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Mixed models for correlated compositional data: applied to microbiome studies in Indonesia

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Part II

Helminth infections on gut microbiome and immune responses

4

The effect of gut microbiome composition on human immune responses - interference of helminth infections

Abstract

Background. Soil transmitted helminths have been shown to have immune regulatory capacity, which they use to enhance their long term survival within their host. As these parasites reside in the gastro-intestinal tract, they might modulate the immune system through altering the gut bacterial composition. Although the relationships between helminth infections or the microbiome with the immune system have been studied separately, their combined interactions are largely unknown. In this study we aim to analyze the relationship between bacterial com-

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munities with cytokine response in the presence or absence of helminth infections.

Results. For 66 subjects from a randomized placebo-controlled trial, stool and blood samples were available at both baseline and 21 months after starting three-monthly albendazole treatment. The stool samples were used to identify the helminth infection status and fecal microbiota composition, while whole blood samples were cultured to obtain cytokine responses to innate and adaptive stimuli. When subjects were free of helminth infection (helminth-negative), increasing proportions of *Bacteroidetes* was associated with lower levels of IL-10 response to LPS (estimate (95% confidence interval (CI)) -1.96 (-3.05, -0.87)). This association was significantly diminished when subjects were helminth-infected (helminth positive) (*p*-value for the difference between helminth-negative versus helminth-positive was 0.002). Higher diversity was associated with greater IFN- γ responses to PHA in helminth-negative (0.95 (0.15, 1.75); versus helminth-positive -0.07 (-0.88, 0.73), *p*-value = 0.056) subjects. Albendazole treatment showed no direct effect in the association between bacterial proportion and cytokine responses, although the *Bacteroidetes*' effect on IL-10 responses to LPS was lower in the albendazole-treated group (-1.74 (-4.08, 0.59) versus placebo (-0.11 (-0.84, 0.62); *p*-value = 0.193).

Conclusion. The differences that we observed in groups of helminth-positive versus helminth-negative supports the hypothesis that helminth are able to modulate the immune system and specifically may alter the relationship between bacterial communities and cytokine response.

Trial registration: ISRCTN, ISRCTN83830814. Registered 27 February 2008 - Retrospectively registered, <http://www.isrctn.com/ISRCTN83830814>

4.1 Introduction

Diseases of modernity, such as allergy, autoinflammatory and metabolic diseases are increasingly observed in industrialized countries. It has been speculated that this growing rate was caused by changes in lifestyle, diet and environmental factors, such as pollutant exposure or hygiene. Hygiene improvement has dramatically decreased the prevalence of certain infectious agents such as parasitic helminths while these may have protective effects against autoinflammatory diseases [Wammes et al. (2016)]. Studies analysing the capacity of helminths to modulate the immune system have been carried out in recent decades. However, it has become clear that this is an interplay with several other factors, such as diet, environment and also other gut inhabitants, such as the microbiota.

Early studies showed that gut microbiota are involved in developmental aspects of the immune system and that disturbance can lead to autoinflammatory disorders [Round and Mazmanian (2009)]. Already in 1963 it was reported that the immune system of germ-free mice failed to respond to molecular patterns of pathogenic and beneficial microorganisms, causing morphological tissue defects in the intestinal wall [Abrams et al. (1963)]. In healthy humans, the role of gut microbiota and immune response was studied more recently. It was found that certain bacteria are beneficial for development and function of the immune system and simultaneously the immune system can influence the composition or function of gut microbiota, all relating to inflammatory disorders (reviewed in Belkaid and Hand (2014)).

The presence of parasitic helminths in the gastro-intestinal tract may exert a direct influence on host's gut microbiome as they share the same niche. Although in animal models helminths were shown to increase microbial abundance and diversity [Reynolds et al. (2015)], the findings in human studies are not consistent. Several studies analysing the effect of helminth on gut microbiota have indicated higher diversity of gut microbiota in helminth-positive subjects compared to helminth-negative subjects [Lee et al. (2014), Ramanan et al. (2016)]. A study in Ecuador showed that this difference in diversity might be related to specific helminth species, since they did not find any alterations in *Trichuris trichiura*-infected children [Cooper et al. (2013)]. This might be influenced by different factors among which are different bacterial profiling techniques or confounders such as ethnicity, anthelmintic treatment and environmental differences.

As it has been shown that changes in both gut microbiota and helminth infection status might affect the host's immune response, it is suspected that the presence of helminth might directly or indirectly affect the immune system by altering the gut microbial community [Zaiss et al. (2015)]. For instance, transfer of the microbiota of *Heligmosomoides polygyrus bakeri*-infected mice to uninfected mice induced similar protection against allergic airway inflammation as observed with helminth infection [Wilson et al. (2005)]. In humans, studies on the triangular relation between helminth with microbiome and immune system are still in infancy. To our knowledge, the number of longitudinal studies analysing the association between gut microbiota and immune responses in helminth-endemic areas is still limited. To understand the interaction of the gut microbial community and helminths and their common effect on immune responses, we used data from a household cluster-randomized, double blind, placebo-controlled trial of albendazole treatment in a helminth-endemic area. In this study, it has been shown that deworming reduced helminth prevalence and consequently increased several whole blood cytokine responses [Wammes et al. (2016)]. Helminth infection and anthelmintic treatment separately did not change the gut microbiota [Martin et al. (2018)]. However, when subjects remained infected while treated with albendazole, a decrease of *Bacteroidetes* : *Firmicutes* ratio and an increase of *Acti-*

nobacteria : Firmicutes ratio were observed, leading to the hypothesis that there is a cross-talk between microbiome composition and immune response which is disrupted by the presence of helminths and that removing helminth by anthelminthic might affect this communication. Our aim was to characterize the association between bacterial relative abundance with the whole blood cytokine responses and the effect of helminth infections and deworming on this interaction.

4.2 Methods

4.2.1 Participants

Stool samples from 150 subjects from immunoSPIN study [Wiria et al. (2010)] were analyzed for the fecal microbiome. From these, 66 subjects were included in this study based on the complete stool data and available cytokine measurements before and 21 months after the first treatment. Four different helminth species were found namely *Ascaris lumbricoides*, hookworms (*Necator americanus* and *Ancylostoma duodenale*) and *Trichuris trichiura*. Details on sample collection and measuring the infection status using PCR are described elsewhere [Wiria et al. (2010)]. *Trichuris trichiura* infection was assessed only by microscopy, since at that time there was no real-time PCR data available for this species. For this manuscript, we defined a helminth-infected subject as participant with a positive real-time PCR (cycle of threshold (Ct) value ≤ 30) and/or positive microscopy for one or more species of helminths, as described previously [Martin et al. (2018)]. Subjects with a positive real-time PCR with a Ct above 30 were regarded as uninfected.

In addition, from the 66 subjects, 20 subjects of 18 years old or older at pre-treatment who were helminth-negative were selected from Nangapanda (Ende) area, as well as 16 subjects who had migrated to Jakarta more than 10 years before and 14 people from the USA (healthy adults from the Human Microbiome Project (HMP) to illustrate the microbiome profile from subjects residing in different geographical environments.

4.2.2 Microbiome composition

The amplification and pyrosequencing of the 16S rRNA gene followed the protocols developed by the Human Microbiome Project (HMP) [HMP (2012)] at the McDonnell Genome Institute, Washington University School of Medicine in St. Louis and have been described previously elsewhere [Martin et al. (2018), Rosa et al. (2018)]. Briefly, the V1 – V3 hypervariable region was PCR – amplified and the PCR products were sequenced on the Genome Sequencer Titanium FLX (Roche Diagnostics, Indianapolis, Indiana), generating on average 6,000 reads per

sample. Details of the filtering and analytical processing of 16S rRNA data for this cohort has been previously described in Rosa et al. (2018). The assembled contigs count data as a result of RDP classification was organized in matrix format with taxa in columns and subjects in row. The entries in the table represent the number of reads for each taxa for each subject. Our work is focused at a phylum level of gut bacterial. Five bacterial phyla have average relative abundances larger than 1%, namely *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and an unclassified category, which consists of sequences which could not be categorized into a phylum. The remaining bacterial phyla which had lower relative abundance were pooled together into a pooled category. In the analysis, we retained the count for the three most abundant bacterial phylum proportions, namely *Actinobacteria*, *Bacteroidetes* and *Firmicutes*. The proportion for each phylum was obtained by dividing each sequence count by the total sequence per person at each time point. Along with bacterial proportions, we computed at a phylum level the bacterial diversity within samples (Shannon index) and between samples (Bray-Curtis dissimilarity) using R package vegan [Oksanen et al. (2017)]. The Shannon index represents not only the presence of taxa but also the abundance of corresponding taxa. The higher diversity index means that there was not a single taxa dominating the community and the total bacterial abundance is spread out over all taxa. The Bray-Curtis dissimilarity measures the percentage of similarity between one sample from the other with values range from 0 (completely similar) to 1 (completely dissimilar).

4.2.3 Whole blood cytokine responses

The method to obtain and assess the cytokines responses were described elsewhere [Wiria et al. (2010)]. In brief, heparinized blood was diluted 1:4 and cultured in 96-well plates. Plates were incubated for 24 (innate responses) or 72 (adaptive responses) hours, after which supernatants were harvested and stored in freezers. Cytokine levels were measured by Luminex bead technology in samples obtained at before and 21 months after start of treatment. The analyses carried in this manuscript are limited to innate responses (interleukin (IL)-10 and tumor necrosis factor-alpha (TNF- α)) to lipopolysaccharide (LPS) from *E. coli* and adaptive responses (interferon-gamma (IFN- γ) and IL-5) to Ascaris antigen (AscAg) and general T cell stimulator phytohemagglutinin (PHA). The AscAg was a homogenate of adult worm *A. lumbricoides* obtained from infected human [Wammes et al. (2014)].

4.2.4 Statistical Methods

The microbiome composition for each group of the different demographical areas was assumed to follow a Dirichlet – multinomial distribution with 6 cate-

gories which represents the 6 most abundant phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, unclassified bacteria and pooled). The difference in the microbiome composition between groups was tested using the likelihood ratio test statistic with 6 degrees of freedom.

All parameters of interest were described as means or frequency (\pm standard deviation). Prevalence rates were calculated and compared using the Pearson chi-square test, while the Student *t*-test was used to compare continuous variables.

To study the relationship between cytokines and microbiome over the two time points, a linear mixed effect regression model was fitted with helminth status and treatment as covariates. All models have been adjusted with age and sex, however, since both covariates were not significantly associated with the cytokine responses in any model, they are not included in the final analysis. The correlation between observations from the same subjects was modelled by including a subject-specific random effect. The microbiome was included in the model either as a bacterial proportion or by the Shannon diversity index. The cytokine responses were \log_{10} -transformed ($\log_{10}(\text{concentration} + 1)$) to obtain normally distributed variables. First, we analyzed the main effect of bacterial proportion and diversity on cytokine responses. Second, to allow for different effect sizes of bacterial proportion or diversity on cytokine responses in helminth-positive versus -negative subjects, an interaction term between bacterial categories and infection was included in the model. The *p*-value for this interaction term indicated the statistical evidence for different effect sizes in helminth-positive or -negative groups.

To allow the estimation of the treatment effect on the relationship between bacterial proportion and cytokine responses, the randomized controlled trial design was used. Since the sample size is too small, we only stratified based on randomization arm. Hence, the effect of treatment cannot be distinguished from the effect of helminth infection. Therefore, we explored the relationship between cytokines and microbiome after anthelminthic treatment. A linear mixed effect model was fitted with bacterial proportion or diversity, and treatment as covariates. This model was able to characterize three different associations, namely the association between bacterial proportion or diversity on cytokines at pre-treatment, the difference of the association at pre-treatment and at post-treatment in the placebo group (time effect), as well as the difference of the association at post-treatment between albendazole and placebo group.

For each outcome separately, these models were fitted on subjects who at least had an observation at pre-treatment. The lme4 package in statistical software R was used for model fitting. The significance of the covariate effect was obtained from the likelihood ratio test. Bonferroni correction was used to adjust for multiple testing. The statistical analyses were performed in R [R Core Team (R Core Team)] with mainly lme4 and lmerTest packages [Bates et al. (2015), Kuznetsova et al. (2017)]. The full record was created using the knitr package in R [Xie (2018)]

and is available online at https://github.com/Helminths_GutMicrobes_Cytokine/Ch4_PhDThesis_StatisticalAnalysisinR.pdf.

4.3 Results

4.3.1 Geographical differences in microbiome composition in a rural to urban gradient

From a subpopulation participating in the ImmunoSPIN study in Flores island, Indonesia, 66 individuals were selected for analysis. To illustrate the difference in gut-bacterial community between different geographical areas, we first compared the microbial composition from a sub-selection of helminth-negative subjects who were 18 years or older from Ende (n=20) with subjects from the same area who had moved to Jakarta (n=16) and healthy adults from the USA (n=14) which were considered as residents of rural, urban area of Indonesia, and Western urban area, respectively. The age ranges were from 18 to 62 years old (Jakarta samples) and 18 to 40 years old according to HMP protocol (USA samples) [HMP (2012)]. The proportions of the five main bacterial phyla and a pooled category of remaining bacteria are depicted in Figure 4.1. Bacteroidetes was dominating in the more urban areas (mean \pm SD $53.23 \pm 2.38\%$ in US to $4\% \pm 0.22\%$ in Ende) while Firmicutes were the most prominent in the rural area ($72.45\% \pm 1.16\%$ in Ende to $32.11 \pm 2.08\%$ in US) (Figure 4.1A). The microbiome compositions among these three geographical areas were significantly different (p -value < 0.001). Furthermore, the distribution of alpha and beta diversity (Shannon index and Bray Curtis dissimilarity) in samples from three different geographical areas were relatively similar (Shannon diversity index: mean \pm SD 0.85 ± 0.21 in Ende, 0.91 ± 0.14 in Jakarta and 0.78 ± 0.16 in US (Figure 4.1B); Bray-Curtis dissimilarity index mean \pm SD 0.21 ± 0.09 in Ende, 0.15 ± 0.08 in Jakarta and 0.31 ± 0.20 in US; Figure 4.1C).

4.3.2 The effect of bacterial proportions and diversity on *in vitro* cytokine responses

We observed a difference in microbial profiles in rural compared to urban areas. Since it is hypothesized that gut bacteria are associated with certain cytokine responses and thereby possibly immune disorders, we went on to explore this relationship by using data from the ImmunoSPIN trial. For 66 subjects, cytokine responses were measured at pre-treatment and 21 months after the start of anthelmintic treatment. At baseline, 40 out of 66 (60.6%) individuals in Ende were infected with one or more helminth species, and hookworm was the most dominant species (31.8%) followed by *A. lumbricoides* (25.7%) and *T. trichiura* (22.7%). The baseline characteristics such as age, gender, BMI and helminth prevalence

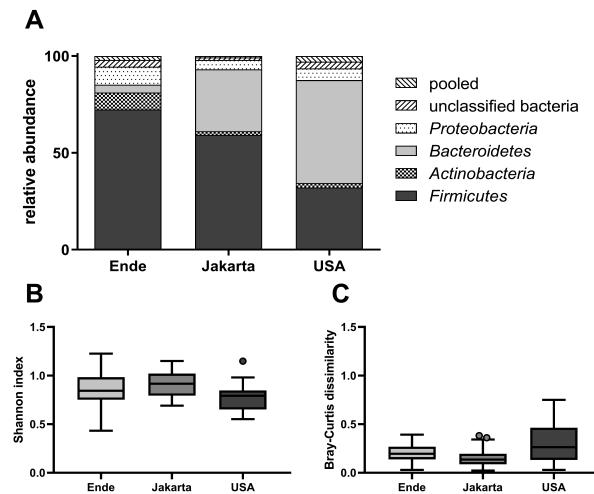


Figure 4.1: The microbiome composition and diversity for subjects in three different geographical areas. Composition and diversity of the fecal microbiota was assessed for subjects from different geographical areas: Ende (n=20), Jakarta (n=16) and USA (n=14). In panel (A) the microbiome composition is depicted in percentages of the six categories, where unclassified bacteria represents the category of sequences that could not be assigned to a phyla, and the pooled category consists of the remaining 13 phyla with average relative abundance less than 1%. Panel B and C show the average \pm SD of the Shannon diversity index and the Bray-Curtis dissimilarity index, respectively, in the different areas.

were similar between the two treatment arms (Table 4.1). Three-monthly albendazole treatment for 21 months reduced the infection prevalence from 65.4% to 19.2% versus a slight increased of helminth infections from 57.5% to 65% in placebo group (Table S4.5.1).

We analyzed proportions of three bacterial phyla (*Actinobacteria*, *Bacteroidetes* and *Firmicutes*) as these were most abundant in our study population. As we analyzed two cytokines for each antigens, we applied conventional Bonferroni correction and used a cut-off level for significance (α) of 0.025. When fitting the linear mixed model, no direct effect was observed of bacterial proportions or Shannon diversity on whole blood cytokine responses (Table 4.2).

4.3.3 Interference by helminth infection in the effect of bacterial proportions and diversity on in vitro cytokine responses

To elucidate the possible role of helminth infections in the interplay of bacteria and immune responses, we conducted analyses in helminth-positive and -negative groups. For this purpose, we used observations at both pre-treatment

Characteristics	albendazole		placebo		
	N	Result	N	Result	
Gender, female (N (%))	26	12 (46.1)	40	22 (55.0)	
Age (mean (SD))	26	27.3 (16.1)	40	26.7 (15.7)	
Children (<= 18 years old; N (%))		10 (38.4)		17 (42.5.0)	
Adults (>18 years old; N (%))		16 (61.5)		23 (57.5)	
zBMI (mean (SD))	10	-0.52 (0.98)	17	-0.83 (0.64)	
BMI (mean (SD))	16	23.39 (3.44)	23	23.49 (4.89)	
Parasite infection (N (%))					
<i>A. lumbricoides</i> ^a	26	9 (34.6)	40	8 (20.0)	
Hookworm	26	11 (42.3)	40	10 (25.0)	
<i>N. americanus</i> ^a	26	10 (38.5)	40	10 (25.0)	
<i>A. duodenale</i> ^a	26	2 (7.7)	40	2 (5.0)	
<i>T. trichiura</i> ^b	26	5 (19.2)	40	10 (25.0)	
Any helminth	26	17 (65.4)	40	23 (57.5)	
Abundance of bacterial phyla (mean % (SD))					
<i>Actinobacteria</i>		8.2 (5.3)		8.6 (6.9)	
<i>Bacteroidetes</i>		7.6 (10.1)		6.7 (11.5)	
<i>Firmicutes</i>	26	66.4 (11.8)	40	65.0 (13.5)	
<i>Proteobacteria</i>		7.3 (5.6)		7.4 (4.6)	
unclassified bacteria#)		1.3 (0.7)		2.1 (2.4)	
pooled*)		9.2 (6.0)		10.1 (5.8)	
Diversity Index, median (IQR)					
Shannon index	26	0.85 (0.71, 0.99)	40	0.84 (0.73, 1.00)	
Bray-Curtis		0.19 (0.12, 0.26)		0.19 (0.13, 0.28)	
Cytokine responses (pg/mL, median, IQR)					
LPS	IL-10	25	242.00 (132.00, 400.00)	40	213.50 (142.00, 380.20)
	TNF- α	25	664.00 (294.00, 1029.00)	40	550.50 (343.00, 840.00)
AscAg	IL-5	22	32.55 (9.55, 58.42)	37	18.90 (12.00, 62.00)
	IFN- γ	23	28.50 (12.10, 111.80)	37	17.40 (7.74, 60.90)
PHA	IL-5	23	490.00 (276.00, 747.50)	37	515.00 (333.00, 870.00)
	IFN- γ	23	2449.00 (354.00, 5424.00)	37	2299.00 (997.00, 3829.00)

Table 4.1: **Characteristics of the participants at baseline.** ^a diagnosed by real-time PCR; ^b diagnosed by microscopy; unclassified bacteria represents the category of sequences that could not be assigned to a phyla, and the; *pooled category consists of the remaining 13 phyla with average relative abundance less than 1%.

		Estimated effect (95 % CI)			
		<i>Actinobacteria</i>	<i>Bacteroidetes</i>	<i>Firmicutes</i>	Shannon
LPS	IL-10	0.20 (-0.58, 0.98)	-0.39 (-0.90, 0.12)	0.24 (-0.23, 0.71)	-0.22 (-0.51, 0.07)
	TNF- α	0.55 (-0.35, 1.44)	-0.06 (-0.66, 0.54)	-0.14 (-0.70, 0.41)	0.03 (-0.31, 0.37)
AscAg	IL-5	-1.02 (-2.78, 0.74)	0.09 (-1.10, 1.28)	0.39 (-0.74, 1.52)	-0.48 (-1.16, 0.20)
	IFN- γ	-1.03 (-2.45, 0.39)	0.15 (-0.80, 1.10)	-0.20 (-1.13, 0.74)	0.14 (-0.44, 0.71)
PHA	IL-5	-0.04 (-1.55, 1.46)	0.32 (-0.67, 1.32)	-0.85 (-1.82, 0.11)	0.61 (0.02, 1.20)
	IFN- γ	-0.57 (-2.12, 0.98)	-0.26 (-1.28, 0.75)	-0.03 (-1.05, 0.99)	0.45 (-0.18, 1.08)

Table 4.2: The association between bacterial proportion and diversity on cytokine responses.

and post-treatment. Regardless of randomization arm, we fitted the linear mixed model on each cytokine responses as outcomes. The predictors were bacterial proportions and its interaction with helminth infection. A similar analysis was performed to estimate the association between bacterial diversity and cytokine responses. Table 4.3 illustrates the associations between bacterial proportions or diversity and cytokine responses when subjects were helminth-positive or - negative.

In the innate immune response to LPS, the *Bacteroidetes* proportion showed a significant negative association with IL-10 levels in helminth-negative subjects (estimated effect (95% confidence interval (CI)): -1.96 (-3.05, -0.87), *p*-value = 0.001; Table 4.3). This association was significantly different from that of helminth-negative subjects (*p*-value for the difference = 0.002, Figure 2A) in which the association was absent (-0.03 (-0.59, 0.53), Table 4.3). The bacterial diversity had no significant association with IL-10 response to LPS (Table 3, Figure 2B). With regard to the helminth-specific cytokine responses, none of IFN- γ and IL-5 responses to AscAg were significantly associated with bacterial proportions or diversity (Table 4.3). In the adaptive responses (PHA), none of the cytokine responses were significantly associated with the bacterial proportion in uninfected subjects (Table 3). Although not significant, we noticed lower levels of IFN- γ to PHA with higher *Firmicutes* proportions (-1.57 (-3.08, -0.05), *p*-value = 0.045; Table 4.3). This association between *Firmicutes* proportion with IFN- γ to PHA in uninfected subjects was however significantly different from that in subjects who were infected (*p*-value for the difference = 0.009, Figure 2C). At the same time, there was a significantly increasing concentration of IFN- γ to PHA among those who were uninfected when bacterial diversity was higher (0.95 (0.15, 1.75), *p*-value 0.022; Table 3), although this association was not significantly different from the helminth-positive group (-0.07 (-0.88, 0.73), *p*-value for the difference = 0.056; Table 3, Figure 2D). A similar negative association of *Firmicutes* was observed in IL-5 responses to PHA in uninfected subjects (-1.52 (-3.02, -0.02), *p*-value = 0.05; Table 4.3). Conversely increasing bacterial diversity led to slightly higher levels

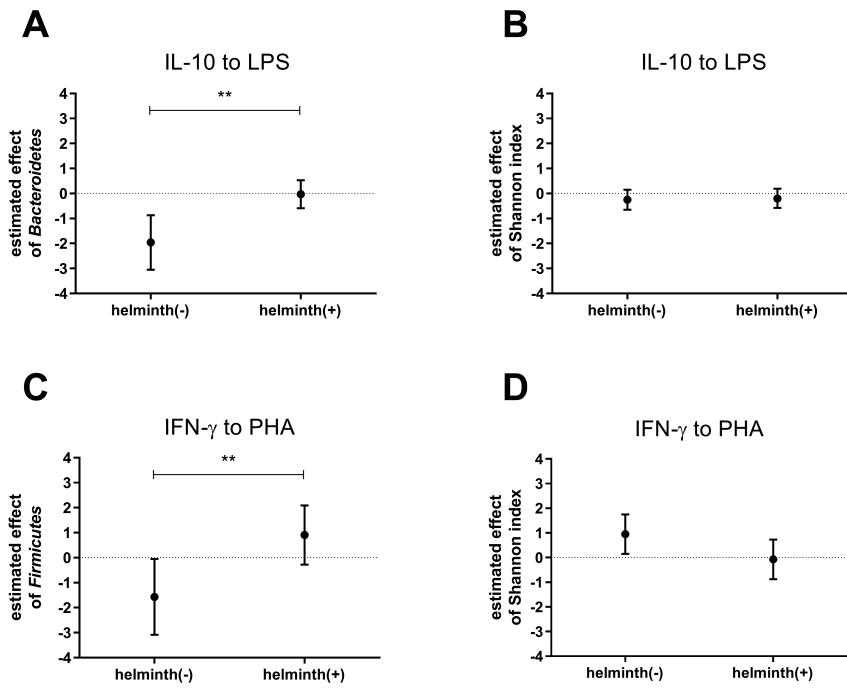


Figure 4.2: The association between bacterial proportion and diversity on certain cytokines in helminth-negative and -positive subjects. The effect of bacterial proportions on cytokine responses was analyzed for helminth-negative (helminth(-)) and helminth-positive (helminth(+)) groups by a linear mixed model. Estimated effects \pm 95% CI are shown for the effect of *Bacteroidetes* proportion on IL-10 responses to LPS (A), diversity on IL-10 to LPS (B) and for the effect of *Firmicutes* (C) and diversity (D) on IFN- γ responses to PHA. For assessing statistical significance conventional Bonferroni correction was applied; * p -value ≤ 0.025 , ** p -value ≤ 0.01 .

of IL-5 to PHA in the uninfected subjects (0.85 (0.07, 1.63), p -value = 0.034; Table 3). Both observations were not significantly different from the effects in those who were helminth-positive.

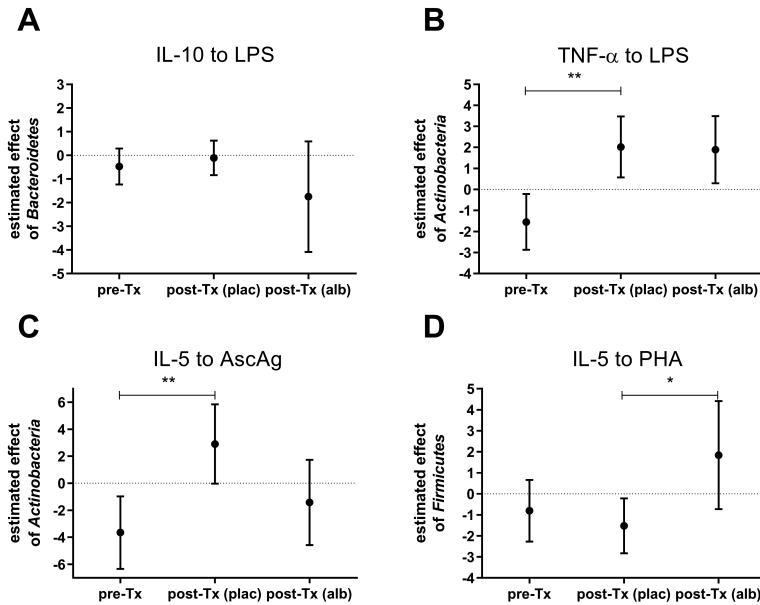


Figure 4.3: The association between bacterial proportion and diversity on certain cytokines at pre-treatment and post-treatment in two randomization arms. After deworming, comparisons were made for all subjects pre-treatment versus post-treatment (placebo group) and post-treatment placebo versus albendazole groups. Estimated effects of a linear mixed model \pm 95% CI are depicted. In panel A, the effect of *Bacteroidetes* proportion on IL-10 responses to LPS is shown for the different groups. Panel B and C depict the effect of *Actinobacteria* on TNF- α levels to LPS and on IL-5 responses to AscAg, respectively. The effect of *Firmicutes* on PHA-induced IL-5 is shown in panel D. * p -value ≤ 0.025 , ** p -value ≤ 0.01 .

		Estimated effect (95 % CI)				Infection status
		<i>Actinobacteria</i>	<i>Bacteroidetes</i>	<i>Firmicutes</i>	Shannon	
LPS	IL-10	0.25 (-0.90, 1.41)	-0.03 (-0.59, 0.53)*	0.14 (-0.45, 0.73)	-0.20 (-0.58, 0.19)	helminth(+)
	TNF- α	0.28 (-0.77, 1.34)	-1.96 (-3.05, -0.87)	0.42 (-0.31, 1.14)	-0.25 (-0.65, 0.15)	helminth(-)
AscAg	IL-5	0.68 (-0.64, 2.00)	-0.18 (-0.86, 0.50)	-0.04 (-0.73, 0.65)	-0.09 (-0.54, 0.36)	helminth(+)
	IFN- γ	0.50 (-0.72, 1.71)	0.35 (-0.97, 1.67)	-0.29 (-1.14, 0.55)	0.16 (-0.31, 0.63)	helminth(-)
PHA	IL-5	-1.69 (-4.34, 0.97)	-0.04 (-1.41, 1.32)	0.60 (-0.81, 2.00)	-0.76 (-1.70, 0.17)	helminth(+)
	IFN- γ	-0.46 (-2.78, 1.86)	0.38 (-2.08, 2.84)	0.10 (-1.69, 1.89)	-0.21 (-1.12, 0.71)	helminth(-)

Table 4.3: The association between bacterial proportion and diversity on cytokine of helminth-infected and -uninfected subjects regardless of treatment allocation. Bold printed numbers represent significant association between corresponding bacterial proportion or diversity and cytokine (p -value ≤ 0.025). *Significant difference in Helminth(+) versus Helminth(-) (p -value ≤ 0.025).

		Estimated effect (95 % CI)			group	
		<i>Actinobacteria</i>	<i>Bacteroidetes</i>	<i>Firmicutes</i>		
LPS	IL-10	-0.36 (-1.60, 0.87)	-0.47 (-1.23, 0.29)	0.44 (-0.28, 1.16)	-0.32 (-0.72, 0.08)	pre
		0.61 (-0.74, 1.96)	-0.11 (-0.84, 0.62)	0.06 (-0.63, 0.75)	-0.14 (-0.57, 0.30)	post-pla
		0.09 (-1.40, 1.58)	-1.74 (-4.08, 0.59)	0.27 (-0.82, 1.35)	-0.20 (-0.91, 0.50)	post-alb
TNF- α		-1.55 (-2.87, -0.22)*	0.10 (-0.80, 1.00)	0.44 (-0.39, 1.27)	-0.41 (-0.87, 0.05)	pre
		2.02 (0.57, 3.47)**	-0.24 (-1.11, 0.62)	-0.35 (-1.14, 0.45)	0.27 (-0.22, 0.76)	post-pla
		1.89 (0.29, 3.49)	1.15 (-1.61, 3.91)	-0.98 (-2.22, 0.27)	0.75 (-0.06, 1.55)	post-alb
AscAg		-3.65 (-6.34, -0.97)*	-0.23 (-2.10, 1.64)	1.52 (-0.19, 3.24)	-0.89 (-1.83, 0.05)	pre
		2.90 (-0.03, 5.84)**	-0.06 (-1.69, 1.58)	-0.76 (-2.29, 0.78)	0.17 (-0.81, 1.16)	post-pla
		-1.42 (-4.58, 1.73)	2.26 (-2.89, 7.41)	1.84 (-1.18, 4.86)	-1.48 (-3.24, 0.28)	post-alb
IFN- γ		0.76 (-1.42, 2.95)	0.13 (-1.37, 1.63)	-0.84 (-2.24, 0.56)	0.49 (-0.29, 1.27)	pre
		-1.61 (-4.00, 0.77)	0.11 (-1.22, 1.43)	-0.21 (-1.46, 1.04)	0.06 (-0.75, 0.87)	post-pla
		-2.95 (-5.52, -0.38)	0.73 (-3.46, 4.91)	1.92 (-0.54, 4.38)	-0.94 (-2.40, 0.51)	post-alb
IL-5		0.67 (-1.66, 3.00)	-0.20 (-1.74, 1.35)	-0.80 (-2.27, 0.66)	0.59 (-0.22, 1.40)	pre
		1.66 (-0.90, 4.21)	0.32 (-1.04, 1.69)	-1.52 (-2.83, -0.22)	1.03 (0.18, 1.87)	post-pla
		-2.54 (-5.29, 0.20)	3.16 (-1.14, 7.47)	1.84 (-0.73, 4.41)***	-0.79 (-2.30, 0.72)	post-alb
PHA		0.32 (-2.09, 2.72)	0.41 (-1.18, 1.99)	-0.72 (-2.25, 0.82)	0.63 (-0.23, 1.48)	pre
		0.90 (-1.72, 3.53)	-1.03 (-2.42, 0.36)	-0.06 (-1.42, 1.31)	0.64 (-0.23, 1.52)	post-pla
		-3.54 (-6.36, -0.71)	2.60 (-1.80, 7.00)	2.19 (-0.50, 4.88)	-0.95 (-2.53, 0.64)	post-alb

Table 4.4: The association between bacterial proportion and diversity on cytokine responses irrespective of infection status at pre- and post-treatment in two randomization arms. pre = pre-treatment (regardless of helminth infection status); post-pla = post-treatment placebo arm; post-alb = post-treatment albendazole arm. * p -value ≤ 0.025 , ** p -value ≤ 0.005 in pre versus post-pla, *** p -value ≤ 0.025 in post-alb versus post-pla.

4.3.4 The effect of albendazole on the relationship between *in vitro* cytokine responses and bacterial proportion and diversity

We further investigated whether deworming affects the relationship between bacterial proportions or diversity and cytokine responses. For this purpose, we fitted the linear mixed model on all subjects ($n = 66$) to characterize the association between bacterial proportions and cytokine responses at two time points and in the two randomization arms. These analyses were irrespective of the infection status. A similar model was applied for the diversity index.

Table 4.4 lists the associations between the proportions of three major bacterial phyla and diversity with cytokine responses, before and after anthelminthic treatment. With regard to the relationship between *Bacteroidetes* and IL-10 response to LPS, no significant differences were observed between pre- versus post-treatment or between treatment groups (Table 4.4). While the estimated association between *Bacteroidetes* proportion and IL-10 to LPS at pre-treatment (estimate (95% CI): -0.47 (-1.23, 0.29)) and post-treatment in placebo group (-0.11 (-0.84, 0.62)) were close to zero, the association at post-treatment in albendazole group was clearly lower (-1.74 (-4.08, 0.59); p -value for the difference between placebo and albendazole was 0.193, Table 4.4 Figure 4.3A). The association between IFN- γ in response to PHA and bacterial diversity was also not significant at post-treatment either in placebo or in albendazole group (Table 4.4).

The association between higher *Actinobacteria* proportion with decreasing response of TNF- α to LPS was borderline significant at pre-treatment (estimate (95% CI): -1.55 (-2.87, -0.22), p -value = 0.024; Table 4.4). This association was significantly different to the effect of *Actinobacteria* at post-treatment when subject received placebo (2.02 (0.57, 3.47); p -value < 0.001; Figure 4.3B), however no difference was observed when comparing placebo and albendazole group (1.89 (0.29, 3.49), p -value for the difference = 0.907; Figure 4.3B). A similar result was obtained from the association between *Actinobacteria* with IL-5 responses to AscAg. At pre-treatment, the increasing *Actinobacteria* proportions were significantly associated with less IL-5 production in response to AscAg (-3.65: (-6.34, -0.97), p -value = 0.009; Table 4.4). This association was significantly different to the effect of *Actinobacteria* at post-treatment in placebo group (2.90 (-0.03, 5.84), p -value = 0.002; Figure 4.3C). Although the estimated association in albendazole group was lower (-1.42 (-4.58, 1.73), this was not significantly different between the treatment groups (p -value = 0.052; Figure 4.3C).

On the other hand, while the association between *Firmicutes* and IL-5 response to PHA at pre-treatment was not significantly different compared to the association at post-treatment in placebo group, there was a significant difference of this association between albendazole and placebo group at post-treatment (estimate (95% CI) for placebo -1.52 (-2.83, -0.22) versus albendazole 1.84 (-0.73, 4.41), p -

value = 0.024; Table 4.4, Figure 4.3D).

4.4 Discussion

This study aimed to analyze the effect of helminth infections on the relationship between gut microbiota and the immune system. Examination of the microbiome composition in rural and urban area of Indonesia as well as USA showed that there were clear gradients in *Bacteroidetes* to *Firmicutes* proportion. This was one of the reason we focused on the three bacterial phyla in this study besides the result of the previous study on these subjects which reveals the associations between helminth infection and the odds of *Bacteroidetes* to *Firmicutes* as well as *Actinobacteria* to *Firmicutes*. When focusing on samples from Ende, we found a negative association between proportions of *Bacteroidetes* and IL-10 response to LPS in helminth-negative subjects and the presence of helminths was shown to dampen this effect. Anthelminthic treatment partly recovered this effect, although not statistically significant. To our knowledge, this is the first time that the association between gut microbiome, presence of parasitic helminths and whole blood cytokine responses was analyzed in a longitudinal study using a randomized placebo-controlled anthelminthic trial.

IL-10 was already marked as a key anti-inflammatory cytokine involved in induction of immune suppression by helminths [Yazdanbakhsh et al. (2002)]. Our observation that helminths counteract the suppressed IL-10 response to LPS in subjects with higher *Bacteroidetes* proportions supports the so called “old friends hypothesis” [Rook (2009)], stating that certain infectious agents such as helminths may have protective effects against immune dysfunction and inflammatory diseases, possibly through IL-10. This is strengthened by our observed gradient of the relative abundance of *Bacteroidetes* from rural to urban areas, where immune-related diseases are more prevalent [Bach (2002)]. In contrast, a recent meta-analysis indicated that inflammatory bowel disease (IBD) patients displayed lower proportions of *Bacteroidetes* [Zhou and Zhi (2016)], however this was only found when measuring by real-time quantitative PCR (not by conventional culture) and mainly in Asian studies. Furthermore, a member of the *Bacteroidetes* family, gut inhabitant *Bacteroides fragilis*, was shown to protect mice from experimental colitis, mediated by polysaccharide A (PSA) possibly through IL-10 induction [Mazmanian et al. (2008)]. However, although *B. fragilis* is the most well-known pathogen of the *Bacteroidetes*, it is the least common species in the *Bacteroidetes* phylum in the human gut [Wexler (2007)]. It could therefore be that other factors or species play a dominant role in the general effect of *Bacteroidetes* on IL-10 responses. Further studies are therefore needed to assess the translation of our findings to a clinical setting, for example prevalence or activity of IBD or other auto-immune diseases. Moreover, since we have measured systemic whole blood cytokine responses, we are not sure whether this is representative for the gut re-

sponses.

A trend of negative association between *Firmicutes* and concentration of IFN- γ to PHA was seen in helminth-negative subjects only. In subjects with helminth, this association was positive, although this difference fell short of statistical significance. Parallel to this trend, the bacterial diversity was positively associated with IFN- γ responses to PHA in subjects who did not carry helminths, and in helminth-positive subjects this association was dampened. Since a similar opposite trend was observed in the relationship between *Firmicutes* compared to bacterial diversity on IL-5 responses to PHA, we may conclude that not the proportion of *Firmicutes*, but the total bacterial diversity drove this association. *Firmicutes* was the most abundant phyla in this population and the increasing proportion of *Firmicutes* will obviously reduce diversity. This indicates that analyzing single bacterial phyla without considering the remaining phyla may lead to biased results as microbiome data is compositional and thus correlated between phyla.

Although deworming removed most helminths, treatment did not significantly alter the effects of bacterial proportions on cytokine responses. Regarding the *Bacteroidetes* effect on LPS to IL-10, we did observe a lower effect in the albendazole group compared to placebo. Although not significant, this might point towards the idea that anthelminthic treatment could restore the -possibly detrimental- interaction of bacteria with immune responses. Surprisingly, we found differences in immune modulation by *Actinobacteria* in the pre- versus post-treatment group. Although there was a significant association of time (in subjects receiving placebo), these associations were not significantly different in the albendazole group. The effect of time could be explained by other factors such as diet and possibly improved hygiene, resulting from increased awareness during the presence of our medical team in the study area. In the analysis of treatment's effect on the association between bacterial proportion and diversity, there was a significant difference between the association of *Firmicutes* on the IL-5 response to PHA in albendazole group compared to placebo group. In subjects receiving albendazole, *Firmicutes* proportions were positively associated with IL-5 levels, while we observed a negative (non-significant) effect in helminth-negative individuals over time. This result seems contradictory, but might be related to the fact that small numbers were analyzed and not everyone in the albendazole group lost their helminth infection. The analysis on subjects who were infected at baseline and cleared their infection would possibly reveal more clearly how the relationship between bacterial communities and immunity are affected by treatment. This analysis lacks statistical power in our study as the sample size was small ($n = 12$ out of 17 subjects who were successfully dewormed). Future research which involves larger sample sizes needs to be conducted. Another relevant thought in this and similar research settings is that although albendazole removes helminths effectively, the immunomodulatory effects of helminths on cytokine responses are long-lasting and cannot be easily corrected by short-term treatment. It was

previously reported by Endara et al. (2010) that the length of periodic treatment is important for altering immune responses, i.e. that studies with a longer period of treatment (up to 30 months) are more likely to show effects of deworming.

As significant associations between bacterial communities and cytokine responses were only observed when subjects were helminth-negative, clearly other factors than helminth and treatment are also involved in the alteration of the microbiome community and their interaction with the immune system. For example, our study data lack information on diet. Dietary intake was clearly shown to affect bacterial communities in the gastro-intestinal tract [Wu et al. (2011)]. This might also be related to changes in social economic status leading towards a more high-fat diet when moving from rural to urbanized areas. Recent articles reported inconsistencies with regard to the direction of *Bacteroidetes* to *Firmicutes* ratio in rural to urban comparisons of microbiome profiles from different geographical areas. Studies comparing children from Bangladesh to USA children showed direction of increasing *Bacteroidetes* : *Firmicutes* in USA, as observed in our data [Lin et al. (2013)], while studies in elderly Korean and children in Burkina Faso showed opposing results, i.e. decreasing *Bacteroidetes* : *Firmicutes* ratios from rural to urban [Park et al. (2015), de Filippo et al. (2017)]. This could be caused by different genera under *Bacteroidetes* or *Firmicutes* phyla which might be affected by certain type of diet. Therefore, it will be beneficial for the future studies to also include dietary factors from the study participants.

A further limitation is related to the statistical tools available in analyzing this relationship. Here, we characterized the association of three single bacterial proportions on cytokine response in the helminth-positive and -negative group. Using this approach, we first ignore the effect of compositional structure in the microbiome data, namely when computing the *p*-value we assumed that these bacterial categories are independent while they are correlated. Secondly, the current statistical model ignores the fact that microbiome is a variable measured with errors at a different scale than the cytokine responses [Teixeira-Pinto et al. (2009)]. In addition, we might as well ignore the possible unobserved confounders. It is therefore important for the future studies in this field to develop a statistical method to characterise the effects of helminth infection on both outcomes simultaneously by accounting these unobserved errors with a joint model.

To conclude, our findings supports the hypothesis for a role of helminths in modulating the immune response, which might be related to bacterial proportion and diversity. Deworming did not show a particular effect on the observed associations. It is therefore important to repeat such studies with a larger sample size as well as using more advanced statistical models to further analyze this relationship by considering the complex structure of microbiome data and other possible confounders.

4.5 Supplementary Materials

Characteristics	albendazole		placebo	
	N	Result	N	Result
Parasite infection (%)				
<i>A. lumbricoides</i>	26	3 (11.5)	40	17 (42.5)
Hookworm	26	0 (0)	40	11 (27.5)
<i>N. americanus</i>	26	0 (0)	40	11 (27.5)
<i>A. duodenale</i>	26	0 (0)	40	2 (5.0)
<i>T. trichiura</i>	26	4 (15.4)	40	13 (32.5)
Any helminths	26	5 (19.2)	40	26 (65.0)
Proportion (in %) of the 6 most abundant bacteria phyla, mean(SD)				
<i>Actinobacteria</i>		14.1 (8.9)		9.2 (7.4)
<i>Bacteroidetes</i>		3.6 (5.8)		7.7 (14.1)
<i>Firmicutes</i>	26	60.1 (13.7)	40	59.2 (16.7)
<i>Proteobacteria</i>		9.0 (6.5)		8.7 (6.8)
Unclassified		1.8 (1.1)		2.6 (2.8)
Pooled		11.5 (6.7)		12.5 (7.4)
Diversity Index, median(IQR)				
Shannon index	26	0.90 (0.85, 1.08)	40	0.97 (0.79, 1.05)
Bray-Curtis		0.19 (0.14, 0.26)		0.24 (0.17, 0.36)

Table S4.5.1: The characteristics of the participants at 21 months after treatment