Probiotics, calcium and acute diarrhea

A randomized trial in Indonesian children

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Especially dedicated to

The health of children in Indonesia and other developing world

My beloved family

my late Ibu, Aslamiah binti Abdul Kohar
who motivated me to become a physician who can help people reach their good
health and wellbeing, especially to those socio-economically marginalized

my ayah, Sanusi Ardidja Ranadipradja
my mamah, Siti Ulyah
my husband, Ahmad Sadariskar
my beautiful daughters, Arini Ayatika Sadariskar
and Dinaka Tatsbita Sadariskar

my brothers and sisters

Abstract

Background

Acute diarrhea and acute respiratory tract infections (ARTIs) continue to lead the infectious cause of morbidity and mortality among children <5 years of age in developing countries, including Indonesia. Efforts to prevent diarrheal disease by probiotics and milk calcium supplementation as alternative strategy are promising. We investigated the efficacy of calcium with or without two probiotic strains, tested independently, on incidence and duration of acute diarrhea and ARTIs among Indonesian children. In addition, cumulative duration and severity of diarrhea due to rotavirus or other causes, growth, and iron and zinc status were tested. The associations of food-hygiene practices with diarrhea prevalence in children were also determined.

Methods

We conducted a 6-month, double-blind, placebo-controlled trial. A total of 494 Indonesian healthy children aged 1 to 6 years randomly received low-lactose milk with low calcium content (LC; ~50 mg/day; n = 124), regular calcium content (RC; ~440 mg/day; n = 126), RC with 5.10^8 colony-forming units per day of *Lactobacillus casei* CRL 431 (casei; n = 120), or RC with 5.10^8 colony-forming units per day of *Lactobacillus reuteri* DSM 17938 (reuteri; n = 124). Incidence and duration of diarrhea were the primary outcomes. Secondary outcomes were incidence and duration of ARTIs, severity of diarrhea (modified Vesikari score and fecal osmolarity, calprotection and mucin), growth, and iron and zinc status. The cross-sectional association between food-hygiene practices and 7-day record period of diarrhea prevalence was assessed among 274 randomly selected children aged 12-59 months in a low socioeconomic urban area of Jatinegara sub-district of East Jakarta, Indonesia.

Results

Incidence of World Health Organization-defined diarrhea (≥3 loose/liquid stools in 24 hours) was not significantly different between RC and LC (relative risk 0.99; 95% confidence interval (CI): 0.62-1.58), between casei and RC (relative risk 1.21; 95% CI: 0.76-1.92), or between reuteri and RC (relative risk 0.76; 95% CI: 0.46-1.25) groups. Incidence of all reported diarrhea (≥2 loose/liquid stools in 24 hours) was significantly lower in the reuteri versus RC group (relative risk 0.68; 95% CI: 0.46-0.99). Irrespective of the definition used, reuteri significantly reduced diarrhea incidence in children with lower nutritional status (below-median height-and-weight-for-age z score). None of the interventions affected ARTIs. The mean total duration (131 children, 190 diarrheal episodes) was 1.35 days shorter in the reuteri group (relative risk 0.60; 95% CI: 0.36-0.99) in a 6-month period, likely by mainly affecting rotavirus-positive diarrhea. Rotavirus prevalence in diarrheal cases (30%) was not significantly different across the groups. None of the supplements affected diarrhea severity based on Vesikari score and fecal markers, except for a higher fecal mucin concentration in the casei group (P = .006). The increase in weight gain, weight-for-age z score (WAZ) changes and monthly weight and height velocities were significantly higher in the reuteri compared with RC group over 6 months period, whereas. *L. casei,* although giving less benefit, modestly improved weight velocity. Changes in underweight and stunting prevalence, anemia prevalence and iron and zinc status were similar among groups. No

serious adverse events related to the interventions were reported. Children living in a house with clean sewage had a significantly lower diarrhea prevalence compared to those who did not have one or had dirty sewage (adjusted odds ratio 0.16; 95% CI: 0.03-0.73). The overall food-hygiene practice score was not significantly associated with diarrhea in the total group, but it was in children aged <2 years (adjusted odds ratio 4.55; 95% CI: 1.08-19.1).

Conclusion

L. reuteri may prevent diarrhea especially in children with lower nutritional status, reduce total duration of diarrheal episodes, and modestly improve growth over 6 months, but does not affect diarrhea severity. *L. casei* modestly improves monthly weight velocity, but does not reduce diarrhea incidence, duration or severity. However, it seems too early to recommend probiotics (e.g. *L.* reuteri) for routine use or for follow-up in public health programs to prevent diarrhea in children in developing countries. Milk calcium alone does not affect any of the outcomes. Moreover, none of the dietary treatments affect incidence and duration of ARTIs, and iron and zinc status in Indonesian children. In addition to other major determinants, poor mother's food-hygiene practices contributes to the occurrence of diarrhea in Indonesian children <2 years.

Contents

Chapter 1

General introduction

Background

Acute diarrhea and acute respiratory tract infections (ARTIs) continue to lead the infectious cause of morbidity and mortality among children <5 years of age in developing countries.[1-3] Despite the substantially declining mortality rate from diarrhea,[4] the overall incidence of this disease in developing countries has remained high and relatively stable over the past two decades with an estimated 2.5 billion cases of diarrhea occurring among children in this age group each year.[1] Whereas ARTIs represent 30-50% of the pediatric medical consultations and 20-40% of the hospitalizations in children.[5] Similar to other developing countries, diarrhea contributes to 25% of mortality and 14% of morbidity,[6] whereas ARTIs (pneumonia) contribute to 16% of mortality rate[7] among younger children in Indonesia. Correspondingly, the prevalence of these diseases and malnutrition among children aged <5 years in low socioeconomic urban communities in Indonesia remains high.[8, 9]

Infection and malnutrition are interrelated.[10] Diarrhea and other infections contribute to malnutrition, and malnutrition increases the susceptibility of children to infection, which constitutes to a vicious cycle.[10, 11] Infection adversely impacts to malnutrition through a decreased nutritional intake, a diminished intestinal absorptive function of vital nutrients, and an increased catabolism and sequestration of nutrients required for tissue synthesis and growth[12, 13] during the acute episode and recovery period. Malnutrition, in turn, decreases the ability of the immune system to fight against infections[12] primarily because of disruption of epithelial integrity and inflammation,[11] causing frequent or repeated infections. Multiple and recurrent infections further cause impaired intestinal absorptive function that lead to malnutrition.[13] Furthermore, impaired innate and adaptive host immune responses and disrupted intestinal barrier function due to both infection and malnutrition together increase children's susceptibility to repeated infections leading to intestinal injury and nutrient malabsorption during the critical stage of child development.[11,13] Malnutrition contributes 53% of childhood deaths[14] and leads to poor health outcomes in childhood that persist into adulthood[13] Therefore, strategies to increase resistance to infections and to break the cycle between diarrhea and malnutrition in this population are needed.

Today, simple, safe, effective and yet relatively inexpensive preventive strategies including provision of safe water and sanitation, exclusive breastfeeding, hand washing with soap, vitamin A and zinc supplementation, and vaccinations are available in developing countries. These prevention strategies are important public health goals that will impact in reducing mortality and better growth and development of children in developing countries.[15] However, these interventions are not always effective to reduce the burden of these diseases.[3] Despite all these efforts, the morbidity from these diseases remains persistently high. The World Health Organization (WHO) identified the following reasons with respect to this problem: suboptimal access to and utilization of available services, lack of focus and coordination on preventive interventions, lack of population-targeted interventions in age groups other than under-five, surveillance, research, monitoring and evaluation, and community mobilization and empowerment, and ineffective intersectoral coordination.[3] In addition, these approaches do not adequately address broader growth and developmental processes that could yield long-term benefits.[16] Moreover, no single intervention seems to be sufficient to eliminate the global burden of these diseases.[16]

Efforts to prevent diarrheal disease by dietary modulation of intestinal host defenses as alternative strategy are promising.[17] These efforts include supplementation of probiotics and milk calcium. This chapter provides the state of the art and background information on the intervention strategy using probiotics and calcium supplementation as means to reduce incidence, duration and severity of diarrhea and respiratory infections among Indonesian children aged 1-6 years. In addition, the potential effects of milk supplemented with probiotics and calcium on growth, iron and zinc status are elaborated. Moreover, important food hygiene determinants associated with diarrhea are addressed. At the end of this chapter, a detailed rationale and aims of the thesis are presented.

Effects of probiotics and calcium on incidence of diarrhea and respiratory tract infections

Calcium

A strictly-controlled human study reported that supplementation of healthy adults with calcium from milk increased resistance to foodborne enterotoxigenic *Escherichia coli* (ETEC) as it reduced infection-induced diarrhea.[17] Several pre-clinical animal infection studies showed protective effects of calcium against Salmonella as well,[18, 19] but verification in humans is missing. Unfortunately, and besides other micronutrient deficiencies, many Indonesian children <5 years have a low dietary calcium intake not meeting their age-specific recommended daily allowances.[20, 21]

> *Whether calcium, previously shown to reduce infection-induced diarrhea in adults, is equally effective in children with a low habitual calcium intake and frequent episodes of intestinal infections, is currently unknown.*

Probiotics

There is a significant expansion of the probiotic market as well as concomitant research in the developed world,[22] particularly in Japan and Europe.[23, 24] However, it remains a scientific curiosity for clinicians, confusing to the general public, and a dilemma for policy makers whether probiotics should be used as a public health strategy to prevent diarrhea in developing countries.[22] Accumulating evidence on probiotics efficacies in the treatment of diarrhea in developed countries has increased expectations of the food industry to expand their probiotics market to developing countries where infectious diarrhea risk is greater. However, this market expansion is not always supported by sound evidence obtained from well-designed RCTs in appropriate human target populations. Clearly, most previous studies on probiotics were mainly in hospital-based settings aiming at treatment of diarrhea in developed countries.[25] So far, very limited published data is available to support that probiotics can be routinely recommended for prevention or reduction of infection in healthy children 1-6 years in developing countries.

a) Probiotics: Strain-specificity

Probiotics are defined by the Food Agriculture Organization (FAO)/WHO in 2001 as "live microorganisms which when administered in adequate amounts confer a health benefit on the host".[26] Probiotics include a large number of species of microorganisms, especially *Lactobacillus* and *Bifidobacterium*.[26] The clinical effects of probiotics are generally known as strain-specific. Strain-specific effects means that the probiotic effects in treatment or prevention, depend on the specific bacteria, which should be defined not only at the genus and species level, but also at strain level.[27]

b) Probiotics: Minimum dosage

It is important to determine the minimum effective dose or the level of viable cells of the probiotic strain in Colony Forming Units (CFU)/day in the carrier food and optimal period of probiotic administration in demonstrating the general or specific health effects or wellbeing in the target population.[26] However, information on minimum dose and optimal period of administration of probiotics remains unclear.[28, 29] Likely, the minimum dose required for a probiotic effect may depend on the probiotic strain used[28] and/or on the food matrix in which the probiotic is ingested.[30] The minimum effective dose of $\geq 10^8$ CFU/day has been recommended for therapeutic effects during the FAO/WHO consultation meeting in 2001.[31] The rationale of this recommendation is derived from some studies reporting a minimum effective dose for therapeutic cultures to be a daily intake of 10^6-10^9 viable CFU,[32] however, other studies showed that the minimum dose of strains such as *L. rhamnosus* GG to yield fecal recovery was 10^{10} CFU/day,[33, 34] and *L. johnsonii* LJ1 to elicit immune effects was 10^9 CFU/day, whereas a dose of $<10^8$ CFU did not.[35] The minimum dose of 10^9 CFU/day and consumption of 100 g or ml (a food containing at least 10^7 cells per gram or milliliter) was recommended for dairy products by the Japanese Fermented Milk and Lactic Acid Bacteria Beverages Association.[28, 31, 36]

c) Probiotics: Clinical effects in pediatrics

Numerous studies in pediatric populations have suggested a potential effect of probiotics in alleviating or preventing acute infectious diarrhea, antibiotic-associated diarrhea (AAD), necrotizing enterocolitis (NEC), allergic and atopic disease, inflammatory bowel disease (IBD), viral infections of the respiratory tract and dental caries.[37] However, clear evidence of benefit was mainly seen in acute viral gastrointestinal tract infections and AAD.[38]

d) Probiotics: Randomized trials on acute diarrhea and ARTIs

Probiotics tested for use in the treatment and prevention of diarrhea in children are from the species of *Lactobacillus rhamnosus, L. casei, L. reuteri, L. acidophilus, L. bulgaricus, Bifidobacterium lactis, B. breve, Streptococcus thermophiles, Enterococcus, E. coli Nissle 1917, and* the yeast *Saccharomyces boulardii*.[39] Among these probiotics, *L. rhamnosus* GG is the most extensively studied strain for the treatment of infectious diarrhea in children. Other probiotic strains, *L. casei* CRL 431 and *L. reuteri* DSM 17938 are related to strains previously suggested to have anti-diarrheal benefits in young children.[40-43]

Several meta-analyses and reviews have concluded that probiotics may prevent or reduce duration of diarrhea in children. However, the beneficial effects are probiotic strain and dose dependent and evidence was obtained mainly in developed countries.[44-47] Moreover, several randomized trials have explored benefits of probiotics in the prevention of ARTIs in children,[15, 48-50] but have yielded contradictory conclusions.[51-53]

So far, recommendations to supplement with calcium or probiotics in community settings in developing countries are not justified.[46]

Effects of probiotics and calcium on duration and severity of diarrhea in Indonesian children

Rotavirus is the leading cause of severe diarrhea-related hospitalization among children in both developed and developing countries.[54, 55] It is estimated to cause >500,000 deaths annually in children under five, with 80% of these deaths occurring in developing countries.[56] In Indonesia, it was estimated that 60% of hospital and 41% of outpatient clinic admissions of children for diarrhea were rotavirus-positive.[57] Yet, bacterial infections are also an important cause of acute diarrhea in developing countries[58] and may lead to severe disease.[59]

Severity of diarrhea

The pathophysiology, etiology, and clinical sequelae of acute diarrhea are well described.[60, 61] So far, there is limited information on severity of diarrhea and the impact of dietary interventions in clinical trials.[60] Some studies assessed severity of gastroenteritis using a single indicator such as diarrheal stool output, diarrhea duration of >7 d, treatment failure, occurrence of dehydration, or death.[62] Other studies scored symptoms of diarrhea using qualitative or semi-quantitative severity scales.[63]

a) Clinical measurement of diarrhea severity: Vesikari score

The most commonly used severity scale is the Vesikari 20-point scale in which an episode of gastroenteritis with a score of ≥11 is considered severe.[63, 64] This scale has been adopted as validated on the basis of its frequent use in clinical trials, and can be used as a measure of diarrhea severity in young children.[60] Despite being commonly used in cohort studies on diarrhea and rotavirus vaccines, the Vesikari scale has not been applied by any trial on probiotics in a community setting in a developing country. A very limited number of probiotic studies in developing countries used single measures to assess severity of diarrhea such as diarrhea duration, treatment failure, dehydration and stool output.[65, 66] A modified

Vesikari score (18-points scale) was recently recommended to be used as a measure of diarrhea severity in outpatient clinical studies of young children.[60]

b) Objective measurement of diarrhea severity: fecal markers

During diarrhea, normal intestinal physiology is disturbed due to the presence and colonization of pathogens resulting in altered intestinal water absorption and secretion,[67] mucosal barrier permeability, mucosal host defense mechanisms and intestinal inflammation[68-71] Currently, there is great interest in non-invasive, objective to measure markers to quantify intestinal infection severity and/or mucosal inflammation in large clinical studies. Candidates of such potential markers are fecal osmolarity, calprotectin and mucin.

Diarrhea severity can be quantified by determination of **fecal osmolarity**. The most obvious characteristic of infectious diarrhea is an increased fecal water excretion due to reduced intestinal absorption or increased secretion of electrolytes,[69] and thus an increased luminal presence of osmolytes attracting water.[67]

Calprotectin is a calcium-binding protein with antimicrobial action,[72] richly found in neutrophils in stool and body fluids.[59] It is the most promising marker due to its resistance to proteolytic degradation and stability in stool.[73] Levels in stool increase upon intestinal infection and other inflammatory diseases of the gastrointestinal tract.[74-76]

Mucins are a family of heavily glycosylated proteins that are the major organic components of the mucus layer covering and protecting epithelial cells in many human organs, including the entire gastrointestinal tract.[77, 78] Mucins are secreted as large aggregates by mucosal goblet cells[79] and are crucial for intestinal mucus formation.[80, 81] The mucus network is an indispensable part of gut barrier function as this viscous layer protects the delicate epithelial cells underneath from aggressive components in the fecal stream, including bacterial pathogens and their toxins.[80] Fecal mucin excretion is enhanced upon intestinal infection and defects in mucus production leading to colonic inflammation.[17, 82]

Probiotic studies on infectious, immune and inflammatory outcomes in humans are inconsistent, appear to be highly probiotic strain-dependent, and are mainly focused on rotavirus diarrhea in children.[39, 44, 46, 83, 84] Dietary calcium improves intestinal resistance to infectious diarrhea due to Salmonella enteritidis[18, 85] and enterotoxigenic E. coli (ETEC)[17] in animal models. Dietary calcium also increases intestinal resistance to ETEC in a human proof-of-principle infection study in adults.[17] However, its effect on duration and severity of acute diarrheal disease due to rotavirus or other causes in children is unknown.

Effects of probiotics and calcium on growth and iron and zinc status in children

Undernutrition and multiple micronutrients (MMNs) deficiencies persist as the most serious nutritional problems among children <5 years in developing countries.[86-88] The Indonesian national prevalence of stunting (37%) and wasting (14%) is higher, and underweight (18%) is similar compared to the estimated overall prevalence of undernutrition among under-five children in the developing world.[7, 89] Deficiencies in vitamin A, iron, zinc and iodine are most prevalent in Indonesian children.[90, 91] Because intake of dairy products in these children is minimal during their growth period and dairy is an excellent source of calcium,[92] calcium deficiency may also be prevalent.

Studies reporting benefits of nutrition interventions on growth and micronutrient status of under-five children used different approaches of supplementation and fortified foods.[88, 93-98] Overall, the results of these studies are conflicting.[99, 100] In addition, some studies indicated that nutrient-dense foods may help to prevent stunting and wasting in young children, but more data are needed to identify the impact of this approach.[101-103] Probiotics are often supplemented to dairy foods and both probiotics[45-47] and calcium in milk[17] may strengthen intestinal infection resistance. However, the impact of probiotics on growth and micronutrient status is uncertain. In addition to a mice study with *L. casei* CRL 431,[104, 105] several human studies showed a positive effect of probiotics on weight gain in children aged 5 months to 5 years.[106-109] Two reviews reported that calcium supplementation in healthy children has no effect on weight, height, body fat, or lean mass in randomized clinical trials (RCTs).[110, 111] However, such evidence in children in developing countries is scarce.

Milk naturally rich in calcium and fortified with probiotics may provide extra energy, high quality protein and micronutrients, but less is known 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[112] and may be beneficial in malnutrition when gut function is impaired and epithelial cell functioning can be improved.[113] Acute inhibitory effects of calcium on iron absorption in adults were shown in some studies,[114-116] but not in others.[117-119] Previous studies reported variable findings on the effects of milk or calcium supplementation on zinc absorption in adults.[120] The possibility that calcium interferes with iron and zinc absorption and affects iron and zinc status in children is an important concern,[121, 122] but very few studies were performed in a pediatric population. Previous studies were conducted mainly in developed countries involving children with adequate calcium and iron intake and showed contradictive results. An RCT in 1-year old children showed that extra calcium resulted in a dose-related decrease in both heme and non-heme iron absorption.[123] However, RCTs conducted in older children and adolescent girls found no effects of calcium supplementation on iron status and the inhibition of iron status may not persist after adaptation to an increased calcium diet.[121, 124, 125]

> *Until now, evidence is inconclusive whether prolonged dietary supplementation with calcium and probiotics in children with low habitual calcium intake in developing countries like Indonesia, affects growth, and iron and zinc status.*

Food-hygiene practices and diarrhea prevalence

Morbidity and overall incidence of diarrhea among children in developing countries has remained high due to multiple determinants[126] such as malnutrition,[127] low socioeconomic status and education of mothers,[128, 129] lack of safe drinking-water, inadequate sanitation and poor hygiene,[130, 131] crowding,[132] and low maternal age.[133] These determinants of diarrheal disease are strongly linked to poverty and social inequities.[3] Furthermore, diarrhea incidence differs greatly with the seasons and is highest in the first two years of life and declines as a child grows older.[1] Diarrhea and malnutrition, alone or acting in concert, constitute major causes of morbidity and mortality among under-five children throughout the tropical world.[1, 127, 134] Many studies showed that prevalence of diarrhea was inversely associated with growth in both height-for-age Z-score (HAZ) and weight-for-age Z-score (WAZ).[135-137]

Various studies conducted in urban settings of developing countries focused on the risk factors of diarrhea related to environmental conditions and utilization of sanitation facilities.[138-140] A previous study on food-hygiene missed some important practices such as food storage, thorough cooking and adequate holding temperature[141] as endorsed by WHO.[142] Mothers and children in low socioeconomic urban areas in East Jakarta with limited hygiene and sanitation facilities tend to have poor hygiene practices such as using dirty cooking or eating utensils for their children.[143] While poor hygiene practices, especially in food preparation and feeding, may increase the risk of having diarrhea, up to 70% of diarrhea episodes are actually caused by water and food contaminated with pathogens.[144] The prevalence and determinants of diarrhea, not only unsafe water, and poor sanitation and hygiene, but also poor food-hygiene practice among children in low socioeconomic urban areas of East Jakarta are important information to design any intervention study, health plans and policies related to mother and child hygienic behavior.

Although the determinants of diarrhea in children are well described, information on the role of food-hygiene practice in the development of diarrhea and malnutrition among children in low socioeconomic urban community is generally lacking.[141]

Rationale, objectives and outline of the thesis

Rationale

As the overview above makes clear, important food-hygiene practices associated with diarrhea are not fully described. The effect of probiotics and calcium supplementation as alternative intervention to reduce incidence, duration and severity of diarrhea and respiratory infections among Indonesian children aged 1-6 years are very relevant to be investigated. Also, the potential effects of calcium in milk and probiotics supplementation on growth, iron and zinc status need to be further elucidated. The rationale of the studies in this thesis are presented below:

Whether calcium, previously shown to reduce infection-induced diarrhea in adults, is equally effective in children with a low habitual calcium intake and frequent episodes of intestinal infections, is currently unknown.

Several meta-analyses and reviews have concluded that probiotics may prevent or reduce duration of diarrhea in children. However, the beneficial effects are probiotic strain and dose dependent and evidence was obtained mainly in developed countries. Moreover, several studies have explored benefits of probiotics in the prevention of ARTIs in children. So far, recommendations to supplement with calcium or probiotics in community settings in developing countries are not justified.

Probiotic studies on infectious, immune and inflammatory outcomes in humans are inconsistent, appear to be highly probiotic strain-dependent, and are mainly focused on rotavirus diarrhea in children. A Cochrane systematic review concluded that probiotics reduced diarrhea duration in infants and children by 29.2 hours in clinical settings, but probiotic effects on diarrhea duration and severity have not been evaluated in community settings.

Calcium increases intestinal resistance to enterotoxigenic E. coli in adults, but an effect on diarrhea duration and severity due to rotavirus or other causes in children is unknown.

Until now, evidence is inconclusive whether prolonged dietary supplementation with calcium and probiotics in children with low habitual calcium intakes in developing countries like Indonesia, affects growth, and iron and zinc status.

Although the determinants of diarrhea in children are well described, information on the role of food-hygiene practice in the development of diarrhea and malnutrition among children in low socioeconomic urban community is generally lacking.

Objectives and outline of thesis

The above mentioned rationale provides us valuable objectives to be studied in this thesis:

(1) Do probiotics and calcium supplementation affect incidence and duration of diarrhea and ARTIs, severity of diarrhea as well as growth, iron and zinc status in Indonesian children? Answers to this question will provide recommendations for nutritional intervention strategies to prevent diarrhea and ARTIs. Moreover, it may show medical professionals, public health and food policy makers how to improve growth, iron and zinc status.

(2) What is the role of poor food-hygiene practices as determinants of diarrhea among children in low socioeconomic urban areas of East Jakarta? The answer to this question will provide important information to design intervention studies, health plans and policies related to mother and child hygienic behavior.

The specific research questions and objectives addressed in this thesis are:

What are the effects of an intervention using probiotics and calcium on reducing the incidence and duration of diarrhea and respiratory infections?
In **chapter 2**, we investigated the efficacy of dietary calcium with or without two probiotic strains, *L. casei* CRL 431 and *L. reuteri* DSM 17938 tested independently, on incidence and duration of acute diarrhea and ARTIs during 6 months of intervention among children aged 1-6 years living in low socioeconomic urban area of East Jakarta, Indonesia.

What are the effects of probiotics and calcium on diarrhea duration and severity in Indonesian children?
In chapter 3, we investigated the efficacy of dietary calcium with or without two probiotic strains on duration and severity of acute diarrheal disease due to rotavirus or other causes. Severity of diarrhea was assessed by the Vesikari score based on the mothers' subjective information and by objective measurement of the markers osmolarity, mucin and calprotection in diarrheal and normal fecal samples collected during the intervention.

What are the potential effects of probiotics and calcium on growth and iron and zinc status in children?
In **chapter 4**, we investigated the hypothesis that milk supplemented with probiotics would improve growth, and iron and zinc status, whereas milk calcium alone would improve growth, but reduce iron and zinc status of Indonesian children.

What is the association between food-hygiene practices and diarrhea prevalence in Indonesian young children from low socioeconomic urban areas?
In **chapter 5**, we determined the prevalence of diarrhea and malnutrition in Indonesian children and assessed the association of food-hygiene practice with the occurrence of diarrheal disease among under-five children living in selected low socioeconomic urban area in Jatinegara sub-districts of East Jakarta.

Finally, **chapter 6** presents the summary of the main findings and discusses the clinical effects of the interventions, public health implications and recommendations, and directions for future research.

Study location in Indonesia

The field work described in this thesis was conducted in urban communities representing flooding (Kampung Melayu village) and non-flooding area (Rawabunga village) of Jatinegara sub district, East Jakarta, Indonesia (**Figure 1.1**). These areas were selected because of their high population density, low socioeconomic status, and high prevalence of diarrhea and underweight in under-five children.[145, 146]

Jakarta is the capital city of Indonesia and is located in the western part of Java Island, which consists of five municipalities (eg. East Jakarta). Jatinegara is one of the sub districts in East Jakarta and is located at low land 0-10 m above sea level, as most of other Jakarta area. It has a tropical climate with two distinctive seasons: a rainy season (December-April) and a dry season (May-November).[147] This sub district is prone to flooding because of three rivers crossing this area together.[147] Ciliwung river is one of the major rivers which used to be the source for water supply in this area of Jakarta. Although floods in Jakarta peak in January and February,[148] floods in Kampung Melayu village can be lasting between November and March almost every year. The population registered in this sub-district in 2002 was 262,699 with approximately 72,166 households living in an area of 10.6 km².[147]
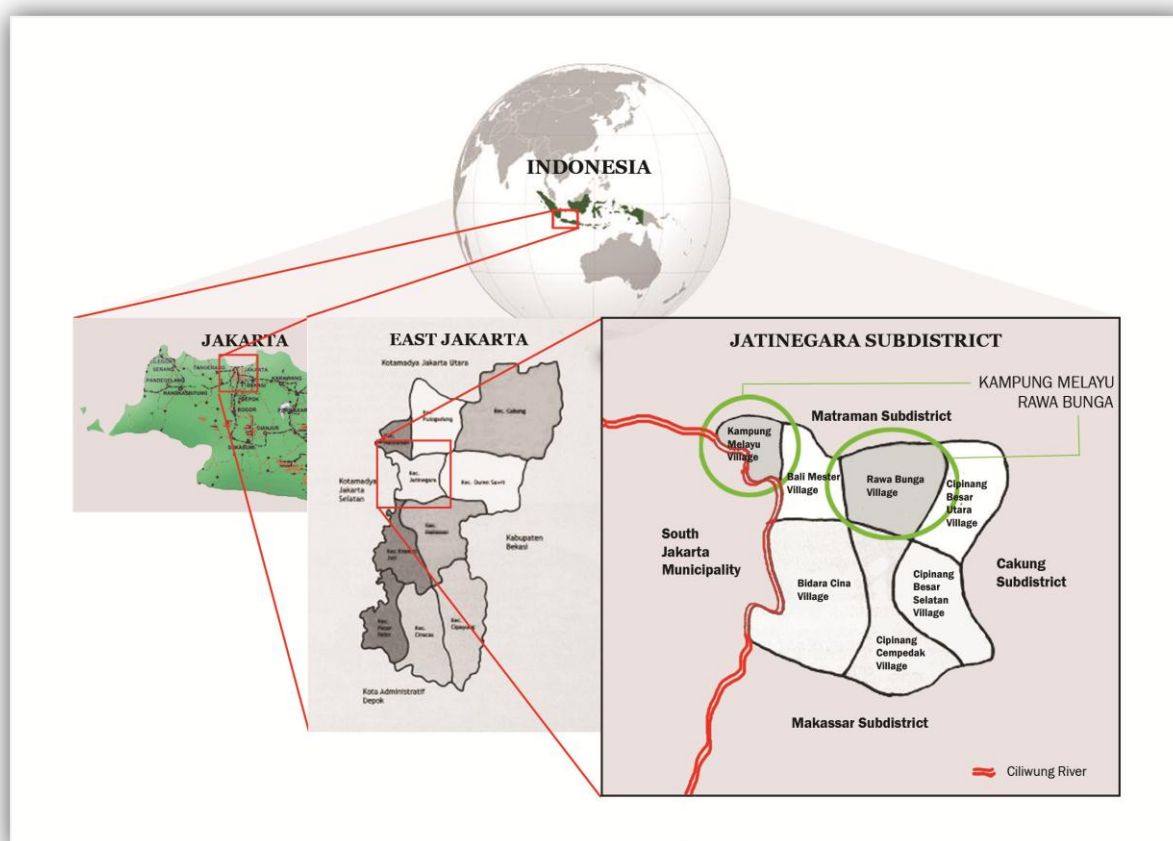

Figure 1.1 Map of study location in East Jakarta

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Chapter 2

Randomized trial of probiotics and calcium on diarrhea and respiratory tract infections in Indonesian children

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Abstract

Objective

To investigate the effects of calcium and probiotics on the incidence and duration of acute diarrhea and acute respiratory tract infections (ARTIs) in low-socioeconomic communities of Jakarta, Indonesia.

Methods

We conducted a 6-month, double-blind, placebo-controlled study in 494 healthy children aged 1 to 6 years who received low-lactose milk with low calcium content (LC; ~50 mg/day; n = 124), regular calcium content (RC; ~440 mg/day; n = 126), RC with 5.10^8 colony-forming units per day of *Lactobacillus casei* CRL 431 (casei; n = 120), or RC with 5.10^8 colony-forming units per day of *Lactobacillus reuteri* DSM 17938 (reuteri; n = 124). Number and duration of diarrhea and ARTIs episodes were primary and secondary outcomes, respectively.

Results

Incidence of World Health Organization–defined diarrhea (≥3 loose/liquid stools in 24 hours) was not significantly different between RC and LC (relative risk [RR]: 0.99 [95% confidence interval (CI): 0.62–1.58]), between casei and RC (RR: 1.21 [95% CI: 0.76–1.92]), or between reuteri and RC (RR: 0.76 [95% CI: 0.46–1.25]) groups. Incidence of all reported diarrhea (≥2 loose/liquid stools in 24 hours) was significantly lower in the reuteri versus RC group (RR: 0.68 [95% CI: 0.46–0.99]). Irrespective of the definition used, reuteri significantly reduced diarrhea incidence in children with lower nutritional status (below-median height-and-weight-for-age z score). None of the interventions affected ARTIs.

Conclusions

RC milk, alone or with *L. casei,* did not reduce diarrhea or ARTIs in Indonesian children. *L. reuteri* may prevent diarrhea, especially in children with lower nutritional status.

Introduction

Acute diarrhea and respiratory tract infections (ARTIs) continue to lead the infectious cause of morbidity and mortality among children <5 years in developing countries.[1-3] In Indonesia, diarrhea and ARTIs (pneumonia) contribute to 25% and 16% of mortality rate among young children, respectively.[4] Moreover, the prevalence of these diseases and malnutrition among children aged <5 years in low socioeconomic urban communities in Indonesia remained high.[5, 6] Infection and malnutrition are interrelated,[7] and strategies to increase resistance to infections in this population are needed.

Preventive strategies (including provision of safe water and sanitation, exclusive breastfeeding, hand washing, vitamin A and zinc supplementation, and vaccinations) are available in developing countries. However, these interventions are not always effective in reducing burden of these diseases.[3]

Efforts to prevent diarrheal disease by dietary modulation of intestinal host defenses as alternative strategy are promising.[8] A strictly-controlled human study reported that supplementation of healthy adults with regular milk, naturally high in calcium, reduced foodborne enterotoxigenic *Escherichia coli* (ETEC)-induced diarrhea.[8] In addition to other micronutrient deficiencies, many Indonesian children aged <5 years unfortunately have a low dietary calcium intake not meeting their age-specific recommended daily allowance.[9, 10] Whether calcium is equally beneficial in children with low calcium intake and frequent episodes of intestinal and respiratory infections is currently unknown.

Several meta-analyses and reviews have concluded that probiotics may prevent or reduce duration of diarrhea in children. However, the beneficial effects depend on the probiotic strain and dose, and evidence was obtained mainly in developed countries.[11-14] Moreover, several studies have explored benefits of probiotics in the prevention of ARTIs in children.[15-18] So far, recommendations to supplement with calcium or probiotics in community settings in developing countries are not justified.[13] Therefore, we investigated the efficacy of dietary calcium with or without two probiotic strains on incidence and duration of acute diarrhea and ARTIs in children. The probiotic strains used are related to strains previously suggested to have antidiarrheal benefits in young children.[19-22]

Methods

Study design

A randomized, double-blind, placebo-controlled trial was conducted between August 2007 and September 2008 in low socioeconomic urban communities representing non-flooding and flooding areas of East Jakarta, Indonesia. The protocol was approved by both the Medical Ethics Committee of the Faculty of Medicine, University of Indonesia and of Wageningen University. All parents provided written informed consent before inclusion.

Subjects

Children aged 1 to 6 years were selected from community registry for the first screening phase to assess eligibility based on the basis of the following inclusion criteria: apparently healthy, not being breastfed, and if consuming milk, calcium intake was <75% of the age-specific recommended daily allowances. In the second phase, registered physicians interviewed mothers and examined the children to check the exclusion criteria: symptoms of chronic/congenital diseases and disabilities, pulmonary tuberculosis, history of allergy, diarrhea on admission, antibiotics use within 2 weeks before study start, severe wasting (less than-3 SD of weight-for-height z score), calcium intake >375 mg/day according to a validated semi-quantitative food-frequency questionnaire, not capable or willing to drink milk with a straw in a 2-day acceptance test, showing allergy or intolerance to the products, and/or sibling of included child (twins excepted).

Interventions

Children were randomly assigned to receive low-lactose milk: with a low calcium content (LC; ~50 mg/day); regular-calcium content (RC; ~440 mg/day); RC plus *Lactobacillus casei* CRL 431 ($5x10^8$ colony-forming units [CFU]/day [casei]), or RC plus *Lactobacillus reuteri* DSM 17938 ($5x10^8$ CFU/day [reuteri]). Milk was sweetened, chocolate-flavored, ambient stable (sterilized by using ultra-high temperatures), and packed in tetra packs (Frisian Flag, Indonesia, Jakarta, Indonesia). Milk was consumed with straws coated inside with the oil drop as placebo (BioGaia AB, Stochkholm, Sweden) or with either *L. casei* CRL 431 (Chr Hanssen, Hørsholm, Denmark) or *L. reuteri* DSM 17938 (BioGaia AB) in vegetable oil. Probiotic dosage was based on supplier's information of efficacy, application in children, safety concerns when dosed for longer periods of time, and technical reasons (ie, straw coating). The different milk drinks and straws were indistinguishable for the investigators and participants. The composition of the milks and straws is described in **Table 2.1**.

Milks and straws were stored cooled (<10ºC) at all times until delivery. Viability of the probiotics was checked each month by selective plating. Field workers distributed milk and straws twice a week to the parents, who were instructed to store products refrigerated and prevent sun exposure. Parents without refrigerators obtained the products from the field workers' house on daily basis and/or children consumed the products directly at the field workers' house.

Mothers were instructed to provide the children 180 mL of milk twice daily (not with a meal) by using the straws provided. Mothers were requested to maintain the child's habitual diet but to exclude probiotic, prebiotic or high calcium foods/drinks other than the supplied ones. The amount of milk consumed was measured by using a calibrated stick put into the tetra-paks to score the remaining volume by using a pretested 5-point scale. The field workers observed the children drinking milk at least once a week and empty packages had to be shown during visits. During diarrheal episodes, children continued or restarted drinking milk as soon as possible but after being rehydrated with oral rehydration solution according to World Health Organization (WHO) guidelines.[23] We followed the local standard for outpatient and hospital care for diarrhea and ARTI, which per WHO guidelines.[23-25] Liability

insurance was provided for the children during the study. Activities with creative and educational contents were implemented to maintain compliance of both mothers and children.

Table 2.1 Composition of LC and RC milk and probiotic straws

Composition	LC	RC	Casei	Reuteri
UHT Milk (per 100 ml)				
Energy, kcal	93.8	98.0	98.0	98.0
Fat, g[a]	3.5	3.9	3.9	3.9
Protein, g[a]	3.9	3.8	3.8	3.8
Total carbohydrate, g[a]	11.7	12.0	12.0	12.0
Lactose, g	0.07	0.09	0.09	0.09
Vitamin A, μg	32	30	30	30
Calcium, mg[a]	15	129	129	129
Phosphor, mg	32	77	77	77
Magnesium, mg	6	6	6	6
Iron, mg	0.30	0.30	0.30	0.30
Zinc, mg	0.14	0.14	0.14	0.14
Straw probiotic (CFU/day)				
Lactobacillus casei CRL 431	-	-	5×10^8	
Lactobacillus reuteri DSM 17938	-	-		5×10^8

UHT, ultra-high temperature; CFU, colony-forming unit.

[a] Based on chemical analyses.

Randomization and blinding

Eligible children were admitted to the study on enrolment basis and stratified according to area of living (flooding and non-flooding), age (<57 and ≥57 months) and gender. A randomization table with treatment codes and block size of eight was generated using SAS version 9.1 (SAS Institute, Inc, Cary, NC) by an independent individual at Wageningen University. Twin siblings of subjects (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. The data safety monitoring board (DSMB) and an independent person at SEAMEO RECFON kept three sets of sealed envelopes allowing deblinding per subject without disclosing other children's treatments.

Outcomes

The primary outcomes were the number and duration of diarrheal episodes. The main secondary outcomes were the number and duration of ARTI episodes. Diarrhea was defined according to the WHO definition (≥3 loose/liquid stools in 24 hours).[23] In addition, all reported diarrhea (broader definition: ≥2 loose/liquid stools in 24 hours) was evaluated. Stool frequency was counted when there was at least 1-hour interval since the previous defecation.[26] An episode was considered to have ended on the last day of diarrhea followed by 2 diarrhea-free days.[27] Duration of diarrhea was defined as number of days from first until

last excretion of the loose or liquid stool that is not followed by another abnormal stool in each episode.[26, 28]

The presence of ARTI was defined when a child had ≥ 1respiratory symptom(s) (runny nose, cough, sore throat) and/or ≥ 1 additional respiratory symptom(s) or 1 constitutional symptom (fever, headache, restless, aphony, shortness of breath, acute ear pain and discharge.[29, 30] These symptoms were confirmed with a physician's diagnosis of acute-upper (rhinitis, pharyngitis, sinusitis, otitis, and common cold) and lower (pneumonia, bronchitis, and bronchiolitis) respiratory tract infection.[31] ARTI duration was the number of consecutive days with ≥ 2defined signs and symptoms, with a 7-day symptom-free interval before a new episode could occur.[32]

Data collection

Field workers collected fecal samples before and at the end of intervention, as well as during diarrheal episodes. Diarrheal samples were collected from onset of diarrhea until maximally 3 days later. Stools contaminated with urine or that had fallen into the toilet or the child's underwear were discarded. Collected stools were kept cool (-20°C) at the field workers' house until storage in a freezer (-70°C) at the laboratory. Stools were freeze-dried and analyzed for calcium[8] and rotavirus (diarrheal samples).[33]

Before and at intervention end, non-fasting venous blood was drawn in the morning by trained phlebotomists. A study physician examined health status of children and field workers performed anthropometric measurements. Lightly clothed children were weighed without shoes using an electronic scale (SECA model 890, SECA, Hamburg, Germany) with a precision of 0.1 kg. Body stature was measured using a microtoise with a precision of 0.1 cm. Routine hematology was performed using an automatic analyzer (ADVIA® 120; Bayer Diagnostics, Tarrytown, NY).[34] A high-sensitivity chemiluminescent assay (Immulite® Dade-Behring, Los Angeles, CA) was used to measure serum high-sensitivity C-reactive protein concentration.[35] Serum α_1-acid glycoprotein was measured by using an enzyme-linked immunosorbent assay.[36]

Follow up observation for diarrhea, ARTIs and adverse events

During the trial, mothers recorded daily defecation patterns (time, frequency and stool's visual appearance)[37] and feces was graded as 1 (normal), 2 (loose), 3 (semi liquid), and 4 (liquid) on a structured form.[38] Field workers verified records twice a week and mothers or caregivers were instructed to report newly observed symptoms of intestinal infection immediately. In addition, the occurrences of ARTIs were determined and recorded by field workers on a structured, pretested form. Final diarrhea and ARTI diagnosis and recording in the trial database were verified by the physicians.

Adverse events were recorded by using International Classification of Diseases, 10th Revision codes.[39] Severity and likelihood of relation to the intervention were scored by the physician and continuously monitored by the DSMB. An independent expert monitored trial conduct and accordance to protocol.

Statistical analysis

Sample size was calculated on the basis of mean episodes and duration of diarrhea, with a preset level of significance of 5% and a power of 80% allowing two-sided testing, and taking 20% dropouts and non-compliant cases into account. A minimum sample size of 480 patients for four treatment groups was required to detect a 21% reduction of mean number of diarrheal episodes and 0.7 day reduction of mean diarrhea duration over a 6-month intervention period. These effect sizes were based on meta-analyses of probiotics.[13, 40]

Intention-to-treat analysis was performed for all outcomes and for all eligible children who were randomly allocated to treatment and had consumed the intervention products at least once. Analyses were conducted according to a predefined data analysis protocol.

The χ^2 test was used for comparison of categorical variables between groups, and Fisher exact test was used when the expected count was <5. Student's t-test was used to identify differences in normally distributed variables between predefined groups (between LC and RC; RC and casei; RC and reuteri). The Mann-Whitney U test was applied when data were not normally distributed. PASW Statistic 17.0.3 for windows (SPSS, Chicago, IL 2009) was used for analyses.

Disease incidence was the number of episodes divided by child-years of observation.[41] For count outcomes, Poisson regression was used, or the negative binomial model in case of excess zeros and overdispersion, to estimate the relative risk (RR) and 95% confidence intervals (CIs) between groups.[42] For this purpose, STATA for windows release 11 (STATA Corp, College Station, TX) was used. The dependent variable was number of episodes, and treatment group the independent variable. The variables area, age, gender, diarrhea and ARTI prevalence within the 2 weeks before study start, household monthly expenditure and weight-for-height z score at baseline were included in the model as covariates. Potential effect modification by age, habitual calcium intake, and baseline nutritional status were assessed by adding interaction terms to the regression model. The adjusted Cox proportional hazards regression model for recurrent events was performed to compare the proportion of children without diarrhea and ARTIs in all groups.

Results

A total of 3,150 children were screened in phase 1 and 1,343 in phase 2. Of 497 eligible children, 3 refused to have baseline measurements taken. In total, 494 children were randomly allocated to 4 treatments groups (**Figure 2.1**) and included in the intention-to-treat analysis.
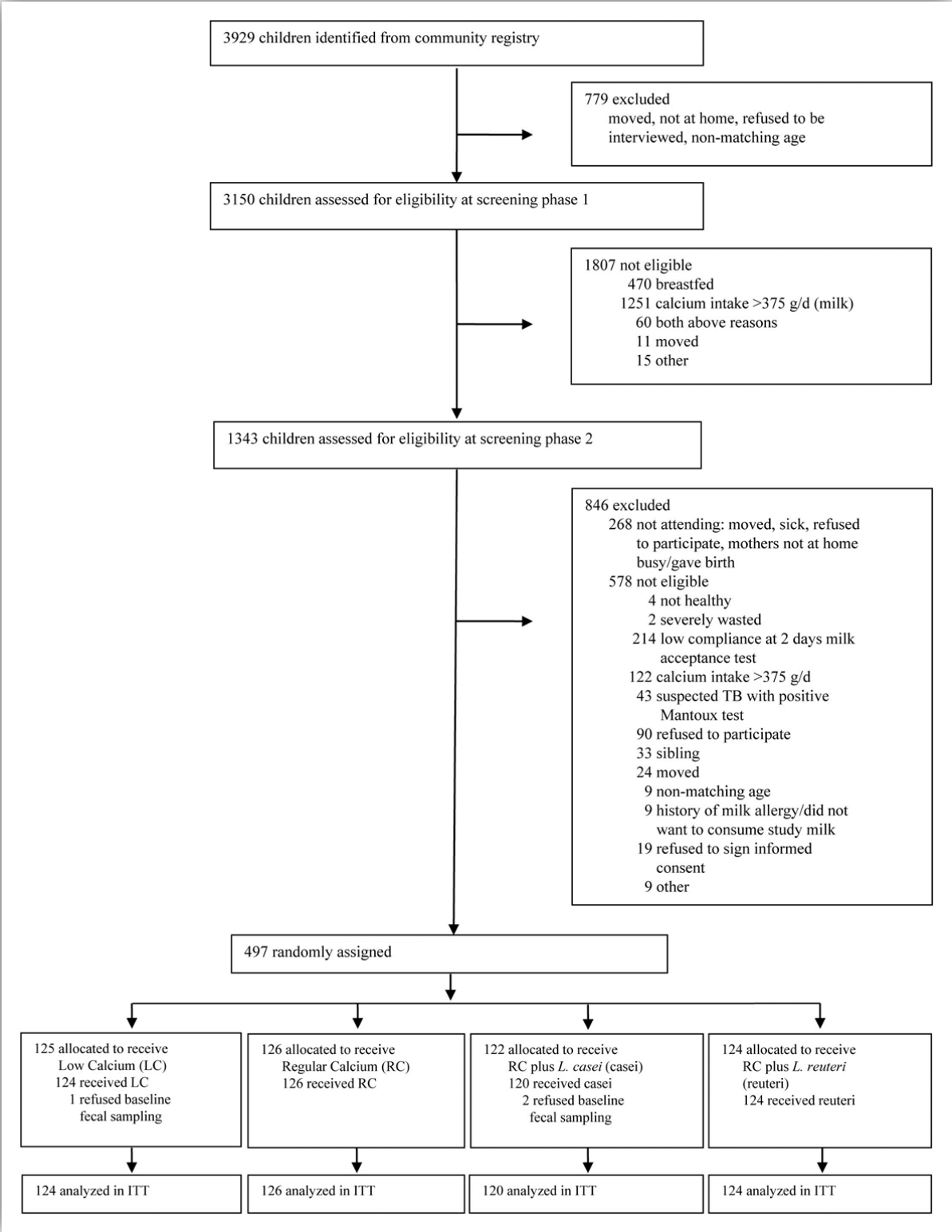
Figure 2.1 Flow diagram of study subjects.
ITT, intention-to-treat; TB, tuberculosis.

Table 2.2 Baseline characteristics of the Indonesian children in according to assigned treatment

Characteristic	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)
Male, n (%)	67 (54)	68 (54)	66 (55)	68 (55)
Age, mean ± SD, mo	59.3 ± 14.3	58.9 ± 14.2	60.3 ±13.7	58.9 ±15.1
Family size, mean ± SD	5.1 ± 1.7	5.4 ±1.7	5.2 ± 1.8	5.0 ±1.8
Household expenditure, mean ± SD, US$/mo[a]	189 ± 97	194 ± 139	159 ± 69	203 ± 181
Mother's education <6 y, n (%)	43 (36)	43 (35)	52 (42)	50 (40)
Diarrhea 2 wk before study, n (%)	20 (16)	13 (10)	24 (20)	15 (12)
ARTI 2 wk before study, n (%)[b]	48 (39)	51 (41)	52 (43)	56 (45)
Serum HS-CRP, median (IQR), 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, median (IQR), 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)
Anemia, n (%)	24 (19)	33 (26)	24 (20)	24 (19)
Nutritional status				
Weight-for-age z score, mean ± SD	-1.27 ± 1.1	-1.40 ± 0.9	-1.15 ± 1.1	-1.26 ±1.2
Height-for-age z score, mean ± SD	-1.53 ± 1.0	-1.65 ± 0.9	-1.39 ± 1.0	-1.47 ± 1.1
Weight-for-height z score, mean ± SD	-0.51 ± 1.0	-0.59 ± 1.2	-0.58 ± 1.0	-0.65 ± 0.9
Fecal calcium, median (IQR), mg/g	7.6 (4.7-11.1)	7.5 (4.8-10.4)	6.6 (4.8-9.3)	7.8 (5.1-11.4)
Habitual dietary intake,[c] mean ± SD				
Energy, kcal/d	1,033 ± 368	1,066 ± 329	1,024 ± 369	976 ± 310
Protein, g/d	34.3 ± 13.5	36.3 ± 12.9	33.5 ± 13.6	32.9 ± 11.0
Carbohydrate, g/d	155 ± 58	157 ± 48	156 ± 58	146 ± 49
Fat, g/d	32.2 ± 13.2	34.5 ± 13.9	31.8 ± 13.7	30.9 ± 11.4
Fiber, g/d	4.5 ± 3.1	5.1 ± 3.5	4.9 ± 3.7	4.6± 2.8
Calcium, mg/d	235 ± 95	241 ± 97	228 ± 105	228 ± 94
Iron, mg/d	6.1 ± 2.7	6.6 ± 2.6	6.2 ± 2.7	6.1 ± 2.4
Zinc, mg/d	4.4 ± 2.0	4.8 ± 1.9	4.4 ± 2.0	4.4 ± 1.6

AGP, α_1-acid glycoprotein; HS-CRP, high-sensitivity C-reactive protein.
[a] Student's t test, significantly different, RC versus casei and reuteri versus casei ($P < .05$).
[b] χ^2 test, significantly different, RC versus reuteri ($P < .05$).
[c] Assessed by using a semiquantitative food-frequency questionnaire.

At admission, all study groups were comparable with respect to socio-demographic, health and nutritional status, and habitual dietary intake (**Table 2.2**). About 21% of children were anemic, 23% underweight, 31% stunted and 3% wasted. The compliance to study products was high (94%) and similar among groups. Both probiotic strains remained >90% viable during the intervention period.

The incidence of WHO-defined diarrhea was not significantly different among groups (**Figure 2.2**, **Table 2.3**). Duration of episodes also did not differ across groups.

For the outcome all reported diarrhea, children receiving RC and *L reuteri* (reuteri group) experienced a significant 32% reduction of diarrheal episodes compared with the RC group (RR: 0.68 [95% CI: 0.46–0.99]) (**Table 2.3**). In addition, the adjusted Cox probability curves showing the proportion of diarrhea-free children was better (*P* = .036) (**Figure 2.3**). For the other treatment groups, results from WHO-defined and all reported diarrhea were comparable.

Importantly, significant interactions with nutritional status were observed ($P < .05$) for both diarrhea outcomes. Stratified analysis showed a strong and significant effect of *L reuteri* in children with below-median weight-for-age z score (RR WHO-defined diarrhea compared to RC group: 0.44 [95% CI: 0.21–0.92]; RR all reported diarrhea: 0.54 [95% CI: 0.31–0.94]) and in children with below-median height-for-age z score (RR WHO-defined diarrhea: 0.44 [95% CI: 0.21–0.90]; RR all reported diarrhea: 0.53 [95% CI 0.30–0.92]). In children above the median z scores the results for the reuteri group were not significantly different from the RC group. The prevalence of underweight and stunting was not significantly changed by the interventions (data not shown).

Table 2.3 Effect of probiotics and calcium on incidence of diarrhea and ARTIs among Indonesian children

Outcome measures	LC (*n*=124)	RC (*n*=126)	Casei (*n*=120)	Reuteri (*n*=124)
WHO-defined diarrhea episodes (≥3 loose/liquid stools in 24 h)				
Mean incidence/child/y	0.91	0.86	1.05	0.67
No. of episodes, mean ± SD	0.40 ± 0.81	0.38 ± 0.78	0.47 ± 0.87	0.30 ± 0.56
Adjusted RR (95% CI)[a]	1.00 (ref)	0.99 (0.62 - 1.58)	-	-
	-	1.00 (ref)	1.21 (0.76 - 1.92)	0.76 (0.46 - 1.25)
Duration of episodes, mean ± SD, d	3.06 ± 4.43	2.94 ± 3.25	2.37 ± 2.68	2.68 ± 3.05
All diarrhea episodes (2 and ≥3 loose/liquid stools in 24 h)				
Mean incidence/child/y	1.73	1.86	2.04	1.28
No. of episodes, mean ± SD	0.73 ± 1.14	0.77 ± 1.38	0.87 ± 1.32	0.56 ± 0.77
Adjusted RR (95% CI)[a]	1.00 (ref)	1.10 (0.77 - 1.59)	-	-
	-	1.00 (ref)	1.06 (0.74 - 1.53)	0.68 (0.46 - 0.99)
Duration of episodes, mean ± SD, d	2.57 ± 4.09	2.03 ± 2.84	2.08 ± 2.40	1.91 ± 2.52
ARTIs episodes				
Mean incidence/child/y	7.22	7.52	7.07	7.45
No. of episodes, mean ± SD	2.41 ± 1.59	2.43 ± 1.61	2.36 ± 1.62	2.48 ± 1.56
Adjusted RR (95% CI)[b]	1.00 (ref)	1.00 (0.86 - 1.18)	-	-
	-	1.00 (ref)	0.97 (0.82 - 1.14)	0.99 (0.84 - 1.16)
Duration of episodes, mean ± SD, d	4.87 ± 4.05	4.90 ± 3.70	4.96 ± 3.71	4.58 ± 3.43

ref, reference group of comparison.
[a] Negative binomial model, adjusted for area of living, gender, age, diarrhea and ARTIs 2 weeks before the study, household expenditure and weight-for-height z score.
[b] Poisson model, adjusted for area of living, gender, age, diarrhea and ARTIs 2 weeks before the study, household expenditure and weight-for-height z score.
- presents an irrelevant comparison, which was not included in the analysis.

The percentages of diarrheal samples positive for rotavirus according to study group were as follows: LC, 28%; RC, 25%; casei, 28%; and reuteri, 19%. Differences were not significant.

The incidence, number of episodes and duration of ARTIs were not significantly different among treatments (**Figure 2.4**, **Table 2.3**).

Reported adverse events (International Classification of Disease, 10th Revision codes) were comparable among groups, except for change in bowel habits (less regular defecation) and asthma. Nine children in the reuteri group experienced change in bowel habits, compared to

two in the RC group. Although based on a few cases, this difference was statistically significant. Three children had asthma in the reuteri group and none in the RC group (*P* < .05). The proportions of antibiotic use during the intervention were 9% in LC, 15% in RC,
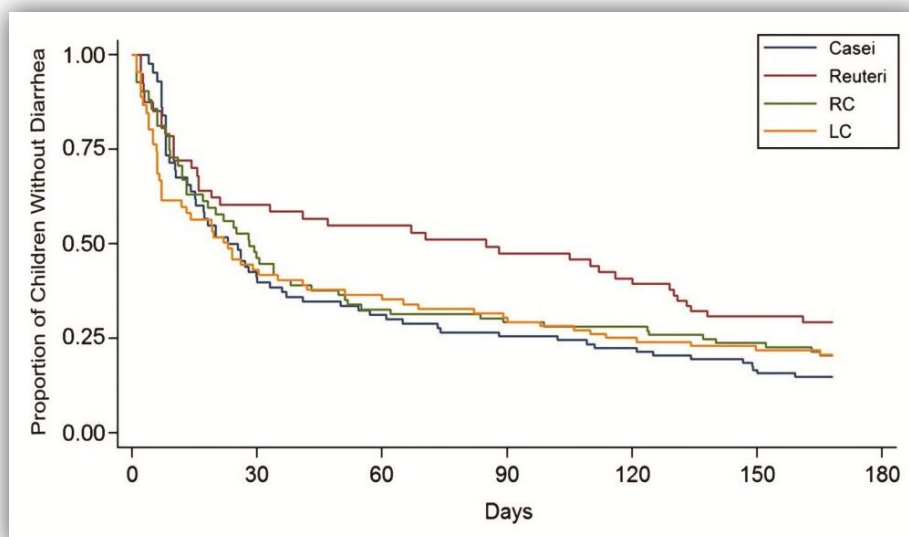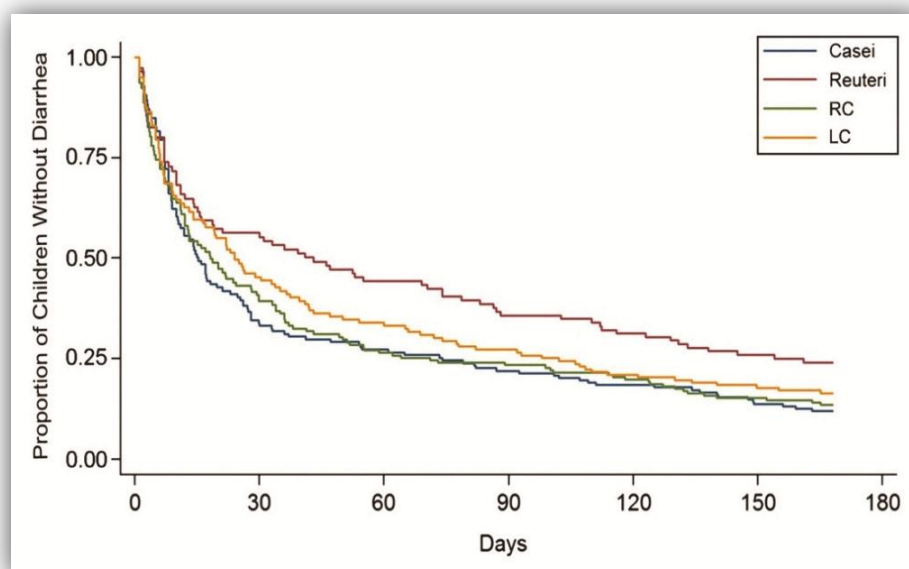

Figure 2.2 Adjusted Cox survival curve of the WHO-defined diarrhea (≥3 loose/liquid stools in 24 hours) episodes. Adjusted for area of living, gender, age, diarrhea and ARTIs 2 weeks before the study, household expenditure. and weight-for-height *z* score. No significant differences between interventions were observed. Probability of survival without diarrhea in relation to duration of diarrheal episodes (days) for 4 groups. No significant differences between interventions were observed.

Figure 2.3 Adjusted Cox survival curve of all reported diarrhea (≥2 loose/liquid stools in 24 hours) episodes. Adjusted for area of living, gender, age, diarrhea and ARTIs 2 weeks before the study, household expenditure, and weight-for-height *z* score. Probability of survival without diarrhea in relation to duration of diarrheal episodes (days) for 4 groups. Significant differences occurred between the RC and reuteri groups (*P* = .036).
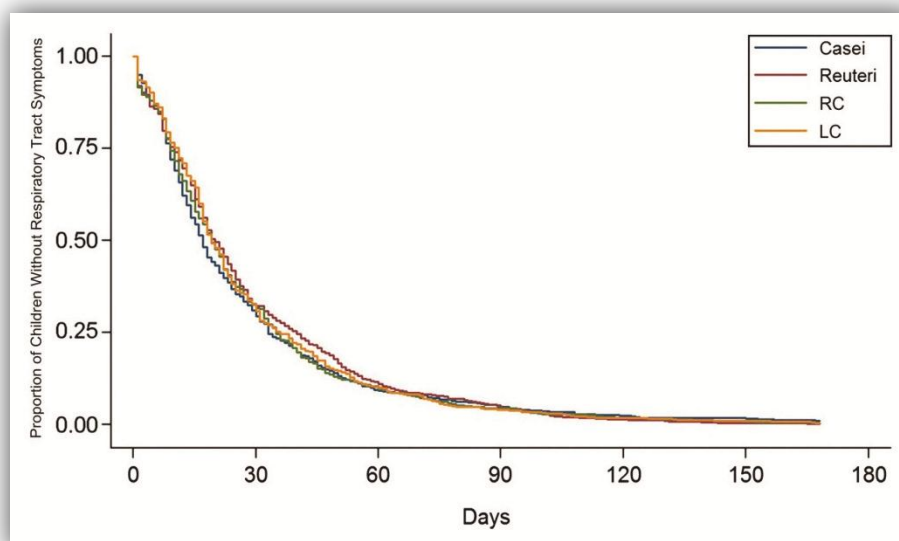
Figure 2.4 Adjusted Cox survival curve of ARTI episodes. Adjusted for area of living, gender, age, diarrhea and ARTIs 2 weeks before the study, household expenditure, and weight-for-height *z* score. Probability of survival without acute respiratory infections in relation to duration of episodes (days) for 4 groups. No significant differences between interventions were observed.

15% in casei and 9% in reuteri group. The median duration of antibiotic use was higher in the RC group (median 10 days, interquartile range (IQR) 4–14) compared to reuteri group (3 days, IQR 2.5–4.5, *P*= .025) but not different from the other groups (LC 4 days, IQR 3–7.5 and casei 5 days, IQR 3–11). One child died from bone tuberculosis 3.5 months after study end, which was unrelated to study participation according to the DSMB.

Discussion

Neither calcium nor *L. casei* CRL 431 affected any of the diarrheal outcomes. In contrast, *L. reuteri* DSM 17938 supplementation significantly reduced the incidence of all reported diarrhea (-32% in ≥2 loose/liquid stools in 24-h) and nonsignificantly reduced the incidence of WHO-defined diarrhea (24% in ≥3 loose/liquid stools in 24 hours). Notably, for both diarrhea outcomes the protective effect of *L reuteri* DSM 17938 was significant in children with lower nutritional status (below-median height- and weight-for-age z score). None of the interventions affected incidence or duration of ARTIs. No serious adverse events related to the interventions were reported.

We applied the WHO definition of diarrhea to collect data on the primary outcome. Because the WHO considers fecal consistency more important than the number of stools[23, 43] and their definition leaves room for registration of any increase in normal stool frequency, we also evaluated the outcome of all reported diarrhea (a broader definition of diarrhea). The WHO definition is the best validated, it may not be generalizable to different settings such as our intervention, which included children of older age and an urban community setting.[44]

Moreover, mothers in the study area usually reported diarrhea when their child defecated ≥2 loose/liquid stools and broader diarrhea definitions were applied by other clinical trials.[45-47]

We did not only rely on mother's perception but implemented an active surveillance to verify mother's daily records with twice-a-week visits of trained field workers and twice-a-month visits of field supervisors. The physician and monitoring expert accompanied the field workers on several of their home visits. All endpoints were assessed using structured and pretested forms as applied by others.[26, 29, 37, 38] The forms were adapted to the local situation and were used by field workers who were rigorously trained and supervised on their application.

Previous evidence on the preventive effect of probiotics on diarrhea and ARTIs has been limited to small studies, mainly hospital or day-care center based, with a short follow-up period and performed in developed countries.[11-13, 40, 48] Therefore, our study in a low-socioeconomic community of a developing country, with a much higher number of subjects and longer follow-up, provides critical data to help establish the relevance of these interventions for the prevention of diarrhea in developing countries. To our knowledge, our study is the first large randomized controlled trial, focusing on the effect of calcium with or without one of two specific probiotics to reduce diarrhea and respiratory tract infections in these settings. Our results indicate that the effect of a probiotic, such as *L. reuteri* DSM 17938, on diarrhea is modified by nutritional status and is confined to children with lower nutritional status.

The rationale for using calcium in children is based on a proof-of-principle study with adults orally challenged with live but attenuated enterotoxigenic *Escherichia coli*. Dietary calcium strongly reduced infection-induced diarrhea in that study.[8] Animal studies show protective effects against Salmonella as well,[49, 50] but human verification for that is still lacking. Rotavirus is responsible for 60% of hospitalized and 41% of outpatient clinics diarrheal cases in Indonesian children.[51] Important bacterial pathogens among children in developing countries are *E coli* (10%–20%), *Salmonella* (<5%), *Shigella* (5%-10%), *Campylobacter* and *Vibrio cholera (*exact %'s unknown).[52] The absence of a beneficial effect of calcium in our trial may indicate a difference in efficacy between children and adults and/or that protective effects are pathogen-dependent.

The application of probiotics to prevent or treat acute diarrhea is based on the assumption that they antagonize intestinal pathogens. Possible mechanisms include the synthesis of antimicrobial substances, competitive inhibition of pathogen adhesion, competition with pathogens for growth substrates, modification of toxin and non-toxin receptors involved in bacterial recognition, and stimulation of the immune responses to pathogens.[53] Thus far, only 3 randomized trials have focused on the role of probiotics in prevention of acute diarrhea in a community setting in developing countries.[17,54,55] These studies found inconsistent effects and differed in probiotic strain and dose, intervention duration, and study subject's age. In our study, the effect size of diarrhea reduction by *L. reuteri* was higher compared with a 14% reduction by supplementing *L. casei* shirota in a comparable study in India,[55] a 6% reduction by *Bifidobacterium lactis* HN019 combined with prebiotic oligosaccharides in India[17] and 6% reduction using *L. rhamnosus* GG in Peru.[54]

Strains of *L reuteri* have been used safely as a probiotic in adults,[56] children,[19] infants,[21,57] and newborns[58] in developed countries. The original strain *L. reuteri* (American Type Culture Collection Strain 55730), from which *L. reuteri* DSM 17938 has been derived by removal of antibiotic resistance gene-carrying plasmids,[59] has been shown to significantly reduce duration of watery diarrhea associated with rotavirus in children aged 6 to 36 months[19,20] and diarrheal episodes in infants in day care centers.[21] In our study, children supplemented with *L. reuteri* experienced a few adverse events, mainly referring to a less regular defecation pattern. *L. reuteri* did not lead to any serious event related to the intervention, and positive results included a lower proportion and shorter duration of antibiotic use.

Milk fermented with *L. casei* CRL 431 and *Lactobacillus acidophilus* reduced the incidence of diarrhea in children,[60] eliminated diarrhea due to post-gastroenteritis syndrome of malnourished hospitalized children,[61] and significantly reduced the number of daily stools, diarrheal duration and vomiting of children with persistent diarrhea.[22] Our results underline that probiotic effects are strain-specific as we found protective effects of *L. reuteri* DSM 17938 against acute diarrhea in children, whereas supplementation of *L. casei* CRL 431 (without other strains) was without effect. The dosage of our probiotics strains ($5x10^8$ CFU/day) is within the effective dosage recommended by Food and Agriculture Organization of the United Nations/WHO.[62]

The major strength of this study was its focus on prevention in contrast to most previous studies, which aimed at treatment of institutionalized children. Additional strengths were its double-blind design, the strict adherence to a rigorous protocol, the use of validated instruments in the assessment of diarrheal episodes, long duration of the intervention, and the excellent compliance rate. Per-protocol analysis, excluding the few noncompliant subjects (6%) and subjects having chronic antibiotic usage, did not change the outcome. A weakness of this study is the lack of microbiological data identifying the diarrhea-inducing pathogens. This subject was not pursued because such stool analysis generally has poor diagnostic yield and incurs high costs.[63] As a consequence, specific effects of calcium or probiotics, if any, against specific diarrheal pathogens may have been missed.

Conclusions

Supplementation of *L reuteri*, at least on a diet including regular calcium milk, is one of the potential interventions to reduce the burden of acute infectious diarrhea in children. These results need to be confirmed by at least one other independent study in a comparable community.

This trial has been registered at www.clinicaltrials.gov (identifier NCT00512824).

What's Known on This Subject:
Some but not all randomized trials have shown effects of probiotics on incidence and duration of diarrhea and respiratory tract infections among children in developing countries. Calcium improves resistance to intestinal infections in adults, but efficacy in children is unknown.

What This Study Adds:
Lactobacillus reuteri DSM 17938 may prevent diarrhea, especially in children with lower nutritional status. Regular calcium milk, alone or with *Lactobacillus casei* CRL 431, did not reduce diarrhea. None of the interventions affected respiratory tract infections in these Indonesian children.

Acknowledgments

Financial disclosure

Specific Author Contribution

Dr Agustina was the principal investigator and responsible for study concept and design, data collection, laboratory analysis, accuracy and completeness of data analysis, and writing the manuscript; Drs Agustina, Kok, van de Rest, Fahmida, Firmansyah, Lukito, and Bovee-Oudenhoven had a major role in study design, interpretation of results, and writing of the report; Dr Feskens was involved in statistical data analyses, interpretation of results, and writing of the report; Drs van den Heuvel and Albers were involved in the study design and trial monitoring; and Drs Kok and Bovee-Oudenhoven coordinated and had final responsibility for the decision to submit for publication.

Funding

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Chapter 3

Randomized trial of *Lactobacillus reuteri* DSM 17938, *Lactobacillus casei* CRL 431 and calcium on diarrhea duration and severity in Indonesian children

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Abstract

Objectives

Diarrheal morbidity and mortality are still prevalent among children in developing countries. We investigated whether supplementation with probiotics and calcium reduces duration and severity of acute diarrhea in Indonesian children.

Methods

We performed a 6-month randomized, double-blind, placebo-controlled trial of 494 healthy children aged 1-6 years living in low-socioeconomic communities of East Jakarta. Children received low-lactose milk with: low calcium content ≈50 mg Ca/day (LC; n=124), regular calcium content ≈440 mg Ca/day (RC; n=126), RC with $5x10^8$ colony-forming units per day *Lactobacillus reuteri* DSM 17938 (n=124), or RC with $5x10^8$ colony-forming units per day *Lactobacillus casei* CRL 431 (n=120). Diarrhea duration and severity were observed during each episode. Severity was assessed using a modified Vesikari score, and diarheal samples were analyzed for osmolarity, mucin, calprotectin and rotavirus presence.

Results

131 children experienced 190 diarrheal episodes. Probiotic strains and calcium did not significantly affect the number of diarrheal episodes, but mean total duration was 1.35 days shorter in the reuteri group (relative risk [RR], 0.60; 95% confidence interval [CI]: 0.36–0.99) and tended to be 0.93 days shorter in the casei group (RR 0.64; 95% CI: 0.39–1.03). Rotavirus prevalence in diarrheal cases was 30% and not significantly different between treatment groups. Rotavirus-positive episodes (36 of 120 analysed samples) were shortened by *L. reuteri* (P = .04) and calcium (P = .009), whereas, *L. casei* shortened duration of rotavirus-negative episodes (P = .04). None of the supplements affected diarrhea severity based on Vesikari score and the fecal markers, except for a higher fecal mucin concentration in the casei group (P = .006).

Conclusions

L. reuteri DSM 17938 reduced total duration of diarrheal episodes. None of the dietary treatments affected diarrhea severity in Indonesian children.

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Introduction

Rotavirus is the leading cause of diarrhea-related hospitalization among children in both developed and developing countries.[1,2] It is estimated to cause >500,000 deaths annually in children under five, with 80% of these deaths occurring in developing countries.[3] In Indonesia, it was estimated that 60% of hospital and 41% of outpatient clinic admissions of children for diarrhea were rotavirus-positive.[4] Yet, bacterial infections are also an important cause of acute diarrhea in developing countries[5] and may lead to severe disease.[6]

The pathophysiology, etiology, and clinical sequelae of acute diarrhea are well described.[7,8] However, information on diarrhea duration and severity and the impact of dietary interventions in clinical trials is limited.[7,9] A Cochrane systematic review indicated that probiotics reduced diarrhea duration in infants and children by 29.2 hours in clinical settings,[10] but probiotics' effect on diarrhea duration and severity have not been evaluated at large scale trial in community settings. Some studies, including probiotics studies, assessed severity of gastroenteritis using a single indicator such as diarrheal stool output, diarrhea duration of >7 days, treatment failure, occurrence of dehydration, or death.[11-13] Other studies scored diarrhea symptoms using severity scales.[14] The most commonly used severity scale is the Vesikari 20-point scale in which an episode of gastroenteritis with a score of ≥11 is considered severe.[14,15] This scale has been adopted as validated on the basis of its frequent use in clinical trials, and can be used as a measure of diarrhea severity in young children.[7] Despite being commonly used by cohort studies on diarrhea and rotavirus vaccines, the Vesikari scale has not been applied by any trial on probiotics in a community setting in a developing country.

During diarrhea, normal intestinal physiology is disturbed due to the presence and colonization of pathogens resulting in altered intestinal water absorption and secretion,[16] mucosal barrier permeability and host defence mechanisms, and intestinal inflammation.[17-20] There is increasing interest in non-invasive, objective biomarkers to quantify intestinal infection severity and/or mucosal inflammation in clinical studies. Candidates of such potential biomarkers are fecal osmolarity, calprotectin and mucin. Calprotectin is a calcium-binding protein with antimicrobial action,[21] richly found in neutrophils in stool and body fluids.[6] It is a promising marker due to its resistance to proteolytic degradation and stability in stool.[22] Levels in stool increase upon intestinal infection and other inflammatory diseases of the gastrointestinal tract.[23, 24] Mucins are a family of heavily glycosylated proteins that are secreted as large aggregates by mucosal goblet cells[25] and is crucial for intestinal mucus formation.[26, 27] The mucus network is an indispensible part of gut barrier function as this viscous layer protects the delicate epithelial cells underneath from aggressive components in the fecal stream, including bacterial pathogens and their toxins.[26] Fecal mucin excretion is enhanced upon intestinal infection and defects in mucus production lead to colonic inflammation.[28,29] Diarrhea severity can also be quantified by determination of fecal osmolarity. The most obvious characteristic of infectious diarrhea is increased fecal water excretion due to reduced absorption or increased secretion of electrolytes,[18] or increased luminal presence of osmolytes attracting water.[16]

Efforts to prevent or reduce diarrheal disease by dietary modulation of intestinal host defenses are promising.[28] However, probiotic studies on infection, immune and inflammatory outcomes in humans are inconsistent, appear to be highly probiotic strain-dependent, and mainly focused on rotavirus diarrhea in children.[30-32] Dietary calcium reduces infectious diarrhea due to *Salmonella enteritidis*[33, 34] and enterotoxigenic *E. coli* (ETEC)[28] in animal models and to ETEC in a human proof-of-principle study in adults.[28] However, its effect in children is unknown. Therefore, we conducted a randomized controlled community trial in which calcium with or without probiotics was supplemented for 6 months to Indonesian children to investigate its effect on duration and severity of acute diarrheal disease due to rotavirus or other causes.

Methods

Subjects

Detailed inclusion and exclusion criteria of subjects has been described elsewhere.[35] Briefly, we included children aged 1–6 years who were apparently healthy, not being breastfed, no symptoms of chronic/congenital diseases and disabilities, not having pulmonary tuberculosis, no history of allergy, not having diarrhea on admission, did not use antibiotics within 2 weeks before study start, not severely wasting, calcium intake ≤ 375 mg/day according to a validated semiquantitative food-frequency questionnaire, capable or willing to drink liquid milk with a straw in a 2-day acceptance test, did show allergy or intolerance to the products, and/or not a sibling of included child (twins excepted). All parents signed the informed consent before inclusion. 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. This study was registered with ClinicalTrials.gov (ID NCT00512824).

Study design

A randomized, double-blind, placebo-controlled trial was conducted in fifteen urban communities representing non-flooding and flooding areas of East Jakarta, Indonesia. These areas were selected on the basis of a number of criteria: high population density, low socioeconomic status, and high prevalence of diarrhea and underweight in under-five children, based on routine data from the primary health care system and local government.

Children were randomly assigned to receive low lactose (0.01 g/L) milk: with a low calcium content (≈50 mg Ca/day, LC); regular calcium content (≈440 mg Ca/day, RC); RC plus *Lactobacillus casei* CRL 431 ($5x10^8$ CFU/day, casei); or RC plus *Lactobacillus reuteri* DSM 17938 ($5x10^8$ CFU/day, reuteri). Milk was sweetened, chocolate-flavored, ambient stable (UHT-sterilized), and packed in tetra packs (Frisian Flag Indonesia, Jakarta). Milk was consumed with straws coated inside with the oil drop as placebo (BioGaia AB, Sweden) or with either *L. casei* CRL 431 (Christian Hanssen, Denmark) or *L. reuteri* DSM 17938 (an antibiotic resistance gene-depleted derivative daughter strain of *L. reuteri* ATCC 55730,

BioGaia)[36] in vegetable oil. Probiotic dosage was based on supplier's information of efficacy, application in children, and safety concerns when dosed for longer time. The different milk drinks and straws were indistinguishable for the investigators and participants. The composition of the milks and straws is described in **Table 2.1**. Viability of the probiotics was checked each month by using selective plating.

Eligible children were admitted to the study on enrolment basis and stratified according to area of living (flooding and non-flooding), age (< 57 months and ≥ 57 months) and sex. A randomization table with treatment codes and block size eight was generated using SAS version 9.1 (SAS Institute, Inc, Cary, NC) by an independent person at Wageningen University. Twin siblings of subjects (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. Faecal calcium at endline was kept blinded from the study team until all other data were fully deblinded. Data Safety Monitoring Board (DSMB) and an independent person at SEAMEO RECFON kept three sets of deblinding envelopes allowing deblinding per subject without disclosing other children's treatments.

Field workers distributed milk and straws twice a week to the parents, who were instructed to store products refrigerated and prevent sun exposure. Parents without refrigerators obtained the products from the field workers' house on daily basis and/or children consumed the products directly at the field workers' house. Mothers were instructed to provide the children 180 mL of milk twice daily (not with a meal) using the straws provided. Field workers observed the children drinking milk at least once a week and empty packages had to be shown during visits. During diarrheal episodes, children continued or restarted drinking milk as soon as possible but after being rehydrated with Oral Rehydration Solution according to WHO guidelines.[37]

Adverse events were recorded by using International Classification of Diseases, 10th Revision codes as described elsewhere.[35] We followed the local standard for outpatient and hospital care for diarrhea and acute respiratory tract infections which were per WHO guidelines as described elsewhere.[35] An independent expert monitored trial conduct and accordance to protocol.

Laboratory measurements

Field workers collected fecal samples before and at the end of intervention, and during diarrheal episodes. Mothers recorded daily defecation pattern (time, frequency and stool's visual appearance).[35] Field workers verified records twice a week and mothers or caregivers were instructed to report newly observed symptoms of intestinal infection immediately. Records of field worker were randomly checked by the fieldsupervisor. Final diarrhea and adverse events diagnoses, and Vesikari scores in the trial database were entered by fieldsupervisors and verified by the study physicians. Stools were freeze-dried and analyzed for calcium (base- and endline samples),[28] rotavirus (diarrheal samples),[38] and fecal mucin, osmolarity and calprotectin (diarrheal and endline fecal samples). Fecal mucin was assessed using a specific fluorimetric assay of O-linked glycoproteins.[28] Fecal osmolites were

measured to assess the relative amount of water present in the original feces.[28] Calprotectin in feces was measured using a commercial ELISA (Calpro, NovaTec Immundiagnostica GmbH, Dietzenbach, Germany).[39]

Outcome assessments

The outcomes presented in this paper were duration of diarrheal episodes, diarrhea severity and the presence of rotavirus in diarrheal samples. Diarrhea was defined according to WHO definition (≥3 loose/liquid stools in 24 hours).[37] Stool frequency was counted when there was at least one hour interval between subsequent defecation moments.[12] An episode was considered to have ended on the last day of diarrhea followed by 2 diarrhea-free days.[11] The number of episodes is the sum of all diarrheal episodes that occurred during the intervention period. Duration of a diarrheal episode was defined as number of days from first until last excretion of the loose or liquid stool that is not followed by another abnormal stool in each of the episodes.[12] The cumulative (total) duration of diarrhea was the sum of all diarrheal days of all episodes per child per 6-month of intervention period.[40, 41]

Severity of diarrhea was assessed by the modified Vesikari score (MVS) based on the mothers' subjective information and by objective measurement of the above-mentioned fecal biomarkers. Vesikari score was slightly adjusted to better fit with the community situation and study population. The original 20-point numerical score includes the assessment of diarrhea duration (3-points), maximal diarrhea frequency in 24-hour (3-points), vomiting duration in days (3-points), maximal vomiting frequency in 24 hour (4-points), fever in degrees Celcius (3-points), dehydration percentage (3-points) and rehydration given or hospitalization (2-points).[15] We graded the severity by using a slightly modified 18-point Vesikari scale and asked a qualitative report of mothers for fever (yes or no) instead of rectal temperature. Also, the percent dehydration was replaced by estimated degree of dehydration,[7,14,38,42] since this variable is not easily assessed in a community setting. We defined mild to moderate disease as scores of <11 and severe disease as scores of ≥11.[7]

Statistical analysis

Sample size was calculated based on the mean episodes and duration of diarrhea, with a pre-set level of significance of 5% and a power of 80% allowing two-sided testing, and taking 20% dropouts and non-compliant cases into account. A minimum sample size of 480 for four treatment groups was required to detect a 21% reduction of the mean number of diarrheal episodes and 0.7 day reduction of mean diarrhea duration over a 6-month intervention period. These effect sizes were estimated based on meta-analyses of probiotics.[32, 43]

Intention-to-treat (ITT) analysis was performed for all outcomes and for all eligible children who were randomly allocated to treatment and had consumed the intervention products at least once. Analyses were carried out according to a predefined data analysis protocol.

For all outcomes, double data entry was applied. Chi-square test was used for comparison of categorical variables between groups and Fisher exact test was used when expected count <5.

Student's t-test was used to identify differences in normally distributed variables between predefined groups (between LC and RC; RC and casei; RC and reuteri). Mann-Whitney U test was applied when data were not normally distributed. We used PASW Statistic 17.0.3 for windows (SPSS, Chicago, IL 2009) for the analyses.

Disease incidence was the number of episodes divided by child-years of observation.[44] For count outcomes (episodes, duration and vesikari score) we used the Negative binomial model in case of excess zeros and over-dispersion, to estimate the relative risk (RR) and 95% Confidence Intervals (CI) between groups.[45] For this purpose, we used STATA for windows release 11 (College Station, Texas 2009). Mann-Whitney U test was performed to evaluate the treatment-effect (median differences) on fecal calprotectin, mucin and osmolarity in children who suffered from diarrhea. Number of episodes, total duration of episodes, Vesikari score and fecal osmolarity, mucin and calprotectin were the dependent variables and treatment group the independent variable. The variables area, age, sex, diarrhea and acute respiratory infections prevalence within 2-week before study start, household monthly expenditure and weight-for-height z-score at baseline were included in the model as covariates. Potential effect modification by baseline age, habitual calcium intake and nutritional status were assessed by adding interaction terms to the regression model.

Results

A total of 3,150 children were screened in phase 1 and 1,343 in phase 2. From the 497 eligible children, 3 refused to have baseline measurements taken. In total, 494 children were randomly allocated to four treatments groups (**Figure 3.1**) and were included in ITT analysis.

At admission, all study groups were comparable regarding socio-demographic characteristics (including environment, and maternal hygiene and caring), health and hematology status, and habitual dietary intake (**Table 3.2**). Changes of habitual dietary intake between baseline and endline were not significantly different between groups. The compliance to study products use was high (94%) and similar among groups. Both probiotic strain remained >90% viable during the intervention.

The incidence and mean number of WHO-defined diarrhea episodes were not significantly different among groups (**Table 3.3**). As reported elsewhere, incidence of all reported diarrhea was significantly lower in the reuteri group particularly among children with lower nutritional status.[35]

Analyses of the diarrheal children (n=131, together having 190 episodes) showed that mean cumulative duration of diarrhea in the reuteri group was significantly shorter (40%) as compared to the RC group (95% CI 0.36–0.99); P<.049). Supplementation with *L. reuteri* shortened the cumulative diarrhea episode duration by 1.35 days. The cumulative duration of diarrheal episodes tended to be shorter (36%) in the casei group as well but the effect did not reach statistical significance (95% CI 0.39–1.03; P=0.07).
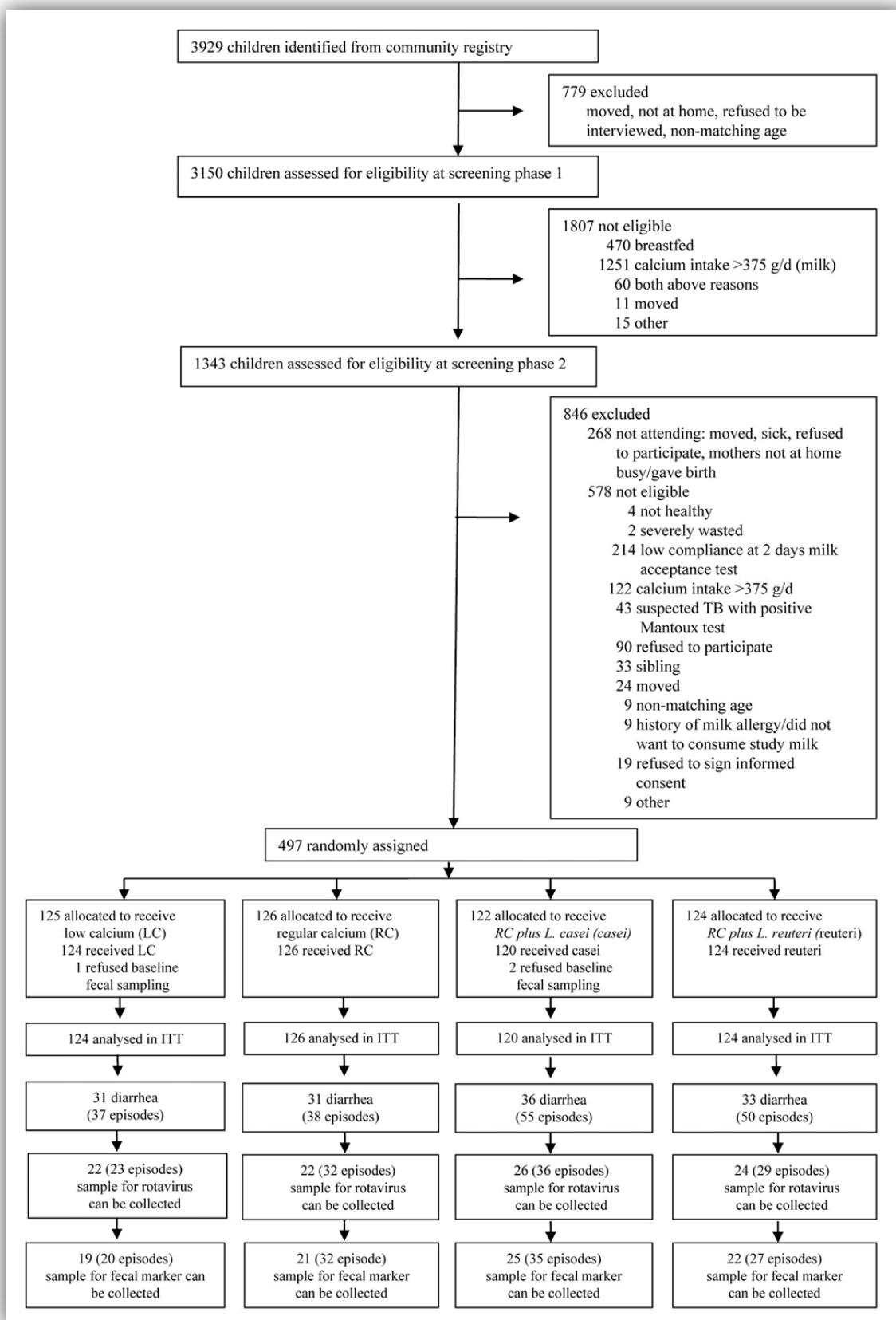
Figure 3.1 Flow diagram of study subjects.
ITT, intention-to-treat; TB, tuberculosis.

Table 3.2 Baseline characteristics of the Indonesian children in four intervention groups

Characteristic	LC (n=124)	RC (n=126)	Casei (n=120)	Reuteri (n=124)
Areas				
Flooding	81 (65%)	82 (65%)	78 (65%)	82 (66%)
Non flooding	43 (35%)	44 (35%)	42 (35%)	41 (34%)
Age (months)	59.3 (14.3)	58.9 (14.2)	60.3 (13.7)	58.9 (15.1)
Sex (male)	67 (54%)	68 (54%)	66 (55%)	68 (55%)
Household expenditure (US$ per months) [a]	189 (97)	194 (139)	159 (69)	203 (181)
Mother's education <6 years	43 (36%)	43 (35%)	52 (42%)	50 (40%)
Health status				
Diarrhea 2 wk prior to study	20 (16%)	13 (10%)	24 (20%)	15 (12%)
ARI 2 wk prior to study[b]	48 (39%)	51 (41%)	52 (43%)	56 (45%)
Hematology profile				
hs-CRP (mg/L)	0.8 (0.2, 1.8)	0.8 (0.3, 2.9)	0.8 (0.3, 2.5)	0.7 (0.3, 3.0)
AGP (g/L)	0.8 (0.7, 0.9)	0.8 (0.7, 1.0)	0.8 (0.7, 1.0)	0.8 (0.7, 0.9)
Anemia	24 (19%)	33 (26%)	24 (20%)	24 (19%)
Nutritional status, mean (SD)				
Weight-for-age	-1.26 (1.2)	-1.15 (1.1)	-1.27 (1.1)	-1.40 (0.9)
Height-for-age	-1.47 (1.1)	-1.39 (1.0)	-1.53 (1.0)	-1.65 (0.9)
Weight-for-height	-0.65 (0.9)	-0.58 (1.0)	-0.51 (1.0)	-0.59 (1.2)
Fecal calcium (mg/g)	7.6 (4.7, 11.1)	7.5 (4.8, 10.4)	6.6 (4.8, 9.3)	7.8 (5.1, 11.4)
Dietary intake,[c] mean (SD)				
Energy (kcal/d)	1033 (368)	1066 (329)	1024 (369)	976 (310)
Protein (g/d)	32.9 (11.0)	33.5 (13.6)	34.3 (13.5)	36.3 (12.9)
Carbohydrate (g/d)	146 (49)	156 (58)	155 (58)	157 (48)
Fat (g/d)	30.9 (11.4)	31.8 (13.7)	32.2 (13.2)	34.5 (13.9)
Fiber (g/d)	4.6 (2.8)	4.9 (3.7)	4.5 (3.1)	5.1 (3.5)
Calcium (mg/d)	228 (94)	228 (105)	235 (95)	241 (97)
Iron (mg/d)	6.1 (2.4)	6.2 (2.7)	6.1 (2.7)	6.6 (2.6)
Zinc (mg/d)	4.4 (1.6)	4.4 (2.0)	4.4 (2.0)	4.8 (1.9)

Note: Data are number, number (%), mean (SD, median (interquartile range).
LC indicates low calcium; RC, regular calcium; ARI, acute respiratory infections; hs-CRP, serum high-sensitivity C-reactive protein; AGP, alpha 1-acid glycoprotein.
[a] Student's t test, significantly different between RC *vs* casei (*P*<.05).
[b] Chi-square test, significantly different between RC *vs* reuteri (*P* <.05).
[c] Defined as the percentage of children with hemoglobin values < 11.0 g/dL (children < 5 years) or < 11.5 gd/L (children 5–11 years).
[c] Assessed using a semi-quantitative food frequency questionnaire.

Table 3.3 Effect of probiotics and calcium on the number of episodes and severity of acute diarrhea assessed by the modified Vesikari score

Variable	LC	RC	Casei	Reuteri
All children, n=494	**124**	**126**	**120**	**124**
Mean incidence/child/year	0.91	0.86	1.05	0.67
Mean number of episode (SD)	0.40 (0.81)	0.38 (0.78)	0.47 (0.87)	0.30 (0.56)
Adjusted RR (95% CI)	1.00 (ref)	0.99 (0.62–1.58)		
		1.00 (ref)	1.21 (0.76–1.92)	0.76 (0.46–1.25)
Diarrheal children, n=131[a]	**33**	**31**	**36**	**31**
Mean number of episodes (SD)	1.52 (0.87)	1.55 (0.81)	1.53 (0.91)	1.19 (0.40)
Adjusted RR (95% CI)	1.00 (ref)	1.08 (0.72–1.62)		
		1.00 (ref)	0.94 (0.63–1.42)	0.74 (0.47–1.14)
Mean cumulative duration in days (SD)	4.64 (5.58)	4.55 (5.06)	3.62 (4.69)	3.20 (3.51)
Adjusted RR (95% CI)	1.00 (ref)	1.18 (0.72–1.92)		
		1.00 (ref)	0.64 (0.39–1.03)	0.60 (0.36–0.99)[b]
Mean Vesikari score (SD)	3.86 (1.37)	3.85 (1.09)	3.96 (1.58)	3.63 (1.19)
Adjusted RR (95% CI)	1.00 (ref)	1.02 (0.79–1.32)		
		1.00 (ref)	1.01 (0.78–1.31)	0.92 (0.71–1.21)

Note: LC indicates low calcium; RC, regular calcium
Data are mean (SD) and Relative Risk (95% Cl); Cl, confidence interval; ref, reference group of comparison.
Adjusted for area of living, sex, age, diarrhea and ARI 2 weeks prior to the study, household expenditure and weight-for-height z-score.
[a] These are all diarrheal children regardless whether fecal samples could be collected. Also, some children had multiple episodes.
[b] Negative binomial model, significantly different between reuteri vs RC ($P < .049$).

Calcium did not affect duration of diarrheal episodes. Six episodes of persistent diarrhea (diarrhea duration >14 days) were recorded: 1 in RC, 3 in LC and 1 in the casei group) (data not shown). It should be noted that, except for one episode, almost all diarrhea in our study was categorized as mild based on the symptom severity scores of the MVS. Vesikari scores were not different between the dietary treatment groups RC and LC (RR 1.02, 95% CI 0.79–1.32), casei and RC (RR 1.01, 95% CI 0.78–1.31) or reuteri and RC (RR 0.92, 95% CI 0.71–1.21) (**Table 3.3**).

From the 190 diarrheal episodes, 120 stool samples could be collected for rotavirus analysis. Failure to collect stool in the remaining episodes was mainly due to unwillingness of children who experienced recurrent diarrhea to retake their stool or difficulty to obtain samples of watery diarrhea. Rotavirus was detected in 36 of 120 (30%) collected stools. The prevalence of rotavirus in collected diarrheal samples was 28% in RC, 31% in LC, 33% in casei and 26% in the reuteri group (**Table 3.4**).

Diarrhea duration of rotavirus-positive episodes was significantly shorter in RC group (RC *vs* LC RR 0.40 95% CI 0.20-0.80; *P* = .009); and shorter in reuteri group (reuteri *vs* RC RR 0.38 95% CI 0.15–0.98; *P* = .04). In rotavirus-negative episodes, diarrhea duration was shorter in the casei group (casei *vs* RC RR 0.52 95% CI 0.28-0.96; *P* = .04). Despite statistical significance, it should be noticed that especially the rotavirus-positive results are based on relatively few cases. Severity of diarrhea, as assessed by the Vesikari score, did not differ between rotavirus-negative and rotavirus–positive episodes on total group level (**Table 3.4**) and within dietary treatment groups (data not shown).
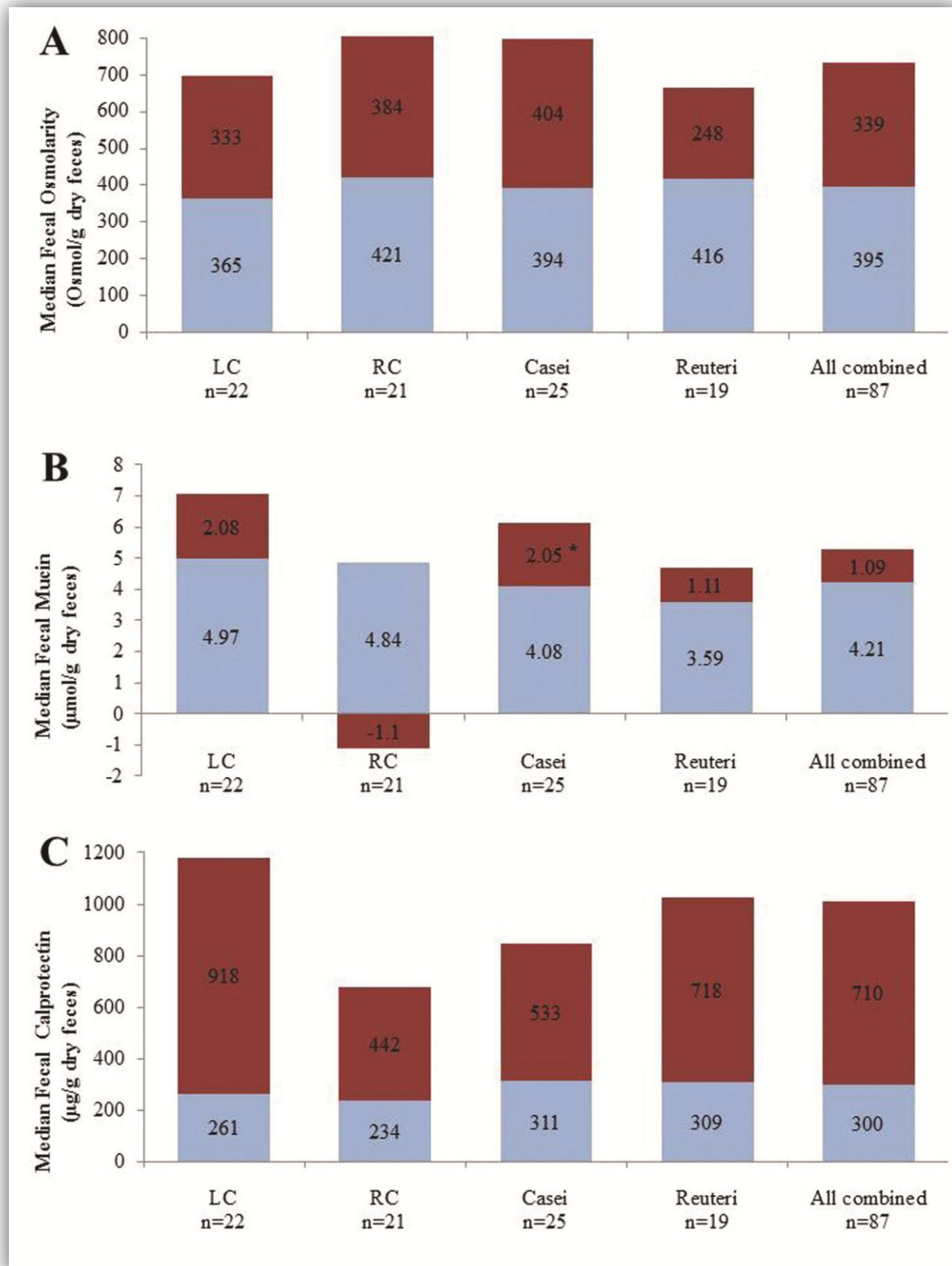
Figure 3.2 Diarrhea-induced changes in fecal *(A)* osmolarity, *(B)* mucin and *(C)* calprotectin in samples collected during diarrheal episodes (red part of the bars) and levels of these markers at endline (normal situation; blue part of the bars) in the intervention groups and all groups combined. Mean values per child were used for children that experienced multiple episodes. RC, regular calcium; LC, low calcium.

Fecal osmolarity, mucin and calprotectin were assessed in 87 children, together having 113 episodes from the total 190 episodes of diarrhea observed during the study. Missing samples were due to practical difficulties in collecting watery diarrhea samples and not fullfilling the minimum volume needed for laboratory analyses. Also, one child missed the endline measurement. Diarrheal episodes were characterized by clear increases in fecal osmolarity, mucin and calprotectin in all groups in comparison with (normal) fecal samples collected at study endline (n=87; **Figure 3.2**). A significantly greater diarrhea-induced change in fecal mucin was observed in the casei group in comparison with the RC group (*P* = .006). Also, fecal mucin tended to increase during diarrhea in the LC group, but the effect was not statistically significant (*P* = 0.09) (**Figure3.2**). There was no significant correlation between Vesikari scores and the fecal biomarkers of diarrhea severity. This may be due to the fact that most diarrheal episodes were classified as mild (mean Vesikari scores were only 3-4 on a total scale of 0-18) and variability in scores was thus rather low (**Table 3.3**). In addition, diarrheal episode duration was not significantly correlated with any diarrhea severity marker or the MVS (data not shown). Reported adverse events (ICD-10) and the proportion and duration of antibiotic use were described elsewhere.[35]

Table 3.4 Prevalence of rotavirus-related diarrhea and severity of diarrheal disease as assessed by the modified Vesikari score among the groups

Variable	LC	RC	Casei	Reuteri	All
Diarrheal children (episode), n	33 (50)	31 (48)	36 (54)	31 (37)	131 (190)
Children (episode) analyzed for rotavirus, n	24 (29)	22 (32)	26 (36)	22 (24)	94 (120)
Rotavirus prevalence, n (%)					
Rotavirus-positive	9 (31%)	9 (28%)	12 (33%)	6 (26%)	36 (30%)
Rotavirus-negative	20 (69%)	23 (72%)	24 (67%)	17 (74%)	84 (70%)
Duration of diarrheal episodes (days), mean (SD)					
Rotavirus-positive	5.4 (6.0)[a]	2.4 (1.7)	2.3 (1.9)	1.9 (2.2)[a]	3.0 (3.5)
Rotavirus-negative	4.1 (5.2)	4.0 (4.2)	2.1 (2.1)[a]	2.6 (3.2)	3.2 (3.9)
Severity of diarrheal episodes (score),[b] mean (SD)					
Rotavirus-positive	4.7 (1.9)	4.1 (1.5)	3.8 (0.7)	4.3 (2.1)	4.2 (1.5)
Rotavirus-negative	3.9 (1.6)	4.5 (1.7)	4.4 (1.9)	3.8 (1.3)	4.2 (1.7)

Note: 120 collected samples for rotavirus analysis from the 190 diarrheal cases.
[a]Negative binomial model, significantly different between: RC *vs* LC (relative risk [RR] 0.40; 95% CI: 0.20−0.80; *P* = .009); reuteri *vs* RC (RR 0.38; 95% CI: 0.15−0.98; *P* = .04); casei *vs* RC (RR 0.52; 95% CI: 0.28−0.96; *P* = .04); Adjusted for area of living, sex, and age.
[b]Total Vesikari score of diarrhea (score range 0-18-point; score<11 is mild; score ≥ 11 is severe). Mean values per child were used for children who experienced multiple episodes.

Discussion

This community-based, randomized controlled trial has shown that the probiotic strains and calcium did not significantly affect the number of WHO-defined diarrheal episodes. Analyses of the diarrheal children (n=131, together having 190 episodes) showed that the mean cumulative duration of diarrhea in the reuteri group was significantly shorter (-40%) as compared to the RC group (*P* = .049). Diarrhea duration tended to be shorter in the casei group (-36%) as well but the effect was not statistically significant (*P* = .07). Stratified analysis discriminating between rotavirus-positive and rotavirus-negative diarrheal episodes

showed that the beneficial effect of *L. reuteri* on diarrhea duration was mainly in rotavirus-positive episodes (*P* = .04), whereas *L. casei* mainly shortened rotavirus-negative diarrhea (*P* = .04). Although, calcium did not affect overall diarrhea duration, it significantly reduced rotavirus-positive episode duration (*P* = .01). Despite statistical significance, we like to stress that these rotavirus-specific effects are based on a relatively small numbers of cases (**Table 3.4**), as rotavirus is just one of the many intestinal pathogens responsible for childhood diarrhea. Confirmation that *L. reuteri,* and also *L. casei* and calcium, can shorten acute diarrheal episodes dependent on the intestinal pathogen involved, needs to come from larger (>500 subjects) controlled intervention studies.

L. reuteri supplementation shorted the cumulative diarrhea duration by 1.35 days in a 6-month period, which is in line with other reported probiotic interventions in children. A recent Cochrane review concluded that diarrhea duration in infants and children was reduced by 29.2 hours in those taking probiotics for <14 days (95% CI 25.14–33.25 hours, fixed effect model; 30.48 hours, 95% CI 18.51–42.46, random effects model, 12 trials, n =970).[10, 46] This effect was larger than the previously reported effect sizes based on meta-analyses studies undertaken by Szajweska in 2001 and McFarland in 2006 for all probiotic strains in children showing a reduction of 18.2 hours[30] and 13.4 hours,[47] respectively. Our study is also consistent with reviews or meta-analyses on efficacy of specific probiotics to reduce diarrhea duration in children: Van Niel et al showed a reduction of 0.7 days by *Lactobacilli* administration,[43] Szajewska et al indicated a reduction of 1.1 days in infants treated with *L. casei* GG[48] and Chmielewska et al showed a reduction of 0.92 days by *L. reuteri* ATCC 55730.[49,50] These effects were more evident in hospital-based settings of developed countries,[51] whereas two studies performed in a community setting in developing countries[52,53] did not explicitly present the effect on diarrhea duration and another study failed to show an effect of probiotics on diarrhea duration.[54] Our observed reduction in rotavirus-induced diarrhea duration of 0.5 days was much lower than the 2.1 days reduction found in a meta-analyses in 2007.[48]

It should be taken into account that effect sizes of nutritional interventions, aimed to prevent disease, are generally of a much smaller magnitude than those obtained by pharmaceutical interventions to cure existing disease. Although, the potential benefit from probiotics supplementation on diarrhea duration is likely small,[55] it might still be clinically relevant in children that need to grow up in a poor environment with low nutritional food intake and high infection pressure. Each additional diarrhea-free day can help to prevent further deterioration of their delicate nutritional status and well-being. It should be remarked that, from public health perspective, the number of diarrheal episodes children experience is more important than durationof the episodes. Occurrence of multiple episodes is more harmful to children's growth, whereas long episode duration is not associated with growth impairment.[56] In that respect, it is important to notice that in the present study *L. reuteri* DSM 17938 supplementation tended to reduce the number of WHO-defined diarrheal episodes by 24% (*P* = .28) and all reported diarrhea episodes by 32% (*P* = .046).[35] It also implies that future probiotic research in developing countries should give more attention to prevention of acute diarrheal disease in children instead of treatment as done in the past.[67]

There was no significant beneficial effect of dietary calcium or one of probiotic strains on diarrhea severity measured by Vesikari score. We applied a MVS similar to Freedman et al.[7] The MVS was considered to be reliable, valid, and easy to administrate by mothers and field workers. This method was pre-tested to ensure its applicability in our study population. In our study population, all diarrheal episodes with the exception of one, had a MVS<11. This score is categorized as mild and is in contrast with the high percentage of severe diarrhea (Vesikari >11) observed in rotavirus vaccine studies in medical care settings in sub-Sahara Africa (>45%)[57] and Asia (>63%).[58] The low MVS in our study (groups mean scores were 3-4) demonstrate that diarrhea in outpatients and/or children in community settings is typically of a milder type.

Considering that expected effect sizes of dietary interventions are generally small, we wanted to include objectively measurable and quantitative fecal biomarkers of diarrhea severity in our study besides the more subjective and semi-quantitative MVS. Although our study population suffered from milder diarrhea than the diarrhea in the above-mentioned studies, there were clear responses in the applied diarrhea severity biomarkers when comparing normal fecal endline samples with samples collected in diarrheal episodes. Fecal calprotectin concentrations increased >2-fold during acute diarrheal episodes in the present study. Application of this biomarker in clinical trials and medical practice is growing as fecal calprotectin is useful in the diagnosis and quantification of gut mucosal inflammation and monitoring of therapeutic efficacy, both in adults and children.[59] However, it is unspecific because multiple causes increase the presence of intestinal neutrophils, leading to increased fecal concentration of this protein.[60] The increased levels in the diarrheal samples of our study likely reflect infection-induced intestinal mucosal inflammation.[6] Analysis of stool osmolarity is helpful in evaluating patients with diarrhea. When gastrointestinal digestion or microbiota-related metabolism is altered, diarrhea can result from osmotic forces in the lumen (e.g. lactose in lactose malabsorbers) or an increased secretory state (e.g. bacterial enterotoxin-induced diarrhea).[61] Though not statistically significant, the reuteri group had the lowest infection-induced change in fecal osmolarity and Vesikari score, which may reflect lower diarrhea severity. There has been scientific debate, whether fecal osmolarity has the potential to differentiate osmotic from secretory diarrhea by evaluation of the stool ion gap.[62] Diarrhea type was not subject of our study, but results showed that acute diarrheal episodes were characterized by increased fecal osmolarity suggestive for the osmotic type of diarrhea.[61] Our fecal sample collection logistics were very strict to ensure quick cooling and storage of samples in freezers to prevent ex-vivo bacterial fermentation and thus artificial ex-vivo osmolyte increases.

The acute infection-induced changes in fecal mucin were not uni-directional among dietary treatment groups and less pronounced. The casei group showed a significant increase (*P*=.006), whereas fecal mucin in the regular calcium group tended to decrease (*P*=.09) during diarrheal. The mucus layer is an important contributor to intestinal host defence and inhibits pathogens adhesion to epithelial cells and subsequent bacterial translocation of invasive strains.[61,62] Mucin excretion is stimulated during infectious diseases.[26,28] So far, studies on intestinal mucin merely focused on qualitative differences in mucin oligosaccharide composition during various intestinal diseases[63,64] whereas quantitative aspects are understudied. Despite responsiveness of fecal calprotectin, osmolarity, and

mucin to less extent, to acute diarrhea, levels were not significantly modified by dietary treatment. The absence of supplement-induced differences in diarrhea severity markers corresponds with the equal Vesikari scores among groups. The relatively mild diarrheal episodes observed in our study population lead to a rather homogeneous dataset of fecal biomarker responses. Unfortunately, this hampers conclusions whether fecal calprotectin, osmolarity and mucin are truely quantitative indicators of diarrhea severity. Confirmation needs to come from future infection studies in subjects with a wider range of diarrhea severity.

Conclusion

L. reuteri DSM 17938 significantly reduced the total duration of diarrheal episodes, likely by mainly affecting rotavirus-positive diarrhea. *L. reuteri, L. casei* CRL 431 and calcium did not affect diarrhea severity in Indonesian children.

Acknowledgments

Conflicts of interest

Specific author contributions

were involved in study design and monitoring. Frans J. Kok and Ingeborg M.J. Bovee-Oudenhoven coordinated and had final responsibility for the decision to submit for publication.

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Chapter 4

Effect of milk calcium with or without probiotics on growth, iron and zinc status of Indonesian children

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Abstract

Objective

Probiotics and milk calcium may increase intestinal infection resistance, but their effect on growth, iron and zinc status of Indonesian children is uncertain. We investigated whether milk supplemented with 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.

Methods

A six-month randomized trial was conducted in low-socioeconomic urban communities of East Jakarta. Healthy children (n=494) were randomly assigned to receive low lactose milk: with low calcium content ≈50 mg Ca/d (LC; n=124), regular calcium content ≈440 mg Ca/d (RC; n=126), RC with $5x10^8$ CFU/d *Lactobacillus casei* CRL 431 (casei; n=120); or RC with $5x10^8$ CFU/d *Lactobacillus reuteri* DSM 17938 (reuteri; n=124). Dietary intake, growth, anemia, iron and zinc status were assessed before and after the intervention.

Results

The increase in weight gain, weight-for-age z score (WAZ) changes and monthly weight and height velocities were significantly higher in the reuteri compared with RC group [0.22 (95% CI: 0.02, 0.42) kg, 0.09 (95% CI: 0.01, 0.17) Z, 0.03 (95% CI: 0.002, 0.05) kg and 0.03 (95% CI: 0.01, 0.05) cm, respectively]. Casei significantly increased average monthly weight velocity [0.03 kg (95% CI: 0.001, 0.05 kg), but not height. However, the changes in underweight and stunting prevalence, anemia prevalence and iron and zinc status were similar among groups.

Conclusions

L. reuteri DSM 17938 modestly improved growth, by increasing weight gain, WAZ changes, and weight and height velocity in Indonesian children. *L. casei* CRL 431 modestly improved weight velocity. Independent from probiotics supplementation, regular milk calcium neither affected growth, nor iron and zinc status. This study was registered at clinicaltrials.gov (registration ID no. NCT00512824).

Introduction

Undernutrition and multiple micronutrients (MMNs) deficiencies persist as the most serious nutritional problems among children <5 years in developing countries.[1,2] The Indonesian national prevalence of stunting (37%) and wasting (14%) is higher, and underweight (18%) is similar compared to the estimated overall prevalence of undernutrition among under-five 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 benefits of nutrition interventions on growth and micronutrient status of under-five children used different approaches of supplementations and, fortified foods, but results are conflicting.[5, 7-11] In addition, some studies indicated that nutrient-dense foods may help to prevent stunting and wasting in young children, but more data are needed to identify the impact of this approach.[12-14] Probiotics are often supplemented to dairy foods and both probiotics.[15,16] and calcium in milk[17] may strengthen intestinal infection resistance. However, the impact of probiotics on growth and micronutrient status is uncertain. In addition to a mice 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 has no effect on weight, height, body fat, or lean mass in randomized clinical trials (RCTs).[24-26] However, such evidence in children in developing countries is scarce.

The combination of regular calcium milk and probiotics may give synergistic benefits, but less is known about the effects on 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 to iron absorption in adults was 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 absorptions 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 were performed in a pediatric population. These studies were conducted mainly in developed countries involving children with adequate calcium and iron intake and showed contradictive results.[36, 38-40]

Until now, evidence is inconclusive whether prolonged dietary supplementation with calcium and probiotics in children with low habitual calcium intakes in developing countries including Indonesia, affects growth, and iron and zinc status. Therefore, we investigated the hypothesis that milk supplemented with probiotics would improve growth, and iron and zinc status, whereas milk calcium alone would improve growth, but reduce iron and zinc status of Indonesian children.

Subjects and methods

Study design

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.

Subjects

Detailed criteria of subjects has been described elsewhere.[41] Briefly, we included children aged 1–6 years who were apparently healthy, not being breastfed, no symptoms of chronic/congenital diseases and disabilities, not having pulmonary tuberculosis, no history of allergy, not having diarrhea on admission, did not use antibiotics within 2 wk before study start, not severely wasting, calcium intake ≤ 375 mg/d according to a validated semiquantitative food-frequency questionnaire, capable or willing to drink liquid milk with a straw in a 2-d acceptance test, did show allergy or intolerance to the products, and/or not a sibling of 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.

Randomization and blinding

Detailed procedure for randomization and blinding procedure has been described elsewhere.[41] Eligible children were admitted to the study on enrollment basis and stratified according to area (flooding and non-flooding), age (<57 and ≥57 mo) and sex. The twin siblings of subjects (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. Fecal calcium at study end was kept blinded from the study team until all data were fully deblinded.

Intervention

Children were randomly assigned to receive low-lactose milk: with a low-calcium content (≈50 mg Ca/day, LC); regular-calcium (≈440 mg Ca/day, RC); RC plus *Lactobacillus casei* CRL 431 (5x10^8 CFU/day, casei); or RC plus *Lactobacillus reuteri* DSM 17938 (5x10^8 CFU/day, reuteri). Milk was sweetened, chocolate-flavored, ambient stable (sterilized by using ultra high temperatures), and packed in tetra-paks (Frisian Flag Indonesia, Jakarta). Milk was consumed with straws coated inside with the oil drop as placebo (BioGaia AB, Sweden) or with either *L. casei* CRL 431 (Chr Hanssen, Hørsholm, Denmark) or *L. reuteri* DSM 17938 (an antibiotic resistance gene-depleted derivative daughter strain of *L. reuteri* ATCC 55730, BioGaia AB)[42] in vegetable oil. Probiotic dosage was based on supplier's information of efficacy, application in children, and safety concerns when dosed for longer time. The different milks and straws were indistinguishable for the investigators and participants. The composition of the milks and straws is described in **Table 4.1**. 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 store products refrigerated and prevent sun exposure. Parents without refrigerators obtained the products from the field workers' house on daily basis and/or children consumed the products directly at the field workers' house. Mothers were instructed to provide the children 180 mL of milk twice daily (not with a meal) using the straws provided. Field workers observed the children drinking milk at least once a week and empty packages had to be shown during visits. During diarrheal episodes, children continued or restarted drinking milk as soon as possible but after being rehydrated with Oral Rehydration Solution according to WHO guidelines.[43] Activities with creative and educational contents were implemented to maintain compliance of mothers and children.

Table 4.1 Composition of LC and RC milk and probiotic straws

Composition	LC	RC	Casei	Reuteri
UHT Milk (per 100 ml)				
Energy, kcal	93.8	98.0	98.0	98.0
Fat, g[a]	3.5	3.9	3.9	3.9
Protein, g[a]	3.9	3.8	3.8	3.8
Total carbohydrate, g[a]	11.7	12.0	12.0	12.0
Lactose, g	0.07	0.09	0.09	0.09
Vitamin A, µg	32	30	30	30
Calcium, mg[a]	15	129	129	129
Phosphor, mg	32	77	77	77
Magnesium, mg	6	6	6	6
Iron, mg	0.30	0.30	0.30	0.30
Zinc, mg	0.14	0.14	0.14	0.14
Straw probiotic (CFU/day)[b]				
Lactobacillus casei CRL 431	-	-	5×10^8	
Lactobacillus reuteri DSM 17938	-	-		5×10^8

UHT, ultra-high temperature; CFU, colony-forming unit.

[a] Based on chemical analyses.

Data collection

Field workers performed anthropometric measurements at baseline (month 0), during intervention (month 1st, 2nd, 3rd, 4th and 5th) and study end (month 6th). Lightly clothed children were weighed without shoes using an electronic scale (SECA model 890, SECA, Hamburg) with a precision of 0.1 kg. Body stature was measured using a microtoise with a precision of 0.1 cm. Habitual dietary intake of the previous 2 months was assessed by using a semiquantitative food-frequency questionnaire (S-FFQ) as described elsewhere.[41]

Before and at intervention end, non-fasting venous blood was drawn in the morning by trained phlebotomists. Four mL whole blood was collected in EDTA tubes for hematology

and plasma preparation, and 7 mL into non-anticoagulant tubes for determination of serum high-sensitivity C-reactive protein (hs-CRP) and alpha-1-acid glycoprotein (AGP). Blood samples were promptly stored and transported to the laboratories in cool boxes. Sera were stored at -70°C in regular microtubes. Procedure for fecal samples collection was described elsewhere.[41]

Adverse events were recorded by using International Classification of Diseases, 10th Revision codes as described elsewhere.[41] We followed the local standard for outpatient and hospital care for diarrhea and ARTI which were per WHO guidelines as described elsewhere.[41] An independent expert monitored trial conduct and accordance to protocol.

Laboratory measurements

Routine hematology testing (ie. hemoglobin (Hb), hematocrite (HCT), red blood cell (RBC), 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, Tarrytown, NY).[44] A high-sensitivity chemiluminescent assay (Immulite® Dade-Behring, Los Angeles, CA) was used to measure serum high-sensitivity C-reactive protein concentration.[45] Serum α_1-acid glycoprotein was measured by using an enzyme-linked immunosorbent assay (ELISA).[46] Iron and zinc status were determined by analyses of serum ferritin, soluble transferrin receptor (sTfR) and zinc concentration. Serum ferritin and sTfR were measured by ELISA.[46] Serum zinc was analyzed by inductively-coupled plasma-optical emission spectrometry (ICP-OES) OPTIMA 2000™ DV (Perkin-Elmer, Norwalk, USA). Fecal calcium (base- and endline samples) was analyzed in freeze-dried feces by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES; Varian, Mulgrave, Australia).[17]

Outcomes

The primary study endpoints concerned the intervention effects on the number and duration of diarrheal episodes as described elsewhere.[41] In this paper, we present data on the secondary outcomes, notably the changes in growth, anemia, and iron and zinc status. The study power calculation was based on the effects on diarrhea.

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 (months 6th intervention) minus baseline (month 0). In addition, using the consecutive monthly anthropometric measurements, the mean change and difference of average monthly weight and height growth velocities as well as WAZ and HAZ were analyzed by using a longitudinal regression model (month 0, 1st 2nd, 3rd, 4th, 5th and 6th).

The anthropometric aspects of nutritional status were determined based on nutritional indices of the WHO Child Growth Standard.[47] The HAZ and WAZ were calculated using WHO anthropometric software 2005 and 2007. Stunting was defined as HAZ <-2 SD, and underweight as WAZ < -2 SD.[2] The changes in the proportion of underweight and stunted children from baseline to study end were calculated.

Iron, and zinc status were assessed by estimating the difference between means at study end to baseline. Iron status was assessed at baseline and after 6 months of intervention by measuring haemoglobin, serum ferritin and sTfR concentrations.[48] Changes in the proportion of anemic, iron deficient anemic and zinc deficient children were calculated. Anemia was defined according to WHO criteria as Hb <11.0 g/dL (children <5 y); Hb <11.5 g/dL (children 5–11 y); iron deficiency was defined as serum ferritin <12 g/L in children <5 y and <15 g/L in children >5 y; and iron deficiency anemia was defined as iron deficiency with anemia.[49] Zinc deficiency was defined as serum Zn concentration <65 mg/dL (9.9 mmol/L).[50] The 6-month 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 *for all eligible children who had consumed the* intervention products at least once. Analyses were carried out according to a predefined data analysis protocol. Normality of the data distribution was analyzed with the Kolmogorov-Smirnov test. Chi-square test was used for comparison of categorical variables between treatment groups and Fisher exact test was used when expected count <5. Student's t-test was used to identify differences in quantitative normally distributed data between predefined groups of comparison (between LC and RC; RC and casei; RC and reuteri). Within-treatment differences of status marker were assessed by paired t test. Mann-Whitney U test was applied when data were not normally distributed. Data are expressed as mean (SD) for normally distributed parameters and median and 25th–75th percentiles for non-normally distributed parameters. We used PSAW Statistic 17.0.3 for windows (SPSS Inc. Chicago, USA 2009) for this analysis.

Random intercept models were applied for statistical analyses of growth outcomes by using SAS version 9.2 (GLIMMIX procedure). We performed a repeated measures logistic regression model using STATA for windows release 11 (XTGEE procedure) for changes in the prevalence of underweight, stunting, anemia, iron and zinc deficiency, and iron deficiency anemia. Growth parameters (weight, height, WAZ, HAZ) and status markers (Hb, serum ferritin, sTfR and zinc) were the dependent variable and treatment group and time of measurement were the independent variable. The variables area, age, sex, HAZ, WAZ, and status markers at baseline were included in the model as covariates dependent on the outcome studied. In additional analyses, potential effect modification by age, habitual calcium intake and baseline nutritional status were assessed by adding interaction terms to the regression model.

Results

A total of 3,150 children were screened in phase 1 and 1,343 in phase 2 (**Figure 4.1**). From the 497 eligible children, 3 refused to have baseline measurements. In total, 494 children were randomly allocated to four treatment groups and included in ITT analysis.
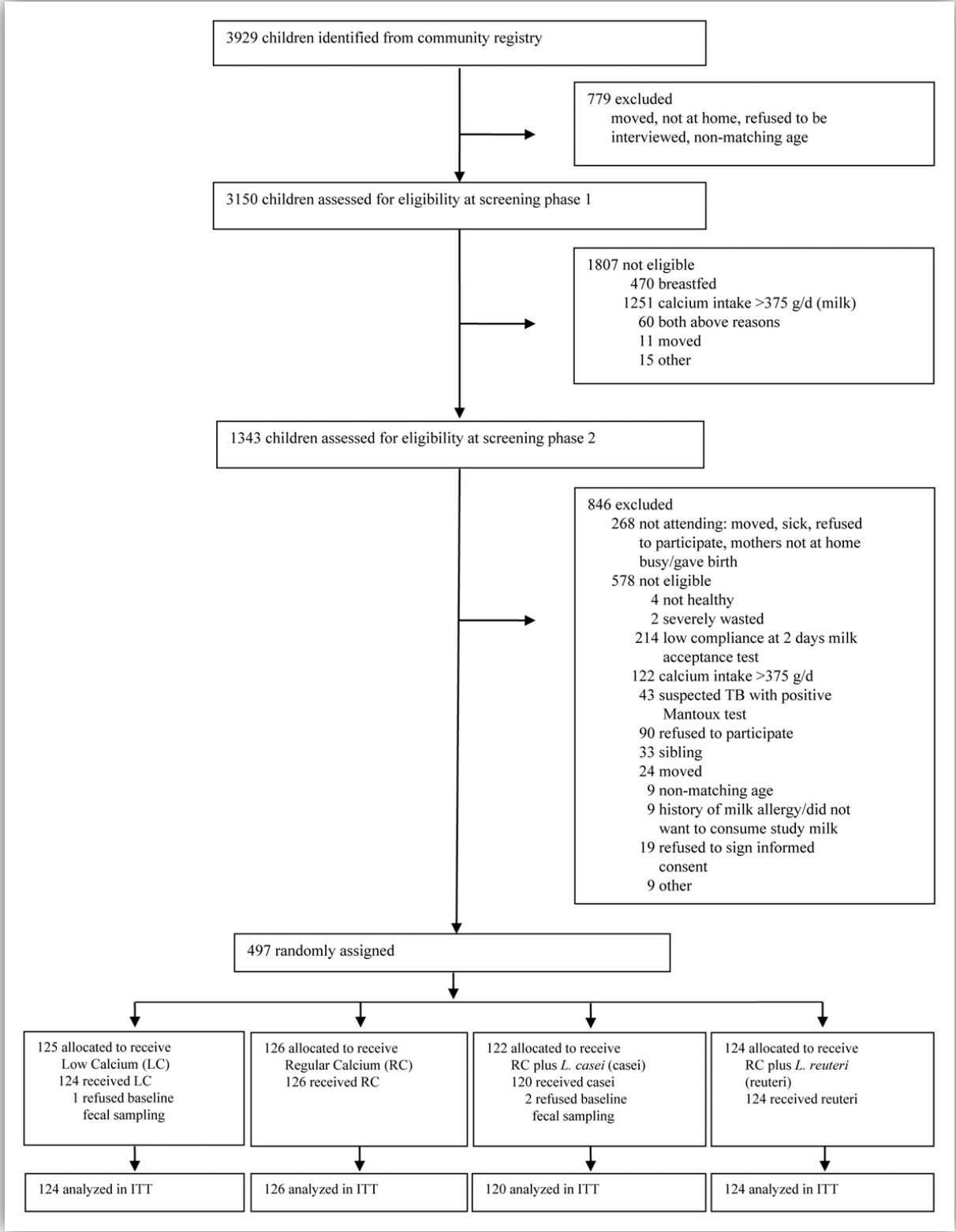
Figure 4.1 Flow diagram of study subjects.
ITT, intention-to-treat; TB, tuberculosis.

Table 4.2 Baseline characteristics of the Indonesian children

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[1]	58.9 ± 14.2	60.3 ±13.7	58.9 ±15.1
Family size	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
Mother's education <6 y [n (%)]	43 (36)	43 (35)	52 (42)	50 (40)
Diarrhea 2-wk before study [n (%)]	20 (16)	13 (10)	24 (20)	15 (12)
ARTI 2-wk before study [n (%)]	48 (39)	51 (41)	52 (43)	56 (45)
Serum hs-CRP (mg/L)	0.79 (0.23, 1.82)[2]	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 Transferrin receptor (μg/L)	9.3 (5.2)	9.3 (4.3)	9.2 (3.8)	9.2 (4.1)
Serum Zinc concentration (μg/dL)	56.0 (16.3)	55.3 (14.6)	57.4 (17.8)	57.1 (14.8)
Anemia[3] [n (%)]	24 (19)	33 (26)	24 (20)	24 (19)
Iron deficiency[4] [n (%)]	40 (32)	41 (33)	32 (27)	37 (30)
Iron deficiency anemia[5] [n (%)]	12 (10)	17 (14)	13 (11)	14 (11)
Zinc deficiency[6] [n (%)]	85 (71)	93 (78)	75 (65)	82 (68)
Anthropometric parameters				
Weight	15.5 ± 3.36	15.3 ± 3.18	16.0 ± 3.66	15.6 ± 4.00
Height	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 [n (%)][7]	24 (19)	36 (29)	21 (18)	34 (27)
Stunted [n (%)][8]	38 (31)	44 (35)	34 (28)	37 (30)
Habitual dietary intake				
Energy (kcal/d)	1,033 ± 368	1,066 ± 329	1,024 ± 369	976 ± 310
Protein (g/d)	34.3 ± 13.5	36.3 ± 12.9	33.5 ± 13.6	32.9 ± 11.0
Carbohydrate (g/d)	155 ± 58	157 ± 48	156 ± 58	146 ± 49
Fat (g/d)	32.2 ± 13.2	34.5 ± 13.9	31.8 ± 13.7	30.9 ± 11.4
Fiber (g/d)	4.5 ± 3.1	5.1 ± 3.5	4.9 ± 3.7	4.6± 2.8
Calcium (mg/d)	235 ± 95	241 ± 97	228 ± 105	228 ± 94
Iron (mg/d)	6.1 ± 2.7	6.6 ± 2.6	6.2 ± 2.7	6.1 ± 2.4
Zinc (mg/d)	4.4 ± 2.0	4.8 ± 1.9	4.4 ± 2.0	4.4 ± 1.6

Note: ARTIs, acute respiratory tract infection; hs-CRP, high sensitivity C-reactive protein; AGP, alpha 1-acid glycoprotein.
[1] Mean ± SD (all such values).
[2] Median (IQR) (all such values).
[3] Defined as a hemoglobin concentration <11 g/dL (children < 5 y) or < 11.5 g/ dL (children 5–11 y).
[4] Defined as a serum ferritin <12 μg/L (children <5 y) or <15 μg/L (children >5 y).
[5] Defined as an iron deficiency with anemia (see above).
[6] Defined as serum zinc concentration < 65mg/dL (9.9 mmol/L) at morning non-fasting .
[7] Defined as a weight-for-age z score < -2 SD.
[8] Defined as a height-for-age z score < -2 SD.

At admission, all study groups were comparable regarding socio-demographic characteristics (including environment, and maternal hygiene and caring), health and hematologic status and habitual dietary intake (**Table 4.2**). About 21% of all children were anemic, 68% zinc deficient, 23% underweight and 31% stunted at baseline. The compliance to study products

use was high (94%) and similar among groups. Both probiotic strains remained >90% viable over the intervention period. As expected, fecal calcium excretion increased significantly in the three groups supplemented with regular calcium milk ($P<0.001$) (**Table 4.6**) indicating good compliance to the study product.

Changes in energy and nutrient intake between baseline and endline were not significantly different among the groups (**Table 4.3**), except for calcium. Regular milk supplementation augmented the daily Ca intake of these children to the RDA (≈500 mg/day) of Indonesian children in this age group, whereas it remained 50% in the LC group (230 mg/day) ($P<0.05$). In addition, daily intake of energy and protein increased by 20% and 30% (up to 11 g), respectively.

Table 4.3 Habitual daily intake of energy and nutrients measured at study baseline and at endline[1,2,3]

Variables	LC (n = 123)	RC (n = 126)	Casei (n = 120)	Reuteri (n = 123)
Energy Intake, kcal/d[4]				
Baseline	1026 ± 365	1066 ± 329	1024 ± 369	977 ± 311
Endline	1240 ± 355	1257 ± 336	1277 ± 380	1238 ± 324
Changes	+212 ± 389	+191 ± 356	+253 ± 414	+261 ± 397
Protein Intake, g/d				
Baseline	34.2 ± 13.5	36.3 ± 12.9	33.5 ± 13.6	32.9 ± 11.0
Endline	45.5 ± 12.3	45.4 ± 12.2	46.2 ± 14.2	44.7 ± 11.6
Changes	+11.4 ± 13.0	+9.1 ± 13.7	+12.8 ± 15.0	+11.8 ± 14.1
Vitamin A, IU/d[5]				
Baseline	286 (127, 533)	283 (154, 283)	231 (119, 563)	268 (140, 606)
Endline	318 (210, 499)	294 (226, 417)	291 (211, 479)	295 (219, 576)
Changes	+70 (-91, 193)	+35 (-242, 164)	+57 (-150, 182)	+62 (-243, 188)
Calcium Intake, mg/d				
Baseline	231(161, 230)	232 (165, 335)	205 (148, 305)	220 (158, 304)
Endline	206 (162, 249)[6]	592 (541, 648)	602 (545, 662)	589 (538, 647)
Changes	+1.25 (-101, 49)[6]	+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)
Endline	6.2 (5.0, 8.6)	6.3 (5.0, 8.4)	6.7 (4.9, 8.2)	6.0 (5.1, 7.9)
Changes	+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)
Endline	4.0 (3.0, 5.0)	4.0 (3.1, 5.4)	4.0 (3.0, 5.1)	3.8 (3.2, 4.9)
Changes	+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] Assessed by Semi-Food Frequency Questionnaire (SFFQ).
[2] Two children did not complete endline measurement (1 in reuteri and 1 in LC group).
[3] Milk supplements were included at mean endline and changes.
[4] Mean ± SD (all such values).
[5] Median (IQR) (all such values).
[6] Significantly different from RC group, P<0.001 (Mann-Witney U test analysis) for calcium intake changes.
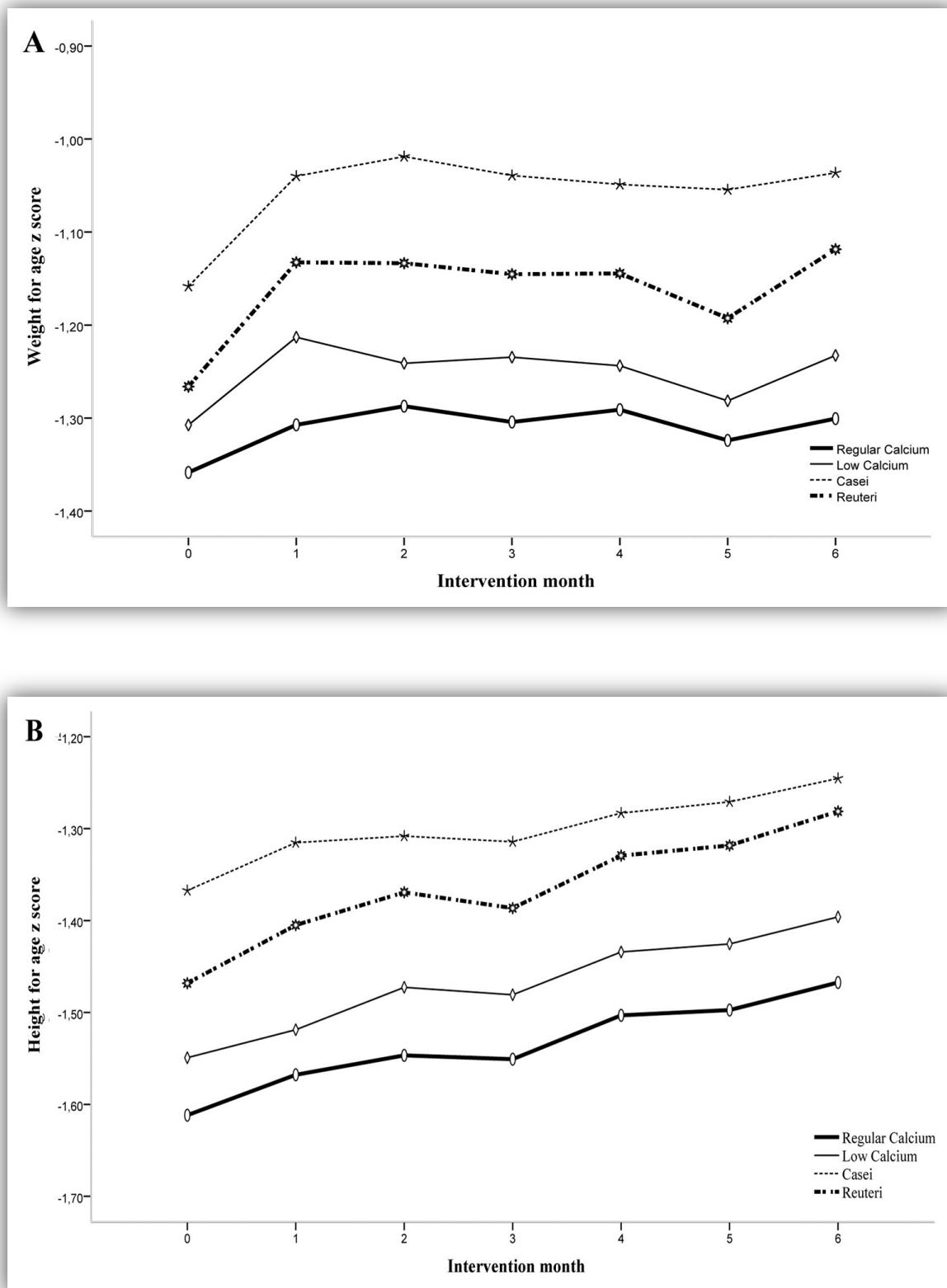
Figure 4.1 Treatment group-specific changes of (*A*) Weight-for-age z score;
(*B*) Height-for-age z score during the 6-months period of intervention.

Table 4.4 Dietary treatment effects on children's body weight, height, and changes in the weight- and height-for-age z scores

Outcomes	LC (n = 124)	RC (n = 126)	Casei (n = 120)	Reuteri (n = 124)	RC vs LC	Casei vs RC	Reuteri vs RC
					Adjusted differences (95% CI)[†]		
Changes in 6 months[1]							
Weight, kg	1.02 ± 0.8[3]	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)[4]
Height, cm	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)
Weight-for-age z score	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)[4]
Height-for-age z score	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 per month[2]							
Weight, kg	0.17 ± 0.13	0.16 ± 0.13	0.20 ± 0.16	0.19 ± 0.11	-0.01 (-0.19, 0.10)	0.03 (0.001, 0.05)[3]	0.03 (0.002, 0.05)[4]
Height, cm	0.56 ± 0.14	0.56 ± 0.14	0.54 ± 0.15	0.58 ± 0.21	-0.02 (-0.04, 0.01)	-0.01 (-0.04, 0.01)	0.03 (0.01, 0.05)[4]
Weight-for-age z score	0.01 ± 0.05	0.01 ± 0.05	0.02 ± 0.06	0.03 ± 0.05	0.0004 (-0.01, 0.01)	0.004 (-0.01, 0.02)	0.01 (-0.01, 0.02)
Height-for-age z score	0.03 ± 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] Three missing values out of 988 observations (2 in RC and 1 reuteri group).
[2] Sixty eight missing values out of 3458 observations (18 in RC, 12 in LC, 19 in casei and 19 in reuteri groups).
[3] Mean ± SD (all such values).
[4] Statistically significant, $P < 0.05$ (repeated measures of general linear model).
[†] Weight and weight-for-age z score (WAZ) were adjusted for area, age, sex, height-for-age z score (HAZ) and iron and zinc status marker at baseline; height and HAZ were adjusted for area, age, sex and WAZ and iron and zinc status marker at baseline.

Table 4.5 Changes in the prevalence of underweight and stunting

Outcomes	Baseline	Month 6	Change	Adjusted change	Adjusted differences	
	[n (%)]	[n (%)]	[n (%)]	OR (95% CI)	OR (95% CI)	OR (95% CI)
Underweight						
LC (*n* = 124)	24 (19)	25 (20)	1 (1)	1.09 (0.71, 1.67)	1.00	-
RC (*n* = 126)	36 (29)	29 (23)	-7 (-5)	0.67 (0.45, 0.99)[5]	0.49 (0.23, 1.04)	1.00
Casei (*n* = 120)	21 (18)	23 (20)	2 (2)	1.20 (0.76, 1.88)	-	1.71 (0.78, 3.74)
Reuteri (*n* = 124)	34 (27)	27 (22)	-7 (-5)	0.63 (0.43, 0.93)[5]	-	0.77 (0.39, 1.55)
Stunting						
LC (*n* = 124)	38 (31)	32 (26)	-6 (-5)	0.67 (0.45, 0.99)[5]	1.00	-
RC (*n* = 126)	44 (35)	38 (31)	-6 (-4)	0.72 (0.49, 1.06)	1.06 (0.53, 2.13)	1.00
Casei (*n* = 120)	34 (28)	26 (22)	-8 (-7)	0.55 (0.36, 0.83)[6]	-	1.15 (0.56, 2.34)
Reuteri (*n* = 124)	37 (30)	25 (20)	-12 (-10)	0.44 (0.29, 0.68)[6]	-	0.70 (0.34, 1.41)

Note: OR, odd ratio; CI, confidence interval.
[1] Defined as a weight-for-age z score < -2 SD.
[2] Adjusted for area, age, sex and HAZ at baseline.
[3] Defined as a height-for-age z score < -2 SD.
[4] Adjusted for area, age, sex and WAZ at baseline.
Statistically significant in changes between baseline and month 6: [5]*P*< 0.05; [6]*P*< 0.01 (repeated measures logistic regression).

Weight, height, WAZ and HAZ increased in all intervention groups over time (**Table 4.4**). Overall, the weight and height velocity of children was 178 g/mo and 0.56 cm/mo (≈7 cm/y), respectively. On average the total group was 1,070 g heavier (0.10 WAZ) and grew up to 3.4 cm taller (0.15 HAZ) during the 6 month intervention (data not shown).The reuteri group experienced significantly higher weight gain (1160± 650 g; *P*=0.03) and WAZ change (0.14 Z ± 0.28; *P*=0.03) after 6 months of intervention as compared to the RC group (960 ± 740 g and 0.06 ± 0.31 Z, respectively). The difference in weight and height gain between reuteri and RC was 220 g (0.09 Z) and 0.19 cm (0.04 Z), respectively. Compared to RC, the average monthly weight velocity was significantly higher in the reuteri group [adjusted difference: 0.03 (95% CI: 0.002, 0.05) kg; *P*=0.04]. Similar significant result was observed for the average monthly height velocity in the reuteri group. In the casei group, the average monthly weight velocity was significantly higher compared to RC [adjusted difference: 0.03 [(95% CI: 0.001, 0.05) kg; *P*=0.04]. However, compared to the RC group, the difference in reductions in underweight (23%) and stunting (30%) in the reuteri group were not significantly different after adjusting for area of living, sex, age and WAZ at baseline (**Table 4.5**). The crude data (**Figure 4.2**) and model results of WAZ (**Table 4.4**) showed that the increases in z score of the reuteri group were most prominent in the first and sixth intervention month. HAZ increased more linearly.

During intervention, Hb, HCT, other hematologic parameters (RBC, RDW, MCV, MCHC), and serum ferritin significantly declined, and the serum transferrin receptor significantly increased in all groups (*P*<0.001). Serum zinc showed a more variable response between groups. However, none of the changes in blood parameters were significantly different among the groups, neither when the RC group was pooled with the probiotic groups as one calcium supplemented group versus the LC group (**Table 4.6**). Because acute or chronic inflammation and infection are potential confounders of serum 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 using both hs-CRP concentration >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 in-or excluded or when serum ferritin was corrected.

Table 4.6 Hematology parameters, serum inflammation biomarkers and the iron, zinc and calcium status changes of children during the 6 months intervention.

Variables	LC (n=123)	RC (n=124)	Casei (n=120)	Reuteri (n=122)
Hemoglobin, g/L				
Baseline	12.0 ± 1.12[1]	11.8 ± 1.35	12.0 ± 1.07	11.9 ± 1.04
Changes	-0.19 ± 0.77	-0.15 ± 0.88	-0.25 ± 0.77	-0.14 ± 0.75
Hematocryte %				
Baseline	37.0 ± 2.67	36.2 ± 3.30	36.8 ± 3.00	36.7 ± 2.76
Changes	-1.00 ± 2.97	-0.55 ± 2.98	-0.93 ± 2.74	-0.62 ± 2.86
RBC, 10^6/μl				
Baseline	4.80 ± 0.39	4.70 ± 0.40	4.76 ± 0.43	4.75 ± 0.41
Changes	0.11 ± 0.33	0.14 ± 0.31	0.12 ± 0.30	0.15 ± 0.28
MCV, fl				
Baseline	77.2 ± 6.1	77.3 ± 6.3	77.6 ± 6.2	77.7 ± 6.2
Changes	-3.8 ± 3.8	-3.4 ± 3.5	-4.0 ± 3.6	-3.7 ± 3.5
RDW, %				
Baseline	14.9 ± 1.5	14.9 ± 1.6	14.9 ± 1.4	14.9 ± 1.4
Changes	-1.2 ± 0.9	-1.2 ± 0.9	-1.1 ± 1.1	-1.3 ± 0.9
Hs-CRP, mg/L				
Baseline	0.79 (0.23, 1.82)[2]	0.75 (0.28, 2.9)	0.75 (0.30, 2.5)	0.66 (0.25, 3.03)
Changes	-0.01 (-1.03, 0.61)	-0.09 (-0.89, 1.0)	-0.05 (-1.42, 0.40)	0 (-1.65, 0.51)
AGP, g/L				
Baseline	0.81 ± 0.18	0.84 ± 0.20	0.84 ± 0.19	0.82 ± 0.17
Changes	-0.01 ± 0.25	-0.02 ± 0.24	-0.03 ± 0.22	-0.02 ± 0.24
sTfR, μg/L[3]				
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)
Changes	0.90 (-0.35, 1.69)	0.87 (-0.64, 2.23)	0.38 (-0.61, 1.89)	0.67 (-0.26, 2.02)
Ferritin, μg/L[4]	n = 109	n = 109	n = 108	n = 102
Baseline	18.3 (9.65, 30.7)	24.4 (9.27, 39.1)	22.8 (12.0, 35.3)	19.5 (11.6, 32.4)
Changes	-3.96 (-11.1, 1.03)	-4.35 (-15.7, 1.58)	-4.21 (-13.5, -0.35)	-3.66 (-11.5, 0.87)
Zinc, umol/L[5]	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
Changes	0.34 ± 2.01	0.30 ± 2.45	-0.03 ± 2.23	-0.04 ± 1.95
Fecal calcium, mg/g[6]	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)
Changes	0.37 (-2.00, 4.26)[7]	15.9 (6.48, 23.9)	14.9 (7.14, 23.4)	15.1 (6.68, 20.7)

Note: RBC, red blood cell, RDW; red cell distribution width, MCV; mean corpuscular volume (MCV); hs-CRP, high sensitivity C-reactive protein; AGP, alpha 1-acid glycoprotein; sTfR, soluble transferrin receptor.
[1] Mean ± SD (all such values).
[2] Median (IQR) (all such values).
[3] Five children did not complete endline measurement (2 in reuteri, 2 in RC and 1 in LC group).
[4] Sixty Six children were not included in the analysis: 5 children did not complete endline measurement (2 in reuteri, 2 in RC and 1 in LC group) and 61 children had hs-CRP baseline and endline > 5mg/L.
[5] Measurement at study end of 30 children were not available (4 in casei, 7 in reuteri, 8 in RC and 9 in LC group).
[6] Four children did not complete endline measurement (2 in reuteri, 2 in RC and 1 in LC group).
[7] Mann-Whitney test, statistically significant between RC vs LC, $P<0.001$.

Table 4.7 Changes in the prevalence of anemia, iron deficiency, iron deficiency anemia and zinc deficiency in Indonesian children

Outcomes	Baseline [n (%)]	Month 6 [n (%)]	Change [n (%)]	Adjusted change OR (95% CI)	Adjusted differences OR (95% CI)	OR (95% CI)
Anemia[1]						
LC (n = 124)	24 (19)	32 (26)	8 (7)	1.52 (0.97, 2.37)	1.00	1.00
RC (n = 126)	33 (26)	40 (32)	7 (6)	1.34 (0.89, 2.02)	0.69 (0.38, 1.27)	
Casei (n = 120)	24 (20)	32 (27)	8 (7)	1.48 (0.94, 2.31)		1.33 (0.72, 2.45)
Reuteri (n = 124)	24 (19)	28 (23)	4 (4)	1.24 (0.78, 1.96)		0.67 (0.37, 1.23)
Iron Deficiency[3]						
LC (n = 124)	40 (32)	60 (49)	20 (17)	2.14 (1.46, 3.14)[6]	1.00	1.00
RC (n = 126)	41 (33)	59 (48)	18 (15)	1.90 (1.30, 2.77)[6]	0.99 (0.58, 1.70)	
Casei (n = 120)	32 (27)	51 (43)	19 (16)	2.07 (1.39, 3.09)[6]		0.78 (0.45, 1.37)
Reuteri (n = 124)	37 (30)	61 (50)	24 (20)	2.43 (1.65, 3.57)[6]		1.12 (0.65, 1.93)
Iron deficiency anemia[4]						
LC (n = 124)	12 (10)	20 (16)	8 (7)	2.04 (1.20, 3.45)[5]	1.00	1.00
RC (n = 126)	17 (14)	28 (23)	11 (9)	1.88 (1.19, 2.97)[5]	1.46 (0.66-3.24)	
Casei (n = 120)	13 (11)	23 (19)	10 (8)	1.99 (1.20, 3.30)		0.81 (0.37, 1.78)
Reuteri (n = 124)	14 (11)	20 (16)	6 (5)	1.55 (0.93, 2.57)[5]		0.80 (0.37, 1.73)
Zinc deficiency[5]						
LC (n = 124)	85 (71)	72 (62)	-13 (-9)	0.65 (0.42, 1.00)	1.00	1.00
RC (n = 126)	93 (78)	85 (71)	-8 (-6)	0.72 (0.45, 1.14)	1.34 (0.74, 2.41)	
Casei (n = 120)	75 (65)	79 (66)	4 (2)	1.08 (0.70, 1.66)		1.79 (1.00, 3.19)
Reuteri (n = 124)	82 (68)	89 (74)	7 (7)	1.34 (0.85, 2.09)		1.57 (0.88, 2.81)

[1] Defined as a hemoglobin concentration <11 g/dL (children < 5 y) or < 11.5 g/ dL (children 5–11 y).
[2] Adjusted for area, age, sex and weight-for-age z score at baseline.
[3] Defined as a serum ferritin < 12 μg/L (children <5 y) or <15 μg/L (children >5 y).
[4] Defined as an iron deficiency with anemia.
[5] Defined as serum zinc concentration < 65mg/dL (9.9 mmol/L) at morning non-fasting blood drawing.   [6]P< 0.01 (repeated measures logistic regression). [5]P< 0.05;
Statistically significant in changes between baseline and month 6,

During the 6-month intervention, the percentage of children with iron deficiency and iron deficiency anemia increased significantly ($P<0.05$) in all groups (**Table 4.7**). Again no significant differences among treatment groups were observed even after adjusting for area of living, seasons and other covariates. So, milk calcium alone or with supplemented probiotic strains did not significantly affect iron status during the 6 months intervention.

Discussion

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

 The weight velocity (178 g/mo) of all treatments in our study children falls 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 ($\approx$7 cm/y) of all children is also within the upper expected average 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 weight parameters (0.24 – 0.40) and casei in weight velocity (0.3) are larger than the results of a meta-analysis and review on MMNs supplementation (<0.15) in under-five children over 1 year 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 size 0.29 and 0.23, respectively) are slightly larger compared to a study in Indian children of similar age (effect size 0.22) supplemented with milk fortified with *Bifidobacterium lactis* HN019 and prebiotic oligosaccharide versus control milk alone [23] and a study in Indonesian 12-mo-old toddlers (effect size 0.23) supplemented with a combination of *B. longum*, *L. rhamnosus*, prebiotics and polyunsaturated fatty acid versus control milk.[56] However, differences in growth outcomes did not affect prevalence of stunting and underweight in the reuteri and other groups. This may implicate that 6-mo intervention is too short to observe reduction of malnutrition prevalences.[57] It may also be that the actual increment of 0.09 for WAZ and 0.05 for HAZ, albeit statistically 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 or combined with prebiotics,[28, 59] did not find any growth effects. Overall, our probiotics interventions, but not milk calcium, showed larger effects on growth than other nutritional interventions.

In our trial, the changes of WAZ sharply increased in the first intervention month. This may reflect a rapid catch-up growth 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 separated by periods of weight loss.[61] This irregular pattern was not seen for HAZ that consistently increased in all intervention months. The beneficial effects observed on growth could be due to enhanced mucosal integrity[62] and reduced intestinal infections in our children.[41]

No effect on growth was observed in the RC (without probiotics) compared to the LC group. This is in line with three reviews of randomized clinical trials (RCT's) in children of mainly developed countries, also reporting 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 claimed to improve digestibility and nutrient uptake by intestinal cells.[27] A study in infant rhesus monkeys given *L. reuteri* showed an improved haematocrite,[63] but so far no information is available about more relevant iron status markers (eg. ferritin, sTfR).

Levels of iron parameters, eg. 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 of iron parameters and prevalence rate changes of anemia, iron-deficiency, and iron-deficiency anemia, or in zinc status. The reduced iron status over 6 months, independent of intervention type, may be due to insufficient iron homeostasis to compensate the fast iron mobilization from storage needed during growth.[22] Chronic blood loss may also contribute to iron deficiencies and anemia in children in developing countries and can be caused by gastrointestinal parasites,[48] *Helicobacter pylori*[64] or allergy to cow's milk protein.[65] No information was available on the parasitic infestation or *Helicobacter pylori* prevalence, neither a deworming program was applied in our study population. No adverse events related to cow's milk protein were present in our study. Also, the considerable prevalence of respiratory (90%) and gastrointestinal infections (27%) in our study children may have negatively affected iron metabolism,[66] known as the anemia of infectious disease.[67] In addition, the polyphenolic compounds of cocoa powder added to our study milk may have inhibited iron absorption.[68, 69]

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 population's age and country.[30, 31, 34, 36, 39, 40] Most studies evaluating iron status were conducted in developed countries involving children and adolescent with adequate iron and calcium intakes and reported no or a small effects on iron status.[36, 39, 40] No difference in iron status of preschool children aged 3-5 y was observed after a 5 wk adaptation to a low- (502 mg/day) versus high- (1180 mg) calcium diet.[39] Also, an RCT in pre-pubertal children found that low or moderate amounts of calcium (78 mg/d or 312 mg/d) of 2 wk did not interfere with iron absorption.[36] A long-term, 1-year-intervention study in adolescent girls showed that daily calcium supplementation of 500 mg did not compromise iron status.[40] With regard

to zinc, calcium per se does not impair zinc absorption in premenopausal women[70] but reduce net zinc absorption in postmenopausal women.[71] So far, evidence from studies in children were missing. In our study children, with a low iron and calcium intake, regular calcium milk did not adversely affect iron and zinc status as compared to low calcium milk.

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 duplo assessment of supervised anthropometric measurements by well-trained field workers. Per-protocol analysis, excluding the few non-compliant subjects (6%) and subjects having chronic antibiotic usage, did not change the outcomes. A study drawback is its primary design to detect differences in intestinal infection incidence and therefore, sample size may have been inadequate to detect group differences in growth or micronutrient status.

Conclusions

L. reuteri DSM 17938 modestly improved growth, by increasing weight gain and WAZ changes over 6 months and monthly weight and height velocity in Indonesian children. *L. casei* CRL 431 modestly improved monthly weight velocity. Regular calcium milk did not affect any growth outcome. Neither the probiotics nor regular calcium milk affected iron and zinc status.

Acknowledgments

Author's contributions

The authors' responsibilities were as follows— R Agustina: the principal investigator and responsible for study concept and design, data collection, laboratory analysis, accuracy and completeness of data analysis and reporting. R Agustina, IMJBO, WL, UF, OvdR, AF, FJK: had a major role in study design, interpretation of results and writing of the report. RW: involved in data collection and laboratory analysis. MBZ: provided advice on the interpretation of study results for micronutrient status markers. EGHMvdH and R Albers: involved in the study design and trial monitoring. All authors evaluated the manuscript and

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Chapter 5

Association of food-hygiene practices and diarrhea prevalence among Indonesian young children from low socioeconomic urban areas

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Abstract

Background

In contrast to determinants such as malnutrition, mother's education, and inadequate sanitation, the role of poor food-hygiene in the development of diarrhea in low socioeconomic urban communities is lacking. This study therefore aimed to assess the contribution of food-hygiene practice to the prevalence of diarrhea among children in Indonesia.

Methods

A cross-sectional study was conducted among 274 randomly selected children aged 12-59 months in selected low socioeconomic urban areas of East Jakarta. The prevalence of diarrhea was assessed during the 7-day record on frequency and consistency of the child's defecation pattern. Food-hygiene practices including mother's and child's hand washing, food preparation, cleanliness of utensils, water source and safe drinking water, habits of buying cooked food, child's bottle feeding hygiene, and housing and environmental condition were obtained through home visit interview and observation by field workers. Thirty six practices were scored and classified into poor (median and below) and better (above median) food-hygiene practices. Nutritional status of children, defined anthropometrically, was measured through height and weight.

Results

Factors found to be significantly associated with diarrhea were age, nutritional and immunization status, family size and number of under-five children living under the same roof. Among the individual food-hygiene practices, children living in a house with clean sewage had a significantly lower diarrhea prevalence compared to those who did not have one and/or had dirty sewage [adjusted odds ratio (OR): 0.16, 95% confidence interval (CI): 0.03-0.73]. The overall food-hygiene practice score was not significantly associated with diarrhea in the total group, but it was in children aged < 2 years (adjusted OR: 4.55, 95% CI: 1.08-19.1).

Conclusions

In addition to other major determinants, poor mother's food-hygiene practices contributed to the occurrence of diarrhea in Indonesian children < 2 years from low socioeconomic urban areas.

Introduction

Despite the substantially declining mortality rate from diarrhea in developing countries,[1] diarrhea still accounts for approximately one-fifth of all mortality in children <5 years, annually.[2,3] Similar to other developing countries, diarrhea contributes to 25% of the mortality rate and 14% of morbidity among children under five in Indonesia.[4,5] The overall incidence of diarrhea in developing countries has remained high due to multiple determinants[6] such as malnutrition,[7] low socioeconomic status and education of mothers,[8,9] lack of safe drinking-water, inadequate sanitation and poor hygiene,[10,11] crowding[12] and low maternal age.[13] These determinants of diarrheal disease are strongly linked to poverty and social inequities.[14] Furthermore, diarrheal incidence differs greatly with the seasons and is highest in the first two years of life and declines as a child grows older.[2]

Although the determinants of diarrhea among children are well described, information on the role of food-hygiene practice in the development of diarrhea and malnutrition among children in low socioeconomic urban communities is lacking.[15] Many studies conducted in this urban settings of developing countries focused on the risk factors of diarrhea related to environmental conditions and utilization of sanitation facilities.[16-18] A previous study on food-hygiene missed some important practices such as food storage, thorough cooking and adequate holding temperature[15] as recommended by World Health Organization (WHO).[19] Mothers and children in low socioeconomic urban areas in East Jakarta with limited hygiene and sanitation facilities tend to have poor hygiene practices such as using dirty cooking or eating utensils for their children.[20] While poor hygiene practices, especially in food preparation and feeding practices, may increase the risk of having diarrhea, up to 70% of diarrhea episodes are actually caused by water and food contaminated with pathogens.[21] Therefore, we hypothesized that the prevalence of diarrhea and malnutrition among children in low socioeconomic urban areas of East Jakarta is high, not only because children and mothers are exposed to the environmental factors that cause diarrhea such as unsafe water, and poor sanitation and hygiene, but also due to poor food-hygiene practice. To address this hypothesis, we assessed the association of food-hygiene practice with the occurrence of diarrheal disease among under-five children in selected urban low socioeconomic areas of East Jakarta, Indonesia. The results of this study can be useful in designing an intervention study, health plans and policies related to mother and child hygienic behavior.

Methods

Study design

A cross sectional study was carried out from October 2004 to February 2005 in an urban area of Jatinegara, East Jakarta district, Indonesia. This district was selected because it has the highest prevalence of diarrhea and underweight (24%) in under-five children in Jakarta province, based on passive surveillance of the local government. In this urban area, purposive sampling was used to target low socioeconomic households: those in which the

housing location was along the river side (flooding area) and households within specific low socioeconomic areas of city centre (non-flooding area).

Subjects

A total of 274 children aged 12 – 59 months were selected randomly from the community health post registry. Only one child was selected for the study if a family had more than one child at this age category. At the time of selection, the children had been living in this district for at least 6 months. Informed consent was obtained from mothers or caregivers after they had received an explanation about the study's objective and method. The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Medicine, University of Indonesia and National Center for Research and Development, Ministry of Health Republic of Indonesia.

Data collection

Data collection in this study was divided into three phases: (a) interview of socio-demographic factors of the family, diarrhea prevalence, food-hygiene practices, (b) observation of environment housing condition and the child's defecation and diarrhea pattern, and (c) anthropometric measurements.

A structured questionnaire, used for conducting interviews, consisted of four sections: (a) general information, (b) diarrhea prevalence, (c) food-hygiene practice, and (d) nutritional status. The questionnaire was developed based on a similar survey, the guidelines set by the WHO, information from staff members of the community health centers and voluntary mothers, and two group discussions in two chosen villages with 8-10 mothers aged less than 45 years old, having children between 1-5 years of age, living in the study area.

The interview was conducted with the mothers or caretakers in their house after anthropometric measurement of their child. During interview, the enumerator observed the environment housing condition. Mothers were asked to fill in the form according to what she had observed in her child's defecation pattern (time, frequency and stool's visual appearance)[22] for seven days, starting at 8.00 AM each day. Every day one enumerator collected the observation form. The enumerator checked whether the child being observed had diarrhea or not based on mother's record presence of three or more watery stools per day.

In addition, mothers or caregivers were interviewed on mother's and child's hand washing, food preparation, cleanliness of utensils, water source and safe drinking water, habits of buying cooked food, child's bottle feeding hygiene and housing and environmental condition, using a structured questionnaire. House and surrounding condition, sewage condition, availability of a latrine, and water source were also observed during interview.

Diarrhea prevalence

Diarrhea was defined as defecation frequency of three or more loose/liquid stools in a day.[23] We calculated the period prevalence of diarrhea as the percentage of children suffering from

diarrhea for three alternative periods: the past 3 months, the past 2 weeks and during a 7-day recording period. Recall periods of more than 48 hours may lead to underreporting of diarrhea cases[24] therefore only the prevalence of diarrhea based on the percentage of children who suffered from diarrhea during the 7-day recording period was used in the analysis.

Food-hygiene practice

All 36 variables that may contribute to food-hygiene practices, i.e. mother's hand washing before preparing food and feeding the child, child's hand washing before eating a meal and after defecating/urinating, food preparation, cleanliness of utensils, water source and safe drinking water, habits of buying cooked food, child's bottle feeding hygiene and housing and environmental condition, were summed up into a total of 36 scores. Each variable was scored as 0 or 1, with 1 representing a positive practice.[25] All individual variables were assigned equal importance. Total score was classified into poor and better food-hygiene practice based on the median score of the population, i.e. food-hygiene practice score ≤ 19 was considered as being poor (n=168), while a score > 19 was considered as being better practice (n=168) (**Table 5.5**).

Nutritional status

Wasted, underweight and stunted were defined as weight-for-height z-score (WHZ), weight-for-age z-score (WAZ), and height-for-age z-score (HAZ) of less than − 2 SD based on nutritional indices of the WHO Child Growth Standard, respectively.[26] Children were weighed lightly clothed without shoes using an electronic scale (SECA platform 770, SECA, Hamburg) with a precision of 0.1 kg. For children who were not able to stand, the child's weight was obtained by subtracting the mother's weight from the measured weight. Body stature was measured using a microtoise for children who could stand erectly, with a precision of 0.1 cm.[27] While for children who could not stand, a SECA length board was used.

Statistical analysis

The minimum study sample size was calculated for estimating the actual prevalence of diarrhea among areas under study. The anticipated proportion of 15% was chosen as average diarrheal prevalence in East Jakarta. With 5% precision and anticipating on 20% missing data, the minimal sample was 245 children.

We used STATA for windows release 11 (College Station, Texas 2009) for data analyses. Data on weight and height of the children were transferred into z-scores using WHO software.[26]

Variables, such as child's age, sex, area of living, nutritional status, breastfeeding practices, utilization of health services, maternal schooling, socioeconomic status (SES), family size and number of under-five children living under the same roof that had a $p<0.25$ based on bivariate analysis by X^2 test were considered as potential confounders.[28] SES was categorised based on the criteria of the local government (ownership of the house, monthly income,

monthly expenditure, type of floor, and availability of latrine). The average score of SES in the study population was 15. SES was classified into binary category as described in other study:[29] households with median score or lower (≤15) as very low SES; or above median score (>15) as medium-low SES.

We next performed single and multiple logistic regression models and used these models to assess the association between diarrhea and food-hygiene practices. First, we calculated the unadjusted odds ratio (OR) and 95% confidence intervals (CI) of each variable using bivariate analysis. Only if the 95% CI did not include one, i.e. $p < 0.05$, we considered the result statistically significant. Secondly, all potential covariates were included in logistic analysis to estimate the adjusted OR and 95% CI. Thus, a multiple logistic regression model was used to account for the effect of several potential confounding factors, i.e., age, weight-for-height z-score, immunization status, family size and number of under-five living under the same roof. Logistic regression was also used to assess whether there was any effect modification ($p < 0.05$, test homogeneity of the OR). Finally, we assessed the unadjusted and adjusted OR and 95% CI of two classifications of food-hygiene practices versus prevalence of diarrhea.

Results

More than half of children resided in the flooding area along the river side. The median age of children was 31 months (12.1 – 59.8 months) with an almost equal number of boys and girls (**Table 5.1**). Half of the children belonged to very low SES families and one third of them were from families consisting of six members. Median age of the fathers was 34 years (range 20 – 66 years) while the mothers had an average age of 30 years (range 17 – 50 years). More than half of mothers had length of schooling ≤ 9 years. The main occupation of fathers was private sectors employee (34%), street vendor and small-scale trader or self-employed (29%), taxi and public transportation driver (12%), construction laborer (10%), factory and day laborer (7%), government employee (3%), while 5% was unemployed. The majority of mothers (83%) were housewives. A few mothers worked as small traders, street vendors, laborers and launderers.

The minimum labor salary rate of Rp. 600,000 (<US$ 65) per month was used to determine the level of family income and expenditure. In one third of the families, the monthly income was less than <US$ 65 indicating that they belonged to a very low-income group. In a second third of the families, the monthly income ranged between US$ 65 – 110, indicating the low income and expenditure group. The last third belonged to the medium income and expenditure group, with a monthly income > US$ 110.

Diarrhea prevalences of 40%, 18% and 10% were observed within the time frames of three months, two weeks preceding the study and during a 7-day record, respectively (**Table 5.2**). There was no significant difference in diarrhea prevalence between children living in flooding and non-flooding areas. The highest diarrhea prevalence was found in children aged 12 to 23 months: 17% of 7-day diarrhea records. Prevalence tended to decrease as child's age

increased, and the difference in the prevalence was significant between children aged 12 to 23 months and older. Diarrhea prevalence was not significantly different between sexes. The prevalence of stunting, wasting and underweight among children aged 12 – 59 months was 32%, 19% and 12%, respectively, and not different between age groups, sexes and living areas.

Table 5.1 General characteristics of the study population in Jatinegara sub-district, East Jakarta (n=274)

Variables	n (%) or median (min-max)
Area of living	
Flooding site	168 (61%)
Non Flooding site	106 (39%)
Children	
Median age (months)	31.1 (12.1 – 59.8)
Age group (months)	
12 – 23	93 (34%)
24 – 35	71 (26%)
36 – 47	65 (24%)
48 – 59	45 (16%)
Gender	
Boy	151 (55%)
Girl	123 (45%)
Mother	
Median age (years)	30 (17-50)
Length of schooling (n=273) [a]	
≤9 years	156 (57%)
>9 years	116 (43%)
Household	
Nuclear/extended family[b]	143 (52%)/131 (48%)
Family size	6 (3-22)
Socioeconomic status[c]	
Medium – low	125 (46%)
Very low	149 (54%)

[a] One mother was illiterate.
[b] Nuclear family consists of only father, mother and children; extended family consisted of a nuclear family and their close relatives living under the same roof
[c] Categorised based on total score of socioeconomic status (SES) criteria of local government (ownership of the house, monthly income, monthly expenditure, type of floor, and availability of latrine).

Factors found to be significantly associated with diarrhea were age, weight-for-height z-score, immunization status, family size and number of under-five children living under the same roof (**Table 5.3**). The odds ratio for children aged < 2 years was 2.9 times (95% CI: 1.32-6.48). Children with complete immunization were protected from diarrhea (OR 0.35 95% CI: 0.16-0.78). Children belonging to families with ≥6 household members had 2.3 times higher risk of suffering from diarrhea (95% CI: 1.03-4.98). Presence of >1 child under five years of age living in the same household increased the risk of suffering from diarrhea 2.8 times (95% CI: 1.26-6.16). The risk of diarrhea was 1.6 times higher when children had a

mother with low education level and family with very low socioeconomic conditions, but this was not significantly different, nor was the association with wasting (OR 2.3 95% CI: 0.70-6.73).

Table 5.2 Prevalence of diarrhea and malnutrition by age and sex of under-five children in Jatinegara sub-district, East Jakarta

Variables	n	Diarrhea prevalence (%)			Undernutrition prevalence (%)		
		Past 3 months	Past 2 weeks	During 7-day record	Stunted (HAZ<-2)	Underweight (WAZ<-2)	Wasted (WHZ<-2)
Area of living							
Flooding	168	38.1	19.6	10.1	36.3	26.8	9.5
Non flooding	106	42.5	15.1	10.4	25.5	17.9	9.5
Age group (months)							
12 - 23	93	58.1*	29.0*	17.2*	32.3	19.4	11.8
24 - 35	71	32.4	11.3	8.5	32.4	25.4	8.6
36 - 47	65	30.8	15.4	6.2	33.8	24.6	6.2
48 - 59	45	26.7	8.9	4.4	28.9	26.7	11.1
All ages	274	39.8	17.9	10.2	32.1	23.4	9.5
Gender							
Boy	151	41.7	16.6	9.9	35.8	25.2	11.9
Girl	123	37.4	19.5	10.6	27.6	21.1	6.6

HAZ, height-for-age z-score; WAZ, weight-for-age z-score; and WHZ, weight-for-height z-score.
* Significantly different between ages, $p < 0.05$, X^2 test.

Among the individual food-hygiene variables, children who lived in the house with clean sewage significantly had lower diarrhea prevalence compared to those who did not have one and/or had dirty sewage (adjusted OR 0.17; 95% CI: 0.04-0.75) (**Table 5.4**). Children whose mother had habits of buying package snacks showed significantly higher diarrhea prevalence compared to those who did not (unadjusted OR 3.00; 95% CI: 1.01-8.93). However, the risk of diarrhea became non-significant after adjustment for age, weight-for-height z-score, immunization status, family size and number of under-five children living under the same roof.

A number of other single food hygiene practices such as mother's and child's hand washing, food holding time <1 hour before eaten, reheating food before eaten, the use of clean utensils, availability of safe water, not frequently buying street food, food bought when were still hot and directly eaten, and proper child's bottle feeding practices seemed to be protective for diarrhea, however, the associations were not statistically significant.

Table 5.3 Risk factors associated with the occurrence of diarrhea in children aged 12 – 59 months (n=274)

Risk factors	Diarrhea		
	Number of cases	**%**	**Unadjusted OR (95% CI)**
Children			
General characteristic			
Living in flooding area	168	10	0.97 (0.44-2.17)
Age ≤ 2 years	93	17	2.93 (1.32-6.48)[*a]
Age ≤ 3 years	164	13	2.69 (1.05-6.86)[*a]
Boy	151	10	0.93 (0.43-2.04)
Nutritional status			
Wasted	36	19	2.32 (0.70-6.73)
Underweight	63	14	1.67 (0.71-3.89)
Stunted	88	8	0.68 (0.28-1.66)
Breastfeeding practice history			
Received breastfeeding after birth (n=273)	170	12	1.58 (0.67-3.74)
Received colostrum at birth	215	10	1.01 (0.39-2.61)
Received exclusive breastfeeding/ No pre-lacteal feeding at birth[†]	103	8	0.64 (0.27-1.50)
Breastfeeding duration ≥12 months (n=273)	224	9	0.62 (0.25-1.55)
Utilization of health services			
Received vitamin A supplementation (n=273)	224	9	0.62 (0.25-1.55)
Received complete immunization	188	7	0.35 (0.16-0.78)[*a]
Mother			
Maternal schooling ≤ 9 years (less or equal to junior high school) (n=273)	157	12	1.64 (0.71-3.76)
Household condition			
Socioeconomic status: very low	149	12	1.58 (0.70-3.56)
Family size ≥ 6 persons	98	15	2.27 (1.03-4.98)[*b]
Under-five children living under the same roof >1	79	18	2.78 (1.26-6.16)[*b]

[†] Prelacteal feeding was any nonhuman milk food or fluids provided to the new-borns before breastfeeding on the first day of life.[58]

[*a] Statistical significant at $p < 0.05$, X^2 test.

[*b] Statistical significant at $p < 0.05$, Fisher's exact test.

Table 5.4 Distribution of diarrhea prevalence by determinant factors of food-hygiene practices among children aged 12 – 59 months

Food-hygiene practices	No. of cases	Diarrhea				
		%	Unadjusted OR (95% CI)	p-value	Adjusted OR (95% CI)[a]	p-value
House and environmental condition (n=274)						
Clean inside the house	134	7	0.46 (0.20-1.05)	0.07	0.68 (0.27-1.68)	0.40
Clean surrounding the house	139	8	0.60 (0.27-1.33)	0.21	0.69 (0.29-1.64)	0.40
Existence and clean sewage	74	3	0.19 (0.04-0.80)*	0.02*	0.16 (0.03-0.73)*	0.02*
Existence of latrine	172	12	1.89 (0.77-4.61)	0.16	1.53 (0.62-3.92)	0.37
Latrine with closet and septic tank	105	13	1.70 (0.78-3.73)	0.18	1.43 (0.62-3.29)	0.41
Child's faeces was thrown in latrine	138	7	0.51 (0.23-1.15)	0.11	0.62 (0.27-1.46)	0.28
Other family defecated in latrine	241	11	1.87 (0.42-8.29)	0.41	1.63 (0.35-7.58)	0.53
Closed domestic waste disposal	145	10	0.87 (0.40-1.90)	0.73	0.96 (0.42-2.21)	0.93
Mother's hand washing (n=274)						
Before preparing food	80	9	0.79 (0.32-1.94)	0.61	0.79 (0.31-2.04)	0.62
Before feeding the child	240	11	4.18 (0.55-31.8)	0.17	4.16 (0.53-32.8)	0.18
Using water and soap	193	9	0.73 (0.32-1.66)	0.45	0.71 (0.30-1.70)	0.45
Child's hand washing (n=274)						
Before eating a meal	185	10	1.02 (0.44-2.35)	0.97	1.08 (0.45-2.61)	0.86
After defecating/urinating	49	10	1.00 (0.36-2.77)	0.99	1.14 (0.39-3.32)	0.81
Using water and soap	154	10	0.89 (0.41-1.95)	0.77	0.87 (0.38-1.99)	0.74
Food preparation (n=274)						
Food cooked by mother	201	10	1.10 (0.45-2.71)	0.84	1.00 (0.38-2.62)	0.99
Cooked special food for child[b]	75	16	2.48 (0.99-6.19)	0.05	2.56 (0.88-7.58)	0.08
Food holding time before eaten <1 hour	151	10	0.93 (0.43-2.04)	0.86	0.78 (0.34-1.78)	0.55
Reheating the food before child eating[c]	191	9	0.46 (1.18-1.13)	0.09	0.48 (0.18-1.28)	0.14
Feeding child with warm/hot food	150	11	1.31 (0.59-2.92)	0.50	1.16 (0.49-2.74)	0.74
Store food covered/closed	254	10	1.03 (0.23-4.67)	0.97	0.93 (0.19-4.41)	0.92
Cleanliness of utensils (n=274)						
Wash utensils inside house	236	11	2.23 (0.51-9.80)	0.29	1.97 (0.43-9.11)	0.38
Wash utensils with flowing tap water	76	9	0.86 (0.35-2.10)	0.73	0.86 (0.33-2.21)	0.75
Change water in the pail	225	9	0.50 (0.21-1.21)	0.13	0.48 (0.18-1.26)	0.14
Water source and safe drinking water(n=274)						
Piped water source	72	10	0.93 (0.38-2.29)	0.87	0.97 (0.37-2.51)	0.94
Existence of septic tank	106	13	1.67 (0.76-3.67)	0.20	1.41 (0.61-3.25)	0.42
Refill/branded drinking water source	57	11	1.04 (0.40-2.71)	0.93	1.02 (0.37-2.80)	0.98
Cover on water storage	270	10	1.32 (0.37-4.76)	0.67	1.06 (0.28-4.07)	0.93
Habits of buying cooked food (n=274)						
Not Frequently bought street food	109	7	0.57 (0.24-1.35)	0.21	0.52 (0.21-1.29)	0.16
Bought hot food from outside	159	13	1.92 (0.82-4.54)	0.14	1.71 (0.69-4.26)	0.25
Food given to child were still hot	235	11	1.43 (0.41-4.98)	0.58	1.48 (0.38-5.73)	0.57
If the food still hot, food directly eaten	193	9	0.61 (0.27-1.38)	0.24	0.71 (0.30-1.67)	0.43
Not frequently bought snack	29	17	2.01 (0.70-5.77)	0.19	1.43 (0.43-4.75)	0.56
Bought package snack	188	13	3.00 (1.01-8.93)*	0.048	2.37 (0.76-7.35)	0.14
Child's bottle feeding hygiene						
Using bottle feeding (n=155)	96	13	2.67 (0.71-9.88)	0.14	3.33 (0.78-14.2)	0.10
Bottle was washed and boiled (n=96)	24	8	0.56 (0.11-2.78)	0.48	0.67 (0.11-3.97)	0.66
Clean bottle milk (n=95)	32	6	0.35 (0.07-1.72)	0.20	0.22 (0.03-1.53)	0.13

Note: OR, odd ratio.
[a] Adjusted for age, weight-for-height z-score, immunization status, family size and number of under-five children living under the same roof.
[b] n=201 ; [c] n=234;
* Statistical significant at $p< 0.05$, X^2 test.

The association between the overall food-hygiene practice score and diarrhea among children aged 12 – 59 months was not significant, neither in the crude nor in the adjusted analyses (**Table 5.5**). However, a significant interaction with age and food-hygiene practices was observed ($p < .001$). Stratified analysis by age showed that a poor food hygiene score was independently associated with diarrhea in children < 2 years, although the confidence interval was very wide.

Table 5.5 Association between food-hygiene practices and diarrhea among children aged 12 – 59 months (n=274)

Determinants	Food-hygiene practice		Diarrhea	
		%	Unadjusted OR (95% CI)	Adjusted[a] OR (95% CI)
All children	Poor	11	1.15 (0.51-2.60)	1.33 (0.57-3.14)
	Better	9	1.00	1.00
	p-value		0.73	0.51
Stratified by age group				
≤ 2 y (n=93)	Poor	23	2.63 (0.78-8.89)	4.55 (1.08-19.1)[*]
	Better	10	1.00	1.00
	p-value		0.12	0.04[*]
> 2 y (n=181)	Poor	5	0.55 (0.17-1.78)	0.62 (0.18-2.14)
	Better	9	1.00	1.00
	p-value		0.32	0.38

Note: Poor food-hygiene practice (score ≤19 of 36 score); good practice (score >19 of 36 score).
[a] Adjusted for age (continuous), weight-for-height z-score (continuous) and number of under-five children living under the same roof (>1/1).
[*]Statistical significant at $p< 0.05$, X^2 test.

Discussion

Our study indicates that the risk of having diarrhea is increased in children aged <2 years whose mother had poor food-hygiene practices. Children who lived in the house with clean sewage had a lower risk of diarrhea.

We found that children aged <2 years were more vulnerable to suffer from diarrhea as supported by previous studies in Vietnam[15] and Bangladesh[30]. This finding is also in line with the previous review that diarrheal diseases were extremely high during the weaning period (6-24 months).[21] The higher prevalence may be explained by the fact that weaning foods prepared under unhygienic conditions to our children are frequently contaminated with pathogens and are an important risk factor in the transmission of diarrhea.[21] Up to 70% of diarrhea episodes were caused by water and food contaminated with pathogens.[21] Enterotoxigenic *Escherichia coli* (ETEC) was the most common identified pathogen constituting about 14% of diarrhea among children in community-based studies.[31] While Enteropathogenic *E. coli* (EPEC), rotavirus, Campylobacter, Cryptosporodium and Shigella (5-9%) were the next common pathogens identified in these studies. Transmission of *E. coli* especially ETEC is known to be specifically associated with contaminated weaning foods among younger children in developing countries.[32,33] Moreover, this pathogen was considered to be responsible for the diarrhea-induced weight faltering.[21] Younger children

are also at risk to be infected by rotavirus because the specific immunity has not yet been developed to fight against this pathogen entering the digestive system. Rotavirus is transmitted primarily person-to-person through the fecal-oral route.[34] Children are infected with rotavirus through their contact with an infected person outside the household and poor food-handling hygiene practice such as contamination of mother's hands by infected fomites or surfaces.[35, 36] Improved water and sanitation is not sufficient to reduce the spread of this virus, as indicated by similar rates of illness in developed and developing countries.[37, 38]

Our study confirmed that children who lived in a house with clean sewage had significant lower risk of having diarrhea than children who did not have one. A previous study conducted in an urban poor setting in Indonesia also reported an increased risk of having diarrhea in children with unavailability of sewage and/or place to dispose the child's stools.[39] Living conditions such as type of housing, population density, sanitation facilities, and water source were observed earlier as the major determinants for enteropathogen contamination.[40-43] Pathogens in feces disposed in sewage near the house can harm the environment and it could contaminate the food eaten by children.[40-43] In developing countries, both *E. coli* and rotavirus were the common pathogens associated with waterborne disease especially for children who live in poor environmental conditions. Outbreaks of viral gastroenteritis resulting from sewage contamination of water supplies have been well described.[44,45] Contamination of drinking water by sewage through pump failure or blockage of a sewage system has been previously described.[46] Unfortunately, information on diarrhea-causing pathogens could not be obtained in our study.

In our study we included a complete set of food-hygiene practices identified in our community. This approach may give advantages because important and specific local practices are not missed. However, we had to rely on recall methods to investigate food-hygiene practices. This may be the reason why we could not observe significant associations between overall and some of single food-hygiene practices Mothers may report social desirable practices which they do not perform.[47] Mothers may also forget some of the practices. For instance, the assessment of handwashing practices seems to require repeatability observations to avoid risks of misclassifying exposure, which reduces the statistical power to identify associations.[48]

Some data in this study which were derived from direct observation, such as observations in the house and its environment, were significantly associated with diarrhea prevalence. Direct observation is most preferred because it allows first hand data collection in a natural setting, and this was used in prospective cohort studies in Nigeria and Brazil.[49, 50] Although there can be potential bias in (any) recall method, this way of data collection is less expensive and relatively fast, and is still considered as a way to find out the extend of the problem in a poor community.[25]

Studies on the effect of seasonal variation on diarrhea prevalence are contradictive. Our study was conducted in a rainy season which was assumed to be the peak of diarrhea prevalence as described in a previous study within a similar community.[51] However, some recent studies in developing countries observed high diarrhea prevalence in dry seasons.[52-56] Another study reported that in a tropical area, rotavirus diarrhea occurs throughout the year,

increasing frequency during the dry season whereas bacterial diarrhea peaks during rainy season.[57]

Conclusions

In conclusion, the poor food-hygiene practice score was not associated with the prevalence of diarrhea among children under five, but was significantly associated with more diarrhea among children aged <2 years. Therefore, food safety education should be especially targeted to this age group, focusing on clean sewage and good food-hygiene practices. Direct observation methods are preferred for future studies to avoid over- or under-reporting by the mothers on their child's feeding practice.

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Chapter 6

General discussion

Acute diarrhea and acute respiratory tract infections (ARTIs) continue to lead the infectious cause of morbidity and mortality among children <5 years of age in developing countries.[1-3] The burden of this problem calls for effective public health intervention as the preventive strategies (including provision of safe water and sanitation, exclusive breastfeeding, hand washing, vitamin A and zinc supplementation, and vaccinations) are available in developing countries, but are not always effective to reduce the burden of these diseases.[3] Efforts to prevent diarrheal disease by probiotics and milk calcium supplementation as alternative strategy are promising.[4] Probiotics are often supplemented to dairy foods and both probiotics[5-7] and calcium in milk[4] may strengthen intestinal infection resistance. Research presented in this thesis consists of an intervention study and a cross sectional study. As presented in the general introduction, the research described in this thesis focused on four major research questions as follows:

What are the effects of an intervention using probiotics and calcium on reducing the incidence and duration of diarrhea and ARTIs among Indonesian children aged 1-6 years?

What are the effects of probiotics and calcium supplementation on severity of diarrhea based on Modified Vesikari Scale and fecal markers?

What are the potential effects of probiotics and calcium on growth, iron and zinc status?

What is the association between food-hygiene practices and prevalence of diarrhea among children in low socioeconomic urban areas of East Jakarta?

The next section presents the main findings of the thesis, methodological issues are discussed and results are placed in a broader perspective. Moreover, implications for public health and future research are given.

6.1. Main Findings

The main findings of this thesis are summarized in **Table 6.1**.

Our community-based RCT involving 494 Indonesian children who were randomized to receive milk with either low or regular calcium as well as two different types of probiotics revealed mixed results (**chapter 2-4**).

We observed a consistent benefit of the probiotic strain *Lactobacillus reuteri* DSM 17938 for many different outcomes. *L. reuteri* may prevent diarrhea especially in children with lower nutritional status (**chapter 2**) and reduce total duration of diarrheal episodes (**chapter 3**). This probiotic modestly improved growth, by increasing weight gain and WAZ changes over 6 months and monthly weight and height velocity in Indonesian children (**chapter 4**). However, *L. reuteri* did not affect diarrhea severity.

The other probiotic strain, *L. casei* CRL 431 modestly improved monthly weight velocity (**chapter 4**), but this strain did not reduce diarrhea incidence (**chapter 2**) and duration or severity of episodes (**chapter 3**).

In contrast to these findings, milk calcium alone did not affect any of the outcomes (**chapter 2-4**). Also, none of the dietary treatments affected incidence and duration of ARTIs (**chapter 2**) and iron and zinc status in Indonesian children (**chapter 4**).

Our cross sectional study showed that in addition to other major determinants, poor mother's food-hygiene practices contributed to the occurrence of diarrhea in Indonesian children <2 years from low socioeconomic urban areas of East Jakarta (**chapter 5**).

Table 6.1 Main findings of the studies described in this thesis

Chapter	Study design	Study population	Objectives	Main findings
Incidence of diarrhea and ARTIs				
2	RCT	494 children aged 1-6 years	Effects of probiotics and calcium on incidence of diarrhea and respiratory tract infections.	*Lactobacillus reuteri* DSM 17938, reduced incidence of WHO-defined diarrhea by 24% (nonsignificant) and all reported diarrhea by 32% (significant)
				Lactobacillus casei CRL 431, no effect
				Regular calcium milk alone, no effect
				None of the interventions affected ARTIs
Total duration and severity of diarrhea				
3	RCT	494 children aged 1-6 years	Effects of probiotics and calcium on duration and severity of diarrhea.	*L. reuteri* reduced the total duration of diarrheal episodes by 1.35 days in a 6-month period, likely by mainly affecting rotavirus-positive diarrhea.
				L. reuteri, *L. casei* and calcium did not affect diarrhea severity based on Vesikari score and fecal osmolarity and calprotectin. A higher diarrhea-induced fecal mucin concentration in children supplemented with *L. casei* was observed.
Growth and iron and zinc status				
4	RCT	494 children aged 1-6 years	Effects of probiotics and calcium on growth and iron and zinc status.	*L. reuteri* modestly improved growth, by increasing weight gain and WAZ changes over 6 months and monthly weight and height velocity.
				L. casei modestly improved monthly weight velocity.
				Regular calcium milk did not affect the measured growth outcomes.
				Neither the probiotics nor regular calcium milk affected iron and zinc status.
Food-hygiene practices and diarrhea prevalence				
5	Cross sectional study	274 children aged 12-59 months	Association of food-hygiene practice with the prevalence of diarrheal disease.	Risk of having diarrhea is increased in children aged <2 years whose mother had poor food-hygiene practices.
				Children who lived in the house with clean sewage had a lower risk of diarrhea.

Abbreviations: RCT, Randomized double-blind placebo-controlled trial; ARTIs, acute respiratory tract infections

6.2. Internal and external validity

This section presents a number of methodological considerations and puts findings in a broader perspective. We first discuss the intervention study and subsequently address the cross sectional study.

6.2.1. Intervention study

A major part of this thesis is based on an intervention study that is subject to certain limitations.

Study population

Apparently healthy children

Because the study aim was to prevent the incidence of diarrhea and ARTIs, we included apparently healthy children without severe illness, pulmonary tuberculosis and severe malnutrition, which have the advantage of less interference with (co) morbidity or drug treatment (e.g. antibiotics). We involved mild and moderately (but not severely) malnourished children that gave the advantage that an additional beneficial effect of probiotics and calcium in malnourished subjects can be observed. Primary and secondary outcomes of this study were adjusted for nutritional status to eliminate the influence of confounding. Moreover, we checked interaction of nutritional status (effect modification).

Subjects with increased susceptibility to infections

We selected young children because the prevalence of infections and malnutrition among children aged <5 years in low socioeconomic urban communities in Indonesia remains high.[8, 9] During our acceptance study, which was conducted before the trial, we observed that the age of children who entered the study was mainly between 2 and 5 years. The main underlying reason was that we included only non-breastfed children in the study. We excluded breastfed children because of the well-known protective effect of breast milk against infection, which may affect the intestinal microbiota,[10] and thus may result in confounding. In addition, for ethical reasons, the intervention must not interfere with the national breastfeeding program for young children. We, therefore, prioritized children with this age range to be included in the intervention study. However, due to the tight eligibility criteria of the study, but to prevent insufficient numbers of children entering and having an underpowered study, children between 1 and 6 years of age were recruited for the study. Higher morbidity of diarrhea in children from families with lower socioeconomic status has been observed in urban areas of some developing countries e.g. Jakarta, Indonesia;[11] Gaziantep, Turkey;[12] Accra, Ghana.[13] It is therefore, that our study was conducted in a low socioeconomic urban area of East Jakarta.

Children with low calcium intake

We chose a study population with a low habitual calcium intake because the assumable benefit of calcium supplementation on resistance to the intestinal infection is likely higher in this population. Therefore, children with daily calcium intake of >75% recommended daily allowances (RDA) (> 375 mg) were excluded. Calcium intake status was assessed during the screening phase by means of a semi-quantitative food-frequency questionnaire (S-FFQ) that was validated and specifically designed to measure calcium intake in less affluent children (unpublished results). Furthermore, we assessed calcium intake from analysis of fecal calcium excretion, which reflects calcium intake over the past days. Both measurements of calcium from S-FFQ and fecal excretion confirmed that our subjects had a low calcium intake as expected, mainly because of low consumption of milk and dairy among children in low socioeconomic urban areas of Indonesia. There are no published data available yet at national scale for calcium intake. Available data suggest that Indonesian children <5 years have a low dietary calcium intake not meeting their age-specific RDA [14-16] with an average intake as low as ≈80 and ≈190 mg/d for non- and breastfed children aged 1-3 years, respectively.[15] Our previous survey in selected urban low socioeconomic East Jakarta revealed that 46% and 61% of the children had a calcium intake less than 75% and 100% of RDA (**Figure 6.1**; unpublished results). This is in line with the reported ≥70% prevalence of inadequate calcium intakes the Filipino, Cambodian and Mongolia children.[17]
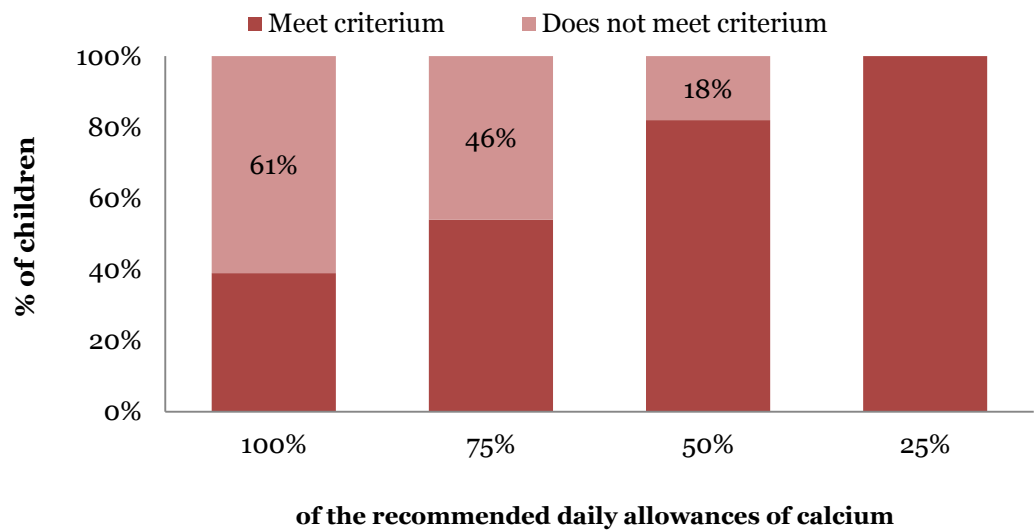
Figure 6.1 Percentage children who had calcium intake meeting 25%, 50%, 75% and 100% of recommended daily of allowances in children aged 1-5 years of selected urban low socioeconomic area of East Jakarta, Indonesia.

Study design

The major strength of our study was our focus on prevention in contrast to most previous studies on probiotics, which aimed at treatment of institutionalized or hospitalized children. There is a difference between hospitalized and community cases in disease severity and it

can be hypothesized that the public health impact of nutritional interventions is expected to be higher in milder, and thus community, cases. Additional strengths were the double-blind, placebo-controlled design: characterized by strict adherence to a rigorous protocol, use of validated instruments in the assessment of outcomes, long duration of the intervention, and excellent compliance rate.

Blinding

RCTs should preferably be double-blind, i.e. subjects and research staff must be unaware about the type of treatment until data-analysis is completed. Studies of probiotics often lack a control group, blinding, validated outcomes, or standards for reporting adverse events.[18] A lack of proper blinding is the most common bias. Our study carefully considered this potential bias by having researchers, mothers, children, and laboratory personnel unaware of the treatment until all biochemical and data analyses were finished, and until after the blind review meeting. Also, the data safety monitoring board (DSMB) and an independent person at SEAMEO RECFON kept three sets of sealed envelopes allowing deblinding per subject without disclosing other children's treatments.

Randomization

To prevent selection bias in a RCT, subjects should be randomly divided over the intervention and placebo groups. To ensure an equal balance of baseline values that may be of influence on the response of subjects to probiotic and calcium supplementation, eligible children were admitted to the study on enrolment basis and stratified according to area of living (flooding and non-flooding), age (<57 and ≥57 months) and gender. A randomization table with treatment codes and block size of eight was generated using SAS version 9.1 (SAS Institute, Inc, Cary, NC) by an independent person at Wageningen University. Randomization in our study was successful, because demographic, anthropometric and lifestyle characteristics of the subjects were well balanced over the four intervention groups. Randomization was maintained throughout the study period since there was no selective drop-out related to the interventions.

Intervention

Important factors concerning our intervention were dose of calcium and probiotics, type of probiotic strains, intervention duration and compliance.

Dosage of calcium

We used a low (50 mg) as well as a high (440 mg) daily dose of calcium. The dosage of 50 mg of calcium per day by providing low calcium milk seems very low. However, to be able to obtain a difference in calcium intake between the control (Low calcium milk, LC) and experimental (Regular milk calcium, RC) group, subjects were asked to exclude dairy from their habitual diet. Normally, milk and milk-based foods are the major sources of calcium in children's diets. However, milk is not the only important dietary calcium source in our study population because milk is considered as an expensive commodity for less fortunate

communities. Local calcium-rich food sources (e.g. tofu and tempeh) are frequently consumed by the children. These common foods could not be excluded from the children's daily diet for ethical reasons. By allowing these non-dairy calcium-rich habitual foods, besides providing LC milk resulting in additional 50 mg calcium/day, the median total daily calcium intake of subjects in the LC group was 206 (interquartile range 162, 249) mg/day. This amount was largely comparable with that provided by their habitual intake (generally low in calcium) as mentioned above (**Figure 6.1**). The Indonesian RDA for calcium for children aged 1-6 y old is set at 500 mg per day,[19] which is roughly equivalent to having two servings of milk per day[20, 21] as part of the existing habitual eating pattern. Calcium is not widely distributed in the food groups and milk as well as milk products are excellent source of calcium.[22] Therefore, an adequately high calcium intake is hardly achievable via dietary intake in the usual eating pattern of our children when dairy is not included.[16] By supplementing regular calcium milk, the median calcium intake of the children increased to 589-602 mg per day, which was presumed of sufficient contrast with the LC group consuming ≈200 mg calcium in total per day.

Probiotic Strains

Food microbes should have a Generally Regarded As Safe (GRAS) status. They have a long history of safe use in foods, are non-pathogenic, and are preferably acid and bile tolerant.[23] Lactobacilli are generally regarded as safe and they have a long history of safe use in foods.[23, 24] The applied *L. reuteri* and *L. casei* strain were selected because of their suggested anti-diarrheal potential in young children in previous studies.[25-28] *L. reuteri* DSM 17938 has been derived from the original strain of *L. reuteri* (American Type Culture Collection strain 55730), by removal of antibiotic resistance gene-carrying plasmids.[29] This strain has over recent years been accepted in the US as GRAS for their intended use.[30] A recent randomized controlled trial indicated that the use of *L. casei* CRL 431 in early infancy is safe, well tolerated and has no adverse effects on growth and infant behavior.[31]

The Food and Agriculture Organization (FAO)/WHO defined in 2001 probiotics as "live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host."[24] It implicates that probiotic strains should remain alive in the product and should survive passage through the human gastrointestinal tract.[32] Also, probiotics must be provided in an adequate dose in order to exert the desirable effects.[24] For a strain to be selected as probiotic, it should preferably meet certain criteria i.e., resistance to gastric and bile acids, adherence to mucus and/or human epithelial cell and cell lines, possess antimicrobial activity against potentially pathogenic bacteria and/or possess the ability to reduce pathogen adhesion to surfaces.[33, 34] Probiotics must be able to exert their benefits to the host through growth and/or activity in the human body.[34, 35] It is the ability to remain viable at the target site and to be effective that should be verified for each potentially probiotic strain. The WHO recommends all strains to be deposited in an internationally recognized culture collection.[33] Many probiotic products were originally developed by and for the dairy industry, but nowadays probiotics are included in a wide range of foods, drinks and supplements.

It is important to maintain high viable survival rates of probiotics during delivery through the gastrointestinal tract. To ensure high survival rates of the probiotic strains, straws containing probiotics in our study were stored cooled (<10ºC) at all times until delivery. Viability of the probiotics cq shelf-life of the probiotic straws was checked each month by selective plating. Both probiotic strains in the straws remained >90% viable during the intervention period. Regarding the ability to survive within the gastrointestinal tract, it is reported that *L. reuteri* DSM 17938 can survive passage through the human gastrointestinal tract after the consumption of a low-fat probiotic spread.[32]

Dosage of probiotics

The probiotic dose is important to achieve clinical effects. The importance of the dose used has been underlined in many studies, it is therefore that we should be more cautious on this aspect. In the past many commercial products claimed to have e.g. 10^9 colony-forming unit (CFU) of probiotics whereas in reality probiotic viability had declined significantly during shelf-life.[36] To prevent consumer misleading, probiotic doses and viable concentration of each probiotic present at the end of shelf-life are necessary to be clearly stated in the labeling of the probiotic product, which can be better monitored and guaranteed.[33,37] The probiotic dosage selected for our intervention was based on supplier's information of efficacy, application in children and safety concerns when dosed for longer periods of time.

There is no single cut-off value for the probiotic dose beyond which clinical effects start to occur. A recent Cochrane review reported the efficacy of probiotics to treat diarrhea: 15 studies used a high probiotic dose (>10^{10} CFU/day) and 26 used a low dose (<10^{10} CFU/day).[5] However, they concluded that the beneficial effects of probiotics to treat acute infectious diarrhea were dependent on the bacterial strain and the dose. Different probiotic strains have different effective dosages. It should be noted that our study was in a community setting and had diarrhea prevention as outcome, which is clearly different from studies in hospital settings applying probiotics for treatment purposes as reviewed by Cochrane. Higher dosages may be needed for treatment of hospitalized severe cases than for occasional and generally milder cases in the community. However, until now, there are no data available indicating "the effective probiotic dose" for prevention of diarrhea in otherwise healthy children given probiotics for a longer time period. Moreover, it can be questioned whether there will be a substantiated single probiotic dose advise in the future. Weizman et al documented that child care-visiting infants fed a formula supplemented with *L. reuteri* ATCC 55730 (sister strain of our study strain) at a dose of 1.2 x 10^9 CFU/day or *Bifidobacterium lactis* 1.2 x 10^9 CFU/day had fewer and shorter episodes of diarrhea, with no effects on respiratory illnesses.[25] They found that effects were more prominent with *L. reuteri* than *B. lactis*.[25] This dosage is within the range of the dose applied in our study as we used a dose of 5 x 10^8 CFU/day for both *L. reuteri* and *L. casei*. The dosage of our probiotics strains is also within the effective dosage recommended by FAO/WHO during their consultation meeting in 2001.[38] As stated in the Introduction section of this thesis, in this consultation, the minimum effective dose of ≥10^8 CFU/day has been recommended for therapeutic effects.[38] The rationale of this recommendation was derived from the reported minimum effective dose for therapeutic cultures from some studies to be 10^6-10^9 viable CFU/day.[39] However, some other studies showed that the minimum dose of some strains

such as *L. rhamnosus* GG to yield fecal recovery was 10^{10} CFU/day,[40, 41] and *L. johnsonii* LJ1 to elicit immune effects was 10^9 CFU/day, whereas dose of <10^8 CFU did not.[42] The minimum dose of 10^9 CFU/day and consumption of 100 g or ml (a food containing at least 10^7 cells per gram or milliliter) was recommended for dairy products as this dosage was also recommended by the Japanese Fermented Milk and Lactic Acid Bacteria Beverages Association.[38, 43, 44]

Study duration

When we designed our 6-month community-based RCT (2006), several meta-analyses, systematic reviews and trials on probiotics and diarrhea were published. These studies reported beneficial effects after respectively very short study durations (<2 weeks) and mainly in hospital-based settings aiming at treatment of diarrhea.[5] Studies on the prevention of diarrhea in healthy children living in the community were mainly in day-care center based studies (6 studies) in Western countries with study duration between 3 to 18 months.[25, 45-53] Thus far, only 1 randomized trial has focused on the role of probiotics in prevention of acute diarrhea in a community setting in a developing country. This study using LGG strain suggested a significant reduction of acute diarrhea with duration of 15 months.[54, 55] However, there is no data available indicating minimal study duration for diarrhea prevention by supplementation with the probiotic strains, *L. reuteri* and *L. casei*, used in our study. Considering the safety aspects and based on our feasibility in predicting the prevalence of diarrhea in the community, we decided for study duration of 6 months. We also considered the logistics arrangement, long holiday after Ramadan (Islamic holiday) and possibility of flooding study sites leading to impaired access. To support our prediction, the sample size was calculated thoroughly on the basis of the expected effect size of 21% reduction of mean number of diarrheal episodes and 0.7 day reduction of mean diarrhea duration over a 6-month intervention period. Effect sizes were based on meta-analyses of probiotics.[7, 56] With a minimum 480 children, we assumed that the 6 months would be sufficient to detect this effect size.

RCTs on probiotics and growth and on other constituents such as prebiotics and LC PUFA showed effects on child growth after 12 months. With a study duration of 6 months we were only able to examine relatively short-term changes in growth outcomes. Therefore, we cannot exclude a possible beneficial effect of long-term intake of probiotics and calcium on growth.

In a small proof-of-concept RCT on calcium supplementation and diarrhea in adults, anti-diarrheal effects were already visible within 3 weeks. In that study all subjects were orally infected with a live but attenuated enterotoxigenic *E. coli* vaccine strain leading to 100% infectivity.[4] This is not the case in a community trial, where the number of diarrheal cases follows less predictable 'natural' incidence rates. Based on the above-mentioned study, an intervention duration as short as a few weeks for calcium might be already effective and it is difficult to think of physiological arguments why efficacy would disappear after (much) longer study duration.

Compliance

Our double-blind randomized placebo-controlled study was conducted with an excellent compliance rate. Subjects in our RCT consumed 180 mL of milk twice daily (not together with a meal) by using the straws provided. Mothers were requested to maintain the child's habitual diet but to exclude probiotic, prebiotic or high calcium foods/drinks other than the supplied ones. Compliance was checked by measuring the amount of milk consumed by using a calibrated stick put into the tetra-packs to score the remaining volume by using a pretested 5-point scale. The field workers observed the children drinking milk at least once a week and empty packages had to be shown during visits. Activities with creative and educational contents were implemented to maintain compliance of both mothers and children. A small acceptance test (n=65 children aged 1-6 years) was conducted before the intervention study to check the acceptance of regular milk and (placebo) straws supplied for 7 days. The acceptance of liquid milk and straw usage was high (83% and 98%, respectively). A 2-day milk drinking acceptance test was part of the screening procedure (to identify potential non-compliant subjects) and only children that didn't refuse to drink milk and did not show allergy or intolerance to the products were included in the intervention.

All measures reflected an excellent compliance rate of 94%; apart from the few subjects who stopped prematurely (n=3 from total n=494), only 6% of subjects consumed less than 70% of the total frequencies (2 consumption units per day x 168 days intervention) for at least ¾ empty packages of 180 ml milk. We performed our primary analysis of the results according to the intention-to-treat (ITT) principle. *Intention-to-treat* analysis was performed for all outcomes and for all eligible children who were randomly allocated to treatment and had consumed the intervention products at least once. Per-protocol analysis, excluding the few noncompliant subjects (6%) and subjects having chronic antibiotic usage, did not change the results.

Outcomes

Definition of diarrhea

There is large variability in defining the outcome measures in probiotic studies on diarrhea.[5, 57] In our study, diarrhea was defined according to the WHO definition (≥3 loose/liquid stools in 24 hours).[58] In addition, all reported diarrhea (broader definition: ≥2 loose/liquid stools in 24 hours) was evaluated.

Our study was performed in a community setting and aimed for diarrhea prevention, which is quite different from clinical or hospital settings focused on treatment. Only children with more severe diarrhea and dehydration symptoms (or additional underlying health problems) are hospitalized, and use of the WHO definition of diarrhea in such settings is highly applicable and valid. There are reasonable arguments to question whether it is equally valid in a community setting with generally milder diarrhea cases. Therefore, it is important to also separately report on all reported diarrhea because of the following reasons: (a) other clinical trials have used broader diarrhea definitions as well;[59-61] (b) the WHO definition considers stool consistency to be more important than frequency;[104,109] and their definition

leaves room for registration of any increase in normal stool frequency; (c) mothers also reported on diarrhea when they considered their children's stool appearance more watery and more frequent than normal, which was often before the WHO-definition of diarrhea was met; and (d) milder forms of diarrhea are more commonly seen by primary pediatricians and are considered a health problem too.[62] Importantly, the WHO definition is the best validated definition, but it was not validated in community settings. It may not be generalizable to different settings such as our intervention, which included children of older age and an urban community setting.[63]

To avoid overestimation of diarrhea frequency using both diarrhea definitions in this study, not every child's defecation can be regarded as one frequency. Stool frequency was counted strictly when there was at least 1-hour interval since the previous defecation.[64] We did not only rely on mother's perception but implemented an active surveillance to verify mother's daily records with twice-a-week visits of trained field workers and twice-a-month visits of field supervisors. The physician and monitoring expert accompanied the field workers on several of their home visits. All endpoints were assessed using structured and pretested forms as applied by others.[64-67] The forms were adapted to the local situation and were used by field workers who were rigorously trained and supervised on their application (**chapter 2**). To avoid over-reporting in using "all reported diarrhea", we restricted the frequency of diarrhea with cut off 2 or more liquid/loose stool. To prevent potential bias of researchers in classifying "all reported diarrhea", all data related to both diarrhea definitions were obtained when all researchers and study subjects were completely blinded to subjects' treatment and study outcome. For both WHO and the broader definition of diarrhea, probably some random misclassification of diarrhea incidence has occurred, but these misclassifications were probably distributed equally across the groups. WHO-defined diarrhea reduction was not statistically significant, whereas the reduction of "all reported diarrhea" was statistically significant, probably because of increased study power due to additional diarrhea cases.

Assessment of incidence and duration

Disease incidence was the number of episodes divided by child-years of observation.[68] An episode was considered to have ended on the last day of diarrhea followed by 2 diarrhea-free days.[69] The number of episodes is the sum of all diarrheal episodes that occurred during the intervention period. To eliminate the effect of seasonal variation on diarrheal incidence, the eligible children were included during two different time periods: December 2007-June 2008 (covering mainly rainy seasons) and March-September 2008 (representing the dry seasons). Children living in flooding areas were admitted in dry seasons and children in non-flooding areas were included in rainy seasons.

We defined duration of diarrhea in two ways: episode duration and cumulative (total) duration of all episodes (**chapter 4**). Episode duration was defined as number of days from first until last excretion of the loose or liquid stool that is not followed by another abnormal stool in each episode.[64,70] In our prospective RCT, some children experienced recurrent episodes of diarrhea (more than one episode). Therefore, besides calculating the duration of each episode, we also calculated the cumulative (total) duration of all diarrhea episodes. The cumulative (total) duration of diarrhea was the sum of all diarrheal days of all episodes per

child per 6-month of the intervention period.[71,72] It reflects the cumulative time spent with diarrhea of each child during 6 months of intervention.

Assessment of severity

Among the numerous methods to assess diarrhea severity, we selected clinical measurement by a modified Vesikari score (MVS) based on the mothers' subjective information. In addition, objective measurements of fecal markers were used i.e. fecal osmolarity, mucin, and calprotectin.

One of the most commonly used severity scales is the Vesikari 20-point scale (an episode of gastroenteritis with a score of ≥11 is considered severe).[73,74] The Vesikari scale has not been formally validated and has not been applied by any trial on probiotics in a community setting in a developing country. However, this method has been commonly used in cohort studies on diarrhea and rotavirus vaccines, and has been adopted as reliable method on the basis of its frequent use in clinical trials.[75]

We slightly adjusted the Vesikari score to better fit with our community situation and study population. We graded the severity by using this slightly modified Vesikari scale (18-point) and asked a qualitative report of the mothers for fever (yes or no) instead of rectal temperature. Also, the percent dehydration was replaced by estimated degree of dehydration[73,75-77] since this variable is not easily assessed in our community. This MVS method was pre-tested to ensure its applicability in our study population. The MVS applied in our study is similar to MVS reported by Freedman et al.[75] In the study of Freedman et al, MVS was considered to be reliable, valid, and easy to administrate by mothers and field workers. Also, the MVS was recently recommended to be used as a measure of diarrhea severity in outpatient clinical studies of young children.[75]

Considering that expected effect sizes of dietary interventions in community setting are generally small, we included objectively measurable and quantitative fecal markers of diarrhea severity in our study besides the more subjective and semi-quantitative MVS. There is increasing interest in non-invasive, objective markers to assess intestinal infection severity and/or mucosal inflammation in clinical studies. Candidates of such potential markers are fecal osmolarity, calprotectin and mucin. Preventing ex-vivo bacterial fermentation and thus artificial osmolyte increases is very important in the measurement of fecal markers. Therefore, we implemented a very strict procedure for fecal sample collection to ensure quick cooling and storage of samples in freezers.

Although, there has been scientific debate whether **fecal osmolarity** has the potential to differentiate osmotic from secretory diarrhea by evaluation of the stool ion gap,[78] this marker is also useful to quantify diarrhea severity in children. When gastrointestinal digestion or microbiota-related metabolism is altered, diarrhea can result from osmotic forces in the lumen (e.g. lactose in lactose malabsorbers) or an increased secretory state (e.g. bacterial enterotoxin-induced diarrhea).[79]

We used **fecal calprotectin** (FC) in our study because this marker is a valuable, non-invasive and easily measured laboratory test in assessing children with acute diarrhea and

reflects infection-induced mucosal inflammation. FC is assumed to act as an early marker in younger children with acute gastroenteritis.[80] However, FC is not specific for gastroenteritis as children with other gastrointestinal disorders i.e. inflammatory bowel disease have high FC concentrations reflecting increased numbers of inflammatory cells in stools.[81-90] There are limited studies on the use of FC as a diagnostic test in pediatric care,[82] especially in acute or chronic diarrhea infections in developing countries. Although the FC median value in our children in healthy state (61 µg/g of wet feces, n=489) is higher than those reported in healthy children in developed countries (range 11-49 µg/g of wet feces),[81, 82, 85, 88, 91-94] this value is comparable with the median value of children aged 1-12 years in a community study in Uganda (52 µg/g of stool, n=302).

We also used **fecal mucin** (FM) as mucin excretion is stimulated during infectious diseases.[4,95] The mucus layer is an important contributor to intestinal host defence and inhibits pathogens adhesion to epithelial cells and subsequent bacterial translocation of invasive strains.[78, 79] Accelerated mucous secretion and mucin release may also contribute to delayed-onset diarrhea.[96] So far, studies on intestinal mucin merely focused on qualitative differences in mucin oligosaccharides composition during various intestinal diseases[97,98] whereas quantitative aspects are understudied.

Ideally, such severity markers can be explored to what extent they may (1) discriminate bacterial from viral infection; (2) associate with the severity classification of gastroenteritis by the above MVS; and (3) monitor the severity and course of diarrhea, which may provide information for disease management.[99] To elaborate on this in relation to FC: recent studies documented that FC varies markedly depending on gut pathogens in acute gastroenteritis as higher FC was observed in children[80] as well as adults[100] with bacterial types of acute gastroenteritis. FC has favorable test characteristics in being sufficiently accurate for early discrimination between bacterial and viral acute gastroenteritis. Although this showed that FC can be used as a screening tool for predicting etiology of acute bacterial diarrhea, our study results so far cannot be used to verify such diagnostic application because, except for rotavirus, microbiological data for (bacterial) pathogen identification in diarrheal episodes is missing.

The severity of gastroenteritis is related to etiology rather than to age, and rotavirus is responsible for the most severe cases.[101] The severity of diarrhea is also closely related to the grade of dehydration; vomiting should be considered an indirect sign of severe acute gastroenteritis, and should be carefully considered in the management of diarrhea. Loss of appetite, fever, vomiting, and mucus in stools are frequently associated with persistent diarrhea. We failed to determine the association of the fecal markers and MVS, or diarrhea duration and severity (both MVS and fecal marker). This may be due to the fact that most diarrheal episodes in our study were classified as mild and the variability in scores was thus rather low (**chapter 4**). The relatively mild diarrheal episodes observed in our study population lead to rather homogeneous fecal marker responses. Unfortunately, this hampers conclusions whether fecal calprotectin, osmolarity and mucin are truly quantitative indicators of diarrhea severity. We are also not convinced whether the subjective MVS is more suitable to predict diarrhea severity in a community setting in contrast to single clinical

diarrhea severity indicators such as fever, vomiting, dehydration and prolonged duration as mentioned above.

Assessment of growth, iron and zinc status

We examined growth, iron and zinc in accordance to the recommended validated methods. Duplo assessment of supervised anthropometric measurements was done by well-trained field workers. Standard protocols and equipment as well as the same field workers to perform the assessment were applied to reduce potential variation in assessment due to intra and inter observer variations. At least 10% of the iron parameters (ferritin, sTfR) measurements assessed by the SEAMEO RECFON laboratory in Indonesia were validated by the laboratory of Wageningen University. The precision (coefficient of variation) of the methods was checked between duplo assessments and plates and was very good (coefficient variation between 2.07-3.68%). Validity and reproducibility methodology used by SEAMEO to analyze ferritin was checked earlier and appeared to be good.[102]

Bias due to incomplete follow-up is unlikely to have influenced our results, because only few children failed to complete the assessment. Under- or overestimation of the effect is therefore unlikely, also because growth and iron and zinc status outcomes were comparable between completers and non-completers. To prevent bias due to selective drop-out, we analyzed the data with a repeated mixed coefficients model (SAS PROC MIXED procedure) for our analyses, which took into account subjects with incomplete follow-up data. As the sample size calculation was based on primary outcomes (diarrhea incidence and duration), we realize that sample size may have been inadequate to detect group differences in growth or micronutrient status. A post-hoc power calculation shows that we have more than 90% power to detect a change in mean over the baseline to study end (within groups) of anthropometric indices (weight, height, WAZ, HAZ) and the level of Hb, serum ferritin, sTfR and zinc concentration. However, our sample size between group of comparisons, on the different secondary outcomes described here, permitted us to detect treatment related differences with an observed small to medium effect size (Cohen's[2]) of <0.29, probability level (α) of 0.05, and a statistical power of <74%.

Standards for reporting adverse events

There is a lack of assessment and systematic reporting of adverse events in probiotic intervention studies.[103] In our study, adverse events were carefully recorded by using International Classification of Diseases, 10th Revision codes. All diagnoses were confirmed by study physicians. Severity and likelihood of relation to the intervention were scored by the physician and continuously monitored by the DSMB.

Interpretation and perspective: intervention study

To our knowledge, our study is the first large randomized controlled trial, focusing on the effect of calcium with or without one of two specific probiotics to reduce incidence and duration of diarrhea and respiratory tract infections, to assess cumulative duration and

severity of diarrhea, and to measure growth and iron and zinc status in young children in a community setting.

Diarrhea incidence

Previous evidence on the preventive effect of probiotics on diarrhea and ARTIs has been limited to hospital or day-care center based studies and performed in developed countries.[6, 7, 56,104,105] In our study, the effect size of diarrhea reduction by *L. reuteri* (20% for WHO-defined diarrhea; and 32% for all reported diarrhea) was higher compared with three previous randomized trials in a community setting in developing countries;[55,106,107] a 14% reduction by supplementing *L. casei* shirota was seen in a comparable study in India,[107] a 6% reduction by *Bifidobacterium lactis* HN019 combined with prebiotic oligosaccharides in India[106] and a 6% reduction using *L. rhamnosus* GG in Peru.[55] It is difficult to provide estimates or draw conclusions of the overall probiotic effect based on these four studies (including our study) on diarrhea prevention in a community setting in developing countries,[54,55,106,108] because of differences in probiotic strains and doses, intervention duration, and study subject's age. An approximate estimate would be a reduction of diarrhea incidence between 6% to 32%, which is in line with observations of diarrhea prevention studies in day-care centers of developed countries using various probiotic strains[25, 46, 47, 49, 51, 53] or multi-micronutrients fortified food or drink.[109] However, these reductions are lower compared to diarrhea risk reductions by hand washing with soap, improved water quality and excreta disposal (48, 17 and 36%, respectively).[110]

Our results underline that probiotic effects are strain-specific as we found protective effects of *L. reuteri* DSM 17938 against acute diarrhea in children, whereas supplementation of *L. casei* CRL 431 (without other strains) was without effect. In our study, children supplemented with *L. reuteri* experienced a few adverse events, mainly referring to a less regular defecation pattern. *L. reuteri* did not lead to any serious events related to the intervention, and positive results included a lower proportion (9% in low calcium, 15% in regular calcium, 15% in *L. casei* and 9% in *L. reuteri*) and shorter duration of antibiotic use (median antibiotic duration: low calcium 4 days, regular calcium 10 days, *L. reuteri* 3 days, and *L. casei* 5 days).

Diarrhea duration

L. reuteri supplementation shortened the cumulative diarrhea duration by 1.35 days in a 6-month period, which is in line with reduction of duration in single episode of other reported probiotic interventions in children. A recent Cochrane review concluded that diarrhea duration in infants and children was reduced by 29.2 hours in those taking probiotics for <14 days for treatment purposes (95% CI 25.14–33.25 hours, fixed effect model; 30.48 hours, 95% CI 18.51–42.46, random effects model, 12 trials, n =970).[5, 111] This effect was larger than the previously reported effect sizes based on systematic review and meta-analyses undertaken by Szajweska in 2001 and McFarland in 2006, respectively. Here for all probiotic strains for the treatment of diarrhea in children, a reduction was seen of 18.2 hours[104] and 13.4 hours,[112] respectively. Our study is also consistent with reviews or meta-analyses on efficacy of specific probiotics to reduce diarrhea duration in children: Van Niel et al showed a reduction of 0.7 days by *Lactobacilli* administration,[56] Szajewska et al indicated

a reduction of 1.1 days in infants treated with *L. casei* GG[113] and Chmielewska et al showed a reduction of 0.92 days by *L. reuteri* ATCC 55730.[114,115] These effects were more evident in hospital-based settings of developed countries, whereas two studies performed in a community setting in developing countries[106,107] did not explicitly present the effect on diarrhea duration and another study failed to show an effect of probiotics on diarrhea duration.[55] Our observed reduction in rotavirus-specific diarrhea duration of 0.5 days was much lower than the 2.1 days reduction found in a meta-analyses in 2007.[113]

In several RCTs on calcium in children, daily doses ranged from 79 mg to 1500 mg calcium per day.[4,116-122] A recent study showed that the desirable fortification levels to achieve in foods for calcium in children of three Asian countries (Philippines n=1374, 6-36 months; Cambodia n=177, 12-36 months; and Mongolia n=179, 12-36 months) is around 313-389 mg per day (530-783 mg per 100 g dry weight).[17] This is based on an Estimated average requirement cut-off point in order to have a prevalence of inadequate intake <2.5% and excessive intake <1% in these children. Effective doses of calcium in RCTs aimed to reduce diarrhea incidence, duration and severity in children are unknown. In a former study with Dutch adults showing improved resistance to ETEC infection-induced diarrhea, study subjects were supplemented with 60 mg (placebo) and 1100 mg calcium per day (treatment group).[4]

Diarrhea severity

There was no significant beneficial effect of dietary calcium or one of probiotic strains on diarrhea severity measured by Vesikari score. In our study population, all diarrheal episodes with the exception of one, had a MVS<11. This score is categorized as mild and is in contrast with the high percentage of severe diarrhea (Vesikari>11) observed in rotavirus vaccine studies in medical care settings in sub-Sahara Africa (>45%)[123] and Asia (>63%).[124] The low MVS in our study (groups mean scores were 3-4) demonstrate that diarrhea in outpatients and/or children in community settings is typically of a milder type.

Although our study population suffered from milder diarrhea than observed in the above-mentioned studies, there were clear responses in the applied diarrhea severity markers when comparing normal fecal samples collected at study end with samples collected in diarrheal episodes. Diarrhea type was not the subject of our study, but results showed that acute diarrheal episodes were characterized by increased fecal osmolarity suggestive for the osmotic type of diarrhea.[79] Fecal calprotectin concentrations increased >2-fold during acute diarrheal episodes in the present study. These increased levels likely reflect infection-induced intestinal mucosal inflammation.[125] In support of this, approximately 50% of our diarrheal samples can be categorized as strongly positive inflammation based on the suggested upper limit cut-off point of FC in pediatric gastrointestinal diseases as previously described.[82, 88] The suggested cutoff point of FC is >50 μg/g (<50 μg/g of stool = negative, 50–100 μg/g of stool = weakly positive, and > 100 ìg/g of stool = strongly positive) for adults that can also be used for children aged from 4-17 years regardless of sex, as a marker of gastrointestinal inflammation.[82] Canani et al used the cut-off of 102.9 μg/g to discriminate patients with active organic/inflammatory disorders from healthy subjects and from patients with functional bowel disorders.[88] The median FC value of our diarrheal samples, 116 (35–

301 interquartile range) μg/g of wet feces, is similar to the median FC value, 110 (0.3-244) μg/g of feces, for acute gastroenteritis in European children.[88] Due to limited publications regarding the clinical applications of FC in acute diarrhea in children[101] and in probiotic studies, it is not possible yet to make a direct comparison with other populations. Though not statistically significant, the reuteri group had the lowest infection-induced change in fecal osmolarity and Vesikari score, which may reflect lower diarrhea severity. On the other hand, *L. reuteri* supplementation might help in mucosal immunity maturation and attenuation of inflammatory responses to dietary and bacterial antigens.[126] There was no attempt to correlate FC concentrations with pathogens as only rotavirus was determined and other pathogen's information is missing. However, this might be interesting for post-hoc exploration.

The acute infection-induced changes in FM were not uni-directional among dietary treatment groups and less pronounced. The similar concentration of FM collected during diarrhea and at normal state (at study end) in our children is consistent with the FM concentration found in Finnish children aged 6-42 months.[127] The casei group showed a significant increase, whereas fecal mucin in the regular calcium group tended to decrease during a diarrheal episode.

Despite responsiveness of fecal calprotectin, osmolarity, and to a less extent mucin, levels of these markers were not significantly modified by dietary treatment. The absence of supplement-induced differences in diarrhea severity markers corresponds with the equal Vesikari scores among groups.

Growth effect

Overall, all treatments in our study resulted in normal growth in weight and height in our children. Our probiotics interventions, but not milk calcium, showed larger effects on growth than other nutritional interventions. A few studies have investigated probiotic effects on children's growth.[128] These studies showed inconsistent effects and differed in probiotic strain and dose, intervention duration and children's age. Our reuteri and casei results on weight gain (effect size 0.29 and 0.23 in 6-month intervention period, respectively) are slightly larger compared to two RCTs investigating growth effects using combined probiotics and other components: *Bifidobacterium lactis* HN019 and prebiotic oligosaccharide versus control milk alone in Indian children of similar age (effect size 0.22 in 12-month period)[129] and *B. longum*, *L. rhamnosus*, prebiotics and polyunsaturated fatty acid versus control milk in Indonesian 12-mo-old toddlers (effect size 0.23 in 12-month period).[130] However, differences in growth outcomes did not affect the prevalence of stunting and underweight in the reuteri and other groups. This may implicate that 6-mo intervention is too short to observe reduction of malnutrition prevalence.[131] It may also be that the actual increment of 0.09 for WAZ and 0.05 for HAZ, albeit statistically significant, were still too small, especially taking the average growth deficit of Asian and African children into account (~−2.0 Z).[132] In contrast to our results, several other studies in infants receiving various probiotic strains, either alone[25] or combined with prebiotics,[48, 133] did not find any effects on growth.

No effect on growth was observed in the RC (without probiotics) compared to the LC group. For growth outcome, the effective dose of calcium in younger children is unknown since

almost no study was performed in this age group. Three reviews on RCTs in mainly Caucasian pre-pubertal children and adolescents indicated a neutral effect (also no adverse effect) of dairy food or calcium consumption on body weight or height, body fat, or lean body mass with doses between 300 mg and 1500 mg calcium per day.[134-136] However, one study in China showed that an increase in milk and thus calcium consumption (560 mg for 2 years) improves bone growth in children aged 10-12 year, especially when calcium intake and vitamin D status are low.[137] This may indicate that increasing the daily calcium intake to support growth might be beneficial in children of lower nutritional status, whereas it might be without significant growth effects in children with higher habitual calcium intakes.

Iron and zinc effect

The present study showed that the probiotics did not affect iron and zinc status. Probiotics are claimed to improve digestibility and nutrient uptake by intestinal cells.[138] A study in infant rhesus monkeys given *L. reuteri* showed an improved haematocrit,[139] but so far no information is available about more relevant iron status markers (e.g. ferritin, sTfR). Levels 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 of iron parameters and prevalence rate changes of anemia, iron-deficiency, and iron-deficiency anemia, or in zinc status. The reduced iron status over 6 months, independent of intervention type, may be due to insufficient iron homeostasis to compensate the fast iron mobilization from storage needed during growth.[140] Chronic blood loss may also contribute to iron deficiencies and anemia in children in developing countries and can be caused by gastrointestinal parasites,[141] *Helicobacter pylori*[142] or allergy to cow's milk protein.[143] No information was available on the parasitic infestation or *Helicobacter pylori* prevalence, neither a deworming program was applied in our study population. No adverse events related to cow's milk protein were present in our study. Also, the considerable prevalence of respiratory (90%) and gastrointestinal infections (27%) in our study children may have negatively affected iron metabolism,[144] known as the anemia of infectious disease.[145] In addition, the polyphenolic compounds of cocoa powder added to our study milk may have inhibited iron absorption.[146, 147] The addition of cocoa powder in our study milk, was intended to mask the discrepancy of the low and regular calcium taste. Besides, chocolate-flavor milk was also the most favorable milk taste in our previous acceptance test (data not shown).

Conflicting results have been reported on the effect of calcium on iron absorption in adults and children, but studies greatly varied in study design, type and duration of supplementation, study population's age and country.[116-118, 148-150] Most studies evaluating iron status were conducted in developed countries involving children and adolescents with adequate iron and calcium intakes and reported no or a small effects on iron status.[116-118] No difference in iron status of preschool children aged 3-5 y was observed after a 5 wk adaptation to a low- (502 mg/day) versus high- (1180 mg) calcium diet.[116] Also, an RCT in pre-pubertal children found that low or moderate amounts of calcium (78 mg/d or 312 mg/d) for 2 weeks did not interfere with iron absorption.[117] A long-term, 1-year-intervention study in adolescent girls showed that daily calcium supplementation of 500 mg did not

compromise iron status.[118] With regard to zinc, there is no reported evidence for a calcium effect on zinc absorption in children.

Rationale of using calcium to influence diarrhea

The rationale for using calcium in children is based on a proof-of-principle study with adults orally challenged with live but attenuated enterotoxigenic *Escherichia coli*. Dietary calcium strongly reduced infection-induced diarrhea in that study.[4] Animal studies show protective effects against Salmonella as well,[151, 152] but human verification for that is still lacking. Rotavirus is responsible for 60% of hospitalized and 41% of outpatient clinics diarrheal cases in Indonesian children.[153] Important bacterial pathogens among children in developing countries are *E. coli* (10%–20%), *Salmonella* (<5%), *Shigella* (5%-10%), *Campylobacter* and *Vibrio cholera (*exact %'s unknown).[154] The absence of a beneficial effect of calcium in our trial may indicate a difference in efficacy between children and adults and/or that protective effects are pathogen-dependent.

How could probiotics influence diarrhea?

The application of probiotics to prevent or treat acute diarrhea is based on three possible mechanisms[155] (**Figure 6.2**):
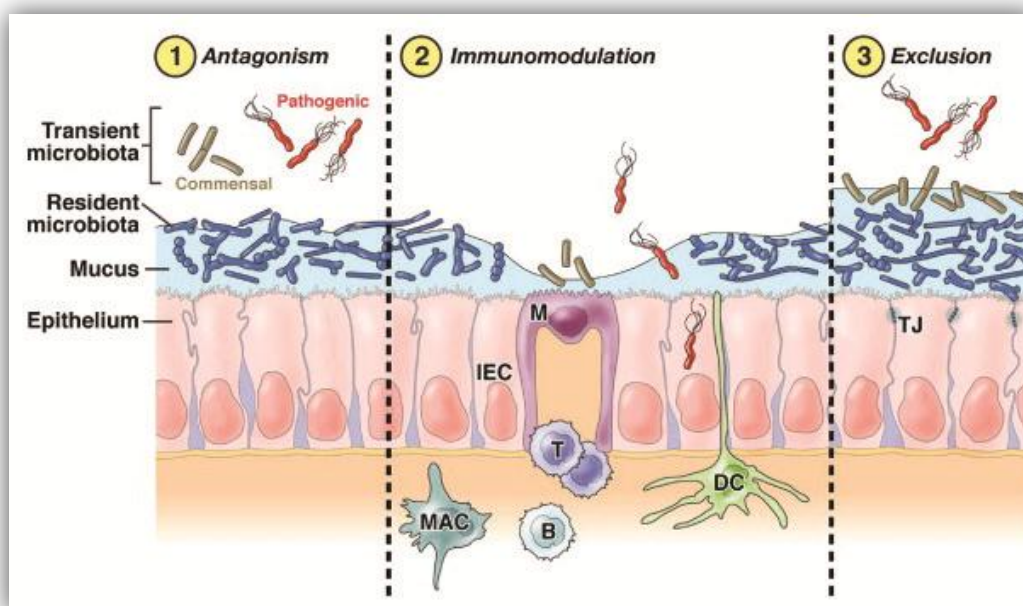
Figure 6.2 Three possible probiotic mechanisms: (1) Direct antagonism; (2) Immunonodulation; (3) Exclusion. B, B cell; DC, dendritic cell; IEC, intestinal epithelial cell; M, M cell; MAC, macrophage; T, T cell; TJ, tight junction.

Sources: Adapted from Preidis et al (2011)[155]

(1) **_Direct antagonism_**. Probiotics are assumed to antagonize intestinal pathogens. Probiotics kill or inhibit the pathogen to limit infection, or they down-regulate the expression of virulence factors, such as adhesins or toxins, required for pathogenesis.[155]

Possible mechanisms include the synthesis of antimicrobial substances, competitive inhibition of pathogen adhesion, and competition with pathogens for growth substrates, modification of toxin and non-toxin receptors involved in bacterial recognition.[156] *L. reuteri* was known to produce a potent antibacterial compound, reuterin, that is capable of inhibiting a wide spectrum of microorganisms.[157]

(2) ***Immunomodulation***. The mechanisms by which probiotic bacteria affect the immune system are unknown yet.[158,159] Probiotics can interact with the immune system to enhance the functionality of innate and/or adaptive immunity, or to limit the ability of the pathogen to initiate or facilitate an immune response.[155] The underlying protective mechanisms of probiotics *L. reuteri* and *L. casei* against gastrointestinal pathogens in children are unknown.[157, 159] In an animal study, it was reported that *L. reuteri* DSM 17938 did not inhibit Liposacharide (LPS)-induced interleukin (IL)-8 production in cultured intestinal cells, in contrast with other *L. reuteri* species.[157] Also in an animal model, Galdeano et al found that the main immune cells activated after oral *L. casei* administration were those of the innate immune response, with an increase in the specific markers of these cells (CD-206 and TLR-2), with no modification in the number of T cells.[159] They also demonstrated that *L. casei* CRL 431 interacts with the epithelial cells of the small intestine and that their fragments can internalize and activate the intestinal epithelial cells.[160]

(3) ***Exclusion***. Probiotic may make the gastrointestinal environment become less favorable for pathogens.[155] Probiotics can alter the microenvironment to prevent pathogens from gaining access to appropriate receptors, to limit pathogen attachment, entry, or translocation, or to improve barrier function.[155] These mechanisms include altering the resident microbiota, decreasing luminal pH, improving epithelial barrier function, interfering with pathogen binding by down-regulating specific host receptors, and stimulating production of defense-associated factors, including mucins and defensins. A beneficial microbe may use a combination of these mechanisms and may employ different actions against different pathogens.

6.2.2. Cross sectional study

Study population

Age-specific risk differences in many studies show that children have a higher overall risk of diarrhea than adults.[161] Children aged less than 5 years are suffering most from diarrhea. We focused on children aged 12-59 months and excluded infants (0-11 months) in our study population because many studies reported about health condition, nutritional status, food intake and feeding practices during infancy that are different from older age. We did not specifically focus on children <2 years, although diarrhea prevalence tends to be higher in this age group and decreases at older age.

Study design

The association of food-hygiene practice and diarrhea was examined based on a cross-sectional study (**chapter 5**), which may have weaknesses related to study design that could affect the validity of the findings. A main limitation of this design is that exposure (food-hygiene practice) and outcomes (diarrhea prevalence) were assessed at the same moment in time which makes it difficult to assess the temporality of the association. It is possible that the presence of diarrhea in children may increase mothers' work load or mothers' attention to children that changed mothers' practices (reverse causation).

The municipality of East Jakarta was purposively selected on the basis of a number of criteria: high population density, low socioeconomic status, and high prevalence of diarrhea and underweight in under-five children, based on routine data from the primary health care system and local government. These criteria were also applied for Jatinegara sub district and four urban administrative villages that were involved in this study. In every village we selected 2–3 hamlets that had a large number of poor people. The list of children of all villages involved was compiled and the children were randomly selected to the study. The number of children in each hamlet in every village was distributed equally but according to the proportion of total children in each hamlet. This approach may introduce a bias in the selection of area, but not in the selection of children. As mentioned in Introduction (**chapter 1**), children were from areas that were selected because of their high population density, low socioeconomic status, and high prevalence of diarrhea and underweight in under-five children, and hence it may not be representative of the general Indonesian children. Random selection of children in this study avoided bias and may provide valid results.

Assessment of exposure (food-hygiene practices)

Information bias may have occurred because mothers may report social desirable practices which they do not perform.[162,163] Moreover, mothers may forget some of the practices. Research has shown that responses tend to be biased towards over reporting of "good" practices.[164]

As indicated previously in **chapter 5**, we included a complete set of food-hygiene practices identified in our community, which consisted of 36 variables. This approach clearly gives advantages because important and specific local practices are not missed. A previous study on food-hygiene missed some important practices such as food storage, thorough cooking and adequate food holding temperature[165] as endorsed by World Health Organization (WHO).[166] All individual variables were assigned equal importance and presented as separate variables reflecting individual dimensions. Breastfeeding between 4 and 9 months of age is known to have a protective effect on children's health and growth. However, for children in the 9-18 months group or older it is not clear whether this is the case. Since scientific evidence on this issue is mixed,[164] we scored breastfeeding equal as other practices.

These 36 single variables are also presented as a summary score reflecting a food-hygiene practice index. This index was classified into poor and better food-hygiene practice based on

the median score of the population. This scoring system for food-hygiene practices can be considered as a preliminary step in the development of a methodology to measure and quantify the different types of food-hygiene practices in a community setting. Many studies with much larger sample sizes classified the total score into tertiles or quartiles. However, in our study the total score was dichotomized: poor and better food-hygiene practice based on the median score of the population because the tertiles classification leads to very large confidence intervals, due to small numbers, which would complicate the interpretation.

Assessment of outcome (diarrhea prevalence)

To avoid information bias in diarrhea prevalence, we choose recalling of diarrhea during a 7-day period. Recall methods may be the reason why we could not observe significant associations between overall and some of the single food-hygiene practices.

Seasonal variation in the incidence of acute diarrhea has previously been identified from surveillance data in many countries.[161] Yet, study results on the effect of seasonal variation on diarrhea prevalence are contradictory. Our study was conducted in a rainy season (November – April), which was assumed to be the peak of diarrhea prevalence as described in a previous study within a similar community.[167] Performing the study in rainy season may lead to an overestimation of diarrhea prevalence because there is a strong link between foodborne and waterborne diarrheal illnesses and weather- and climate-related events. The incidence of diarrhea generally rises in the rainy season in developing countries, partly because of direct effects of temperature and rainfall on the growth and spread of pathogens.[161] However, some recent studies in developing countries observed high diarrhea prevalence in dry seasons.[168-172] The prevalence of diarrhea in our study (10-20%) corresponds with the known prevalence, thus an effect of season was not clearly seen. This is supported by the findings that although worldwide the seasonality of rotavirus may vary broadly, the lack of seasonal variations of rotavirus is more common in tropical developing countries than in temperate climates (zones in some western country that have warm/hot temperature but not as hot as the subtropical/tropical countries) in developed countries.[173]

Confounding

The overall incidence of diarrhea in developing countries has remained high due to multiple determinants.[174] Besides lack of safe drinking-water and inadequate sanitation and poor hygiene,[175,176] many studies in developing countries identified other important factors that were significantly associated with diarrhea in children such as younger age,[161,165,177,178] malnutrition,[179] low socioeconomic status and education of mothers,[180-182] low maternal age,[183] overcrowding (family size),[184,185] lack of completeness of immunization,[186]and number of under-five children living under the same roof.[187] These factors may become potential confounding variables. To reduce the influence of confounding, we adjusted for several variables i.e. child's age, malnutrition, completeness of immunization, family size and number of under-five children living under the same roof.

Interpretation and perspective: cross sectional Study

Despite the described limitations i.e., reverse causation, selection and information bias, and confounding the outcome-specific findings of diarrhea prevalence were consistent with many studies in developing countries (Vietnam,[165] Thailand[177] and Bangladesh[178]) and in similar settings confirming that children aged <2 years were more vulnerable to suffer from diarrhea. This finding is also in line with a previous review that diarrheal diseases are extremely high during the weaning period (6-24 months).[188]

The association between food-hygiene practices and diarrhea prevalence in children has been examined in several epidemiologic studies in developing countries e.g. Vietnam,[165] Bangladesh,[178, 189] Nigeria,[190] Gambia,[191] Nicaragua,[192] Brazil,[193] and Congo.[194] The practices were evaluated either as combined or individual practices such as hand washing, food preparation and storage, clean sewage, and safe water source and storage.[191] These studies generally suggested a direct association between food-hygiene practice and diarrhea prevalence, but findings are not conclusive.[195] Our study, using more complete set of local practices than other studies, indicates that the poor food-hygiene practice score was not associated with the prevalence of diarrhea among children under five, but was significantly associated with more diarrhea among children aged <2 years (**chapter 2**). Our present study typically: (1) provides a comprehensive and more complete set of local practices; (2) presents not only single variables, but also a summary score of practices; and (3) focus its findings on diarrhea of children aged<2 years.

We speculate that the increased risk of having diarrhea in children aged <2 years whose mother had poor food-hygiene practices in our study may be explained by the fact that weaning foods prepared under unhygienic conditions are frequently contaminated with pathogens and are an important risk factor in the transmission of diarrhea (**chapter 2**). This observation is also reported in a peri-urban district of Guinea-Bissau.[196] However, our study was not able to demonstrate an association between the contamination of weaning foods and diarrhea due to a lack of assessment of specific enteropathogens. Although food-borne infection is the main route of transmission of gastrointestinal infections in developed countries, their contribution to the burden of diarrhea in low-income settings is still unclear.[195] Contaminated weaning food has been suggested as a major contributor to diarrhea in low-income settings as up to 70% of diarrhea episodes are actually caused by water and food contaminated with pathogens,[188] although observational studies gave inconclusive results.[197] A study in Gambia failed to document an association between water or weaning food contamination and higher rates of diarrheal morbidity.[191]Two more recent studies have found an increased risk of diarrhea associated with the consumption of maize-based weaning foods.[191, 194] However, in one of these studies, this association was only significant in children living in rural communities.[194] Therefore, association between contaminated weaning foods and diarrheal diseases in young children living in urban setting of developing countries remains lacking.[197]

The best studied hygiene practice in developing countries is that of hand washing. Evidence from all four types of sources is consistent, with RCTs on hand washing showing reductions in diarrhea of around 30%, and of 43–47% if soap is used.[195] Our study found that mother's and child's hand washing, seemed to be protective against diarrhea, however, our study failed to demonstrate statistically significant associations. We must remark that the lack of

association between hand washing and diarrhea in our study may be due to application of single instead of repeated observation methods to assess hand washing practices.

Our study confirmed that children who lived in a house with clean sewage had significantly lower risk of having diarrhea than children who did not have one. A previous study conducted in an urban poor setting in Indonesia also reported an increased risk of having diarrhea in children with unavailability of sewage and/or place to dispose the child's stools.[198] Contamination of drinking water by sewage through pump failure or blockage of a sewage system has been previously described.[199] Overwhelmed sewage systems might be an important contributor to the increased prevalence of acute gastrointestinal illness during the rainy season in Cuba[161] and also in many other studies conducted in a community setting. In developing countries, both *E. coli* and rotavirus were the common pathogens associated with waterborne disease especially for children who live in poor environmental conditions. Also, outbreaks of viral gastroenteritis resulting from sewage contamination of water supplies have been well described.[200,201] Unfortunately, information on diarrhea-causing pathogens could not be obtained in our study.

6.3. Implication for public health

In this section, we will focus on the consequences of our research for public health on the basis of the four major research questions that were addressed in this thesis.

Probiotics as an alternative prevention strategy

Public health perspective

According to WHO South-East Asia, many countries in the region are facing difficulties in achieving the Millennium Development Goal 4 which calls for the reduction of child mortality by two third by the year 2015 due to high morbidity and mortality from acute diarrhea and ARTIs in the region.[3] Simultaneously, most of developing countries including Indonesia are facing a public health threat due to the overuse and misuse of antibiotics[202] in the treatment of diarrhea which yields progressive increase in antibiotic resistance of enteric pathogens in the communities.[203] Therefore, evidence on probiotics efficacy for treating diarrhea in children,[5] administered in addition to rehydration therapy, has become an alternative solution for medical purposes. Based on our present findings, probiotics may serve as an alternative solution beyond treatment. As presented earlier in this chapter, there is suggestive evidence for a beneficial effect of *L. reuteri* for Indonesian children in reducing incidence (24-32%) especially in lower nutritional status ones, shortening total duration of diarrhea (1.35 days) and modestly improving growth over 6 months period, whereas. *L. casei,* although giving less benefit, modestly improved weight velocity. These beneficial microbes offer potential alternatives for the prevention of diarrhea in vulnerable children and improvement of child growth in developing countries.

In practice, this proven probiotics efficacy may provide additional information to health professionals in choosing the right strain for preventing the progression of acute and persistent diarrhea associated malnutrition in the future. Reduction of illness will

significantly impact on family dynamics and economic situation because parents could work regularly[204] and do not spend money for extra health care costs. Children may grow normally and will become a productive generation for the nation. Therefore, the role of probiotics should not be limited to simply addressing a narrow range of unsubstantiated individual effects.[156] There should be room for probiotics to be seen as an alternative strategy for diarrhea prevention. However, to recommend policy makers to adopt probiotics in a diarrhea prevention program in a community setting in developing countries, it is important to explore how strong its disease reduction evidence would be as compared to other existing preventive measures. Moreover the acceptability in a community, affordability, cost-effectiveness and the risk of adverse effects for long term use need to be faced.[7]

Looking at the reduction of diarrhea incidence and duration, *L. reuteri* in our trial is better than other probiotic strains. However, it seems too early to recommend probiotics (e.g. *L. reuteri*) for routine use or for follow-up in public health programs to prevent diarrhea in children in developing countries. It is not only because there is limited data on probiotic studies in a community setting, but also the current effect size of probiotics for the reduction of diarrhea incidence is rather similar with nutritional intervention (non-probiotic based) and lower than the non-nutritional intervention (hand washing with soap, improved water quality and excreta disposal).[110] The child growth outcome in our study using *L. reuteri* showed a promising effect, but probably it is not massive enough to really overcome the high stunting and underweight problem in children in the developing countries. In addition, no data is available for the assessment of mortality reduction due to diarrhea by probiotics. Until now, no single intervention is sufficient to eliminate the persistent burden of diarrheal diseases.[155] Therefore, probiotics intervention should not be introduced alone into prevention strategy because it may be difficult to contribute to public health goals, and will not simply replace the existing health strategy. Probiotic intervention should be part of an integral system of existing prevention strategies. Lesson learned from the past showed there has been temporary or delayed improvements of diarrhea morbidity using various interventions especially those that required behavior changes.[205] Multiple interventions could work synergistically, such as the combination of existing improved water and sanitation, vaccines, micro- and macronutrient provision, and probiotics for this specific age group. These combined efforts might be also able to address broader and long-term benefits to growth and developmental processes of the children.[155] Multiple approaches will be a good solution if each single approach has been shown efficacious and effective. It is possible that part of single approaches is not proven efficacious and therefore, the combination of interventions should be evaluated for its efficacy and effectiveness. Effective promotion strategy should be indentified to encourage individuals and the community to comply towards several interventions (including probiotic) and to sustain their positive behavior change.

Clearly substantial savings and reduction of health care costs have been predicted by the routine use of probiotics in diarrhea treatment.[206] So far, there is no published data to support that probiotics in children 1-6 years provide a better cost-effective program as compared to other existing health intervention programs. In terms of affordability, policy makers in developing countries like Indonesia should carefully evaluate the economic implications before applying probiotics, e.g. *L. reuteri* in diarrhea prevention programs.

Policy makers should also encourage probiotics manufacturers to voluntary produce a mass probiotic food product affordable to most vulnerable children in Indonesia who mostly live in poverty. An existing affordable probiotic drink product (e.g. using *L. casei* shirota) is widely consumed by Indonesian children. This product was not specifically studied in Indonesian children but was shown effective in reducing diarrhea in India.[54] Other probiotic products, in the form of yogurt are introduced in the Indonesian market, but information on the scientific benefit for diarrhea reduction are not available. The formulation of affordable products has to be accompanied with quality control, good manufacturing practices, sufficient dose and proper storage of probiotic strain from production line until consumer's home. The feasibility to develop such products in the Indonesian situation with its tropical climate should be evaluated. In addition, manufacturers are obliged to serve better education on probiotics and avoid misleading information or too promising advertisement.

Fermented products are widely consumed by people in developing countries like Indonesia. However, the acceptability towards probiotic products (and its food carrier) will be another critical challenge and may face cultural barriers. *L. reuteri* is a non-indigenous/overseas type of microbe that has to be proven safe for long-term use in Indonesian children. If *L. reuteri* will be introduced in Indonesian children using probiotic dairy products, specific acceptance evaluation and efforts should be made to convince the community to consume this product. Not only because milk is considered as an exotic and expensive product, but also because of potential lactose intolerance in Asian children.[207] On the other hand, dairy is well accepted because it has been believed as "nutritious product" (e.g. due to calcium). Innovative technology using straw coating for the delivery of *L. reuteri* like in our study in combination with Tetra Pak milk was proven useful for Indonesian children. The acceptability of these products is excellent as shown in our acceptability and intervention study. Many factors influenced the success story of the probiotic and calcium milk acceptability in our study such as low lactose milk with good sensory aspects for children, intensive communication, creativity program, simple packaging, community involvement, solid team workers, role of health volunteers (kaders), local physicians and leaders. Therefore, these types of strategy are essential for the success of probiotic acceptance in the future. Also, appropriate probiotic storage systems have to be feasible and well instructed to the community. The introduction of milk in the community may raise ethical and political issues because the enhancement of this commodity will stimulate a change in dietary habits and resistance towards overseas/imported products. The national production of probiotic product may be an option in the near future.

The reported risk of adverse effects of probiotics use is very minimal. For public health intervention, the use of probiotics should be targeted to healthy and non-severely malnourished children with specific dose and duration as it was used in our intervention. Caution has to be made for the immuno-compromised children and/or with severe barrier dysfunction such as critically ill patients because these populations might be at higher risk for adverse effects such as the development of septic conditions due to their reduced capability for microbial clearance.[208]

We failed to show beneficial effects of calcium in milk in this study. Calcium intake was increased to meet the specific recommended daily allowance for calcium. This calcium

increase did not negatively influence iron or zinc status in children when not consumed together with meals. Benefits beyond diarrhea prevention might exist but were not assessed. Although we did not find specific adverse effects of calcium on iron absorption, we did find an iron status reduction in all intervention groups. It is unclear whether this is due to consumption of the supplied milk products or to other confounding factors as described earlier in this chapter (e.g. increased iron requirement during growth,[140] chronic blood loss due to parasites[141] or *Helicobacter pylori,*[142] allergy to cow's milk protein,[143] high prevalence of infections,[144] presence of polyphenolic compounds of cocoa powder[146,147] or increasing food crisis). Addition of a fifth trial arm receiving no dietary intervention at all could have discriminated whether it was due to milk consumption or to other causes. For now, it is important for the policy maker that milk interventions need specific attention as assurance of an adequate iron and zinc intake in children in the growing phase is as important as a sufficient calcium intake.

Regulatory standpoint on probiotic claims

Probiotic products have received significant attention from the public, industries and scientific community due to their perceived potential health benefits.[156, 209-211] Indonesia, and other developing countries, are currently bombarded with strong marketing and advertisement of probiotic bacteria in food products. These products are mainly probiotic dairy for young children such as fermented milks, baby-food and milk powder, probiotic drinks and milk powder for children. However, this market expansion is not supported by a similar increase in scientific evidence and is insufficiently regulated in most of the developing countries. At present, regulatory issues on probiotics are unclear and subject to misinterpretation.[212] In Europe, the European Food Safety Authority (EFSA) and the Panel on Dietetic Products, Nutrition, and Allergies (NDA) have rejected the majority of claims of the benefits of probiotic products submitted to EFSA ($\approx$260 out of 300 claims)[213,214] due to insufficiency in the characterization of microorganisms. This situation has raised concern from the International Scientific Association for Probiotics and Prebiotics (ISAPP) because in their opinion that claims supported by solid scientific evidence are also being rejected.[215] The Indonesian National Drug and Food Control Agency (NADFC) regulates probiotics through a pre-market evaluation of Functional Food products. However, most probiotic products registered or rejected by this agency give insufficient efficacy and safety information, and in some cases, studies were not performed with the intended target population. Moreover, based on a report of the NADFC, post-market evaluation showed that unsubstantiated and non-approved claims were used. Good Manufacturing Practices producers were not always maintained and handling practices during storage and retailing were not in place.[216] This situation raises concerns not only from regulatory agency and industries in providing quality and substantiated probiotic products to protect consumers, but also from the scientific community in performing high quality probiotic studies. From a regulatory perspective, it is important that agencies improve their existing regulation and as soon as possible provide specific and clear probiotic regulation guidelines and standards that should be adhered by industries. On the other hand, the scientific community in Indonesia and developing countries should carefully perform probiotic studies in the right target population, with appropriate strain selection, a sound study design, with validated outcomes and results published in peer reviewed journals.

Innovative technology

Efforts in developing innovative technology on probiotics should be advocated for Indonesia and many other developing countries. As one of the mega biodiversity countries in the world, Indonesia is also unique with typical multiple-ethnicities that maybe potentially rich in genetic resources, traditional fermented foods, and abundance of microbiota variations. This give a great opportunity to develop indigenous probiotic products through innovation[216] without excluding or becoming exclusive to the potential probiotic products from other countries. The potential local probiotic strains are probably more effective in fighting against local pathogens adhesion. The beneficial effect of some Indonesian traditional fermented products such as tempeh (fermented soy-bean from Java)[217] and dadih (yogurt-like product from West Sumatera)[218] on reduction of diarrhea in Indonesian children has been explored, and their potential isolated microbes need to be further researched. Public-private collaboration between Indonesian academia, industries, and government should strengthen the development of probiotics in Indonesia. Furthermore, international collaboration should be encouraged among developed and developing countries in applying innovative technology in probiotics with commitment mutual benefit for both continents.

Food-hygiene practices

Proper food-hygiene practice of mothers is important for the prevention of diarrhea in children <2 years (especially non-breastfed infants and young children, or those who receiving breast-milk in combination with complementary feeding), in line with the results of present and previous studies on food-hygiene practice and diarrhea.

A program on hand washing with soap has been introduced as a simple and cost effective means to reduce the incidence of food-borne disease. However, more comprehensive food safety education to mothers of young children with special emphasis on good food-hygiene practices and clean sewage seems to be more effective than only one single food safety message to prevent diarrhea among children in these areas. For a sustained program, the implementation of food safety education should involve community participation and integrated stakeholders such as local government, academia, health authorities, non-governmental organization, and industries. Based on our study, refinement of the sewage system is a promising measure for the prevention of diarrhea. However, this program will be difficult to be implemented in the Jakarta urban slum area especially in flooding areas. The urban slum areas of Jakarta are not only lacking proper sewages, as Jakarta's municipality sewage system serves only 2% of the population,[219] but are often overcrowded settlements. Both are known as risk factors of diarrhea among children. Infrastructure refinement plans to improve sewage system and slum dweller families relocation (especially those who live in flooding area) should be advocated to local government. A combination of an existing diarrhea prevention program and allocating resources for a food safety program and infrastructure refinement for sewage system might lower the prevalence of acute diarrhea disease in these populations.[161]

Promotion of hygiene might be the single most cost-effective way of reducing the global burden of infectious disease. Governments and funding agencies increasingly accept that

hygiene promotion should be an integral part of health investments across the wider community, not just in health-care settings. Policy makers are also realising that the health benefits of increased investment in water and sanitation infrastructure refinement are largely delivered through improvements in personal and domestic hygiene. Original approaches using new insights are modernising the hygiene sector, making it more attractive to investors. Improved water supplies and sanitation facilities make it easier to practice food-hygiene, keeping children from infection. It might be almost impossible in an area near the river site.[195] But even without improved facilities, better food-hygiene can still make a huge difference to health and may contribute to the achievement of Millennium Development Goals (MDGs) and beyond.

6.4. Recommendations for future research

This section gives suggestions for future research. **Figure 6.3** (A and B) are used as a framework for some potential interventions and propose new research questions. **Figure 6.3** (A) shows the potential of various interventions that may inhibit progression to a further development of the infection-malnutrition cycle, and minimize both acute and chronic morbidities: including probiotics, micronutrients (calcium, iron and zinc) and food hygiene practices that were highlighted in our study (**orange boxes**). **Figure 6.3** (B) presents a recommendation for future research: employing a spectrum of disease outcome measures for new research questions. Although we studied three elements of possible interventions, in this thesis we specifically focus on the proposed research questions to the probiotic intervention (**orange box)**.

6.4.1. Multicenter study on probiotics using *L. reuteri* (and/or other strains): longer duration, larger sample size, higher prevalence of severe diarrhea in community-based trial in South East Asia Region.

Reduction of diarrhea incidence

Further investigations are needed to confirm whether our documented successful prevention strategy using probiotics (*L. reuteri*) can be confirmed and replicated by studies in similar settings in South East Asia Region. A multicenter community trial should be initiated for further studyparticularly in the apparently healthy population, with larger sample size, longer duration, and in a population with higher prevalence of (severe) diarrhea. Population with more severe diarrhea is indeed important due to some reasons e.g. high placebo response and the possible "floor effect"[221] which means the severity of symptoms is too low to measure any improvement. This doesn't mean that *L. reuteri* or *L. casei* cannot work against incidence or severity of diarrhea in a community setting, but the relatively low severity cases and the room for improvement is low compared to the diarrhea patient in a hospital setting, where symptom severity is much higher. We like to stress that definitions of all reported diarrhea, besides the strict one of WHO, need to be applied in follow up trials to investigate dietary intervention effects in community settings. It is also encouraged to include comparative strains and to evaluate the most effective probiotic organisms and optimal dosage. Financial and logistic arrangements, community acceptance, laboratory infrastructure and capacity as well as human resources involved for proper study conduct
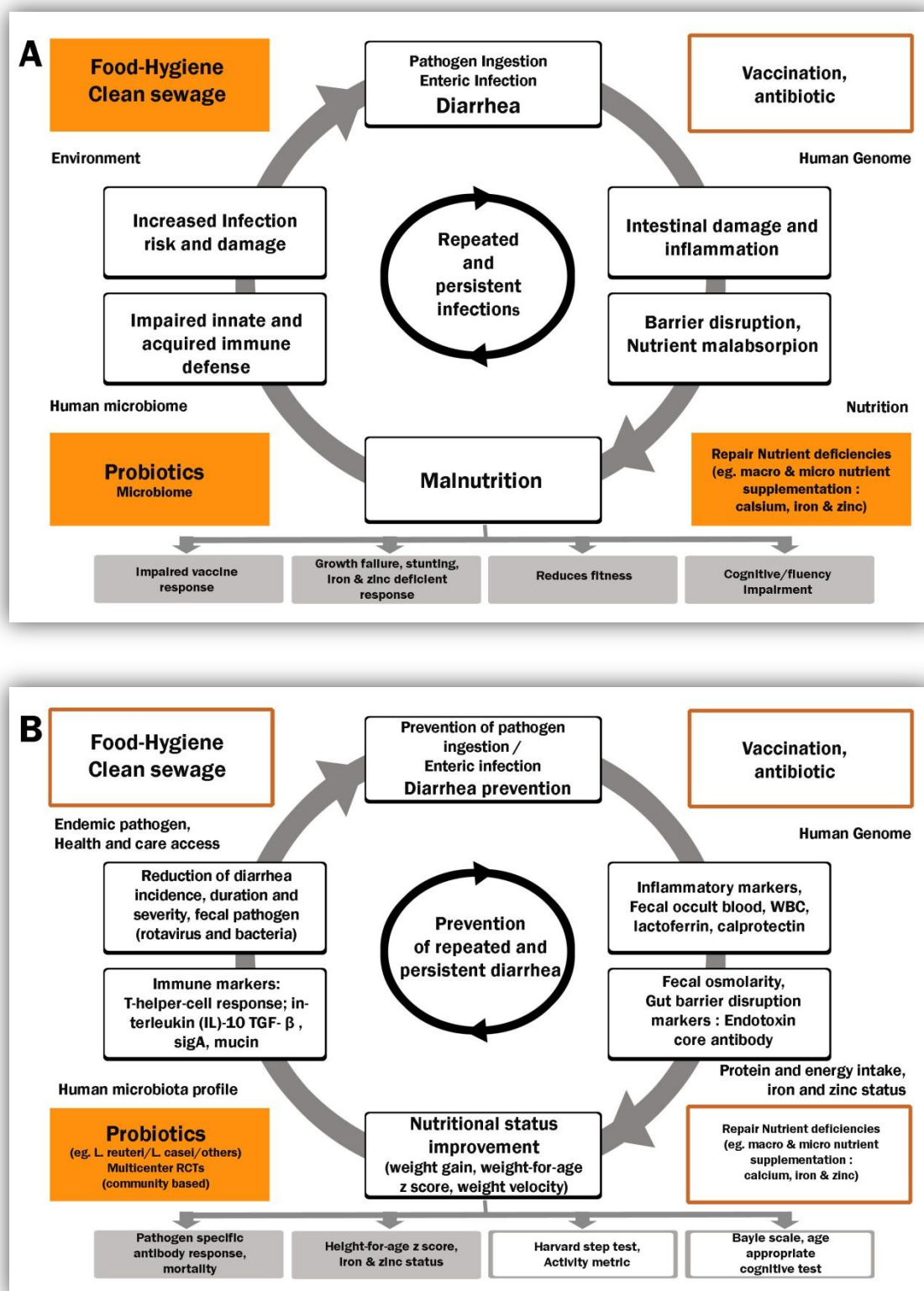
Figure 6.3 The vicious cycle of diarrhea and undernutrition in susceptible children. (**A**) Various interventions: probiotics, micronutrients (calcium, iron and zinc) and food hygiene practices that were highlighted in our study (**orange boxes**); (**B**) Recommendation for future study: employing a spectrum of disease outcome measures. Adapted from Guerrant et al, Nutrition Reviews copyright 2008;[220] and Preidis et al, Gastroenterology, copyright 2011[155]

be crucial factors to get high-quality data and this can be improved in developing countries. Such studies are important to further advice policy makers and regulators on applying probiotics into diarrheal prevention programs in developing countries.

Reduction of duration and severity of diarrhea

In our present study, we were not able to conclude: (a) whether the subjective modified Vesicari scale is an appropriate diarrhea severity measure in a community setting in contrast to single clinical diarrhea severity parameters such as fever, vomiting, dehydration and prolonged duration; or (b) whether fecal calprotectin, osmolarity and mucin are truly quantitative indicators of diarrhea severity. Further study to confirm whether *L. reuteri, L. casei* and calcium, can shorten acute diarrheal episodes dependent on the intestinal pathogen involved, needs to come from larger (>500 subjects) controlled intervention studies. Confirmation needs to come from future infection studies in subjects with a wider range of diarrhea severity. It is clinically important to find means to reduce diarrhea severity in addition to reduction of diarrhea incidence in the community because diarrhea severity might affect prolongation or chronic diarrhea and other functional disorders (eg. immune status, gut integrity, malabsorption, severe malnutrition) leading to diarrhea-related mortality and malnutrition. Identification of etiologic pathogens (rotavirus and other common bacteria) may also be useful to be included in planning further study.

Growth, iron and zinc status effect

Following the above mentioned outcomes, it is always useful to include growth and micronutrient status assessment in future studies in developing countries. Attention has to be made on longer study duration (6-24 months). Meta-analysis or systematic reviews of probiotic effects (versus other nutritional interventions) needs to be specifically carried out to evaluate what the best interventions are to reduce chronic stunting and underweight in children in developing countries.

6.4.2. Mechanistic study

Diarrhea severity, immuno-modulatory and intestinal barrier status markers

Further studies are needed to investigate the effect of probiotics on diarrhea severity markers and other functional outcomes, such as immune and gut integrity parameters. Besides three fecal markers presented in our study, it is scientifically crucial to further identify markers for outcomes of diarrhea reduction in a community settings. The usefulness of other rapid stool tests such as fecal occult blood, fecal lactoferrin, fecal leukocytes for the evaluation of diarrhea severity in community setting requires attention. This will prevent physicians to rely on expensive fecal cultures to determine severity and etiology of diarrhea, but to make use of objective and inexpensive markers. Besides feces and blood that was collected in our study, future research should also explore the use of other non-invasively obtainable biological samples such as urine and saliva. For fecal calprotectin it needs to be explored whether this marker truly aids in assessment of diarrhea severity or pathogen identification or only reflects intestinal immune response.[81] The underlying mechanism of the protective effect of L reuteri was unclear. The potential of this strain to enhance the

production of a balanced T-helper-cell response, stimulate production of interleukin (IL)-10, transforming growth factor (TGF)-β and stimulate the synthesis and secretion of immunoglobulin A (sIgA) are scientifically interesting to explore.[222]

In our study, we included measurement of serum antibodies to the conserved core of endotoxin (EndoCab) as a newly proposed marker of intestinal barrier function. Circulating immunoglobulin levels can go up following translocation of gut-derived endotoxins. Further validation of this marker and the assay, and exploration of other methods for assessment of intestinal barrier integrity status and function loss, are needed to obtain more advanced insight on the role of probiotics on the gut wall integrity.

Motivation: To improve understanding on main mechanism of probiotic actions in the small and large intestine (bacterial-epithelial "cross talk") in the prevention of infectious *diarrhea*. Disturbed intestinal barrier integrity and function is linked to a range of diseases.[223] The role of probiotics or calcium in the improvement of the gut barrier integrity and function may therefore be imperative for clinical practice and important to explore. It will result in better understanding of disease etiology and pathophysiology.

Molecular mechanism

As mentioned above, Indonesia is one of the mega biodiversity countries that are rich in genetic resources. It is crucial to identify specific microbial genes and their interaction that mediate the probiotic beneficial functions in this country.[155] The International human microbiome consortium studies and human microbiome project published a catalogs reference on human genomes, but these genomes are mostly from human microbiome of subjects in developed countries.[155, 224] We are not sure about country-specific microbial genes or microbiota or probiotics, but diarrhea, for instance, disturbs commensal microbiota, and the pathogens responsible to this are geographical dependent. Microbioma profile may depend on several factors such as environmental condition (hygiene-hypothesis), lifestyle, food intake, cultures and ethnicity,[225-227] which are nearly country specific. Looking at the biodiversity in Indonesia, it is worth to put this potential field into the future research agenda and a wide range of studies can be done in this field. It is also important to identify whether there is a great difference of microbial composition in children with different nutritional status and from different ethnicities in Indonesia. This study will help to have separate unique data of microbiome that could be different from developed countries. Further, isolation of new probiotics can be done by selecting the most potential microbes from these various ethnicities. Studies on the link between gut microbiota and health are very popular nowadays. This may have gone way too far and draws attention away from more obvious and direct nutritional intervention strategies to achieve health. However, we may consider an innovative research on a potential role of gut microbiota to the most emerging disease in the country.

6.4.3. Innovative technology and efficacy study for indigenous probiotic strain development

It is necessary for the government of Indonesia and other developing countries to seriously put their investment on innovative technology studies and to establish efficacy of existing or new indigenous probiotic strains through public-private cooperation. This investment should be planned on a long term basis. Microbial culture collection should be initiated at national as well as regional level. The majority of probiotic strains are currently owned by profit companies in Western countries who have the right to control who has access to their strains and what studies can be performed using their products.[228] The accumulating scientific evidence on benefits to reduce diarrhea in developing countries (such as in our study) will have two sides of a coin. On the one hand, it gives a bright future support for the prevention of diarrhea in children. But on the other hand, it will raise a problematic challenge because these strains are not available in the developing world. It might cause dependence on the Western profit companies. The role of probiotics in public health (specifically diarrhea) will stimulate the benefit for business partners. It can also cause a publication bias, because most data are derived from Western countries, and researchers from the developing world might not have much freedom to publish their findings or having the possibility of patent ownership when using overseas strains.[228] FAO/WHO has to take a role in providing more guidelines to enhance the development of local probiotic strains for non-profit usage when probiotics will be applied in large scale programs, although the private ownership of probiotic strain may not be prevented.

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Summary

Acute diarrhea and acute respiratory tract infections (ARTIs) continue to lead the infectious cause of morbidity and mortality among children <5 years of age in developing countries, including Indonesia. The burden of this problem calls for effective public health intervention to support the existing preventive strategies (including provision of safe water and sanitation, exclusive breastfeeding, hand washing, vitamin A and zinc supplementation, and vaccinations) that are available, but not always effective to reduce the burden of these diseases. Efforts to prevent diarrheal disease by probiotics and milk calcium supplementation as alternative strategy are promising. Probiotics are often supplemented to dairy foods and both probiotics and calcium in milk may strengthen intestinal infection resistance.

Several meta-analyses, systematic reviews and trials on probiotics and diarrhea were published. These studies concluded that probiotics may prevent or reduce duration of diarrhea in children. However, the reported beneficial effects are probiotic strain and dose dependent and evidence was obtained mainly in hospital-based settings in developed countries aiming at treatment of diarrhea. Studies on the prevention of diarrhea in apparently healthy children living in the community were mainly in day-care center based studies in Western countries. Currently, only three randomized trials have focused on the role of probiotics in the prevention of acute diarrhea in a community setting in developing countries. Moreover, several randomized trials have explored benefits of probiotics in the prevention of ARTIs in children, but have yielded contradictory conclusions. Calcium has previously shown to reduce infection-induced diarrhea in adults, but the efficacy in children with a low habitual calcium intake and frequent episodes of intestinal infections is currently unknown. So far, recommendations to supplement with calcium or probiotics in community settings in developing countries are not justified.

Currently, it is unclear, whether probiotics and calcium supplementation do affect incidence and duration of diarrhea and ARTIs, severity of diarrhea as well as growth, iron and zinc status in Indonesian children. For this purpose, we conducted an intervention study and answers to this question will provide recommendations for nutritional intervention strategies to prevent diarrhea and ARTIs. Moreover, it may show medical professionals, public health and food policy makers how to improve growth, iron and zinc status. Although the determinants of diarrhea in children are well described, information on whether poor food-hygiene practices are associated with diarrhea in children in low socioeconomic urban areas of East Jakarta is generally lacking. The answer to this question will provide important information to design intervention studies, health plans and policies related to mother and child hygienic behavior, which was described in our cross sectional study.

In **chapter 2**, we investigated the efficacy of milk calcium with or without two probiotic strains, *Lactobacillus casei* CRL 431 and *Lactobacillus reuteri* DSM 17938 tested independently, on incidence and duration of acute diarrhea and acute respiratory tract infections in a 6-month, double-blind, placebo-controlled trial. A total of 494 healthy Indonesian children aged 1 to 6 years from a low socioeconomic urban area of East Jakarta randomly received low-lactose milk with low calcium content (LC; ~50 mg/day; n = 124),

regular calcium content (RC; ~440 mg/day; *n* = 126), RC with 5.10^8 colony-forming units per day of *L. casei* CRL 431 (casei; *n* = 120), or RC with 5.10^8 colony-forming units per day of

L. reuteri DSM 17938 (reuteri; *n* = 124). Number and duration of diarrhea and ARTIs episodes were primary and secondary outcomes, respectively. Our study showed an excellent compliance rate (94%), which was similar among the four groups. The incidence of World Health Organization-defined diarrhea (≥3 loose/liquid stools in 24 hours) was not significantly different between RC and LC (relative risk 0.99; 95% confidence interval [CI]: 0.62-1.58), between casei and RC (relative risk 1.21; 95% CI: 0.76-1.92), or between reuteri and RC (relative risk 0.76; 95% CI: 0.46-1.25) groups. Incidence of all reported diarrhea (≥2 loose/liquid stools in 24 hours) was significantly lower in the reuteri versus RC group (relative risk 0.68; 95% CI: 0.46-0.99). Irrespective of the definition used, reuteri significantly reduced diarrhea incidence in children with lower nutritional status (below-median height-and-weight-for-age *z* score). None of the interventions affected ARTIs.

To investigate further the efficacy of milk calcium with or without two probiotic strains, in **chapter 3**, we investigated their effects on cumulative (total) duration and severity of acute diarrheal disease due to rotavirus or other causes. Diarrhea duration and severity were observed during each episode. The cumulative (total) duration of diarrhea was assessed by summing up of all diarrheal days of all episodes per child per 6-month of intervention period. Severity of diarrhea was assessed by using a modified Vesikari score based on the mothers' subjective information and by using objective measurement of the markers osmolarity, mucin and calprotection in diarrheal and normal fecal samples collected during the intervention. A total of 131 children experienced 190 diarrheal episodes. Probiotic strains and calcium did not significantly affect the number of diarrheal episodes, but mean total duration was 1.35 days shorter in the reuteri group (relative risk 0.60; 95% CI: 0.36-0.99) and tended to be 0.93 days shorter in the casei group (relative risk 0.64; 95% CI: 0.39-1.03). Rotavirus prevalence in diarrheal cases was 30% and not significantly different between treatment groups. Rotavirus-positive episodes (36 of 120 analyzed samples) were shortened by *L. reuteri* (*P* = .04) and calcium (*P* = .009), whereas, *L. casei* shortened duration of rotavirus-negative episodes (*P* = .04). None of the supplements affected diarrhea severity based on Vesikari score and the fecal markers, except for a higher fecal mucin concentration in the casei group (*P* = .006).

In **chapter 4**, we investigated whether milk supplemented with 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. Dietary intake, growth, anemia, iron and zinc status were assessed before and after the intervention. The increase in weight gain, weight-for-age z score (WAZ) changes and monthly weight and height velocities were significantly higher in the reuteri compared with RC group [0.22 (95% CI: 0.02-0.42) kg, 0.09 (95% CI: 0.01-0.17) Z, 0.03 (95% CI: 0.002-0.05) kg and 0.03 (95% CI: 0.01-0.05) cm, respectively]. Casei significantly increased average monthly weight velocity [0.03 (95% CI: 0.001-0.05) kg], but not height. However, the changes in underweight and stunting prevalence, anemia prevalence and iron and zinc status were similar among groups.

In **chapter 5**, we reported on the prevalence of diarrhea and malnutrition in Indonesian children and assessed the association of food-hygiene practice with the occurrence of diarrheal disease. We conducted a cross-sectional study among 274 randomly selected children aged 12-59 months in selected low socioeconomic urban areas in Jatinegara sub-

district of East Jakarta. The prevalence of diarrhea was assessed by using a 7-day record of frequency and consistency of the child's defecation pattern. Food-hygiene practices including mother's and child's hand washing, food preparation, cleanliness of utensils, water source and safe drinking water, habits of buying cooked food, child's bottle feeding hygiene, and housing and environmental condition were obtained through home visit interview and observation by field workers. Thirty six practices were scored and classified into poor (median and below) and better (above median) food-hygiene practices. Nutritional status of children, defined anthropometrically, was measured through height and weight. Factors found to be significantly associated with diarrhea were age, nutritional and immunization status, family size and number of under-five children living under the same roof. Among the individual food-hygiene practices, children living in a house with clean sewage had a significantly lower diarrhea prevalence compared to those who did not have one or had dirty sewage (adjusted odds ratio 0.16; 95% CI: 0.03-0.73). The overall food-hygiene practice score was not significantly associated with diarrhea in the total group, but it was in children aged < 2 years (adjusted odds ratio 4.55; 95% CI: 1.08-19.1).

Finally, the main findings and clinical effects of the interventions, methodological issues, interpretation and perspective of the study findings are discussed in **chapter 6**. The public health implications and recommendations, and directions for future research are also presented.

To our knowledge, this Indonesian study is the first large randomized controlled trial, focusing on the effect of calcium with or without one of two specific probiotics on incidence and duration of diarrhea and respiratory tract infections, diarrhea severity, growth, and iron and zinc status in these settings. **In conclusion**, we observed a consistent benefit of the probiotic strain *L. reuteri* DSM 17938 for several outcomes. *L. reuteri* may prevent diarrhea especially in children with lower nutritional status and reduce total duration of diarrheal episodes. This probiotic modestly improved growth, by increasing weight gain and WAZ changes over 6 months and monthly weight and height velocity in Indonesian children. However, *L. reuteri* did not affect diarrhea severity. The other probiotic strain, *L. casei* CRL 431 modestly improved monthly weight velocity, but this strain did not reduce diarrhea incidence and duration or severity of episodes. In contrast to these findings, milk calcium alone did not affect any of the outcomes. Also, none of the dietary treatments affected incidence and duration of ARTIs and iron and zinc status in Indonesian children. However, it seems too early to recommend probiotics (e.g. *L. reu*teri) for routine use or for follow-up in public health programs to prevent diarrhea in children in developing countries. Further investigations are needed to confirm whether our intervention results using probiotics and calcium can be confirmed and replicated by studies in similar settings in developing countries. Several important factors should be taken into consideration in conducting studies in a community such as ways to maintain compliance, cultural, ethical issues in recruiting subjects, and cooperation with local university, government, leaders and health

care facilities. Our cross sectional study showed that in addition to other major determinants, poor mother's food-hygiene practices contributed to the occurrence of diarrhea in Indonesian children <2 years from low socioeconomic urban areas of East Jakarta, Indonesia.

Samenvatting

Acute diarree en acute luchtweginfecties zijn nog steeds de grootste infectieuze veroorzakers van morbiditeit en mortaliteit bij kinderen <5 jaar in ontwikkelingslanden, zoals Indonesië. De ziektelast van deze infecties vraagt om een effectieve volksgezondheidsinterventie om de bestaande preventieve strategieën (waaronder de voorziening van veilig water en sanitair, het exclusief geven van borstvoeding, handen wassen, suppletie met vitamine A en zink en vaccinaties) te ondersteunen, aangezien deze wel beschikbaar zijn, maar niet altijd effectief zijn om de ziektelast te verminderen. Suppletie met probiotica en calcium in melk zijn veelbelovend als alternatieve strategie ter preventie van diarree. Probiotica worden vaak toegevoegd aan zuivelproducten en zowel probiotica als calcium in melk zouden de resistentie van de darmen tegen infecties kunnen verhogen.

Er zijn verscheidene meta-analyses, systematische reviews en interventiestudies over de relatie tussen probiotica en diarree gepubliceerd. Deze studies concludeerden dat diarree bij kinderen zou kunnen worden voorkomen of de duur ervan zou kunnen worden verkort door probiotica. De gerapporteerde gunstige effecten zijn echter afhankelijk van de stam en dosis probiotica en de bewijslast is veelal verkregen uit onderzoeken in ziekenhuizen in ontwikkelde landen waarbij het doel van het onderzoek de behandeling van diarree was. Onderzoeken naar de preventie van diarree in ogenschijnlijk gezonde kinderen in de open populatie zijn met name uitgevoerd bij kinderdagverblijven in Westerse landen. Op dit moment zijn er slechts drie gerandomiseerde interventiestudies die de rol van probiotica ter preventie van acute diarree in ontwikkelingslanden hebben onderzocht. Daarnaast hebben verscheidene gerandomiseerde interventiestudies de gunstige effecten van probiotica ter preventie van luchtweginfecties bij kinderen onderzocht, maar deze lieten tegenstrijdige resultaten zien. In het verleden is aangetoond dat calcium door infectie geïnduceerde diarree in volwassenen kan verminderen, maar het effect bij kinderen met een lage calcium inname en frequente episodes van darminfecties is op dit moment niet bekend. Tot op heden zijn aanbevelingen om te suppleren met calcium of probiotica in ontwikkelingslanden dan ook niet gerechtvaardigd.

Op dit moment is het onduidelijk of suppletie met probiotica en calcium de incidentie en duur van diarree en luchtweginfecties, de ernst van diarree alsmede groei, ijzer- en zinkstatus van Indonesische kinderen gunstig zou kunnen beïnvloedt. Daarom hebben wij een interventiestudie uitgevoerd, waarvan de resultaten kunnen leiden tot aanbevelingen voor voedingsinterventie strategieën ter preventie van diarree en luchtweginfecties. Daarnaast kunnen de resultaten medische professionals, beleidsmakers van volksgezondheid en voedingsbeleid inzicht geven in hoe groei, ijzer- en zinkstatus verbeterd zouden kunnen worden. Hoewel de determinanten van diarree bij kinderen bekend zijn, ontbreekt over het algemeen informatie over hoe een slechte voedselhygiëne gerelateerd is aan diarree bij kinderen in stedelijke gebieden van Oost Jakarta met een lage sociaal economische status. Het antwoord op deze vraag zal belangrijke informatie geven om interventiestudies, gezondheidsplannen en beleid gerelateerd aan het hygiëne gedrag van moeder en kind, zoals beschreven in onze cross-sectionele studie, op te zetten.

In **hoofdstuk 2**, zijn de effecten van calcium in melk met of zonder twee verschillende stammen probiotica, *Lactobacillus casei* CRL 431 en *Lactobacillus reuteri* DSM 17938, op incidentie en duur van acute diarree en acute luchtweginfecties, onafhankelijk van elkaar onderzocht in een dubbelblinde, placebo gecontroleerde interventiestudie gedurende 6 maanden. In totaal kregen 494 gezonde Indonesische kinderen in de leeftijd van 1 tot 6 jaar

wonende in een stedelijk gebied van Oost Jakarta met een lage sociaal economische status, op basis van loting melk met een laag lactose gehalte met een laag calcium gehalte (LC; ~50 mg/dag; n = 124), regulier calcium gehalte (RC; ~440 mg/dag; n = 126), RC met 5.10^8 kolonievormende eenheden per dag van *L. casei* CRL 431 (casei; n = 120), of RC met 5.10^8 kolonievormende eenheden per dag van *L. reuteri* DSM 17938 (reuteri; n = 124). Frequentie en duur van diarree en luchtweginfecties waren respectievelijk de primaire en secondaire uitkomstmaten. Onze interventiestudie liet een excellente therapietrouw zien (94%), die vergelijkbaar was tussen de vier groepen. De incidentie van diarree, zoals gedefinieerd door de Wereld Gezondheidsorganisatie, ($\geq$3 losse/vloeibare defecaties binnen 24 uur) was niet significant verschillend tussen RC en LC (relatief risico 0.99; 95% betrouwbaarheidsinterval [CI]: 0.62-1.58), tussen casei en RC (relatief risico 1.21; 95% CI: 0.76-1.92), of tussen reuteri en RC (relatief risico 0.76; 95% CI: 0.46-1.25). Incidentie van alle gerapporteerde diarree ($\geq$2 losse/vloeibare defecaties binnen 24 uur) was significant lager in de reuteri vergeleken met de RC groep (relatief risico 0.68; 95% CI: 0.46-0.99). Reuteri reduceerde significant de incidentie van diarree bij kinderen met een relatief lage voedingsstatus (onder de mediane lengte-en-gewicht-voor-leeftijd z score), ongeacht de gebruikte definitie. Geen van de interventies had effect op luchtweginfecties.

In **hoofdstuk 3** hebben we het effect van calcium in melk met of zonder twee verschillende stammen probiotica op cumulatieve (totale) duur en ernst van acute diarree veroorzaakt door rotavirus of andere oorzaken verder onderzocht. De duur en ernst van de diarree zijn iedere episode vastgesteld. De cumulatieve (totale) duur van diarree is bepaald door alle dagen met diarree van alle episodes per kind per zes maanden interventie periode bij elkaar op te tellen. Ernst van diarree is bepaald door middel van een gemodificeerde Vesikari score, gebaseerd op de subjectieve informatie van de moeders en door middel van een objectieve meting van de markers osmolariteit, mucine en calprotection in monsters van diarree en van normale feces die verzameld zijn gedurende de interventie. In totaal 131 kinderen hadden 190 episodes van diarree. Probiotica en calcium hadden geen significant effect op het aantal episodes diarree, maar de gemiddelde totale duur was 1,35 dagen korter in de reuteri groep (relatief risico 0.60; 95% CI: 0.36-0.99) en neigde tot 0.93 dagen korter in de casei groep (relatief risico 0.64; 95% CI: 0.39-1.03). De prevalentie van rotavirus in de gevallen van diarree was 30% en niet significant verschillend tussen de interventie groepen. Rotavirus-positieve episodes (36 van de 120 geanalyseerde monsters) waren korter door *L. reuteri* (P = .04) en calcium (P = .009), terwijl, *L. casei* de duur van rotavirus-negatieve episodes verkortte (P = .04). Geen van de supplementen had effect op de ernst van diarree gebaseerd op de Vesikari score en de fecale markers, behalve voor een hogere fecale mucine concentratie in de casei groep (P = .006).

In **hoofstuk 4,** hebben we onderzocht of melk gesuppleerd met probiotica de groei en ijzer- en zinkstatus van Indonesische kinderen zou verbeteren, terwijl alleen calcium in melk de groei zou verbeteren, maar ijzer- en zinkstatus zou verlagen. Voedingsinname, groei, anemie, ijzer- en zinkstatus zijn zowel voor als na afloop van de interventie bepaald. Toename in gewicht, veranderingen in gewicht-voor-leeftijd (WAZ) score en de maandelijkse groeisnelheid voor gewicht en lengte waren significant hoger in de reuteri groep vergeleken met de RC groep [0.22 (95% CI: 0.02-0.42) kg, 0.09 (95% CI: 0.01-0.17) Z, 0.03 (95% CI: 0.002-0.05) kg en 0.03 (95% CI: 0.01-0.05) cm, respectievelijk]. Casei verhoogde de gemiddelde snelheid van maandelijkse gewichtstoename significant [0.03 (95% CI: 0.001-0.05) kg], maar niet die van lengte. De veranderingen in de prevalentie van ondergewicht en

achterstand in lengtegroei, de prevalentie van anemie en ijzer- en zinkstatus waren echter gelijk tussen de groepen.

In **hoofdstuk 5** wordt gerapporteerd over de prevalentie van diarree en ondervoeding in Indonesische kinderen en over de associatie tussen voedselhygiëne gewoontes en het ontstaan van diarree. We hebben een cross-sectioneel onderzoek verricht bij 274 random geselecteerde kinderen tussen de 12 en 59 maanden oud wonend in geselecteerde stedelijke gebieden met een lage sociaal economische status in het Jatinegara district in Oost Jakarta. De prevalentie van diarree is gemeten middels een 7-daagse vragenlijst naar de frequentie en consistentie van het defecatie patroon van de kinderen. Informatie over de gewoontes met betrekking tot voedselhygiëne, zoals handen wassen door moeder en kind, de bereiding van het eten, de properheid van kookgerei, waterbron en veilig drinkwater, gewoontes met betrekking tot het kopen van gekookt eten, hygiëne bij het flesvoeden van kinderen en woon- en omgevingscondities is verkregen door interviews en observatie tijdens huisbezoeken door veldwerkers. Zesendertig gewoontes zijn gescoord en geclassificeerd naar lage (gemiddeld en lager dan gemiddeld) en betere (boven gemiddeld) voedselhygiëne. De voedingsstatus van kinderen, antropometrisch gedefinieerd, is bepaald door het meten van lengte en gewicht. Factoren die significant gerelateerd waren aan diarree waren leeftijd, voedings- en immunisatiestatus, gezinsgrootte en aantal kinderen onder de vijf jaar die onder hetzelfde dak woonden. Kinderen die woonden in een huis met een schone riolering hadden een significante lagere diarree prevalentie vergeleken met kinderen die geen of een bevuilde riolering hadden (gecorrigeerde odds ratio 0.16; 95% CI: 0.03-0.73). De totale voedselhygiëne score was niet significant gerelateerd met diarree in de totale groep, maar wel in kinderen onder de 2 jaar oud (gecorrigeerde odds ratio 4.55; 95% CI: 1.08-19.1).

Ten slotte worden de belangrijkste resultaten en klinische effecten van de interventies, methodologische aspecten, interpretatie en perspectief van de bevindingen bediscussieerd in **hoofdstuk 6**. De implicaties en aanbevelingen voor de volksgezondheid en suggesties voor toekomstig onderzoek worden hier ook beschreven.

Deze Indonesische studie is, voor zover bij ons bekend, de eerste grote gerandomiseerde, gecontroleerde interventiestudie naar het effect van calcium met of zonder één of twee specifieke stammen probiotica op de incidentie en duur van diarree en luchtweginfecties, de ernst van diarree, groei en ijzer- en zinkstatus in deze leefomstandigheden. **Concluderend**, vonden we een consistent gunstig effect van de probiotische stam *L. reuteri* DSM 17938 op verschillende uitkomstmaten. *L. reuteri* kan preventief werken tegen diarree, met name in kinderen met een lage voedingsstatus en kan de totale duur van episodes van diarree verkorten. Deze probiotica lieten ook een bescheiden verbetering zien in groei, door een stijgende gewichtstoename en gewichts-voor-lengte veranderingen over 6 maanden en snelheid van maandelijkse stijging in gewicht en lengte in Indonesische kinderen. *L. reuteri* had echter geen effect op de ernst van de diarree. De andere probiotische stam, *L. casei* CRL 431 liet een kleine verbetering zien in snelheid van maandelijkse gewichtstoename, maar deze stam verminderde de incidentie, duur of ernst van de diarree episodes niet. In tegenstelling tot deze bevindingen had calcium in melk op zichzelf op geen van de uitkomstmaten effect. Ook had geen van de interventies effect op de incidentie en duur van luchtweginfecties en ijzer- en zinkstatus bij Indonesische kinderen. Echter, het is te vroeg om probiotica (zoals bijvoorbeeld *L. reu*teri) aan te bevelen voor dagelijks gebruik of om te gebruiken in volksgezondheidsprogramma's om diarree te voorkomen bij kinderen in

ontwikkelingslanden. Meer onderzoek is nodig om te bevestigen of de resultaten van onze interventie met probiotica en calcium gerepliceerd kunnen worden in studies in vergelijkbare situaties in ontwikkelingslanden. Bij het uitvoeren van studies in de open gemeenschap moeten rekening worden gehouden met een aantal belangrijke factoren, zoals manieren om therapietrouw te behouden, culturele en ethische aspecten bij het werven van deelnemers en samenwerking met lokale universiteiten, regeringen, leiders en de faciliteiten voor de gezondheidszorg. Ons cross-sectionele onderzoek heeft aangetoond dat, naast andere belangrijke determinanten, een slechte voedselhygiëne van de moeder bijdroeg aan het ontstaan van diarree bij Indonesische kinderen onder de 2 jaar oud in stedelijke gebieden met een lage sociaal economische status in Oost Jakarta, Indonesië.

Ringkasan

Diare akut dan infeksi saluran pernapasan akut (ISPA) senantiasa menjadi penyebab morbiditas dan kematian pada anak-anak <5 tahun di negara berkembang, termasuk Indonesia. Beban permasalahan kesehatan ini membutuhkan intervensi kesehatan masyarakat yang efektif untuk mendukung strategi pencegahan yang telah ada sebelumnya (termasuk penyediaan air bersih dan sanitasi, pemberian ASI eksklusif, mencuci tangan, suplementasi vitamin A dan zink, serta vaksinasi) tetapi belum efektif untuk mengurangi beban penyakit ini. Upaya untuk mencegah penyakit diare dengan probiotik dan susu berkalsium sebagai strategi alternatif cukup menjanjikan. Probiotik sering ditambahkan dalam susu dan baik probiotik maupun kalsium yang terkandung dalam susu dapat memperkuat ketahanan usus terhadap infeksi.

Beberapa meta-analisis, penelaahan secara sistematik dan penelitian probiotik dan diare telah dipublikasikan. Studi ini menyimpulkan bahwa probiotik dapat mencegah atau mengurangi durasi diare pada anak. Namun, efek positif penggunaan probiotik sangat tergantung pada jenis dan dosis strain probiotiknya, dan studi probiotik tersebut pada umumnya dilakukan di negara maju dalam rangka pengobatan diare di rumah sakit. Studi probiotik yang memfokuskan pada pencegahan diare pada anak-anak sehat yang hidup di masyarakat umumnya dilakukan di pusat penitipan anak yang dilakukan di negara-negara Barat. Hingga saat ini, hanya ada tiga penelitian di komunitas yang berfokus pada peran probiotik dalam pencegahan diare akut di negara-negara berkembang. Lebih jauh, beberapa studi telah melakukan penelitian mengenai manfaat probiotik dalam pencegahan ISPA pada anak, namun masih menghasilkan kesimpulan yang bertentangan. Kalsium, sebelumnya telah terbukti mengurangi infeksi yang menyebabkan diare pada orang dewasa, tetapi manfaatnya pada anak-anak yang memiliki asupan kalsium yang rendah dan episode penyakit infeksi usus berulang sampai saat ini belum dapat diketahui. Sejauh ini, rekomendasi untuk memberikan suplementasi kalsium atau probiotik pada anak dalam masyarakat di negara berkembang belum teruji.

Saat ini, belum jelas, apakah pemberian asupan probiotik dan kalsium berdampak positif terhadap kejadian dan durasi penyakit diare serta ISPA, tingkat keparahan, tingkat pertumbuhan, status zat besi dan zink pada anak-anak Indonesia. Untuk tujuan inilah, kami melakukan studi intervensi. Sebagai jawaban dari pertanyaan diatas, studi ini akan memberikan rekomendasi untuk strategi intervensi gizi dalam pencegahan diare dan ISPA. Selain itu, studi ini dapat memperlihatkan kepada para profesional bidang medis, kesehatan masyarakat dan pembuat kebijakan pangan mengenai bagaimana meningkatkan pertumbuhan serta status zat besi dan zink. Meskipun faktor-faktor penyebab diare pada anak telah diketahui dengan jelas, pengetahuan mengenai hubungan praktik perilaku higienis pada penanganan makanan dengan tingginya prevalensi diare pada anak-anak di daerah perkotaan dengan tingkat sosial ekonomi rendah di Jakarta Timur, umumnya masih kurang. Jawaban atas pertanyaan ini akan memberikan informasi penting untuk merancang studi intervensi, perencanaan kesehatan dan kebijakan yang berkaitan dengan perilaku higienis ibu dan anak, yang merupakan bagian dalam penelitian potong lintang (*cross sectional)* kami.

Dalam **bab 2**, kami meneliti manfaat susu berkalsium dengan atau tanpa dua jenis probiotik, *Lactobacillus casei* 431 dan *Lactobacillus reuteri* DSM 17938 yang diuji secara

terpisah, terhadap kejadian dan durasi diare akut serta ISPA dalam penelitian *double-blind, placebo-controlled trial* selama 6 bulan. Sebanyak 494 anak Indonesia sehat berusia 1-6 tahun berasal dari daerah perkotaan dengan tingkat sosial ekonomi yang rendah di Jakarta Timur secara acak menerima susu rendah laktosa dengan kandungan kalsium rendah (LC; ~ 50 mg/hari, n = 124), kandungan kalsium biasa (RC; ~440 mg / hari, n = 126), RC dengan 5.10^8 *colony-forming unit* per hari *L. casei* CRL 431 (casei, n = 120), atau RC dengan 5.10^8 *colony-forming unit* per hari *L. reuteri* DSM 17938 (reuteri, n = 124). Jumlah episode dan durasi diare merupakan hasil akhir utama, sedangkan insiden dan durasi ISPA adalah hasil akhir sekunder. Studi kami menunjukkan tingkat kepatuhan yang sangat baik (94%), yang tidak berbeda di antara empat kelompok. Kejadian diare berdasar definisi *World Health Organization* (≥ 3 tinja lembek/cair dalam 24 jam) tidak berbeda secara signifikan antara kelompok RC dan LC (*relative risk* 0,99; 95% confidence interval (CI): 0,62-1,58), antara kelompok casei dan RC (*relative risk* 1,21; 95% CI: 0,76-1,92), atau antara kelompok reuteri dan RC (*relative risk* 0,76; 95% CI: 0,46-1,25). Kejadian semua diare (≥2 tinja lembek/cair tinja dalam 24 jam) secara signifikan lebih rendah pada kelompok reuteri dibandingkan RC (*relative risk* 0,68; 95% CI: 0,46-0,99). Terlepas dari definisi yang digunakan, reuteri secara signifikan mengurangi kejadian diare pada anak dengan status gizi rendah [di bawah median-*z score* tinggi badan terhadap umur (TB/U) dan berat badan terhadap umur (BB/U)]. Tak satu pun dari intervensi mempengaruhi ISPA.

Untuk meneliti lebih lanjut manfaat kalsium susu dengan atau tanpa dua jenis probiotik, dalam **bab 3**, kami meneliti pengaruhnya terhadap kumulatif (total) durasi dan tingkat keparahan penyakit diare akut dikarenakan rotavirus atau penyebab lainnya. Durasi dan tingkat keparahan diare diobservasi setiap episode. Kumulatif (total) durasi diare dinilai dengan menjumlahkan semua hari diare dari semua episode per anak dalam 6 bulan periode intervensi. Tingkat keparahan diare dinilai dengan menggunakan modifikasi skor Vesikari berdasarkan informasi subyektif ibu dan dengan menggunakan pengukuran objektif berupa uji osmolaritas, *calprotection* dan *mucin* dalam sampel tinja yang dikumpulkan selama intervensi. Sebanyak 131 anak mengalami 190 episode. Strain probiotik dan kalsium tidak terlalu berpengaruh pada jumlah episode diare, tetapi rerata durasi diare menjadi 1,35 hari lebih pendek pada kelompok reuteri (*relative risk* 0,60; 95% CI: 0,36-0,99) dan cenderung 0,93 hari lebih pendek pada kelompok casei (*relative risk* 0,64; 95% CI: 0,39-1,03). Prevalensi rotavirus pada kasus diare adalah 30% dan tidak berbeda secara signifikan antara semua kelompok penelitian. Durasi episode rotavirus positif (36 dari 120 sampel yang dianalisa) menjadi lebih singkat dengan pemberian *L. reuteri* (*P* = 0,04) dan kalsium saja (*P* = 0,009), sedangkan, durasi episode rotavirus-negatif menjadi lebih pendek dengan pemberian *L. casei* (*P* = 0,04). Tak satu pun dari keempat intervensi tersebut berpengaruh pada tingkat keparahan diare berdasarkan skor Vesikari dan *fecal markers*, kecuali adanya konsentrasi yang lebih tinggi pada pemeriksaan mucin tinja pada kelompok *L. casei* (*P* = 0,006).

Dalam **bab 4**, kami meneliti apakah susu yang dilengkapi dengan probiotik akan meningkatkan pertumbuhan dan status zat besi dan zink pada anak-anak Indonesia, sedangkan susu berkalsium saja (tanpa probiotik) akan meningkatkan pertumbuhan tetapi mengurangi status zat besi dan zink. Asupan makanan, pertumbuhan, anemia, status zat besi dan zink dinilai sebelum dan sesudah intervensi. Peningkatan berat badan, perubahan

BB/U, *velocity* berat badan dan tinggi badan bulanan secara signifikan lebih tinggi pada kelompok reuteri dibandingkan pada kelompok RC [0,22 (95% CI: 0,02, 0,42) kg, 0,09 (95% CI: 0,01, 0,17) Z, 0,03 (95% CI: 0,002, 0,05) kg dan 0,03 (95% CI: 0,01, 0,05) cm, secara berurutan]. *Casei* secara signifikan meningkatkan rerata *velocity* berat badan bulanan [0,03 (95% CI: 0,001, 0,05) kg], tetapi tidak tinggi badan. Perubahan prevalensi kurus dan pendek, prevalensi anemia dan status zat besi dan zink tidak berbeda di antara kelompok intervensi.

Dalam **bab 5**, kami melaporkan prevalensi diare dan gizi kurang pada anak-anak Indonesia dan menilai hubungan antara praktik kebiasaan higienis pada penanganan makanan dengan terjadinya penyakit diare. Kami melakukan studi *cross-sectional* di antara 274 anak usia 12-59 bulan yang dipilih secara acak dari daerah perkotaan dengan tingkat sosial ekonomi rendah terpilih di kecamatan Jatinegara, Jakarta Timur. Prevalensi diare dinilai dengan menggunakan catatan 7 hari frekuensi dan konsistensi pola buang air besar anak. Kebiasaan higienis makanan termasuk kebiasaan mencuci tangan anak, persiapan makanan, kebersihan peralatan makan, air dan sumber air minum yang aman, kebiasaan membeli makanan yang dimasak, kebersihan botol susu anak, dan kondisi rumah dan lingkungan dilakukan dengan wawancara dan observasi rumah oleh peneliti lapangan. Tiga puluh enam kebiasaan diberi skor dan dikelompokkan dalam rendah (rata-rata dan di bawah) dan lebih baik (di atas rata-rata). Status gizi anak, yang didefinisikan secara antropometrik, diukur melalui tinggi dan berat badan. Faktor-faktor yang secara signifikan terkait dengan diare adalah usia, status gizi dan imunisasi, jumlah anggota keluarga dan jumlah balita. Di antara kebiasaan higienis makanan secara perorangan, anak-anak yang tinggal di rumah dengan saluran pembuangan limbah yang bersih memiliki prevalensi diare jauh lebih rendah dibandingkan dengan mereka yang tidak memiliki atau memiliki saluran pembuangan limbah yang kotor (*adjusted odds ratio* 0,16; 95% CI: 0,03-0,73). Hubungan antara skor perilaku higienis pada penanganan makanan dan diare secara keseluruhan tidak signifikan, tapi berpengaruh pada anak usia <2 tahun (*adjusted odds ratio* 4,55; CI 95%: 1,08-19,1).

Akhirnya, temuan utama dan dampak klinis dari intervensi, isu-isu metodologis, interpretasi dan perspektif temuan penelitian dibahas dalam **bab 6**. Implikasi kesehatan masyarakat dan rekomendasi, serta arah untuk penelitian di masa depan juga disampaikan pada bab ini.

Sepanjang pengetahuan kami, studi terhadap anak Indonesia ini adalah studi pertama berbasis komunitas yang terbesar dengan fokus pada efek kalsium dengan atau tanpa salah satu dari dua probiotik pada kejadian dan durasi diare dan ISPA, tingkat keparahan diare, pertumbuhan, serta status zat besi dan zink. Sebagai kesimpulan, kami mengamati manfaat yang konsisten dari jenis probiotik *L. reuteri* DSM 17938 untuk beberapa hasil akhir (*outcomes*). *L. reuteri* dapat mencegah diare terutama pada anak dengan status gizi rendah dan mengurangi total durasi dari semua episode diare. Probiotik ini dapat meningkatkan pertumbuhan dengan meningkatkan pertambahan berat badan dan perubahan BB/U serta *velocity* berat badan dan tinggi badan bulanan dalam 6 bulan intervensi. Namun, *L. reuteri* tidak berpengaruh terhadap tingkat keparahan diare. Jenis probiotik lainnya, *L. casei* CRL 431 meningkatkan *velocity* berat badan bulanan, tetapi jenis probiotik ini tidak mengurangi kejadian, durasi, ataupun tingkat keparahan diare. Berbeda dengan temuan ini, susu berkalsium saja tidak berpengaruh apapun. Juga, tidak ada satupun dari intervensi tersebut

berpengaruh pada angka kejadian dan durasi ISPA serta status zat besi dan zink. Namun demikian, tampaknya masih sangat dini untuk merekomendasikan probiotik (misalnya. *L. reuteri*) untuk penggunaan secara rutin atau untuk tindak lanjut dalam program kesehatan masyarakat dalam pencegahan diare pada anak-anak di negara-negara berkembang. Penelitian lanjutan dari penelitian ini diperlukan untuk mengonfirmasi apakah hasil studi intervensi kami menggunakan probiotik dan kalsium dapat dikonfirmasikan dan direplikasi oleh studi-studi lain dengan desain yang sama di negara-negara berkembang. Beberapa faktor penting yang harus dipertimbangkan dalam studi yang dilakukan di masyarakat seperti studi ini adalah cara untuk menjaga kepatuhan, masalah etika dalam merekrut subyek penelitian, dan kerjasama dengan akademisi, pemerintah daerah, para pemimpin serta petugas kesehatan. Studi *cross sectional* kami menunjukkan bahwa selain faktor penentu utama lainnya, kebiasaan kebersihan makanan pada ibu berkontribusi pada terjadinya diare pada anak-anak Indonesia <2 tahun di daerah perkotaan dengan tingkat sosial ekonomi yang rendah di Jakarta Timur, Indonesia.

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My PhD project had been a very long journey, which had involved various collaborators and sponsors, had steps of No-Go decisions and clearances, involved a large sample size with a long study duration, screened almost 4,000 children, faced complicated fields organizations, administrative and financial arrangements and logistics, involved about 100 co-workers who were trained extensively to meet the requirements of research standard quality, been in a very difficult environment, had a large dataset, collected a lot of human samples and performed many laboratory analyses. I also faced some unfortunate times with my health conditions during this period. Thanks to Allah, finally all the hard work came to a final end. At the end of this long and special PhD journey, I would like to express my sincere gratitude to all of those who have contributed and supported me during the study and thesis writing.

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About the author

Curriculum Vitae

Rina Agustina was born on the 27th of August, 1970 in Palembang, South Sumatera, Indonesia. She graduated from "Upland High School" in Upland, California, USA in 1988 when she represented Indonesia as an exchange student through the American Field Service (AFS) program. In the following year, she re-graduated from her senior high school at "SMA Muhammadiyah I" Yogyakarta, Indonesia. She completed her medical degree (Dra.Med and MD) at the Faculty of Medicine, Gadjah Mada University, Yogyakarta (1997) and worked as a physician at private clinics in East Jakarta area. In the same year, she received a fellowship from the German Technical Cooperation (Deutsche Gesellschaft für Technische Zusammenarbeit-GTZ) to continue her Master (MSc) degree program in Nutrition at SEAMEO-TROPMED, Faculty of Medicine, University of Indonesia. For her MSc thesis, she conducted a one year clinical trial on the effect of a combined probiotic, prebiotics and micronutrient for the treatment of moderately dehydrated diarrhea children in two hospitals (Harapan Kita and Sulianti Saroso) in Jakarta, Indonesia. In 1999, Rina served the Ministry of Health, Republic of Indonesia to work as a physician at a primary health center in Menteng, Central Jakarta. After finishing her Master Degree in 2000, she started to work as a lecturer at the SEAMEO-TROPMED University of Indonesia and as an associate for the Industry Council for Development (ICD) cooperative program with SEAMEO and WHO. Her major research at the SEAMEO was on probiotics, diarrhea, food safety, pediatric nutrition and micronutrient. She received awards from the Rector of University of Indonesia for the International Publication (2007) and International Handbook (2010). In 2003, she received a fellowship from the International Nutrition Foundation/Ellison Medical Foundation USA to pursue her PhD degree. In 2004, she was admitted for a PhD program at Division of Human Nutrition, Wageningen University supervised by Prof. Frans J Kok, Prof. Agus Firmansyah, Dr. Ingeborg Bovee-Oudenhoven and Dr. Widjaja Lukito. That year, she started her PhD research on the effect of probiotics and calcium on incidence of diarrhea in Indonesian children, of which the results are described in this thesis. This project was part of Dr. Ingeborg's project and funded by the Top Institute Food and Nutrition, FrieslandCampina Research, and Unilever Research and Development, Netherlands. During this period she performed series of studies including a feasibility study (cross sectional and methodological study) in 2004-2005, an acceptance trial in 2006 and finally a large intervention study in 2007-2008 which involved 494 children with almost 100 co-workers in East Jakarta, Indonesia. During her thesis writing period, she stayed in Wageningen with her husband, Ahmad Sadariskar and her two daughters, Arini Ayatika Sadariskar and Dinaka Tatsbita Sadariskar. Her research entitled "Randomized trial of probiotics and calcium on diarrhea and respiratory tract infections in Indonesian children" has been presented in an international conference and published in high-ranked and prestigious peer reviewed journal. Rina joined international conferences, courses and discussion groups of Division of Human Nutrition. In her final phase of writing, she was selected to receive a fellowship from the Higher Education Network Ring Initiative and Endang Rahayu Sedyaningsih Scholarship Program to attend a summer course program and to write two papers at the Harvard School of Public Health, Harvard University, Boston, MA, USA.

Publications

Publications in peer-reviewed journals

Agustina R, Kok FJ, Van de Rest O, Fahmida U, Firmansyah A, Lukito W, Feskens EJM, Van den Heuvel EGHM, Albers R and Bovee-Oudenhoven I. Randomized trial of probiotics and calcium on diarrhea and respiratory tract infections in Indonesian children. Pediatrics 2012;129:e1155-64.

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Agustina R, Bovee-Oudenhoven IMJ, Lukito W, Fahmida U, van de Rest O, Zimmermann M, Firmansyah A, Wulanti R, Albers R, van den Heuvel EGHM, Frans J Kok. Effect of milk calcium with or without probiotics on growth, iron and zinc status of Indonesian children. Submitted.

Agustina R, Tirta Prawita Sari, Soemilah Satroamidjojo, Ingeborg MJ Bovee-Oudenhoven, Edith JM Feskens, and Frans J Kok. Association of food-hygiene practices and diarrhea prevalence among Indonesian young children from low socioeconomic urban areas. Submitted.

Agustina R, Shankar AV, Ayuningtyas A, Achadi EL, Shankar AH. Multiple maternal factors associated with diarrhoea and acute respiratory tract infections among Indonesian children. Submitted.

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Wiradnyani, LA, Muslimatun, S, **Agustina, R**. Effect of Yogurt enriched with *Lactobacillus acidophilus* in lipid profile among Indonesian adult male**:** The proceedings of the international conference on functional and health foods: market, technology & health benefit, Gadjah Mada University, 2003

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Agustina R and Surono I. Nutrition in Food Industry. Student's Handbook. Jakarta: SEAMEO-TROPMED Regional Center for Community Nutrition University of Indonesia, 2009. ISBN 978-979-98157-9-8

Agustina R and Wijaya M. New Challenge on Food Safety in the South East Asia Region: The proceedings of the 10th anniversary of ICD and SEAMEO Cooperative Program. 2001

New Perspective on Regional Opportunities for Community Nutrition": The proceedings of the 10th anniversary of SEAMEO and GTZ cooperation: Asia Pacific J Clin Nutr 2002; 11 (Suppl 4)

Training and education program

Discipline specific activities	Organizers and location	Year
Courses		
Analyses and interpretation of nutrition and health program data from low income countries	HSPH, HARVARD University, Boston (USA)	2012
Advance course in clinical nutrition and metabolism	European Society for Clinical Nutrition and Metabolism, Maastricht (NL)	2011
Clinical nutrition and metabolism	Medical Faculty Joseph Forrier University France and University of Indonesia, Jakarta (Indonesia)	2004
Clinical nutrition	Human Nutrition, WUR, Wageningen (NL)	2004
Public health intervention in real-life settings: the AGORA experience	VLAG, WUR, Graduate School Mansholt and AGORA Academic Collaborative Centre, Wageningen (NL)	2010
Nutrition and infection; Health promotion; Nutritional assessment updates	SEAMEO-TROPMED RCCN UI, Jakarta (Indonesia)	2009; 2006
Basic immunology	Medical Faculty Gadjah Mada University, Yogyakarta	2007
Interplay between innate and adaptive immunity	Nederlandse Vereniging Voor Immunologie, Lunteren (NL)	2006
Fundamental of nutrigenomics	ILSI SEAR (Singapore)	2006
Diet application in hospital (Nutriclin)	Ministry of Health Indonesia, Bogor (Indonesia)	2005
Management of microbiological hazards in foods	VLAG, European Chair in Food Safety, Food Microbiology, WUR, Wageningen (NL)	2004
Conferences and meetings		
Probiotics prebiotics in pediatrics (*oral presenter*)	Medical Faculty Eskisehir Osmangazi (INF Scholarship), Istanbul (Turkey)	2012
The 6th probiotics prebiotics & new foods	ESPGHAN and Italian Academy Study of Intestinal Microbiota, Rome (Italy)	2011
Probiotic, prebiotic and constipation; Probiotic and health (*invited speaker*)	Medical Faculty UI, Jakarta (Indonesia)	2010 2009
Probiotics prebiotics as functional food for human health promotion	Indonesian Scientific Society for Probiotics and Prebiotics, Jakarta (Indonesia)	2010
The 1st Danone Asian probiotics convention: Gut Microbiota and Probiotics (*invited speaker*)	Danone China, Shanghai (China)	2009
Gut flora in health and disease-potential role of probiotics; What is scientific evidence for nutrition and health claims	KNAW, Afdeling Genootschap, Royal Netherlands Academy of Arts and Scienceherlands, Amsterdam (NL)	2004; 2012
Mini-symposium: Fatty acids, ventricular arrhythmias and sudden death	Human Nutrition, WUR, Wageningen (NL)	2011
International Congress of Nutrition	IUNS, Bangkok (Thailand)	2009
Food and Nutrition (Widya karya Nasional Pangan dan Gizi IX)	Indonesian Institute of Science, Jakarta (Indonesia)	2008
Controlling a major risk for cardiovascular disease: cholesterol	Indonesian Nutrition Medical Association, Jakarta (Indonesia)	2007
Inulin and oligofructose (prebiotic)	ILSI SEAR and INMU Mahidol University, Bangkok (Thailand)	2006
The 4th Asian Congress of Dietetics	Dietetic Association South East Asia, Manila (Philippines)	2006
The role of clinical nutrition specialist nutritional support team	Medical Faculty University of Indonesia, Jakarta (Indonesia)	2006
Nutrigenomics, opportunities in Asia	ILSI SEAR (Singapore)	2005

Training and education program

Discipline specific activities	Organizers and location	Year
Conferences and meetings		
Regional expert consultation on functional foods in South East Asia Region;	ILSI SEAR (Singapore)	2005
Functional Food (*invited speaker*)	National Agency for Food and Drug Control Indonesia, Jakarta (Indonesia)	2005
The role of surveilance in integrated Food Safety System; Development of networking mechanism for microbiological risk assessment (*invited speaker*)	National Agency for Food and Drug Control Indonesia, and WHO, Jakarta (Indonesia)	2004; 2005
The 3rd Asian conference of for lactic acid bacteria (*invited speaker*)	ISLAB, AFSLAB, ISAPP, Denpasar (Indonesia)	2005
International congress of clinical nutrition	IUNS and APCNS, Brisbane (Australia)	2004
General course and workshop		
Analysis using R Multilevel analysis	VLAG and Human Nutrition, WUR, Netherlands (NL)	2012; 2011
Concept and methods in epidemiology: Introduction of Rothman book	Human Nutrition, WUR, Wageningen (NL)	2010-2011
Scientific writing in english	Language Services WUR, Wageningen (NL)	2011
How to write a world-class article	WUR Library and Elsevier, Wageningen (NL)	2011
Training: Marketing your education institution	Value Consult, Jakarta (Indonesia)	2009
Philosophy and ethics of food science and technology	VLAG WUR, Wageningen (NL)	2006
Applied epidemiology with special reference to nutrition	SEAMEO Network, Mahidol University Bangkok and SEAMEO-TROPMED RCCN UI, Jakarta (Indonesia)	2005
WHO standard management for severe malnutrition in hospital and feeding center; Health related field	Ministry of Health Indonesia, Jakarta (Indonesia)	2006
Nutrisurvey program in application dietary Assessment; Evaluating health and nutrition projects/programs	SEAMEO TROPMED RCCN UI, Jakarta (Indonesia)	2007; 2006
Online course: Human participants protection education for research teams	National Institutes of Health (USA)	2006
Quantitative methods in clinical research; Advance topics in epidemiology research; Basic epidemiology, and statistical methods	NIHES and Medical Faculty UI, Jakarta (Indonesa)	2004
The 2nd South East Asian Nutrition Leadership Program (SEA-NLP)	SEAMEO-TROPMED RCCN UI, Sukabumi (Indonesia)	2003
Optional courses and activities		
Preparation of PhD research proposals	Human Nutrition, WUR, Wageningen (NL)	2004, 2006
PhD study tour USA	TUFT, John Hopkins, HARVARD, Yale, Penn State, NIH, Cornell University (USA)	2007
PhD study tour Scandinavia	Karolinska Institute (Sweden), Univ. Copenhagen (Denmark), Univ. Helsinski, Univ. Upsala, THL, Valio company (Finland)	2008
International nutrition meeting	Human Nutrition, WUR, Wageningen (NL)	2010-2011
Life Science Company Visit: Human and veterinary infectious disease	Postdoc Career Initiative and Amsterdam Biomed Cluster, Lelystad (NL)	2011