

Letter to the Editor

Effect of probiotics in prevention of atopic dermatitis is dependent on the intrinsic microbiota at early infancy

To the Editor:

Atopic dermatitis (AD) represents an allergic inflammatory disease, and is often the first disease present in a series of allergic diseases.^{E1} There has been a dramatic increase in the prevalence of AD, with a tripling rate over the last 30 to 40 years in developed countries.^{E2} Changes in environmental factors represent a likely driver.^{E3} Bacterial exposure has been suggested as a key factor in atopic diseases, potentially through intestinal colonization and immunomodulation.^{E4} Several trials have evaluated the role of probiotics in the prevention of atopic diseases and atopic sensitization, and there is compelling evidence for the effect on AD in early life.¹⁻³ Our mechanistic understanding of the effect, however, is still limited.

In line with the current conception, we have recently reported a 40% reduction in AD among 2-year-old children with maternal probiotic supplementation from the Prevention of Allergy among Children in Trondheim (ProPACT) cohort,⁴ which is a randomized double-blind placebo-controlled study. Despite the major effect on AD, there was little or no effect of the probiotic on the gut microbiota composition through the first 2 years of life.^{5,6} Therefore, it is unlikely that the AD effect is through gut microbiota modulation. A hypothesis that has not yet been explored, however, is that there are intrinsic differences in the gut microbiota that impact the effect of probiotics on AD.

The aim of the current work was to test the intrinsic microbiota hypothesis through reanalysis of the ProPACT 16S rRNA gene gut microbiota data set. We did this through analyses of 4 separate groups: probiotic intervention and AD (PA), probiotic intervention and no AD (PN), no probiotic intervention and AD (NA), and no probiotic intervention and no AD (NN).

We present results showing that the children developing AD not prevented by probiotic intervention (PA) have a divergent microbiota with an overrepresentation of *Bifidobacterium dentium* as compared with children from the other categories. The implications of these findings are that the intrinsic microbiota can have an association with the probiotic intervention effect.

ProPACT, a randomized double-blind intervention study, was started in 2003 and is described in detail in Dotterud et al.⁴ In short, women received daily doses of probiotic-containing fermented milk starting from 36th gestation week until 3 months after birth. Women from the control group received corresponding amounts of placebo milk, which was fermented skimmed milk, pasteurized after fermentation and contained no probiotic bacteria.

Most children were born at term and breast-fed during at least first 3 months of age.⁵ Stool samples were collected longitudinally—twice from mothers at start and end of the probiotic consumption period and 4 times from children (at the age of 10 days, 3 months, and 1 and 2 years). Stool samples were frozen at -20°C after defecation and stored at -80°C upon delivery to the laboratory.

The 16S rRNA gene sequence data have previously been reported.⁶ Briefly, stool samples were homogenized and

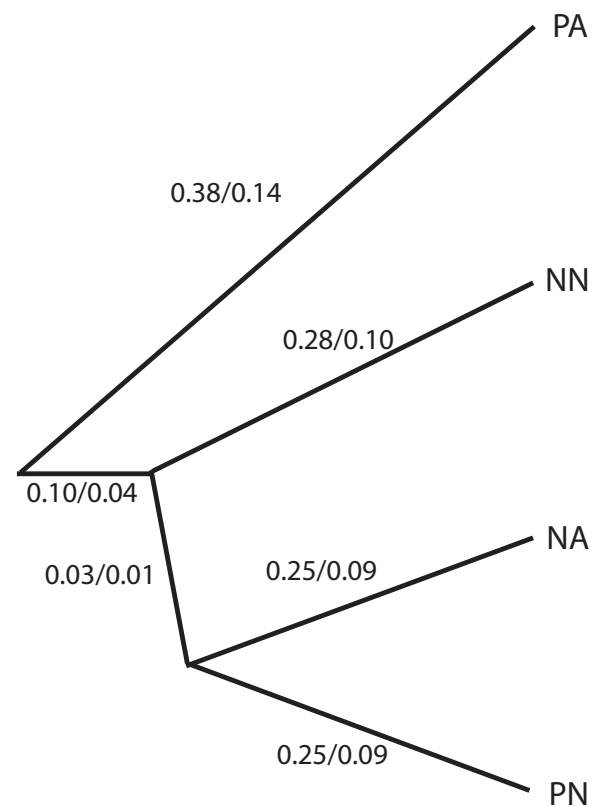


FIG 1. Divergence in microbiota between the categories (PN, PA, NN, and NA) at 10 days. Both weighted (quantitative, account for abundance) and unweighted (qualitative, considering presence or absence) Unifrac distances (first and second values) are shown for the corresponding branches.

processed using mechanical lysis. DNA was isolated using magnetic beads; 16S rRNA gene was PCR amplified using prokaryotes-targeting primers, and resulting amplicons were sequenced on Illumina MiSeq platform using v3 sequencing chemistry with 600 cycles (300 bp paired-end reads). Sequences were further paired-end joined, quality-filtered using QIIME, and clustered with 97% identity level using closed-reference *usearch* v7.0 algorithm against Greengenes database v13.8. QIIME was also used for the diversity measures.

Final data set after normalization comprised 1125 samples with a sequencing depth of 2000 sequences per sample belonging to 287 mother-child pairs of which 278 had end-point results at 2 years (see Table E1 in this article's Online Repository at www.jacionline.org). Samples included in each time point were representative of the whole data set with regard to probiotic intake, atopy incidence, and C-section (binomial $P > .05$).

No significant Benjamini-Hochberg false discovery rate (BH-FDR)-corrected differences were observed between the 4 categories for alpha diversity (observed species; Simpson diversity index $1/D$, and Shannon diversity index) for any of the age categories analyzed. For beta diversity analyses using Unifrac distances (ie, taking phylogeny into account), we found that children in the PA group showed the most distinct separate clustering from PN at 10 days (Fig 1). The 90-day samples showed

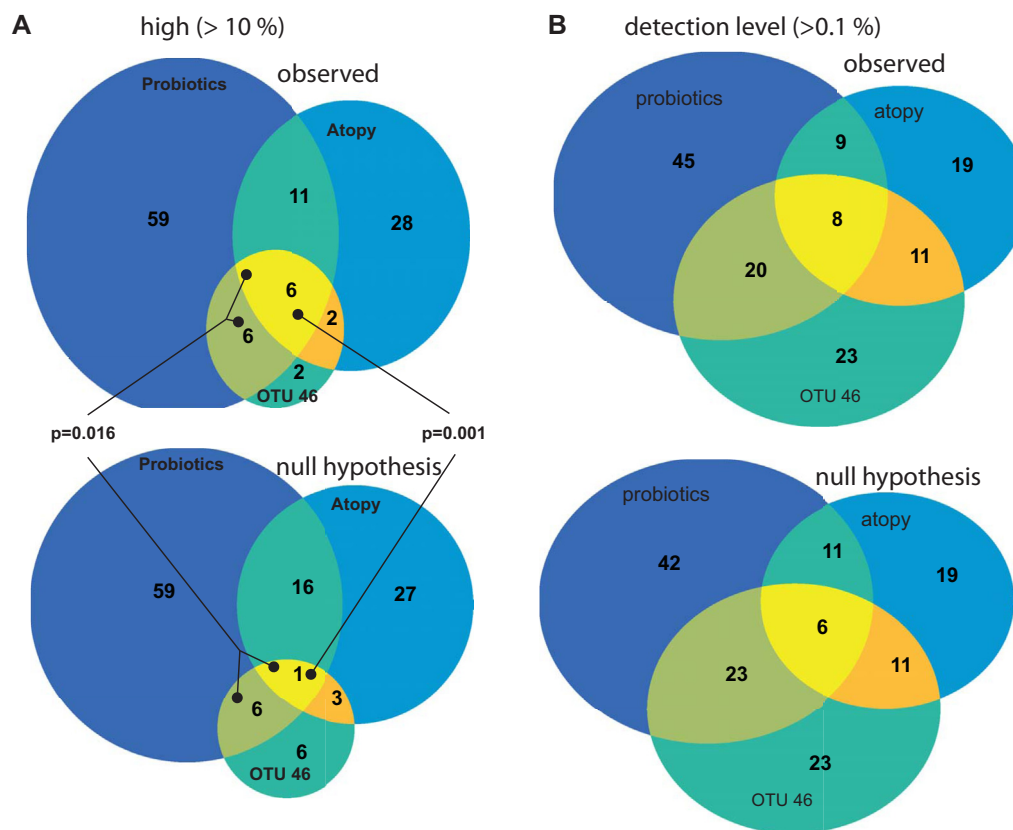


FIG 2. Prevalence of OTU 46 *B dentium* for high (A) and detection (B) levels. The prevalence data are represented as Venn diagrams, comparing the observed distribution with that expected under the null hypothesis with no associations of OTU 46. Statistical testings were based on permutations.

separate branching of PA, but nonconsistent branches between unweighted and weighted Unifrac for the other categories. For 1- and 2-year samples, there was no consistent clustering between unweighted and weighted Unifrac for any of the categories. Bray Curtis distances (considering abundance) also confirmed the separate clustering of PA from PN at 10 days ($P = .028$, BH-FDR-corrected Kruskal Wallis), but not at any other time point ($P > .05$).

We first determined the direct association of the microbiota with probiotic intervention and AD status using the multivariate statistical approach partial least squares discriminant analysis. Partial least squares discriminant analysis revealed that the 10-day PA group showed the best classification based on the microbiota (accuracy of classification = 0.81 and 0.63, validated and calibrated, respectively). The Variable Importance in Projection score (a measure for the importance in the classification model) showed that operational taxonomic unit (OTU) 46 classified as *B dentium* was clearly the most important in the PA model (see Fig E1 in this article's Online Repository at www.jacionline.org). However, no single OTU showed significant false discovery-corrected correlations to any of the categories when taking bacterial levels into account. We therefore analyzed the direct association of OTUs based on binarized data with thresholds of 0.1% (detection limit) and 10% (this threshold was set to investigate bacteria that tend to have high levels in the population; see Fig E2 in this article's Online Repository at www.jacionline.org). Chi-square analyses at the 10%

level revealed that OTU 46 classified as *B dentium* was significantly different at 10 days for the 4-category data ($P = .039$, BH-FDR-corrected), while no OTUs were significant at the detection level (see Fig E3 in this article's Online Repository at www.jacionline.org).

We visualized the 10% level association of *B dentium* for the category data using a Venn diagram (Fig 2, A). Compared with the distribution expected by chance, there was a 6-fold overrepresentation of *B dentium* for the PA group ($P = .001$, permutation test). In addition, there was a slight overrepresentation of *B dentium* in the combined PA and PN groups ($P = .016$, permutation test). For the detection level, however, there was no divergence from that expected by chance (Fig 2, B).

Bifidobacteria levels were previously associated with birth mode. We therefore investigated whether *B dentium* levels were affected by mode of delivery. Twenty-six of 204 children for whom we had information on the delivery mode were delivered by Cesarean section. We did not detect any significant differences in either prevalence or abundance of *B dentium* between 2 birth modes at any time point (Fig E3). In addition, no association between detection of *B dentium* in mothers and in their children was found (data not shown).

We also had information on mother's age at birth, child's birth weight, sex, history of atopy in the family, and possession of pets and smoking in family. None of these factors was significantly associated with either abundance or prevalence of *B dentium* in children (Spearman $c = -0.05$; $P = .43$ between *B dentium*

abundance and both mother's birth age and child's birth weight; for other factors, see Table E2 in this article's Online Repository at www.jacionline.org).

We found that for the detection level, OTU 104 belonging to *Lactobacillus* showed a significant overrepresentation in the probiotic group (PN and PA combined) at 10 days, and OTU 87 belonging to *Bifidobacteria* in mothers at 90 days postpartum (see Figs E4 and E5 in this article's Online Repository at www.jacionline.org). BLAST search against the National Center for Biotechnology Information database identified the closest match to OTU 104 as *Lactobacillus rhamnosus* GG and to OTU87 as *Bifidobacterium animalis* subsp. *lactis*. Because there was a potential confounding association of OTU 104 and 46 related to probiotic intervention, we tested the direct association between these OTUs. We found no associations between OTU 104 and OTU 46.

Our results support the hypothesis of the probiotic intervention effect being dependent on the intrinsic microbiota, with high levels of *B dentium* in combination with a deviating microbiota at 10 days being significantly associated with lack of probiotic intervention effect on AD, or even potentially and increased risk of AD (the opposite effect as for the whole population⁴).

The probiotic intervention had no effect on the presence of *B dentium*. The only OTUs showing significant association with the probiotic intervention were OTUs closely related to *L rhamnosus* GG and *B animalis* subsp. *lactis* BB12. Both these bacteria were used in the intervention, and their presence in stool samples has previously been confirmed by quantitative PCR.⁵ The colonization of the child with *L rhamnosus* GG, however, was not directly associated either with AD or with *B dentium*.

All the probiotic strains are lactic acid producers. Therefore, the combination of a deviating microbiota and low pH could be contributing factors to *B dentium* outgrowth, if present. *B dentium* is able to colonize both the oral cavity and the gut, in addition to withstanding low pH and produce gamma-aminobutyric acid (GABA).^{7,8} The potential production of GABA is of particular interest. It has recently been shown that GABA is important not only as a neurotransmitter inhibitor but also as an important immunomodulator.⁹ GABA has been associated with reduced levels of AD-like skin lesions in adult mice.^{E5} Given that *B dentium*-produced GABA has an immunomodulatory role in the infant gut, the potential effect in the infant gut is opposite of what is expected from the mouse experiment. This discrepancy can potentially be explained by the fact that the timing of immunomodulatory processes is crucial for a normal immune development.^{E6} Future research, however, is clearly needed to unveil potential mechanistic roles of *B dentium* and GABA in AD development.

In conclusion, our results contribute to the importance of early colonization for diseases later in life. Furthermore, our results point toward the hypothesis that the effect of probiotics

in the prevention of AD can be dependent on the intrinsic microbiota.

We thank Norwegian University of Life Sciences and Norwegian University of Science and Technology for the financial support. Travels and stays in Spain and Norway for this study were supported by EEA Coordinated Mobility of Researchers NILS Science and Sustainability Project 017-CM-01-2013.

Ekaterina Avershina, PhD^a

Raul Cabrera Rubio, PhD^b

Krister Lundgård, MSc^a

Gaspar Perez Martinez, PhD^b

Maria Carmen Collado, PhD^b

Ola Storrø, MD, PhD^c

Torbjørn Øien, MD, PhD^c

Christian Kvikne Dotterud, MD, PhD^{c,d}

Roar Johnsen, MD, PhD^c

Knut Rudi, PhD^a

From ^athe Department of Chemistry, Biotechnology and Food Science, University of Life Sciences, Ås, Norway; ^bthe Department of Biotechnology, Institute of Agrochemistry and Food Technology, Spanish National Research Council, Valencia, Spain; ^cthe Department of Public Health and General Practice, Norwegian University of Science and Technology, Trondheim, Norway; and ^dthe Department of Dermatology, St Olavs Hospital, Trondheim, Norway. E-mail: knut.rudi@nmbu.no.

Disclosure of potential conflict of interest: T. Øien has received a grant and travel support from Tine BA. C. K. Dotterud has received a grant from Tine BA. K. Rudi has a board membership with Genetic Analysis AS; receives royalties from Nofima AS and Invent2, UiO; and owns stock in Genetic Analysis AS.

REFERENCES

1. Pelucchi C, Chatenoud L, Turati F, Galeone C, Moja L, Bach JF, et al. Probiotics supplementation during pregnancy or infancy for the prevention of atopic dermatitis: a meta-analysis. *Epidemiology* 2012;23:402-14.
2. Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 2001;357:1076-9.
3. Panduru M, Panduru NM, Salavastru CM, Tiplica GS. Probiotics and primary prevention of atopic dermatitis: a meta-analysis of randomized controlled studies. *J Eur Acad Dermatol Venereol* 2015;29:232-42.
4. Dotterud CK, Storro O, Johnsen R, Oien T. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. *Br J Dermatol* 2010;163:616-23.
5. Dotterud CK, Avershina E, Sekelja M, Simpson MR, Rudi K, Storrø O, et al. Does maternal perinatal probiotic supplementation alter the intestinal microbiota of mother and child? A randomized controlled trial. *J Pediatr Gastroenterol Nutr* 2015;61:200-7.
6. Avershina E, Lundgård K, Sekelja M, Dotterud C, Storrø O, Øien T, et al. Transition from infant- to adult-like gut microbiota. *Environ Microbiol* 2016;18:2226-36.
7. Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C. Gamma-aminobutyric acid production by culturable bacteria from the human intestine. *J Appl Microbiol* 2012;113:411-7.
8. Andriantsoanirina V, Allano S, Butel MJ, Aires J. Tolerance of *Bifidobacterium human* isolates to bile, acid and oxygen. *Anaerobe* 2013;21:39-42.
9. Prud'homme GJ, Glinka Y, Wang Q. Immunological GABAergic interactions and therapeutic applications in autoimmune diseases. *Autoimmun Rev* 2015;14:1048-56.

<http://dx.doi.org/10.1016/j.jaci.2016.09.056>

REFERENCES

- E1. Spergel JM. From atopic dermatitis to asthma: the atopic march. *Ann Allergy Asthma Immunol* 2010;105:99-106; quiz 107-9, 117.
- E2. Devenny A, Wassall H, Ninan T, Omran M, Khan SD, Russell G, et al. Respiratory symptoms and atopy in children in Aberdeen: questionnaire studies of a defined school population repeated over 35 years. *BMJ* 2004;329:489-90.
- E3. Bieber T. Atopic dermatitis. *Ann Dermatol* 2010;22:125-37.
- E4. Wold AE. The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? *Allergy* 1998;53:20-5.
- E5. Hokazono H, Omori T, Ono K. Effects of single and combined administration of fermented barley extract and gamma-aminobutyric acid on the development of atopic dermatitis in NC/Nga mice. *Biosci Biotechnol Biochem* 2010;74:135-9.
- E6. Storro O, Avershina E, Rudi K. Diversity of intestinal microbiota in infancy and the risk of allergic disease in childhood. *Curr Opin Allergy Clin Immunol* 2013;13:257-62.

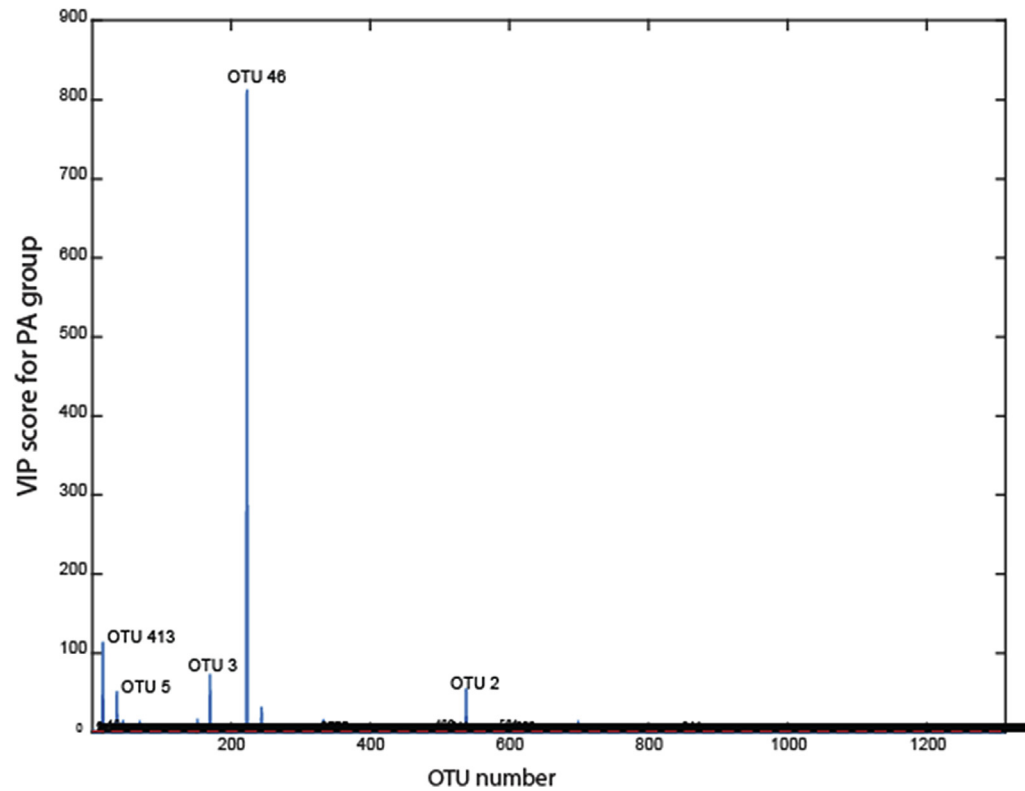


FIG E1. OTUs important for explaining the association between the PA group at 10 days and the microbiota by partial least squares discriminant analysis. *VIP*, Importance of each OTU in fitting both OTUs and PA groups for partial least square discriminant analysis.

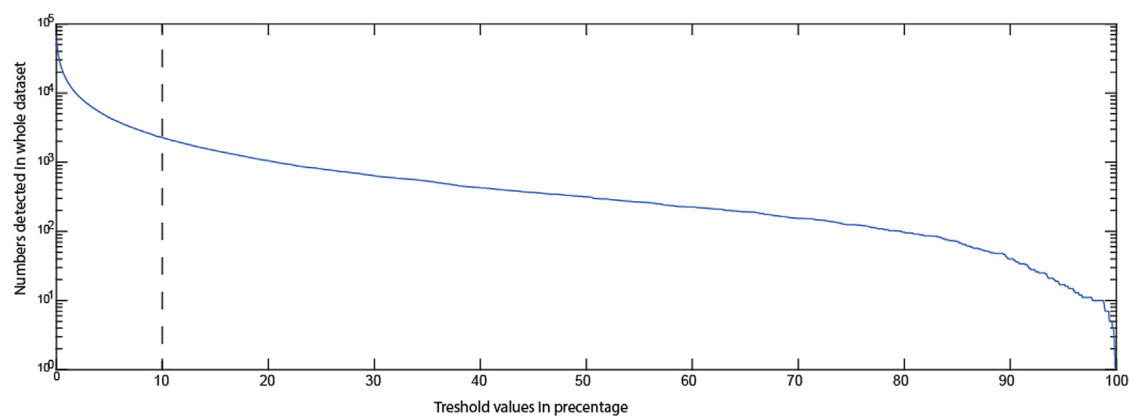


FIG E2. Number of occurrences of OTUs above the different thresholds. The stippled line at 10% represents the lower log-linear range for the high-abundant OTUs. This threshold was therefore used to define high high-abundant OTUs.

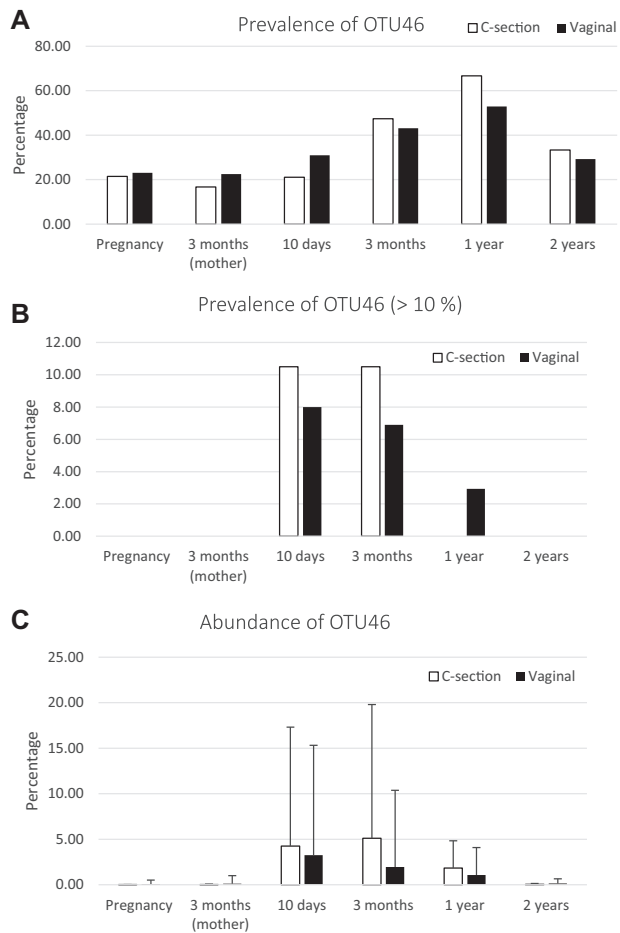


FIG E3. Prevalence of OTU46 (*B dentium*) at detection (A), 10% level (B), and its average abundance (C) in stool samples from children born vaginally or by C-section, and in stool samples from their mothers.

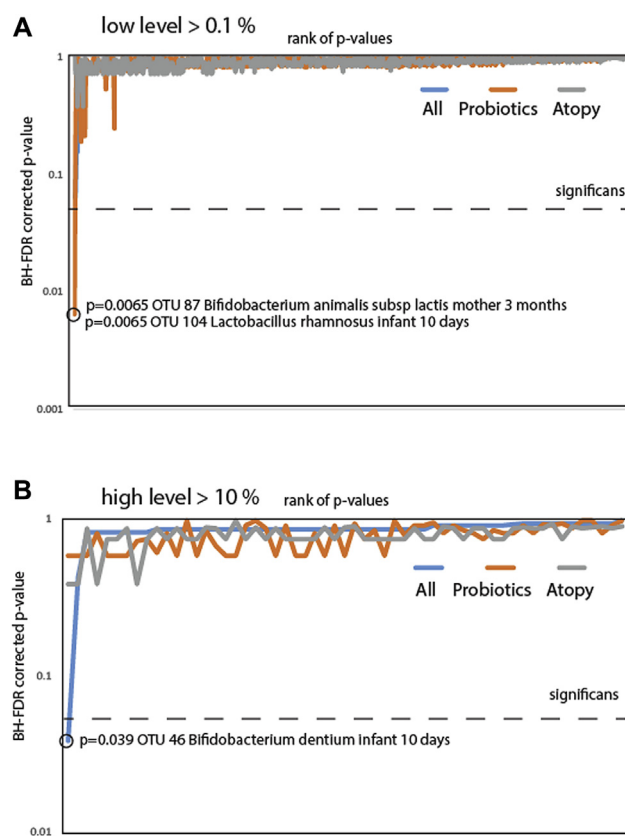


FIG E4. BH-FDR-corrected P values for detected (**A**) and high-level (**B**) OTUs. Fig E4, A and B, Tests for all age categories were included in the FDR correction. A, Singletons were excluded for detection. B, A relative abundance of more than 10% was used as a threshold for determining high levels.

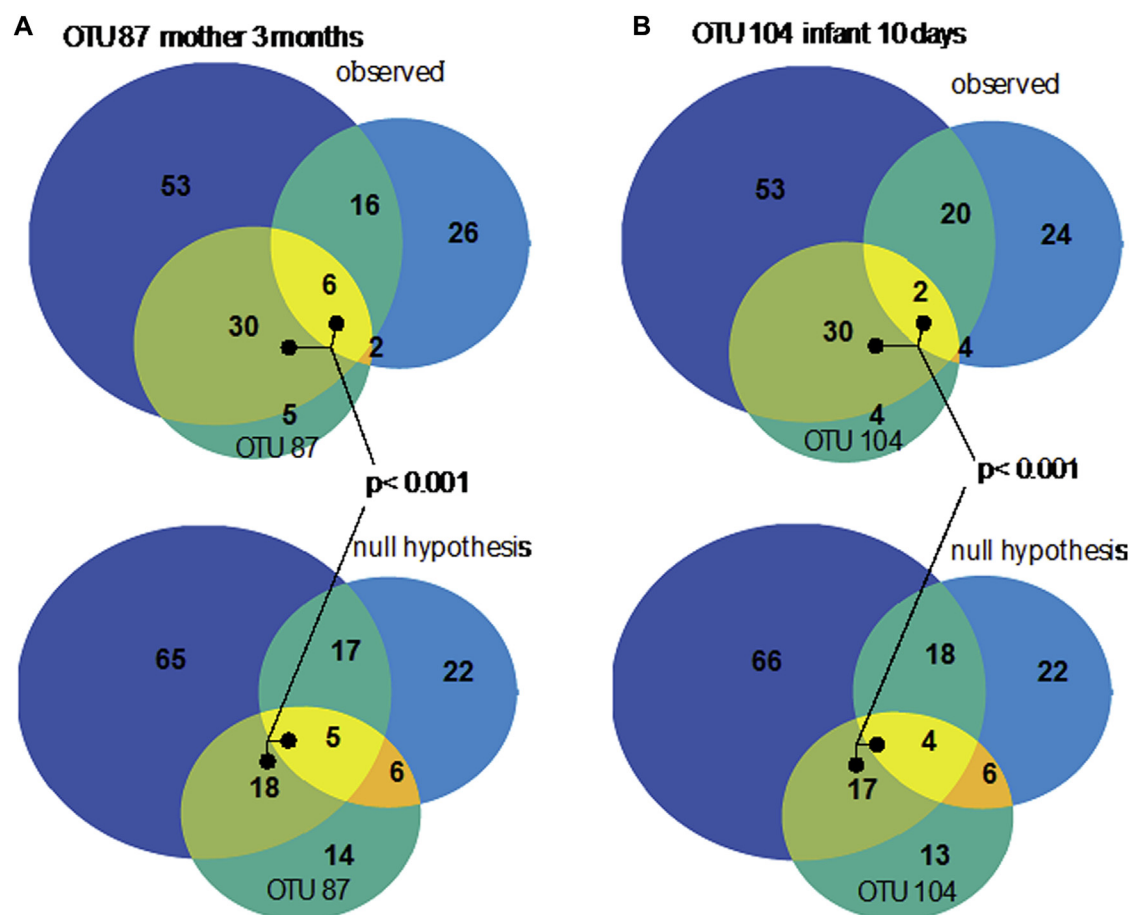


FIG E5. Prevalence of OTU 87 for mothers 3 months postdelivery (**A**) and OTU 104 for 10-day children (**B**). The prevalence data are represented as Venn diagrams, comparing the observed distribution with that expected under the null hypothesis with no associations of OTUs. Statistical testings were based on permutations.

TABLE E1. Number of samples at each time point

Age	Total no. of samples	Probiotic		Placebo	
		Atopy (PA)	No atopy (PN)	Atopy (NA)	No atopy (NN)
MotherPreg	167	18	61	27	57
3 mo	200	22	83	28	66
10 d	178	17	65	30	64
3 mo	193	22	74	29	66
1 y	216	24	82	40	70
2 y	171	18	65	29	57

TABLE E2. Abundance of *B dentium* in 10-day-old infants with regard to various demographic factors

Demographic factor	Mean $\pm$ SD, %		<i>P</i> value	FDR- <i>P</i>
	Group 1	Group 2		
Sex	2.5 $\pm$ 10.3	4.3 $\pm$ 12.9	.97	.40
Breast-feeding at 3 mo	3.6 $\pm$ 12.1	0.05 $\pm$ 1.1	.77	.51
Atopy in family	3.8 $\pm$ 12.8	12.7 $\pm$ 8.5	.21	.35
Pets in family	5.1 $\pm$ 14.4	2.3 $\pm$ 9.2	.79	.44
Smoking father	7.0 $\pm$ 18.3	2.5 $\pm$ 9.2	.63	.70
Smoking mother	21.6 $\pm$ 29.4	2.2 $\pm$ 8.3	.04	.15