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SEVERITY OF ATOPIC DISEASE INVERSELY CORRELATES WITH INTESTINAL MICROBIOTA DIVERSITY AND BUTYRATE-PRODUCING BACTERIA

Running title: Correlation of eczema severity and microbiota characteristics

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Abstract

The reports on atopic diseases and microbiota in early childhood remain contradictory and both decreased and increased microbiota diversity have been associated with atopic eczema. In this study, the intestinal microbiota signatures associated with the severity of eczema in 6-month-old infants were characterized. Further, the changes in intestinal microbiota composition related to the improvement of this disease 3 months later were assessed. The severity of eczema correlated inversely with microbiota diversity ($r=-0.54$, $P=0.002$) and with the abundance of butyrate-producing bacteria ($r=-0.52$, $P=0.005$). During the 3 months follow-up, microbiota diversity increased ($P<0.001$) and SCORAD values decreased ($P<0.001$) in all infants. This decrease coincided with the increase in bacteria related to butyrate-producing *Coprococcus eutactus* ($r=-0.59$, $P=0.02$). In conclusion, the high diversity of microbiota and high abundance of butyrate-producing bacteria were associated with milder eczema, thus suggesting they have a role in alleviating symptoms of atopic eczema.

KEY WORDS: butyrate-producing bacteria, eczema severity, microbiota diversity, SCORAD

ABBREVIATIONS:

SCORAD: SCORing Atopic Dermatitis

RDA: Redundancy analysis

MCPP: Monte Carlo Permutation Procedure

Introduction

Decrease in microbial contacts in affluent societies during last decades is considered to lie behind the rise in allergic and other chronic inflammatory diseases. In addition, associations between the composition and diversity of intestinal microbiota and different diseases have been studied extensively and decreased total microbiota diversity has been linked to several disorders (1). A special focus has been on atopic diseases and intestinal microbiota modulation by probiotics. The reports on atopic eczema and microbiota in early childhood are contradictory and there is no consensus on what would constitute an “atopy-promoting” or “atopy-preventing” microbiota. Confusingly, both decreased and increased microbiota

diversity have been associated with the atopic eczema (2). However, thus far the relationship between intestinal microbiota and the severity of eczema has not been considered.

Materials and methods

Our aim was to characterize the intestinal microbiota signatures associated with the severity of eczema in 6-month-old infants and to assess the changes in intestinal microbiota composition related to the improvement of this disease 3 months later. We characterized the faecal microbiota diversity and composition from healthy infants (n=11) and infants with varying extent of eczema severity (n=28) as determined by SCORAD-values. Study subjects represent a sub-population from two prospective follow-up trials at Turku University Central Hospital, Finland, which have been described in detail previously (3,4) (see the supplementary text and Table S1 in this article's Online Supplementary material for additional information on the subject characteristics and methodology).

Results

The severity of eczema correlated inversely with microbiota diversity ($r=-0.54$, $P=0.002$, Figure 1A) and with the abundance of butyrate-producing bacteria ($r=-0.52$, $P=0.005$, Figure 1B). Infants with mild eczema (SCORAD <15) had 1.6-fold higher diversity index values ($P=0.002$, Figure 1C) and two-fold higher abundance of butyrate-producing bacteria when compared to the infants with more severe eczema (SCORAD 15—45.5) ($P=0.03$, Figure 1D). In healthy infants, both the microbiota diversity and the abundance of butyrate-producing bacteria were between the corresponding values of infants with mild or moderate eczema (Figures 1C and 1D). Furthermore, the severity of eczema was the most discriminating factor in the clustering of infants based on the microbiota profiles as assessed by RDA analysis (MCP, $P=0.03$, see Figures S1 and S2 in the Online Supplementary material). Mode of delivery and whether the infant got breast-milk at the time of sampling or not did not have statistically significant effect on the microbiota composition ((MCP, $p=0.51$ and $p=0.11$, respectively) or diversity ($p=0.93$ and $p=0.68$, respectively), see Figures S3 and S4 in the Online Supplementary material).

We followed the alleviation of eczema symptoms and microbiota changes within the more severe eczema group (SCORAD >15, n=15). These infants participated in an intervention trial where they received extensively hydrolysed casein formula with or without *Lactobacillus rhamnosus* GG supplementation. The follow-up faecal samples were collected 3 months after the baseline. Microbiota composition showed significant development within the study period as assessed by RDA analysis (MCP, $P=0.002$, see Figures S5 and S6 in Online Supplementary material), but there were no differences in the microbiota composition between the intervention groups (RDA and MCP, $p=0.54$, see Figure S7 in Online Supplementary material). During the intervention, SCORAD values decreased ($P<0.001$, Figure 2A) and microbiota diversity increased ($P<0.001$, Figure 2B) in all infants. The decrease in SCORAD values correlated with the increase in bacteria related to *Coprococcus eutactus* ($r=-0.59$, $P=0.02$, Figure 2C), the predominating butyrate-producing bacteria (see Figure S8 in Online Supplementary material).

Discussion

Subjects with atopic eczema have been suggested to have an impaired intestinal barrier function and increased intestinal permeability (5). Butyrate-producing bacteria are a functional group of phylogenetically diverse Gram-positive anaerobes that are able to produce butyrate as metabolic end-product from carbohydrate fermentation. These bacteria enhance the intestinal barrier function by up-regulating the expression of tight-junction proteins and by inhibiting pro-inflammatory signalling pathways such as nuclear factor kappa B (NF- κ B) activation (6). Moreover, butyrate regulates the proliferation and activation of regulatory T cell (Treg) in the colon (7) and increases the capacity of Tregs to suppress the proliferation of effector CD4⁺ T cells in mice (8). Thereby, butyrate-producing bacteria may play an important role in alleviating eczema symptoms in early childhood. The promotion of microbiota diversity and especially butyrate-producing bacteria can provide new tools to control eczema symptoms. Microbiota diversity can be supported by avoiding disturbances in the normal microbiota development (such as administration of antibiotics) and butyrate-producing bacteria by providing a diet rich in plant-derived polysaccharides after weaning (9).

Recent microbiota studies have shown that characteristics of the intestinal microbiota are associated with atopic eczema in early childhood (4,10,11). Both lower total microbiota diversity and declined diversity or abundance of Bacteroidetes phylum have been reported in infants with atopic eczema (4,10). In addition, increased prevalence or abundance of different *Clostridium* clusters has been associated with eczema (4,11). Further, the present study revealed that the high diversity of total microbiota and high abundance of butyrate-producing bacteria are associated with milder symptoms in infants with atopic eczema. In conclusion, the results suggest that intestinal microbiota has a role in alleviating symptoms of atopic eczema.

Acknowledgements

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Author contributions

LN performed experiments, data analyses, interpretation of results and wrote the manuscript. RS and WMdV designed and supervised the study, performed interpretation of results and contributed to the writing of manuscript. MN made the clinical evaluations of infants and contributed to the writing of manuscript. EI and SS designed the original intervention trials and contributed to the writing of manuscript.

Conflict of interests

The authors report no conflict of interests.

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Figure legends

Figure 1. **A**, The severity of eczema (SCORAD values) correlates inversely with microbiota diversity and **B**, with the abundance of butyrate-producing bacteria in 6-month-old infants. **C**, Microbiota diversity and **D**, abundance of butyrate-producing bacteria were significantly different between infants with mild (SCORAD <15) and moderate eczema (SCORAD >15), whereas showed non-significant difference when compared with healthy infants.

Figure 2. **A**, In infants with moderate eczema, the SCORAD values were significantly decreased within the 3 months study period. **B**, This decrease in eczema symptoms was accompanied by an increase in microbiota diversity. **C**, The decrease in SCORAD values correlated with the increase in the predominant butyrate-producer *Coprococcus eutactus et rel.* (*C. eutactus*).

Figure 1.

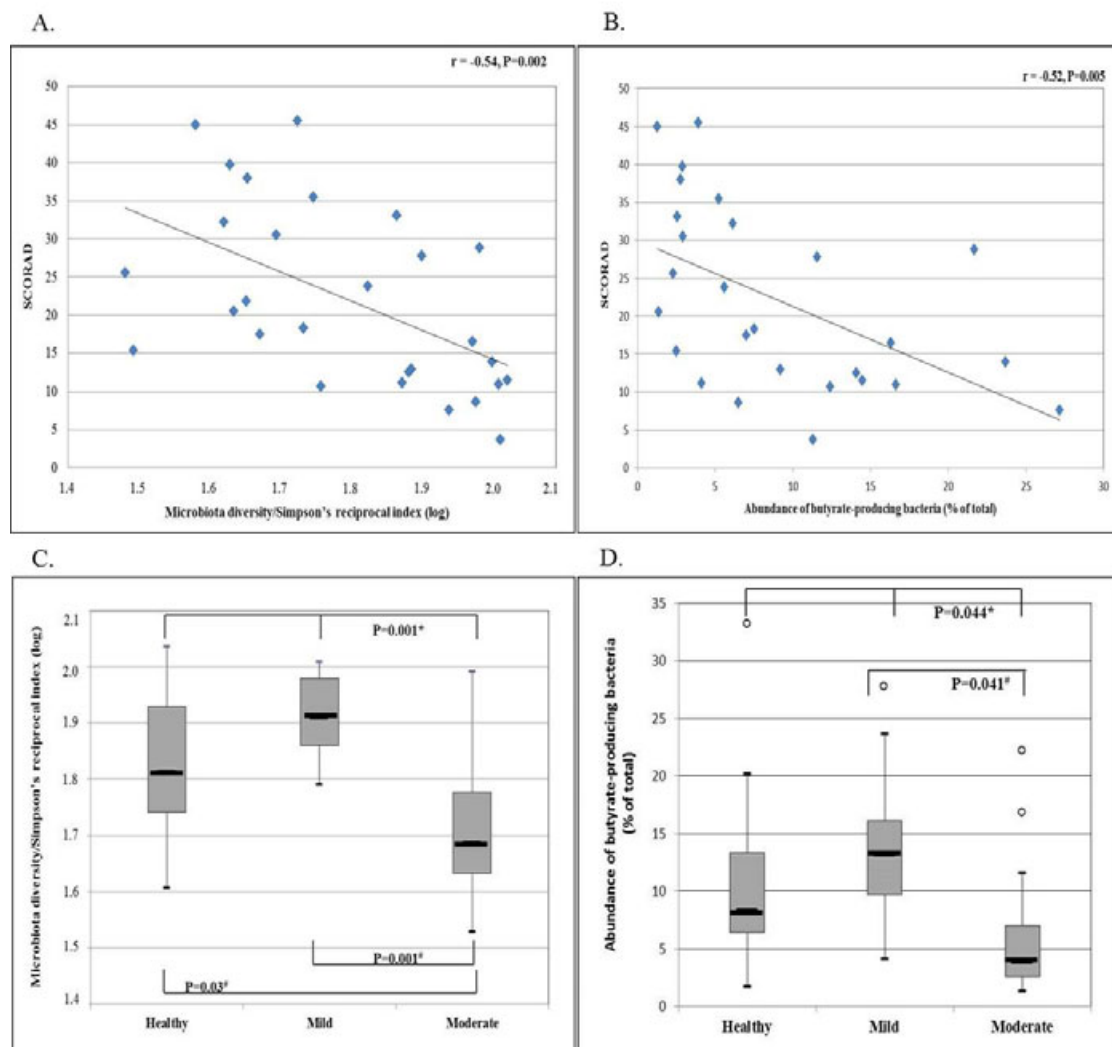


Figure 2.

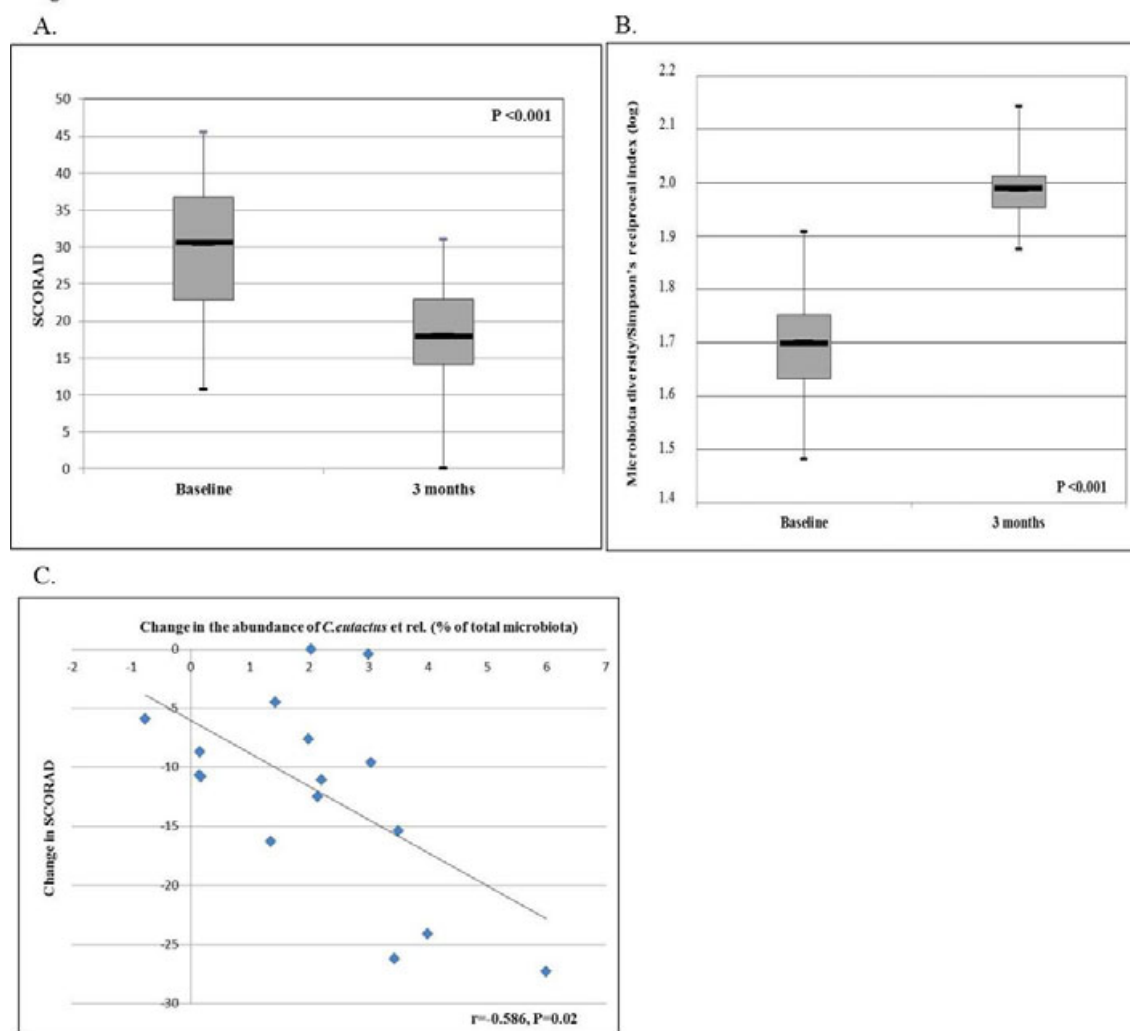


Figure S1

MCPP=0.03

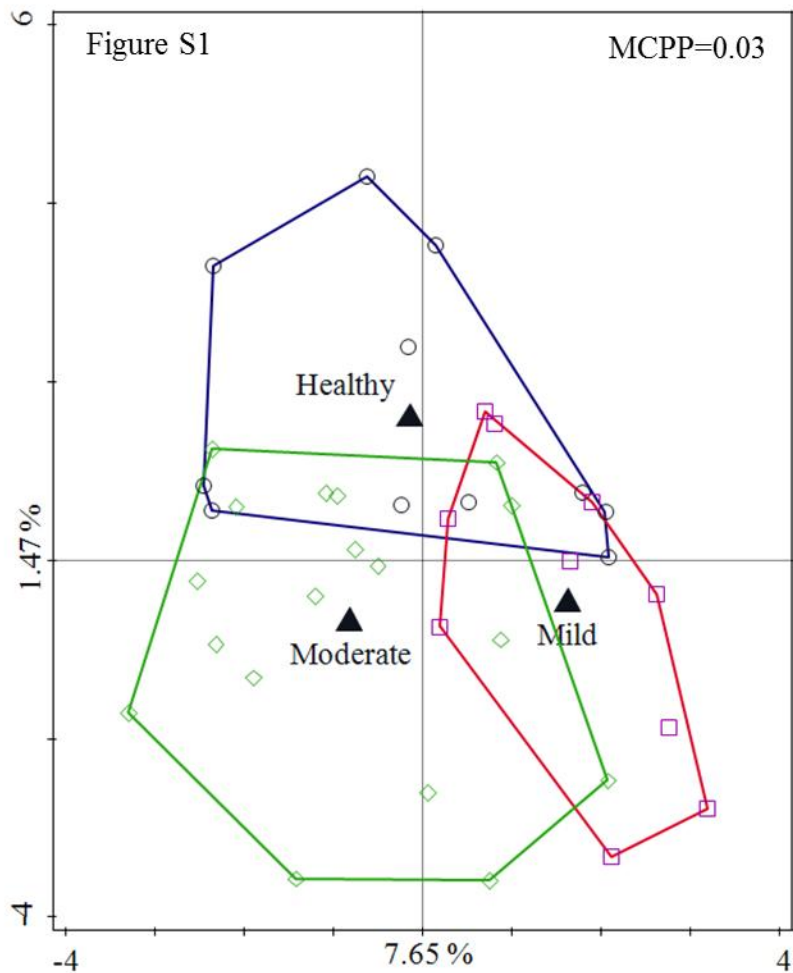


Figure S2

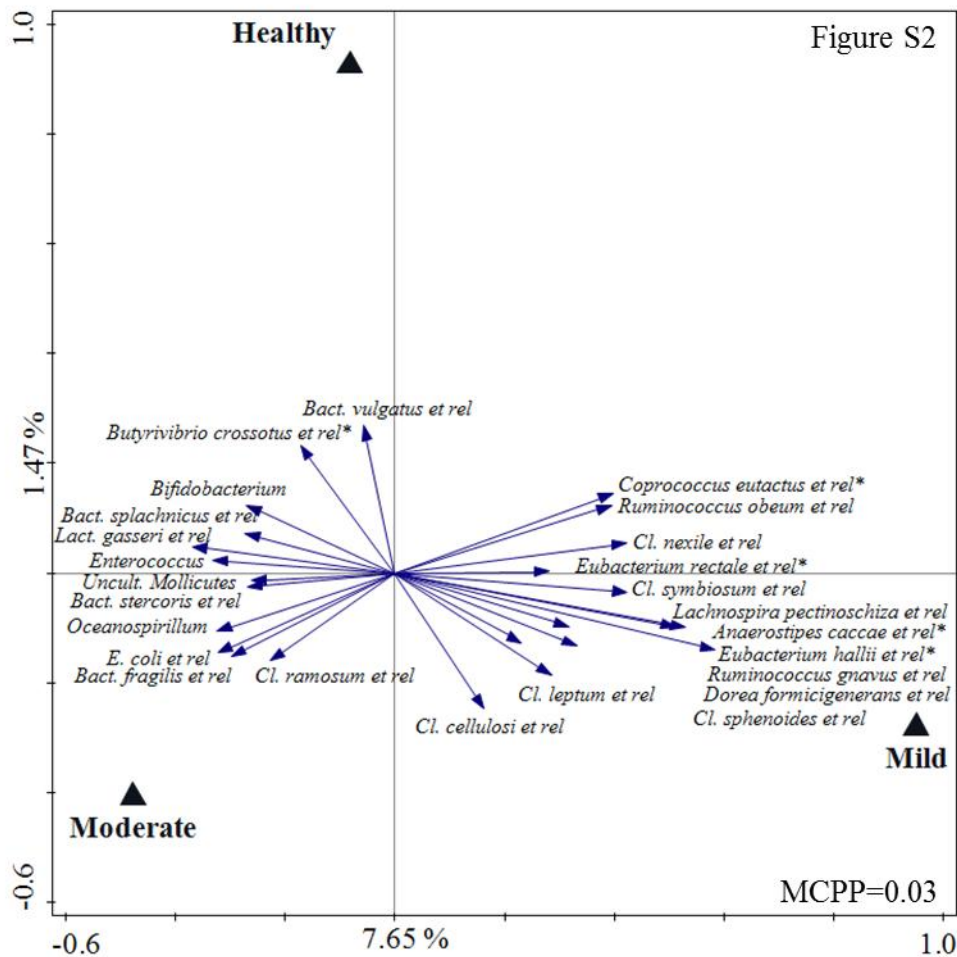
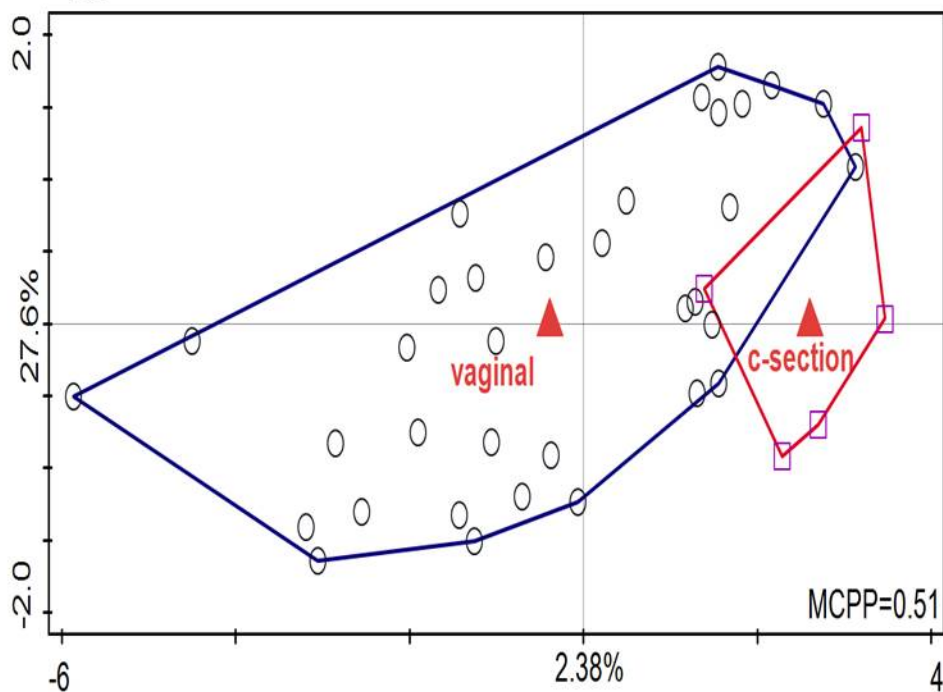


Figure S3.

A.



B.

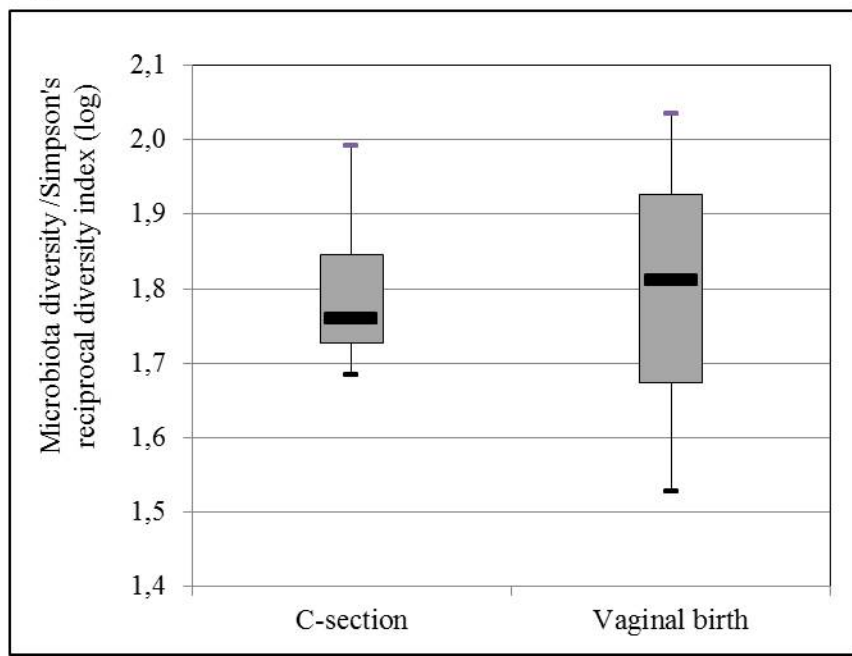
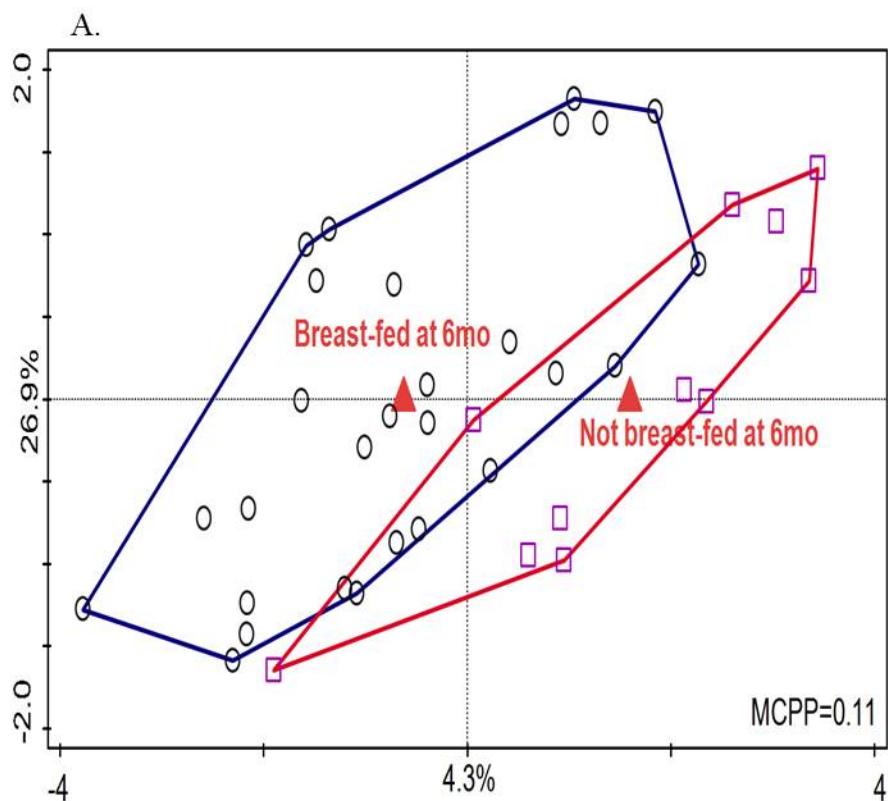


Figure S4.



B.

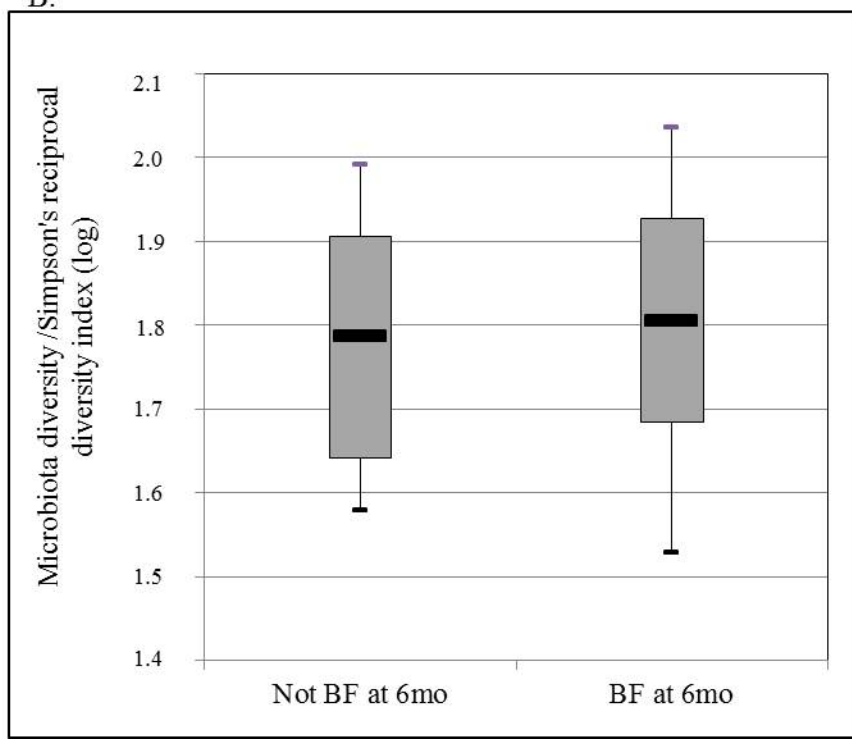
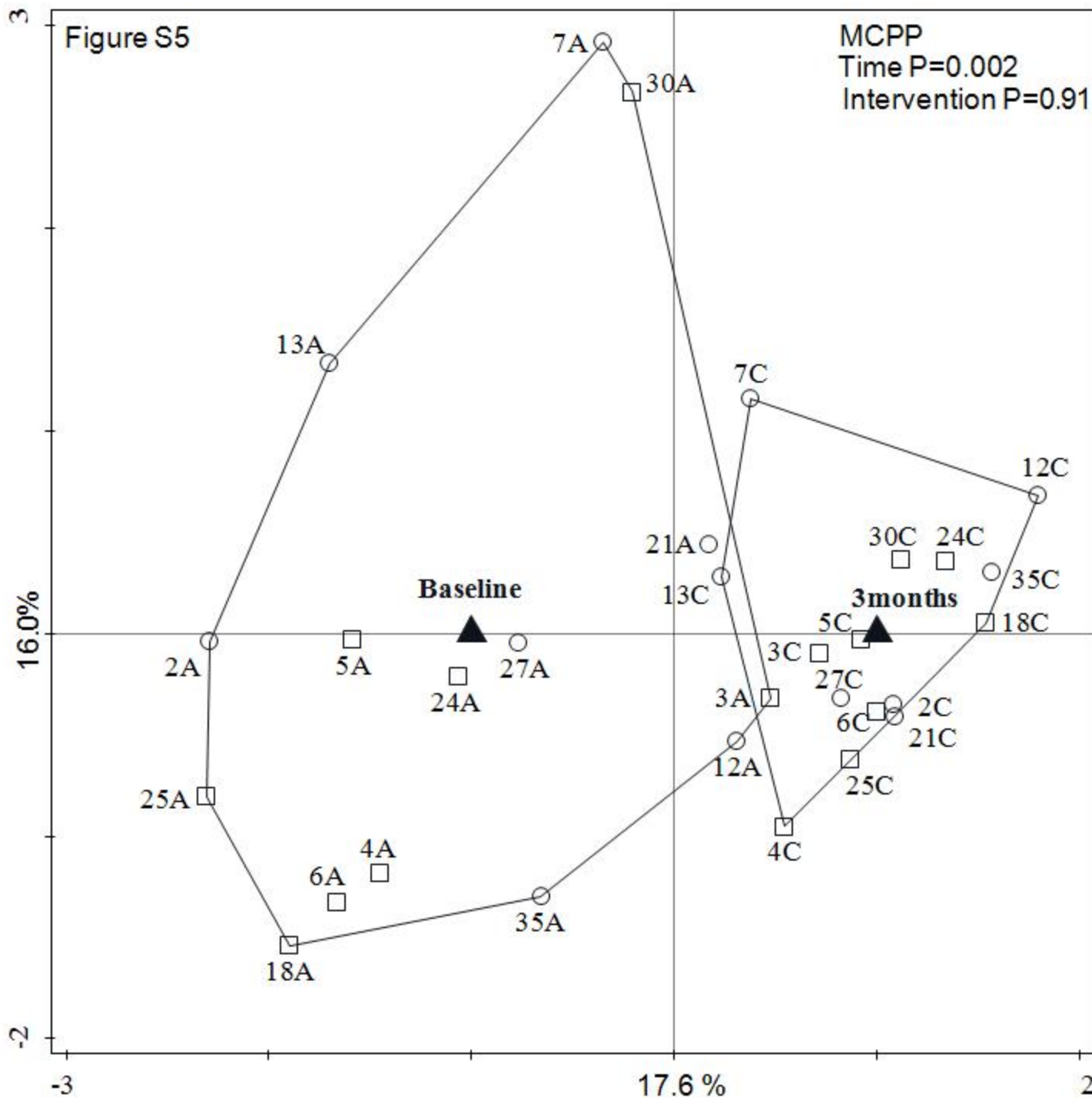


Figure S5

MCP
Time P=0.002
Intervention P=0.91



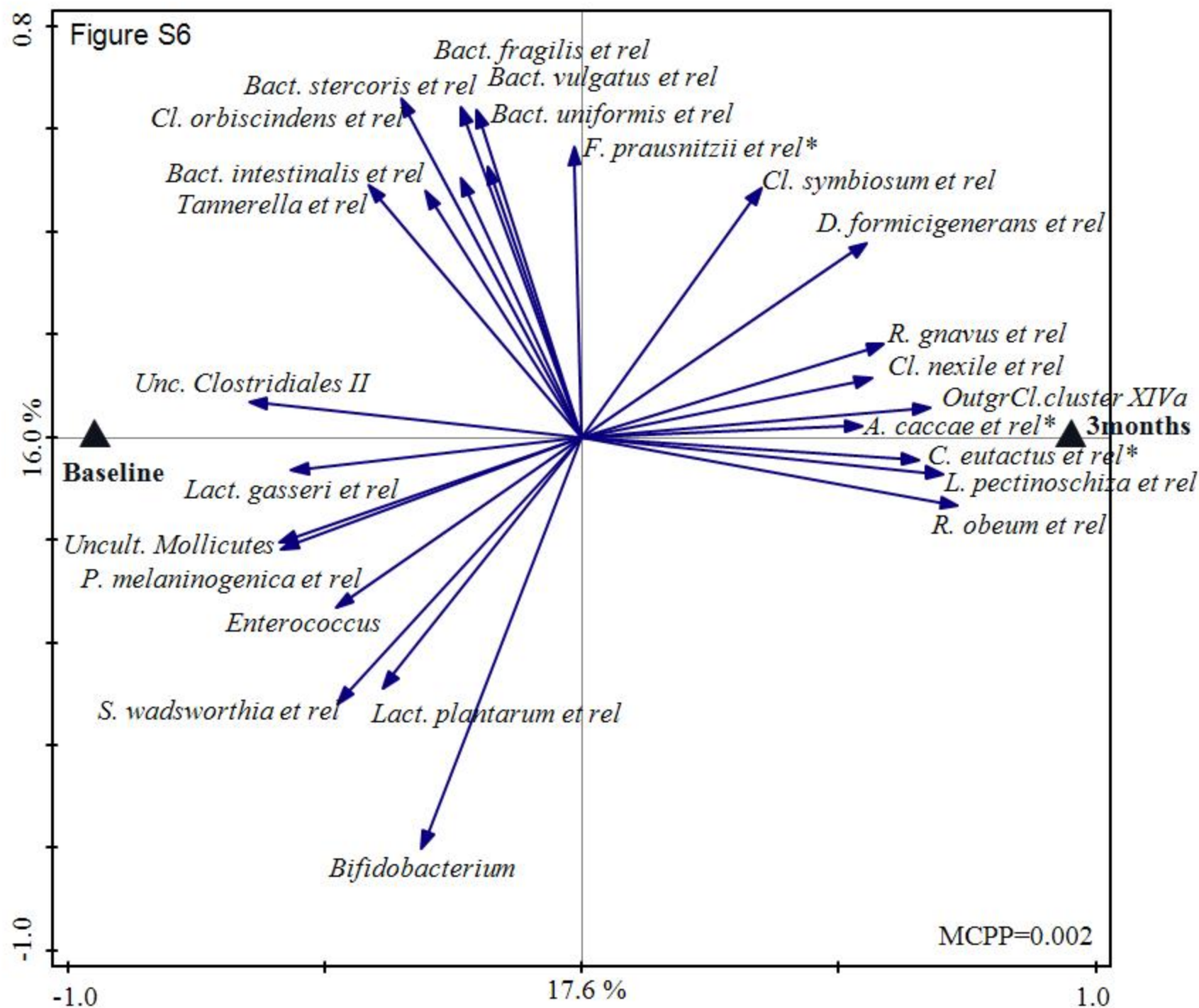


Figure S7

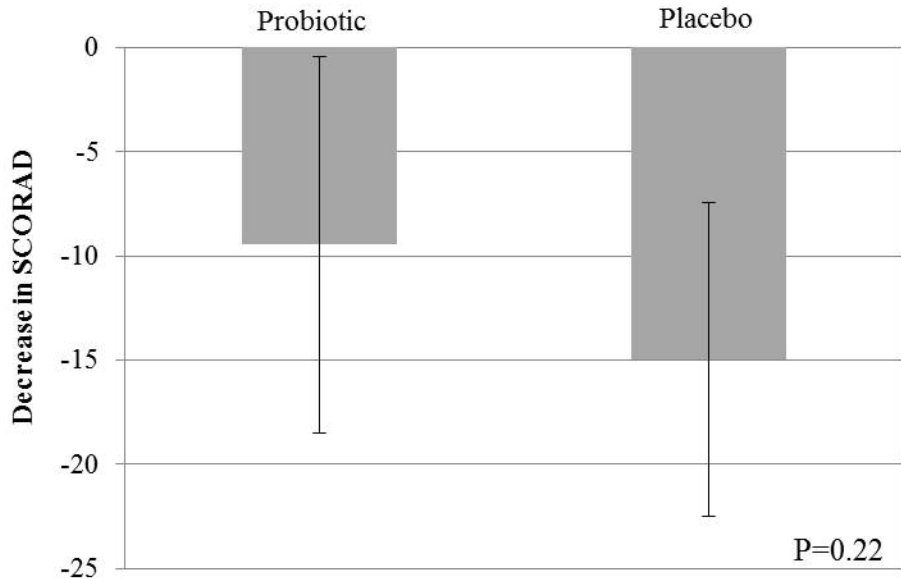


Figure S8

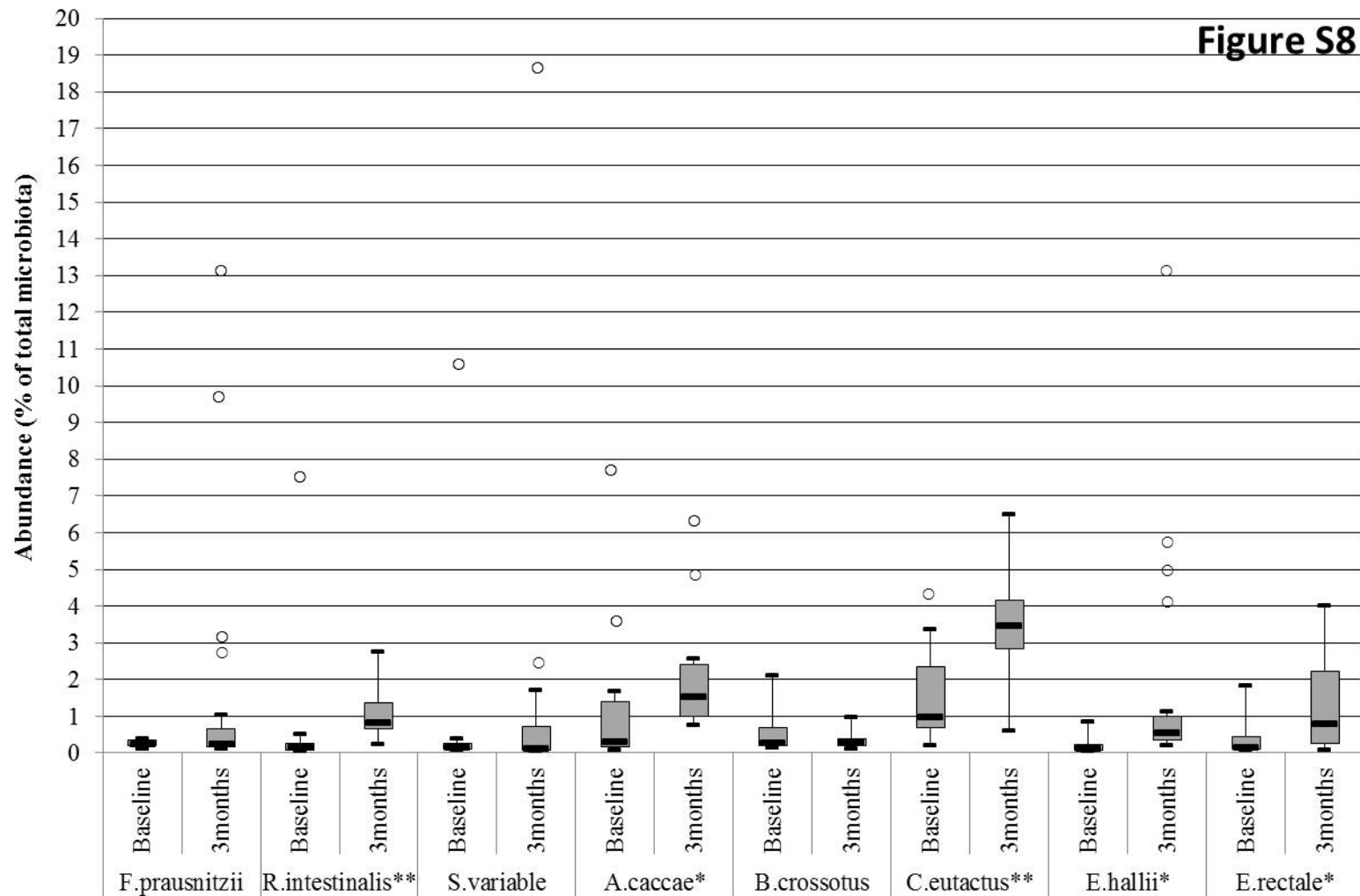


Table S1. Basic characteristics of the study subjects

<u>Sample code</u>	<u>Subject</u>	<u>Severity of eczema group</u>	<u>SCORAD</u>	<u>Age</u>	<u>Delivery mode</u>	<u>Breast-fed at the time of sampling</u>
101	101	Healthy	0	6.0	vaginal	yes
152	152	Healthy	0	7.6	vaginal	yes
159	159	Healthy	0	6.2	vaginal	yes
164	164	Healthy	0	7.0	vaginal	yes
181	181	Healthy	0	7.0	vaginal	yes
194	194	Healthy	0	6.1	vaginal	no
206	206	Healthy	0	6.3	vaginal	yes
208	208	Healthy	0	5.4	vaginal	yes
214	214	Healthy	0	6.1	vaginal	yes
215	215	Healthy	0	7.6	vaginal	no
225	225	Healthy	0	6.2	vaginal	yes
123	123	Mild	13.9	6.2	vaginal	yes
250	250	Mild	11.1	5.7	vaginal	yes
105	105	Mild	12.9	6.0	vaginal	yes
127	127	Mild	7.6	5.9	vaginal	yes
149	149	Mild	11.5	5.6	vaginal	yes
187	187	Mild	10.9	6.2	vaginal	yes
243	243	Mild	12.5	6.0	vaginal	yes
245	245	Mild	8.6	6.1	vaginal	yes
252	252	Mild	3.7	6.2	vaginal	yes
20A	20	Mild	10.7	6.5	vaginal	yes
109	109	Moderate	16.5	8.1	vaginal	yes
128	128	Moderate	17.5	6.9	vaginal	yes
151	151	Moderate	20.5	7.0	vaginal	no
2A	2	Moderate	39.7	5.4	vaginal	yes
3A	3	Moderate	23.8	5.2	C-section	no
4A	4	Moderate	25.6	4.5	vaginal	yes
5A	5	Moderate	35.5	8.5	vaginal	yes
6A	6	Moderate	15.4	7.3	vaginal	no
7A	7	Moderate	21.8	8.8	vaginal	yes
12A	12	Moderate	28.8	9.3	C-section	no
13A	13	Moderate	32.2	5.5	vaginal	yes
18A	18	Severe	45.0	4.2	vaginal	no
21A	21	Severe	45.5	4.5	vaginal	yes
24A	24	Moderate	18.3	9.0	C-section	yes
25A	25	Moderate	38.0	6.5	C-section	yes
27A	27	Moderate	33.1	8.9	vaginal	no
30A	30	Moderate	27.8	8.8	vaginal	no
35A	35	Moderate	30.5	5.4	C-section	no
2C	2	3 month follow-up	13.5	8.4	vaginal	no
3C	3	3 month follow-up	17.9	8.2	C-section	no
4C	4	3 month follow-up	25.7	7.5	vaginal	no
5C	5	3 month follow-up	31.0	11.5	vaginal	no

6C	6	3 month follow-up	0	10.3	vaginal	no
7C	7	3 month follow-up	14.2	11.8	vaginal	no
12C	12	3 month follow-up	17.7	12.3	C-section	no
13C	13	3 month follow-up	21.4	8.5	vaginal	no
18C	18	3 month follow-up	17.7	7.2	vaginal	no
21C	21	3 month follow-up	21.4	7.5	vaginal	no
24C	24	3 month follow-up	17.9	12.0	C-section	no
25C	25	3 month follow-up	25.5	9.5	C-section	no
27C	27	3 month follow-up	24.4	11.9	vaginal	no
30C	30	3 month follow-up	18.2	11.8	vaginal	no
35C	35	3 month follow-up	14.2	8.4	C-section	no

SUPPLEMENTARY TEXT

Methods

Study design, subjects and faecal samples. The severity of atopic eczema was classified in three categories as described previously (1). According to this classification, ten infants with mild eczema (SCORAD <15), 16 infants with moderate (15<SCORAD >40) and two infants with severe eczema (SCORAD >40) were included in this study. Due to the low amount of subjects with severe eczema, these samples were analysed as a part of the moderate eczema group. Eleven children without any eczema symptoms (SCORAD 0) before or at the time of sampling were included as healthy controls. All infants were born full-term (≥ 37 weeks) with normal birth weight (≥ 2500 g). Five infants were born by caesarean section and 33 were delivered vaginally. At the time of faecal sample collection, all infants received some solid food(s). Atopic eczema was diagnosed and SCORAD values determined as described before (2) and infants with other diseases except atopic eczema were excluded.

Subjects of this study represent a sub-population from two prospective follow-up trials at Turku University Central Hospital, Finland, which have been described in detail previously (2-4). Healthy infants (n=11), infants with mild atopic eczema (n=10) and two infants with moderate atopic eczema are a sub-population from a follow-up trial which has been described previously (3,4). Briefly, the inclusion criterion for these children was that they had a high risk of atopic diseases, i.e. they had at least one close relative (mother, father and/or sibling) with atopic eczema, allergic rhinitis or asthma. Mothers were randomized to receive capsules containing either placebo or 1×10^{10} colony-forming units of *Lactobacillus rhamnosus* GG (ATCC 53103) daily for 2–4 weeks before expected delivery. The intervention continued 6 months postnatally. Infants with moderate atopic eczema represent a sub-population from another probiotic intervention study where infants with atopic eczema were randomized in a double-blind design to receive extensively hydrolysed casein formula supplemented with or without *Lactobacillus rhamnosus* GG (ATCC 53103). The duration of intervention was 3 months and faecal samples were collected at the study entry and at the end of the intervention. Based on the availability of two faecal samples and other criteria mentioned above, 16 children from the original study population (n=37) (2) were included. The basic characteristics of the study subjects are presented in Table S1.

The study protocols of original interventions were approved by the Ethics Committee of the Hospital District of South-Western Finland and subjects were enrolled in the study after written informed consent was obtained from the parents (ClinicalTrials.gov ID NCT01148667).

Faecal samples and DNA extraction. The faecal samples were taken from children at the age of 6 months (mean age 6.6 months, Table S1). A follow-up sample was available from 15 infants, representing the microbiota composition 3 months after the baseline. The samples were aliquoted and frozen immediately after collection and stored in -70 °C. DNA was extracted from faecal samples using the repeated bead-beating method as described previously (5,6).

16S rRNA gene microarray analysis. The composition of total microbiota was assessed by using the phylogenetic Human Intestinal Tract chip (HITChip) as described previously (7). Microarray analysis of all samples were performed in at least two independent hybridizations until satisfactory reproducibility was achieved (> 96%). Array normalization and calculation of cut-off values for positive responding probes were performed as described previously (4,7). The HITChip genus-like level data are summary values of the intensities from all the probes assigned to the respective genus. This genus-like level data was used in correlation analysis, in the RDA clustering and in the between-group comparisons of microbial abundances.

Statistical analyses. Statistical analyses of HITChip data was carried out with log-transformed data using the IBM SPSS Statistics 21 –software. The data is presented as medians with 25th and 75th percentiles. Redundancy analysis (RDA) was performed by using the multivariate statistical analysis package Canoco 5 for Windows (Microcomputer Power, Ithaca, USA). The Monte Carlo Permutation Procedure from Canoco 5 was used to assess the statistical significance of the variation in large datasets. The diversity of the microbial community assessed by HITChip was expressed as Simpson's reciprocal index of diversity (1/D) as described before (8) by using the probe-level data. Differences in the microbial abundance and diversity data were analysed by using ANOVA with Tukey's HSD post hoc test. Correlations between diversity indexes, bacterial groups and SCORAD values were calculated using Pearson's coefficient (r).

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1. The clustering of infant microbiota profiles at 6 months as assessed by RDA analysis. P-value obtained by Monte Carlo Permutation Procedure was 0.03.

Healthy: SCORAD =0

Mild: SCORAD <15

Moderate: SCORAD >15

Figure S2. RDA plot of the microbiota composition of healthy infants and infants with a varying extent of eczema severity. 25 bacterial groups that contributed most to the variability of the samples are indicated as black arrows. P-value obtained by Monte Carlo Permutation Procedure was 0.03.

*Butyrate-producing bacteria

Healthy: SCORAD =0

Mild: SCORAD <15

Moderate: SCORAD >15

Abbreviations: Bact. *Bacteroides*, Cl. *Clostridium*, E. *Escherichia*, et rel. and relatives, Lact. *Lactobacillus*, Uncult. Uncultured.

Figure S3. The composition (A) and diversity (B) of microbiota did not differ statistically significantly between the infants born by Caesarean section (n=5) or vaginally (n=33) (p=0.51 and p=0.93, respectively) at the age of 6 months.

Figure S4. At the age of 6 months, the composition (A) and diversity (B) of microbiota did not differ statistically significantly between the infants who were partially breast-fed and infants who did not receive any breast-milk at the time of sampling (p= 0.11 and p=0.68, respectively.).

Figure S5. The clustering of microbiota profiles of infants with moderate/severe eczema (SCORAD >15) at baseline and 3 months later as assessed by RDA analysis. The samples clustered by age (Monte Carlo Permutation Procedure (MCP), P= 0.002) whereas this clustering was not affected by the intervention group (MCP, P=0.91). Number indicates the subject and letter A and C refer to the baseline and 3 months samples, respectively. Samples from infants who received extensively hydrolysed casein formula with or without

Lactobacillus rhamnosus GG supplementation are indicated as squares and circles, respectively.

Figure S6. RDA plot of the microbiota composition of infants with moderate/severe eczema (SCORAD >15) at baseline and 3 months later. 25 bacterial groups that contributed most to the variability of the samples are indicated as black arrows. P-value obtained by Monte Carlo Permutation Procedure was 0.002.

*Butyrate-producing bacteria

Abbreviations: A. *Anaerostipes*, Bact. *Bacteroides*, Cl. *Clostridium*, C. *Coprococcus*, D. *Dorea*, et rel. and relatives, F. *Faecalibacterium*, L. *Lachnospira*, Lact. *Lactobacillus*, P. *Prevotella*, R. *Ruminococcus*, S. *Sutterella*, Uncult. Uncultured

Figure S7. The decrease in SCORAD values during 3 months consumption of the extensively hydrolysed casein formula with or without *Lactobacillus rhamnosus* GG supplementation was comparable in both intervention groups (P=0.22).

Figure S8. Relative abundances of butyrate-producing bacteria (% of total microbiota) in infants with moderate/severe eczema at study entry (Baseline) and at the end of the intervention (3 months after). Outlier samples (values 1.5 times above the 3rd quartile) are marked with white circles. Statistically significant change: * P<0.05, **P<0.001.

Abbreviations: F. *Faecalibacterium*, R. *Roseburia*, S. *Subdoligranulum*, A. *Anaerostipes*, B. *Butyrivibrio*, C. *Coprococcus*, E. *Eubacterium*