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Long-term benefits of probiotics and calcium supplementation during childhood, and other biomedical and socioenvironmental factors, on adolescent neurodevelopmental outcomes

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ABSTRACT

We evaluated the impact of 6-month probiotics and calcium supplementation at 1–6 years of age on neurodevelopment in adolescence, along with the effects of other biomedical and socioenvironmental factors. We re-enrolled 238 adolescents 10-years after supplementation with low-lactose milk with either low calcium (LC; ~50 mg/d; n = 53), regular calcium (RC; ~440 mg/d; n = 70), RC with $5x10^8$ CFU/d *Lactobacillus reuteri* DSM17938 (reuteri; n = 55), or RC with $5x10^8$ CFU/d *L. casei* CRL431 (casei; n = 60). Compared to RC, the casei group scored 0.38 SD (effect size, 0.04–0.72) higher on the Raven's Progressive Matrices; the reuteri group was 0.38 SD (0.01–0.75) lower on the Children's Depression Inventory; and the LC and younger adolescents in the reuteri group were 0.36 (0.01–0.71) and 0.49 SD (0.02–0.95) lower in brain-derived neurotrophic factor. Diet quality, physical activity, and home environment contributed similar effect sizes. Probiotics supplementation in childhood have strain-specific long-term neurodevelopmental benefits and integration with socioenvironmental interventions are warranted.

1. Introduction

Promoting child and adolescent neurodevelopment is a global priority, especially in low-to-middle-income countries (LMICs). Risk factors for poor neurodevelopment include recurrent gastrointestinal and other infections, poor nutritional status, and a suboptimal home environment, all of which are prominent in LMICs (Grantham-McGregor et al., 2007). A recent report on 35 LMICs between 2005 and 2015 estimated that 80.8 million children aged 3–4 years experienced low cognitive or socio-emotional development, with large proportions from sub-Saharan Africa, South Asia, East Asia, and the Pacific (McCoy et al., 2016). Moreover, about 37% of all children from LMICs performed poorly in at least one developmental domain. This raises concerns for both childhood and throughout life. Given the emerging evidence of probiotics on reduced morbidity in children, and the interplay between the childhood gut microbiome and brain development, probiotics may promote

Abbreviations: ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; BDNF, brain-derived neurotrophic factor; BMI, body mass index; CDI, Children's Depression Inventory; CFU, colony-forming unit; CREB, cAMP response element-binding protein; CRS-I, calcium response sequence-I; DQI-A, Diet Quality Index for Adolescents; EA-HOME-A, Abbreviated Early Adolescent Home Observation and Measurement of the Environment; GLM, general linear model; LC, low calcium; LMICs, low-to-middle-income countries; LPS, lipopolysaccharides; PAQ-C, Physical Activity Questionnaire for Older Children; RC, regular calcium; RCTs, randomized controlled trials; RPM, Raven's Progressive Matrices; SCFAs, short-chain fatty acids; SD, standard deviation; SDQ, Strength and Difficulties Questionnaire.

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healthy gut and brain development in childhood and into adolescence and later life (Cusick & Georgieff, 2016; Furnham & Cheng, 2016).

Current studies suggest the existence of a gut-brain axis wherein the gut microbiome influences brain development and function across the lifespan (Jasarevic et al., 2016; Liang et al., 2018). The dynamics of the gut-brain axis in early childhood underscore this symbiosis; and because gut microbiota colonization occurs in childhood, targeted intervention at this critical time could affect short and long-term neurodevelopment (Diaz Heijtz, 2016; Gensollen et al., 2016). To promote a robust gut-brain axis, interventions have been explored including childhood probiotics and calcium supplementation. Probiotics bacteria can produce short-chain fatty acids (SCFAs) with a broad range of effects, including immunomodulation, and regulation of brain-derived neurotrophic factors (BDNF) through inhibition of histone deacetylase (Moens et al., 2019; Stilling et al., 2016). In addition, calcium intake may increase resistance to enterotoxigenic bacteria (Bovee-Oudenhoven et al., 1997; Bovee-Oudenhoven et al., 2003), thereby reducing the risk of diarrhea and adverse effects of lipopolysaccharide (LPS) translocation to the bloodstream, which can disrupt the blood–brain barrier and increase neuroinflammation, leading to suboptimal brain development (Noble et al., 2017). However, randomized controlled trials (RCTs) of probiotics in children and their effects on neurodevelopmental outcomes are limited.

A recent systematic review of probiotics supplementation on child cognition found only 7 RCTs, with one demonstrating benefits of reduced risk of attention deficit hyperactivity disorder (ADHD) or Asperger syndrome (i.e. autism) (Rianda et al., 2019). However, while children in LMICs are at higher risk of suboptimal neurodevelopment, only a few properly designed RCTs were conducted in LMICs, and the majority of the trials did not examine long-term effects. Perhaps most important, studies may not have used probiotics strains that affect neurodevelopment (Fernald et al., 2017; Prado et al., 2017). For example, a previous follow-up study of maternal and child supplementation of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium animalis* subsp. *lactis* HN019 given until 2 years of age in New Zealand found no effect on child cognition, behavior, and mood after 11 years (Slykerman et al., 2018). In contrast, a 13-year follow-up study in Finland observed a long-term benefit of maternal *L. rhamnosus* GG supplementation on reduced risk for ADHD or autism (Pärtty et al., 2015).

In 2007–2008, Agustina et al. (Agustina et al., 2012, 2013) conducted a double-blind RCT of probiotic *L. reuteri* DSM17938 or *L. casei* CRL 431 and calcium supplementation in Indonesian children1–6 years old. The *L. reuteri* DSM17938 reduced the risk of diarrhea, particularly among children with low nutritional status. Both *L. reuteri* DSM17938 and *L. casei* CRL431 demonstrated modest effects on improved child growth. These effects portend long-term neurodevelopmental impact mediated by better nutritional status, fewer infections, and potentiation of the gut-brain axis. Hence, this study aimed to evaluate the long-term effect of probiotics and calcium supplementation during childhood, and other biomedical and socioenvironmental factors, on cognition, mood, behavior, and serum BDNF levels in adolescents aged 11–18 years old in a LMIC.

2. Materials and methods

2.1. Study design

The 6-month RCT of probiotics and calcium supplementation in children has been described elsewhere (Agustina et al., 2012, 2013). In brief, a randomized double-blind, controlled trial was conducted in two low socioeconomic urban areas in East Jakarta, Indonesia, representing flooding and non-flooding areas. Subjects were stratified by dwelling location, flooding vs. non-flooding, age, and gender at enrollment and randomly assigned to receive one of four types of milk. The four types of milk, all of which were low-lactose ultra-high temperature sterilized milk, differed (a) in their calcium content and (b) whether they contained *Lactobacillus,* and (c) the strain of *Lactobacillus* they contained. The four groups were: LC, low calcium providing ~50 mg/day of calcium and no *Lactobacillus*, (2) RC, regular calcium providing 440 mg/day and no *Lactobacillus*, (3) reuteri, regular calcium providing 440 mg/day along with $5x10^8$ colony-forming unit (CFU)/day *L. reuteri* DSM17938, and (4) casei, providing 440 mg/day and $5x10^8$ CFU/day *L. casei* CRL431 (casei group). The products given to the four groups were indistinguishable to the subjects and investigators, and their composition has been detailed elsewhere (Agustina et al., 2012, 2013).

The original trial is registered at clinicaltrials.gov (NCT00512824) and was approved, along with the re-enrollment study (NCT04046289), by the Medical Ethics Committee of the Faculty of Medicine, Universitas Indonesia, and the local government (Agustina et al., 2012, 2013). All participants and the parents submitted written informed consent and assent for the original and follow-up studies.

2.2. Participants

From the 494 children enrolled and analyzed for primary outcomes of the RCT in 2007–2008, we re-enrolled 238 (48.2%) subjects in adolescence for the 10-year follow-up study (Fig. 1). Of 256 adolescents who were not re-enrolled, 41 (8.3%) had moved outside Jakarta province, 46 (9.3%) refused to participate, 3 (0.6%) died, and 166 (33.6%) could not be traced. These proportions were similar across intervention groups. Of 238 subjects tested for the neurodevelopmental outcomes and serum BDNF, 53 (22.3%) were in the LC group, 70 (29.4%) in the RC group, 60 (25.2%) in the casei group, and 55 (23.1%) in the reuteri group. Among them, 1 participant in the RC group refused to perform the Raven's Progressive Matrices (RPM) test and 1 in LC group refused to take the serum BDNF measurement. All participants, parents, field workers, laboratory personnel, and investigators, except the principal investigator and team of the original study (R.A., F.J.K.), were blinded to the treatment group until data analyses were finished and the blinded review of results was completed.

2.3. Procedures

Biomedical (i.e. anemia, diet quality) and socioenvironmental (i.e. living area, home environment) factors related to neurodevelopmental outcomes were assessed in the re-enrollment study. The data for exclusive breastfeeding, the proportion of stunting at the end-line of the trial, and age at enrollment were from the original study. Parents or legal guardians were interviewed to obtain the data related to the socioeconomic status of the family, participants provided the history of antibiotic consumption $\geq$ 10 days and the presence of chronic infection (e.g. tuberculosis, leprosy, chronic suppurative otitis media, chronic rhinosinusitis) in the past 10 years.

Anthropometry at re-enrollment was performed by certified nutritionists (Setiawan et al., 2021). Adolescents were weighed without shoes using an electronic scale (Seca model 876) and the mean of two measurements to the nearest 0.1 kg was used. Height was assessed with a wooden board (ShorrBoard) and the mean of two measurements to the nearest 0.1 cm was used. Hemoglobin was measured from venous blood using the HemoCue 201. Anemia was defined according to WHO recommendations (WHO, 2011).

We evaluated dietary quality using the Diet Quality Index for Adolescents (DQI-A) (Vyncke et al., 2013). Dietary data were collected by trained personnel using two non-consecutive 24-h food recalls representing weekends and weekdays. Food models and a book displaying portion sizes of the foods were used to guide the estimation of portion sizes. Data obtained were grouped into 9 recommended food groups consisting of (1) water, (2) bread and cereals, (3) grains and potatoes, (4) vegetables, (5) fruit, (6) milk products, (7) cheese, (8) meat, fish, eggs and substitutes, and (9) fat and oils. Furthermore, dietary quality, dietary diversity, and dietary equilibrium scores based on the grouping were calculated to obtain DQI-A for each food recall. The mean of daily

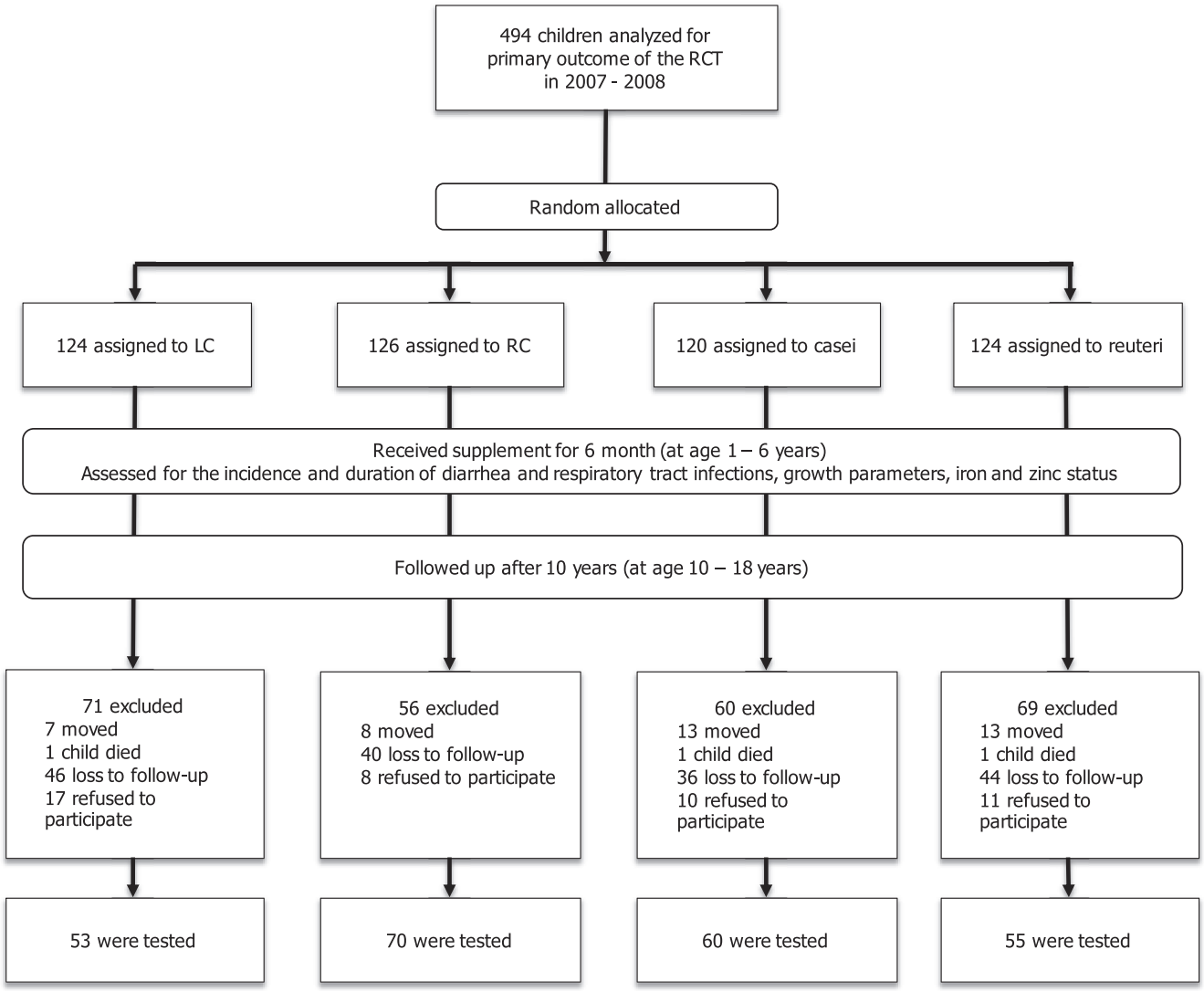

Fig. 1. CONSORT diagram of the 10-year follow-up study.

DQI-A scores for each respondent was calculated and ranged from –33 to 100%, in which a higher score reflects better diet quality.

Physical activity was quantified by the Physical Activity Questionnaire for Older Children (PAQ-C), which consists of 9 items of 7-day activity recalls designed to assess elementary and middle school children's activity in a field-based setting (Wang et al., 2016). Adolescents were asked to recall their activities in the past seven days to answer all 9 items to calculate the PAQ-C score in which a higher score represents higher engagement to physical activity. If respondents were prevented from engaging in regular physical activity (e.g. due to an illness) or reported that their physical activity would change after the initial contact, the questionnaire was re-administered the following week.

Home environment nurturing, support, and stimulation was assessed using the Abbreviated Early Adolescent Home Observation and Measurement of the Environment (EA-HOME-A) Inventory (Green et al., 2018). Trained assessors with a Bachelor's of Psychology or Communication degree visited participants' homes and evaluated the 44-items of the EA-HOME-A Inventory through both interview and observation. The six domains measured were (1) physical environment, (2) learning materials/enriched surroundings, (3) variety of experiences and family social engagement, (4) acceptance and responsivity, (5) regulatory activities: risk tasking, and (6) regulatory activities: rules and routines. Binary (Yes = 1; No = 0) scoring was used to calculate the total score, which ranged from 0 to 44.

2.4. Outcomes

The RPM test was administered by experienced psychologists who were otherwise not involved in the original nor follow-up study and were blinded to the randomization. RPM is designed to measure non-verbal general intelligence in field-based settings using the progressive matrices method which is purported to be independent of formal schooling and language. The RPM test has been widely used in many studies across Asia, including Indonesia (Sandjaja et al., 2013). Tests were conducted in a well-lit comfortable place that was free of noise. Data from the RPM was obtained as raw scores which were converted into age and sex-adjusted z-scores before final analysis. A higher RPM score indicates better performance on the test.

Children's Depression Inventory (CDI) was a 27-item self-administered questionnaire that is sensitive to changes in depressive symptoms over time and intended to assess children's and adolescents' levels of depression (Bang et al., 2015; Yusuf, 2019). Participants were asked to choose 1 of 3 provided statements for each item, resulting in a 3-point ordinal scale, based on what they felt in the last two weeks related to the depressive state, such as anhedonia, irritability, indecisiveness, loneliness, and feeling unloved. Total scores were adjusted

with age and sex and converted to z-scores. The lower depressive state was indicated by a lower CDI score.

Participants were required to administer the Strength and Difficulties Questionnaire (SDQ) to evaluate their emotional and behavioral problems (Wiguna et al., 2010; Yusuf, 2019). SDQ comprises 25 items in which each item is scored as 0,1, or 2. The total difficulties score was quantified by summing the domain scores for hyperactivity, conduct problems, emotional symptoms, and peer problems. A lower total difficulties score is interpreted as having lower emotional and behavioral problems.

We evaluated the serum brain-derived neurotrophic factor (BDNF) as a tracer neurotrophin for the gut-brain axis. Previous reports have associated reduced BDNF levels with depression (Aydemir et al., 2007; Galvez-contreras et al., 2016). BDNF was measured in venous blood samples of the subjects obtained at 08.00–10.00 AM after overnight fasting. About 2 mL of blood were placed into a clean dry tube, left to clot at room temperature, and serum was collected after 15-min centrifugation. The serum was stored at –80 °C until analysis. We avoided repeated freeze–thaw cycles to prevent the loss of bioactive substances. Free serum BDNF was measured by sandwich-ELISA using a commercial kit according to the manufacturer's instructions (Quantikine ELISA, R&D Systems, Inc., USA) with a lower detection limit of < 20 pg/mL, and recorded in ng/mL.

2.5. Statistics

We used SPSS version 20.0 to analyze all data. We evaluated whether baseline characteristics were comparable between the found and unfound (i.e. subjects who moved, died, lost to follow-up, refused to participate) children within each intervention group, in addition to comparing the biomedical and socioenvironmental characteristics between groups in the follow-up study. BMI of each subject was converted into a z-score using WHO AnthroPlus software based on the WHO Reference 2007 (WHO, 2009). We converted our outcome variables, RPM, CDI, and SDQ scores, measured in adolescence into z-scores adjusted for age at follow-up and sex due to the associations of the outcome scores with those variables. DQI-A score, PAQ-C score, and EA-HOME-A score, risk factors measured in adolescence, were converted into z-scores based on age at which the outcome was measured and sex.

General linear models (GLM) were used to investigate the effect of probiotics and calcium supplementation, and the associations with biomedical and socioenvironmental factors, on our outcome variables, RPM score, CDI score, and SDQ score. Higher RPM, lower CDI, and lower SDQ scores indicated better adolescent status. Living area, history of stunting, PAQ-C score, and EA-HOME-A inventory z-score were included in the model analyzing the effect of the supplementation on RPM, CDI, and SDQ scores. For the outcome of BDNF, results were presented as effect sizes calculated by dividing the B coefficient with the pooled standard deviation.

To understand other factors that may have affected each neurodevelopmental outcome in this extended 10-year interval study, we included covariates from the original trial and those collected at the follow-up into the model and compared their effect sizes on outcomes. The covariates from the original trial were exclusive breastfeeding and stunted at the end-line of the trial, while from the follow-up study covariates were anemia, DQI-A score, PAQ-C score, living area, family financial distress, maternal education, and EA-HOME-A Inventory score. These were categorized as biomedical or socioenvironmental factors.

We evaluated the effect of the supplementation during childhood on serum BDNF with an adjustment for age, sex, and body mass index-for-age z-score. To explore whether the effect was consistent in any subgroup among the subjects, age and sex-based stratification were performed. Furthermore, we analyzed the determinants predicting serum BDNF among adolescents by multivariable regression analysis. Because of its nature as a biological outcome as compared to the other outcomes, different covariates were used, which included age, sex, exclusive breastfeeding, stunting at the end-line of the trial, anemia, BMI-for-age z-score, DQI-A score, PAQ-C score, and living area of the adolescents.

3. Results

Baseline characteristics of the 238 found and 256 unfound children within each intervention group were relatively similar, except for the gender proportion in reuteri group, which was accommodated by using age and sex-adjusted z-score for the neurodevelopmental outcomes. Among the 238 re-enrolled participants, adolescents in each intervention group were comparable with regard to the biomedical and socioenvironmental factors, except for the proportion of stunting at the end of the trial which was the outcome of the original trial (Table 1), and was taken into account in the multivariable analysis of the long-term effects of supplementation.

Table 2 presents the effects of probiotics and calcium supplementation in childhood on the RPM score, CDI score, and total difficulties score as measured by SDQ at the age of 11–18 years. The effect of *L. casei* CRL431 (casei group) on RPM score was 0.38 SD (95 %CI 0.04–0.72; p = 0.03) higher as compared with the RC group. However, we found no significant effect on this score in the other groups. On the outcome of CDI score in which a lower score indicates lower depressive state, children in the reuteri group scored 0.38 SD (95% CI 0.01–0.75; p = 0.044) lower than the RC group. Neither children in the casei group nor the LC group demonstrated significant differences compared to the RC group. For SDQ there was no effect found across all comparisons.

Multiple regression models using GLM to assess biomedical and socioenvironmental factors on RPM score, CDI score, and total difficulties score of SDQ are presented in Table 3. Specifically, better diet quality, higher engagement in physical activity, and EA-HOME-A inventory at follow-up were associated with better scores for RPM and CDI. The biomedical factor significantly associated with a better RPM score was diet quality, with an effect size of 0.13 SD. Meanwhile, a higher EA-HOME-A inventory score as part of socioenvironmental factors accounted for a similarly higher RPM score. In predicting CDI score, only biomedical factors showed significant association at the 95% CI level. Higher PAQ-C score at follow-up, which illustrates a higher physical activity, had effect sizes of 0.14 SD in lowering the CDI score at the age of 11–18 years. However, no covariate was found to be associated with SDQ.

When we evaluated the effect of probiotics and calcium supplementation on the serum BDNF in all subjects, a higher mean of 0.36 SD (95 %CI 0.01–0.71; p = 0.048) was observed in the RC group as compared to LC (Table 4). In adolescents above 15 years old and male, the RC group consistently showed higher serum BDNF level of 0.75 SD (95 %CI 0.28 to 1.23 SD; p = 0.002) and 0.48 SD (95 %CI 0.08–0.89; p = 0.02), respectively. Although children in the casei and reuteri groups had lower serum BDNF concentration as compared to the RC group, the effects were not significant. However, when stratified by age, *L. reuteri* DSM17938 demonstrated a lower mean of 0.49 SD (95 %CI 0.02–0.95; p = 0.04) compared to RC in subjects 15 years old and below. Finally, among covariates included in regression analysis, increased BMI-for-age z-score and higher DQI-A score at follow-up were associated with higher serum BDNF among adolescents as shown in Fig. 2.

4. Discussion

In this study, the addition of probiotics of *L. casei* CRL431 and *L. reuteri* DSM17938 to regular-calcium milk in childhood demonstrated potential long-term benefits on neurodevelopmental outcomes, and with strain-specific effects. To the best of our knowledge, this is the first study for *L. casei* CRL431 and the second for *L. reuteri* DSM17938, to evaluate the effects of childhood supplementation on neurodevelopmental outcomes (Akar et al., 2017; Rianda et al., 2019).

Several factors support the validity of these findings. First, the original trial showed balance at baseline, high compliance for its 6-

Table 1
Baseline characteristics of adolescents by treatment group.

	Intervention group							
	LC		RC		Casei		Reuteri	
	Found (n = 53)	Unfound[1] (n = 73)	Found (n = 70)	Unfound[1] (n = 54)	Found (n = 60)	Unfound[1] (n = 60)	Found (n = 55)	Unfound[1] (n = 69)
Baseline characteristics of original trial								
Aged < 5 years old	31 (58.5)	29 (40.8)	34 (48.6)	26 (46.4)	30 (50)	25 (41.7)	27 (49.1)	32 (46.4)
Living in flooding area, n (%)	36 (67.9)	45 (63.4)	43 (61.4)	39 (69.6)	40 (66.7)	38 (63.3)	39 (70.9)	43 (62.3)
Male, n (%)	25 (47.2)	42 (59.2)	34 (48.6)	34 (60.7)	33 (55)	33 (55)	20 (36.4)	48 (69.6)
Born by vaginal delivery[2], n (%)	48 (90.6)	69 (97.2)	66 (94.3)	55 (98.2)	58 (96.7)	54 (91.5)	51 (92.7)	64 (92.8)
Exclusively breastfed[3], n (%)	10 (18.9)	14 (19.2)	14 (20)	11 (20.8)	8 (13.3)	8 (13.3)	11 (20)	11 (15.9)
Without anemia[4], n (%)	40 (75.5)	60 (84.5)	51 (73.9)	41 (73.2)	45 (75)	51 (85)	42 (76.4)	58 (84.1)
Follow-up characteristics								
Age at follow-up assessment, years	15.3 ± 1.25	–	15.3 ± 1.21	–	15.4 ± 1.34	–	15.3 ± 1.22	–
Living in flooding area, n (%)	35 (66)	–	43 (61.4)	–	40 (66.7)	–	39 (70.9)	–
Stunted at the end-line of the trial[5], n (%)	14 (26.4)	–	22 (31.4)	–	9 (15)	–	7 (12.7)	–
Without anemia at follow-up[6], n (%)	36 (67.9)	–	50 (71.4)	–	48 (80)	–	41 (74.5)	–
BMI-for-age z-score	−0.45 (1.1)	–	−0.2 (1.4)	–	−0.2 (1.3)	–	−0.08 (1.3)	–
History of antibiotic consumption ≥ 10 days or presence of chronic infection, n (%)	11 (20.8)	–	16 (22.9)	–	11 (18.3)	–	8 (14.5)	–
Maternal education < 9 years, n (%)	22 (41.5)	–	23 (32.9)	–	17 (28.3)	–	15 (27.3)	–
No financial distress[7], n (%)	39 (73.6)	–	57 (81.4)	–	50 (83.3)	–	44 (80)	–

Values are mean ± standard deviation or median (min – max), or n (%). BMI, body mass index.
[1] Including subjects who moved, died, loss to follow-up, and refused to participate in the follow-up study.
[2] 1 unfound children in LC group did not have complete information on this variable.
[3] 1 unfound children in RC group did not have complete information on this variable.
[4] Defined as hemoglobin > 11 g/dL for child < 5 years or hemoglobin > 11.5 g/dL for child ≥ 5 years. One found children in RC group did not have complete information on this variable.
[5] Defined as height-for-age Z-score < -2 SDs at the end-line of the original trial.
[6] Defined as hemoglobin ≥ 11.5 g/dL (child < 12 years) or ≥ 12.0 g/dL (child aged 12 to < 15 years and female adolescent aged ≥ 15 years) or ≥ 13.0 g/dL (male adolescent aged ≥ 15 years).
[7] Defined as a condition of a family whose monthly income met their financial outlay.

Table 2
Effects of childhood probiotics and calcium supplements on cognitive function, mood, and behavior in adolescence.

	Intervention group					Effect size[1] (95% CI)				
	LC (n = 53)	RC[2] (n = 70)	Casei (n = 60)	Reuteri (n = 55)	RC vs LC	p^3	Casei vs RC	p^3	Reuteri vs RC	p^3
RPM z-score[4]	−0.11 ± 0.95	−0.18 ± 0.99	0.25 ± 0.98	0.06 ± 1.01	−0.03 (−0.38 to 0.31)	0.85	0.38 (0.04 to 0.72)	0.03	0.21 (−0.15 to 0.56)	0.26
CDI z-score[5]	−0.003 ± 0.91	0.18 ± 1.15	−0.04 ± 0.90	−0.19 ± 0.94	0.18 (−0.19 to 0.55)	0.34	−0.22 (−0.58 to 0.14)	0.23	−0.38 (−0.75 to −0.01)	0.044
Total difficulties z-score[6]	0.05 ± 0.99	−0.004 ± 0.97	−0.001 ± 1.07	−0.05 ± 0.98	−0.05 (−0.40 to 0.30)	0.77	−0.02 (−0.38 to 0.33)	0.89	−0.15 (−0.50 to 0.19)	0.39

Z-score are adjusted for age and gender. BDNF, brain-derived neurotrophic factor; CDI, Children Depression Inventory; LC, low calcium. RC, regular calcium; RPM, Raven's Progressive Matrices.
[1] Adjusted for living area, history of stunting, PAQ-C score, and EA-HOME-A inventory score.
[2] n = 69 for RPM score in RC group.
[3] *P* values were calculated using general linear model.
[4] Higher RPM z-score indicates better performance on the test.
[5] Lower CDI z-score indicates lower depressive state.
[6] Lower total difficulties z-score as assessed by the Strength and Difficulties Questionnaire indicates lower emotional and behavioral problems.

months duration, limited loss to follow-up, and clear impact (Agustina et al., 2012, 2013). Second, in this 10-year follow-up study, while about half were re-enrolled, subject characteristics across intervention groups were comparable with the exception of gender in one group, which we could adjust for, and stunting in the casei and reuteri groups which were part of the original impact, and potentially part of the mechanism of the observed effects. Third, assessment of outcomes and other covariates, such as home environment, was done by trained and standardized graduate students with relevant expertise. Lastly, our data confirmed known associations between socioenvironmental factors and cognitive performance, thereby supporting the validity of our methods.

In addition, our results are in line with studies that investigated the effect of *L. casei* on various outcomes, including neurocognition. Multi-strain probiotics supplementation which included *L. casei* was shown to improve cognitive reactivity and the Mini-Mental State Examination score in adult populations (Akbari et al., 2016; Steenbergen et al., 2015). However, only a few studies specifically evaluated the effect of the strain *L. casei* CRL431. In the original study by Agustina et al. (Agustina et al., 2013), *L. casei* CRL-431 supplementation modestly increased monthly weight gain velocity. Interestingly, animal and transcriptomic studies have demonstrated the specific role of *L. casei* CRL431 in immune activation in the intestinal and respiratory tract through mucosal gene-

Table 3
Adjusted effect sizes of determinants on neurodevelopmental outcomes from multivariable regression analysis.

	RPM z-score (n = 237)	p^1	CDI z-score (n = 238)	p^1	Total difficulties z-score (n = 238)	p^1
Biomedical factors						
Exclusively breastfed	−0.11 (−0.43 to 0.22)	0.52	−0.16 (−0.35 to 0.32)	0.92	0.24 (−0.10 to 0.57)	0.17
Not stunted at the end-line of the trial	−0.11 (−0.42 to 0.21)	0.50	−0.003 (−0.32 to 0.32)	0.99	0.05 (−0.27 to 0.38)	0.76
Without anemia at follow-up	−0.18 (−0.46 to 0.10)	0.22	0.08 (−0.21 to 0.37)	0.61	0.22 (−0.07 to 0.51)	0.14
DQI-A score at follow-up (z-score)	0.13 (0.01 to 0.26)	0.03	−0.03 (−0.16 to 0.09)	0.60	−0.05 (−0.18 to 0.08)	0.42
PAQ-C score at follow-up (z-score)	−0.05 (−0.18 to 0.07)	0.42	−0.14 (−0.27 to −0.01)	0.03	−0.03 (−0.16 to 0.10)	0.64
Socioenvironmental factors						
Living in non-flooding area	0.25 (−0.02 to 0.51)	0.07	0.06 (−0.21 to 0.34)	0.66	0.02 (−0.26 to 0.30)	0.90
No financial distress	−0.07 (−0.38 to 0.23)	0.64	0.22 (−0.10 to 0.54)	0.17	0.14 (−0.18 to 0.47)	0.38
Higher maternal education (≥6 years)	0.17 (−0.10 to 0.44)	0.22	0.07 (−0.20 to 0.35)	0.60	−0.05 (−0.33 to 0.23)	0.73
EA-HOME-A inventory z-score	0.14 (0.01 to 0.26)	0.03	−0.02 (−0.15 to 0.11)	0.75	0.04 (−0.09 to 0.17)	0.52

Values are effect sizes in standard deviation. CDI, Children Depression Inventory. DQI-A, Diet Quality Index for Adolescents; EA-HOME-A, Abbreviated Early Adolescent Home Observation and Measurement of the Environment; PAQ-C, Physical Activity Questionnaire for Older Children; RPM, Raven's Progressive Matrices.
[1] *P* values were calculated using general linear model.

Table 4
Effects of childhood probiotics and calcium supplements on serum BDNF by age and sex of the adolescents.

	Mean serum BDNF in ng/mL				Effect size[1] (95% CI)					
	RC (n = 69)	LC (n = 53)	Casei (n = 60)	Reuteri (n = 55)	RC vs LC	p[2]	Casei vs RC	p[2]	Reuteri vs RC	p[2]
Overall	31.7 ± 7.6	28.6 ± 7.9	30.3 ± 8.2	29.6 ± 7.7	0.36 (0.01 to 0.71)	0.048	−0.19 (−0.52 to 0.15)	0.27	−0.27 (−0.61 to 0.08)	0.14
Age										
≤ 15 years	31.8 ± 8.3	30.7 ± 8.0	29.9 ± 6.8	28.3 ± 7.2	0.1 (−0.38 to 0.59)	0.67	−0.24 (−0.69 to 0.21)	0.30	−0.49 (−0.95 to −0.02)	0.04
> 15 years	31.6 ± 7.0	25.6 ± 6.6	30.6 ± 9.5	31.1 ± 8.0	0.75 (0.28 to 1.23)	0.002	−0.12 (−0.63 to 0.38)	0.63	−0.07 (−0.56 to 0.41)	0.77
Sex										
Male	31.6 ± 6.4	30.0 ± 6.6	31.7 ± 7.6	29.6 ± 8.2	0.48 (0.08 to 0.89)	0.02	−0.02 (−0.44 to 0.4)	0.93	−0.29 (−0.78 to 0.19)	0.24
Female	31.7 ± 8.8	29.3 ± 9.1	28.5 ± 8.7	29.6 ± 7.5	0.02 (−0.56 to 0.6)	0.94	−0.27 (−0.82 to 0.28)	0.34	−0.24 (−0.72 to 0.24)	0.32

BDNF, brain-derived neurotrophic factor; LC, low calcium; RC, regular calcium.
[1] Adjusted for age, sex, and BMI-for-age z-score.
[2] *P* values were calculated using general linear model.

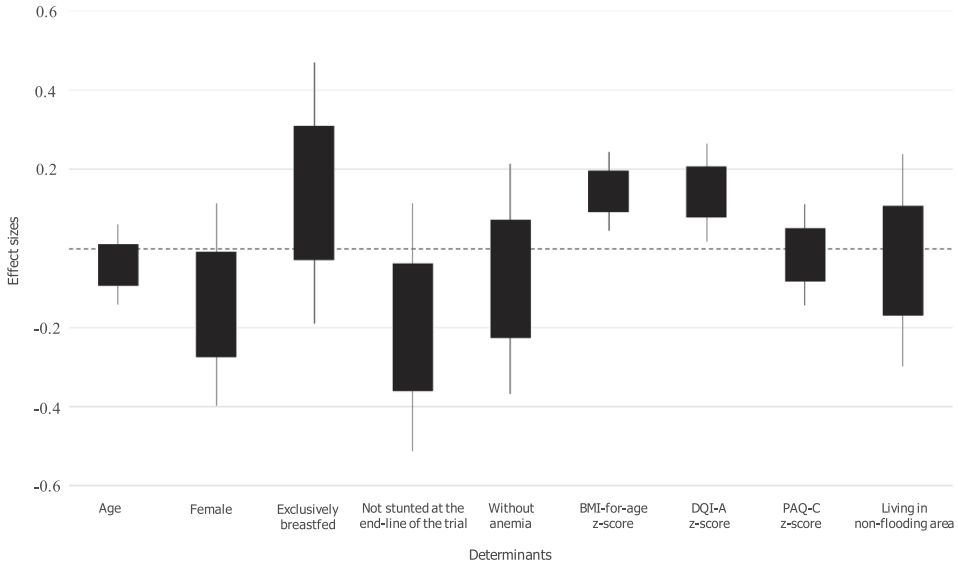

Fig. 2. Adjusted effect sizes of serum BDNF determinants from multivariable regression analysis.

expression networks regulating the balance between Th1 and Th2 (Marranzino et al., 2012; Van Baarlen et al., 2011), which resulted in a greater increase of IFN-γ and mobilization of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells from the gut to the lungs; an observation not seen for other strains of probiotics. However, there has been no previous study investigating whether the strain-specific capability of *L. casei* CRL431 on immune

activation extends to improved cognition through the 'gut-immune-brain axis' (van Sadelhoff et al., 2019).

Our study also identified the long-term impact of *L. reuteri* DSM17938 in reducing depressive state measured using CDI. We note a previous study by Akar et al. wherein supplementation of preterm infants with $1x10^8$ CFU/d *L. reuteri* DSM17938 did not demonstrate any effect on neurosensory, neuromotor and cognitive outcomes at 18–24 months (Akar et al., 2017). However, the younger subjects, lower dosage, and shorter duration of intervention compared to our study limits comparability. In several studies, *L. reuteri* DSM17938 has consistently shown benefits in reducing the diarrhea duration and incidence in children (Agustina et al., 2012; Gutierrez-Castrellon et al., 2014; Urbanska et al., 2016). Moreover, the beneficial impact on growth as indicated by greater weight gain, weight-for-age Z-score, and monthly weight and height velocities was also observed in our original study (Agustina et al., 2013). These effects of *L. reuteri* DSM17938 support improved intestinal tight junction integrity, which might contribute to reduced lipopolysaccharides translocation and prevent disruption of the blood–brain barrier (Noble et al., 2017). However, to the best of our knowledge, no previous study had linked the role of *L. reuteri* DSM17938 in supporting intestinal integrity to better neurodevelopment.

Our study supports the idea that specific strains of probiotics may impact brain function and specific outcomes. *L. casei* CRL431 provided an effect on cognition as measured by RPM but not on the depressive state (i.e. mood). Conversely, the benefit on mood using CDI was observed in *L. reuteri* DSM17938 with no effect on cognitive abilities. To explain strain-and outcome-specific effects, further studies are needed that integrate the transcriptomic and proteomic profiles of strains and their effect in the gut and brain. This will require sensitive markers of changes in brain activity within specific regions related to particular neurodevelopmental outcomes. Hence, the distinctive characteristic of specific brain regions should be considered in designing studies to explore pathways of the gut-brain axis. For instance, a recent *meta*-analysis reported that divergent nerve clusters were activated under emotional versus cognitive stimulation. Cognitively demanding conditions altered the right medial frontal and insula regions, whereas emotional (cognitively undemanding) conditions were associated with greater activation of the bilateral amygdala (Palmer et al., 2015). Biological perturbations that easily impact these regions could be manifested in specific neurodevelopmental outcomes. For instance, brain regions related to cognition such as the hippocampus, the dorsolateral caudate nucleus, and the reticular nucleus of the thalamus are sensitive to reduction in blood flow and disturbances in brain energy metabolism (Hossmann, 1999). Considering this, certain strains of probiotics which are beneficial in supporting the blood flow and balance of energy might improve cognition. Interestingly, a study of human mucosal *in vivo* transcriptome responses to *L. casei* CRL431 demonstrated an up-regulation of genes involved in blood-vessel development, such as endothelin-1 (Van Baarlen et al., 2011). Thus, the distinctive nature of each strain in influencing the gut-brain axis (e.g. through the immune system, vagal nerve, SCFA production), and specific regions in the brain, warrants exploration.

Long-term benefits of milk consumption with regular calcium content as compared to low calcium during childhood on serum BDNF was observed in our study, wherein this effect was more pronounced in males and $>$ 15 years of age. This is consistent with recent studies that reported the association between variants of the BDNF gene with lower calcium intake (Dušátková et al., 2015; Marcos-Pasero et al., 2019). BDNF has a core role in synaptic plasticity by regulating its structure and function. Furthermore, this role may be modulated by changes in feeding and fasting (Locke et al., 2015). For example, some BDNF variants have been associated with energy homeostasis in relation to obesity in childhood and adolescence (Zhao et al., 2009). A few studies have described the role of calcium influx in regulating the transcription of BDNF in cortical neurons through the calcium response sequence, CRS-I, and activation of the cAMP response element-binding protein (CREB) (Shieh et al., 1998). Although we found a more profound benefit of calcium supplementation on serum BDNF in male and older adolescents (i.e. older age at enrollment), it remains unknown whether the finding is related to varied fractional calcium absorption during the lifespan and between genders, thereby enabling a particular group to gain benefits from better calcium intake and absorption (Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium, 2011). Along with calcium supplementation, better diet quality and higher BMI-for-age z-score were associated with higher serum BDNF, in which the former has been known to maintain membrane integrity for BDNF signaling through TrkB receptor (Gomez-Pinilla & Tyagi, 2013), whereas the association with the latter might be due to the increased fat mass accompanied by elevation of leptin level and further stimulation of BDNF production (Noble et al., 2011).

However, the results on serum BDNF level should be interpreted carefully as some conflicting results were found in studies investigating the association between serum BDNF level with neuropsychiatric outcomes. Declining levels of serum BDNF in children were associated with depression (Sun et al., 2017). Whereas other studies observed elevated serum BDNF in children with autism spectrum disorder (ASD), including attention deficit hyperactivity disorder (Zhang et al., 2018). In the current study, we found that *L. reuteri* DSM17938 supplementation resulted in lower serum BDNF among adolescents aged 15 years and below, which may indicate a better impact of probiotics when given at a younger age when the development of the gut microbiota is more dynamic (Jasarevic et al., 2016). The observed impact of *L. reuteri* DSM17938 on a reduced level of serum BDNF is similar to the result of a randomized trial by Riezzo et al. (Riezzo et al., 2019) that found a significantly lower serum BDNF concentration after 105 days of *L. reuteri* DSM17938 administration. The clinical outcomes of lower serum BDNF demonstrated by *L. reuteri* DSM17938 supplementation have not been extensively explored. However, it is worth mentioning that gut microbiota dysbiosis is prominent in ASD, a disorder that has been associated with higher BDNF concentration as mentioned previously. The underlying mechanism might be related to the BDNF hyperactivity resulting in the synaptic overgrowth and deficits of synaptic pruning, which are commonly found in ASD children (Chomiak & Hu, 2013; Tang et al., 2014). However, we did not evaluate the effect of *L. reuteri* DSM17938 on any autism-related outcomes. Thus, it is still unclear whether the lower BDNF serum found in the reuteri group as compared to the RC group is related to any clinical outcomes. Similarly, the impact of higher BDNF serum through regular-calcium milk consumption as compared to low-calcium content on clinical benefits is still unknown and should be addressed in further studies. Nonetheless, our findings on both reduced and increased serum BDNF via probiotic and calcium supplementation might hint at the importance of maintaining the level in a physiologically optimum range (i.e. not too high and not too low), as this balance is observed in other biological substances, such as leptin, through feedback mechanisms (Jequier, 2002).

Our study highlights the importance of combined biomedical (i.e. diet quality, physical activity) and socioenvironmental (i.e. home environment) factors in promoting better child and adolescent neurodevelopment. With similar effect sizes, both factors notably demonstrated their associations with cognition and depressive state among adolescents. Better diet quality, as reflected by high fiber consumption and low intake of saturated fat and refined sugar, may promote the growth of beneficial intestinal bacteria and enhance a balanced gut-brain axis environment (Graf et al., 2015; Noble et al., 2017). In a previous study involving 5,200 grade 5 students (Florence et al., 2008), children with decreased diet quality performed poorly on academic performance assessments, a finding consistent with our study. Moreover, a better home environment to promote child development has consistently been a core determinant of child cognition, as reported here and elsewhere (Orri et al., 2019; Prado et al., 2017). Lastly, higher engagement in physical activity, which was associated with a lower state of depression in this study, should be encouraged among adolescents since

it may improve the neuroplastic processes altered in depression, and promote better self-esteem and social support (Kandola et al., 2019).

The long-term benefits of probiotic and calcium supplementation along with the significant association of the socioenvironmental factors suggest the need for innovative and integrated interventions to achieve child and adolescent well-being. Still, some limitations have been noted in our study, such as the absence of other time points of observation in the cohort prior to the 10-year follow-up, and the relatively high number of unfound adolescents. However, we have included the essential covariates known to be associated with child and adolescent neurodevelopment obtained from the original study, and the recent data collection, to address the 10-year gap. Moreover, we have compared the found adolescents with unfound ones to accommodate for potential bias. While previous studies demonstrated that dramatic biological development of the human brain still exists until the age of six years of age (Brown & Jernigan, 2012; Dobbing & Sands, 1973; Vértes & Bullmore, 2015), we recommend providing intervention at a younger age when the development of gut microbiome is highly dynamic and assessing the microbiome profile to evaluate the long term changes in intestinal colonization (Derrien et al., 2019). While the results for probiotics and calcium supplementation warrant future larger RCTs with thorough longitudinal data collection, this study suggests the importance of identifying strains affecting child neurodevelopment to accelerate the improvement of future generations.

5. Conclusions

Probiotics *L. casei* CRL431 and *L. reuteri* DSM17938 supplementation in childhood have long-term benefits on neurodevelopmental outcomes with strain-specific effects. *L. casei* CRL431 supplementation showed a beneficial effect on cognition as measured by RPM, while *L. reuteri* DSM17983 supplementation demonstrated benefit on reducing depressive state as assessed with CDI. Higher calcium intake through regular-calcium milk provision as compared to low-calcium milk resulted in higher serum BDNF. Biomedical (i.e. diet quality, physical activity) and socioenvironmental factors (i.e. home environment) were significant determinants in predicting RPM and CDI scores, whereas serum BDNF was only associated with biomedical factors, such as BMI and diet quality. The cumulative findings on the effects of *L. casei* CRL431, *L. reuteri* DSM17938, and higher calcium intake through regular-calcium milk consumption in childhood on particular neurodevelopmental outcomes warrant a future RCT using similar strains, and a thorough longitudinal study, and include other biomedical and socioenvironmental determinants to improve child and adolescent well-being.

Ethical statement

The original trial is registered at clinicaltrials.gov (NCT00512824) and was approved, along with the re-enrollment study (NCT04046289), by the Medical Ethics Committee of the Faculty of Medicine study (protocol code 18–10-1170; October 22, 2018), Universitas Indonesia, and the local government.

CRediT authorship contribution statement

Davrina Rianda: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft. **Sri Hartati R. Suradijono:** Methodology. **Evania A. Setiawan:** Funding acquisition, Investigation. **Fenny Susanto:** Funding acquisition, Investigation. **Meilianawati Meilianawati:** Funding acquisition, Investigation. **Erfi Prafiantini:** Funding acquisition, Project administration. **Frans J. Kok:** Supervision. **Anuraj H. Shankar:** Writing – original draft. **Rina Agustina:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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