Gut environment and socioeconomic status: a study of children in urban area of Makassar

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About the cover: The front cover illustrates two areas of study described in this thesis, the lower part represents the area with lower socioeconomic status and the upper parts represents the area with high socioeconomic status. The lower part illustrates some traditional houses with soil rich in worms and parasite eggs. The upper part illustrates the silhouettes of modern buildings and houses. Also, below the title, there is a small boat namely Katinting, a symbol of Makassar.

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To my beloved family

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CHAPTER ONE
GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

Indonesia oscillates between being graded as upper middle or lower middle-income country [1], with great variation not only in the socioeconomic status of its population but also the degree of urbanization that its population faces. Socioeconomic status (SES) is a well-known determinant of health [2]. However, SES indicators often do not impact health directly, but instead serve as proxies for other determinants. Indeed, many intermediate factors have been highlighted in the relationship between SES and health outcomes, such as poor environmental conditions that lead to high exposure to microorganisms and parasites. In addition, factors such as gut microbiota composition or diversity can also be intermediates. One mechanism by which SES may put forth its effects on health is through differential activation of the immune system. Several studies have shown the maternal environment such as exposure to helminth infection during pregnancy and maternal gut microbiota might affect child immune responses soon after birth [3-5]. This in turn is thought to determine the subsequent immune responses of a child later in life against pathogens, vaccines, venoms or allergens.

In Indonesia, low socioeconomic status or residence in rural areas have been shown to increase the risk of getting helminth infections compared to those with higher SES living in urban centers [6, 7]. Poor sanitation, low degree of hygienic lifestyle, low levels of maternal education, distance from clean water source are important contributors to infection with helminths [8, 9]. Although there are reports that helminth infections can be associated with poor nutritional status [10, 11], anemia [12] and cognitive development [13], there are also reports from meta-analysis that refute this [14, 15]. There are even reports of helminth infections having protective effects against allergy or diabetes [16, 17]. "The Old Friends" hypothesis that was coined by Rook in 2003 stated that humans co-evolved with their "old friends", among them parasitic helminths, can act as inducers of immunoregulatory circuits [18, 19]. Hygienic lifestyles and western medicine lessen the exposure to the "old-friends" and other organisms from the natural environments that humans co-evolved with. Therefore, the human immune system must have evolved to act optimally under a very different condition and any change in these conditions might alter the immune system and its downstream effects on disease outcomes.

Over the past decades, it is becoming increasingly clear that the microbiome can be important for the gut function and human health [20-26]. The gut microbiota profile is predominantly affected by environmental factors such as diet and exposure to infection [27, 28]. The gut microbiota is considered as an important factor as derived metabolites might maintain gut barrier function. Short chain fatty acids, including butyrate, which are produced by certain gut microbiota are thought to have a protective effect on the intestinal lining and thus associate with intestinal barrier health [29]. Therefore, imbalance of gut microbiota composition might aggravate gut inflammation, affect nutritional absorption and consequently lead to growth faltering in children [30-32]. Therefore, one of the ways human health is wired by the socioeconomic status, environment and lifestyle factors can be through the variation in the microbiome.

Environmental factors are thought to affect immune responses to vaccines. With respect to economic status, the response to *Bacille Calmette-Guérin* (BCG) vaccine [33] and the effica-

cy of rotavirus [34] are lower in the low compared to high-income countries. These differences might be due to varied exposure to infection, and host characteristics, including diet and nutritional status.

The geographical differences are also observed in the prevalence of allergic diseases [35]. In this context, most intensively studied allergic diseases are asthma, rhinitis, atopic dermatitis and food allergies. Allergic reactions to bee- and wasp-venom can be life-threatening [36], but they are less studied. Most studies of venom allergy, have so far been conducted in developed western countries. Prevalence of Hymenoptera sensitization varies among regions, with some of the studies performed in the community while others were performed in groups at risk. There is, however, very little information on the magnitude of venom allergy in tropical parts of the world. The location of Indonesia near the equator provides a warm and humid climate which is favorable for Hymenopterans like bees and wasps to live. Therefore, further information of the sensitization to Hymenoptera in low-to-middle-income countries such as Indonesia would aid understand the extent of Hymenoptera allergy not only in the temperate but also in tropical areas and also the risk factors associated with such allergic disease [6].

Scope of the thesis

The main objective of this thesis is to improve our understanding of the impact of life style and socioeconomic status on the immune response of children living in an urban area of Indonesia. We started by studying responses to a vaccine. To this end, we assessed lifestyle and socioeconomic factors that determine the response to BCG vaccination. Data on maternal infection status, demographic and socioeconomic characteristics, as well as their infant's nutritional status, total IgE and leptin levels of newborn were studied and pathway analysis was performed to understand the effect of different variables on response to BCG vaccine. Next, we conducted a study in schoolchildren with different socioeconomic backgrounds living in the same urban area and assessed their allergic response to bee and wasp venom. Baseline data on sociodemographic, anthropometric and intestinal parasitic infection status were collected. The skin prick test reactivity and reported sting-related symptoms to bee and wasp were recorded. The profile of bacterial gut microbiota and its association with environmental factors were also assessed as a function of SES. The markers of intestinal permeability and intestinal damage before and after albendazole treatment were measured to assess the association between SES, intestinal parasitic infections and markers of intestinal barrier function and taking into account the alteration of gut microbiota after treatment.

The following questions were addressed:

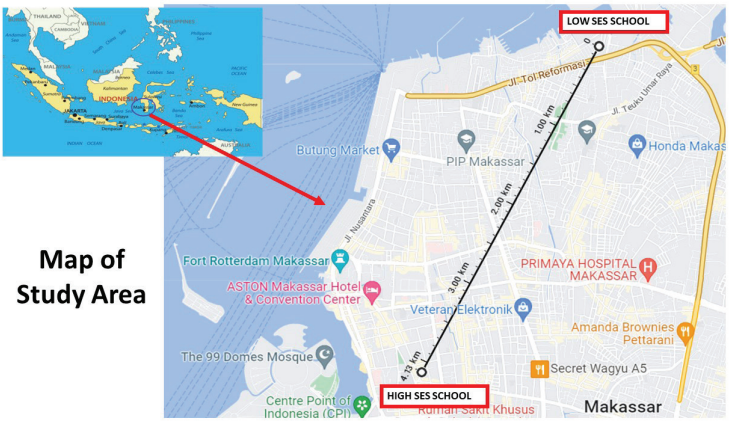
What is the effect of SES on the outcome of BCG vaccination in young infants?

BCG is one of the vaccines that is given early in life to infants in Indonesia. This vaccine has been known to have protective effect against meningitis and miliary tuberculosis (TB) in children [37] and also known to have non-specific effects unrelated to TB during childhood [38]. One way to measure the strength of the immune response to BCG vaccine is by assessing BCG scarification. In **Chapter Two**, differences on BCG scar size and factors contributing to the development of the scar were investigated in high and low-SES newborns. To this end, we recruited pregnant mothers at the third trimester of pregnancy and followed up the babies at birth and at 10 months of age. Pathway analysis was used to investigate factors contributing to the development of BCG scar, in particular, exposure to infection during gestational period (third trimester) and nutritional status of the baby at birth. In addition, we

also explored whether the size of BCG scar is affected by nutritional status at birth through measuring adipokines.

What is the impact of SES on atopic sensitization, bacterial gut microbiota and intestinal barrier function in schoolchildren?

Study area



Map of Study Area

In **Chapter Three**, Allergic reactions and sensitization to Hymenoptera venoms and aeroallergens were compared between high and low-SES schoolchildren and the question whether helminth infection might be involved in these reactions. In addition, we also assessed the agreement between SPT and sIgE positivity to allergens.

Together, **Chapter two** and **Chapter three** investigate how SES, nutritional status and exposure to infection might affect the immune responses of Indonesian children living in an urban area.

It is important to investigate how intestinal parasites [39], gut microbiota [40], and gut permeability [41], which can influence the immune system, differ in high versus low SES schoolchildren. In **Chapter Four, an** exploratory analysis was conducted of gut microbiota of Indonesian schoolchildren living in an urban area but with distinct SES. We compared the diversity and composition of bacterial gut microbiota in children from high and low-SES schools, taking into consideration the intestinal parasitic infections and the nutritional status of the children. In **Chapter Five,** the differences in intestinal permeability (determined by LMR, lactulose-mannitol ratio) between high and low-SES children was investigated. In addition, markers of intestinal cell injury (I-FABP, intestinal fatty acid binding protein), bacterial translocation and inflammatory markers (LBP, LPS-binding protein), were measured. Furthermore, we also asked whether presence of intestinal parasites and composition of bacterial gut microbiota contributed to the association between SES and intestinal permeability.

In **Chapter Six**, the findings of this thesis are discussed and can be used as a starting point for further research to understand the complex association between SES, intestinal microorganisms and parasites, gut barrier function, and immune responses in children.

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CHAPTER TWO

BCG SCAR, SOCIOECONOMIC AND NUTRITIONAL STATUS: A STUDY OF NEWBORNS IN URBAN AREA OF MAKASSAR, INDONESIA

(TROPICAL MEDICINE AND INTERNATIONAL HEALTH, 2019)

BCG SCAR, SOCIOECONOMIC AND NUTRITIONAL STATUS: A STUDY OF NEW-BORNS IN URBAN AREA OF MAKASSAR, INDONESIA

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ABSTRACT

Objective. To investigate factors that determine the response to BCG vaccination in urban environments with respect to socioeconomic status (SES), prenatal exposure to infections or new-born's nutritional status.

Methods. The study was conducted in an urban area, in Makassar, Indonesia. At baseline, 100 mother and new-borns pair from high and low SES communities were included. Intestinal protozoa, soil transmitted helminths, total IgE, anti-Hepatitis A Virus IgG and anti-*Toxoplasma* IgG were measured to determine exposure to infections. Information on gestational age, birth weight/ height, and delivery status were collected. Weight-for-length z-score, a proxy for new-borns adiposity, was calculated. Leptin and adiponectin from cord sera were also measured. At 10 months of age, BCG scar size was measured from 59 infants. Statistical modelling was performed using multiple linear regression.

Results. Both SES and birth nutritional status shape the response towards BCG vaccination at 10 months of age. Infants born to low SES families have smaller BCG scar size compared to infants born from high SES families and total IgE contributed to the reduced scar size. On the other hand, infants born with better nutritional status were found to have bigger BCG scar size but this association was abolished by leptin levels at birth.

Conclusion. This study provides new insights into the importance of SES and leptin levels at birth on the development of BCG scar in 10 months old infants.

Keywords : BCG scar, socioeconomic status, leptin, new-borns

`Introduction

Tuberculosis (TB) is known as one of the top 10 diseases causing high mortality worldwide. In 2017, with 391 new cases per 100.000 population, Indonesia was among the top 3 countries with absolute numbers of incident TB cases (1).

Bacille Calmette-Guérin (BCG) is a live attenuated *Mycobacterium bovis* vaccine. It is the only available vaccine used to protect against TB disease, in particular meningitis and disseminated TB in children (2). BCG is one of the most widely used vaccines worldwide. In Indonesia, BCG vaccination is included in the Indonesian national immunization program and it is given to new-borns at the age of 4-6 weeks. Beside its protective effects against TB, BCG vaccination has also shown to result in non-specific lower mortality and morbidity during childhood (3-5). BCG vaccination induces a memory T-helper-1 (Th-1) type response irrespective of when in life it is given (6). Studies have shown that reactions at the site of the BCG vaccination are associated with the production of Interferon gamma (IFN-γ) in response to the mycobacterial antigens. BCG scarification has been mentioned as a marker to a better survival and stronger immune response among BCG-vaccinated children living in countries with higher mortality rates (7, 8).

Immune responses to vaccines are associated with multiple factors such as economic status, parasite infestation and nutrition. Nutritional status at birth reflects new-borns adiposity and this might affect BCG vaccine response in these babies (9, 10). Adipocytes influence not only the endocrine system but also the immune response through several cytokine-like mediators known as adipokines, which include leptin and adiponectin (11, 12). Adiponectin and leptin are considered the most important hormones related to adipose depots in modulating metabolism and energy homeostasis. It is thought that leptin can directly link nutritional status and pro-inflammatory Th-1 immune responses, while adiponectin possesses *anti-inflammatory* properties (13-16).

With respect to economic status, a study on rotavirus vaccine showed that the efficacy of this vaccine was lower in low- compared to high-income-countries (17). Moreover, within low-income countries, a gradient in reduced efficacy was shown with decreasing Gross Domestic Products (GDP) (18). Low socioeconomic status (SES) has been linked with bad sanitation, hygiene (19), and higher exposure to infections (19-22). All these factors can have an impact on the immune system as shown by a study of twins in the USA where it was discerned that not only genetic but very importantly environmental factors can affect the immune system (23). This is confirmed by several studies involving low-middle-income-countries where geographical differences in immune profiles have been examined (24-27).

Indonesia with its rapid economic development bears a large diversity in population life style and SES. This study is aimed to better understand factors that determine the response to BCG vaccination. To this end, a study was conducted in three hospitals that serving high and low SES population. Maternal demographic data, socioeconomic characteristic, and infection status as well as their new-borns nutritional status, total IgE, and leptin levels were used for performing pathway analysis in order to better understand how BCG vaccine responses are shaped.

Methods

Ethics statement

The study was approved by the Health Research Ethical Committee, Faculty of Medicine, Hasanuddin University (ref.: 0685/H4.8.4.5.31/PP36-KOMETIK/2014). This study was executed based on codes stated in Declaration of Helsinki and International Ethical Guidelines for Epidemiological Studies. Written informed consent was obtained from mothers for the collection of their samples and their new-born samples.

Study population

The study population consisted of pregnant mothers and their born infants living in urban city of Makassar between January 2015 and May 2016. Pregnant women in the last trimester were recruited in government hospitals and private hospitals. Questionnaires were used to gather information regarding demographic, socioeconomic status and education. Based on Makassar minimum city wages, family income of IDR 60 million/year is used to define high (income equal/more than IDR 60 million/year) and low (income less than IDR 60 million/year) SES. Maternal characteristics such as gravidity, parity, miscarriage history or other medical conditions were also collected. Information about gestational age was estimated from last menstrual date.

Child information such as sex and delivery status were collected via the midwives or obstetricians through a questionnaire. Only infants born full term, delivered vaginally and healthy were included in the study. Birth weight and height were assessed of new-born wearing the minimum clothing, using baby weighing scale (GEA, Megapratama Medikalindo, Indonesia) and new-born length board. The weighing scale was calibrated using standardized weight as part of routine care. Weight for length z-score (Wflz) at birth, a proxy of new-born adiposity and nutritional status was calculated according to the WHO references value (28, 29).

BCG vaccination and scar measurement

Vaccination program in Indonesia requires every infant to be vaccinated with BCG at 6-weeks of age by staff from local primary health care center. The vaccine given contains live-attenuated *Mycobacterium bovis* Paris 1173-P2 strain (Biofarma, Bandung, Indonesia). The resulting BCG scar was measured at 10 months of age by same person from the research team. Mean diameter of scar size was calculated from diameters perpendicular to each other. Immunization card which proved that the newborns were BCG-vaccinated were recorded.

Blood and stool samples collection

Maternal blood samples were collected in the last trimester of pregnancy while cord blood samples were collected right after delivery. All blood samples were transported to the laboratory at Hasanuddin University Medical Research Center (HUM-RC) and processed within 4 hours after collection. Maternal stool samples were collected and stored at -80°C until further analysis.

Parasitological examination

DNA isolation from stool was performed as described elsewhere (30, 31). Two different panels of multiplex real-time PCR were used to detect and quantify soil transmitted helminths and intestinal protozoa. Panel 1 targets hookworm (*Ancylostoma duodenale, Necator americanus*), *Ascaris lumbricoides*, *Trichuris trichuria*, and *Strongyloides stercoralis* (30-33) while panel 2 targets *Entamoeba histolytica, Dientamoeba fragilis, Giardia lamblia,* and *Cryptosporidium* spp. (33-36).

Measurements of total IgE, anti-Hepatitis A Virus IgG, anti-*Toxoplasma gondii* IgG, leptin and adiponectin

Total IgE level was measured using ELISA technique as described previously (30). For this assay, maternal and cord sera were diluted 50 and 2 times in PBS containing 0.05% Tween20, respectively (Tween-20, Sigma-Aldrich, St. Louis, MO, USA). The results were expressed in International Units per millilitre (IU/ mL).

Anti-Hepatitis A Virus IgG (anti-HAV IgG) and anti-*Toxoplasma gondii* IgG (anti-Toxoplasma IgG) were measured at the clinical microbiology laboratory of the Department of Microbiology at Leiden University Medical Center as part of routine diagnostic procedures. Samples were considered to be positive for anti-HAV IgG if the signal-to-cut-off (S/CO) ratio was ≥ 1.00 while for Anti-Toxoplasma Ig-G, the samples were considered to be positive if the titre was ≥ 8 IU/ml.

Adiponectin and leptin levels were quantified using Human Adiponectin Duo Set and Human Leptin Duo Set (R&D System, Abingdon, UK) at Department of Parasitology in LUMC, according to manufacturer's guidelines (37). Cord sera were diluted 40 and 10000 times for measurement of leptin and adiponectin, respectively. The values were expressed in ng/mL and µg /mL for leptin and adiponectin, respectively.

Statistical analysis and conceptual framework

Descriptive data was presented in mean ± standard deviation for normally distributed data and median (Interquartile range, IQR) for non-normally distributed data. The correlation between two continuous data was done using Pearson correlation for normally distributed data or Spearman's rank correlation for non-normally distributed data. Prevalence was calculated as percentage of collected data. Pearson chi-square test was used to compare the prevalence of infection between two groups.

To obtain normally distributed data, maternal total IgE, cord leptin and adiponectin level were log10-transformed. Student t-test was then used to compare the mean differences between two groups. Since the levels of cord blood total IgE, anti-HAV IgG and anti-Toxoplasma IgG were not normally distributed even after transformation, the comparison between two groups was performed using Mann-Whitney U test.

The conceptual framework in Figure 1 was used to answer our research question. First, (A) we assessed whether the effect of SES to the size of BCG scar is through exposure to infections. Second, (B) we assessed the contribution of SES to the size of BCG scar is through the nutritional status pathway and whether leptin or adiponectin have a mediatory role in this path.

We primarily performed a linear regression to obtain estimate coefficients of SES on the size of BCG scar. Next, to test the contribution of SES on BCG scar through infection pathway (A), this model was adjusted for total IgE, anti-HAV IgG or anti-Toxoplasma IgG as a proxy of prenatal exposure to infection. In pathway (B), we first assessed association between SES and WfLz, a proxy of adiposity at birth. Subsequently, the effect of WfLz on BCG outcome was modelled by adjusting with leptin and adiponectin. Mediation was considered to occur if (1) all the crude association between variables tested in this pathway were significant; (2) the association disappeared and the effects attenuated when the model was adjusted for total IgE, leptin or adiponectin.

All models above were analysed using linear regression with adjustments for gestational age, sex and other variable that we found relevant to be account as confounder. Statistical test was considered significant at $p<0.05$. All data were analysed using IBM Statistical Package for the Social Sciences (SPSS) Statistics version (IBM-SPSS Inc., Chicago, IL, USA).

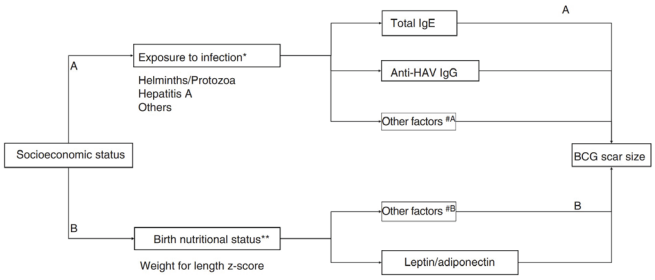

Figure 1. Conceptual framework of the study. The analysis of the association between Bacille Calmette–Guérin (BCG) scar size and SES through two different pathways. The analysis asked whether SES contributes to the outcome of BCG vaccination through (A) prenatal exposure to infection or (B) birth nutritional status pathway, using linear regression adjusted with gestational age and child gender for both pathways. *total IgE and anti-Hepatitis A IgG were measured as proxy of current or previous exposure to infections. **weight-for-length *z*-score was measured as a proxy of nutritional status at birth. $^{\#A/\#B}$ Other factors that were not assessed in pathway A or B.

Results

Characteristics of the study population

Maternal characteristics in low and high SES are presented in Table 1. The prevalence of any intestinal protozoa was not different between low and high SES. Although no significant differences in the prevalence of soil transmitted helminths was seen between two groups, the prevalence of *A. lumbricoides* (12% vs 4%) and *T. trichiura* (12% vs 6%) was slightly higher in low than in high SES mothers, respectively. The prevalence of hookworm (2%) and *S. stercoralis (2%)* was very low.

The levels of total IgE were significantly higher in low compared to high SES mothers [geomean (95% confidence interval, CI), 248.08 IU/ml (175.36-350.96 IU/ml) and 131.80 IU/ml (95.94-181.06 IU/ml), respectively; p=0.008] (Table 1).

Table 1. Baseline characteristics of the study population of Low (low SES) and High (high SES) socio-economic status

	Total		Low SES		High SES		P value[$]
	N	Results	N	Results	N	Results	
Mothers							
Age, years, mean ± SD	100	27.44 ± 4.90	50	26.71 ± 5.27	50	28.18 ± 4.42	0.135
Primigravida, N, n (%)	100	53 (53)	50	30 (60)	50	23 (46)	0.229
Intestinal parasites infection							
Any helminth, N, n (%)	100	16 (16)	50	9 (18)	50	7 (14)	0.585
Ascaris lumbricoides, N, n (%)	100	8 (8)	50	6 (12)	50	2 (4)	0.140
Trichuris trichiura, N, n (%)	100	9 (9)	50	6 (12)	50	3 (6)	0.295
Any protozoa, N, n (%)	100	20 (20)	50	17 (34)	50	13 (26)	0.517
Entamoeba histolytica, N, n (%)	100	3 (3)	50	2 (4)	50	1 (2)	0.558
Dientamoeba fragilis, N, n (%)	100	26 (26)	50	14 (28)	50	12 (24)	0.648
Giardia lamblia, N, n (%)	100	4 (4)	50	2 (4)	50	2 (4)	1.000
Cryptosporidium. spp N, n (%)	100	1 (2)	50	1 (2)	50	0 (0)	0.315
Any intestinal parasites, N, n (%)	100	42 (42)	50	23 (46)	50	19 (38)	0.418
Total IgE, IU/ ml, geomean (95%CI)	100	180.82 (142.31-229.75)	50	248.08 (175.36-350.96)	50	131.80(95.94-181.06)	**0.008**
Leptin, ng/ml, geomean (95%CI)	100	30.28 (26.40-34.73)	50	29.45 (24.00 - 36.12)	50	31.15 (25.77 - 37.65)	0.687
Adiponectin, µg/ml, geomean (95%CI)	100	3.41 (3.00-3.87)	50	3.58(3.04 - 4.23)	50	3.25 (2.67 - 3.96)	0.453

New-borns							
Sex, female N, n (%)	100	50 (50)	50	28 (56)	50	22 (44)	0.317
First born, N, n (%)	100	52 (52)	50	30 (60)	50	22 (44)	0.161
Gestational age, weeks, mean ± SD	100	39.71±1.6	50	39.69 ± 1.68	50	39.73 ± 1.53	0.902
Birth weight, grams, mean ± SD	100	3130.5±402.76	50	3194.64 ± 368.26	50	3156.67 ± 415.79	0.666
Weight-for-length z- score at birth, mean ± SD	100	-0·50±0·63	50	-0·47 ± 0·52	50	-0·46 ± 0·65	0.887
Total IgE, IU/ml, median (IQR)	100	0.07(0.07-0.21)	50	0.12(0.07-0.76)	50	0.07(0.07-0.07)	**<0.001**
Ratio S/CO anti-Hepatitis A Virus IgG, median(IQR)	98	9.60 (7.77-10.37)	49	9.93 (9.07-10.46)	49	9.33 (0.30 - 10.28)	0.088
Anti-Hepatitis A Virus IgG seroposi-tivity, N, n (%)	98	77 (78.6)	49	42 (85.7)	49	35 (71.4)	0.139
Anti-*Toxoplasma* IgG titres, IU/ml, median(IQR)	100	0.00 (0.00-41.25)	50	0.00 (0.00 - 44.25)	50	0.00 (0.00 - 37.00)	0.846
Anti-*Toxoplasma* IgG seropositivity, N, n (%)	100	35(35)	50	20 (40)	50	15 (30)	0.402
Leptin, ng/ml, geomean (95%CI)	100	10.04(8.30-12.14)	50	9.15 (7.17-11.68)	50	11.01 (8.17-14.84)	0.336
Adiponectin, µg/ml, geomean (95%CI)	100	16.44(14.35-18.83)	50	17.73 (14.68-21.41)	50	15.24 (12.48-18.61)	0.271

Data presented as number of positives (n) of the total population (N) and as percentage of total population (%), SD: standard deviation, IU: international unit; S/CO: signal-to-cut-off. [$]unadjusted; Bold: p value<0.05.

Anti-HAV IgG S/CO ratio was slightly higher in mother with low compared to high SES [median (IQR), 9.93 (9.07-10.46) and 9.33 (0.30 - 10.28), respectively; p=0.088] (Table 1). There were no differences in the levels of IgG antibody against *T. gondii* between two groups. Similarly, adiponectin or leptin levels were not different between low and high SES mothers.

Of 100 new-borns that were included in the study, no significant differences in sex ratio, nor gestational age between low and high SES group was seen. The birth weight, height and Wflz were not different between low and high SES new-borns (Table 1). The level of total IgE in cord blood was significantly higher in low (median [IQR], 0.12 IU/ml [0.07-0.76]) compared to high SES (median [IQR], 0. 07 IU/ml [0.07-0.07]) new-borns (p< 0.001) (Table 1).

The levels of leptin and adiponectin were not different between high and low SES new-borns. When gender was considered, girls either form high or low socioeconomic status tended to have higher leptin levels compared to boys (High SES: geomean [95%CI] = 15.25 ng/ml [9.76-23.84] vs 8.52 ng/ml [5.74-12.69], p=0.103; Low SES: 10.90 [8.09-14.70] vs 7.31 ng/ml [4.84-11.03], p=0.051), in girls and boys, respectively.

During follow up at 10 months of age, we were able to collect data from 59 out of 100 infants (29 from low SES and 30 from high SES). There were no differences in sex ratio between these high and low SES groups. In terms of weight, high SES infants gained more weight compare to low SES infants (mean ±SD, 5103.33 ± 534.65 vs 4777.58 ± 466.28; p=0.016). The major reason of loss to follow up was families moving out of the city. There were no differences in gestational age, sex, birth weight, Wflz, total IgE, leptin, nor adiponectin levels between those who remained in the study and those who were lost to follow up.

The effect of SES and exposure to infection on the size of BCG scar

All 59 infants that participated in the follow up time points were vaccinated. Among these infants, 89.3% (n=53/59) had developed a BCG scar, while 11.7% (n=6/59; 5 from low SES and 1 from high SES) infants did not develop BCG scar. Comparison of BCG scar size between the two groups (Figure 2) revealed that infants from high SES have bigger scar size compared to low SES (2,2 ± 0,98 mm vs 1,65 ± 1,01mm, p=0.041, respectively).

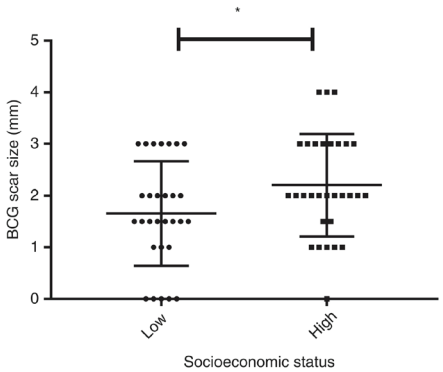

Figure 2. The BCG scar size in low and high socioeconomic status infants measured at 10 months of age. Data presented as Mean ± SD. * indicates p<0.05 (adjusted for gestational age, sex, and weight-for-length z-score)

To investigate whether prenatal exposure to infection has an effect on the size of BCG scar, we analysed the association between the size of BCG scar and total IgE, anti-HAV IgG and anti-Toxoplasma IgG. We found an inverse relationship between BCG scar size and total IgE (estimates [95%CI] = -0.33 [-0.67 - -0.004] p=0.048) but no association with either anti-HAV IgG or anti-Toxoplasma IgG (-0.015 [-0.079 – 0.048], p=0.625; and -0.003 [-0.002 – 0.008], p=0.243; respectively).

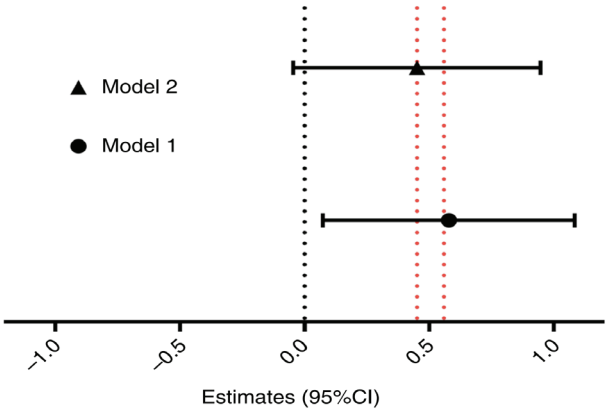

Figure 3. Effects of socioeconomic status on BCG scar size at 10 months. Model 1: crude (adjusted for gestational age, sex, and birth weight-for-length z-score); Model 2: Model 1+ cord total IgE. Data presented as beta estimate with 95% confidence interval.

Next, the association between SES and BCG scar size were analysed in multivariate analysis, adjusted with gestational age, sex, and Wflz. The results showed that the size of BCG scar remained larger in high SES compared to low SES infants (estimate [95%CI] = 0.559 [0.083-1.094], p=0.022) (Figure 3 Model 1). Interestingly, when the model was adjusted with total IgE (Figure 3 Model 2), the effect of SES on the size of BCG scar was slightly attenuated (estimates [95%CI] = 0.451 [-0.045-0.947], p=0.074) and fell short of significance in terms of predictor of BCG scar size.

Nutritional status at birth and its association with cord blood adipokines and BCG scar size

To find out whether the size of BCG scar is affected by birth nutritional status through adipokines (Figure 4), we first analysed the association between Wflz and adipokine levels. The results showed that the levels of leptin and adiponectin increased with increasing Wflz (estimates [95%CI] = 1.54 [1.16-2.06], p=0.003 and 1.43 [1.16-1.77] p=0.001, respectively). The analysis on the association between BCG scar size and adipokine levels revealed that the BCG scar size increased with increasing leptin and adiponectin levels (estimates [95%CI] = [9.46 [2.40 – 37.32], p=0.002]; [13.33 [1.82 – 97.27], p=0.012]; respectively).

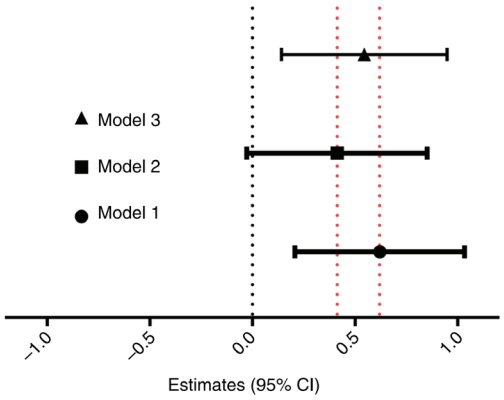

Figure 4. Pathway analysis of nutritional status at birth and BCG scar size at 10 months. Model 1: Crude (adjusted for gestational age, sex, and SES). Model 2: Model 1 + Leptin. Model 3: Model 1 + Adiponectin. Data presented as beta estimate with 95% confidence interval.

Pathway analysis on Figure 4 model 1 showed that higher Wflz was associated with larger size of BCG scar (estimates [95%CI] = 0.620 [0.206-1.034], p=0.004). Interestingly, when leptin level was also considered (model 2), the association between nutritional status at birth and BCG scar size is no longer significant and the Wflz effects to BCG scar size was attenuated from 0.620 [0.206-1.034] to 0.412 [-0.027-0.852]. However, when adiponectin level was considered (model 3), this had less effect on the effect of birth Wflz effects on BCG scar size; from 0.620 [0.206-1.034] to 0.545 [0.141-0.949] and Wflz remained as a significant predictor of BCG scar size.

Discussion

In the present study which involved mothers and their new-borns residing in an urban area of Makassar, Indonesia, we reported that both SES and nutritional status at birth determine the response towards BCG vaccination measured at 10 months of age. Regarding SES, our study found that infants born to low SES families have smaller BCG scar size compared to infants born to high SES families and that total IgE partly contributed to reducing the size of scars. Conversely, infants born with better nutritional status were found to have bigger BCG scar size but this association was abrogated by leptin levels at birth.

Similar to the finding in the current study, a study in school-aged children in Dominican Republic found positive correlation between BCG scar prevalence and an index of socio-economic factors (38). In epidemiological studies, low SES has been associated with poorer hygiene practices which in turn could increase exposure to other microorganism or parasites (39). Infection with parasitic helminths is known to induce a strong Th-2 immune response that can lead to elevated levels of IgE (40, 41). In the current study, we found no statistical significant differences in the prevalence of current helminth infections between mothers with low and high SES, however, the levels of total IgE in mothers/ new-borns with low SES were significantly higher than mothers/ new-borns with high SES. This finding indicates that previous helminth infections, or other factors might contribute to the high levels of total IgE. Our previous study in Indonesia, which looked at the development of Th-2 responses from

infancy to 4 years of age reported that children born to mothers with low education or low SES showed stronger development of total IgE responses over time compared to children born to mothers with high SES or high education. In the study, maternal helminth infection status was not the strongest factor determining the Th-2 polarisation in their children (42). Like infection with *M. tuberculosis*, BCG vaccination induces Th-1 type immune responses and cause suppression of Th-2 type responses.(43) Here we found that BCG scar size is inversely associated with the levels of total IgE which is in an agreement with Soysal A *et al* who reported that the presence of scar was associated with lower levels of total IgE (44).

The nutritional status at birth in our study population were within the normal range and no differences as a function of SES were found. Furthermore, adipokine levels were observed to be positively associated with nutritional status which is consistent with previous studies showed adiponectin and leptin levels increases with increasing body composition at birth (45-48). The finding that leptin, but not adiponectin, strongly attenuated the relationship between nutritional status and the BCG scar size suggests that leptin levels and not nutritional status at birth determine the response toward BCG scar formation. To our knowledge, our study is the first to investigate the relationship between leptin levels and BCG scar size. Regarding nutritional status, a recent study in 6-12 months old babies from Guinea Bissau found that BCG scarification was not associated with nutritional status determined by mid-upper-arm-circumference as well as weight-for-age (49).

Leptin is a 16-kD hormone mainly secreted from adipocytes (50, 51) and has been reported to be positively associated with intrauterine foetal growth (52), birth weight and total body fat content of neonates (53). Leptin is released in the circulation in proportion to the number of adipocytes and acts at the hypothalamus receptor to maintain metabolic homeostasis (51, 54, 55). Besides its role in energy homeostasis, there is also evidence for an immunomodulatory role of leptin. Leptin may shift immune responses towards Th-1 phenotype (15, 55, 56). In this study, for the first time, we observed that infants with higher neonatal leptin levels had bigger BCG scar size. Although no studies in human have been reported on the role of leptin in vaccine induced protection, in experimental mouse model, Wehrens and colleagues reported that functional leptin receptor signalling is required for mediating an effective protective response against *Helicobacter pylori* (57).

Among infants in this study, 10.16% did not develop BCG scar. The presence or absence of a scar is often used as an indicator of BCG vaccination in a clinical context as well as in health surveys to assess vaccine coverage (58). Our finding was quite similar to that observed by Rani *et al* (59) and Sara *et al* (60) where 8.6 – 9.8 % of BCG-vaccinated infants did not develop any scar. Previous studies have been reported that the development of BCG scar in BCG-vaccinated children is also influenced by several factors such as sex, seasons at vaccination, sequence of vaccinations, BCG administration techniques and BCG strain (4, 7, 61, 62).

Some limitations are worth noting. The small sample size and considerable loss to follow up, however, some interesting data were generated that should be followed up in larger studies powered to discern the role of leptin in modulating BCG scar size.

In conclusion, BCG scar size was influenced by SES and leptin levels at birth. Furthermore, total IgE partly contributed to reducing the size of BCG scar. This study would need to be followed up to determine whether leptin and total IgE affect BCG vaccine efficacy and it also provides a departure point for thinking of strategies whereby leptin levels are increased while total IgE levels are decreased in order to improve responses to BCG vaccine.

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Conflict of interest statement

We declare that we have no conflict of interest.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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CHAPTER THREE

BEE AND WASP VENOM SENSITIZATION IN SCHOOLCHILDREN OF HIGH AND LOW SOCIO-ECONOMIC STATUS LIVING IN AN URBAN AREA OF INDONESIA

(INTERNATIONAL ARCHIVES OF ALLERGY AND IMMUNOLOGY, 2021)

CHAPTER 3

BEE- AND WASP-VENOM SENSITIZATION IN SCHOOLCHILDREN OF HIGH AND LOW SOCIO-ECONOMIC STATUS LIVING IN AN URBAN AREA OF INDONESIA

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ABSTRACT

Background. There is not much known about venom allergy in tropical regions. Here, we studied the prevalence of specific IgE (sIgE), skin prick test (SPT) reactivity and reported sting-related symptoms, in high- and low socio-economic status (SES) schoolchildren living in urban city of Makassar in Indonesia.
Methods. Children from high- (n=160) and low- (n=165) SES schools were recruited. Standardized questionnaires were used to record information on allergic disorders as well as sting-related symptoms. Parasitic infection, SPT reactivity and sIgE to *Apis mellifera* (bee-venom) as well as *Vespula spp* (wasp-venom) were assessed.
Results. SPT reactivity to bee- and wasp-venom were 14.3% and 12.7%; while the prevalence of sIgE was 26.5% and 28.5%, respectively. When SES was considered, prevalence of SPT to bee- and wasp-venom was higher in high-SES compared to low-SES schoolchildren (bee: 22.8% vs 5.7%, p<0.001; and wasp: 19.6% vs 5.7%, p<0.001). Conversely, sIgE to both venoms was lower in high-SES compared to low-SES (bee: 19% vs 34%, p=0.016; and wasp: 19% vs 38%, p=0.003). Furthermore, among SPT positive subjects, considerable proportion had no detectable sIgE to bee- (65.85%) or wasp-venom (66.67%). Altogether the sensitizations were rarely translated into clinical reaction, as only 1 child reported significant local reaction after being stung. No associations with parasitic infections was found.
Conclusions and Clinical Relevance. Sensitization against bee- or wasp-venom is quite prevalent amongst schoolchildren in Indonesia. The discordance between SPT and sIgE might suggest the direct (non-IgE) effect of venoms in skin reactivity. Recorded sensitizations had poor clinical relevance as they rarely translated into clinical symptoms.

Keywords: venom and insect allergy, epidemiology, pediatrics, urban, developing country

Introduction

The global magnitude of venom allergy is not completely established as the majority of studies have been conducted in temperate or sub-tropical countries. The prevalence of *Hymenoptera* venom sensitization has been reported to vary from 3.66 % to 41.6% [1-5], whereas prevalence of systemic allergic reactions to venoms has been estimated to be between 0.34% to 16% [3, 6-9]. A single *Hymenoptera* sting may induce large local reactions (LLR) and/or systemic reactions (SR) in venom-allergic individuals, and in some instances, SR can be fatal [10]. Altogether, there is not much information on the extent of venom allergy and symptoms in tropical countries.

Indonesia, a tropical country, located across the equator with a warm climate and high humidity, provides favorable conditions for Hymenopterans like bees and wasps. To gather information on venom allergy in Indonesia, we conducted a study amongst schoolchildren of high and low socioeconomic status (SES) living in Makassar, Sulawesi. We investigated the prevalence of reported *Hymenoptera* sting reactions and their correlation with *Hymenoptera* venom-specific IgE and skin reactivity. As the prevalence of parasitic infections can be high in children of low SES [11] and inverse relationship between aeroallergens and helminth infections have been reported [12], we also assessed whether the same is true for venom allergy.

Material and Methods

Study area and design

This study was approved by Health Research Ethics Committee of Faculty of Medicine, Hasanuddin University (Ref: 1504/H04.8.4.5.31/PP36-KOMETIK/2016). Of 500 children (low SES: n= 250; high SES: n=250) invited, 325 children (65%) participated in the study.

The study was conducted in two elementary schools in Makassar, Indonesia. Two elementary schools that were distinct in SES were selected. The low SES school was located near the sea port where majority of parents earned wages by working in fishing or other low-education labor. Students from this school mainly lived in the school neighborhood in a densely populated area which is located near a landfill. The high SES school was situated in the city center and all students were living scattered over the city. The majority of the students' parents worked as a moderate-to-high level civil servants or as professionals in business sector.

After discussion with the school management team and obtaining their agreement to participate in the study, a letter with detailed information on the study was sent to parents of all children in 3rd and 5th grade in both high and low SES schools. The parents could call the study team with any questions regarding the study. We asked the parents' permission to include their children in the study, which was indicated by signing the informed consent letter. Only children who returned the signed letters were enrolled into the study.

Based on availability of sufficient serum samples and considering budgetary restriction, all 70 samples of subjects who were SPT positive to any venom and 130 samples of randomly selected subjects who were SPT negative to any venom, were selected for measurements of specific IgE against *D. pteronyssinus*, bee- and wasp-venom.

Questionnaires

Standardised questionnaire to gather data regarding demographic and socioeconomic status [11] as well as information on *Hymenoptera* sting allergy [13] were administered to the parents. In addition, questions about self-reported asthma, rhinitis, and eczema were asked using a questionnaire which was modified from ISAAC questionnaires when translated into Bahasa Indonesia (Supplementary Questionnaires).

The parents were asked whether the children ever helped their parents with beekeeping or gardening, and some sting-related questions such as, whether their children had ever been stung by bee or wasp, and whether they noticed any response after the sting such as: a large local reaction (LLR), characterized by a swelling defined to exceed 10 cm that lasts for more than 24 hours; and a Systemic Reaction (SR), characterized as generalized hives or angioedema after the sting, or breathing difficulties, or loss of consciousness after the sting [14]. The questionnaires were all translated into Bahasa Indonesia and answered as a Yes or No.

Skin prick testing (SPT)

SPT reactivity to bee (*Apis mellifera*), and wasp (*Vespula spp.*) venom were tested using allergen extract at concentrations 300 µg/ ml for each venom (Soluprick-SQ, ALK-Abello, Madrid, Spain). SPT reactivity to mould (*Aspergillus spp.*), cockroach (*Blattella germanica*) and house dust mites such as *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* were also tested (Soluprick-SQ, ALK-Abello, Madrid, Spain). Histamine dihydrochloride (10mg/ml) was used as positive control, meanwhile, allergen diluents as the negative control (Soluprick-SQ, ALK-Abello, Madrid, Spain). This measurement was performed using standard protocol as described previously [15, 11]. SPT was carried out by one trained research staff and measured by another staff for whole study population. Wheal size were assessed at 15 minutes by measuring mean of perpendicular diameter. Wheal size ≥ 3 mm was considered as positive. Anthropometric measurements such as body weight and height were also performed.

Specific and total IgE measurements

Based on availability of sufficient serum samples and considering budgetary restriction, all 70 samples of subjects who were SPT positive to any venom and 130 samples of randomly selected subjects negative for SPT to any venom, were selected for measurements of specific IgE against *D. pteronyssinus*, bee- and wasp-venom.

Total IgE levels in serum were measured using ELISA as previously described with minor modification [16]. For this assay, serum sample was diluted 50 times using PBS containing 0.05% Tween-20. The results were expressed as IU/ml. Allergen specific IgE (sIgE) antibodies against house dust mite *D. pteronyssinus* (d1), bee (i1) and wasp-venom (i3) from serum samples were determined by the ImmunoCAP system according to the manufacturers recommendation (Thermo-Fisher Scientific, Uppsala, Sweden). Specific IgE levels ≥0.35 kU/L was interpreted as positive.

Parasitological examination

Stool samples collected from children were used to assess intestinal helminths and protozoa infection. Kato-Katz method on stool samples was performed to quantify eggs from

soil-transmitted helminths such as *Ascaris lumbricoides* and *Trichuris trichiura.* Aliquots of fresh stool samples were preserved in -80°C for further analysis. DNA extraction was performed from frozen stool sample as described elsewhere [17]. PCR was used for detection *of Entamoeba histolytica, Dientamoeba fragilis, Giardia lamblia* and *Cryptosporidium spp.* as has been described in detail previously [18-20].

Statistical analysis

Central distribution of continuous variables was presented as mean ± SD if the data was normally distributed and as median (IQR) if not normally distributed. Total IgE level was log-transformed and the value was presented as geometric mean (95% CI). Categorical variables were presented as proportion. Differences between two groups was tested using student t-test or Mann-Whitney U for continuous data or chi square for categorical data. Logistic regression was used to analyse potential factors that might contribute to the development of sensitization to allergens tested. In multivariate analysis, we included age, sex, zBMI as a priori confounders, as well as other variables that were significant in univariate analysis.

The agreement between skin test and IgE positivity was analysed using Cohen Kappa test while the Spearman rank correlation was used to assess the correlation between skin wheal size and the levels of sIgE [21]. Agreement is defined as having SPT positive with corresponding sIgE positive or having SPT negative with corresponding sIgE negative.

All statistical measurements considered to be significant if p-value <0.05. Statistical analysis was performed using IBM Statistical Package for the Social Sciences (SPSS) Statistics version 25 (IBM-SPSS Inc., Armonk, NY, USA).

Results

Characteristics of the study populations

In total, 325 children were recruited from low (n=165) and high (n=160) SES schools. No differences in terms of age and sex were observed between the two groups (Table 1). Low SES children had significantly lower z-BMI compared to children from high SES school (mean ± SD: -0.96 ± 1.18 vs 0.26 ±1.48; respectively; p-value<0.001). Stool samples were collected from 255 (low SES, n=128; high SES, n=127) children. There was no difference between children who provided stool samples and those who did not in terms of age, sex, and zBMI. Helminths (65.6% vs 1.6%; p-value<0.001) and protozoa infections (72.8% vs 39.2%; p-value<0.001) were more prevalent in low compared to high SES schoolchildren (Table 1). The levels of total IgE were higher in low compared to the high SES schoolchildren (geomean (95% CI): 422.08 (342.52 - 520.12) vs 164.06 (135.38 - 198.81); p-value<0.001)

Sensitization to aeroallergens and venoms

Skin prick tests were performed in 322 children (low SES=164 and high SES=158). After excluding 7 children of the low SES group with a histamine negative response, we found no differences in histamine wheal size between low (n= 157) and high (n=158) SES schoolchildren (mean ± SD, 6.1 ± 0.9 mm vs 6.3 ± 0.9 mm, respectively). Next, when we compared the wheal size of SPT to each *D. pteronyssinus, D. farinae,* cockroach, mould, bee- and wasp-venom allergen, between low and high SES and observed no differences in wheal size of skin response to allergens tested, except for bee-venom which is slightly larger in the

low SES compared to the high SES (median (IQR): 4.0 mm (3.25-4.25) and 3.0 mm (3.0-3.5), respectively) (Supplementary Table 1).

The highest percentage of positive SPT was to bee-venom (14.3%), followed by cockroach (13.3%), wasp-venom (12.7%), *D. pteronyssinus* (11.8%), *D. farinae* (7.9%) and mould (5.4%). Strikingly, for all allergens tested the percentage positive SPT was significantly higher in high compared to low SES schoolchildren. Positivity for any SPT was 51.3% vs 22.9%, in high versus low SES group (p-value<0.001) and for specific allergens the values were 22.8% vs 5.7% to bee-venom (p-value<0.001); 17.1% vs 9.6% to cockroach (p-value= 0.049); 19.6 % vs 5.7% to wasp-venom (p-value<0.001); 18.4% vs 5.1% to *D. pteronyssinus* (p-value<0.001); 12.7% vs 3.2% to *D. farinae* (p-value=0.002); and 8.9% vs 1.9% to mould (p-value=0.006).

In the low SES school, there was no difference in zBMI of children who were positive or negative for allergen SPT (Supplementary Table 2). Moreover, no differences were found in the prevalence of parasitic infection between subjects positive and negative skin test reactivity (Supplementary Table 3).

In contrast to skin test reactivity, analysis of allergen specific IgE revealed that the prevalence of IgE positivity to both venoms was higher in low compared to high SES school (34.0% vs 19.0%, p-value=0.016, for bee-venom; and 38% vs 19%, p-value=0.003, for wasp-venom). The percentage of children with IgE positivity to both bee- and wasp-venom was 17.5% (25% in the low SES and 10% in the high SES, p-value=0.005). There were no differences in the specific IgE positivity to *D. pteronyssinus* between the two groups (Table 1).

Potential factors associated with sIgE positivity and SPT reactivity to any venom

In univariate analysis, skin reactivity was positively associated with high SES (OR, 4.52; 95%CI, 2.28-7.59) while negatively associated with the presence of helminth infection (OR, 0.26; 95%CI, 0.12-0.59). No association with zBMI was observed. In contrast to SPT positivity, sIgE was negatively associated with high SES and high zBMI (OR, 0.45; 95% CI, 0.25-0.81 and OR, 0.77; 95% CI, 0.62-0.96; respectively). No association was observed between sIgE positivity and current helminth infection.

Multivariate analysis adjusted with age, sex, and zBMI revealed that skin reactivity to any venom remained associated with high SES (OR (95%CI), adjusted p-value: 5.15 (2.66-9.97); p.adj<0.001), Similarly, following adjustment with age, sex, and zBMI, the negative association between high SES and sIgE remained intact (0.52 (0.27-0.98); p.adj=0.042)

Reported clinical symptoms

The sting-related questionnaire was assessed in 151 children with completed data on SPT and sIgE to any venom. Of these children, 17 (11.3%) had been stung by Hymenopterans at least once in their lifetime and experienced a local reaction such as sharp burning pain, redness, and slight swelling at the location after being stung and among them, 1 (5.9%) child reported to have a history of LLR while none did report SR after being stung. The child who has reported to have LLR, had a positive skin test but negative sIgE to any venom. Among the remaining 16 children who had been stung but had no reported LLR, 7 were positive for both SPT and sIgE to any venom while 9 children had positive SPT but were negative for sIgE to any venom.

Next, we analyzed the association between sIgE and skin sensitization to *D. pteronyssinus* and self-reported clinical symptoms of allergy among 151 subjects who had completed data. The Venn diagram in Supplementary Figure S1 shows the overlap between sensitization and self-reported and/or ever-diagnosed allergic asthma, rhinitis, or eczema. Among 22 children who reported clinical symptoms of allergy, 16 (72%) were positive in specific IgE to *D. pteronyssinus*, of which, 10 children were also positive for SPT against *D. pteronyssinus*.

Discordance between specific IgE and skin test reactivity

To assess discordance between SPT and sIgE, we selected individuals with positive SPT among 197 subjects, for whom we had both SPT and IgE data. When analysing 41 subjects with positive SPT to bee-venom, we found 27 (65.9%) were sIgE negative. Similarly, among 39 subjects that were SPT positive to wasp-venom, 26 (66.7%) were sIgE negative. However, of 30 children with positive SPT to *D. pteronyssinus*, there were only 5 children (16.7%) that were negative for sIgE to *D. pteronyssinus* (Table 2A and Supplementary Figure S2).

Furthermore, the proportion of SPT negative among sIgE positive to bee- and wasp-venom were, 72.6% and 77.2%, respectively. When we stratified the analysis based on SES, the proportion of discordance was higher in the low SES compare to the high SES and was statistically significant for bee-venom (bee-venom: 84.4% and 52.6%, p=0,01; wasp-venom: 81.6% and 68.4%, p=0.27, respectively). The proportion of SPT negatives among sIgE positive to *D. pteronyssinus* were also assessed. Among 61 children with positive sIgE, 36 children (59.0%) were negative for SPT; with higher discordance in the low SES (77.8%) compared to the high SES (44.1%) (Table 2B and Supplementary Figure S2).

In Figure 1, we` plotted the wheal size of skin reactivity against the specific IgE levels. The results showed a “none to slight” agreement for bee- or wasp-venom sensitization (Kappa = 0.096 (-0.051 – 0.243), p-value = 0.175, Figure 1B and Kappa = 0.047 (-0.092 – 0.186), p = 0.499, Figure 1C, respectively), while a “moderate” agreement (Kappa = 0.434 (0.317-0.550); p-value<0.001, Figure 1A) was found for sensitization to *D. pteronysssinus*

Analysing the correlation between the wheal size and sIgE levels revealed a correlation for sensitization to *D. pteronysssinus* (rho = 0.431, p-value<0.001, Figure 1A) but neither to bee- nor to wasp-venom (rho: 0.055; p-value=0.440, Figure 1B; and rho: 0.071 p-value=0.321, Figure 1C respectively). When socioeconomic status was considered, no difference was found on the agreement analysis for sensitization to *Hymenoptera* venom between high and low SES. We also found no differences on the agreement analysis between helminth-infected vs helminth-uninfected neither between protozoa-infected and protozoa-uninfected as shown in Supplementary Figure S3.

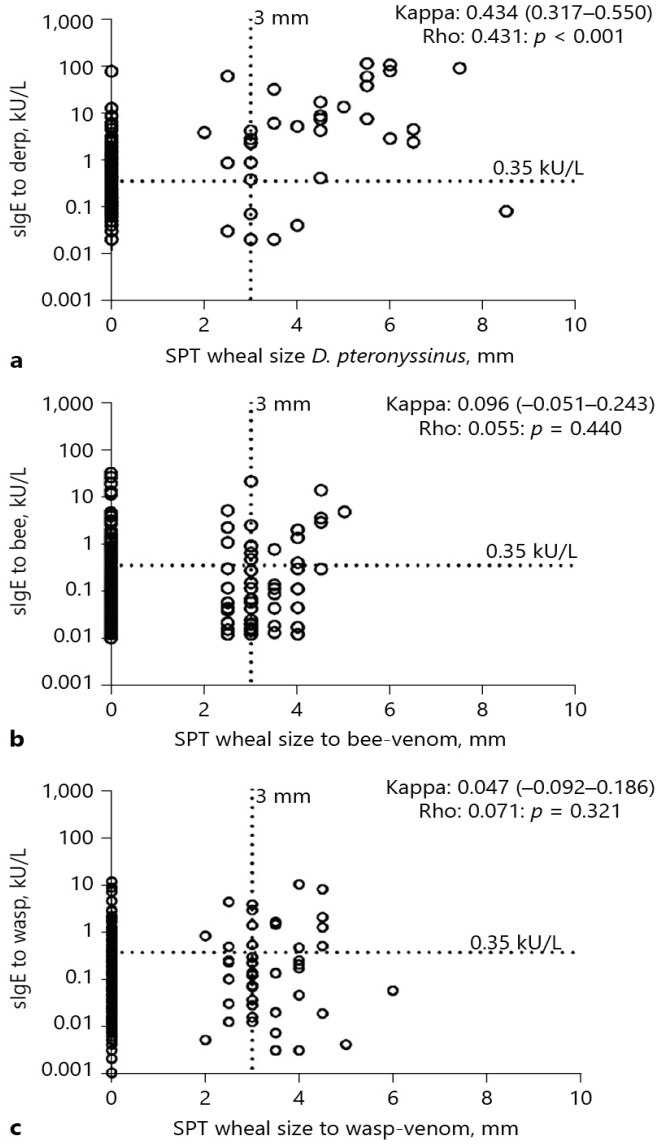

Figure 1. Comparison between SPT wheal size and sIgE to A) *D. pteronysssinus*; b) bee-venom; and C) wasp-venom. Dotted lines show sensitization cut off of 0.35 kU/L for sIgE and 3 mm for SPT wheal size. *Agreement between SPT positivity and sIgE positivity were tested using Kappa Cohen's test and presented as Kappa (95% CI). Correlation between SPT wheal size and level of sIgE to allergen tested presented as Spearman correlation coefficients (rho) and p-values.*

Table 1. Characteristic of study population

Variables	All		Low SES		High SES		*p-value*
	N	Results	N	Results	N	Results	
Age, years, (mean, SD)	325	10.26 ± 0.89	165	10.21 ± 1.08	160	10.32 ± 0.64	0.259
Sex, N, n%	325						
Male	144	44.31	73	44.2	71	44.4	0.981
Female	181	55.69	92	55.8	89	55.6	
z-BMI, mean ± SD	325	-0.36 ± 1.47	165	-0.96 ± 1.18	160	0.26 ± 1.48	<0.001
Parasites infection, N, n%							
Any intestinal parasites	234	150 (64.1)	114	101 (88.6)	120	49 (40.8)	<0.001
Any helminths	255	86 (33.7)	128	84 (65.6)	127	2 (1.6)	<0.001
Ascaris lumbricoides	255	59 (23.1)	128	59 (46.1)	127	0	<0.001
Trichuris trichiura	255	54 (21.2)	128	52 (40.6)	127	2 (1.6)	<0.001
Hymenolepis diminuta	255	2 (0.8)	128	2 (1.6)	127	0	
Any protozoa	234	110 (47.0)	114	83 (72.8)	120	47 (39.2)	<0.001
Entamoeba histolytica	234	18 (7.7)	114	16 (14.0)	120	2 (1.7)	<0.001
Dientamoeba fragilis	234	66 (28.2)	114	41 (36.0)	120	25 (20.8)	0.010
Giardia lamblia	234	87 (37.2)	114	59 (51.8)	120	28 (23.3)	<0.001
Cryptospridium parvum	234	3 (1.3)	114	3 (2.6)	120	0	
Skin prick test reactivity							
Any skin prick test reactivity	315	117 (37.1)	157	36 (22.9)	158	81 (51.3)	<0.001
Any venom	315	70 (22.2)	157	17 (10.8)	158	53 (33.5)	<0.001
Apis mellifera	315	45 (14.3)	157	9 (5.7)	158	36 (22.8)	<0.001

Vespula spp.	315	40 (12.7)	157	9 (5.7)	158	31 (19.6)	<0.001
Any aeroallergen	315	76 (24.1)	157	23 (14.6)	158	53 (33.5)	<0.001
House dust mite (HDM)	315	46 (14.6)	157	12 (7.6)	158	34 (21.5)	<0.001
Dermatophagoides pteronys-sinus	315	37 (11.8)	157	8 (5.1)	158	29 (18.4)	<0.001
D. farinae	315	25 (7.9)	157	5 (3.2)	158	20 (12.7)	0.002
Blattella germanica	315	42 (13.3)	157	15 (9.6)	158	27 (17.1)	0.049
Aspergillus spp.	315	17 (5.4)	157	3 (1.9)	158	14 (8.9)	0.006
sIgE (kU_A/L), median (IQR)							
A. mellifera	200	0.08 (0.03 - 0.37)	100	0.15 (0.04 - 0.67)	100	0.05 (0.02 - 0.22)	<0.001
Vespula spp.	200	0.10 (0.01 - 0.45)	100	0.19 (0.04 - 0.70)	100	0.04 (0.01 - 0.26)	<0.001
D. pteronyssinus	200	0.09 (0.04 - 0.66)	100	0.10 (0.05-0.39)	100	0.07 (0.03-1.48)	0.282
sIgE (cut off ≥0.35 kU_A/L)							
Any venom	200	75 (37.5)	100	47 (47.0)	100	28 (28.0)	0.006
A. mellifera	200	53 (26.5)	100	34 (34.0)	100	19 (19.0)	0.016
Vespula spp.	200	57 (28.5)	100	38 (38.0)	100	19 (19.0)	0.003
D. pteronyssinus	200	61 (30.5)	100	27 (27.0)	100	34 (34.0)	0.285
Total IgE (IU/ml), geomean (95%CI)	311	269.21 (231.40 - 313.19)	163	422.08 (342.52 - 520.12)	148	164.06 (135.38 - 198.81)	<0.001

The number of positives (n) of the total population examined (N). SD: standard deviation. z-BMI: z-score of body mass index. CI: Confidential intervals

Table 2. Proportion of tests with discordant results between sIgE and skin reactivity to *D. pteronyssinus*, bee- and wasp-venom in low- and high- SES schoolchildren.

(A) Proportion of sIgE negative among SPT positive schoolchildren			
	D. pteronyssinus	**Bee-venom**	**Wasp-venom**
All, n, N (%)	5/30 (16.7)	27/41 (65.9)	26/39 (66.7)
low-SES, n, N (%)	1/7 (14.3)	4/9 (44.4)	2/9 (22.2)
high-SES, n, N (%)	4/23 (17.4)	23/32 (71.9)	24/30 (80.0)
(B) Proportion of SPT negative among sIgE positive schoolchildren			
	D. pteronyssinus	**Bee-venom**	**Wasp-venom**
All, n, N (%)	36/61 (59.0)	37/51 (72.6)	44/57 (77.2)
low-SES, n, N (%)	21/27 (77.8)	27/32 (84.4)	31/38 (81.6)
high-SES, n, N (%)	15/34 (44.1)	10/19 (52.6)	13/19 (68.4)

Data presented as **(A)** percentage of sIgE negative (n) among sIgE positive schoolchildren (N) and **(B)** percentage of SPT negative (n) among sIgE positive (N) schoolchildren

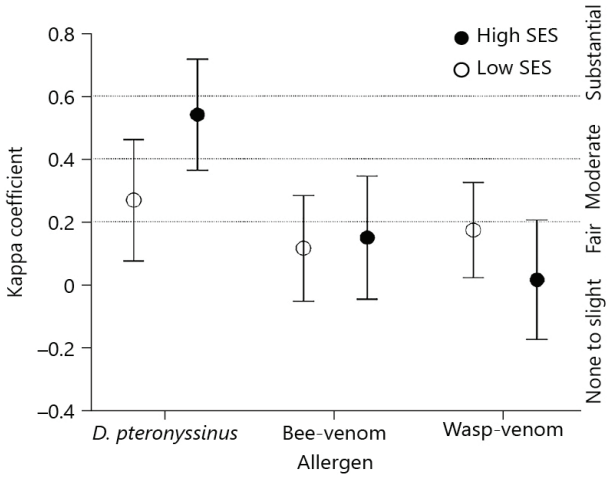

Figure 2. Agreement between SPT and sIgE to *D. pteronyssinus*, bee- and wasp-venom in high and low SES schoolchildren. *Kappa coefficient: ≤0.20 (none to slight agreement); 0.21-0.40 (fair agreement); 0.41-0.60 (moderate agreement); 0.61-0.80 (substantial agreement).*

Regarding *D. pteronyssinus* sensitization, the kappa agreement was stronger in the high compared to low SES ("moderate", 0.541 (0.364-0.717) vs "fair", 0.269 (0.076 – 0.461) for high and low SES, respectively) (Figure 2). Similarly, when we stratify the population into helminth infected and uninfected, as shown in Supplementary Figure S3, weaker agreement also observed in the helminth-infected (fair agreement: 0.228 (-0.041-0.497) compared to helminth-uninfected group (moderate agreement: 0.550 (0.383-0.716). However, no differences in the agreement analysis between protozoa infected vs uninfected groups was found.

Discussion

To the best of our knowledge, this is the first report of a study into the prevalence of *Hymenoptera* venom sensitization in schoolchildren living in an urban area of a tropical country. Studies on the *Hymenoptera* venom sensitization so far were performed in temperate or subtropical areas, where exposure to bee and wasp stings are frequent [3, 9] and these studies involved a high-risk population such as beekeepers and their relatives [22].

Here, we found the prevalence of skin reactivity to bee- and wasp-venom was 14.3% and 12.7%, respectively. A cross-sectional study in Italy reported that 2.98% and 1.45% of primary schoolchildren was positive for skin test reactivity [3]. It should be noted that the venom concentration used in the Italian study was three times lower than in our study. In addition, the test material was purchased from ALK-Abello, while the Italian study used material from Lofarma, which might also contribute to the differences in the prevalence of skin reactivity in the two studies. When specific IgE to venom was considered, we found the prevalence of positive sIgE to bee- and wasp-venom were 26.5% and 28.5%, respectively, which are similar to the findings in an adult German cohort that reported 23.1% and 31.7% IgE positivity to bee- and wasp-venom [23].

In our study, majority (88.7%) of children reported no history of having been stung. From 17 children (11.3%) who had a history of a sting, only one child (5.9%) reported LLR but none SSR. The child who reported LLR had a positive skin test, which could suggest a recent exposure to a sting. Proportion of loss of sensitization to Hymenoptera venom in skin tests has been reported to be 12% per year [24]. The prevalence of clinical reaction in our population is much lower compared to a questionnaire-based survey in Turkish children [9], which reported 24.3% and 9.9% for LLR and SSR, respectively. The prevalence of LLR in our study is comparable to a study in Irish children that reported LLR in 5.8% of the participants [7].

Studies in temperate climates have reported that asymptomatic sensitization to bee- and wasp-venom is a common phenomenon in the general population [25, 26]. Therefore, it was concluded that detected sensitization to both venoms are clinically irrelevant as the presence of sensitization to both venoms did not translate into clinical reactions. In our study, all children with positive sIgE to either bee- or wasp-venom reported no LLR nor SR after being stung. This finding is higher compared to 69.3% reported in Denmark [5].

We observed considerable skin reactivity in the absence of specific IgE to bee- and wasp-venom. This might suggest that skin reactivity to *Hymenoptera* venom is not necessarily through IgE but through IgE-independent mechanisms [27, 28]. Bee and wasp [29] venom may contain several substances that could provoke toxic reactions [30] by inducing mast cell degranulation directly resulting in positive skin reaction in some children. The low molecular weight components of venoms often mediate local toxic and non-allergic reactions while components such as hyaluronidases, phospholipase A1 and A2, antigen 5, serine proteases, and acid phosphatases do so through IgE [31].

The absence of skin reactivity despite the presence of sIgE in our population may suggest the presence of IgE with poor biologic activity, which has been described for cross reactive IgE [32]. The cross-reactive IgE might arise through recognition of *Hymenoptera* venom components, such as hyaluronidases [4, 33]. Interestingly, a 44-kD protein similar to hyaluronidase has been shown in mosquito extracts [34, 35] and might be responsible for the high sIgE that we observe to venoms. This is in line with our finding of higher sIgE positivity in the low SES, which live in conditions associated with inadequate sewage and poor sanitation, and therefore increased population of mosquitos. There is also a possibility that higher total IgE might contribute to the overall elevation of sIgE as reported by Dold et al [36].

Similar to our study, the lack of association between sIgE and skin sensitization to venoms has also been reported in a previous study [37]. However, the study was on selected subjects with SR to insect sting which reported that 32% of participants had negative skin test response. Interestingly, among these subjects, 43% had venom specific IgE antibodies. Discordance between RAST and skin test in their study may reflect the different sensitivity of the two tests for diagnosing the venom reactions and it could also be due to differences in the material used in the two tests [37].

The asymptomatic sensitization is not only seen to venom allergens, but also to other allergens as already reported in plethora of studies conducted in developing countries [38-40]. In our study, most of the children with detected sIgE or positive skin reactivity to *D. pteronyssinus* did not report clinical symptoms. When stratified according to SES, we observed discordant results in *D. pteronyssinus* sensitization where the correlation and agreement between SPT and sIgE was weaker in low SES compared to the high SES children in particular when considering the proportion of SPT negative among sIgE positive children. Skin sensitization to aeroallergens including house dust mites were higher in the high SES compared to the low SES, however, no differences were observed in the prevalence of specific IgE to *D. pteronyssinus* between high and low SES group. This finding was similar to our previous study in the same urban area [11]. In a previous study, thin children have been shown to have less skin sensitivity [41], however this is not the case in our study as the zBMI was similar between those positive and negative for SPT to any allergen tested in the low SES group. In line with our study, Keller-Franco et al [42], showed that nutritional status which was measured by BMI did not affect the skin reactivity to either histamine or *D. pteronyssinus*.

Low SES has been reported to be associated with lower prevalence of skin reactivity to house dust mite despite the presence of sIgE [40]. This we have suggested to be due to the down-modulation of skin reactions to allergens by induction of IL-10 [43]. Parasitic infections, in particular helminths are often highly prevalent in the less affluent populations, leading to expansion of regulatory T cells [44]. However, even though the SPT to venom was lower in low SES schoolchildren, when we tested the agreement between SPT and sIgE, we observed “poor” agreement in both low and high SES groups.

One of the limitations of our study is its cross-sectional design which does not allow us to determine causality and time of exposure. It is known that sensitization to venom peaks few weeks after a sting and recedes over time [24, 45]. In this study we studied past reactions to venom and current sensitization instead of following up sensitization and reactions after being stung. Moreover, the questionnaire-based approach data on allergy and sting-related reaction is restricted by recall bias. In addition, the lack of component resolved diagnostic methods in this study may have hampered the evaluation of true sensitization to venom allergy.

Our findings, however, provide evidence that sensitization against bee- or wasp-venom are

quite prevalent in Indonesian schoolchildren living in an urban area of a tropical region. Higher prevalence of skin reactivity was observed in high SES compare to the low SES children, in contrast, sIgE-positive was more prevalent in the low SES compare to the high SES. These sensitizations to Hymenoptera venom appear to have poor clinical relevance as they rarely translated into clinical symptoms. Moreover, we also observed discordance between SPT and sIgE to bee- and wasp-venom, in particular the high proportions of positive SPT in absence of sIgE, which suggests the direct (non-IgE) effect of venoms resulting in skin reactivity. However, further studies are needed to determine the possible mechanisms underlying this which could be through component resolved diagnostic methods.

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Statement of Ethics

This study was approved by Health Research Ethics Committee of Faculty of Medicine, Hasanuddin University (Ref: 1504/H04.8.4.5.31/PP36-KOMETIK/2016). Written informed consent was obtained from all parents prior to data collection.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conception and design of the experiments: R.R., M.Y., F.H. and E.S. Data collection and experimentation: A.I.A, J.P.R.K., M.M, S.A.V., S.W., F.H., and E.S. Interpretation of data and statistical analysis: A.I.A, J.P.R.K. Writing of this paper: A.I.A, J.P.R.K., M.M, S.A.V, S.W., F.H., E.S., R.R., and M.Y. The corresponding author had full access to all of the data in this study and takes final responsibility for the decision to submit it for publication.

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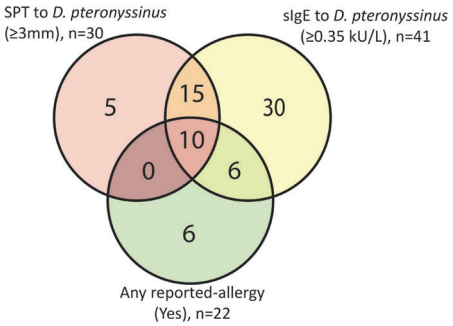
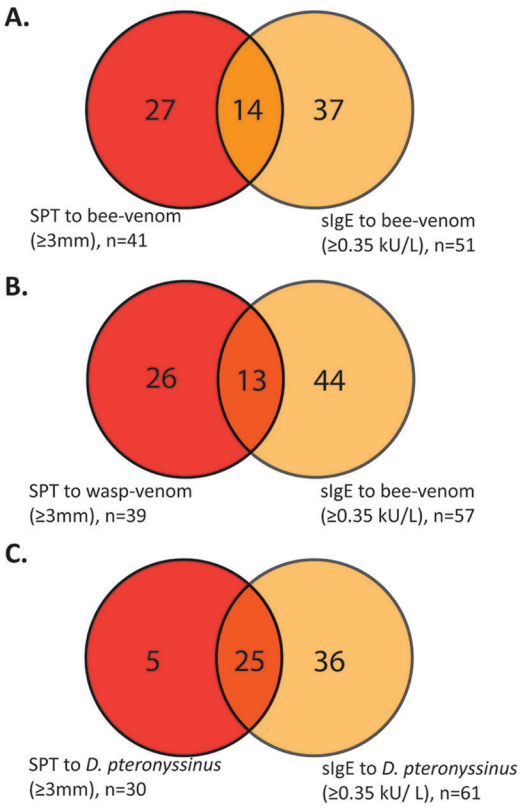
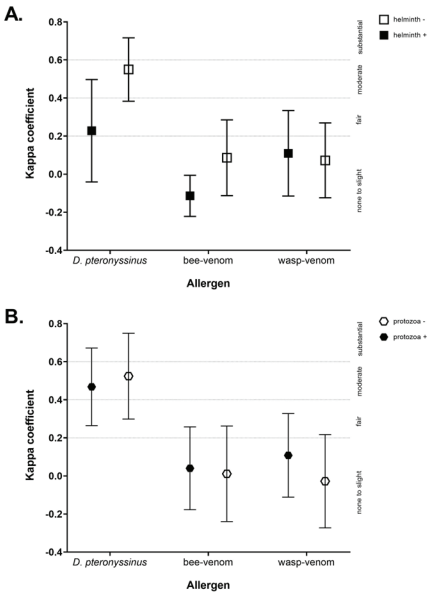
SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure S1. Overlap between sensitization to *D. pteronyssinus* and self-reported diagnosis of any allergy for subjects (N=151) with completed data of SPT, sIgE and allergy-related questionnaire. SPT: skin SPT: skin prick test. sIgE: specific Immunoglobulin E.



Supplementary Figure S2. Dis-/Concordance of tests between SPT and sIgE to (A) bee-venom, (B) wasp-venom, and (C) *D. pteronyssinus* of all subjects (n=197) with match SPT and sIgE data.



Supplementary Figure S3. Agreement between SPT and sIgE to *D. pteronyssinus*, bee-venom and wasp-venom in A. helminth infected and uninfected schoolchildren, and B. protozoa infected and uninfected schoolchildren. Kappa coefficient: 0.01-0.20 (none to slight agreement); 0.21-0.40 (fair agreement); 0.41-0.60 (moderate agreement); 0.61-0.80 (substantial agreement).

Supplementary Table 1. Wheal size of skin prick test (SPT) among children with positive SPT to each allergen tested

Allergen	Wheal size in mm						*p-value*
	All		**Low SES**		**High SES**		
	N	**median (IQR)**	**N**	**median (IQR)**	**N**	**median (IQR)**	
Aeroallergens							
D. pteronyssinus	37	4.50 (3.50-6.00)	8	4.25 (3.25-6.25)	29	4.50 (3.50-6.00)	0.71
D. farinae	25	5.00 (4.00-6.00)	5	4.00 (3.25-5.00)	20	5.00 (4.00-6.00)	0.11
B. germanica	42	4.00 (3.50-4.50)	15	4.00 (3.50-4.50)	27	4.5 (3.50-5.00)	0.69
Aspergillus spp.	17	3.50 (3.00-4.50)	3	4.50 (4.50-4.50)	14	3.25 (3.00-4.12)	0.54
Venoms							
A. mellifera	45	3.50 (3.00-4.00)	9	4.00 (3.25-4.25)	36	3.00 (3.00-3.50)	0.03
Vespula spp.	40	3.50 (3.00-4.00)	9	3.50 (3.00-4.50)	31	3.50 (3.00-4.00)	0.31

N: total population. IQR: interquartile range. *p-value* derived by Mann-Whitney U test

Supplementary Table 2. Body mass index (zBMI) of SPT positive and SPT negative children living in the low SES

Allergen	zBMI				*p-value*
	SPT positive		**SPT Negative**		
	N	**mean ± SD**	**N**	**mean ± SD**	
Any aeroallergen	23	-1.13±1.19	134	-0.93±1.21	0.45
House dust mite	12	-1.09±1.46	145	-0.95±1.18	0.69
Any venom	17	-0.74±1.80	140	-0.98±1.11	0.60

zBMI: z-score of Body Mass Index. *p-value* derived from student t-test

Supplementary Table 3. Comparison of intestinal parasitic infection prevalence between SPT positive and SPT negative among low SES schoolchildren

Parasites infection n/ N (%)	Any allergen			Any aeroallergen			Any venom		
	SPT positive	**SPT negative**	***p-value***	**SPT positive**	**SPT negative**	***p-value***	**SPT positive**	**SPT negative**	***p-value***
Any intestinal parasites	22/24 (91.7)	73/84 (86.9)	0.53	14/14 (100)	81/94 (86.2)	0.14	11/13 (84.6)	84/95 (88.4)	0.69
Any helminths	16/26 (61.5)	62/95 (65.3)	0.72	10/16 (62.5)	68/105 (64.8)	0.86	7/14 (50)	71/107 (66.4)	0.23
Any protozoa	18/24 (75)	59/84 (70.2)	0.65	12/14 (85.7)	65/94 (69.1)	0.20	9/13 (69.2)	68/95 (71.6)	0.86

Total infected (n) among total population (N) in each group. SPT: skin prick test

SUPPLEMENTARY QUESTIONNAIRES

A. **Core questionnaires for Asthma, Rhinitis and Eczema (The questionnaire was modified from The International Study of Asthma and Allergies in Childhood (ISAAC) core questionnaires)**

1. **Questionnaire for wheezing and asthma**

No	Question	Answer
Q1	Has your child ever had asthma (asma)? Diagnosed by doctor	[ ] Yes [ ] No
Q2	Has your child had wheezing or whistling (mengik/ poso/asma) in the chest in the past 12 months?	[ ] Yes [ ] No If no skip to Questionnaire about Rhinitis
Q3	How many attacks of wheezing (mengik/poso/ asma) has your child had in the past 12 months?	[ ] None [ ] 4-12 [ ] 1-3 [ ] > 12
Q4	In the past 12 months, how often, on average, has your child's sleep been disturbed due to wheezing (mengik/poso/asma)?	[ ] Never woken with wheezing (mengik/ poso/asma) [ ] Less than one night per week [ ] One or more nights per week
Q5	In the past 12 months, has wheezing (mengik/ poso/asma) ever been severe enough to limit your child's speech to only one or two words at a time between breaths?	[ ] Yes [ ] No
Q6	In the past 12 months, has your child's chest sounded wheezy (mengik/poso/asma) during or after exercise?	[ ] Yes [ ] No
Q7	In the past 12 months, has your child had a dry cough at night, apart from a cough associated with a cold or chest infection?	[ ] Yes [ ] No

2. **Questionnaire for allergic rhinitis**

No	Question	Answer
Q1	Has your child ever had problem with sneezing or a runny or blocked nose (nose problem) without cold or the flu?	[ ] Yes [ ] No If no skip to Q6
Q2	In the past 12 months, has your child had a problem with sneezing, or a runny, or blocked nose when you DID NOT have a cold or the flu?	[ ] Yes [ ] No If no skip to Q6

Q3	In the past 12 months, have itchy-watery eyes accompanied this nose problem?	[ ] Yes [ ] No
Q4	In which of the past 12 months did this nose problem occur? (Please tick any which apply)	[ ] Rainy season [ ] Dry season [ ] Anytime [ ] No idea
Q5	In the past 12 months, how much did this nose problem interfere with your child's daily activities?	[ ] Not at all [ ] A Moderate [ ] A little [ ] A lot
Q6	Has your child ever been diagnosed by doctor to had allergic rhinitis?	[ ] Yes [ ] No If no, STOP here

3. **Questionnaire for eczema (allergic dermatitis) (show the pictures to the subject)**

No	Question	Answer
Q1	Has your child ever had an itchy rash (like in the picture) which was coming and going for at least six months?	[ ] Yes [ ] No If no skip to Q6
Q2	Have your child had this itchy rash at any time in the past 12 months?	[ ] Yes [ ] No If no skip to Q6
Q3	Has this itchy rash at any time affected any of the following places? The folds of the elbows, behind the knees, in front of ankles, under the buttocks or around the neck, ears or eyes?	[ ] Yes [ ] No
Q4	Has this rash completely disappear at any time during the past 12 months?	[ ] Yes [ ] No
Q5	In the past 12 months, how often, on average, have your child been kept awake by this itchy rash?	[ ] Never in the past 12 months [ ] Less than one night per week [ ] One or more nights per week
Q6	Has your child ever been diagnosed by doctor to had allergic dermatitis?	[ ] Yes [ ] No

B. **Sting-related Questionnaire**

Questionnaire for venom allergy (show the pictures to the subject).

No	Question	Answer
Q1	Has your child, currently or within the last 5 years occupationally or as a hobby done any of the following?	1. Bee-keeping 2. Farming 3. Gardening 4. None
Q2	Has your child ever been stung?	1. Yes 2. No
Q3	Being stung by an insect, did you notice that your child had a swelling at the site of the sting?	1. Yes 2. No
Q4	Being stung by an insect, did you notice that your child had a swelling with diameter of more than 10 cm, lasting over 24 hours in certain part of his/her body?	1. Yes 2. No
Q5	Being stung by an insect, did you notice that your child had an urticarial reaction on the whole body?	1. Yes 2. No
Q6	Being stung by an insect, did you notice that your child had swelling of the face, tongue or lips?	1. Yes 2. No
Q7	Being stung by an insect, did you notice that your child had an experience with difficulties of breathing, dyspnoea?	1. Yes 2. No
Q8	Being stung by an insect, have you child ever lost consciousness?	1. Yes 2. No

CHAPTER FOUR

GUT MICROBIOME IN INDONESIAN SCHOOLCHILDREN LIVING IN URBAN AREA OF MAKASSAR, SOUTH SULAWESI

(MICROORGANISMS, 2020)

CHAPTER 4

THE BACTERIAL GUT MICROBIOTA OF SCHOOLCHILDREN FROM HIGH AND LOW SOCIO-ECONOMIC STATUS: A STUDY IN AN URBAN AREA OF MAKASSAR, INDONESIA

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Abstract: To understand the relationship between the gut microbiota and the health profile of Indonesians, it is important to elucidate the characteristics of the bacterial communities that prevail in this population. To this end, we profiled the faecal bacterial community of 140 Indonesian schoolchildren in urban Makassar. The core microbiota of Indonesian schoolchildren consisted of *Bifidobacterium*, *Collinsella* and multiple members of the *Lachnospiraceae* and *Ruminicoccaceae* families, but the relative abundance of these taxa varied greatly among children. Socioeconomic status (SES) was the main driver for differences in microbiota composition. Multiple bacterial genera were differentially abundant between high and low SES children, among others *Bifidobacterium, Lactobacillus, Prevotella* and *Escherichia-Shigella*. In addition, the microbiota of high SES children was less diverse and strongly associated with BMI. In low SES children, helminth infection was prevalent and positively associated with *Olsenella, Enterohabdus, Lactobacillus* and *Mogibacterium* abundance, while negatively associated with relative abundance of *Prevotella*. Protozoa infection was also prevalent, and positively associated with *Rikenellaceae,* while negatively associated with relative abundance of *Romboutsia* and *Prevotella*. In conclusion, Indonesian schoolchildren living in urban Makassar share a core microbiota, but their microbiota varies in diversity and relative abundance of specific bacterial taxa depending on socioeconomic status, nutritional status and intestinal parasites infection.

Keywords: gut microbiota; socioeconomic status; intestinal parasites; nutritional status; schoolchildren.

Introduction

In the past decades, several studies have established the role of the gut microbiota in maintaining host physiological states, including immune responses, metabolism, mental and physical development [1]. The imbalance of gut microbiota composition may aggravate inflammation, metabolic diseases, or other health problems [2-5].

Gut microbiota composition is predominantly driven by environmental factors, such as diet, physical activity level, hygiene, disease and medication use, instead of genetics [6,7]. Previous studies revealed notable differences in gut microbiota profile among different ethnic and geographical areas, sometimes representing socioeconomic inequalities between groups [8-11]. A study involving European and African children has shown that the microbiota of African children is more diverse and contains more fibre-degrading, short-chain fatty acid producing bacteria than European children, attributable to differences in diet and lifestyle [12].

Indonesia is a developing country with enormous economic growth, but with great socioeconomic disparities in its population. This inequality is also reflected by the wide gap in health status between people from high and low socioeconomic status (SES). Low SES has been associated with lack of sanitation and bad hygiene and, as consequence, higher exposure to soil-transmitted-helminths or other intestinal parasites, especially in schoolchildren. Recently, we investigated gut microbiota composition in a helminth endemic area in rural Indonesia. When comparing helminth infected with uninfected individuals, we observed no difference in bacterial composition nor diversity of the gut microbiota [13]. Since there are no large disparities in lifestyle and SES in that area, these factors were not studied therein.

Given the role of the gut microbiota in health and susceptibility to diseases, it is important to investigate the gut microbiota in a specific population and determine what factors affect its composition. Here, we studied the bacterial gut microbiota, and its association with environmental factors, of Indonesian schoolchildren living in urban Makassar.

Methods

Study design and ethics approval

This study was a cross-sectional study, involving Indonesian schoolchildren living in an urban area of Makassar. Children from two public schools, that were distinct in socioeconomic level, were included in this study. The study was approved by the ethics committee for medical research of Faculty of Medicine, Universitas Hasanuddin, Indonesia (approval number: 1504/H04.8.4.5.31/PP36-KOMETIK/2016). Written, signed and dated informed consent was obtained from parents or guardian of each child prior to the study.

Study population and data collection

One hundred and forty children were recruited from one high (n=74) and one low SES (n=66) school. The high SES school is located in the city centre and is considered of high status, with majority of the parents working as a high-skilled worker or professional with higher education. Meanwhile, the low SES school is located near a landfill and port area, where most of the parents are low-educated and work on low-skilled labour jobs.

Table 1. Characteristics of study populations. The number of positives (n) of the total population examined (n=140). Statistical testing was performed using student t-test for continuous variables and using chi-square test for categorical variables. SD: standard deviation.

Characteristics	All children (n=140)	High SES children (n=74)	Low SES children (n=66)	p value
Age, years, mean ± SD	10.33 ± 0.85	10.40 ± 0.56	10.25 ± 1.04	0.294
Female: n (%)	85 (60.71)	41 (62.1)	44 (59.5)	0.748
zBMI, mean ± SD	-0.31 ± 1.46	0.33 ± 1.5	-0.89 ± 1.14	<0.001
Nutritional status (WHO): n (%)				<0.001
Thinness	13 (9.29)	2 (3.0)	11 (14.9)	
Normal	99 (70.71)	39 (59.1)	60 (81.1)	
Overweight and Obese	28 (20.00)	25 (37.9)	3 (4.1)	
Intestinal parasitic infection: n (%)				
Any helminth	47 (33.57)	2 (3.0)	45 (60.8)	<0.001
Ascaris lumbricoides	34 (24.29)	0	34 (45.9)	
Trichuris trichiura	25 (17.86)	2 (3.0)	23 (31.1)	<0.001
Hymenolepis diminuta	1 (0.71)	0	1 (1.4)	
Any protozoa	66 (47.14)	18 (27.3)	48 (65.8)	<0.001
Entamoeba histolytica,	14 (10.0)	0	14 (19.2)	
Dientamoeba fragilis	30 (21.43)	10 (15.2)	20 (27.4)	0.080
Giardia lamblia	39 (27.86)	9 (13.6)	30 (41.1)	<0.001
Cryptosporidium parvum	2 (1.43)	0	2 (2.7)	

A standardised questionnaire was used to gather demographic information of the children including age and sex. To determine the nutritional status, anthropometric measurement was performed. To assess the intestinal parasitic infection and gut microbiota composition,

stool samples were collected using a stool container with enclosed spoon (Sarstedt AG&Co. KG, Nümbrecht, Germany). As soon as samples were collected by research staff, samples were stored inside an ice bag and transported to Laboratory of Parasitology Department at Hasanuddin University to be aliquoted and stored at -80C. Children characteristics are presented in Table 1.

Anthropometric measurements

Height and weight of the participant were measured using a portable stadiometer (SECA GmbH & Co., Hamburg, Germany) and digital scale (GEA, Megapratama Medikalindo, Indonesia). The weighing scale was calibrated using standardised weight as part of routine care. BMI-for-age (zBMI) was calculated according to the WHO references value to determine nutritional status of the participants [14]. Children were categorised as thinness if zBMI < -2SD, normal if zBMI between -2SD to 1SD and overweight/obese if zBMI>1SD.

Parasitological examination

A single-slide of each freshly-collected stool sample was examined by the Katokatz methods for detection of soil transmitted helminths eggs. DNA was extracted from cryopreserved stool for intestinal protozoa identification identification using QIAamp Spin Columns/Mini Kit (Qiagen, Germany) [15]. In each sample, a fixed amount of Phocine Herpes Virus 1 was included within the isolation lysis buffer as an internal control [16]. A panel of multiplex real-time PCR was used to detect and quantify intestinal protozoa in which targeting *Entamoeba histolytica, Dientamoeba fragilis, Giardia lamblia* and *Cryptosporidium parvum.* The procedure has been described elsewhere [15,17].

Microbiota analysis

DNA was extracted from approximately 0.1 gram cryopreserved stool using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (ZymoResearch, CA, USA) according to manufacturer instructions. Quality control, library preparation and 16S rRNA gene amplicon sequencing were performed by BaseClear (Leiden, The Netherlands), targeting the V3-V4 region (F: CCTACGGGNGGCWGCAG, R: GACTACHVGGGTATCTAATCC), using the Illumina MiSeq platform (300bp, paired-end, Illumina, CA, USA). Raw sequencing data are available in the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under study accession PRJEB38465.

Read filtering, operational taxonomic unit (OTU)-picking and taxonomic assignment were performed using the NG-Tax 0.4 pipeline with following settings: forward and reverse read length of 120, ratio OTU abundance of 2.0, classify ratio of 0.9, minimum threshold of $1*10^{-7}$, identity level of 100%, error correction of 98.5, using the Silva_132_SSU Ref database [18,19]. The obtained OTU-table was filtered for OTUs with a number of sequences less than 0.005% of the total number of sequences [20].

Six positive controls (ZymoBiomics Microbial Community Standard, ZymoResearch, Leiden, the Netherlands) and six negative controls (empty extractions) were taken along from DNA extraction onwards, meaning one control per DNA extraction kit. In addition, four positive

controls (ZymoBiomics Microbial Community DNA Standard, ZymoResearch, Leiden, The Netherlands) were taken along from QC onwards, meaning one control per sequencing run. The eight bacterial species present in the included positive controls were all identified. The relative abundance of these species was on average 1.04 ± 0.20 and 1.06 ± 0.30 fold different from theoretical abundances for the sequencing control and DNA extraction control, respectively. This indicates that minor variation is induced by the sequencing and DNA extraction procedures. Four additional bacterial taxa were identified in the positive controls, namely *Collinsella*, *Bifidobacterium*, *Enterobacteriaceae* and *Catenibacterium*, but their relative abundance only accounted for 0.017 ± 0.013 percent of total relative abundance. The included negative controls resulted in non-quantifiable DNA concentrations using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher, Landsmeer, the Netherlands) on a Qubit 3.0 Fluorometer (Invitrogen, Breda, the Netherlands), but they were sequenced nevertheless, resulting in approximately ten times less reads than the stool samples, with *Delftia* and *Staphylococcus* as most abundant contaminants.

Statistical analysis

Prevalence rates were calculated as percentage of collected data and compared between schools using Pearson chi-square test. Comparison between groups for continuous data was analysed using Student t-test for normally distributed data and using Mann-Whitney-U test if the data was not normally distributed. This analysis was performed using IBM SPPS Statistics version 25. (IBM-SPSS Inc., Chicago, IL, USA).

Statistical analysis and data visualisation for microbiota data were performed in R (v3.6.1) using the packages phyloseq (v1.30.0) [21], vegan (v2.5-6) [22], ggplot2 (v3.2.1) [23], DESeq2 (v1.22.2)[24] and microbiome (v1.8.0) [25]. All analyses were performed on genus-level, except for alpha-diversity measures (Shannon diversity and observed richness). For differential abundance testing by DESeq2, genera present in less than 25% of the samples were removed to minimize zero-variance errors and spurious significance. Outcomes were considered significant when the Benjamini-Hochberg corrected p-value was ≤ 0.05. Bacterial taxa were considered part of the core microbiota when present in 95% of the samples from the specified group. Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis function with 999 permutations and Bray-Curtis dissimilarity to determine associations between microbiota composition and clinical variables. Outcomes were considered significant when the p-value was ≤ 0.05.

Results

The bacterial gut microbiota of Indonesian schoolchildren

To obtain a comprehensive overview of the bacterial gut microbiota of Indonesian school children, microbiota composition, as well as factors influencing their microbiota composition, were determined. The core microbiota consisted of *Bifidobacterium*, *Collinsella* and multiple members of the *Lachnospiraceae* and *Ruminicoccaceae* families (Table 2), together constituting an average relative abundance of 56.3 ± 19.8%. With an average relative abundance of 23.5% and 13.4%, *Bifidobacterium* and *Collinsella* were the most abundant members of the bacterial community (Table 3). However, their relative abundance varied greatly between children, ranging from 0.8 to 78.6% and 0.0 to 65.2%, respectively (Table 3). Large variation in relative abundance was also observed for the other most abundant bacterial taxa (Table 3). Variation in overall microbiota composition was significantly associated with socioeconomic status, as determined by Bray-Curtis dissimilarity-based multivariate analysis

(PERMANOVA; R2=0.049; p=0.001).

To explore the relation between SES and the bacterial gut microbiota, microbiota composition and bacterial richness/diversity were compared between high and low SES children (Figure 1). Taxonomic profiles and principal coordinate analysis confirmed the difference, but also showed the similarity, in overall microbiota composition between high and low SES children (Figure 1a,b). Differential abundance analysis revealed that, among others, *Bifidobacterium*, *Lactobacillus* and various *Lachnospiraceae* and *Ruminococcaeae* members were more abundant in high SES children, while *Prevotella*, *Mogibacterium*, *Escherichia-Shigella* and other members of the *Lachnospiraceae* and *Ruminococcaceae* families were more abundant in low SES children (Figure 1c). While bacterial richness was comparable, diversity was significantly higher in low SES children (Figure 1d).

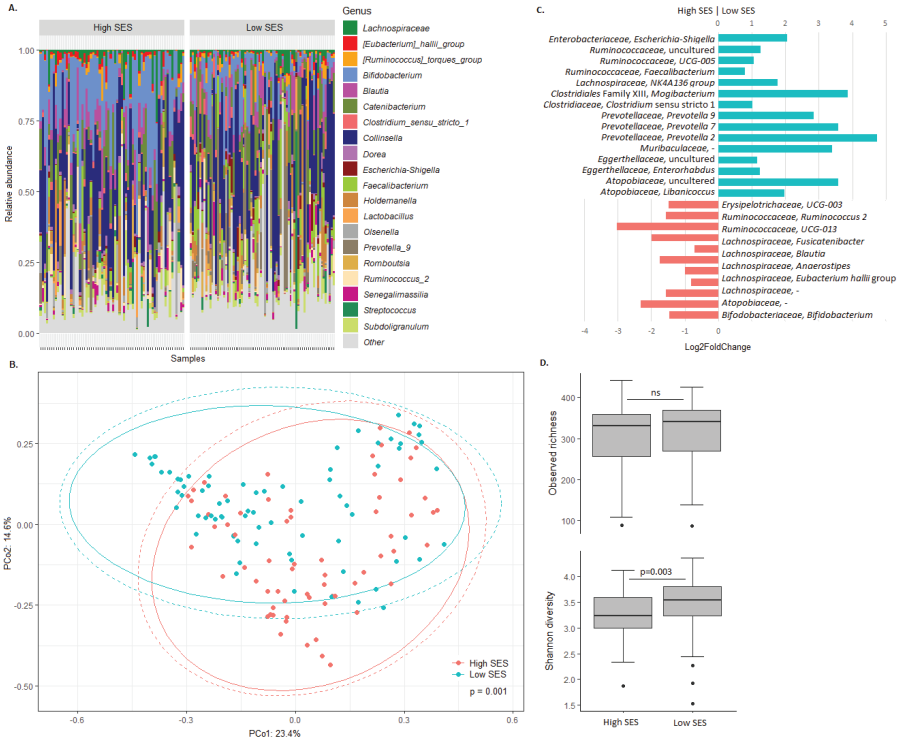

Figure 1. Association between the bacterial gut microbiota and SES. A) Taxonomic profiles of the gut microbiota of high and low SES children. The 20 most abundant bacterial taxa are shown. Relative abundance of all other taxa is summed and labelled as 'other'. B) Bray-Curtis dissimilarity-based principal coordinate analysis plot labelled according to SES. Ellipses indicate confidence assuming a multivariate t-distribution (solid line) or a multivariate normal distribution (dotted line). C) Differential abundance of bacterial taxa between high and low SES children. Taxa with Benjamini-Hochberg corrected p-value below 0.05 are shown. D) Alpha diversity in high and low SES children.

The bacterial gut microbiota of high SES Indonesian schoolchildren

To obtain a comprehensive overview of the bacterial gut microbiota of high SES children (n=66), microbiota composition, as well as factors influencing their microbiota composition,

were determined. The core microbiota of high SES children consisted of *Bifidobacterium*, *Collinsella*, *Clostridium* sensu stricto 1, *Romboutsia* and multiple members of the *Lachnospiraceae* and *Ruminicoccaceae* families (Table 2), together constituting an average relative abundance of 65.6 ± 15.2%, with *Bifidobacterium* and *Collinsella* being most abundant (Table 3). Variation in overall microbiota composition was significantly associated with nutritional status (zBMI; PERMANOVA; R2=0.036; p=0.011).

To explore the relation between nutritional status and the bacterial gut microbiota, microbiota composition and bacterial richness/diversity were compared between normal weight and overweight/obese high SES children (Figure 2). Despite the association of zBMI with overall microbiota composition (Figure 2a), bacterial richness and diversity were similar between normal weight and overweight/obese children (Figure 2b), and only *Eggerthella* was significantly differentially abundant (Log2FoldChange = -3.49; padj = 0.005), with higher relative abundance in normal weight children.

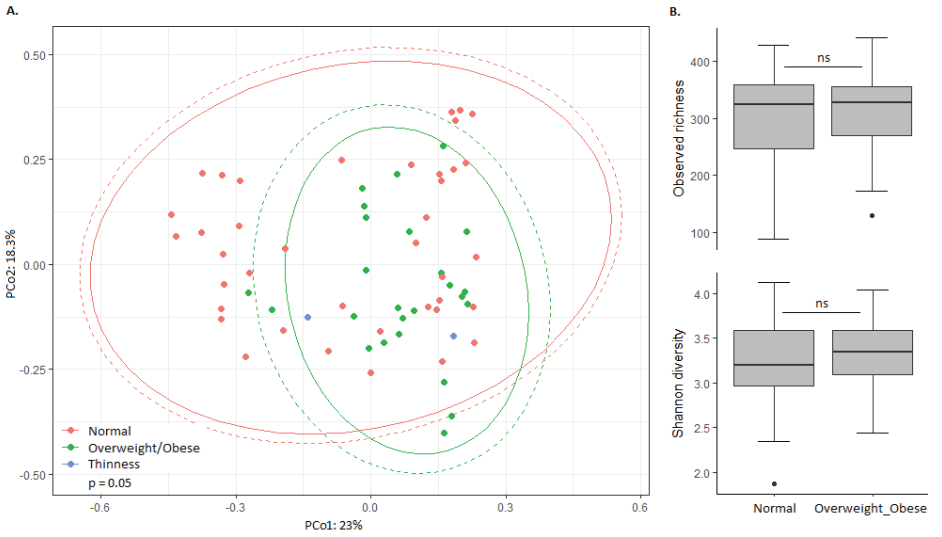

Figure 2. Association between the bacterial gut microbiota and nutritional status in high SES children. A) Bray-Curtis dissimilarity-based principal coordinate analysis plot labelled according to nutritional status. Ellipses indicate confidence assuming a multivariate t-distribution (solid line) or a multivariate normal distribution (dotted line). B) Alpha diversity in high SES children with normal weight and overweight/obesity.

The bacterial gut microbiota of low SES Indonesian schoolchildren

To obtain a comprehensive overview of the bacterial gut microbiota of low SES children (n=74), microbiota composition, as well as factors influencing their microbiota composition, were determined. The core microbiota of low SES children consisted of *Bifidobacterium*, *Collinsella*, *Senegalimassilia*, *Holdemanella* and multiple members of the *Lachnospiraceae* and *Ruminicoccaceae* families (Table 2), together constituting an average relative abundance of 54.4 ± 20.8%, with *Collinsella* being most abundant (Table 3). None of the included variables were significantly associated with overall microbiota composition in low SES children. Nevertheless, the high prevalence of helminth infection (60.8%) and protozoa infection (65.8%) in this group prompted studying their association with microbiota composition.

To explore the relation between helminth or protozoa infection and the bacterial gut microbiota, microbiota composition and bacterial richness/diversity were compared between low SES children with and without these infections (Figure 3). Principal coordinate analysis confirmed that overall microbiota composition was not associated with helminth nor protozoa status (Figure 3a, c). In addition, bacterial richness and diversity were similar between children with and without helminth or protozoa infection (Figure 3b,d). Despite these commonalities, various bacterial taxa were differentially abundant. Relative abundance of *Olsenella*, *Enterorhabdus*, *Lactobacillus* and *Mogibacterium* was higher in helminth infected children, whereas the relative abundance of *Prevotella* and unclassified *Lachnospiraceae* was higher in children without helminth infection (Figure 3e). In addition, relative abundance of *Rikenellaceae* RC9 gut group was higher in protozoa infected children, whereas the relative abundance of *Prevotella* and *Romboutsia* was higher in children without protozoa infection (Figure 3f).

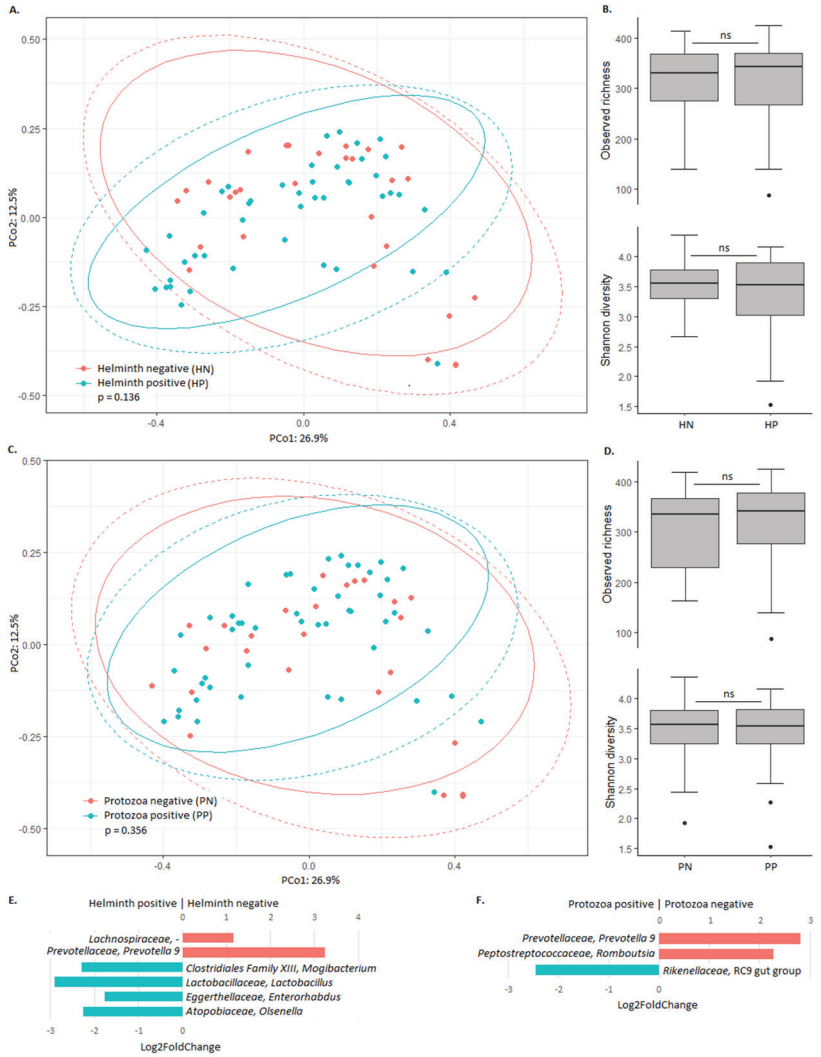

Figure 3. Association between the bacterial gut microbiota and helminth and protozoa infection in low SES children. A) Bray-Curtis dissimilarity-based principal coordinate analysis plot labelled according to helminth infection. Ellipses indicate confidence assuming a multi-

variate t-distribution (solid line) or a multivariate normal distribution (dotted line). B) Alpha diversity in low SES children that are helminth negative (HN) and helminth positive (HP). C) Bray-Curtis dissimilarity-based principal coordinate analysis plot labelled according to protozoa infection. Ellipses indicate confidence assuming a multivariate t-distribution (solid line) or a multivariate normal distribution (dotted line). D) Alpha diversity in low SES children that are protozoa negative (PN) and protozoa positive (PP). E) Differential abundance of bacterial taxa between helminth positive and helminth negative low SES children. Taxa with Benjamini-Hochberg corrected p-value below 0.05 are shown. F) Differential abundance of bacterial taxa between protozoa positive and protozoa negative low SES children. Taxa with Benjamini-Hochberg corrected p-value below 0.05 are shown.

Table 2. Core microbiota in all, high SES, and low SES children. Core taxa (x) are considered those present in 95% of the samples from the specified group.

Family	Genus	All children	High SES children	Low SES children
Bifidobacteriaceae	*Bifidobacterium*	x	x	x
Coriobacteriaceae	*Collinsella*	x	x	x
Ruminococcaceae	*Faecalibacterium*	x	x	x
Ruminococcaceae	*Subdoligranulum*	x	x	x
Lachnospiraceae	-	x	x	x
Lachnospiraceae	*Blautia*	x	x	x
Lachnospiraceae	*Dorea*	x	x	x
Lachnospiraceae	*Fusicatenibacter*	x	x	x
Lachnospiraceae	*Eubacterium hallii* group	x	x	x
Lachnospiraceae	*Ruminococcus torques* group	x		x
Peptostreptococcaceae	*Romboutsia*		x	
Clostridiaceae 1	*Clostridium* sensu stricto 1		x	
Erysipelotrichaceae	*Holdemanella*			x
Eggerthellaceae	*Senegalimassilia*			x

Discussion

Gut microbiota composition is largely influenced by environmental factors. In this study, socioeconomic status was the main factor associated with bacterial gut microbiota composition of Indonesian schoolchildren. Bacterial diversity was lower in children from high socioeconomic status and their microbiota contains higher relative abundance of *Bifidobacterium* and *Lactobacillus*, while containing lower relative abundance of *Prevotella* and *Escherichia-Shigella*, among others. The gut microbiome plays a key role in host metabolism and immune functioning, and microbial dysbiosis during early life has been associated with the development of several diseases in later life, including atopy, obesity, chronic inflammatory conditions and infections [26]. Alterations in microbiota composition as observed in our study may therefore contribute to the inequalities in health status and life expectancy of children from low and high socioeconomic status. Vice versa, early life exposures are likely to be affected by socioeconomic status-associated differences in lifestyle, education and healthcare, all which influence gut microbiota composition and may explain the observed differences in gut microbiota composition between low and high SES children.

Table 3. The ten most abundant bacterial taxa in all, high SES and low SES children. Relative abundances (average, min, max) are presented as fraction of total relative abundance. SD: Standard deviation.

	Family	Genus	Average ± SD	Min - max
All children	*Coriobacteriaceae*	*Collinsella*	0.235 ± 0.188	0.009 - 0.786
	Bifidobacteriaceae	*Bifidobacterium*	0.134 ± 0.139	0.000 - 0.652
	Erysipelotrichidae	*Catenibacterium*	0.077 ± 0.090	0.000 - 0.425
	Lachnospiraceae	*Blautia*	0.049 ± 0.047	0.001 - 0.208
	Prevotellaceae	*Prevotella 9*	0.044 ± 0.118	0.000 - 0.638
	Ruminococcaceae	*Faecalibacterium*	0.043 ± 0.056	0.000 - 0.322
	Erysipelotrichaceae	*Holdemanella*	0.031 ± 0.055	0.000 - 0.337
	Ruminococcaceae	*Subdoligranulum*	0.030 ± 0.035	0.000 - 0.224
	Lachnospiraceae	-	0.024 ± 0.038	0.000 - 0.255
	Coriobacteriaceae	*Olsenella*	0.024 ± 0.077	0.000 - 0.729
High SES children	*Coriobacteriaceae*	*Collinsella*	0.246 ± 0.185	0.013 ± 0.786
	Bifidobacteriaceae	*Bifidobacterium*	0.198 ± 0.152	0.004 ± 0.652
	Erysipelotrichidae	*Catenibacterium*	0.065 ± 0.082	0.000 ± 0.416
	Lachnospiraceae	*Blautia*	0.055 ± 0.050	0.001 ± 0.208
	Ruminococcaceae	*Subdoligranulum*	0.033 ± 0.038	0.000 ± 0.224
	Lachnospiraceae	-	0.030 ± 0.047	0.000 ± 0.255
	Ruminococcaceae	*Faecalibacterium*	0.029 ± 0.044	0.000 ± 0.266
	Ruminococcaceae	*Ruminococcus 2*	0.029 ± 0.059	0.000 ± 0.292
	Erysipelotrichaceae	*Holdemanella*	0.028 ± 0.059	0.000 ± 0.337

	Coriobacteriaceae	*Olsenella*	0.025 ± 0.064	0.000 ± 0.456
Low SES children	*Coriobacteriaceae*	*Collinsella*	0.225 ± 0.190	0.009 ± 0.737
	Erysipelotrichidae	*Catenibacterium*	0.088 ± 0.095	0.000 ± 0.425
	Bifidobacteriaceae	*Bifidobacterium*	0.077 ± 0.095	0.000 ± 0.403
	Prevotellaceae	*Prevotella 9*	0.071 ± 0.150	0.000 ± 0.638
	Ruminococcaceae	*Faecalibacterium*	0.055 ± 0.062	0.000 ± 0.322
	Lachnospiraceae	*Blautia*	0.043 ± 0.043	0.001 ± 0.170
	Erysipelotrichaceae	*Holdemanella*	0.035 ± 0.051	0.000 ± 0.267
	Ruminococcaceae	*Subdoligranulum*	0.029 ± 0.031	0.001 ± 0.183
	Coriobacteriaceae	*Olsenella*	0.023 ± 0.087	0.000 ± 0.729
	Lachnospiraceae	-	0.019 ± 0.026	0.000 ± 0.119

Similar to our study, Chong CW *et al*. showed lower microbial diversity in wealthier children as compared to economically deprived children living in the same rural area in Malaysia [27]. Such differences in microbiota structure most likely result from lifestyle differences. Children from low SES are expected to have higher exposure to microorganisms and parasites [28]. In addition, diet is considered as a main driver of gut microbiota composition [12]. As such, observed differences in microbiota composition between high and low SES children may be reflecting differences in their diet. High SES children are more likely to be exposed to high-fat diet products as compared to low SES children [29]. The high abundance of *Bifidobacterium* and *Lactobacillus* in high SES children could be associated with higher probiotics intake [30]. High SES children consume dairy products regularly, and probiotic drinks were available in the canteen of the high SES school, but not in the low SES school. *Prevotella*, observed to be of higher relative abundance in low SES children, has been associated with higher intake of vegetables and fibre [31]. However, the influence of diet on microbiota composition remains speculative herein, as food intake was not determined in our study.

In high SES children, *Bifidobacterium*, *Collinsella*, *Clostridium* sensu stricto 1, *Romboutsia* and multiple members of the *Lachospiraceae* and *Ruminicoccaceae* families were prevalent. Overall microbiota composition was mostly associated with nutritional status, but except for differential abundance of *Eggerthella*, relative abundance of individual bacterial taxa did not significantly differ between normal weight and overweight/obese children, nor did bacterial richness and diversity. A recent study investigating Asian children from 5 different countries also found *Bifidobacterium* as the most abundant bacterium, among others [32]. In addition, nutritional status has previously been associated with microbiota composition in Mexican children [33]. It is important to realise that these differences may be a result of underlying variations in diet and environmental, geographical, demographic and clinical factors. Also after correction for such factors, however, a significant association between microbiota and BMI has been shown in children who were part of the American Gut Project [34].

In low SES children, the core microbiota consisted of *Bifidobacterium*, *Collinsella*, *Senegalimassilia*, *Holdemanella* and multiple members of the *Lachnospiraceae* and *Ruminicoccaceae* families. Helminth infection was prevalent in low SES children and was positively associated with *Olsenella, Enterorhabdus, Lactobacillus* and *Mogibacterium* abundance, while negatively associated with relative abundance of *Prevotella*. In addition, protozoa infection was prevalent in low SES children, and was also negatively associated with relative abundance of *Prevotella*. We observed no apparent association between helminth or protozoa infection and microbiota diversity. Recently, there is much interest in the relationship between helminth infection and the gut microbiota as they shared the same niche in the human body. Infection with intestinal helminths can impact the intestinal microbiome, with important consequences for each player in the tripartite relationship between host, helminth and the microbiota [35]. In a previous study conducted in rural Flores, Indonesia, where helminth infection is highly prevalent, no association between helminth infection and gut microbiota diversity was observed [13]. In contrast to our study, increased diversity of gut microbiota has been observed in helminth colonised people in rural Malaysia [36]. In a previous study conducted in rural Cameroon, higher alpha diversity was observed in people infected by *Entamoeba* (*E. histolytica*, *E. dispar*, or both). Furthermore, *Entamoeba* presence also associated with gut microbiota composition [37]. Discrepancies in findings most likely result from differences in the included population, population size and the specific intestinal parasite species someone is infected with. Further studies are needed to elucidate the complex "three-way" relationship between intestinal parasites, microbiota and the human host.

Conclusions

We demonstrated that Indonesian schoolchildren living in urban Makassar share a core microbiota consisting of *Bifidobacterium*, *Collinsella* and multiple members of the *Lachnospiraceae* and *Ruminicoccaceae* families, but that their microbiota varies in diversity and relative abundance of specific bacterial taxa depending on socioeconomic status, nutritional status and helminth and protozoa infection. These findings contribute to increased understanding of environmental factors shaping gut microbiota composition in Indonesian schoolchildren.

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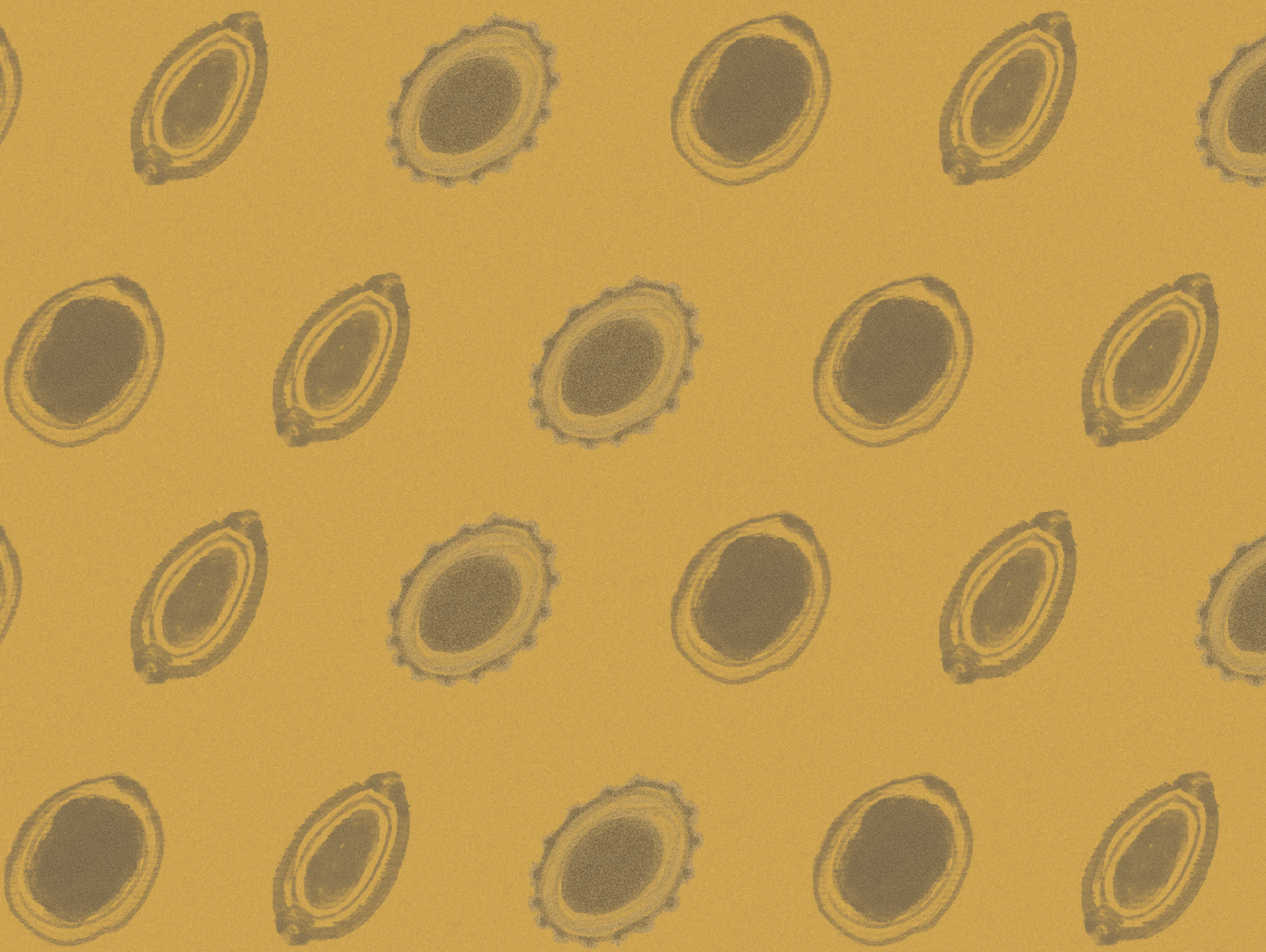
CHAPTER FIVE

INTESTINAL PERMEABILITY BEFORE AND AFTER ALBENDAZOLE TREATMENT IN LOW AND HIGH SOCIOECONOMIC STATUS SCHOOLCHILDREN IN MAKASSAR, INDONESIA

(SCIENTIFIC REPORTS, 2022)



Intestinal permeability before and after albendazole treatment in low and high socioeconomic status schoolchildren in Makassar, Indonesia

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ABSTRACT

Background. In Indonesia, there are discrepancies in the health status of children from high- and low- socioeconomic status (SES) background. Intestinal helminths are highly prevalent in low-SES children and could contribute to poor health outcomes either directly or via alteration of the gut microbiome and gut barrier function.

Methods. We analysed parasitic infections and gut microbiota composition in 325 children attending high- and low-SES schools in Makassar, Indonesia before and after albendazole treatment. Lactulose/Mannitol Ratio (LMR, a marker of gut permeability); as well I-FABP (a surrogate marker of intestinal damage) as well as inflammatory markers (LBP) were measured.

Results. Helminth infections were highly prevalent in low-SES children. LMR and I-FABP levels were higher in low-SES children while LBP levels were lower. Albendazole reduced helminth infections and also decreased LMR but only in helminth-uninfected children. Following treatment, I-FABP decreased in high- but increased in low-SES children. Albendazole did not alter the levels of LBP. Microbiota analysis showed no contribution from specific bacterial-taxa to the changes observed.

Conclusions. Intestinal permeability and epithelial damage are higher while peripheral blood inflammatory marker is lower in children of low-SES in Indonesia. However, the involvement of helminth or gut microbiota could not be discerned.

Keywords: intestinal parasitic infection; socioeconomic status; albendazole; gut microbiome; intestinal permeability

Introduction

Approximately 1.5 billion people suffer from soil-transmitted helminth (STH) infections worldwide [1]. These infections are caused by different species of worms including *Ascaris lumbricoides, Trichuris trichiura, Necator americanus* and *Ancylostoma duodenale* [2]. Children from lower socioeconomic status (SES) backgrounds are often highly infected with parasitic helminths because of poor sanitation and limited access to clean water facilities [3]. Untreated, STH infection can cause malnutrition, impaired growth and physical development [2,4].

Elevated intestinal permeability, and therefore impaired barrier function, along with gut inflammation and dysbiosis have been observed in various pathological conditions such as in stunting, obesity, and metabolic diseases [5-8]. The human intestine, which essentially allows absorptions of dietary products while maintaining a barrier function with selective permeability, prevents intrusion of pathogens or translocation of harmful products [9]. The intestinal lining is at the interface of interaction between helminths and protozoa that reside in the gastrointestinal tract and their human host and if damaged by parasites, could lead to poor barrier function and poor health outcomes.

To quantify the intestinal permeability *in vivo*, assays can be used that utilise the absorptive properties of differently sized carbohydrate probes [5]. The lactulose/mannitol ratio (LMR) is the most commonly used probe combination. As a result of increased intestinal permeability, bacterial products may be able to cross the barrier more easily and end up in the systemic circulation. Therefore, another way to characterize the intestinal permeability is by looking at markers for bacterial translocation and subsequent immune-activation. Examples of these include lipopolysaccharide binding protein (LBP) [6]. Compromised intestinal epithelial integrity and epithelial cell damage can also be assessed by measuring markers of intestinal injury such as intestinal-fatty acid binding protein (I-FABP) [10].

Previous studies in low to middle income countries, have shown a difference in gut permeability of children of high and low SES [11]. Although an association between helminth infections and increased intestinal permeability [12] was found in other study, there was no confirmation of causality through treatment. In the current study, we assessed the association between socioeconomic status (SES), intestinal parasitic infections and markers of intestinal barrier function. To this end, the presence of intestinal parasitic infections and the levels of LMR, I-FABP, and LBP were determined in schoolchildren of low- and high-SES, before and after albendazole treatment. In addition, this population has been characterized for baseline gut microbiota to assess the effect of socioeconomic status [13]. Here we assessed the alteration of the gut microbiota after albendazole treatment to delineate any contribution to intestinal permeability and barrier function.

Results

Study participants

A total of 325 children (165 and 160 children from low- and high-SES schools, respectively) were recruited. Fifty-four children were lost to follow-up due to migrating out of the study area, absence from school for an extended period of time, or withdrawal of consent as indicated in consort diagram in Supplementary Figure S1. There were no differences in age, sex, SES, or z-BMI between those who remained in the study and those who were lost to follow-up (Supplementary Table S1).

The characteristics of children from low- and high-SES schools are shown in Table 1. The mean age and sex were comparable in both groups. The z-BMI was higher in the high- compared to low-SES children (z-BMI, mean ± SD: 0.27 ± 1.48 vs -0.97 ± 1.19, p<0.001). Prevalence of any helminth infection in low- and high-SES was 65.6% and 1.6%, respectively (p<0.001). In high-SES group, only 2 children (1.6%) were infected with *T. trichiura* and no other STH infections were detected. In low-SES children, the prevalence of *T. trichiura* and *A. lumbricoides* was 65.6% and 39.8%, respectively. No hookworm infection was detected, but 2 children (1.6%) were infected with *Hymenolepis diminuta* (Table 1).

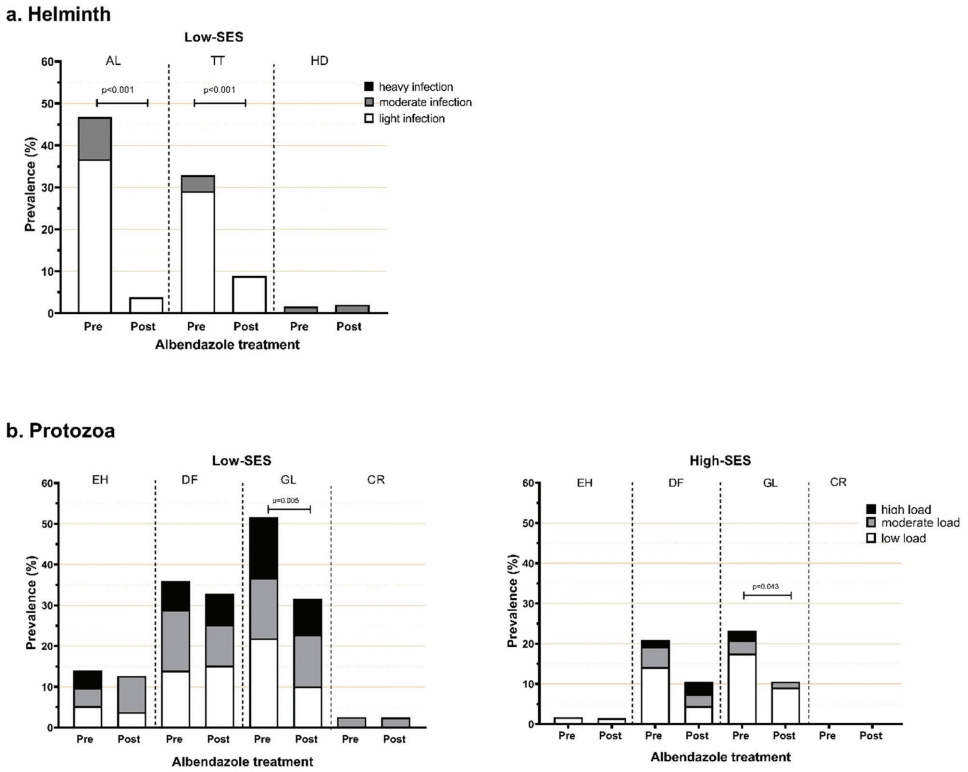

Figure 1. The effect of treatment on the proportion infected with (A) intestinal helminths (by microscopy) and (B) intestinal protozoa (by PCR). SES: socioeconomic status. AL: *Ascaris lumbricoides,* TT: *Trichuris trichiura,* HD: *Hymenolepis diminuta,* EH: *Entamoeba histolytica*, DF: *Dientamoeba fragilis*, GL: *Giardia lamblia*, CR: *Cryptosporidium parvum.* P-values were calculated using a mixed effects logistic model fitted with subject random effects and adjusted for sex, age, and z-BMI.

Similar to STH infections, intestinal protozoa prevalence was higher in low- compared to high-SES children (72.8% vs 39.2%, respectively, p<0.001). The most prevalent species was *G. lamblia* followed by *D. fragilis* and *E. histolytica*. Infection with *Cryptosporidium parvum* was only detected in low-SES children (Table 1).

Intestinal barrier function in low-SES and high-SES children at baseline

Markers for intestinal permeability and acute intestinal injury exhibited substantial differences between low- and high-SES children (Figure 2). LMR was significantly higher in low- com-

pared to high-SES children (geomean(95%CI): 4.03(3.67-4.42) vs. 3.22(2.91-3.57), respectively; adjusted p-value (p.adj) <0.001). I-FABP was also higher in low-SES (1.57(1.42-1.74) vs. 1.25(1.13-1.38); p.adj=0.02). In contrast, LBP was lower in low- compared to high-SES (19.39(17.09-22.01) vs. 22.74(20.07-26.12); p.adj=0.01). Additionally, we observed no correlation between any of these measurements (Supplementary Figure S2). To assess the role of parasitic infections in intestinal barrier function, we performed further analysis. Although univariate analysis showed that the presence of *A. lumbricoides* infection was associated with higher LMR levels (Supplementary Table S2), a subsequent linear regression model including both SES and *A. lumbricoides* demonstrated that the effect is mainly through SES (Table 2). Infection with *D. fragilis* was associated with higher LBP levels (Supplementary Table S2) but adding this variable into the model did not alter the effect of SES on LBP.

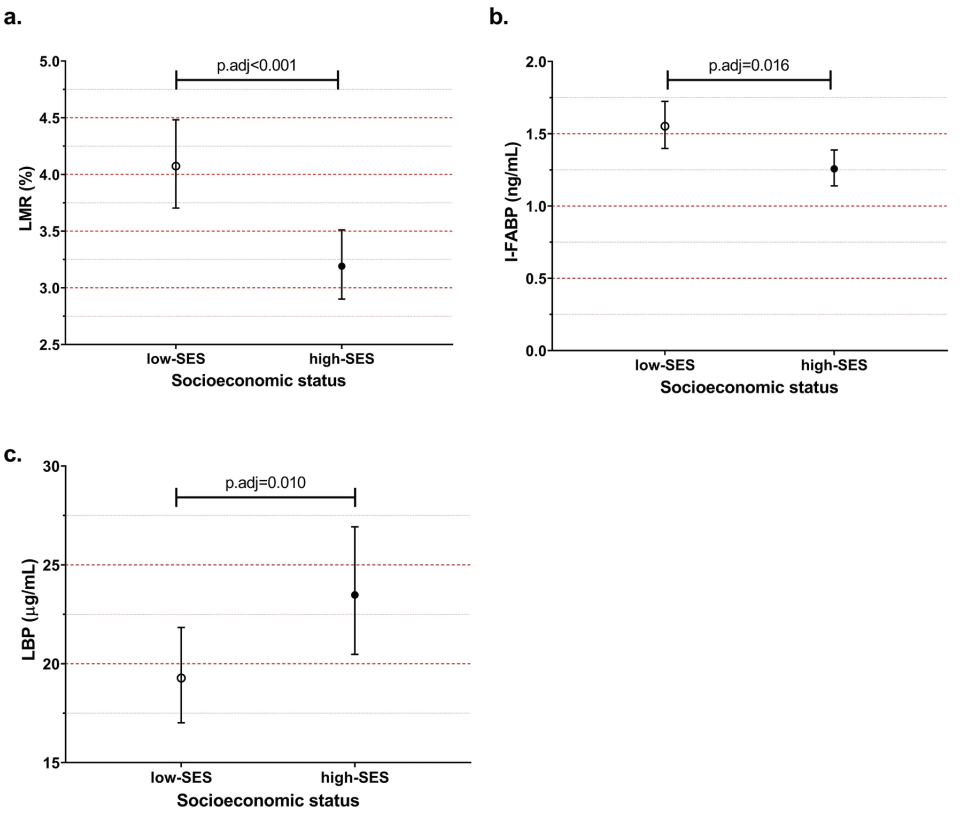

Figure 2. Geometric means and their 95% confidence intervals for different gut permeability markers at baseline, LMR: Lactulose Mannitol Ratio (A), I-FABP: Intestinal fatty acid binding protein (B), LBP: Lipopolysaccharide binding protein (C) and soluble CD14 (D). SES: socioeconomic status. P-values derived from linear regression models after adjusting for age, sex, and zBMI.

Furthermore, we observed no association between z-BMI and any of the gut biomarkers (Supplementary Table S3). In addition, the effect of SES on all these markers did not change after adjusting for z-BMI (Table 2).

In low-SES group, albendazole treatment resulted in a reduction of proportion of subjects infected with helminths and in infection intensity. Albendazole had the largest effect on *A. lumbricoides*, followed by *T. trichiura* (Figure 1A). Before treatment, the percentage of moderate and light infection intensity for *A. lumbricoides* were 10.1% and 36.7% while 3.8% and 29.1% for *T. trichiura*, respectively. Following treatment, only light infections were seen for these two parasites and the proportion of those infected with *A. lumbricoides* and *T. trichiura* was reduced to 3.8% and 8.9%, respectively. In high-SES group, we found no helminth-infected children following albendazole administration. Treatment also led to a reduction in the prevalence of *G. lamblia* (from 51.7% to 31.7%, p.adj=0.005 in low-SES; from 23.3% to 10.6%, p.adj=0.04 in high-SES) but changes were not observed for other protozoa (Figure 1B).

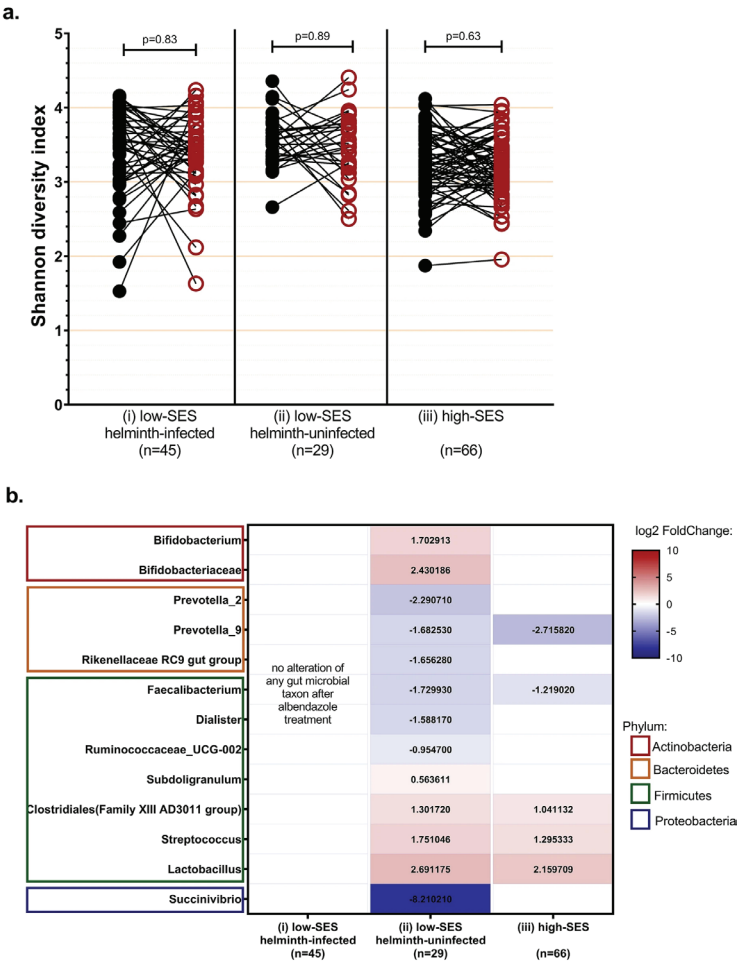

Figure 3. Effect of triple-dose albendazole treatment on A) gut microbiota diversity; and B) gut microbiota composition in (i) low-SES helminth-infected, (ii) low-SE helminth-uninfected, and (iii) high-SES children. A) Shannon diversity index measurements at both timepoints were compared using Wilcoxon signed rank test. Black closed-dots: before treatment; red open-dots: after treatment. B) Data plotted as $\log_2$ fold change derived from differential

abundance analysis by DESeq2. Cell colours indicate taxa changes after albendazole treatment: red colour indicate increased relative abundance after treatment and blue colour indicate decreased relative abundance after treatment. Only taxa detected to have significant difference in abundance (adjusted p-value <0.05) are displayed; adjusted p-value were determined using Benjamini-Hochberg method. Row annotation showed specific taxa that were assigned under 4 different phylum. SES = Socioeconomic status

The differences in the diversity and composition of bacterial gut microbiota at baseline between high and low-SES children of this study have been published [13]. These children shared a core microbiota consisting of *Bifidobacterium*, *Collinsella*, and multiple members of *Lachnospiraceae* and *Ruminicoccaceae* families, but the diversity and the relative abundance of several taxa differed depending on SES [13]. At baseline, Shannon diversity index was higher in the low- compared to the high-SES; however, no differences were seen when we compared helminth-infected vs -uninfected in low-SES [13]. In line with this, after treatment, there were no changes in Shannon diversity index in low-SES children, whether these were helminth-infected or not, nor in high-SES children group (Figure 3A). Similarly, we observed no alteration in gut microbiota composition in helminth-infected low-SES children. However, we observed some alteration of several short chain fatty acid (SCFA)-producing bacteria in the uninfected low- and high-SES children. We found decreased *Faecalibacterium* and *Prevotella*, but an increased *Lactobacillus, Streptococcus, and Clostridiales* relative abundance in the low-SES uninfected and high-SES children (Figure 3B). In addition, some other bacteria changed in the same group: a decreased relative abundance of *Succinivibrio*, *Dialister,* and *Rikenellaceae,* and increased relative abundance of *Bifidobacteriaceae* and *Bifidobacterium* in low-SES helminth-uninfected children (Figure 3B).

Effect of albendazole treatment on intestinal barrier function

As shown in Figure 4A, albendazole significantly decreased LMR in helminth-uninfected children. Estimated treatment effect was largest in high-SES children, with a 13% reduction (p.adj<0.001), while in low-SES uninfected children there was a 11% reduction (p.adj=0.01). No significant reduction was observed in low-SES children who were helminth-infected at baseline.

Similar analysis was performed for I-FABP, marker of intestinal damage (Figure 4B). Firstly, we observed a 9% decrease in I-FABP in high-SES children (p.adj<0.001). In contrast, there was an increase of 12% (p.adj=0.004) in I-FABP following albendazole treatment in low-SES helminth-infected children and although a similar increase was seen in the I-FABP in uninfected low-SES children, this fell short of statistical significance (p.adj=0.08).

Regarding the protozoa, when the model was adjusted for these infections, the effect of albendazole on LMR and I-FABP did not change. Albendazole treatment did not alter the levels of LBP.

To answer the question whether the effect of treatment on LMR and I-FABP was mediated by a specific gut microbiota, we analysed the correlation between the relative abundance of specific taxa and these biomarkers. We could not pinpoint one specific bacterial taxon that might contribute to markers of gut barrier function nor to intestinal damage (Supplementary Figure S3).

Table 1. Baseline characteristics of study population for low- and high-SES schoolchildren

Characteristics	**Low-SES**		**High-SES**		**P-value**
	N	**Result**	**N**	**Result**	
Age in years (mean, SD)	165	10.2 ± 1.08	160	10.3 ± 0.65	0.26
Sex (%)					
Male	73	44.2	71	44.4	0.98
Female	92	55.8	89	55.6	
z-BMI (mean, SD)	165	-0.97 ± 1.19	160	0.27 ± 1. 48	<0.001
z-HA (mean, SD)	165	-2.05 ± 1.08	160	-0.66 ± 1.00	<0.001
Helminth infection (N, n, %)					
Any intestinal helminth	128	84 (65.6)	127	2 (1.6)	<0.001
Ascaris lumbricoides	128	59 (46.1)	127	0	<0.001
Trichuris trichiura	128	51 (39.8)	127	2 (1.6)	<0.001
Hymenolepis diminuta	128	2 (1.6)	127	0	0.16
Intestinal protozoal infection (N, n %)					
Any intestinal protozoa	114	83 (72.8)	120	47 (39.2)	<0.001
Entamoeba histolytica	114	16 (14.0)	120	2 (1.7)	<0.001
Dientamoeba fragilis	114	41 (36.0)	120	25 (20.8)	0.01
Giardia lamblia	114	59 (51.8)	120	28 (23.3)	<0.001
Cryptosporidium parvum	114	3 (2.6)	120	0	0.07

The number of positives (n) of the total population examined (N). SD: standard deviation. Statistical testing was performed using student t-test for continuous variables and using chi-square test for categorical variables.

Table 2. Association between SES and gut permeability markers (at baseline)

Outcomes **Model 1** GMR (95%CI); p-value		**Effect of SES on LMR, I-FABP, and LBP (GMR, 95% CI, p-value)**			
		Model 2	**Model 3**	**Model 4**	
		GMR (95%CI); p-value	GMR (95%CI); p-value	GMR (95%CI); p-value	
LMR	Low-SES	reference	reference	reference	reference
	high-SES	0.78 (0.68-0.90); p<0.001	0.76 (0.65-0.88); p<0.001	0.82 (0.67-1.00); p=0.049	0.73 (0.61-0.88); p=0.001
I-FABP	Low-SES	reference	reference		
	High-SES	0.81 (0.7-0.94); p=0.004	0.83 (0.71-0.97); p=0.016		
LBP	Low-SES	reference	reference		reference
	High-SES	1.22 (1.01-1.47); p=0.036	1.30 (1.07-1.60); p=0.010		1.40 (1.12-1.73); p=0.003

Multivariate analysis using linear regression models. Model 1: crude. Model 2: adjusted for age, sex, z-BMI; Model 3: Model 2 + A. lumbricoides infection. Model 4: Model 2 + D. fragilis infection. SES: socioeconomic status. GMR: Geometric Mean Ratio. CI: Confidence Interval. LMR: Lactulose Mannitol Ratio; I-FABP: Intestinal Fatty Acid Binding Protein; LBP: LPS Binding Protein.

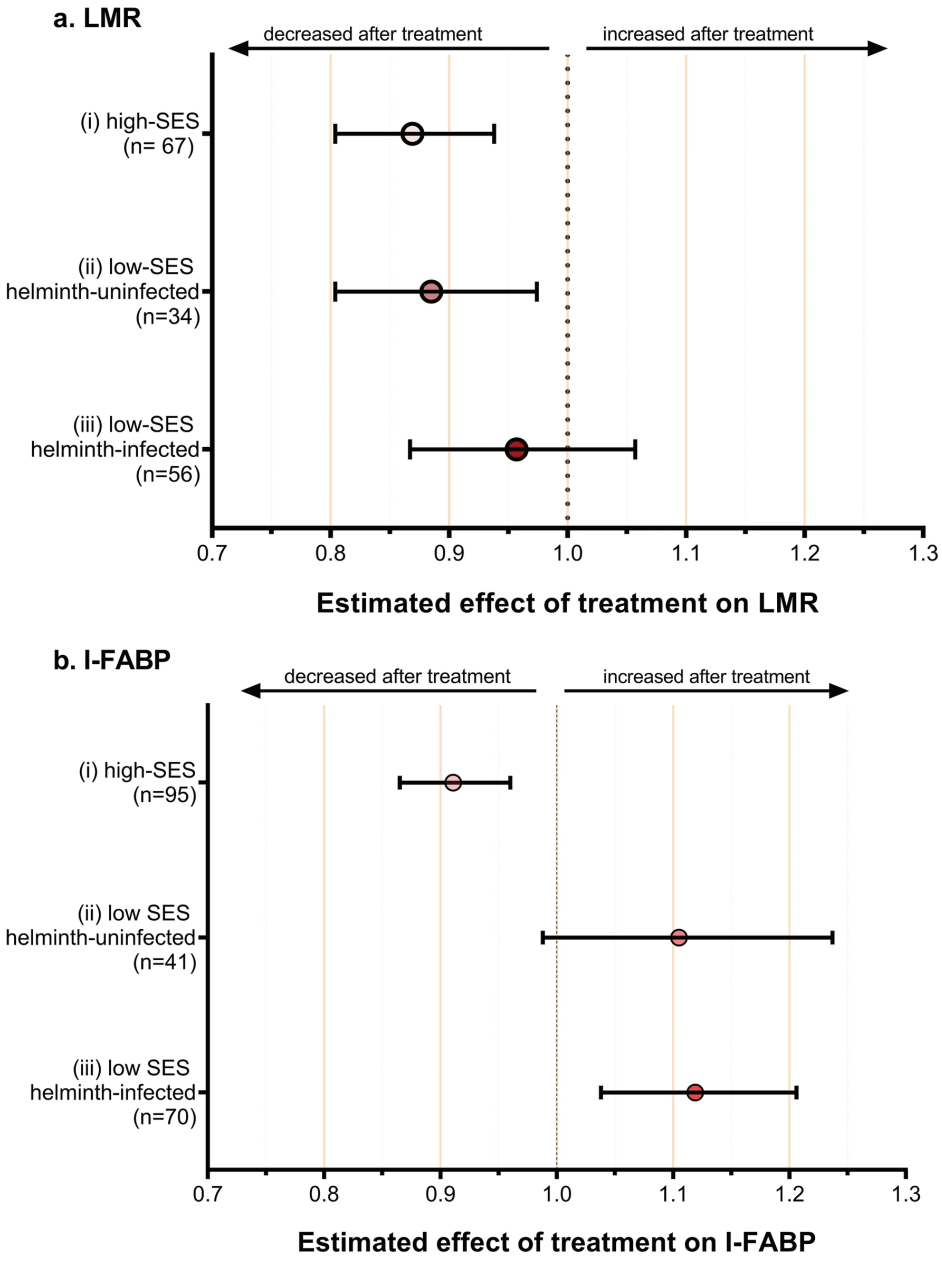

Figure 4. Effect of albendazole treatment on A) LMR and B) I-FABP in study children stratified by SES and helminth infection at baseline (i) high-SES (ii) low-SES helminth-uninfected; and (iii) low-SES helminth-infected. Analysis was using linear mixed model and adjusted for age, sex, and zBMI. The estimated treatment effects are presented as geometric means ratios with the corresponding 95% confidence interval.

Discussion

In this study, we demonstrated that intestinal barrier function, measured by LMR, I-FABP, and LBP differs between low- and high-SES schoolchildren living in an urban center of Makassar, Indonesia. Low-SES children exhibited higher LMR and I-FABP, yet lower LBP. The higher LMR, indicating increased intestinal permeability and higher I-FABP, a marker of epithelial damage, show that the intestinal barrier health and function might be compromised in low- compared to the high-SES children. We had hypothesized that high intestinal permeability might allow bacterial translocation and result in higher LBP levels. Contrary to this, we observed higher LBP values in high SES children that were not associated with zBMI, which could mean that LBP is a better marker of inflammation associated with other factors, such as macronutrient intake. A longitudinal st udy of healthy lean subjects has shown an increase in LPS and LBP concentrations in subjects given a high-fat, high-carbohydrate meal but not in subjects given a high-fiber and fruit meal [14].

In answer to our question whether the differences were due to intestinal parasitic helminths, which were different between low- and high-SES children, we assessed the effect of reducing helminth infections through albendazole treatment. The differences in LMR or I-FABP could not be attributed to current intestinal helminth infections. While LMR decreased after treatment with albendazole, this was restricted to children who were uninfected at baseline. In contrast, I-FABP, increased after albendazole treatment, which might indicate that killing and expulsion of worms, leads to more epithelial damage. In addition, although we observed some changes in microbiota composition after the administration of albendazole, the differences in gut integrity markers could not be explained by differences in microbiota composition. These data indicate that although there are significant differences in gut barrier function of low and high SES children, this could not be accounted for by current parasitic infections.

Despite deworming programs in Indonesia, helminth infections are still widespread, especially in low-SES children. In addition, several parasitic protozoa, such as *G. lamblia*, are more common in low-SES compared to high-SES children in our urban Indonesian population, in line with other studies [15-17]. Several studies have explored the effect of helminth infections on intestinal permeability, however only one has considered SES as a contributing factor [11]. *A. lumbricoides* infection was associated with elevated LMR in Bangladeshi and Malaysian children [11,12], consistent with our findings in the crude analysis. However, multivariate analysis including both SES and *A. lumbricoides* indicated that SES is the main driver of the difference in LMR. Other factors than current *A. lumbricoides* infections could contribute to increased LMR, for example recurrent gastrointestinal infections that lead to diarrhea [18].

It should be noted that exercise can also induce intestinal damage or intestinal permeability, especially after a long and intense physical activity such as running or cycling for 90 minutes [19]. Several studies have shown that active school transport such as walking have been associated with higher physical activity in general [20], which might contribute to the higher LMR in low-SES children.

I-FABP, expressed in mature intestinal epithelial cells, is released into circulation if the cell membranes are damaged [21,22]. I-FABP has been used as a non-invasive marker for acute intestinal damage or integrity loss [23]. In our study, we found no influence of *Ascaris* and *Trichuris* infections on I-FABP. This is in contrast to studies in subjects infected with hookworm and *Strongyloides stercoralis,* which reported elevated levels of I-FABP [24,25]. It is possible that the latter two helminths are more pathogenic, for example, hookworms feed on intestinal mucosa and blood [26], which can indeed result in more damage. In our study, the marked

increased I-FABP after treatment in helminth-infected children, might suggest that removal of worms leads to local inflammation and damage to epithelial cells, however, as there was a tendency for a similar effect in the helminth-uninfected low-SES children, it is possible that albendazole either directly or indirectly affects gut epithelial homeostasis and damage.

One of the biomarkers generated in response to bacterial translocation is LBP, a class 1 acute phase protein. Unexpectedly, in our study, LBP was found to be lower in low-SES where the intestinal barrier was more disrupted compared to high-SES children. The higher LBP levels in high-SES schoolchildren might be in line with previous observations where elevated LBP levels were linked to obesity, weight gain [27,28], high fat and high carbohydrate diet [14], as in the high-SES, the children have a different nutritional status and some are obese. However, these differences persisted after controlling for zBMI, suggesting there may be other factors at play, potentially high-fat and high-carbohydrate intake.

As published already, gut microbiota composition of Indonesian schoolchildren in our study is associated with socioeconomic status, even when living in the same urban area [13]. Not only sanitation[29] but also differences in diet, hygiene, or helminth infections might be causes of the differences in the gut microbiota profile [29-32]. Regarding the diversity of microbiota in our population, we have reported that low-SES children have higher microbial diversity compared to high-SES children, independently of helminth-infection status [13]. Easton and colleagues showed that 3 weeks after albendazole treatment gut microbiota diversity did not change, yet, microbiota composition was altered with decreased *Aeromonodales (Gammaproteobacteria)*. However, their study did not distinguish the effect of albendazole, between helminth infected and uninfected subjects [33]. In line with finding of Easton and colleagues, we showed no change in microbiota diversity while microbiota composition was altered 4 weeks after albendazole administration. Nonetheless, larger sample size in our study allowed us to stratify the population based on their SES and helminth status, where the relative abundance of several taxa was altered but only in the uninfected subjects and not in helminth-infected subjects of low-SES group. Moreover, we also observed changes in the high-SES children, indicating that alteration in bacterial taxa was more SES-related and not associated with helminth infections. Further studies should shed light on whether the altered microbiota composition, in a SES-specific manner, is albendazole-related or reflects natural oscillations over time.

An important limitation of our study is the substantial loss to follow-up. Despite our efforts to retain children within the study, 48 % of children could not be followed up. However, we report no difference in baseline characteristics between the children that were lost to follow up and those who remained in the study. Furthermore, although we controlled for pre-identified confounders, dietary intake and physical activity was not surveyed, thus, their effect on intestinal permeability and microbiota composition could not be explored. No placebo was used in our study, consequently, we do not have the benefits of controlling for the changes related to time rather than treatment. Due to the lack of data regarding urine volume in this study, we are not able to compare the LMR result with published data.

In conclusion, the level of intestinal permeability and acute intestinal injury as measured by LMR, as well as LBP and I-FABP, differed between high- and low-SES children, and these differences were not associated with intestinal parasitic infections. Further research is needed to elucidate the exact mechanisms responsible for the elevated intestinal permeability in low-SES children as well as the off-targets effect of albendazole.

Material and Methods

Study population and design

The study was conducted in Makassar, South Sulawesi, Indonesia. Ethical approval was obtained through the local ethics committee of Hasanuddin University (approval number: 1504/H04.8.4.5.31/PP36-KOMETIK/2016). Study participants were recruited from two elementary schools that are distinct in SES. The low-SES school was located near the port area where mostly low-skilled labourers lived and worked. Children generally lived in the area surrounding the school site and travelled to school on foot. The high-SES school was located in the city centre. Children attending this school lived in distinct parts of the city, mostly in residential compounds and travelled to school by privately chartered school buses or by private vehicles.

Prior to the start of the study, an information letter concerning the study was given to the parents seeking permission for their children to participate in the study. Only children who returned the signed informed consent were included in the study. At baseline, anthropometry data were collected. Blood sample was obtained from median cubital vein by a venipuncturist. The day before, a stool container with enclosed spoon (Sarstedt AG&Co.KG, Nümbrecht, Germany) was given to these children. They were asked to collect stool samples in the morning before school, the same day when the study was conducted.

As soon as samples were gathered by research staff on site, stool, blood and urine samples were stored inside an ice box and transported to the Laboratory of Parasitology Department at Hasanuddin University and Hasanuddin University Medical Research Center laboratory to be aliquoted and kept at -80˚C for further analyses.

After completion of the baseline visit, all participants received a single albendazole dose (400 mg, PT HoliPharma, Cimahi, Indonesia) given for three consecutive days regardless of their helminth infection status. The follow-up visit took place 4 weeks after treatment at which collection of blood, stool, and urine samples were repeated.

Parasitological examination

A single Kato-Katz slide was prepared from each stool sample [34] and examined for the detection of STH Infection. Intensity was determined for each species according to WHO guidelines [35]. PCR was performed to identify intestinal protozoa. Briefly, DNA was extracted from stool samples followed by a multiplex real-time PCR used for the specific amplification and detection of *Entamoeba histolytica*, *Dientamoeba fragilis*, *Giardia lamblia*, and *Cryptosporidium parvum*. The procedure has been described elsewhere [36-38]. PCR output was expressed as the cycle threshold (Ct)-value reflecting the load of specific DNA in the sample tested. Protozoa specific DNA load were categorized into low load ($35 \leq Ct < 50$), moderate load ($30 \leq Ct < 35$), or high load ($Ct < 30$). Negative DNA results were recoded as $Ct = 50$.

Urinary lactulose-mannitol ratio (LMR)

Following overnight fasting, a lactulose/mannitol drink, containing 2 g mannitol and 5 g lactulose dissolved in 100 mL drinking water, was administered at school. The following three hours, all urine was collected in a large container together with 1 mL chlorhexidine 2% as a preservative. Urine samples were analysed using liquid chromatography mass spectrometry

(LC-MS) as described previously [39,40]. LMR was calculated by dividing the lactulose concentration by the mannitol concentration in absence of data on the collected urine volumes. These values were multiplied with 100 to create percentages.

ELISA for measurements of I-FABP, LBP and sCD14

ELISA techniques were used to quantify the concentrations of I-FABP and LBP (Duoset, R&D system, UK), according to the manufacturer's instruction. For these assays, serum was diluted 8 and 2000 times for I-FABP and LBP, respectively. The results were expressed in µg/ml for LBP and ng/ml for I-FABP.

Microbiota analysis

Microbiota analysis was performed in 140 children from whom sufficient stool samples were available before and after treatment. The procedure for sample processing and microbiota analysis is already described elsewhere [13]. Raw sequencing data are available in the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under study accession PRJEB38465 (baseline) and PRJEB40889 (follow up).

Statistical Analysis

In accordance with the WHO guidelines, age standardized of z-scores of body mass index (z-BMI) were calculated. For the crude analyses, categorical data were compared using chi-square tests, whereas normally distributed continuous data was compared using the student t-test. Correlation between variables was tested using Pearson or Spearman correlation and we considered $\rho \geq 0.4$ suggestive for a relevant correlation. To help explore the complex interplay between SES and helminth infection, the analysis was stratified into i) high-SES, ii) low-SES helminth-uninfected, and iii) low-SES helminth-infected. Linear regression models were used to adjust for a priori confounders such as age, sex, and z-BMI in addition to the identified explanatory variables. The data was analysed using IBM SPSS Statistics version 25 (IBM-SPSS Inc., Chicago, USA), and GraphPad Prism (GraphPad Software, Inc., CA, US) was used for visualisation. Longitudinal data were analysed using mixed models with subject random effects, and fitted using lmerTest package (R software) [41]. LMR, I-FABP, sCD14, and LBP were log10-transformed before analysis. Parameter estimates of treatment effects (95%CI) were then back transformed to obtain the geometric mean ratios (GMR). The reported p-values were obtained using a likelihood ratio test comparing the model with and without a time effect.

Microbiota data were analysed in R-software (v3.5.1) using the packages phyloseq (v1.26.1)45, vegan (v2.5–4)46, ggplot2 (v3.1.0)47, DESeq2 (v1.22.2)48 and microbiome (v1.4.2)49. Wilcoxon signed rank tests were performed to compare Shannon diversity index before and after treatment groups. For differential abundance testing by DESeq2, the OTU-table was filtered for OTUs present in less than 25% of the samples to minimize zero-variance errors and spurious significance. Outcomes were considered significant when the Benjamini–Hochberg corrected p value was ≤ 0.05. To analyse albendazole-altered taxa in this population, paired analysis was done.in this population, paired analysis was done.

Conflict of interest

We declare that we have no conflict of interest.

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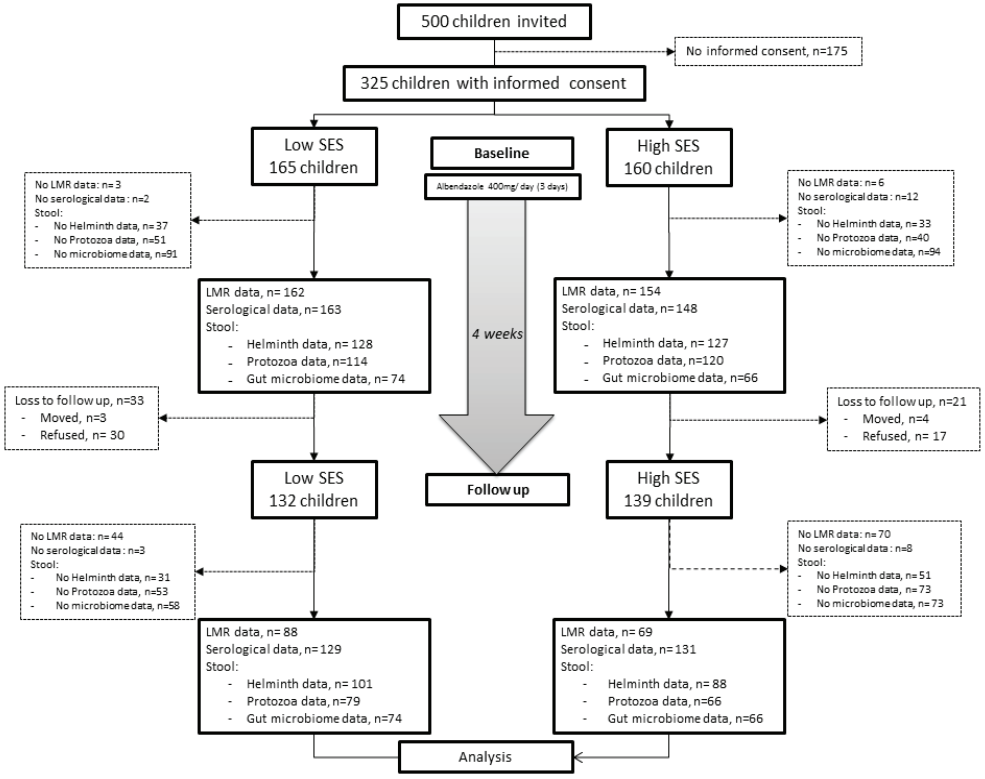
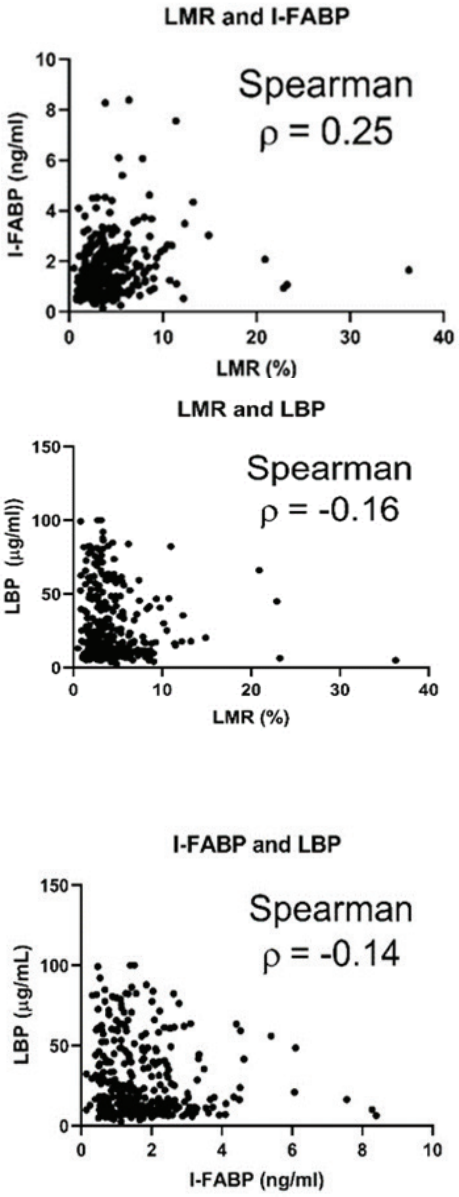
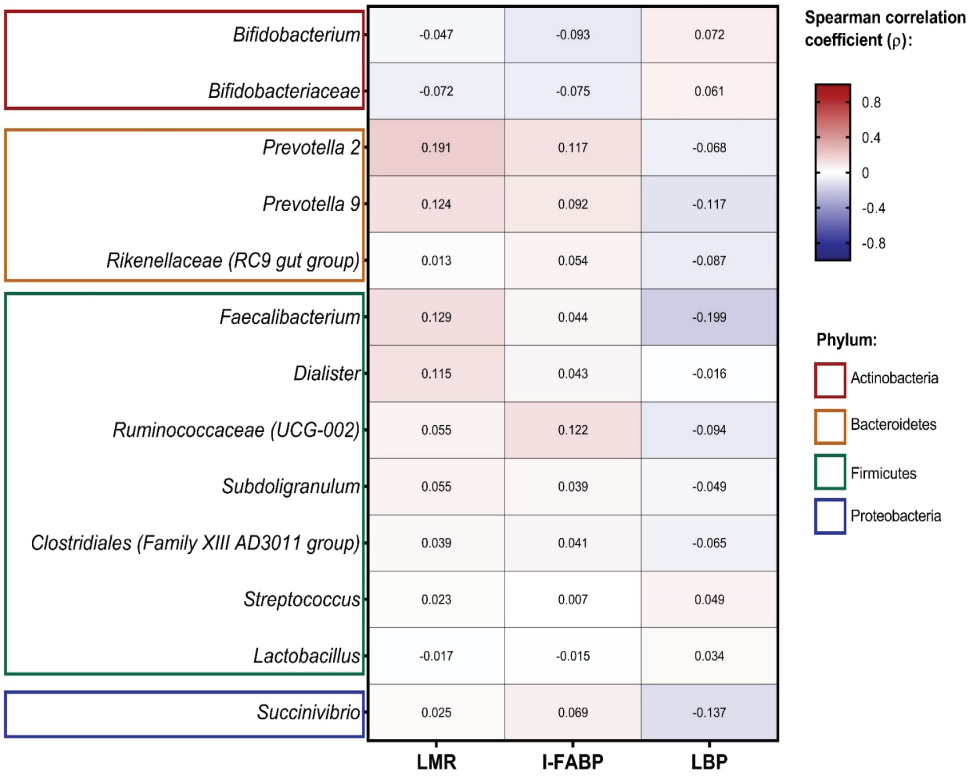
SUPPORTING INFORMATION



Supplementary Figure S1. Consort Diagram. After collecting baseline data all participant (n=325) were treated with single dose albendazole for three consecutive days. Follow up data were collected 4 weeks after albendazole treatment. SES: socioeconomic status. LMR = lactulose mannitol ratio.



Supplementary Figure S2. Correlation between paired biomarkers at baseline. Data presented as ρ (spearman correlation) and considered relevant correlation if ρ≥0.4. LMR: Lactulose Mannitol Ratio; I-FABP: Intestinal Fatty Acid Binding Protein; LBP: LPS Binding Protein.



Supplementary Figure S3. Correlation between albendazole-altered taxa and LMR, I-FABP, and LBP. Data presented as ρ (spearman correlation) and considered relevant correlation if ρ≥0.4. LMR: Lactulose Mannitol Ratio; I-FABP: Intestinal Fatty Acid Binding Protein; LBP: LPS Binding Protein

Characteristics of study population remained in the study and loss to follow-up

Characteristics	remained in the study	lost to follow up	p-value
SES (low-SES), n/N, %	88/157 (56.1)	74/159 (46.5)	0.093
Any helminths, n/N, %	58/157 (36.9)	27/90 (30.0)	0.330
Sex (female), n/N, %	93/157 (59.2)	81/159 (50.9)	0.143
Age, mean ± SD (N)	10.30 ± 0.87 (157)	10.23 ± 0.90 (159)	0.501
zBMI, mean ± SD (N)	-0.36 ± 1.47 (157)	-0.31 ± 1.47 (159)	0.755

The number of positives (n) of the total population examined (N). SD: standard deviation. Statistical testing was performed using student t-test for continuous variables and using chi-square test for categorical variables.

Supplementary Table S2. Geometric means and 95% confidence intervals for gut permeability markers in relation to different explanatory variables

		LMR (%)		I-FABP (ng/ mL)		LBP (µg/mL)
	N	Geomean (95%CI)		Geomean (95%CI)	N	Geomean (95%CI)
Any intestinal helminth						
Positive	85	4.15 (3.60 - 4.78)	85	1.55 (1.33 – 1.79)	85	15.91 (13.46 – 18.81)
Negative	162	3.56 (3.24 – 3.93	158	1.35 (1.22 – 1.49)	159	18.80 (16.64 – 21.23)
Ascaris lumbricoides						
Positive	58	**4.44 (3.74 – 5.28)**	59	1.51 (1.25 – 1.83)	59	17.10 (13.86 – 21.10)
Negative	189	**3.57 (3.26 – 3.90)**	184	1.38 (1.26 – 1.52)	185	17.94 (16.04 – 20.07)
Trichuris trichiura						
Positive	53	4.16 (3.40 – 5.08)	53	1.54 (1.27 – 1.88)	53	16.55 (13.27 – 20.64)
Negative	194	3.65 (3.35 – 3.98)	190	1.38 (1.26 – 1.51)	191	18.08 (16.19 – 20.19)
Any intestinal protozoa						
Positive	127	3.83 (3.42 - 4.30)	126	1.38 (1.22 – 1.56)	125	19.03 (16.63 – 21.78)
Negative	101	3.60 (3.18 – 4.06)	97	1.46 (1.28 – 1.65)	99	15.89 (13.60 – 18.54)
Entamoeba histolytica						
Positive	18	4.07 (3.09 – 5.38)	18	1.43 (0.99 – 2.08)	18	15.60 (10.60 – 19.74)
Negative	210	3.70 (3.39 – 4.04)	205	1.41 (1.29 – 1.55)	206	17.75 (15.97 – 19.74)
Dientamoeba fragilis						
Positive	64	3.64 (3.05 – 4.36)	64	1.31 (1.10 -1.56)	63	**20.84 (17.19 – 25.28)**
Negative	164	3.76 (3.42 – 4.13)	159	1.45 (1.31 – 1.66)	161	**16.44 (14.58 – 18.51)**
Giardia lamblia						
Positive	144	3.86 (3.35 – 4.46)	140	1.44 (1.23 – 1.70)	142	19.50 (16.51 – 23.05)
Negative	84	3.65 (3.29 – 4.04)	83	1.40 (1.26 – 1.55)	82	16.54 (15.55 – 18.81)

CI: confidence intervals. Testing performed using unpaired student t-test on log transformed data. Bold: p-value<0.05. LMR: Lactulose Mannitol Ratio; I-FABP: Intestinal Fatty Acid Binding Protein; LBP: LPS Binding Protein

Supplementary Table S3. Association between anthropometric measurement and LMR, I-FABP, and LBP.

		LMR	I-FABP	LBP
zBMI	**GMR (95%CI)**	1.02 (0.97-1.07)	0.97 (0.92-1.03)	0.95 (0.88-1.01)
	p.adj	0.497	0.371	0.119
zHA	**GMR (95%CI)**	1.04 (0.97-1.11)	0.96 (0.90-1.03)	0.95 (0.86-1.04)
	p.adj	0.320	0.304	0.231

Data presented as geometric mean ratio (GMR) with 95% confidential interval (95%CI). Adjusted p-value (p.adj) was derived from linear regression analysis and adjusted for SES, age and sex. LMR: Lactulose Mannitol Ratio; I-FABP: Intestinal Fatty Acid Binding Protein; LBP: LPS Binding Protein.

CHAPTER SIX
SUMMARIZING DISCUSSION

CHAPTER 6

SUMMARIZING DISCUSSION

WHAT WAS ALREADY KNOWN ABOUT THE RELATIONSHIP BETWEEN CHILD HEALTH AND SOCIOECONOMIC STATUS

The rise of communicable and non-communicable diseases, together with socioeconomic health inequalities are still a problem in developing countries. Indonesia is now facing the triple burden of disease, namely, communicable diseases, non-communicable diseases, and re-emerging diseases [1]. The upsurge of Indonesian economic development is also widening the gap in the health inequality between the rich and the poor.

A large study examined development and growth in young children across socioeconomic status (SES) in four developing countries (Indonesia, India, Peru and Senegal) and reported that children from wealthiest households had higher development scores, determined by EASQ (Extended Ages and Stages Questionnaire) and better growth, indicated by LAZ (length for z-scores) than children from the poorest households, while controlling for maternal education and relevant covariates [2]. The association between SES and child health is also reported in developed countries, for example, a study in Germany showed that children from low-SES report lower quality of life and adopt less healthy lifestyle, while wealthiest children have better health [3]. In this thesis, the focus has been on studying vaccination, allergies, the microbiome and gut health in children of high and low SES in Indonesia to gain more insight into how socioeconomic discrepancy might affect certain biological processes. Therefore, in the following sections, the background to these focus areas will be sketched.

Vaccination

Tuberculosis, one of vaccine-preventable diseases, is found to be more prevalent among the poor. Despite massive efforts to prevent this disease, tuberculosis remains a significant cause of morbidity and even mortality especially in the low-income countries [4]. Until now, BCG is the only vaccine licensed for prevention of tuberculosis. Vaccine response and effectiveness has been reported to be lower in low- compared to high-income countries [5]. A comparative study on immune responses following BCG vaccination in the UK versus Malawian infants reported that immune responses induced by BCG vaccination differ in both profile and magnitude between the two settings and these might be due to factors related to maternal, nutritional and environmental factors [6]. One explanation of the weakened protection from the vaccine has been suggested to be the influence of chronic helminth infection of the mother before [7] or during pregnancy [8] which may affect the developing immune system of the child. However, a double-blind placebo-controlled trial performed in Ugandan pregnant mothers showed that anthelmintic treatment had no effect on the immune response of their infants to BCG, tetanus or measles immunization [9]. Indeed, more factors other than helminth infections that occur in similar environments might be responsible for poor BCG responses.

Allergies

The changes in lifestyle and environment, characterized by increasing sanitation, hygienic measures and urbanization in developed countries in the past decades have been linked to the increase in the prevalence of allergic disease [10, 11]. Developing countries, such as India [12] and China [13] are now also rapidly facing the allergic epidemic. With regards to

the urban and rural differences, there is strong evidence from many parts of the world reporting higher prevalence of allergic disorders in urban compared to rural areas [14-16]. Different environmental exposures along with genetic factors could account for the observed variability [17]. Environmental exposure can include exposures to microbes, parasites such as helminths, and different lifestyles [18]. Helminths have coevolved with human immune system and they have developed numerous survival strategies which can modulate the host immune system through direct secretion of excretory/secretory molecules [19] [20]. These molecules then interact with the immune system and influence it directly or indirectly, which can be through the regulation of the microbiome [21, 22]. One type of allergy that is life-threatening is venom allergy, which has mostly been studied in temperate or subtropical countries. The prevalence of Hymenoptera venom sensitization has been reported to vary from 3.66 to 41.6% [23-27], whereas prevalence of systemic allergic reactions to venoms has been estimated to be between 0.3 and 16.0% [25, 28-31]. Little information is available regarding the prevalence of venom allergies in the tropics. Several epidemiological studies in Indonesia have reported an inverse relationship between aeroallergens and helminth infections [32, 33], which spark the question whether the same relationship applies to venom allergy in Indonesia.

The microbiome

In many observational studies it has been shown that the gut microbiota affects numerous aspects of human physiology [34]. The imbalance of microbiota composition, richness or diversity, also known as dysbiosis, may induce inflammation, metabolic condition, or other pathologies [35, 36]. Microbiota diversity is a measure of how many different species exist in the community and, depending on the diversity indices, how evenly distributed they are. In the tropics, intestinal parasites, such as helminths or protozoa co-exist in the gut with gut microbiota. There are several factors that might determine gut microbiota profile in a population. Environmental factors are known to predominantly shape the composition of gut microbiota instead of genetic factors [37]. Large amount of data has been gathered by a study across ethnic groups in the United States [38] as well as studies of populations with varied ethnic origins either living in different geographical areas [39] or in the same location [40]. However, little is known about the gut microbiota profile of Indonesian children living in urban areas where socioeconomic disparities exist with the resulting differences in exposure to helminth infections, hygienic practices and diet. So far, few investigations have been conducted regarding the interaction between SES, intestinal parasites, and gut microbiota. A study in a group of indigenous people in rural Malaysia has observed that helminth colonization is associated with higher microbiome diversity and that *Trichuris trichiura* infection drives the higher abundance of *Paraprevotellacae* in this population [41]. Moreover, among this indigenous population in Malaysia, it was reported that serum zinc and iron levels were affected by helminth infection status and also associated with an abundance of specific microbial taxa. It was also mentioned that the majority of microbiota that were associated with the changes in zinc levels, belonged to *Bacteroidales* order while the predominant microbiota associated with changes in iron levels belonged to *Clostridiales* order [42]. Since helminths and intestinal microbiota share the same niche, it would be interesting to investigate their relationship in populations living in urban Indonesia with inequalities in SES.

Gut health

Several studies have established the importance of gut barrier function including intestinal permeability in gut health and development of diseases [43, 44]. The intestinal barrier is semipermeable with critical function for nutrient absorption and as a barrier against pathogens [45]. To maintain this intestinal barrier function, short chain fatty acids (SCFAs) and

other microbial metabolites, end-products resulting from fermentative metabolism of fibers or complex polysaccharides by intestinal microbiota, are needed [46-48]. Therefore, the interaction between intestinal parasitic infections, gut microbiota and intestinal cells should be investigated.

As far as we know, very little data are available on the direct effects of intestinal parasites on human gut permeability [45]. A previous study indicated that residents of a tropical country have higher level of gut permeability compared to residents living in temperate or subtropical areas suggesting the importance of environmental factors [49]. Exposure to microorganisms and parasites as well as hygienic lifestyle might contribute to this variation but further research is needed to disentangle the relationships that influence intestinal permeability, as a determinant of health disparity in populations with distinct SES.

The gap

Most studies on child health and SES have been performed by comparing health status of low- and high-income countries or by assessing rural versus urban areas within one country. However, very few studies have been conducted on the health status of children within one urban area but with different SES. In order to investigate the impact of SES on child health we conducted studies in Makassar, an urban center in Indonesia with a population living under very different socioeconomic conditions.

HOW DID OUR STUDIES ADVANCE THE FIELD?

In this thesis, the complex association between SES and several outcomes that can affect health, such as responses to BCG vaccine, venom sensitization and gut barrier function have been investigated. Here, we conducted a study in children with different socioeconomic backgrounds living in the same urban area in Makassar, Indonesia. Information regarding the presence of intestinal helminths and protozoa as well as gut microbiota characteristics were included to gain information on factors that might play a role in how SES shapes child health.

The impact of socioeconomic status on responses to BCG vaccination

Vaccines are among the most cost-effective preventions against the rise of infectious diseases. However, it is known that vaccine responses can vary across populations [50, 51]. For example, BCG vaccine has shown very distinct immunogenicity and efficacy in different parts of the world, in particular between high-income countries and low- and middle-income countries [5, 6] including Indonesia.

In **Chapter 2**, we provide evidence that both SES and nutritional status at birth determine the response to BCG vaccination measured at 10 months of age in Indonesian children. Our results showed that low-SES children have smaller BCG scar size compared to high-SES children in the same city, which is in line with another study in children from Dominican Republic [52], where lower socioeconomic index was associated with smaller BCG scar size. We went further to investigate whether exposure to helminths and/or nutritional status might be responsible for this observation.

Infections with helminths is associated with Th-2 immune response characterized by increased production of IL-4, IL-5 and IL-13 cytokines, polyclonal and specific IgE as well as eosinophilia [53, 54]. BCG vaccination induces a Th-1 type immune response and can be in an equilibrium with Th-2 type responses [55] and therefore a strong Th-2 response could be inversely associated with response to BCG. In agreement with this notion and a study conducted in Turkey [56], in **Chapter 2,** we report that the larger size of BCG scar was associated with lower IgE levels observed in high-SES infants. However, here through examining helminth infections we found that although IgE levels were higher in low- compared to high-SES mothers and their newborns, this could not be related to the presence of helminth infections in pregnant women taking part in the study. This could either be due to previous helminth infections of mothers that had an imprinted immune system towards Th-2, or contribution of other environmental factors to skewing of the immune system and elevated IgE antibodies.

As nutritional status might affect responses to BCG vaccine [52], we also assessed whether BCG scar size was influenced by z-weight-for-height at birth as a proxy of newborns' nutritional status and adiposity. We observed z-weight-for-height to be positively associated with larger size of BCG scar through the leptin pathway. Leptin has been shown to drive immune responses towards Th-1, [57, 58], for example, Mattioli et. al [57] have shown that leptin down-regulates IL-10 production by dendritic cells and drives naive T cell polarization toward Th1 phenotype. In **Chapter 2,** we found that leptin, and not adiponectin, strongly attenuated the relationship between nutritional status and BCG scar size in infants. This finding suggests that higher leptin levels at birth determine the development of larger BCG scar in vaccinated-infants. Emerging evidence shows that lower leptin levels can be associated with poor vaccine responses in the general population by reducing the differentiation of human follicular T helper cells (T_{FH}), which are a subset of T cells that facilitate B cell antibody production [59]. Thus, leptin levels might not only be important in B cell dependent vaccines but also in vaccine responses such as BCG that hav an important cell mediated efficacy against *Mycobacterium tuberculosis*. [60, 61].

In summary, the size of BCG scar as a proxy of immune response to BCG vaccination, was affected by SES and leptin levels at birth. Additionally, total IgE, partly contributed to the reduction in BCG scar size.

Bee- and wasp venom sensitization in schoolchildren with different socioeconomic status

The global magnitude of venom allergy is not completely mapped as the majority of studies have been conducted in temperate or subtropical regions of the world. Despite the high sensitization to bee and wasp venoms in Europe, most of those individual have had no systemic reactions to bee and wasp stings [62] and this could be due to cross-reactive carbohydrate determinants (CCDs, alpha 1,3-fucosylated N-glycans) [63]. Apart from true sensitization, IgE against CCD has been seen in plants and invertebrate glycoproteins and show low clinical relevance [64-66]. In areas where helminth infections are prevalent, a number of studies have shown that high levels of cross reactive IgE to allergens are not biologically functional [67]. Therefore, we aimed to gather more information on bee venom allergy in Indonesia, to assess how SES and factors associated with it, affect the prevalence of this allergic sensitization.

In **Chapter Three,** we showed that skin sensitization against bee- (14.3%) or wasp-venom (12.7%) is quite prevalent among schoolchildren in Indonesia and that the prevalence was different when SES of the children was considered. Similar to the responses against aeroallergens, SPT reactivity to bee- and wasp venom was more prevalent in high compared to

the low-SES children, while the proportion of specific IgE positivity to venom allergens was higher in the low compared to the high-SES children. Interestingly, the sensitizations to Hymenoptera venoms appeared to have poor clinical relevance as they rarely translated into clinical symptoms.

The observation of discordance between SPT and sIgE to venom allergens in our study can have two reasons. Firstly, when SPT reactivity is seen in the absence of sIgE, it might suggest that skin reactivity to Hymenoptera venom is not through IgE but might be through non-IgE mediated mechanisms [68, 69] since venom components such as peptide-401 may provoke mast cell degranulation [70] and directly result in a positive skin reaction in some children [71]. Secondly, the lack of skin reaction with detectable sIgE, may suggest the presence of sIgE with poor function, for example cross reactive IgE, unable to induce mast cell degranulation [72, 73]. However, the low report of clinical venom allergy and SPT, might also be the result of desensitization as consequence of frequent stinging [74, 75].

Despite interesting results provided in this thesis; some limitations are worth noting. Cross sectional design used in **Chapter Three** does not allow us to determine causality and time of exposure. It is known that sensitization peaks several weeks after the sting and subsides gradually. [62, 76]. This chapter examined past responses to venom and current sensitization, instead of tracing responses after sensitization and sting. In addition, the involvement of helminths in the lack of skin reaction despite the presence of sIgE in the low-SES could not be discerned.

Bacterial gut microbiota and socioeconomic status

In the past decades, plethora of studies have tried to disentangle the association between environmental influences and human health through examining gut microbiota [77-79]. Several reports showed that the gut microbiota is highly variable among individuals and is determined primarily by environmental factors such as diet, hygiene level, physical activity, disease status and medication, rather than genetics [37, 80]. In **Chapter Four**, we showed that schoolchildren living in urban Makassar share a core microbiota irrespective of their SES. These core microbiotas consisted of *Bifidobacterium, Collinsella*, and multiple members of the *Lachnospiraceae* and *Ruminicoccaceae* families, but the relative abundance of these taxa varied greatly among children. SES has been found to be the main driver of differences in gut microbiota composition. Several genera of bacteria showed different abundance in children with high and low SES, including *Escherichia-Shigella, Prevotella,* and *Lactobacillus.* Similar to our study, Chong and co-workers [81] showed lower microbial diversity in wealthier children as compared to economically deprived children living in the same rural area in Malaysia. Furthermore, the study also reported that the presence of parasitic infections exerted a significant but only a small influence (explained 5% variance) on the elevated gut microbial diversity [41]. The finding in our **chapter four,** presented that helminth infections were prevalent in low-SES children, and although this was positively associated with *Olsenella, Enterorhabdus, Lactobacillus*, and *Mogibacterium* abundance, it was negatively associated with the relative abundance of *Prevotella*. Additionally, infection with protozoa was prevalent in low-SES children, and was also negatively associated with the relative abundance of *Prevotella*. In contrast to the study by Chong and co-workers [81], our results observed no clear association between helminths or protozoa with microbiota diversity. Such variations in microbiota structure at high and low-SES are most likely caused by lifestyle differences.

Diet has been considered as a major driver of the composition of bacterial gut microbiota [82]. Thus, observed variations in microbiota composition between high- and low-SES children can be reflecting variations in their diet. The high abundance of *Bifidobacterium* and

Lactobacillus among the wealthier children could be related with dairy products and probiotics consumption [83]. Meanwhile the higher relative abundance of *Prevotella* observed in the low-SES has been linked with vegetable-rich diet [81]. A study in Thai schoolchildren reported a contrasting microbiota type between urban (Bangkok) and rural (Khon Kaen) population. Using dietary intake questionnaires, they found that children in Bangkok who eat much less vegetables and fruits, tended to have the BB- (*Bifidobacterium/Bacteroides*) type microbiota while children living in rural have the P- (*Prevotella*) type microbiota [84].

The study performed in **chapter four** did not collect information on food intake, therefore we are not able to assess the influence of diet on microbiota composition

In **Chapter Five**, we showed that irrespective of helminth infection status, or SES, albendazole did not affect bacterial gut microbiota diversity. However, the composition of gut microbiota was altered 4 weeks after albendazole administration in low-SES uninfected and high-SES groups. This finding is similar to a study in Kenya where no changes were observed in gut microbiota diversity 3 weeks after albendazole treatment, but the microbiota composition was altered with a significantly decreased *Aeromonodales (Gammaproteobacteria)* [85]. It should be noted that the study did not distinguish the effect of albendazole between helminth infected and uninfected groups. In our study, following albendazole treatment, we observed changes in the relative abundance of several gut microbiota taxa, but specifically in the uninfected children of low-SES and the high-SES children. We also observed some alteration in several short chain fatty acid (SCFA)-producing bacteria in the uninfected low- and high-SES children. *Faecalibacterium* and *Prevotella* wer found to be decreased, meanwhile the relative abundance of *Lactobacillus, Streptococcus, and Clostridiales* were increased in the low-SES uninfected and high-SES children following treatment. Moreover, some alteration also observed in the uninfected group of low-SES only with a decreased relative abundance of *Dialister, Succinivibrio*, and *Rikenellaceae,* and increased relative abundance of *Bifidobacteriaceae* and *Bifidobacterium*. This indicates that these alterations were more SES-related and not associated with helminth infection status of the children. The data would suggest that albendazole has an effect on the microbiome, either directly or indirectly through influencing protozoa. However, as our study was not placebo-controlled, the changes might reflect natural variation over time in the uninfected group.

Soil-transmitted helminth infections still remain a burden especially in the tropics, and despite the high efficacy of albendazole in reducing *Ascaris lumbricoides*, it is less effective against *Trichuris trichiura* [86], even our approach by administering 400 mg albendazole given on 3 consecutive days, did not eliminate *T. trichiura* (Chapter five). The inability of albendazole to reduce helminths might be one reason why no alteration was observed in the gut microbiota composition in the helminth-infected group. Several strategies are currently being investigated to lessen the prevalence of *T. trichiura* infections, such as increasing the dose of albendazole to 800 mg [87] or by combination of ivermectin and albendazole [88]. Interestingly, a recent study has suggested that the gut microbiota composition can affect the efficacy of anthelminthic treatment on hookworms and *T. trichiura* [89]. The complexity of microbiome studies in their own right, let alone when considering the effect of helminths and treatment, indicates that better designs and larger studies are needed to fully address the question we tried to tackle.

Intestinal permeability, parasite infections and socioeconomic status

The gut barrier consists of epithelial cells and a mucus layer which create a barrier between tThe gut barrier consists of epithelial cells and a mucus layer which create a barrier between the lumen of the digestive tract and systemic circulation [90]. Under certain circumstances,

gut barrier could be impaired leading to a disrupted permeability in the nutrient absorbing areas of the intestine. Therefore intestinal integrity can be assessed by measuring the transcellular and paracellular transport of orally administered high- and low-molecular-weight sugars across the gastrointestinal tract [91]. Lactulose and mannitol, of all sugars, are most frequently used in studies of gut permeability [90] and can measure the permeability of the small intestine [92]. The Lactulose-Mannitol Ratio (LMR) is beneficial to use as both sugars are not actively absorbed from the intestine, not metabolized, and excreted unaltered in urine corresponding to the quantities absorbed [93]. Mannitol, the smaller molecule, is presumed to permeate transcellularly through the water pores of the membrane, whereas lactulose, the larger molecule, is assumed to have paracellular permeation that it transverse through the tight junctions [94]. With increased intestinal permeability, lactulose would passes through the paracellular spaces, cleared by glomerular filtration, not undergo tubular reabsorption, and later on presented in the urine at high concentrations, leading to an elevated LMR. Other markers for compromised intestinal barrier integrity are LBP and I-FABP. LBP is a protein manufactured by enterocytes and liver cells [95] in response to bacterial translocation of endotoxin (lipopolysaccharide, LPS) [96] from the intestinal microbiota to the bloodstream [97]. LBP binds LPS and presents LPS to CD14, promoting immune responses [98] that in turn activate Toll Like Receptor 4 (TLR-4). Higher LBP levels may reflect the leakage of LPS out of the gut and into the bloodstream [96]. The other marker, I-FABP is a protein which is expressed in the small intestinal epithelium. When intestinal damage or injury occurs, I-FABP is released into the circulation. Both LBP and I-FABP markers has been used as a non-invasive predictor of intestinal injury [99, 100].

In **Chapter five,** we observed different levels of gut barrier function differed between high- and low-SES schoolchildren. Both LMR and I-FABP were higher, while LBP was lower in the low-SES group. High LMR and I-FABP indicate that intestinal barrier function and integrity may be compromised in children with low-SES compared to those with high-SES.

The differences in LMR could not be attributed to the high prevalence of helminth infections in the low-SES. In previous studies, *A. lumbricoides* has been associated with high LMR [101, 102] and this is similar to what we observed in the crude analysis. Yet, when we include SES in the multivariate analysis, SES is the main driver in determining the LMR level. Moreover, after anthelminthic administration, LMR was decreased in the group of children who were uninfected at baseline. Therefore, factors other than *A. lumbricoides* might contribute to the high level of LMR, such as recurrent gastrointestinal infections [103] that are not surveyed in this study. Another study conducted in several developing countries discovered that a higher LMR was associated with low-SES, recent diarrheal illness, and enteropathogen load in infants [104]. High physical activity such as walking to school might also contribute to the higher LMR [105] in our low-SES population. Following albendazole administration, I-FABP was increased in the low-SES-infected group which might suggest that worm expulsion induces gut inflammation and therefore leads to the release of I-FABP into the circulation.

The LBP level, a marker for microbial translocation, was shown to be lower in the low-SES children, where the intestinal barrier was more compromised, compared to the high-SES group. Helminth infections are thought to be associated with elevated levels of several microbial translocation markers [106], however, in **chapter five**, we observed no differences in LBP levels between helminth infected and uninfected group. Furthermore, no alteration in LBP levels was observed after albendazole administration. Similar findings have been reported in a previous study conducted in Southern India where they observed no significant differences in LBP levels between hookworm-infected and uninfected and no alteration in LBP levels after anthelminthic administration [107]. This suggests that LBP is not linked to helminth infection. The higher LBP levels in children from higher socioeconomic backgrounds

may be consistent with other findings linking elevated LBP levels to obesity, weight gain, or a high carbohydrate and high fat diet [108-110]. However, in our study, the differences were maintained even after adjusting for zBMI, indicating that additional variables play a role. Another factor to consider is that LPS translocation occurs not only via paracellular leakage but also via transcellular transport [111]; however, the clinical significance of this transcellular pathway remains unknown. Importantly, while we observed some changes in microbiota composition following albendazole administration, the differences in gut integrity markers could not be explained by the composition of intestinal microbiota.

DIRECTION FOR FUTURE RESEARCH

This thesis contributes to our current knowledge of the extent to which SES and a number of associated factors affect BCG vaccine response, venom and aeroallergen sensitization, gut microbiome and gut permeability, which might affect child health. The necessity of larger well designed cohort studies to establish the complex relationship between intestinal parasitic infections, gut microbiota and host responses is needed. Specifically, a number of directions to focus on in the near future are indicated below:

Innovative and alternative approaches to TB prevention, including vaccine development and improving their efficacy, are necessary. Therefore, a larger scale study would be needed to investigate whether leptin and total IgE have an effect on the efficacy of BCG vaccine. The data might help develop a strategy whereby leptin levels are increased while tIgE levels are decreased in order to improve responses to BCG and any future vaccines.

Previous studies have shown the important role of gut microbiota in effectiveness of immunotherapy by modulating the tumour immuno-microenvironment [112, 113]. In the field of neglected tropical diseases, the first step has been taken towards understanding possible interaction between gut microbiota and anthelminthic drugs [89], but we also need to look at its effects on vaccine responses. Given that gut microbiota is highly dynamic and demonstrates substantial inter and intra-individual variation, larger and more in-depth studies in different geographical settings are needed. Such studies should also take into account SES and other indicators such as food intake in terms of food quality, quantity and diversity; hygienic lifestyle, and also physical activity, which could be driving the associations observed. Any intervention, would benefit from including a placebo arm in the trials, if possible.

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APPENDIX

SUMMARY

SAMENVATTING

CURRICULUM VITAE

LIST OF PUBLICATION

ACKNOWLEDGEMENTS

SUMMARY

Although urbanization has long been associated with human development and progress, recent research has revealed that urban environments can also result in significant inequalities in many areas including health. In particular, urban areas in low and middle income countries (LMIC), often see a widening gap in economic growth which contributes to health disparities between wealthy and underprivileged children. Several studies have established the association between socioeconomic status (SES) and child health, showing that children of low-income parents had poorer health status. The rise of non-communicable diseases, the persistence of communicable disease, and the challenge of re-emerging diseases are currently a burden in developing countries. Given the rapid increases of urbanization and urban poverty in several developing countries including Indonesia, it is important to elaborate to what extent SES affects child health. We have done so, in relation to outcome of BCG vaccination, atopic sensitization, and intestinal barrier function. We have also investigated the interaction between intestinal parasitic infections and bacterial gut microbiota in order to be able to delineate the contribution of this interaction on the function of intestinal barrier.

Chapter One, provides a general introduction to the research topics in the thesis including the background to why we perform the studies described in this thesis. This chapter also covers the study area, study population, and the scope of the thesis

Chapter Two establishes the association between the size of BCG scar, which indicates the degree of response to the vaccine, and SES. We included maternal helminth infection status and nutritional status of the newborn to assess their roles in the development of BCG scar in infants. We observed that children from the low-SES have smaller BCG scar size and total IgE (a marker of exposure to helminth infection) moderately contributes to reducing the size of the scar. At the same time, high-SES children have larger size of BCG scar, and leptin levels, a hormone that is released by fat cells, contributed to this association. This finding suggests that SES is the major determinant of BCG scar size and that leptin levels at birth together with total IgE can contribute to the development of BCG scar in Indonesian infants.

In **Chapter Three**, we reported for the first time Hymenoptera venom sensitizations in Indonesian schoolchildren. Higher prevalence of skin prick test reactivity (SPT), a measure of functional sensitization, was observed in high-SES compared to the low-SES children; in contrast, sIgE-positivity, a measure of allergen exposure that causes sensitization, was more prevalent in the low SES than the high SES. These sensitizations appear to have poor clinical relevance as they rarely translated into clinical symptoms. The finding that there is a discordance between SPT and sIgE in our study, suggests the presence of IgE (high levels of sIgE) with poor biologic activity (low SPT). We also found a considerable number of subjects positive for skin reactivity but negative for sIgE, indicating a non-IgE dependent reaction to venom allergen. Further studies are needed to determine the true sensitization of Indonesian children by using component-resolved diagnostic (CRD) methods. The CRD method has been widely used in affluent countries, but less used in low middle income countries. This method allows the detection of specific IgE against individual purified native or recombinant allergens, instead of against allergen extracts comprised of mixtures of allergenic and non-allergenic components, which are commonly used in SPT and conventional specific allergy testing.

In **Chapter Four**, we profiled fecal bacterial microbiota of Indonesian children to elucidate the characteristics of bacterial microbiota. The core microbiota of the children consisted of *Bifidobacterium, Collinsella*, and multiple members of the *Lachnospiraceae* and *Ruminicoccaceae* families. Here we show that bacterial gut microbiota was predominantly driven by children's SES. Bacterial diversity was higher in the low-SES and among others, *Prevotella* and *Escherichia-Shigella*, were more abundant, while in the high-SES, bacterial diversity was lower and we observed higher relative abundance *Bifidobacterium* and *Lactobacillus.* These differences might be associated with different diet and lifestyle which are distinct between high- and low-SES, however, we can not discern this association between gut microbiota composition and diet as food intake was not surveyed. To delineate the association between helminth infections and gut microbiota, in **Chapter five**, the alteration of the gut microbiota was assessed 4 weeks after albendazole administration and no changes in gut microbiota diversity were observed, however, the alteration of bacterial microbiota composition was more SES-related and not associated with helminth infections as the changes observed was found in the uninfected group only.

In **Chapter Five**, we assessed the intestinal barrier function of Indonesian schoolchildren living in the same urban area but distinct in SES. Low-SES children exhibited higher LMR, indicating increased intestinal permeability and higher I-FABP, a marker of epithelial damage. This indicates that the intestinal barrier function and integrity might be compromised in low- compared to the high-SES children. The high LMR in the low-SES is not due to higher prevalence of intestinal parasitic infections in the low-SES as the changes in the level of LMR after albendazole treatment were only altered in the uninfected children. In the future, placebo should also be used, to be able to discern whether the changes are albendazole related or time-related.

Chapter Six summarizes and discusses the main findings of this thesis together with previous studies on the relationship between child health and SES. We highlighted the effect of SES on BCG scar development in young infants. In addition, we also discussed the impact of SES on venom sensitization, bacterial gut microbiota and intestinal permeability function in schoolchildren. In the end, this thesis sparks some directions for future research and to this end how our findings might affect policies for child health especially in Indonesia where there are wide socioeconomic disparities.

SAMENVATTING

Hoewel verstedelijking lange tijd geassocieerd werd met ontwikkeling en vooruitgang, laat recent onderzoek zien dat stedelijke omgevingen ook kunnen leiden tot ongelijkheden op verschillende gebieden, zoals gezondheid. Met name in stedelijke gebieden in lage- en middeninkomenslanden is er een groeiende kloof in economische groei die bijdraagt aan verschillen in gezondheid tussen welvarende en achtergestelde kinderen. Verschillende studies hebben aangetoond dat er een associatie is tussen de sociaal-economische status (SES) en gezondheid van kinderen, waarbij kinderen van ouders met een laag inkomen een slechtere gezondheid hebben. De toename van niet-overdraagbare ziektes, het persisteren van overdraagbare ziektes en de uitdaging van weer opduikende ziektes zijn momenteel een grote uitdaging in ontwikkelingslanden. Gegeven de snelle toename van verstedelijking en stedelijke armoede in verschillende ontwikkelingslanden waaronder Indonesië, is het daarom belangrijk om te onderzoeken in welke mate SES de gezondheid van kinderen beïnvloedt. Dit hebben we gedaan, in relatie tot vaccinatie met BCG, atopische sensitisatie en functie van de darmbarrière. Ook hebben we onderzoek gedaan naar de interactie tussen intestinale parasitaire infecties en de bacteriële darm microbiota om te bepalen van de bijdrage is van deze interactie aan de darmbarrière.

Hoofdstuk één, geeft een algemene inleiding van de onderwerpen van het proefschrift, waaronder de achtergrond van de uitgevoerde onderzoeken die in dit proefschrift zijn beschreven. Dit hoofdstuk behandelt ook het onderzoeksgebied, de onderzoekspopulatie en het kader van het proefschrift.

Hoofdstuk twee, legt het verband tussen de grootte van het BCG-litteken, dat de mate van response op het vaccin aangeeft, en SES. Ook worminfectiestatus van de moeder en de voedingsstatus van de pasgeborene werden meegenomen, om de rol van deze factoren in de ontwikkeling van het BCG-litteken te bepalen. De resultaten laten zien dat kinderen met een lage SES een kleiner BCG-litteken hadden en dat totaal IgE (een marker voor de blootstelling aan worminfecties) matig bijdraagt aan het verkleinen van de maat van het litteken. Tegelijkertijd hadden kinderen met een hoge SES een groter BCG-litteken en droegen de niveaus van leptine, een hormoon dat wordt afgegeven door vetcellen, bij aan deze associatie. Deze bevindingen suggereren dat SES een belangrijke bepalende factor is voor de grootte van het BCG-litteken en dat het niveau van leptine bij geboorte samen met totaal IgE kunnen bijdragen aan de ontwikkeling van het BCG-litteken bij Indonesische zuigelingen.

In **Hoofdstuk drie**, rapporteren we voor de eerste keer Hymenoptera gif sensitisatie bij Indonesische schoolkinderen. Een hogere prevalentie van huidreacties (SPT), een maat voor functionele sensitisatie, werd waargenomen bij kinderen met een hoge SES in vergelijking tot kinderen met een lage SES. Daarentegen, was sIgE-positiviteit, een maat voor blootstelling aan allergenen die sensitisatie veroorzaken, vaker aanwezig bij kinderen met een lage SES dan bij kinderen met een hoge SES. Deze sensitisaties lijken weinig klinische relevantie te hebben, omdat ze zelden leiden tot klinische symptomen. De bevinding dat er een discrepantie is tussen SPT en sIgE in onze studie, suggereert de aanwezigheid van IgE (hoge niveaus van sIgE) met een slechte biologische activiteit (lage SPT). We vonden ook dat een aanzienlijke hoeveelheid deelnemers van de studie positief was voor de huidreactie, maar negatief voor sIgE, wat er op wijst dat de reactie gif allergenen die onafhankelijk is van sIgE. Verder onderzoek is nodig om de daadwerkelijke sensitisatie van Indonesische kinderen te bepalen door gebruik te maken van diagnostische methoden met componentoplossing (CRD). De CRD-methode is een veelgebruikte methode in welvarende landen,

maar veel minder in lage en middeninkomenslanden. Deze methode maakt de detectie van specifiek IgE tegen individuele gezuiverde inheemse of recombinante allergenen mogelijk, in plaats van tegen allergenenextracten die bestaan uit mengsels van allergene en niet-allergene componenten, die doorgaans worden gebruikt bij SPT en conventionele specifieke allergietesten

In **Hoofdstuk vier**, hebben we de fecale bacteriële microbiota van Indonesische kinderen geanalyseerd om de kenmerken van de bacteriële microbiota op te helderen. De kern microbiota van de kinderen bestond uit *Bifidobacterium, Collinsella*, en meerdere leden van de *Lachnospiraceae* en *Ruminicoccaceae* families. Hier laten we zien dat de bacteriële darm microbiota voornamelijk werd beïnvloed door de SES van de kinderen. Bacteriële diversiteit was groter in kinderen met een lage SES, en onder andere *Prevotella* en *Escherichia-Shigella* waren meer aanwezig, terwijl in kinderen met een hoge SES, de bacteriële diversiteit kleiner was en *Bifidobacterium* en *Lactobacillus* relatief meer voorkwamen. Deze verschillen zouden verband kunnen houden met verschillende voedingspatronen en levensstijlen die variëren tussen kinderen met hoge en lage SES, al kan de associatie tussen darm microbiota en voedingspatronen niet onderzocht worden aangezien voedselinname niet uitgevraagd is in deze studie. Om de associatie te bepalen tussen worminfecties en de darm microbiota, is in **Hoofdstuk vijf**, de verandering in de darm microbiota beoordeeld vier weken na Albendazol toediening. Er werden geen verschillen in de darm microbiota diversiteit waargenomen gerelateerd aan de Albendazol toediening, maar we observeerde dat de veranderingen in bacteriële microbiota compositie meer SES-gerelateerd zijn, aangezien de veranderingen alleen gevonden werden in de groep zonder infectie.

In **Hoofdstuk vijf**, hebben we de functie van de darmbarrière beoordeeld in kinderen die in hetzelfde stedelijke gebied woonden, maar verschillend waren in SES. Kinderen met een lage SES vertoonden een hogere LMR (wat wijst op een verhoogde darmdoorlaatbaarheid) en hogere I-FABP, een marker voor epitheliale schade. Dit wijst erop dat de functie en integriteit van de darmbarrière mogelijk is aangetast in kinderen met een lage SES in vergelijking tot kinderen met een hoge SES. De hoge LMRI in kinderen met een lage SES is niet te wijten aan de hogere prevalentie van intestinale parasitaire infecties in deze kinderen, aangezien veranderingen in het LMR-niveau na behandeling met Albendazol, een middel tegen worminfecties, alleen werden waargenomen bij kinderen zonder infectie. In de toekomst, is het gebruik van een placebo nodig om te kunnen bepalen of de veranderingen verband houden met Albendazol of tijd.

Hoofdstuk zes, bevat een samenvatting en discussie van de belangrijkste bevindingen van dit proefschrift samen met eerdere studies over de relatie tussen gezondheid van kinderen en SES. We benadrukken het effect van SES op de ontwikkeling van het BCG-litteken bij jonge zuigelingen. Daarnaast wordt de impact van SES op de mate van sensitisatie bij blootstelling aan gif, bacteriële darm microbiota en de functie van de darmbarrière bij schoolkinderen besproken. Tot slot, geeft dit proefschrift enkele richtingen voor toekomstig onderzoek en laat het zien hoe onze bevindingen van invloed kunnen zijn op het beleid voor gezondheid van kinderen, vooral in Indonesië waar grote sociaal-economische verschillen zijn.

CURRICULUM VITAE

Aldian Irma Amaruddin was born in Ujung Pandang, Indonesia, on 21st of October 1985. She completed her secondary education in SMUN 17 Makassar in 2003, and in the same year she enrolled in the Faculty of Medicine at Hasanuddin University (FKUH), Indonesia. In December 2008, she obtained her medical degree. And in June 2009, she started working as a GP in a remote area under the PTT Program of Ministry of Health. Under this program, she was appointed as GP in Puskesmas Pembantu Kaledupa in Wakatobi Island (June-December 2009) and in Puskesmas Poli-polia, Kolaka Regency (December 2009 – November 2010). In December 2010, she moved back to FKUH and was employed as a lecturer at the Faculty of Medicine.

During the first two years of her academic career, she was involved in the Medical Education Unit and the Student Assessment Unit. In 2012, she also became involved in the development of Quality Assurance Unit of the Faculty of Medicine. In 2014, she was assigned as a lecturer to the Department of Parasitology at FKUH.

In December 2013, she was selected as a PhD candidate at Leiden University, which was funded by the Indonesian DIKTI program (2013-2016) and by Leiden University (2017). For her PhD, she worked on two projects in urban Makassar: 1) BCG scar responses in infants from high and low socioeconomic status families (2014-2016), and 2) intestinal permeability in helminth infected children (2017-2021). During her PhD, she was supervised by Professor Maria Yazdanbakhsh and Dr. Erliyani Sartono of Leiden University Medical Center and by Dr. Sitti Wahyuni and Dr. Firdaus Hamid.

In conducting these projects, she collaborated with Dr Romy Zwittink and Professor Ed Kuiper from Center for Microbiome Analyses and Therapeutics (CMAT), Dr. Kaatje Lenaerts and Dr Hans HM Eijk from Maastricht University and Professor Ronald Van Ree from University of Amsterdam.

After completion and submission of her PhD thesis, she returned to her post as a lecturer and will direct research at the Department of Parasitology. Currently, she is also involved in Clinical Epidemiology, Research Development and Publication (CRP) Unit of FKUH. In addition, to ensure academic quality at FKUH, she was appointed as Secretary of Quality Assurance Unit of FKUH (2021-2022). She was recently appointed as the Manager of Vice Dean of Planning, Finance and Resources at the Faculty (2022 till present).

This year, she would like to pursue a new path as a clinician and therefore she is starting her residency at the Department of Internal Medicine of Faculty of Medicine, Hasanuddin University.

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