THE EFFECTS OF MULTI-NUTRIENT FORTIFIED DAIRY-BASED FORMULAS ON GROWTH, ANAEMIA, AND MICRONUTRIENT STATUS IN NIGERIAN LATE PRETERM INFANTS AND MODERATELY MALNOURISHED TODDLERS

Adedotun Joshua Owolabi

PROPOSITIONS

1. Unfortified breastmilk is sufficient to support growth in late preterm infants. (this thesis)

2. Daily consumption of iron-fortified dairy-based formula improves anaemia in malnourished toddlers. (this thesis)

3. Allowing smoking in care homes violates the human rights of the inhabitants.

4. The food industry is largely responsible for the unaffordability of nutritious foods in developing countries.

5. The first step to moving forward is forgetting about the past.

6. You cannot live in the Netherlands without learning how to ride a bicycle.

Propositions belonging to the thesis, entitled

The effects of multi-nutrient fortified dairy-based formulas on growth, anaemia, and micronutrient status in Nigerian late preterm infants and moderately malnourished toddlers

Adedotun Joshua Owolabi
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CHAPTER 1

Introduction and thesis outline

Adequate nutrition in the first 1000 days of life

Nutrition plays a critical role in optimum growth, development, and health maintenance throughout every stage of a child's life, especially in the first 1000 days (1,2). From conception through pregnancy, birth, childhood and into older age, good nutrition supports health, well-being and quality of life (3). The consequences of poor nutrition are already manifested in the womb where epigenetic changes that impact future health are initiated. Good nutrition during fetal development, early childhood and throughout adolescence is essential since it has direct and indirect effects on physical and mental growth, development and immunity, which in turn impact the child's long-term cognitive performance and physical health. These processes depend on the adequacy and timeliness of intake of specific nutrients during early life (4). Currently, there are important gaps in knowledge regarding the nutritional management of late preterm infants, as well as in addressing anaemia in young children, especially in low-resource settings, which are the topics of this thesis.

Nutritional management of late preterm infants

Nigeria ranks third in the world for its incidence rate of preterm births (5). Preterm complications contribute to up to 31% of under-five mortality (6). Every year, 854,400 babies are born before 37 weeks of pregnancy are completed (5,7). About 85% of these preterm births are born between 32 and 37 weeks of gestational age (GA), referred to as moderate-to-late preterm infants (7–9). Similar to extreme preterm (EP; <28 weeks of GA) and very preterm (VP; 28 to 31 weeks of GA) infants, moderately preterm (MP; 32- 33 weeks of GA) and late preterm (LP; 34 -37 weeks of GA) infants also have unique medical vulnerabilities and nutritional needs that predispose them to greater rates of morbidity. Breastmilk from healthy and well-nourished women is the globally accepted standard for feeding infants (10–12). The World Health Organization (WHO) recommends exclusive breastfeeding for term-born infants in the first six months of life (13). Breastmilk provides adequate nutrients such as energy, fatty acids and protein in the appropriate amount to facilitate growth, support cognitive development, and offer a beneficial immunological advantage to young infants (14,15). Children that are born preterm, i.e. before the 37th week of gestation, require optimized nutritional support for their rapid growth rate and nutrient accretion, approximating that of the third-trimester foetus, without imposing stress on the developing metabolic and excretory systems (16,17). Immaturity of the gastrointestinal tract appears to be one of the most critical problems, resulting in feeding intolerance in low-birth-weight preterm infants, which hampers achieving satisfactory energy intake (18,19). Growth failure among small

infants is often attributable to the failure to ingest recommended dietary intakes (20–23). Compared to formula milk, human milk (mother's own breast milk or donor breast milk) is well tolerated by EP and VP infants, and is associated with lower risk of necrotizing enterocolitis, sepsis, retinopathy of prematurity, and better neurocognitive development (24). However, human milk alone is not able to meet the requirements of VP infants for some essential nutrients, such as protein (3.5 to 4.5 g/kg/d), energy (110 to 135 kcal/kg/d), minerals, vitamins, and trace elements (16, 25–27). For these infants, human milk should be fortified with multi-nutrient fortifiers (powder or liquid supplements of protein, energy from carbohydrates or fat, and other nutrients) (26,28–33). For moderately (32- 33 weeks GA) and LP infants (34 -37 weeks GA), unfortified human milk appears to be sufficient to meet requirements for energy (115 to 130 kcal/kg/d) and protein (2.5 to 3.5 g/kg/d) (10, 34). Globally, there are no standardized nutritional recommendations and guidelines for the nutritional management of LP infants in terms of required nutrient intake, recommended volume of milk consumption, growth standards and monitoring, and desired physiological status at hospital discharge (10, 16). Most guidelines have focused attention on EP or VP infants with low birth weight. Available guidelines for the nutritional management of preterm infants include the "Guideline for the enteral nutrient supply for preterm infants" of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), but this guideline applies specifically for stable-growing preterm infants weighing approximately 1800g (16). In 2013, the national perinatal association published multidisciplinary guidelines for the care of LP infants in the USA (35). More recently, the "National Guideline for comprehensive newborn care" was published in Nigeria, which also provides some nutritional guidance for newborns, but t not specifically focused on LP infants. For monitoring the growth of newborns, the national guideline recommend to use the Fenton growth chart for preterm infants (6). In Singapore, Malaysia and Taiwan, healthcare professionals have adopted their local (hospital) or national guidelines, which also are not specifically for the management of LP infants and may not be appropriate (34). The outcome of a recent multi-country survey conducted among healthcare professionals in seven countries, including Nigeria, showed that there are no standardized and agreed-upon nutritional practices, guidelines and expected growth outcomes for LP infants in the countries studied (34). This further justifies the need to assess the outcomes of current nutritional management and practices and to advocate for a country-specific guideline for the feeding and management of LP infants.

Specific nutrients of importance for LP infants

Because of the shorter duration of pregnancy and the generally lower birth weight, certain nutrients are essential in the nutritional management of LP infants. The specific nutritional requirements of this group of infants are described in this section.

Protein and energy intake

For many healthcare professionals, the utmost goal of nutrition intervention for preterm infants is growth, and in particular rapid weight gain. However, rapid weight gain during infancy may also lead to long-term adverse metabolic health consequences (36, 37). The rate of weight gain is dependent on the absolute intake of energy and protein (38). Therefore, a balanced protein and energy ratio is important to ensure that preterm infants attain optimal growth in alignment with their required body composition. The desired outcome of a balanced energy and protein ratio is to ensure optimal lean mass accretion. Small for gestational age (SGA) and appropriate for gestational age (AGA) preterm infants require an energy intake >100 kcal/kg/d to gain weight at rates that are similar to in-utero growth (39). On the other hand, excessive intake of energy (in the range of 140–150 kcal/kg/d), which seems safe in the short term, has also been shown to promote fat mass deposition in preterm infants with low birth weight, and yet, with limited evidence of improvement in linear growth as a proxy for lean mass (39–42). Some studies suggest that a protein intake of > 3–3.6 g/100 kcal, with a lower limit of energy of 100 kcal/kg/d, is well accepted by infants, promotes growth, and leads to relatively lower fat deposition during the infant's stay in the hospital (38, 39). This lower intake of protein and energy may also be better tolerated than a higher intake, resulting in a fat mass percentage close to intrauterine references of normal-terms (43). A recent Cochrane study found a higher weight gain of 2 g/kg/day in a comparison between a protein intake of 3-4 g/100 kcal versus <3 g/100 kcal, while no harmful effects of higher protein intake were reported (44).

Iron

Preterm infants have smaller iron reserves due to their preterm birth since two-thirds of the total body iron is transported towards the foetus in the third trimester. Also, as a result of catch-up growth, strongly activated erythropoiesis, a shorter life span of (foetal and premature) red blood cells and frequent blood sampling, preterm-born infants have a higher requirement for iron (45,46). LP infants were even found to have double the iron need of term infants (47). At birth, LP infants are at risk of iron deficiency and iron deficiency anaemia due to lower iron stores, especially LP infants who are small-for-gestational-age(SGA) and infants born to diabetic mothers, and to have increased erythropoiesis at birth (48,49). Serum ferritin (SF)

concentration in cord blood at birth is often used to determine iron status *in utero* (50). Delayed umbilical cord clamping (DCC) or umbilical cord milking (UCM) has been described to be effective in preventing iron deficiency, and increasing haemoglobin concentration and mean arterial pressure level in LP infants in the first few weeks of life, though optimal delay time of cord clamping is not certain (51–53). Iron requirements for LP infants are not exactly known. Iron supplementation is essential for LP infants, although studies have shown variable practices regarding iron supplementation regimens. Iron deficiency can lead to decreased cognitive and psychomotor development (45–47). Well-designed studies are needed to fill these knowledge gaps.

Long-chain polyunsaturated fatty acids: DHA and AA

Long-chain polyunsaturated fatty acids (LCPUFAs) have been recognized to be particularly important for early infant development, cell function, immunity, epigenetics or visual acuity for all preterm as well as children of older ages (54). These LCPFAs, i.e. docosahexaenoic (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA), are important across the entire life cycle, but in particular for preterm infants who are generally born with lower stores (55). Due to rapid brain growth in the last trimester of pregnancy up to two years postpartum, sizeable amounts of LCPFA are required, which are most efficiently obtained by transplacental transfer, and via human milk after birth (56). The long-term neurodevelopmental consequences of low LCPFA, notably DHA and EPA, accretion in newborn brain tissue is as yet unclear. Meta-analyses of randomized controlled trials in premature and term babies are inconclusive, but various recommendations for the addition of both DHA and AA to infant formulae for preterm and term infants have been issued. For instance, in 2001, Koletzko et al recommended that (in-hospital) formulae for preterm infants should contain at least 0.35% DHA and 0.4% AA of total fatty acids (57). However, this ratio in DHA and AA is far from what might be considered to be normal from an evolutionary point of view (58). In 2020, the charitable Child Health Foundation (Stiftung Kindergesundheit, www.kindergesundheit.de), in collaboration with the European Academy of Paediatrics (www.eapaediatrics.eu) published a position paper in which they recommend that infant formulae include both AA and DHA at a ratio of DHA at 0.64%, and AA at least equal to DHA (59). Well-designed studies are still required to determine optimal intake of DHA and AA in preterm infants at different ages.

Vitamin A

Vitamin A plays important roles in growth, vision, immunity, reproduction and gene transcription (60). Deficiency of vitamin A results in xerophthalmia, severe anaemia, wasting, reproductive and infectious morbidity, and increased risk of mortality (61).

As a result of low hepatic stores and decreased vitamin A absorption, preterm infants require a higher daily intake of Vitamin A (16, 62). There is no particular recommended daily intake (RDIs) for LP infants (63). According to WHO, preterm infants weighing more than 1000g at birth should consume 180 to 420 μg/kg/day of Vitamin A until one year of age, which differs from the recommendation of 400 to 1000 μg/kg/day for stable preterm infants weighing 1000 g to 1800 g by ESPGHAN(16, 64). Vitamin A concentrations in mature human milk (of mothers who gave birth at term as well as preterm) range from 18-60 μg/100 ml. These concentrations are considered to be too low for preterm infants. Serum vitamin A concentrations <20 μg/100ml (0.7 μmol/L) are considered to indicate deficiency, whereas <10 μg/100ml (0.35 μmol/L) indicates severe deficiency and depleted liver stores (65, 66).

Vitamin D

Serum 25-hydroxy-vitamin D [25(OH)D] concentration is widely accepted as a biomarker for monitoring vitamin D status. Placental transfer of vitamin D occurs during the third trimester of pregnancy. Compared to full-term infants, preterm infants < 32 weeks GA were shown to have lower serum vitamin D concentration, and more so especially in case of low maternal vitamin D status (67). For healthy preterm and full-term infants, there is no agreed cut-off to indicate vitamin D deficiency, although most authorities have defined serum 25(OH) D concentrations < 20 ng/ml (50 nmol/L) as vitamin D deficiency (68, 69). Several studies on the relation of serum 25(OH)D concentration with intact parathyroid hormone (iPTH), calcium absorption and bone mineral density (BMD), indicate that hypovitaminosis D may be defined as serum 25(OH)D concentrations μ80 nmol/L while sufficiency may be defined as concentrations of 90-100 nmol/L (68,70,71). Since body stores at birth are inversely related to length of gestation and linearly to maternal vitamin D status, preterm born infants are at risk for vitamin D deficiency (72,73). Intrauterine growth, brain development, infantile vitamin D deficiency rickets, early childhood wheezing and asthma, and reduced bone mineral content at the age of 9 years are found to be related to low maternal vitamin D status during pregnancy (72,74,75). Unsupplemented breastmilk will supply <100 IU/day, which is not sufficient to meet the recommended Vitamin D intake of 400 IU/day for preterm infants (76,77). Certain authorities such as the American Academy of Pediatrics (AAP) and ESPGHAN recommend breastmilk supplementation of 200–400 IU/d and 800–1000 IU/d vitamin D, respectively, for preterm infants (16,78). These supplementation recommendations are also quite divergent and not specific for LP infants, hence the need for an agreed optimal vitamin D intake of LP infants in particular, which is currently missing.

Undernutrition, a threat to growth and development of Nigerian children under 5 years

In the second half of infant life and beyond, when breastmilk is no longer sufficient to meet the nutritional requirements of an infant, the WHO recommends the introduction of safe and appropriate complementary feeding, with continued breastfeeding up till the age of two years (79). This will allow children to meet evolving nutritional requirements necessary for optimal growth, development and good health (13). The transition from exclusive breastfeeding to complementary foods is a very vulnerable period, especially for children growing up in low- and middle-income countries (LMIC). At the age of 6–23 months, many children become malnourished, manifesting in the form of wasting, stunting and underweight (80). Globally, 149.2 million children (20%, or one in five) are stunted, and 45.4 million (6.7%) are wasted (81). An estimated number of 3.1 million children less than 5 years of age die annually due to undernutrition. Undernutrition is the outcome of inadequate intake of essential nutrients such as energy, high-quality protein, vitamins and minerals, thus not meeting the nutritional needs to facilitate physical growth, mental development, maintenance, and specific metabolic functions (82,83). Undernutrition is a risk factor for adult short height and a key marker for diminished survival, susceptibility to infection, reduced learning capacity and productivity (84).

Currently, the total number of Nigerian children under the age of 5 stands at nearly 31 million, while annually at least 7 million babies are born (85). Nigeria falters in all measurable indicators of nutrition, with a high percentage of poorly nourished children. Malnutrition, in all its forms, is both a cause and consequence of Nigeria's devastating burden of poverty, disease and mortality. Annually, Nigeria loses over US$1.5 billion in GDP to vitamin and mineral deficiencies (86). According to the 2018 National Demographic Health Survey (NDHS), 37% of children less than 5 years are stunted with a particularly high prevalence of stunting in the north-east and north-west; 7% and 22% are wasted and underweight respectively, with higher prevalence in the rural areas and among children of women in the poorest quintile with no or non-formal education (87). The prevalence of any type of anaemia (<11.0 g/dl) in children 6-59 months is 68% (mild anaemia (10.0-10.9 g/dl), 27%; moderate anaemia (7.0-9.9 g/dl), 38%; and severe anaemia (<7.0 g/dl), 3%). In addition, 29.5% and 21% are vitamin A and zinc deficient, respectively (88).

Based on the 2018 NDHS assessment of infant and young child feeding (IYCF), only 29% of children in the first six months of life were exclusively breastfed in accordance with the WHO recommendations (79,87). Thirty-nine per cent of children in their first six months already received plain water, 4% consumed non-milk liquids while 4%

were given other milk in addition to breastmilk. Appropriate complementary feeding was present in 22%. Only 11% of children aged 6-23 months were fed a minimum acceptable diet (ranges from 5% in the North Central zone to 16% in the South East), 33% of children had an adequately diverse diet in which they had been given foods from at least four food groups, and 42% had been fed the minimum number of times appropriate for their age. Similarly, the proportion of children receiving a minimum acceptable diet increases with increasing household wealth, from 8% to 19%. Children in urban areas (14%) are more likely to be fed according to the minimum acceptable dietary standards than those in rural areas (9%) (87). The NDHS 2018 reported foods made from grains as the most commonly consumed foods among children aged 6-23 months (78% breastfeeding and 90% non-breastfeeding children). Only 27% and 59% of breastfeeding and non-breastfeeding children, respectively, often consume meat, fish, and poultry. Thirty-seven per cent of breastfeeding children consumed fruit and vegetables compared to 57% of non-breastfeeding children. Eggs were only consumed by 13% and 28% of breastfeeding and non-breastfeeding children respectively (87).

Preventing undernutrition typically involves implementing nutritional interventions. Studies suggest that correcting nutritional deficits is more effective with multi-micronutrient interventions providing relevant amounts of iron, zinc, vitamin A, folic acid, and B vitamins in a food-based format compared to single-nutrient interventions (89,90). Food fortification programmes in which essential micronutrients such as iron, zinc, folic acid and vitamin A are added to food vehicles during food processing are important to address micronutrient deficiency (MND). Fortification of processed food vehicles is widely accepted as one of the most cost-effective strategies for delivering additional relevant micronutrients to improve iron status, reduce anaemia, and support growth in children. Fortificants such as micronutrient powders, lipid-based nutrient supplements and iron and vitamin A in targeted fortifiable food vehicles can be impactful in countries with nutritional deficiencies such as Nigeria. Fortification programmes have been implemented for nearly three decades such as the successful salt iodization program achieving 98% household coverage, low prevalence of Iodine Deficiency Disorders (IDD) at 6%, and a median urinary iodine concentration of > 130 µg/l between 1999 and 2004. Iodized salt monitoring became irregular, leading to a decline in household coverage with iodized salt to 52% (91–93). Recent data records that iodized salt coverage at household levels amounts to 97% (87). In 2002, the fortification of cooking oil, sugar and flour (wheat flour, maize flour and semolina flour) with vitamin A and other multiple micronutrients also became mandatory (94,95). The initiation and implementation of these interventions were successful, however, compliance by the industry leaders is a challenge. So far, vitamin A compliance with national standards stands at 25% for vegetable oil, 29% for sugar,

to 56% for cereal flour (96). Most of these interventions are highly efficacious, but implementation and coverage are notoriously poor, and they should be accountably monitored and scaled up.

Micronutrient fortification of familiar beverages, though not widely implemented, may be an effective option to cover gaps in other existing nutrition strategies. Some advantages of such beverages as vehicles for fortification include high consumer acceptance and flexible delivery as a ready-to-consume beverage product or as a powder which can be reconstituted (97). According to the Food and Agriculture Organization of the United Nations (FAO), milk contains all the nutrients needed to support growth and development in children and does not contain antinutrients; therefore, it can be successfully used in the prevention and treatment of moderate and severe malnutrition in children (98). Milk and dairy-based products are nutrient-rich foods providing essential nutrients such as energy, protein, and micronutrients (calcium, potassium, phosphorus vitamin B2 and B12) that support growth and development, with in addition bioactive compounds and fatty acids such as caseins, whey proteins (99,100), milk polar lipids (MPL), α-linolenic acid (ALA), conjugated linoleic acids (CLA), palmitic acid (16:0), lactose and other minor constituents (i.e., calcium, phosphorous, magnesium, and vitamin D), which have an important impact on human metabolism and health (101).

A cross-sectional study revealed that the risk of stunting for children who consumed dairy at least once per day was 28% lower than for children without dairy intake in the last week (102). Fortifying milk with multiple micronutrients including iron, zinc, and vitamins A and D seems a viable strategy to deliver essential nutrients to children who are already used to milk consumption (in differing formats and usage patterns). However, the affordability of such fortified products then becomes a matter of concern in developing countries such as Nigeria, where about 39.1% of Nigerian households are estimated to be living on $1 a day or less (103,104). Therefore, there is a need for research to determine the minimal effective volume of intake that maintains optimal nutrient status and growth.

Specific nutrients of importance for toddlers

All nutrients are essential for the growth and development of children. In this section, the specific requirements for iron, zinc, iodine, protein, fatty acids, as well as multiple vitamins and co-factors of growing children are discussed. These nutrients would fill the most important gaps in the current diets of Nigerian children in relation to their growth and development.

Iron

Growing children require more iron during their early childhood compared to adults. They require more iron for ongoing neurodevelopment, and increases in blood volume, muscle and brain tissue mass. An adequate supply of iron is thus required to support these physiological functions (105,106). When the iron supply is inadequate, children will gradually become iron deficient. Different authorities such as WHO, Institute of Medicine, German Nutrition Society (GNS), United Kingdom Ministry of Health, Australian and New Zealand Ministry of Health and Nordic nutrition recommendations have recommended daily intake for children between the ages of 1-3 years to be between 5.8-9.0 mg/day (107–110). Iron deficiency is a common cause of anaemia and is estimated to be responsible for half of all anaemia cases in women and children globally (111). There are limited recent nationally available data on the prevalence of iron deficiency among Nigerian children less than 5 years; however, a previous study shows that 36% of children less than 5 years were iron deficient, with 13% of them having depleted iron stores (serum ferritin value of less than 20 ng/ml) (112). Anaemia can also be caused by malaria infection, hookworm and other helminth infestations, other nutritional deficiencies, chronic infections, and genetic conditions such as thalassemia. A secondary analysis of two recent national survey datasets from Nigeria, i.e. the Nigeria Demographic and Health Survey (NDHS 2018) and the National Human Development Index (NHDR 2018), was conducted to quantify predictor probabilities of anaemia among Nigerian children aged 6-59 months The findings showed that age, sex, duration of breastfeeding, deworming status, intake of iron pills/syrup, comorbidities of malaria, malnutrition, fever and acute respiratory infection are significant predictors of anaemia status among children aged 6–59 months in Nigeria. Interestingly deworming did not result in a child being less likely to have anaemia (113). The recently published National food consumption and micronutrient survey, conducted in 2022, shows that only 28% of Nigerian children aged 6-59 months received deworming treatment in the last six months. As expected, children who were malnourished were more likely to be anaemic. Iron supplementation was also shown to reduce the risk of anaemia (114).

Inadequate intake of iron can increase a child's risk of infections, behavioural deficits, and cognitive dysfunction resulting in poor school performance and lower productivity (115,116). According to the recent national food consumption survey, the intake of iron and micronutrient powder in the last six months was 7% among children under 5 years, with the lowest intake in the south-east zone (2%) and highest (10%) in the south-west among caregivers with tertiary education (9%) (114). While it has been shown that iron supplements and consumption of fortified foods can improve anaemia status and reduce iron deficiency, some studies have also questioned the safety of high doses of supplemental iron, since it may negatively affect the composition of the intestinal microbiota (117,118). Iron is an essential, growth-limiting nutrient for many

gut bacteria, especially for gram-negative pathogenic bacteria such as *E. coli*, and they will compete for unabsorbed dietary iron. In the case of low bioavailable Fortificants iron salts or excessive iron intake, unabsorbed iron will pass onto the colon and may stimulate (probably dose-dependently) the overgrowth of pathogenic bacteria (119). An upper boundary for safe iron dosage for young children has not yet been set.

High-Quality Protein

Growth and development in all life stages, from infancy through childhood and adolescence, is characterized by a rapid increase in height, weight, neurocognitive development, and physical function maturation, which require an adequate intake of high-quality protein (120). Proteins are made up of amino acids which are needed for growth, building up tissues, protection against infections, and as a source of energy (120). Inadequate intake of protein has far-reaching health consequences, such as growth faltering due to protein-energy malnutrition (PEM), a form of severe malnutrition that is caused by a lack of supply or under-utilization of protein and energy. This form of malnutrition is common in low- and middle-income countries, such as Nigeria (121). Some studies conducted in two regions of Nigeria (south-west and south-east) documented an average PEM mortality rate of between 22- 40% among children less than 5 years of age (122,123). This is caused by poor diets, with the majority of the foods eaten by Nigerian children being predominantly plant-based, i.e. local staples such as maize, cassava, sorghum, corn, and millet, which have a low protein quality and are lacking in the amino acid lysine, followed by deficits in other essential amino acids (EAA) (124).

For children aged 1-3 years, the Food and Nutrition Board of the Institute of Medicine, recommends a dietary protein intake of 1.05 $g \cdot kg^{-1} \cdot day^{-1}$ (13 g/day) (125). However, it is not enough to simply meet a certain amount of protein intake, but it should be ensured that the protein consumed is of high quality and contributes to a balanced intake of EAA (126,127).

Calcium and Vitamin D

Calcium and vitamin D are essential nutrients for bone growth, and many metabolic processes in all body cells, such as maintenance of normal blood pressure, nerve conduction, and regulatory and building elements in the body (128,129). Calcium activates blood coagulation processes involved in the production of haemoglobin, and is important in maintaining the acid–base balance (130). Stronger bones at early age support healthy bones and mobility later in life. Inadequate dietary calcium intake during infancy, childhood, and adolescence affects bone mass acquisition (128). Children aged 1-3 years are required to ingest between 500 -700 mg/day of dietary calcium. Milk and dairy products have long been recognized as an important

dietary source of calcium (131). Milk is well-known for its high concentration of calcium (132). Dietary calcium intake among Nigerian children was found to be approximately 200 mg, which is regarded to be very low compared to the requirements. More children may have sufficient calcium intake if they would consume more milk and dairy products, which is currently not the case (133). To facilitate the efficient absorption and metabolization of calcium, dairy products such as milk have to be fortified with Vitamin D. Vitamin D is also made in the skin after exposure to sunlight (134). Without sufficient vitamin D, only 10-15% of dietary calcium is absorbed (68). Children are considered 25OH-vitamin D insufficient when serum concentrations are between 20-29 ng/mL, and deficient when below 20 ng/mL (135,136). An intake of 15 ug/day of vitamin D is regarded as adequate for children between 1-3 years of age to support physiological processes (137).

Vitamin A

Vitamin A deficiency (VAD) in children is a major public health problem and the leading cause of preventable visual impairment and blindness in low-income countries such as Nigeria (112). Vitamin A is important for DNA synthesis, immune response, and visual development. The dietary reference intake of vitamin A for children aged 1-3 years according to the Institute of Medicine is 300 ug per day (110). Among Nigerian children under 5 years, VAD is 29.5% (112) With VAD, growth deficits worsen in children and their vulnerability to iron-deficiency anaemia is increased (112,138). Insufficient vitamin A intake predisposes children to have decreased immunity, thereby causing frequent illnesses from common childhood infections such as diarrheal diseases and measles that prevent them from attending school (139,140). Occasionally, this can lead to increased mortality among this target group (141). In Nigeria, children between the ages of 6-59 months receive a periodical high dose of vitamin A through the national vitamin A supplementation program (142). According to the recent NDHS, forty-five per cent of children aged 6-59 months received vitamin A supplements in the past 6 months (87). The effectiveness of a high-dose vitamin A supplementation programme for children versus smaller daily doses still has to be investigated.

Vitamin B12

Vitamin B12 is an essential B vitamin that plays an integral role in mental and memory performance, methylation reactions, and maintenance of genomic stability (143,144). The recommended daily requirement for children between the ages of 1-3 years is 0.9 ug/day. Dietary Vitamin B12 can only be derived from animal-sourced foods such as meat, and cow's milk is one of the most important sources of vitamin B12 (145,146). Vitamin B12 deficiency among children is usually caused by a maternal deficiency or pernicious anaemia, and symptoms include fatigue, shortness of breath, weakness or palpitations (147). Poor vitamin B12 status is common among young children in

LMICs, but data on intake and status of vitamin B12 among children in Nigeria is limited (148–152). Studies conducted in countries with a similar socio-demography compared to Nigeria have shown that increased intake of vitamin B12 results in improved cognitive performance, school performance and developmental indexes in early childhood (153–158).

Aim and outline of the thesis

Milk and dairy-based drinks are nutrient-rich foods (159), which, especially when fortified, could be a cost-effective nutritional strategy to increase the intake and status of essential nutrients such as iron, zinc, vitamins A and D, and polyunsaturated fatty acids in children under 5 years old, thereby reducing the prevalence of anaemia and supporting both physical and cognitive development in anaemia endemic countries such as Nigeria.

The overall aim of this thesis is to determine the effects of multi-nutrient fortified dairy-based formulae on growth, anaemia, micronutrient status, cognitive development and gut microbiota in Nigerian LP infants and moderately malnourished toddlers.

Specific objectives:

1. To evaluate growth and nutritional status in Nigerian LP infants receiving a preterm formula or breastmilk.
2. To gain more insight into the perspectives of healthcare professionals on the current nutritional management and practices of LP infants in Nigeria.
3. To determine which dose of iron-fortified dairy-based drink would improve anaemia in anaemic Nigerian toddlers, without stimulating intestinal pathogenic bacteria.
4. To study the effect of a multi-nutrient fortified dairy-based drink on micronutrient status, growth, and cognitive development in malnourished Nigerian toddlers

Chapter 2 explores the effects of preterm infant formula on growth, iron, vitamin D and vitamin A status in apparently healthy Nigerian LP infants (gestational age 32-34 weeks), who could not breastfeed by medical indication, compared to a group of LP infants who were fed according to the ‘golden standard’ of feeding: breastmilk.

Chapter 3 describes a formative research study conducted among Nigerian healthcare professionals who provide nutritional care for LP infants, to gain more insight into

their perspective on their current practices and the guidelines on which these are based. We also identified gaps between current practices, the Nigerian National Guideline for Comprehensive Newborn Care (released in November 2021), and international best practices used for the management of LP infants in Nigeria.

Chapters 4 and 5 describe the results of a 6-month open (partly blind: statistics, biochemical analyses), randomized dose-response trial on the effect of a multi-nutrient fortified dairy-based formula on anaemia, haemoglobin concentration, and intestinal microbiota composition (chapter 4), and anthropometrics, cognitive performance and micronutrient status (chapter 5) in moderately malnourished anaemic Nigerian toddlers, aged 12 – 36 months. In this study, the test product was provided daily in different amounts (200, 400 or 600 mL), supplying 2.24, 4.48 and 6.72 mg of elemental iron, respectively.

Chapter 6 provides a general discussion based on the results reported in this thesis, its public health implications, and suggestions for future research.

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CHAPTER 2

Growth and micronutrient status of Nigerian preterm infants consuming preterm formula or breastmilk

Submitted to Pediatric Research

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Abstract

Background

Moderate-to-late preterm infants have an increased risk of neonatal morbidities compared to term infants, but dedicated nutritional guidelines are lacking.

Methods

Moderate-to-late preterm infants received a preterm formula (n=17) or breastmilk (n=24) from age 2-10 weeks in a non-randomized, open-label observational study. Anthropometric measurements were assessed bi-weekly. Blood concentrations of haemoglobin, ferritin, serum retinol, and 25-hydroxy-vitamin D (25OHD) were analysed at age 2 and 10 weeks.

Result

Average growth per kg of body weight (BW) per day was higher in formula-fed (14.7 g/kg BW/day) than in breastmilk-fed infants (12.8 g/kg BW/day, p=0.001). Length and head circumference in both groups were in line with the median reference values of the Fenton growth chart. At 10 weeks of age, haemoglobin tended to be higher in the formula-fed group (10.2 g/dL vs. 9.6 g/dL, p=0.053). 25OHD increased in formula- and breastmilk-fed infants from 73.8 to 180.9 nmol/L and from 70.7 to 97.6 nmol/L, respectively. Serum retinol only increased in the formula-fed group (0.63 to 1.02 μmol/L, p<0.001).

Conclusion

Breastfeeding resulted in adequate growth in moderate-to-late preterm infants, but was limiting in some micronutrients. The preterm formula provided adequate micronutrients, but growth velocity was high.

Introduction

Annually, 800,000 preterm infants are born in Nigeria, of whom 85% are moderate preterm (32-33 weeks of gestational age (GA)) or late preterm (LP, 34-36 weeks of GA). Although being considered relatively healthy, these moderate-to-late preterm infants still have an increased risk of neonatal morbidities and retarded development as compared to term-born infants (1–7). However, only for the extremely and very preterm infants dedicated nutritional guidelines have been developed (8,9). The main target for feeding of preterm infants is to maintain, or catch up with, normal fetal growth rates without metabolic stress, and to prevent micronutrient deficiencies. For monitoring growth, the Fenton preterm growth chart is the most widely used, allowing growth monitoring of preterm infants from as early as 22 weeks GA to 10 weeks of post-term age (10,11). Recently a survey among healthcare professionals from seven countries showed that a majority (~60%) of respondents rated unfortified human milk as adequate for growth of LP infants, and >70% of them considered good growth as a weight gain of 15-20 g/kg BW/day in-hospital and >20 g/kg BW/day after discharge (12). However, according to the median values of the Fenton preterm growth chart, normal growth velocity for LP infants at the age of 34 weeks is 15 g/kg BW/day, decreasing to approximately 8 g/kg BW/day at term age, and 4-5 g/kg BW/day at 50 weeks (10,13).

The European Society of Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) also considers unfortified breastmilk as sufficient for appropriate for gestational age (AGA) LP infants. The increased nutritional needs of these infants can be compensated by an increased intake of breastmilk (14). With regard to formula feeding, preterm infant formulas used in hospitals are based on the recommendations as set for extremely and very preterm infants (8). Use of these typical preterm infant formulas for a longer period may lead to increased weight gain in the form of fat mass which is associated with health risks at later age (15). Regarding nutrient requirements, adequate intake of protein is particularly important for preterm infants to cope with accelerated growth. The recommended amount of protein intake for infants with a GA of 32-37 weeks is 2.5-3.5 g/kg/day, decreasing to 2-3 g/kg BW/day at term (15–17). The protein-energy ratio (2.5-3 g/100 kcal) is considered to be important to realize qualitative growth instead of stimulating fat mass deposition, (15,17,18) although this ratio is not achieved in human milk. Concerning micronutrients, iron, vitamin D, and vitamin A are often limited in preterm born infants (8,14). The risk of developing iron deficiency is mainly caused by limited iron stores at birth and fast postnatal growth (14). Vitamin D status at birth is positively related to the length of gestation and maternal vitamin D status (19,20). For both iron and vitamin D the concentrations in breastmilk are low (21,22). Of the reported normal vitamin A

range (18-60 μg-RE/100 ml) in mature human milk (term as well as preterm) only the higher concentrations can be considered as sufficient for term infants (390 μg-RE per day) (8,23).

In the present study, growth and the nutritional status of iron, and vitamins A and D were studied in moderate-to-late Nigerian preterm infants fed with a preterm infant formula or unfortified breastmilk from 2-10 weeks of age.

Methods

Study design

For this open (only blinded for analytical measurements), non-randomized parallel observational study, healthy moderate-to-late preterm-born infants were recruited at the Department of Paediatrics of the University College Hospital (Ibadan, Oyo State), the Adeoyo Maternity Teaching Hospital (Ibadan, Oyo state) and the Sacred Heart Hospital (Lantoro, Abeokuta, Ogun State) in Nigeria. During the first 14 postnatal days, parents were informed by the medical research teams about the study, and when agreeing on the participation of their infants the informed consent was signed. Eligible infants were recruited and received their own mother's milk or, when medically indicated, a preterm formula (Peak Baby Preterm, FrieslandCampina, Nigeria). The formula contained per 100 kcal: 3.2 g hydrolysed protein, 5.5 g fat, 9.5 g carbohydrates, 1.67 mg iron (as iron sulphate), 6.7 μg vitamin D_3, and 360 µg-RE (full analysis in supplementary materials **Table s2.1**). The medical indication was judged by the local paediatricians or neonatologist according to the standard of care, including ≥2 births at delivery leading to insufficient breastmilk, or being an orphan. The intervention period lasted for 8 weeks (age 2-10 weeks) or longer until a body weight of 3,500 g was reached. The study was approved by the ethics committee of the University of Ibadan College Hospital, with the assigned number UI/EC/16/0418, and registered in the Netherlands trials register: NTR6156 at https://trialsearch.who.int/

Subjects

Eligible healthy moderate-to-late preterm-born infants had to be 2-3 weeks of age at inclusion, AGA, and on full enteral feeding. In the formula group, preterm infants must be able to consume at least 50% of daily preterm formula milk daily at inclusion and 100% at 4 weeks of age. In the case of breastmilk, at least 75% of daily milk intake had to be breastmilk. Infants with congenital malformations, conditions known to affect growth (e.g., severe bronchopulmonary dysplasia, inborn errors of metabolism, cardiac or renal disease, necrotizing enterocolitis with substantial gut

loss, and grade IV intraventricular haemorrhage), family history of impaired iron metabolism (haptoglobin Hp2-2, hemochromatosis, sickle cell anaemia, thalassemia), or medications that could affect digestion, absorption of food or sleep were not included in the study. Furthermore, blood transfusions and vitamin supplements were not allowed during the intervention period.

Body weight, recumbent length and head circumference (HC) were measured by trained paediatricians and research assistants using calibrated equipment. Growth data were collected at the start of the study (age 14±2 days), and every 14 days thereafter until age 75±2 days. Most of these measurements took place at the homes of participants. The equipment for body weight (Seca 834 Electronic baby and child scales) and recumbent body length (Seca 417 mobile Infantometer) were calibrated every day by using a known weight of 2 kg or a 40 cm length standard. Infants were weighed wearing only a dry diaper, whereas length was measured naked and knees were gently pressed towards the board in order to fully extend the feet. Each measurement was done three times and the mean value was recorded. The difference between two measurements had to be <10 g or <0.5 cm. For growth velocity, the Average 2-point method (Avg2pt) was used with initial weight at baseline (W1) and weight at endline (W2) as a function of time: *((W2 –W1) / ([(W2 + W1)/2] /1000))/ number of days* (13). Growth in gram per day was calculated by: (weight endline-weight start)/56 days. Z-scores and small for gestational age (SGA) or AGA classifications were calculated using the Fenton z-score calculator (http://ucalgary.ca/fenton) (10). For HC, a flexible, non-stretchable, measurement tape (Seca 212 Measuring tape) was used. The tape was placed around the largest part of the head while positioning the lower edge of the tape just above the eyebrows and ears, and around the biggest part of the back of the head. Measurements were recorded to the nearest 0.1 cm. Each infant was measured three times and the mean value was recorded. All outcomes were compared with median growth values as extracted from the Fenton preterm growth chart or data provided in this manuscript (13).

At the start and end of the study, venous blood samples (total 5 ml) were taken to assess Hb, serum ferritin, 25OHD and serum retinol. Frozen serum and plasma samples were transported to the Netherlands by courier on dry ice with temperature monitoring and delivered at the Amsterdam University Medical Centre for analysis. Vitamin D (plasma $25OHD_2$ and $25OHD_3$) was analysed in EDTA plasma using an optimized LC-MS/MS method, as described by Dirks et al (referring to method E) (24). For this study, two cut-off values for 25OHD are considered: 50 (skeletal metabolism) and 75 nmol/L (extra-skeletal activities) (25–27). Heparin plasma was used for the analysis of retinol (vitamin A). Retinol was determined using isocratic high-performance liquid chromatography with UV detection (28), with a sufficiency

cut-off value of 0.7 μmol/L (200 μg/L) (29). Serum ferritin was analysed using Particle Enhanced Immunoturbidimetric Assay (Cobas c 502 analyser, Roche/Hitachi, Mannheim, Germany), with a cut-off value of 76 μg/L (30). Hb was measured in whole blood according to standard procedures in the Ibadan University Clinical Laboratory (Ibadan, Nigeria) with a cut-off value of 10 g/dL (31).

The daily amount of formula milk consumed was recorded by the nurse or mother. Furthermore, the number of hospital days was monitored.

Breast milk samples (30 ml in total) were taken at lactation day 45±2, between 12-14 h o'clock, from a full expression of one breast. Aliquots were frozen at -20°C and transported to the Netherlands on dry ice, and analysed for fatty acids and vitamin D (University Medical Center Groningen, Groningen, the Netherlands), iron, and vitamin A, using methodology described earlier (21,32). Vitamin D is expressed as international units of anti-rachitic activity (ARA) in which 25 ng/L parent vitamin D2-D3, or 5 ng/L 25OHD are calculated as 1 IU ARA.(33) Retinol and iron concentrations were analysed by the European Laboratory of Nutrients (Bunnik, The Netherlands), using ICP-MS for iron and LC-MS-MS for retinol.

Finally, tolerance parameters such as reflux (any, >teaspoon, >25% of milk intake, within 10 min of intake), cramps/colic (any, how often, when, belly distension & noise), and stool (any, consistency according to Bristol Stool chart, & colour: 1) brown, 2) brown-green, 3) green, 4) green-yellow, 5) yellow) (reflux, cramps/colic, stool consistency/frequency/colour) were recorded at the start of the study, and at 45±2 and 75±2 days of age, using a 3-day recall questionnaire (filled in just before the scheduled home or hospital visits).

Statistics

The sample size estimation was based on findings from previous research (34), in which a comparable group of Dutch preterm formula-fed infants showed a higher increase in body weight (4.6 g/kg BW/d, $p<0.015$) when the protein content of the formula was higher (1.6 vs. 1.9 g/100 ml, 75 kcal). In breastmilk the protein content is about 1 g/100 ml (~65 kcal), whereas the present study formula provided 2.56 g protein per 100 ml (80 kcal), hence the differences in energy and protein intake in the current study were considerably larger and 30 infants per group, as in the previous study, was considered sufficient (estimated power >90%).

Growth data, absolute values and delta values with 5 and 4 repeated measurements respectively, were analysed using three-way mixed ANOVA (BBW) with time as within-group variable, and sex and group as between-group variables. Interactions

were studied for time*group*gender, time*group, time*gender, and group*gender. GLM univariate analysis of variance was used to study the effects per time point for each significant interaction. For growth velocities, generalized estimated equations (GEE) was used since these data were often nonparametric.

All other normally distributed data were tested with paired or unpaired T-tests to study differences within or between groups. When co-variables had to be considered, one-way ANCOVA was used. In case of skewed data, comparable non-parametric tests were used. The statistical evaluation took place by using IBM SPSS 26 (IBM, IBM, Armonk, NY, USA). A p-value of ≤ 0.05 was considered statistically significant, while a p-value of <0.1 was considered to show a trend.

Results

Participants

The main reasons for recruiting fewer infants than expected (**Figure 2.1)** were three long hospital strikes that restricted neonatal admissions, refusal of parents because of blood sampling, several SGA infants at birth, and an extension to other hospitals that came too late. A strict breastfeeding policy and providing formula only on medical prescription did limit the inclusion of formula-fed infants. Reasons for the formula prescription were maternal death (n=1), no or insufficient breast milk available (n=16), especially in case of multiple births (n=12; **Table 2.1**). Five SGA infants (2 on breastmilk, 3 on formula) were included based on the advice of the principal investigator. Many participants, however, became SGA during the first 2 weeks of life (**Table 2.1**). All infants finalized the study.

Product intake

Median milk intake in the formula group was 115 ml/kg BW/day at age 2 weeks, increasing to 152 ml at age 4 weeks, and to 165 ml at 8 weeks of age. At 10 weeks of age, the intake dropped again to 147 ml. Average consumption of formula tended to be higher in girls than in boys at 4 (p=0.060) and 10 weeks (p=0.056) of age. Girls in the formula group consumed 15-35% more volume of formula than advised by the manufacturer, which may explain faster growth than their breastmilk-fed counterparts. None of the formula-fed infants received breastmilk during the intervention period. Breastmilk-fed infants did not receive any formula. Although vitamin supplements were not allowed during the intervention period, most infants did receive one or a combination of supplements that included iron, vitamin A and/or vitamin D2 (**Table s2.2**).

Figure 2.1: Flow-chart of screening and allocation to study groups of moderate-late preterm Nigerian infants

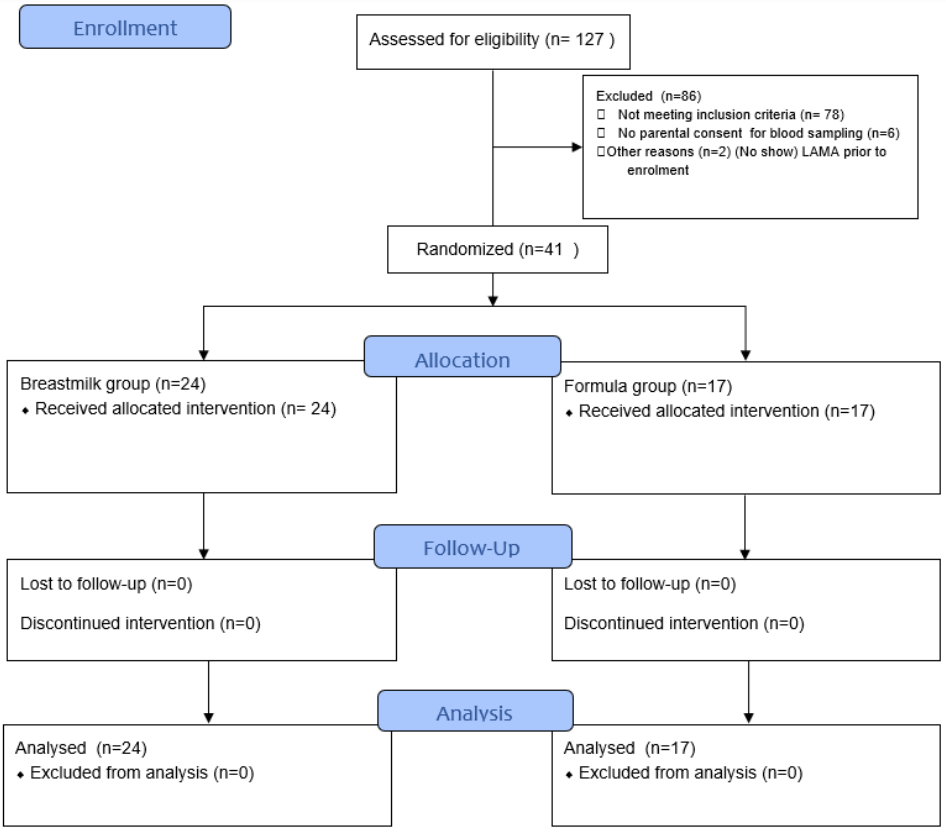

Table 2.1: General characteristics of study participants

Parameter	Formula-fed infants (N=17)	Breastmilk-fed infants (N=24)	P-value
Boys/girls	8/9	8/16	0.518*
Gestational age	33 ± 0.8	32.6 ± 0.7	
Birthweight (g)			
All	1650 ± 233.9	1702 ± 167.1	0.438**
Boys	1600 ± 223.6	1768.7 ± 166.8	0.747**
Girls	1694 ± 246.8	1668.7 ± 162.1	0.098**
SGA[a]			
birth	5 (2 girls, 3 boys)	2 (2 girls)	
age 2 weeks	14 (6 girls, 8 boys)	17 (12 girls, 5 boys	
age 10 weeks	5 (1 girl, 4 boys)	6 (5 girls, 1 boy)	
Age at inclusion (weeks)	1.96 ± 0.8	2 ± 0.20	0.142**

Table 2.1: Continued

Parameter		Formula-fed infants (N=17)	Breastmilk-fed infants (N=24)	P-value
Singletons		5	20	0.001*
Twins		1	2	
Triplets		2		
Quadruplets		1		
Number of hospital days: mean ± SD (median; range)				
	All	12.4 ± 2.8 (13; 5-17)	13.4 ± 7.8 (12; 5-47)	0.523**
	Boys	13 ± 2 (13; 11-17	12 ± 3.6 (12; 5-18)	0.456**
	Girls	11.8 ± 3.5 (14; 5-14)	14.2 ± 9.3 (12; 8-47)	0.344**

Data are provided as mean ± SD, or absolute numbers
* Fisher's exact test, 2-sided. ** Unpaired T-test.
*SGA: small for gestational age. Z-score calculator based on the Fenton preterm growth chart (10).

Anthropometry

Body weight

Within each group, a significant linear improvement of weight was shown ($p<0.001$) during the intervention period (**Figure 2.2**). At the end of the study, body weight between groups tended to be different from each other (3973 g formula-fed vs. 3681 g breastfed, $p=0.080$). When body weight at 2 weeks of age was taken as a co-variable, the adjusted body weights (4026.9 ± 75.6 SE versus 3643.0 ± 90.0 SE) were different at 10 weeks of age ($p=0.002$), which was in particular an effect of adjusted body weights of the girls. In the breast milk group, sex affected body weight in week 10 ($p=0.046$) and tended to do so in week 6 ($p=0.057$): boys weighed 446 ± 216 (SE) g less than girls in week 10, and 400±203 g less in week 6. The three-way interaction between time, group and sex (data not shown) was statistically significant ($p=0.029$), whereas a significant two-way interaction was found for sex and group ($p= 0.017$). A trend towards significance was found for time*group ($p=0.053$). Body weights at the start of the study (age 2 weeks) and after the intervention period of 8 weeks, as well as the average growth in g/day during this period, are reported in **Table 2.2**

Average growth velocity in both groups was at all times well above the median values reported for the specific postconceptional ages (13). For the average growth velocity during the total intervention period of 8 weeks, 94.1% of formula-fed infants (average growth 14.7 ± 1.53 g/kg BW/day) and 87.5 % of breastmilk-fed infants (average growth 12.8 ± 1.77 g/kg BW/day) were above the expected median growth velocity of 10.5 g/kg BW/day for 35-42 weeks of postconceptional age. Average growth velocity was higher in the formula group than in the breast-fed group ($p=0.001$). Girls in the formula group grew faster than their breastmilk-fed counterparts, whereas formula-fed boys showed an almost equal growth as compared

to boys in the breastmilk group.

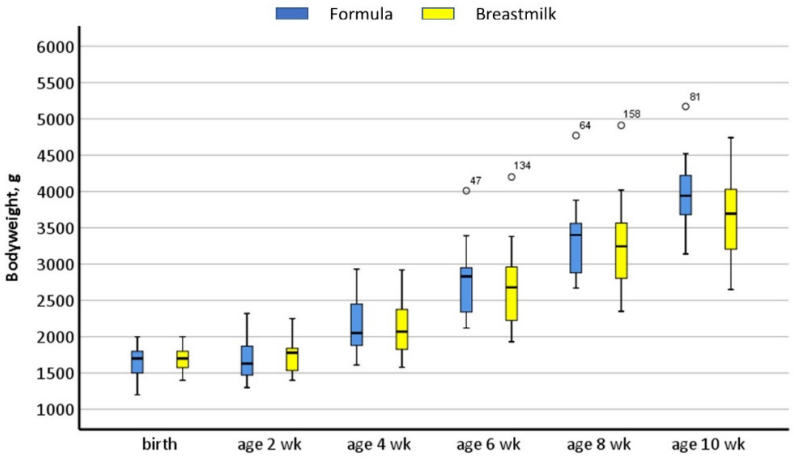

Figure 2.2: Body weight development (g) of Nigerian moderate-to-late preterm born infants on full enteral feeding with breast milk (n=24) or special preterm formula (n=17), from 2-10 weeks of age. Data are presented as boxplots (median, 1st quartile at the lower boundary, 3rd quartile at upper boundary, and whiskers representing the minimum and maximum values within a distance of 1.5 inter-quartile range value. Values outside this range are reported separately).

Table 2.2: Body weights, and changes in body weights (total weight gain, g/day), in Nigerian moderate-to-late preterm infants who were either breastmilk or formula-fed.

Parameter	Formula N=17	Breastmilk N=24	*P* value (between groups)**
Body weight at 2 weeks of age (g)			
All	1668 ±283.8	1730.0 ± 227.3	*0.463*
Boys*	1561.2 ± 247.8	1833.8 ± 242.0	***0.043***
Girls*	1763.3 ± 292.8	1678.1 ± 208.0	*0.455*
P* value *(within group)	*0.148*	*0.116*	
Body weight at 10 weeks of age (g)			
All	3973 ± 479	3681.3 ± 552.4	*0.080*
Boys*	3802.5 ± 432.7	3978.8 ± 488.5	*0.458*
Girls*	4124.4 ± 490.3	3532.5 ± 534.5	***0.012***
Within the group**, p=	*0.174*	*0.060*	
Weight gain (g) from 2-10 weeks of age			
All	2308 ± 305.2	1951.3 ± 431.9	***0.004***
Boys*	2241.3 ±266.4	2145 ±382.6	*0.570*
Girls*	2361 ± 341.4	1854 ± 433.2	***0.004***

Table 2.2: Continued

Parameter	Formula N=17	Breastmilk N=24	*P* value (between groups)**
Within the group**, p=	*0.437*	*0.122*	
Growth in g/day			
All	41.2 ± 5.5	34.8 ± 7.7	***0.004***
Boys*	40 ± 4.8	38.3 ± 6.8	*0.570*
Girls*	42.2 ± 6.1	33.1 ± 7.7	***0.004***
Within the group**, p=	*0.437*	*0.122*	

* Data are mean ± SD. In the formula group, 8 boys and 9 girls participated, whereas in the breastmilk these figures were 8 and 17, respectively. ** Independent Samples T-test.

Both groups showed a recovery or increase in weight-for-age z-score (WAZ) during the intervention period. Breastmilk-fed infants with a WAZ at birth of -0.63 ± 0.50, decreased to WAZ -1.71 ± 0.58 at 2 weeks of age, but ended up with a WAZ of -0.81 ± 1.06 at 10 weeks of age. For the formula-fed infants, these WAZ were -1.02 ± 0.80, -2.08 ± 0.93, and -0.49 ± 1.12, respectively.

Body length

Both groups showed a significant linear improvement ($p<0.001$) in length (formula-fed infants from 42.94 ± 2.08 to 52.47 ± 2.24 cm, and breastmilk-fed infants from 42.58 ± 2.39 to 52.42 ± 1.60 cm) without differences between the groups. At the ages of 2 and 10 weeks, boys in the formula-fed group were shorter than their breast-fed counterparts ($p<0.042$), whereas girls in the formula-fed group were taller than breastfed girls ($p<0.017$). The average increase in length per week (formula-fed: 1.19 ± 0.08, breastmilk-fed: 1.23 ± 0.20 cm/week) was not different between the groups (for all infants as well as for each of the sexes), and slightly higher than the expected median growth value (1.03 cm/week) for 35-42 weeks postconceptional age (13).

Head circumference

Both groups of infants showed a significant linear improvement in HC ($p<0.001$) for the total intervention period (formula-fed from 30.88 ± 1.50 to 37.18 ± 1.29 cm, and breastmilk-fed from 30.63 ± 1.35 to 36.88 ± 1.42 cm). At the age of 2 and 10 weeks, the HC of girls in the formula-fed group was larger as compared to girls in the breastfed group ($p=0.037$). The average increases in HC (cm/week) during the study were not different between groups (formula-fed: 0.79 ± 0.09, breastmilk-fed: 0.78 ± 0.11 cm/week) and higher than the expected median growth value (0.58 cm/week) for 35-42 weeks postconceptional age (13).

Nutritional parameters in blood

In both feeding groups, concentrations of Hb and serum ferritin (as parameters of iron status), decreased during the study period of 8 weeks. At the age of 10 weeks, mean concentrations of ferritin had decreased from 427.1 ± 174.5 to 158.5 ± 121.1 μg/L in breastmilk-fed infants ($p=0.001$), and from 452.3 ± 177.8 to 115.8 μg/L in formula-fed infants ($p=0.003$). In the breastmilk group, 67% of the infants were still above the minimum value of the reference range (≥76 μg/L ferritin) at the end of the study. For the formula-fed infants this percentage was 75%. Hb decreased from 13.3 ± 1.3 to 9.6 ± 1.3 g/dL in the breastmilk group ($p<0.001$), and from 13.4 ± 2.2 to 10.2 ± 0.75 g/dL in the formula group ($p=0.001$). Hb concentrations in the formula-fed group tended ($p=0.053$) to be slightly higher at the end of the study than in the breastfed group. At 10 weeks of age, 62% of breastfed infants and 94% of formula-fed infants were at or above the minimum value of the reference range (≥10 g/dL). In the breastfed group, 25OHD improved from 70.7 ± 17.4 nmol/L at 2 weeks of age to 97.6 ± 39.9 nmol/L at 10 weeks of age, with 75% of the infants above the minimum reference value (≥75 nmol/L 25OHD) at 10 weeks of age. Serum retinol did not improve in the breastmilk group, with 79% of infants being below the reference value of 0.7 μmol/L serum retinol at 10 weeks of age. Formula-fed infants significantly improved their vitamin D (25OHD) and vitamin A (serum retinol) status, with 100% and 95% of the infants being above minimum reference values at 10 weeks of age. For 25OHD, the concentration increased from 73.8 ± 12.6 nmol/L at 2 weeks of age to 180.9 ± 173.5 nmol/L at 10 weeks of age ($p<0.001$), while serum retinol increased from 0.63 ± 0.29 μmol/L to 1.02 ± 0.25 μmol/L ($p<0.001$).

Breastmilk composition

The measured concentrations of vitamin D (calculated as ARA), iron, and vitamin A in breastmilk were lower than those in the special preterm formula: ARA 13.1 ± 6.0 vs. 268 IU/100 ml, iron 0.025 ± 0.046 vs. 1.3 mg/100 ml, and retinol 6.8 ± 2.1 vs. 288 μg/100 ml. Also docosahexaenoic acid (DHA) and arachidonic acid (AA) were measured (breastmilk vs. formula): DHA 0.66 ± 0.40 vs. 0.48 g% of total fatty acids, and AA 0.40 ± 0.14 vs. 0.48 g% of total fatty acids.

Markers of food tolerance

No differences were seen in tolerance indicators between the groups (% reflux, number of defecations, faeces colour and consistency, % colic or cramps, belly distention, and % belly noise (**Supplemental materials Figure s2.1**).

Number of hospital days

Most infants (16/17 in the formula group and 20/24 in the breastmilk group) were discharged from the hospital within 14 days postnatal. Body weight of most infants

(15/17 in the formula group and 22/24 in the breastmilk group) at 14 days of age or at discharge was <2,000 g (range at 14 days of age in the formula group 1,300-2,320 g, in the breast milk group 1,400-2,250 g).

Discussion

Unfortified breastmilk sufficiently supported adequate growth in weight (12.8 ± 1.8 g/kg BW/day), length (1.23 ± 0.20 cm/week) and HC (0.78 ± 0.11 cm/week) in moderately-to-late preterm Nigerian infants during the intervention period at age 2-10 weeks. Preterm formula resulted in an average weight gain velocity (14.7 ± 1.53 g/kg BW/day) that was significantly higher than the reference median weight gain of 10.5 g/kg BW/day for infants with a postconceptional age of 35-42 weeks. Due to the higher weight gain velocity in formula-fed infants, body weight at 10 weeks tended to be higher as compared to breastmilk-fed infants. This difference was significant when body weight at 2 weeks of age was used as co-variable. Average growth in length was not different between the groups, and slightly higher than the reference median growth reference values (1.03 cm/week for length and 0.58 cm/week for HC). Vitamin D and vitamin A status improved in the formula-fed infants, and, whereas Hb and ferritin serum concentrations decreased, the majority (>75%) still had ≥10 g/dL Hb and ≥76 μ/L serum ferritin. In breastmilk-fed infants, vitamin D status also improved (likely due to supplements) but to a lesser extent as compared to the formula-fed infants. Vitamin A status in breastmilk-fed infants did not change, whereas Hb and ferritin concentrations decreased (>62% had ≥10 g/dl Hb and ≥76 μ/L ferritin). Tolerance parameters were comparable between the groups. Most infants (90%) were discharged from the hospital with a BW of <2,000 g, and before the intervention started (age 2-3 weeks).

The observed high gain in body weight (mainly girls), as well as growth velocity (mainly boys), in the formula-fed infants needs attention. According to literature this is more often seen in formula-fed moderate-to-late preterm infants and is considered to be in particular an increase in fat mass (14,35). A high contribution of non-protein calories (>90 kcal/kg BW/day) could be an important cause (17). In general, breast milk would reach this level of non-protein calories (>90 kcal/kg BW/day) at an intake of 150 ml/kg BW/day), whereas in the formula of the present study this is reached at an intake of 130 ml/kg BW/day. Overfeeding is common, and caregivers should be aware of and adhere to the recommended intakes unless there is a medical indication to do differently. Long-term effects of an early excessive increase in fat mass are not known, but may lead to an increased risk for obesity (36).

Earlier reported growth rates of preterm low birth weight (LBW) Nigerian babies were 26.8–34 g/day, 0.86–0.96 cm/week for length, and 0.48–0.50 cm/week for HC in early infancy (37). Although the exact ages of these LBW infants are not reported, the average outcomes in the present study are within the upper range of these published figures, or higher (for formula and breastmilk fed infants respectively: weight 41.2 ± 5.4 and 35.0 ± 7.7 g/day; length 1.2±0.1 and 1.2 ± 0.2 cm/week; HC 0.8±0.1 and 0.8 ± 0.1 cm/week). A study in LBW infants from Chile, the UK and USA, showed that those fed a preterm formula weighed approximately 500 g more at term age than infants fed predominantly human milk. This absolute difference persisted until 6 months of corrected gestational age. Preterm formula infants were also longer and had larger HC at term than human milk-fed infants (38). Finally, it has been recommended that preterm born infants should follow their growth trajectory indicated by the birth weight percentile (39). When WAZ at birth should be regained postnatally, this was the case for the breastmilk group in the present study, but the formula-fed group ended up at a higher WAZ indicating growth beyond their ideal growth profile.

The formula used in the present study is in accordance with the ESPGHAN guidelines for apparently healthy and stable preterm-born infants up to a body weight of 1800 g. In other words, for moderate-to-late preterm infants (≥32 weeks GA) these guidelines may only account for the first 2-4 weeks of life. At higher body weights, current preterm formulae probably provide too much energy for this target population (15). Although the breastfed group showed a growth profile more in line with the reference values, the weight gain velocity during weeks 4-8 was still quite high. This might have been caused by the Nigerian policy to feed breastfed infants at high frequency (at least every 3 hours, at least 8 times a day, until satisfied), and over 1,500 g of breastmilk per day.

Deficiencies of vitamin D and vitamin A were corrected in most formula-fed moderate-to-late preterm infants. In breastmilk-fed infants, vitamin D status improved mainly due to an increase in serum $25OHD_2$ which may have been derived from the vitamin D_2 in the supplement Abidec (200-400 IU vitamin D_2/day) (21). In formula-fed infants, the increase was mainly in $25OHD_3$, as provided by the formula. Although Abidec also contains vitamin A (2000-4000 IU/day), this was not reflected in serum retinol of breastfed infants.

Despite a considerable iron intake (also due to additional supplement intake), formula-fed preterm infants showed a decrease in ferritin concentration (to ~100 μg/L) and Hb (to ~10 g/dL) during the first 10 weeks of life. These decreases were also seen in the breastmilk-fed infants, of whom surprisingly fewer (42% vs 82% in

the formula group) received iron supplements at the age of 10 weeks (**Table s2.2**). A postnatal (10-12 weeks) decline in Hb values to about 10 g/dL appears to be normal for most newborn infants and requires no therapy (physiological anaemia of infancy). A fast decline (i.e., nadir at 4–6 weeks of age) to approximately 8 g/dL (anaemia of prematurity) is associated with abnormal clinical signs and needs treatment (31). In the present study, none of the formula-fed infants reached this 8 g/dL, whereas 4 breastmilk-fed infants showed to have anaemia of prematurity at the age of 10 weeks. The transient drop in Hb is caused by increased hepcidin (limiting iron absorption and release from stores) transcription due to a higher ferritin and Hb status at birth (39). Besides, newborns have to switch from foetal (greater oxygen affinity) to adult Hb (from birth onwards) (40). Hb status will improve at a later age (from 3-6 months onwards), as shown in LP infants (39,40) and Dutch post-discharge very preterm-born infants (41).

As expected, concentrations of vitamin A, vitamin D and iron were low in the breast milk samples. Based on the vitamin A concentration in the Nigerian breastmilk (0.24 ± 0.08 μmol/L), all mothers were vitamin A deficient (cut-off value of 1.0 μmol/L), explaining the low vitamin A status of their newborns (42). The concentration of DHA suggests that the mothers' diets contained a close to an adequate amount of fish (or other DHA sources).

The strength of the study is the described growth outcomes of breastmilk- and preterm formula-feeding in Nigerian moderate-to-late preterm infants under real-life Nigerian conditions (including supplements, limited restrictions to milk intake, formula only on medical indication, and being at home). At the same time, all these practical conditions are limitations of this study. Nevertheless, the results are in line with expectations from the literature and the study provides a clear overview on current practice in Nigeria. The higher than recommended intake of formula, contributing to excessive intake of calories, is unfortunately general practice. The provision of vitamin and mineral supplements is both a limitation and a worry. The preterm formula provides all vitamins and minerals in sufficient amounts, and additional supplementation should only be based on biochemical parameters. Supplementation with vitamin D and K is generally recommended for breastmilk-fed infants, however, in the present study also vitamin A was limited. The finding that breastmilk-fed infants received less often iron supplements than formula-fed infants is surprising and unwanted (41). Finally, this study was not randomized which resulted in boys in the formula-fed group being lighter as compared to their breastmilk-fed counterparts at the start of the study, as well as in more multiple births in the formula group.

Conclusion

Overall, this study shows that breastmilk feeding results in adequate growth of moderate-to-late preterm born infants. When feeding these infants with a special preterm formula (for very preterm infants), weight gain is considerably faster than expected, whereas growth in length and HC are in line with the expectations. This outcome indicates that, following discharge, there is a need for adapted formulae to warrant normal growth of moderate-to-late preterm born infants who cannot be breastfed. The results show that regular growth monitoring is necessary to check on the individual growth trajectory of preterm infants and to adjust the nutritional plan when necessary. The provision of vitamin D, vitamin A and iron via fortified formula appears to be sufficient. Vitamin A via breastmilk was too low for the present target population, probably as a result of maternal vitamin A deficiency. Vitamin D and iron are always low in breastmilk and should therefore be supplemented. Finally, overfeeding of formula-fed preterm infants, with supplements on top of that, appears to be common, even in a research setting, but should be prevented.

Data Availability Statement

The data are not publicly available but can be provided upon reasonable request.

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Author contributions
A.J.O., and A.S. designed the study, I.A.A., O.O.A., A.G.F., G.B.A., O.O.C., G.O.A., A.O.A and A.J.O. conducted the study, A.S., & A.J.O analysed the data, all authors contributed to writing and editing, A.M.-B. and E.J.M.F. supervised the study. All authors have read and agreed to the final version of the manuscript.

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Competing interests
At the time of submission, A.S just retired as an employee of FrieslandCampina. He was involved in the design of the study but not in the recruitment and execution. The product for the study was provided by FrieslandCampina.

A.A.I as an employee of University College Hospital Ibadan implemented the study on behalf of her institution. Her institution received the grant for the project implementation.

Consent statement
Parents and legal guardians of study participants (Preterm infant) received detailed information about the study, the requirements, and procedures, and all their questions were answered after which a signed informed consent was obtained from all parents before any baseline measurement was taken.

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Supplementary Materials

Table s2.1: Composition of Peak Baby Preterm per 100 kcal, as well as the recommended intakes per kg per day from the ESPGHAN (8).

	ESPGHAN 2010[1]	Peak Baby Preterm
Protein hydrolysate (eq)	3.5 – 4.0	3.2
Fat (g)	4.8 – 6.6	5.5
α-linolenic acid (mg)	>55	78
linoleic acid (mg)	385 – 1540	560
docosahexaenoic acid (mg)	12-30	26.5
arachidonic acid (mg)	18-42	18.0
Carbohydrates (g)	11.6 – 13.2	9.5
lactose (g)		7.8
galacto-oligosaccharides (g)		0.1
maltodextrin (g)		1.6
Energy (kcal)	110-135/kg BW	
Na (mg)	69 - 115	63
K (mg)	66 - 132	120
Cl (mg)	105 - 177	100
Ca (mg)	120 -140	124
Mg (mg)	8 - 15	10
P (mg)	60 - 90	69
Fe (mg)	2 - 3	1.67
Cu (µg)	100 - 132	94
Mn (µg)	≤27.5	24
Zn (mg)	1.1 – 2.0	1.1
I (µg)	11 - 55	31.4
Se (µg)	5 - 10	4.5
F (µg)	1.5 - 60	5.9
Cr (ng)	30 - 1230	90
Mb (µg)	0.3 – 5.0	3.6
Vitamin A (µg-RE)	400 - 1000	360
Vitamin D (µg)	20 – 25	6.7
Vitamin E (mg α-TE)	2.2 – 11	5.1
Vitamin K (µg)	4.4 – 28	9
Vitamin B1 (µg)	140 – 300	150
Vitamin B2 (µg)	200 – 400	220
Niacin (µg-NE)	380 – 5500	3750
Vitamin B6 (µg)	45 – 300	150
Vitamin B12 (µg)	0.1 – 0.77	0.27
Folic acid (µg)	35 – 100	45
Pantothenic acid (µg)	330 – 2100	900
Biotin (µg)	1.7 – 16.5	4.1
Vitamin C (mg)	11 – 46	20

Table s2.1: Continued.

	ESPGHAN 2010[1]	Peak Baby Preterm
Choline (mg)	8 – 55	20
Inositol (mg)	4.4 – 55	30
Taurine (mg)		8
Carnitine (mg)		2.6
Nucleotides (mg)		5
Osmolarity (mOsmol/L)		≤300

[1] Agostoni et al. Enteral nutrient supply for preterm infants. A comment of the ESPGHAN Committee of Nutrition. Journal of Pediatric Gastroenterology and Nutrition 2010. BW: body weight

Table s2.2 Number of infants using additional supplements besides the breast milk or special preterm formula.

	Formula fed group (n=17)			Breast milk group (n=24)		
	Age 2 wk	Age 6 wk	Age 10 wk	Age 2 wk	Age 6 wk	Age 10 wk
Abidec[1]	9 (53)	15 (88)	10 (59)	19 (79)	23 (96)	23 (96)
Calcimax	7 (41)	6 (35)	5 (29)	18 (75)	20 (83)	21 (88)
Ferrofer[2]	3 (17)	9 (53)	14 (82)	1 (4)	4 (17)	10 (42)
Folic acid	11 (65)	15 (88)	15 (88)	19 (79)	22 (92)	22 (92)

Data presented as number and (%) of infants using supplements.
[1] Including Afrab Vite Multivitamins. [2] Including Astyfer, Ranferron and Orofer.
Abidec Multivitamin drops for babies (Medana Pharma SA, Sireadz, Poland) providing (in 0.3 ml/day): 2000 IU vitamin A, 200 IU vitamin D2, 0.5 mg vitamin B1, 0.2 mg vitamin B2, 0.25 mg vitamin B6, 2.5 mg niacin, and 25 mg vitamin C. In some cases Afrab Vite (Afrab-Chem Ltd., Lagos, Nigeria) multivitamin drops were used (0.6 ml/day): 4000 IU vitamin A, 400 IU vitamin D2, 1 mg vitamin B1, 0.4 mg vitamin B2, 0.5 mg vitamin B6, 5 mg niacin, and 25 mg vitamin C.
Calcimax Syrup (Vitabiotics Ltd, Lagos, Nigeria) providing (in 2.5 ml/day): 75 mg calcium, 12.5 mg magnesium, 0.75 mg zinc, and 100 IU vitamin D3.
Ferofer Syrup (Kwality Pharmaceuticals Ltd, Amritsar, India) providing (in 5 ml/day: 2x2.5 ml): 50 mg iron. In some cases Astyfer (per 10 ml: 47 mg iron, 5 mg vitamin B1, 3 mg vitamin B2, 2.5 μg vitamin B12, 0.5 mg folic acid, 25 mg niacin) or Ranferron-12 (per 5 ml: 41 mg iron, 5 μg vitamin B12, 0.5 mg folic acid) were used. Folic acid supplement, 2.5 mg daily.

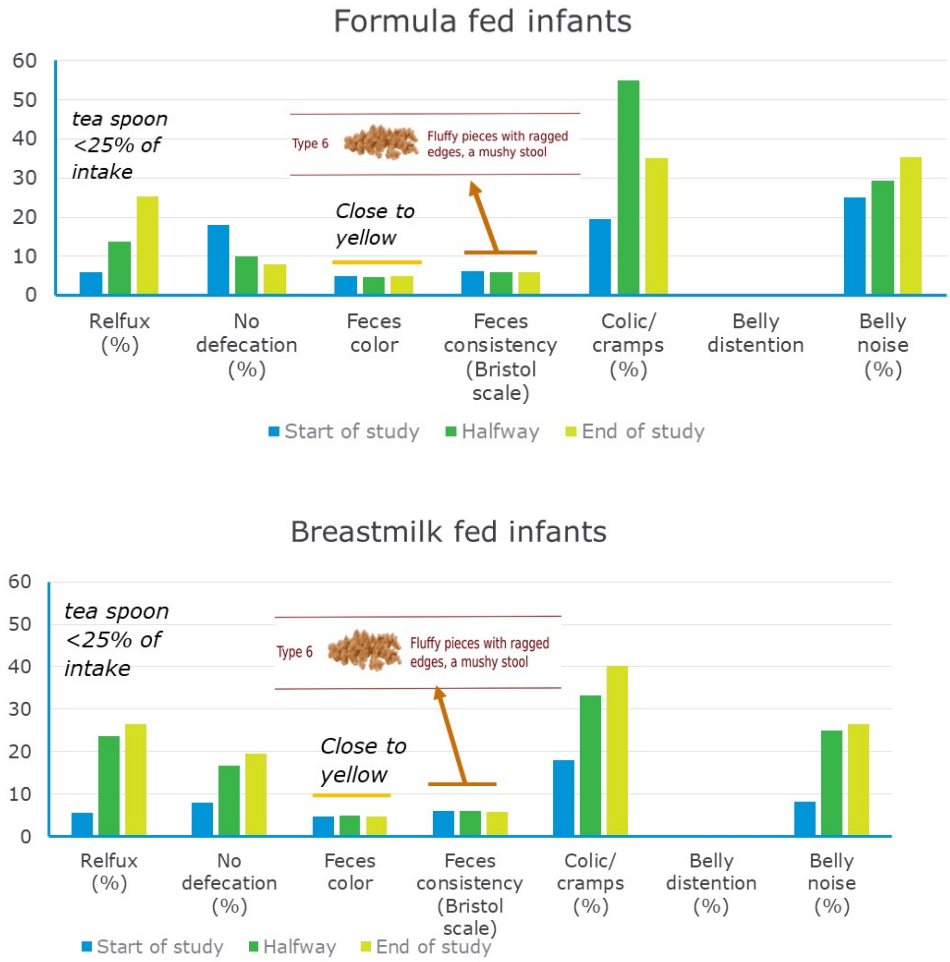

Figure s2.1: Tolerance indicators in exclusive breastmilk or preterm formula-fed Nigerian moderate-late preterm born infants at the age of 2 weeks (start of the study), 4 weeks (halfway) and 10 weeks of age (end).

CHAPTER 3

Insights into feeding practices and guidelines used for the management of late preterm infants among healthcare professionals in Nigeria

Submitted to Acta Pediatrica journal

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Abstract

Aim

We aimed to gain insights into current nutritional management practices of late preterm (LP) infants in Nigeria.

Methods

Purposive sampling was employed to recruit 19 healthcare professionals (HCP: neonatologists, paediatricians, general practitioners and nurses) involved in the care and nutritional management of LP infants in Lagos and Ogun states, Nigeria. Data were collected using interviews, either individually or in small focus groups. Thematic analysis of interview transcripts was carried out to interpret the data.

Results

Ten distinct themes emerged across the research questions and objectives. For growth monitoring, 11 (58%), 6 (31.5%), 1(5%) and 1(5%) of our participants preferred to use the 2006 WHO growth standards, Fenton preterm growth chart, Ballard score and Intergrowth-21, respectively. Regarding growth velocity of LP infants, most HCPs aimed for 15 g/kg BW/day or more during hospitalization. Breastmilk was unanimously the primary feeding option for LP infants. Most HCP preferred to use international guidelines over local guidelines.

Conclusion

Our study shows that there is a wide divergence in the nutritional guidelines used in managing LP infants in Nigeria. Regarding growth monitoring, HCPs tended to aim for a growth velocity higher than necessary for LP infants, which may be disadvantageous for their long-term health.

Introduction

To date, considerable attention has been drawn to support the nutritional management of preterm infants, to improve their survival and quality of life. The World Health Organization (WHO) defines preterm infants as babies delivered at a gestational age of less than 37 completed weeks of pregnancy (1). Preterm birth is recognized as a major clinical risk and is associated with perinatal mortality, severe neonatal morbidity and moderate to severe childhood disability (2,3). Based on gestational age (GA), preterm birth can be categorised into the following sub-categories: extremely preterm (<28 weeks of GA), very preterm (28-31 weeks of GA), moderate preterm (32-33 weeks of GA), and late preterm (LP, 34-36 weeks of GA) (4). In Nigeria, close to 85% of preterm babies are born between 32 and 37 weeks of GA (moderate-to-late preterm infants) (5,6). In 2010, the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition Committee on Nutrition (ESPGHAN) published recommendations on the quantity and quality of enteral feeding for preterm infants weighing 1000-1800 g, to achieve growth that is similar to foetal growth and satisfactory functional development (7).

Since guidelines have been developed primarily for very preterm and moderate preterm infants(7), they are not necessarily fit for LP infants (8). LP infants have unique and often-unrecognized medical vulnerabilities and nutritional needs, predisposing them to greater morbidity rates and even hospital readmissions (9,10) Contrary to the popular opinion that LP infants are relatively "healthy" compared to babies born <34 weeks of GA, LP infants also have a degree of immaturity that poses them with a higher risk of clinical complications and long-term health consequences, such as overweight/obesity, cognitive and neurodevelopmental delay. All of these outcomes are inversely correlated with gestational age(11,12). According to (Johnson et.al 2015), LP infants have twice the risk for neurodevelopmental disability (13). They are equally as vulnerable as other premature infants to acute health risks such as hypoglycaemia and hyperbilirubinemia during hospitalization (14,15). Late-preterm birth presents a challenge to healthcare providers, especially when decisions must be made about their in-hospital nutrient requirement, required volume of milk consumption, growth standards and monitoring, and desired physiological status at hospital discharge. Providing optimal nutritional support for LP infants may improve survival and quality of life, as it does for other preterm infants. However, detailed guidelines that specify how to feed LP infants, either during hospitalization or after discharge, are notably absent (8,16).

Recently, a multi-country survey was conducted among neonatologists, paediatricians and neonatal nurses to inquire about the nutritional practices of LP infants in seven

low to-high and very-high Human Development Index countries, namely Malaysia, Bangladesh, Indonesia, Singapore, Taiwan, Mexico, and Nigeria . For Nigeria, the interviewed health care professionals (HCP) were involved in the care and nutritional management of LP babies in several specific tertiary hospitals. The survey showed that there are no standardized and agreed nutritional practices or expected growth outcomes for LP infants in any of the seven countries, including Nigeria (17). This calls for the development of country-specific nutritional guidelines for LP infants, especially for developing nations such as Nigeria. Building on the Nigerian part of the survey, in the present study we aimed to gain more insights into the current nutritional management of LP infants in Nigeria. Specifically, we were interested in the views of Nigerian HCP on the implementation of existing (inter)national and local guidelines for preterm infants in their daily practice.

Methodology

For this study, we used qualitative study methods comprising in-depth interviews (IDI) and focus group discussions (FGD). We used both IDI and FGD because in some instances it proved difficult to plan FGD with our participants due to their busy and rather unpredictable working schedules, inherent to their medical profession. In those instances, we performed IDI. The standards for reporting on qualitative studies were used in the preparation of this manuscript (18).

Recruitment and eligibility criteria

Eligible participants were HCPs who were involved in the care and nutritional management of LP infants, working either at government hospitals (General Hospitals, University Teaching Hospitals, Maternity Hospitals or Primary Healthcare Centres) or private hospitals in Lagos and Ogun State, Nigeria. To accommodate different levels of medical practice in the study, we recruited four groups of respondents according to their specializations: neonatologists, paediatricians, general practitioners and nurses. This classification was similar to that in the previously conducted survey (19). An invitation email to participate in the study was sent to eighty-four HCPs, after which we followed up with phone calls.

Data collection

Before the interviews, all potential participants received the interview guide (see suppl. 1) by email. To ensure the quality, comprehensibility, relevance, context and suitability of the questions before they were administered, we piloted the interview guide among two neonatologists and two qualitative research experts who were not participating or involved in the study. Unclear questions were identified and then revised accordingly by the first author. Interviews (IDI and FGD) were conducted online in Microsoft Teams version 1.5.00.33362 (Microsoft, Redmond, WA, USA) by

the first author, with support from a research assistant affiliated with the Department of Human Nutrition and Dietetics, University of Ibadan, Nigeria. The responsibility of the research assistant was mainly to support the logistics of following up with potential participants and taking notes during the interviews. After obtaining verbal consent, all the interviewees were asked to find a quiet environment to answer the interview questions. On average, each interview lasted 50 minutes, and the total interview time was 900 minutes.

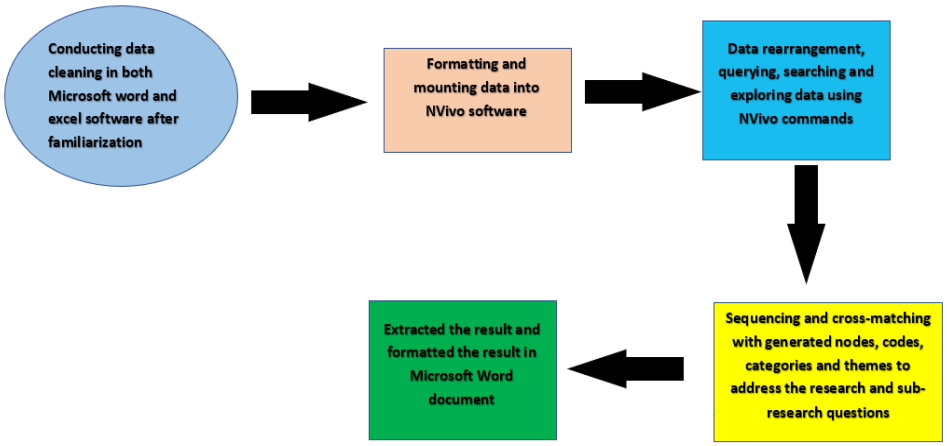
Instrument for data collection

The interview questions were tailored towards three key areas: (i) Growth monitoring of LP infants during hospitalization and following discharge; (ii) Preferred feeding options and enteral feeding initiation for LP infants; and (iii) Guidelines used in the feeding management of LP infants in Nigeria. The interview guide was used to prompt responses from the respondents. Interviews were recorded in Microsoft Teams, after asking interviewees for permission to record, and then transcribed in full. Both the transcripts and the supporting notes were used to code and analyse the interviews using NVivo software, version 12 (QSR International, Burlington, MA, USA). Text data were matched into sequential nodes, categories and themes that were then used to answer the research questions.

Data analysis and management

After each interview session, recordings obtained were transcribed *verbatim* and curated for easy navigation during analysis by two persons (the first author and one of the research assistants). Inter-transcript reliability of the transcriptions was reviewed by an independent researcher (Department of Human Nutrition and Dietetics, University of Ibadan, Nigeria) not otherwise involved in the study. The thematic analytical method was used for the data analysis and was aided by the NVivo software. The transcribed data were first rearranged in Excel and Microsoft Word documents before being imported into the software. Themes and sub-themes were generated from the participant's responses to the interview questions, and were then categorized and matched with the sub-research topics that were asked of all participants. Data-generated and a priori themes were linked with the research objectives to aid data interpretation and conclusions (see flow diagram in figure 3.1).

Figure 3.1: Diagram of the data analysis process



Ethical considerations

This study obtained ethical clearance from the Lagos University Teaching Hospital Health Research Ethics Committee (Reference number: ADM/DCST/HREC/APP/3847). All participants were assured during the interview that their information would be treated with utmost anonymity and confidentiality. Participants were aware that they could opt out when they were no longer interested to continue with the interview. Acceptance to be part of the study through email was regarded as written consent, while verbal consent was also taken before the start of each interview.

Results

A total number of 13 interviews (9 IDIs and 4 FGDs) were conducted with 19 HCPs from Lagos and Ogun states; 84.2% of the respondents were HCPs working in hospitals in Lagos state (**Table 3.1**). Interviews and FGD continued until a sense of saturation was reached, i.e. the point when similar responses are being collected with no new or additional information (20). The majority of our respondents were females (73.7%). Based on their hospital affiliations, about (84.1%) of the respondents were employed in government-owned hospitals (Table 1).

Ten distinct themes were generated and cross-matched with the research questions and objectives as shown in **Table 3.2**.

Table 3.1 Socio-demographic characteristics of interviewed HCPs[1] (N= 19)

Characteristics	IDI[2]	FGD[3]	N[4] (%)
Sex			
Male	2	3	5 (26.3)
Female	7	7	14 (73.7)
Medical Profession			
Neonatologist	2	1	3 (15.7)
Paediatrician	3	5	8 (42.1)
General Practitioner	2	1	3 (15.7)
Nurses	2	3	5 (26.3)
State of practice			
Lagos State	5	11	16 (84.2)
Ogun State	2	1	3 (15.7)
Type of Hospital			
General Hospital	4	4	8
University Teaching Hospital	1	3	4
Maternity Centre	1	2	3
Private Hospital	1	3	4

[1]Healthcare Professional [2]In-depth interview [3]Focus group discussion [4]number of respondents

Table 3.2: Thematic summary representation

Objective 1	**Growth monitoring of LP infants**
Theme 1	Specific growth chart used during hospitalization *Sub-themes:* *i- The gold standard: WHO, Fenton, Intergrowth-21, or Ballard* *ii- Observed growth velocity*
Theme 2	Specific growth chart used following hospital discharge of LP infants *Sub-themes:* *i- The gold standard: WHO, Fenton, Intergrowth-21, or Ballard*
Objective 2	**Preferred feeding options for LP infants**
Theme 3	Feeding Practices *Sub-themes:* *i- Preferred feeding options (breastfeeding, preterm formula, wet nursing)* *ii- Initiation of enteral feeding*
Objective 3	**Nutritional guidelines used in the feeding management of LP infants**
Theme 4	International, national and local guidelines
Theme 5	The Nigerian local context
Theme 6	Maternal and neonatal specificity
Theme 7	Rigid physiological principles and conventional practices
Objective 4	**Gaps between current nutritional management practices and international guidelines**
Theme 8	Poor circulation and inadequacy of local and national guidelines
Theme 9	Differential credence on guidelines developed by various healthcare professional associations
Theme 10	Challenges encountered in the management of LP infants in Nigeria *Sub-themes:* *i- Maternal socio-economic conditions* *ii- Available clinical infrastructure* *iii- Lack of awareness and evidence-based convictions among the HCPs* *iv- Poor attitude to change among HCPs*

Intergrowth chart(28), *WHO growth chart*(29), *Ballard chart*(30), *Fenton growth chart*(31)

The findings presented below were by the curated responses. They match the categorisation of responses in line with each sub-research question and address pertinent themes and sub-themes. This thematic analysis shows the current nutritional management of LP infants in Nigeria, based on the three identified research questions.

Objective 1: Growth monitoring of LP infants

Theme 1: Specific growth chart used during hospitalization

Sub-theme 1-i: The gold standard growth chart for monitoring the growth of LP infants

Regarding care given to LP infants during hospitalization, all the participants emphasized that they keep LP infants only in the hospital when they require urgent and immediate medical care, such as in case of birth complications, inability to feed on their own, and underweight. Majority of the participants described in detail how those three activities require dedicated clinical principles and continuous monitoring through routine ward rounds for patient management. This is usually done by a team of professionals that manage neonates, and their activities are determined by the specific requirements of both the baby and the mother at that moment. Immediately after birth, hospital care centres around three major clinical care services: assessment of the baby's state of health, feeding and growth monitoring. As part of the gold standard clinical practices that they follow, all nineteen participants responded "yes" to doing a ward round, and they all explained what they do during such ward rounds including assessing the case file of each LP infant along with their growth monitoring charts, that may be attached to the case file or as electronic records that are shared across the facilities.

Most of the participants (11 HCPs) mentioned that they use the WHO growth chart, six reported using the Fenton chart, one participant mentioned using the Intergrowth-21 charts and one participant the Ballard score.

Some comments illustrate participants' perceptions of the WHO growth chart and why they use it.

> *"We followed the WHO standard in monitoring this late preterm. I've been taking care of them since 2003 and that is what we've been following. Though, at times, using that WHO standard may not work for you or work for all babies. It depends on the situation of the baby or when the baby is brought in. Then most of the time we follow the WHO standard for those units that I have worked with or I've worked in. That's just my answer."* (Participant 1, FGD 1)

A similar response was provided by Participant 2, FGD 4, who said:

> "*We use the WHO growth chart, and we use this growth chart for us to know how the preterm is progressing because without this growth chart, there is no way we can know if the preterm is thriving the way they are supposed to thrive, so the growth chart is what we use to monitor how they are thriving if they are feeding well and then if they are approaching the developmental milestone the way it's supposed to be, so that's what we use where I practice."*
>
> *"Well, because in my centre, at least that is what is known to us and that is what is more commonly used. Then even though most hospitals preferred to use the WHO growth chart as a reference for their studies and the WHO growth chart is there, I can see it widely accepted than the other chart that you talked about"* (Participant 2, FGD 3)

Participant 1 in FGD 4 said:

> *"With regards to growth monitoring, the charts used for growth monitoring. I believe that you have many more people using the WHO growth standards because I don't want to say it's popular, but it's, you know, WHO is the one that gives us most of the guidelines that we use and people are more aware of it".*

Participant IDI 8 also said:

> *"Where I trained at the University of Lagos, there we use the WHO growth chart, and we used to have this Lagos local one, a Lagos growth chart by a Professor, he was one of the professors at University of Lagos, College of Medicine . So, whether while in the hospital or at the well-baby clinic where we discharge our babies and they come for follow-up and reviews, it is usually the WHO mainly growth chart that is circulated and that we use. The Fenton chart? No, we don't use that and even at the hospital where I work it is still the same WHO growth chart we use. But we all know the deficiencies of that, because the more localized the growth chart is, where the child is growing, and where the child lives, the circumstances will usually be the same. So, comparing that child's parameters to the standard in that locality would be ideal, but for now, to be direct it's the WHO growth chart."*

The quotes show that the participants follow the standard guidelines, but that some improvised with the charts.

One of the participants (Participant 3, FGD 4), who preferred the Fenton chart, explained that:

> *"When the child is born and I will say okay this child is born, preterm 30 weeks, 32 weeks, so, we want to find out is this small for gestational age (GA), appropriate for GA, large for GA, yes, at that point we will be using the Fenton… but by the time we get to postconceptional age 37 weeks and above we tend to switch to WHO growth charts."* This participant provided reasons for her preference for Fenton and also corroborated the reasons why the WHO chart is preferred.

Another participant (Participant 2, FGD 2) said:

> *"…for us in my unit, what we use currently is the Fenton chart. Also, that is what we have recommended in the national guideline for comprehensive newborn care."* The same expression was made by Participant 2, FGD 1: "*So Fenton chart is what we used for even almost all our preterm because it is first easy to get for a consultant, a personal preference, then easy to follow as we use it. But I know that you are talking about late preterm so that's what you use."*

Participant IDI 3 asserted that intergrowth-21 charts are now being adopted by tertiary facilities across the country in addition to the WHO chart due to its detailed parameters that allow for them to assess other anthropometric measurements, like the head or occipital frontal circumference (OFC). He said:

> *"There are current adaptations in Nigeria, I'm sure I'm aware PAN (Pediatric Association of Nigeria) have recently advocated for its use generally in Nigeria now. So that's what most centres are using. Most not all, mind you…I'm aware some still use WHO centiles and all that, but intergrowth in most tertiary centres now… because, for the intergrowth, you can check all parameters; you can measure the head circumference, the OFC, you can measure the lengths, the weights and plot them appropriately. Then you can follow up."*

IDI 4, said:

> *"What we use currently is the Fenton chart. You know, when there's no guideline, people will just use what is easily available to them. OK, and also because the WHO intergrowth chart was done in so many countries, including African countries and low and middle-income countries were involved in that. However, what the national guideline recommends is for us to use the Fenton chart which adequately covers these growth parameters."*

Additionally, the Ballard score was preferred by one participant (IDI 6), a neonatologist, who reported that she improvised the use of the Ballard score in her affiliated hospital. She said:

> " *…When I am in that unit, it is my standard. When I am doing a round, the first thing I ask is what the Ballard score of this child is, when I am on call I will say Ballard score, so they know me as Ballard score. So that's why I always talk about the Ballard score.*"

Sub-theme 1-ii: Observed growth velocity

With regards to what growth velocity they would like to achieve during hospitalisation, our findings showed that the majority of the participants also followed the WHO standards for this and would like to achieve a growth velocity of 15-20 g/kg/BW/day.

They said:

Participant IDI 4:

> *"At least when they are in the hospital, we still use 15-20 g/kg BW/day. That's what we use in my unit [..] This is the standard that is recommended and if I'm not wrong, I think that is what the guideline also says, 15 g/kg BW/day."*

Participant 3, FGD 4, mentioned:

> *"So it's the standard ones, the 15 g/kg BW/day minimum, that's what we expect, and they must show that they can do that consecutively."*

Participant 3, FGD 3, explained that:

> *"…15 g/kg BW/day is usually the target, and of course we want babies to gain weight faster because weight for them is an index of survival. So, we want them to gain weight faster as much as they can."*

Participant IDI 9, explained:

> *"Our target is usually 15 g/kg BW/day, but I put a bracket; 10-20 g/kg BW/day."*

Participant 1, FGD 2, said:

> *"Well, the recommended is a range of 10-15 g/kg BW/day. Well, I would say the maximum attainable, I mean recommended now has 15 g/kg BW/day. Why would I want to go more than that, you know (laughs) […] So, they more or less behave like term babies as per their life, as per feeding, as per growth rates eventually. You know, so of course, the target is 15g/kg BW/day by the books."*

According to IDI 1 participant:

> *"We use 15-20 g/kg BW/day in my unit. This is the standard that is recommended and if I'm not wrong, I think that is what the guideline also says: 15g/kg BW/day"*

In his exact words, IDI 3 participant:

> *"If you're going to put a cut-off, let's say 15g/kg BW/day and it's fair enough and is achievable for late preterm babies that are stable relatively and that's our target actually, in our units."*

Responses from five participants showed they desire that LP gain more weight during hospitalization.

They said:

> *"Well, I am used to targeting 20 to 25 to 30 g/kg BW/day , but if I have a client that is doing 15 g/day and there seems to be no problem; the parameters, the vital signs are fine, then I would not worry, once it is within the range."* (Participant IDI 2)

> *"But for me, I can target 20 to 25 to reach 30 g/kg BW/day. I encourage the mothers that this is what I want you to do[..] that's what am used to actually."* (Participant 2, FGD 1)

> *"Once we are sure the baby is crying well, and all the reflexes are intact, you start feeding. So, we do about 30 to 50 grams per day."* (Participant 3, FGD 1)

> *"The issue is once it is steady if the baby can get 500 grams in like 2-3 days or four days. We are okay, we want it steady." (Participant IDI 5)*

> *"For follow-up, what you just want to see is that there is progress. If you are seeing them every week and I will check over this last week, have I been able to get 20-30 g/kg BW/day."* (Participant IDI 7)

Theme 2: Specific growth chart used following hospital discharge

Following discharge and post-hospital discharge, the majority of the participants mentioned that they have a standard weight requirement before the baby can be discharged from the ward. Post-discharge, parents are requested to bring their babies back to the facilities every week for routine checks and weight monitoring. Apart from one HCP, the majority of our participants mentioned that after discharge they

continue to use the same preterm growth chart as they used during hospitalization.

All the participants mentioned that the standard practice of asking the mothers to come back on specific days is to ensure that the mothers can adequately maintain a steady growth requirement of the LP infants and proper feeding practices after discharge. Participant 2, FGD 4 supported this assertion by saying:

> *"They come to the hospital. Yes, we follow them up in the out-patient clinic, in the outpatient clinic we still follow them up with the growth chart, which is still the WHO growth chart that we still use. At least we use that one to monitor if they are gaining weight and then if their mothers are compliant with feeding, then to also check if there are any other anomalies, maybe medical anomalies or maybe social anomalies or family issues that might be affecting the well-being of the child."*

Similarly, Participant IDI 4, explained the procedures that they follow for post-discharge care by saying:

> *"They come in for vaccinations once they are at least 2.5 kilograms, but usually, we would not discharge any child under 2 kilograms, first of all. So once they hit that weight, you can give the child maybe a week or two weeks follow-up appointment. [...] I continue with the Fenton, the one I have already gotten their charts."*

Contrary to other opinions, Participant 2, FGD 2, who would use the Fenton growth chart for LP infants during hospitalization, said:

> *"Well, following discharge, once the babies are up to 42 weeks, we use Intergrowth when they are with us, Intergrowth charts. When they reach 42 completed weeks which is when Intergrowth charts end, we go on to use WHO growth standard."*

Objective 2: Preferred feeding options for LP infants

Theme 3: Preferred feeding options

Sub-theme 3-i and 3-ii: Preferred feeding options and initiation of enteral feeding

Regarding preferred feeding options, the majority of the participants shared a common expression on the need for enteral feeding to be introduced early as LP infants usually require more feeding to be able to recover the weight loss after delivery or to be able to meet up with their expected growth curve. Also, they unanimously preferred breastmilk as the best feeding option for feeding LP infants because it consists of all the essential vitamins and minerals, it is easily affordable and economical for the family, accessible, it's always in the right temperature and helps in stimulating the gut. This special feeding practice involves a whole lot of clinical management

practices including enteral and parenteral using nasogastric or orogastric and IV-fluid feeding, cup feeding and syringe feeding. In addition to these, the majority of the participant also mentioned that feeding usually starts within the first 30 minutes after an uncomplicated delivery and in situations when both mother and child are stable. When asked about the initiation of enteral feeding and preferred feeding options considered appropriate for LP infants, participant 2, FGD 2, shared her experience:

> *"OK, just like what I said earlier that on that admission day in the first 24 hours, we go on parenteral. Then after that, we commence once we see that the baby is ready, from 2.5 mls, then you start with tropic feeding, you can start with colostrum rub, from colostrum rub to 2.5 mls. From 2.5 mls, then you graduate next to 5 mls and so on and so on like that, depending on how that baby tolerates the feeds. Then once that child sucking reflex, swallow reflex you can see or while you are starting with the nasogastric tube, you will feel, see, you will observe that child, the way it sucks that tube because as you are passing some will be sucking the tube, telling you they are ready"*

Participant IDI 7, asserted that:

> *"The preferred feeding option is breast milk. There is no controversy, you know, yes, we don't use donor milk, it's just the mother's breast milk, yes. So, if she's not lactating at all or probably for RVD (Retroviral disease). Because of what I've noticed about mothers with preterm, they usually suffer from lactation failure, a lot of them, and most especially those who had a difficult delivery or had CS (caesarean section). And that they have decided not to breastfeed their children, the mother can, so we go for formula. And then those babies or preterm that are very sick and unable to suck for a long time their mothers suffer from lactation failure so the breast milk lactation is usually not adequate."*

Participant IDI 8, shared a similar opinion:

> *"And so what we do is we have a policy of starting them on breast milk almost immediately. So as soon as they are delivered, they are sorted out in terms of resuscitation, they are cleaned and they are examined, and they are provided warmth, the next thing is for us to feed and of course, we try as much as possible to start with breast milk."*

Participant 1, FGD 2, like all other participants, also shared similar insights which buttressed the uniformity of the preferred feeding options uncovered by this study. She said:

> *"So, for all babies including late preterm, the preferred feeding option is*

the mother's breast milk. That's the best, but you know that like I said, women that have preterm babies are usually would be ill. Something led up to the preterm birth. So, if the mother cannot breastfeed for any reason, we move to infant formula, while we try to work on different forms of feeding. A woman, maybe you can re-lactate a grandma and make her wet nurse the baby."

Objective 3: Nutritional guidelines used in the feeding management of LP infants

When Nigerian HCPs follow guidelines, the following guidelines are preferred over local guidelines: Recommendations from the WHO, European Society for Paediatric Gastroenterology Hepatology And Nutrition (ESPGHAN), [8] American Academy of Pediatrics (AAP), Royal College of Pediatrics and Child Health United Kingdom (RCPCH, UK) and Neonatal guidelines from the NHS (National Health Services).

Theme 4: International, National and Local Guidelines

Participant 2, FGD 2, mentioned that:

"In my hospital, since 2019 of October or November, we started following the WHO guidelines with regards feeding…(And) I think the Federal Minister of Health and some NISONM Members, NISONM being the Nigerian Society of Neonatal Medicine, also developed guidelines in late 2021.

Participant IDI 5, mentioned in her explanation:

"Recently, new guidelines for enteral feedings in premature infants were issued by the American Academy of Pediatrics, European Society for Pediatric Gastroenterology Hepatology, that's ESPGHAN. Okay, so what I am trying to say is that most people take their guidelines from those places. ESPGHAN, there is NISPGHAN too. Nigerian Society for Pediatric, Hepatology, Gastroenterology and Nutrition."

She further asserted that:

"Many facilities and institutions try to have their facility-based guidelines. Then, all of us are covered by an international body like American Academic Pediatrics or by the National Health Service (NHS) or by the Royal College of Pediatrics and Child Health in England and all of that."

Additionally, Participant IDI 3, responded:

"There's a comprehensive newborn care guideline on feeding, I think from WHO if am right, that we follow. There are local adaptations for peculiarities but by and large, we refer to the central, comprehensive

guideline for newborn care. (And)… Yes, there are, there is PAN guideline (Pediatric Association of Nigeria), there is a newborn guideline in Nigeria and there is one developed locally in my centre"

Theme 5: The Nigerian Local Context

Throughout the entire interviews, a topic that kept reoccurring was how the Nigerian local context influenced the clinical management practices of LP infants. This issue of local context cuts across all the aspects of nutritional management, i.e. feeding practices, growth monitoring, and follow-up, as well as the overall management guidelines.

Participant 3, FGD 2, for instance, explained how the local environment influences the growth monitoring and velocity post-discharge in her clinic:

"As per the kind of environment we have now, as I told you most of the people I deal with, most of them are not educated. They are these people from the rural areas; all these local mothers selling fish and the rest, so, I just ensure that a minimum of 0.5 kg weight addition is okay for me every week…Actually, if it is more than that, I would have preferred it, but you know some of them, we want to really deal with them, putting them on exclusive breastfeeding, not trying to use infant formulas to grow them up."

Another piece of supporting evidence was from the response of Participant IDI 3, who said:

"What it means is to have local guidelines that you can you use. For the hospital, you would understand your peculiarities, you know your constraints, you know, so the hospital guidelines are tailored in a way to fit into the peculiarities of the particular hospital. Take for instance, just, for instance, if the comprehensive guideline says to feed babies late preterm within 30 minutes if the mother delivers spontaneous vertex delivery. You know your peculiarities that might not be feasible before you move the babies and all that."

Participant IDI 1 also explained how the environment where her affiliated clinic is located influences every practice in the facility. She said

"Epe is a rural place, and a lot of the women there are so ignorant. Most of them do not come to the hospital until things get out of hand, yes, I can say a lot of them that deliver preterm are unbooked patients, I can say 85- 90% of them are unbooked patients, and like I said, most of them they come out of delivery with asphyxia and if that happens, you know the

reflexes may not be there, you may not come up almost immediately. So, the first thing we do is nasogastric." She further commented the same thing on feeding practices too where she said *"We don't use donor milk in my facility, even I don't know if donor milk is now acceptable in Nigeria, but you know the mentality "how would I allow someone else to be giving my baby milk, no"*

Theme 6: Maternal and neonatal specificity

The findings from this study also showed that the nutritional guidelines used for the management of LP infants in Nigeria depend on the mother and child in question.

For instance, Participant 2, FGD 4, said:

"Well like my hospital, there are no guidelines. We don't follow any particular guidelines. What we do is we don't generalize about every patient; we just take the neonate as who it is. So, because this neonate now can tolerate this phase, this other one cannot. So, each neonate has its peculiarity. So, we treat each neonate based on its peculiarity and how he or she can tolerate the feeds. So, in terms of the neonate start tolerating breast milk from day one. You know in a day now they might not be able to tolerate it. So, it depends on each neonate. So, each baby is taken based on his peculiarities."

On follow-up, Participant 1, FGD 2, said:

"It depends on where they stay. At times, if they stay very far from the hospital, we ask them to go to the health centres close to their houses for them to do the follow-up care there. But if they stay close to the hospital, they come to the hospital and then do their follow-up" Similarly, participant IDI 2 supported this assertion by saying: *"The follow-up has to be a shorter one depending on that child's condition. At times you give three days if their house is so close to the hospital, or at times we give one week. At most it's a week, they should come after a week to see how that child is doing. Then another date will be given like one or two weeks just to check that baby, that he's doing fine at home."*

Additionally, Participant 2, FGD 4, explained how follow-up is determined:

"They come to the hospital (for follow-up) when the child is initially discharged, every week. Once the child is doing well, gaining weight, and the mother knows how to feed well, the child is gaining weight, we increase up to two weeks, a lot of times from 2 weeks, we really don't go to 3 weeks. From 2 weeks we go to one month, we move to every two months. Of

course, when there are issues, in fact, sometimes a child comes to see you today, there is no need for admission, nothing, but there are some things you may say to come back in two days, I want to review something in a very much shorter appointment…Once they are stable, and the mother and baby are coping well we move to two weeklies, after two weeks."

Furthermore, the health condition of the baby is used to determine the number of days LP infants stay at the hospital. For instance, from the account of Participant IDI 5, who revealed that:

"Some of them have sepsis then what else? Some of them have jaundice. If it's jaundice, by the time it is cleared they are gone. If it's sepsis, that one will take about six, seven to eight days. But the observation was just two-three days. By the time is average, we are looking at five-six days"

Theme 7: Rigid physiological principles and conventional practices

Some of the participants also explained that their background knowledge in physiology and their long years of practice determined how they manage their cases. This assertion was more common among medical consultants that specialize in LP infants who have tried and tested established guidelines over the years. One of these senior professionals included Participant 1, FGD 1, who expressed this by saying:

"I think for us, we just follow our protocol, we have a standard operating protocol (SOP). You know in the neonatal units as we have in all other wards and that protocol has the guidelines for all conditions we see, but the majority of the conditions we see and there is a section for prematurity. So that's really what we follow. So, we follow our SOP, our SOP would have been put forth by the head of the department and most times you know looking through the SOPs of other hospitals around and looking at what you know, and putting all together, you know so more or less SOP in one secondary health facility more or less is the same in the other secondary health facilities in Lagos State."

Participant IDI 7 also shared his experience by stating how he and people in his affiliated facilities follow physiologic principles in their practices of LP infants:

"So, those, I don't think they've come out with any guideline about the feeding of late preterm. You know everybody just goes by the physiologic principles that, for example, a baby from 34 weeks will have good suck, swallow, no respiratory distress, then you introduce the feed. The type of feed you use depends on what is available to you. If you have breast milk, you do. If you don't have breast milk and the baby is in distress or something, you give intravenous fluids and the baby is fine, then if the

baby has any congenital anomaly or what they may decide not to feed and give intravenous fluids instead. If the baby is fine, active, for example, a 34-weeker will be like 2.8 to 2.9 kg and that baby is able to suck soon."

Another participant (Participant IDI 6), who is an experienced professional, explained how she managed by leveraging the experience of HCPs that she worked with and also by using her own wealth of experience and self-developed guidelines based on her successful years of practice and as a trainer of HCPs. She said:

"I have a guideline, but I also follow the nurses, there are guidelines, I facilitate for Lagos State Ministry of Health on neonatal resuscitation, Essential newborn care, putting the baby to the skin, skin to skin, feed immediately, those things are not always possible. So, I listen to the nurses. I consider the nurse working with me. Only by working with her can I have the best outcome. If I insist on my rights as a doctor, if I insist on my right for my protocols, I insist on WHO this, Fenton that, Ballard this, am just going to have a dead baby at the end of the day. And of course, all the entries will be correct, they did Ballard. They are on the back, but it will be a dead baby. So, I listen to the nurses."

Objective 4: Gaps between current nutritional management practices and international guidelines

Theme 8: Poor circulation and adequacy of available local and national guidelines

One of the major challenges faced by Nigerian HCPs is the poor circulation of locally available nutritional national guidelines for the management of preterm infants. Specifically, the majority of the participants said that they are either unaware of any change in the nutritional management practices for preterm infants, that copies of the guidelines are yet to be widely distributed, or that they are too bulky. The assertion about the poor circulation of existing and new guidelines was further strengthened by the response from Participant 1, FGD 4, who stated:

"Then the other thing is us ourselves, the doctors, particularly the consultant if you are aware of the guidelines like I said the WHO guidelines have been here since 2011. I qualified in 2008 and honestly, I didn't know anything about it until I went for my master's in 2018. I mean in practice I found out that I can feed these babies faster, I can move faster but how fast and all of that.."

Participant 2, FGD 3, also said:

"(For the localized version that was created by a Professor), it was during the residency training program that we had that, but it's not

widely circulated. Am not even sure they have that same chart in other universities. So, it might have been done for a project and then left in the neonatal ward and we used it then. But out of the university circles, no it's not widely used. But I know it's available because I used it then and that's why I mentioned it just so you know there are other local ones too"

Participant IDI 6 thought that the existing guidelines are what she recognizes, and she did not have time to check if there is a new update when saying:

"Yes. What I know are all these essential newborn cares which we used to do. Yes, as in skin-to-skin, Kangaroo Mother Care (KMC), that feed on time and how much you feed, temperature, giving vitamin K, all this, yes, I am aware of that. If there's an update, I've not checked it this year, but we have always followed that, I even teach that guideline."

On the contrary, some of the professionals mentioned they often feel that there is nothing new that the new guideline can add as they feel that guidelines are being recycled. Participant 2, FGD 2, justified this assertion when she reacted to a new national and local professional association guideline that was newly introduced:

"*In fact, I kind of think, the WHO guidelines are maybe more compact, for I believe that WHO guideline for feeding preterm and low birth weight babies was like their backbone (NISONM), you know, in developing the guidelines."*

Additionally, Participant 2, FGD 4, also expressed that

"There's nothing much, not much different. But I went through some parts of it. As I said, it's over a 200-page document. And it's difficult to go through that thing, but I try to read through it when I got it. Like my colleague said, I have it on three different platforms for Paediatricians. And going through some parts of it I realize that we really do not have differences, really? Nothing different that's the truth. Nothing different except maybe they were able to put up tables and they are saying that some preterm should be fed every two hours, some preterm should be fed every three hours.

Participant 1, FGD 2, acknowledged that she was aware of new national guidelines since 2021 but she did not take a look at them.

"Federal Minister of Health and some NISONM Members, NISONM being Nigerian Society of Neonatal Medicine, developed guidelines in late 2021, which they've tried to disseminate. In all honesty, I know I have received it on two different platforms. Have I sat down to read it? No."

Theme 9: Differential credence on guidelines developed by various healthcare professional associations

According to some study participants, the gaps in the current preterm management practices were due to the inherent differences in the guidelines being developed and introduced by different professionals and those introduced by international healthcare agencies like WHO, NHS and others. Differences were identified in the growth chart measurement calibration, growth velocity and feeding practices.

Theme 10: Challenges encountered in the management of LP infants in Nigeria

All the participants mentioned that there are some challenges inhibiting the adequate management of LP infants in Nigeria. Each participant mentioned at least two or three challenges in their responses, including maternal socio-economic conditions, inadequate clinical infrastructure, lack of awareness and evidence-based convictions of HCPs, and poor attitude to change among HCPs. The quotes below show some of the responses from the participants.

Participant 1, FGD 2, mentioned:

> *"Hmm, we have one thousand and one reasons, one thousand and one points, you know. Honestly, the list is endless, I must say, you know, apart from the facilities we have in Nigeria is nothing to write to me about. Like one of us just said now, we improvise a lot, the only thing we are yet to improvise is a human being."*

On inadequate clinical infrastructure, Participant IDI 5, expressed:

> *"So when you look at the international standard you know, it's difficult for us to start comparing apples with onions if I can use that. So, by international standards, your preterm baby comes in that of parenteral nutrition, we do not have facilities for that here in Nigeria. The most we do is some form of partial parenteral nutrition, we give amino acids, we give glucose".*

Participant 3, FGD 2, also expressed how the maternal socioeconomic situation poses a big challenge to the effective management of LP infants in her facility and the country

> *"Most of the time, you look at your situation, you look at the part of the country you are in to determine because you can't just enforce a guideline or a line of or a pattern of something on the family. They will tell you. Are you going to sponsor it? Do you understand? Or do I know what I'm going through? I look at the situation of a family, because most of the time, those patients that we have, nurses and healthcare practitioners*

in general, we do help, we will be the ones to give these mothers things for baby care when some of their husbands abandon them. For most of them, their husband abandoned them. They tell you, why this, I don't have money, I don't have this, then you will need to help financially. So, you cannot impose on them."

Discussion

Our study shows that there is no consistency in growth monitoring protocols used in the nutritional management of LP infants in Nigeria. For growth velocity, most HCPs aim for a growth velocity of 15 g/kg BW/day or more during hospitalization. Nigerian HCPs strive to initiate enteral feeding in the first hour of birth in stable LP infants, with breastmilk as the unanimously preferred feeding mode. Many respondents indicated deviating from guidelines based on clinical assessment guided by their expertise, but sometimes also due to the local context. With regards to national guidelines, most HCPs are more inclined to use international guidelines, and mostly those issued by WHO, rather than local or national guidelines.

In the monitoring of infants, growth charts play a major role by providing the basis for growth assessment in comparison to a reference. The WHO growth standards (21), the Fenton growth monitoring chart (22), and the Intergrowth-21 (23) are the most commonly used growth charts among HCPs in Nigeria. About 58% of our participants preferred to use the 2006 WHO growth standards because these growth chart are specifically made for preterm growth monitoring up to 40 weeks, are easy to interpret, have good representation and detailed inclusion of monitoring from 0-5 years of age. Furthermore, we found that all neonatologists in our sample preferred to use the Fenton infant growth chart, while the WHO standards were largely used by paediatricians, general practitioners and nurses. This disparity in the type of growth chart used by the different practitioners may be the result of first exposure during medical school, as asserted by some of the participants, or of the growth chart adopted by the respective medical association.

Contrary to clinical practice, some scientists think that the 2006 WHO growth standards are not appropriate for LP infants (24–26). A comparison of the 2006 WHO growth standards with the Infant Health Development Program (IHDP) growth reference, specifically developed for preterm infants <37 GA (27), showed that "there was only a moderate agreement between the two growth curves for two clinically relevant classifications of growth (< 5th and ≥ 95th percentiles). Early gestational age, especially under 30 weeks of gestation, was the primary risk factor

for unequal classification of weight and length (28). The Fenton growth charts, on the other hand, are specifically designed for growth monitoring of preterm infants with a GA at the birth of fewer than 37 weeks. Also, the Intergrowth-21 chart is based on growth patterns of preterm infants between GA 33-34 weeks with "an uncomplicated intrauterine life and low neonatal and infant morbidity". Fenton growth charts have reportedly been used in 45 developed countries, with South Africa being the only African country. In our study, six (36%) HCPs reported using the Fenton growth charts for monitoring the growth of LP infants because of their completeness (representing all anthropometric indicators) and their specificity for preterm age. In addition, the Fenton growth charts were specifically recommended as the gold standard by Nigerian national guidelines.

Using different types of growth charts not specifically designed for monitoring the growth of LP infants, in particular, might negatively influence how HCPs interpret growth and might not provide adequate or expected improved outcomes for the infants (29,30). It is not clear which population and what medical and nutritional condition should a particular growth chart be used for. A validation study of existing infant growth charts in low- and middle-income African countries should be conducted. An example of such a validation study is the preterm infant multicentre growth study (PreMGS) conducted in six developed countries (Germany, Italy, USA, Canada, Australia, and Scotland). The study compared a growth chart review, foetal-infant growth reference (FIGR) and the WHO Growth Standard (31) among preterm infants of GA 19 – 40 weeks (32–38).

Growth velocity illustrates the difference in anthropometric measurement of infants between two time periods (39). The recommended intrauterine growth rate for preterm infants during hospitalization is 15 g/kg BW/day (40,41). Most HCPs (74%) in our study wanted to achieve a growth velocity of 15-20 g/kg BW/day because it is the global recommendation. About 26% of our respondents would even prefer a higher growth velocity of 20-30 g/kg BW/day. A growth velocity of 20-30 g/kg BW/day has been associated with LP infants maintaining or surpassing their birth weight z-score, with growth rates similar to infants with lower GA at birth (42). A growth velocity above the recommended growth rate may result in an increase in fat mass as a result of overfeeding (43). Overfeeding can result in preterm infants surpassing their birth weight z-score as was recently shown with formula-fed LP infants in an 8-week study in Nigeria with an average growth velocity of 14.7 ± 1.53 g/kg BW/day, while breastfed LP infants had a growth velocity of 12.8 ± 1.77 g/kg BW/day in the same study (44). A higher plasma insulin concentration stimulated by fat deposition and early development of adipocytes has been reported to increase growth velocity more in formula fed infants than breastfed infants (45,46). Infant formulas

for LP infants should therefore be designed in such a way it delivers balanced and appropriate nutrients, with exactly the amount needed to maintain normal growth. Regarding preferred feeding practices, all 19 participants in this study unanimously considered breastmilk and breastfeeding as the most preferred feeding option. Their basis for preferring breastfeeding is to achieve growth similar to foetal growth coupled with satisfactory functional development (8). Breastfeeding is indeed strongly recommended for the feeding of LP infants during hospitalisation (16). As a result of their developmental maturity, and physiological and psychological maternal factors, LP infants have been reportedly linked with higher breastfeeding challenges such as breastfeeding initiation and reduced breastfeeding duration compared to term infants (47,48). HCPs have been identified as an essential factor in enhancing successful breastfeeding practices among the preterm infant population (49). Mothers of LP infants who do not receive sufficient breastfeeding support during their hospital days were not likely to breastfeed for up to 10 days following discharge (50). Baby-Friendly Hospital Initiative support from HCPs such as postnatal breastfeeding support, maternal education, kangaroo mother care, and skin to skin care has been shown to improve breastfeeding initiation and duration in LP infants (16). All study participants recommended preterm baby formula only when the mother is not available, in case of birth complications or HIV, or if the baby is unable to suckle directly from the mother. Unlike some developed countries, the acceptability of donor milk is still very low in Nigeria. About 61% of women in Jos, Nigeria were found willing to donate their breastmilk, but would not feed their infants with donor milk from another woman owing to the fear of transfer of diseases such as human immunodeficiency virus (HIV), genetic traits transfer, and religious and cultural taboos (50). In South Africa, there are almost 24 milk banks in operation, and some initiative has been taken in other African countries such as Kenya, Uganda and Zimbabwe. The same perception is reported in Kenya (51), Zimbabwe (52) and Uganda (53). South Africa, on the other hand, has recently reported a substantial improvement in the operation of their milk banks with almost 24 milk banks in seven out of nine provinces (54). According to data from the European Milk Bank Association, there are 281 active human milk banks (HMBs) in Europe, including 38 in Central and Eastern Europe where donated breastmilk is stored and dispensed when needed for feeding infants including preterm infants (55). In the absence of donor milk through milk banks, Nigerian HCPs do explore possibilities for wet nursing, where a known family member capable of lactating is encouraged to feed the baby.

With regards to guidelines and recommendations used for the management of LP infants, our observations show that guidelines and recommendations are not strictly followed, irrespective of whether there is valid scientific evidence or not. More than five international guidelines were in use across different facilities captured in

this study for the same target group. Although Nigerian HCPs were aware of local guidelines, they preferred to follow international guidelines. Besides, the basis for their current practice is largely dependent on the peculiarity of LP infants' health conditions. The special health conditions of patients affect all aspects of care including growth monitoring, type of feeding practices to be adopted, and guidelines to follow. However, all guidelines mentioned by HCPs focus on support for preterm infants in general, and not specifically for LP infants. The local national guideline for comprehensive newborn care released in 2021 was perceived by HCPs as too bulky and thus not easy to follow, or not well circulated as most of the HCPs interviewed were either not aware of the guideline or did not yet receive a copy. Besides, the information contained in this guideline was said to be the same over the years. However, they all agreed that there are currently no local and international guidelines specifically for LP infants.

A strength of this study was the participation of different layers of medical practitioners (neonatologists, paediatricians, general practitioners and nurses), which gave rich insights into the different perspectives on the management of LP infants in the Nigerian clinical setting. The outcome of this study is not representative of the whole nation, since only participants from two states in the southwest of Nigeria were included in the study. Also, another limitation is the high number of no-response and no show of over 58 HCPs.

In conclusion, our study has further shown that there is a wide divergence in the nutritional management of LP infants in Nigeria. A collaborative effort between different medical practitioners is required to come to a proper nutritional management system for Nigerian LP infants. Lastly, there is an urgent need for a harmonised (inter) national guideline on LP infant growth to prevent overfeeding.

Author contributions

A.J.O., A.M.-B, F.O.S and A.S. designed the study, A.J.O. conducted the study, A.J.O analysed the data, all authors contributed to writing and editing, A.M.-B. and E.J.M.F. supervised the study. All authors have read and agreed to the final version of the manuscript.

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SUPPLEMENTARY MATERIALS

INTERVIEW GUIDE

Insights into nutritional guidelines and feeding practices used for the management of late preterm infants among healthcare professionals in Nigeria

STRUCTURE OF THE INTERVIEW

Introduction

Good Day Sir/Ma! My name is Adedotun Owolabi, a PhD student at the Department of Human Nutrition and Health at Wageningen University and Research, Wageningen, The Netherlands under the supervision of Ass. Prof Alida Melse and Ass Prof Folake Samuel. Thank you so much for taking the time to be here today.

Purpose of the interview

Today's interview aims to gain more understanding into the basis for the current nutritional practices of LP infants in Nigeria and what you perceive as the gaps between your practice, national guidelines (new National Guideline for Comprehensive Newborn Care (released in November 2021) and international best practices used for the management of LP infants are. Please be reminded that there are no right or wrong answers, we are only interested in knowing and understanding the basis for the current practices.

Interview dynamics

- This interview will take approx. 45 minutes. Each question is based on key findings from the literature on nutritional management of LP infants in Nigeria.

- Please be assured of your anonymity and confidentiality. All information gathered in this focus group discussion will be solely used for my PhD thesis, presentation and publications in peer-reviewed journals. If at any point you do not want to continue participating in this discussion, you are free to leave the group and we will no longer be asking you any more questions.

- We would like to record this discussion. Even though we will be taking notes, we are not able to write everything down and want to be able to go back and listen to any information we might have missed. All notes and recording will be kept safely and securely. Is everyone comfortable with recording this conversation? (Confirm that all participants consent). We ask that you kindly use the 'raising hand' tool of this platform *TEAMS* should you have a comment. We are interested in what all of you have to say and will appreciate your participation.

BACKGROUND

LP infants are born between 34 and 36 completed weeks. They constitute around 74% of all preterm births, and this segment of neonates is increasing globally. Like very premature infants, also these LP infants have increased risks for health issues in early life. Also, LPT are at higher risk for long-term health complications such as overweight, and cognitive and neurodevelopmental delay as compared to term infants.

There's been a lot of attention on extremely preterm (less than 28 weeks) and very preterm (28 to 32 weeks) but little attention on LP infants despite the fact that LP infants constitutes about 74% of all preterm and are also at higher risk of both early and later health issues.

It is generally agreed that in-hospital nutrient requirements of LP infants are likely higher than that of term infants. Also, there are human milk can meet these requirements especially when the volume of consumption is high enough. However, there are no detailed guidelines as those available for very preterm infants or very low birth weight neonates.

Lately, several attempts were made to propose some forms of nutritional guidance/ recommendations for LP infants.

I. Growth monitoring of LP infants during hospitalization In monitoring the growth of LP infants during hospitalization, a recent study shows that 38% of Nigerian Healthcare professionals prefer to use the WHO child growth study chart, 31 % Fenton chart for preterm while 22% use both the Fenton chart/WHO Child growth

Let's discuss why you think any of these charts is preferred over another. (Probe for reasons for preference).

I. Following hospital discharge, studies have shown that 39% of Nigerian Health Care Professionals (HCPs) who manage LP infants prefer to monitor their growth using WHO child growth standards, 28% will use the Fenton chart for preterm, and 22% chose to use both the Fenton chart and WHO child growth 2006.
    a. Do you think anything might be responsible for this trend among Nigerian HCPs?
    b. Why are any of these charts preferred over another? (Probe for reasons for preference).

II. With regards to the weight gain during hospitalisation of LP infants, a recent

survey indicates that most (44%) HCPs managing LP infants in Nigeria observed 15 g/kg BW/day weight gain among LP infants while 25.5-32% would like to see higher weight gain.

a. What weight gain would you like to achieve?
b. What is the reason why you like to achieve these growth rates?
a. Do you think this reported observed growth velocity is in line with what is expected based on the Fenton growth chart?

Preferred Feeding Options:

What are your perspectives on preferred feeding options for LP infants (probe for different options such as breastmilk, donor milk, infant formula, surrogate breastfeeding, and human milk fortifiers)?

a. What is your perspective about the **adequacy** of human breastfeeding for the growth of LP infants (Probe for reasons for answers)
b. Discuss HCPs' access, at their places of practice, to different feeding options (probe for access to Donor milk, access to human milk fortifiers, etc)

Enteral feeding Initiation:

a. With regards to the initiation of enteral feeding, how soon after birth will you typically initiate enteral feeding in a stable LP infant? (Probe for factors which influence)
b. What are the reasons for the timing of this initiation? Why do you introduce enteral feeding at the time you introduce it?
c. Are there factors that influence your timing of initiation of enteral feeding?

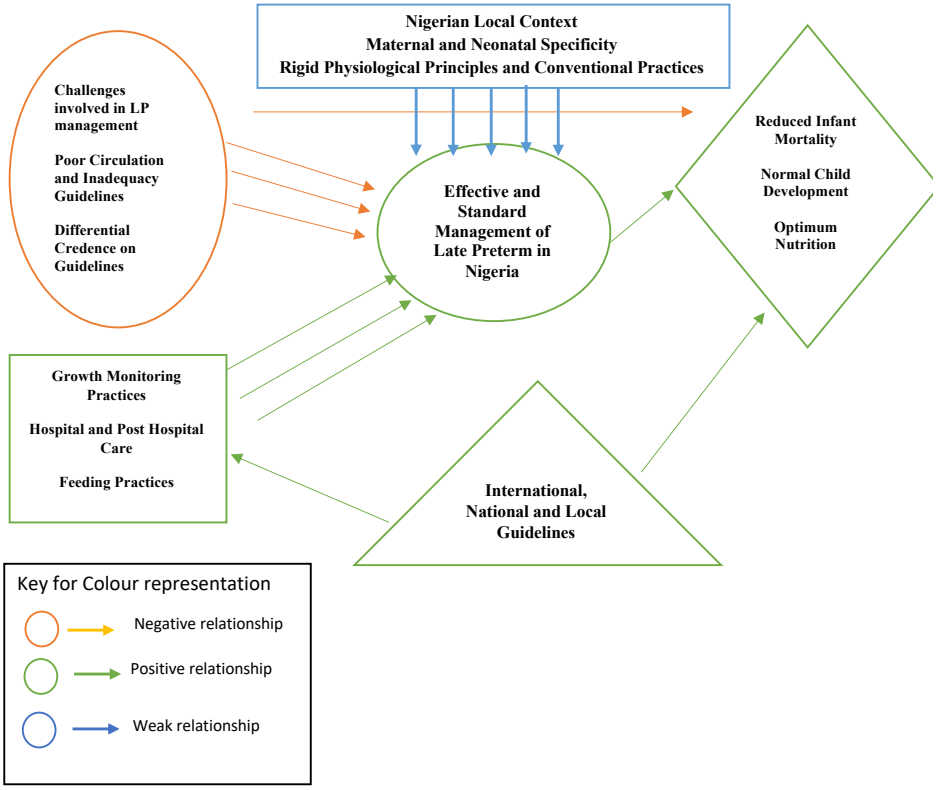
Guidelines used in the feeding management of LP infants

a. Are there any specific guidelines that you follow currently in feeding LP infants? guidelines of feeding LP in the hospital?
b. Please, describe to me a hospital guideline (Probe for a discussion for the features of the different guidelines, probe for why they choose hospital guidelines instead of national and international guidelines).
c. Are you aware of the new National Guidelines for Comprehensive Newborn Care? Probe for correctness (The publishers, year of publication etc), If YES,
d. How did you get to know about the new guidelines?
e. what are your perspectives on the New National Guidelines for Comprehensive Newborn Care (probe for the content- are there any gaps unaddressed), user-friendliness, coverage, adaptability to local

context and comparability with international best practices)

f. What do you think will be the main challenges for implementing the New National Guidelines for Comprehensive Newborn Care and what would need to support the implementation of the new guidelines on Comprehensive Newborn Care?

If NO, probe for reasons for the lack of awareness among HCPs. Is there any pattern associated with this lack of awareness? (eg, common among old staff, etc).



Conceptual Framework of the Thematic Summary

CHAPTER 4

Multi-nutrient fortified dairy-based drink reduces anaemia without observed adverse effects on gut microbiota in anaemic malnourished Nigerian toddlers: A randomised dose–response study

This chapter has been published as:

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Abstract

Prevalence of anaemia among Nigerian toddlers is reported to be high, and may cause significant morbidity, affects brain development and function, and results in weakness and fatigue. Although, iron fortification can reduce anaemia, yet the effect on gut microbiota is unclear. This open-label randomised study in anaemic malnourished Nigerian toddlers aimed to decrease anaemia without affecting pathogenic gut bacteria using a multi-nutrient fortified dairy-based drink. The test product was provided daily in different amounts (200, 400 or 600 mL, supplying 2.24, 4.48 and 6.72 mg of elemental iron, respectively) for 6 months. Haemoglobin, ferritin, and C-reactive protein concentrations were measured to determine anaemia, iron deficiency (ID) and iron deficiency anaemia (IDA) prevalence. Faecal samples were collected to analyse gut microbiota composition. All three dosages reduced anaemia prevalence, to 47%, 27% and 18%, respectively. ID and IDA prevalence was low and did not significantly decrease over time. Regarding gut microbiota, *Enterobacteriaceae* decreased over time without differences between groups, whereas *Bifidobacteriaceae* and pathogenic *E. coli* were not affected. In conclusion, the multi-nutrient fortified dairy-based drink reduced anaemia in a dose-dependent way, without stimulating intestinal potential pathogenic bacteria, and thus appears to be safe and effective in treating anaemia in Nigerian toddlers.

Keywords

Anaemia; malnourished; iron deficiency; iron deficiency anaemia; microbiota; Nigeria; toddler; multi-nutrient fortified dairy-based drink.

Introduction

Anaemia, which is characterised by a haemoglobin level below 11.0 g/dL, continues to be a serious global public health problem that particularly affects young children and pregnant women (1). The World Health Organization (WHO) reported that an estimated 42% of children aged <5 years are anaemic worldwide whereas the burden is even higher in Africa, reaching 62.3% (2). Anaemia is associated with significant morbidity, affects normal brain development and function, and results in weakness and fatigue (2). Literature (3–6) indicates that iron deficiency (ID), when serum ferritin levels are low, is the most common cause of anaemia (5–7) and a result of low iron intake and or iron malabsorption, decreased iron uptake into the blood, increased requirements, or blood loss.

Dietary iron absorption occurs almost exclusively in the duodenum, where it can be absorbed as haem, iron chelates, or as ferrous iron (e.g., from ferrous sulphate) (1,8). In the body, the total amount of iron, and free iron, in particular, is guarded by a controlled absorption (via iron stores in the enterocyte), body iron status (via hepcidin blockade of iron transport into the blood), and by transport and storage proteins such as lactoferrin, transferrin, ferritin and hemosiderin in circulation and organs (9–12). Synthesis of both hepcidin and ferritin (acute phase protein) is increased during infection and inflammation. In this way, the transport of iron into the circulation is limited and iron is sequestered in the reticuloendothelial system, which in the end may lead to anaemia (2,13,14). Increased levels of ferritin, due to inflammation may overestimate iron status, and that is why the ferritin cut-off value for iron deficiency is increased when CRP is increased.

Multiple micronutrient powders (MNP) are strongly recommended in areas with anaemia prevalence in children of 20% or higher (15). These supplements contain 10-12.5 mg of elemental iron, often supplied as ferrous fumarate (FeFum) or ferrous sulphate heptahydrate, per sachet (15). A recent Cochrane review concluded that MNP is an effective intervention to reduce the risk of ID and anaemia in infants and young children, 6–23 months of age (16). Lower iron doses have also been proved successful in increasing Hb. In a 9-month iron-fortified complementary foods intervention study, the efficacy of 2 mg of NaFeEDTA plus 3.8 mg of either FeFum or ferric pyrophosphate (FePP) was explored in toddlers (12-36 months of age) in a highly malaria-endemic region. Following the treatment (6 days/week), Hb increased in the FeFum group from 9.9 to 10.4 g/dL and in the FePP group from 9.6 to 10.5 g/dL (10). Iron-deficiency anaemia (IDA), the most severe degree of ID (17), sharply decreased from 32.8–1.2% (FeFum) and from 23.6–3.4% (FePP) (18).

Besides MNP there is another way to introduce higher levels of iron in the diet of young children, via young child formula (YCF). In a study conducted in New Zealand and Australia, daily provision of 300 mL iron-fortified (1.7 mg/100 mL elemental iron [source not mentioned]) YCF significantly improved the iron status of children as compared to unfortified cow's milk (19). Iron-fortified YCF is also mentioned by the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) Committee on Nutrition as an efficacious option to increase iron status in children (20).

Although iron fortification has positive effects on anaemia and iron status, some studies indicate that oral iron may negatively affect the composition of the intestinal microorganisms (microbiota) (21,22). Except for *Lactobacilli* and *Borrelia burgdorferi*, all microorganisms require iron to survive (8). Many pathogens have acquired strong mechanisms for acquiring iron from the environment, e.g., by secreting iron chelators (e.g., siderophores) that facilitate the bacterial uptake of iron (22,23). In contrast, beneficial commensal gut bacteria from the genera *Lactobacillus* and *Bifidobacterium* require little or no iron (22). In the case of low iron intake, the availability of iron for the microbiota is even stimulated by bacterial metabolites (in particular produced by a few *Lactobacillus* strains) that reduce the absorption of iron by decreasing the activity of hypoxia-inducible factor (HIF-2α) in the enterocyte. HIF-2α regulates directly the three key intestinal iron transporters (24). However, in case of sufficient iron intake, or the use of iron-fortified food and supplements, the amount of iron available for the microbiota is more than enough since absorption is relatively low (5–20%); typically, 20% of the iron is absorbed in the duodenum (6,7). Meaning that most of the iron passes unabsorbed into the colon and may, in particular, stimulate (probably dose-dependently) the more pathogenic bacteria.

In 6-month-old Kenyan infants consumption of MNP with either 2.5 mg (NaFeEDTA) or 12.5 mg (FeFum) iron daily for 4 months adversely affected the gut microbiota composition (21). For both fortifications, an increase in *Enterobacteriaceae* (including pathogenic *E. coli*) and a decrease in *Bifidobacteriaceae* abundances were observed. Furthermore, the incidence of diarrhoea that required treatment was increased in the higher iron dose group, as compared to no iron fortification (27.3% vs. 8.3%, respectively). This study indicates that iron fortification should be balanced: high enough to treat anaemia, but without affecting the gut microbiota. In the present study, a multi-nutrient fortified dairy-based drink was provided daily in different amounts (200, 400 and 600 mL, supplying 2.24, 4.48 and 6.72 mg of elemental iron, respectively, provided as ferrous sulphate) to anaemic, malnourished Nigerian toddlers (12–36 months of age) for 6 months. This study aimed to investigate the effect of different doses of multi-nutrient fortified dairy-based drink (including iron) on the reduction of anaemia prevalence in the target population without stimulating potential pathogenic bacteria in the gut.

Materials and Methods

Subjects and Study Design

In this three-arm, open (blind for biochemical analyses) randomised intervention trial, apparently healthy Nigerian toddlers ($n = 184$), but having mild-moderate anaemia (Hb $\geq$ 7.0 g/dL and $\leq$10.9 g/dL) and mild-moderate acute malnutrition (Height-for-age Z score (HAZ) and/or Weight-for-age Z score (WAZ) < -1 SD and > -3 SD), were recruited in Ijora-Badia community in Apapa-Iganmu Local Council Development Area (LCDA), Lagos, South-West Nigeria. Children with severe malnutrition and anaemia were excluded since they require additional measures such as hospital admission and blood transfusion. At birth, all subjects were born vaginally (since the way of birth may affect microbiota composition (25)) and term, and should be able to consume a maximum of 600 mL of product per day at the time of inclusion in this study. Children were not included when they (I) had a chronic or severe illness requiring hospitalisation and/or special treatment, (II) had a recent medical history (past 3 months) of serious infections, injuries and/or surgeries, (III) had any known allergies or intolerances to milk or milk ingredients, (IV) were predominantly breastfed toddlers, (V) consumed any other fortified foods or supplements, (VI) participated in micronutrient supplementation programs, (VII) participated in any other nutritional study in the last 6 months, (VIII) participated in another clinical study or received an investigational drug in the last 30 days, (IX) were likely to move within the period of intervention, (X) were related or employed by the sponsor or the university, (XI) used any prescription medications before and/or during the study period for more than or equal to two weeks. No restrictions were set for regular food intake.

For recruitment, all subjects gave their informed consent for inclusion before they participated in the study. All families that were permanent residents of Ijora-Badia and with toddlers were informed about the study by a mobilisation team working in the Ijora Badia community from the Lagos State Ministry of Health a few weeks before the commencement of the study. During information meetings, parents, or legal guardians of potential candidates (toddlers) were fully informed about the study, the requirements, and procedures, and all their questions were answered. At the screening, when a signed informed consent was obtained from parents or legal guardians, trained researchers verified age (by birth certificate confirmation or caregiver), and took anthropometric measurements (weight, height, waist-, head-, and mid-upper arm circumference). Eligible children were directly enrolled by trained researchers and randomly (computer-generated block-randomisation), based on the order of screening and stratified for gender and age (12–27, and 28–36 months of age) assigned by the principal investigator to one of the three study groups, with an allocation ratio of 1:1:1. Following inclusion, study participants received deworming treatment (10 mg/kg

bodyweight pyrantel pamoate), to assure that expected worm infections would not play a role during the study. Afterwards, baseline measurements were performed. Venous blood samples (10 mL) were taken for the assessment of iron status and inflammation parameters. Samples of faeces and early morning urine were collected before the start of the intervention. All measurements were repeated at the end of the 6 months intervention period. WHO Anthro 2007 (26) was used to generate z-score values for weight-for-age, height-for-age, weight-for-height, and BMI-for-age.

Ethics

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Lagos State University Teaching Hospital and the Lagos State Government (LREC/10/06/829). The trial was registered at ClinicalTrials.gov: NCT03411590.

Study Products

The three groups received a multi-nutrient fortified dairy-based drink (Peak 123, FrieslandCampina WAMCO, Lagos, Nigeria), in amounts of 200, 400 or 600 mL per day. In the case of 400 and 600 mL, parents were requested to spread the portions (200 mL each) during the day. The time of intake was not monitored. The composition of the drink is shown in **Table 4.1**. The ingredient list is presented in Table S1. Airtight packed powder sachets (for 200 mL each) were delivered weekly to the families by trained researchers who also provided instructions for use. Consumption of test products started as soon as all baseline examinations were completed and baseline blood, early morning urine, and faecal samples were collected. In the case of twins and siblings, only the child who met the inclusion criteria participated in the study officially, however, the other child received the same treatment to prevent sharing and noncompliance with the study protocol.

Table 4.1. Composition of the multi-nutrient fortified dairy-based drink intervention product in the volumes of 200, 400 and 600 mL.

Nutrient	Unit	Per 200 mL	Per 400 mL	Per 600 mL
Energy	kcal	149	297	446
Protein	g	5	11	16
Carbohydrates	g	20	41	61
Sucrose	g	2.9	5.8	8.7
Lactose	g	7	14.5	21.7
Fat	g	5	10	15
DHA	mg	14	28	42
Calcium	mg	188	376	564

Table 4.1. Continued.

Nutrient	Unit	Per 200 mL	Per 400 mL	Per 600 mL
Phosphorus	mg	152	304	455
Potassium	mg	244	488	733
Magnesium	mg	17	33	50
Sodium	mg	63	125	188
Iron	mg	2.24	4.48	6.72
Copper	ug	58	116	173
Zinc	mg	1	2	3
Iodine	ug	40	79	119
Selenium	ug	3.6	7.3	11
Vitamin A	ug-RE	128	255	383
Vitamin D3	ug	2	4	6
Vitamin E	mg	3	5	8
Vitamin B1	ug	155	310	465
Vitamin B2	ug	158	317	475
Vitamin B6	ug	157	314	470
Folic acid	ug	24	48	71
Vitamin B12	ug	0.4	0.8	1,2
Vitamin K1	ug	9.2	18.5	28
Biotin	ug	5.3	10.6	16
Niacin	mg	2.0	4.0	6
Pantothenic acid	mg	0.7	1.3	2
Vitamin C	mg	38	76	114

Blood Sampling and Sample Preparation

Venous blood sampling was performed in the morning between 9:00 and 11:00 am at baseline and endline, obtaining a maximum of 10 mL blood, collected in 3 different types of test tubes: EDTA microtainer (4 mL), heparin gel microtainer (4 mL), and a serum microtainer (2 mL). The EDTA and heparin microtainers were kept at 4 °C and transferred (on ice) to a local laboratory on the day of collection. In the laboratory, tubes were directly centrifuged (HaematoSpin 1400, Hawksley, UK) at 3300 g for 15 min and the extracted EDTA and heparin plasma was pipetted into aliquots of 200 µL. Serum microtainers were kept at room temperature for at least 60 min to allow clotting. Clotted blood was centrifuged at 2000 g for at least 3 min and the extracted serum was pipetted into aliquots of 200 µL. All serum and plasma aliquots were stored at −20°C and transported on dry ice to the Amsterdam University Medical Center (Location Vumc, Amsterdam, The Netherlands) for biochemical analyses.

Biochemical Parameters

Hb was determined in whole blood using the HemoCue Hb 301 system kit (HemoCue AB, Ängelholm, Sweden). Anaemia was defined as Hb < 11.0 g/dL (27). CRP was determined in heparin plasma using an immunoturbidimetric assay on Roche/Hitachi Cobas c systems (measuring range: 0.3–350 mg/L; interassay CV < 20% in low CRP samples; repeatability CV < 5%, intermediate precision CV < 12%). Serum ferritin was determined in heparin plasma using an immunoturbidimetric assay on Roche/Hitachi Cobas c systems (measuring range: 5-1000 μg/L; interassay CV < 20% in low ferritin samples; repeatability CV < 10%, intermediate precision CV < 14%). CRP and ferritin concentrations were used to determine ID and IDA. In the present study, ID was defined as <12 μg/L serum ferritin when CRP ≤ 5 mg/L (no inflammation) or <30 μg/L serum ferritin when CRP > 5 mg/L (inflammation), and IDA as the combination of ID and anaemia (2,27). Folate was analysed in heparin plasma using the Elecsys Folate III binding assay and Cobas-e immunoassay analyser (Roche/Hitachi). Measuring range: 0.6–20.0 ng/mL or 1.36–45.4 nmol/L. Vitamin B12 was determined in serum using the Elecsys Vitamin B12 binding assay and Cobas-e immunoassay analyser. Measuring range: 50.0–2000 pg/mL or 36.9–1476 pmol/L. The cut-off values for folate and vitamin B12 used in this study were 10 nmol/L and 150 pmol/L, respectively.

Faecal Samples and Microbiota Analysis

Parents/caregivers were asked to collect 5-10 g early morning faeces in stool collection stubs with a spoon attached to the lid (Greiner Bio-One, Vilvoorde, Belgium), at baseline and after the intervention. The samples were brought to the collection centres by the parents and caregivers and stored at (5–7 °C) within 1 h, and then transferred on ice to the nearest freezer (−20 °C) that same day. Samples were transported to the laboratory (NIZO, Ede, the Netherlands) on dry ice, where faecal pathogenic *Escherichia coli* was determined using targeted qPCR (n = 88; 200 mL n = 26, 400 mL n = 27, 600mL n = 35). Furthermore, 16S rRNA gene sequencing was performed in a subset of 60 samples (200 mL n = 19, 400 mL n = 20, 600 mL n = 21) to determine faecal microbiota composition as previously described(28). A full description of the materials and methods used for DNA extraction and gut microbiome analysis is available as Supplementary Text.

Urine Samples and Iodine Analysis

The iodine status of the children was assessed using spot urinary samples. The parents were asked to collect the child's early morning urine (5 mL) into a 10mL universal laboratory bottle at baseline and after the intervention, before any food or drink consumption, for determining urinary iodine concentrations. The samples were brought to the collection centres by the parents and caregivers and stored at 5–7 °C within 1 h, and then transferred on ice to the nearest freezer (−20 °C). Samples were

transported on dry ice to the Central Clinical Laboratory of the University Medical Center Groningen, the Netherlands. Iodine in urine was analysed using ICP-MS (Varian, Varian Inc., Palo Alto, USA; lowest level of quantification (LLOQ) 25 μg/L). The cut-off value used for iodine deficiency in this study was <100 ug/L (29).

Sample Size Calculation

Although the change in anaemia prevalence was the primary objective of the study, the sample size calculation was based on an expected change over time in Hb (baseline versus endline) as at baseline all included children were anaemic. The expected change in the 600 mL group (6.72 mg Fe/day) was 1.0 ± 1.5 g/dL, based on the finding of Sazawal et al., 2010 (30). A total sample size of 35 toddlers per treatment arm was considered adequate to detect the estimated increase in Hb (two-sided-power: 80%; $\alpha = 0.05$). To compensate for a possible drop-out of about 30%, the sample size was increased to 45 toddlers per treatment arm, however, during recruitment, 184 children were included in the study.

Statistical Analysis

All participants with at least weight measurement at baseline were included in the intention to treat (ITT) population. Subjects were included in the Per Protocol (PP) population when Hb measurements were available at both baseline and endline. Baseline subject characteristics of the three groups within the PP population were compared using the Pearson Chi-square test, Fischer's exact test, independent t-test, one-way ANOVA, Mann–Whitney U test or Kruskal–Wallis H test depending on the type and distribution of the parameter. All analyses were performed using IBM SPSS Statistics version 24 (IBM Corp, Armonk, NY, USA).

Evaluation of Biochemical and Related Parameters

The post-intervention prevalence of anaemia of the three groups was compared using a Pearson Chi-Square test, with post hoc pairwise comparisons using the z-test of two proportions with a Bonferroni correction. ANCOVA was used to determine the effect of the different interventions on Hb and ferritin, including baseline concentrations as a covariate. Post hoc analyses were performed with a Bonferroni adjustment. Differences in Hb and ferritin between baseline and endline within intervention groups were tested with either a paired t-test or Wilcoxon signed-rank test, depending on the distribution of the data. ID, IDA, and inflammation prevalence between groups were compared using Fisher's Exact test. Within-group differences in ID, IDA, and inflammation prevalence were tested using McNemar's test. A p-value < 0.05 was considered significant. All analyses were performed using IBM SPSS Statistics version 24 (IBM Corp, Armonk NY, USA).

Evaluation of Faecal Microbiota

Between-group differences in alpha diversity (variance within a particular sample) and beta diversity (variation between samples; phylogenetic distance metric weighted UniFrac) were tested by the nonparametric Kruskal–Wallis test with Dunn's post hoc test, as implemented in GraphPad Prism 5.01. Between-group differences of single taxa were assessed using the nonparametric Mann–Whitney U test with FDR correction for multiple testing unless stated otherwise. Comparisons of targets of our primary interest (*Lactobacillaceae, Bifidobacteriaceae, Enterobacteriaceae*) were not corrected for multiple testing. For comparisons of more than 2 groups, the nonparametric Kruskal–Wallis test with Dunn's post hoc test was applied. In the longitudinal analysis, change of taxon relative abundance over time, 2log ratios were calculated, in which the relative abundance of a taxon at the second or later time point was divided by the relative abundance of the same taxon at an earlier time point. Ratios were compared among groups by Mann–Whitney U tests with FDR correction for multiple testing, and for comparisons of more than 2 groups by the nonparametric Kruskal–Wallis test with Dunn's posthoc test. Redundancy analyses (RDAs) on the gut microbiota composition as assessed by 16S rRNA gene sequencing were performed in Canoco version 5.11 using default settings of the analysis type "Constrained". Relative abundance values of genera or operational taxonomic unit (OTUs) were used as response data and metadata as an explanatory variable. For visualisation purposes, families (and not OTUs) were plotted as supplementary variables. Longitudinal effects of the intervention were assessed by calculating 2log ratios in which the relative abundance of an OTU or genus at endline was divided by the relative abundance of the same OTU or genus at baseline. These ratios were used as response variables in RDAs and were weighted based on the average relative abundance of each OTU or genus in all subjects. RDA calculates *p*-values by permutating (Monte Carlo) the sample status. qPCR gene copy counts of each target (total bacterial counts, EPEC *eaeA* gene, ETEC *lt* gene, ETEC *st* gene) were compared between test product dose groups by Kruskal–Wallis test with Dunn's posthoc test and changes over time were calculated by subtracting the counts at endline from the counts at baseline. Pathogenic *E. coli* was defined as the sum of the gene copies of EPEC, ETEC lt and ETEC st.

Results

Baseline Characteristics

In total 184 Nigerian toddlers were recruited, between February and June 2018, and allocated to one of the study groups: 200 mL $n = 62$, 400 mL $n = 61$, and 600 mL $n = 61$. Nine children without any anthropometric and/or no iron-status biochemical data at baseline, seven children who did not comply with the malnutrition criteria (false

inclusions), and three children with very high ferritin but low CRP levels (suspected iron metabolic disease) were excluded from evaluation. Meaning that 165 children were finally included in the ITT analysis, as shown in **Figure 4.1**. We experienced a high loss to follow-up, mainly due to the traditional travelling of families during the festive periods such as Id el Kabir and Christmas (n = 42). For 18 toddlers Hb concentrations at endline were missing. This resulted in a population of 105 toddlers for PP analysis 105 subjects. The baseline characteristics of the three different intervention groups of the PP population are shown in **Table 4.2**. No significant differences were found between the three groups, except for height which was lower in the 600 mL group compared to the 200 mL group (p = 0.03). The baseline characteristics of both ITT and PP populations can be found in Table S2 and were comparable between populations except for inflammation prevalence, which was higher in the ITT population (30.6%) compared to the PP population (17.6%).

Figure 4.1. Flow-chart of screening and randomisation process.

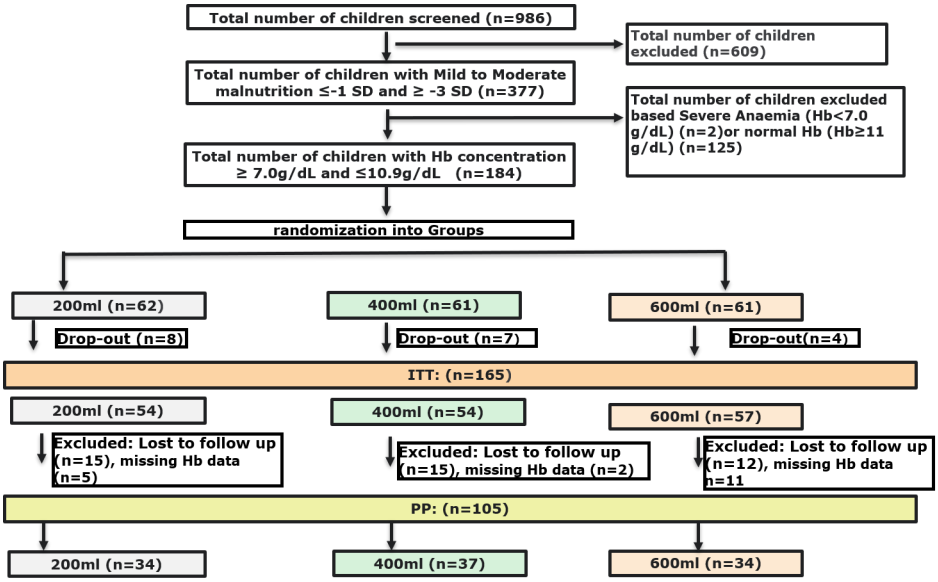

Table 4.2. Baseline characteristics of the three intervention groups (PP population).

	200 mL	400 mL	600 mL	*p*-Value
n	34	37	34	
Age (months)	20.0, 8.5	20.0, 7.5	18.0, 8.5	0.48 °
Gender (boys/girls) (%)	47.1/52.9	51.4/48.6	38.2/61.8	0.53 #
Social class (upper/middle/lower) (%)	0.0/21.2/78.8	2.8/19.4/77.8	0.0/17.6/82.3	0.57 #
Religion (Muslim/Christian) (%)	72.7/27.3	63.9/36.1	70.6/29.4	0.71 #
Weight (kg)	9.2, 2.3	8,9, 1.1	8.7, 1.8	0.42 °
Height (cm)	78.9 ± 5.5 [a]	78.2 ± 4.8 [a,b]	75.8 ± 4.4 [b]	0.03 *
Weight for age Z-score	−1.78 ± 0.60	−1.71 ± 0.55	−1.78 ± 0.54	0.84 *
Height for age Z-score	−1.60 ± 0.61	−1.74 ± 0.53	−1.96 ± 0.62	0.06 *
Weight for height Z-score	−1.34 ± 0.77	−1.15 ± 0.72	−1.07 ± 0.72	0.30 *
Hb (g/dL)	10.4, 0.8	10.1, 1.7	10.0, 1.6	0.08 °
Ferritin (μg/L)	37.6, 36.9	35.7, 46.9	38.2, 52.1	0.83 °
CRP (mg/L)	1.7, 5.6	2.1, 7.2	2.1, 4.8	0.60 °
Inflammation prevalence (%)	18.2	19.4	15.2	0.89 #
Vitamin B12 deficiency (%)	0.0	7.1	0.0	0.32 *
Folate deficiency (%)	9.1	8.0	21.1	0.40 *
Iodine (μg/L)	268.3, 405.7	327.6, 458.5	315.8, 467.5	0.99 °

Data are presented as median, IQR, percentages, or mean ± SD. Output of ° Kruskal–Wallis H test, # Chi-square test, or * one-way ANOVA. Different letters in superscript (a and b) indicate differences between treatment groups.

Anaemia Prevalence and Hb

After the intervention, anaemia prevalence was reduced for all treatment groups, but the effect was significantly different between the treatments ($p = 0.03$), see Table 3. The intervention with 600 mL resulted in a significantly lower anaemia prevalence compared to the 200 mL intervention ($p = 0.03$). The anaemia prevalence of the 400 mL group was not different from the other groups. The intervention also improved Hb concentrations in each group ($p < 0.0005$), see Table 3. After adjustment for Hb concentration at baseline, there was a significant difference in post-intervention Hb concentrations between the three treatments ($p = 0.005$), being higher for the 400 and 600 mL groups as compared to the 200 mL group ($p = 0.01$ and $p = 0.01$, respectively).

Table 4.3. Anaemia prevalence (%), Hb (mean ± SD) and ferritin concentrations (median, IQR) of the different treatment groups after 6 months of daily consumption of the multi-nutrient fortified dairy-based drink.

	200 mL	400 mL	600 mL	Treatment Effect *p*-Value
Anaemia prevalence (%)	47.1 [a]	27.0 [a,b]	17.6 [b]	0.03
Hb (g/dL)	11.2 ± 0.9 [a,*]	11.6 ± 0.9 [b,*]	11.7 ± 1.0 [b,*]	0.005
Ferritin (μg/L)	37.0, 19.3	34.7, 35.1	39.5, 28.4	0.84

* Significantly higher compared to baseline concentration ($p < 0.05$). Chi-square test was used for anaemia prevalence, one-way ANCOVA for Hb and ferritin with corresponding baseline concentrations taken along as covariate. In contrast to baseline concentrations, Hb at endline was normally distributed, and although this was not the case for ferritin ANCOVA was considered robust enough to apply for ferritin as well. Within treatment groups, concentrations before and after the intervention were compared using either paired t-test or Wilcoxon signed-rank test. Different letters in superscript (a and b) indicate differences between treatment groups.

Median ferritin concentrations did not significantly increase during the intervention. Post-intervention concentrations were not different between treatment groups ($p = 0.84$). The CRP concentrations in the 600 mL group significantly decreased compared to baseline (median 0.7, IQR 1.7, $p = 0.02$). This effect was not found for the 200 and 400 mL groups (median 2.0, IQR 4.0 and median 2.1, IQR 3.1, respectively). A one-way ANCOVA, with baseline CRP as a covariate, did not show any difference in post-intervention CRP concentrations between the three groups ($p = 0.19$). Inflammation prevalence following treatment was not different between treatment groups ($p = 0.27$). Within treatments, there seemed to be a reduction in the 400 and 600mL groups (from 19.4% to 11.1% and from 15.2% to 6.1%, respectively), but this was not significantly different ($p = 0.29$ and $p = 0.38$, respectively). For the 200mL group, the inflammation prevalence at endline was 20.0%, not different from baseline ($p = 1.00$).

Iron Deficiency and Iron-Deficiency Anaemia

The prevalence of ID at baseline was not statistically different between treatment groups ($p = 0.25$) (**Table 4.5**). As all children were anaemic at baseline, the results for IDA at baseline are similar to ID. Following treatment, a trend for a decrease in ID prevalence was found for the 200 mL group ($p = 0.06$). No significant differences were found in the 400 and 600 mL groups ($p = 0.50$ and $p = 1.00$ respectively). Taking the whole PP population into account (independent of consumption volumes), ID significantly reduced from 12.1% at baseline to 3.0% at endline ($p = 0.04$). Similar results were found for IDA. The iron status parameters of the iron and non-iron deficient toddlers at baseline are presented in Table S3. This indicates that the toddlers who were not iron deficient at baseline recovered faster from anaemia, as prevalence in the iron-deficient at baseline group prevalence after the intervention was 41.7%, while for the non-iron deficient at baseline the prevalence was 28.7% after the intervention.

Table 4.4. Prevalence of iron deficiency (ID) based on different ferritin cut-off levels depending on CRP levels (27), before and after the 6-month daily consumption of different amounts of multi-nutrient fortified dairy-based drink.

		200 mL	400 mL	600 mL	*p*-Value *
ID prevalence	**pre**	20.0%	11.1%	6.1%	0.25
	post	0%	2.8%	6.1%	0.53

* The prevalence between study groups has been compared with Fisher's exact test. Iron deficiency (ID): <12 μg/L serum ferritin when CRP ≤ 5 mg/L or <30 μg/L serum ferritin when CRP > 5 mg/L.

Faecal Microbiota

For qPCR, 81 paired (base- and endline) samples, four single baseline and three single endline samples were studied. Faecal samples from other children were not suitable for analysis, due to insufficient amounts, or were missing. For 16S rRNA gene sequencing 34 paired samples, and 26 single endline samples were used. Based on quality control, one sample was removed because of a very low read count. On average 32,926 (25,930–48,354) bacterial 16S rDNA sequences per sample were analysed. The average gut microbiota composition at baseline is shown in **Figure S4.1**. There was no significant link between microbiota composition for any of the parameters gender, CRP, serum vitamin B12 and serum ferritin, as determined through RDA at both time points, i.e., they were not confounders. With regard to alpha diversity, no significant difference was found at baseline between the groups. At endline phylogenetic diversity was different between the groups ($p = 0.0138$) and lowest within the 400 mL group ($p < 0.05$) as compared to the 600 mL group (**Figure S4.2**). The change in diversity within individuals over time was not significantly different.

RDA indicated a clear effect of time (variation explained by time point was 16.8%, $p = 0.002$) (Figure 2). Baseline samples were associated with e.g., *Veillonellaceae* and *Prevotellaceae*. Endline samples were associated with e.g., *Actinomycetaceae* and *Erysipelotrichaceae*, but not with *Enterobacteriaceae*.

Based on RDA at endline, there is a significant treatment effect (variation explained 2.2%; $p = 0.006$), but without a linear dose–response (**Figure S4.3**). The gut microbiota composition of toddlers on 400 mL appeared different compared with those on 200 or 600 mL. More specifically, *Enterobacteriaceae* were different between the groups ($p = 0.041$) at endline with a higher relative abundance associated with the 400 mL dose and lowest abundance with the 600 mL dose ($p < 0.05$) (Figure 3A). *Enterobacteriaceae* decreased over time (baseline to endline, **Figure 4.3B**), and the decrease was not significantly different between the groups. The higher level of *Enterobacteriaceae* at endline in the 400 mL group was reflected in a higher ratio of *Enterobacteriaceae-Bifidobacteriaceae* as compared to the 600 mL group ($p < 0.01$)

(Figure S4). The relative abundance of *Bifidobacteriaceae* at endline and change over time did not differ between groups (**Figure 4.3E,F**). Pathogenic *E. coli* qPCR, targeted at EPEC ETEC *lt* and ETEC *st*, revealed no differences between the groups (**Figure S4.5**). At endline the sum of pathogenic *E. coli* (sum of EPEC, ETEC *lt* and ETEC *st*) was slightly lower compared to baseline, but the change was not significantly different between the test product doses (**Figure 4.3C,D**).

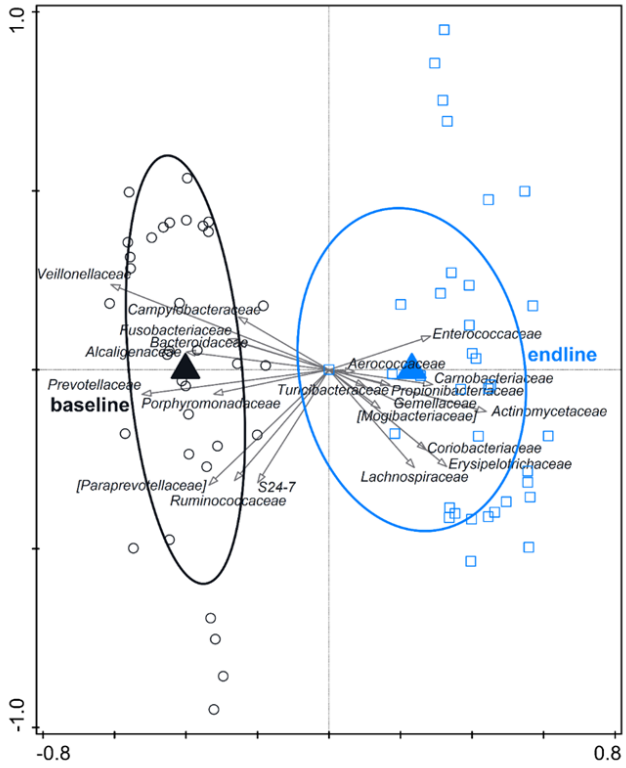

Figure 4.2. RDA on the operational taxonomic unit (OTU) level, assessing the effect of time (including test formula treatment) on gut microbiota composition. The covariance attributable to the subject was first fitted by regression and then partially out (removed) from the ordination. OTUs were used as response data and the time point was explanatory data, the bacterial families that contributed most were plotted supplementary. Variation explained by time point was 16.8%, $p = 0.002$.

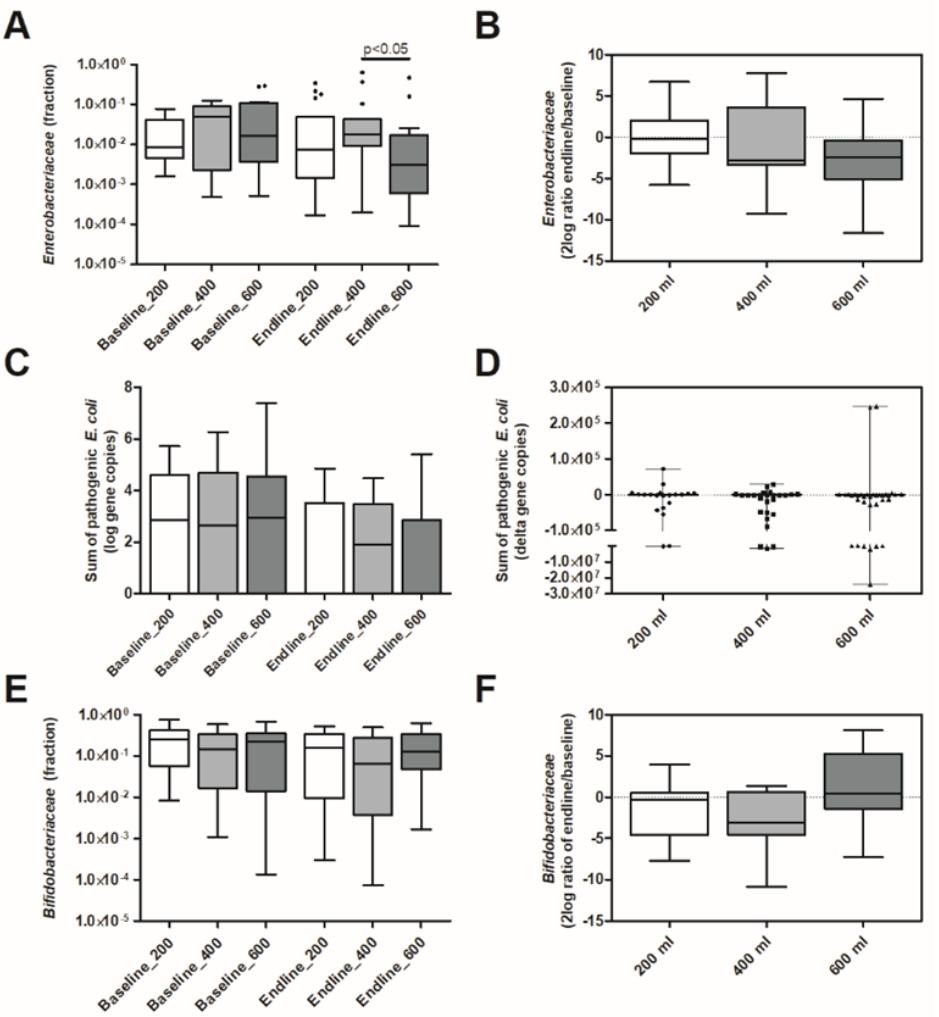

Figure 4.3. Effect of daily intakes of 200, 400, or 600 mL of YCF during 6 mo. on Enterobacteriaceae, pathogenic E. coli and Bifidobacteriaceae. Boxplots are displayed as Tukey whiskers. (**A**) Relative abundance of Enterobacteriaceae at baseline and endline. At endline relative abundance was significantly higher in the 400 mL group compared to the 600 mL group ($p < 0.05$ based on Dunn's posthoc test). (**B**) Change in the relative abundance of Enterobacteriaceae over time (2log ratio of relative abundance at endline and baseline). (**C**) Abundance of pathogenic E. coli at baseline and endline (pathogenic E. coli represents the sum of the log gene copies of EPEC, ETEC lt and ETEC st). (**D**) Change in abundance of pathogenic E. coli over time (delta gene copies). (**E**) Relative abundance of Bifidobacteriaceae at baseline and endline. (**F**) Change in the relative abundance of Bifidobacteriaceae over time (2log ratio of relative abundance at endline and baseline).

Discussion

This study showed that daily consumption of different doses (200, 400 or 600 mL) of multi-nutrient fortified dairy-based drink for 6 months dose-dependently reduced anaemia prevalence in anaemic malnourished Nigerian children aged 1–3 years. Hb concentrations increased with all interventions, but the effect was more pronounced in the 400 and 600 mL groups as compared to 200 mL. Furthermore, this study showed that daily consumption of 200–600 mL multi-nutrient fortified dairy-based drink did not cause an increase in the potentially pathogenic gut bacteria.

Anaemia Prevalence Reduction

In the current study, anaemic toddlers received different dosages of iron fortification (2.24, 4.48, and 6.72 mg/day) in the form of a multi-nutrient fortified dairy-based drink. All three dosages reduced anaemia prevalence from 100% at baseline to 47%, 27% and 17.6%, respectively, at endline. A similar trial conducted by Rivera et al. studied the effect of 400 mL iron-fortified milk (5.28 mg elemental iron from ferrous gluconate), administered as two 200 mL servings per day for 12 months, on anaemia and ID prevalence in Mexican children aged 12–30 months with mild-to-moderate anaemia (Hb < 11g/dL) (31). After 6 months of intervention, anaemia prevalence in the fortified milk group decreased from 44.5% at baseline to 12.7% (reduction of 72%), while the nonfortified milk group (0.16 mg Fe/400 mL;) showed a 54% reduction in anaemia prevalence, from 42.6% to 19.7% (31). The effect of the iron-fortified milk is in line with the results of the 400 mL group in the present study, showing a 73% reduction in anaemia prevalence, whereas the 600 mL (6.7 mg iron) group showed an 82.5% reduction. Surprisingly, the effect of unfortified milk in the study of Rivera et al. is equal to the effect as seen in our 200 mL group. Although intervention durations were different, 12 versus 6 months in the present study, in general, it is assumed that the contribution of cow's milk to total iron intake is not relevant. Moreover, the consumption of cow's milk by toddlers is reported to be associated with declining iron stores (32). Rivera et al. did not discuss this finding in the nonfortified group in detail but the improvement suggests an effect of other nutrients, possibly protein coming with the milk (33), change in diet, or other features associated with the program. De-worming, which is known to reduce anaemia, did not take place in the Mexican study(31).

ID is Not the Most Important Causes of Anaemia

ID is present when serum ferritin levels are lower than the defined cut-off values: <12 μg/L when CRP ≤ 5 mg/L or <30 μg/L when CRP > 5 mg/L for children 0-59 months [2]. Low Hb (<11 g/dL) in combination with ID results in IDA. In the present study, with only anaemic children enrolled, the mean prevalence of ID and IDA in the

PP population at baseline were similar, on average 12.1%. These prevalences reduced to 3% after 6 months of intervention. This low ID outcome conflicts with the general statement that ID is the major cause of anaemia (34). However, studies in Akwa Ibom State (Nigeria) and Côte d'Ivoire also indicated a low ID prevalence in anaemic children of 6.7% and 13.2%, respectively (35,36). In a Mexican study, ID prevalence at baseline was 37% and 30% in the nonfortified and fortified group, respectively (31). Although ID in the Mexican study contributed more to the cause of anaemia as compared to our study, it was not the major contributor to anaemia either. Other reported major causes of anaemia are concurrent infection (including parasites), RBC- and/or iron metabolic diseases, acquired- and autoimmune-haemolytic anaemia, hypersplenism, transient erythroblastopenia, hypothyroidism, vitamin B12 or folate shortcomings, and protein deficiency (33,37). From this list, it is unlikely that vitamin B12 and folate deficiencies were major causes in the present study since their prevalence at baseline were 3% and 12%, respectively. Additionally, hypothyroidism can be excluded since urinary iodine excretion was 2–3 times higher than the cut-off value of 100 µg/L: being >300 µg/L in most children at baseline (median 309 µg/L, range: 22–5622). Additionally, protein deficiency is a possible cause of non-ID anaemia as protein is necessary for Hb synthesis. This type of anaemia is reversible by feeding a complete protein (33) such as the cow's milk protein in the multi-nutrient fortified dairy-based drink in the present study. Therefore, the observed effect of this study might be partly due to increased protein intake. Worm infestations are associated with (ID) anaemia and these infections probably are tolerated by the immune system meaning that parameters of inflammation will not increase (in contrast, worm infections may be anti-inflammatory) (38–40). The effect of anthelmintic treatment in toddlers is unknown but in a literature review and meta-analysis conducted on the effects of deworming on child and maternal health, deworming did not show consistent benefits for indicators of mortality, anaemia, or growth in children younger than five or women of reproductive age (41). Before starting the intervention of the present study all children received deworming treatment. As no control group was included, it cannot be excluded that the observed reduction in anaemia prevalence was partly related to this treatment. However, as anaemia prevalence was further reduced in the 400- and 600-mL groups compared to the 200 mL group, the multi-nutrient fortified dairy-based drink seems to affect anaemia on its own. In addition, malaria is a possible cause of anaemia in children, without causing ID, and supplementation with iron following malaria has been reported to promote recovery from anaemia in 4-6 weeks (42). Malaria is endemic in Nigeria and Lagos State(43). A review article on the epidemiology of malaria in endemic areas shows an illustration of the start/end of malaria transmission season in Lagos being from April till December (44) which perfectly coincides with the intervention period of the present project. This could imply that malaria at least in part was a cause of anaemia without ID. When antimalaria drugs were prescribed (not recorded in this

study but assumed by a local paediatrician), recovery might have been stimulated by iron supplementation. Taken together, several factors or combinations of factors other than ID may cause anaemia. For the present study, anthelmintic infection and malaria are plausible factors, and their treatments may have contributed to anaemia reduction. However, as a dose–response effect was observed in this study it can be concluded that the multi-nutrient fortified dairy-based drink relevantly contributed to the reduced anaemia. The total composition of multi-nutrient fortified dairy-based drink, but in particular the combination of a good available iron source (ferrous sulphate), sufficient vitamin C (to further stimulate iron availability), and likely also the cow's milk protein, probably contributed to this beneficial effect on anaemia (33,45,46).

Effect of Multi-Nutrient Fortified Dairy-Based Drink on Gut Microbiota

Food fortification programs are usually considered the most cost-effective and sustainable approach to combat iron deficiency, especially in sub-Saharan African countries. However, most of the iron will not be absorbed and passes into the colon (46,47) where it may be used in particular by potential pathogenic gut bacteria such as *Salmonella*, *Shigella* or pathogenic *Escherichia coli* (48,49). This adverse effect has been shown in two double-blind randomised controlled trials. In 6-month-old Kenyan infants consuming iron-fortified (2.5 or 12.5 mg) maize porridge daily for 4 months, the growth of potentially pathogenic bacteria was stimulated whereas the relative abundance of beneficial 'barrier' *bifidobacteria* decreased (21). This shift towards a potentially more pathogenic profile was associated with intestinal inflammation, as was reflected in an increased faecal calprotectin concentration (21). Furthermore, the increase in *enterobacteria* correlated with an increase in faecal calprotectin concentration (49). Additionally, in a 6-months, randomised, double-blind, controlled trial, in 6–14-y-old Ivorian children, consumption of iron-fortified biscuits (20 mg/day, 4 times/week) increased the number of faecal *enterobacteria* and decreased the number of *lactobacilli*. At least one study has shown that the negative effect of iron on gut microbiota can be corrected by the concurrent addition of galactooligosaccharides (28). Two additional fortification trials in infants with an MNP containing 12.5 mg FeFum have raised safety concerns: in Ghana, there was an increased rate of hospitalisation and based on data from the outpatient register, 83% of the additional cases in the iron group were due to diarrhoea, but this was not significant (50), and in Pakistan, a small but significant increase in overall diarrhoea prevalence, bloody diarrhoea, and respiratory illness was reported (51). In the present study, we did not find any adverse effects of the intervention on or related to the gut microbiota, such as diarrhoea. *Enterobacteriaceae* relative abundance decreased over time and the magnitude of decrease was not different between groups. Moreover, after the intervention, the lowest abundance of *Enterobacteriaceae* was found in the 600 mL group. Furthermore, pathogenic *E. coli* decreased over time and did not differ between groups, and the slight

decrease in *Bifidobacteriaceae* over time was not different between the groups. Based on this information, it appears that feeding a multi-nutrient fortified dairy-based drink, containing low levels of relatively high bioavailable iron per serving (iron sulphate, vitamin C, no phytic acid) (46,47) is unlikely to significantly disturb the gut microbiota in toddlers. The food matrix may have contributed to the non-observed adverse effect, as well as the relatively small amount of iron provided per serving. Whether and how high thresholds are at which iron disrupts gut microbiota should be investigated.

Strengths and Limitations

By using anaemia as an inclusion criterion, the effect on improving anaemia could be studied. The omission of a placebo group is a limitation, such that the effect on anaemia cannot be attributed to the multi-nutrient dairy-based drink alone. A placebo group would have been very helpful in determining the effect of deworming on anaemia prevalence. Furthermore, as the morbidity profile of the study subjects was not monitored, we do not know whether malaria might have played a role, and how the iron doses affected malaria recovery or severity.

The loss of quite a lot of children for follow-up ($n = 42$) was a pity and possibly could have been prevented in part when midpoint follow-ups would have been organised. We cannot exclude loss of information due to faecal 16S rRNA analysis in only a subgroup of children. For the purpose of the study, however, the analysis appears to be sufficient.

Since medically diagnosed allergies, not having anaemia, intolerances to milk or milk ingredients were exclusion criteria, and children were recruited from a poor environment, study results cannot directly be generalised to children with different characteristics.

A strength of this study is the food-based dose–response approach, showing that already 200 mL of product improves anaemia. Therefore, this amount could be a good starting point when discussing the possibilities of cheaper though effective products for those at the bottom of the pyramid.

Conclusions

This study shows that daily consumption of 200–600 mL of iron-fortified multi-nutrient fortified dairy-based drink reduces anaemia without stimulating potential pathogenic gut bacteria in Nigerian toddlers. Although the main cause of anaemia in the study population is not clear (protein deficiency, malaria or worm infestations might be contributors, besides a minor role for ID), the study shows a dose–response effect

of the multi-nutrient fortified dairy-based drink in reducing anaemia which is likely attributable to the well-available iron and possibly also to the high-quality protein. The finding that 200 mL of the multi-nutrient dairy-based drink (2.24 mg iron daily) already results in a relevant improvement of anaemia in 6 months is of interest since this amount is more affordable, for many households, than 400 or 600 mL.

Author Contributions

A.J.O.: A.S. and I.O.S. designed the study, I.O.S.: K.O. and A.J.O. conducted the study, J.B., R.T.E., G.A.M.K. conducted the microbial analysis, J.H.J.H. and A.J.O. analysed the data, all authors contributed to writing and editing, A.M.-B. and F.S. supervised the study; all authors have read and agreed on the submitted version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the health research and ethic committee of Lagos State University Teaching Hospital and Lagos state Government ((LREC/10/06/829). The trial was registered at ClinicalTrials.gov: NCT03411590 (protocol code 2017-GAGA-GND, signed on the 9 January 2018.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study, including consent to publish the outcome of this study.

Data Availability Statement

The data in this study are not publicly available but could be requested from the corresponding author.

Acknowledgments

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Conflicts of Interest

At the time of this study, A.J.O. was employed by FrieslandCampina WAMCO Nigeria, A.S. and J.H.J.H. are employees of FrieslandCampina, no other conflict of interest.

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Supplementary materials to:

Multi-nutrient fortified dairy-based drink reduces anaemia without observed adverse effects on gut microbiota in anaemic malnourished Nigerian toddlers: a dose-response study.

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1 Supplemental text: Faecal samples and microbiota analysis

Faecal samples were collected (5-10 g) in stool collection stubs with a spoon attached to the lid (Greiner Bio-One, Vilvoorde, Belgium), at the start and the end of the intervention. The samples were stored at a cold temperature within 1 hour, and transported on ice to the nearest freezer (-20°C) that same day. Samples were shipped on dry ice with temperature control to The Netherlands, where faecal microbiota was studied in a sub-sample of children (n= 88; group A n=26, group B n=27, group C n=35), by NIZO (Ede, The Netherlands) using 16S rRNA gene sequencing (in 60 samples; group A n=19, group B n=20, group C n=21) and targeted qPCR (in 88 samples).

Bacterial DNA extraction, PCR amplification and 16S rRNA gene Illumina sequencing

Faecal samples were first thawed at 4 °C. Then in a 2.0 mL screw-cap tube containing 0.5 g of 0.1 mm sterilized zirconia beads, 250 (± 10%) mg of faeces and 700 μL S.T.A.R. buffer (Roche, Indianapolis, IN, USA) were added. The FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA) was used for lysis at 5.5 m/s for 3 times 1 min at room temperature. Thereafter samples were incubated while shaking at 100 rpm and 95 °C for 15 min. The samples were then centrifuged at 16000 g for 5 min at 4 °C. The collected supernatant was kept on ice, while another lysis round as described above, except that only 350 μl S.T.A.R. buffer was added, was done with the remaining stool pellet. The supernatant kept on ice was then pooled with the supernatant from the second lysis round. Purification of DNA was performed on the automated Maxwell instrument (Promega, Madison, WI, USA) by applying the Maxwell 16 Tissue LEV Total RNA Purification Kit (Promega) according to the manufacturer's instructions. To the first well of the Maxwell cartridge 250 μL of the supernatant was added and finally, DNA was eluted with 50 μL of RNAse/DNAse free water.

Using a 2-step PCR, barcoded amplicons from the V3–V4 region of 16S rRNA genes were generated (see library PCR below for a description of second PCR step). For initial amplification of the V3–V4 part of the 16S rRNA

we used universal primers with the following sequences: forward primer, '5-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG***CCTACGGGAGGCAGCAG'** (broadly conserved bacterial primer 357F in bold and underlined); reverse primer, '5-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG***TACNVGGGTATCTAAKCC**' (broadly conserved bacterial primer 802R (with adaptations) in bold and underlined), appended with Illumina adaptor sequences (in italics). The PCR amplification mixture contained: 1 µL faecal sample DNA, 1 µL barcoded forward primer (10 μM), 14 µL master mix (1 µL KOD Hot Start DNA Polymerase (1 U/µL; Novagen, Madison, WI, USA), 5 µL KOD-buffer (10×), 3 µL $MgSO_4$ (25 mM), 5 µL dNTP mix (2 mM each)), 1 µL (10 µM) of reverse primer and 33 µL sterile water (total volume 50 µL). PCR conditions were: 95 °C for 2 min followed by 30 cycles of 95 °C for 20 sec, 55 °C for 10 sec, and 70 °C for 15 sec. We then purified the approximately 500 bp PCR amplicons using the MSB Spin PCRapace kit (Invitek, Berlin, Germany).

For the library PCR step in combination with sample-specific barcoded primers, purified PCR products were shipped to BaseClear BV (Leiden, The Netherlands). PCR products were checked on a Bioanalyzer (Agilent) and quantified. This was followed by multiplexing, clustering and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing. The sequencing run was analysed with the Illumina CASAVA pipeline (v1.8.3) with de-multiplexing based on sample-specific barcodes. From the raw sequencing data, we removed the sequence reads of too low quality (only "passing filter" reads were selected) and discarded reads containing adaptor sequences or PhiX control with an in-house filtering protocol. On the remaining reads, a quality assessment was performed using FASTQC version 0.10.0. (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)

Supplemental table 4.1: **Detection and quantification of pathogenic *E. coli* by qPCR**
The following quantitative real-time polymerase chain reaction (qPCR) assays were applied on isolated DNA from faecal samples:

Bacterial target	Gene	Primers	reference
EPEC	*eaeA*	Fwd: 5'- GCCTTCATCATTTCGCTTTC' Rev: 5'- GCCTTCATCATTTCGCTTTC'	(52)
ETEC	In-house method; *st* and *lt* genes	Fwd *stla:* 5' TTTCCCCTCTTTTAGTCAGTCAA' *Fwd stlb:* 5' TGCTAAACCAGTAGAGTCTTCAAAA' Rev *stl*: 5'-GCAGGATTACAACACAATTCACAGCAG' Fwd *lt*: 5'- TCTCTATGTGCATACGGAGC' Rev *lt*: 5'- CCATACTGATTGCCGCAAT'	(52)
Total counts	In-house method; 16S rRNA gene	Fwd: 5'- CGGTGAATACGTTCYCGG' Rev: 5'- GGWTACCTTGTTACGACTT'	(53)

Validation of the qPCR assays

The *E. coli* qPCR assays were adapted from (52) and were first validated in a faecal matrix background (i.e. total microbial DNA isolated from faecal samples) using the following strains of *E. coli*:

- DSM8699 (EPEC)
- ATCC43887 (EPEC)
- DSM10937 (ETEC)
- H10407 (ETEC)

The following control samples were implemented for the validation:

- Target gene amplified DNA product (using bacterial genomic DNA);
  - Faecal DNA (isolated from an adult faecal sample, diluted 10-fold) spiked with target gene amplified DNA product (positive control) (to determine potential inhibition of the assay);
  - Purified water (negative control);
  - Non-spiked faecal DNA (diluted 10-fold, negative control);
  - Faecal DNA spiked with chromosomal DNA from *Campylobacter coli* DSM4689, *Campylobacter jejuni* DSM4688 (ATCC 35560), *Clostridium difficile* C630derm, *Clostridium perfringens* SM101, *Klebsiella pneumoniae* DSM30104 (ATCC 13883), *Salmonella enterica* DSM17058 (LT2) (negative control) (specificity testing)

The slope, correlation coefficient and PCR efficiency were determined using values of three calibration curves on one plate. The Cq_{min} and Cq_{max} values (minimal and maximal number of amplification cycles) for each assay were determined using these calibration curves. Accuracy and intermediate precision of the assays were determined using three replicate measurements of three different dilutions (10x, 100x and 1000x) on three replicate plates. Accuracy was calculated as the difference between the experimentally measured value and the true value and is indicated in fold-change differences. The intermediate precision was calculated as a measure of the variation between plates using average and standard deviation values of quadruple measurements in two dilutions (100x and 1000x) of faecal matrix DNA background with spiked target gene amplified product in six concentrations (10^2 – 10^7 copies/mL). The limit of detection and quantification (LOD/LOQ) was estimated using the lowest and the highest reliable Cq values of the standard curves of 10 replicate measurements. Any reliable measurement obtained with a value above 1 but below the LOD was still interpreted to reflect a probable quantification of the bacterial DNA present in the total DNA isolated from the faecal sample. Because the value is a true value (>1) but below the LOD, the value was included in the analyses, but renumbered to ½ LOD

Supplemental table 4.2: **Validation values for three qPCR assays in a faecal matrix background.** The following criteria were adjusted for acceptance of the validation outcome: slope; -3.1 to -3.8 (perfect slope is -3.3), correlation coefficient (R^2); ≥0.98 (perfect R^2 is 1.0), PCR efficiency; 90 – 110% (perfect is 100%), accuracy; <10-fold (perfect is 1.0-fold), precision; <35%.

	EPEC	ETEC lt	ETEC st
Linear dynamic range	1E8 – 1E1	1E8 – 1E1	1E8 – 1E1
Slope	-3.371	-3,450	3,312
Correlation coefficient (R^2)	0.982	0,993	0.987
Cq_{min} and Cq_{max}	9.4 - 33	11.1 - 35	10 – 32,9
PCR efficiency	98%	95%	100%
Accuracy	1.2	2.3	1.3
Intermediate precision	11 – 34%	10 – 25%	5 – 33%
LOD/LOQ	10^2 copies/μL (=750 copies/mg)	10^2 copies/μL (=750 copies/mg)	10^2 copies/μL (=750 copies/mg)

No positive signals >LOD were obtained against negative control species for any of the assays, meaning that the qPCR assays are specific against *E. coli* EPEC and ETEC.

Supplemental table 4.3: **Execution of the qPCR assays**
The following PCR conditions were applied on three dilutions (10x, 100x and 1000x) of total microbial DNA isolated from faecal samples, in single measurements:

Bacterial target	T_{ann}	Primer dilution	Mastermix	Amplification curve slope (ΔC_q) limits
EPEC				
eaeA gene	60 ˚C	Fwd: 200 nM Rev: 200 nM	Primer + SYBR Green	Min: 2.2 Max: 4.6
ETEC				
st gene	60 ˚C	Fwd: 250 nM Rev: 250 nM	Primer + SYBR Green Primer + SYBR Green	Min: 2.2 Max: 4.6
lt gene	60 ˚C	Fwd: 150 nM Rev: 150 nM		Min: 2.2 Max: 4.6
Total counts				
16S rRNA gene	56°C	Fwd: 500 nM Rev: 500 nM	Primer + SYBR Green	Min: 2.2 Max: 4.6

Values were deemed reliable when within the Cq_{min} and Cq_{max} values of the assay (Table XXX) and within the ΔCq limits (2.2 – 4.6). The total number of copies per μl was calculated using the standard curve of the assay and used for calculation of the total number of copies of target-specific DNA present in the total microbial DNA isolated from the faecal samples using the following formula: (copies per μl/2)*200/250 = copies per mg.

16S rRNA gene sequence analysis, qPCR analysis and statistics

16S rRNA gene sequences were analyzed using a workflow based on Qiime 1.8 (54). We performed operational taxonomic unit (OTU) clustering (open reference), taxonomic assignment and reference alignment with the pick_open_reference_otus. py workflow script of Qiime, using uclust as clustering method (97% identity) and GreenGenes v13.8 as a reference database for taxonomic assignment. Reference-based chimera removal was done with Uchime(55). The RDP classifier version 2.2

was performed for taxonomic classification (56)(ref Cole). Statistical tests were performed as implemented in SciPy (https://www.scipy.org/), downstream of the Qiime-based workflow.

We tested for between-group differences in alpha diversity and beta diversity (phylogenetic distance metric weighted UniFrac) by the non-parametric Kruskal-Wallis test with Dunn's posthoc test, as implemented in Graphpad Prism 5.01. Between-group differences of single taxa were assessed using the non-parametric Mann-Whitney U test with FDR correction for multiple testing; unless stated otherwise. Comparisons of targets of our primary interest (*Lactobacillaceae*, *Bifidobacteriaceae, Enterobacteria*ceae) were not corrected for multiple testing. For comparisons of more than 2 groups, the non-parametric Kruskal-Wallis test with Dunn's posthoc test was applied. In the longitudinal analysis, change of taxon relative abundance over time, 2log ratios were calculated, in which the relative abundance of a taxon at the second or later time point was divided by the relative abundance of the same taxon at an earlier time point. Ratios were compared among groups by Mann-Whitney U tests with FDR correction for multiple testing, and for comparisons of more than 2 groups by the non-parametric Kruskal-Wallis test with Dunn's posthoc test.

We performed redundancy analyses (RDAs) on the gut microbiota composition as assessed by 16S rRNA gene sequencing in Canoco version 5.11 using default settings of the analysis type "Constrained(57)". Relative abundance values of genera or OTUs were used as response data and metadata as an explanatory variable. For visualization purposes, families (and not OTUs) were plotted as supplementary variables. Longitudinal effects of the intervention were assessed by calculating 2log ratios in which the relative abundance of an OTU or genus at endline was divided by the relative abundance of the same OTU or genus at baseline. These ratios were used as response variables in RDAs and were weighted based on the average relative abundance of each OTU or genus in all subjects. RDA calculates p-values by permutating (Monte Carlo) the sample status.

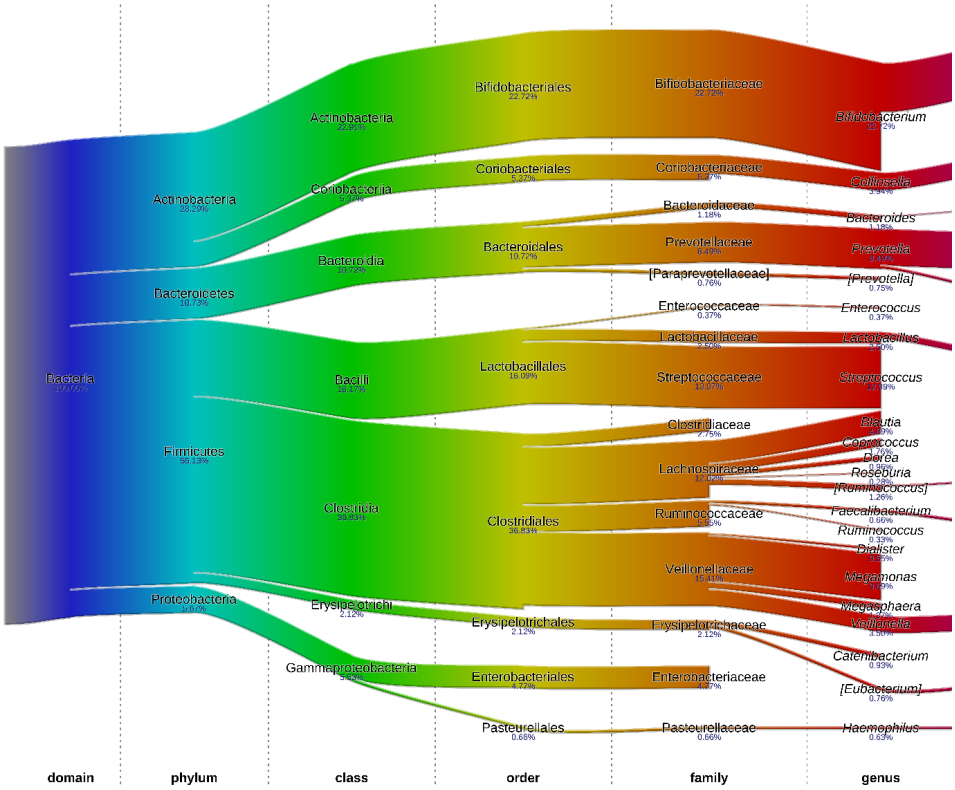
qPCR gene copy counts of each target (total bacterial counts, EPEC *eaeA* gene, ETEC *lt* gene, ETEC *st* gene) were compared between test product dose groups by Kruskal-Wallis test with Dunn's posthoc test and change over time were calculated by subtracting the counts at end-line from the counts at baseline. Pathogenic *E. coli* was defined as the sum of the gene copies of EPEC, ETEC lt and ETEC st).

Supplemental table S4.4: *Baseline characteristics of the ITT and PP population*

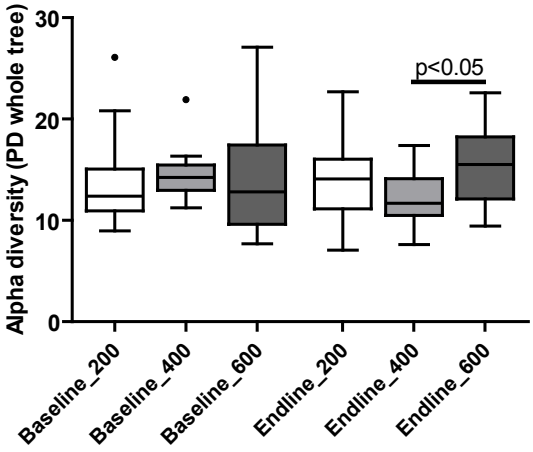
	ITT	PP
n	165	105
Age (months)	20.15 ± 6.27	20.13 ± 6.24
Gender (boys/girls) (%)	44.8 / 55.2	45.7 / 54.3
Social class (upper/middle/lower) (%)	0.6 / 18.4 / 81.0	1.0 / 19.4 / 79.7
Religion (Muslim/Christian) (%)	71.0 / 29.0	68.9 / 31.1
Weight (kg)	8.9 ± 1.2	8.9 ± 1.2
Height (cm)	77.5 ± 4.9	77.6 ± 4.8
Weight for age Z- score	-1.80 ± 0.56	-1.78 ± 0.56
Height for age Z- score	-1.80 ± 0.65	-1.79 ± 0.59
Weight for height Z-score	-1.24 ± 0.78	-1.21 ± 0.74
Hb (g/dL)	10.1, 1.1	10.1, 1.3
Ferritin (μg/L)	38.6, 41.7	37.0, 42.6
CRP (mg/L)	1.7, 6.7	1.8, 6.4
Inflammation prevalence (n) (%)	30.6% (49)	17.6% (18)
Vitamin B12 deficiency (n) (%)	2.5% (3)	2.7% (2)
Folate deficiency (n) (%)	13.5% (14)	12.1% (8)

Data are presented as median¸IQR¸ percentages¸ or mean±SD.

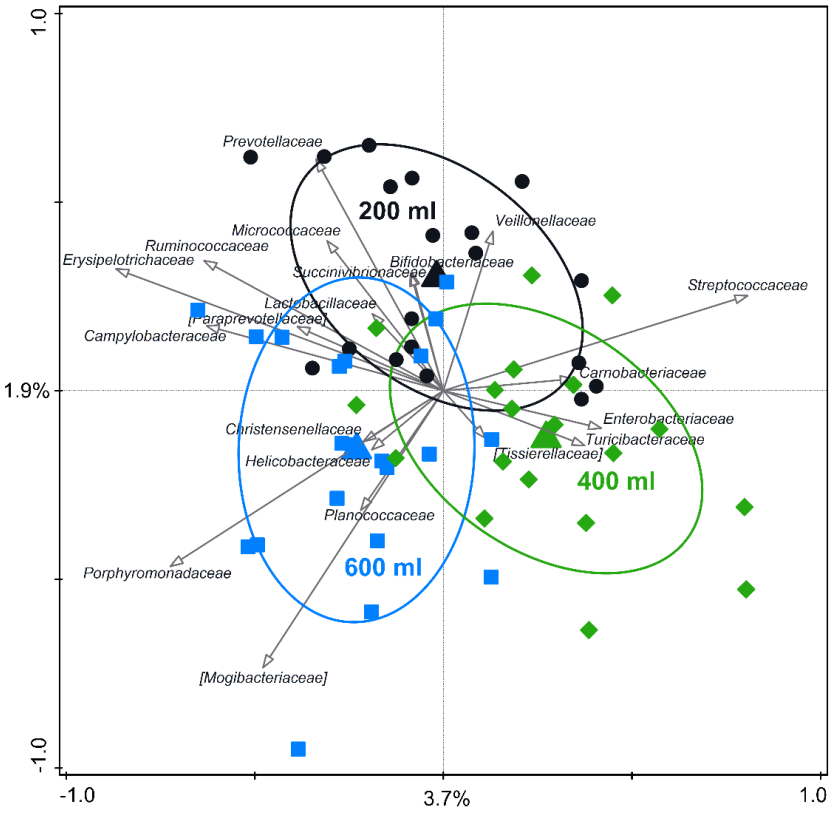
Supplemental figure S4.1: Average gut microbiota composition of the studied toddler population at baseline, from the phylum to the genus level. The figure was generated using the software described by Sundquist *et al.*, 2007 (58).



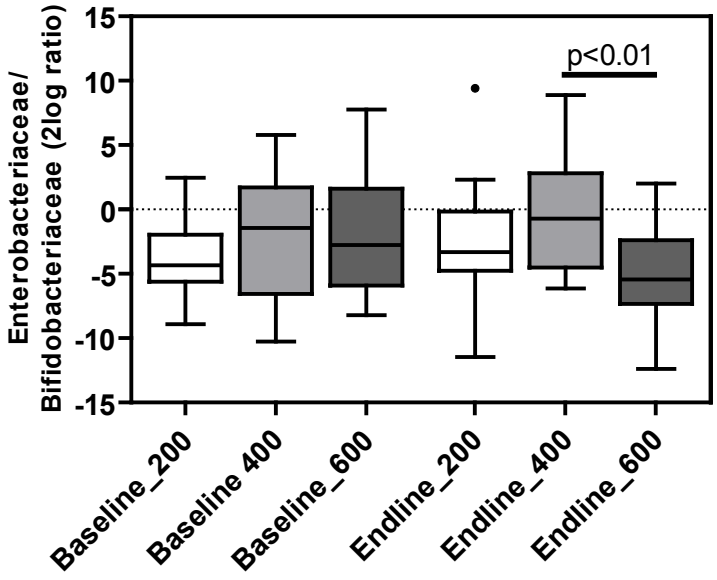
***Supplemental figure S4.2*:** Alpha diversity (PD whole tree metric) in the multi-nutrient fortified dairy-based drink dose groups at baseline and endline. At endline the diversity was significantly lower in the 400 mL group compared to the 600 mL group ($p<0.05$ based on Dunn's posthoc test). Boxplots are displayed as Tukey whiskers.



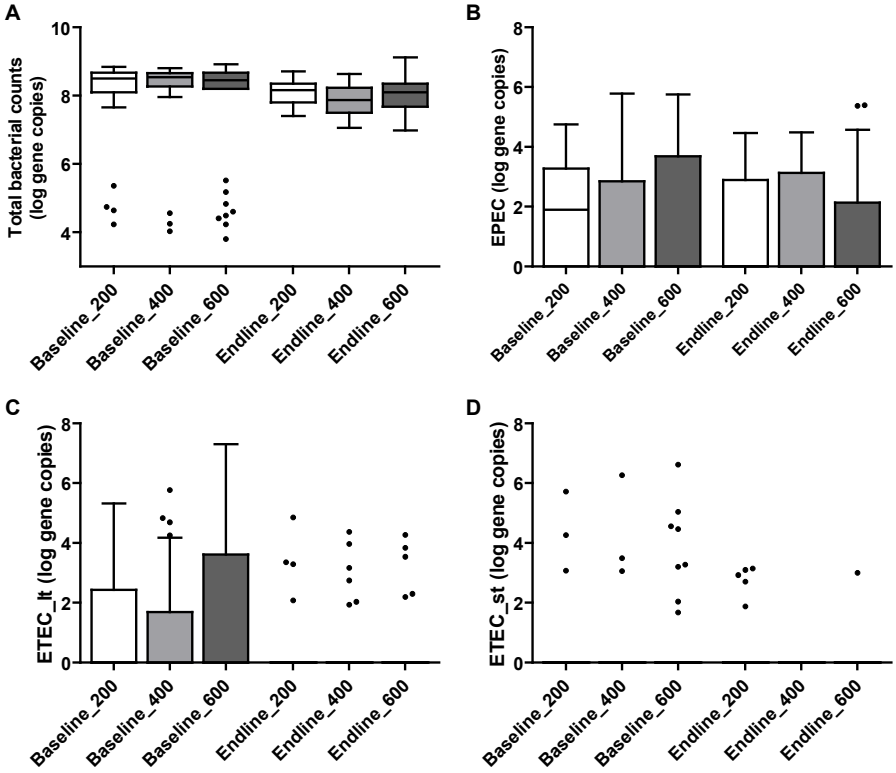
***Supplemental figure S4.3*:** Redundancy analysis (RDA) on the OTU level, assessing the effect of test product dose on gut microbiota composition at endline. OTUs were used as response data and test product dose was explanatory data, the bacterial families that contributed most were plotted supplementary. Variation explained by test product dose was 2.2%, p=0.006. Test product was a multi-nutrient fortified dairy-based drink



Supplemental figure S4.4:
Enterobacteriaceae/Bifidobacteriaceae ratio in the test product dose groups at baseline and endline. At endline the ratio was significantly higher in the 400 mL group compared to the 600 mL group ($p<0.01$ based on Dunn's posthoc test). Boxplots are displayed as Tukey whiskers. Test product was a multi-nutrient fortified dairy-based drink.



***Supplemental figure S4.5*:** Effect of daily intakes of 200 mL, 400 mL, or 600 mL of test product during 6 months on total bacterial counts (**A**) and pathogenic *E. coli*; EPEC (**B**), ETEC_lt (**C**), ETEC st (**D**) at baseline and endline. Test product was a multi-nutrient fortified dairy-based drink



CHAPTER 5

Effect of a fortified dairy-based drink on micronutrient status, growth, and cognitive development of Nigerian toddlers- A dose-response study

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Abstract

Malnutrition results in a high prevalence of stunting, underweight, and micronutrient deficiencies. This study investigated the effect of a multi-nutrient fortified dairy-based drink on micronutrient status, growth, and cognitive development in malnourished height-for-age z-score (HAZ) and/or weight-for-age z-score (WAZ) <-1 SD and > −3 SD] Nigerian toddlers (n = 184, 1–3 years). The product was provided in different daily amounts (200, 400, or 600 mL) for 6 months. At baseline and endline, venous blood and urine samples were collected to determine micronutrient status. Bodyweight, height, waist, and head circumference were measured, and corresponding Z-scores were calculated. The Bayley-III Screening Test was used to classify the cognitive development of the children. In a modified per-protocol (PP) population, the highest prevalence of micronutrient deficiencies was found for vitamin A (35.5%) and selenium (17.9%). At endline, there were no significant improvements in iodine, zinc, vitamin B12, and folate status in any of the three groups. Regarding vitamin D status (25OHD), consumption of 600 and 400mL resulted in an improved status as compared to baseline, and in a difference between the 600- and 200-mL groups. Consumption of 600 mL also increased vitamin A and selenium status as compared to baseline, but no differences were found between groups. Within the groups, WAZ, weight-for-height z-score (WHZ), and BMI-for-age z-score (BAZ) improved, but without differences between the groups. For HAZ, only the 600 mL group showed improvement within the group, but it was not different between groups. For the absolute weight, height, and head circumference only trends for differences between groups were indicated. Cognition results did not differ between the groups. Within groups, all showed a decline in the per cent of competent children for receptive language. To study the effects of a nutritional intervention on linear growth and cognition, a longer study duration might be necessary. Regarding the improvement of micronutrient status, 600 mL of fortified dairy-based drink seems most effective.

Keywords

Undernutrition, toddlers, Nigeria, multi-nutrient fortified dairy-based drink, growth, micronutrient status, cognition

Introduction

Undernutrition remains one of the leading causes of mortality in children under 5 years in developing countries (1,2) and will have a negative impact on development over generations (3). Acute undernutrition, often referred to as wasting, is primarily caused by protein–energy malnutrition (PEM), whereas chronic undernutrition is mostly the result of long-term deprivation from sufficient amounts of calories and essential nutrients and is marked by stunted vertical growth. Poor nutrition during the early developing years is associated with morbidity, mortality, growth retardation, impaired immunological functioning, and delayed mental and motor developments (4,5). In the long term, there is an increased risk of non-communicable diseases including skeletal, cardiovascular, and metabolic disorders, as well as impaired intellectual performance and work capacity (6,7). Annually, Nigeria loses over US$1.5 billion in Gross Domestic Product to vitamin and mineral deficiencies (8). That is why the World Health Organization (WHO) states the necessity of adequate nutrition of good quality in order to significantly reduce stunting and wasting by 2025 in the early stages of life (9). Therefore, nutrient deficiencies have to be addressed (10,11) Micronutrients, though required in minute quantities, are important to ensure adequate growth and development. The inadequate intake of these essential nutrients can result into major health complications such as poor health, blindness, stunted growth, mental retardation, and learning disabilities (12). In Nigeria, the most common deficiencies exist for vitamin A, folate, iron, iodine, and zinc. The estimated prevalence of vitamin A deficiency especially in children aged 6 to 59 months is reported between 40-50% (13). Vitamin A supplementation is associated with a clinically meaningful reduction in mortality in children by about 24% (14). Other vitamins of importance are vitamin D, vitamin B12 and folic acid for their roles in growth, brain function, and immunity. Despite its low latitude, vitamin D can also be deficient (<30 nmol/L 25OHD) in Nigeria, as reported for 50% of the children from the region of Jos, and may affect bone formation, immunity and brain function (15,16). However in other regions vitamin D status was found to be sufficient (~ 50 nmol/L 25OHD according the WHO), or even high (~ 125 nmol/L) (17,18). Vitamin B12 is essential for brain development (19), and linear growth (20). deficiency In Nigeria, about 8-36% pregnant Nigerian women from Jos, and 9% adolescent girls in Northern Nigeria were reported to be vitamin B12 deficient (21,22). Folic acid is recognized as crucial for nervous system development and maintenance, and brain maturation (23,24). Although folic acid deficiency was low in adolescent girls from Maiduguri in Nigeria, (24), 60% of children in Ibadan had an inadequate dietary intake of folate (25).

With regard to minerals, zinc status is associated with incidence, severity, and duration of childhood diarrhoea, as well as with growth (26–28). In Nigeria, zinc

deficiency is reported in 20-99% of children aged <5 years, depending on the region (29–31). Besides zinc, iodine supplementation is rare in children, however, the deficiency is most often indicated and translated in fortification programmes (in particular salt iodisation) (32). Iodine plays an important role in thyroid function, and as such also in brain development and function (33). Based on food intake, 1-10% of the population of Nigeria has a risk of low iodine status (32). When talking about iodine, an adequate selenium status is necessary to control hydrogen peroxide that is released in the production of thyroid hormones (34). On average, selenium deficiency is estimated to be 0-10% in the Nigerian population (32).

In a systematic review, a positive, although non-significant, impact of multi-micronutrient fortification on height-for-age (HAZ), weight-for-age (WAZ), and weight-for-height Z-scores (WHZ) was reported (35). However, energy-rich lipid-based nutrient supplements (LNS), providing lipids, essential fatty acids, protein, and micronutrients, might be more effective in improving growth in malnourished children (36–38). With regard to protein, intake appears to be adequate in Nigerian children, though, the quality of protein in developing countries may be questioned (39). Additional high-quality protein could support neurodevelopment and catch-up growth is stunted or wasted children (40–42).

Based on the above, a multi-micronutrient fortified food or beverage containing micronutrients, fat, carbohydrates, and high-quality protein is potentially a good option to support the growth and development of children. The affordability of such fortified products is still a matter of concern; hence it is necessary to assess daily effective volumes of intake. We reported earlier on the efficacy of different volumes of a multi-micronutrient fortified dairy-based drink on anaemia and gut microbiota in malnourished Nigerian toddlers aged 12-36 months (43). In this paper, we report on the efficacy of the drink on the biochemical status of other micronutrients, as well as possible effects on growth and cognition.

Materials and Methods

Subjects and Study Design

In this three-arm, open (blind for biochemical analyses) randomized intervention trial, apparently healthy Nigerian toddlers (1-3 years) (n = 184) with mild-moderate anaemia (Hb ≥7.0 g/dL and ≤10.9 g/dL) and mild-moderate malnutrition (HAZ and/or WAZ <-1 SD and >-3 SD), were recruited in Ijora-Badia community in Apapa-Iganmu Local Council Development Area (LCDA), Lagos, South-West Nigeria. Children with severe malnutrition and anaemia were excluded since they require

additional measures such as hospital admission and blood transfusion. All subjects were able to consume a maximum of 600 mL of product per day at the time of inclusion. Children were not included when they (I) had a chronic or severe illness requiring hospitalization and/or special treatment, (II) had a recent medical history (past 3 months) of serious infections, injuries and/or surgeries, (III) had any known allergies or intolerances to milk or milk ingredients, (IV) were predominantly breastfed toddlers, (V) consumed any other fortified foods or supplements, (VI) participated in micronutrient supplementation programs, (VII) participated in any other nutritional study in the last 6 months, (VIII) were likely to move within the period of intervention, (IX) when parents or guardians were related to or employed by the sponsor or the university, (X) used any prescription medications before and/or during the study period for ≥2 weeks. No restrictions were set for regular food intake. Families with toddlers that were permanent residents of Ijora-Badia were informed about the study by a mobilization team from the State Ministry of Health working in the Ijora Badia community a few weeks before the commencement of the study. During information meetings, parents, or legal guardians of potential candidates (toddlers) received detailed information about the study, the requirements, and procedures, and all their questions were answered. At screening, after a signed informed consent was obtained from parents or legal guardians, trained researchers verified age (by birth certificate confirmation or caregiver) and took anthropometric measurements. Eligible children were directly enrolled and randomLy assigned to one of the three study groups by the principal investigator. For randomization, a computer-generated block-randomization, based on the order of screening and stratified for gender and age (12–27, and 28–36 months of age) was used, with an allocation ratio of 1:1:1. Following inclusion, study participants received deworming treatment (10 mg/kg bodyweight pyrantel pamoate), to ensure that expected worm infections would not interfere with the study treatment. Afterwards, baseline measurements were performed. Venous blood samples (10 mL) were taken for the assessment of nutritional status parameters. Samples of early morning urine were collected before the start of the intervention. All measurements were repeated at the end of the 6-month intervention period. WHO Anthro 2007 (44) was used to generate WAZ, HAZ, WHZ, and BMI-for-age (BAZ) scores.

Ethics

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Lagos State University Teaching Hospital (LREC/10/06/829). The trial was registered at ClinicalTrials.gov: NCT03411590.

Study Products

The three groups received a multi-nutrient fortified dairy-based drink (Peak 123, FrieslandCampina WAMCO, Lagos, Nigeria), in amounts of 200, 400 or 600 mL per day. In the case of 400 and 600 mL, parents were requested to spread the portions (200 mL each) over the day. The time of intake was not monitored. The composition of the drink is shown in **Table 5.1**. The ingredient list is presented in **Supplemental Table 5.1**. Airtight packed powder sachets (for 200 mL each) were delivered weekly to the families by trained researchers who also provided instructions for use. Consumption of test products started as soon as all baseline examinations were completed and baseline blood, early morning urine, and faecal samples were collected. In the case of twins and siblings, only the child who met the inclusion criteria was enrolled in the study, however, the other child received the same treatment to prevent sharing and noncompliance with the study protocol.

Table 5.1. Composition of the multi-nutrient fortified dairy-based drink, provided to Nigerian toddlers with malnutrition in daily amounts of 200, 400 or 600 mL, during 6 months.

Nutrient	Unit	Per 200 mL	Per 400 mL	per 600 mL
Energy	kcal	149	297	446
Protein	g	5	11	16
Carbohydrates	g	20	41	61
Sucrose	g	13	27	40
Fat	g	5	10	15
DHA	mg	14	28	42
Calcium	mg	188	376	564
Phosphorus	mg	152	304	455
Potassium	mg	244	488	733
Magnesium	mg	17	33	50
Sodium	mg	63	125	188
Iron	mg	2.24	4.48	6.72
Copper	ug	58	116	173
Zinc	mg	1	2	3
Iodine	ug	40	79	119
Selenium	ug	3.6	7.3	11
Vitamin A	ug-RE	128	255	383
Vitamin D3	ug	2	4	6
Vitamin E	mg	3	5	8
Vitamin B1	ug	155	310	465
Vitamin B2	ug	158	317	475
Vitamin B6	ug	157	314	470
Folic acid	ug	24	48	71

Table 5.1. Continued.

Nutrient	Unit	Per 200 mL	Per 400 mL	per 600 mL
Vitamin B12	ug	0.4	0.8	1,2
Vitamin K1	ug	9.2	18.5	28
Biotin	ug	5.3	10.6	16
Niacin	mg	2.0	4.0	6
Pantothenic acid	mg	0.7	1.3	2
Vitamin C	mg	38	76	114

Micronutrient status parameters in blood

Venous blood sampling (10 mL in total) was performed in the morning between 9:00 and 11:00 a.m. following an overnight fast of at least 12 hrs. The blood was collected in an EDTA microtainer (4 mL), heparin gel microtainer (4 mL), and a serum microtainer (2 mL). The EDTA and heparin microtainers were kept at 4˚C and transferred (on ice) to a local laboratory on the day of collection. In the laboratory, tubes were directly centrifuged (HaematoSpin 1400, Hawksley, UK) at 3300 g for 15 min and the extracted EDTA and heparin plasma was pipetted into aliquots of 200 μL. Serum microtainers were kept at room temperature for at least 60 min to allow clotting. Clotted blood was centrifuged at 2,000 g for at least 3 min and the extracted serum was pipetted into aliquots of 200 μL. All serum and plasma aliquots were stored at -20˚C and transported on dry ice to the Amsterdam University Medical Center (Location Vumc, Amsterdam, The Netherlands), and Medlon B.V. (Enschede, The Netherlands), for biochemical analyses.

EDTA plasma was used for the analysis of zinc, selenium and 25-hydroxyvitamin D ($25OHD_3$ and $25OHD_2$). For zinc and selenium analyses, samples were diluted 30× using 0.2% v/v HNO3 0.05% v/v Triton 1% v/v Methanol and analysed by inductively coupled plasma mass spectrometry (ICP-MS) using a kinetic energy discrimination procedure on the Perkin Elmer Nexion 300× ICP-MS. Cut-off values used for zinc and selenium sufficiency were 10 μmol/l (45) and 0.8 μmol/L (46). Vitamin D was analysed using an optimized LC-MS/MS method, as described by Dirks et al (referring to method E) (47). For this study 2, cut-off values for 25OHD are considered: 50 and 75 nmol/L; 50 nmol to be sufficient for skeletal metabolism and 75 nmol for extra-skeletal activities (15,48,49).

Heparin plasma was used for the analysis of folate and vitamin A. Folate was analysed using the Elecsys Folate III binding assay and Cobas-e immunoassay analyser (Roche/Hitachi); measuring range: 0.6–20.0 ng/mL or 1.36–45.4 nmol/L. The cut-off value for folate sufficiency used in this study was 10 nmol/L (46). Vitamin A was determined

using isocratic high-performance liquid chromatography with UV detection. Intra-assay CV was 0.6 - 0.9%, whereas the inter-assay CV was 1.0 - 1.3%, with a lowest detection limit of 0.1μM (50). The cut-off value for vitamin A sufficiency was 0.7 μmol/L (51).

Vitamin B12 was determined in serum using the Elecsys Vitamin B12 binding assay and Cobas-e immunoassay analyser. Measuring range: 50.0–2000 pg/mL or 36.9–1476 pmol/L. The cut-off value for vitamin B12 sufficiency used in this study was 150 pmol/L (46).

Urinary iodine analysis

The iodine status of the children was assessed by measuring the iodine concentration in early morning spot urinary samples (5 mL) before any food or drink was consumed, collected into a 10-mL universal laboratory bottle, at baseline and after the intervention. The samples were brought to the collection centres by parents and/or caregivers within 1 h, stored at 5–7 ˚C, and transferred on ice to the nearest freezer (-20˚C) at the end of the day. For analysis, frozen samples were transported on dry ice to the Central Clinical Laboratory of the University Medical Center Groningen, the Netherlands. Iodine in urine was analysed using ICP-MS (Varian, Varian Inc., Palo Alto, USA; the lowest level of quantification (LLOQ) 25 μg/L). The cut-off value used for iodine deficiency in the study population sample was a median value of <100 ug/L, whereas ≥300 μg/L was used as a cut-off for iodine excess (52)

Anthropometry

Bodyweight, recumbent length or standing height (for toddlers up to 24 months old or 24 to 36 months respectively), head circumference (HC), and mid-upper arm circumference (MUAC) were measured in triplicate at baseline (screening) and after 6 months (at home). All measurements were taken and recorded by well-trained team members. For bodyweight, a SECA electronic scale (Seca gmbh & co, Hamburg, Germany) appropriate for infants and toddlers was used having a precision of ±20 g for weights below 20 kg and ±50 g for weights up to 50 kg. The scale was placed on a flat, stable surface and every effort was made to ensure that restless toddlers were calm during the weighing procedure. When the child was not calm enough, the child was weighed together with the parent or caregiver. The weighing scale was calibrated daily using a weight standard of 10 kg. All children were weighed undressed, without a diaper, jewellery, or other ornaments. Recumbent length (children 12-24 months of age) was measured to the nearest 0.1 cm using Seca 417 Light and stable measuring board (Seca gmbh & co, Hamburg, Germany) with a stationary headpiece, a sliding vertical foot piece and a horizontal back piece with a measuring tape mounted on it. All children were measured without shoes or any other footwear. Any haircut

influencing length was considered. The measuring board was calibrated daily using a length standard of 40 cm.

In 24- to 36-month-old toddlers, standing height was measured to the nearest 0.1 cm using Seca 213 Mobile stadiometer for measuring height (Seca gmbh & co, Hamburg, Germany). The measurement of height was conducted without shoes and with children keeping their shoulders in a relaxed position, their arms hanging freely, and their heads aligned in Frankfurt plane. Any haircut influencing length was considered. The stadiometer was calibrated daily using a length standard of 80 cm. Weight and length were used to calculate Body Mass Index (BMI; kg/m^2). WHO Anthro 2007(53) was used to generate WAZ, HAZ, WHZ, and BAZ scores.

HC and MUAC were measured to the nearest 0.1 cm with the use of a flexible, non-stretchable, measurement tape (Lufkin W606PM tape, Hoechstmass Ballzer GmbH, Sulzbach (Taunus), Hessen, Germany) and with the toddler at a sitting (HC) and standing position (MUAC). HC was measured after aligning the head in Frankfurt plane and passing the measuring tape around the head, just above the eyebrows, above the ears on each side and over the occipital prominence at the back of the head to its maximal circumference. MUAC of the left upper arm was measured at the mid-point between the tip of the shoulder and the tip of the elbow (acromion and the olecranon process respectively). MUAC is a precise, sensitive and accurate method and parameter for the identification of undernutrition among children aged under 5 years (54).

Cognitive development

Cognitive assessment of children was done using the short (screening) version of the Bayley-III (BSID, Bayley Scales of Infant and Toddler Development®, Third Edition, Bayley–III) (Bayley, 2006). The BSID is an internationally, a multi-scale neuro-developmental battery designed for use in infants and young children from 0 to 42 months (55). The test has been used in some African cultures and is said to be a valid developmental assessment scale for Nigerian children (56). Items in the subtest are particularly valuable in quick screening high-risk infants for developmental delay with regard to five domains, namely expressive and receptive language, cognition, and fine and gross motor areas (55). The test results in a score per development area, and based on cut-off scores, as provided by the BSID, the children can be classified as At Risk, Emerging, or Competent for each cognitive area (**Supplemental materials Table S5.2**). Good reliability coefficients (>0.9) have been established for all the Bayley-III tests, both in the general population and special groups. The BSID takes 15-25 minutes to complete (55).

The test was carried out by two trained licensed neuro-developmental psychologists. These experts attended a 3-day refresher course on how to administer the BSID, including a 2-day practical exposure in the field. The mean interrater reliability of the test between the trainer and the psychologist was 0.98, while the interrater reliability between the two psychologists was 0.97. All tests took place within the study Ijora-Badia community at both baseline and endline on a one-on-one basis. The test was administered to children in the presence of their mothers, or caregivers in a quiet room that was well-lit, well-ventilated, and free from distractions according to the standard of the testing of Bayley. The parents were asked to sit beside their toddlers as it helped the child to concentrate. The test used a set of standardized toys and a detailed scoring sheet to provide a quick assessment of the five key developmental domains.

Sample size calculation

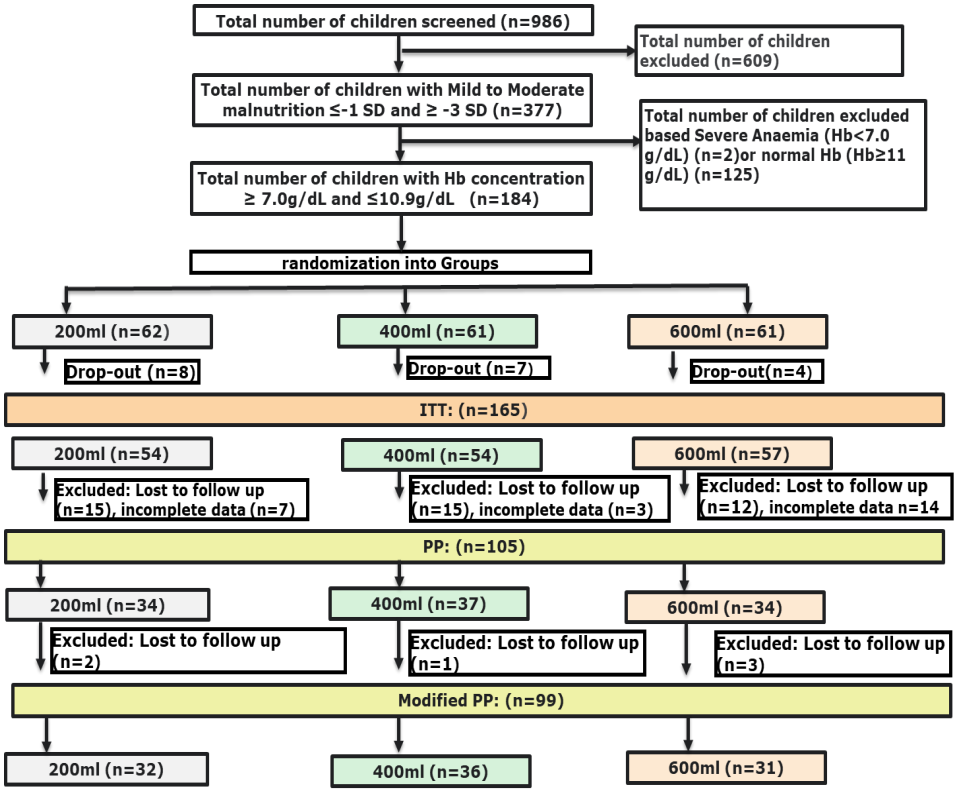
The initial sample size calculation, based on a reduction in anaemia has been described previously (43). To indicate that this sample size would also be suitable for other micronutrients, the calculation was repeated for vitamin D. Depending on the region in Nigeria, the level of 25OHD in children differs considerably. In this calculation we used an average level of 51.2 ± 15.5 nmol/L 25OHD (18). Furthermore, based on the provided amount of vitamin D, the increase in 25OHD can be estimated by using a response factor of 1.2 or 2 nmol/L per μg of daily oral vitamin D during 5 months of supplementation (57). A high response factor will be likely in malnourished children. Using a power of 0.8 and α of 0.05, and for finding a significant difference with baseline, the number of children to be included in the 600 mL group was 47 or 26 (for response factors 1.2 and 2 respectively). With a dropout of 30% these numbers increase up to 61 and 32.

Statistical analysis

We used a modified PP population with at least 50% of the cases with micronutrient data at endline, and with weight and height measurements available at both base- and endline, which resulted in a population that was slightly smaller (105 versus 99 subjects) as compared to the initial PP population described earlier (43) (**Figure 5.1**). ANCOVA was used to determine the differences in effect between the interventions on the micronutrients measured. Post hoc analyses were performed with a Bonferroni adjustment. Although not all micronutrients were normally distributed, ANCOVA was considered robust enough to be applied for these micronutrients as well. The change in micronutrient status from baseline to endline within intervention groups was tested with either a paired t-test or a related-samples Wilcoxon signed-rank test per study group. Differences in growth outcomes between intervention groups were compared using Generalized Linear Models with the study arm as a factor, and the

corresponding baseline outcome as a covariate. To determine changes from baseline to endline within intervention groups, growth outcomes were tested with a paired t-test. The main outcomes of the Bayley-III Screening Test were the absolute numbers and the percentages of participants in each of the 3 subtest classifications: at risk, emerging or competent, for each of the five domains. From the absolute scores of the subtests, delta-values were calculated to study the effect of the intervention period. Differences in delta-values were compared between groups with one-way ANOVA. Changes in the percentages from baseline to endline within each intervention group and differences between intervention groups at endline were tested with a Fisher's Exact test. A p-value <0.05 was considered significant, whereas p-values <0.1 were considered trends. All analyses were performed using IBM SPSS Statistics version 24 (IBM Corp, Armonk, NY, USA).

Figure 5.1. Flow-chart of screening and randomization process.



Results

Baseline characteristics of the three groups within the modified PP population are presented in Table 2. No significant differences were found between the three groups, except for height and a borderline difference for HAZ, which were lower in the 600 mL group compared to the 200 mL group (p = 0.016 and p=0.05, respectively). An overview of baseline characteristics of the ITT population can be found in **Supplemental Table S5.3**.

Table 5.2. Baseline characteristics of the modified PP population, a study in which Nigerian toddlers with malnutrition were provided with a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL, during 6 months (presented as mean ± SD or median, IQR).

	200 mL	**400 mL**	**600 mL**	***p*-value**
N	**32**	**36**	**31**	
Age (months)	21.0, 9	20.0, 8	18.0, 8	0.281°
Sex (% male/female)	50.0/50.0	52.8/47.2	38.7/61.3	0.486#
Religion (% Christian/Muslim)	29.0/71.0	34.3/65.7	29.0/71.0	0.865#
Family setting (% Monogamy/ Polygamy)	79.3/20.7	73.5/26.5	75.0/25.0	0.861#
Social class (upper/middle/lower/ lowest)	0/22.6/77.4/0	2.9/20.0/71.4/5.7	0/16.1/77.4/6.5	0.661#
Weight (kg)	9.3, 2.5	8.9, 1.1	8.6, 1.7	0.238°
Height (cm)	79.2 ± 5.4[a]	77.9 ± 4.6[a, b]	75.7 ± 4.3[b]	**0.016***
BMI (kg/m^2)	14.6 ± 0.9	14.9 ± 0.9	15.0 ± 1.1	0.212*
Head circumference (cm)	46.7 ± 1.8	46.6 ± 1.3	46.5 ± 1.5	0.858*
Mid-upper arm circumference (cm)	13.7 ± 1.1	13.8 ± 0.7	13.8 ± 0.9	0.843*
Weight-for Age z-score (WAZ)	-1.8 ± 0.6	-1.7 ± 0.6	-1.8 ± 0.5	0.842*
Height-for-age z-score (HAZ)	-1.6 ± 0.6	-1.8 ± 0.5	-2.0 ± 0.6	0.050*
Weight-for height z-score (WHZ)	-1.3 ± 0.8	-1.2 ± 0.7	-1.1 ± 0.7	0.428*
BMI-for-age z-score (BAZ)	-1.1 ± 0.8	-0.9 ± 0.7	-0.8 ± 0.8	0.291*
Iodine (ug/L)	252.6, 402.0	323.0, 426.5	315.8, 492.1	0.968°
Zinc (mmol/L)	12.1, 4.0	11.0, 3.1	11.8, 3.0	0.076°
Selenium (μmol/L)	1.0, 0.2	0.9, 0.2	0.9, 0.2	0.314°
Vitamin A (umol/L)	0.9 ± 0.3	0.8 ± 0.3	0.7 ± 0.2	0.134*
Vitamin B12 (pmol/L)	620.0 ± 291.6	560.9 ± 269.0	618.1 ± 281.4	0.704*
Folate (nmol/L)	19.7 ± 7.2	21.7 ± 7.4	21.5 ± 13.0	0.663*
Vitamin D3 (nmol/L)	68.0, 20	65.0, 21	67.0, 24	0.850°

° Kruskal-Wallis test, # Chi-square test, or * one-way ANOVA. Different letters in superscripts [a,b] indicate differences between intervention groups.

Micronutrients

Micronutrient status was determined in blood and urine before and after the intervention. **Table 5.3** shows no differences at endline between the three interventions for iodine, zinc, selenium, vitamin B12, folate, and vitamin A. Only vitamin D3 was higher in the 600 mL group as compared to the 200 mL group ($p=0.012$). C-reactive protein concentrations, as an indicator of acute inflammation, were reported previously and considered to be normal (<5 mg/L) for the majority of the study participants (43).

Table 5.3. Micronutrient status of the modified PP population, a study in which Nigerian toddlers with malnutrition were provided a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL, during 6 months (data are presented as mean ± SD or median, IQR).

	200 mL (n=32)	400 mL (n=36)	600 mL (n=31)	*p*-value[1]
Iodine (ug/L)	377.0, 331.0	394.5, 344.5	337.0, 544.5	0.782
Zinc (μmol/L)	11.4 ± 1.7	11.2 ± 1.9	11.4 ± 2.4	0.922
Selenium (μmol/L)	0.9, 0.2	1.0, 0.1	1.0, 0.2	0.695
Vitamin A (μmol/L)	0.8, 0.3	0.8, 0.2	0.9, 0.4	0.093
Vitamin B12 (pmol/L)	521.0, 229	495.0, 317	463.5, 198	0.734
Folate (nmol/L)	19.3 ± 6.3	18.9 ± 7.5	17.1 ± 6.4	0.449
Vitamin D3 (nmol/L)	73.0, 15[a]	79.0, 21[a,b]	80.0, 21[b]	**0.012**

[1] The p-value represents the outcome of the ANCOVA, controlling for baseline values.
superscripts [a,b] indicate differences between intervention groups.

In **Figure 5.2** the base- and endline values of the micronutrients and the outcomes of the within-group comparisons are shown. A small decrease in zinc concentration was found after the intervention with 200 mL compared to baseline ($p=0.047$). Consumption of the multi-nutrient fortified formula increased vitamin D3 status ($p<0.0001$) in both the 400- and 600-mL group, but not in the 200 mL group. The intervention with 600 mL of multi-nutrient fortified formula per day also increased selenium ($p=0.022$) and vitamin A ($p=0.003$) levels. Table 4 shows the prevalence of deficiencies for the different micronutrients per intervention group at baseline and after 6 months of intervention. The median urinary iodine excretion was 309 μg/L (range: 22–5622) at baseline indicating a population with an excessive iodine intake. A minority of 13.2% of the subjects had an iodine excretion of <100 μg/L (low iodine status), which decreased to 10.9% after the intervention. Zinc deficiency increased during the study from 18.2% at the start to 26.0% at the end of the study. Of the total group, 27.4% had selenium deficiency before starting with the intervention, and this decreased to 10.4%. More than one-third of the study subjects (39.4%) suffered from vitamin A deficiency, this decreased to 26.6%. Vitamin B12 deficiency was only

present in 2.9% of the study population before the intervention, and this hardly changed during the study (2.3% at endline). A deficiency of folate was found in 11.3% of the subjects, which decreased to 6.1%. Vitamin D deficiency was present in 16.7% of the study population (using the WHO cut-off value of 50 nmol/L 25OHD), which decreased to 3.1% after the intervention.

Figure 5.2. Micronutrient levels in blood or urine, sampled at baseline and after 6 months of intervention with a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL, in Nigerian toddlers with malnutrition: **A)** Iodine (median + IQR), **B)** zinc (mean + SD), **C)** selenium (median + IQR), **D)** vitamin A (median + IQR), **E)** vitamin B12 (median + IQR), **F)** folate (mean + SD), and **G)** vitamin D3 (median + IQR) for the three study groups at baseline and after the 6 months intervention. The difference between baseline and endline was tested with either a paired t-test or a related-samples Wilcoxon signed-rank test per study group. *p<0.05, **p<0.01, ****p<0.0001

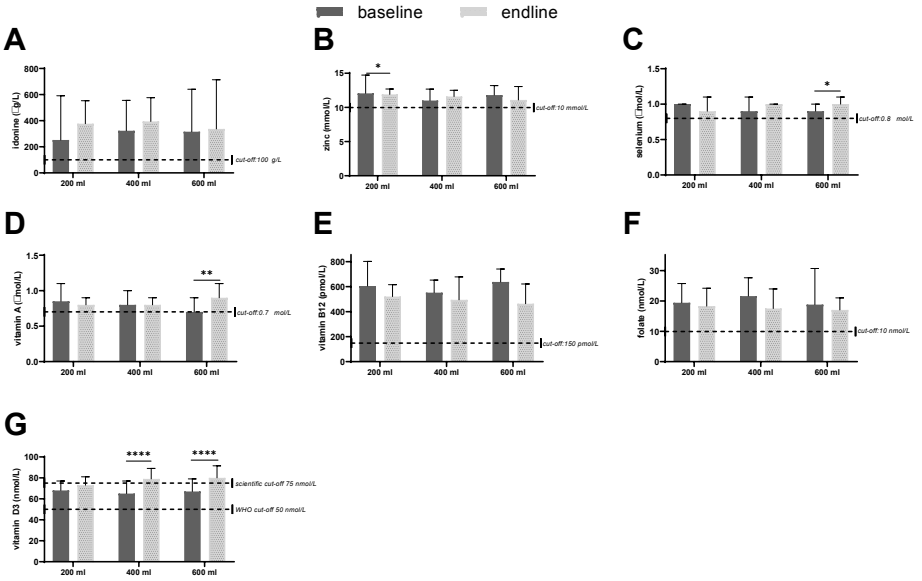


Table 5.4. Prevalence (%) of base- and end-line micronutrient status of modified PP population Nigerian toddlers provided with a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL, during 6 months.

	Reference value*	200 mL		400 mL		600 mL	
		baseline	endline	baseline	endline	baseline	endline
Iodine	100 ug/L	n=28 7.1 (2.0-22.6)	n=29 10.3 (3.6-26.4)	n=33 15.1 (6.7-30.0)	n=34 8.8 (3.0-23.0)	n=30 16.7 (7.3-33.6)	n=29 13.8 (5.5-30.6)
Zinc	10 μmol/L	n=32 6.3 (1.7-20.1)	n=31 25.8 (13.7-43.2)	n=36 25.0 (13.8-41.1)	n=35 28.6 (13.6-45.1)	n=31 19.4 (9.2-36.3)	n=30 16.7 (7.3-33.6)
Selenium	0.8 μmol/L	n=32 18.8 (8.9-35.3)	n=30 6.7 (1.8-21.3)	n=32 12.5 (5.0-28.1)	n=35 2.9 (0.5-14.5)	n=31 22.6 (11.4-39.8)	n=31 6.5 (1.8-20.7)
Vitamin A	0.7 μmol/L	n=29 34.5 (19.9-52.7)	n=30 26.7 (14.2-44.4)	n=34 32.4 (19.1-49.2)	n=34 23.5 (12.4-40.0)	n=30 40.0 (24.6-57.7)	n=30 16.7 (7.3-33.6)
Vitamin B12	150 pmol/L	n=23 0.0 (0.0-14.3)	n=27 0.0 (0.0-12.5)	n=27 7.4 (2.1-23.4)	n=31 3.2 (0.6-16.2)	n=18 0.0 (0.0-17.6)	n=28 0.0 (0.0-12.1)
Folate	10 nmol/L	n=20 5.0 (0.0-16.1)	n=27 3.7 (0.7-18.3)	n=25 8.0 (2.2-25.0)	n=29 6.9 (1.9-22.0)	n=17 23.5 (9.6-47.3)	n=26 7.7 (2.1-24.1)
Vitamin D	50 nmol/L	n=29 13.8 (5.5-30.6)	n=32 3.1 (0.6-15.7)	n=36 16.7 (7.9-31.9)	n=35 0.0 (0.0-9.9)	n=31 19.35 (9.2-36.3)	n=30 3.3 (0.6-16.7)

*Reference value for zinc, selenium, vitamin B12, folate and vitamin D3 (46), iodine (52), vitamin A (51)

Anthropometry

Table 5.5 shows the estimated means of the anthropometric outcomes after the intervention. The three study groups all show an increase in weight and height during the study. Following the intervention, weight and height showed a (dose-responded) trend to differ between the three study groups (p=0.081 and p=0.062, respectively). BMI, head-circumference and MUAC improved in all groups compared to baseline, but no differences were found between groups. Also, for the Z-scores, no differences were found between the groups. Only the WAZ showed a (dose-response) trend to differ between groups (p=0.079). Within groups WAZ-, WHZ-, and BAZ scores significantly improved during the intervention (Figure 3). With regard to HAZ, only the 600 mL group improved from baseline to endline (p<0.0001). When growth parameters were plotted in the WHO growth curves (44): 1) the absolute weights gradually moved from the 3rd to the 15th percentile line (Figure S1A-B); 2) length or height followed more or less the 3rd percentile line (Figure S1C-D), at least for boys, whereas older girls (24-29 months) tended to improve their length towards the 15th percentile; 3) BMI went from the 15th percentile toward median values, in particular for the girls in the 600 mL group (Figure S1E-F); and 4) head circumference followed the curve in between the 15th percentile and median (Figure S1G-H).

Table 5.5 Endline anthropometric data of the modified PP population of Nigerian toddlers with malnutrition who were provided with a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL, during 6 months (data presented as means ± SE).

	200 mL (n=32)	400 mL (n=36)	600 mL (n=31)	*p*-value[1]
Weight (kg)	10.36±0.14	10.49±0.13	10.79±0.14	0.081
Height (cm)	82.39±0.27	82.62±0.25	83.29±0.27	0.062
BMI (kg/m^2)	15.16±0.17	15.34±0.16	15.60±0.18	0.203
Head circumference (cm)	47.42±0.17	47.69±0.16	47.15±0.17	0.071
Mid-upper arm circumference (MUAC) (cm)	14.32±0.18	14.61±0.17	14.56±0.18	0.455
Weight-for Age z-score (WAZ)	-1.37±0.10	-1.29±0.10	-1.05±0.11	0.079
Height-for-age z-score (HAZ)	-1.70±0.09	-1.65±0.08	-1.53±0.09	0.395
Weight-for height z-score (WHZ)	-0.70±0.13	-0.60±0.12	-0.35±0.13	0.150
BMI-for-age z-score (BAZ)	-0.48±0.14	-0.37±0.13	-0.12±0.14	0.161
MUAC-z-score	-0.77±0.15	-0.50±0.14	-0.46±0.15	0.278

[1] The p-value represents the outcome of the Generalized Linear Model with the study arm as a factor and the corresponding baseline value for the outcome as a covariate.

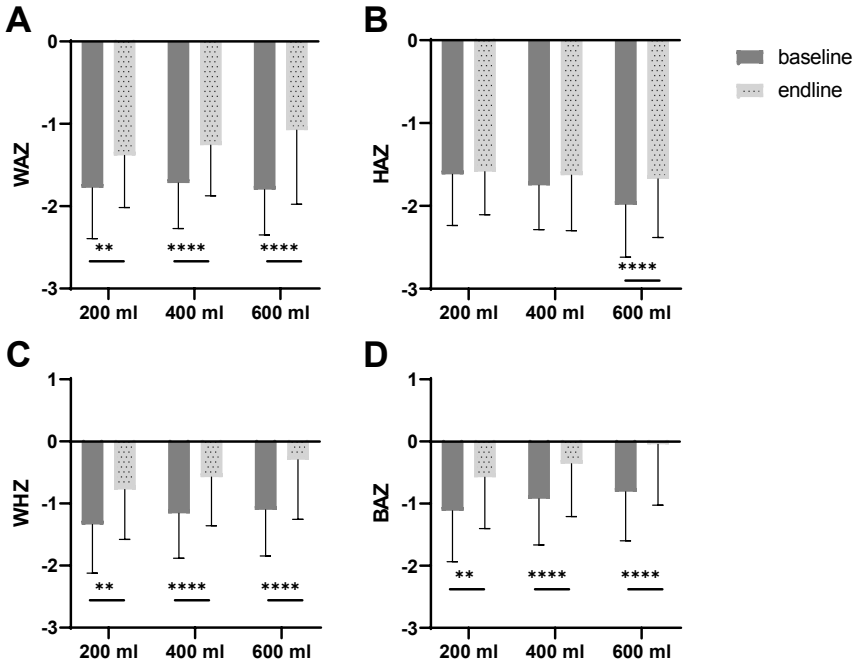


Figure 5.3. Weight-for-age z-scores (WAZ), height-for-age z-scores (HAZ), weight-for-height z-scores (WHZ), and BMI-for-age z-scores BAZ), at baseline and after 6 months of intervention, in Nigerian toddlers with malnutrition, provided with a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL: **A)** WAZ, **B)** HAZ, **C)** WHZ, and **D)** BAZ. The difference between baseline and endline was tested with a paired t-test per study group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Average MUAC outcomes at base- and end-line, in combination with reference values, are presented in the supplementary materials (Supplemental Table S4). At baseline, the average calculated Z-scores for the 200-, 400- and 600 mL groups were -1.12, -0.98 and -0.78 SD, which are slightly better than Z-scores for weight and height (Table 2). At endline, the calculated Z-scores improved to -0.79, -0.52 and -0.39 SD, but groups were not different from each other.

Cognition

Twenty-six children in the modified-PP population did not perform the Bayles-III Screening Test, due to no show or lack of cooperation of children. Seventy-three children at baseline and 72 at endline had data available for at least one sub-score of the test. Sixty-six subjects completed all subtests. Most missing values (n= 6) for subtests were seen in the 400 mL group at endline. Only 1 child in the 600 mL group did not perform a subtest at baseline. No missing values were seen in the 200 mL group. The reason for not completing all tests was a lack of concentration or willingness during the test. The absolute score values are presented in Supplemental **Table S5.5**. As indicated by the positive delta-values, all study groups improved their average scores for the different subtests. No differences were found between the study groups. Groups did not differ in the per cent of children per classification per development category after the intervention for any of the subtests (Supplemental Table S6). The only change observed within study groups was a decline in the per cent of competent children for receptive language ($p=0.03$, $p=0.01$, and $p=0.01$ for the 200mL, 400mL and 600 mL groups respectively).

Discussion

In the present study, 200, 400 or 600 mL of a multi-nutrient fortified dairy-based drink was provided daily for 6 months to malnourished and anaemic Nigerian toddlers. The intake of 600 mL improved selenium ($p<0.05$), vitamin A ($p<0.01$), and vitamin D3 ($p<0.0001$) status and consequently reduced the percentages of children who are deficient of these micronutrients. For vitamin D, these effects were also seen in the 400 mL group. Groups did not differ in growth parameters, however, a trend towards differences between groups for height ($p=0.062$) and weight ($p=0.081$), suggests a dose-response effect. No differences were seen between interventions on cognitive subscores of the Bayley-III Screening Test.

Effect of multi-fortified Dairy-based drink on micronutrient Status

The present study shows that at baseline most children were not deficient in any of the nutrients studied. For vitamin B12 and folate this is in accordance with limited

literature available for Northern Nigeria (24), but it is not in line with the reported low folate intake in the Ibadan region (25). The prevalence of vitamin D deficiency was 16.7% when <50 nmol/L was taken as a cut-off value, however, this cut-off is mainly supportive for skeletal metabolism (49,58) For extra-skeletal activities, e.g., the immune system, a cut-off value of at least 75 nmol/L 25OHD is suggested (48,49,59–61). Vitamin D status is importantly determined by cutaneous synthesis following exposure to sunlight. For a pigmented skin, 1 to 1.5 hour of sunlight on 25% of unprotected skin should be enough to ensure an sufficient vitamin D synthesis (62–64). However, the present study shows that close to 70% of the study population, with sufficient possibilities to be exposed to the sun in the absence of seasonality in cutaneous vitamin D synthesis (64), had less than 75 nmol/l 25OHD at baseline. The study shows that oral vitamin D intake resulted in an improvement of vitamin D status in all study groups. Based on the improvement in the 600 mL group from 67.0 nmol/L 25OHD at baseline to 78 nmol/L after 6 months, the response factor appears to be high (1.8 nmol/l per μg oral vitamin D) as might be expected in malnourished children (57). In healthy European children aged 1-3 years, receiving 8.5 µg additional vitamin D daily (65), and in Australian and New Zealand 1-y-old participants receiving 1.4 µg additional vitamin D daily (66), the response factor were around 1.1. Vitamin A deficiency (VAD) in Nigerian children under 5 years of age was reported to be about 29.5% (67) despite the mandatory fortification of vegetable oil, wheat flour and sugar with vitamin A (68,69), Although, plausible reason might be repeated malaria infections with malaria being endemic in the study region. Repeated malaria infection has been associated with reduced vitamin A status in children(70). Baseline prevalence in the present study (39.4%) indicates that vitamin A deficiency has hardly improved. Restoring vitamin A , however, has shown to reduce all-cause and diarrhoea specific mortality in children under 5 years of age quite significantly (71). The reduction in the vitamin A deficiency in the 400 mL and 600 mL groups from 38.2% to 23.5% and from 46.7% to 23.3%, respectively, therefore shows that daily supplementation with relatively low doses of vitamin A (255-383 μg-RE) might be very supportive for general health.

With regard to the minerals, plasma selenium concentrations in the present study show that 27.4% of children at baseline were selenium insufficient (<0.8 µmol/L), which is higher than estimated (1-10%) based on selenium intake (32). The prevalence of selenium deficiency decreased to 13.3%, 11.4% and 6.5% in the 200-, 400- and 600-mL group respectively, at the end of the study. Plasma concentrations of 0.9 to 1.3 µmol/L (70 to 100 µg/L) are proposed to reflect selenium adequacy (72), whereas maximal platelet glutathione peroxidase activity is achieved at a plasma concentration of about 1.25–1.45 µmol/L (100–115 µg/L) (73). This might indicate that the currently used cut-off value for children is too low. An important role of

selenium is being part of selenocysteine in the catalytic centre of enzymes protecting the thyroid from H_2O_2 which is released when iodine is used in the synthesis of thyroid hormones (34). A higher selenium status therefore would be very useful in the toddlers of the present study to cope with the relatively high iodine intake. Urinary median iodine excretion was 309 μg/L (range: 22–5622) at baseline, indicating an excessive iodine intake by these children. The high intake may be a result of the iodine-salt-fortification program, recommending 50 mg iodine fortification per kg of salt (11). The WHO recommends <5 g salt intake per day for adults (52), which is, based on energy requirements, and about 3 g for children 3 years of age providing 150 μg iodine per day to toddlers whose recommendation is 90 μg/day (74), assuming that all salt is consumed as discretionary salt and not from processed foods. Zinc is important in stimulating growth in length and weight, with a stronger effect after 2 years of age (26). However, plasma zinc concentrations may be normal when it is already limiting growth (a so-called "type 2 nutrient"). When under zinc limiting conditions, more energy is provided to stimulate growth, zinc availability will be stressed which may result in a plasma zinc decrease (58). According to the National Food Consumption Survey 2003, 20% of children aged <5 years were found to be zinc deficient in Nigeria (29), which is in agreement with the present study (average deficiency of 18%.2 at baseline). Although plasma zinc responds to supplementation, as shown in children supplemented with 7 or 10 mg/day (75), the present study did not show this effect (supplementing 1, 2 or 3 mg/day). For the latter finding, it is hypothesized that the low levels of zinc provided are not enough to compensate for the zinc requirement associated with stimulated growth by the dairy-based product, while for zinc adequate children no effect on zinc plasma levels might be seen due to a plateauing effect when zinc intakes are higher than the requirements, as suggested in healthy men (75).

Effect of multi-fortified dairy-based drink on physical growth

In developing countries, milk intake is associated with linear growth, in which at least the high-quality protein and bone-friendly components such as calcium play a role (42,76,77). Although protein intake might not be limited in many Nigerian children, the protein quality might not be optimal (42). The present study suggests that an increasing amount of daily multi-fortified dairy-based could be beneficial for linear growth. Children consuming 600 mL daily were 0.9 cm and 0.67 cm taller than those from the 200 mL and 400 mL groups, respectively. The negative anthropometric Z-scores at baseline improved during the intervention period in all groups. A possible effect of supplementation on height in the present study is consistent with a study conducted in 1002 preschool-age children (1-5 years) from the National Health and Nutrition Examination Survey (NHANES). This study showed that children who drank milk daily were taller (1.0 cm; $p<0.02$) than those with less frequent intake (77). A

prospective cohort study among premenarchal girls who drank >3 servings per day of milk grew 0.28 cm more the following year than girls consuming <1 serving per day. Of the foods and nutrients studied, dairy protein had the strongest association with linear growth while non-dairy animal protein and vegetable protein were never significant, nor were non-dairy animal fat and vegetable fat (78). It is suggested that milk components stimulate IGF-I concentrations and, thereby, growth (77,79–86).

Cognition

With the use of the Screening Test of the Bayley-III, no effects of the intervention were found between or within groups. The per cent of children at risk (for any of the subtests) varied considerably from 0-38.1%. For receptive language (how the child understands language) a decline in classification was seen in all three study groups. Important factors associated with poor development are non-stimulating home environments, a limited role of the father in child-raising, or low social-economic status of the family (associated with poor nutrition and stunting) (87–89). The reported decline in receptive language might be a consequence of the low social and economic conditions (80% of the households in the studied population) in combination with undernutrition (all were malnourished and anaemic children) (3). Also, the fact that many children are exposed to more than one language at home (more than 250 native languages spoken in Nigeria) might have played a role, though English is the official language. The improvements in nutritional status and anthropometry apparently could not prevent a decrease in receptive language development, at least not within the 6-month study period.

Strengths and limitations

Although it was not the goal of the study, the absence of a placebo group is a limitation, preventing the insight into the effect of the lowest amount of product intake. Therefore, in a future study, it would be of interest to follow up a non-supplemented control group as well. In this study we had an intervention period of 6 months; while this was sufficient to see improvements in micronutrient status, a longer study period appears to be necessary to see real improvements in cognition. For a future study, an intervention duration of 12 months would be advised. With regard to the cognitive data, the screening version of the Bayley-III tool does not allow to make diagnostic interpretations. Besides, the test had quite a lot of dropouts and faced a lack of concentration and willingness to do or complete the test. Therefore, it is difficult to draw any firm conclusions from this dataset. The absence of food intake data in this manuscript makes it difficult to attribute the improvements observed to the fortified dairy-based drink only. Since children with medically diagnosed allergies, not having anaemia, intolerances to milk or milk ingredients were excluded, and since children were recruited from a poor environment, study results cannot directly be generalized

to children with different characteristics. A strength of this study is the food-based dose-response approach, showing that an intake of 200 mL of the study product already can have beneficial effects on growth (improved z-scores).

Conclusions

This study showed that daily consumption of a multi-nutrient fortified dairy-based drink by toddlers improved their nutritional status of vitamin A (600 mL), vitamin D (400 and 600 mL) and selenium (600 mL). The latter can be important to cope with an excessive intake of iodine. The effect on zinc status is not clear due to the absence of a placebo, the amount provided and/or study duration. With regard to growth, z-scores of weights, height, and BMI improved as compared to baseline, but no difference was seen between groups. No effects were seen on cognitive development. For anthropometry and cognition, a longer study duration might be necessary. The most beneficial daily amount of fortified diary-based drink appears to be 600 mL.

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Supplementary Material

Effect of a fortified dairy-based drink on micronutrient status, growth, and cognitive development of Nigerian toddlers- A dose-response study

Supplemental Table 5.1: List of Ingredients

List of ingredients
Skimmed milk, Glucose syrup solids, Vegetable oils, Palm oil, Canola oil (low erucic acid type), Palm kernel oil, Sunflower oil, Saccharose, Fish oil, Sodium L-ascorbate, Emulsifier (Lecithin), Lactose, Taurine, Meso-inositol, Choline chloride, Ferrous sulphate, Vanilla flavour, DL M-tocopheryl acetate, Zinc sulphate, L-Ascorbyl palmitate, Nicotinamide, Manganese sulphate, Calcium D-pantothenate, Thiamin hydrochloride, Cupric sulphate, Retinyl-acetate, Pyridoxine hydrochloride, ß-carotene, Folic acid, Potassium iodide, Phytomenadione, D-Biotin, Cholecalciferol, Sodium selenite.

Supplemental Table 5.2: Bayley-III Screening Test: an overview of the subtest scores used for classification

		Total Raw Score		
Age category	**Subtests**	**At risk**	**Emerging**	**Competent**
Ages 12 months 16 days – 18 months 15 days	Cognitive	0-13	14-16	17-33
	Receptive Communication	0-9	10-11	12-24
	Expressive Communication	0-9	10-12	13-24
	Fine Motor	0-10	11-13	14-27
	Gross Motor	0-12	13-16	17-28
Ages 18 months 16 days – 24 months 15 days	Cognitive	0-16	17-20	21-33
	Receptive Communication	0-11	12-15	16-24
	Expressive Communication	0-11	12-15	16-24
	Fine Motor	0-11	12-16	17-27
	Gross Motor	0-16	17-18	19-28
Ages 24 months 16 days – 30 months 15 days	Cognitive	0-20	21-24	25-33
	Receptive Communication	0-12	13-18	19-24
	Expressive Communication	0-12	13-18	19-24
	Fine Motor	0-14	15-18	19-27
	Gross Motor	0-18	19-21	22-28
Ages 30 months 16 days – 36 months 15 days	Cognitive	0-22	23-27	28-33
	Receptive Communication	0-12	13-20	21-24
	Expressive Communication	0-14	15-20	21-24
	Fine Motor	0-17	18-22	23-27
	Gross Motor	0-18	19-23	24-28
Ages 36 months 16 days – 42 months 15 days	Cognitive	0-24	25-31	32-33
	Receptive Communication	0-14	15-22	23-24
	Expressive Communication	0-15	16-23	24
	Fine Motor	0-17	18-25	26-27
	Gross Motor	0-20	21-26	27-28

Supplemental Table S5.3: Baseline characteristics of ITT and modified PP populations of malnourished Nigerian toddlers provided with a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL, during 6 months.

	ITT	Modified PP
N	165	99
Age (months)	20.2 ± 6.3	20.0 ± 6.2
Gender (boys/girls) (%)	44.8 / 55.2	47.5 / 52.5
Social class (upper/middle/lower) (%)	0.6 / 18.4 / 81.0	1.0 / 19.6 / 79.4
Religion (Muslim/Christian) (%)	71.0 / 29.0	69.1 / 30.9
Weight (kg)	8.9 ± 1.2	8.9 ± 1.2
Height (cm)	77.5 ± 4.9	77.6 ± 4.9
Head circumference (cm)	46.6 ± 1.6	46.6 ± 1.6
Waist circumference (cm)	44.8 ± 3.1	44.8 ± 3.2
Mid-upper arm circumference (cm)	13.7 ± 0.9	13.8 ± 0.9
Weight for age Z- score	-1.80 ± 0.56	-1.76 ± 0.57
Height for age Z- score	-1.80 ± 0.65	-1.78 ± 0.61
Weight for height Z-score	-1.24 ± 0.78	-1.20 ± 0.75
BMI for age Z-score	-0.95 ± 0.78	-0.95 ± 0.79
Iodine	311.0, 434.0	297.2, 424.4
Selenium	0.9, 0.3	0.9, 0.2
Zinc	11.4, 2.6	11.3, 2.7
Vitamin A (umol/L)	0.8, 0.4	0.8, 0.4
Vitamin B12 (pmol/L)	579.0, 341	602.5, 324
Folate (nmol/L)	19.3, 12.9	21.0 ± 9.1
Vitamin D3 (nmol/L)	66.0, 25	67.0, 23

Data are presented as mean ± SD or median, IQR.

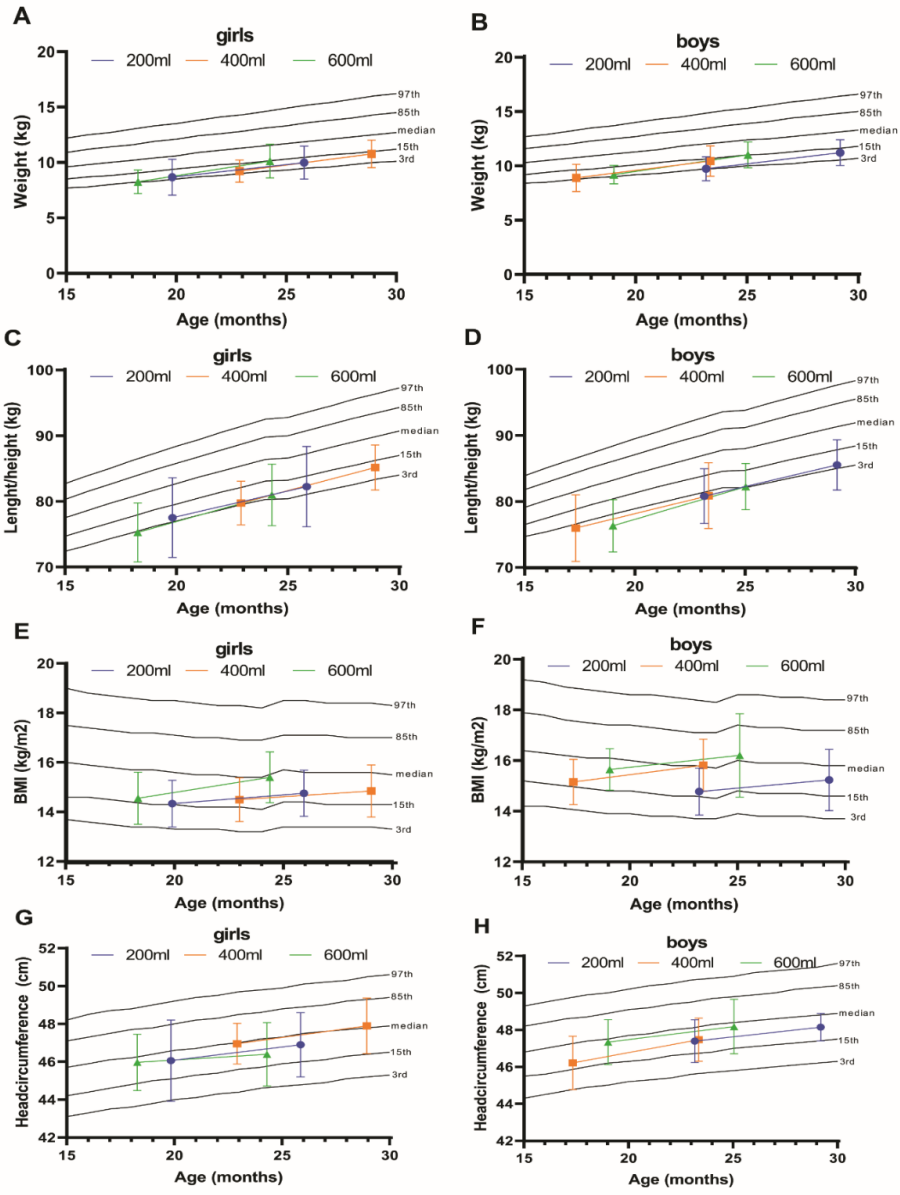

Figure S5.1: **A)** Weight of girls, **B)** weight of boys, **C)** length/height of girls, **D)** length/height of boys, **E)** BMI of girls, **F)** BMI of boys, **G)** head circumference of girls and **F)** head circumference of boys of the different intervention groups (200, 400 and 600 mL) in the modified PP population of malnourished Nigerian toddlers, at the start and after the intervention period. Data are plotted in WHO growth curves presenting with the 3rd, 15th, median, 85th and 97th percentiles of the growth curves.

Supplemental Table S5.4: MUAC age-reference values for boys and girls aged 6-59 months (90), and mean outcomes (at base- and end-line) in Nigerian toddles provided with a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL, during 6 months.

	Current study		MUAC reference values and Z-scores (90)							
Product group	Mean age (months)	Average MUAC (cm) (min-max)	-4SD	-3SD	-2SD	-1SD	MEAN	1SD	2SD	3SD
Baseline										
200mL	21	13.7 (12.0-15.5)	10.8	12.1	13.4	14.7	16	17.3	18.7	20
400mL	20	13.7 (12.3-15.1)	10.7	12.1	13.4	14.7	16	17.3	18.6	19.9
600 mL	18	13.8 (12.5-15.7)	10.7	12	13.3	14.6	15.9	17.2	18.5	19.8
Endline										
200mL	27	14.3 (12.5-17.0)	10.8	12.2	13.5	14.9	16.2	17.6	18.9	20.3
400mL	26	14.6 (13.0-18.8)	10.8	12.2	13.5	14.9	16.2	17.5	18.9	20.3
600 mL	24	14.6 (12.5-16.5)	10.8	12.1	13.5	14.8	16.1	17.5	18.8	20.2

Supplemental Table S5.5. Average absolute values of the subtest scores of the Bayley Screening III test at baseline and after the intervention in the modified PP population of Nigerian toddlers provided with a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL, during 6 months. (mean ± SD or median, IQR).

		200 mL		400 mL				*p*-value*
Subtest		**baseline**	**endline**	**baseline**	**endline**	**Baseline**	**endline**	
Cognition	Absolute average (mean±SD)	21.0 ± 2.9 (n=23)	23.4 ± 3.0 (n=23)	20.3 ± 3.4 (n=29)	23.0 ± 2.7 (n=24)	18.6 ± 2.8 (n=21)	21.8 ± 2.3 (n=21)	
	Delta (mean±SD)	2.8 ± 2.6 (n=23)		3.2 ± 2.9 (n=24)		3.2 ± 2.1 (n=21)		0.851
Receptive language	Absolute average (mean±SD or median,IQR)	14.0, 3 (n=23)	16.2 ± 2.6 (n=23)	14.0, 2 (n=29)	14.9 ± 2.9 (n=24)	13.0, 2 (n=21)	14.5 ± 1.9 (n=21)	
	Delta (mean±SD)	1.7 ± 2.0 (n=23)		0.9 ± 2.4 (n=24)		1.4 ± 2.1 (n=21)		0.424
Expressive language	Absolute average (mean±SD or median,IQR)	14.0, 3 (n=23)	15.7 ± 3.4 (n=23)	12.0, 5 (n=29)	15.5 ± 4.1 (n=28)	13.0, 3 (n=21)	13.3 ± 2.7 (n=21)	
	Delta (mean±SD)	1.4 ± 2.6 (n=23)		2.4 ± 3.5 (n=28)		0.7 ± 2.1 (n=21)		0.116
Fine motor skills	Absolute average (median,IQR)	13.0, 4 (n=23)	16.0, 8 (n=23)	13.0, 3 (n=29)	15.0, 5 (n=28)	12.0, 3 (n=20)	14.0, 3 (n=21)	
	Delta (mean±SD)	2.3 ± 2.9 (n=23)		2.4 ± 3.4 (n=28)		1.8 ± 2.2 (n=20)		0.744
Gross motor skills	Absolute average (median,IQR)	19.0, 5 (n=23)	23.0, 5 (n=23)	19.0, 2 (n=24)	20.0, 5 (n=28)	18.0, 5 (n=21)	20.0, 3 (n=21)	
	Delta (mean±SD)	2.3 ± 2.3 (n=23)		2.5 ± 3.3 (n=24)		2.8 ± 3.5 (n=21)		0.873

**The p-value represents the outcome of a one-way ANOVA which was used to compare the delta-values of the subtests of the different study groups*

Supplemental Table S5.6: Percentages of classification groups of the subtest scores of the Bayley Screening III test at baseline and after the intervention in the modified PP population of Nigerian toddlers provided with a fortified dairy-based drink in daily amounts of 200, 400 or 600 mL, during 6 months.

		200 mL		400 mL		600 mL		*p*-value*
Subtest		**baseline**	**endline**	**baseline**	**endline**	**baseline**	**Endline**	
Cognition	at risk (%)	17.4	13.0	17.2	12.5	28.6	28.6	0.347
	emergent (%)	34.8	34.8	20.7	37.5	23.8	14.3	
	competent (%)	47.8	52.2	62.1	50.0	47.6	57.1	
	p-value^	1.00		0.38		0.78		
Receptive language	at risk (%)	0.0	13.0	3.4	20.8	4.8	4.8	0.452
	emergent (%)	65.2	78.3	44.8	62.5	42.9	85.7	
	competent (%)	34.8	8.7	51.7	16.7	52.4	9.5	
	p-value^	**0.03**		**0.01**		**0.01**		
Expressive language	at risk (%)	13.0	21.7	24.1	28.6	19.0	38.1	0.612
	emergent (%)	65.2	60.9	48.3	42.9	47.6	42.9	
	competent (%)	21.7	17.4	27.6	28.6	33.3	19.0	
	p-value^	0.83		0.94		0.39		
Fine motor skills	at risk (%)	8.7	17.4	13.8	35.7	35.0	33.3	0.292
	emergent (%)	69.6	52.2	62.1	39.3	45.0	57.1	
	competent (%)	21.7	30.4	24.1	25.0	20.0	9.5	
	p-value^	0.52		0.12		0.58		
Gross motor skills	at risk (%)	8.7	8.7	12.5	17.9	14.3	9.5	0.684
	emergent (%)	13.0	17.4	16.7	28.6	42.9	23.8	
	competent (%)	78.3	73.9	70.8	53.6	42.9	66.7	
	p-value^	1.00		0.49		0.32		

**these p-values represent the outcome of a Fisher's Exact test in which the prevalences of the classification groups were tested between study groups at endline, ^ these p-values represent the outcome of the Fisher's exact test that was used to compare the baseline and endline percentages within study groups.*

CHAPTER 6

General discussion

General Discussion

This thesis describes the effects of multi-nutrient fortified dairy-based formulas in Nigerian late preterm infants (growth, micronutrient status) and moderately malnourished toddlers (growth, micronutrient status, cognitive development and faecal microbiota). In addition, a qualitative exploration into feeding practices and nutritional guidelines used by Nigerian healthcare professionals for late preterm infants was conducted among HCPs who were involved in the management of later preterm infants. In this chapter, the main findings are summarised, followed by some critical notes regarding the methodologies employed, the public health implications of this work, and suggestions for future research.

Summary of the main findings

Two of our studies were performed in Nigeria: the first study is a non-randomized parallel observational study conducted among healthy moderate-to-late preterm infants fed on either preterm formula or breastmilk during the 2nd to 10th week of life. The second study describes a randomised intervention trial on the effect of a multi-fortified dairy-based drink on anaemia, haemoglobin concentration, and intestinal microbiota composition in moderately malnourished and anaemic Nigerian toddlers, aged 12 – 36 months, receiving either 200, 400 or 600 ml of a dairy-based drink for 6 months. Additionally, in-depth interviews and focus group discussions with 19 Nigerian healthcare professionals were organized to get insight into some aspects of the nutritional management of LP infants. The main findings and characteristics of the studies are captured in **Table 6.1**.

Table 6.1. Summary of main findings

Thesis Chapter	Study design, population	Objectives	Main findings
Chapter 2	Open, non-randomized parallel, observational study among 41 healthy moderate-to-late preterm-born infants.	To evaluate growth and nutritional status from 2-10 weeks post-delivery in Nigerian moderate-to-late preterm infants receiving a preterm formula or breastmilk.	1. Unfortified breastmilk supported adequate growth but was inadequate for providing vitamins A and D, and iron. 2. Feeding with preterm formula showed accelerated growth in weight.
Chapter 3	In-depth interviews and focus group discussions with 19 Nigerian healthcare professionals involved in the nutritional management of LP infants.	To gain insight into LP infants' current nutritional management (growth monitoring, feeding practices, and guidelines) in Nigeria.	1. There is no consistency in growth monitoring protocols used in the nutritional management of LP infants in Nigeria. 2. Breastmilk is the preferred method of feeding. 3. Nigerian HCPs generally tended to aim for higher weight gain than necessary according to international growth charts
Chapter 4	A randomized controlled trial with 200, 400 and 600 mL of a multi-nutrient fortified dairy-based drink in 105 moderately malnourished, anaemic Nigerian toddlers aged 1-3 years.	To determine which dose of iron from a multi-nutrient fortified dairy-based drink would improve anaemia without stimulating pathogenic gut bacteria.	1. Anaemia prevalence was reduced to 47% (200 mL), 27% (400 mL) and 18% (600 mL) in the three dose groups, respectively, in a dose-response manner ($p=0.03$). 2. There were no differences in abundance of gram-negative pathogenic gut bacteria before and after treatments, or between the three treatment groups.
Chapter 5	A randomized controlled trial with 200, 400 and 600 mL of a multi-nutrient fortified dairy-based drink in 99 moderately malnourished, anaemic Nigerian toddlers aged 1-3 years.	To study the effect of different doses of a multi-nutrient fortified dairy-based drink on micronutrient status, growth, and cognitive development.	1. Consumption of 600 mL increased vitamin A, D and selenium status as compared to baseline, but no differences were found between groups. 2. Consumption of 600 mL improved HAZ, WAZ, WHZ, and BAZ, but there were no significant differences between groups. 3. There were no relevant improvements in iodine, zinc, vitamin B12, and folate status in any of the three groups.

Late preterm (LP), Weight-for Age z-score (WAZ), Height-for-age z-score (HAZ), Weight-for height z-score (WHZ), BMI-for-age z-score (BAZ).

Methodological reflections

The individual research chapters already contain a discussion on the strengths and limitations of the different studies in this thesis. Nevertheless, in this section, a more integrated critical review of the methodologies used in this thesis will be provided, which is important for the interpretation of the study findings and their dissemination.

Potential threats, limitations, and strengths of our study methods, and how these were addressed

Selection Bias

Our study in **Chapter 2** included a higher number of multiple births in the formula-fed group (1 twin, 2 triplets and 1 quadruplet) compared to the breastfed group (2 twins). This is a form of confounding by indication [1, 2]. This might have possibly biased our outcome. However, no child included in the study was lost to follow-up and no family opted out of the study. Therefore, we can conclude that the effect of loss to follow-up as a bias did not affect our study outcome. Also, in the breastfed group, more girls (n=16) were recruited than boys (n=8), whereas the ratio was 1:1 in the formula-fed group. This is a disbalance in gender distribution which could lead to a confounding bias. In **Chapter 3**, a homogenous purposive sampling method [3] was employed by compiling a list of Nigerian HCPs involved in managing LP infants. In total, 89 healthcare professionals (HCPs) in Southwest Nigeria were listed and invited to participate in the study. Only 19 of them accepted the invitation and were included in the study. This group of HCPs who attended the interviews were only from Lagos and Ogun states; thus the outcome of the study is not completely representative of the whole nation.

Our randomised controlled trial **(chapters 4 and 5)** was conducted among malnourished toddlers between the ages of 1-3 years. To rule out the possibilities of selection bias, the randomization of study participants to one of the 3 study groups (200 mL, 400 mL or 600 mL) was based on a computer-generated sequence stratifying for gender and age thereby avoiding potential confounders from other factors [4]. Block randomisation was used to assign study participants to study groups of equal size. Children were excluded when they had medically diagnosed allergies, did not have anaemia, were intolerant to milk or milk ingredients, and since children were recruited from a poor environment, study results cannot directly be generalised to children with characteristics different from our inclusion and exclusion criteria. Since selection bias can also occur when participants decide to discontinue the study: during the study, about 60 participants (36%) were lost to follow-up for reasons

such as Christmas period travels, no show and missing data. These numbers were almost equally distributed in all three groups with 20 (200mL), 17 (400mL) and 23 (600mL) groups respectively. Therefore, we do not expect that this loss to follow-up affected our results to a large degree. However, we can conclude that the result of this study, especially the effect of formula on anaemia, can be generalised to anaemic Nigerian toddlers in general.

Measurement bias

In **chapters 2, 4 and 5,** where we measured the effect of treatments on parameters of growth, haemoglobin, micronutrients, cognitive development and gut microbiota outcomes, all research assistants were adequately trained before the commencement of the studies. For measurements such as anthropometrics, digital scales and other equipment for measurement of body weight, head circumference, recumbent length, waist circumference, and MUAC were calibrated daily, and measurements were conducted in triplicate. Haemoglobin was assessed both at baseline and endline using an HemoCue instrument, which has been validated against the international reference method for haemoglobin determination [5]. When Hb results were unusually high, or low or when an error message was shown, a control test was performed to ensure the HemoCue instrument worked properly. If the control test confirmed that the system was working properly, a second measurement was taken from the same child. For cognitive development (**chapter 5)**, an experienced neuro-developmental expert with relevant certification, credentials, and licensure to administer neuro-developmental screening instruments was engaged to train the psychologists who took the measurements. With regard to the cognitive data, the screening version of the Bayley-III tool does not allow to make diagnostic interpretations. Also, it was a 6-month intervention, which may have been too short and is possibly the reason why we did not find any improvement in cognitive development. Besides, the test had quite a high number of dropouts as a lot of children lacked concentration and willingness to complete the test.

With regards to data collection and entry, data were collected on paper, and information from the hard copies of CRFs was entered into the digital CRF system by trained research assistants. All case report forms (CRFs) and questionnaires related to the trials **(chapters 2, 4 and 5)** were pre-tested before they were administered. Any unclarity in the protocol was cleared up with each member of the team. Statistical analysis plans (SAP), which describe the data analysis specifications for each study, were developed and approved by all members of the scientific team.

To prevent missing data, the SOP instructed to enter data into the digital CRF within one week of data collection from the community, which was not realised for some

of the data (**chapters 4 and 5**) and resulted in n=5 (200 mL), n=15 (400 mL) and n=11 (600 mL) missing data for Hb, thereby reducing the sample size that could be analysed. Since it was unknown what treatment effect to expect for the different doses before the study, the power of the study was calculated per protocol analysis when 600 mL of the formula is provided per day, for 6 months. Therefore, the sample size may not have been sufficient to detect treatment effects in the lower dosing groups. However, in **Chapter 4** we found a dose-response effect on anaemia with the intervention resulting in a significantly lower anaemia prevalence compared to the 200 mL intervention ($p = 0.03$). Although we had smaller data set to analyse as a result of loss to follow-up, we believe this bias did not affect the outcome of the primary objective of the study.

Confounding bias

One of the possible confounders in our study described in **chapters 4 and 5** is the lack of a placebo or no milk group. Although it was not originally part of the objective of the study to include a placebo group, its absence hampers to exclude the contribution of seasonal or other factors to the observed effects. The absence of dietary intake data in **chapters 4 and 5** makes it difficult to attribute the improvements observed to the multi-fortified dairy-based drink only. Also, since the intervention products were different in volume and calories, this may have led to the replacement of foods in the background diet, leading to differences between groups. An increase in rainfall can lead to an increase in the amount of standing water, which provides a breeding ground for mosquitoes [6]. Nigeria is a malaria-endemic region with a malaria prevalence of 23% among children less than 5 years old [7]. About 36% of childhood mortality is attributed to malaria [8]. Our study commenced at the peak of rainfall in June 2018, which might have contributed to malaria episodes and the use of antimalarial drugs. According to the NDHS 2018, 52% of children under five received the recommended Artemisinin Combination Therapy (ACT) two weeks before the NDHS survey [7]. Neither our study subjects' morbidity profile nor drug use was monitored, and we did not assess if seasonal malaria played a role in the reduction of anaemia prevalence among these participants. Regarding helminths, all children in our study were dewormed two weeks before study commencement, to reduce any effect of this potential confounder. Previous studies have shown that deworming every six months prevents anaemia among children up to the age of 5 years [9-11]. Therefore it cannot be excluded that the improvement we observed in the prevalence of anaemia at the end of our study in **Chapters 4 and 5** is attributable to other factors than the treatment, such as a change in season, anti-malaria drugs or anti helminths. This is of importance since a season can be related to differences in morbidity, specifically infectious diseases such as malaria and worm infestations, which in their turn can have an impact on anaemia.

Public Health Implications

Observational and qualitative studies: Chapters Two and Three

Following the findings from our study in **Chapter 2**, unfortified breastmilk adequately improved the physical growth of Nigerian moderate-to-late preterm infants. Growth in weight among our formula-fed infants was higher than the reference median weight gain of 10.5 g/kg BW/day. This too-fast increase in body weight has become a common phenomenon among preterm infants fed on the formula [12-14]. The observed high increase in body weight and growth velocity among formula-fed moderate-to-late preterm infants is similar and in line with what is seen in other studies conducted among formula-fed preterm infants [15, 16]. Also, previous studies in Nigeria showed a high weight gain of 26.8–34 g/day, 0.86–0.96 cm/week for length, and 0.48–0.50 cm/week for HC among preterm infants fed on formula in early infancy [17, 18]. Moreover, our study in **Chapter 3** shows that 26% of HCPs interviewed would like LP infants to have a growth velocity of 20-30 g/kg BW/day, which is even higher than the recommendation (15 g/kg BW/day) for preterm infants at GA 32 weeks and can predispose preterm infants to risk of obesity and metabolic problems later in life [19, 20]. Also, overfeeding of LP infants after discharge needs more attention as HCPs pointed out in **Chapter 3** that following the discharge of LP infants, mothers and caregivers are tempted to overfeed their infants for faster weight gain. Medical staff who conduct home visits must educate mothers/caregivers and monitor them more closely to ensure that feeding instructions are strictly adhered to. Healthcare professionals' intention to strive for a higher weight gain can be attributed to the lack of a specific guideline or appropriate growth chart for monitoring of moderate-to-late preterm infants in Nigeria. Higher weight gain above the reference value can be attributed to an imbalance in energy from protein, or overfeeding. Preterm formula with a high amount of non-protein calories (>90 kcal/kg BW/day) has been recognised as a major concern in preventing unnecessary increases in body weight [21].

Our findings show that concentrations of iron and vitamins A and D in breastfed infants were low when compared to formula-fed moderate-late preterm infants [22]. However, the improvement we observed in vitamin A and D status of the formula-fed LP infants could also be attributed to the additional multivitamin supplements received by both breastfed and formula-fed participants. Due to low body stores at birth and maternal deficiencies, preterm infants are usually at high risk of micronutrient deficiencies thus the need for breastmilk fortification or provision of multivitamins to increase the concentration of nutrients to meet requirements [23, 24]. The administration of multivitamin supplements to preterm infants is a common practice. In the United Kingdom, all preterm infants (formula fed and breastfed)

born at <34 weeks gestation receive multivitamins containing vitamins A, B, C and D after full feeds are established [25]. In Australia and New Zealand, most preterm infants with GA less than 37 weeks (in and outpatients) receive around 400 IU/day of vitamin D [26]. The recently published kangaroo mother care (KMC) operational guidelines in Nigeria recommends 400-1000 IU/day of vitamin D to low birth weight infants but not specifically for late preterm infants. In the present study, the provision of supplements containing vitamins A, B, C, and D (200-400 IU/day), and niacin was 'a standard practice' as part of treatment. Some studies have shown that vitamin D supplementation up to the maximum of 400 IU/day supports adequate bone mineralisation in preterm infants. However, our study also shows that in the case of fortified formula feeding, supplementation is likely not needed [27, 28].

Randomised Clinical Study: Chapters four and five

The outcome of our study in **chapters 4 and 5** confirms that multi-nutrient fortified dairy-based drink improves anaemia in malnourished Nigerian toddlers. Our finding aligns with similar studies in Mexico [29], the UK [30], Thailand [31], Indonesia [32], New Zealand, and Australia [33], which all showed major decreases in the prevalence of anaemia with fortified milk fed to growing children. Food fortification and supplementation are considered to be powerful, evidence-based and cost-effective interventions to fight vitamin and mineral deficiencies, including iodine deficiency disorders, anaemia and iron deficiency, either in general or specific populations [34]. There are currently five major food vehicles enlisted in the mandatory large-scale food fortification programme in Nigeria, namely: wheat flour (vitamin A, zinc, folic acid, iron as NaFeEDTA, B Vitamins [35]), edible oil (vitamin A), sugar (vitamin A) and salt (iodine) [36]. Concerning the type of iron source best suited for the fortification of milk, studies have shown that ferrous sulphate based on bioavailability and cost is usually the first choice in the fortification of milk and dairy products [37-41]. Interestingly, the multi-nutrient fortified dairy-based formula used for this study (**chapters 4 and 5**) was fortified with ferrous sulphate. Voluntary or mandatory fortification of milk could be added to this fortification portfolio as a complementary source of nutrients.

While the improvement in weight, height, and BMI z-scores in all groups is a positive finding, it does not necessarily indicate that toddlers are at risk for overweight or obesity in the future. However, it is worth noting that there is some evidence to suggest that excessive consumption of dairy products, including fortified dairy drinks, may be associated with an increased risk of overweight and obesity in children and adults. A systematic review and meta-analysis of 14 randomized controlled trials found that higher dairy intake was associated with modest increases in body weight and body mass index (BMI) in children and adolescents [42]. Another study found

that the consumption of high-fat dairy products was associated with an increased risk of overweight and obesity in a sample of children aged 4-10 years [43]. It is important to consider the potential impact of any dietary intervention on the long-term health outcomes of children, including the risk of overweight and obesity. However, it is not possible to determine the impact of the intervention product on overweight and obesity in isolation of the dietary intake data which is missing in our study. Also, considering the ultra-processing nature of the study product, one might question the public health implications of later obesity and overweight among the study population. Ultra processing foods (UPFs) can be, but not necessarily, energy-dense, high in added sugars, unhealthy fats, and salt, and low in fibre and micronutrients, which can lead to excessive calorie intake and suboptimal nutrient intake. However, ultra processing may also be necessary to guarantee the safety of certain products (e.g., infant and toddler feeding) whereas such products often are in line with nutritional guidelines [44]. However, in the case of this multi-fortified dairy-based drink, the addition of essential micronutrients may counterbalance the potential negative effects of ultra-processing. Long-term negative health effects are not expected because this mainly involves heat treatment to improve food safety.

Affordability is a major determining factor in ensuring food security and adequate nutrition for growing children. In many regions of the world, healthy foods have been reported to be two to five times more expensive than foods that only meet energy needs [45, 46]. Children from poorer households have a higher risk of anaemia compared to children of higher socioeconomic status [47-52]. In Nigeria, approximately 39.1% of Nigerian households are estimated to be living on $1 a day or less [53, 54]. About 75.4% of our study participants belong to the lower class of the demography. Affordability is important since the cost of large volumes of milk formula can be a major barrier among low-income earners. Daily consumption of 600 mL of milk seems unrealistic considering the current average per capita milk consumption being only 10 litres per year [55]. Apart from the behavioural change it would require and the costs for the consumers, also the current limitations of production, processing and functioning of the supply chain for the manufacturers need to be taken into account. Interestingly, the outcome of this study shows that 200 mL of the multi-fortified dairy-based product already improves anaemia from 100% to 47%, which indicates that even the addition of a smaller volume of milk to the diet of toddlers can already make an impact.

Given that animal-source foods, including dairy products, can have significant positive impacts on human health, but also have a larger environmental footprint, the EAT-Lancet report recommends consuming dairy products in moderation as part of a balanced diet [56]. The EAT-Lancet healthy diet reference recommends a range

of 0-500g/day for dairy consumption as part of a 2500 kcal daily energy requirement for generally healthy individuals aged 2 years and older. However, the report acknowledges that young children aged 0-2 years have unique dietary requirements to support rapid growth and development. Young children's diets are important with only minor effects on food systems due to their small population proportion and low absolute food requirements. We, therefore, think that small amounts (200 mL) of fortified milk contribute to better health, and could be a low-cost and effective food-based option for tackling anaemia among children of low-income earners.

Recommendations and future studies

Breastfeeding is the best option for feeding infants. However, following our findings, feeding moderate -late preterm infants with regular preterm infant formulas in Nigeria should be discouraged and faced out. Instead, an age-specific adaptation of available preterm formulas is necessary to ensure balanced growth outcomes among moderate-to-late preterm infants. This type of adaptation can be facilitated by the Federal Ministry of Health, medical societies, academia and preterm formula manufacturers. We believe that when the preterm formula provides adequate protein and energy, protein utilization towards accretion would be promoted, thereby supporting adequate growth in weight, height and head circumference comparable to intra-uterine growth rates [57]. Further studies with a larger sample size of both breastfed and formula-fed infants are still needed to further validate our findings.

To prevent faster weight gain as a result of overfeeding, there is an urgent need for a harmonised (inter)national nutritional guideline on LP infant growth. First, validation studies of existing infant growth charts should be conducted among Nigerian moderate-to-late preterm infants, after which a localised version specifically tailored to Nigerian infants can be designed.

Our findings indicate that concentrations of iron, vitamins A, and D in breastfed infants are lower in comparison to formula-fed moderate-late preterm infants. This suggests that moderate-to-late preterm infants who are exclusively breastfed may require specific nutrient supplementation in addition to breastmilk to promote their development [58]. The current guideline of administering between 400-1000 IU/day of vitamin D to low birth weight infants might be unsafe for moderate-late preterm infants, therefore more studies on the vitamin D status in these infants are needed. Also, the national medical practice of administering this amount of vitamin D and possibly of other supplements to LP infants must also be reviewed to prevent toxicity and overdosing of nutrients.

Our study in **Chapter 4** shows that the adoption of a multi-fortified dairy-based drink among low-income earners in Nigeria could have significant public health benefits, particularly in reducing anaemia. However, for future studies, it is essential to improve on our current methodology and to ensure including a group receiving unfortified formula or a no-milk group, as this will exclude some sources of confounding. Also, the study design should include other missing parameters such as dietary intake, morbidity profile, and list of drugs used during the study. Future studies can also explore the possibility of comparing the fortification of animal-source milk versus plant-based source (soya) replacers to ensure environmental sustainability while achieving healthy diets.

Following the outcome of our studies, we think it is prudent to include the iron fortification of milk and all foods marketed to children with a highly bioavailable iron compound in Nigeria's mandatory food fortification programme. This will positively promote the reduction and prevention of anaemia and iron deficiency among vulnerable target groups such as children.

Additional product development may be needed to certify the type of cost-effective iron source best suits dairy-based food fortification.

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English summary

Undernutrition among children under 5years is still major public health concern in low middle income countries such as Nigeria. Micronutrient fortification of food based vehicles such as dairy products fortified with essential micronutrients of public health relevance can be a cost effective, strategic approach to tackling undernutrition. This thesis described the effect multi-nutrient fortified dairy-based formulas on growth, anaemia, and micronutrient status in Nigerian LP infants and moderately malnourished toddlers.

Chapter 1 of this thesis describes a general introduction into the importance of adequate nutrition in the first 100 days of life, provides an overview of nutritional management of LP infants in Nigeria and specific nutrients to support adequate growth and development of LP infants. Furthermore, it presented a comprehensive nutritional situation of Nigerian children ages 0-5 years followed by an explanation of specific nutrient of importance for toddlers. It also present the aim and objectives of the studies contained in this thesis.

Chapter 2 describes the effect of breastmilk or preterm formula on growth and micronutrient status of Nigerian moderate – LP infants. This study was an open parallel non-randomized observational. The primary objective of this study is to investigate growth and the nutritional status of iron, and vitamins A and D in moderate-to-late Nigerian preterm infants fed with a preterm infant formula (n=17) or unfortified breastmilk (n=24) from 2-10 weeks of age. At the end of the study, unfortified breastmilk sufficiently supported adequate growth in weight (12.8 ± 1.8 g/kg BW/day), length (1.23 ± 0.20 cm/week) and HC (0.78 ± 0.11 cm/week) in moderately-to-late preterm Nigerian infants during the intervention period at age 2-10 weeks. Preterm formula resulted in an average weight gain velocity (14.7 ± 1.53 g/kg BW/day) which was significantly higher than the reference median weight gain of 10.5 g/kg BW/day for infants with a postconceptional age of 35-42 weeks. With regards to micronutrient status, Vitamin D and vitamin A status improved in the formula-fed infants whereas in breastmilk-fed infants, vitamin D status also improved (likely due to additional supplements received by preterm infants) but to a lesser extent as compared to the formula-fed infants. Vitamin A status in breastmilk-fed infants did not change despite the intake of additional multivitamin supplements.

Chapter 3 presented the perspective of Nigerian HCPs on the nutritional management of LP infants in a qualitative study. Healthcare professionals (neonatologists, paediatricians, general practitioners and nurses) who are involved in the care and nutritional management of LP infants in Lagos and Ogun states, Nigeria. The aim of the study was to gain more insight into the current nutritional management of LP infants in Nigeria with specific interest in growth monitoring practices, preferred

feeding options for feeding LP infants and nutritional guidelines used in managing LP infants. Nineteen healthcare professionals were purposively included either in an in-depth interview or focus group discussion using a semi-structured questionnaire. Audio recordings of interviews were transcribed and subjected to thematic analysis using NVivo software. The result of the study showed that that there is no consistency in growth monitoring protocols used in the nutritional management of LP infants in Nigeria. For growth velocity, most HCPs aim for a growth velocity of 15 g/kg BW/day or more during hospitalization. Nigerian HCPs strive to initiate enteral feeding in the first hour of birth in stable LP infants, with breastmilk as the unanimously preferred feeding mode. Many respondents indicated deviating from guidelines based on clinical assessment guided by their expertise, but sometimes also due to the local context. With regards to national guidelines, most HCPs are more inclined to use international guidelines, and mostly those issued by WHO, rather than local or national guidelines.

In **chapter 4,** we provided a multi-nutrient fortified dairy-based drink daily in different amounts (200, 400 and 600 mL, supplying 2.24, 4.48 and 6.72 mg of elemental iron respectively, provided as ferrous sulphate) to anaemic, malnourished Nigerian toddlers (12–36 months of age) for 6 months. The primary aim of the study was to investigate the effect of these different doses of multi-nutrient fortified dairy-based drink (including iron) on the reduction of anaemia prevalence in the target population without stimulating potential pathogenic bacteria in the gut. One hundred and eighty four toddlers ages having mild-moderate anaemia (Hb $\geq$ 7.0 g/dL and $\leq$10.9 g/dL) and mild-moderate acute malnutrition (Height-for-age Z score (HAZ) and/or Weight-for-age Z score (WAZ) <-1 SD and >-3 SD) were recruited into our study. Parameters of anaemia, haemoglobin (Hb) and gut microbiota were studied. After the intervention, anaemia prevalence was reduced in all treatment groups with a significant effect difference between the treatment groups ($p = 0.03$). The intervention with 600 mL resulted in a significantly lower anaemia prevalence compared to the 200 mL intervention ($p = 0.03$). The intervention improved haemoglobin concentration in each group ($p < 0.0005$). With regards to gut microbiota, the study showed that daily consumption of 200–600 mL multi-nutrient fortified dairy-based drink did not cause an increase in the potentially pathogenic gut bacteria.

In **chapter 5**, parameters of growth, micronutrients status and cognition were investigated in the same malnourished toddlers. Our study showed that the intake of 600ml multi-fortified dairy-based drink improved selenium ($p < 0.05$), vitamin A ($p < 0.01$), and vitamin D3 ($p < 0.0001$) status and therefore reduced the percentages of children who were deficient or had a sub-normal status for these micronutrients.

At the end of the study, vitamin A deficiency reduced in the 400ml and 600ml groups from 32.3 to 23.5% and from 40.0 to 16.7%, respectively. The prevalence of selenium deficiency decreased to 6.7%, 2.9% and 6.5% in the 200-, 400-, and 600-ml group respectively, at the end of the study. For vitamin D, these effects were also seen in the 400ml group. The present study suggests that an increasing amount of daily multi-fortified dairy-based drink could be beneficial for linear growth as seen in children consuming 600ml daily were 0.9 cm and 0.67 cm taller than those from the 200 and 400ml groups, respectively. There was no significant difference between the groups in growth parameters, however, a trend towards differences between groups for height ($p = 0.062$) and weight ($p = 0.081$) was observed. With regards to cognition, no differences were seen between interventions on cognitive sub scores of the Bayley-III Screening Test.

Chapter 6 of this thesis summarised all the main findings followed by a reflection of validity of our methodology including possible biases, potential confounders and the generalizability of the study outcomes. This thesis also enumerated public health implications of our studies, and provided suggestions for future research.

In conclusion, we believe that multi-fortified dairy based formula improves anaemia, selenium, Vitamins A and D status in and moderately malnourished Nigerian toddlers. Also unfortified breastmilk is sufficient to support adequate growth in LP infants.

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There are moments when the urge to capture my PhD journey in writing arises, but each time I make an attempt, I find myself overcome with a whirlwind of emotions. My PhD encountered both exhilarating milestones and several minor setbacks. The road to completing my thesis has been a challenging and demanding journey that has exacted a substantial toll. If, in the midst of my overwhelming gratitude, I have unintentionally overlooked acknowledging any of the support I received throughout my PhD journey, I humbly offer my sincerest apologies.

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My parents have continuously prayed for me and provided encouragement. Mom and Dad, you have been my pillars of strength, always believing in me and pushing me to reach for the stars. Your unwavering support and sacrifices have been the driving force behind my academic achievements. From the early years of my education to this very moment, you have been my biggest cheerleaders, inspiring me to pursue knowledge and never settle for mediocrity.

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valuable input, discussions, and critical feedback that significantly enriched the content and quality of this thesis. Your insights and perspectives have challenged me to think more deeply and approach my research from a different angle.

Curriculum Vitae

Adedotun Owolabi was born on May 23, 1983, in Lagos State, Nigeria. He completed his primary education at Adesola Nursery & Primary School and his secondary education at Ebenezer Comprehensive High School, both located in Lagos. In 2004, he gained admission to the Federal University of Technology, Akure, where he pursued a degree in Food Science and Technology. During his undergraduate studies, Adedotun focused on developing a nutritious and healthy snack for diabetic patients using locally sourced root produce, specifically Cocoyam. His project received high praise from the external examiner for its innovative approach and potential for scaling up. Adedotun successfully completed his bachelor's program in 2009. Following his undergraduate studies, Adedotun participated in the compulsory National Youth Service Program (NYSC) from 2009 to 2010.

In 2013, Adedotun was admitted to the Department of Human Nutrition at the University of Ibadan in Oyo State, where he obtained a master's degree in Human Nutrition. For his master's project, he continued his research on nutritious snacks for diabetic patients, this time conducting a laboratory rat assay to evaluate the hypoglycaemic effects of the product on alloxan-induced diabetes in Wistar rats. His work demonstrated promising results.

Due to his outstanding performance and potential, Adedotun was employed as a Nutrition Advisor by FrieslandCampina WAMCO in Nigeria and subsequently promoted to the position of Country Nutrition Manager . In January 2018, he was awarded a PhD scholarship by the organization to pursue his doctoral studies at Wageningen University and Research in the Netherlands. He commenced his PhD journey in November 2018.

At Wageningen University and Research, Adedotun conducted his research within the Global Nutrition Chair, under the Division of Human Nutrition and Health. The primary objective of his thesis was to assess the efficacy of various multi-nutrient fortified dairy-based products in improving the nutritional status of vulnerable populations in Nigeria, with a specific focus on preterm infants and moderately

malnourished toddlers. Throughout his PhD, he actively participated in various training sessions and conferences to enhance his knowledge and skills.

Adedotun's contributions to the field of nutrition have been recognized internationally. In 2015, his abstract presentation at the 3rd World Congress of Public Health Nutrition in Spain earned him five Best Abstract awards out of 870 oral presentations. Furthermore, in 2021, his abstract presentation at the 6th World Congress of Paediatric Gastroenterology, Hepatology, and Nutrition was honoured with a top ten FISPGHAN abstract award. He has also received an excellence award from Nigerian Top Executives in the medicine and Pharmaceuticals industry, recognizing his strong international network, as highlighted in the 2015 publication and rating.

List of Publications

1. **Adedotun J. Owolabi**, Idowu O. Senbanjo, Kazeem A. Oshikoya, Jos Boekhorst,Robyn T. Eijlander,Guus A. M. Kortman, Jeske H. J. Hageman, Folake Samuel, Alida Melse-Boonstra and Anne Schaafsma, Multi-Nutrient Fortified Dairy-Based Drink Reduces Anaemia without Observed Adverse Effects on Gut Microbiota in Anaemic Malnourished Nigerian Toddlers: A Randomised Dose–Response Study. Nutrients, https://www.mdpi.com/2072-6643/13/5/1566 10.3390/nu13051566
2. Idowu O. Senbanjo*, **Adedotun J. Owolabi*,** Kazeem A. Oshikoya, Jeske Hageman, Yetunde Adeniyi, Folake Samuel, Alida Melse-Boonstra and Anne Schaafsma. (2022) Effect of a Fortified Dairy-Based Drink on Micronutrient Status, Growth, and Cognitive Development of Nigerian Toddlers- A Dose-Response Study. Front. Nutr. 9:864856. https://doi.org/10.3389/fnut.2022.864856
3. Rodas-Moya S, Giudici FM, **Adedotun Joshua Owolabi**, Samuel F, Kodish SR, Lachat C, Abreu TC, van het Hof KH, Osendarp S, Brouwer ID, Feskens EJM and Melse-Boonstra A (2023) A generic theory of change-based framework with core indicators for monitoring the effectiveness of large-scale food fortification programs in low- and middle-income countries. Front. Nutr. 10:1163273. doi: https://doi.org/10.3389/fnut.2023.1163273
4. Brai BIC, Afolabi WA, Ariyo O, Oloyede J, Anjorin F, **Owolabi A.** Large scale food fortification in Nigeria: Opportunities and challenges: A position of the Nutrition Society of Nigeria. Niger J Nutr Sci [Internet]. 2022 Nov 11 [cited 2023 Jan 31];43(2):1–8. Available from: https://www.ajol.info/index.php/njns/article/view/236042
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6. Cheang Hon Kit, Chun-Yan Yeung, Irene Cheah, Guslihan Dasa Tjipta, Bugis Mardiana Lubis, Raul Garza-Bulnes, Dagoberto Delgado, Adejumoke Idowu Ayede, Chinyere V.Ezeaka, Mohammad Abdullah Al Mamun, **Adedotun Owolabi,** Anne Schaafsma, Urszula Kudla, Leilani Muhardi, Low Jia Ming, Lee Le Ye. A survey among healthcare professionals from seven countries reported diverse nutritional practices of late preterm infants Acta Paeditrica https://doi.org/10.1111/apa.16344
7. Judith de Vries-ten Have, **Adedotun Owolabi**, Jan Steijns, Urszula Kudla and Alida Melse-Boonstra Protein intake adequacy among Nigerian infants, children, adolescents and women and protein quality of commonly consumed

foods. Nutrition Research Reviews, 1-19. https://doi.org/10.1017/S0954422419000222

8. Olayinka A. Oridupa, Oluyemisi F. Folasire, **Adedotun J. Owolabi**, Oluwasanmi Aina. Effect of Traditional Treatment of Diabetes Mellitus with Xanthosoma sagittifolium on the Male Reproductive System of Alloxan-Induced Diabetic Wistar Rats. DOI http://dx.doi.org/10.1055/s-0043-103575. Drug Res 2017; 67: 1–5 © Georg Thieme Verlag KG Stuttgart · New York. ISSN 2194-9379
9. Oluyemisi Folake FOLASIRE, Olayinka Ayotunde ORIDUPA, **Adedotun Joshua OWOLABI** and Oladejo Thomas ADEPOJU. Anti-hyperglycemic effect of cocoyam (Xanthosoma sagittifollium) corm in alloxan-induced diabetic albino rats. Vol. 8(4), pp. 24-29, July 2016. DOI: 10.5897/IJNAM2016.0200 Article Number: 2E14C4659996 ISSN 2141-2332 Copyright © 2016. http://dx.doi.org/10.1055/s-0043-103575
10. Olayinka A. Oridupa, Oluyemisi F. Folasire, **Adedotun Owolabi**. Evaluation of the sub-chronic toxicity profile of the corm of Xanthosoma sagittifolium on haematology and biochemistry of alloxan-induced diabetic Wistar rats. Journal of Complementary and Integrative Medicine. 2017; 20160072. DOI: 10.1515/jcim-2016-0072 Received: July 19, 2016; Accepted: January 12, 2017

Manuscripts under review

11. **Adedotun Joshua Owolabi**, Idowu Adejumoke Ayede, Olugbenga Oyewumi Akinrinoye, Adegoke Gbadegesin Falade, Gboyega Bosun Ajibola, Ologunore Olufisayo Christopher, Gregory Olawole Arifalo, Ayodele Oladejo Abiona, Edith J.M. Feskens, Alida Melse-Boonstra AS. Growth and Micronutrient status of Nigerian preterm infants consuming preterm formula or breastmilk. Pediatr Res. 2023;
12. **Adedotun Joshua Owolabi,** Folake Samuel, Anne Schaafsma, Edith J. M. Feskens, Alida Melse-Boonstra. Insight into feeding practices and guidelines used for the management of late preterm infants among healthcare professionals in Nigeria

Overview of completed training activities

A: Discipline specific activities		
Name	**Organizer and location**	**Year**
Global Nutrition Development meeting	FrieslandCampina, Netherlands	2018
Nutrition Community Days	FrieslandCampina, Netherlands	2019
Abuja 2019, NSN Conference	Nutrition Society of Nigeria	2019
International Master Class on healthy and Sustainable diets: Synergies and trade-offs	VLAG, Netherlands	2019
Nutrition Community Days	FrieslandCampina, Netherlands	2020
Nutrition Society of Nigeria Conference	NSN Conference, Nigeria	2020
3rd Global Conference Sustainable Food System	Virtual	2020
Independent Food System dialogue	Choices International, Nigeria	2021
Nigeria Food Systems National Fortification Dialogue)	Global Alliance for Improved Nutrition, Nigeria	2021
Nutrition and Growth Conference	N & G 2021, Virtual	2021
6th World Congress of Paediatric Gastroenterology, Hepatology and Nutrition	WCPGHAN 2021, Virtual	2021
Risk Assessment of Regulated Products (Parma summer School)	European Food Safety Authority, Universita DI Parma	2022
The problem with the formula milk industry-should health professional associations refuse industry funding	World Health Organization, Virtual	2022
B: General courses	**Organizer and location**	**Year**
Research Data Management (1,2,3)	Wageningen Graduate School, Netherlands	2018
Interpersonal Communication for PhD Students	Wageningen Graduate School, Netherlands	2018
Ethics for social sciences Research	Wageningen Graduate School, Netherlands	2018
Stakeholder Management Course	FrieslandCampina, Netherlands	2020
Project Management for Project managers	FrieslandCampina, Netherlands	2020
Presentation Skills for R & D - Effective presentation	FrieslandCampina, Netherlands	2020
A systemic approach to financial inclusion	The World Bank online learning platform– CGAP	2021
Effective Listening	LinkedIn	2021
WASH in Emergency	The International Federation of Red Cross and Red Crescent Societies	2021
Project/programme planning	The International Federation of Red Cross and Red Crescent Societies	2021
Project Management Foundations	Project Management Institute/LinkedIn	2022
Advanced Statistics for Nutritionists	Wageningen, Netherlands	2020
Food System for Healthier diets Masterclass	A4NH/IITA/WUR, Nigeria	2021
C: Other activities	**Organizing institute**	**Year**
Preparation of research proposal	Human Nutrition, WUR	2018
PhD study tour	Human Nutrition, WUR (Switzerland)	2022

Colophon

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