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Investigating the effects of biofortified wheat flour on plasma zinc, selenium, and enterobacteria in pediatric populations

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ABSTRACT

INTRODUCTION. Zinc and Selenium deficiency affects 39% of children in Pakistan, according to the recent National Nutritional Survey 2011. Considering the deficiency states of zinc in our population, interventional strategies have been employed, such as fortification of cereals, food products, and zinc preparations in suspension forms. Zinc biofortification is a better option as wheat flour is the most common and easily assessed food in resource-poor settings compared to the other fortification methods. Although zinc has been used to prevent and treat diarrhoea, the relationship of plasma zinc status with potentially pathogenic bacteria has not been studied.

OBJECTIVE. The primary objective of this study was to assess the impact of zinc biofortified wheat flour on plasma zinc and selenium status in resource-poor rural settings of Peshawar where wheat is a major food staple. Furthermore, the secondary objective was to observe the relationship of change in zinc and selenium concentration (with biofortified wheat flour) with absolute quantities of potentially pathogenic gut bacteria such as *Campylobacter jejuni*.

STUDY DESIGN AND SETTING. Children aged 5-10 years (n=10) were randomly allocated into a control group (n=5) or an intervention group (n=5). The study was a pilot randomized control parallel trial in a rural area of Peshawar.

METHODS. Dietary zinc intake was assessed using 24 hours' dietary recall; plasma zinc and selenium status were analyzed through Inductively Coupled Plasma Mass Spectrometry ICP-MS, and Enterobacteria (*Campylobacter jejuni*) was amplified with TaqMan probes and primers through 7500 Real-Time qPCR.

RESULTS. No difference in the plasma zinc and selenium levels was observed in pre- and post-intervention (918.0 µg/L and 90.7 µg/L VS 880.9 µg/L and 78.5 µg/L). Similarly, no significant difference in the change in plasma zinc (ΔZn = 37.1 µg/L) and selenium (ΔSe = 12.2 µg/L) was observed between the two groups. 16S RNA gene qPCR amplification of *Campylobacter jejuni* was negative in all the samples from both the groups before and after the intervention.

CONCLUSIONS. Biofortified flour consumption did not affect plasma zinc and selenium status, although this may be due to a small sample size. Large community-based studies are needed to determine the effectiveness of this intervention.

KEYWORDS

ZINC DEFICIENCY
GUT MICROBIOME
ENTEROBACTERIA
CAMPYLOBACTER JEJUNI
BIOFORTIFICATION

INTRODUCTION

Micronutrients are essential substances required by the body in small quantities, playing pivotal roles in metabolism and tissue functions. Among these, zinc stands out as a critical micronutrient, integral to various bodily functions, including maintenance of barrier function under malnutrition conditions, management of alcoholic liver disease, chronic inflammatory bowel diseases like Crohn's disease, and promotion of growth. The Recommended Dietary Allowance (RDA) for zinc is 11 mg/day for men and 8 mg/day for women[1]. Insufficient zinc intake can lead to a range of deficiency symptoms affecting gastrointestinal, skeletal, immune, reproductive, and central nervous systems, manifesting as disorders such as diarrhea, pneumonia, and acrodermatitis enteropathica. The recognition of zinc deficiency dates back to 1961, characterized by symptoms like growth retardation, hypogonadism, skin abnormalities, and mental lethargy[2].

Various factors contribute to zinc deficiency, including inadequate dietary intake, malabsorption, increased losses, and impaired utilization. Notably, regions with a high cereal-based diet are more prone to zinc deficiency compared to those with a diet rich in animal products, as red meat is a potent source of bioavailable zinc[3]. Studies in Pakistan have highlighted a significant prevalence of zinc deficiency, particularly among children, with provincial variations[4].

Interventional strategies, such as the fortification of cereals and food products with zinc preparations, aim to address this deficiency. Despite inhibitors like phytate in cereals, fortification remains a viable option, given the widespread consumption of cereals like wheat in resource-poor settings. Dietary modifications can enhance zinc bioavailability, including genetically modified plants with reduced phytate content and techniques like sourdough fermentation[5].

Considering the intricate relationship between zinc and gut microbiota, particularly in the context of diarrheal diseases, understanding the impact of zinc supplementation on gut microbial diversity, specifically Enterobacteria like *Campylobacter jejuni*, is crucial. While studies on animal models suggest significant alterations in gut microbiota composition with zinc deficiency, limited research exists on humans, particularly children[6]. Hence, our ongoing population-based study in Peshawar aims to elucidate the effects of zinc-supplemented flour on Enterobacteria and plasma zinc status, emphasizing the need for further exploration in this area.

SUBJECTS AND METHODS

Study Design: The study was a single-blind, randomized control parallel group trial to observe the impact of biofortified wheat flour (wheat was conventionally grown in soil that was artificially enriched with zinc concentration. The zinc content of this variety (ZN-col/NR-421) is 35 ppm, which is higher than the commercially grown wheat, which is 25 ppm elemental zinc) on plasma zinc, selenium, and Enterobacteria in children in rural areas of Peshawar.

Subjects: Ten children (aged 5-10 years) were recruited from 10 families who consumed zinc-biofortified wheat flour in the Baghbanan area of rural Peshawar. Children who were apparently healthy, not having any chronic or acute malabsorption, inflammatory bowel disease, lactose intolerance, and celiac disease, were not on the regular use of zinc supplements, and were from a specific community of rural Peshawar were included in the study.

Trial Protocol: The research work was approved by the Ethical Review Committee of the Khyber Medical University Peshawar (DIR/KMU-EB/1B/000360) and followed the Helsinki Declaration of Good Clinical Practice. With the help of the Abaseen Foundation, 10 children were recruited from families receiving zinc-biofortified wheat flour in the study area. An information sheet (Annexure I) was given to the parents of each participant; moreover, the purpose and components of the study were explained verbally at the study site. Once agreed, the parents of the children were asked to give written informed consent (Annexure II) on behalf of their children. The consent form was filled out by the parents, and it was indicated by signature or thumbprint. Participants were then assigned with an anonymous study number. At the Pre-intervention stage, control wheat flour was provided to all the families for a period of two weeks (washout period). They were asked to keep a compliance diary in case they missed eating the flour during this period. Blood and fecal samples, along with dietary and anthropometric data, were collected from each child. At the end of the washout period, each child was randomly assigned to the intervention (consuming Zncol-NR/421 wheat flour) or control group (consuming control flour) for a period of 4 weeks. Blood and fecal samples, along with dietary and anthropometric data, were collected from each child at the end of 4 weeks. Questions regarding socioeconomic status, family size, number of siblings, and personal hygiene were also asked through questionnaires. The schema of the trial is given below (Figure 1).

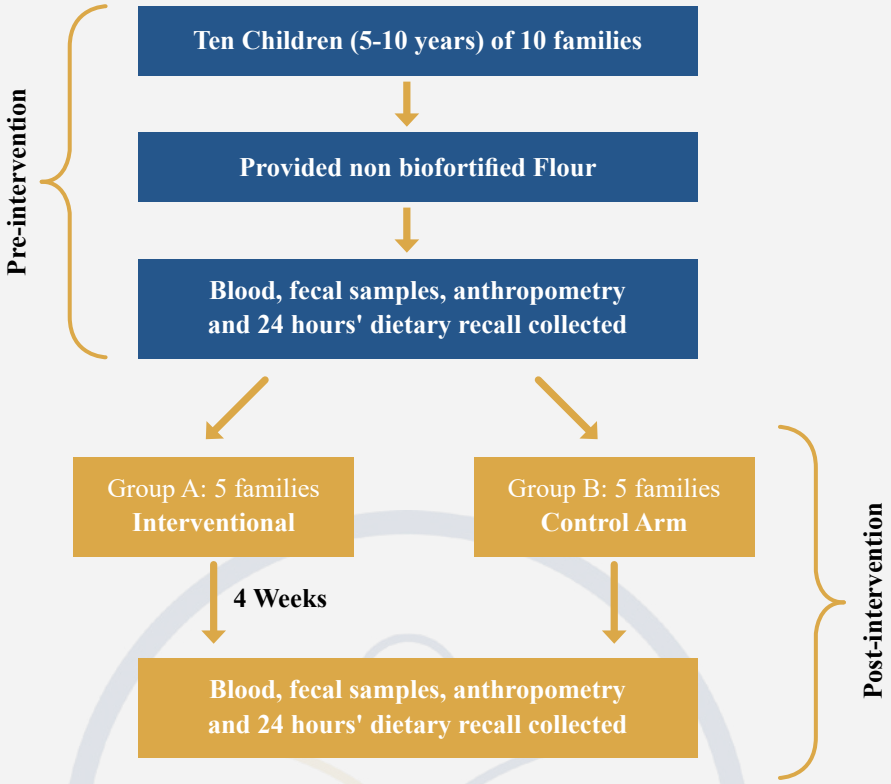

Figure 1. Trial's schema.

Data Collection

During bio-fortified wheat flour distribution, every child was assessed, and data were collected using a data collection sheet (Annexure III). This included data regarding children, parental care, Public Health, and Anthropometric data. This data was then Statistically processed using Minitab(R) Version 17.

Anthropometric data

Height: Height of young children was measured using a height stadiometer. The participant was asked to stand still with his/her back towards the scale. They were asked to touch their heels, the back of the calves, the upper back, and the back of their head with the board of the scale. The head was positioned so that the Frankfurt line was straight (the arbitrary line between the tragus of the ear and the outer canthus of the eye on the same side). The measuring device was lowered gently on the hair until it rested gently on the scalp without putting pressure on the scalp.

Weight: The child was asked to relax for 5 minutes before being asked to remove added clothes, shoes, socks, anything in the pocket, watch or necklace, etc. The child was asked to stand on the marked position on the scale until the digital reading or the reading needle stabilized. The child was asked to look straight and stand still.

Mid-upper arm circumference (MUAC): The participant was asked to roll his/her sleeves upwards above the mid-arm. The participant was then asked to sit with a straight back or stand still. The mid-point between the tip of the elbow and the shoulder was considered for the placement of the tape, and the reading was taken to the nearest cm.

Head Circumference (HC)

With the child on the couch or in the lap of the mother, any head-wearing was removed from the child's head. The tape was placed on the head and forehead along the maximum diameter of the head.

Waist Circumference (WC)

The participant was asked to remove any clothing from his/her waist. With the participant standing, a point midway between the highest point of the iliac crest and the lowermost rib on both sides was considered for positioning the measuring tape around the waist. The circumference of the waist was then measured to the nearest cm. The above procedure was followed to measure hip circumference.

Waist-Hip Ratio (WHR): WHR was calculated using the following formula:

$$WHR=(Waist\ (cm))/(Hip\ (cm))$$

24-Hours Dietary Recall: The dietary intake of the children was assessed using 24-hours dietary recall (Annexure IV) and then analyzed through Windiet 2005 version software. Data on caloric intake, fats, proteins, carbohydrates, zinc, and selenium consumption were estimated using the given portion sizes according to Pakistan's food composition table. Foods that were not present in the software, such as specific brands of foods, were added from the nutritional information available on the respective websites. Foods such as local snacks, traditional curry, traditional sweets, and desserts were added manually to the software.

Sample Collection

The samples, such as stool and blood, were collected during the whole procedure two times, first at the end of two weeks after providing wheat flour for the washout period and second at the end of four weeks after the children were randomly assigned to the control and intervention groups.

Stool Sample: A stool collection kit was provided to each child for the collection of stools at baseline and at the end of four weeks of intervention. Once collected, each stool sample was transported to IBMS in cool bags within 3 hours after collection. Once in the lab, each sample was thoroughly homogenized with the help of a sterile wooden spatula. Approximately 200 mg of homogenized stool sample was added to 2 mL screw top tubes in triplicates and immediately stored at -80 °C until further analysis.

Blood Sample: Blood sample was collected using zinc-free needles, syringes, and vacutainers. Further handling of these samples was ensured to prevent zinc contamination using zinc-free centrifuge tubes, storage vials, and transfer pipettes. After that, they were transported to IBMS lab and centrifuged for 10 minutes at 2500 rpm. Supernatant plasma was carefully pipetted out and transferred to a screw top tube. Furthermore, tubes were stored at -20 °C for further zinc and selenium analysis.

Sample Analysis

Zinc and selenium analysis in blood plasma

Blood plasma samples stored at -20 °C were defrosted at room temperature. For the analysis of plasma zinc and selenium through ICP-MS, these samples were then transferred into fresh prelabelled 2 mL screw-top tubes and stored in an alcohol-sprayed plastic box in dry ice for transportation to the University of Nottingham, where it was analyzed through Inductively Coupled Plasma Mass Spectrometry ICP-MS.

Quantification of Enterobacteria (Campylobacter jejuni) in stool samples

DNA extraction: Total DNA was extracted from the fecal samples using Favor Prep™ stool DNA isolation mini kit (cat. No FASTI 001, 50 Preps, Favorgen® Biotech Corp, Taiwan). An aliquot of the standardized 200 mg stool sample was collected in a 2 ml bead tube and was thawed on the ice. After the addition of SDE1 buffer and proteinase k, the sample mixture was vortexed at maximum speed and incubated at 60 °C for 20 minutes. All other extraction steps were followed using the manufacturer's guidelines. For centrifugation steps, Eppendorf mini-spin centrifuge was used. The final elution volume of DNA was 200 µl. Extracted DNA samples were stored at –20 °C until qPCR analysis.

qPCR Quantification of *Campylobacter jejuni*: Amplification of *Campylobacter jejuni* was performed on an ABI Prism 7500 sequence detector. The forward primer, reverse primer, and TaqMan probe (Table 1) were used to quantify a representative member of Enterobacteria, such as *C. jejuni*. The following sequence has been chosen from the previous study of Sails et al (2003)[7].

Preparation of qPCR Plate: Real-Time qPCR machine was switched on for 30 minutes before the run. The biological cabinet and pipettes were sprayed and cleaned with 70% ethanol and then sterilized by UV light for 15 minutes before the start of work. qPCR plate was pipetted with the following (Table 2) volumes. The adhesive

Table 1. Primers sequence for targeting 16s rRNA of *C. jejuni*.

ID	5'……3' Sequence	BP	GC%	MT
CJTP_2F (Forward Primer)	TTGGTATGGCTATAGGAACTCTTATAGCT	29	37.9	66.2
CJTP_2R (Reverse Primer)	CACACCTGAAGTATGAAGTGGTCTAAGT	28	42.8	67.2
CJTP_Probe (TaqMan Probe)	TGGCATATCCTAATTTAAATTATTTACCAGGAC	33	30.3	66.5

BP: Base Pair length; GC%: Percent of guanine and cytosine content in DNA; MT: Melting Temperature

Table 2. Volumes of different components of qPCR Plate volume.

S. No.	Reaction chemicals	Vol. for 1 reaction
1	Master Mix	5.0 µl
2	NF H2O	9.5 µl
3	Probe	2.5 µl
4	Forward primer	2.5 µl
5	Reverse primer	2.5 µl
6	DNA template	3.0 µl
	Total Volume	**25 µl**

NF: Nuclease-Free

film was applied firmly to the plate, and each corner of the plate was vortexed gently to make sure the contents were mixed and had no bubbles. The top of the plate was cleaned with a lint-free tissue.
Amplification was carried out under the following cycle conditions on ABI 7500 series; 50 °C for 2 min, 95°C for 10 min, and 45 cycles of 95 °C for 15 secs and 60 °C for 1 min. Sequence detection system software was used for the analysis of data.

Statistical Analysis
Observational analysis: The data collected was first organized in Microsoft Excel® (Microsoft office® 2016) and checked for any personal or random errors.
Statistical analysis: Data was then analyzed using Minitab® Version 17. Anderson Darling test of normality showed that the plasma zinc data were normally distributed ($p = 0.267$). Hence, parametric analysis was applied. Data were expressed as mean and standard deviation. A comparison between control and intervention groups was made using a two-sample t-test. The association of plasma zinc status with age, BMI, nutritional intake, socioeconomic status, and Enterobacteria could not be calculated through multiple regression analysis due to the low sample size.

RESULTS

Subjects and anthropometric characteristics
All children (n=10, 6.900 ± 1.483) completed the trial. Of these, 3(30%) were females (mean age; 6.66), while 7(70%) were males (mean age; 7.00). Parents of the children reported 20% of births in hospital. Parents were mostly uneducated [9(90%)] and were from the lower income group (mean income; 15,000 PKR). Most of the participants were shorter (Ht-SDS -2.36 ± 2.38) of good weight range (SDS-weight; 1.65 ± 1.72) and had normal mid-upper arm circumference (MUAC; 15.40 ± 1.31 cm). The anthropometric characteristics of participants are further outlined in Table 3.

Table 3. Anthropometric characteristics of study participants (n=10).

Variable	Mean ± SD	Range
Age (years)	6.900 ± 1.48	5.00
No of people	7.22 ± 5.86	19.00
Height (cm)	108.20 ± 18.94	66.00
SDS_Height	-2.361 ± 2.38	8.12
Weight (kg)	19.50 ± 5.59	19.00
SDS_Weight	-1.650 ± 1.72	6.17
BMI	16.194 ± 2.02	6.24
SDS-BMI	0.250 ± 1.52	4.29
Mid Upper Arm Circumference (cm)	15.400 ± 1.31	4.00
Head Circumference (cm)	49.300 ± 2.34	7.00
Waist Circumference (cm)	55.300 ± 3.59	10.00
Waist to Hip ratio	0.95700 ± 0.01	0.06

SDS: Standard Deviation Scores, BMI: Body Mass Index.

Table 4. 24-hour dietary recall intake data and comparison between two groups (control and intervention arm).

	Control (n=5) Mean	SD	Intervention (n=5) Mean	SD	*p-value*
Pre-intervention					
Energy (KJ)	7650	2451	7007	2611	0.700
Fats (g)	75.50	30.40	57.60	33.80	0.410
Proteins (g)	47.50	20.90	49.70	24.10	0.881
Carbohydrates (g)	253.60	78.10	251.50	59.70	0.050
Zinc (mg)	6.68	4.16	6.50	4.99	0.952
Selenium (µg)	9.20	4.92	9.80	5.17	0.856
Post-intervention					
Energy (KJ)	4585	487	5623	1597	0.237
Fats (g)	38.60	12.30	50.90	17.4	0.238
Proteins (g)	29.30	2.86	38.40	9.37	0.106
Carbohydrates (g)	166.50	17.50	192.60	54.80	0.368
Zinc (mg)	3.58	0.75	4.28	0.94	0.235
Selenium (µg)	7.80	6.68	5.80	4.02	0.587

SD=Standard Deviation

24 hr-dietary recall

The data reported was normally distributed; hence, parametric analysis and two-sample t-test were applied to compare the two groups. There was no significant difference in the total energy intake in KJ, fats (g), proteins (g), carbohydrates (g), zinc (mg), and selenium (µg) intake between pre- and post-intervention periods in both control and intervention groups. Table 4 estimates the variable intake comparison between the two groups, such as control and intervention, with respect to their randomization.

Plasma zinc and selenium

Statistical comparison for the difference between plasma zinc and selenium between control and intervention group by two-sample t-test could not be calculated because the amount of plasma volume obtained from some of the children or poor quality of plasma extracted from the obtained samples reduced the number of samples for comparison to less than 5 in the control group. Data is therefore shown only as mean values of the analysed samples.

Change in plasma zinc (ΔZn) and selenium (ΔSe) was calculated as the difference pre- and post-intervention plasma zinc and selenium. In case of zinc, there was a non-significant decrease in zinc level post intervention (35.7 µg/dL in the control group and 40.5 µg/dL in the intervention group) (Table 5).

A similar trend was observed in the change in plasma selenium (14.3 µg/dL in the control group and 11.0 µg/dL in the intervention group) (Table 6).

Enterobacteria in children

Out of the twenty, none of the genomic bacterial DNA samples (extracted from faeces of participants) reading was seen over the threshold designated Ct value[55]. None of the samples, therefore, yielded any amplicon of *C jejuni*. Therefore, all the faecal samples were observed to be *C. jejuni* negative.

Table 5. Plasma zinc status of the participants.

	Mean Control (*n=10*)	Mean Intervention (*n=10*)	Total Mean
Plasma Zn pre-intervention (µg/L)	925.4	914.3	918.0
Plasma Zn post-intervention (µg/L)	889.7	873.8	880.9
Total difference (ΔZn)	35.7	40.5	37.1

Table 6. Plasma selenium status of the participants.

	Mean Control (*n=10*)	Mean Intervention (*n=10*)	Total Mean
Plasma Se pre-intervention (µg/L)	89.5	92.3	90.7
Plasma Se post-intervention (µg/L)	75.2	81.3	78.5
Total Difference (ΔSe)	14.3	11.0	12.2

DISCUSSION

Zinc is among the essential micronutrients for the metabolism of the human body. A deficiency of zinc may lead to different types of serious complications in human health[3]. About 40% of children in Pakistan are zinc deficient[4]; therefore, it is of serious concern to raise their zinc status with simple, affordable, and socially accepted strategies such as biofortified wheat (35 ppm Zn). Although zinc elemental interaction with the body gut microbiome is extensively studied with diarrhea[8], little is known about the relationship of change in zinc concentration (with biofortified wheat flour) with absolute quantities of potentially pathogenic gut bacteria such as *Campylobacter jejuni*.

Agronomic biofortification is considered to be the most feasible, cost-effective and sustainable approach to eradicate the "hidden hunger"[9]. In humans, other interventional strategies, such as sprinkling and supplements, are broadly utilized as a part of clinical trials for micronutrient deficiencies[10]. As our study trial was in the rural area of Peshawar henceforth, agronomic biofortification was the most ideal, easily assessable, and cost-effective approach[11].

Agronomic biofortification can expand the yield of the dietary nature of Zn and Se in staple products, such as wheat or maize[12]. However, there is a lack of further evidence suggesting the increase of plasma zinc micronutrient levels by interventional strategies such as wheat biofortification[13]. Moreover, the relationship between the zinc micronutrient levels by agronomic biofortification in order to improve the human health is very rare[14]. A study conducted in Finland, suggests 15 folds of the average increase in plasma Se levels, nationwide with the addition of Se in cereal crops[10]. This intervention served as a basis for our pilot study trial, as granular zinc may arise from 25 ppm (conventional wheat) up to 35 ppm (biofortified wheat)[15].

Herein, in this pilot randomized control trial, we assess the impact of zinc biofortified wheat flour on plasma zinc status, but the results failed to support our hypothesis. No difference in the plasma zinc and selenium levels is observed in pre- and post-intervention stages (918.0 µg/L and 90.7 µg/L vs. 880.9 µg/L and 78.5 µg/L). However, a study conducted in Zambia showed conflicted results with our study [16]. In that study (n=29) 340 µg Zn in staple food of maize biofortification, plasma zinc levels increased by 0.4% in the rural settings [16]. Unexpected findings may be due to the low sample size in our study, the short duration of intervention, and a limited supply of biofortified wheat. In addition, we also faced issues with collecting enough blood from children to separate enough plasma for trace element analysis using ICP-MS.

We observed a slight decrease in the mean plasma zinc and selenium with intervention, although this was not significantly different between the two groups. This might be due to the different tastes of the intervention flour. This could also be due to the lower consumption of the "new" flour introduced into the child's diet, as some participants reported sand in the chapatti made out of the intervention flour, and this may not have been acceptable to the child. However, the decrease in zinc levels in both groups indicates that there could be other reasons, such as taking food from outside the home, mothers not feeding the new flour to the children or disease during the intervention period. However, no such evidence was reported.

In this study, zinc intake measured using 24 hr. dietary recall in control versus intervention (3.58 mg/d vs. 4.28 mg/d) was higher; however, no significant difference was detected in post-intervention period (p-value=0.235). This was expected as we included healthy children in our study, and their intake was likely to remain the same. It also shows that the diet and the diversity of the diet in this resource-poor population are pretty monotonous and less diverse and do not change over long periods. Therefore, this may be a major contributing factor to the higher prevalence of malnutrition and micronutrient deficiencies in this area. This is supported by previous studies in the same area. Twenty-four hours of dietary recall is a good way to assess the dietary intake of children and adults alike. However, recording accurate information from mothers regarding the dietary intake of the child is sometimes unreliable given the age range of children (5-10 years) in our study who are not only school-going but also independent (playing outside the home most of the

time). Moreover, it also needs trained personnel; there is a possibility of portion recall bias and memory recall from mothers who may not be able to remember the diet of their child, especially foods taken from outside[17]. It is well known that eating a routine zinc-rich dietary pattern is fundamental to the rise of plasma zinc status compared to supplements[13].

Quantitative PCR using TaqMan primers and probes did not detect *C. jejuni* in the stool samples, which were collected between December 2016 and January 2017. These findings are in line with previous studies undertaken in the developing countries, in which *C. jejuni* species of Campylobacter were negative in spite of a larger sample size[18]. This is because the relative abundance of *C. jejuni* is below 5% in the normal gut and may not be detectable in most of the healthy guts with the help of qPCR. A recent study conducted in China found that around 4.38% of 3,061 diarrhoeal patients are *C. jejuni* positive[19]. In addition, 60-70% of diarrhoea is due to viruses and the rest is due to bacteria, in the most common cause is *C. jejuni*. Differences in habitual dietary intake may also be one of the contributing factors to the variations in the quantities of this group of bacteria in the gut when compared to the Western diet[20]. The studies have documented that about 10% of the world's acute diarrhoeal patients are generally Campylobacter positive[21,22]. Hence, we may not have been able to capture Campylobacter because all our participants were healthy and had no diarrhoea or diarrheal complications. qPCR amplifies only primer-specific bacteria, and the product is limited by the DNA quality, presence of inhibitors in the sample, and technique for DNA extraction. Amplifying genomic bacterial DNA using a more robust technique using next-generation sequencing is therefore suggested to allow for the sequencing of all the members of the Enterobacteria, including *C. jejuni*. The study, therefore, suggests further large community-based studies for this intervention's effectiveness, including participants with diarrheal cases.

CONCLUSIONS

Consumption of biofortified wheat flour did not affect plasma zinc and selenium status, although this may be because of the small sample size in this study. It is recommended that further larger community-based trials be conducted to establish the impact of biofortified zinc flour on plasma zinc and gut microbiome (through state-of-the-art technologies such as next-generation sequencing).

Conflict of interest

The author declares no conflict of interest.

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