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The effects of regular consumption of a multiple micronutrient fortified milk beverage on the micronutrient status of school children and on their mental and physical performance

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Abstract

Multiple micronutrient deficiencies exist in school going children in India and bridging the gap between nutrient intake and requirements is an effective way to combat the deficiencies. This study aimed to test the effect of a multi-micronutrient fortified malt and cocoa based milk beverage on the micronutrient status, cognition, physical performance and nutritional deficiencies of 7-10 years old south Indian children. A randomized, double blind placebo controlled study design was used with normal healthy children from low to middle income families, aged 7-10 years randomly assigned to receive either a multi-micronutrient fortified or an unfortified milk based control drink. The drinks were provided 6 days/week for 5 months. Assessments included anthropometry, blood biochemistry, physical performance and cognition at baseline and endline.The baseline characteristics of the study groups were similar. The changes in body weight and height were similar between the groups at the end of the study. Levels of vitamin B_{12}, red cell folate and vitamin B_2 significantly improved in the intervention group, while vitamin D, selenium and body iron showed no difference. The Hemoglobin (Hb) and serum ferritin levels of the control group decreased at endline, while those in the intervention group maintained their levels. The serum transferrin receptor levels increased in

both the groups. The prevalence of iron deficiency and Vitamin B_2 deficiency were significantly lower in the intervention group at endline. Overall improvement in cognitive and physical performance was seen in both the groups at endline, with no significant differences between the groups. The micronutrient fortified milk based drink was efficacious in improving the micronutrient status of Vitamin B_2, Vitamin B_{12} and red cell folate and in preventing a decline in Hb level compared to an unfortified milk based drink. It also reduced anemia and the risk of deficiencies of iron, and B_{12}, in apparently healthy children.

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Introduction

Micronutrient (MN) deficiencies are often observed in young school children of the developing world, where the quantity and quality of dietary intakes are compromised. Multiple, concurrent deficiencies can exist in these populations since the causative dietary factors are the same for deficiencies of a number of micronutrients (1). Additionally, the predominantly cereal-based diets commonly consumed are rich in phytate, low in animal products and absorption enhancers (2). The common micronutrient deficiencies prevalent in low income countries are iron, vitamin A, iodine, B vitamins and zinc; the sub-optimal supply of the MN's could affect the children's optimal growth, as well as their functional and neurobehavioral development (3).

Iron deficiency anaemia (IDA) is a major public health problem in India. The third National Family Health Survey (4,5) found the prevalence of anaemia among children below 3 years of age to be 78%, while the prevalence of IDA in school-aged children was 64–68% (6,7). A recent study reported the prevalence of IDA in south Indian children aged 6-12 years to be 19% (8). The observed prevalence of blood biochemical deficiency in school aged children for other micronutrients were folic acid (99%), Vitamin A (44%), B1 (12%), B2 (66%), B6 (66%), B12 (67%) and Vitamin C (60%) (9).The Indian National Nutrition Monitoring Bureau (NNMB) found the diets of children aged 7-9 years to be deficient in vitamin A (33% RDA), riboflavin (33% RDA), vitamin C (73% RDA) and folic acid (58% RDA) (10). The variation in the levels of micronutrient intake and deficiency across India could be due to various existing state-led and locally led intervention and fortification programs.

Bridging the gap between nutrient intake and requirements is an effective strategy to combat MN deficiencies and their impact on growth, cognition and physical performance. For example, memory and/or attention span improved in young south Indian children provided with

multiple micronutrient food supplements (11,12). Physical performance outcomes of whole body endurance, aerobic capacity, speed, muscle strength, endurance capacity, forearm endurance and visual reaction time improved with multiple micronutrients fortification in Indian children aged between 6-15 years (13,14), while in rural adolescent girls it was observed that normal/adequate haemoglobin levels were positively related to endurance capacity(15).

The aim of the present study was primarily to test the effect of a multi micronutrient fortified malt and cocoa based milk beverage as compared to an energy matched non fortified milk based drink over a 5 months period, on the micronutrient status of children aged 7-10 years. Secondarily, the study aimed to assess the effect of the multi micronutrient fortified malt and cocoa based milk beverage on cognition, physical performance and nutritional deficiencies.

Materials and Methods

The present study was conducted among school children aged 7-10 years, attending St. Joseph's Convent School, Kolar, Karnataka, India. The parents of these children were agricultural workers, carpenters, domestic workers, or labourers and therefore were in the low to middle socio-economic bracket. Normal healthy children in the age range of 7-10 years were eligible for the study. The exclusion criteria included girls who had attained menarche, presence of severe anaemia (Hb < 8 g/dl), moderately/severely undernourished children (BMI for age z-score <-2SD, WHO) (16) organ failure (as assessed by medical history), physical disability, recent history of serious infections, food allergies or intolerance, children consuming nutritional supplements and or health food drinks, participating in any nutritional study in the previous year and child's family likely to

move out of the study area within the period of study intervention. The study protocol was approved by the Ethical Committee of St. John's Medical College, Bangalore, India. The details of the study were explained to parents with the help of teachers, either in English, Kannada or Tamil and informed written consent was obtained from the parents of the children along with oral assent from the children. The children who participated in the present study were not part of any State fortification program.

Study design

The study was a randomized, double blind, placebo-controlled study with two parallel groups (Control and Intervention group). Eligible children (n=227) were randomly allocated into one of the two groups to receive either a multi micronutrient fortified drink, or a similar energy matched placebo unfortified drink. A block randomisation sequence with block size of 20 was generated such that the eligible children were randomized equally to the study groups within each block. The randomization sequence was generated by GraphPad program (http://www.graphpad.com/quickcalcs/randomize1.cfm)

At baseline before the start of the intervention, all the selected children were subjected to measurements of anthropometry, blood biochemistry, physical performance and cognitive assessments. These measurements were conducted by the study staff members and they were trained on the relevant study methods and testing procedures.

Test drink preparation and administration

The products for both groups were provided in color coded individual-serve sachets each of which was mixed with 200 ml of un-fortified double toned milk, and poured into color coded plastic glass before serving to the children. The drink was served twice daily, once in the morning (between 9.30 -10.30am) and again in the afternoon (between 2.30-3.30 pm), 6 days a

week for a period of 5 months. The children consumed their drink under supervision and the quantity of the leftover drink, if any from each child was noted by the research staff using a standard measuring glass to assess the level of compliance. The participant child who consumed the drink first in the morning was provided the drink first in the afternoon, to ensure that the time interval was constant between drinks.The nutrient composition of the micronutrient fortified beverage and placebo is provided in Table 1. The placebo product was made similar to fortified product (malt and chocolate beverage base) but was not fortified with micro nutrients.

The fortified and placebo formulae were coded using different color markers (blue and green). These color codes were used throughout the manufacturing chain (product manufacturing site, product quality evaluation, sensory clearance) and were maintained till the end of the study to maintain blinding. Details of the code were only known to the product developer, who was not directly involved in the study. The addition of 400 ml of milk per day to both drinks provided an additional 268 kcals, 12.8 g proteins, 16.4g fats and 17.6 g carbohydrates.

Anthropometry

Body height was measured using a portable stadio-meter (built in-house and calibrated against a standard length measure), with the subject standing bare-foot, to the nearest 0.1cm. Weight was measured in minimal clothing using a calibrated portable digital weighing scale (Salter, India) to the nearest 0.1 kg. The mid-upper arm circumference (MUAC) was measured in all the subjects using standard procedures (17) in duplicates, to the nearest 0.1 cm. All study personnel received appropriate training for conducting anthropometric measurements.

Blood Biochemistry

Collection of blood specimens were carried out by trained phlebotomist to avoid participant discomfort and to ensure the quality of the sample. The serum and plasma samples were separated

within 4 hours of collection, aliquoted in vials and stored at -80° C until analysis. None of the blood samples were hemolyzed. The time gap between the blood collection and serum separation was a maximum of 4 hours for all the samples. Biochemical measurements were carried out at baseline and endline for hemoglobin (Hb), Serum Ferritin (SF), Soluble Transferrin Receptor (sTfR), C-reactive protein (CRP), Vitamin B2, Vitamin B12, Vitamin D, red cell folate and Serum Selenium. Hemoglobin concentrations were measured using ABX Pentra 60 C+ hematology analyzer (Horiba group, France). SF, Vitamin B12, Vitamin D, and red cell folate were measured using electro-chemiluminescence technology (Elecsys 2010, Roche Diagnostics). CRP and sTfR were measured using immunoturbidometry (Hitachi-902, Roche Diagnostics). Vitamin B2 was measured by the Erythrocyte Glutathione Reductase Activity Coefficient (EGRAC) method using UV-VIS 1800 spectrophotometer by the modified ascorbic acid methodology (18)**.** All biochemical analyses except for selenium were carried out at the Micronutrient laboratory, Division of Nutrition, St John's Research Institute, Bangalore. The coefficient of variation for the assays were 3% for Vitamin D, 2.5% for vitamin B_{12}, 3% for folate, 1.5% for CRP, 1.5%, 3.8% for SF, 1.6% for sTfR, 0.6% for Hb and 2.6% for vitamin B_2. Selenium analysis was conducted using ICP Mass Spectrometry at Bureau Veritas Consumer Product Services (India) Pvt. Ltd, Bangalore. Body iron was calculated (19) as

Body Iron (mg/kg) = [log (R/F ratio) - 2.8229]/0.1207

Where R/F ratio = serum transferrin receptor/serum ferritin.

Dietary Assessments

Dietary intake of each child was assessed using a 3-day dietary recall method and was collected at the beginning of the study and included two weekdays and a weekend day. It was performed by interviewing the children along with their parent using standard weights and measures to aid in

quantifying the portion size of the food items consumed. Energy and nutrient intake was computed using a nutrient database for Indian foods (20) on the dietary information obtained from 225 children.

Assessment of morbidity

The incidence of morbidity was assessed weekly using a questionnaire administered to the children, recording the occurrence and duration of common illnesses such as diarrhea, vomiting, fever, runny nose, visit to physician and details of any hospitalization. There was a physician always on site to monitor the children.

Physical Performance

Physical endurance was measured in the children using the shuttle test, which is reliable, valid, non-invasive and requires limited facilities (21). The Illinois agility test is a fitness test which is easy to administer and requires little equipment. It tests the ability of the child to turn in different directions and at different angles (22). In the present study, the test was carried out in the school grounds, where the required length and width was marked using plastic cones. The test was standardized in terms of translating the instructions into the local language, and ensuring that this was understood, so that the test was performed correctly. All the physical performance tests were first demonstrated to the study children in order to ensure that the study children understood the needs of the tests. Practice tests were conducted on the participants. The physical performance tests were conducted at baseline and endline (once in the morning and once in the evening). The evening measurement was taken on the following day to avoid fatigue effects from the morning test.

Cognitive Assessments

All randomized children were administered a battery of cognitive tests (both at baseline and endline) that assessed the broad domains of attention (mental alertness), short term memory and executive

functions (working memory, planning, cognitive flexibility, creativity). The cognitive assessments were performed once in the morning between 9.00-11.00 am and in the evening between 3:00 -5:00 pm to assess the changes in cognitive abilities during the course of the day, both at baseline and endline. The tests used included the color cancellation test (CCT) which is a measure of visual scanning and sustained attention, the color trails test (CTT), which is a measure of focused attention and conceptual tracking (23,24). Short term memory was assessed using the word order test (25,26), while Porteus maze test (27) was used to measure planning, motivation and adaptability (i.e, choosing, trying, rejecting, and adopting alternative courses of conduct or thought). The Visual Analogue Scale (VAS) developed for the present study assessed mood states (represented by smiley faces) of the children before and after performance on cognitive tests. Three dimensions of mood states were assessed: Happy/Sad, Relaxed/Worried, and Alert/Tired. The cognitive assessments were carried out by trained psychologists and each child worked with the same psychologist at baseline and endline. Ten trained psychologists conducted the cognitive assessments. Intra and inter-rate reliability was assessed periodically to minimize test takerss bias and ranged from 90 - 95%. A supervisor monitored the psychologists regularly to maintain the quality of data.

Statistical analysis

Descriptive statistics were used to examine the distributions of all variables. A sample size of 107 children per arm (accounting for 20% drop outs) was sufficient to observe a difference of 0.1 in standardized short term memory at 5% level of significance (12). Continuous variables that were not normally distributed were log transformed. Baseline characteristics were compared between groups using independent t test, chi-square tests and Fisher's exact test. All efficacy analyses were carried out using "Intention to Treat" (ITT) principle where all subjects

randomized into the study were considered for statistical analyses. For the three subjects who dropped out at endline, the baseline observation was carried forward for anthropometric and biochemical parameters. Anthropometric and biochemical parameters were analyzed using analysis of covariance (ANCOVA) to assess the effect of intervention at endline, adjusting for baseline measurements as a covariate. For the cognition and physical performance parameters, mixed model analysis was performed considering study groups, baseline/ end-line and morning/evening status as factors where the last two were repeated factors. Within each treatment group, biochemical and anthropometric status, as well as cognitive and physical performances were compared between baseline and endpoint, using paired t tests. Prevalence of deficiencies between groups at endpoint were compared using Chi-square test. McNemar Chi square test was used to compare the prevalence of deficiencies between baseline and endpoint within each group. The changes in deficiency status between the groups were examined after controlling for baseline deficiency status using logistic regression. Statistical analysis of serum ferritin was carried out only for children with CRP<10 mg/L. An alpha level of less than 0.05 was considered statistically significant.All analyses were carried out using SPSS version 17 (SPSS Inc, Chicago, USA). An external monitor, not involved in the study, visited the study site regularly to ensure that the study was being carried out as per the recommendations of Good Clinical Practice (GCP) and the protocol.

Results

Of the 227 children who were enrolled, 224 (98.7%) completed the study (Figure 1).The mean age of the children at the time of enrollment in the study was 8.2 ± 0.9 y, and 77% of them were girls **(**Table 2).Forty nine percent of the parents (head of the family) who responded to the

demographic questionnaires, had completed high school education or below, while 36 % and 9% had completed pre-university and graduate degrees respectively. The monthly family income and educational status were similar between the two groups. Education status, occupation and monthly income were considered as surrogates for socio-economic status. Since all children consumed the drink with no leftovers, the compliance was estimated based on the number of days they were present in the school during the study period. Taking into account the absenteeism, the overall compliance (days of intervention as a percentage of total days of planned intervention) was comparable between the study groups, which was 85% for both groups. The dietary intake (before supplementation) assessed by the 3 day 24 dietary recall method (2 weekdays & 1 weekend) of the children, was comparable between the study groups. The mean daily energy, protein, carbohydrate, fat and iron intake was 1073 ± 290 kcal, 34±9 g, 155± 42 g, 35 ± 12 g and 5.4 ± 2.0 mg in the control group and 1095 ± 325 kcal, 34 ± 10. g, 160± 47 g, 36 ± 12 g and 5.3 ± 1.7 mg in the intervention group. The energy and micronutrient intake in children of both study groups were below the recommended dietary allowance (28).

Anthropometry

The baseline anthropometric characteristics of the children were comparable between the study groups (Table 2). Twenty-five children (11.1%) demonstrated stunting with a height-for-age Z-score of less than –2 SD from the WHO child growth standards 2006 (11 in the intervention group and 14 in the control group) and twenty three subjects (10.2%) were wasted, with weight-for-age Z-scores of less than –2 SD (11 in the intervention group and 12 in the control group). At the end of the study, the changes in the body weight and MUAC were similar with an average increase of , 3kg in weight and 1.5 cm in MUAC for both the groups. The mean change in height of children in the control group (3.7± 0.8 cm) and the intervention group (4.0 ± 0.9 cm), at the end of

the study showed a trend toward significance, with a better effect observed in the intervention group (p=0.07).

Blood Biochemistry

The baseline biochemical characteristics of the children were comparable between the study groups (Table 3). The concentrations of hemoglobin, serum ferritin, and soluble transferrin receptor were significantly different between the groups at the end of the study period, which was the result of a decrease in levels of hemoglobin, serum ferritin and soluble transferrin receptor levels in the control group. In paired analysis, hemoglobin and serum ferritin levels significantly decreased over the study period in control group subjects (P<0.001) while in the intervention group subjects, they were comparable to baseline values. At the end of the intervention, levels of Vitamin B_{12}, RCF and Vitamin B_2 were significantly different (P<0.001) between the study groups, in favor of the intervention group. Vitamin D, Selenium levels and body iron did not differ between the two groups at endline. However in the paired analysis, there was a significant decline in total Vitamin D levels for the intervention group (p<0.01) and for body iron in the control group (p=0.005) (Table 3).

The prevalence of deficiencies was similar in both the groups at baseline. At the end of the study, the prevalence of iron deficiency (ID) and IDA were significantly lower in the intervention group as compared to the control group (ID 9.0% vs. 21.1%; IDA 2.7% vs. 9.6%:p<0.05). Prevalence of Vitamin B2 deficiency was also significantly lower in the intervention group as compared to the control group (p<0.001). After adjusting for baseline prevalence, the statistical significance remained the same for all deficiency analysis except for IDA (p= 0.12). There was no significant difference in the prevalence of anemia, Vitamin D, Vitamin B12 and selenium deficiencies between the study groups (Table 4).

Cognition and Physical Performance

For cognition and physical performance, since there was an additional 'assessment time' factor, the effect of intervention was examined using main effect of study group and its interaction with time and study status. Baseline to endline improvement was examined using the main effect of study status, and effect of assessment time (morning/evening) was examined using main effect of time. At baseline, subjects in both the study groups were comparable for both time points for all the cognitive measures using mixed model analysis. However the evening performance was relatively better than that of the morning in both the study groups, this could be partly attributed to practice effect. This effect was similar at endline as well. The endline cognitive performance was significantly greater than that at baseline for both the study groups. There was no significant interaction effect between the study group and time/status. In the paired analyses of morning and evening cognitive measures, there was a significant improvement from baseline to endline for most of the cognitive measures (($p<0.01$), except for Trial A – no of correct responses of evening assessment in control group and Trial B – no of correct responses of evening assessment in both groups (Table 5).

At the end of the study, there was an improvement in the physical endurance and agility in both the groups with no significant interaction between the study groups with time and status (Table 6).

There were no serious adverse events observed that were related to the study product. The occurrence of common morbidity symptoms between the two groups were comparable throughout the intervention period.

Discussion

This study was designed to test the efficacy of a micronutrient fortified milk based drink versus a non-fortified milk drink given twice daily for a period of 5 months, in improving micronutrient status and reducing the prevalence of micronutrient deficiencies, as well as in

316 improving cognitive and physical performance in apparently healthy school children. Normal,
317 otherwise healthy children were recruited for the study since we wanted to study the efficacy of
318 the micronutrient fortified drink in the context of real life, where no obvious MN deficiencies
319 were present. In addition, the majority of the children were not anemic, with higher than
320 expected Hb levels; this may have been due to a secular trend of improving iron status in
321 Bangalore, South India due to well-run school programs of supplementation (29). The children
322 who participated in the present study did not receive any supplementation for at least a year
323 before the start of the study. The micronutrient fortified drink had a beneficial effect on the
324 status of Vitamin B_{12}, Vitamin B_2 and red cell folate levels. The intervention group children
325 were able to maintain their Hb levels during the study period and showed a slight though not
326 statistically significant improvement in their serum ferritin levels at the end of the study in
327 comparison to their baseline values, while in the control group Hb and serum ferritin levels
328 showed a significant decline. There was however no improvement in measured Vitamin D levels
329 in the children of the intervention group. A possible reason could be the fact that we analyzed
330 total vitamin D using the chemiluminescence method, which measures total 25 hydroxy Vitamin
331 D and does not partition to vitamin D2 and D3 levels specifically. The intervention product
332 contained Vitamin D2, and thus measuring D2 may have shown better results. It is also possible
333 that these rural children had adequate sunlight exposure since they spent more time playing
334 outside. Additionally the prevalence of Vitamin D deficiency was low (4.9%) at baseline.
335 Children in both groups had improvements in anthropometric measures, cognitive and physical
336 performance, with no significant differences observed between the groups at the end of the
337 intervention period. The increase in height of the children at the end of the study was similar to
338 another study in Indian children aged 6-12 years (30). Although the dietary intake in the present

study was measured only once, it is less likely to have changed during the intervention period of 6 months in this group of children from low to middle income families. The lack of statistical significance in the change in height of children between the two groups at the end of the study could be attributed to relatively short study duration

Deficiencies of micronutrients are emerging as the major bottleneck to optimal mental development and physical growth. Deficiencies of thiamine, folate and vitamin B_{12} affect episodic memory and language ability while iron deficiency affects memory and impairs learning, causing low cognitive scores and development in school age children (31). Moderate and severe iron deficiency anemia also adversely affects motor development and physical performance in Indian children (32), and Hb concentration was positively associated with cardio-respiratory and muscular fitness in adolescents (33). Impaired psychomotor performance in school children was shown to be associated with low riboflavin levels (33,34). Decreases in physical performance measures of aerobic power, onset of blood lactate accumulation, and oxygen consumption have been associated with low intakes of thiamine, riboflavin and vitamin B_6 (35).

From an interventional viewpoint, beneficial effects of micronutrient intervention on levels of iron, zinc, vitamins B_1, B_2, B_6, B_{12}, folate and vitamins A and C (,36, 37, 38, 39,30, 13) have been reported in healthy children. Studies exploring effects of micronutrients on cognitive function have shown improved short-term memory (12) and those focusing on physical performance demonstrated improvements in aerobic capacity and physical endurance (30, 13). A meta-analysis of trials that assessed micronutrient supplementation and cognitive outcomes showed that micronutrients significantly improved immediate free recall memory, but not other

362 cognitive measures like delayed free recall memory or verbal fluency (40), while a recent review
363 highlighted the positive effects of multiple micronutrient interventions on fluid intelligence and
364 academic performance but found no significant effects on crystallized intelligence in healthy
365 school children (41). Based on these outcomes, the provision of multiple micronutrient fortified
366 beverage is an attractive option, since it also has shown to be effective in Indian school children
367 aged 6-12 years, when provided for 8 weeks, to improve micronutrient status and decrease the
368 prevalence of iron deficiency, IDA, vitamin C and B_{12} deficiency among children with low iron
369 stores (8).

370 The fortified intervention product of the present study did not show improvement on the
371 mean Hb levels of the children in the intervention group, whereas the Hb level of control group
372 decreased. This could have been due to several reasons a) the children included into the study
373 were normal healthy children with moderate to good Hb levels, who may not have responded to
374 the intervention and b) the iron fortificant used in this intervention product was ferric
375 pyrophosphate (FEPP), which is known to have a relatively low bio-availability, and may not
376 have been adequate to overcome the inhibitory nature of the food vehicle (whole milk) for iron
377 absorption used in this study. However, even though the supplemented iron was not enough to
378 increase the Hb levels in the intervention group, it was adequate to maintain their Hb levels,
379 allow for significant growth, and to decrease the risk of iron deficiency (though not statistically
380 significant). These are important considerations in terms of the additional benefit of
381 supplementing with an iron fortified drink versus milk only and have been observed elsewhere in
382 younger children (42).

383 Improvements in the functional measurements of physical and cognition performance and
384 growth were observed in both the groups. This could have been primarily due to the relatively

385 substantial amount of 400 ml milk per day that was offered to all children who participated in the
386 study. Milk by itself provided them with good quality protein and energy. Previous studies have
387 demonstrated that milk has positive effects on linear growth of children from low income
388 countries and also in situations where the nutrient intake is adequate (43,44). Since the aim of
389 the study was to assess the effect of a multiple micronutrient fortified beverage powder provided
390 in milk, it was decided to retain milk as the vehicle for both groups in that it provided for a more
391 realistic study setting as parents in India where possible give milk as a drink to their children. It
392 is entirely possible that the differences and trends of the present study could have been amplified
393 if the placebo drink did not contain milk but this would also have resulted in a less than
394 satisfactory double blind placebo. Since there was some influence on linear growth in the present
395 study, it is possible that the children in both the groups used the substantial intake of calcium
396 (480mg/day) and protein from the milk provided and utilized it for growth. Another reason for
397 the sub-significant trends observed could be that the intervention period of 5 months was
398 potentially not sufficient to show significant differences between the groups.

399 There have been very few studies on selenium status in Indian populations, particularly in
400 children and as selenium has many important functions it was considered a useful nutrient to
401 prioritise for investigation (45). The serum selenium levels of the children were not different
402 between the two groups at endline. The RDA for selenium in children is about 30 mcg/day (46).
403 The children in the intervention group received about 27.4 mcg/day (12.6 mcg from the product
404 and 14.8 mcg from milk), while those in the placebo received about 14.8 mcg (from milk). The
405 dietary intake data assessed by the 3 day dietary recall method suggested selenium intake of 35.9
406 ± 14.4 mcg in the intervention group and 37.4 ± 14.1 mcg in the placebo group. Additionally,
407 the serum selenium levels of children from both study groups at both time points were similar to

408 those obtained in healthy individuals in Tehran from other countries **(**47). Thus it is possible that
409 since the dietary intake and serum levels of children participating in the study were normal, the
410 additional 12.6 mcg/day from the product did not result in any difference in the selenium levels
411 between the study groups at endline.

412 One of the novel aspect of the present study was to investigate, within the framework of
413 an efficacy trial, both the short term (morning vs evening) and long effects (baseline vs endline)
414 of multi micronutrient fortification in physical performance and cognition. Within a day, it is
415 clear that the cognitive performance of children can vary. Other functional indexes, such as
416 physical performance could also vary, and in that sense, providing nutrients at different
417 timepoints for the purpose of evaluating functional outcomes is novel. Most cognitive studies
418 have been performed to assess acute effects: however, these acute effects could also wear off
419 over time. Our study design permitted this to be evaluated as well. This has not been done
420 before and the authors felt that these results will help in understanding the short and long term
421 benefits of micronutrient fortification on physical performance and cognition.

422 The study has some limitations. Due to ethical consideration, only normal, healthy
423 children were recruited in the study, which was a slight disadvantage as the children had normal
424 Hb levels and no obvious MN deficiencies. It is highly likely that more evident and promising
425 results for the supplemented group as against the placebo-control group would have been
426 observed if undernourished children with the presence of clear iron deficiency anemia were
427 recruited. Milk, which was the vehicle used for the study could have had some inhibitory effects,
428 In addition, there is the possibility that the similar amount of milk provided to both groups, could
429 have masked some of the effects of the intervention product. However, the aim was to test for
430 the efficacy of the fortified intervention in a realistic study setting and as milk is commonly

provided to children in India it represents a realistic placebo. Thus we preferred to use milk rather than water in the present study. It is also possible that due to the short duration of the study, significant changes in the height of the children were not observed**.**

In conclusion, this study shows that the micronutrient fortified milk based drink is efficacious in improving the micronutrient status of Vitamin B_2, Vitamin B_{12} and red cell folate. It also was efficacious in preventing a decline in Hb & Serum ferritin with normal diets, and reduces the risk of deficiencies of certain micronutrients (iron, anemia, B12), in apparently healthy children from lower to middle socio-economic groups.

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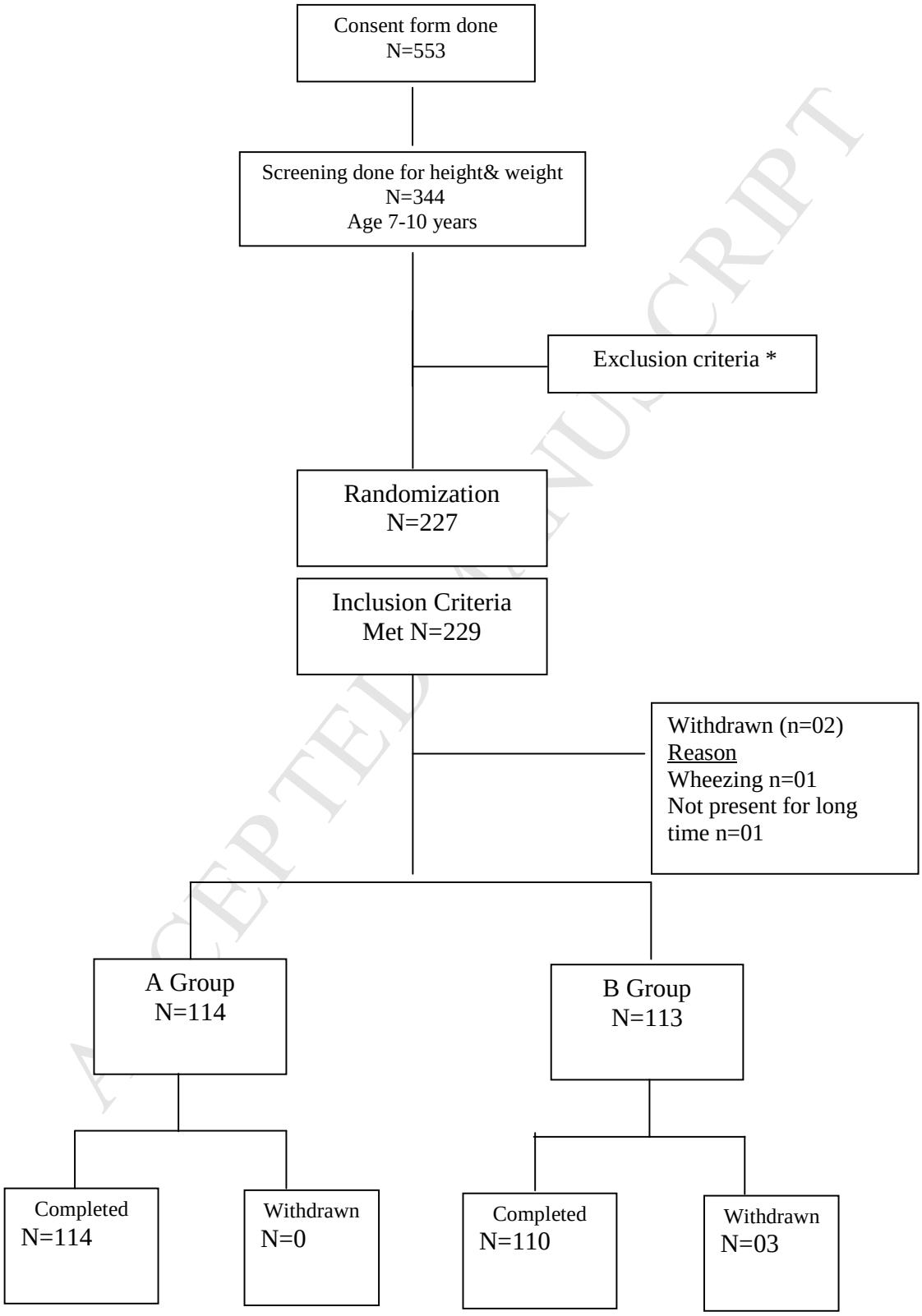
Statement of Authors Contribution to Manuscript

All authors have read, provided input on the manuscript content and approved the final manuscript. Rebecca Kuriyan was involved in the planning of the study, interpretation of results and writing of the manuscript. Prashanth Thankachan was involved in the study design, protocol development and conduct of the study. Sumithra Selvam was involved in the data analysis and interpretation. Maria Pauline was involved in the planning and conduct of the study. K Srinivasan helped plan and interpret the cognitive assessments. Shilpa Kamath-Jha was involved in study planning monitoring, process management, results review and discussion. Situn Misra was involved in study product formulation, planning, monitoring, results review and discussion. Sophie Vinoy was involved in results review and discussion. Yvonne Finnegan was involved in study design, protocol development, study planning and monitoring

Figure 1: Disposition of subjects



Reason
Taking medications n=01(Before Intervention)
Left School n=01 (Before Intervention)
Not present for long time n=01

*Exclusion criteria

Hb < 8 g/dl ; Moderately/severely undernourished children (BMI for age z-score <-2SD, WHO) (15); Organ failure (assessed by medical history), physical disability; Recent history of serious infections, Food allergies or intolerance ; Children consuming nutritional supplements and or health food drinks Participating in any nutritional study in the previous year and child's family likely to move out of the study area within the period of study intervention

Table1 .Nutrient composition of the multiple micronutrient fortified beverage

Nutrients	Units	FAO/WHO 2004 RNI 7-9y IOM 2006 RDA/AI 4-8y Reference Table*	Cow's milk per 200ml*	Per 2 serves intervention product	Per 2 serves intervention product with milk	Per 2 serves placebo product (analytical values)	Per 2 serves placebo product with milk
Vitamin A	mcg	500	90	300	480	-	180
Vitamin C	mg	35	0	54	54	-	0
Vitamin D	mcg	5	0.2	2.5	2.9	-	0.4
Thiamin	mg	0.9	0.1	0.54	0.74	-	0.2
Riboflavin	mg	0.9	0.34	0.54	1.22	-	0.68
Niacin	mg	12	0.16	7.2	7.52	-	0.32
Vitamin B6	mg	1	0.08	1	1.16	-	0.16
Folic Acid	mcg	300	0	179.6	179.6	19	19
Vitamin B12	mcg	1.8	0.9	1.08	2.88	0.36	2.16
Pantothenic acid	mg	4	0.74	2	3.48	0.06	1.54
Biotin	mcg	20	0	10	10	0.08	0.08
MINERALS							
Calcium	mg	700	226	40	492	21	473
Copper	mg	0.44	0.05	0.264	0.364	0.3	0.4
Iodine	mcg	100	0	60	60	38.4	38.4
Iron	mg	18	0.06	18	18.1	3.5	3.62
Manganese	mg	1.5	0.008	0.3	0.316	0.2	0.216
Phosphorous	mg	500	168	82	418	72	408
Selenium	mcg	21	7.4	12.6	27.4	5.2	20
Zinc	mg	5	0.74	1.8	3.28	0.4	1.88
Potassium	mg	3800	264	64	592	125.4	653.4

(- refers to negligible values)

*** Source: Values are from USDA National Nutrient Database for Standard Reference Release 26**

* Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), 2004. Vitamins and Mineral Requirements in Human Nutrition. Second edition

* Institute of Medicine, 2006. Dietary Reference Intakes: Recommended Dietary Allowances and Adequate Intakes, Vitamins and Elements.

Table 2. Baseline demographic and anthropometric characteristics of children in the control and intervention groups[1]

	Control	Intervention
n	114	111
Age, *y*	8.4 ± 0.9	8.1 ± 0.8
Male, No (*%)*	29 (25.4)	23 (20.7)
Female, No (%)	85 (74.6)	88 (79.3)
Baseline anthropometry		
Height, *cm*	124.8 ± 6.5	125.1 ± 7.2
Weight, *kg*	22.9 ± 2.9	22.9 ± 3.4
MUAC, cm	17.3 ± 1.3	17.3 ± 1.2
Stunting[2], *%*	14(12.3)	11 (9.9)
Underweight[2], *%*	12 (10.5)	11 (9.9)

[1] Values are means ±SD or number within parenthesis percent; Variables did not differ between the groups

2 Stunting and –underweight (low weight-for-age)were defined as height-for-age and weight-for-age Z-scores <-2 from WHO child growth standards 2006 respectively

Table 3. Micronutrient status in children at baseline and endline between control and intervention groups[1]

Biochemical Variables	Control		Intervention		
	Baseline	Endline	Baseline	Endline	P value
Hemoglobin, g/dL	n (114) 12.5 ± 0.8	n (111) 12.2 ± 1.0	n (114) 12.7 ± 0.8	n (111) 12.6 ± 0.9	0.01
Serum ferritin, ng/mL	n (111) 31.9 (18.7, 47.1)	n (108) 26.7 (16.6, 39.9)	n (111) 31.2 (21.7, 42.9)	n (108) 34.9 (22.6, 43.9)	0.001
Serum transferrin receptors, *mg/dL*	n (114) 3.2 ± 1.4	110 5.1± 1.4	n (114) 3.1 ± 1.2	n (110) 4.7± 1.3	0.04
Folate ng/mL	n (114) 626.2 (497.5, 798.9)	n (111) 520.1 (471.3, 593.9)	n (114) 560.9 (462.6, 722.3)	n (111) 953.5 (820.8, 1058.4)	<0.001
Vitamin B_2*(AC)*	n (114) 1.39 ± 0.12	n (111) 1.24 ± 0.08	n (114) 1.35 ± 0.10	n (111) 1.16± 0.07	<0.001
Vitamin D	n (114)	n (109)	n (114)	n (109)	

ng/mL	21.9 ± 6.8	21.4 ± 7.0	21.6 ± 6.3	20.4 ± 6.0	0.15
Vitamin	n (111)	n (109)	n (111)	n (109)	
B_{12}pg/mL	360.9	409.8	341.0	606.8	<0.001
	(273.7, 468.8)	(317.8, 535.5)	(285.3, 426.4)	(491.2, 816.8)	
Selenium	n (114)	n (109)	n (114)	n (109)	
µg/L	85.3 ± 11.7	87.0 ± 10.7	84.3 ± 11.9	88.0± 11.7	0.34
Body Iron	n (114)	n (111)	n (114)	n (111)	
mg/kg	6.73 ± 3.68	4.39 ± 3.16	6.99 ± 3.22	5.49 ± 2.47	0.001

[1] Values are means ± SD or median within parenthesis (25^{th} percentile, 75^{th} percentile)

Analyzed using Analysis of covariance

Table 4. Prevalence of anemia, iron deficiency, iron deficiency anemia, vitamin A, zinc and selenium deficiencies in children at baseline and endline between control and intervention groups[1]

	Control	Intervention	*P* value[2]
Anemia[3]			
Baseline	8 (7.0)	7 (6.3)	
Endpoint	18 (15.8)	9 (8.1)	0.05
Iron deficiency[4]			
Baseline	22 (19.3)	16 (14.4)	
Endpoint	24 (21.1)	10(9.0)	0.01
Iron deficiency anemia[5]			
Baseline	6 (5.3)	2 (1.8)	
Endpoint	11 (9.6)	3 (2.7)	0.12
Vitamin D deficiency[6]			
Baseline	4 (3.5)	7 (6.4)	
Endpoint	6 (5.3)	6 (5.5)	0.49
Vitamin B_{12} deficiency[7]			
Baseline	8 (7.2)	6 (5.5)	
Endpoint	1 (0.9)	1 (0.9)	0.98
Vitamin B_2 deficiency[8]			

	Baseline	108 (94.7)	103 (92.8)	
	Endpoint	72 (63.7)	21 (19.1)	<0.001
Selenium deficiency[9]				
	Baseline	53 (46.5)	57 (52.3)	
	Endpoint	43 (39.1)	45 (42.9)	0.77

[1] Values are *number and within parenthesis percentages*;

[2] *P* value for comparing deficiency prevalence among study groups at endline using logistic regression adjusted for baseline % deficiency

[3] Hb levels <115 g/L

[4] Serum ferritin <15 µg/L or serum transferrin receptor > 8.5 mg/L

[5] Iron deficiency along with anemia by the above mentioned criteria

[6] Vitamin D <11 ng/mL

[7] Vitamin B2 >1.2 AC

[8] Vitamin B12 <203 pg/ml

[9] Selenium <84.9 µg/mL

Table 5. Cognitive measures in children at baseline and endline between control and intervention groups[1]

	Morning		Evening		P value
	Control	Intervention	Control	Intervention	
CCT – No of correct responses					
Baseline	56.5 ± 3.4	55.8 ± 3.9	58.0 ± 3.2	57.7 ± 4.6	
Endpoint	58.4 ± 2.5	57.8 ± 4.5	58.8 ± 2.3	58.8 ± 2.0	0.72
CCT – time taken for correct response (seconds)					
Baseline	109.8 ± 36.9	106.5 ± 27.7	96.7± 26.9	95.1 ± 23.9	
Endpoint	86.8 ± 27.8	88.3 ± 23.4	77.7 ± 19.4	79.4 ± 21.4	0.57
CTT – Trial A No of correct Response					
Baseline	24.4 ± 1.9	24.2 ± 2.1	24.7 ± 1.2	24.7 ± 1.2	
Endpoint	24.9 ± 0.4	24.8 ± 0.6	24.9 ± 0.4	24.9 ± 0.3	0.74
CTT – Trial B No of correct Response					
Baseline	22.9 ± 4.5	22.1 ± 5.3	23.5 ± 3.8	23.0 ± 3.8	
Endpoint	24.3 ± 2.0	23.4 ± 4.1	24.1 ± 2.7	23.8 ± 3.5	0.68
Time taken Trial A correct Response (seconds)	160.9 ± 72.1	163.3 ± 73.3	138.1 ± 61.3	133.5 ± 45.3	

Baseline	109.7 ± 49.1	111.1 ± 45.7	91.6 ± 36.7	100.8 ± 41.9	0.07
Endpoint					
Time taken Trial B correct Response (seconds)	267.5 ± 99.4	271.7 ± 99.5	217.4 ± 76.3	226.5 ± 75.7	
Baseline	188.6 ± 77.8	194.3 ± 73.1	164.4 ± 65.4	179.1 ± 68.6	0.71
Endpoint					
Word Order Test No of Responses					
Baseline	16.3 ± 3.4	16.3 ± 3.3	17.5 ± 7.2	17.3 ± 3.6	
Endpoint	17.7 ± 3.7	17.2 ± 3.2	18.4 ± 3.8	18.2 ± 3.7	0.45
Portues Maze test –Test Age (months)					
Baseline	165.9 ± 43.0	161.0 ± 44.9	174.9 ± 40.7	175.9 ± 39.1	
Endpoint	191.9 ± 22.5	188.1 ± 25.2	195.1 ± 17.7	192.9 ± 18.0	0.41

[1] Values are means ± SD

Table 6. Physical performance in children at baseline and endline between control and interventiongroups[1]

	Morning		Evening		
	Control	Intervention	Control	Intervention	*P value*
No of shuttles completed					
Baseline	55.7 ± 13.6	57.8 ± 13.4	56.7 ± 14.6	57.3 ± 15.7	
Endpoint	62.6 ± 15.5	62.7 ± 14.6	61.6 ± 16.2	61.1± 16.0	0.65
Test agility					
Baseline	23.2 ± 1.4	23.2 ± 1.3	23.4 ± 1.4	23.5 ± 1.4	
Endpoint	22.8 ± 1.2	22.7 ± 1.1	23.0 ± 1.3	22.9 ± 1.2	0.58

[1] Values are means ± SD