ORIGINAL ARTICLE

No interplay between gut microbiota composition and the lipopolysaccharide-induced innate immune response in humans *in vivo*

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Abstract

Objective. Animal studies have demonstrated the extensive interplay between the gut microbiota and immunity. Moreover, in critically ill patients, who almost invariably suffer from a pronounced immune response, a shift in gut microbiota composition is associated with infectious complications and mortality. We examined the relationship between interindividual differences in gut microbiota composition and variation in the *in vivo* cytokine response induced by bacterial lipopolysaccharide (LPS). Furthermore, we evaluated whether an LPS challenge alters the composition of the gut microbiota. **Methods.** Healthy male volunteers received an intravenous bolus of 2 ng kg^{-1} LPS ($n = 70$) or placebo ($n = 8$). Serial plasma concentrations of tumor necrosis factor-α, interleukin (IL)-6, IL-8 and IL-10 were measured, and subjects were divided into high and low cytokine responders. Gut microbiota composition was determined using 16s RNA gene sequencing of faecal samples obtained 1 day before (baseline) and 1 day and 7 days following the LPS challenge. **Results.** Baseline microbiota composition, analysed by principal coordinate analysis and random forest analysis, did not differ between high and low responders for any of the four measured cytokines. Furthermore, baseline microbiota diversity (Shannon and Chao indices) was similar in high and low responders. No changes in microbiota composition or diversity were observed at 1 and 7 days following the LPS challenge. **Conclusion.** Our results indicate that existing variation in gut microbiota composition does not explain the observed variability in the LPS-induced innate immune response. These findings strongly argue against the interplay between the gut microbiota composition and the innate immune response in humans.

Keywords: 16s RNA, gut microbiota, human endotoxaemia, innate immunity, LPS

INTRODUCTION

Over the last decades, it has become evident that commensal microorganisms in the gut may influence host physiology and pathology. Much work in this field has been dedicated to the role of the gut microbiota in host defence. For instance, bone marrow-derived neutrophils obtained from mice treated with broad-spectrum antibiotics to deplete the gut microbiota exhibited reduced killing of *Streptococcus pneumonia* and *Staphylococcus aureus ex vivo*.[1] In accordance, the depletion of the gut microbiota in mice resulted in reduced pulmonary cytokine levels and increased bacterial dissemination following infection with *S. pneumonia*, demonstrating the protective role of a healthy gut microbiota composition in pneumococcal pneumonia.[2] Human data are sparse, although there are some indications that variations in the gut microbiota correlate with cytokine production by leucocytes *ex vivo* stimulated with specific microbial stimuli.[3] Furthermore, a decrease in total obligate anaerobes in the gut was shown to be associated with infectious complications and mortality in critically ill patients.[4] However, these results are likely biased by several other factors, such as more antibiotic administration in the sickest patients. Evidence for a limited role of the microbiota in regulation of the host response is emerging as well. For instance, acute changes in bacterial composition caused by a 7-day course of broad-spectrum antibiotics in healthy volunteers exerted only limited effects on cytokine release by mononuclear cells upon *ex vivo* stimulation with bacterial lipopolysaccharide (LPS)[5], and a similar course of antibiotics did not affect the *in vivo* systemic cytokine response during experimental endotoxaemia, a standardised, controlled and reproducible model of systemic inflammation consisting of intravenous LPS administration in healthy volunteers.[6] However, the question remains whether naturally occurring interindividual differences in microbiota composition are associated with variation in the *in vivo* immune response.

In addition to the above-mentioned putative influence of the gut microbiota composition on the systemic immune response, there are several indications that the immune response may alter the gut microbiota composition. For instance, critical illness, which almost invariably involves a pronounced immune response, is associated with alterations in gut microbiota composition[7,8], which is in turn associated with increased susceptibility to develop hospital-acquired infections, sepsis and organ failure.[9,10] Again, many of these patients received antibiotics, and it remains unknown whether an acute systemic immune response by itself influences the gut microbiota composition.

In this study, we investigated whether naturally occurring interindividual differences in intestinal microbiota composition are associated with variation in the cytokine response during experimental human endotoxaemia. Second, we explored whether experimental endotoxaemia causes alterations in the intestinal microbiota composition up to 7 days following the LPS challenge.

RESULTS

Subjects, symptoms and plasma cytokine levels

There were no differences in baseline demographic characteristics between the experimental endotoxaemia and placebo groups (Table 1). All subjects in the experimental endotoxaemia group experienced flu-like symptoms, reflected by an increase in total symptom score from 0 [0–1] just before LPS administration to a peak of 5 [3–6] at 1.5 h post-LPS. Furthermore, both high and low responders developed a transient systemic immune response, reflected by an increase in plasma levels of pro-inflammatory [tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8] and anti-inflammatory (IL-10) cytokines following the LPS administration, which reverted to normal levels in the ensuing hours (Figure 1 and Supplementary figure 1). Subjects in the placebo group did not experience any symptoms (median total symptom score of 0 at all time points) and did not display an increase in plasma cytokine levels (Figure 1 and Supplementary figure 1).

Associations between the baseline gut microbiota composition and the LPS-induced cytokine response

Baseline composition of the microbiota, analysed using the Bray–Curtis dissimilarity index and

Table 1. Baseline demographic and clinical data

Parameter	All subjects (n = 78)	LPS (n = 70)	Placebo (n = 8)	P-value
Age (years)	22 [20–24]	22 [20–24]	21 [19–23]	0.22
Temperature (°C)	36.6 [36.4–37]	36.6 [36.4–37]	36.6 [36.2–37]	0.74
Weight (kg)	76.8 [70–83]	76.9 [70.3–82.3]	75.7 [69.0–89.8]	0.90
MAP (mmHg)	98 [93–109]	98 [93–103]	98 [94–99]	0.31

Data are presented as median [IQR]; P-values are calculated using Mann–Whitney U-tests (LPS vs placebo group).
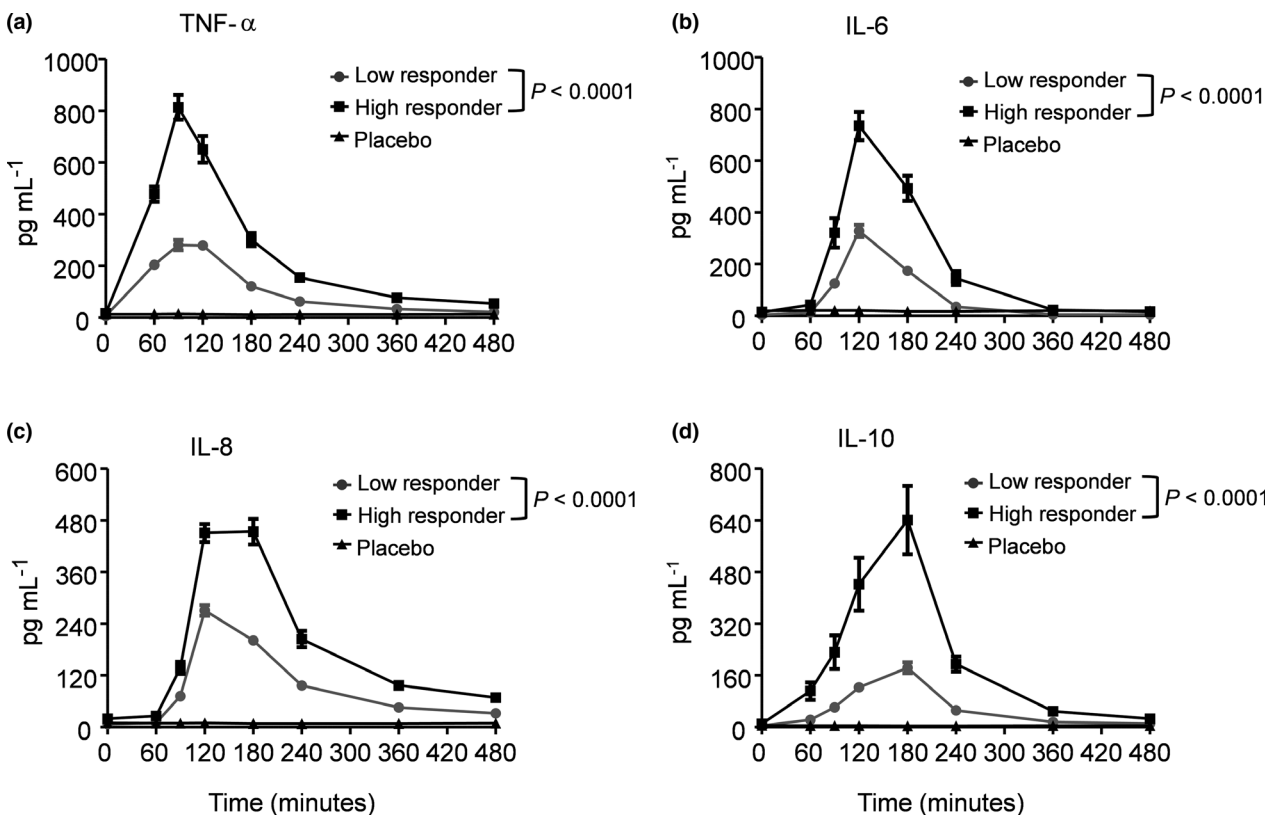MAP, mean arterial blood pressure.

Figure 1. Plasma cytokine levels of subjects intravenously challenged with lipopolysaccharide (LPS) or receiving placebo. **(a)** Tumor necrosis factor (TNF)-α. **(b)** Interleukin (IL)-6. **(c)** IL-8. **(d)** IL-10. Subjects who were challenged with LPS were divided into two groups based on their area under the concentration–time curve plasma cytokine levels (AUC, reflecting total cytokine production over time). Low responders have AUC plasma cytokine levels below the median, and high responders have AUC plasma cytokine levels above the median. Only subjects who, apart from LPS, received a placebo or an intervention that did not significantly affect the cytokine response were included, on a per-cytokine base. Data are presented as mean ± SEM. P-values were calculated by two-way repeated-measures ANOVA (time*group interaction term).

plotted using principal coordinate analysis, did not differ between high and low responders for any of the four measured cytokines (Figure 2). Performing the same analysis, but using quartiles instead of median area under the concentration–time curve (AUC) cytokine levels, also revealed no relationship between baseline composition of the gut microbiota and the cytokine response (Supplementary figure 2). For microbiota diversity, no differences were found either between high and low cytokine responders (Figure 3 and Supplementary figure 3). Finally, random forest analysis showed no difference between high and low cytokine responders in abundance of taxa present in the gut (Table 2).

The influence of the LPS-induced systemic immune response on the intestinal microbiota composition

Experimental human endotoxaemia induced a profound systemic inflammatory response, reflected by the aforementioned increase in circulating cytokines and the development of flu-like symptoms and fever, but no changes in microbiota composition or diversity were observed 1 and 7 days following endotoxaemia (phylum level shown in Figure 4 and genus level in Supplementary figure 4).

DISCUSSION

In the present study, we demonstrate that existing interindividual differences in gut microbiota composition are *not* associated with variation in *in vivo* cytokine responses during experimental human endotoxaemia. Second, we demonstrate that the LPS-induced systemic immune response does *not* alter the composition of the gut microbiota in the following week. These results strongly argue against a relevant interplay between the gut microbiota composition and the innate immune response in humans.
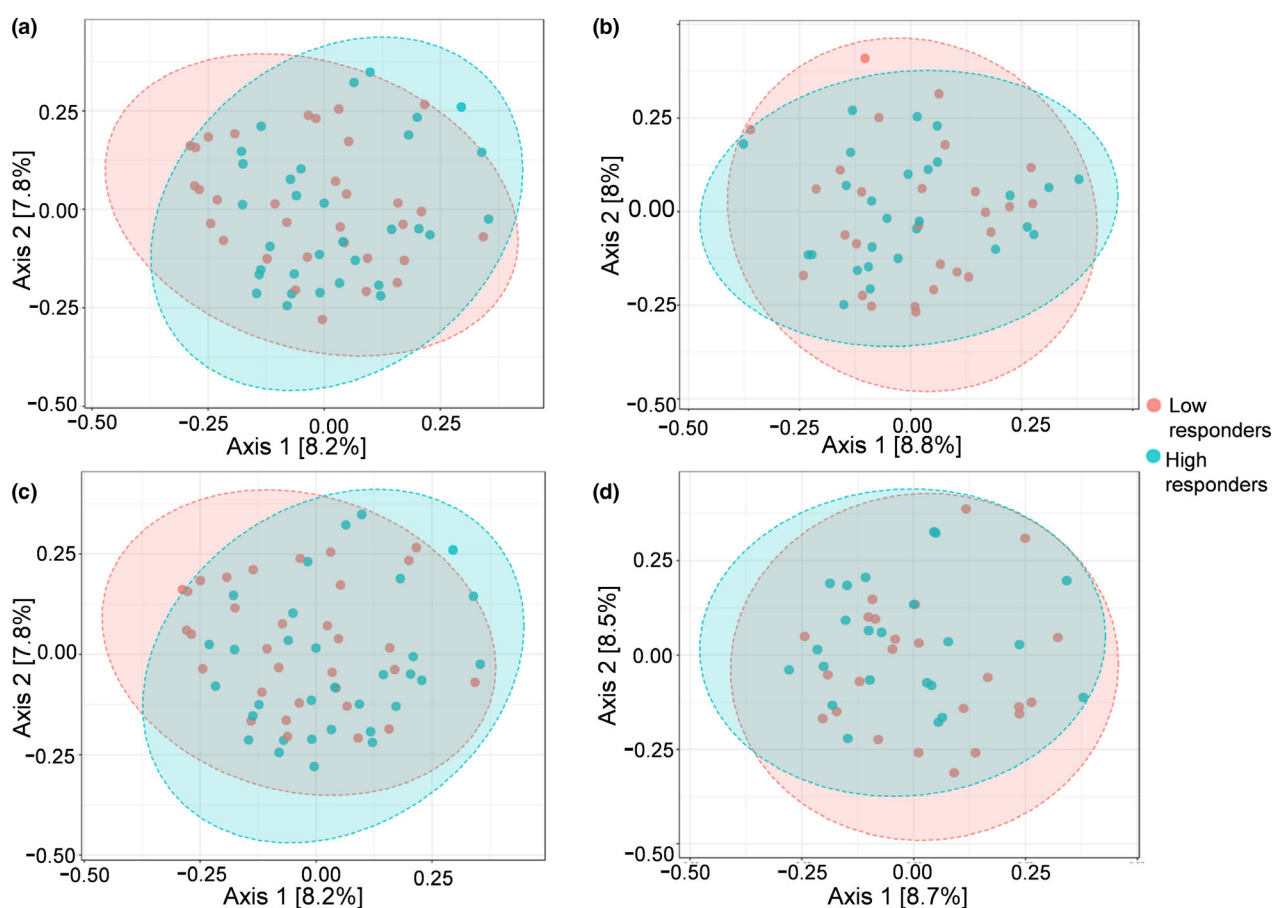

Figure 2. Principal coordinate analysis (PCoA) of baseline gut microbiota composition in high (blue dots) and low (red dots) cytokine responders upon lipopolysaccharide (LPS) challenge. **(a)** Tumor necrosis factor (TNF)-α (high responder n = 32, low responder n = 32). **(b)** Interleukin (IL)-6 (high responder n = 27, low responder n = 27). **(c)** IL-8 (high responder n = 32, low responder n = 32). **(d)** IL-10 (high responder n = 23, low responder n = 24). Subjects were divided into two groups based on their area under the concentration–time curve plasma cytokine levels (AUC, reflecting total cytokine production over time). Low responders have AUC plasma cytokine levels below the median, and high responders have AUC plasma cytokine levels above the median. Only subjects who, apart from LPS, received a placebo or an intervention that did not significantly affect the cytokine response were included, on a per-cytokine base. The horizontal axis reflects principal coordinate 1, and the vertical axis reflects principal coordinate 2 with their corresponding percentages of explained variance. PERMANOVA on the Bray–Curtis dissimilarity index: TNF-α: F-value = 1.14, R^2 = 0.02, P = 0.21. IL-6: F-value = 1.01, R^2 = 0.02, P = 0.46. IL-8: F-value = 1.29, R^2 = 0.02, P = 0.08. IL-10: F-value = 1.17, R^2 = 0.023, P = 0.19.
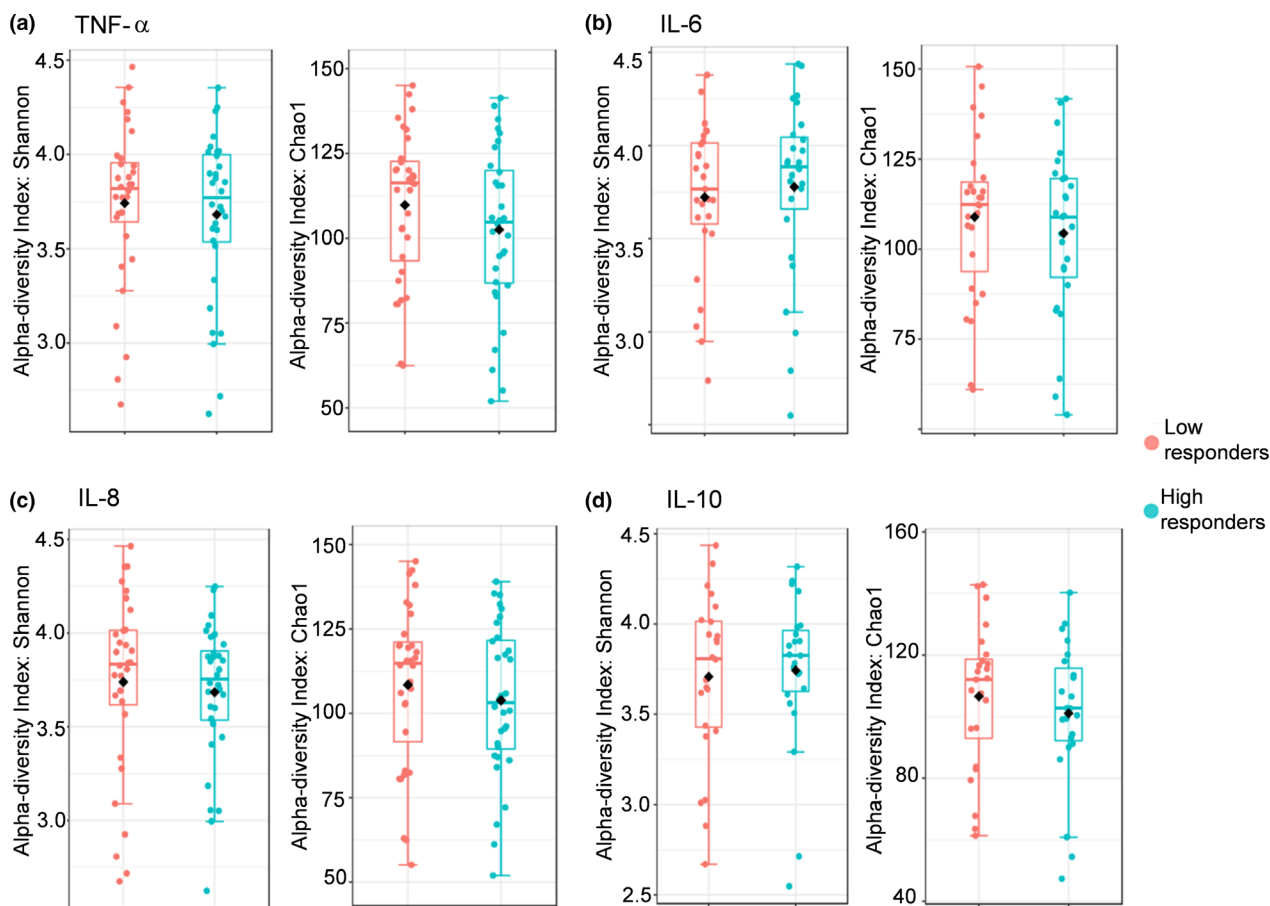
Figure 3. Alpha diversity in gut microbiota at baseline, reflected by the Shannon diversity index and the Chao1 index, in high (blue dots) and low (red dots) cytokine responders upon lipopolysaccharide (LPS) challenge. **(a)** Tumor necrosis factor (TNF)-α (high responder $n = 32$, low responder $n = 32$). **(b)** Interleukin (IL)-6 (high responder $n = 27$, low responder $n = 27$). **(c)** IL-8 (high responder $n = 32$, low responder $n = 32$). **(d)** IL-10 (high responder $n = 23$, low responder $n = 24$). Subjects were divided into two groups based on their area under the concentration–time curve plasma cytokine levels (AUC, reflecting total cytokine production over time). Low responders have AUC plasma cytokine levels below the median, and high responders have AUC plasma cytokine levels above the median. Only subjects who, apart from LPS, received a placebo or an intervention that did not significantly affect the cytokine response were included, on a per-cytokine base. Data are presented as box-and-whisker plots, with whiskers representing the lowest and highest values within 1.5*interquartile range (IQR). *P*-values were calculated by Mann–Whitney *U*-tests.

Table 2. Area under the receiver operating characteristic curve values using microbiota composition at baseline as the independent variable and high vs low responders (based on AUC cytokine levels) as the dependent variable

	Area under the curve	
Groups	OTU	Genus
IL-6 high vs low responder	0.596	0.476
IL-8 high vs low responder	0.551	0.609
IL-10 high vs low responder	0.525	0.548
TNF-α high vs low responder	0.495	0.565

There were no differences in taxa at baseline, which explain the extent of the cytokine response.
OTU, operational taxonomic unit.

Results obtained in various animal models demonstrated that microbiota dysbiosis is associated with increased susceptibility to infections.[11,12] However, whereas broad-spectrum antibiotic-induced depletion of the microbiota in healthy volunteers drastically lowered diversity and decreased absolute numbers of bacteria, no effects on the cytokine response were observed during experimental human endotoxaemia.[6] This suggests that *acute* changes in microbiota composition do not influence the innate immune response in humans. In contrast, chronically existing differences in microbiota composition between individuals may impact susceptibility to
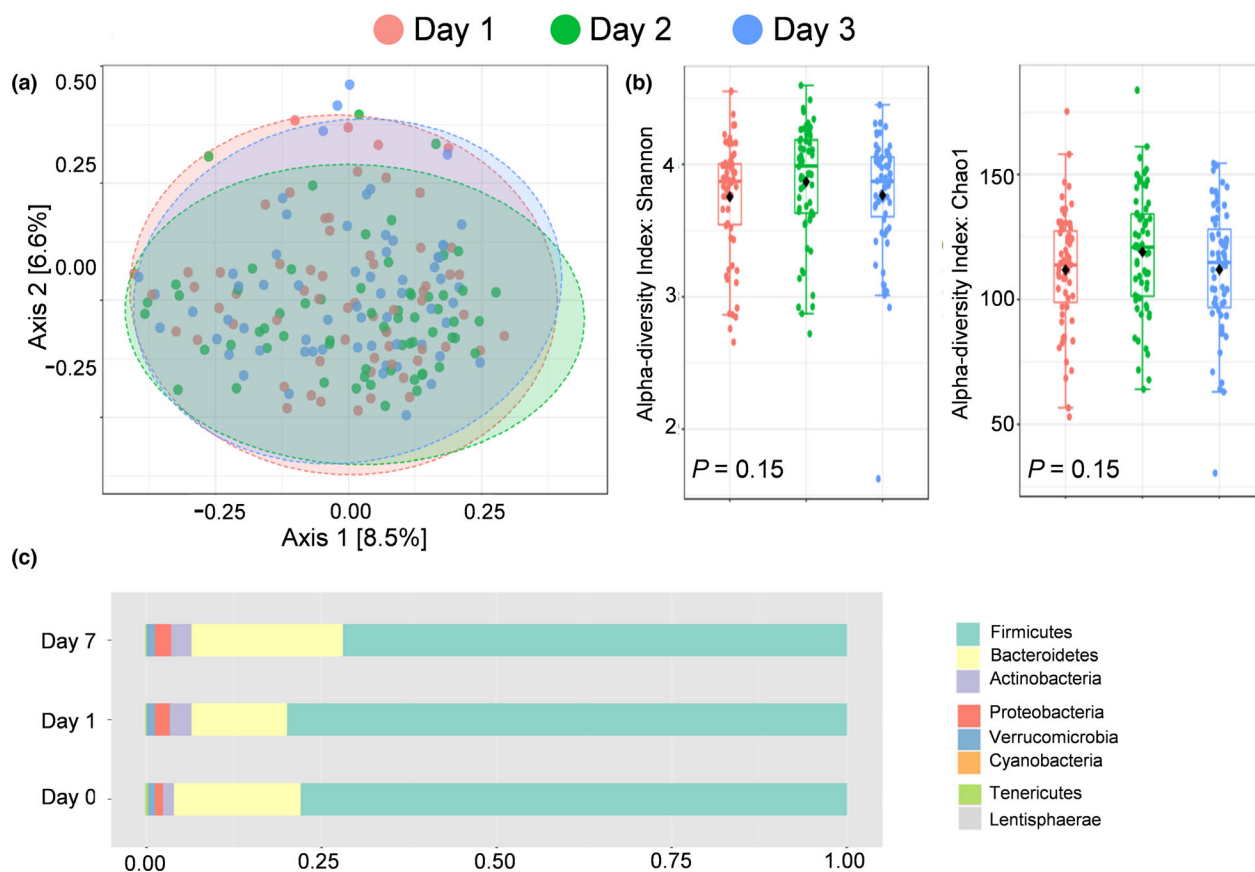
Figure 4. Effect of lipopolysaccharide (LPS) challenge on gut microbiota composition. **(a)** Principal coordinate analysis (PCoA) of microbiota composition over time following the LPS challenge ($n = 63$, paired samples across time points). PERMANOVA on the Bray–Curtis dissimilarity index: F-value = 0.44, $R^2 = 0.01$, $P = 1.00$. **(b)** Gut microbiota diversity over time following the LPS challenge ($n = 63$, paired samples across time points), reflected by the Shannon index and the Chao1 index. P-values were calculated by Friedman tests. **(c)** Relative abundance of gut microbiota (phylum level) over time following the LPS challenge ($n = 63$, paired samples across time points).

auto-immune diseases[13,14], as well as the risk to develop other diseases.[15–19] For example, it is argued that the more pro-inflammatory phenotype observed in elderly or obese individuals can be explained by differences in their microbiota composition compared with young and lean individuals.[15,20,21] Although these observations might suggest that these chronically existing interindividual differences in microbiota composition influence human immunity, these associations are likely to be confounded by various other factors. Furthermore, the effect size is likely small, as variation in microbiota composition was found to explain only 10% of the variation in *ex vivo* cytokine production by leucocytes of healthy volunteers.[3] In our study, all subjects developed a transient systemic immune response. However, this immune response evoked by LPS, a microbiota-derived

compound, is not influenced by an individual's gut microbiota composition. So, in addition to the previously reported absence of effects of acute depletion of the microbiota by broad-spectrum antibiotics on the LPS-induced innate immune response[6], our findings indicate the absence of an association between chronically existing interindividual differences in microbiota and the immune response. To our knowledge, the present study is the first to investigate the proposed interplay between the variability of existing intestinal microbiota and the systemic innate immune system in humans under controlled circumstances. These findings are not in line with previous animal studies or observational patient studies, and illustrate the importance of translating findings obtained in animals to humans and from uncontrolled to controlled settings.

In addition, we demonstrate that, despite induction of a pronounced systemic immune response by LPS administration, the gut microbiota composition remains unaltered for up to a week. These findings in healthy volunteers contradict results obtained in septic and non-septic critically ill patients admitted to the ICU, where a shift in microbiota composition was observed in the days after ICU admission.[7,8,22] This may be because of the fact that experimental human endotoxaemia mimics a short-lived systemic inflammatory response and cannot recapitulate the entire disease spectrum observed in patients admitted to the ICU with sepsis or other conditions. Furthermore, the findings in ICU patients were obtained in an uncontrolled setting, where not only systemic inflammation but also the use of antibiotics, severity of organ dysfunction and many other confounding factors are likely to play a role. Irrespective of the cause of microbiota disruption in critically ill patients, restoration of a healthy microbiota may nevertheless still be important for improving outcomes of these patients.

Our study has several limitations. First, our study population consisted of healthy Caucasian young men, with little variation in age and weight and a relatively limited diversity in gut microbiota at baseline. For example, in patients with or without Alzheimer's disease, the difference in the Chao1 index was more than 50[18], compared to differences of less than 25 in our study population. Therefore, the interindividual differences in microbiota composition in our relatively homogenous group of young volunteers may have been too small to cause differences in the immune response. As such, the possibility remains that more pronounced differences in diversity indices, for instance related to health parameters[15], may be associated with differences in immunity. Second, as briefly touched on before, experimental human endotoxaemia does not fully recapitulate the entire disease spectrum observed in ICU patients with sepsis or other conditions, and is also not fully comparable with the often severe animal models used in the sepsis research field. However, our human model captures many hallmarks of the early septic immune response. These similarities are not confined to the inflammatory response alone, but also comprise clinical parameters and organ-specific changes, such as cardiovascular (reduced left ventricular contractility, hyperdynamic circulation), haematological (reduced platelet count and function) and endocrinological (catecholamine and cortisol stress response) alterations. Especially of relevance to the current study, experimental human endotoxaemia results in increased gut permeability[23], which is also observed in sepsis patients. Strengths of our study include the large sample size for an endotoxaemia study and the highly standardised, controlled and reproducible set-up. As such, the absence of confounding factors enables meticulous dissection of the interplay between the intestinal microbiota composition and the innate immune response.

In conclusion, we demonstrate that no relationship exists between existing gut microbiota composition and the cytokine response induced by an LPS challenge in healthy volunteers. Furthermore, the LPS-induced systemic innate immune response does not alter the gut microbiota composition in the following week. Our data suggest that it is unlikely that interindividual differences in microbiota composition determine the extent of the immune response and that the systemic immune response per se does not account for the change in microbiota composition observed in critically ill patients. These observations strongly argue against the relevant interplay between the gut microbiota composition and the innate immune response in humans, indicating that the therapeutic value of modulation of the gut microbiota composition in critically ill patients may be limited.

METHODS

Subjects

During the conduct of four experimental human endotoxaemia studies, data were obtained from a total of 78 healthy male volunteers.[24–28] All studies were approved by the local ethics committee (CMO Arnhem-Nijmegen, registration numbers 2013-041, 2013-290, 2015-2058 and 2015-2079) and registered at ClinicalTrials.gov: NCT01944228, NCT01978158, NCT02642237 and NCT02675868. Written informed consent was obtained from all study participants, and all procedures were in accordance with the Declaration of Helsinki, including latest revisions. Seventy subjects received LPS (further detailed below), whereas 8 subjects received placebo (saline). For the analyses aimed at determining the relationship between variation in the baseline microbiota composition and the cytokine response, we only included subjects who, apart from LPS, received a placebo or an intervention that did not significantly affect the cytokine response. The latter was determined on a per-cytokine basis.

For the analyses aimed at determining the effects of the LPS-induced systemic immune response on microbiota composition, we included all subjects who received LPS. Seven subjects were excluded because of insufficient amounts of faecal material to extract bacterial DNA from, resulting in a total of 63 remaining subjects for these analyses.

Subjects were healthy, non-smoking, male volunteers, 18–35 years old, without febrile illness or use of any medication (including antibiotics) during the month before the experiment. They had a normal physical examination, routine blood investigation and electrocardiography upon screening before the start of the experiments. Caffeine and alcohol intake was not allowed within 24 h before the start of the experiments and subjects refrained from food 12 h before the start of the experiment.

Experimental human endotoxaemia procedures

Endotoxaemia experiments were conducted at the research unit of the intensive care department of the Radboud University Medical Center according to our standardised protocol.[23] In all subjects, an arterial line was placed in the radial artery for blood sampling and continuous monitoring of blood pressure, and a venous cannula was placed for hydration and LPS administration. Heart rate was monitored using a 3-lead electrocardiogram, and body temperature was measured every 30 min. The severity of flu-like symptoms (headache, nausea, shivering, muscle and back pain) was scored every 30 min on a scale ranging from 0 to 5 per symptom, resulting in a total score of 0–25 points.

Subjects received an intravenous bolus of 2 ng kg^{-1} purified LPS (US Standard Reference Endotoxin *Escherichia coli* O:113, obtained from the Pharmaceutical Development Section of the National Institutes of Health, Bethesda, MD, USA) or matched bolus volume of NaCl 0.9% (placebo group) at $T = 0$. All subjects received 1.5 L of 2.5% glucose/ 0.45% saline solution during 1 h before LPS/placebo administration, followed by 150 mL h^{-1} until 6 h after LPS/placebo administration and 75 mL h^{-1} until the end of the experiment.

Cytokine and leucocyte analysis

Before ($T = 0$) and at 60, 90, 120, 180, 240, 360 and 480 min after LPS administration, blood samples were obtained from the arterial line and collected in ethylenediaminetetraacetic acid (EDTA)-containing vacutainers. Blood was immediately centrifuged at 2000 g for 10 min at 4°C, and plasma was stored at −80°C until analysis. Concentrations of TNF-α, IL-6, IL-8 and IL-10 in all samples used for the analyses described in the current study were measured in one batch using a Luminex assay (R&D Systems; Abingdon Science Park, UK). The lower detection limit was 2 pg mL^{-1} for all cytokines. Leucocyte counts were determined in EDTA-anticoagulated blood by the Department of Laboratory Medicine of the Radboud University Medical Center (Sysmex XE-5000; Sysmex Nederland B.V., Etten-Leur, the Netherlands).

Microbiota analysis

Subjects collected faecal samples at home 1 day before and 1 day and 7 days after the endotoxaemia experiment. These samples were stored at −20°C and transported to the study centre for storage at −80°C. Bacterial DNA was extracted using a bead-beating protocol, and the extracted DNA was used for 16s rRNA gene sequencing (Illumina MiSeq sequencing) as described previously.[29] Data were processed using the NG-Tax software package.[30]

Statistical analysis

The distribution of data was tested using the Shapiro–Wilk tests. Parametric data are presented as mean ± SEM, whereas non-parametric data are presented as median [interquartile range]. The differences between groups were analysed using two-way repeated-measures analysis of variance (two-way ANOVA, time*group interaction term). To increase sensitivity to detect possible differences in microbiota composition at baseline between high and low cytokine responders, several analyses were performed: subjects were divided into two (above and below median) or four (quartiles) groups based on their AUC (reflecting total cytokine production over time) plasma cytokine levels. To analyse microbial beta diversity, which describes the diversity in a microbial community between different samples, principal coordinate analysis (PCoA) of the different groups was performed in an unconstrained way and permutational multivariate analysis of variance (PERMANOVA) on the Bray–Curtis dissimilarity index was performed in MicrobiomeAnalyst.[31,32] Furthermore, random forest analyses using microbiota composition at baseline as the independent variable and AUC cytokine levels as dependent variables were performed to identify taxa that may be differently abundant in the gut of high cytokine responders compared with low cytokine responders. The Shannon diversity index and the Chao1 index were used as measures of alpha diversity of the gut microbiota.[33,34] The Chao1 estimates the numbers of unique operational taxonomic unit (OTUs) in a sample (microbial richness). The Shannon index takes into account richness and evenness. To analyse the differences in alpha diversity between high and low responders, the Mann–Whitney U-tests were used. For our second objective – determining whether an LPS challenge alters the gut microbiota composition – the above-described principal coordinate analysis was used to compare gut microbiota composition at baseline vs days 1 and 7. Additionally, the differences in alpha diversity at baseline vs days 1 and 7 were assessed using Friedman tests.

Statistical analyses were performed using GraphPad Prism 5.03 (GraphPad Software Inc, San Diego, California, USA), SPSS for Windows 22.0 (SPSS Inc, Chicago, IL, USA), R (package 'randomForest', version 4.6-14) and MicrobiomeAnalyst.[31,32]

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Quirine Habes: Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing-original draft; Writing-review & editing. **Prokopis Konstanti:** Formal analysis; Methodology; Software; Supervision. **Harmke Kiers:** Data curation; Investigation; Writing-review & editing. **Rebecca Koch:** Data curation; Investigation; Writing-review & editing. **Roeland Stolk:** Data curation; Investigation; Writing-review & editing. **Clara Belzer:** Formal analysis; Software; Supervision; Writing-review & editing. **Matthijs Kox:** Formal analysis; Investigation; Methodology; Software; Supervision; Visualization; Writing-original draft; Writing-review & editing. **Peter Pickkers:** Supervision; Writing-original draft; Writing-review & editing.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.