The potential benefits and challenges of micronutrient interventions for public health nutrition

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Abstract

The purpose of this dissertation is to evaluate the landscape of currently-recommended public health interventions to address micronutrient deficiencies and associated conditions, and to describe the effectiveness of those interventions to successfully reduce or eliminate micronutrient deficiencies. Further, this dissertation aims to understand the effect of one specific micronutrient intervention, large-scale food fortification, on the microbiome of women of reproductive age and their children. The dissertation is divided into four chapters with the corresponding objectives:

1. The first chapter is a literature review of current public health interventions with micronutrients, and a summary of their estimated impacts, and challenges associated with each intervention strategy.
2. The second chapter is a secondary analysis of a larger clinical trial, the Zinc in Powders trial (ZiPT), which aims to evaluate the risk factors for anemia among Bangladeshi children who received micronutrient powders (MNPs) for 24 weeks as a part of the clinical trial.
3. The third chapter aims to evaluate the effect of LSFF with quintuply-fortified salt (QFS) on microbiome outcomes (diversity and relative abundance of taxa) among a subgroup of women of reproductive age in Punjab, India who participated in the Multiply-Fortified Salt (MFS) trial.
4. The fourth chapter aims to evaluate the effect of LSFF with QFS on microbiome outcomes (diversity and relative abundance of taxa) among a subgroup of children in Punjab, India who participated in the Multiply-Fortified Salt (MFS) trial.

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Literature Review: The Benefits and Challenges of Public Health Micronutrient Interventions for Improving Population-Level Intake

1.1 Abstract

Micronutrient deficiencies remain one of the most widespread forms of malnutrition globally, posing significant public health challenges. Several evidence-based intervention strategies have been developed to address and prevent micronutrient deficiencies in vulnerable subpopulations, as well as the general population. These strategies include supplementation, fortification of staple foods and condiments, diet diversification, and biofortification. This review summarizes evidence on the effectiveness and expected outcomes of the various micronutrient interventions with a specific emphasis on supplementation, fortification and diet diversity interventions, and considers the benefits and challenges of each strategy to effectively and sustainably address micronutrient deficiencies. The review concludes with a discussion of future directions for optimizing micronutrient strategies to achieve sustainable improvements in global nutrition and contribute to broader development goals.

1.2 Introduction

Micronutrient deficiencies are among the most prevalent forms of malnutrition globally. In 2021, it was estimated that over half of children under five years of age worldwide are deficient in either vitamin A, iron, and/or zinc. In addition, two-thirds of women of reproductive age (15-49 years) are deficient in folate, iron, and/or zinc (1). Deficiencies in these essential nutrients contribute to significant morbidity and mortality, resulting in economic consequences in a low- or middle-income country.

A significant risk factor for micronutrient deficiencies includes inadequate intake of micronutrients from the diet. It is estimated that nearly 4.9 billion people (65% of the global population) have inadequate intakes of iron, 3.5 billion people (46% of the population) have inadequate intakes of zinc, and 3.6 billion people (48% of the population) have inadequate intakes of vitamin A from food alone (2). Several evidence-based, complementary intervention strategies have been developed to increase micronutrient intake and prevent deficiencies in the general population, or among populations with increased requirements such as young children and women of reproductive age. These interventions include supplementation, fortification of staple foods and condiments, diet diversification, and biofortification.

Although each of these strategies have been effective in improving population-level micronutrient status in systematic reviews, they each have trade-offs in a given context, including cost, feasibility, and potential adverse effects that can limit the benefits and success of

the intervention. In this paper, we will summarize various micronutrient interventions with a specific focus on supplementation, fortification and dietary diversity interventions, discuss the expected outcomes of each intervention, consider reported challenges of specific interventions, and discuss future directions for addressing micronutrient malnutrition on a global scale.

1.3 Micronutrient Intervention Strategies

1.4 Supplementation

Supplementation is an intervention strategy to increase micronutrient intake in nutritionally vulnerable subgroups (3). Supplementation interventions aim to meet daily micronutrient needs to address and prevent micronutrient deficiencies. Supplementation can take many forms, including the provision of single micronutrients or two or more micronutrients as a capsule, tablet, or in drops or syrups. Multiple micronutrient supplements are defined as a single administration of more than three or more micronutrients. Additional products that fall within the category of supplementation include lipid-based nutrient supplements (LNS), which are micronutrients embedded in a food matrix that also provides energy, essential fatty acids, protein (4). Finally, point-of-use micronutrient powders (MNPs) are single-serving sachets of encapsulated micronutrients that are used to enhance the nutritional quality of complementary foods of young children (5).

Vitamin A supplementation for children 6 months to 5 years of age

Vitamin A deficiency is a major public health problem among young children and is associated with a wide range of preventable health and developmental consequences including respiratory diseases, diarrhea, measles, vision problems including blindness, and mortality. To prevent vitamin A deficiency, vitamin A supplementation programs provide vitamin A in the form of a capsule every four to six months. These programs are often integrated into routine immunization services and vaccination campaigns (6).

Supplementation with vitamin A among children 6 months to 5 years of age has been associated with a 12% reduction in all-cause mortality (RR 0.88, 95% CI 0.83 to 0.93, 9 studies, high-certainty evidence) and a reduction in vitamin A deficiency of nearly 30% (RR 0.71, 95% CI 0.65 to 0.78, 4 studies, moderate-quality evidence) (7) compared to no intervention. Vitamin A supplementation (8) is also associated with a reduction in measles incidence (RR 0.50, 95% CI 0.37 to 0.67, 6 studies, moderate-certainty evidence), diarrhea incidence (RR 0.85, 95% CI 0.82 to 0.87, 15 studies, low-certainty evidence), mortality due to diarrhea (RR 0.88, 95% CI 0.79 to 0.98, 9 studies, high-certainty evidence), Bitot's spots incidence (RR 0.42, 95% CI 0.33 to 0.53, 5 studies, moderate-certainty evidence), and night blindness incidence (RR 0.32, 95% CI 0.21 to 0.50, 2 studies, moderate- certainty evidence) (7). It is noted that few studies have reported adverse effects of VAS, however, VAS was associated with an increased risk of vomiting (RR 1.97, 95% CI 1.44 to 2.69, 4 studies, moderate-certainty evidence).

The World Health Organization (WHO) recommends the provision of a one-time does of 100,000 IU of vitamin A among infants 6-11 months of age, and 200,000 IU of vitamin A among children 12-59 months every 4-6 months where the prevalence of night blindness is >1% or where the prevalence of vitamin A deficiency is 20% or higher among children 6-59 months of age (6).

Zinc supplementation for children 6 months to 12 years of age

Zinc deficiency remains an elusive public health challenge among children. Zinc deficiency can result in growth restriction and immune system dysfunction, leading to significant childhood morbidity and mortality (9). Because of zinc's role in promoting mucosal barrier integrity and immune function in the gastrointestinal tract, and the WHO recommends zinc supplementation in combination with ORS for treatment of diarrhea in young children (10) (11).

From a meta-analysis of 33 studies, therapeutic zinc supplementation may reduce the duration of diarrhea (MD -11.46 hours, -19.72 to -3.19, 9 studies, low-certainty evidence) and reduce the risk of diarrhea persisting until day 7 (RR 0.73, 95% CI 0.61 to 0.88, 6 studies, moderate-certainty evidence) among children older than 6 months. However, these effects were not observed among children younger than 6 months (10). No studies reported serious adverse events, however, zinc supplementation increased the risk of vomiting in children greater than 6 months (RR 1.57, 95% CI 1.32 to 1.86, 6 studies, moderate-certainty evidence) and children less than 6 months of age (RR 1.54, 95% CI 1.05 to 2.24, 2 trials, moderate-certainty evidence).

The evidence for the effectiveness of preventative zinc supplementation on growth, morbidity and mortality is less consistent. In a meta-analysis of over 90 studies including children 6 months to 12 years of age, zinc supplementation was associated with a 44% reduction (RR 0.56, 95% CI 0.52 to 0.60, 24 studies, moderate-certainty evidence) in zinc deficiency, and a small reduction in the incidence of all-cause diarrhea (RR 0.91, 95% CI 0.90 to 0.93, 39 studies, moderate-certainty evidence), but no difference in other morbidity or mortality. Zinc supplementation is also associated with a small increase in height (standardized mean difference (SMD) 0.12, 95% CI 0.09 to 0.14, 74 studies, moderate-certainty evidence). Zinc supplementation also results in a slight increase in vomiting within 48 hours (RR 1.29, 95% CI 1.14 to 1.46, 5 studies, high-certainty evidence) (9). Studies provided zinc supplements for 2 months to 12 months, and doses ranged from 5 mg to more than 20 mg. There are currently no WHO guidelines for preventative zinc supplementation as a stand-alone intervention among children; however, multi-micronutrient interventions including MNPs and SQ-LNS include zinc.

Iron supplementation for children 6 months to 12 years of age

Iron deficiency is prevalent among young children and is a major cause of anemia. Iron deficiency in childhood can also result in irreversible cognitive deficits. Iron supplementation among children is premised on the potential to reduce iron deficiency anemia and also improve

cognitive development (12). Several systematic reviews have been conducted among different age strata in children to inform recommendations for iron supplementation.

In a systematic review of randomized, controlled clinical trials, daily iron supplementation among young children 4-23 months was associated a reduced risk of anemia of nearly 40% (RR 0.61, 95% CI 0.50 to 0.74), a reduction in iron deficiency of 70% (RR 0.30 (95% CI 0.15 to 0.60) and a reduction in iron deficiency anemia of 86% (RR 0.14, (95% CI 0.10–0.22) (13). Iron supplementation was also associated with improved hematological parameters (hemoglobin (MD 7.22 g/L, 95% CI 4.87 to 9.57) and mean cell volume (MD 2.81 fL, 1.20 to 4.42) and iron indices (ferritin (MD 21.42 ng/mL, 17.25 to 25.58), transferrin saturation (MD 6.00 %, 95% CI 2.65 to 9.35) and transferrin receptor (SMD -0.99, -1.60 to -0.39). Interestingly, daily iron supplementation resulted in a reduced length gain (SMD -0.83, 95% CI -1.53 to -0.12) and weight gain (SMD -1.12, 95% CI -1.91 to -0.33), however, there was no effect on final length, length-for-age *Z* score, final weight, weight-for-age *Z* score, or weight-for-length *Z* score. Daily iron supplementation was also associated with vomiting (RR 1.38, 95% CI 1.10 to 1.73) and prevalence of fever (RR 1.08, 0.79 to 1.47). Among the 17 studies contributing data for meta-analysis of effects on anemia, 13 provided <12.5 mg of iron. The duration of supplementation ranged from two weeks to 14 months (13).

A systematic review of 15 studies of daily iron supplementation among children 2-5 years of age found an improvement in hemoglobin (MD 6.97 g/dL, 95% CI 4.21 to 9.72), ferritin (MD 11.64 µg/L, 95% CI 6.02 to 17.25). Only one study contributed data on anemia; this study found no

effect on anemia. There was also no effect on transferrin saturation, hematocrit, or mean cell volume. There was a small effect on cognition (MD 0.25, 95% CI 0.06 to 0.45), and no effects on height or weight (14). The dose or iron where reported ranged from 10-65 mg/day, with intervention duration of 28 days to 12 months. Another systematic review of 32 studies of daily iron supplementation among children 5 to 12 years of age identified a reduction in anemia of 50% (RR 0.50, 95% CI 0.39 to 0.64, 7 studies), a reduction in iron deficiency of 79% (RR 0.21, 0.07 to 0.63, 4 studies), improved ferritin concentrations (MD 28.45 g/L, 95% CI 18.03 to 38.86, 14 studies) and improved hemoglobin concentrations (MD 8.38 g/L, 6.21 to 10.56, 28 studies) (15). There was also a small effect on global cognitive performance (SMD 0.50, 0.11 to 0.90, 9 studies) and height-for-age Z-score (MD 0.09, 95% CI -0.01 to 0.17, 6 studies). There were no reported effects on safety parameters (malaria prevalence, rate of positive malaria test), gastrointestinal upset, constipation or vomiting (15). Most studies provided iron as ferrous sulfate and the doses ranged from 5 to 120 mg/day, for a duration of 1-12 months.

A systematic review of 33 trials of intermittent iron supplementation found that children under 12 years of age had a 49% lower risk of anemia (RR 0.51, 95% CI 0.37 to 0.72, 10 studies, moderate-quality evidence), a 76% lower risk of iron deficiency (RR 0.24, 95% CI 0.06 to 0.91, 3 studies, very-low quality evidence) and higher hemoglobin (MD 5.20 g/L, 95% CI 2.51 to 7.88, 19 studies, low-quality evidence) and ferritin concentrations (MD 14.17 µg/L, 95% CI 3.53 to 24.81, 5 studies, low-quality evidence) (16). In comparison to daily iron supplementation, children receiving intermittent iron supplementation were more likely to develop anemia (RR 1.23, 95% CI, 1.04 to 1.47, 6 studies, low-quality evidence) but do difference in hemoglobin and

ferritin concentrations. This review did not report adverse effects. Lastly, an additional systematic review found that iron supplementation was not associated with an increased risk of clinical malaria (RR 0.93, 95% CI 0.87 to 0.97, 14 studies, high-quality evidence) among children living in malaria-endemic areas if malaria control measures are in place (17).

Based on the evidence presented above, the World Health Organization currently recommends the daily provision of 10-12.5 mg of elemental iron to children 6-23 months of age, 30 mg of elemental iron to children 24-59 months, and 30-60 mg to children 5-12 years of age for three consecutive months in a year in settings where the prevalence of anemia in infants and children is 40% or higher. In addition, the WHO recommends that oral iron supplements should only be given to infants and children in malaria-endemic areas with access to preventative care and treatment for malaria.

Multiple micronutrient powders (MNPs) for children 6-23 months and 2-12 years of age

Given that micronutrient deficiencies rarely occur in isolation, multiple micronutrient powders (MNPs) were developed to increase vitamin and mineral intake among young children, and school-age children (18). MNPs are sachets that contain up to 22 micronutrients in powder form and can be mixed directly into a semi-solid complimentary food for children 6-23 months of age, or energy-containing foods consumed at home for children 2-12 years of age(19, 20).

In a systematic review of 26 studies, MNP provision reduced the risk of anemia by 18% (RR 0.82, 95% CI 0.76 to 0.90, 16 studies, moderate-certainty evidence) and iron deficiency by 53% (RR 0.47, 95% CI 0.39 to 0.56, 7 studies, high-certainty evidence) compared to no intervention. There were also significant improvements in hemoglobin and iron parameters. There was no effect on weight-for-age Z-score or risk of diarrhea (19). In a systematic review among pre-school and school-age children, MNPs reduced the risk of anemia by 34% (RR 0.66, 95% CI 0.49 to 0.88, 10 studies, moderate-certainty evidence) and iron deficiency by 65% (RR 0.35, 95% CI 0.27 to 0.47, 5 studies, moderate-certainty evidence). There was no effect on all-cause mortality, diarrhea, or adverse effects (20). MNPs contained 2-18 vitamins and minerals, and iron doses ranged between 2.5 to 30 mg of elemental iron.

It should be noted that another systematic review and meta-analysis among children under 5 years, MNPs produced a similar effect on anemia reduction (RR 0.76, 95% CI 0.69 to 0.84) and risk of iron deficiency (RR 0.50, 95% CI 0.40 to 0.63), as well as hemoglobin and iron biomarkers, however, the authors found a significant increase in the risk of diarrhea (RR 1.30, 95% CI 1.11 to 1.53) with the inclusion of additional studies in their meta-analysis (18).

The WHO recommends the point-of-use fortification with iron-containing MNPs for children 6 months-23 months, 10-12.5 mg of elemental iron) to improve iron status and reduce anemia where the prevalence of anemia is greater than 20% among children under 2 years or under 5 years. Similarly, the WHO recommends the provision of MNPs among children 2-12 years (10-12.5 mg of elemental iron for children 2-4 years, and 12.5-30 mg elemental iron for children 5-

12 years) to improve iron status and reduce anemia in areas where anemia where the prevalence of anemia among children under 5 years of age is >20%) (strong recommendation, moderate-quality evidence). The program target for both recommendations is the provision of 90 sachets/doses over a 6-month period (21).

Lipid-based nutrient supplements

Lipid-based nutrient supplements (LNS) are food-based supplements that contain lipids as the main source of energy, as well as protein and micronutrients. These products were designed to fill nutrient gaps during the complementary feeding period in order to prevent malnutrition and poor growth among young children (4, 22). Although LNS products contain ingredients beyond just micronutrients, they are becoming an increasingly important strategy for delivering micronutrients to the diets of young children.

A systematic review identified that compared to no intervention, the provision of SQ-LNS or MQ-LNS plus complementary feeding among children 6-23 months reduced the prevalence of moderate stunting by 7% (RR 0.93, 95% CI 0.88 to 0.98, moderate-quality evidence), severe stunting by 15% (RR 0.85, 95% 0.74 to 0.91, moderate-quality evidence), moderate wasting by 18% (RR 0.82, 95% CI 0.74 to 0.91, moderate-quality evidence), moderate underweight by 15% (0.85, 95% CI 0.81 to 0.91, moderate-quality evidence) and anemia by 21% (0.79, 95% CI 0.54 to 1.13, low-quality evidence) (23). Adverse effects did not differ between groups. Compared to MNPs, LNS plus complementary feeding reduced the prevalence of underweight (RR 0.88, 95%

CI 0.78 to 0.99, moderate-quality evidence) and anemia (RR 0.38, 95% CI 0.21 to 0.68, low-quality evidence). However, this evidence should be interpreted with caution as only two studies contributed data to the meta-analysis.

An individual participant data meta-analysis expanded upon the Cochrane systematic review above, and included 15 randomized, controlled trials of SQ-LNS provision among children 6-23 months (24). Children who received SQ-LNS had a 12% reduction in stunting, 14% reduction in wasting, and 13% reduction in underweight. Children who received SQ-LNS experienced a 16% reduction in anemia, a 56% reduction in iron deficiency, 64% reduction in iron-deficiency anemia, a 56% reduction in vitamin A deficiency, and a 27% reduction in mortality. There were also improvements in cognitive development. Most trials began child supplementation with SQ-LNS at 6 months of age, and the trial duration ranged from 6 to 18 months.

Currently, in the WHO guideline for complementary feeding of infants and young children 6-23 months, the WHO recommends the provision of LNS in contexts where nutrient requirements cannot be met with unfortified foods alone, or in food insecure populations facing significant nutritional deficiencies (25). In the WHO guideline on the prevention and management of wasting and nutritional edema (acute malnutrition) (26), the WHO recommends the provision of MQ-LNS or SQ-LNS may be considered for the prevention of wasting and nutritional edema for a limited duration for all infants 6-23 months. In addition, during periods of high food insecurity, children in vulnerable households should be targeted for receipt of LNS, or when targeting is not possible, provided via a blanket approach.

Iron supplementation for menstruating women and adolescent girls

Iron-deficiency anemia continues to be highly prevalent among non-pregnant women of reproductive age (menstruating women) globally. Iron deficiency has a range of adverse health consequences among women, and the provision of iron supplements is intended to prevent iron losses and deficiency among menstruating women.

In a systematic review of 67 studies, women receiving iron were significantly less likely to be anemic following the intervention compared to control (RR 0.39, 95% CI 0.25 to 0.60, 10 studies, moderate-quality evidence), and have a higher hemoglobin concentration (MD 5.30, 95% CI 4.14 to 6.45, 10 studies, high-quality evidence) (27). Women also had a lower risk of iron deficiency (RR 0.62, 95% CI 0.50 to 0.76, 7 studies, moderate-quality evidence). There was no overall effect on 'any side effect' among women receiving iron (RR 2.14, 95% CI 0.94 to 4.86, 7 studies, low-quality evidence), however women experienced an increased prevalence of gastrointestinal side-effects (RR 1.99, 95% CI 1.26 to 3.12, 5 studies, low-quality evidence), increased loose stools/diarrhea (RR 2.13, 95% CI 1.10 to 4.11, 6 studies, high-quality evidence) hard stools/constipation (RR 2.07, 95% CI 1.35 to 3.17, 8 studies, high-quality evidence) and increased prevalence of abdominal pain (RR 1.55, 95% CI 0.99 to 2.41, 10 studies, low-quality evidence). Most trials lasted between 1 and 3 months, and iron doses ranged from 1-300 mg of elemental iron. Ferrous sulfate was the most commonly used iron compound. There is also evidence that iron supplementation increases exercise performance among women(28).

The World Health Organization currently recommends daily iron supplementation of 30-60 mg of iron among menstruation women and adolescent girls living in settings where the prevalence of anemia is 40% or higher in this group to prevent anemia and iron deficiency (29).

Iron or iron and folic acid (IFA) supplementation for pregnant women

Anemia is common among pregnant women as nutritional needs elevate during pregnancy. Iron deficiency is a predominate cause of anemia, as well as folate deficiency. Anemia during pregnancy can put the mother and child at risk for adverse birth outcomes.

In a systematic review of iron supplementation among pregnant women reduced maternal anemia (RR 0.30, 95% CI 0.20 to 0.47, 14 studies, low-certainty evidence) and iron deficiency at term (RR 0.51, 95% CI 0.38 to 0.68, 8 studies, low-certainty evidence) and iron deficiency anemia at term (RR 0.41, 95% CI 0.26 to 0.63, 7 studies, moderate-certainty evidence) compared to placebo or no intervention. Women may be less likely have low birthweight infants (RR 0.84, 95% CI 0.72 to 0.99, 12 studies, moderate-certainty evidence). Effects on maternal anemia and iron deficiency at term were similar among women provided iron-folic acid supplements, however there was no effect on low birthweight (RR 1.07, 95% CI 0.31 to 3.74, 2 studies, low-quality evidence). The World Health Organization currently recommends daily iron and folic acid supplementation (30-60 mg elemental iron and 400 µg folic acid) throughout

pregnancy for the prevention of maternal anemia, iron deficiency, low birthweight, and neural tube defects in all settings (30).

Multiple micronutrient supplements (MMS) for pregnant women

Combining multiple micronutrients into one supplement during pregnancy has been suggested as a cost-effective approach to achieve additional benefits for women during pregnancy. In a systematic review that compared MMS to iron supplements with or without folic acid, MMS led to a slight reduction in babies considered small for gestational age (SGA) (RR 0.92, 95% CI 0.88 to 0.97, 17 studies, moderate-quality evidence) and low birthweight (RR 0.88, 95% CI 0.85 to 0.91, 18 studies, high-quality evidence). There was no difference in pre-term births, stillbirths, perinatal mortality, or neonatal mortality. Currently, the World Health Organization recommends the use of antenatal MMS in the context of rigorous research but does not recommend the replacement of IFA supplements with MMS in the context of routine antenatal care (31).

Benefits of supplementation

Micronutrient supplementation has emerged as an important public health strategy, as robust evidence has demonstrated significant reductions in micronutrient deficiencies, as well as functional health outcomes in vulnerable subgroups across diverse populations. Vitamin A supplementation, for example, has long been recognized as a cornerstone intervention for

preventing childhood blindness and reducing child mortality, especially in low-resource settings where deficiency rates are particularly high. Similarly, zinc supplementation has been an important intervention as part of the treatment of diarrhea in young children. Iron supplementation is associated with modest but significant improvements in cognitive development among children and has proven to be effective in reducing anemia among children and women of reproductive age. Furthermore, when combined with folic acid, iron supplementation in pregnancy has been found to reduce the risk of maternal anemia, low birthweight, and other adverse birth outcomes. Multiple micronutrient supplements, like MNPs, provide a method to meet daily micronutrient needs and are associated with significant reductions in anemia among children. LNS represent a very promising intervention not only for preventing micronutrient deficiencies, but also for promoting child growth, a benefit that may traditional supplementation interventions have not consistently achieved.

The relative ease of use and distribution of supplementation interventions have facilitated rapid deployment and high coverage, even in areas with limited health care infrastructure (32, 33). These interventions are frequency integrated into routine health services, such as antenatal care and child health programs, enhancing their accessibility and overall impact. Furthermore, these interventions are generally cost-effective (34-36), making them an attractive strategy for policy-makers. The cumulative benefits of these interventions extend beyond individual health outcomes and have contributed to broader public health improvements and economic gains (37). Micronutrient supplementation interventions have contributed to an overall decline in

micronutrient deficiencies among vulnerable groups globally and been pivotal in advancing progress toward sustainable development goals related to health (37, 38).

Challenges with supplementation

Although there are consistent and significant benefits observed with micronutrient supplementation interventions, there are inherent challenges with supplementation programs at scale. First, data scarcity can present challenges to identify and target supplementation programs to geographic areas and subpopulations that are experiencing micronutrient deficiencies or anemia (39). National household surveys are a commonly used data source for nutrition indicators, however, very few collect biological samples needed to confirm deficiency. Simulation and modeling data is an emerging strategy to address data gaps (2). However, often these data are not disaggregated by geographic location or at-risk population groups and use secondary data sources to estimate micronutrient intake and risk of deficiency. This data sparsity makes it a challenge to map, identify and prioritize micronutrient supplementation programs.

Second, supplementation suffers from issues with adherence as well as supply-chain issues. Limited resources at the individual, health system and governmental levels can impact access to supplements, further exacerbated by poor infrastructure, political unrest, and natural disasters, depending on the context. Barriers to adherence may arise due to women's education, workload, beliefs and attitudes towards supplements for themselves and their children (40-43).

Since the benefits of supplementation depend on regular and sustained intake, any interruption due to supply or adherence can significantly diminish the effectiveness of the intervention.

Supplementation can also have undesirable side-effects. As summarized above, zinc, iron and MNP interventions have been associated with increased risk morbidity such as vomiting, fever, diarrhea, and malaria. Micronutrients like iron and folic acid are essential for parasitic and bacterial pathogens which are likely to be more prevalent in low-income and low-resource settings where hygiene is suboptimal. Furthermore, there have been several reports of adverse effects of iron supplementation on the gut microbiome in women and in children who have consumed iron in the form of MNPs (44-49). The supplemental iron has been found to increase abundance of enteric pathogens like *Escherichia-Shigella* and adversely affect gut microbial diversity (45, 48). While there is indeed the potential for benefit from supplementation interventions like MNPs, this increased risk for morbidity could incur potential harm in certain contexts, reducing the cost-effectiveness of the intervention at scale. Therefore, careful of planning supplementation interventions is necessary in order to reduce potential risks of undue harm.

Lastly, supplementation is also not a long-term solution. Supplements provides a bridge to address acute micronutrient deficiency in the short-term, however, this does not resolve the issue of micronutrient inadequacy in the diet. Nutritional adequacy should ideally be achieved through sustained improvements to diet quality and food systems, rather than reliance on supplementation alone.

1.5 Fortification of Staple Foods and Condiments

Food fortification is an intervention strategy that has been used to increase micronutrient intake at the population-level. Large-scale food fortification is defined as the mandatory or voluntary addition of micronutrients to widely consumed staple foods or condiments (such as flour, salt, sugar and cooking oil) during production. Several systematic reviews have evaluated the effectiveness of this approach to improve micronutrient status and other functional outcomes in several contexts.

Fortification of staple foods with vitamin A

Vitamin A deficiency is a widespread public health problem in many lower- and middle-income country settings. Fortification of staple foods with vitamin A has been used to increase dietary vitamin A at the population-level. In a systematic review of 10 randomized, controlled tials, fortification of staple foods (sugar, edible oils, edible fats, maize flour or corn meal, wheat flour, milk and dairy products, and condiments and seasonings) with vitamin A made little or no difference on serum retinol concentration (MD 0.03 µmol/L, 95% CI -0.06 to 0.12, 3 studies, very low-certainty evidence) or on subclinical vitamin A deficiency (RR 0.45, 95% CI 0.19 to 1.05, 2 studies, very low-certainty evidence) versus the unfortified staple food. Fortification of staple foods with vitamin A plus other micronutrients may increase serum retinol concentrations (MD 0.22 µmol/L, 95% CI 0.15 to 0.30, 2 studies, low-certainty evidence) and reduce the risk for

subclinical vitamin A deficiency (RR 0.71, 95% CI 0.52 to 0.98, 2 studies, very low-certainty evidence) versus no intervention. Effects are similar between fortification of staple foods with vitamin A versus the unfortified staple food. The duration of the studies ranged from 3 to 9 months, and the study populations were children and adolescents between 2 and 19 years of age. Studies varied in terms of fortification vehicle used, and the overall quality of evidence was low (50).

Fortification of rice with vitamin A alone or in combination with other nutrients may improve serum retinol concentration (MD 10.00, 95% CI 8.79 to 11.21, 1 study, low-certainty evidence), though it may make little or no difference in vitamin A deficiency (RR 0.68, 95% CI 0.36 to 1.29, 4 studies, low-certainty evidence) (50).

The WHO recommends the fortification of rice with vitamin A as a strategy to improve iron status and vitamin A status of populations (conditional recommendation, low-quality evidence) (51).

Fortification of staple foods with zinc

Zinc deficiency is a widespread global public health problem. Fortification of staple foods with zinc has been proposed as an intervention in parts of the world where diets mainly consist of cereal grains. In a systematic review of 59 studies, zinc fortification with and without other micronutrients increased plasma zinc concentrations (efficacy studies, *n* = 27: MD 4.68 µg/dL,

95% CI 2.62 to 6.75, low-quality evidence; effectiveness studies, *n* = 13, MD 6.28 µg/dL, 95% CI 5.03 to 7.77 µg/dL, low-quality evidence) and reduced the prevalence of zinc deficiency (efficacy studies, *n* = 11, OR 0.76, 95% CI 0.60 to 0.96, very-low quality evidence; effectiveness studies, *n* = 10, OR 0.45, 95% CI 0.31 to 0.64, very-low quality evidence). There review also identified statistically significant increases in child weight (efficacy, *n* = 11, MD 0.43 kg, 95% CI 0.11 to 0.75 kg, low-quality evidence), improvements in short-term auditory memory (efficacy, *n* = 3, MD 0.32 point, 95% CI 0.13 to 0.50 point, moderate-quality evidence), and decreased incidence of diarrhea (efficacy studies, *n* = 3, RR 0.79, 95% CI 0.68 to 0.92, low-quality evidence) and fever (efficacy studies, *n* = 2, RR 0.85, 95% CI 0.74 to 0.97, low quality evidence. There were a wide range in zinc doses, from 0.7 mg/d to 54.4 mg/d (median, 4.37 mg/d). A majority of studies were conducted in lower- and middle-income countries. The greatest number of studies (25) were conducted in school-age children (5–11 years), 20 studies were conducted among WRA, 8 studies were conducted among pre-school age children (2-5 years), and 3 studies were among children aged under 2 years of age. (52)

The World Health Organization recommends fortification of wheat flour with zinc may be used as a public health strategy to improve zinc status (conditional recommendation, low-certainty evidence) (53).

Fortification of staple foods with iron

As anemia is a widespread condition that affects many age strata, fortification of staple foods with iron, as well as other micronutrients, is a proposed solution to address anemia. In a systematic review of 10 trials, iron-fortified wheat flour with or without other micronutrients may reduce the risk of anemia by 27% (RR 0.73, 95% CI 0.55 to 0.97, 5 studies, low-certainty evidence) compared to wheat flour with the same other micronutrients but no iron. There was no effect on hemoglobin and iron deficiency. There was no reported data on adverse outcomes except for inflammation measured by C-reactive protein (CRP), and there was no effect on CRP. It was unclear whether iron-fortified wheat flour with other micronutrients decreases anemia compared to unfortified wheat flour (RR 0.77, 95% CI 0.41 to 1.46, 2 studies, very-low certainty evidence). However, the intervention may reduce iron deficiency (RR 0.73, 95% CI 0.54 to 0.99, 3 studies, moderate-certainty evidence). There was no effect on hemoglobin. The level of fortification ranged from 10 mg/kg iron to 200 mg/kg iron, with most studies using ≥ 40 mg/kg. Duration of studies varied from 3 to 24 months among the general population of all age groups over 2 years of age (54).

In a systematic review of rice fortification, rice fortified with iron alone, or in combination with other micronutrients, may make little difference in the risk of anemia (RR 0.72, 95% CI 0.54 to 0.97, 7 studies, low-certainty evidence), and may reduce iron deficiency (RR 0.66, 95% CI 0.51 to 0.84, 8 studies, low-certainty evidence). Rice fortification may also increase hemoglobin (MD 1.83, 95% CI 0.66 to 3.00, 11 studies, low-certainty evidence). There was no effect on the risk of diarrhea (RR 3.52, 95% CI 0.18 to 67.39, 1 study, very-low quality evidence (55). However, one study of reported an increase in hookworm infection (RR 1.78, 95% CI 1.18 to 2.70, low-

certainty evidence). Elemental iron varied from 0.2 to 112.8 mg/g uncooked rice, and study duration ranged from 2 weeks to 48 months (55).

In a systematic review of 3 studies, it was uncertain whether maize flour or corn meal fortification with iron and other micronutrients has an effect on anemia (RR 0.90, 95% CI 0.58 to 1.40, 2 studies, very low-quality evidence), iron deficiency, hemoglobin concentration or ferritin concentration when compared to unfortified maize flour. No adverse effects were assessed. Studies included children 2-11 years of age, and women. Study duration ranged from 6-10 months. Studies used different flour types, and iron concentrations ranged between 28 to 140 mg/kg. Evidence from this review is very uncertain due to few studies and high risk of bias (56).

In a systematic review of 12 studies, fortification of condiments and seasonings with iron reduces anemia (RR 0.34, 95% CI 0.18 to 0.65, 4 studies, low-certainty evidence) and probably reduces iron deficiency (RR 0.33, 95% CI 0.11 to 1.01, 2 studies, moderate-quality evidence) when compared to the same unfortified condiment. It is uncertain whether fortified condiments and seasonings increases hemoglobin or ferritin concentrations. When condiments and seasonings are fortified with iron plus other micronutrients, risk of anemia declines (RR 0.59, 95% CI 0.40 to 0.89, 5 studies, very low-certainty evidence) compared to seasonings fortified with other nutrients excluding iron. The effect on hemoglobin (MD 6.22 g/dL, 95% CI 1.60 to 10.83, 5 studies, very low-certainty evidence), iron deficiency (RR 0.36, 95% CI 0.19 to 0.69, 4 studies, low certainty evidence), and ferritin concentrations (MD 10.63 ug/L, 95% CI 2.40 to 18.85, 5 studies, very low-certainty evidence). No studies reported adverse effects. The dose or

iron received by participants ranged between 4.4 mg to 55 mg/day, and study duration was between 3 months to 2 years (57).

The WHO recommends fortification of wheat flour with highly bioavailable iron as a public health approach to improve hemoglobin concentrations and iron status and prevent anemia in populations (strong recommendation, low-certainty evidence) (53). The WHO also recommends fortification of maize flour and cornmeal to prevent iron deficiency in populations, especially vulnerable subgroups like women and children (very low-quality evidence, conditional recommendation) (58). Lastly, the WHO recommends the fortification of rice with iron in settings where rice is a staple food (strong recommendation, moderate-certainty evidence) (51).

Fortification with folic acid

Folate is an essential nutrient for DNA repair and cell division and is particularly crucial for fetal development during pregnancy (59). Over 80 countries have introduced folic acid fortification programs to prevent neural tube defects and prevent folate deficiency in the general population (60).

In a systematic review of 10 studies of fortification of wheat or maize flour with folic acid alone or with other micronutrients, pregnant women who received folic-acid fortified maize porridge had significantly higher erythrocyte folate concentrations (MD 238.90 nmol/L, 95% CI 149.40 to 328.40, 1 study, very low-certainty evidence) and higher plasma folate (MD 14.98, 95% CI 9.63,

20.33, 1 study, very low-certainty evidence) compared to no intervention. Women of reproductive age consuming maize flour did not have higher erythrocyte or plasma folate compared to women consuming unfortified flour. However, women who consumed flour fortified with folic acid and other micronutrients had higher serum folate (MD 2.92 nmol/L, 95% CI 1.99 to 3.85, 2 studies, very low-certainty evidence) compared to women consuming unfortified flour. Adults consuming folic acid-fortified wheat flour bread rolls had higher erythrocyte folate (MD 0.66 nmol/L, 95% CI 0.13 to 1.19, 1 study, very low-certainty evidence) and plasma folate (MD 27.00 nmol/L, 95% CI 15.63 to 38.37; 1 study, very low-certainty evidence), versus unfortified flour. In one RCT, wheat flour fortified with folic acid and other micronutrients was associated with lower risk of neural tube defects (RR 0.32, 95% CI 0.21 to 0.48, 1 study, low-certainty evidence). The duration of interventions lasted 2 weeks to 36 months, up to 7 years post-intervention (60).

Fortification of wheat flour with folic acid is a recommended approach by the WHO to reduce the risk of neural tube defects in pregnancy and improve folate status of the population (strong recommendation, low and very low-certainty evidence) (53). The WHO also recommends the fortification of maize flour and corn meal with folic acid to reduce NTDs and improve folate status in the general population (conditional recommendation, very low-quality evidence) (58). Fortification of rice with folic acid may also be used as a public health approach to improve folate status (conditional recommendation, very low-certainty evidence) (51). Lastly, there is emerging research to suggest the effectiveness of folic acid fortification of salt and bouillon.

Food fortification with multiple micronutrients

In comparison to food fortification with single micronutrients, fortification with multiple micronutrients may be a cost-effective way to reduce the risk of multiple micronutrient deficiencies.

In a systematic review of 43 studies, when compared to no intervention or placebo, micronutrient fortification of staple foods and condiments with multiple micronutrients may reduce anemia by 32% (RR 0.68, 95% CI 0.56 to 0.84, 11 studies, low-quality evidence), iron deficiency anemia by 72% (RR 0.28, 95% CI 0.19 to 0.39, 6 studies, low-quality evidence), iron deficiency by 56% (RR 0.44, 95% CI 0.32 to 0.60, 11 studies, low-quality evidence), vitamin A deficiency by 58% (RR 0.42, 95% CI 0.28 to 0.62, 6 studies, low-quality evidence), vitamin B2 deficiency by 64% (RR 0.36, 95% CI 0.19 to 0.68, 1 study, low-quality evidence), vitamin B6 deficiency by 91% (RR 0.09, 95% CI 0.02 to 0.38, 2 studies, low-quality evidence) and vitamin B12 deficiency by 58% (RR 0.42, 95% CI 0.25 to 0.71, 3 studies, low-quality evidence). Multiple micronutrient fortification may improve serum ferritin (MD 8.27 µg/mL, 95% CI 3.26 to 13.27), vitamin B6 (MD 35.02 nmol/L, 95% CI 22.95 to 47.09), vitamin B9 (folate) (MD 12.41 nmol/L, 95% CI 6.55) and vitamin B12 (MD 61.90 pmol/L, 95% CI 53.56 to 70.23). This approach may also improve child growth in terms of weight for age Z-score (MD 0.1, 95% CI 0.02 to 0.17, 8 studies, low-quality evidence), and weight for length/height Z-score (MD 0.1, 95% CI 0.02 to 018, 6 studies, low-quality evidence). Effects on zinc deficiency, and length/height for age Z-score were uncertain. Effects on other outcomes such as cognition, morbidity and mortality

continue to be limited. No studies reported on adverse effects. Food vehicles in both systemic reviews such as rice and flour; dairy products, including milk and yogurt; non-dairy beverages; biscuits; spreads; and salt (61).

Benefits of fortification

Fortification of staple foods and condiments has consistently been shown to be effective to improve micronutrient status and functional outcomes in populations across various contexts. The evidence from systematic reviews summarized above suggests that fortification with single or multiple micronutrients results in reductions in anemia, iron deficiency, vitamin A deficiency, zinc deficiency, improved folate status, and reduced risk for neural tube defects (9, 50, 54-57, 60, 61). There is even some evidence to suggest that this intervention results in improved growth and cognition among children (61).

Food fortification has led to a substantial increase in the nutrient quality of the food supply in many countries since fortification programs it began in the 1920s, with fortification of wheat flour with iron and B vitamins, to fortification of sugar with vitamin A (62). The historical widespread success of fortification programs has promoted further research and implementation in lower and middle-income countries (63). Currently, nearly 140 countries globally have guidelines in place for implementing fortification programs, a majority of which are mandatory (64).

Beyond the health and nutritional benefits of food fortification, additional benefits include its cost-effectiveness. Food fortification is consistently ranked as one of the most cost-effective strategies to improve the nutritional status of a population (35, 64-67). Cost per person is as low as cents on the dollar per person to implement programs, and returns on investments include gains in productivity and healthcare savings, as well as lives saved (68-73). Another advantage of this intervention is its feasibility. By adding a micronutrient premix during industrial processing of staple foods, producers can offer nutritionally enhanced products without requiring individuals to change their consumption habits (67). This intervention also takes advantage of existing supply chains and infrastructure to deliver micronutrients (67). Thus, this intervention has the potential for widespread coverage and impact. Innovations in food technology has expanded the range of products that can be fortified with multiple micronutrients (74), from flour, oil and salt to rice and bouillon cubes. This offers new opportunities to target deficiencies in vulnerable populations.

Challenges with fortification

Although fortification programs can effectively reduce micronutrient deficiencies, they also face several limitations and potential risks that underscore the need for continued research and careful implementation. For example, evidence supporting the effectiveness of certain food vehicles, including rice, maize flour, and certain condiments and seasonings is inconsistent and limited (55-57), underscoring the need for additional, and more rigorous, research. Another critical concern is the lack of clarity regarding possible adverse effects, such as diarrhea, changes

in the gut microbiome, inflammation, and increased vulnerability to infections like malaria, which may be linked to supplemental iron and folic acid intake (17, 19, 45, 49, 75). While these risks are not definitively established for fortification interventions, the absence of rigorous data highlights an urgent need for more comprehensive studies that evaluate not only efficacy but also potential harms.

Large-scale fortification programs require careful planning and context-specific analysis of needs and benefits. Barriers to adoption of new fortification programs often hinges on overcoming challenges such as political instability and limited government support, which can impede the establishment of legislation, regulatory frameworks, and funding streams needed to guide and sustain fortification initiatives (62). Detailed cost-benefit analyses are critical not only for justifying the program's expense to policymakers and stakeholders but also for clarifying its long-term impact (35, 67, 76). Furthermore, robust monitoring and evaluation systems are essential to ensure adherence to standards, document improvements in health metrics, and make any necessary adjustments (39).

Private-sector engagement is another key challenge. Companies play a direct role in implementing fortification programs, so buy-in and incentives for compliance are crucial (62). Industry stakeholders need to be provided with clear guidelines, technical support, and reliable supply chains for premixes. A further complication is the fact that micronutrients may degrade during processing and storage (74). Ensuring the stability of these nutrients over time requires

regular quality assurance, from selecting appropriate fortificants and formulations to monitoring how well micronutrient content holds up on shelves.

For planning fortification programs, accurate data that quantify the prevalence and severity of dietary inadequacy and micronutrient deficiencies within a target population can be difficult to collect (74). This information helps policy-makers and program designers predict the potential impact of a fortification program and ensures that it addresses the specific nutritional gaps in the population (67). In contrast to supplementation programs, where the dose (e.g., a pill, syrup or sachet) is more straightforward to measure, fortification relies on knowing how much of a particular food vehicle people regularly consume (67). This becomes especially important in vulnerable groups whose dietary patterns may differ considerably from the general population. Without adequate consumption data guide the choice of fortification vehicle and the level of fortificants to add, it can be a challenge to strike the right balance between correcting deficiencies and avoiding excessive intakes that exceed tolerable upper limits. Lastly, data on quality and coverage of ongoing fortification programs are not routinely collected, limiting the ability to track progress and barriers to success (64).

1.6 Dietary diversification

In many lower- and middle-income countries, diets are heavily dependent on cereal grains and starchy staples, with minimal consumption of nutrient-dense fruits, vegetables and animal-

source foods (77). This reliance on low nutrient-dense foods is a major contributor to micronutrient deficiencies, as well as other functional health consequences like anemia and poor growth in children (78). Increasing dietary diversity helps improve the bioavailability of micronutrients by promoting the availability, access, and consumption of a greater range of high-quality, nutritious foods.

A variety of interventions have been designed with the intention to improve dietary diversity (79, 80). Home gardens, for example, enable households to cultivate nutrient-dense fruits and vegetables (80-82), and animal husbandry interventions can provide access to animal-source foods which are rich in bioavailable micronutrients like iron and zinc (80, 83). Nutrition education programs further empower communities by teaching the importance of incorporation of a variety food groups in the diet (84, 85). Women's empowerment may improve household diet diversity, as women often play a central role in purchasing preparing and allocating food within households (86-88). Finally, direct provision of nutrient-dense foods, such as eggs or meat, may bridge nutritional gaps in the short-term (89) and complement other interventions to promote sustained improvements in dietary diversity.

Although dietary diversity interventions are regarded as a promising strategy for improving micronutrient intake, no systematic reviews have evaluated their effectiveness to improve micronutrient status or other functional health outcomes. A recent systematic scoping review (90) indicates that dietary diversity indicators generally correlate with dietary quality, as evidenced by micronutrient intakes and biomarkers of micronutrient status; however, the

strength of this relationship varies, and findings across studies are mixed. In addition, the same review found that dietary diversity indicators are often not associated with health outcomes or body composition. However, individual studies of interventions aimed to improve dietary diversity have shown moderate effects on micronutrient status and health outcomes including child growth (79, 81, 83, 84, 88, 91-93). Because dietary diversity interventions are highly heterogeneous, systematically measuring their impacts remains challenging.

These results suggest that while dietary diversity interventions can play an important role in addressing micronutrient gaps, more rigorous research, including controlled trials, replication studies in other contexts, and comprehensive reviews, are needed to clarify how different dietary diversity interventions affect a range of health outcomes and to identify the most effective strategies for scaling up interventions.

Benefits and challenges of dietary diversification

Whole foods generally provide the most bioavailable sources of essential micronutrients (94), making dietary diversification a promising strategy for reducing micronutrient deficiencies. The primary goal of dietary diversification is to promote the regular intake of a wide variety of food groups, each offering a distinct array of nutrients necessary to meet dietary needs (77). In lower- and middle-income countries where diets are often centered around starchy staples, such as rice, maize, and wheat, there is a lack of nutrient density, which contributes to widespread deficiencies in micronutrients like zinc, iron and vitamin A. Additionally, starchy

staples tend to be high in phytates-naturally occurring compounds that can bind to minerals like zinc and iron and impair their absorption (95, 96). By diversifying diets to include a range of nutrient-dense foods, individuals can access a broader spectrum of bioavailable micronutrients, supporting improvements in both nutritional status and functional health outcomes.

Furthermore, food diversification strategies can be tailored to fit local dietary patterns and cultural preferences, ensuring that interventions are not only effective but also acceptable to the populations they aim to support (79). This helps to increase the likelihood of sustained behavior change and improve nutrient intake without requiring changes to traditional eating patterns. Thus, dietary diversification may offer a practical and more sustainable solution for improving micronutrient bioavailability and addressing the underlying causes of malnutrition in resource-constrained settings.

However, this strategy has several disadvantages. As alluded to above, there is a lack of a strong evidence-base for successful dietary diversity interventions (79). Variables such as cultural acceptability, affordability, and adherence play significant roles in determining whether dietary diversification interventions with achieve sustained success (97-100). Cost and infrastructure requirements of diet diversity interventions are a particular challenge, as many nutrient-dense foods tend to be expensive and require cold storage and reliable supply-chains (79). Approaches that include home gardening, small-scale animal husbandry, and women's empowerment can help mitigate these challenges by enabling families to produce their own nutrient-rich foods. However, such programs must be designed with careful attention to local cultural norms and

household roles to avoid imposing excessive labor demands on women or other vulnerable household members (86, 93, 99). Equally important is the need for clear, measurable impact indicators to track whether increased availability of diverse foods is actually improving micronutrient intake and health status (79, 101).

1.7 Future Directions

Micronutrient interventions have demonstrably reduced deficiencies and improved health outcomes among vulnerable populations, including children, women of reproductive age, and the broader community, through strategies such as supplementation (10, 13-16, 19, 20, 22, 27, 102, 103), food fortification (50, 54-56, 60, 61) and promotion of dietary diversity (79, 81, 83, 84, 88, 90-93). However, despite these achievements, the long-term sustainability of these interventions remains limited when each is implemented in isolation. Future research calls for rigorous evaluation of multisectoral interventions that can target at-risk groups for sustained improvements in micronutrient status (39, 104-106).

Currently, research examining the combined effects of multi-pronged interventions on micronutrient status is limited (107). Although WHO guidelines emphasize that interventions should complement additional or existing programs, few studies have rigorously evaluated the effectiveness of implementing multiple interventions simultaneously (108, 109). This gap largely stems from the challenges and high costs associated with such research. Implementing several strategies concurrently and evaluating their combined impact in a randomized controlled trial

demands significant resources and coordinated efforts. Nevertheless, prioritizing this type of research is critical for identifying sustainable strategies that reduce reliance on short-term solutions, such as supplementation, and promote long-term nutritional improvements.

Future research may also need to prioritize the underlying factors that influence the biological response to micronutrient interventions. Conflicting evidence about the effectiveness of interventions may suggest unknown mechanisms affect how individuals respond to micronutrient supplementation or fortification. Investigating the interactions between different micronutrients and the form in which they are delivered (e.g. powders vs. food matrices), and individual characteristics, such as age, health status, and genetic variation, will be crucial for optimizing micronutrient strategies in at-risk subpopulations (110-114). Moreover, the integration of advanced methodologies, such as multi-omics approaches, may further clarify the biological systems underlying responses to micronutrient interventions across diverse populations (115).

In addition, it is important to balance the benefits and potential risks of micronutrient interventions. There is evidence to suggest that strategies that include iron and folic acid supplementation and fortification can increase susceptibility to infection and inflammation in especially high-risk settings, including those with high rates of malaria and low sanitation (44-46, 48, 49, 114, 116-119). Consequently, the local context, including dietary patterns, health system capacity, cultural practices, environmental conditions, and the impacts of climate change, must be thoroughly evaluated to determine the appropriateness and sustainability of

specific interventions (41, 105, 107, 108, 120). Without a strong fundamental understanding of the local context, long-term improvements in micronutrient status will be challenging to achieve. To that end, continued investment in the collection of robust dietary data and accurate measures of micronutrient status is essential for identifying at-risk populations and designing effective interventions (39). These data sources enable more precise identification of vulnerable groups, whether defined by age, sex, socioeconomic status, or geographic region, so that interventions can be better tailored to local contexts. Beyond guiding intervention design, comprehensive dietary and deficiency data are essential for monitoring the impact of ongoing programs and adjusting strategies in response to changing nutritional landscapes.

Equally important is the use of cost-benefit analyses to inform and influence policymakers. By quantifying the return on investment, whether in terms of reduced healthcare costs, improvements in productivity, disability-adjusted life years averted, or lives saved, decision-makers can better understand the economic and social advantages of nutrition interventions (34, 36, 57, 68, 121-132). Evidence from multiple countries indicates that well-targeted micronutrient programs often yield high cost-benefit ratios, supporting the case for sustained or increased funding (51, 64, 65, 73, 133). In resource-limited settings, these analyses can be particularly persuasive, guiding the allocation of funds toward the most impactful interventions and ensuring that investments are grounded in evidence-based estimates of efficacy, cost-effectiveness, and long-term sustainability.

Fundamentally, governments will need to invest in the future of their populations through improvements to nutrition and health. Policies that support social programs and infrastructure to reduce poverty, increase food and healthcare access and availability will be essential for any nutrition intervention to have its intended effect (134). Without prioritizing these factors in society, nutrition interventions will not be enough to solve micronutrient deficiencies, and the consequences associated with them. Ultimately, the global community must assess the value and long-term impact of investing in developing nations. In many instances, micronutrient intervention programs in LMICs depend heavily on external funding from higher-income countries and philanthropic organizations, leading to inconsistent research priorities and temporary interventions. Achieving sustainable reductions in micronutrient deficiencies requires a long-term commitment to development in these regions, including capacity-building for local research and program implementation, strengthening of health systems, and empowerment of communities to become self-sufficient in addressing nutritional challenges. Such efforts necessitate coordinated actions among international stakeholders, governments, and local communities, ensuring that micronutrient initiatives are integrated into broader public health and development goals.

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Chapter 2. Predictors of anemia among young children receiving daily micronutrient powders (MNPs) for 24 weeks in Bangladesh: a secondary analysis of the Zinc in Powders Trial[1]

[1] This paper has been published previously [Thompson L, Arnold C, Peerson J, Long JM, Westcott JLE, Islam MM, et al. Predictors of Anaemia Among Young Children Receiving Daily Micronutrient Powders (MNPs) for 24 Weeks in Bangladesh: A Secondary Analysis of the Zinc in Powders Trial. Matern Child Nutr. 2025:e13806. doi: 10.1111/mcn.13806.] Reprinted with permission. This version may contain minor differences, as it does not contain changes made during copyediting.

2.1 Abstract

In Bangladesh, anemia is estimated to affect 52% of children 6-59 months, with the youngest children (6-23 months) experiencing the highest levels of anemia (71%). Micronutrient powders (MNPs) are designed to increase micronutrient intake in young children; however, in some settings, the prevalence of anemia may remain elevated despite high coverage of MNPs. In a secondary analysis of the Zinc in Powders trial (ZiPT), we identified risk factors that were associated with anemia among Bangladeshi children 9-11 months of age who received standard 15-component MNPs, including 10 mg of iron, daily for 24 weeks. At enrollment, socio-demographic characteristics were collected. Morbidity symptoms were assessed on a semi-weekly basis. Hemoglobin (measured via single-drop capillary blood using Hemocue 301+) and child anthropometry were assessed at enrollment and endline (24 weeks). Risk factors for anemia at endline (24 weeks) were identified using minimally-adjusted (age and sex) logistic regression models. Multivariate models were subsequently constructed, controlling for age, sex, and significant risk factors. Of the 481 children randomized to the MNP arm, *442 completed the* trial and had hemoglobin data available at endline. Anemia (hemoglobin <10.5 g/dL) prevalence declined from 54.1% at baseline to 32.6% at endline. In minimally-adjusted models, season of enrollment, underweight at enrollment, asset score, hygiene score and frequent morbidity symptoms were associated with the odds of anemia at endline. However, some factors lost statistical significance in multivariate models. MNPs are an important tool for anemia prevention, however, they should be part of an integrated approach for anemia control.

2.2 Introduction

Anemia affected 40%, or 372 million children under 5 years of age worldwide in 2019 (1), and minimal progress has been made to alleviate the burden of anemia among young children in recent years (2, 3). Anemia in early childhood can have severe heath consequences, including poor immune function, impaired cognitive development, and suboptimal physical growth, which may be irreversible and have lasting impacts into adulthood (4, 5). Iron deficiency is considered a major cause of anemia, however, other micronutrient deficiencies, as well as various environmental, socioeconomic, maternal and genetic risk factors may contribute to the development of anemia in childhood (6).

Meeting the nutritional needs of young children in low-income settings is challenging due to limited availability and access to nutrient-rich foods (7). Since 2011, the World Health Organization (WHO) has recommended the provision of micronutrient powders (MNPs) as an approach to prevent anemia and iron deficiency among young children 6-23 months by enhancing the micronutrient composition of complementary foods. It is recommended that MNPs be distributed in areas where anemia prevalence among children under 2 years age is >20% (8). Standard MNP sachets contain 15 vitamins and minerals, including 10-12.5 mg of iron (8). Currently, over 65 countries are implementing MNP programs as a part of national policies for reducing anemia and micronutrient deficiencies among young children (9).

Although MNPs have successfully decreased the prevalence of anemia in certain settings (10, 11), some randomized controlled trials (RCTs) have demonstrated that anemia prevalence can remain elevated despite MNP provision. In a cluster RCT of daily MNP provision for 6 months among children 6 months of age in Cambodia (N = 925), 77% remained anemic at endline (12). Likewise, in a large RCT in Pakistan, 72% of children 6-23 months (N = 638) who received a daily MNP for 12 months remained anemic at endline (13). In a large, community-based cluster RCT of MNP provision on alternating days for 12 months in Burkina Faso, the prevalence of anemia among children 6-59 months (N = 1,318) increased from 75% at baseline to 88% at endline (14).

This suggests that MNPs alone may be inadequate to address anemia to the greatest extent possible in certain settings. Further evidence is needed to understand key risk factors for anemia among children who have received MNPs to improve the design and context-specific targeting of MNP interventions for effective anemia control.

The recently completed Zinc in Powders Trial (ZiPT) evaluated the effects of various doses, forms, and frequencies of zinc supplementation, including MNPs, on diarrhea incidence and linear growth among young children in Dhaka, Bangladesh (15, 16). Despite high (>80%) levels of adherence, at the end of the 24-week intervention period, the prevalence of anemia among children 9-11 months of age at enrollment who had received standard, 15-component MNPs containing 10 mg of iron for 24 weeks, was nearly 33% according to the newly revised WHO cutoff (hemoglobin <10.5 g/dL) and 46% using the previous cutoffs (hemoglobin <11 g/dL)(15). The objective of this secondary analysis was to identify risk factors for anemia at endline (24 weeks) among children enrolled in the ZiPT trial who had received daily MNPs.

2.3 Methods

Study design, setting and population

The ZiPT trial was a randomized, partially double-blind, controlled, community-based efficacy trial conducted in the peri-urban, low-income area of Mirpur in Dhaka, Bangladesh. The study protocol has been published previously (McDonald et al, 2018). This area was chosen due to its approximation to the International Center for Diarrheal Disease Research, Bangladesh (icddr,b), large population size, and high prevalences of zinc deficiency, diarrhea and stunting among preschool-aged children. The trial had six intervention arms with varying doses, forms and/or frequencies of zinc supplementation. A summary of the intervention groups is provided in Supporting Information: Table 1. Between October 2017 and July 29, 2019, a total of 5,567 children 9-11 months of age were screened for eligibility and 2,886 children were enrolled into the trial and randomized to one of the six study groups. The analyses presented here are restricted to children who received a standard 15-component MNP containing 4.1 mg zinc and 10 mg iron (8) (manufactured by DSM India Private Limited) that was intended to be consumed daily. The complete composition of the MNP can be found in Supplementary Information: Table 2.

A detailed description of the study participants has been published previously (16). Briefly, children were eligible for enrollment if they had a weight-for-length z-score (WLZ) ≥−3 according to the 2006 WHO Growth Standards. Children were excluded from the trial if they

had any of the following conditions: (1) Severe Acute Malnutrition, defined as WLZ <−3 and/or the presence of bipedal edema and/or mid-upper arm circumference (MUAC) <115 mm; (2) Severe anemia (hemoglobin concentration < 8 g/dL, as measured by single-drop capillary blood collected via fingerprick using Hemocue 301+) (3) Congenital anomalies (e.g., cardiac defects, cleft lip or palate) or any other conditions that interfere with feeding; and (4) Chromosomal anomalies and other organic problems (e.g., jaundice, tuberculosis, etc.). Children who were identified as severely anemic (hemoglobin < 8 g/dL) according to Hemocue 301+ measurement of a fingerprick blood sample after 12 weeks of follow-up were dropped from the study and referred for treatment.

Data collection

At enrollment, self-reported data on background, socioeconomic status, education level, household characteristics, sanitation and handwashing practices, source of drinking water, ownership of assets, and food security were collected using pretested questionnaires. Certain variables were used to estimate composite household asset scores and hygiene scores. Asset score was estimated using the sum of 2 subscores: asset score 1, which was the sum of the following possessions: iron, chair/bench, sofa, table, computer, fridge, motorcycle, and bank account; asset score 2, which was the sum of the following possessions: electric fan, television, mattress, mobile phone. Asset score could range from 0–12. Hygiene score was calculated as the sum of the scores following variables: “Wash hands after helping child defecate”; “Wash hands before preparing food”, “Wash hands after using toilet”; and “Uses toilet paper”. Each

variable was scored as follows: 1 = never, 2 = rarely, 3 = sometimes, 4 = always. Asset score could range from 4–16. Household food security was assessed using the Household Food Insecurity Access Scale (17).

Trained Field Research Assistants (FRAs) visited the child participant and his/her caregiver at their home twice per week throughout the 24-week study period. At one of these twice-weekly visits, the FRA provided the child's caregiver with the MNP for seven days and instructed the caregiver on proper use. During these home visits, FRAs inquired about MNP consumption over the past 3-4 days, used MNP sachets were collected, and the number consumed was recorded. FRA's also inquired about the presence of diarrhea, respiratory illness, fever and other morbidity symptoms in the preceding 3-4 days. Definitions of morbidity symptoms can be found in Supplementary Table 2. If the study child developed diarrhea, MNPs were temporarily suspended and he/she received standard treatment, including a 20 mg therapeutic zinc supplement in the form of a dispersible tablet for 10 days.

Body weight and length of each child was measured at enrollment and at 12 and 24 weeks. Study anthropometrists conducted all measurements. Body weight was measured on a balance sensitive to 2 g (SECA, model No. 7281321009, Hamburg, Germany) and length was measured to 0.1 cm using an infantometer (SECA, model No. 4161721009, Hamburg, Germany). The measurements were performed in triplicate following standard procedures (18). Stunting, underweight and wasting were defined as length-for-age z-scores, weight-for-age z-scores and weight-for-length z-scores <-2 SD, respectively (WHO Multicenter Growth Reference Study

Group, 2006). Hemoglobin measurements were conducted on all study participants at baseline, 12 weeks, and at the end of the 24-week intervention period. Hemoglobin concentration was measured from a fingerprick blood sample using a Hemocue, HB 301+ Analyzer (Hemocue AB, Angelholm, Sweden).

Statistical analysis

The primary outcome variable was anemia at endline (following the 24-week intervention period). Recently, the WHO released new guidelines on hemoglobin cutoffs to define anemia in individuals and populations, which recommend that anemia be defined as hemoglobin <10.5 g/dL among children 6-23 months (19). Therefore, anemia was defined as hemoglobin <10.5 g/dL for this analysis. However, we also replicated the analyses using the previously-recommended cutoff (hemoglobin <11 g/dL). Analyses were restricted to the children who completed the trial and had hemoglobin concentrations available at endline (24 weeks).

Logistic regression models were used to identify socioeconomic, maternal, environmental and morbidity risk factors that were significantly associated with anemia at endline in minimally adjusted bivariate models, controlling for child sex and age at enrollment. Multivariable models were subsequently fit including all predictors that were associated with anemia at endline in bivariate models at a significance level of *P-value* <0.1. Because we defined our outcome as

anemia at endline and not change in anemia status from baseline to endline, we did not control for baseline anemia status in regression models. However, regression analyses were replicated to include baseline anemia status and results were reported separately. Collinearity was assessed using variance inflation factors (VIF), and if risk factors were significantly correlated, one risk factor was selected for inclusion in the multivariable model to represent the relevant information. If continuous risk factor variables were significantly associated with the outcome, analyses were repeated with categorical variables for ease of interpretation. Results are presented as odds ratios (OR) and 95% confidence intervals (CI). Statistical analyses were performed using R (Version 2023.12.1+402).

2.3 Results

Characteristics of participant children and their families

Of the 2886 children were enrolled into the ZiPT trial, 481 children were randomized into the standard MNP daily group (16). Of these, 442 children completed the trial and had hemoglobin concentration data available at endline (i.e. 24 weeks). At enrollment, mean ± SD child age was *9.7* ± 0.8 months, and 91% were breastfeeding. Only 23.3% of children were considered healthy at enrollment (had no morbidity symptoms in the past two weeks as reported by the child's caregiver). Mean ± SD hemoglobin concentration among the children was 10.4 ± 1.2 g/dL. The prevalences of anemia according to the recently revised (hemoglobin <10.5 g/dL) and

previously-recommended WHO cutoffs (hemoglobin <11 g/dL) were 54.1% and 68.8%, respectively. Mothers of children enrolled in the standard MNP daily group were 24.6 ± 5.4 years of age on average at enrollment. Most (93.2%) women did not work outside the home and completed 6.6 ± 4.1 years of education on average. Mean ± SD household size was 4.5 ± 1.4 people. More than 70% of households had drinking water piped directly into the home, and mean household hygiene score was considered relatively favorable (12.2 ± 2.4 SD out of 16). Conversely, mean ± SD asset score was relatively low, 5.8 ± 2.4 out of 12. Nearly 22% of households were classified as food insecure at the time of enrollment (**Table 2.1**).

Risk factors for anemia among children enrolled in the standard MNP daily group at endline (24 weeks)

At the end of the 24-week intervention period, the prevalence estimates of anemia according to WHO's newly-recommended (hemoglobin <10.5 g/dL) and previously-recommended (<11.0 g/dL) cutoffs were 32.6% and 45.9%, respectively. In bivariate logistic regression models, children living in households with a higher asset score were less likely to be anemic (*P-value* for trend = 0.04) at endline following the receipt of standard MNPs daily for 24 weeks (**Table 2.2, Figure 2.1**). Children whose households had a hygiene score above the median had a lower risk of anemia at endline (OR 0.63, 95% CI 0.42-0.94).

Children who were underweight at enrollment had a significantly increased risk of anemia at endline (OR 1.80, CI 1.06-3.02) in bivariate models. Children who were enrolled in the summer season were significantly less likely to be anemic at endline as compared to children enrolled in the winter season (OR 0.61, CI 0.37-1.01). Several morbidity symptoms were associated with an increased risk of anemia at endline, including frequent low appetite (number of days above the median) (OR 1.68, 95% CI 1.08-2.62), shortness of breath (OR 3.32, 95% CI 1.28-9.23) and runny nose (OR 1.48, CI 0.99-2.22) across the 24-week intervention period. Among the risk factors that were associated with anemia at endline in bivariate logistic regression models, only season of enrollment, number of days with low appetite and number of days with shortness of breath above the median remained significant in multivariable models (**Table 2.2, Figure 2.1**).

When anemia was defined by the previously WHO-recommended cutoffs for children 6-23 months (hemoglobin <11 g/dL), maternal education (*P-value* for trend = 0.01), hygiene score (*P-value* for trend = 0.01) and household food security (OR 0.47, 95% CI 0.30-0.75) were significantly associated with anemia at endline in bivariate logistic regression models. Household drinking water source (*P-value* for trend = 0.06) and asset score (*P-value* for trend = 0.07) were marginally significant in bivariate models. In addition, frequent low appetite (OR 1.60, 95% CI 1.04-2.47), cough (OR 1.46, 95% CI 1.00-2.14) and shortness of breath (OR 3.08, 1.14-9.77) across the 24-week intervention period were significantly associated with anemia at endline in bivariate models. However, in multivariate models, only household food security and number of days with shortness of breath remained marginally significant (*P-value* <0.1) (**Supplementary Table 2.3 and Supplementary Figure 2.1**). When baseline anemia status was

included in regression models (**Supplementary Table 2.4 and Supplementary Figure 2.3**), it was found to be a significant predictor of anemia at endline in bivariate models (OR 2.90, 1.80-4.83) and multivariate models (OR 2.91, (1.91-4.59). Season of enrollment (*P-value* for trend = 0.04), frequent with low appetite (OR 2.00, 1.23-3.26) and shortness of breath (OR 2.91, 1.06-8.43) were also significant predictors of anemia at endline in multivariate models that included baseline anemia status (**Supplementary Table 2.4 and Supplementary Figure 2.3**).

2.4 Discussion

In this secondary analysis of a *randomized, partially double-blind, controlled, community-based efficacy trial of various forms and frequencies of zinc supplementation, we* found many young children in Dhaka, Bangladesh were anemic despite receiving standard, 15-component MNPs containing 10 mg of iron daily for 24 weeks. In bivariate analyses, several socioeconomic, environmental, anthropometric and morbidity risk factors were associated with an increased odds of anemia. However, some risk factor variables lost their statistical significance in multivariate analyses. This is one of few studies to evaluate risk factors for anemia among children receiving MNPs.

In the most recent DHS survey in Bangladesh (2011), the national prevalence of anemia (hemoglobin <11 g/dL) among children 6-59 months was estimated to be 52%, with the highest prevalence observed among children 6-23 months (71%) (20). This is comparable to what we

observed in our study, suggesting limited progress toward reducing anemia among young children in recent years in Bangladesh. In the ZiPT trial, we observed a reduction in anemia prevalence of about 18 percentage points from baseline in the standard MNP group (21). However, we were surprised that nearly 33% (hemoglobin <10.5 g/dL) [46% (hemoglobin <11 g/dL)] of children were anemic at endline despite the provision of MNPs daily for nearly 6 months and high adherence (81.7%). This is twice the WHO-recommended frequency of MNP provision (90 sachets delivered over a 6-month period, or one sachet every other day). This suggests that anemia remained a significant public health problem, as defined by the WHO as an anemia prevalence of >20% among children 6-23 months.

It is not completely unexpected that anemia rates may remain high; anemia is a multifactorial issue, and the causes of anemia have been described as a complex ecology of internal and external factors (22). MNPs were designed to prevent the nutritional causes of anemia by meeting children's daily micronutrient needs, however, MNPs do not address non-nutritional causes (22, 23). Consistent with conceptual models of anemia and previous studies in South Asia (23-25), in bivariate models we observed that children who had an increased risk of anemia resided in households with lower socioeconomic status. Poor socioeconomic status is associated with an increased risk of anemia through multiple pathways, such as poor water, sanitation and hygiene practices and more limited intake of nutrient-dense foods (6). In bivariate models, poor household hygiene practices were associated with anemia. Furthermore, children living in poor sanitary conditions are more likely to experience more morbidity and mortality (26), and we observed that frequent morbidity symptoms were associated with an increased odds of anemia

in this study. Infection increases inflammation, which triggers the release of hepcidin into circulation which can reduce absorption of iron and limit mobilization of iron from body tissues (27, 28). Children who have frequent illnesses are also likely to experience undernutrition and poor growth (29), and we observed that children who were underweight at enrollment were more likely to be anemic at endline.

In multivariate models, many correlates lost their statistically significant association with anemia. This may be explained by the complex interactions between the various underlying risk factors of anemia, as the most vulnerable children are likely to experience several risk factors simultaneously (30, 31). Therefore, although we assessed collinearity, associations with anemia were likely influenced by other underlying and unmeasured risk factor variables in the multivariate models.

It is important to note that baseline anemia status was also a significant risk factor for anemia at endline (**Supplementary Table 2.4 and Supplementary Figure 2.3**). We did not include baseline anemia as a predictor in our primary regression analyses. Because MNP programs are intended to be initiated without assessment of anemia status at the individual level before the intervention is initiated, our goal was to identify the predictors of anemia among children who had received the MNP intervention irrespective of their baseline anemia status. However, we note that when baseline anemia was included as a predictor in multivariate models, the

strength and direction of associations of other predictors with anemia at endline did not change appreciably (**Supplementary Table 2.4**).

Although micronutrient biomarkers were unavailable for all children in the ZiPT study, micronutrient status was measured in a small subset of 58 children enrolled in the standard MNP group (21). At baseline, the prevalence of IDA (anemia defined as hemoglobin < 10.5 g/dL) and inflammation-adjusted low serum ferritin (ferritin < 12/ugL) among these children was 47%, and at endline, the prevalence of IDA was 16% (Supplementary Information, Table 2.5). This suggests that that MNPs were important for reducing the prevalence of IDA from baseline to endline, however, a considerable proportion of anemia at endline may have been due to remaining iron deficiency. IDA is especially common among children 6-23 months, whose iron stores deplete rapidly after birth and iron needs increase substantially during the first two years of life (32).

Our findings highlight that children who experience multiple risk factors for anemia likely need more than MNPs alone. We note that in the control group in the main trial, where children received a placebo powder for 24 weeks, anemia (hemoglobin < 11 g/dL) prevalence was 75% (hemoglobin < 10.5 g/dL; 62%) at endline (24-weeks) compared to a baseline prevalence of 72% (hemoglobin < 10.5 g/dL; 56%) (Islam et al., 2022b). Thus, anemia prevalence persisted over time in the absence of any intervention. This suggests that MNPs were a critical tool for anemia reduction and/or prevention in this population. This also demonstrates the importance of

targeting MNP programs to the children at greatest risk and considering multi-sectoral interventions to address underlying causes of anemia to further reduce anemia in this context. For example, in this study, vulnerable children may have benefited from additional interventions to improve hygiene practices and infectious disease control (33) alongside the provision of MNPs. Furthermore, targeting these interventions among children who were underweight prior to the intervention may have been important for improving anemia outcomes. In addition, prioritizing initiation of MNP provision in the summer months (February to June) season as opposed to the winter (November to February) or Monsoon season (July to October) may have improved anemia outcomes in this setting. This is consistent with evidence suggesting there is a higher prevalence household food insecurity in the boro harvest season (April-June) in Bangladesh (34).

In addition, although children who experienced severe anemia (hemoglobin < 8 g/dL) were referred for further diagnosis and treatment in this study, children who experienced any anemia may have benefitted from additional screening for iron deficiency and potential treatment for IDA (35, 36). The WHO indicates that use of MNPs is intended to be preventative strategy that is implemented at the population level without screening for any condition or disease (8), as laboratory testing used for diagnostic screening is often impractical in programmatic or field settings. However, MNPs provided only 10 mg of iron, and this dose is insufficient to treat IDA (37). Identifying children with IDA may be critical to successfully and sustainably reducing rates of anemia by repleting iron stores via treatment with therapeutic iron supplementation. In addition, interventions aimed toward dietary modification to improve iron intake should be

considered, alongside MNP provision, to promote improvement in iron status. Furthermore, as noted above, these approaches should ideally be combined with infectious disease control so that iron may be absorbed and mobilized from tissues (28).

In this study, we found that a large proportion (13%) of children were no longer considered anemic when the cutoff shifted from 11 g/dL to 10.5 g/dL (**Supplementary Figure 2.1**). The WHO recently updated its guidelines for anemia cutoffs to suggest separate cutoffs for the 6-23 months age group based on consistent reported differences in hemoglobin concentrations among child 6-23 months versus children 24-59 months, as well as differences in nutrient requirements between these two age strata, along with other rationale (19). The updated cutoffs were derived from the 5^{th} percentiles of hemoglobin distributions derived from apparently healthy populations in the USA, Canada, Ecuador and Bangladesh (38). Among the studies used to inform the guidelines, hemoglobin was measured via venous blood and using a hematology analyzer, and we note that the guidelines do not provide adjustments for hemoglobin using single-drop capillary blood using a Hemocue instrument. Very few studies to date have used these new hemoglobin cutoffs for this age group, and we consider this a strength of our analysis. We note that household food security and maternal education were significant predictors of anemia when classified as hemoglobin <11 g/dL (Supplementary Information, Table 2.3 and Figure 2.2), however these variables were non-significantly associated with anemia when classified as hemoglobin <10.5 g/dL in bivariate models.

Additional strengths of this study include the large sample size, the randomized, controlled design, and rigorous data on adherence. In addition, we collected frequent, continuous data on morbidity symptoms throughout the intervention period. However, we acknowledge that this secondary analysis had several limitations. While we included a wide range of risk factors in our analysis, we did not assess the primary causes of anemia, including micronutrient and inflammatory biomarkers, data on genetic hemoglobinopathies and other red blood cell disorders, and infections such as soil-transmitted helminthiasis. We also did not capture data on dietary intake or complementary feeding practices. Finally, we were limited to hemoglobin measurements using single-drop capillary blood collected via fingerprick and measured using a HemoCue 301+ device. Although this method of hemoglobin measurement is frequently used in field settings, our estimates of anemia may have been subject to measurement error, resulting in inaccurate estimates of anemia (39). The gold-standard method of hemoglobin measurement is the assessment of venous blood using an automated hematology analyzer. This method is challenging in field contexts, as well as costly, and there is no consensus regarding the acceptable level of error for population-based assessments performed using single-drop capillary blood and a point-of-care hemoglobinometer. However, using venous blood in a point-of-care hemoglobinometer may introduce less measurement error. We acknowledge this as a limitation of our analysis, and future studies should consider the use of venous blood instead of single-drop capillary blood where possible (40).

In conclusion, this study allowed us to evaluate modifiable risk factors among children who were vulnerable to anemia regardless of MNP provision. Anemia is a complex condition that

requires coordinated efforts to effectively and sustainably reduce it (41). Although MNPs are an important tool for anemia prevention, in isolation, they are insufficient to resolve anemia completely and should be part of an integrated and comprehensive approach for anemia control. This study highlighted areas for implementation of co-interventions that, together with MNPs, may more effectively manage anemia among vulnerable children in this region in Bangladesh. Future studies that leverage multiple data sources, including biomarker data, to elucidate context-specific causes and underlying determinants anemia will be important for MNP program planning.

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Table 2.1: Enrollment characteristics of children randomized to standard micronutrient powder (MNP) daily group

	Standard MNP (N=442)
Child Characteristics	
Age, months	9.74 ± 0.83
Female	51.13% (226)
Breastfeeding	90.95% (402)
Length (1)	69.30 (2.80)
Weight (kg)	7.93 (1.07)
Length-for-age Z-score	-1.14 ± 1.05
Weight-for-age Z-score	-0.92 ± 1.06
Weight-for-length Z-score	-0.38 ± 1.02
Hemoglobin (g/dL)	10.40 ± 1.16
Anemic (Hb <11 g/dL)	68.78% (304)
Anemic (Hb < 10.5 g/dL[1])	54.07% (239)
Child is healthy at enrollment	23.30% (103)
Maternal Characteristics	
Age, years	24.63 ± 5.36
Occupation	
Housewife	93.21% (412)
Work outside/inside the home	6.79% (30)
Years of education completed	6.63 ± 4.14
Socioeconomic Characteristics	
Number of household members	4.50 ± 1.40
Source of drinking water	
Piped into dwelling	72.85% (322)
Piped into yard/plot	22.17% (98)

Other	4.98% (22)
Hygiene score[2]	12.22 ± 2.43
Asset score[3]	5.76 ± 2.37
HFIAS Classification	
Food-secure	78.28% (346)
Food-insecure	21.72% (96)

Note: Values are means ± SD or % (n). Abbreviations: Hb; hemoglobin, HFIAS; Household Food Insecurity Access Scale

[1] Anemia was estimated using revised WHO cutoffs for children 6-23 months. [2]Hygiene score was calculated as the sum of the scores for the following variables: "Wash hands after helping child defecate"; "Wash hands before preparing food"; "Wash hands after using toilet"; and "Uses toilet paper". Each variable was scored as follows: 1 = never, 2 = rarely, 3 = sometimes, 4 = always. [3]Asset scores were the sum of two sub-scores: asset score 1, which was the sum of the following possessions: iron, chair/bench, sofa, table, computer, fridge, motorcycle, and bank account; asset score 2, which was the sum of the following possessions: electric fan, television, mattress, mobile phone. Asset score could range from 4 to 16.

			Minimally-adjusted[1] bivariate model		Multivariate model[2]	
Predictor variable	**N**	**Anemia prevalence at endline (24 weeks) (%)**	**OR (95% CI)**	***P-value***	**OR (95% CI)**	***P-value***
Maternal age, years						
≤20	123	38.21	1	0.22		
21-24	114	32.46	0.76 (0.44-1.30)			
≥25	205	29.27	0.66 (0.41-1.06)			
Maternal Education						
<1 year	60	40.00	1	0.13		
2-7 years	197	35.03	0.80 (0.44-1.47)			
≥8 years	185	27.57	0.57 (0.31-1.05)			
Maternal occupation						
Housewife	412	32.52	1	0.88		
Employed (Works inside or outside of home)	30	33.33	1.06 (0.46-2.29)			
Household size						
4-5	329	31.91	1	0.63		
6-7	113	34.51	1.12 (0.71-1.75)			
Asset Score[3]						
1-3	86	37.21	1	**0.04**	1	0.15
4-7	243	35.39	0.92 (0.56-1.55)		0.99 (0.58-1.69)	
8-12	113	23.01	0.50 (0.27-0.93)		0.59 (0.30-1.13)	
Hygiene Score[4]						
Below median	223	37.34	1	**0.02**	1	0.28
Above median	209	26.27	0.63 (0.42-0.94)		0.79 (0.51-1.22)	
Drinking Water Source						
Piped into dwelling	322	30.43	1	0.11		
Piped into yard/plot	98	35.71	1.27 (0.78-2.03)			
Other	22	50.00	2.41 (0.99-5.89)			

Food Secure[5]						
No	96	38.54	1	0.17		
Yes	346	30.92	0.72 (0.45-1.16)			
Child age at baseline	442		0.99 (0.78-1.26)	0.92	1.03 (0.79-1.32)	0.84
Child sex						
Male	216	35.19	1	0.25	1	0.49
Female	226	30.09	0.79 (0.53-1.18)		0.86 (0.57-1.31)	
Wasted at baseline[6]						
No	421	32.78	1	0.64		
Yes	21	28.57	0.79 (0.28-2.00)			
Stunted at baseline[7]						
No	352	31.25	1	0.26		
Yes	90	37.78	1.80 (1.06-3.02)			
Underweight at baseline[8]						
No	372	30.38	1	**0.03**	1	0.10
Yes	70	44.29	1.80 (1.06-3.02)		1.59 (0.91-2.76)	
Season of enrollment						
Winter (November-February)	82	36.79	1	**0.03**	1	**0.03**
Summer (February-June)	107	26.24	0.61 (0.37-1.01)		0.55 (0.32-0.95)	
Monsoon (July-October)	106	38.81	1.09 (0.64-1.85)		0.99 (0.57-1.72)	
Number of days with low appetite[9]						
Below the median	329	29.48	1	**0.02**	1	**0.01**
Above the median	113	41.49	1.68 (1.08-2.62)		1.94 (1.21-3.13)	
Number of days with acute upper respiratory infection[9]						
Below the median	220	30.00	1	0.29		
Above the median	222	35.14	1.24 (0.83-1.86)			

Number of days with cough[9]						
Below the median	218	35.27	1	0.26		
Above the median	224	29.82	1.26 (0.84-1.89)			
Number of days with any illness[9]						
Below the median	224	31.25	1	0.62		
Above the median	218	33.94	1.11 (0.74-1.66)			
Number of days with runny nose[9]						
Below the median	229	28.38	1	**0.05**	1	0.48
Above the median	213	37.09	1.48 (0.99-2.22)		1.17 (0.76-1.81)	
Number of days with shortness of breath[9]						
Below the median	424	31.37	1	**0.02**	1	**0.02**
Above the median	18	61.11	3.32 (1.28-9.23)		3.36 (1.22-9.79)	
Number of days with elevated respiratory rate[9]						
Below the median	405	32.10	1	0.50		
Above the median	37	37.84	1.27 (0.62-2.55)			
Number of days with vomiting[9]						
Below the median	243	33.74	1	0.52		
Above the median	199	31.16	0.88 (0.58-1.31)			
Number of days with fever[9]						
Below the median	354	31.64	1	0.41		
Above the median	88	36.36	1.23 (0.75-2.00)			
Number of days with ORS use[9]						
Below the median	237	32.07	1	0.83		
Above the median	205	33.17	1.04 (0.70-1.56)			

Number of episodes of acute lower respiratory infection[9]						
Below the median	405	32.10	1	0.50		
Above the median	37	37.84	1.27 (0.62-2.55)			
Number of episodes of diarrhea[9]						
Below the median	307	30.48	1	0.41		
Above the median	135	34.48	1.18 (0.79-1.77)			
Number of episodes of diarrhea with dehydration[9]						
Below the median	422	32.46	1	0.81		
Above the median	20	35.00	1.13 (0.41-2.84)			
Number of episodes of diarrhea with dysentery[9]						
Below the median	420	32.62	1	0.89		
Above the median	22	31.82	0.94 (0.35-2.29)			
Number of episodes of severe diarrhea[9]						
Below the median	329	31.00	1	0.26		
Above the median	113	37.17	1.29 (0.82-2.02)			
Number of hospital episodes[9]						
Below the median	427	32.32	1	0.59		
Above the median	15	40.00	1.34 (0.44-3.80)			

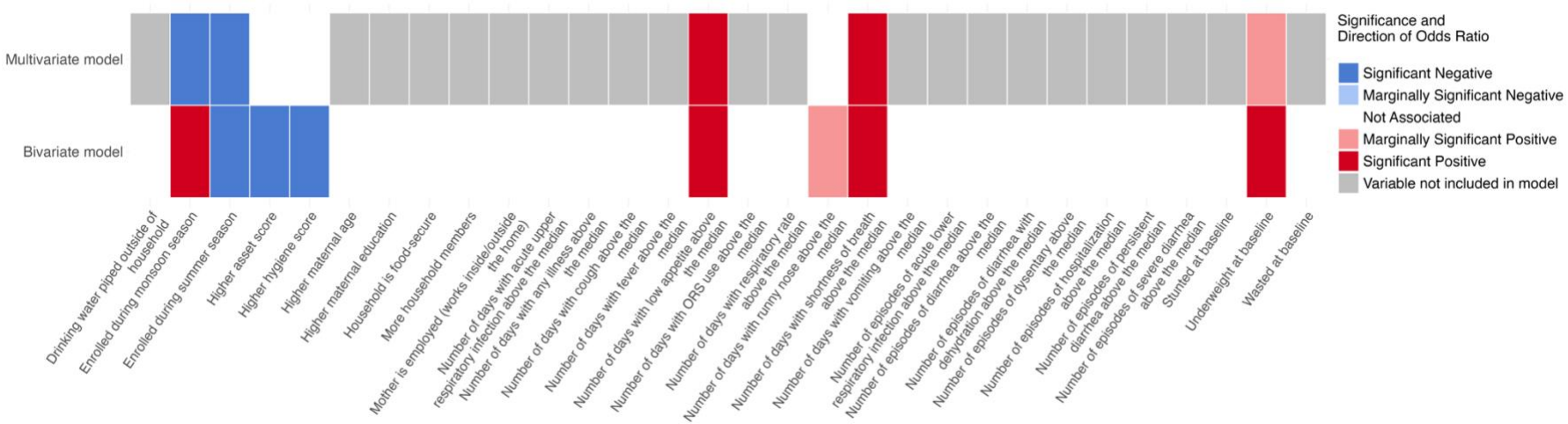
Abbreviations: OR; Odds Ratio, CI; Confidence Interval, ORS; Oral rehydration salts.

[1]Minimally-adjusted models are adjusted for age at baseline and child sex. [2]Multivariate models are adjusted for child age at enrollment, child sex, and all predictor variables that were significantly (*P-value* <0.05) associated with anemia at endline in minimally-adjusted models (asset score, hygiene score, underweight at baseline, season of enrollment, number of days with low appetite, runny nose, and shortness of breath). [3]Hygiene score was calculated as the sum of the scores for the following variables: “Wash hands after helping child defecate”; “Wash hands before preparing food”; “Wash hands after using toilet”; and “Uses toilet paper”. Each variable was scored as follows: 1 = never, 2 = rarely, 3 =

sometimes, 4 = always. [4]Asset scores were the sum of two sub-scores: asset score 1, which was the sum of the following possessions: iron, chair/bench, sofa, table, computer, fridge, motorcycle, and bank account; asset score 2, which was the sum of the following possessions: electric fan, television, mattress, mobile phone. Asset score could range from 4 to 16. [5]As defined by the Household Food Insecurity Asset Scale (HFIAS). [6]Wasted defined as weight-for-length z-score <-2 standard deviations. [7]Stunted defined as length-for-age z-score <-2 standard deviations. [8]Underweight defined as weight-for-age z-score <-2. [9]Morbidity variable definitions can be found Supporting Information: Table 2

1. ICMR-NIN Expert Group on Nutrient Requirements for Indians. Recommended Dietary Allowances (RDA) and Estimated Average Requirements (EAR). Hyderabad: ICMR-National institute of Nutrition, 2020.

Figure 2.1: Heatmap of predictors of anemia (hemoglobin <10.5 g/dL) among children enrolled in the standard MNP daily group[1,2]



[1]Variables are only shown if they are associated (*P-value* <0.1) with anemia at endline (24 weeks) in either minimally-adjusted bivariate or multivariate logistic regression models. [2]Variables described as lower assume the value of the lower (binary) or lowest variable category. Variables described as higher assume the value of the higher (binary) or highest variable category.

Supplementary Table 2.1: Characteristics of Zinc in Powders Trial (ZiPT) Study Interventions

Study Group	Description	Form	Micronutrient Content	Frequency of Supplementation
1	Standard 15-component MNP	Powder	Vitamin A: 400 µg Vitamin D: 5 µg Vitamin E: 5 mg Vitamin C: 30 mg Thiamine: 0.5 mg Riboflavin: 0.5 mg Niacin: 6 mg Pyridoxine: 0.5 mg Vitamin B12: 0.9 mg Folate: 150 µg Iron: 10 mg Zinc: 4.1 mg Copper: 0.56 mg Selenium: 17.0 µg Iodine: 90 µg	Daily for 24 weeks
2	High zinc, low iron MNP	Powder	Same as study group 1, except with 10 mg zinc and 6 mg iron	Daily for 24 weeks
3	High zinc, low iron MNP; high-zinc, no-iron MNP on alternating days	Powder	High zinc, low iron MNP; high-zinc, no-iron MNP on alternating days	Daily for 24 weeks
4	Dispersible zinc supplement	Dispersible tablet	10 mg zinc	Daily for 24 weeks
5	Intermittent zinc supplement	Dispersible tablet	10 mg zinc	Daily for 14 days at enrolment and 3 months, placebo tablet on all other days
6	Placebo powder	Powder	None	Daily for 24 weeks

Supplementary Table 2.2: Definitions of Morbidity Variables

Morbidity Variable	Definition
Diarrhea	Any three or more loose, liquid, or watery stools over a 24-hour period, separated in time from an earlier or subsequent episode for at least 2 consecutive diarrhea-free days since last visit (yes/no) and number of days/episodes.
Severe diarrhea	Severe diarrhea was defined as an episode of diarrhea with dehydration, hospitalization (for any reason), or 6 or more non-bloody loose stools in a single day occurring during the episode since last visit (yes/no) and number of days/episodes.
Dysentery	Any diarrheal episode in which the loose or watery stools contain visible blood since last visit (yes/no) and number of days/episodes.
Diarrhea with dehydration	Diarrhea with dehydration was defined as an episode of diarrhea in which dehydration (reported) occurred at least once since last visit (yes/no) and number of days/episodes.
Fever	Axillary temperature above 38.3°C reported either by the caregiver or the study workers, who carried thermometers, since last visit (yes/no) and number of days.
Acute upper respiratory infection	Any pharyngitis or rhinitis, both without rapid respiratory rate or chest indrawing since last visit (yes/no) and number of days.
Acute lower respiratory infection	Any cough or difficulty breathing, rapid respiratory rate (>50 breaths/minute in infants 9–11 months of age and >40 breaths per minute among infants 12 months of age and older), and either a fever of >38.3°C or chest retractions since last visit (yes/no) and number of days.
Hospitalization	Any overnight stay in the hospital due to illness since last visit (yes/no) and number of days.
Cough	Any cough since last visit (yes/no) and number of days.
Vomiting	Any vomiting since last visit (yes/no) and number of days.
Runny nose	Any runny nose since last visit (yes/no) and number of days.
Low appetite	Any low appetite since last visit (yes/no) and number of days.
Skin rash	Any skin rash since last visit (yes/no) and number of days.
Shortness of breath	Any shortness of breath since last visit (yes/no) and number of days.
Elevated respiratory rate	Rapid respiratory rate (>50 breaths/minute in infants 9–11 months of age and >40 breaths per minute among infants 12 months of age and older) since last visit (yes/no) and number of days.
Any illness	Any morbidity since last visit (yes/no) and number of days (including persistent diarrhea, severe diarrhea, diarrhea with dehydration, dysentery, acute lower respiratory infection, hospitalization, fever, cough, vomiting, runny nose, any illness, low appetite, skin rash, acute upper respiratory infection).

Supplementary Table 2.3: Predictors of anemia (hemoglobin <11 g/dL) among children enrolled in the standard micronutrient powder (MNP) daily group

			Minimally-Adjusted[1] Models		Multivariate Model	
Predictor Variable	**N**	**Anemia Prevalence at Endline (24 Weeks) (%)**	**OR (95% CI)**	**P-Value**	**OR (95% CI)**	**P-Value**
Maternal age (years)						
≤20	123	50.41	1	0.32		
21-24	114	47.37	0.88 (0.52-1.47)			
≥25	205	42.44	0.71 (0.45-1.12)			
Maternal education						
<1 year	60	58.33	1	**0.01**	1	0.28
2-7 years	197	49.75	0.69 (0.38-1.24)		0.84 (0.55-1.28)	
≥8 years	185	37.84	0.43 (0.23-0.77)			
Maternal occupation						
Housewife	412	46.36	1	0.56		
Employed (Works inside or outside of home)	30	40.00	0.80 (0.36-1.69)			
Household size						
4-5	329	45.90	1	1.00		
6-7	113	46.02	1.00 (0.65-1.54)			
Asset Score[2]						
1-3	86	50.00	1	**0.06**	1	0.52
4-7	243	48.97	0.96 (0.59-1.58)		1.14 (0.68-1.93)	
8-12	113	36.28	0.57 (0.32-1.01)		0.086 (0.45-1.65)	
Hygiene Score[3]						
Below median	223	51.93	1	**0.01**	1	0.42
Above median	209	39.23	0.60 (0.41-0.88)		0.84 (0.55-1.28)	
Drinking Water Source						
Piped into dwelling	322	43.17	1	**0.07**	1	0.47
Piped into yard/plot	98	51.02	1.36 (0.86-2.14)		1.07 (0.66-1.74)	
Other	22	63.64	2.53 (1.04-6.56)		1.80 (0.71-4.85)	
Food Secure[4]						
No	96	60.42	1	**0.00**	1	**0.09**
Yes	346	41.91	0.47 (0.30-0.75)		0.64 (0.38-1.07)	
Child age at baseline	442		0.99 (0.78-1.26)	0.92	0.89 (0.70-1.14)	0.37
Child sex						
Male	216	48.61	1	0.79	1	0.47
Female	226	43.36	0.79 (0.53-1.18)		0.87 (0.58-1.28)	
Wasted at baseline[5]						
No	421	46.32	1	0.42		
Yes	21	38.10	0.69 (0.27-1.67)			
Stunted at baseline[6]						
No	352	44.32	1	0.19		
Yes	90	52.22	1.37 (0.86-2.19)			
Underweight at baseline[7]						
No	372	44.09	1	**0.08**	1	0.30
Yes	70	55.71	1.58 (0.95-2.67)		1.33 (0.77-2.33)	

Season of enrollment						
Winter	82	47.17	1	0.21		
Summer	107	41.58	0.80 (0.50-1.28)			
Monsoon	106	51.49	1.18 (0.71-1.97)			
Number of days with low appetite[8]						
Below the median	329	42.86	1	**0.03**	1	**0.05**
Above the median	113	54.87	1.60 (1.04-2.47)		1.57 (0.99-2.48)	
Number of days with acute upper respiratory infection[8]						
Below the median	220	41.82	1	0.51		
Above the median	222	50.00	1.35 (0.93-1.98)			
Number of days with cough[8]						
Below the median	218	40.83	1	**0.05**	1	0.52
Above the median	224	50.89	1.46 (1.00-2.14)		1.16 (0.74-1.80)	
Number of days with any illness[8]						
Below the median	224	42.86	1	0.27		
Above the median	218	49.08	1.24 (0.85-1.82)			
Number of days with runny nose[8]						
Below the median	229	41.48	1	**0.07**	1	0.43
Above the median	213	50.70	1.42 (0.98-2.08)		1.19 (0.77-1.84)	
Number of days with shortness of breath[8]						
Below the median	424	44.81	1	**0.04**	1	**0.10**
Above the median	18	72.22	3.08 (1.14-9.77)		2.48 (0.88-8.13)	
Number of days with elevated respiratory rate[8]						
Below the median	405	45.43	1	0.45		
Above the median	37	51.35	1.30 (0.66-2.59)			
Number of days with vomiting[8]						
Below the median	243	46.91	1	0.60		
Above the median	199	44.72	0.90 (0.62-1.32)			
Number of days with fever[8]						
Below the median	354	45.76	1	0.97		
Above the median	88	46.59	0.99 (0.66-1.50)			
Number of days with ORS use[8]						
Below the median	237	43.04	1	0.21		
Above the median	205	49.27	1.27 (0.87-1.85)			
Number of episodes of acute lower respiratory infection[8]						
Below the median	405	45.43	1	0.45		
Above the median	37	51.35	1.30 (0.66-2.59)			
Number of episodes of diarrhea[8]						
Below the median	307	42.38	1	0.19		
Above the median	135	49.14	1.29 (0.88-1.88)			
Number of episodes of diarrhea with dehydration[8]						
Below the median	422	45.26	1	0.23		
Above the median	20	60.00	1.76 (0.71-4.60)			

Number of episodes of diarrhea with dysentery[8]						
Below the median	420	45.95	1	0.91		
Above the median	22	45.45	0.95 (0.39-2.26)			
Number of episodes of severe diarrhea[8]						
Below the median	329	44.98	1	0.57		
Above the median	113	48.67	1.13 (0.74-1.74)			
Number of hospital episodes[8]						
Below the median	427	45.90	1	0.96		
Above the median	15	46.67	0.97 (0.33-2.76)			

Abbreviations: OR; Odds Ratio, CI; Confidence Interval, ORS; Oral rehydration salts.

[1]Minimally-adjusted models are adjusted for age at baseline and child sex. [2]Hygiene score was calculated as the sum of the scores for the following variables: “Wash hands after helping child defecate”; “Wash hands before preparing food”; “Wash hands after using toilet”; and “Uses toilet paper”. Each variable was scored as follows: 1 = never, 2 = rarely, 3 = sometimes, 4 = always. [3]Asset scores were the sum of two sub-scores: asset score 1, which was the sum of the following possessions: iron, chair/bench, sofa, table, computer, fridge, motorcycle, and bank account; asset score 2, which was the sum of the following possessions: electric fan, television, mattress, mobile phone. Asset score could range from 4 to 16. [4]As defined by the Household Food Insecurity Asset Scale (HFIAS). [5]Wasted defined as weight-for-length z-score <-2 standard deviations. [6]Stunted defined as length-for-age z-score <-2 standard deviations. [7]Underweight defined as weight-for-age z-score <-2. [8]Morbidity variable definitions can be found Supporting Information: Table 2

Supplementary Table 2.4: Predictors of anemia (hemoglobin <10.5 g/dL) among children enrolled in the standard micronutrient powder (MNP) daily group including baseline

			Minimally-adjusted[1] bivariate model		Multivariate model	
Predictor variable	**N**	**Anemia prevalence at endline (24 weeks) (%)**	**OR (95% CI)**	**P-Value**	**OR (95% CI)**	**P-Value**
Maternal age, years						
≤20	123	38.21	1	0.22		
21-24	114	32.46	0.76 (0.44-1.30)			
≥25	205	29.27	0.66 (0.41-1.06)			
Maternal education						
<1 year	60	40.00	1	0.13		
2-7 years	197	35.03	0.80 (0.44-1.47)			
≥8 years	185	27.57	0.57 (0.31-1.05)			
Maternal occupation						
Housewife	412	32.52	1	0.88		
Employed (Works inside or outside of home)	30	33.33	1.06 (0.46-2.29)			
Household size						
4-5	329	31.91	1	0.63		
6-7	113	34.51	1.12 (0.71-1.75)			
Asset Score[2]						
1-3	86	37.21	1	**0.04**	1	0.46
4-7	243	35.39	0.92 (0.56-1.55)		0.99 (0.58-1.72)	
8-12	113	23.01	0.50 (0.27-0.93)		0.55 (0.29-1.15)	
Hygiene Score[3]						
Below median	223	37.34	1	**0.02**	1	0.46
Above median	209	26.27	0.63 (0.42-0.94)		0.84 (0.51-1.33)	
Drinking Water Source						
Piped into dwelling	322	30.43	1	0.11		
Piped into yard/plot	98	35.71	1.27 (0.78-2.03)			
Other	22	50.00	2.41 (0.99-5.89)			
Food Secure[4]						
No	96	38.54	1	0.17		
Yes	346	30.92	0.72 (0.45-1.16)			
Child age at baseline	442		0.99 (0.78-1.26)	0.92	0.97 (0.75-1.26)	0.83
Anemic at baseline (Hb < 10.5 g/dL)						
No	138	62.08	1	**0.00**	1	**0.00**
Yes	304	82.64	2.90 (1.80-4.83)		2.91 (1.91-4.59)	
Child sex						
Male	216	35.19	1	0.25	1	0.61
Female	226	30.09	0.79 (0.53-1.18)		0.89 (0.58-1.37)	
Wasted at baseline[5]						
No	421	32.78	1	0.64		
Yes	21	28.57	0.79 (0.28-2.00)			
Stunted at baseline[6]						
No	352	31.25	1	0.26		
Yes	90	37.78	1.80 (1.06-3.02)			

Underweight at baseline[7]						
No	372	30.38	1	**0.03**	1	0.12
Yes	70	44.29	1.80 (1.06-3.02)		1.58 (0.89-2.78)	
Season of enrollment						
Winter	82	36.79	1	**0.03**	1	**0.04**
Summer	107	26.24	0.61 (0.37-1.01)		0.59 (0.33-1.03)	
Monsoon	106	38.81	1.09 (0.64-1.85)		1.10 (0.62-1.94)	
Number of days with low appetite[8]						
Below the median	329	29.48	1	**0.02**	1	**0.01**
Above the median	113	41.49	1.68 (1.08-2.62)		2.00 (1.23-3.26)	
Number of days with acute upper respiratory infection[8]						
Below the median	220	30.00	1	0.29		
Above the median	222	35.14	1.24 (0.83-1.86)			
Number of days with cough[8]						
Below the median	218	35.27	1	0.26		
Above the median	224	29.82	1.26 (0.84-1.89)			
Number of days with any illness[8]						
Below the median	224	31.25	1	0.62		
Above the median	218	33.94	1.11 (0.74-1.66)			
Number of days with runny nose[8]						
Below the median	229	28.38	1	**0.05**	1	0.47
Above the median	213	37.09	1.48 (0.99-2.22)		1.18 (0.75-1.85)	
Number of days with shortness of breath[8]						
Below the median	424	31.37	1	**0.02**	1	**0.04**
Above the median	18	61.11	3.32 (1.28-9.23)		2.91 (1.06-8.43)	
Number of days with elevated respiratory rate[8]						
Below the median	405	32.10	1	0.50		
Above the median	37	37.84	1.27 (0.62-2.55)			
Number of days with vomiting[8]						
Below the median	243	33.74	1	0.52		
Above the median	199	31.16	0.88 (0.58-1.31)			
Number of days with fever[8]						
Below the median	354	31.64	1	0.41		
Above the median	88	36.36	1.23 (0.75-2.00)			
Number of days with ORS use[8]						
Below the median	237	32.07	1	0.83		
Above the median	205	33.17	1.04 (0.70-1.56)			
Number of episodes of acute lower respiratory infection[8]						
Below the median	405	32.10	1	0.50		
Above the median	37	37.84	1.27 (0.62-2.55)			
Number of episodes of diarrhea[8]						
Below the median	307	30.48	1	0.41		
Above the median	135	34.48	1.18 (0.79-1.77)			

Number of episodes of diarrhea with dehydration[8]						
Below the median	422	32.46	1	0.81		
Above the median	20	35.00	1.13 (0.41-2.84)			
Number of episodes of diarrhea with dysentery[8]						
Below the median	420	32.62	1	0.89		
Above the median	22	31.82	0.94 (0.35-2.29)			
Number of episodes of severe diarrhea[8]						
Below the median	329	31.00	1	0.26		
Above the median	113	37.17	1.29 (0.82-2.02)			
Number of hospital episodes[8]						
Below the median	427	32.32	1	0.59		
Above the median	15	40.00	1.34 (0.44-3.80)			

Abbreviations: OR; Odds Ratio, CI; Confidence Interval, ORS; Oral rehydration salts.

[1]Minimally-adjusted models are adjusted for age at baseline and child sex. [2]Hygiene score was calculated as the sum of the scores for the following variables: “Wash hands after helping child defecate”; “Wash hands before preparing food”; “Wash hands after using toilet”; and “Uses toilet paper”. Each variable was scored as follows: 1 = never, 2 = rarely, 3 = sometimes, 4 = always. [3]Asset scores were the sum of two sub-scores: asset score 1, which was the sum of the following possessions: iron, chair/bench, sofa, table, computer, fridge, motorcycle, and bank account; asset score 2, which was the sum of the following possessions: electric fan, television, mattress, mobile phone. Asset score could range from 4 to 16. [4]As defined by the Household Food Insecurity Asset Scale (HFIAS). [5]Wasted defined as weight-for-length z-score <-2 standard deviations. [6]Stunted defined as length-for-age z-score <-2 standard deviations. [7]Underweight defined as weight-for-age z-score <-2. [8]Morbidity variable definitions can be found Supporting Information: Table 2

Supplementary Table 2.5: Prevalence of Iron Deficiency Anemia (1) among children enrolled in the biochemistry subgroup of the Zinc in Powders trial (ZiPT) using updated hemoglobin cutoffs for anemia (hemoglobin <10.5 d/dL)

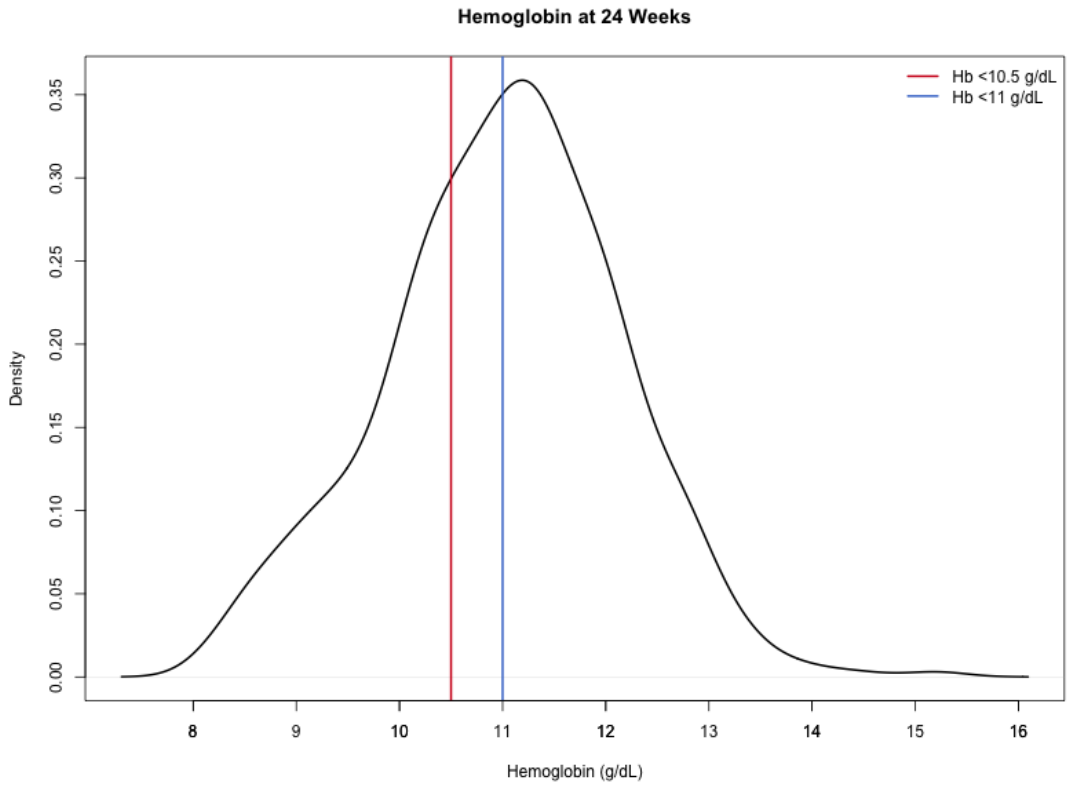
Biochemistry Subgroup: Standard MNP	Prevalence of IDA[1]
Baseline (N=58)	46.55% (27)
24 weeks (N=50)	16.00% (8)

[1]IDA defined as the presence of both anemia (hemoglobin < 10.5 g/dL) and low serum ferritin (ferritin < 12/ugL). Ferritin values were adjusted for inflammation (C-reactive protein (CRP) and alpha(1)-Acid glycoprotein (AGP)) using the BRINDA regression correction approach.

Reference:

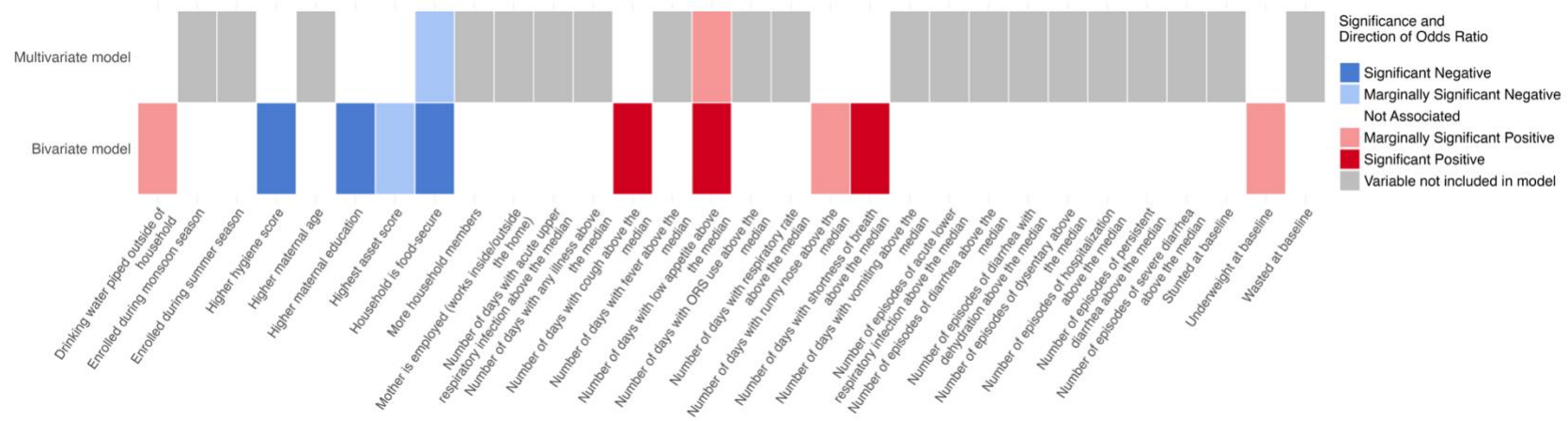
Islam, M. M., Black, R. E., Krebs, N. F., Westcott, J., Long, J. M., Islam, K. M., Peerson, J. M., Sthity, R. A., Khandaker, A. M., Hasan, M., El Arifeen, S., Ahmed, T., King, J. C., & McDonald, C. M. (2022, Dec 15). Effects of Different Doses, Forms, and Frequencies of Zinc Supplementation on Biomarkers of Iron and Zinc Status among Young Children in Dhaka, Bangladesh. *Nutrients, 14*(24). https://doi.org/10.3390/nu14245334

Supplementary Figure 2.1: Density plot of hemoglobin concentrations at 24 weeks



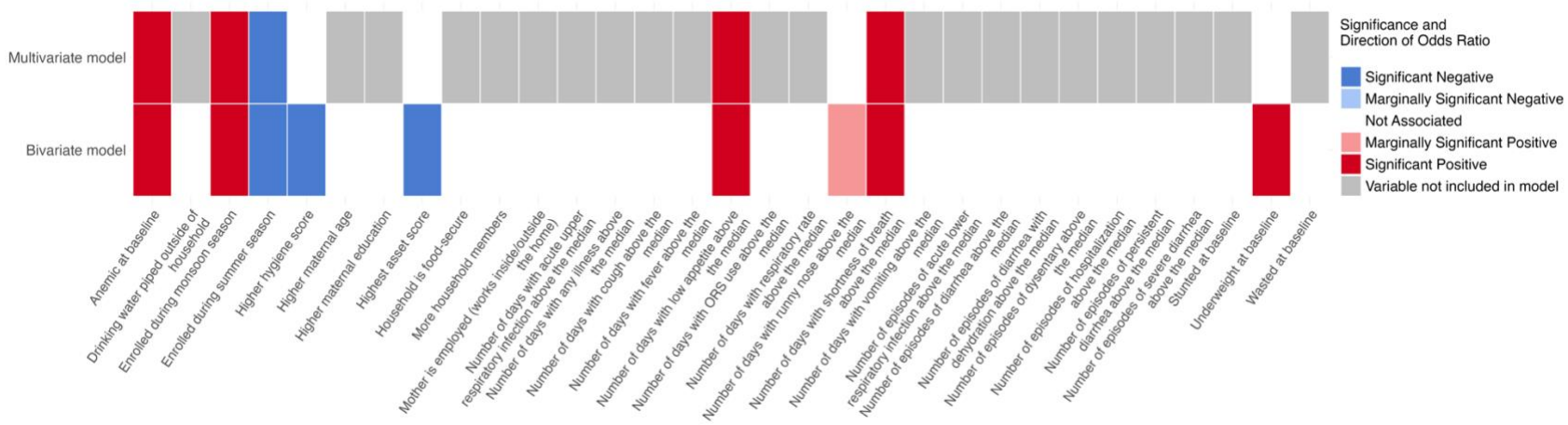
1. Ogawa F, Takachi R, Ishihara J, Yamagishi M, Maruya S, Ishii Y, et al. Dietary sodium sources according to four 3-d weighed food records and their association with multiple 24-h urinary excretions among middle-aged and elderly Japanese participants in rural areas. Br J Nutr. 2023;129(11):1955-63. doi: 10.1017/S0007114522002653.

Supplementary Figure 2.2: Heatmap of predictors of anemia (hemoglobin <11 g/dL) among children enrolled in the standard MNP daily group



[1]Variables are only shown if they are associated (p<0.1) with anemia at endline (24 weeks) in either minimally-adjusted bivariate or multivariate logistic regression models. [2]Variables described as lower assume the value of the lower (binary) or lowest variable category. Variables described as higher assume the value of the higher (binary) or highest variable category.

Supplementary Figure 2.3: Heatmap of predictors of anemia (hemoglobin <10.5 g/dL) among children enrolled in the standard micronutrient powder (MNP) daily group including baseline anemia status as a predictor



[1]Variables are only shown if they are associated (p<0.1) with anemia at endline (24 weeks) in either minimally-adjusted bivariate or multivariate logistic regression models. [2]Variables described as lower assume the value of the lower (binary) or lowest variable category. Variables described as higher assume the value of the higher (binary) or highest variable category.

Chapter 3. The Effect of Quintuply-Fortified Salt on the Gut Microbiome of Non-Pregnant Women of Reproductive Age in Punjab, India; A Substudy of a Randomized, Community-Based Trial[2]

[2]This paper has been published previously [Thompson L, Goh YE, Jamwal M, Singh BL, Brar GK, Arnold CD, et al. The Effect of Quintuply-Fortified Salt on the Gut Microbiome of Nonpregnant Women of Reproductive Age in Punjab, India: A Substudy of a Randomized, Community-Based Trial. The Journal of Nutrition. 2025. doi: https://doi.org/10.1016/j.tjnut.2025.04.012.] Reprinted with permission. This version may contain minor differences, as it does not contain changes made during copyediting.

3.1 Abstract

Background: Women of reproductive age (WRA) in India are vulnerable to multiple micronutrient deficiencies. Large-scale food fortification (LSFF) of staple foods and condiments offers a cost-effective approach to improving micronutrient intake. However, the impact of LSFF on the gut microbiome remains poorly understood.

Objectives: To determine whether quintuply-fortified salt (QFS) alters the gut microbiome of non-pregnant WRA (NPWRA) after 12 months.

Methods: A double-blind, randomized, controlled community-based trial was conducted among 998 NPWRA (18-49 years) in Punjab, India. Eligible participants were randomized to receive 1) QFS with iron as encapsulated ferrous fumarate (eFF), zinc, vitamin B12, folic acid, and iodine (eFF-QFS); 2) QFS with the same micronutrients, but iron as encapsulated ferric pyrophosphate (eFePP) plus ethylenediaminetetraacetic acid (EDTA) (eFePP-QFS); or 3) standard iodized salt. Stool samples were collected from a subsample of NPWRA at baseline and 12 months and subjected to 16S rRNA gene sequencing. Outcomes included intervention effects on alpha diversity (Shannon index and abundance-based estimator (ACE) index) assessed via linear mixed regression models, Bray-Curtis dissimilarity (beta diversity) assessed via permutational multivariate analysis of variance (PERMANOVA), and relative abundance of *Enterobacteriaceae*, *Lactobacillus*, and *Bifidobacterium*, *Prevotella or Streptococcus* modeled using zero-inflated negative binomial mixed regression.

Results: Among the 129 NPWRA who provided both a baseline and 12-month stool sample, 86 had sufficient read depth following sequencing (eFF-Q5S, n = 33; eFePP-Q5S, n = 26; iodized salt, n= 27). Neither alpha diversity nor beta diversity differed significantly at baseline or following the 12-month intervention. There was no intervention effect on relative abundance of individual taxa (*q*-value > 0.05).

Conclusions: QFS did not appear to alter the gut microbiome of NPWRA in Punjab, India.

3.2 Introduction

Multiple micronutrient deficiencies are widespread in northern India, especially among non-pregnant women of reproductive age (NPWRA). A recent survey conducted in selected communities of Punjab, India indicates that 37% of NPWRA are anemic, 67% are iron deficient, and 35% have iron deficiency-anemia (1). Furthermore, 70% of NPWRA experienced RBC folate insufficiency, 34% were zinc deficient, and 37% experience vitamin B12 insufficiency (1). Micronutrient deficiencies among NPWRA are of specific concern, as inadequate periconceptual micronutrient status increases risk of poor pregnancy outcomes, including birth defects and other developmental consequences (2-4). Furthermore, inadequate micronutrient status may increase risk for infectious and chronic diseases (5) and may have educational and economic consequences for NPWRA (6).

Various strategies have been recommended to address micronutrient deficiencies. Supplementation is a targeted strategy designed to close dietary micronutrient gaps among nutritionally vulnerable subgroups of the population. For example, the World Health Organization recommends the provision of iron supplements (30-60 mg per day) to women of reproductive age in areas where anemia prevalence is greater than 40% (7). However, some drawbacks of supplementation include supply chain issues and low rates of adherence (8). This is especially true in India, where adherence to iron and folic acid supplements has been found to be relatively low (<26 %)(9, 10). Furthermore, there is an ongoing concern that oral iron supplements may increase the abundance of enteric pathogens. Iron is an essential, growth-

limiting nutrient for many pathogenic bacteria, and because oral iron has poor bioavailability, unabsorbed luminal iron can be readily metabolized by iron-dependent enteric pathogens (11-13). This may especially be a concern in low-resource settings where the burden of enteropathogens is high (11).

Several animal models and clinical studies among children and adults have demonstrated a proliferation of pathogenic bacteria in the presence of excess luminal iron (13). Specifically, numerous studies investigating the effect of supplemental iron in the form of micronutrient powders (MNPs) on the microbiomes of young children in low-resource settings have found increases in relative abundance of *Enterobacteriaceae*, a bacterial family that includes many known enteric pathogens (14-17). In particular, increases in relative abundance of pathogenic *Escherichia-Coli*, *Campylobacter*, and *Clostridium*, which are frequently responsible for diarrheal diseases, have been observed in response to MNPs (14, 15). These increases in enteric pathogens were often accompanied by relative decreases in bacteria considered to be beneficial, including *Bifidobacterium* and *Lactobacillus* (14, 15). Studies have been more limited among women of reproductive age (WRA), however, a recent study among WRA in Cambodia observed a significant increase in the relative abundance of *Enterobacteriaceae* and *Escherichia-Shigella* in response to daily supplementation with 18 mg or 60 mg of elemental iron (18). These microbiome alterations may result in adverse consequences, including intestinal inflammation, infection, and nutrient malabsorption (11), which may, in-turn, reduce the effectiveness of micronutrient supplementation interventions.

Large-scale food fortification (LSFF) refers to the addition of one or more vitamins and minerals to commonly consumed staple foods or condiments and is designed to improve micronutrient intake across a population at risk for deficiencies (19). This approach can be cost-effective, requires no behavior change, and relies on existing delivery platforms to provide micronutrients, mitigating supply chain issues often observed with supplementation interventions (4, 20). LSFF with multiple micronutrients provides the opportunity to address several micronutrient deficiencies and has consistently proven to improve biomarkers of micronutrient status (21). LSFF with iron effectively delivers a relatively lower dose of iron across a longer duration and may be an important strategy for avoiding potential negative effects of higher iron doses. However, limited research to date has evaluated the effect of LSFF on the microbiome.

This study used 16S ribosomal RNA (rRNA) gene sequencing to identify changes in the microbiome following exposure to quintuply-fortified salt (QFS) with iron, zinc, folic acid, vitamin B12 and iodine among NPWRA in Punjab, India. We aimed to determine whether exposure to QFS for 12 months altered the stool microbiome among NPWRA.

3.3 Methods

Study design

This study is a substudy of a double-blind, randomized, controlled, community-based, trial that took place in the Mohali District of Punjab, India. The full details of the study design can be

found in the study protocol (22). Briefly, NPWRA and their households were randomized to one of three intervention groups 1) QFS with iron in the form of encapsulated ferrous fumarate (eFF), zinc as zinc oxide, vitamin B12, folic acid, and iodine as potassium iodate (eFF-QFS); 2) QFS with the same five micronutrients, but with iron in the form of ferric pyrophosphate plus ethylenediaminetetraacetic acid (EDTA) (eFePP-QFS); 3) iodized salt. Fortification levels for eFF-QFS and eFePP-QFS per gram of salt were: 1.3 mg iron; 1.4 mg zinc; 0.6 µg vitamin B12; 52 µg folic acid, and 30 µg iodine. These levels were based on formative research among women in the study area, which found that mean discretionary salt intake was 4.7 g (1). Prior to randomization, a researcher who was independent of the study assigned two color codes to each of the three salt types and shared these with JVS Foods/Wella Nutralogicals in Jaipur, India, who produced the micronutrient premixes which were blended with iodized salt at the appropriate ratios. Randomization of women to one of the three study groups was implemented via a computer-generated block-randomized assignment, which was computed by the study statistician and shared with the field team. Sealed opaque envelopes containing the color-coded group assignment were prepared in ordered stacks and women were asked to select one envelope. Women were then randomly selected to participate in the microbiome subgroup. The study team remained blinded to the group assignment until after data analysis. The primary outcomes of the main trial included impacts on biomarkers of iron, zinc, vitamin B12, folate and iodine following exposure to the study salt for 12 months.

To participate in the study, women must have met the following criteria: 1) 18-49 years of age; 2) not pregnant (confirmed by a urinary human chorionic gonadotropin (HCG) test); 3) not

severely anemic (defined as a hemoglobin concentration <8.0 g/dL, as measured by fingerprick blood sample and HemoCue® 301+); 4) permanent resident of the study village with no plans to move or travel outside the village for more than 4 weeks over the next 12 months; 5) no serious health problems that require hospitalization or interfere with eating practices; and 6) willingness to use refined salt provided by the study as a primary source of discretionary salt for the household. Written and oral informed consent was obtained from women prior to participation in the study, and the trial protocol was approved by the Institutional Review Board of the University of California San Francisco, the Institutional Ethics Committee of the Post Graduate Institute of Medical Education and Research (23), and India's Health Ministry's Screening Committee. The study was registered at ClinicalTrials.gov (NCT05166980) and in the Clinical Trials Registry-India (CTRI/2022/02/040333).

Following confirmation of eligibility, study staff collected data socio-demographic status and women were scheduled for biochemical and anthropometric assessments in the following 2-3 days. Trained phlebotomists drew 10 mL of whole blood following International Zinc Consultative Group (IZiNCG) protocols for the avoidance of zinc contamination (24). Tubes were stored in coolers at 4°C and then transported to the laboratory at PGIMER within 4 hours of collection. Two trained anthropometrists measured each woman's height to the nearest 0.1 cm using a stadiometer and weight to the nearest 0.1 kg using a digital scale. If the blood draw was successful, women were randomized to one of the three study groups, and an initial disbursement of the study salt was provided for the woman and her household. Additional study salt was provided via monthly home visits for 12 months. Women were instructed to use

the assigned salt to fulfill the cooking and consumption needs of all household members for the duration of the study, and to maintain habitual discretionary salt consumption. Women were then visited on a monthly basis at home to provide additional disbursements of study salt, as well as to weigh and collect any unused and assess acceptability. Salt utilization was calculated from the household salt disappearance data collected at monthly visits and converted into adult female equivalents using household roster data and standard assumptions of weight and total energy expenditure (25, 26). Biochemical and anthropometric assessments were repeated at endline (12 months). Micronutrient and inflammatory biomarkers (serum zinc, serum ferritin, vitamin B12, thyroglobulin, serum soluble transferrin receptor (sTfR), C-reactive protein (CRP), alpha-1-glycoprotein (AGP), urinary iodine, red blood cell and serum folate, plasma homocysteine, serum holotranscobalamin (holoTC), and serum methylmalonic acid were assessed using standard protocols which have been published previously (27, 28). Methods are described in further detail in the study protocol (22) and in the primary endpoints publication(29).

Stool sample collection and sequencing

Stool samples were collected from women enrolled in the microbiome subgroup at home following randomization (baseline) and endline (12 months) using stool sample collection and preservation tubes (Norgen Biotek). Women were instructed to collect stool samples in the morning, and samples were subsequently retrieved by study staff within 4 hours and transported to the PGIMER central laboratory for storage at -20° C until analysis and shipment

to Medgenome in Bangalore, India for sequencing.

DNA extraction was performed using QIAamp PowerFecal Pro DNA Kit and quantified using Qubit DNA High sensitivity Assay (Invitrogen), Qubit RNA Broad range Assay (Invitrogen) and QIAxpert. Samples were diluted to 5 ng and were amplified for the 16S region (1500bp) followed by the Variable 3 Variable 4 (V3V4) region (460-480bp) using 16S and V3V4 primers respectively in a nested PCR approach. PCR Primer sequences are provided in the Supplemental Methods (**Supplementary Table 1**). All PCR products were further processed for DNA library preparation using the Twist MF Library Prep Kit for Illumina. All prepared libraries were checked for fragment distribution using the 5300 Fragment Analyzer system. Prepared libraries were sequenced using the Illumina NextSeq2000 platform. Quantitative Insights into Microbial Ecology 2 (QIIME 2) was used to cluster sequence reads into operational taxonomic units (OTUs) at 99% sequence identity. One representative sequence from each OTU was selected and classified using the Ribosomal Database Project classifier against the SILVA-138 database at 99% similarity.

Statistical analysis

For this substudy, a sample size of at least 50 women per intervention arm was estimated to provide 80% power to detect an effect size of 0.54 standard deviations (SDs) in each outcome between two groups with a level of significance of α=0.05, using ANCOVA models controlling for baseline values (assuming a weak correlation of 0.1 between baseline and endline). Accounting

for an attrition rate of 25%, we aimed to enroll 185 women, or ~62 women per study group to the microbiome subgroup. Due to loss to follow-up and insufficient sequencing depth, the final analyzed sample size was 86 women (eFePP-QFS, n = 33; eFF-QFS, n = 27; iodized salt, n = 26). A sample size of at least 26 per group was estimated to provide 80% power to detect an effect size of 0.79 SDs in each outcome between two groups.

All statistical analyses were performed using R software (Version 2024.09.0+375). All tests were conducted as two-tailed tests with a *p*-value <0.05 were statistically significant. Covariates were selected a priori based on biological plausibility and prior evidence linking them to gut microbiome composition. In all models, only those covariates that showed evidence of association with the outcome at *p-value* < 0.1 were retained to balance model parsimony with adequate adjustment for confounders. Selected covariates included: age, continuous serum AGP values at baseline, continuous serum CRP values at baseline, continuous serum ferritin values at baseline, continuous serum zinc values at baseline, continuous hemoglobin values at baseline, continuous Household Food Insecurity Access (HFIAS) score (30) at baseline, and household assts index measured at baseline. Log-transformations were performed on CRP, AGP, and ferritin to address skewness and approximate normal distributions, and ferritin values were corrected for inflammation (31).

Shannon Diversity and ACE indices were used to determine overall microbial diversity within each sample (alpha diversity). Shannon diversity and ACE indices were estimated using the vegan package in R (32). As Shannon diversity and ACE metric values were normally distributed,

linear mixed models were used to estimate intervention effects on each alpha diversity metric. An (intervention group*endline) interaction term was used to quantify the intervention effect, and participant ID was included as a random effect. Shannon diversity models were adjusted for serum AGP values at baseline, hemoglobin at baseline, serum ferritin values at baseline, and serum zinc at baseline. ACE metric models were adjusted for AGP values at baseline, CRP values at baseline, and serum zinc values at baseline. Residual diagnostic plots (residual plots and quantile-quantile (QQ) plots) were inspected to assess model fit and normality of residuals.

The distribution of microbial diversity between samples (beta diversity) at endline was evaluated using Bray-Curtis distances, which were estimated using normalized OTU counts via the vegan package in R (32). OTU counts were normalized via cumulative sum scaling (CSS) using the metagenome package in R (33). Linear regression models were used to estimate intervention effects on Bray-Curtis pairwise dissimilarity between baseline and endline measurements with covariate adjustment where the pre-selected covariates mentioned above were associated with the outcome at p-value <0.1. No pre-specified covariates were associated with Bray-Curtis pairwise distance at p-value <0.1, thus the linear regression models were not covariate-adjusted. Residual diagnostic plots (residual plots and QQ plots) were inspected to assess model fit and normality of residuals. Permutational multivariate analysis of variance (PERMANOVA) was also used to estimate Bray-Curtis dissimilarity between intervention groups. The PERMANOVA model included Bray-Curtis matrix at endline as the outcome, with 999 permutations to assess statistical significance. The Bray-Curtis distance matrix was also used in principal coordinates analysis (PCoA) to visualize clustering by intervention group at endline.

Intervention effects on abundance of prespecified bacterial taxa of interest (*Enterobacteriaceae, Lactobacillus, and Bifidobacterium, Prevotella, Streptococcus*) were estimated using zero-inflated negative binomial mixed models (glmmTMB package in R (34)). These taxa were chosen based on their abundance in this population (within the top 25 most abundant genera), and the documented interaction between iron interventions and alterations in abundance of these taxa. The zero-inflated negative binomial mixed model was chosen to accommodate the distribution of the abundance data, which are count data and were over-dispersed and zero-inflated. The intervention effect was quantified using a (intervention group*endline) interaction term in the fixed-effects portion of the model. Total OTU counts were included as an offset term to account for varying total counts in the fixed effects portion of the model, and participant ID was included as a random effect. Models were adjusted for pre-specified covariates described above that were associated with each taxon separately at a p-value < 0.1. The model with *Bifidobacterium* as the outcome was adjusted for serum CRP values at baseline, serum zinc values at baseline, serum AGP at baseline, and asset index at baseline. Model with *Lactobacillus* as the outcome was adjusted for ferritin values at baseline. Model with *Prevotella* as the outcome was not covariate-adjusted. Model with *Streptococcus* specified as the outcome was not covariate-adjusted. Model with *Enterobacteriaceae* as the outcome was adjusted for serum zinc values at baseline, hemoglobin values at baseline, and household assets index. Model fit was assessed via the DHARMa package in R (35), which provides a simulation-based approach to generating standardized residuals for generalized linear mixed models. Diagnostic plots of residuals versus predicted values and QQ-plots were generated and visually inspected. Statistical tests for

overdispersion were conducted using the testDispersion() function, and for zero-inflation using the testZeroInflation() function. *Q*-values were calculated from *p*-values corrected for false discovery rate (FDR) using Benjamini-Hochberg correction. A *q*-value <0.05 was considered statistically significant. Lastly, an exploratory differential abundance analysis was performed on all bacterial genus groups using Analysis of Microbiomes with Bias Correction 2 (36) (ANCOMBC 2) via the phyloseq and ANCOMBC R packages and also assessed via *q*-values.

3.4 Results

Between August 2022-July 2023, 1,204 were screened and 998 women were enrolled and randomized to one of the three intervention groups. Following randomization, a subsample of 189 women were randomly selected to the microbiome subgroup (eFePP-QFS, n = 65; eFF-QFS, n = 63; iodized salt, n = 59). 19 women in the eFePP-QFS and iodized salt subgroups, and 20 women in the eFF-QFS subgroup were excluded as they did not complete the study and/or provide an endline stool sample. Further, 13 women in the eFePP-QFS subgroup, 16 women in the eFF-QFS subgroup, and 14 women in the iodized salt subgroup were excluded due to inadequate sequencing depth in either the baseline or endline sample (final classified read depth below 10,000 reads). Ultimately, 86 women with paired baseline and endline samples with sufficient sequencing depth were included in the analysis (eFePP-QFS, n = 33; eFF-QFS, n = 27; iodized salt, n = 26). Loss to follow-up was not statistically different by group. A participant flow diagram is shown in **Supplementary Figure 3.1**.

Baseline characteristics were similar between groups (**Table 3.1**). Overall, women were 30 years old on average. Levels of education were high, with more than 80% of women completing middle school or higher. More than 94% of households were food secure, and over 62% of women achieved minimum dietary diversity (MDD). Over 52% of women were overweight or obese, over 31% had iron-deficiency anemia and nearly 20% had hypozincemia. There were no statistical or substantive differences in baseline characteristics among the women who were randomly selected to the microbiome but were excluded due to inadequate sequencing depth (n = 43), and those who were included in the analysis (n = 86) (**Supplementary Table 3.2**). Average household salt intake was estimated to be about 6 grams per adult female equivalent (AFE) per day across all three groups.

At baseline, *Prevotella, Bifidobacterium* and *Lactobacillus*, were among the most abundant genera among women. In addition, these genera frequently appeared together in individual microbiome communities (**Figure 3.1**). There were no differences in alpha diversity (Shannon diversity and ACE index) between groups at baseline nor at the end of the 12-month intervention period (group-by-time interaction, *p*-value >0.05) (**Figure 3.2A**). Changes in pairwise Bray-Curtis distances between baseline and endline (12-months) did not differ significantly between groups (**Figure 3.2B**). Bray-Curtis dissimilarity did not significantly differ between intervention groups after visual inspection with PCOA plots (**Figure 3.2C**) and PERMANOVA testing (*p*-value = 0.17, R^2 = 0.03). At baseline and after 12 months, there were no differences in the relative abundance of *Prevotella, Bifidobacteria, Lactobacillus, Streptococcus, Bacteroides* nor *Enterobacteriaceae* between groups (**Figure 3.3**). Differential abundance testing

using ANCOMBC revealed differences in the relative abundance of 10 genus groups at endline between at least two intervention groups. After FDR correction, no taxa remained significantly different (group-by-time interaction, *q*-value >0.05) between groups at endline.

All analyses were replicated in the full sample (n = 129) to ascertain whether our findings were altered by exclusion of women with inadequate sequencing depth. Effect estimates were similar in terms of magnitude and direction, indicating that exclusion of these women did not meaningfully alter our findings. A summary of the baseline characteristics stratified by included and excluded women can be found in **Supplementary Table 3.2**.

3.5 Discussion

This study is the first to our knowledge to evaluate the effect of large-scale food fortification (LSFF) in the form of QFS on microbiome outcomes among NPWRA. We observed no significant alterations in the overall structure and composition of the microbiome in terms of diversity, relative abundance of taxa, or potential pathogens belonging to the *Enterobacteriaceae* family. We observed no differential effects by the form of iron (eFePP plus EDTA or eFF). Our findings mirrored other studies of microbiome composition among individuals in Northern India (37-40). We saw a dominance of the genus *Prevotella* among women in this study, which is commonly observed among those consuming a predominately lacto-vegetarian dietary pattern high in carbohydrate, dietary fiber and fermented dairy products (39).

Studies examining the effects of oral iron supplementation in low- and middle-income country (LMIC) settings frequently report negative effects on the gut microbiome (11, 41, 42). In our study, we were specifically interested in whether the level of iron provided in QFS would elicit adverse changes to the composition of the microbiome among women similar to those observed in oral iron supplementation interventions, as no other studies to our knowledge have investigated the effect of LSFF with iron among NPWRA. QFS contained 1.3 mg of iron per gram of salt; at this level of fortification, we observed no alterations to the microbiome in terms of microbial diversity or relative abundance of potential pathogens, or beneficial microbes belonging to the *Lactobacillus* or *Bifidobacteria* genus groups which require no or little iron. These results differ from a recent study of iron supplementation among women who received either 18 mg (n=46) of ferrous bisgycinate, 60 mg of ferrous sulfate (n = 40) or placebo (n = 47) for 12 weeks in Cambodia. In this study, the authors noted an increase in the relative abundance of *Enterobacteriaceae* among the women receiving ferrous bisgycinate (18). In addition, the authors found an increase in the relative abundance of the *Escherichia-Shigella* genus among WRA in the ferrous bisgycinate group and the ferrous sulfate group (18). Furthermore, several randomized controlled trials among young children in Africa and South Asia have demonstrated negative effects of iron interventions, such as the provision of MNPs containing 2.5 to 12.5 mg of iron on both clinical and microbiome outcomes, including diarrhea, increased intestinal inflammation and increased enteropathogen burden (14-17, 43, 44).

In contrast, adverse effects of iron supplementation are less frequently reported in high-income settings, or settings with low enteropathogen burden is low. For example, in a randomized

controlled trial of iron supplementation (65.7 mg ferrous fumarate, n = 38) versus placebo (n = 41) for 21 days among NPWRA in the high-income setting in South Australia, the authors observed no changes to the microbiome in terms of beta diversity or relative abundance of *Escherichia-Shigella* (45). Additionally, studies of iron supplementation among children in the USA and Canada found no impact on the microbiome (46, 47). Lastly, in a randomized trial of iron supplements (50 mg/d for 4 days a week) among South African children from an area with an improved water supply and better hygiene results showed no significant effects on gut inflammation (measured by fecal calprotectin) or on the microbiome. This evidence suggests that context is crucial, especially with regard to baseline carriage of enteric pathogens. Individuals living in poor sanitary conditions are more likely to be exposed to opportunistic pathogens, and unabsorbed dietary iron from supplements can further promote the proliferation of these existing pathogens in the intestine (11). Therefore, interventions that deliver a lower effective daily iron dose, such as LSFF, may be favorable with respect to microbiome outcomes in LMIC settings.

We note that in this study, women resided in relatively hygienic households with access to piped water and flush-toilets. Women reported no diarrhea at the time of sample collection and the incidence of diarrhea across the intervention period was very low. Prevalence of genus groups such as *Escherichia-Shigella, Vibrio,* and *Clostridium* were low or not detected in this population, indicating low baseline prevalence of potential enteric pathogens. One potential pathogen, *Streptococcus*, a bacterial genus group that contains strains known to cause disease in humans (48), was found in 99% of samples in this population with a median relative abundance of 0.24%

(**Supplementary Table 3.3**). However, we observed no increases in *Streptococcus* in response to the intervention. These favorable sanitation and hygiene conditions and low baseline carriage of enteric pathogens may partly explain the absence of adverse microbiome outcomes in this study. It is important to also note that in the main trial, the odds of iron deficiency trended lower (*p*-value <0.1) in the eFF-QFS vs. IS group at 12-months (geometric mean ratio: 0.58 (95% CI, 0.35, 0.95)). However, this effect was not observed in the eFePP-QFS vs. IS group (29). This suggests that eFF-QFS may have been more efficacious to improve iron status, and women may have benefitted from a higher iron level in the QFS.

QFS also contained additional nutrients beyond iron, including zinc, vitamin B12, folic acid, and iodine, and we observed significant improvements in parameters of B12, folate and zinc status among all women enrolled in the main trial (29). Multiple micronutrient deficiencies frequently coexist with enteric infections, and addressing these issues through LSFF may provide synergistic benefits. Zinc is essential for immune function and intestinal wall integrity, as evidenced by negative alterations to the microbiome in the setting of zinc deficiency (49-51). Vitamin B12, an essential nutrient produced and utilized by the microbiome, has been linked to increased alpha diversity and higher relative abundances of *Bacteroides* and *Bifidobacteria* in both in-vitro and in-vivo studies (52). It is difficult to determine whether the presence of additional micronutrients may have mitigated potential alterations to the microbiome by iron, however, some evidence suggests that the addition of zinc to iron-containing interventions can improve microbiome outcomes (17, 53). Further research is needed to explore potential synergistic effects of multiple micronutrients on the microbiome.

Our study has a number of strengths, including the double-blind, randomized, controlled design. We used baseline-adjusted zero-inflated negative binomial linear mixed models to enable rigorous assessment of longitudinal changes in relative abundance of taxa due to the intervention, and to account for inter-individual variability. We also adjusted models for baseline inflammation and micronutrient deficiencies, which are known to influence the microbiome (49). We conducted differential abundance analysis using ANCOMBC, which takes into account the compositional nature of microbiome data (36), on all taxa to identify changes in rare or low-abundance genera. We acknowledge that this study also has several limitations. First, the use of 16S rRNA gene sequencing limited taxonomic resolution to the genus level, precluding species-level analysis. Furthermore, we did not include metagenomic or metatranscripomic data in our analysis, limiting our ability to draw conclusions regarding functional changes to the microbiome in response to the intervention. Sample quality was another limitation, as we were unable to include 43 women due to poor sample integrity and insufficient DNA for sequencing, possibly due to issues with home-based stool sample collection. Thus, our final sample size was smaller than our original intended sample size, which resulted in less statistical power to detect an effect, thus limiting our ability to identify modest changes to the microbiome. Although we found no substantive differences in our interpretation of the results when we replicated our analyses in our full sample, our power to detect small effect sizes was undoubtedly diminished. Consequently, we emphasize that our non-significant findings should be interpreted with caution. In addition, we were limited to two collection timepoints (baseline and 12-month endline), restricting our ability to capture temporal microbiome dynamics. Lastly, although we

collected markers of systemic inflammation, we did not collect any additional markers of intestinal inflammation or enteric dysfunction, such as fecal calprotectin.

To our knowledge, this is the first study to examine microbiome changes in response to LSFF. We observed no significant alterations to the gut microbiome among NPWRA who received QFS for 12 months in Northern India. Additional studies among NPWRA in other settings and with larger sample sizes, especially where enteropathogen burden is high, are needed in order to confirm these findings.

3.6 References

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Table 3.1: Baseline characteristics of women randomly selected to the microbiome subgroup with sufficient read depth following sequencing (n = 86)

	Mean ± SD or n (%)		
Characteristics	**FePP-QFS (n = 33)**	**eFF-QFS (n = 27)**	**Iodized Salt (n = 26)**
Age (years)	30.9 ± 5.2	30.3 ± 4.2	29.9 ± 5.1
Married	32 (97%)	27 (100%)	26 (100%)
Religion			
Hindu	6 (18%)	12 (44%)	6 (23%)
Muslim	2 (6%)	1 (4%)	2 (18%)
Sikh	25 (76%)	14(52%)	18 (69%)
Educational attainment			
None	1 (3%)	0 (0%)	0 (0%)
Primary	3 (9%)	2 (7%)	1 (4%)
Middle/Secondary	22 (67%)	20 (74%)	17 (65%)
Diploma/Post-graduate	6 (18%)	4 (15%)	7 (27%)
Employed outside of household	1 (3%)	1 (4%)	3 (12%)
Number of household members	6.1 ± 1.9	6.7 ± 2.4	6.7 ± 2.4
Household income (rupees)			
< 10,000	3 (9%)	2 (7%)	2 (8%)
10,000-12,000	8 (24%)	7(26%)	10 (38%)
13,000-25,000	15 (45%)	11 (41%)	8 (31%)
26,000-50,000	7 (21%)	2 (11%)	5 (19%)
>50,000	0 (%)	4 (15%)	1 (4%)
Household is food-secure[1]	31 (94%)	26 (96%)	25 (96%)
Average household salt intake (g/AFE)[2]	6.2 ± 1.3	5.9 ± 1.6	5.7 ± 1.1
Minimum Dietary Diversity	25 (76%)	17 (63%)	16 (62%)
BMI (kg/m^2)			
Underweight (<18.5)	1 (3%)	3 (11%)	1 (4%)
Normal (18.5-24.9)	15 (45%)	9 (33%)	8 (31%)
Overweight (25.0-29.9)	17 (52%)	15 (56%)	17 (65%)
Taking antibiotics	0 (0%)	0 (0%)	0 (0%)
Anemia (hemoglobin < 12 g/dL)	13 (39%)	15 (56%)	8 (31%)
Iron deficiency (serum ferritin[1] < 15 ug/L)	22 (67%)	17 (63%)	16 (62%)
Iron deficiency anemia (hemoglobin < 12 g/dL and serum ferritin[3] < 15 ug/L)	13 (39%)	12 (44%)	8 (31%)
Hypozincemia (serum zinc < 70 ug/dL)	8 (24%)	9 (33%)	5 (19%)
Inflammation			
CRP ≥ 5 mg/L	4 (12%)	5(19%)	5 (19%)
AGP ≥ 1 g/L	8 (24%)	11 (41%)	8 (31%)

Definitions: QFS; quintuply fortified salt, eFF-QFS; QFS with iron in the form of eFF, eFePP-QFS; QFS with iron in the form of ferric pyrophosphate plus ethylenediaminetetraacetic acid (EDTA), AFE; Adult female equivalent, CRP; C-reactive protein, AGP; Alpha-1 glycoprotein

[1]Household food security was estimated using the Household Food Insecurity Access Scale (HFIAS)(1)

[2]Salt utilization was calculated from the household salt disappearance data collected at monthly visits and converted into adult female equivalents using household roster data and standard assumptions of weight and total energy expenditure(2, 3)

[3]Serum ferritin values were adjusted for inflammation using the BRINDA regression equations (4, 5)

References:

1. Coates J, Swindale A, Bilinsky P. Household Food Insecurity Access Scale (HFIAS) for measurement of food access: indicator guide: version 3. 2007.
2. Human energy requirements: report of a joint FAO/ WHO/UNU Expert Consultation. Food Nutr Bull. 2005;26(1):166.
3. Weisell R, Dop MC. The adult male equivalent concept and its application to Household Consumption and Expenditures Surveys (HCES). Food Nutr Bull. 2012;33(3 Suppl):S157-62. doi: 10.1177/15648265120333s203.
4. Rohner F, Namaste SM, Larson LM, Addo OY, Mei Z, Suchdev PS, et al. Adjusting soluble transferrin receptor concentrations for inflammation: Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project. Am J Clin Nutr. 2017;106(Suppl 1):372s-82s. doi: 10.3945/ajcn.116.142232.
5. Namaste SM, Rohner F, Huang J, Bhushan NL, Flores-Ayala R, Kupka R, et al. Adjusting ferritin concentrations for inflammation: Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project. Am J Clin Nutr. 2017;106(Suppl 1):359s-71s. doi: 10.3945/ajcn.116.141762.

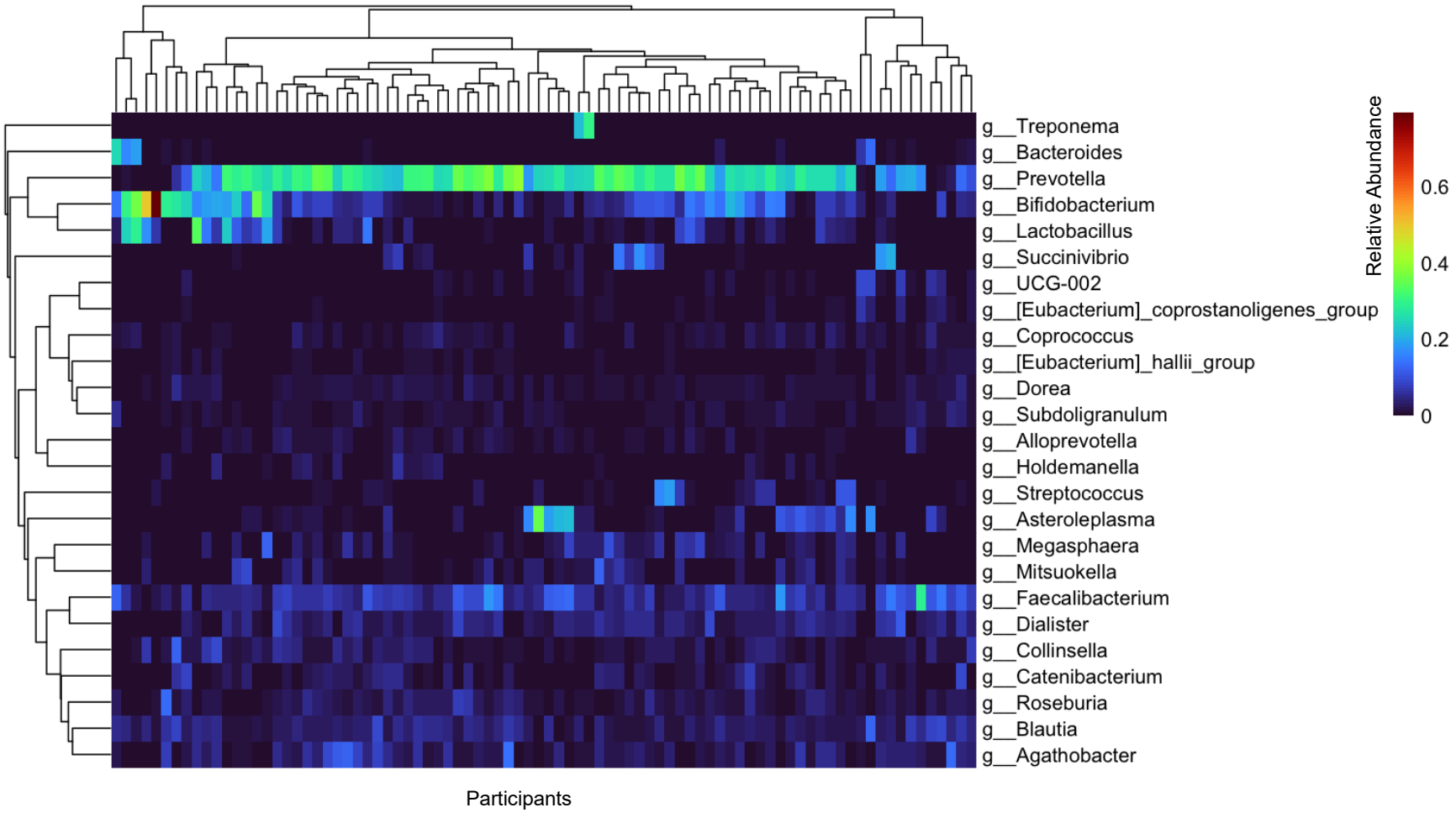


Figure 3.1: Genus-level microbiota composition at baseline among non-pregnant women of reproductive age at baseline. Heatmap representing the 25 most abundant genus groups in baseline stool samples based on relative abundance; dendrograms illustrated on the x and y axes are based on Bray-Curtis distances and represent which genera (rows) or samples (columns) cluster together based on similarity in their community composition.

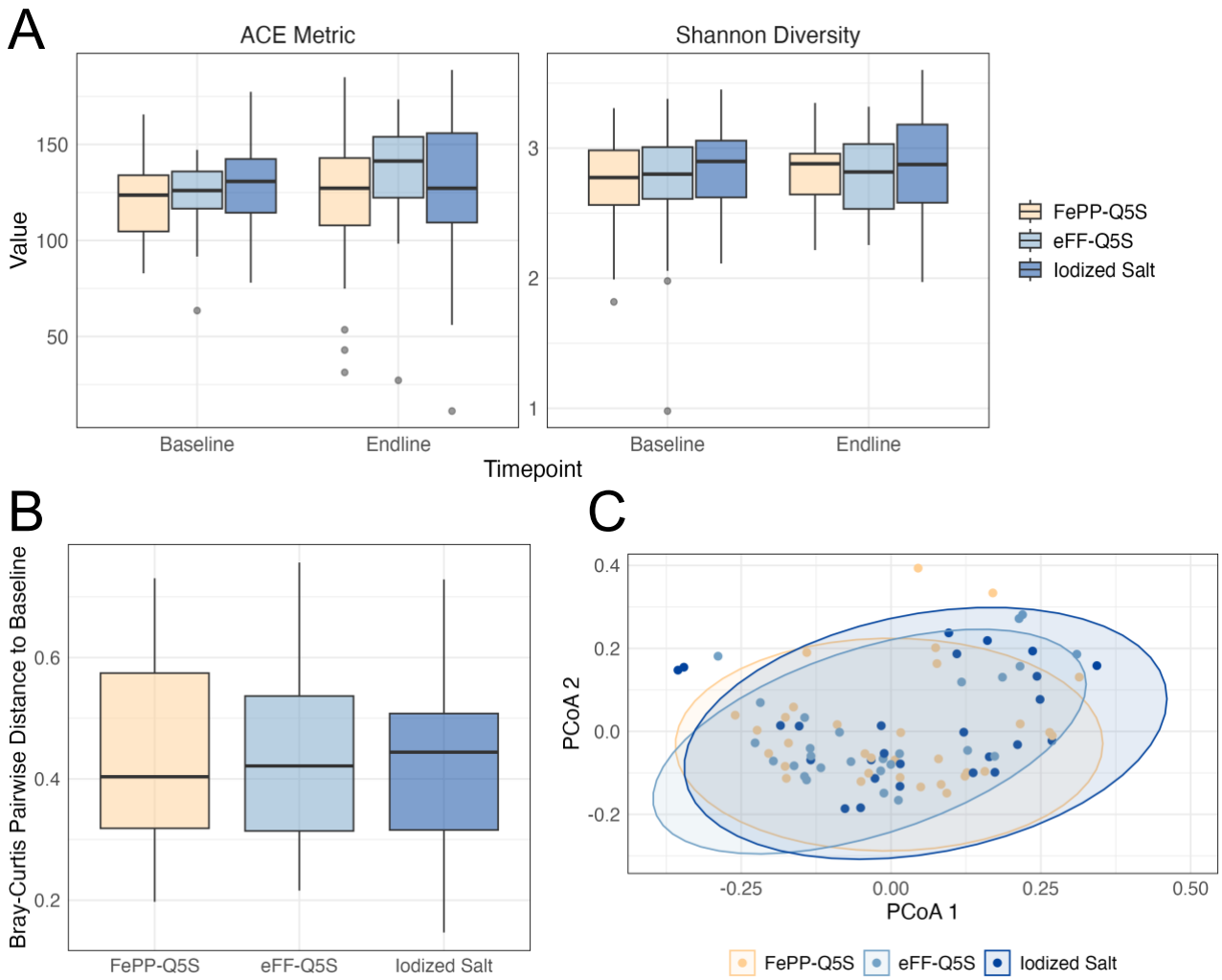


Figure 3.2: Impact of quintuply fortified salt (QFS; QFS with iron in the form of encapsulated ferrous fumarate (eFF-Q5S) and QFS with iron in the form of ferric pyrophosphate plus ethylenediaminetetraacetic acid (EDTA) (FePP-Q5S)) on the fecal microbiome in terms of alpha and beta diversity, compared to iodized salt control. **(A)** Effect of QFS on measures of alpha diversity (Abundance-Based Estimator (ACE) metric and Shannon Diversity) measured at baseline and endline (12 months). Intervention effects were measured using linear mixed models evaluated using a (intervention group*endline) interaction term in the fixed effects portion of the model. A random effect of participant ID was also included. Covariate adjustment was only applied if baseline covariates specified a-priori were associated (*p-value* < 0.1) with alpha diversity in bivariate models (pre-specified covariates: continuous, log-transformed serum alpha-glyco protein (AGP) at

baseline, continuous, log-transformed serum C-reactive protein (CRP) values at baseline, continuous serum zinc values at baseline, continuous hemoglobin values at baseline, inflammation-adjusted serum ferritin values at baseline, household food insecurity access score (HFIAS) score at baseline and household asset index at baseline). Models with ACE metric specified as the outcome were adjusted for serum AGP and CRP values at baseline and baseline serum zinc values. Models with Shannon Diversity specified as the outcome were adjusted for serum AGP at baseline, hemoglobin values at baseline, serum ferritin values at baseline, and serum zinc values at baseline. No significant intervention effects were identified for either alpha diversity measure. **(B)** Boxplot of Bray-Curtis pairwise distances to baseline to identify structural changes within individuals from baseline to endline. Intervention effects were measured using linear regression models without covariate adjustment, as none of the pre-specified covariates measured at baseline were associated with Bray-Curtis pairwise distance. No statistical differences in pairwise distances between any two intervention groups were identified. **(C)** A principal coordinate (PCoA) plot of Bray-Curtis distances at the endline timepoint. No statistical differences between intervention groups were identified using Permutational multivariate analysis of variance (PERMANOVA) testing on the Bray-Curtis matrix at endline with 999 permutations to assess statistical significance.

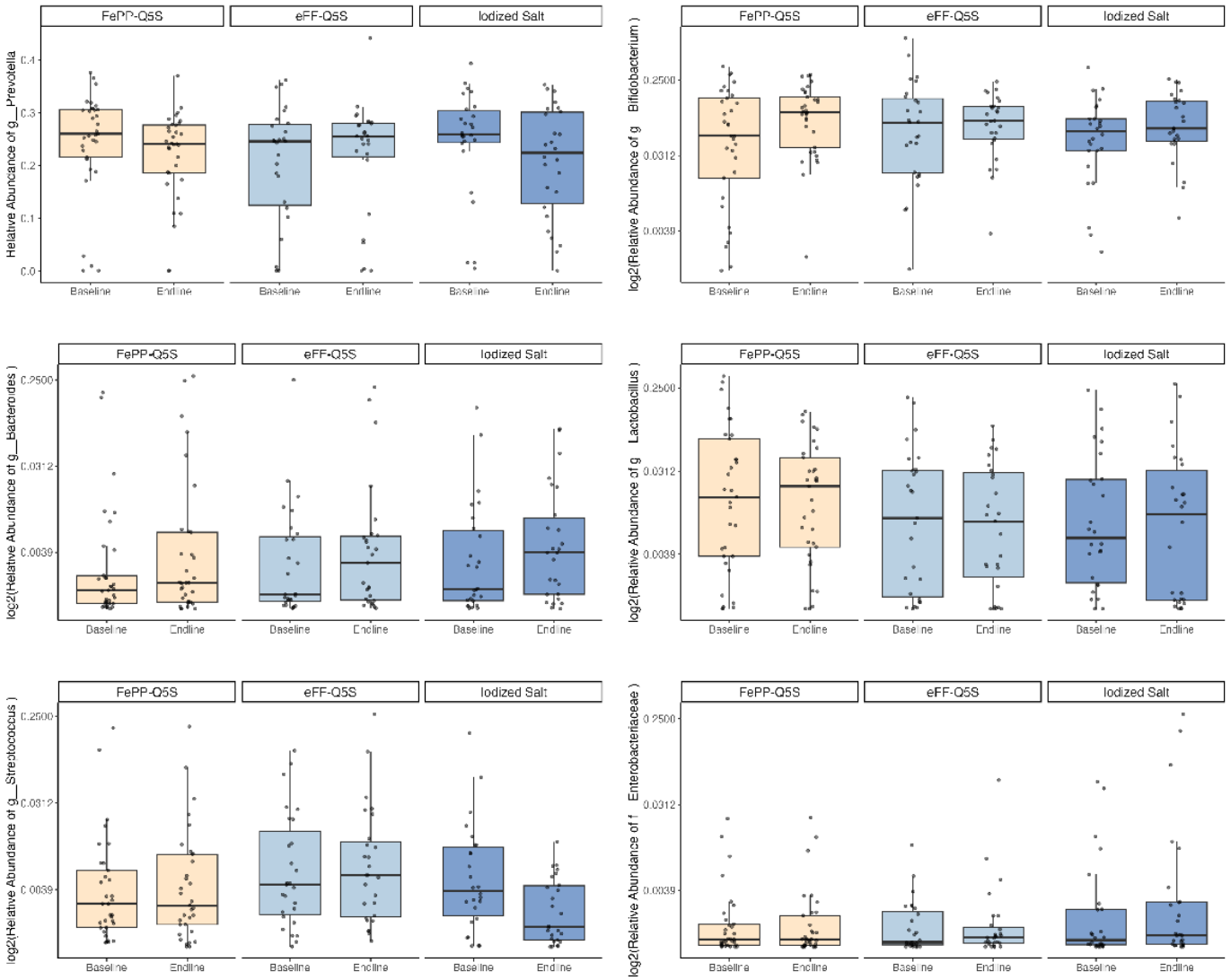
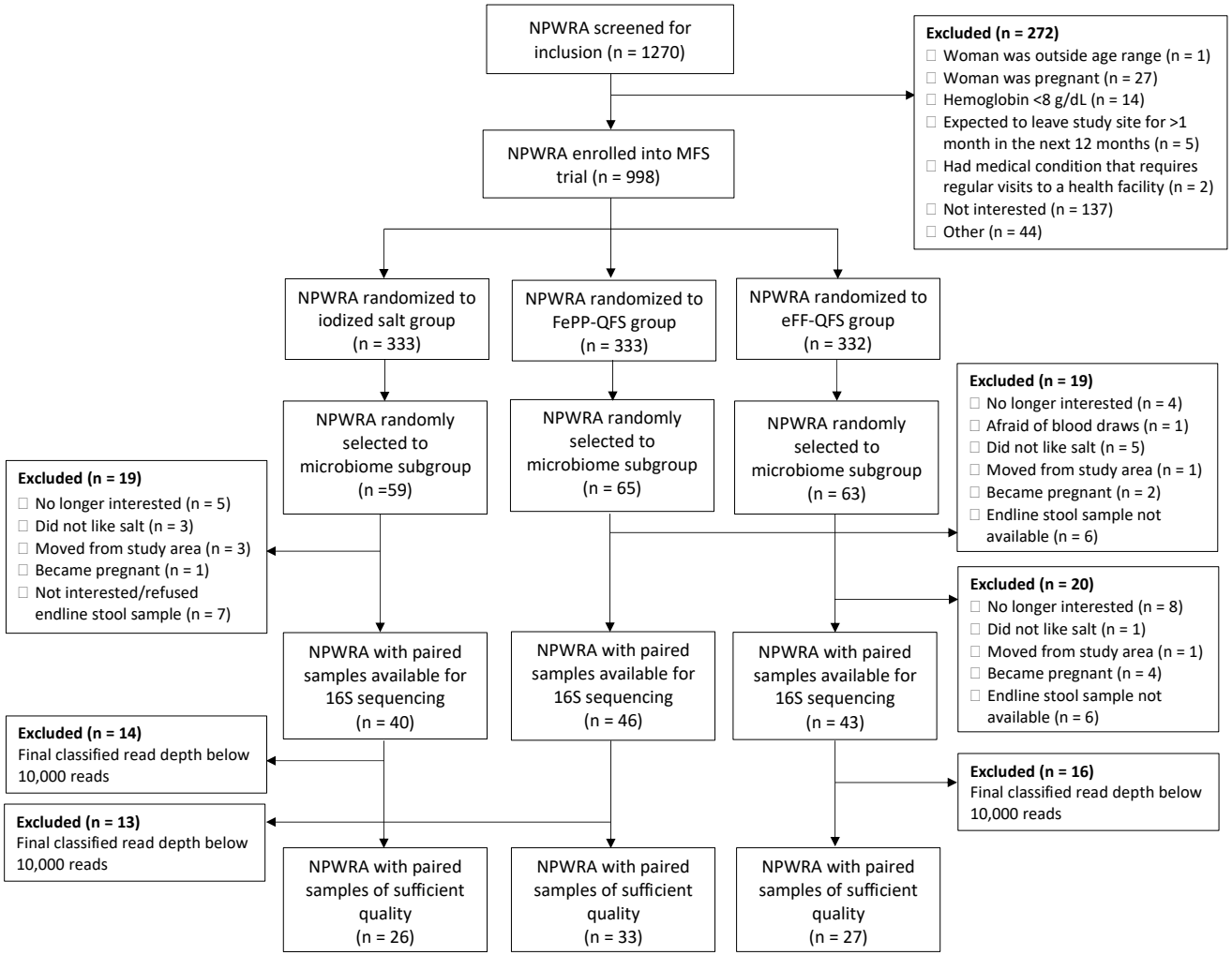


Figure 3.3: Box plots of abundance of bacterial genus groups (*Bifidobacterium, Lactobacillus, Prevotella, Streptococcus*) and the bacterial family *Enterobacteriaceae* among all three intervention groups (Iodized salt control, (quintuply fortified salt (QFS) with iron in the form of encapsulated ferrous fumarate (eFF-Q5S) and QFS with iron in the form of ferric pyrophosphate plus ethylenediaminetetraacetic acid (EDTA) (eFePP-Q5S)). Intervention effects on

abundance were evaluated using zero-inflated negative binomial mixed models. The intervention effect was quantified using a (intervention group*endline) interaction term in the fixed-effects portion of the model. Total OTU counts were included as an offset term to account for varying total counts in the fixed effects portion of the model. Participant ID was included as a random effect. Covariate adjustment was only applied if pre-specified covariates collected at baseline were associated (*p-value* < 0.1) with abundance (pre-specified covariates: continuous, log-transformed serum alpha-1-glycoprotein (AGP) at baseline, continuous, log-transformed serum C-reactive protein (CRP) values at baseline, continuous serum zinc values at baseline, continuous hemoglobin values at baseline, inflammation-adjusted serum ferritin values at baseline, household food insecurity access score (HFIAS) score at baseline and household asset index at baseline). Model with *Bifidobacterium* as the outcome was adjusted for serum CRP values at baseline, serum zinc values at baseline, serum AGP at baseline, and asset index at baseline. Model with *Lactobacillus* as the outcome was adjusted for ferritin values at baseline. *Prevotella* model was not covariate-adjusted. *Streptococcus* model was not covariate-adjusted*. Enterobacteriaceae* model was adjusted for serum zinc values at baseline, hemoglobin values at baseline, and household assets index*. P-values* were corrected for false-discovery rate (FDR) using Benjamini-Hochberg correction. No statistically significant differences in relative abundance at endline were identified between any two intervention groups.



Supplementary Figure 3.1: Flow diagram depicting the allocation of non-pregnant women of reproductive age (WRA) to the different arms of the Multiply Fortified Salt (MFS) trial. A subset of WRA (n = 186) were randomized to the microbiome subgroup (n = 59 in the iodized salt group, n = 65 in the QFS with iron in the form of encapsulated ferrous fumarate (eFF-QFS); group, and n = 63 in the QFS with iron in the form of ferric

pyrophosphate plus ethylenediaminetetraacetic acid (EDTA) (FePP-QFS) group). Samples were excluded from the analysis if NPWRA did not provide both a baseline and stool sample (iodized salt n = 19, FePP-Q5S n = 19, eFF-QFS n = 20). Samples were further excluded if the final classified read depth was below 10,000 reads following 16S sequencing (iodized salt n = 14, FePP-Q5S n = 13, eFF-Q5S n = 16).

Supplementary Table 3.1**:** PCR Primer Sequences

Primer	**Sequence**
16s rRNA FP	5'AGAGTTTGATCCTGGCTCAG'3
16s rRNA RP	5'GGTTACCTTGTTACGACTT'3
V3V4 FP	CCTACGGGNGGCWGCAG
V3V4 RP	GACTACHVGGGTATCTAATCC

Supplementary Table 3.2**:** Baseline characteristics of women that were randomly selected to the microbiome subgroup and included in the final analysis (n = 86) and women that were excluded due to insufficient sample quality (n = 43)

Characteristics	**Analyzed (n = 86)**	**Excluded (n = 43)**	***P*-value**
Age (years)	30.4	30.7	0.77
Married	85 (99%)	43 (100%)	1.00
Religion			0.84
Hindu	24 (28%)	12 (28%)	
Muslim	5 (6%)	1 (2%)	
Sikh	57 (66%)	30 (70%)	
Educational attainment			0.74
None	1 (1%)	0 (0%)	
Primary	6 (7%)	4 (9%)	
Middle/Secondary	59 (69%)	32 (74%)	
Diploma/Post-graduate	17 (20%)	7 (16%)	
Number of household members	6.43 (2.22)	6.56 (1.92)	0.74
Household income (rupees)			0.43
< 10,000	7 (8%)	1 (2%)	
10,000-12,000	25 (29%)	8 (19%)	
13,000-25,000	34 (40%)	21 (49%)	
26,000-50,000	15 (17%)	10 (23%)	
>50,000	5 (6%)	3 (7%)	
Household is food-secure	82 (95%)	43 (100%)	0.74
Average household salt intake (g/AFE)	5.96 (1.34)	5.66 (1.18)	0.19
Minimum Dietary Diversity (MDD)	58 (67%)	31 (72%)	0.74
BMI (kg/m^2)			0.95
Underweight (<18.5)	5 (6%)	2 (5%)	
Normal (18.5-24.9)	32 (37%)	15 (35%)	
Overweight (25.0-29.9)	49 (57%)	26 (60%)	
Taking antibiotics	0 (0%)	0 (0%)	1.00
Anemia (hemoglobin < 12 mg/dL)	36 (42%)	21 (49%)	0.57
Iron deficiency (serum ferritin[1] < 15 ug/L)	55 (64%)	27 (63%)	1.00
Iron deficiency anemia (hemoglobin < 12 g/dL and serum ferritin[1] < 15 ug/L)	33 (38%)	15 (35%)	0.85
Zinc deficiency (Plasma zinc < 70 ug/dL)	22 (26%)	14 (33%)	0.50
Inflammation			
CRP ≥ 5 mg/L	14 (16%)	13 (30%)	0.11
AGP ≥ 1 g/L	27 (31%)	13 (30%)	1.00

Supplementary Table 3.3: Median relative abundance and detection of top 25 taxa present in baseline samples

Taxon	Median Relative Abundance (%)	Presence In Samples (%)
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	25.52%	98.85%
d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Bifidobacterium	6.50%	100.00%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__Faecalibacterium	5.92%	100.00%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;__	5.68%	100.00%
d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	0.87%	95.40%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Blautia	3.04%	100.00%
d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__Veillonellaceae;g__Dialister	3.19%	95.40%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Agathobacter	2.23%	100.00%
d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelatoclostridiaceae;g__Asteroleplasma	0.12%	66.67%
d__Bacteria;p__Actinobacteriota;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Collinsella	1.54%	98.85%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Roseburia	1.81%	100.00%
d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__Veillonellaceae;g__Megasphaera	1.03%	70.11%
d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelatoclostridiaceae;g__Catenibacterium	1.38%	93.10%
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae;g__Succinivibrio	0.00%	36.78%
d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__Selenomonadaceae;g__Mitsuokella	0.08%	66.67%
d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus	0.24%	98.85%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Dorea	1.14%	98.85%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Coprococcus	1.12%	97.70%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__Subdoligranulum	0.95%	100.00%
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	0.06%	98.85%
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Alloprevotella	0.48%	81.61%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__UCG-002	0.30%	96.55%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__[Eubacterium]_hallii_group	0.63%	97.70%
d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Holdemanella	0.25%	80.46%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__[Eubacterium]_coprostanoligenes_group;g__[Eubacterium]_coprostanoligenes_group	0.45%	96.55%
d__Bacteria;p__Spirochaetota;c__Spirochaetia;o__Spirochaetales;f__Spirochaetaceae;g__Treponema	0.00%	3.45%
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Rikenellaceae_RC9_gut_group	0.00%	47.13%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium_sensu_stricto_1	0.24%	90.80%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__Ruminococcus	0.46%	95.40%

d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Fusicatenibacter	0.37%	100.00%

Chapter 4. The Effect of Quintuply-Fortified Salt on the Gut Microbiome of Young Children 1-5 Years of Age in Punjab, India; A Substudy of a Randomized, Community-Based Trial

4.2 Abstract

Background: Young children in India often face multiple micronutrient deficiencies, and interventions such as micronutrient powders (MNPs) have raised concerns about potential adverse effects of supplemental iron on the gut microbiome. Large-scale food fortification (LSFF) is a cost-effective strategy to improve micronutrient intake, yet its impact on the gut microbiome of young children remains unclear.

Objective: To determine whether intake of quintuply-fortified salt (QFS) for 12 months alters gut microbial diversity and relative abundance of taxa in children aged 1-5 years.

Methods: In a double-blind, randomized, controlled trial in Punjab, India, children 1-5 years old and non-pregnant women received: 1) QFS with iron as encapsulated ferrous fumarate, zinc, vitamin B12, folic acid, and iodine (eFF-QFS); 2) QFS with the same micronutrients, but iron as encapsulated ferric pyrophosphate plus EDTA (eFePP-QFS); or 3) standard iodized salt for 12 months. Stool samples were collected from 124 children (eFF-QFS, n = 43; eFePP-QFS, n = 45; iodized salt, n= 37) at baseline and 12 months, and were analyzed via 16S rRNA gene sequencing. Changes in alpha diversity (Shannon, ACE index) between groups was assessed with linear mixed models, beta diversity (Bray-Curtis dissimilarity) with linear regression and PERMANOVA, and relative abundance of *Enterobacteriaceae*, *Lactobacillus*, *Bifidobacterium*, *Prevotella*, and *Escherichia–Shigella* with zero-inflated negative binomial mixed models.

Results: At 12 months, there were no differences in Shannon diversity across groups. The iodized salt group showed a higher ACE index than the eFePP-QFS group but not the eFF-QFS. Although PERMANOVA revealed no group-wise differences, pairwise Bray-Curtis distances to baseline were larger in eFF-Q5S versus the eFF-QFS and iodized salt groups. No differences in relative abundance were identified across groups for *Enterobacteriaceae, Lactobacillus, Bifidobacterium, Prevotella, or Escherichia-Shigella* for QFS compared with iodized salt, although eFePP-Q5S demonstrated a greater increase in *Lactobacillus* compared to eFF-Q5S.

Conclusions: After 12 months, exposure to QFS resulted in minimal, inconsistent shifts in certain diversity metrics and no major changes in key taxa compared to the iodized salt control, suggesting no adverse effects of QFS on the gut microbiome of young children.

4.3 Introduction

Micronutrient deficiencies are widespread in Northern India, particularly among young children. The most recent National Family Health Survey (NFHS), conducted between 2019 and 2021, found that 67% of children aged 6-59 months were anemic; an increase from 59% in the 2015-2016 survey (1). In the northern Indian state of Punjab, the prevalence of anemia was even higher, at 71% (1). According to the most recent Comprehensive National Nutrition Survey (2016-2018) (2), the national prevalence of iron deficiency in the same age group was 32%, rising to 67.2% in Punjab (2). Anemia and iron deficiency can result in severe health, growth and developmental consequences among young children (3).

Multiple strategies have been recommended to address micronutrient deficiencies among individuals and populations at risk for deficiencies. Supplementation and home fortification, including provision of iron supplements and point-of-use micronutrient powders (MNPs) are targeted strategies to close dietary micronutrient gaps among children (4). Large-scale food fortification (LSFF), or the addition of one or more micronutrients to commonly consumed staple foods or condiments, is another strategy which is designed to improve micronutrient intake at the population level. Both approaches have consistently proven to improve biomarkers of micronutrient status (5, 6).

However, there is concern that the level of iron provided in many micronutrient interventions could inadvertently harm gut health. Due to the low fractional absorption (<20%) of iron from

iron fortificants and supplements, the majority of ingested iron remains unabsorbed in the colon (7). For many gram-negative bacteria, iron is essential for colonization and virulence, and excess colonic iron may produce an environment conducive to pathogenic growth (8, 9). This can result in adverse shifts in the gut microbiome, promote intestinal inflammation, and increase the risk of diarrhea (7). Specifically, iron-containing micronutrient powders (MNPs) have come under scrutiny, with several studies linking their use to increased diarrhea incidence among young children (7, 10-14). Some studies have reported increases in the relative abundance of *Enterobacteriaceae*, a bacterial family that includes several known enteric pathogens (13, 15), as well as rises in *Escherichia-Shigella, Campylobacter, and Clostridium* (13, 16) all of which are commonly associated with diarrheal diseases. These shifts have also been accompanied by declines in beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* (13). This is especially important in children, as microbiome alterations have been associated with poor growth (17, 18), and diarrhea carries an elevated risk of mortality (19-21).

Micronutrient interventions that deliver a lower effective level of iron may help mitigate the potential negative effects that have been associated with higher iron doses and maximize efficiency in preventing micronutrient deficiencies and anemia. LSFF is a promising strategy, offering a cost-effective, sustainable approach to increase micronutrient intake of the entire population that requires no behavior change and leverages existing delivery platforms. By delivering smaller amounts of iron over an extended period of time, LSFF could help minimize potential adverse effects on the gut. However, the impact of LSFF on the gut microbiome is not well understood, underscoring the need for additional research.

To address this evidence gap, we aim to leverage data from a double-blind randomized, controlled, community-based trial in Punjab, India that compared quintuply-fortified salt (QFS) with iron as either encapsulated ferrous fumarate (eFF) or encapsulated ferric pyrophosphate (eFFeP) plus EDTA, in addition to zinc, folic acid, vitamin B12 and iodine vs. an iodized salt control group for the improvement of micronutrient status among women and their children. The present study used 16S ribosomal RNA (rRNA) gene sequencing to identify changes in the gut microbiome of children 1-5 years of age in Punjab, India following exposure to QFS for 12 months.

4.4 Methods

Study design

The present study was designed as a substudy of a double-blind, randomized, controlled, community-based, trial that took place in the Mohali District of Punjab, India. The full details of the parent study design can be found in the study protocol (22). Briefly, non-pregnant women of reproductive age were recruited to participate in the study. One of the women's children 12-59 months of age was also eligible to participate in the study. Eligible women, children, and their households were randomized to one of three study groups 1) QFS with iron in the form of encapsulated ferrous fumarate (eFF), zinc as zinc oxide, vitamin B12, folic acid, and iodine as potassium iodate (eFF-QFS); 2) QFS with the same five micronutrients, but with iron in the form

of encapsulated ferric pyrophosphate plus ethylenediaminetetraacetic acid (EDTA) (eFePP-QFS); 3) iodized salt. Fortification levels for eFF-QFS and eFePP-QFS per gram of salt were: 1.3 mg iron; 1.4 mg zinc; 0.6 µg vitamin B12; 52 µg folic acid, and 30 µg iodine. These fortification levels were based on formative research among women in the study area, which found that mean discretionary salt intake was 4.7 g (23). The primary outcomes of the main trial included impacts on biomarkers of iron, zinc, vitamin B12, folate and iodine following exposure to the study salt for 12 months.

To be eligible for participation in the parent study and the microbiome substudy, children must have met the following criteria: 1) 12-59 months of age; 2) child's mother or primary caregiver has been enrolled into the trial; 3) not severely anemic (defined as a hemoglobin concentration <7.0 g/dL, as measured by fingerprick blood sample and HemoCue® 301+); 4) not severely acutely malnourished (defined according to a weight-for-length/height Z-score <-3 or mid-upper arm circumference (MUAC) <115 mm); 5) no serious health problems that interfere with the child's eating practices. Written and oral informed assent was obtained from the child's mother prior to participation in the study. The trial protocol was approved by the Institutional Review Board of the University of California San Francisco, the Institutional Ethics Committee of the Post Graduate Institute of Medical Education and Research (24) and India's Health Ministry's Screening Committee. The study was registered at ClinicalTrials.gov (NCT05166980) and in the Clinical Trials Registry-India (CTRI/2022/02/040333).

Following confirmation of eligibility and provision of consent, study staff collected

sociodemographic data from the child's mother, and children were subsequently scheduled for biochemical and anthropometric assessments in the following 2-3 days. If baseline data collection was completed and the blood draw was successful, women and children were randomized to receive FePP-QFS, eFF-QFS or iodized salt.

Randomization of women and their children to one of the three study groups was implemented via a computer-generated block-randomized assignment. Sealed opaque envelopes containing the color-coded group assignment were prepared in ordered stacks and women were asked to select one envelope which contained a group assignment. Women were then asked to select an additional opaque envelope from a separate ordered stack which assigned the women and her child to the microbiome subgroup. The study team remained blinded to the group assignment until after data analysis.

Following randomization, initial disbursement of the study salt was provided for the mother and child, and their household. Additional study salt was provided via monthly home visits for 12 months. Women were instructed to use the assigned salt to fulfill the cooking and consumption needs of all household members, including children, for the duration of the study, and to maintain habitual discretionary salt consumption. Households were then visited on a monthly basis at home to provide additional disbursements of study salt, as well as to weigh and collect any unused and assess acceptability. Salt utilization was calculated from the household salt disappearance data collected at monthly visits and converted into adult female equivalents using household roster data and standard assumptions of weight and total energy expenditure

(25, 26).

Biochemical and anthropometric assessments completed at enrollment were repeated at endline (12 months) using the same protocols described above. Laboratory assessment of micronutrient and inflammatory biomarkers (serum ferritin, hemoglobin, C-reactive protein (CRP), alpha-1-glycoprotein (AGP)) were performed using standard protocols which have been published previously (27, 28). Laboratory methods are described in further detail in the study protocol (22) and in the primary endpoints publication (29).

Stool sample collection and sequencing

Stool samples were collected from children enrolled in the microbiome subgroup following randomization (baseline) and endline (12 months) using stool sample collection and preservation tubes (Norgen Biotek). Women were instructed to collect stool samples from their child at home using the provided collection kit. Women were asked to collect a stool sample in the morning, and sample collection tubes were subsequently retrieved by study staff within 4 hours and transported to the PGIMER central laboratory for storage at -20° C. Stool samples were then shipped on dry ice to Medgenome in Bangalore, India for sequencing.

DNA extraction from stool samples was performed using QIAamp PowerFecal Pro DNA Kit and quantified using Qubit DNA High sensitivity Assay (Invitrogen), Qubit RNA Broad Range Assay (Invitrogen) and QIAxpert. Samples were diluted to 5 ng and were amplified for the 16S region

(1500bp) followed by the Variable 3 Variable 4 (V3V4) region (460-480bp) using 16S and V3V4 primers respectively in a nested PCR approach. PCR Primer sequences are provided in the Supplemental Methods (**Supplementary Table 1**). All PCR products were further processed for DNA library preparation using the Twist MF Library Prep Kit for Illumina. All prepared libraries were checked for fragment distribution using the 5300 Fragment Analyzer system. Prepared libraries were sequenced using the Illumina NextSeq2000 platform. Quantitative Insights into Microbial Ecology 2 (QIIME 2) was used to cluster sequence reads into operational taxonomic units (OTUs) at 99% sequence identity. One representative sequence from each OTU was selected and classified using the Ribosomal Database Project classifier against the SILVA-138 database at 99% similarity.

Statistical analysis

For this substudy, a sample size of at least 50 child per group was estimated to provide 80% power to detect an effect size of 0.54 standard deviations (SDs) in each outcome between two groups with a level of significance of α=0.05, using ANCOVA models controlling for baseline values (assuming a weak correlation of 0.1 between baseline and endline). Accounting for an attrition rate of 25%, we aimed to enroll 185 children, or ~62 children per study group to the microbiome subgroup.

All statistical analyses were performed using R software (Version 2024.09.0+375). All statistical tests with a *p*-value <0.05 were statistically significant. All regression models were minimally-

adjusted for child age and breastfeeding ("ever breastfed" (yes/no) variable). Additional baseline covariates were selected a priori based on biological plausibility and prior evidence linking them to gut microbiome composition. Selected covariates included: continuous serum AGP values at baseline, continuous serum CRP values at baseline, continuous serum ferritin values at baseline, continuous hemoglobin values at baseline, length-for-age Z-score at baseline, weight-for-age Z-score at baseline, and weight-for-length Z-score at baseline, continuous Household Food Insecurity Access (HFIAS) score (30) at baseline, and household assts index measured at baseline. In all models, only those baseline covariates that showed evidence of association with the outcome at *p-value* < 0.1 were retained to balance model parsimony with adequate adjustment for confounders. Log-transformations were performed on CRP, AGP, and ferritin to address skewness and approximate normal distributions, and ferritin values were corrected for inflammation (31).

Shannon Diversity and ACE indices were used to determine overall microbial diversity within each individual (alpha diversity). Shannon diversity and ACE indices were estimated using the vegan package in R (32). As Shannon diversity and ACE metric values were normally distributed, linear mixed models were used to estimate intervention effects on each alpha diversity metric. An (intervention group*endline) interaction term was used to quantify the intervention effect, and participant ID was included as a random effect. Residual diagnostic plots (residual plots and quantile-quantile (QQ) plots) were inspected to assess model fit and normality of residuals.

The distribution of microbial diversity between samples (beta diversity) was evaluated using

Bray-Curtis distances, which were estimated using normalized OTU counts via the vegan package in R (32). OTU counts were normalized via cumulative sum scaling (CSS) using the metagenome package in R (33). Linear regression models were used to estimate intervention effects on Bray-Curtis pairwise dissimilarity between baseline and endline measurements within individual children. Residual diagnostic plots (residual plots and QQ plots) were inspected to assess model fit and normality of residuals. Permutational multivariate analysis of variance (PERMANOVA) was also used to estimate Bray-Curtis dissimilarity between intervention groups. The intervention effect on Bray-Curtis dissimilarity was quantified in the PERMANOVA model as (intervention group*endline), with 999 permutations to assess statistical significance. The Bray-Curtis distance matrix was also used in principal coordinates analysis (PCoA) to visualize clustering by intervention group at baseline and endline.

Intervention effects on abundance of prespecified bacterial taxa of interest (*Enterobacteriaceae, Lactobacillus, Bacteroides, Bifidobacterium, Prevotella, Escherichia-Shigella*) were estimated using zero-inflated negative binomial mixed models (glmmTMB package in R (34)). These taxa were chosen based on their abundance in this population (within the top 25 most abundant genera), and the documented interaction between iron interventions and alterations in abundance of these taxa. The zero-inflated negative binomial mixed model was chosen to accommodate the distribution of the abundance data, which are count data and were over-dispersed and zero-inflated. The intervention effect was quantified using a (intervention group*endline) interaction term in the fixed-effects portion of the model. Total OTU counts were included as an offset term to account for varying total counts in the fixed effects portion of

the model, and participant ID was included as a random effect. Model fit was assessed via the DHARMa package in R (35), which provides a simulation-based approach to generating standardized residuals for generalized linear mixed models. Diagnostic plots of residuals versus predicted values and QQ-plots were generated and visually inspected. Statistical tests for overdispersion were conducted using the testDispersion() function, and for zero-inflation using the testZeroInflation() function. If the zero-inflation test was non-significant, the zero-inflation portion of the model was removed. Lastly, an exploratory differential abundance analysis was performed on all bacterial genus groups using Analysis of Microbiomes with Bias Correction 2 (36) (ANCOMBC 2) via the phyloseq and ANCOMBC R packages. *Q*-values were calculated from *p*-values corrected for false discovery rate (FDR) using Benjamini-Hochberg correction. If taxa were identified to be significantly different according to the ANCOMBC model (*q-value* <0.05), taxa were also assessed using zero-inflated negative binomial mixed models to incorporate additional covariate adjustment and obtain regression coefficients.

4.5 Results

Between August 2022-July 2023, 798 children were enrolled and randomized to one of the three intervention groups. Following randomization, a subsample of 171 children were randomly selected to participate in the microbiome subgroup (eFePP-QFS, n = 64; eFF-QFS, n = 57; iodized salt, n = 55). 17 children in the iodized salt subgroup, and 16 children in the eFePP-QFS subgroup, and 13 children in the eFF-QFS subgroup were excluded as their household did not complete the study, the child did not provide an endline stool sample, or the stool sample

was excluded due to inadequate sequencing depth. Ultimately, 124 children with paired baseline and endline samples with sufficient sequencing depth were included in the analysis (eFePP-QFS, n = 45; eFF-QFS, n = 43; iodized salt, n = 37). Rates of attrition were not statistically different by group. A participant flow diagram is shown in **Supplementary Figure 4.1**.

Baseline characteristics were similar across groups (**Table 4.1**). Overall, average child age was nearly 40 months. Mean (SD) length-for-age Z-score was -0.80 (1.31) across all three groups, and the prevalence of stunting ranged from 7% to 16.2%. More than than 80% of mothers completed a middle school education or higher. More than 95% of households were food secure. However, over one-third of children were anemic, over 57% had iron deficiency, and over 24% had iron deficiency-anemia. Average household salt intake was estimated to be about 6 grams per adult female equivalent (AFE) per day across all three groups.

At baseline, *Prevotella, Bifidobacterium* and *Bacteroides*, were among the most abundant genera among children (**Figure 4.1**). There were no differences in Shannon diversity between groups at baseline. Shannon diversity also did not differ according to group at the end of the 12-month intervention period (group-by-time interaction, *p*-value > 0.05) (**Figure 4.2A**). Similarly, there were no differences in ACE index according across groups at baseline. However, at 12 months, the iodized salt group had a greater increase in diversity as measured by ACE index versus the eFePP-QFS group (group-by-time interaction, *p*-value = 0.03). Changes in pairwise Bray-Curtis distances between baseline and 12 months differed significantly between iodized salt vs. eFF-QFS groups (*p-value* = 0.02), and eFePP-QFS vs. eFF-QFS groups (*p-value* = 0.01),

(**Figure 4.2B**). Visual inspection with PCOA plots and PERMANOVA testing showed that Bray-Curtis dissimilarity did not differ according to group (group-by-time interaction, *p*-value = 0.93) (**Figure 4.2C**).

At baseline and after 12 months, there were no differences in the relative abundance of *Prevotella, Bifidobacteria, Lactobacillus, Bacteroides, Escherichia-Shigella* nor *Enterobacteriaceae* between QFS and iodized salt groups (group-by-time interaction, *q*-value > 0.05). The eFePP-QFS group demonstrated a marginally significantly greater increase in relative abundance of *Lactobacillus* compared to the eFF-QFS group (group-by-time interaction, *q*-value = 0.054) (**Figure 4.3**). Differential abundance testing via ANCOMBC identified no genus or family groups that were differentially abundant between groups (group-by-time interaction, *q*-value > 0.05).

4.6 Discussion

To our knowledge, this study is one of the first to evaluate the effects of LSFF in the form of multiply-fortified salt on gut microbiome outcomes among young children. At baseline, children's gut microbiomes closely matched findings from other studies in Northern India (37-40). We observed a dominance of *Prevotella*, which is common in populations consuming a predominately lacto-vegetarian dietary pattern high in carbohydrate, dietary fiber and fermented dairy products (39, 41). Our analysis revealed some groupwise differences in diversity metrics. However, these differences did not consistently suggest a detrimental impact

of iron fortification on gut microbial diversity or composition, despite concerns about potential adverse effects of iron on the microbiome.

At endline, the iodized salt group showed a moderate increase in ACE index (~14 ACE units) relative to eFePP-QFS, however, no significant difference in Shannon diversity was observed between these two groups. The Shannon diversity index accounts for both richness and evenness of taxa (42, 43), whereas the ACE index is more sensitive to low abundance taxa (44, 45). The stability of Shannon diversity over time suggests that the overall composition and dominant taxa remained similar across intervention groups, and the shift between eFePP-QFS and the iodized salt group may have occurred among rare taxa. Further, the eFF-QFS group did not differ from the iodized salt group in terms of the ACE index, suggesting the eFePP formulation may interact with the microbiome differently than eFF. However, differential abundance testing of all genera and families did not identify significant differences in abundance of any specific bacterial taxa between the iodized salt and eFePP-Q5S group, implying no harmful or pathogenic shift.

Linear regression models showed that the eFF-QFS group experienced a significantly greater change in pairwise Bray-Curtis dissimilarity from baseline to endline compared to both the iodized salt and eFePP-QFS groups. This suggests a somewhat larger compositional change over time among the children in the eFF-QFS group, as this metric examines the difference in Bray-Curtis distance from baseline to endline within individual children. However, when Bray-Curtis distance was compared between children using PERMAVOA testing on the Bray-Curtis matrix,

there were no statistical differences between groups. In addition, the there were no differences in alpha diversity in between the eFF-Q5S group and the other two groups. Therefore, this effect may also be attributable to unmeasured variables that were not accounted for, such as dietary changes and current breastfeeding status, rather than the intervention.

In the present study, we did not detect any changes in the relative abundance of key genera, including *Prevotella, Bifidobacterium, Lactobacillus, Bacteroides*, or *Escherichia-Shigella* in QFS compared to the iodized salt control. Despite studies demonstrating that oral iron can reduce beneficial *Lactobacillus* and promote potential pathogens like *Escherichia-Shigella* (11-13, 46), we observed no increase in *Enterobacteriaceae* or *Escherichia-Shigella* in any group. This finding suggests that QFS did not adversely affect dominant bacterial groups or increase the abundance of known enteric pathogens among children.

Adverse effects of iron interventions can depend on baseline iron status. Studies in young children indicate that providing excess iron to those who are iron-replete may lead to growth deficits (47-50) and diarrhea (14, 50, 51), potentially due to iron-dependent pathogens proliferating and altering the microbiome (52). In contrast, negative outcomes may be less pronounced in children who are iron-deficient, as supplementation may help restore immune function compromised by deficiency (52). In our study, over 58% of participants across all groups were iron-deficient at baseline. Furthermore, QFS also contained additional nutrients beyond iron, including zinc, vitamin B12, folic acid, and we observed significant improvements in parameters of B12 and folate status among all children enrolled in the main trial.

Micronutrient deficiencies beyond iron deficiency are associated with adverse microbiome outcomes (53, 54), thus, addressing multiple deficiencies through QFS may have provided synergistic benefits.

Environmental factors, particularly hygiene and sanitation, can be important predictors of adverse microbiome outcomes. Under poor sanitary conditions, individuals are more likely to be exposed to opportunistic pathogens, and any unabsorbed dietary iron may further promote their proliferation (19, 52). However, improved sanitation may reduce baseline pathogen burden and may prevent overgrowth due to oral iron. For instance, a randomized trial of iron supplements (50 mg/d, 4 days/week) in South African children with access to an improved water supply and better hygiene found no significant effects on the microbiome or gut inflammation (fecal calprotectin) (55). By comparison, a study of children 6-14 years in Côte d'Ivoire who had high systemic inflammation and hookworm infections saw declines in both *Bifidobacteria* and *Lactobacillus* and elevated enteric inflammation after consuming iron-fortified (20 mg) biscuits 4 times per week (13). Thus, interventions that deliver a lower effective daily iron dose, such as LSFF, may be favorable with respect to microbiome outcomes in LMIC settings where hygiene is suboptimal by minimizing daily iron exposure.

We note that in our study, children lived in relatively hygienic households, as all children lived in households with piped water and flush toilets, reported no diarrhea at sample collection, and had minimal diarrhea throughout the intervention. Potentially pathogenic genera like *Vibrio*, *Clostridium, Campylobacter* were rare or undetected, indicating a low baseline pathogen

burden. Although *Escherichia–Shigella* was present at low abundance in 89% of samples (Supplemental Table 3), abundance did not increase in any intervention group. We did note a relatively high prevalence of inflammation among children at baseline, especially reflected in elevated levels of AGP. We accounted for this by adjusting for inflammatory markers at baseline (AGP and CRP) in our statistical models, and we found that adjustment for inflammation did not appreciably change our results or interpretation.

Strengths of this study include its double-blind, randomized, controlled design and the use of baseline-adjusted, zero-inflated negative binomial mixed models, allowing a robust assessment of longitudinal changes in taxa while accounting for inter-individual variability. We further adjusted for baseline inflammation and micronutrient deficiencies; factors known to influence the microbiome (54). To detect shifts in low-abundance taxa, we conducted differential abundance analysis using ANCOMBC, which appropriately handles the compositional nature of microbiome data (36). However, the study also has limitations. We relied on 16S rRNA gene sequencing, which restricts taxonomic resolution to the genus level and prevents species- or strain-level inferences. We did not include metagenomic or metatranscriptomic analyses, limiting our ability to identify functional changes in microbial communities. Only two collection timepoints (baseline and 12-month endline) were used, reducing the opportunity to capture more nuanced temporal dynamics or acute shifts. The children in our sample represented a large range of ages between 1 to 5 years. This variability may have affected our ability to determine whether changes were due to the intervention itself, or due to age-related changes in the developing microbiome. However, age was not statistically different across intervention

groups, and the median age in our sample was over 3 years of age, suggesting that most children were beyond the more rapid early-life shifts, likely having relatively stable microbiomes (56). In addition, all statistical models were adjusted for age and breastfeeding to account for age-related variation. Although we measured systemic inflammatory markers, we did not collect fecal markers of intestinal inflammation or enteric dysfunction (e.g., fecal calprotectin), limiting insights into gut inflammatory processes. Lastly, the study was conducted in a single region in Punjab, India with relatively hygienic conditions, which may limit generalizability to populations with different environmental exposures or baseline pathogen burden.

To our knowledge, this is the first study to examine microbiome changes in response to LSFF in the form of multiply-fortified salt among children 1-5 years of age. We noted minimal and inconsistent shifts in certain microbial diversity metrics, and no major changes in abundance of taxa. Importantly, we did not observe increases in *Escherichia-Shigella* or Enterobacteriaceae, suggesting that QFS did not induce substantial or adverse microbiome alterations related to increased abundance of potentially pathogenic bacteria. Future studies should confirm these findings by examining children in regions with higher enteropathogen burdens and incorporating additional assessments (functional metagenomics, strain-level analyses, or more frequent sampling) to capture subtle temporal changes and confirm the safety and efficacy of LSFF interventions across diverse settings.

4.7 References

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Table 4.1: Baseline characteristics of children randomly selected to the microbiome subgroup with sufficient read depth following sequencing (n = 125)

	Mean (SD) or n (%)		
Characteristics	**eFF-QFS (n = 43)**	**FePP-QFS (n = 45)**	**Iodized Salt (n = 37)**
Age, months	40.0 (11.5)	42.0 (10.7)	38.5 (11.9)
Sex, female	19 (44.2%)	24 (53.3%)	12 (32.4%)
Ever breastfed	36 (83.7%)	36 (80.0%)	29 (78.4%)
Length-for-age Z-score	-0.89 (0.9)	-0.54 (1.6)	-1.03 (1.2)
Weight-for-age Z-score	-0.57 (0.9)	-0.69 (1.2)	-0.87 (1.0)
Weight-for-length Z-score	-0.09 (1.0)	-0.55 (1.2)	-0.43 (1.1)
Stunted	3 (7.0%)	7 (15.6%)	6 (16.2%)
Wasted	1 (2.3%)	8 (17.8%)	6 (16.2%)
Underweight	1 (2.3%)	5 (11.1%)	3 (8.1%)
Maternal age, years	30.6 (4.6)	31.0 (4.9)	29.8 (4.5)
Maternal BMI, kg/m^2	25.6 (4.6)	25.2 (4.5)	26.3 (3.9)
Maternal educational attainment			
None	0 (0.0%)	1 (2.3%)	0 (0.0%)
Primary	4 (9.5%)	3 (6.8%)	2 (5.6%)
Middle/Secondary	31 (73.8%)	33 (75.0%)	24 (66.7%)
Diploma/Post-graduate	7 (16.7%)	7 (15.9%)	10 (27.8%)
Annual household income (INR)			
< 10,000	4 (9.3%)	3 (6.7%)	2 (5.4%)
10,000-12,000	9 (20.9%)	10 (22.2%)	12 (32.4%)
13,000-25,000	18 (41.9%)	20 (44.4%)	13 (35.1%)
26,000-50,000	6 (14.0%)	12 (26.7%)	8 (21.6%)
>50,000	6 (14.0%)	0 (0.0%)	2 (5.4%)
Religion			
Hindu	14 (32.6%)	12 (26.7%)	10 (27.0%)
Muslim	1 (2.3%)	3 (6.7%)	1 (2.7%)
Sikh	28 (65.1%)	30 (66.7%)	26 (70.3%)
Household is food-secure[1]	42 (97.7%)	43 (95.6%)	36 (97.3%)
Average household salt intake (g/AFE)[2]	5.93 (1.44)	6.04 (1.33)	5.58 (1.14)
Anemia (hemoglobin < 11 g/dL)	19 (44.2%)	15 (33.3%)	19 (51.4%)
Iron deficiency (serum ferritin[1] < 12 ug/L)	26 (61.9%)	26 (57.8%)	27 (73.0%)
Iron deficiency anemia (hemoglobin < 11 g/dL and serum ferritin[3] < 12 ug/L)	16 (37.2%)	11 (24.4%)	16 (43.2%)
Inflammation			
CRP ≥ 5 mg/L	5 (11.9%)	5 (11.1%)	1 (2.7%)
AGP ≥ 1 g/L	17 (40.5%)	12 (26.7%)	14 (37.8%)

Definitions: QFS; quintuply fortified salt, eFF-QFS; QFS with iron in the form of encapsulated ferrous fumarate, FePP-QFS; QFS with iron in the form of ferric pyrophosphate plus ethylenediaminetetraacetic acid (EDTA), AFE; Adult female equivalent, CRP; C-reactive protein, AGP; Alpha-1 glycoprotein

[1]Household food security was estimated using the Household Food Insecurity Access Scale (HFIAS)

[2]Salt utilization was calculated from the household salt disappearance data collected at monthly visits and converted into adult female equivalents using household roster data and standard assumptions of weight and total energy expenditure

[3]Serum ferritin values were adjusted for inflammation using the BRINDA regression equations

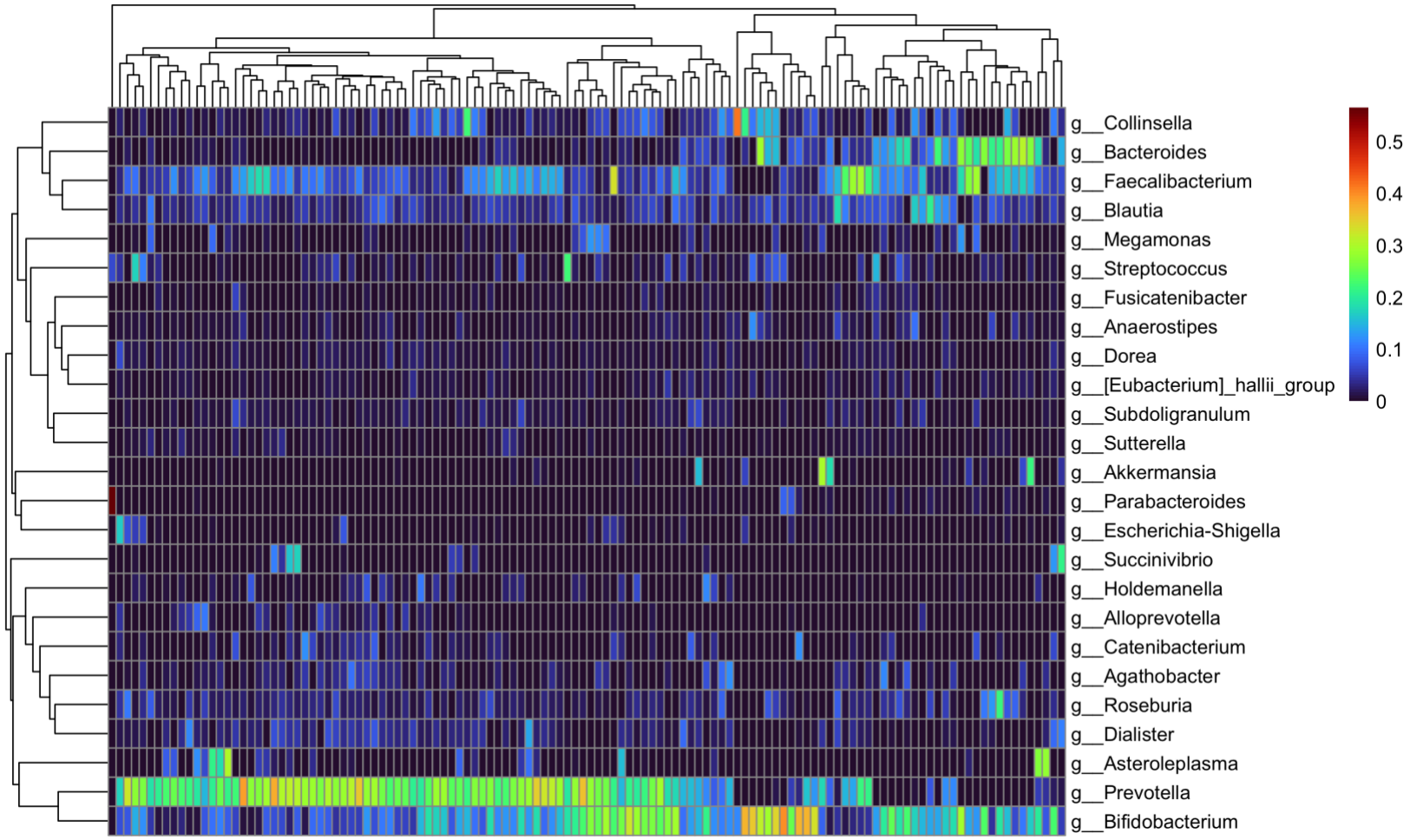


Figure 4.1: Genus-level microbiota composition at baseline among children at baseline. Heatmap representing the 25 most abundant genus groups in baseline stool samples based on relative abundance; dendrograms illustrated on the x and y axes are based on Bray-Curtis distances and represent which genera (rows) or samples (columns) cluster together based on

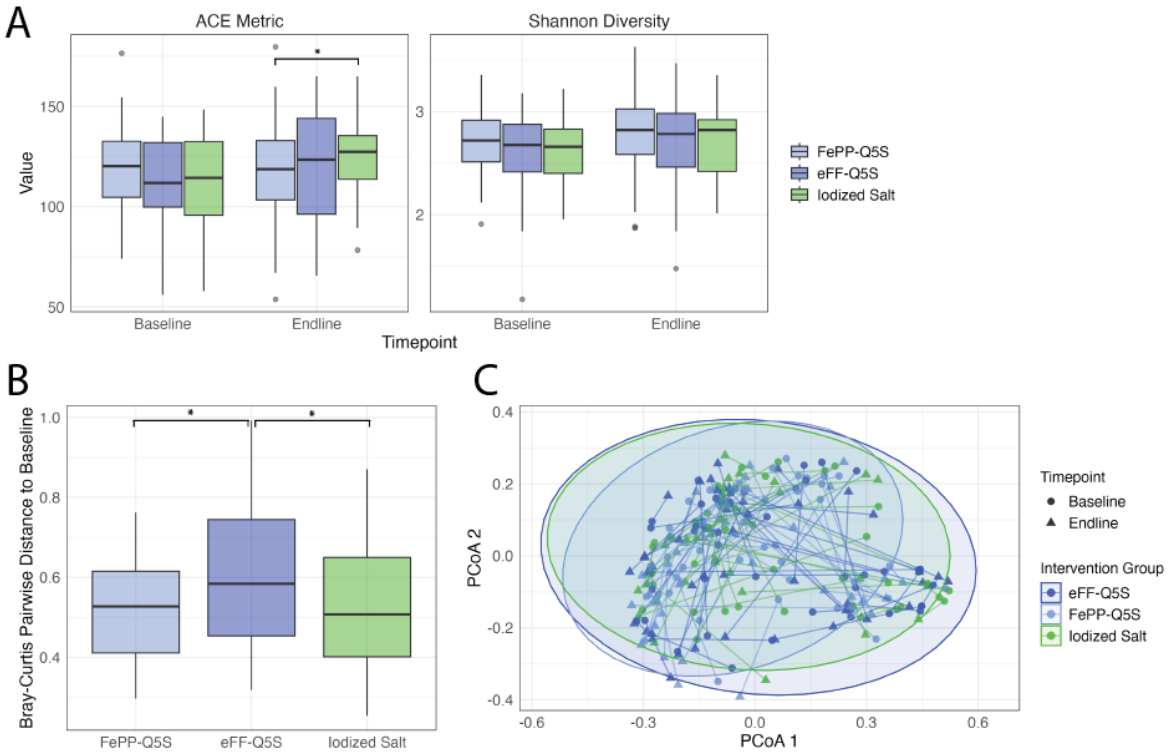


Figure 4.2: Impact of quintuply fortified salt (QFS; QFS with iron in the form of encapsulated ferrous fumarate (eFF-Q5S) and QFS with iron in the form of ferric pyrophosphate plus ethylenediaminetetraacetic acid (EDTA) (FePP-Q5S)) on the fecal microbiome in terms of alpha and beta diversity, compared to iodized salt control. **(A)** Effect of QFS on measures of alpha diversity (Abundance-Based Estimator (ACE) metric and Shannon Diversity) measured at baseline and endline (12 months). Intervention effects were measured using linear mixed models evaluated using a (intervention group*endline) interaction term in the fixed effects portion of the model. A random effect of participant ID was also included. Models were adjusted for age and additional covariates (ever breastfed, assets index, baseline hemoglobin, length-for-age Z-score, weight-for-age Z-score, baseline alpha-1 acid glycoprotein, and baseline inflammation-adjusted ferritin). **(B)** Boxplot of Bray-Curtis pairwise distances to baseline to identify structural changes within individuals from baseline to endline. Intervention effects were measured using linear regression models adjusting for age and additional covariates (sex, ever breastfed, baseline hemoglobin, HFIAS score, length-for-age Z-score, weight-for-age Z-score, baseline AGP). **(C)** A principal coordinate (PCoA) plot of Bray-Curtis distances at baseline and endline. Differences between intervention groups were identified using Permutational multivariate analysis of variance (PERMANOVA) testing on the Bray-Curtis matrix using a (intervention group*endline) interaction term, adjusting for age and additional covariates ((sex, ever breastfed, baseline hemoglobin, HFIAS score, length-for-age Z-score, weight-for-age Z-score, baseline AGP), with 999 permutations to assess statistical significance.

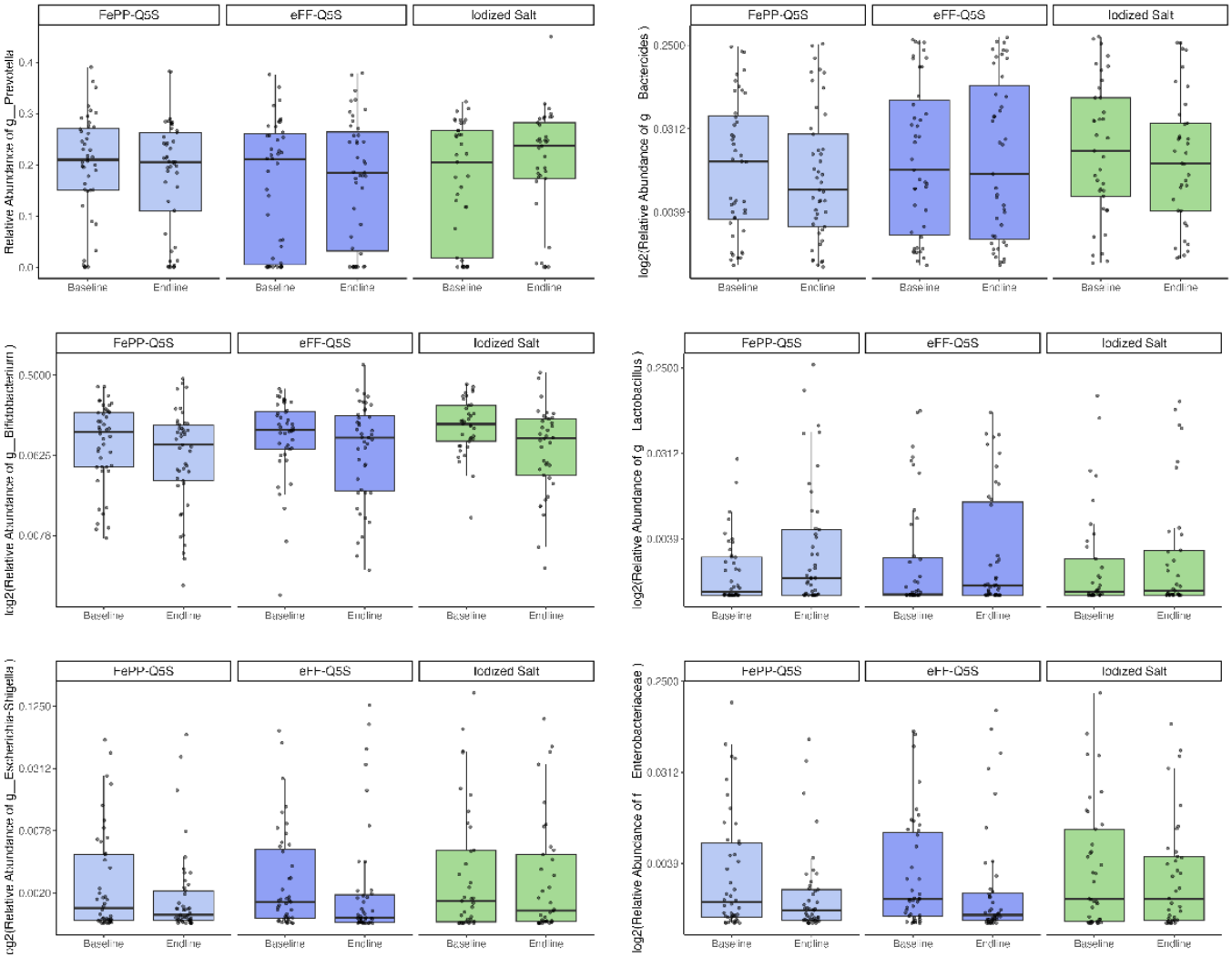
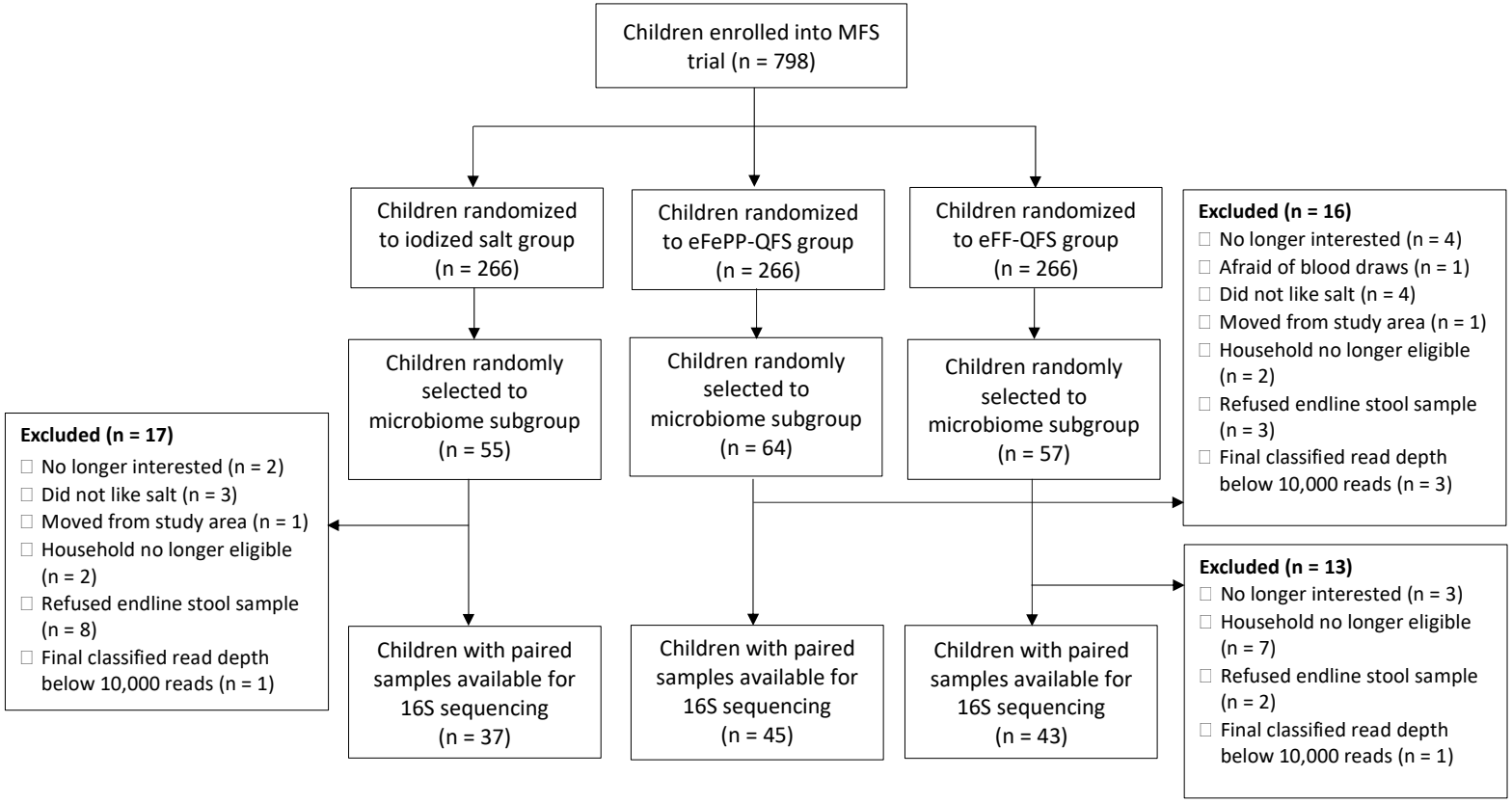


Figure 4.3: Box plots of abundance of bacterial genus groups (*Bifidobacterium, Lactobacillus, Prevotella, Bacteroides, Escherichia-Shigella*) and the bacterial family *Enterobacteriaceae* among all three intervention groups (Iodized salt control, (quintuply fortified salt (QFS) with iron in the form of encapsulated ferrous fumarate (eFF-Q5S) and QFS with iron in the form of ferric pyrophosphate plus ethylenediaminetetraacetic acid (EDTA) (FePP-Q5S)). Intervention effects on abundance were evaluated using zero-inflated negative binomial mixed models. The intervention effect was quantified using a (intervention group*endline) interaction term in the fixed-effects portion of the model. Total OTU counts were included as an offset term to account for varying total counts in the fixed

effects portion of the model. Participant ID was included as a random effect. Models were adjusted for age and additional covariates: *Prevotella* model adjusted for ever breastfed and assets index, *Lactobacillus* model adjusted for ever breastfed, baseline hemoglobin, length-for-age Z-score, weight-for-age Z-score, baseline alpha-1 acid glycoprotein (AGP), and baseline inflammation-adjusted ferritin, *Bacteroides* model adjusted for ever breastfed, HFIAS score, assets index, length-for-age Z-score, weight-for-age Z-score, baseline AGP, baseline C-reactive protein (CRP), and baseline inflammation-adjusted ferritin, *Escherichia-Shigella* model adjusted for ever breastfed, baseline hemoglobin, length-for-age Z-score, baseline AGP, baseline inflammation-adjusted ferritin, Enterobacteriaceae model adjusted for ever breastfed, baseline hemoglobin, length-for-age Z-score, baseline hemoglobin and baseline inflammation-adjusted ferritin. *P-values* were corrected for false-discovery rate (FDR) using Benjamini-Hochberg correction.



Supplementary Figure 4.1: Flow diagram depicting the allocation of children 1-5 years of age to the different arms of the Multiply Fortified Salt (MFS) trial. A subset of children (n = 171) were randomized to the microbiome subgroup (n = 55 in the iodized salt group, n = 64 in the QFS with iron in the form of encapsulated ferrous fumarate (eFF-QFS); group, and n = 57 in the QFS with iron in the form of ferric pyrophosphate plus ethylenediaminetetraacetic acid (EDTA) (FePP-QFS) group). Samples were excluded from the analysis if children did not provide both a baseline and stool sample or did not meet eligibility criteria to

Supplementary Table 4.1: PCR Primer Sequences

Primer	**Sequence**
16s rRNA FP	5'AGAGTTTGATCCTGGCTCAG'3
16s rRNA RP	5'GGTTACCTTGTTACGACTT'3
V3V4 FP	CCTACGGGNGGCWGCAG
V3V4 RP	GACTACHVGGGTATCTAATCC

Supplementary Table 4.3: Median relative abundance and detection of top 25 taxa present in baseline samples

Taxon	Median Abundance (%)	Presence in Samples (%)
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	20.9%	93.55%
d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Bifidobacterium	12.5%	100.00%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__Faecalibacterium	8.1%	98.39%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;__	5.4%	100.00%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Blautia	4.1%	100.00%
d__Bacteria;p__Actinobacteriota;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Collinsella	2.2%	93.55%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Roseburia	1.9%	97.58%
d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__Veillonellaceae;g__Dialister	1.6%	83.87%
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	1.3%	100.00%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__Subdoligranulum	0.9%	92.74%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Dorea	0.9%	96.77%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__[Eubacterium]_hallii_group	0.8%	92.74%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Anaerostipes	0.7%	97.58%
d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus	0.7%	100.00%
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Agathobacter	0.5%	98.39%

d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Sutterellaceae;g__Sutterella	0.5%	95.16%
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Tannerellaceae;g__Parabacteroides	0.1%	91.13%
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacterales;f__Enterobacteriaceae;g__Escherichia-Shigella	0.1%	88.71%
d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__Selenomonadaceae;g__Megamonas	0.0%	66.94%
d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelatoclostridiaceae;g__Catenibacterium	0.0%	62.90%
d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Akkermansiaceae;g__Akkermansia	0.0%	57.26%
d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Holdemanella	0.0%	51.61%
d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelatoclostridiaceae;g__Asteroleplasma	0.0%	51.61%
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae;g__Succinivibrio	0.0%	24.19%
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Alloprevotella	0.0%	49.19%