13, 14). But considering the lower bioavailability of Fe and Zn in lentils, RDAs of Fe and Zn, existing consumption rate (12 g/d per person) compared with recommended consumption rate (50 g/d per person) of pulses (15), and improvement in micronutrient concentration using other approaches may improve the concentration of these 2 micronutrients in lentils.

Among all of 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 the 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 1 industry-milled cereal grain (17). Wheat flour fortification with B vitamins is mandatory in 14 countries (18). The US FDA established a requirement to fortify an appropriate food vehicle for fortification in 1995 (19). Fortified rice is mandatory in 6 countries, and several subnational 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); nondairy 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 (LPTs), 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 (PA) concentrations, 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. The 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 dialyzability (26). A study reported that in vitro dialyzability data had a strong correlation coefficient (0.93; $P < 0.0001$) with in vivo human absorption data (26). 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 lentils, 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 lentils 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 lentils is shown in **Figure 1.**

Selection of appropriate red and yellow genotypes and 3 milled LPTs for dual fortification

Several red and yellow lentil cultivars/genotypes were analyzed to estimate the Fe and Zn concentration (micrograms/gram) 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) LPTs were used for dual fortification. For yellow cotyledon lentils, the cultivar "CDC Greenstar" (yellow-split product; YS) 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, Canada, and they are widely grown in Canada due to their high yield potential and resistance to biotic and abiotic stresses. All LPTs [red football (RF), red-split (RS), and 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 3 LPTs are manufactured as 2 subtypes, 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 3 LPTs were evaluated to assess the differences in intrinsic Fe and Zn concentration before fortifying them with Fe and Zn fortificants. Fe and Zn concentrations were assessed using an Inductively Coupled Plasma (ICP)–mass spectrometer (iCAP 6500 series; Thermo Jarrell Ash Corp.).

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 with other bioavailable fortificants. In general, fortificants added

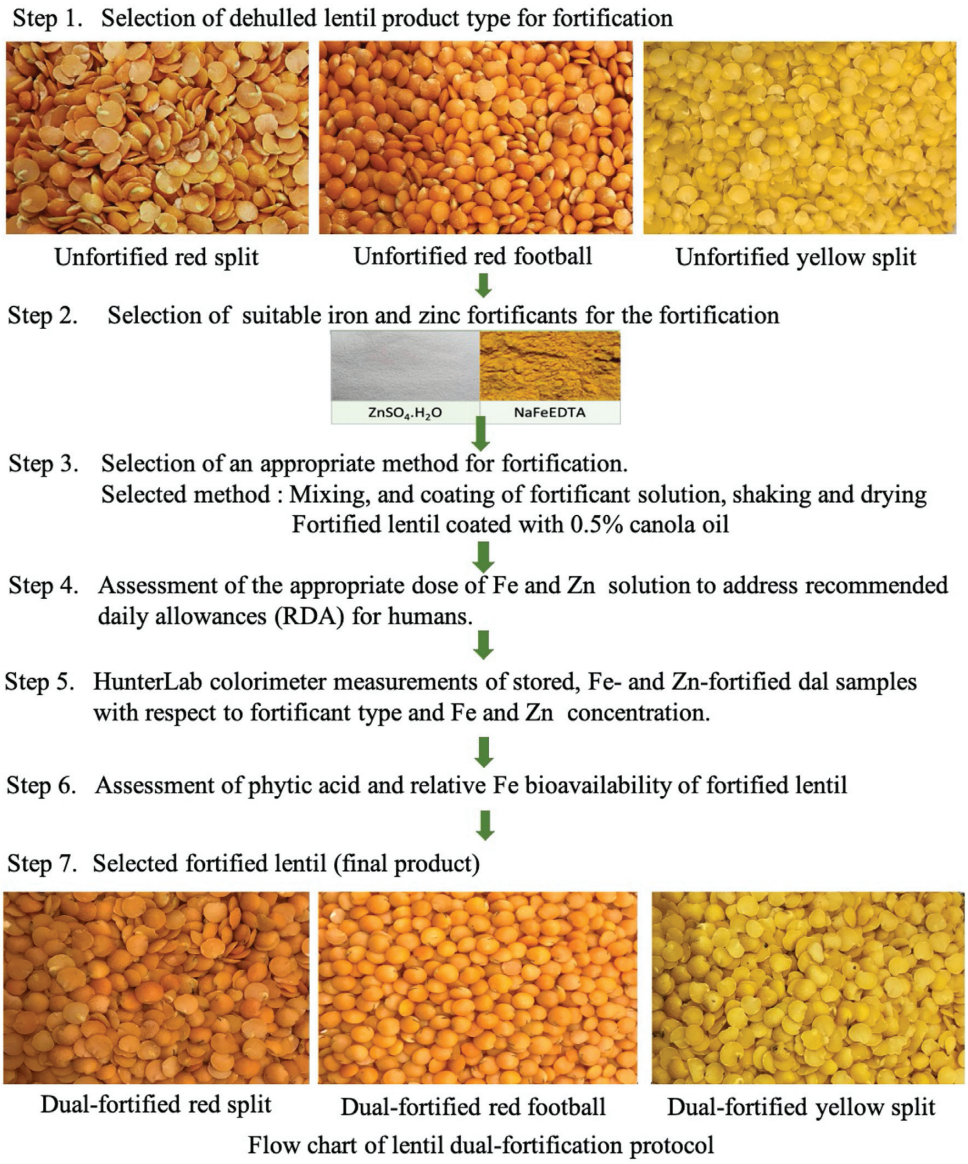

FIGURE 1 Dual-fortification protocol to fortify red and yellow cotyledon dehulled lentil products with both iron and zinc fortificants.

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 lentils (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 in organoleptic characteristics compared with unfortified lentils (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, 2 food-grade Zn fortificants, zinc-sulfate monohydrate ($ZnSO_4.H_2O$) 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 fortificants, $ZnSO_4.H_2O$ and ZnO were compared, and $ZnSO_4.H_2O$ 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, Illinois, and Spectrum Chemical, Gandora, California, 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) 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

TABLE 1 Nine dehulled lentil samples from each of the 3 lentil product types (red football, red split, and yellow split) used for single [either iron (Fe) or zinc (Zn) fortified] and dual (both Fe and Zn) fortification with different doses of Fe and Zn from NaFeEDTA and $ZnSO_4.H_2O$, respectively

	Fortificant dose(s) added per 100 g of lentils, mg	
Samples with their fortification status	Fe from NaFeEDTA	Zn from $ZnSO_4.H_2O$
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

solution, thereby reducing the amount of fortificant solution to help maintain the acceptable moisture content of the fortified lentils. Unpolished dehulled lentils were fortified with fortificants for 10 min followed by polishing with 0.5% canola oil for 5 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.) for another 10 min. A 250-W electric heat lamp (NOMA incandescent, clear, 130 V heat lamp; Trileaf Distributors) and a mini portable desk fan (model 043–5498-4; Trileaf Distributors) were used to provide both heat and air to achieve the desired 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 3 replications with 2 repeats. The fortification protocol was repeated 10 times and samples were analyzed to determine the Fe and Zn concentrations.

Selection of appropriate dose of iron and zinc fortificants

Seven samples from each of the 3 LPTs (**Table 1**) were prepared using either $ZnSO_4.H_2O$ (single) or NaFeEDTA (single) or both $ZnSO_4.H_2O$ 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 the WHO and FAO (2). Two control samples (unpolished and polished with 0.5% canola oil) from each of the 3 LPTs were used to compare the Fe and Zn concentration with fortified samples. Fe and Zn concentrations 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 3 LPTs was measured using a HunterLab (Hunter Associates Laboratory, Inc.) instrument, and compared the changes with 2 unfortified control lentil samples (12, 33). The HunterLab L∗, a∗, and b∗ scales were measured 3 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 mo, and after 12 mo of storage at 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), similar to methods typically used to store dal products. The 1-y storage period was considered an approximate maximum storage period from processing to consumption by dal consumers.

Assessment of relative Fe bioavailability and PA concentration of dual-fortified lentils

Five samples from each of the 3 LPTs, 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 2 h followed by freezing at −80°C for 24 h. Frozen samples were freeze-dried using a Freezone 12-L Console Freeze Dry System with Stoppering rays (model 7,759,040; Labconco) for 72 h and then stored at room temperature (25, 31). Fifteen grams of freeze-dried lentils from each sample were finely ground and sent to the USDA–Agricultural Research Service Robert Holley Center for Agriculture and Health (Ithaca, NY) 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) were seeded at a density of 50,000 cells/cm^2 in collagen-treated 6-well plates (Costar Corp.). The cells were then grown for 13 d in DMEM (GIBCO) with 10% vol:vol 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 medium (no. 41,500; GIBCO, Inc.) at pH 7.0. Then the 6-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 piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), at pH 7.0. 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), 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). A 1-stage, 2-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Ferritin Assay; RAMCO Laboratories). 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.).

Ferritin values from all fortified samples of the 3 LPTs were compared with the control lentils (unfortified and unpolished) to calculate the relative Fe bioavailability (RFeB%) using the following 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 percentage of increase or decrease in bioavailability compared with the control. Sample 1 (unfortified lentils) from each of the 3 groups of LPTs was used to calculate the RFeB% of the other 4 samples from each of the groups. Zn bioaccessibility was assessed using in vitro dialyzability (26). PA content was measured for all samples used for bioavailability assessment, using the PA (total phosphorus) test kit (Megazyme International), a simple, quantitative, colorimetric, and high-throughput method (25, 36).

Statistical methods

One-factor ANOVA in SAS version 9.4 (SAS Institute) was used to determine differences in Fe and Zn concentration of fortified red and yellow lentil genotypes of each of the 3 LPTs at concentrations ranging from 6 to 24 mg/100 g of lentils. The initial HunterLab colorimetric L∗, a∗, and b∗ and effect of storage time on colorimetric changes in stored control and fortified lentil samples were analyzed using ANOVA in SAS version 9.4. Similarly, ANOVA was used to analyze RFeB% and PA concentration differences between the samples. In all analyses, Fisher's least significant difference was calculated with the level of significance set at $P < 0.05$.

Results

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

Supplemental Table 2 shows Fe and Zn concentrations of the 2 subtypes of 3 LPTs. Significant differences were observed between 3 LPTs for both Fe and Zn concentration but there was no significant difference between the 2 subtypes of all 3 LPTs. RF had a significantly higher Fe concentration than RS and YS lentils. Unlike Fe, RF and RS lentils had significantly similar Zn concentrations, and was higher than YS lentils.

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 LPTs by using 50% less solvent, which helped maintain an acceptable moisture content of the fortified lentils. Coating with 0.5% canola oil after fortification helps protect the fortificants from washing out during rinsing of fortified lentils before cooking. Moreover, nonsignificant differences were observed for Fe and Zn concentrations for all of the 3 LPT samples, indicating that the protocol was repeatable and reproducible (**Supplemental Table 3**). 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 concentrations in 3 LPT samples that were either single- or dual-fortified at different concentrations are shown in **Table 2**. Fe concentration ranged from 7.5–28.6 mg, 7.1–31.6 mg, and 5.9–32.9 mg/100 g of lentils 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 lentils in RF, RS, and YS lentil samples, respectively. No significant differences were found for Fe concentration within 2 control and 2 Zn-fortified samples (samples 3 and 4) of each of the 3 LPTs. These 4 samples had significantly different Fe concentrations when Fe was added in either the Fe-fortified (samples 5 and 6) or in 3 dual-fortified (samples 7–9) samples for all 3 LPTs. Similarly, nonsignificant differences were observed for Zn concentration among 2 controls and 2 Fe-fortified samples (samples 5 and 6) in each of the 3 LPTs. These 4 samples had significant differences for Zn concentration compared with 2 Zn-fortified (samples 3 and 4) and all 3 dual-fortified samples for all 3 LPTs. Overall, among the 3 dual-fortified lentil samples, RS had a significantly higher Fe concentration than RF and YS, when similar amounts of Fe and Zn (12 mg) were used for fortification (sample 7). In samples 8 and 9, YS had a significantly higher Fe concentration than RS and RF lentil samples. Unlike Fe concentrations, all of the 9 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 3 scales (L, a∗, and b∗) in all 3 LPTs: RF (**Supplemental Table 4**), RS (**Supplemental Table 5**), and YS (**Supplemental Table 6**). In all 3 LPTs, significantly higher and lower L values were observed for Zn-fortified samples (sample 3) and in both Fe-fortified lentil samples (samples 5 and 6), respectively. The range of L values in RF, RS, and YS samples were 50.8–53.2, 53.2–54.1, and 59.2–62.1, respectively.

Among all 3 LPTs, the a∗ value was significantly higher in unpolished control samples and was also significantly different from the pol-

TABLE 2 Iron (Fe) and zinc (Zn) concentration in 3 milled lentil product types (red football, red split, and yellow split) samples ($n = 3$), fortified with either NaFeEDTA (single-fortified) or $ZnSO_4.H_2O$ (single-fortified) or both NaFeEDTA and $ZnSO_4.H_2O$ (dual-fortified) at different concentrations[1]

	Fe, mg/100 g lentils			Zn, mg/100 g lentils		
Sample no.	Red football	Red split	Yellow split	Red football	Red split	Yellow split
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)^{a,b}	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)^{e}	15.1 (0.2)^{e}	14.6 (0.1)^{d}	13.2 (0.3)^{d}

[1]Values are means (95% CIs). Means with different superscript letters within columns are significantly different, $P < 0.0001$.

ished control samples (sample 2). The ranges of the 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 3 LPTs, significantly higher and lower b∗ values were observed in unpolished control (sample 1) and in dual-fortified lentil samples fortified with 12 mg Zn and 24 mg 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 are shown in **Supplemental Text 1**.

Effect of storage time on colorimetric changes of stored dual-fortified lentils

Results of changes in L, a∗, and b∗ values of 3 LPTs with storage time are shown in Supplemental Tables 4–6. The L value increased and a∗ and b∗ values decreased for all 3 LPTs over time. The ranges of L, a∗, and b∗ values were wider in initial samples than the 8-mo and 1-y stored samples in all 3 LPTs. A similar trend of increment or decrement of L, a∗, and b∗ scores with changes in Fe and Zn doses was observed for all 8-mo and 1-y stored samples.

Assessment of relative Fe bioavailability and PA concentration of dual-fortified lentils

The average Fe and Zn concentration, nanogram ferritin/milligram protein, and PA concentration of 5 samples from each of the 3 LPTs are shown in **Table 3**. Significant differences were observed between fortified and unfortified lentil samples for all 4 attributes. A nonsignificant difference for Fe concentration was observed between the control and Zn-fortified sample (sample 2) in all LPTs, indicating that there was no influence on Fe concentration from Zn-fortified lentils. Zn concentration was similar between the control (sample 1) and the Fe-fortified sample (sample 3). These 2 samples had significantly different Zn concentrations when compared with the other 3 samples (samples 2, 4, and 5) in all 3 LPTs.

Significant differences were found for ferritin (nanogram ferritin/milligram protein) concentration between control (sample 1) and fortified lentil (samples 2–5) samples for all 3 LPTs. Ferritin concentration increased with the increase in Fe concentration and the highest ferritin concentration was found in dual-fortified sample 5 in all 3 LPTs. Dual-fortified samples had higher ferritin concentrations compared with single fortified samples (samples 2 and 3). Comparing samples 3, 4, and 5 from all 3 LPTs, both dual-fortified samples had higher RFeB% than that of the Fe-fortified sample (sample 3). RFeB% decreased in the Zn-fortified RF and YS sample (sample 2) by 43.4% and 36%, respectively. An RFeB% increase of only 2% was observed in the RS sample 2. PA concentration was reduced significantly from the control in all the fortified lentil samples of the 3 LPTs. The 2 dual-fortified samples had similar PA concentrations compared with the other 3 samples in all 3 LPTs.

Discussion

The aim of the dual-fortification investigations with Fe and Zn was to improve the bioavailable Fe and Zn intake of the human population who consume lentils as a major or partially staple food in their diets and are also deficient in these 2 micronutrients. Among the 2 market classes of lentils 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 lentils were 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 with 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 lentils/h 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., 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 accommodate the preferences for different LPTs by consumers in most lentil-consuming regions.

In favor of selecting NaFeEDTA and $ZnSO_4.H_2O$ as Fe and Zn fortificants, respectively, recommendations from the WHO were used as a reference (2). The combination of Fe and Zn doses for dual fortification was selected based on the RDAs of micronutrients, men-

TABLE 3 Mean iron (Fe) and zinc (Zn) concentration, ng ferritin/mg protein, relative iron bioavailability, and phytic acid concentration of 5 cooked freeze-dried lentil samples ($n = 3$) from each of the 3 milled lentil product types (red football, red split, and yellow split)[1]

Fortified and unfortified cooked lentil samples			Mean (95% CI)					
Sample no.	Fe added from NaFeEDTA, mg	Zn added from $ZnSO_4.H_2O$, mg	Fe, mg/100 g of lentils	Zn, mg/100 g of lentils	ng Ferritin/mg protein	RFeB%	RFeB% increase/decrease compared with control	Phytic acid, mean (95% CI), mg/g
Red football								
1[2]	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)^{a,b}
2[3]	0.0	12	7.7 (0.3)^{a}	15.5 (0.3)^{e}	6.1 (0.5)^{a}	51.7	-43.4	0.62 (0.09)^{b}
3[4]	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}
4[5]	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}
5[5]	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								
1[2]	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}
2[3]	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}
3[4]	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}
4[5]	16	8	28.0 (2.0)^{b}	13.4 (0.6)^{b}	32.3 (7.0)^{b,c}	459.4	304.3	0.73 (0.07)^{c}
5[5]	24	12	31.6 (1.2)^{c}	14.6 (0.1)^{c}	36.7 (10.1)^{c}	521.8	359.2	0.71 (0.18)^{c}
Yellow split								
1[2]	Unfortified control		5.9 (0.3)^{a}	3.9 (0.1)^{a}	19.9 (7.5)^{a}	122.0	0.00	0.96 (0.16)^{a}
2[3]	0.0	12	6.1 (0.3)^{a}	13.9 (0.3)^{d}	12.8 (8.8)^{a}	78.1	-36.00	0.92 (0.04)^{b}
3[4]	24	0.0	21.6 (0.5)^{b}	3.9 (0.1)^{a}	40.1 (12.1)^{b}	245.5	101.16	0.87 (0.19)^{c}
4[5]	16	8	29.9 (1.1)^{c}	12.1 (0.4)^{b}	70.0 (31.7)^{c}	428.9	251.47	0.79 (0.11)^{d}
5[5]	24	12	32.9 (1.0)^{d}	13.2 (0.3)^{c}	84.8 (19.0)^{d}	519.5	325.67	0.79 (0.11)^{d}

[1]Means with different superscript letters within columns are significantly different, $P < 0.0001$. RFeB%, relative iron bioavailability.
[2]Unfortified control lentils polished with 0.5% canola oil.
[3]Zn-fortified lentils with $ZnSO_4.H_2O$.
[4]Fe-fortified lentils with NaFeEDTA.
[5]Dual-fortified lentils with NaFeEDTA and $ZnSO_4.H_2O$.

tioned in the WHO fortification guide (2). The Fe concentration used was higher than that of the Zn concentration based on the RDAs for these 2 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 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 also has 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, which acts as a coating material to protect the fortificants after the 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 lentils) in sample 2 and sample 5, Zn concentration was significantly lower in the dual-fortified sample. This 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 by only 2% in RS lentils. This 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 in Fe absorption from $ZnSO_4.H_2O$-fortified wheat flour dumplings was also reported in a previous study with Indonesian children (40). This result indicated that dual-fortified lentils can provide more bioavailable Fe than single-fortified Fe- or Zn-fortified lentils. Some ingredients may help 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 with the sole use of these fortificants. Inclusion of EDTA with Zn salt increased Zn bioaccessibility by 3-fold from fortified millet flour (43). Since in vitro bioaccessibility was not measured in this study, it will be used to estimate Zn absorption in future studies. PA concentration was significantly higher in control lentils than in the fortified lentil samples of the 3 LPTs. This could be due to the dephytinization that can activate phytases, and reduce the content of phytate in fortified lentils. (44). The PA-to-Fe molar ratio was also reduced in fortified lentils compared with the control lentils. A previous study with Fe-fortified lentils showed that PA concentration was reduced due to the fortification process. Another study also reported that, for Fe-fortified fonio porridge, dephytinization and fortification reduced the PA-to-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 min-

imum possible. In this study, the L, a∗, and b∗ values of 2 control samples of 3 LPTs were significantly different, indicating that polishing has a significant effect on colorimetric changes. Again, the 3 dual-fortified samples, even with the highest dose (24 mg Fe and 12 mg Zn/100 g lentils) of Fe and Zn, showed significantly lower L values 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 with the Zn fortificant. The previous study with Fe-fortified lentils 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 the presence of humidity, temperature fluctuation, and light. Lentils have a small amount of lipid (1.52–2.95%) (48) and lipid oxidation may influence the colorimetric changes in fortified samples. More discussion on colorimetric results are shown in Supplemental Text 1.

Lentils are primarily consumed in dehulled form and have the potential to be used as a vehicle for micronutrient fortification. Globally, lentils are consumed in 120 countries and consumption rates vary from region to region. For example, in Bangladesh, lentils are considered a staple to partially staple food. The FAO-recommended consumption rate of lentils in Bangladesh is 50 g/person per day, but the actual consumption rate is only 12 g/person per day (15, 49). Results from this dual-fortification study show that lentils can be used as a potential vehicle for dual fortification, and NaFeEDTA and $ZnSO_4.H_2O$ were found to be 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 Zn and 24 mg Fe/100 g can provide ∼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 per day) and Zn (40 mg/person per day) for adults (50, 51).

In 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 with unfortified lentils. 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 lentils recently, and results were published in a manuscript (12) that showed that the consumers widely accepted dual-fortified lentils. The stability of added micronutrients and changes in 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 subtropical regions. Additional research to choose a suitable packaging system for dual-fortified lentils 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 lentils are frequently consumed as a staple or partially staple food.

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