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Probiotics *Lactobacillus reuteri* DSM 17938 and *Lactobacillus casei* CRL 431 Modestly Increase Growth, but Not Iron and Zinc Status, among Indonesian Children Aged 1–6 Years[1–4]

Rina Agustina,[5,6,9]* Ingeborg M. J. Bovee-Oudenhoven,[7,8] Widjaja Lukito,[5] Umi Fahmida,[5] Ondine van de Rest,[9] Michael B. Zimmermann,[10] Agus Firmansyah,[11] Ratna Wulanti,[5] Ruud Albers,[12] Ellen G. H. M. van den Heuvel,[13] and Frans J. Kok[9]

[5]Southeast Asian Ministers of Education Organization Regional Center for Food and Nutrition (SEAMEO RECFON), Jakarta, Indonesia; [6]Department of Nutrition, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia; [7]Top Institute Food and Nutrition, Wageningen, The Netherlands; [8]NIZO Food Research, Ede, The Netherlands; [9]Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands; [10]Human Nutrition Laboratory, Swiss Federal Institute of Technology, Zurich, Switzerland; [11]Division of Pediatric Gastroenterology, Department of Child Health, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia; [12]Unilever Research and Development, Vlaardingen, The Netherlands; and [13]FrieslandCampina, Amersfoort, The Netherlands

Abstract

Probiotics and milk calcium may increase resistance to intestinal infection, but their effect on growth and iron and zinc status of Indonesian children is uncertain. We investigated the hypotheses that cow milk with added probiotics would improve growth and iron and zinc status of Indonesian children, whereas milk calcium alone would improve growth but reduce iron and zinc status. A 6-mo randomized trial was conducted in low-socioeconomic urban communities of Jakarta. Healthy children (n = 494) were randomly assigned to receive low-lactose milk with a low calcium content of ~50 mg/d (LC; n = 124), a regular calcium content of ~440 mg/d (RC group; n = 126), regular calcium with 5×10^8 CFU/d *Lactobacillus casei* CRL 431 (casei; n = 120), or regular calcium with 5×10^8 CFU/d *Lactobacillus reuteri* DSM 17938 (reuteri; n = 124). Growth, anemia, and iron and zinc status were assessed before and after the intervention. Compared with the RC group, the reuteri group had significantly greater weight gain [0.22 (95% CI: 0.02, 0.42) kg], weight-for-age Z-score (WAZ) changes [0.09 (95% CI: 0.01, 0.17)], and monthly weight [0.03 (95% CI: 0.002, 0.05) kg/mo] and height [0.03 (95% CI: 0.01, 0.05) cm/mo] velocities. Casei significantly increased monthly weight velocity [0.03 (95% CI: 0.001, 0.05) kg/mo], but not height. However, the changes in underweight, stunting, anemia prevalence, and iron and zinc status were similar between groups. In conclusion, *L. reuteri* DSM 17938 modestly improved growth by increasing weight gain, WAZ changes, and weight and height velocity, whereas *L. casei* CRL 431 modestly improved weight velocity. Independent from probiotics supplementation, regular milk calcium did not affect growth or iron and zinc status. J. Nutr. doi: 10.3945/jn.112.166397.

Introduction

Undernutrition and multiple micronutrient deficiencies persist as the most serious nutritional problems among children aged <5 y in developing countries (1,2). The Indonesian national prevalence of stunting (37%) and wasting (14%) is higher and underweight (18%) is similar compared with the estimated overall prevalence of undernutrition among under-5 children in the developing world (3,4). Deficiencies in vitamin A, iron, zinc, and iodine are the most prevalent in Indonesian children (5). Because intake of dairy products in these children is minimal during their growth period (6), calcium deficiency may also be prevalent.

Studies reporting the benefits of nutrition interventions on growth and micronutrient status of under-5 children used different approaches such as supplementation and fortified foods, but results are conflicting (5,7–11). In addition, some studies indicated that nutrient-dense foods may help prevent stunting and wasting in young children, but more data are needed to identify the impact of this approach (12–14). Probiotics are often added to dairy foods, and both probiotics (15,16) and calcium in milk (17) may strengthen intestinal infection resistance. However, the

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[3] This trial was registered at clinicaltrials.gov as NCT00512824.
[4] Supplemental Figure 1 and Supplemental Tables 1–2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
* To whom correspondence should be addressed. E-mail: dr.rinaagustina@gmail.com.

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impact of probiotics on growth and micronutrient status is uncertain. In addition to a mouse study with *Lactobacillus casei* CRL 431 (18,19), several human studies showed a positive effect of probiotics on weight gain in children aged 5 mo to 5 y (20–23). Three reviews reported that calcium supplementation in healthy children had no effect on weight, height, body fat, or lean mass in randomized clinical trials (RCTs)[14] (24–26). However, such evidence is scarce in children in developing countries where undernutrition is prevalent.

Milk naturally rich in calcium and fortified with probiotics may provide extra energy, high-quality protein, and micronutrients, but less is known about whether the combination of milk and probiotics may influence the absorption of iron and zinc. Probiotics are often used to improve digestibility and uptake of nutrients by intestinal cells (27) and may be beneficial in malnutrition when gut function is impaired (28). Acute inhibitory effect of calcium on iron absorption in adults has been shown in some studies (29–31), but not in others (32–34). Previous studies reported variable findings on the effects of milk or calcium supplementation on zinc absorption in adults (35). The possibility that calcium interferes with iron and zinc absorption and thus affects iron and zinc status in children is an important concern (36,37), but very few studies have been performed in a pediatric population. These studies conducted to date were in children with adequate calcium and iron intake and showed mixed results (36,38–40).

Until now, evidence has been inconclusive whether prolonged dietary supplementation with calcium and probiotics in children with low habitual calcium intakes affects growth and iron and zinc status. Therefore, we investigated the hypotheses that milk with added probiotics would improve growth and iron and zinc status, whereas milk alone would improve growth but reduce iron and zinc status of Indonesian children.

Participants and Methods

Study design and participants. A randomized, double-blind, placebo-controlled trial was conducted between August 2007 and September 2008 in low-socioeconomic urban communities representing flooding and nonflooding areas of East Jakarta, Indonesia.

Detailed criteria for participation have been described elsewhere (41). Briefly, we included children aged 1–6 y who were apparently healthy, were not being breastfed, did not have symptoms of chronic and/or congenital diseases and disabilities, did not have pulmonary tuberculosis, did not have a history of allergy, did not have diarrhea on admission, were not taking antibiotics within 2 wk before the study start, did not have severe wasting, had a calcium intake of ≤ 375 mg/d according to a validated semiquantitative FFQ, were capable or willing to drink liquid milk with a straw in a 2-d acceptance test and did not show allergy or intolerance to the products, and/or were not a sibling of another included child (twins excepted). All parents signed the informed consent. The protocol was approved by the Medical Ethics Committee of the Faculty of Medicine of the University of Indonesia and of Wageningen University, The Netherlands.

Intervention. Eligible children were admitted to the study and stratified according to area (flooding and nonflooding), age (<57 and ≥57 mo), and sex. The twin siblings of participants (n = 3) were allocated to the same treatment group. Researchers, mothers, children, and laboratory personnel were unaware of the treatment until all biochemical and data analyses were finished and until after the blind review meeting. Postintervention fecal calcium results were not reviewed until the data results were fully analyzed.

Children were randomly assigned to receive 180 mL of low-lactose milk twice daily (not with a meal) using the straws provided according to the following milk groups: a low calcium content of ~50 mg/d (LC group), a regular calcium content of ~440 mg/d (RC group), regular calcium plus 5×10^8 CFU/d *Lactobacillus casei* CRL 431 (casei group), or regular calcium plus 5×10^8 CFU/d *Lactobacillus reuteri* DSM 17938 (reuteri group). Milk was sweetened, chocolate-flavored, ambient stable (sterilized by using ultrahigh temperatures), and packed in Tetra Paks (Frisian Flag Indonesia). Milk was consumed with straws coated inside with the oil drop without probiotic strain as placebo (BioGaia AB) or with either *L. casei* CRL 431 (Chr Hanssen) or *L. reuteri* DSM 17938 (an antibiotic-resistant, gene-depleted derivative daughter strain of *L. reuteri* ATCC 55730; BioGaia AB) (42) in vegetable oil. The different milks and straws were indistinguishable for the investigators and participants. The composition of the milk and straws has been described elsewhere (41). Milk and straws were stored cooled (<10°C) at all times until delivery. Viability of the probiotics was checked each month by using selective plating.

Field-workers distributed milk and straws twice a week to the parents, who were instructed to keep the products refrigerated and to prevent sun exposure. Parents without refrigerators obtained the products from the field-workers' house on a daily basis and/or children consumed the products directly at the field-workers' house. The amount of milk consumed was measured by using a calibrated stick put into the Tetra Pak to score the remaining volume with a pretested 5-point scale (4 = empty, 3 = three-fourths empty, 2 = one-half empty, 1 = one-fourth empty, and 0 = not consuming). The measured consumption was scored and recorded daily on a compliance card by both mothers and field-workers. Field-workers observed the children drinking milk at least once a week, and empty packages had to be shown during visits. Compliance was determined by calculating the percentage of total frequencies of milk consumed and total consumption of 2 units/d × 168 d of intervention with a maximum score of 1344. The acceptable compliance was defined as a minimum score of 756 (equal to 70% × 2 units/d × 168 d × score of 3). Activities with creative and educational contents were implemented to maintain the compliance of mothers and children. Adverse events were recorded as described elsewhere (41).We followed the local standards for outpatient and hospital care for diarrhea and acute respiratory tract infection, which were per WHO guidelines, as described elsewhere (41). An independent expert monitored trial conduct and adherence to protocol.

Data collection. Field-workers performed anthropometric measurements at baseline (month 0), during the intervention (months 1, 2, 3, 4, and 5), and at study end (month 6). Lightly clothed children were weighed without shoes by using an electronic scale (Seca model 890; Seca GmbH) with a precision of 0.1 kg. The recumbent length for children aged <2 y and standing height for children aged ≥2 y were measured twice by trained personnel by using a wooden length board with a precision of 0.1 cm (43). Habitual dietary intake over the previous 2 mo was assessed by using a semiquantitative FFQ with the use of a list of locally available food items. Food models and calibrated household utensils were used to visualize and aid in estimation of portion sizes.
Before and at the intervention end, nonfasting venous blood was drawn in the morning by trained phlebotomists. Two milliliters of whole blood was collected in EDTA tubes for hematology preparation, and 7 mL was collected into non-anticoagulant tubes for determination of serum high-sensitivity C-reactive protein (hs-CRP) and α1-acid glycoprotein (AGP) ferritin, soluble transferrin receptor (sTfR), and zinc. Blood samples were promptly stored and transported to the laboratories in cool boxes. Sera were stored at −70°C in regular (for hs-CRP, AGP, ferritin, and sTfR assessment) and acid-washed microtubes (for zinc analysis). The procedure for fecal sample collection has been described in detail elsewhere (41).

Laboratory measurements. Iron and zinc status were determined at baseline and after 6 mo of intervention by measuring hemoglobin and serum zinc and ferritin and sTfR concentrations (44). Routine hematology

[14] Abbreviations used: AGP, α1-acid glycoprotein; HAZ, height-for-age Z-score; Hb, hemoglobin; Hct, hematocrit; hs-CRP, high-sensitivity C-reactive protein; LC, low-calcium (group); RC, regular-calcium (group); RCT, randomized clinical trial; sTfR, soluble transferrin receptor; WAZ, weight-for-age Z-score.

testing [i.e., hemoglobin (Hb), hematocrit (Hct), RBCs, red cell distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC)] was performed by using an automatic analyzer (Advia 120; Bayer Diagnostics) (45). Serum ferritin and sTfR were measured by ELISA (46). Serum zinc was analyzed by inductively coupled plasma-optical emission spectrometry (OPTIMA 2000 DV; Perkin-Elmer). A high-sensitivity chemiluminescent assay (Immulite; Dade-Behring) was used to measure serum hs-CRP concentration (47). Serum AGP was measured by using an ELISA (46). Fecal calcium (baseline and endline samples) was analyzed in freeze-dried feces by inductively coupled plasma atomic emission spectrometry (Varian) (17).

Outcomes. The primary endpoints of the study focused on the intervention effects on the number and duration of diarrheal episodes as described elsewhere (41). In this article, we present data on the secondary outcomes, notably the changes in growth, anemia, and iron and zinc status. Therefore, the study power calculation was based on the primary outcomes as described elsewhere (41). A post hoc power calculation shows that our sample size between groups of comparisons permitted us to detect treatment-related differences in the different outcomes described here, with an observed small to medium effect size (Cohen's d) of 0.01–0.29, a probability level (α) of 0.05, and a statistical power of 1–74%.

The effect on growth was evaluated by calculating differences in weight, height, weight-for-age Z-score (WAZ), and height-for-age Z-score (HAZ) at the study end (6-mo intervention) minus baseline (month 0). The HAZ and WAZ were calculated on the basis of the WHO Child Growth Standards (48) by using the WHO anthropometric software 2005 and 2007. In addition, using the consecutive monthly anthropometric measurements, the mean change and difference in monthly weight and height growth velocities as well as WAZ and HAZ were analyzed by using a longitudinal regression model (months 0, 1, 2, 3, 4, 5, and 6). Stunting was defined as HAZ < −2 SD and underweight as WAZ < −2 SD (2). Changes in the proportion of underweight and stunted children from baseline to study end were calculated.

Iron and zinc status was assessed by comparing the estimated means of the differences (end − baseline) between and within treatment groups. Changes in the proportion of anemic, iron-deficient anemic, and zinc-deficient children were calculated. Anemia was defined according to WHO criteria as Hb <110 g/L (children aged <5 y) and Hb <115 g/L (children aged 5–11 y); iron deficiency was defined as serum ferritin <12 g/L in children aged <5 y and <15 g/L in children aged >5 y; and iron deficiency anemia was defined as anemia with iron deficiency (49). Zinc deficiency was defined as a serum zinc concentration <9.9 μmol/L (<65 mg/dL) (50). The 6-mo changes in fecal calcium were used to verify the differences in calcium intake during the intervention (51).

Statistical analysis. Intention-to-treat analysis was performed according to a predefined data analysis protocol for all eligible children who had consumed the intervention products at least once. Student's t test was used to identify differences in quantitative normally distributed data (or Mann-Whitney U test when data were not normally distributed) between predefined groups of comparison (between LC and RC, RC and casei, RC and reuteri). For the outcomes on blood variables, the RC group was pooled with the probiotic groups (casei and reuteri) as 1 calcium-supplemented group and compared with the LC group. Within-treatment differences in status markers were assessed by paired t test. Values are expressed as means ± SDs for normally distributed variables and as medians (25th, 75th percentiles) for nonnormally distributed variables. We used PASW Statistics 17.0.3 for Windows (SPSS, Inc.) for this analysis.

Random intercept models were applied for statistical analyses of growth outcomes by using SAS version 9.2 (GLIMMIX procedure; SAS Institute). A general linear model for repeated measures was used to observe the difference in linear trend of WAZ and HAZ at baseline; at months 1, 2, 3, 4, and 5; and after 6 mo of intervention. We performed repeated-measures logistic regression model by using Stata for Windows, release 11 (XTGEE procedure; StataCorp) for changes in the prevalence of underweight, stunting, anemia, iron and zinc deficiency, and iron deficiency anemia. Growth variables (weight, height, WAZ, HAZ) and status markers (Hb, serum ferritin, sTfR, and zinc) were the dependent variables, and treatment group and time of measurement were the independent variables. The variables area, age, sex, HAZ, WAZ, and status markers at baseline were included in the model as covariates dependent on the outcome studied.

Results

A total of 3150 children were screened in phase 1 and 1343 were screened in phase 2. From the 497 eligible children, 3 refused to undergo baseline measurements. In total, 494 children were randomly allocated to LC (n = 124), RC (n = 126), casei (n = 120), or reuteri (n = 124) groups and included in the analysis (**Supplemental Fig. 1**).

At admission, all study groups were comparable with regard to sociodemographic characteristics (including environment and maternal hygiene and caring), age group distribution, birth weight, feeding history in the first year of life, health, hematologic and nutritional status (**Table 1**), and habitual dietary intake (**Table 2**). The mean age of children at enrollment was 59.4 ± 14.3 mo. Compliance to the intervention was high (94%) and similar among groups. The mean total compliance score was 1324 out of 1344 (95% CI: 1230, 1342). Both probiotic strains remained >90% viable over the intervention period. As expected, fecal calcium excretion increased significantly in the 3 groups given regular-calcium milk (P < 0.001) (**Table 3**), indicating good acceptance of the study product.

Changes in energy and nutrient intake between the baseline and endpoint were not significantly different among the groups (Table 2), except for calcium. The nutrient contents of nonmilk food intake most related to growth (e.g., energy, protein, and zinc) at the end of the study were not significantly different among the groups (data not shown). Regular milk supplementation augmented the daily calcium intake of these children to the recommended daily allowances (~500 mg/d) of Indonesian children in this age group, whereas it remained 50% in the LC group (230 mg/d) (P < 0.05). In addition, daily intakes of energy and protein increased in all groups up to 20% and 30%, respectively.

Weight, height, WAZ, and HAZ increased in all intervention groups over time (**Table 4**). Overall, the mean weight and height velocity of children was 178 ± 131 g/mo and 0.56 ± 0.16 cm/mo (~7cm/y), respectively. On average, the total group was 1070 g heavier (WAZ of 0.10) and grew up to 3.4 cm taller (HAZ of 0.15) during the 6-mo intervention (data not shown).

The reuteri group experienced significantly greater weight gain (1160 ± 650 g; P = 0.03) and WAZ change (0.14 ± 0.28 Z; P = 0.03) after 6 mo of intervention compared with the RC group (960 ± 740 g and 0.06 ± 0.31 Z, respectively). The significant mean difference in weight gain and WAZ change between reuteri and RC groups was 220 g and 0.09 Z, respectively. Compared with the RC group, the reuteri group had a significantly higher mean monthly weight [adjusted difference: 0.03 (95% CI: 0.002, 0.05) kg/mo; P = 0.04] and height velocity (P = 0.04) (Table 4). In the casei group, the mean monthly weight velocity was significantly higher compared with the RC group [adjusted difference: 0.03 (95% CI: 0.001, 0.05) kg; P = 0.04]. However, the differences in the reduction in underweight (23%) and stunting (30%) in the reuteri group were not significant compared with the RC group after adjusting for area of living, sex, age, and WAZ at baseline (**Supplemental Table 1**). The crude data (**Fig. 1**) and model results of WAZ (Table 4) showed that the increases in Z-score of the reuteri group were most prominent in the first and sixth intervention month.

TABLE 1 Baseline characteristics of the Indonesian children who consumed milk with a low calcium concentration or a regular calcium concentration with or without probiotics[1]

	Group			
Characteristics	LC (n = 124)	RC (n = 126)	Casei (n = 120)	Reuteri (n = 124)
Living in flooding area, *n (%)*	81 (65)	82 (66)	78 (65)	82 (66)
Sex (male), *n (%)*	67 (54)	68 (54)	66 (55)	68 (55)
Age, *mo*	59.3 ± 14.3	58.9 ± 14.2	60.3 ± 13.7	58.9 ± 15.1
Age group, *n (%)*				
12–47 mo	25 (20)	32 (25)	23 (19)	29 (24)
48–59 mo	35 (28)	28 (22)	32 (27)	31 (25)
60–71 mo	37 (30)	42 (33)	38 (32)	41 (33)
72–83 mo	27 (22)	24 (19)	27 (23)	23 (19)
Family size, *n*	5.1 ± 1.7	5.4 ± 1.7	5.2 ± 1.8	5.0 ± 1.8
Household expenditure, *US$/mo*	189 ± 97	194 ± 139	159 ± 69	203 ± 181
Maternal education of <6 y, *n (%)*	43 (36)	43 (35)	52 (42)	50 (40)
Weight at birth,[2] *g*	3109 ± 511	3082 ± 416	3126 ± 434	3066 ± 482
Ever breastfed, *n (%)*	120 (97)	121 (96)	115 (96)	120 (97)
Breastfeeding duration, *n (%)*				
<24 mo	55 (44)	48 (38)	58 (48)	63 (51)
≥24 mo	65 (52)	73 (58)	57 (48)	57 (46)
Exclusively breastfed ≥6 mo,[3] *n (%)*	22 (20)	25 (21)	16 (14)	21 (17)
Given liquid/solid food at birth, *n (%)*	52 (42)	47 (37)	43 (36)	52 (42)
Started complementary food at ≥6 mo	18 (23)	16 (21)	16 (14)	20 (16)
Diarrhea 2 wk before study, *n (%)*	20 (16)	13 (10)	24 (20)	15 (12)
ARI 2 wk before study, *n (%)*	48 (39)	51 (41)	52 (43)	56 (45)
Serum hs-CRP, *mg/L*	0.79 (0.23, 1.82)	0.75 (0.28, 2.90)	0.75 (0.30, 2.50)	0.66 (0.25, 3.03)
Serum AGP, *g/L*	0.79 (0.69, 0.93)	0.82 (0.70, 0.95)	0.83 (0.70, 0.97)	0.81 (0.71, 0.94)
Serum ferritin, *μg/L*	16.2 (9.56, 27.7)	18.4 (9.2, 36.4)	18.9 (10.6, 34.3)	16.5 (10.9, 30.4)
Serum sTfR, *μg/L*	9.3 ± 5.2	9.3 ± 4.3	9.2 ± 3.8	9.2 ± 4.1
Serum zinc,[4] *μmol/L*	8.6 ± 2.5	8.5 ± 2.2	8.8 ± 2.7	8.7 ± 2.3
Anemia,[5] *n (%)*	24 (19)	33 (26)	24 (20)	24 (19)
Iron deficiency,[6] *n (%)*	40 (32)	41 (33)	32 (27)	37 (30)
Iron deficiency anemia,[7] *n (%)*	12 (10)	17 (14)	13 (11)	14 (11)
Zinc deficiency,[8] *n (%)*	85 (71)	93 (78)	75 (65)	82 (68)
Weight, *kg*	15.5 ± 3.36	15.3 ± 3.18	16.0 ± 3.66	15.6 ± 4.00
Height, *cm*	101.7 ± 8.87	101.2 ± 8.94	103.0 ± 8.94	101.9 ± 9.70
Weight-for-age Z-score	−1.27 ± 1.05	−1.39 ± 0.94	−1.15 ± 1.07	−1.26 ± 1.24
Height-for-age Z-score	−1.53 ± 0.98	−1.65 ± 0.94	−1.39 ± 1.00	−1.46 ± 1.09
Underweight,[9] *n (%)*	24 (19)	36 (29)	21 (18)	34 (27)
Stunted,[10] *n (%)*	38 (31)	44 (35)	34 (28)	37 (30)

[1] Values are means ± SDs, median (IQRs), or n (%). AGP, α1-acid glycoprotein; ARI, acute respiratory tract infection; hs-CRP, high-sensitivity C-reactive protein; LC, low-calcium; RC, regular-calcium; sTfR, soluble transferrin receptor.
[2] Forty-three children did not have complete information on this variable (11 in the LC, 11 in the RC, 13 in the casei, and 9 in the reuteri group).
[3] Twenty-seven children did not have complete information on this variable (11 in the LC, 8 in the RC, 4 in the casei, and 4 in the reuteri group).
[4] Eighteen children did not have complete sampling collection on this assessment (5 in the LC, 6 in the RC, 4 in the casei, and 3 in the reuteri group).
[5] Defined as a hemoglobin concentration <110 g/L (children aged <5 y) or <115 g/L (children aged 5–11 y).
[6] Defined as serum ferritin <12 μg/L (children aged <5 y) or <15 μg/L (children aged >5 y).
[7] Defined as an iron deficiency with anemia (see footnote 6).
[8] Defined as a morning, nonfasting serum zinc concentration <9.9 μmol/L.
[9] Defined as a weight-for-age Z-score < −2 SDs.
[10] Defined as a height-for-age Z-score < −2 SDs.

During the intervention, Hb, Hct, other hematologic variables (RBCs, RDW, MCV, MCHC), and serum ferritin significantly declined, and sTfR significantly increased in each of the groups (or all combined) ($P < 0.001$). However, none of the changes in these blood variables, including serum zinc, were significantly different among the groups, even when the RC group was pooled with the probiotic groups as 1 calcium-supplemented group versus the LC group (Table 3).

Because acute or chronic inflammation and infection potentially confound the association with ferritin, an indicator of iron stores, we repeated the analysis excluding children with hs-CRP concentrations >5 mg/L. Serum ferritin values were also corrected by using both hs-CRP concentrations >5 mg/L and/or AGP >1 g/L to classify children into incubation, early, or late convalescence groups (52). However, results were not different when children with elevated CRP and/or AGP were included or excluded or when serum ferritin was corrected.

During the 6-mo intervention, the percentage of children with iron deficiency and iron deficiency anemia increased significantly in all groups ($P < 0.05$) (**Supplemental Table 2**). No significant differences among treatment groups were observed for iron status even after adjusting for area of living, season, and

TABLE 2 Habitual daily intake of energy and nutrients in children who consumed milk with a low calcium concentration or a regular calcium concentration with or without probiotics at baseline and after 6 mo of intervention[1]

	Group			
Variable	LC (n = 123)	RC (n = 126)	Casei (n = 120)	Reuteri (n = 123)
Energy intake, *kJ/d*				
Baseline	4310 ± 1530	4460 ± 1380	4290 ± 1550	4090 ± 1300
Endpoint	5190 ± 1490	5260 ± 1410	5350 ± 1590	5180 ± 1360
Change	890 ± 1630	800 ± 1490	1060 ± 1730	1090 ± 1660
Protein intake, *g/d*				
Baseline	34.2 ± 13.5	36.3 ± 12.9	33.5 ± 13.6	32.9 ± 11.0
Endpoint	45.5 ± 12.3	45.4 ± 12.2	46.2 ± 14.2	44.7 ± 11.6
Change	11.4 ± 13.0	9.1 ± 13.7	12.8 ± 15.0	11.8 ± 14.1
Vitamin A, *μg RAE/d*				
Baseline	86 (38, 160)	85 (46, 179)	69 (36, 169)	80 (42, 182)
Endpoint	95 (63, 150)	88 (68, 125)	87 (63, 144)	88 (66, 173)
Change	21 (−28, 58)	11(−73, 49)	17 (−45, 57)	19 (−73, 57)
Calcium intake, *mg/d*				
Baseline	231(161, 230)	232 (165, 335)	205 (148, 305)	220 (158, 304)
Endpoint	206 (162, 249)*	592 (541, 648)	602 (545, 662)	589 (538, 647)
Change	1 (−101, 49)*	354 (267, 440)	394 (292, 458)	382 (275, 455)
Iron intake, *mg/d*				
Baseline	5.9 (4.0, 7.6)	6.1 (4.7, 8.0)	5.5 (4.2, 7.3)	6.0 (4.6, 7.4)
Endpoint	6.2 (5.0, 8.6)	6.3 (5.0, 8.4)	6.7 (4.9, 8.2)	6.0 (5.1, 7.9)
Change	0.9 (−1.1, 2.5)	0.4 (−1.3, 1.8)	1.1(−0.9, 3.0)	0.2 (−1.3, 2.3)
Zinc intake, *mg/d*				
Baseline	4.0 (2.9, 5.5)	4.8 (3.5, 5.7)	3.6 (2.9, 5.9)	4.3 (3.1, 5.4)
Endpoint	4.0 (3.0, 5.0)	4.0 (3.1, 5.4)	4.0 (3.0, 5.1)	3.8 (3.2, 4.9)
Change	0.1 (−1.3, 1.0)	−0.5 (−1.5, 0.8)	0.1 (−1.0, 1.4)	−0.1 (−1.6, 1.1)

[1] Values are means ± SDs or median (IQRs) assessed by semiquantitative FFQ. Two children did not complete endpoint measurements (1 in the reuteri and 1 in the LC group). Milk supplements were included at the mean endpoint and for changes. *Different from the RC group, $P < 0.001$ (Mann-Whitney U test). LC, low-calcium; RAE, retinol activity equivalents; RC, regular-calcium.

other covariates. Thus, milk calcium alone or added with probiotic strains did not significantly affect iron status during the 6-mo intervention.

Discussion

Our study showed that supplementing the habitual diet of Indonesian children aged 1 to 6 y with regular-calcium milk with added *L. reuteri* DSM 17938 modestly improved growth by increasing weight gain, WAZ changes over 6 mo, and monthly weight and height velocity compared with regular-calcium milk alone. Regular-calcium milk with *L. casei* CRL 431 modestly improved monthly weight velocity compared with regular-calcium milk. Regular-calcium milk did not significantly affect any of the growth variables compared with low-calcium milk. None of the treatments affected iron and zinc status. All groups declined in iron and zinc status over time in the study.

The weight velocity (178 g/mo) of all treatments in our study children was within the 50th percentile of the weight velocity (138–192 g/mo) of children in this age group on the CDC growth chart (53). The height velocity (~7 cm/y) of all children was also within the upper expected mean velocity (5–6 cm/y) (54). Overall, all treatments resulted in normal growth in weight and height in our children.

The modest effect sizes (calculated by dividing the difference between the mean change in treatment and control groups by the pooled SD for the 2 groups) (7) of *L. reuteri* in all significant growth variables (0.17–0.29) and of casei in weight velocity (0.27) were larger than the results of a meta-analysis and review on multiple micronutrient supplementation (<0.15) in under-5 children over a 1-y period (7,8).

A few studies have investigated probiotic effects on children's growth (55). These studies showed inconsistent effects and differed in probiotic strain and dose, intervention duration, and children's age. Our reuteri and casei results in weight gain (effect sizes of 0.29 and 0.23, respectively, in a 6-mo period) were slightly larger compared with 2 RCTs investigating effects on growth using combined probiotics and other components: *Bifidobacterium lactis* HN019 and prebiotic oligosaccharide versus control milk alone in Indian children of similar age (effect size of 0.22 in a 12-mo period) (23) and *B. longum*, *L. rhamnosus*, prebiotics, and PUFAs versus control milk in 12-mo-old Indonesian toddlers (effect size of 0.23 in 12-mo period) (56). However, differences in growth outcomes did not affect the prevalence of stunting and underweight in the reuteri and other groups. This may be due to the fact that 6 mo of intervention may have been too short to observe a reduction in malnutrition prevalence (57). It may also be that the actual increments of 0.09 for WAZ and 0.05 for HAZ, albeit significant, were still too small, especially taking the average growth deficit of Asian and African children into account (~ –2.0 Z) (58). In contrast to our results, several other studies in infants receiving various probiotic strains, either alone (58,59) or combined with prebiotics (28,59), did not find beneficial effects on growth. Overall, our probiotics interventions, but not milk calcium, showed larger effects on growth than other nutritional interventions.

In our trial, the changes in WAZ sharply increased in the first intervention month. This may reflect a rapid catch-up growth

TABLE 3 Hematology variables, serum inflammation markers, and iron, zinc, and calcium status changes in children who consumed milk with a low calcium concentration or a regular calcium concentration with or without probiotics at baseline and after 6 mo of intervention[1]

	Group			
Variable	LC (n = 123)	RC (n = 124)	Casei (n = 120)	Reuteri (n = 122)
Hb,[2] *g/L*				
Baseline	120 ± 11	118 ± 14	120 ± 11	119 ± 10
Change	−1.9 ± 7.7	−1.5 ± 8.8	−2.5 ± 7.7	−1.4 ± 7.5
Hct[2]				
Baseline	0.37 ± 0.27	0.36 ± 0.33	0.37 ± 0.03	0.37 ± 0.27
Change	−0.01 ± 0.30	−0.005 ± 0.30	−0.009 ± 0.27	−0.006 ± 0.29
RBCs,[2] 10^{12}/L				
Baseline	4.80 ± 0.39	4.70 ± 0.40	4.76 ± 0.43	4.75 ± 0.41
Change	0.11 ± 0.33	0.14 ± 0.31	0.12 ± 0.30	0.15 ± 0.28
MCV,[2] *fL*				
Baseline	77.2 ± 6.1	77.3 ± 6.3	77.6 ± 6.2	77.7 ± 6.2
Change	−3.8 ± 3.8	−3.4 ± 3.5	−4.0 ± 3.6	−3.7 ± 3.5
RDW,[2] %				
Baseline	14.9 ± 1.5	14.9 ± 1.6	14.9 ± 1.4	14.9 ± 1.4
Change	−1.2 ± 0.9	−1.2 ± 0.9	−1.1 ± 1.1	−1.3 ± 0.9
Serum hs-CRP,[2] *mg/L*				
Baseline	0.79 (0.23, 1.82)	0.75 (0.28, 2.9)	0.75 (0.30, 2.5)	0.66 (0.25, 3.03)
Change	−0.01 (-1.03, 0.61)	−0.09 (-0.89, 1.0)	−0.05 (-1.42, 0.40)	0 (-1.65, 0.51)
Serum AGP,[2] *g/L*				
Baseline	0.81 ± 0.18	0.84 ± 0.20	0.84 ± 0.19	0.82 ± 0.17
Change	−0.01 ± 0.25	−0.02 ± 0.24	−0.03 ± 0.22	−0.02 ± 0.24
Serum sTfR,[2] μ*g/L*				
Baseline	8.07 (6.54, 10.4)	8.46 (6.64, 10.4)	8.44 (6.89, 10.6)	8.54 (6.76, 10.2)
Change	0.90 (-0.35, 1.69)	0.87 (-0.64, 2.23)	0.38 (-0.61, 1.89)	0.67 (-0.26, 2.02)
Serum ferritin,[3] μ*g/L*	(n = 109)	(n = 109)	(n = 108)	(n = 103)
Baseline	18.3 (9.65, 30.7)	24.4 (9.27, 39.1)	22.8 (12.0, 35.3)	19.4 (10.9, 32.3)
Change	−3.96 (-11.1, 1.03)	−4.35 (-15.7, 1.58)	−4.21 (-13.5, -0.35)	−3.51 (-11.2, 0.82)
Serum zinc,[4] μ*mol/L*	(n = 115)	(n = 116)	(n = 116)	(n = 117)
Baseline	8.50 ± 2.47	8.42 ± 2.24	8.78 ± 2.72	8.70 ± 2.28
Change	0.34 ± 2.01	0.30 ± 2.45	−0.03 ± 2.23	−0.04 ± 1.95
Fecal calcium,[5] *mg/g*	(n = 124)	(n = 123)	(n = 120)	(n = 122)
Baseline	7.6 (4.7, 11.1)	7.5 (4.8, 10.4)	6.6 (4.8, 9.3)	7.8 (5.1, 11.4)
Change	0.37 (-2.00, 4.26)*	15.9 (6.48, 23.9)	14.9 (7.14, 23.4)	15.1 (6.68, 20.7)

[1] Values are means ± SDs or median (IQRs). *Different from the RC group, $P < 0.001$ (Mann-Whitney U test). AGP, α1-acid glycoprotein; Hb, hemoglobin; Hct, hematocrit; hs-CRP, high-sensitivity C-reactive protein; LC, low-calcium; MCV, mean corpuscular volume; RC, regular-calcium; RDW, red cell distribution width; sTfR, soluble transferrin receptor.
[2] Five children did not complete sampling collection at study end (1 in the LC, 2 in the RC, and 2 in the reuteri group).
[3] Six children did not complete sampling collection at study end (2 in the LC, 2 in the RC, and 2 in the reuteri group) and 59 children had an hs-CRP concentration >10 mg/L at baseline and endpoint.
[4] Thirty children did not complete sampling collection at study end (9 in the LC, 10 in the RC, 4 in the casei, and 7 in the reuteri group).
[5] Five children did not complete sampling collection at study end (3 in the RC and 2 in the reuteri group).

due to the extra energy and protein in the supplied milks (60). The stasis of WAZ in all groups between the 2nd and 5th intervention month may reflect the inability to continue the catch-up. Growth is a nonlinear process within short periods, which are characterized by periods of weight gain and of weight loss (61). This irregular pattern was not seen for HAZ, which consistently increased in all intervention months. Although speculative, the beneficial effects of probiotics observed on growth could be due to enhanced mucosal integrity (62) and reduced intestinal infections (diarrhea incidence, especially in children with lower nutritional status supplemented with probiotics, i.e., *L. reuteri*) (41).

No effect on growth was observed in the RC group (without probiotics) compared with the LC group. This is in line with 3 reviews of RCTs in children of mainly developed countries, which also reported no benefit of calcium supplementation on weight, height, body fat, or lean mass (24–26).

The present study showed that the probiotics did not affect iron and zinc status. Probiotics are reported to improve digestibility and nutrient uptake by intestinal cells (27). A study in infant rhesus monkeys given *L. reuteri* showed an improved Hct (63), but so far no information is available about more relevant iron status markers (e.g., ferritin, sTfR).

Concentrations of iron parameters (e.g., Hb, Hct, and serum ferritin) decreased and sTfR increased over the 6-mo intervention in all groups, whereas serum zinc remained constant. No treatment-specific differences were observed in mean change in iron parameters and prevalence changes in anemia, iron deficiency, and iron deficiency anemia, or in zinc status. The reduced iron status over 6 mo, independent of intervention type, may be due to insufficient iron homeostasis to compensate for the fast iron mobilization from storage needed during growth (22). Chronic blood loss may also contribute to iron deficiencies and

TABLE 4 Dietary treatment effects on body weight, height, and changes in the weight- and height-for-age Z-scores in children who consumed milk with a low calcium concentration or a regular calcium concentration with or without probiotics at baseline and after 6 mo of intervention[1]

	Group				Adjusted differences (95% CI)[2]		
Outcome	LC (n = 124)	RC (n = 126)	Casei (n = 120)	Reuteri (n = 124)	RC vs. LC	Casei vs. RC	Reuteri vs. RC
Change[3]							
Weight, *kg/6 mo*	1.02 ± 0.8	0.96 ± 0.74	1.15 ± 0.93	1.16 ± 0.65	−0.09 (−0.29, 0.11)	0.20 (−0.01, 0.40)	0.22 (0.02, 0.42)*
Height, *cm/6 mo*	3.38 ± 0.85	3.32 ± 0.87	3.23 ± 0.9	3.49 ± 1.23	−0.07 (−0.32, 0.18)	−0.08 (−0.33, 0.17)	0.19 (−0.06, 0.43)
WAZ, *score/6 mo*	0.07 ± 0.31	0.06 ± 0.31	0.11 ± 0.37	0.14 ± 0.28	−0.02 (−0.01, 0.06)	0.05 (−0.03, 0.14)	0.09 (0.01, 0.17)*
HAZ, *score/6 mo*	0.15 ± 0.19	0.14 ± 0.17	0.12 ± 0.21	0.18 ± 0.32	−0.02 (−0.08, 0.04)	−0.01 (−0.07, 0.05)	0.05 (−0.01, 0.11)
Velocity[4]							
Weight, *kg/mo*	0.15 ± 0.13	0.14 ± 0.13	0.17 ± 0.16	0.17 ± 0.11	−0.01 (−0.19, 0.10)	0.03 (0.001, 0.05)*	0.03 (0.002, 0.05)*
Height, *cm/mo*	0.56 ± 0.14	0.54 ± 0.14	0.53 ± 0.15	0.57 ± 0.21	−0.02 (−0.04, 0.01)	−0.01 (−0.04, 0.01)	0.03 (0.01, 0.05)*
WAZ, *score/mo*	0.003 ± 0.05	0.005 ± 0.05	0.01 ± 0.06	0.01 ± 0.05	0.0004 (−0.01, 0.01)	0.004 (−0.01, 0.02)	0.01 (−0.01, 0.02)
HAZ, *score/mo*	0.02 ± 0.03	0.02 ± 0.03	0.02 ± 0.04	0.03 ± 0.05	−0.004 (−0.01, 0.004)	−0.003 (−0.01, 0.01)	0.01 (−0.002, 0.01)

[1] Values are means ± SDs unless otherwise indicated. *Different from the RC group, $P < 0.05$ (repeated measures of general linear model). HAZ, height-for-age Z-score; LC, low-calcium; RC, regular-calcium; WAZ, weight-for-age Z-score.
[2] Weight and WAZ were adjusted for area, age, sex, HAZ, and iron and zinc status markers at baseline; height and HAZ were adjusted for area, age, sex, and WAZ and iron and zinc status markers at baseline.
[3] There were 3 missing values out of 988 observations (2 in the RC and 1 in the reuteri group).
[4] There were 68 missing values out of 3458 observations (12 in the LC, 18 in the RC, 19 in the casei, and 19 in the reuteri group).

anemia in children in developing countries and can be caused by gastrointestinal parasites (44), *Helicobacter pylori* (64), or allergy to cow milk protein (65). No information was available on the parasitic infestation or *H. pylori* prevalence, and no deworming program was applied in our study population. No adverse events related to cow milk protein were present in our study. Also, the considerable prevalence of respiratory (90%) and gastrointestinal (27%) infections in our study children may have negatively affected iron metabolism, known as the anemia of infectious disease (66,67). In addition, the polyphenolic compounds of cocoa powder added to our study milk may have inhibited iron absorption (68,69). A 5th group receiving no dietary intervention at all could have discriminated whether the decline in iron status was due the supplied milk products or to other causes as described above. Unfortunately, we did not include this group in our intervention as it was out of our main scope.

Conflicting results have been reported on the effect of calcium on iron absorption in adults and children, with great variability in study design, type and duration of supplementation, study sample by age, and setting (30,31,34,36,39,40). Most studies evaluating iron status were conducted in developed countries involving children and adolescents with adequate iron and calcium intakes and reported little or no effects on iron status (36,39,40). A long-term, 1-y intervention study in adolescent girls showed that daily calcium supplementation of 500 mg did not compromise iron status (40). With regard to zinc, to date, evidence from studies in children is lacking. In our study children, who had a low iron and calcium intake, regular-calcium milk did not adversely affect iron and zinc status compared with the LC group.

Strengths of our study were its double-blind design, long intervention duration, the excellent compliance and high response rate, the strict adherence to a rigorous protocol, and supervised anthropometric measurements by well-trained field-workers. Per-protocol analysis, excluding the few noncompliant participants (6%) and participants with chronic antibiotic usage, did not change the outcomes. A study drawback is its primary design to detect differences in intestinal infection incidence, and therefore the power to detect differences in growth and micronutrient status were not primary considerations in the design of the study. We were able to detect significant differences in the effect of *L. reuteri* on weight gain, WAZ changes, weight and height velocity, and *L. casei* on weight velocity even with a power of 21–73%. However, it is likely that the differences in other growth variables (height gain, HAZ changes, WAZ and HAZ velocity) in this study reflected low statistical power to detect significant improvements and would require a larger sample size to provide a more definitive conclusion. Moreover, the study was underpowered (1–46%) to detect significant differences in mean changes in iron and zinc status and prevalence of underweight, stunting, anemia, and iron and zinc deficiencies.

We included only nonbreastfed children in the study because of the following considerations: *1*) the well-known protective effect of breast milk against infection, possibly via influencing intestinal microbiota composition and/or activity (70), which may result in confounding with our probiotics intervention; *2*) difficulties in quantifying the amount of calcium supplied from breast milk to the children, leading to unknown variability in calcium intake and thus interpretation problems of the calcium intervention effect; and *3*) most importantly, the intervention should not interfere with the national breastfeeding program for young children and avoid mothers in refraining their children from breastfeeding.

In conclusion, *L. reuteri* DSM 17938 modestly, but significantly, improved growth, as shown by increasing weight gain and WAZ scores over 6 mo and greater mean monthly weight and height velocity in Indonesian children. Also, *L. casei* CRL 431 modestly improved monthly weight velocity. Regular-calcium milk did not affect any of the growth outcomes. Neither the probiotics nor regular-calcium milk affected iron and zinc status. The modest effect on growth of older children (mostly 4 to 6 y olds) supplemented with the probiotic *L. reuteri* in this study supports our previous finding on the reduced diarrhea incidence, especially in children with lower nutritional status, by this strain (41). These cumulative findings, all in the same positive direction, strengthen our conclusion that the reported effects of probiotics, especially *L. reuteri*, in the present study are truly beneficial and not just a coincidental finding. Nevertheless, before implementing the routine use of probiotics for child growth improvement, we suggest conducting another efficacy study in a similar setting to confirm our findings. Separate effectiveness

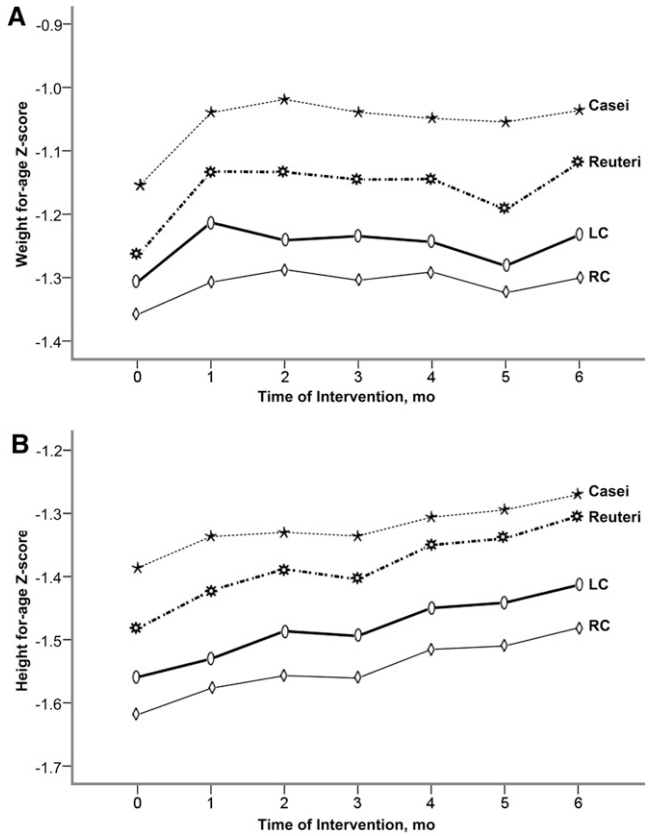

FIGURE 1 General linear model for repeated measurements showing treatment group–specific changes in (A) weight-for-age Z-score and (B) height-for-age Z-score in children who consumed milk with a low calcium concentration or a regular calcium concentration with or without probiotics at baseline; at months 1, 2, 3, 4, and 5; and after 6 mo of intervention. Each point represents the means ± SDs Z-score (n = 117 for the LC, n = 118 for the RC, n = 114 for the casei, and n = 117 for reuteri groups). LC, low-calcium; RC, regular-calcium.

studies are also needed to explore the acceptability, affordability, cost-effectiveness, practicality, feasibility, sustainability, and the potential risk of adverse effects for long-term use of probiotics in a community.

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